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COLD-INDUCIBLE RNA BINDING PROTEIN (CIRP) IMPEDES PROLIFERATION AND INFLAMMATION IN THE PYMT MOUSE MODEL FOR BREAST CANCER

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BY

DANIEL ALBINO LUJAN

B.S. Biology, University of New Mexico, 2013

DISSERTATION

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DEDICATION

This dissertation is dedicated to my mom, for when she believed I was smart enough to achieve big things. To my dad for being my supporter and instilling me with my tenacity. To my grandmas for teaching me resilience and emotional intelligence. To Noelle, for being my rock and partner through our biggest obstacles and achievements.

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ABSTRACT

RNA binding proteins (RBPs) regulate gene expression by controlling mRNA export, translation, and stability. When altered, some RBPs allow cancer cells to grow, survive, and metastasize. Cold-inducible RNA binding protein (CIRP) is overexpressed in a subset of breast cancers, induces proliferation in breast cancer cell lines, and inhibits apoptosis. We generated a transgenic mouse model overexpressing human CIRP in the mammary epithelium to ask if it plays a role in mammary gland development. We also assessed the effects of CIRP on breast tumorigenesis using breeding crosses with the PyMT mouse model for breast cancer. CIRP decreased proliferation at the lactational switch during mammary gland development, but no differences in morphology were found compared to wild-type mice. In the PyMT model, CIRP decreased tumor growth, pulmonary metastasis, and several protumorigenic cytokines. CIRP also decreased $CD3^+CD4^+$ T-cells while increasing $CD3^+CD8^+$ T-cells, suggesting CIRP decreased tumorigenesis by altering inflammation.

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Chapter 1 - Introduction: Cold-Inducible RNA Binding Protein in Cancer and Inflammation

Abstract

RNA binding proteins (RBPs) play key roles in RNA dynamics, including subcellular localization, translational efficiency and metabolism. Cold-inducible RNA binding protein (CIRP) is a stress-induced protein that was initially described as a DNA damage-induced transcript (A18 hnRNP), as well as a cold-shock domain containing cold-stress response protein (CIRBP) that alters the translational efficiency of its target messenger RNAs (mRNAs). This introduction summarizes recent work on the roles of CIRP in the context of inflammation and cancer. The function of CIRP in cancer appeared to be solely driven though its functions as an RBP that targeted cancer-associated mRNAs, but it is increasingly clear that CIRP also modulates inflammation. Several recent studies highlight roles for CIRP in immune responses, ranging from sepsis to wound healing and tumor-promoting inflammation. While modulating inflammation is an established role for RBPs that target cytokine mRNAs, CIRP appears to modulate inflammation by several different mechanisms. CIRP has been found in serum, where it binds the TLR4-MD2 complex, acting as a Damage-associated molecular pattern (DAMP). CIRP activates the NF-κB pathway, increasing phosphorylation of Iκκ and IκBα, and stabilizes mRNAs encoding pro-inflammatory cytokines. While CIRP promotes higher levels of pro-inflammatory cytokines in certain cancers, it also decreases inflammation to accelerate wound healing. This dichotomy suggests that the influence of CIRP on inflammation is context dependent and highlights the importance of detailing the mechanisms by which CIRP modulates inflammation.

CIRP AS AN RNA BINDING PROTEIN

RNA binding proteins (RBPs) play key roles in RNA dynamics, including subcellular localization, translational efficiency and metabolism [1, 2]. As these diverse roles suggest, RBPs have been identified as key molecules in many diseases, including neurodegenerative disorders, cardiovascular disease, genetic disease, developmental disorders and several cancers [2].

Cold-inducible RNA binding protein (CIRP, also known as CIRBP and A18 hnRNP) belongs to the glycine-rich RNA-binding protein family, which possesses an RNA recognition motif (RRM), and a carboxyl-terminal domain containing several arginine glycine glycine (RGG) motifs which facilitate protein-protein interactions (Figure 1.1) [3]. CIRP is expressed in wide variety of tissues and cells and can be induced in response to cellular stress, translocating from the nucleus to the cytosol [4, 5]. In the cytosol, CIRP binds the 3' untranslated regions (UTRs) of target mRNAs using its RNA-recognition motif (RRM) and can increase or suppress their translation [3, 6, 7]. Originally described as a DNA damage-induced transcript [6], and named heterogeneous nuclear ribonucleoprotein A18 (A18 hnRNP) [7], CIRP was later characterized as a cold-stress response protein. Upon moderate cold stress, CIRP is expressed and translocates from the nucleus to the cytoplasm, where it binds to poly(U) polypyrimidine tracks at the 3' ends of introns as well as to 5' and 3' regions of mRNAs [8]. Its binding has been suggested to be important for 3'end cleavage and polyadenylation, as well as for regulating translation of specific mRNAs helping the cell to adapt to cold stress [9]. CIRP's initial roles as a tumor suppressor was established during hypothermic stress and DNA damage [3, 7].

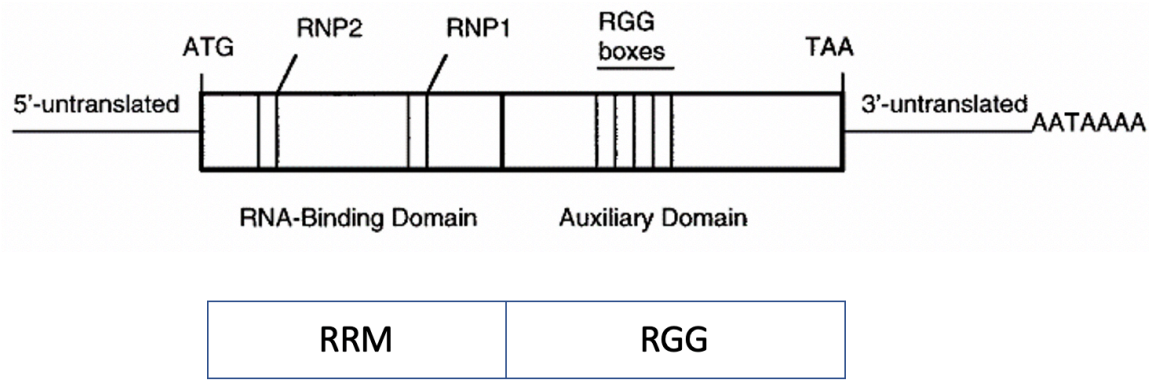

Figure 1.1 Schematic of CIRP mRNA. CIRP is ~18kD in size and contains an RNA-recognition motif (RRM) and an auxiliary domain containing several arginine-glycine-glycine (RGG) boxes, which facilitates protein-protein interactions.

Recent studies have implicated CIRP in human disease, including several types of cancer, as well as a modulator of inflammation [10-17]. In this introduction, we summarize what is known about the role of CIRP in human cancers, where it has been implicated in tumor suppression and promotion, as well as its emerging role in inflammation in human disease, including its role in cancer-related inflammation.

CIRP AS A TUMOR SUPPRESSOR

One of the earliest reported functions of CIRP was suppression of mammalian cell growth *in vitro* in response to mild hypothermia [3]. In this study, CIRP overexpression in NIH3T3 cells slowed cell growth by prolonging the G1 phase of the cell cycle. These effects were abolished upon siRNA mediated knockdown of CIRP. In response to DNA damage, CIRP was upregulated and increased the translational efficiency of the mRNAs for thioredoxin (Trx-1), replication protein A (RPA2) and ATR, by binding both their 3'UTR and eukaryotic translation initiation factor 4 gamma (eIF4G) [18, 19]. Trx-1 quenches reactive oxygen species while RPA2 is involved in repair of

damaged DNA. ATR signals cell cycle arrest and initiates response to DNA damage by recruiting RPA2 and other repair proteins. This ability to inhibit proliferation and protect cells from genotoxic damage during cellular stress *in vitro* is consistent with circumstantial evidence for a tumor suppressor role for CIRP *in vivo*.

In agreement with a role in suppressing proliferation, in normal endometrium CIRP expression inversely correlated with the proliferation marker Ki-67 during the menstrual cycle. CIRP expression was consistently highest in normal endometrium, variable in endometrial hyperplasias and significantly reduced in carcinomas [20]. This hints that loss of CIRP expression could play a role in endometrial carcinogenesis. Loss of CIRP expression was also implicated in the progression of benign ovarian cancer to malignancy. A large microarray analysis of benign and malignant human ovarian tumors identified CIRP as an upregulated gene in benign vs. malignant tumors. This finding was confirmed using CIRP-transfected ovarian cancer cells, where CIRP transfected cells slowed doubling time [21]. Lastly, higher CIRP levels in breast tissue were part of the gene signature identified for parity, a condition with reduced lifetime risk for breast cancer for post-menopausal women [22].These studies suggest that CIRP may function as a tumor suppressor via suppressing proliferation, potentially via its function in the DNA damage response. CIRP's reported roles as a tumor suppressor are summarized in Table 1 and Figure 1.2.

Table 1.1 Roles of CIRP as a Tumor Suppressor			
Role	**Possible Mechanisms/Conclusions**	**Experimental Model(s)**	**References**
Slows cell growth via prolonging G1 phase	Possible interaction with G1 regulators	Mouse Fibroblasts (BALB/3T3 Cells)	[3]
Inhibits proliferation through its function as a stress-induced RBP	Binds and increases translational efficiency of DNA damage response genes ATR, Trx-1, RPA2 via 3' UTR binding	Human Rectal carcinoma cells (RKO cells)	[18, 19]
Inversely correlates with proliferation/reduced in endometrial cancer compared to normal endometrium	Maintains normal endometrial function, aside from its role as a stress response protein	Human Normal Endometrium and Endometrial Carcinoma Tissues	[20]
Slows doubling time in ovarian cancer cells/reduced in malignant compared to benign ovarian tumors	Prevents malignancy in ovarian tumors	Human Malignant and Benign Ovarian Tumors, Ovarian Cancer Cells	[21]
Decreases proliferation during mammary gland development	Halts proliferation when it is no longer needed during development	Transgenic mice expressing hCIRP in mammary glands	[23]
Part of transcriptomic signature for parity	Breast differentiation leads to transcriptomic changes that decrease the lifetime risk for breast cancer in parous women	Human breast biopsies	[24]

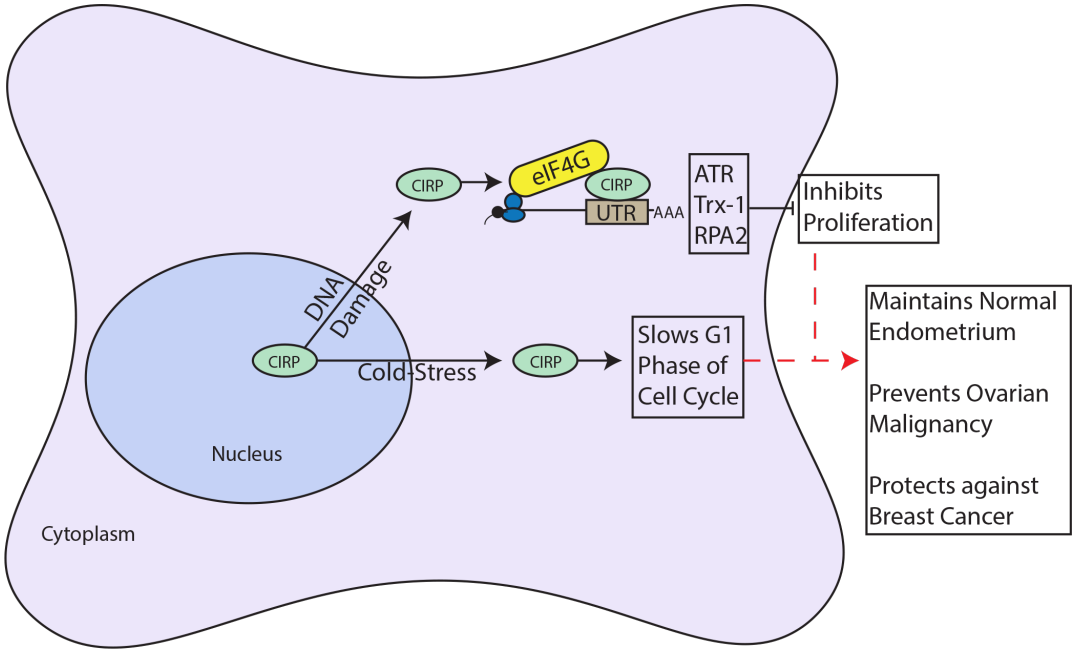

Figure 1.2 CIRP as a Tumor Suppressor. CIRP moves from the nucleus to the cytoplasm in response to stress such as DNA damage and cold-stress. In the cytoplasm, CIRP binds the 3'UTR of mRNAs encoding ATR, Trx-1 and RPA2, and interacts with eIF4G to increase translational efficiency of these mRNAs. The end result is inhibition of proliferation. In response to cold-stress, CIRP slows G1 phase of the cell cycle by an unknown mechanism. Red dashed arrows indicate potential links to suggested CIRP functions.

CIRP AS A TUMOR PROMOTER

Other studies provide evidence for CIRP as a tumor promoter. CIRP displayed the ability to bypass replicative senescence in primary mouse embryonic fibroblasts through activation of the ERK1/2 signaling pathway [25]. In this study, CIRP was overexpressed in subsets of prostate, breast, and colon cancer, correlative evidence for an *in vivo* role as a tumor promoter. Downregulation of CIRP enhanced chemosensitivity and impaired survival of prostate cancer cells [26]. CIRP downregulation was thought to mimic the

molecular effects of heat stress on prostate cancer cells, which is known to boost the efficacy of chemotherapeutics. CIRP was overexpressed in multiple breast cancer cell lines, with its overexpression contributing to upregulation of cyclin E1, increasing proliferation and decreasing apoptosis [27]. Cyclin E1 positively regulates the cell cycle and is a marker of poor prognosis in breast cancer [28, 29]. Also through its function as an RBP, CIRP upregulated HIF-1α to promote tumor growth in ectopic mouse xenograft models of human breast cancer and melanoma [30]. As further evidence for a role in breast tumorigenesis, CIRP was identified as part of a serum autoantibody signature in breast cancer progression from ductal carcinoma *in situ* to invasive breast cancer [31]. This suggests that CIRP may be targeted by the immune system during breast cancer development.

In addition to its upregulation in carcinoma, CIRP was upregulated in pituitary corticotroph adenoma, where its expression correlated with Cushing's disease recurrence [32]. CIRP overexpression in pituitary corticotroph cells increased cell proliferation as well as proopiomelanocortin transcription, a marker of aberrant pituitary function. Similar to studies with primary mouse embryonic fibroblasts, CIRP promoted proliferation via ERK-signaling, in this case by downregulating the CDK-inhibitor p21, and inducing cyclin D1, which promotes G1 progression. This study identified CIRP as a marker of poor prognosis in pituitary adenoma and as a possible marker for recurrence.

Further evidence for CIRP's oncogenic function is its upregulation of telomerase activity in a temperature dependent fashion (mild hypothermia) in several human cell lines of different origins including HEK293T (embryonic kidney), HeLa (ovarian cancer),

HTC75 (T lymphocytes), and U2OS (osteosarcoma) [33]. Telomerase is responsible for adding telomeric repeats to chromosomal ends and consists of the reverse transcriptase TERT and the RNA subunit TERC. Telomere shortening due to lack of or decreased telomerase activity causes cellular senescence, while aberrant activation of telomerase has been observed in >85% of human cancers [34]. CIRP regulated telomerase through its function as an RBP, binding to TERC mRNA to increase its stability. CIRP also regulated telomerase assembly via protein-protein interaction in Cajal bodies, increasing telomerase complex stability during assembly [33]. These studies show that CIRP can directly bypass senescence by boosting telomerase function, as well as indirectly by promoting signaling pathways that promote proliferation.

Recent mechanistic studies show that CIRP promotes epithelial to mesenchymal transition (EMT) [35] and inhibits apoptosis via ERK activation [36, 37], both established hallmarks of cancer [38], by downregulating p53. TGF-β treatment of human lung carcinoma cells (A549 cells) overexpressing CIRP upregulated mesenchymal markers, downregulated epithelial markers, and increased migration and invasion. Knockdown of CIRP abrogated these effects as well as downregulated the EMT marker Snail, which suggests that CIRP likely upregulates Snail through activation of ERK and p38MAPK pathways. Table 1.2 summarizes CIRP's purported roles in promoting tumorigenesis.

Table 1.2 Roles of CIRP as a Tumor Promoter			
Role	**Possible Mechanisms/Conclusions**	**Model**	**References**
Bypasses replicative senescence *in vitro;* overexpressed in breast, prostate, colon and pituitary cancers	Enhances translation via ribosomal protein S6 and 4E-BP1 interactions; increases ERK activation, cyclin D1 and proliferation; decreases p27 via Erk1/2 signaling pathway	Mouse Embryo Fibroblasts and Human Tumors	[25, 32]
Downregulation impairs prostate cancer cell survival	Downregulation mimics heat stress and impairs cancer cell survival	Human Prostate Cancer Cell Lines (PC3 and LNCaP Cells)	[26]
Upregulates HIF-1α in breast cancer and melanoma xenografts, cyclin E1 in breast cancer cells, and is part of autoantibody signature in breast cancer	Stabilizes HIF-1α and cyclin E1 mRNAs, increasing their translation	Human Breast Cancer cells (MCF7 Cells), Human Melanoma Cells (HEMa-LP) and Human Breast Tumors	[27, 30, 31]
Knockdown results in decreased telomerase activity *in vitro*	Stabilizes TERC mRNA, increasing its translation; stabilizes telomerase complex assembly in Cajal bodies	Cancer Cell Lines (HTC75, HeLa, U2OS, HEK293)	[33]
Upregulation decreases p53 levels and activity, decreasing apoptosis; induced EMT markers	Unknown mechanism of p53 regulation; function in EMT relies on its RRM motif	Lung Carcinoma Cells (A549); Hepatoma cells (HepG2, SK-HEP-1)	[35-37]

Taken as a whole, these studies provide evidence for CIRP's role as an oncogene through its actions in various contexts, including bypassing replicative senescence, upregulating telomerase activity, promoting proliferation, inhibiting apoptosis, and promoting EMT (Figure 1.3). Although not yet experimentally linked, these roles are likely interdependent. For example, CIRP's role in p53 regulation and EMT suggests that it could be affecting senescence and telomerase maintenance via its regulation of p53, as the link between p53, replicative senescence and telomerase maintenance has been

established in many cancer types [39-42]. Links between the known roles of CIRP in activating ERK signaling pathways and potential downstream effects on p53 have not been explored.

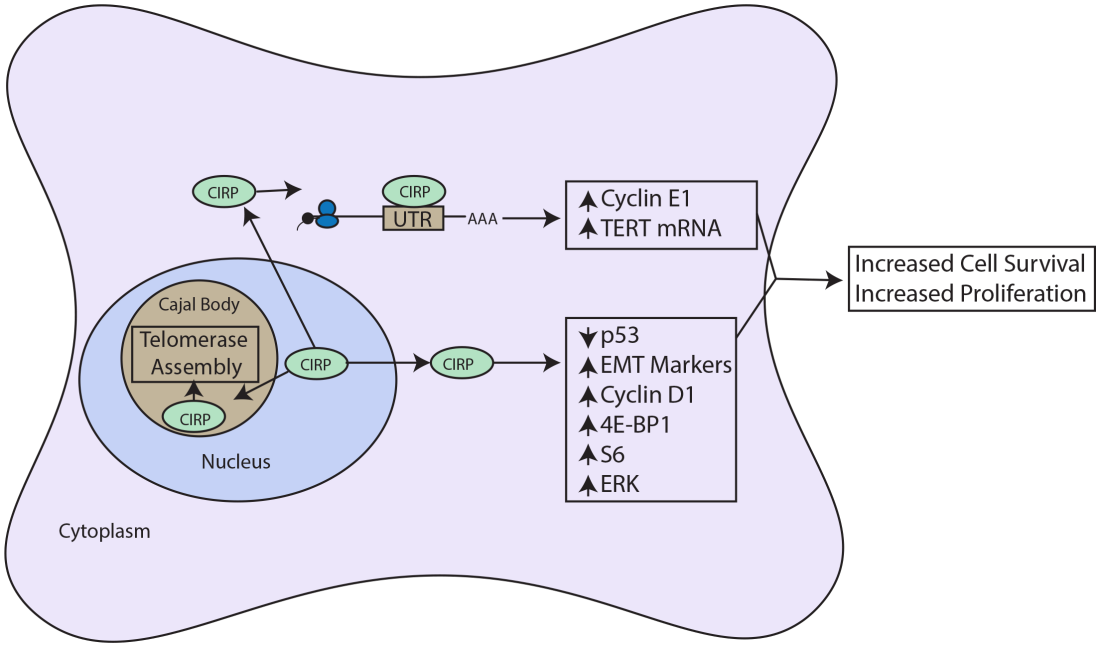

Figure 1.3 Roles of CIRP as a tumor promoter. CIRP moves from the nucleoplasm into Cajal bodies where it stabilizes hTERC and telomerase complex assembly via protein-protein interaction. CIRP also moves from the nucleus to the cytoplasm where it binds and stabilizes cyclin E1 mRNA, and binds hTERT mRNA, increasing both of these proteins. CIRP also downregulates p53 and upregulates EMT markers, cyclin D1, 4E-BP1, S6 and ERK1/2 by unknown mechanisms. The overall result is increased cell proliferation and cell survival. Arrows and boxes in black represent known roles from literature and red dashed arrows and boxes represent possible mechanisms or connections.

CIRP AS A MEDIATOR OF INFLAMMATION

Several recent studies highlight roles for CIRP in immune responses, ranging from sepsis and pulmonary inflammation to wound healing and tumor-promoting

inflammation The first study to implicate CIRP in inflammation showed that it was upregulated in the serum of patients undergoing hemorrhagic shock and sepsis [14]. Patients with elevated levels of CIRP had a significantly higher mortality rate than patients without elevated CIRP levels. This finding was noteworthy because it was the first time CIRP was observed extracellularly. CIRP was also upregulated in the heart and liver of mice undergoing hemorrhagic shock and was released via lysosomal secretion; neutralizing antibodies against CIRP ameliorated the hemorrhagic effects. CIRP was shown to function as a DAMP in this study, as it bound to the TLR4-MD2 complex on professional antigen presenting cells (APCs) and stimulated release of pro-inflammatory cytokines TNFα and IL-6. Although this study first hinted that CIRP could serve as a marker for poor prognosis in sepsis and hemorrhagic shock, a more recent study corroborated and confirmed their findings [43]. The mechanism of CIRP's extracellular localization was not explored in either of these studies, nor was its function as an RBP. Bolognese et al. recently established that CIRP also activates splenic T-cells via TLR-4 [44]. CIRP stimulated both $CD8^+$ and $CD4^+$ T-cells and drove $CD4^+$ to a Th1 type response profile, which could explain its abilities as a tumor suppressor in specific contexts [44]. CIRP may also play a role in synovial inflammation, as it is upregulated in the serum and synovial fluid of patients with rheumatoid arthritis and is correlated with inflammatory disease activity, which implicates CIRP as a marker for chronic synovial inflammation [17].

In a model for pulmonary cold-stress, CIRP mediated increased tissue injury, inflammation and increased mucus secretion through the TLR4/NF-κB pathway [12]. These effects were further supported by the finding that CIRP was increased in both

patients with chronic obstructive pulmonary disease (COPD) and mice exposed to cold air. Blocking of CIRP expression, TLR4 function, or NF-κB function, each attenuated an increase in inflammation, tissue damage and mucus secretion. CIRP can also increase mucus secretion and airway inflammation though a TLR4 independent mechanism. Using rats exposed to either cigarette smoke or cold air, CIRP was increased in a TLR4-independent fashion [13]. This study also showed that CIRP overexpression in normal human bronchial epithelial cells promoted the formation of stress granules and that CIRP bound to the 3'UTR of MUC5AC to increase its translational efficiency and thus increases mucus production and secretion. These studies show that CIRP can modulate airway inflammation by triggering TLR4, further establishing CIRP as a DAMP, as well function as an RBP to increase respiratory mucus.

Further elucidating CIRP's function in pulmonary inflammation, CIRP stimulated activation and assembly of the Nlrp3 inflammasome in mouse lung vascular endothelial cells (MLVECs) [45]. Treatment of MLVECs with CIRP led to increased caspase-1 and IL-1β and induction of pyroptosis, while intravenous CIRP injection into wild-type mice led to endothelial cell activation and significant lung damage. These findings provide a possible mechanism for lung damage under conditions of hemorrhagic shock and sepsis [46].

CIRP likely stimulates release of proinflammatory cytokines through activation of the NF-κB pathway [11]. Using UVC radiation to induce upregulation of CIRP in neonatal foreskin fibroblasts (NFhTrt cells), Brochu et al. screened for novel CIRP induced transcripts and identified IL-1β, IL-8 and TNFα mRNAs[11]. These increases were mediated by the NF-κB pathway, as blockade of CIRP decreased Iκκ phosphorylation. Additional evidence for CIRP as a positive regulator of the NF-κB pathway was provided

by Zhou et al. when they found that CIRP activated NF-κB in microglia, which caused neuroinflammation *in vivo* and apoptosis in neurons *in vitro* [47]. Although the direct mechanism is not known, these studies provide strong support for CIRP as a positive regulator of the NF-κB pathway. While CIRP can upregulate TNFα through the NF-κB pathway, TNFα has also been shown to be a negative regulator of CIRP through the non-canonical RelB NF-κB pathway, suggesting a negative feedback loop [48, 49].

Although the studies above provide several contexts in which CIRP functions as a positive inflammatory modulator, CIRP has also been shown to decrease inflammation [50]. In a study of CIRP in wound healing, wild-type and CIRP knockout mice were subjected to a full thickness wound. When measured post-injury, TNFα expression was increased 65-fold in CIRP knockout mice and only 16-fold in wild-type mice, which suggests CIRP could be decreasing wound-associated inflammation [50]. In addition, during the initial (inflammatory) phase of wound healing, TNFα was significantly decreased in wild-type mice when compared to CIRP knockout mice. Wounds in CIRP knockout mice healed significantly faster with shorter initial inflammatory phases when compared to wild-type mice. Taken together, these studies show that the function of CIRP in inflammatory signalling appears to be context dependent (Table 1.3 and Figure 1.4), as further illustrated by studies of CIRP-influenced inflammation in cancer, discussed in the next section.

Table 1.3 Roles of CIRP as a Mediator of Inflammation

Role	Possible Mechanisms/Conclusions	Model	References

Stimulates inflammation in sepsis and hemorrhagic shock; where it serves as a marker for poor prognosis	Secreted through lysosomal export and binds the TLR4/MD2 complex on APCs to stimulate release of TNFα and IL-6	Human serum, mouse and rat models of sepsis and hemorrhagic shock	[14, 43]
Activates splenic T-cells during sepsis, contributing to T-cell dysregulation	Binds TLR4/MD2 complex on $CD4^+$ and $CD8^+$ T-cells to induce activation and Th1 hyperinflammatory response	Mice with induced sepsis	[44]
Mediates lung damage, inflammation and increased mucus secretion; elevated in COPD patients and cold-air exposed mice and rats	Increases inflammation and tissue damage by activation of TLR4/NFκB pathway; stabilizes MUC5AC mRNA in stress granules to increase mucus secretion	Human bronchial biopsies, bronchial epithelial cells, mice exposed to cold-air, rats exposed to cold-air or cigarette smoke	[12, 13]
Elevated in serum and synovial fluid from patients with rheumatoid arthritis	Levels in synovial fluid strongly correlated with markers for rheumatoid arthritis disease activity; potential marker for rheumatoid arthritis	Human serum and synovial fluid	[17]
Increases caspase-1, IL-1β and induced pyroptosis; CIRP injections into mice causes endothelial cell activation and significant lung damage	Activates assembly of the Nlrp3 inflammasome; possible mechanism for lung damage in sepsis and hemorrhagic shock	Mouse lung vascular endothelial cells (MLVEC), WT Mice	[45, 46]
Induces IL-1β, IL-8 and TNFα transcripts	Increases Iκκ phosphorylation, activating inflammation through NFκB pathway	Neonatal foreskin fibroblasts (NFhTrt cells)	[11]
Activates NFκB *in vivo* and apoptosis in neurons	Positively regulates NFκB causing neuroinflammation	Mouse Fibroblasts (NIH3T3 cells), mouse models of neuroinflammation	[47, 49]

Knockout mice have higher TNFα expression during initial wound healing phase	Decreases TNFα during the initial (inflammatory) phase of wound healing	Wild-type and CIRP knockout mice subjected to wounds	[50]

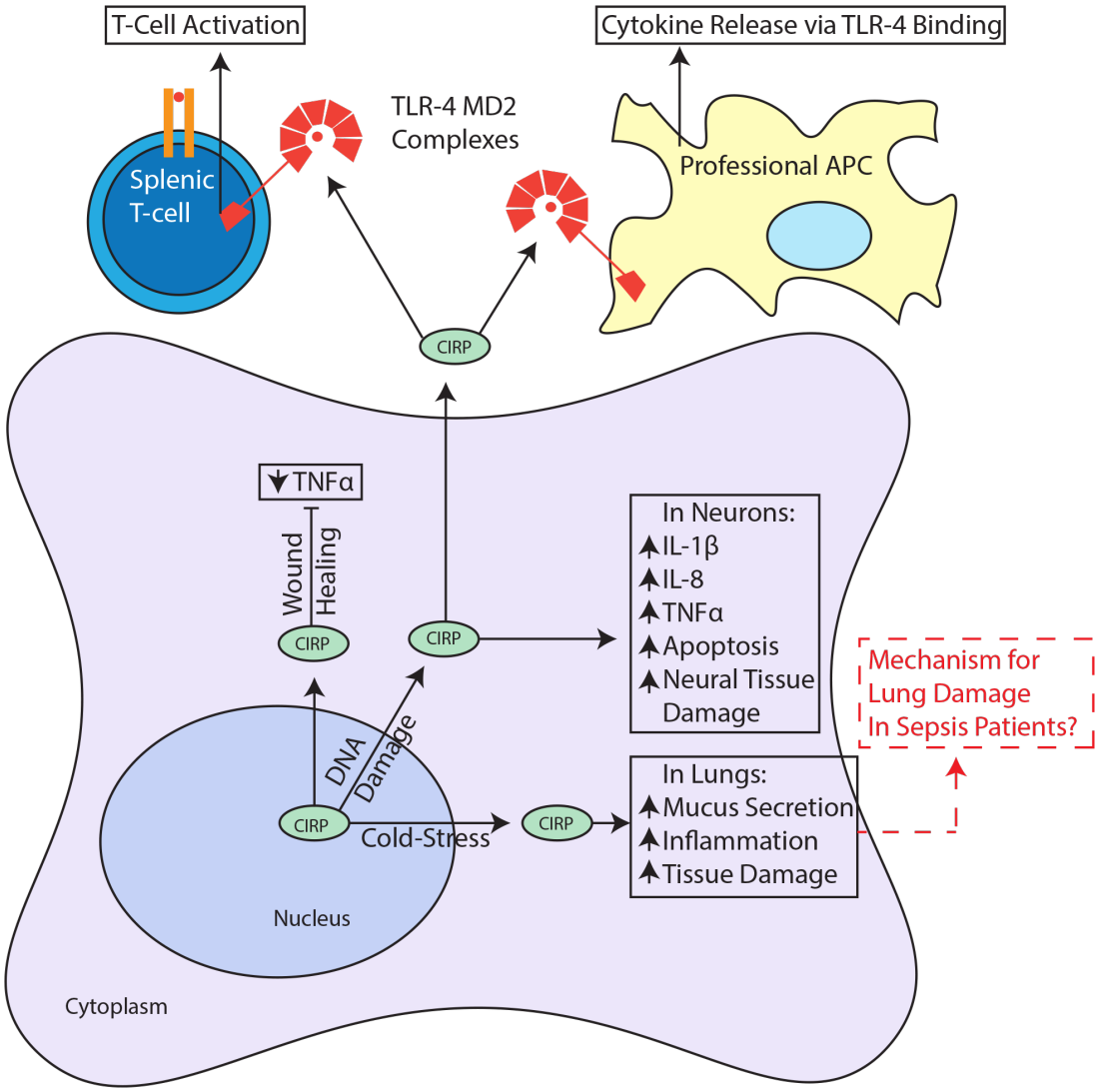

Figure 1.4. Roles of CIRP as a mediator of inflammation. In response to cold-stress CIRP stimulates mucus secretion, inflammation and tissue damage in the lung. In response to DNA damage, CIRP decreases TNFα to promote wound healing and increases IL1β, IL-8 and TNFα in neurons as well as inducing apoptosis and neural tissue damage. Also, in response to DNA damage, CIRP is found extracellularly where it binds MD2 in TLR-4

complexes of professional antigen presenting cells (APC) and splenic T-cells. This results in cytokine release and T-cell activation. Red dashed arrows indicate potential links to suggested CIRP functions. Arrows and boxes in black represent known roles from literature and red dashed arrows and boxes represent possible mechanisms or connections.

CIRP IN INFLAMMATION AND CANCER

Recent studies have begun to highlight the mechanistic link between CIRP's function as an inflammatory molecule and its oncogenic function. In a mouse model for colitis-associated cancer (CAC), CIRP promoted an increase of TNFα and IL-23 expression in inflammatory cells [16]. CIRP knockout mice were less susceptible to CAC development and had reduced expression of chronic inflammation markers TNFα, IL-23 and anti-apoptotic proteins Bcl-2 and Bcl-XL in colonic lamina propria cells. Reduced expression of anti-apoptotic proteins Bcl-2 and Bcl-XL in these mice stimulated inflammatory cell apoptosis in the lamina propria. CIRP deficiency also decreased expression of stem cell marker Sox2 and the number of $Dclk1^{+}$ cells, which is a gut associated cancer stem cell marker. Lastly, bone marrow transplants from CIRP knockout mice into wild-type mice reduced tumorigenesis, indicating that CIRP may function at the stem cell level in CAC. This study concluded that CIRP likely increased expression of IL-17/23 and TNFα via the NF-κB pathway, as CIRP knockout mice had lower levels of IκBα phosphorylation. Interestingly, CIRP levels are elevated in colonic mucosae of patients diagnosed with refractory ulcerative colitis [16]. These patients are considered to be "at risk" for CAC development.

In a study of oral squamous cell carcinoma (OSCC), expression of TLR4 and CIRP were both elevated in OSCC tissues when compared to matched normal tissues. Both CIRP and TLR4 expression correlated with poor outcomes, implicating that both are markers of poor prognosis [15]. Since CIRP is a known trigger of TLR4 through its function as a DAMP, it is possible that CIRP and TLR4 activity may lead to increased levels of pro-inflammatory cytokines in OSCC, although it has yet to be studied. Although CIRP is capable of triggering TLR4, upon TLR4 binding, CIRP was shown to promote a Th1 type of response, which is typically effective in eliminating cancer [51].

Reinforcing CIRP's promotion of inflammation in cancer is a study of hepatocellular carcinoma (HCC) [52]. In this study, CIRP was found to increase expression of IL-1β and IL-6 in Kupffer cells, liver specific macrophages. In addition, CIRP expression correlated with levels of reactive oxygen species (ROS) as well as with HCC recurrence. CIRP knockout mice exhibited attenuated tumorigenesis, reduced ROS accumulation and reduced expression of IL-1β and IL-6. CIRP deficiency also decreased expression of the cancer stem cell marker CD133, providing further evidence that CIRP may play a role in promoting stemness in HCC as well as in CAC and possibly in other forms of cancer. CIRP was shown to promote inflammation in the liver during ischemia/reperfusion injury, which promotes liver metastasis of colon cancer. This suggests a role for CIRP in metastasis [53, 54]. These studies firmly establish CIRP's role as a molecular modulator of inflammation and cancer (Figure 1.5), providing a rationale for the work proposed and completed for this dissertation.

Summary

This introduction summarizes historical and recent studies into the roles of CIRP in cancer, inflammation, and cancer-associated inflammation. One of the most notable recent developments is CIRP's influence on cancer through its effects on inflammation. The function of CIRP in cancer appeared to be mainly driven though its functions as an RBP, promoting the stability and translation of specific mRNAs encoding cancer-associated proteins [55]. It is becoming clear that CIRP also functions as a modulator of inflammation in several forms of cancer as well as in other diseases. Playing a role in inflammation is not a new role for RBP's in general [10, 55, 56]. A recent study integrated diverse public domain datasets to catalogue 1344 RBPs and perform a meta-analysis of the RBPome [57]. An important finding was that RBPs are significantly associated with inflammatory diseases and immune responses, as well as all major types of cancer. CIRP was noted in this report. Recent findings expand the scope of the CIRP's capabilities and provoke several new questions. For instance, it is now known that CIRP is capable of promoting higher levels of inflammatory cytokines in certain cancers, but also appears to be capable of decreasing inflammation to accelerate wound healing. This dichotomy suggests that the influence of CIRP on inflammation is context dependent and highlights the importance of detailing the mechanisms by which CIRP modulates inflammation in various contexts. Since it is now clear that CIRP is capable of influencing cancer as both an RBP and as an inflammatory modulator, it is possible that CIRP plays more than one role.

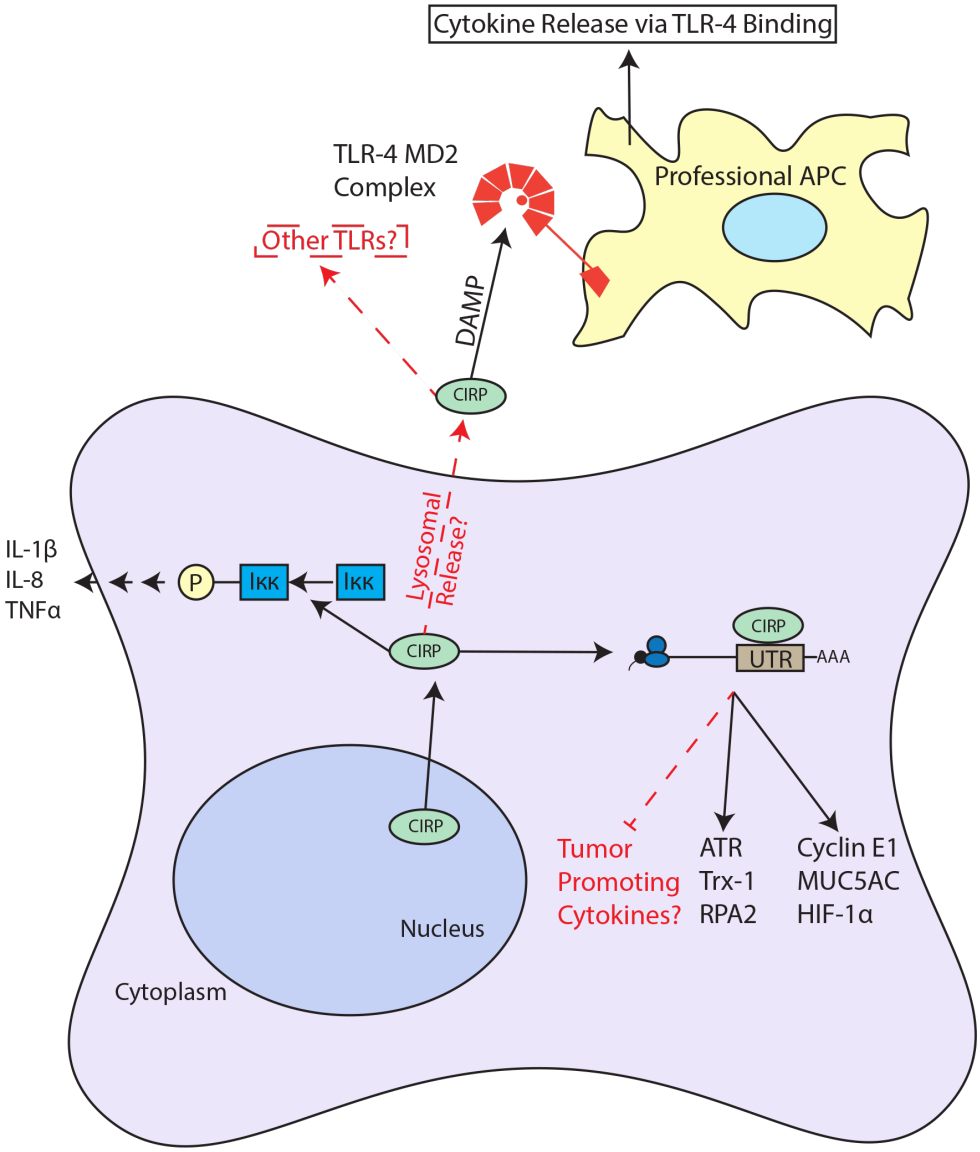

Figure 1.5. Summary of CIRP Roles in Inflammation and Cancer. CIRP shuttles from the nucleus to the cytoplasm to bind the 3' UTRs of target mRNA to increase their translation. It is possible that CIRP can bind tumor promoting cytokines in certain contexts. CIRP in found extracellularly in patients with sepsis (likely through lysosomal secretion) where it binds the TLR-4 MD2 complex, functioning as a DAMP and stimulating cytokine release from APCs. Also, CIRP increases Iκκ phosphorylation through an unknown mechanism. Arrows and boxes in black represent known roles from

literature and red dashed arrows and boxes represent possible mechanisms or connections.

Goal of Dissertation

Previous studies in our laboratory focused on the post-transcriptional regulation of the cell-cycle protein cyclin E1 in breast cancer. Cyclin E1 is a positive regulator of the cell cycle. In breast cancer cell lines, we showed that HuR, a stress induced RBP, binds and upregulates cyclin E1 [27]. In addition, we found that when HuR is downregulated via siRNA, CIRP is upregulated. We also found that CIRP binds the cyclin E1 3'UTR in association with HuR, and that when CIRP is overexpressed, HuR expression increases by an unknown mechanism, leading to downstream increases in cyclin E1. A follow-up study in our laboratory showed that CIRP is overexpressed in several breast cancer cell lines when compared to nontumorigenic breast epithelial cells, including both cell lines and non-transformed cells [58]. In this study, overexpression of exogenous CIRP increased cyclin E1 and breast cancer cell proliferation and decreased apoptosis. We also showed that culturing invasive breast cancer cells in 3-dimensional collagen I inhibits proliferation through direct negative regulation of cyclin E1, and dual effects on CIRP, which is both downregulated and translocated to the cytoplasm [59]. These studies along with others that showed CIRP is upregulated in some human breast cancers [25] led to our current focus on the role of CIRP in normal breast epithelial cells and breast cancer.

CIRP is expressed in breast epithelial cells, overexpressed in breast cancer cells [27] and some human breast cancers [25, 32], and has been shown to modulate

inflammation, CIRP is one of the first RNA binding proteins that has the potential to modulate the immune response in breast cancer. We and others have shown that CIRP has pro-tumorigenic effects *in vitro*, such as increased expression of cancer-associated proteins, inhibition of apoptosis, and promotion of cell division [26, 33, 37]. Paradoxically, as discussed earlier in this introduction, there is also strong evidence to suggest that CIRP can act as a tumor suppressor. One potential explanation for this paradox is that the immune system may be driving the anti-tumor effects *in vivo*, while *in vitro* experiments do not have an immune component. In support of this hypothesis, recent studies have shown CIRP as a capable driver of both local and systemic inflammatory immune responses in the brain, in hemorrhagic shock and sepsis, in colon cancer, and in liver cancer [14, 16, 47, 52].

Our long-term goal is to understand CIRP functions in normal breast epithelial cells and in breast cancer *in vivo*. Based on our preliminary data and work by others, our central hypothesis is that CIRP functions in both and may either increase or impede breast tumorigenesis, possibly through promotion of an acute inflammatory response and/or by a pro-tumorigenic inflammatory response. The following aims test our hypothesis:

Aim 1: Determine the role of CIRP in normal mammary gland development

The first aim of this dissertation will query the role of CIRP in normal breast epithelial cells during breast (mammary gland) development, when they actively proliferate (puberty and pregnancy), produce milk, and then undergo apoptosis during involution (following weaning). Since CIRP is expressed in mammary epithelial cells but has not been studied in this context, it is important to first establish an understanding of

CIRP in a normal physiological setting. Using a novel transgenic mouse that expresses human CIRP (hCIRP) in mammary epithelial cells under control of the mouse mammary tumor virus (MMTV) regulatory sequences, we investigate the effects on mammary gland development, which begins at puberty, through pregnancy, lactation and involution. Prior to this investigation, the majority of studies on CIRP focused on CIRP in various contexts of disease, providing rationale for exploring the role of CIRP in normal development. Results from this Aim are presented in Chapter 2.

Aim 2: Determine the effects of CIRP overexpression on breast tumorigenesis and inflammation

The second aim of this dissertation will investigate the role of CIRP overexpression on mouse mammary tumorigenesis and inflammation. Effects on CIRP overexpression will be assessed by crossing the CIRP transgenic mouse with the MMTV-PyMT mouse model for breast cancer. We will measure differences in tumor growth and inflammation at both early and late stages of tumorigenesis between CIRP/PyMT mice and PyMT/+ mice. Based on previous findings that show CIRP is overexpressed in breast cancer cell lines, and was shown to alter inflammation in liver and colorectal cancers, CIRP overexpression may either decrease or increase tumor latency, while affecting tumor size and metastases by influencing the inflammatory environment. Results from this aim are presented in Chapter 3.

Aim 3: Determine the effects of CIRP loss in a PyMT graft model

Studies show that CIRP may have a role in maintaining normal homeostasis in certain tissues types. For example, evidence suggests that CIRP could have a role in

maintaining normal endometrium, as expression of CIRP is significantly lower in endometrial carcinoma when compared to normal endometrium [20]. Similarly, CIRP expression is also significantly lower in malignant ovarian tumors when compared to benign ovarian tumors [21]. Due to these and previous studies, this aim will investigate the effects of CIRP loss on mammary tumorigenesis using Py2T tumor cell grafts. Py2T cells are a cell line established from a tumor resected from an MMTV-PyMT mouse that can be grafted into the mammary fat pads of wild-type mice to assess their tumorigenicity. Py2T cells will be treated with CRISPR-Cas9 ribonucleocomplexes with guide RNAs targeting the coding sequence of the CIRP gene. Control treatments will be completed using non-specific guide RNAs. CIRP depleted and control Py2T cells will be injected into contralateral mammary fat pads of wild-type mice at 7 weeks of age. We predict that CIRP loss may lead to an increase in tumorigenesis. Results from this aim are presented in Chapter 3.

Altogether, results from these aims will show the normal physiological function of CIRP *in vivo* during mammary gland development and how CIRP functions in breast tumorigenesis, including if CIRP affects tumor associated inflammation. Despite recent therapeutic advancements, breast cancer remains the most common cancer in women and causes ~40,000 deaths in the US each year. With a better understanding of the function of CIRP in both normal breast development and breast cancer, we can rationally design new therapies targeting CIRP for either prevention or therapy and determine if CIRP can serve as a biomarkers for early detection and prognosis.

Chapter 2: Cold-inducible RNA binding protein in mouse mammary gland development

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Abstract

RNA binding proteins (RBPs) regulate gene expression by controlling mRNA export, translation, and stability. When altered, some RBPs allow cancer cells to grow, survive, and metastasize. Cold-inducible RNA binding protein (CIRP) is overexpressed in a subset of breast cancers, induces proliferation in breast cancer cell lines, and inhibits apoptosis. Although studies have begun to examine the role of CIRP in breast and other cancers, its role in normal breast development has not been assessed. We generated a transgenic mouse model overexpressing human CIRP in the mammary epithelium to ask if it plays a role in mammary gland development. Effects of CIRP overexpression on mammary gland morphology, cell proliferation, and apoptosis were studied from puberty through pregnancy, lactation and weaning. There were no gross effects on mammary gland morphology as shown by whole mounts. Immunohistochemistry for the proliferation marker Ki67 showed decreased proliferation during the lactational switch (the transition from pregnancy to lactation) in mammary glands from CIRP transgenic mice. Two markers of apoptosis showed that the transgene did not affect apoptosis during mammary gland involution. These results suggest a potential *in vivo* function in suppressing proliferation during a specific developmental transition.

Introduction

RNA binding proteins (RBPs) are key players in RNA metabolism, influencing the expression of many genes by regulating mRNA splicing, export, localization, translation, stability, and surveillance [60-62]. RBPs bind to RNA and other proteins to form ribonucleoprotein (RNP) complexes [62]. While RBPs typically associate with specific regulatory sequences in the 5'- and 3'- untranslated regions (UTRs) of mRNA, association of some RBPs to RNA is structure-dependent [62]. Some RBPs, like cold-inducible RBP (CIRP), also known as hnRNP A18 [63], have been implicated in the etiology of cancer and other diseases as their target mRNAs facilitate proliferation, migration, and angiogenesis and inhibit apoptosis [9, 62].

CIRP is predominantly found in the nucleus with low amounts in the cytosol of cells [63]. However, cytosolic levels of CIRP increase in response to stressors such as cold, UV irradiation, hypoxia, and oxidation [63, 64]. Cytosolic CIRP increases mRNA translation by binding to the 3'UTR of target mRNAs [65]. Some studies suggest that CIRP suppresses tumorigenesis because it binds and upregulates tumor suppressor genes, one example being ATR, which inhibits proliferation and protects cells from cellular stress [63, 65, 66]. CIRP expression has also been found to be decreased in endometrial cancer as compared to normal endometrial tissue, where it inversely correlates with proliferation [67]. CIRP has also been identified as a part of the genomic signature for multiparity, which is a condition associated with a reduced lifetime risk of developing breast cancer[68]. However, other studies suggest CIRP acts as a proto-oncogene because it is overexpressed in some cancers, including colon and breast cancers [69]. CIRP's proto-oncogenic effects include increasing general translation, immortalizing

primary cells, inhibiting apoptosis, promoting proliferation, and promoting inflammation [14, 47, 69-73].

Our previous studies showed that CIRP is upregulated in several different breast cancer cell lines as compared to normal and nontransformed breast epithelial cells [74, 75]. The overexpression of CIRP upregulated another RBP, the stress-induced Human antigen R (HuR) and its target, cyclin E1, a positive regulator of the G1/S cell cycle transition [75]. HuR, like CIRP shuttles from the nucleus to the cytosol in response to stress. Overexpression of HuR and its cytosolic localization has been linked to poor outcomes in several forms of cancer, including breast cancer [76-80]. Both HuR and CIRP contribute to deregulation of cyclin E1 in breast cancer cell lines [74, 75]. Our unpublished work has shown that CIRP is expressed in the normal mammary epithelium of both humans and mice (T.H. and R.S.H.).

Based both on published studies and our unpublished observation of CIRP expression in normal mammary epithelium, this study sought to determine whether CIRP plays a role in mammary gland development. To do this, we generated a hemizygous transgenic mouse expressing the human CIRP (hCIRP) gene in the mammary epithelium. We show that hCIRP decreases proliferation in the mammary gland during the lactational switch, suggesting a specific developmental role.

Materials and Methods

Creation of transgenic mice

cDNA of the full-length human CIRP coding sequence was inserted into the EcoRI site of the MKbpAII vector, generously provided by Dr. Jeffrey Rosen, Baylor College of

Medicine [81]. The vector contains the mouse mammary tumor virus long terminal repeat (MMTV-LTR), which restricts transgene expression primarily to the mammary gland. Transgenic FVB mice were generated by the MD Anderson Genetically Engineered Mouse Facility. Offspring were tested for integration of the transgene by PCR of genomic DNA isolated from tail clips, using primers specific for the transgene (CIRP genotyping F, Table 1) and the vector (CIRP genotyping R, Table 1). Founder mice were mated to wild-type mice to generate five individual lines of transgenic mice. qRT-PCR was used to measure hCIRP expression after normalizing expression to that of 18s ribosomal RNA or the epithelial marker cytokeratin 18, which gave identical results. Figure 1 shows CIRP normalized to 18s as it is a general housekeeping gene that has been shown to have the least variability in the mammary glands of FVB mice when compared to other reference genes [82]. Two transgenic lines designated 559 and 1459 were considered for further analysis based on robust expression of the transgene in the mammary glands.

Table 2.1 Primers used for creation of CIRP transgenic mice and for qRT-PCR			
Primer	Source	Sequence	Final Concentration
Human CIRP F2	IDT	5'—CGG GCG GGT CCT ACA GA—3'	20 micromolar
Human CIRP R2	IDT	5'—CAC TTG TCA TCG TCG TCC TTG T—3'	20 micromolar
Mouse CIRP F	IDT	5'—GGC GGC AGA TCA GAG TTG A—3'	20 micromolar
Mouse CIRP R	IDT	5'—TCG GGA CCG GTT GTC AGA—3'	20 micromolar
CIRP genotyping F	IDT	5'—TAC TAT AGC AGC CGG AGT CAG AGT—3'	20 micromolar
CIRP genotyping R	IDT	5'—AAC AGA TGG CTG GCA ACT AGA AGG—3'	20 micromolar
Cytokeratin 18 F	IDT	5'—CAA GTC TGC CGA AAT CAG GGA C—3'	20 micromolar
Cytokeratin 18 R	IDT	5'—G TCC CTG ATT TCG GCA GAC TTG—3'	20 micromolar
18S ribosomal RNA F	IDT	5'-GTA ACC CGT TGA ACC CCA TT-3'	20 micromolar
18S ribosomal RNA R	IDT	5'-CCA TCC AAT CGG TAG TAG CG-3'	20 micromolar

Collection of mammary tissue

Female mice were euthanized and the skin with the mammary glands still attached was removed. One mammary gland #4 was removed and cut in half lengthwise. One half was frozen in liquid nitrogen for protein analysis and the other half was homogenized in Trizol (Invitrogen, Carlsbad, CA) and frozen at -80ºC for RNA extraction. The skin with the remaining mammary glands was pinned flat onto paraffin wax and fixed in 4% paraformaldehyde overnight. After rinsing in cold 1x phosphate buffered saline (PBS), mammary glands 2, 3 or 4 were removed from the skin with a scalpel and used for whole mounts and immunostaining.

Whole mounts

Whole mounts were prepared by rinsing paraformaldehyde-fixed number 4 mammary glands two times with acetone over an 8-24 hour period followed by a series of water rinses over an hour and then staining with carmine alum stain solution, consisting of 0.2% carmine red and 0.5% aluminum potassium sulfate, overnight with

gentle shaking [83]. Glands were destained in a graded series of ethanol and stored in methyl salicylate. All steps were performed at room temperature. Whole mounts were imaged using a stereomicroscope and Motic camera on Motic Images Plus 2.0 ML software. Multiple images of the mammary gland were taken and composite images of whole mounts were created using Adobe Photoshop. For 5-week pubertal mammary gland (day 35 of age or D35) whole mounts, terminal end bud number and size (8 CIRP and 7 wild type) were quantitated using Image J software (Version 1.46r).

Immunostaining

Mammary glands were prepared from 5 week-old pubertal mice (D35), pregnant mice at day 16 of gestation (G16), lactating mice (1 day after birth or lactation day 1, L1) and day 3 after forced weaning (involution day 3, I3). Formaldehyde-fixed tissues were dehydrated through an ascending series of ethanol, cleared with Hemo-De and then embedded in Paraplast Plus. Paraffin sections were cut at 4μm and stained with hematoxylin and eosin for histological examination, or used for immunohistochemistry, immunofluorescence, or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Antibodies are detailed in Table 2.

For histological examination, sections were briefly stained with a 30 second hematoxylin dip, followed by 1-10 acid alcohol dips and a 30 second-2 minute Eosin Y dip. The sections were then dehydrated in an ascending series of ethanol rinses and mounted with DPX and coverslipped.

For immunohistochemistry (IHC) and immunofluorescence analysis (IFA), paraffin sections were rehydrated to 1x PBS (IFA) or 1x PBS with 0.1% Tween 20 (PBST) (IHC), and

subjected to antigen retrieval for 15-20 minutes at 90-95°C in a waterbath in 10mM Tris pH 9.0, 1mM EDTA and 0.05% Tween20. For IHC, endogenous peroxidase activity was suppressed by incubation with 3% H_2O_2 in PBS for 30 minutes with agitation, followed by 1x PBS + 3% normal goat serum (NGS) + 0.1% Triton X-100 for 30 minutes to prevent non-specific staining. Sections were incubated in primary antibody (as indicated in Table 2) diluted in 1xPBS + 3% NGS + 0.1% Tween20 and overnight at 4ºC. Following the primary antibody, sections were washed 3 times in 1x PBST for 10 minutes each and then incubated in donkey anti-rabbit IgG HRP secondary antibody for 1 hour at room temperature. Sections were developed using DAB as chromagen and washed in 1x PBS twice for 5 minutes, followed by 1 wash with diH_2O. Sections were then counterstained in Mayer's progressive hematoxylin for 15 seconds, dehydrated and mounted using DPX mount (Electron Microscopy Science, Hatfield, PA) and a cover slip. Slides were viewed and photographed using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera and analyzed using Image-J software (1.46r). For Ki-67 staining, bar graphs were produced using representative images to count HRP stained nuclei against non-HRP stained nuclei in mammary glands from 8 CIRP and 6 WT D35, 7 CIRP and 5 WT G16, and 6 CIRP and 4 WT L1 mice. For cleaved caspase 3 staining, mammary glands from 4 CIRP and 4 WT mice were assessed.

For double IFA, sections were blocked and permeabilized in 1x PBS + 2% BSA (Fraction V; Sigma A-4503) + 0.1% Triton X-100 for 30 minutes and incubated overnight at 4°C with primary antibodies diluted in 1x PBS + 2% BSA (V) + 0.1% Tween20 to the following concentrations: rabbit-anti-CIRBP, 0.75 μg/ml (stock 0.3 μg/μl) and goat anti-actin 2 μg/ml (Santa Cruz sc-1616, 100μg/ml stock). After washing 4 x 10 min with PBST,

sections were incubated in secondaries for 2 hours at room temperature in the same antibody diluent (DyLight 549-donkey-anti-rabbit, Jackson ImmunoResearch 711-505-052 and Cy5 donkey-anti-goat, Jackson 705-175-147). Sections were then washed 2 x 10 minutes PBST and 2 x10 minutes PBS, nuclei counterstained with Sytox Green at 0.25 μM and mounted in Gelvatol. Slides were viewed on a Leica TCS SP5 Confocal Microscope.

For TUNEL, slides were stained using the Roche In Situ Cell Death Detection Kit (Fluorescein 11-684-795-910) according to the instructions except that 0.5x TUNEL Dilution Buffer with 25μl Enzymatic Solution from the kit were added to the 450μl label mix. Slides were then mounted using Mowiol + DABCO and a cover slip. TUNEL slides were photographed using a Zeiss Axioscop upright microscope equipped for epifluorescence with a Roper Scientific Photometrics CoolSNAP ES camera using MetaMorph 6.2r4 software and analyzed using Image-J software (1.46r). Mammary glands from 4 CIRP and 4 WT mice were assessed.

Table 2.2 Description of Antibodies

Antibody	Source	Catalogue Number	Dilution	Stock Concentration
Ki67	NeoMarkers	RM-9106-S0	1:300	Not provided
Cleaved Caspase-3	Cell Signaling Technology	9661	1:250	Not provided
CIRBP	Proteintech Group	10209-2-AP	1:400 IFA 1:200 IHC	0.3 mg/ml IFA 1 mg/ml IHC
Actin	Santa Cruz	sc-1616	1:50 IFA 1:1000 IHC	100 μg/ml

qRT-PCR

Total RNA was extracted from #4 mammary glands that were homogenized and stored in Trizol at -80°C, according to the manufacturer's specifications (Invitrogen by Life Technologies, Grand Island, NY,). Briefly, chloroform was added to thawed homogenates and incubated at room temperature for 3 minutes. The sample was centrifuged at 4ºC for 15 minutes at 12,000g. The upper aqueous phase was removed, and RNA precipitated by adding 2 volumes of 100% ethanol and incubating at room temperature for 10 minutes or at -80º overnight. The sample was centrifuged at 4ºC for 10 minutes at 12,000g. The RNA pellet was washed in 80% ethanol, vortexed and centrifuged at 4ºC for 5 minutes at 7,500g. The ethanol was removed, and the pellet was air-dried. The pellet was re-dissolved in 10μl of RNase free water. RNA quality and concentration was assessed using a Nanodrop 2000 (Thermo Scientific, Waltham, MA). cDNA was generated following the SuperScript II RT Protocol (Invitrogen, Carlsbad, CA). qRT-PCR was performed following the SYBR Green PCR Master Mix protocol (Applied Biosystems, Foster City, CA) on Applied Biosystems 7500 Fast Real-Time PCR System for hCIRP, mouse CIRP and 18S ribosomal RNA as a housekeeping gene or cytokeratin 18 an epithelial marker (Table 1). Analysis was completed using the $\Delta\Delta C_T$ method using 18S ribosomal RNA for normalization as it has the least variability in the mammary glands of FVB mice when compared to other reference genes [82]. hCIRP expression in age-matched wild type mice was used as baseline for both human and mouse CIRP expression in order to directly compare their expression. Numbers of mice were 8 CIRP and 8 WT for D35, 10 CIRP and 13 WT for G16, 7 CIRP and 6 WT for L1, and 5 CIRP and 10 WT for I3. We used the maximum number of available mice for each genotype for each development time point.

Statistics

Statistical analysis was performed using GraphPad Prism. A minimum of 5 mammary glands from 5 separate mice were assessed for each condition and an unpaired student's t-test was used to compare CIRP transgenic FVB to wild type FVB mice. All statistical studies had a range from 5-10 CIRP and 6-13 wild type mammary glands from separate mice. Differences were considered significant at a p-value of 0.05 or lower.

Results

CIRP Transgenic Mice

In order to query the function of CIRP in the mammary epithelium, we utilized the mouse mammary tumor virus regulatory sequences (MMTV-LTR) to drive expression of the human CIRP (hCIRP) gene in the mouse mammary epithelium. Human and mouse CIRP are 99% identical at the amino acid level, thus we expect to see a conservation of function. Mammary gland development consists of puberty, pregnancy, lactation, and involution following weaning. The MMTV-LTR drives the expression of transgenes beginning at puberty (35 days of age, D35) through pregnancy and lactation. Transgene expression patterns under the control of the MMTV-LTR have been described in many studies [81, 84]. Out of six initial founders, two lines were chosen for further analysis. Figure 1 shows expression of both hCIRP and mCIRP as quantitated by RT-qPCR. hCIRP expression was detected at 40-fold relative to baseline during puberty at D35 of age, increased during gestation (gestation day 16 or G16), peaked by lactation day 1 (L1, one day after birth of pups), and then gradually decreased. mCIRP was expressed robustly in the pubertal gland, decreased during lactation (L1), and increased again during early involution (involution day 3, I3). The difference in expression between the hCIRP and

mCIRP are due to the MMTV-LTR, which drives the expression of the hCIRP transgene, while mCIRP expression is driven by the endogenous wild-type promoter.

CIRP protein in the developing mammary gland

We next assessed CIRP protein in mammary glands from CIRP and wild type mice using immunohistochemistry. Immunostaining for CIRP showed qualitatively higher levels in mammary glands from CIRP transgenic mice when compared to mammary glands from wild type mice during gestation (G16), lactation (L1) and involution (I3), but similar levels at puberty (D35) (Fig 2, and Fig S1). Interestingly, despite its predominantly nuclear localization in mammary epithelial cell lines (Wu et al 2009, Guo et al 2010), CIRP was predominantly cytosolic in the mammary epithelial cells of both transgenic and wild type mice. Distinct nuclear staining was present in occasional cells in mammary glands from CIRP mice (Figs 2 and S1). These cells appeared to be mainly stromal, were sometimes in a position basal to the ductal cells, and were not seen in wild type mice. The basal position of some of these cells suggested a possible myoepithelial identity.

Notably, cells with similar nuclear staining were seen in the lymph node of number 4 mammary glands (Fig 3A-D), suggesting the possibility that some of the cells with nuclear CIRP near the mammary epithelial ducts could be immune cells. Nuclear CIRP was also seen in lymph nodes from wild type mice as well as in peripheral nerves within the mammary gland of both CIRP and wild type mice (not shown). This was endogenous mouse CIRP as the MMTV promoter is not active in these tissue types in transgenic mice, and is not present in wild type mice.

To determine if the cells with nuclear CIRP near the ductal structures could be myoepithelial, we stained adjacent sections of mammary glands for CIRP and actin,

which is present in myoepithelial cells. Fig. 3E shows a D35 mammary gland in which cells with nuclear CIRP do not obviously contain actin (Fig. 3F). Fig 3H shows cells in an L1 mammary gland in a myoepithelial position that contain actin (arrows) but do not have obviously nuclear CIRP (Fig. 3G). The CIRP antibody used detects both human and mouse CIRP. Therefore, nuclear CIRP in mammary epithelial cells could be either from the transgene, endogenous, mouse protein or both.

Since it is difficult to determine if cells on two adjacent sections are the same cells, we performed double immunofluorescence analysis to further investigate co-localization of CIRP and actin (Fig. 4). Fig. 4A shows CIRP L1 and Fig. 4B-E shows I3 (B, C are CIRP and D, E are wild type) mammary glands co-labeled for CIRP (red) and actin (blue). Nuclei are counterstained green. Column E shows a no primary control taken with a 63x objective, while columns A-D are 2x zooms with this objective. Consistent with IHC results, nuclear and cytosolic CIRP was seen in mammary glands from CIRP mice (A-C) but only cytosolic CIRP in mammary glands from wild type mice (D). CIRP and actin could be seen in the same cell for both CIRP and wild type mammary glands (B and D), but cells with obviously nuclear CIRP did not contain actin (A and C). These results suggest that cells with nuclear CIRP are not myoepithelial cells but rather, based on their location, may be ductal epithelial cells. The brighter points of red seen in the no primary control (E) result from autofluorescence of red blood cells in capillaries throughout the gland.

CIRP does not affect mammary gland morphology

CIRP has been shown to increase proliferation in human breast cancer cell lines [69, 70, 75], other cell lines [64], as well as in male germ cells and in both mouse and human testes *in vivo* [64]. Other studies suggest CIRP suppresses proliferation as CIRP expression is inversely correlated with proliferation in endometrial cancer [67]. CIRP

expression is also positively correlated with reduced malignancy in ovarian cancer [21]. Therefore, we asked whether expression of hCIRP altered proliferation in the mammary gland. During puberty, the ductal system expands within the adipose stroma, with proliferation occurring in cells of the terminal end buds (TEBs), found at the tips of the ductal branches. To determine if proliferation was affected at a gross level, enough to affect mammary gland morphology, the morphology of D35 mammary glands as well as the number and size of the TEBs were compared in whole mount mammary glands from pubertal CIRP and wild type mice (Fig 5A, F, G). Whole mount staining of D35 mammary glands showed similar morphology, with no apparent difference in TEB number or size (Fig 5A). Quantitation of TEB number and size confirmed that there were no significant differences between the CIRP and wild type mammary glands (Figure 5F, G). Consistent with these results, mammary glands from D35 pubertal CIRP females were not microscopically distinguishable from mammary glands from wild type mice (Figs 2, 6). This lack of effect on pubertal mammary glands could be due to the relatively low level of expression at this time of transgenes driven by the MMTV-LTR (Fig 1).

Mammary gland morphology was also assessed at G16, L1, I3, and in multiparous mice that had two or three litters (MP). No apparent morphological differences were seen in mammary glands from CIRP and WT mice at G16 when the epithelial tree has undergone extensive branching (Fig 5B), at L1 when the gland is full of milk (Fig 5C), at I3 when apoptosis has begun but gross morphology is similar to the lactating gland (Fig 5D), or in glands from multiparous mice (Fig 5E).

hCIRP expression decreases proliferation at the onset of lactation

To directly study the effect of the human transgene on proliferation within the mammary epithelium, we performed immunohistochemistry for the proliferation marker Ki67. Ki67 is expressed in proliferating cells in any phase of the cell cycle, but is not present in non-proliferating or G_0 cells [85]. Proliferation index (percentage of mammary epithelial cells expressing Ki67) was determined at stages in which mammary epithelial cells are actively proliferating: puberty (D35), gestation day 16 (G16), and lactation day (L1). The majority of mammary epithelial cell growth occurs during puberty and pregnancy. Mammary epithelial cell proliferation continues into early lactation, after which it decreases [86]. Proliferation marker Ki67 was not assessed during involution because proliferation is minimal during this developmental time point [86].

Consistent with the morphological and TEB data, there was no significant difference in proliferation between transgenic and wild type mammary glands during puberty or at gestation day 16 (Figure 6A). In contrast, proliferation was significantly lower in CIRP mice compared to wild type mice at lactation day 1 ($p=0.002$). During this period, proliferation of mammary epithelial cells is normally decreasing, as the gland shifts from a proliferative phase to a glandular phase, where its primary function is to lactate [86]. These results suggest that CIRP can decrease proliferation in the mammary gland *in vivo* during the lactational switch.

hCIRP does not affect apoptosis during early involution

hCIRP was previously reported to inhibit apoptosis in mouse fibroblasts, neural stem cells, and in cortical neurons *in vivo* [70, 87-89]. The mammary gland undergoes a

process of involution characterized by two stages, 1) apoptosis of the epithelial cells and 2) the breakdown of the alveolar structures, which result in return of the gland to its resting state. We chose to examine mammary glands 3 days after forced weaning (involution day 3, I3) because it is during this part of involution that the mammary epithelial cells are undergoing peak apoptosis. Specifically, peak cleaved-caspase 3 staining is seen 3 days following forced weaning in mammary glands from FVB mice [90]. As mammary epithelial cells have been shown to undergo both caspase 3-dependent and caspase 3-independent apoptosis [91], we used two methods to assess apoptosis, immunohistochemistry for cleaved caspase 3 (CC3) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Figure 7 shows that there was no significant difference between levels of apoptosis in I3 mammary glands from CIRP or wild type mice, when assessed with either marker.

Discussion

We previously showed that CIRP functions to promote proliferation in breast cancer cell lines [75], consistent with other studies showing that CIRP positively regulates proliferation in cultured cells [72]. Another study reported that CIRP was upregulated in 11 of 33 human breast tumors assessed [69], as well as in other tumor types. This study also showed that CIRP stimulates proliferation in primary cells, allowing them to bypass replicative senescence [69]. Unpublished studies by us (TH and RSH) as well as the current study show that CIRP is expressed in both human and mouse normal mammary epithelium. Several studies have found CIRP to have oncogenic functions, either by suppressing apoptosis or promoting proliferation [70, 88, 89]. Sakurai et al. [70] have shown that CIRP can inhibit tumor necrosis factor alpha induced apoptosis *in vitro*.

Furthermore, Li et al. [88] demonstrated that CIRP has neuroprotective effects in primary cortical rat neurons by significantly reducing apoptosis.

In order to assess the function of CIRP *in vivo,* we created a transgenic mouse model that overexpresses human CIRP (hCIRP) in the mammary epithelium. We show that hCIRP overexpression leads to a decrease in proliferation in the mammary epithelium during the lactational switch, but not during puberty or gestation. We also show that hCIRP does not affect apoptosis during weaning, nor does it affect mammary gland morphology.

Decreased proliferation was seen in mammary glands from CIRP transgenic mice one day after birth of pups, at lactation day 1, when the mammary gland is switching from growth to lactation. Despite this result, there were no morphological differences between CIRP and wild-type mammary glands at this time point. Interestingly, proliferation was not affected in pubertal glands or gestation day 16 glands, developmental stages with normally high proliferation rates. While the percentage of proliferating cells seen in our study seemed high, a literature search showed that proliferation is most often assessed in the mouse mammary gland with either BrdU or Phospho-histone H3, which only assess cells in S phase or mitosis, respectively [92, 93]. We chose Ki67 to assess proliferation as it is present in all proliferating cells, in all phases of the cell cycle. Ki67 is also used clinically to assess proliferation rates in breast cancer [94]. The lack of effect on pubertal mammary glands could be due to the low level of activity of the MMTV-LTR at this developmental time, but the MMTV-LTR is highly expressed during gestation [81, 84]. It is possible that peak levels of proliferation are already present at day 16 of gestation. It may also be possible that the *in vivo* function of

CIRP changes, depending on the relative abundance of its target mRNAs. Assessment of known CIRP target genes (at the mRNA, protein and activity levels) is underway in our transgenic mice in order to accurately dissect the *in vivo* role of CIRP at different developmental times.

Our results suggest that CIRP may function in the mammary epithelium to decrease proliferation at a specific developmental time, when high levels of proliferation are no longer needed. It is also possible that CIRP could regulate proliferation only in lactating mammary glands. Our results are consistent with previous *in vitro* studies, which showed a function for CIRP in protecting cells from cellular stress by decreasing proliferation [63, 65, 66]. These studies showed that CIRP decreases proliferation through upregulation of the tumor suppressor gene ATR (as well as others) in response to DNA damage. It is possible that CIRP may be decreasing proliferation at the lactational switch through upregulation of ATR. Experiments to assess expression and activity of ATR at this developmental timepoint will resolve this. Also consistent with our results, a study of human endometrial tissue showed that CIRP level was lower in endometrial cancer compared to normal endometrium [67], where CIRP level inversely correlated with proliferation. Taken together, evidence suggests that CIRP responds to a changing environment to downregulate proliferation when it is no longer needed or when it may be detrimental to a cell or tissue.

We found that the expression of hCIRP in mammary epithelial cells did not affect apoptosis at involution day 3, which is the peak of apoptosis during mammary involution [86]. Apoptosis was assessed using both a TUNEL assay and by the assessment of cleaved caspase-3 because a previous study reported that apoptosis occurs through

both caspase-dependent and independent mechanisms during mammary gland involution [91]. CIRP was found to inhibit apoptosis in mouse fibroblasts and neural stem cells [70, 89]. Interestingly, an *in vivo* study by Masuda et al. (2012) showed that apoptotic levels do not change when CIRP levels change in mouse testicular cells, consistent with our results [95]. However, it is possible that apoptosis levels could be affected in other stages of involution or during puberty, another period of mammary gland remodelling. Assessment of apoptosis during both puberty and additional times during involution should lend further insight into CIRP's potential role in apoptosis during mammary gland development.

Immunohistochemistry for CIRP showed a qualitative increase in mammary epithelial cells from CIRP mice as compared to wild type mice. The increased CIRP seen in epithelial ducts is likely from the transgene, as its expression is limited to mammary epithelial cells under control of the MMTV regulatory sequences. CIRP staining was predominantly cytosolic in both CIRP and wild type mice. This is in contrast to the predominantly nuclear expression seen in previous *in vitro* studies [70-72, 75, 95, 96]. We showed that when mammary epithelial cells were cultured in a 3D collagen matrix, CIRP translocated from the nucleus to the cytosol [59]. CIRP localization is likely affected by the cellular three-dimensional microenvironment. Distinct nuclear staining is also present in mammary glands from CIRP mice. While the identity of the cells containing nuclear CIRP is not known, their position suggests that at least some are mammary epithelial cells. Since CIRP has a known role in signaling [96], it is possible that the hCIRP transgene is inducing expression of endogenous CIRP in neighbouring cells within the stroma. Future studies will query the identity of these cells, as well as any role hCIRP

may play in signaling during mammary gland development. A study by Peng et al. showed that during Xenopus development CIRP is required for the expression of developmentally vital cell adhesion molecules (CAMs), including E-cadherin [96]. Therefore, we will probe roles for CIRP in maintaining expression of CAMs in breast development and breast cancer.

Nuclear staining was also observed in mammary gland lymph nodes and peripheral nerves from both CIRP and wild type mice. CIRP presence in immune cells and nervous tissue is consistent with reported expression of CIRP in neurons [31-33], glial cells [16,17] and immune cells [97].

In summary, the current study shows that CIRP can play an *in vivo* role in the mammary epithelium, decreasing proliferation during the lactational switch. We also show that CIRP does not appear to have a role in apoptosis during the early stages of involution. Consistent with these results, mammary gland morphology is not affected during puberty or gestation, or in the fully involuted, resting or multiparous mammary gland. These results indicate that CIRP may have a role in damping down proliferation when it is no longer needed. CIRP may have an important role in breast cancer, as it was found to be upregulated in 11 of 33 breast tumors studied [69]. According to studies on CIRP's role in other cancers or cancer cell lines, this upregulation could be either tumor promoting or tumor inhibiting [52, 67-69, 96, 97]. However, any correlation between breast cancer patient outcomes and CIRP expression remains unknown. Future experiments will further dissect out the role of CIRP *in vivo* including how CIRP affects the immune environment during mammary gland development [98] and tumor

development, as CIRP has recently been implicated in both local and systemic inflammation [97, 99-101].

Acknowledgments

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Figures

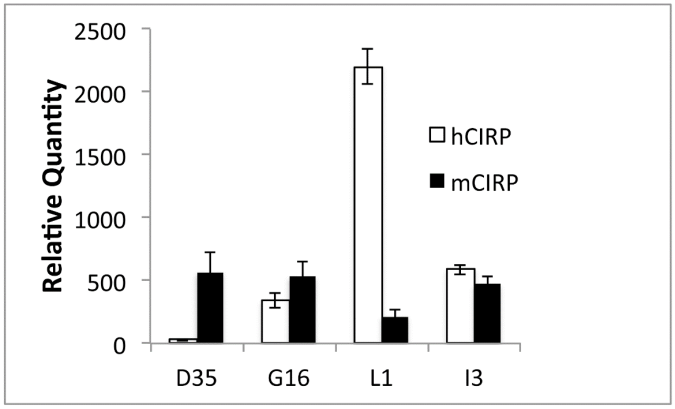

Fig 2.1. Human CIRP (hCIRP) and mouse CIRP (mCIRP) expression during mammary gland development. RT-qPCR of RNA extracted from CIRP and wild type (WT) mouse mammary glands at the developmental stage indicated: day 35 (D35) pubertal mice, gestation day 16 (G16), lactation day 1 (L1), and involution day 3 (I3). RNA level was normalized to 18S ribosomal RNA and quantitated relative to hCIRP expression in age-matched wild type (WT) mice. Numbers of mice were: 8 CIRP and 8 WT for D35, 10 CIRP and 13 WT for G16, 7 CIRP and 6 WT for L1, and 5 CIRP and 10 WT for I3.

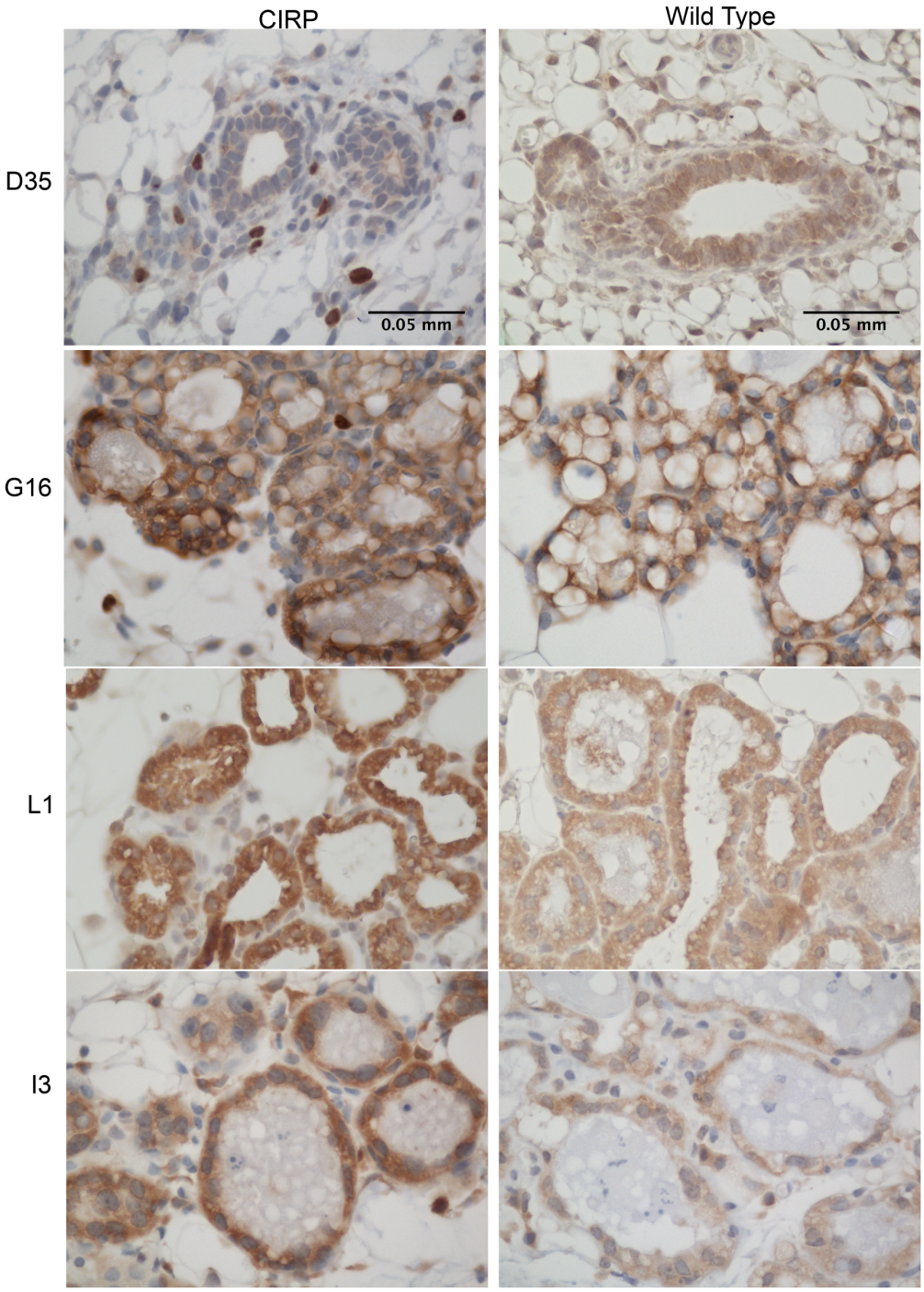

Fig 2.2. Immunohistochemistry for CIRP in mammary glands from CIRP and wild type mice. Representative images from longitudinally sectioned mammary glands at the developmental time point indicated. Brown represents CIRP staining and

blue nuclei were counterstained with hematoxylin. Pictures were taken at a 40X magnification using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera. Supplemental Figure 1 shows CIRP staining of mammary glands from 3 representative CIRP and wild type mice for each time point.

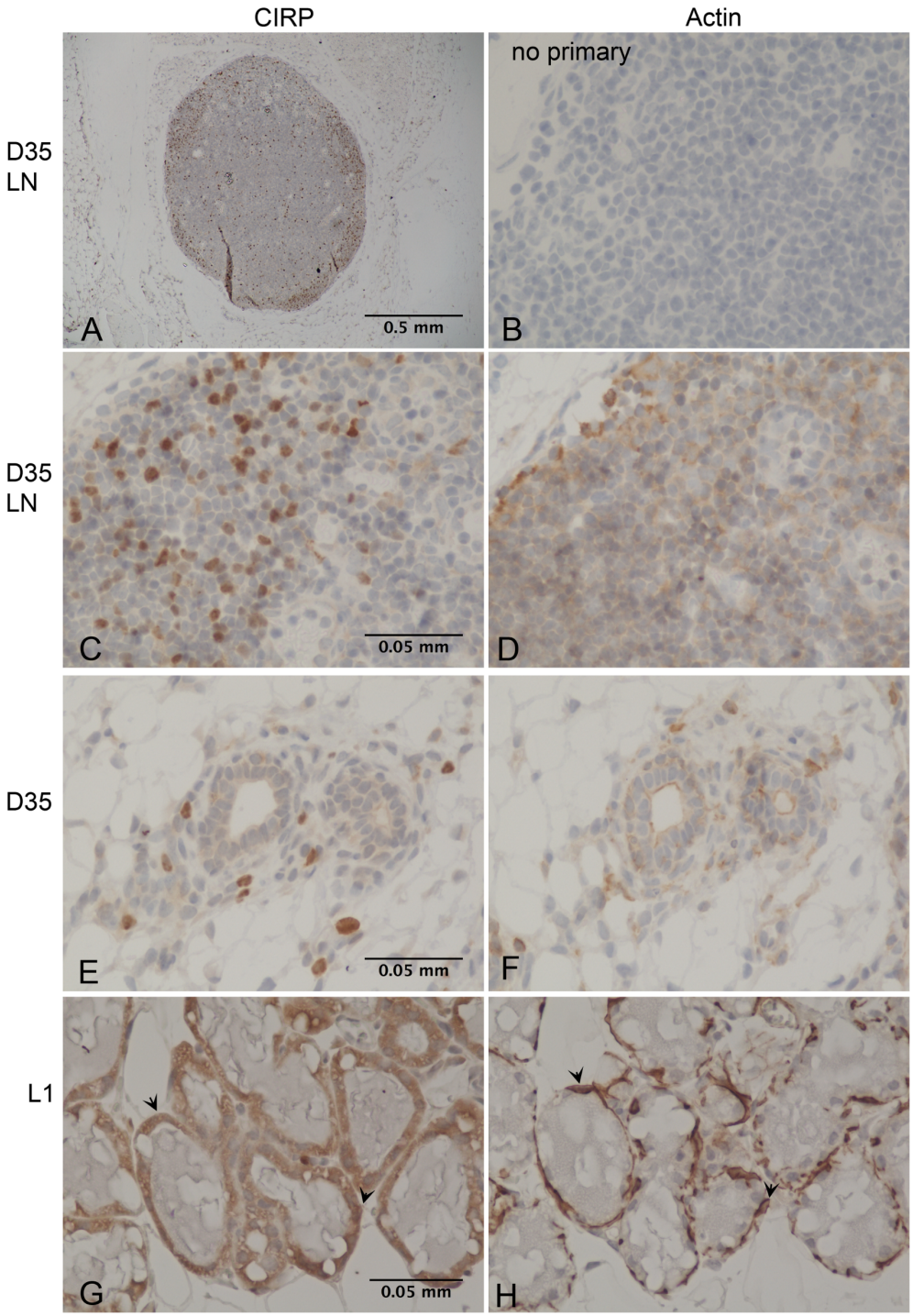

Fig 2.3. Immunohistochemistry for CIRP and actin in mammary glands from CIRP mice.

Representative images are shown from a longitudinally sectioned CIRP mammary gland at D35 and L1. Brown represents CIRP (A, C, E, G) or actin (D, F, H) staining and blue

nuclei were counterstained with hematoxylin. B is a no primary control. A-D, lymph node (LN) in a D35 mammary gland at 10X (A) or 40X magnification (B-D). E-H, epithelial ductal structures from D35 (E, F) or L1 (G, H) taken at 40X magnification. Arrows in G and H indicate cells in a myoepithelial position containing actin that do not contain nuclear CIRP. Pictures were taken with a Nikon Eclipse e400 microscope equipped with Nikon DS-Fi1 camera.

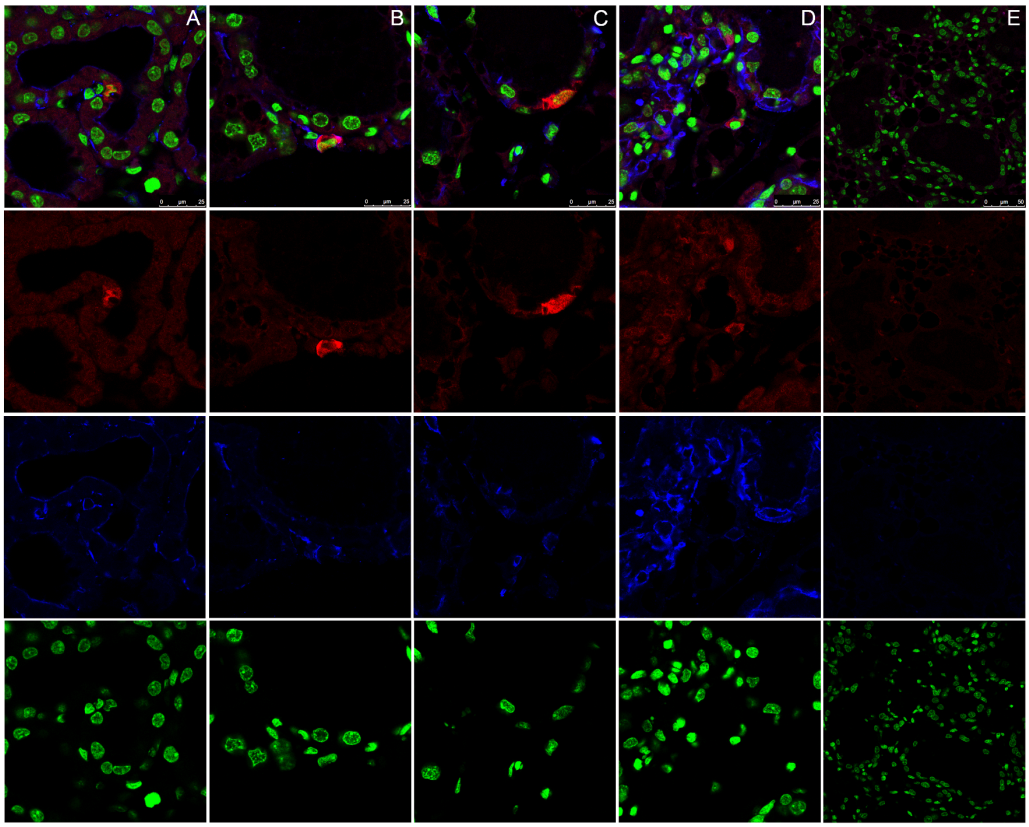

Fig 2.4. Double immunofluorescence for CIRP and actin. Representative images from longitudinally sectioned L1 and I3 mammary glands doubly stained for CIRP (red) and actin (blue). Nuclei are stained with Sytox green. The top row is a merge of the channels shown in row 2 (red), 3 (blue), and 4 (green). Column A shows images from a CIRP L1 mammary gland, Columns B and C show two different fields from CIRP I3 mammary glands, D shows a wild type I3 mammary gland, and E, a wild type I3 no primary control. Slides were viewed on a Leica TCS SP5 Confocal Microscope using a 63x objective. Columns A-D are 2X zoom images to better visualize nuclear versus cytosolic staining.

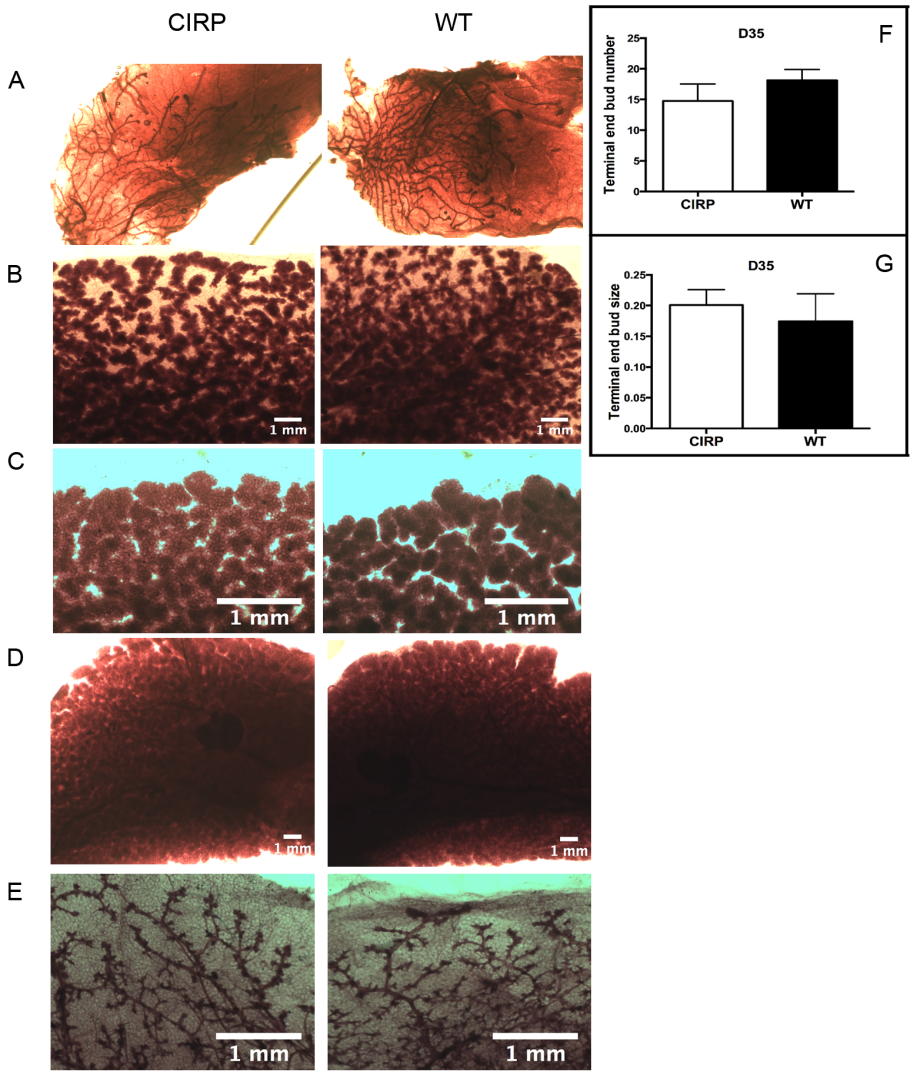

Fig 2.5. Developmental time course of mouse mammary glands (**a**-**e**)

Representative images of whole mounted mouse mammary glands from CIRP and wild type (WT) mice. Shown are mammary glands from **(a)** day 35 (D35) pubertal mice, **(b)** gestation day 16 (G16), **(c)** lactation day 1 (L1), **(d)** involution day 3 (I3) and **(e)** multiparous mice (MP). Scale bar represents 1 mm for all panels (different magnifications). (**f, g**) Terminal end bud (TEB) number and size in mammary glands from D35 CIRP and WT pubertal mice (8 CIRP and 7 WT). (**f**) The average number of terminal end buds, p = 0.361 (unpaired t-test). (**g**) The average size of terminal

end buds (relative units), p= 0.467 (unpaired t-test). Whole mounts were imaged using a stereomicroscope and Motic camera on Motic Images Plus 2.0 ML software. TEB number and size were assessed using ImageJ software.

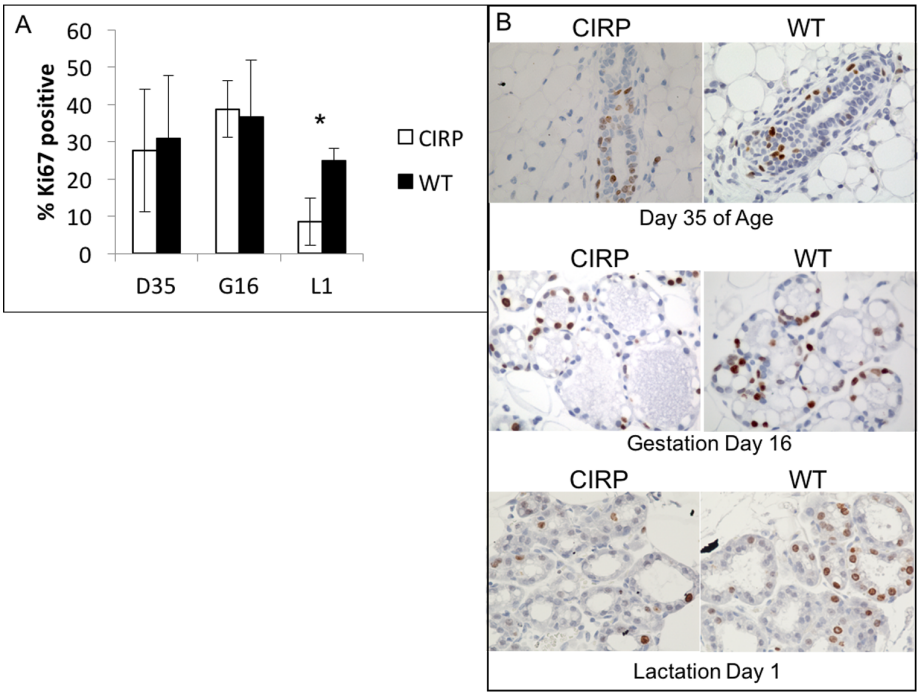

Fig 2.6. Proliferation in the mammary epithelium of CIRP and wild type mice.

(**a**) Percentage of Ki67 positive cells in the mammary epithelium at the time points indicated. D35, 8 CIRP and 6 WT mice. G16, 7 CIRP and 5 WT mice. L1, 6 CIRP and 4 WT mice. p = 0.002, unpaired t-test. (**b)** Representative pictures of Ki67 stained mammary glands. All pictures were taken at 40X magnification using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera and analyzed using Image-J software (1.46r). Brown represents Ki67 staining while nuclei are blue. Bar graphs were produced using representative images to count stained nuclei against non-stained nuclei.

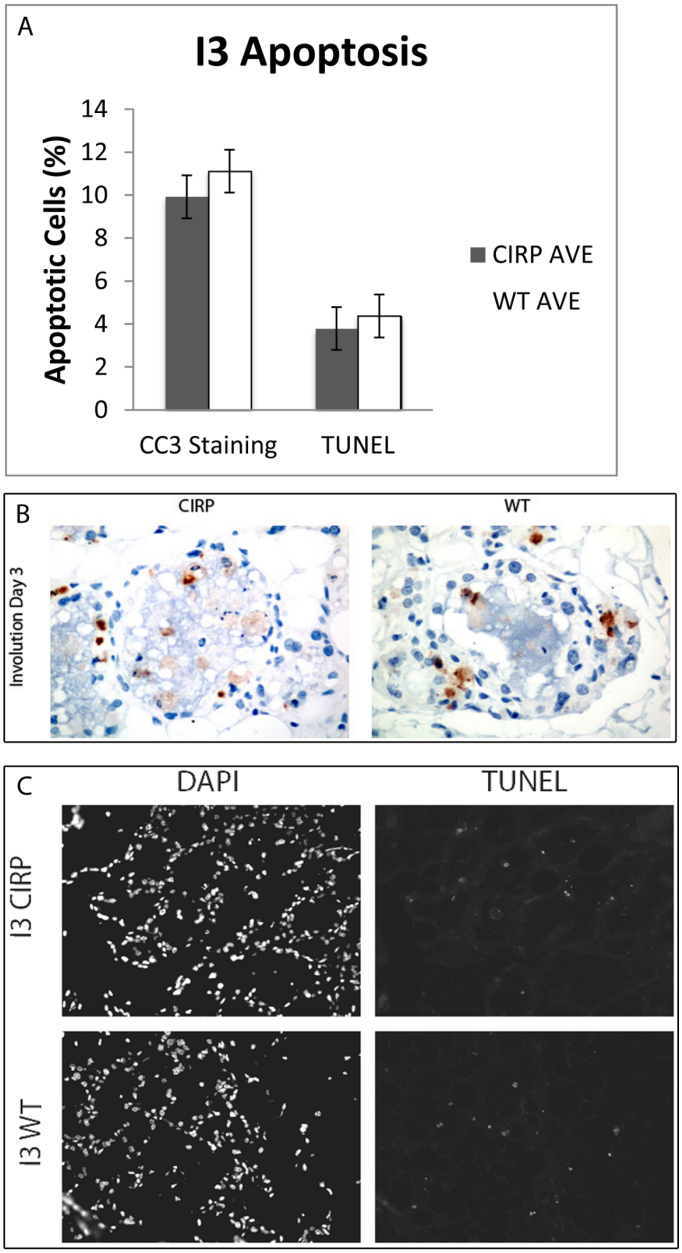

Fig 2.7. Apoptosis in the mammary epithelium of involuting mammary glands from CIRP and wild type mice. Mammary glands were assessed 3 days after forced weaning (involution day 3, I3). (**a**) Quantitation of immunohistochemistry for the apoptotic marker cleaved caspase 3 (CC3) and of TUNEL staining. n = 4 CIRP and 4 WT mice; p = 0.652 for CC3 and p = 0.375 for TUNEL (unpaired t-test). (**b**)

Representative photographs of CC3 immunohistochemistry (brown) in I3 mammary glands. (**c**) Representative photographs of TUNEL staining in I3 mammary glands. The left panels show DAPI staining of nuclei while the right panels show fluorescent TUNEL staining. Magnification is 40X. CC3 stained slides were photographed using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera. TUNEL slides were photographed using a Zeiss Axioscop upright microscope equipped for epifluorescence with a Roper Scientific Photometrics CoolSNAP ES camera using MetaMorph 6.2r4 software. Positive cells were counted and analyzed using Image-J software (1.46r). Bar graphs were produced using representative images to count stained nuclei against non-stained nuclei.

Supplemental Figures and Tables

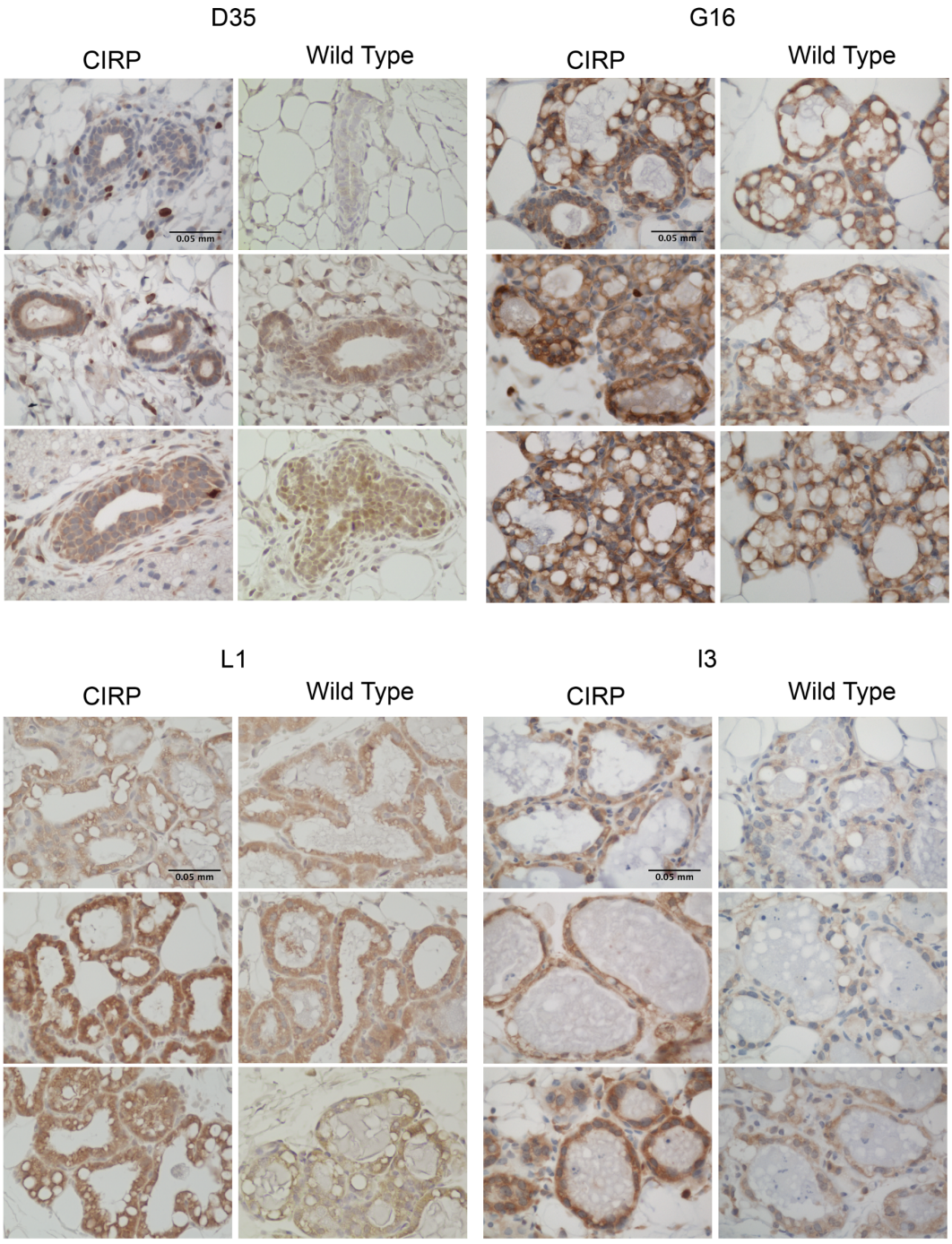

Figure S2.1. Immunohistochemistry for CIRP in mammary glands from CIRP and wild type mice. Representative images from longitudinally sectioned mammary glands at the developmental time point indicated. Brown represents CIRP staining and blue nuclei were counterstained with hematoxylin. Pictures were taken at a 40X magnification using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera. CIRP staining of mammary glands from 3 representative CIRP and wild type mice for each time point are shown.

Chapter 3: Cold-inducible RNA binding protein impedes breast tumor growth in the PyMT murine model for breast cancer

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Abstract

RNA binding proteins (RBPs) post-transcriptionally regulate gene expression by associating with regulatory sequences in the untranslated regions of mRNAs. Cold-inducible RBP (CIRP) is a stress-induced RBP that was recently shown to modulate inflammation in response to cellular stress, where it increases or decreases pro-tumorigenic (proinflammatory) cytokines in different contexts. CIRP expression is altered in several cancers, including breast cancer, but the effects of CIRP on inflammation in breast cancer is not known. Here we investigate if CIRP alters growth and the inflammatory profile of breast tumors. Transgenic mice overexpressing CIRP in the mammary epithelium were crossed with the PyMT mouse model of breast cancer and effects on both early and late tumorigenesis and inflammation were assessed. The effects of CIRP knockdown were also assessed in Py2T cell grafts. We found that overexpression of CIRP leads to decreased tumorigenesis at both early and late stages in the PyMT mouse model. Conversely, knockdown of CIRP in Py2T cell grafts led to increased tumor growth when compared to control Py2T cell grafts. Luminex cytokine assays assessed effects on the inflammatory environment. CIRP/PyMT mammary glands/mammary tumors and serum had decreased cytokines that promote inflammation, angiogenesis and metastasis compared to PyMT mammary glands and serum. Additionally, mammary tumors from CIRP/PyMT contained significantly lower levels of $CD4^+$ helper T-cells and higher levels of $CD8^+$ cytotoxic T-cells when compared to PyMT mammary tumors via flow cytometry. Overall, these data support a role for CIRP as a potent anti-tumor molecule that suppresses both local and systemic pro-tumorigenic inflammation.

Introduction

RNA binding proteins (RBPs) play key roles in RNA dynamics, including subcellular localization, translational efficiency and metabolism [3, 4, 6, 7]. Given the numerous physiological roles of RBPs, they are also key molecules in disease, including cancer [11, 13-17, 52, 102]. Cold-inducible RNA binding protein (CIRP) also known as hnRNPA18 and CIRBP, is a glycine-rich RBP that contains an RNA-recognition motif (RRM) and a carboxyl-terminal domain with several RGG motifs that facilitate protein-protein interactions [3]. CIRP is expressed in a wide variety of tissues and is induced in response to various cellular stresses, where it translocates from the nucleus to the cytosol and binds target mRNAs [4, 5]. Recent studies have detailed the role of CIRP in modulating inflammation in a variety of contexts, including several types of cancer [15, 16, 52, 53]. CIRP acts as tumor suppressor in some cases [3, 18-21, 23, 24, 103], but also promotes tumor growth by increasing pro-tumorigenic inflammation [25, 26, 31-33, 35, 36]. Collectively, the role CIRP plays in inflammation and cancer is likely context dependent. CIRP was previously shown to be a positive regulator of cyclin E1 and HIF-1α in breast cancer [27, 30]. We have previously studied the effects of CIRP overexpression in normal mammary gland development [23]. However, CIRP's function in breast cancer has largely been based on *in vitro* studies. Here, we show that CIRP decreases tumor growth and metastasis *in vivo* while decreasing pro-tumorigenic inflammation.

Materials and Methods

Transgenic Mice

MMTV-PyMT (FVB/N-Tg (MMTV-PyMT) 634Mul/J) mouse colonies were seeded from mice generously donated from Dr. Helen J. Hathaway at the University of New Mexico Health Sciences Center Animal Research Facility (ARF). Mice overexpressing the full-length human coding sequence of CIRP (FVB/N-Tg MMTV-hCIRP) were generated and characterized as previously described [23]. Animals were housed at the ARF in a temperature-controlled environment (23°C) with a 12 hour light-12 hour dark cycle. All animal procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals (protocol number 18-200805-HSC). Hemizygous CIRP females (CIRP/+) were crossed with hemizygous PyMT males (PyMT/+) to generate CIRP/PyMT (PyMT/+, CIRP/+) female mice. Mice were genotyped using DNA extracted from a 0.2cm tail clipping taken at weaning. Genotyping qPCR reactions were carried out using the Applied Biosystems Fast PCR 7500 system (Applied Biosystems, Foster City, CA). 10μl reactions were used consisting of 5μl Fast-SYBR Green (Applied Biosystems, Foster City, CA), 1μl of mixed 20μM forward and reverse genotyping primers, 3μl of RNAse-free water and 1μl of template DNA. Genotyping primers are described in Table S1. For all mouse studies described below, CIRP/PyMT mice were compared to PyMT mice.

Collection of Tissue

Mouse tumors were dissected from PyMT or CIRP/PyMT mice at either 7 weeks or 14 weeks of age. Mammary glands, mammary tumors, and lungs were collected and fixed in either 4% paraformaldehyde or 10% buffered formalin for

24-48 hours. Serum was also collected for cytokine analyses. One mammary gland #3 was cut in half lengthwise, and one half homogenized in Trizol (Invitrogen, Carlsbad, CA) and frozen at -80°C for subsequent RNA extraction and one half frozen at -80°C for subsequent protein extraction. Tissue supernatants were obtained by incubating a 6-10 mg portion of mammary tumor or mammary gland (containing epithelial ducts from the proximal end of the mammary gland) in 500μl of complete tissue culture media overnight (16 hours) at 37°C with 5% CO_2. Supernatants were then used in either Luminex cytokine arrays or in ELISA kits to quantify CIRP protein levels. All other tissues were dehydrated through ascending concentrations of ethanol, cleared with Hemo-De and embedded in Paraplast Plus. For mice with syngeneic Py2T cell grafts, only mammary tumors were harvested at 14 weeks of age for their final weight.

Whole Mounts

Whole mounts of number 4 mammary glands from 7-week mice were prepared by rinsing paraformaldehyde-fixed mammary glands two times with acetone over an 8-24 hour period, followed by a series of water rinses over an hour, and staining with carmine alum (0.2% carmine red and 0.5% aluminum potassium sulfate), overnight with gentle shaking [83]. Glands were destained in a graded series of ethanol and stored in methyl salicylate. All steps were performed at room temperature. Whole mounts were imaged using a stereomicroscope and Motic camera on Motic Images Plus 2.0 ML software. Multiple images of the mammary gland were taken, and composite images of whole mounts were created using Adobe Photoshop. For whole mounts of mammary glands from 7-

week mice (early tumorigenesis), mammary intraepithelial neoplasias (MIN) within these glands were quantitated using Image J software (Version 1.46r). A grid of 10mm squares was superimposed onto the whole mounts, with the squares first being counted for those that contained mammary epithelial tissue and then those containing mammary epithelial tissue and neoplasia. A percentage of MIN burden was calculated by dividing the MIN count by the total mammary epithelial tissue count and multiplying by 100.

Histology and Immunostaining

Formalin-fixed paraffin-embedded (FFPE) mouse mammary glands and lungs were sectioned in 50μm levels for 20 levels with three slides taken per level. Sections were 5μm in thickness. One slide from each level was stained with hematoxylin and eosin (H&E) for histological examination, and one was used for immunohistochemistry (IHC). For histological examination, sections were stained in Harris' hematoxylin (Sigma-Aldrich) for 30-45 seconds followed by 1-10 dips in acid alcohol and a 45 second dip in Eosin Y. Sections were then dehydrated through two-minute dips in ascending concentrations of ethanol. Coverslips were mounted on slides using DPX mounting medium (Electron Microscopy Services, Hatfield PA) and slides were analyzed for histopathology. For assessment of 7-week old mammary glands, five 60x fields in the central portion of the most advanced lesion were assessed. Tumors (14 weeks) and glands containing MINs (7 weeks) were both sectioned and processed as described above. Lung sections were also analyzed to determine the number of metastatic foci. Only lungs taken

from mice at 14 weeks of age were sectioned to assess pulmonary metastases. Lungs were H&E stained using the same method described above.

For immunostaining, paraffin sections were rehydrated to 1x PBS (IFA) or 1x PBS with 0.1% Tween 20 (PBST) and subjected to antigen retrieval for 15–20 minute at 90–95°C in a water bath in 10 mM Tris pH 9.0, 1 mM EDTA and 0.05% Tween20. Endogenous peroxidase activity was suppressed by incubation with 3% H_2O_2 in PBS for 30 minutes with agitation, followed by 1x PBS + 3% normal goat serum (NGS) + 0.1% Triton X-100 for 30 minutes to prevent non-specific staining. Sections were incubated in Ki67 primary antibody (as indicated in Table S2) diluted in 1x PBS + 3% NGS + 0.1% Tween20 and overnight at 4°C. Following the primary antibody, sections were washed 3 times in 1x PBST for 10 minute each and then incubated in donkey anti-rabbit IgG HRP secondary antibody for 1 hour at room temperature. Sections were developed using DAB as chromogen and washed in 1x PBS twice for 5 minutes, followed by a wash with diH_2O. Sections were then counterstained in Harris' progressive haematoxylin for 15 seconds, dehydrated and mounted using DPX mount (Electron Microscopy Science, Hatfield, PA) and a cover slip. Slides were viewed and photographed using a Nikon Eclipse e400 microscope equipped with a Nikon DS-Fi1 camera and analyzed using ImageJ software (1.46r). For Ki67 staining, bar graphs were produced using representative images to count HRP stained nuclei against non-HRP stained nuclei in mammary glands from PyMT and CIRP/PyMT mice.

Syngeneic Py2T Tumor Cell Grafts

Py2T cells were generously provided by co-author Dr. Helen Hathaway. Cells were originally derived from a primary mammary tumor from a transgenic MMTV-PyMT mouse on an FVB background. Cells were characterized using the method described by Waldmeier et al. [104]. Cells were maintained in DMEM/F-12 media with 5% fetal bovine serum and 1% penicillin/streptomycin and cultured at 37°C and 5% CO_2. Cells were used between passages 3 to 12.

Control Py2T cells or CIRP knockdown Py2T cells (1×10^6) were injected into the number 4 mammary fat pads of 7-week-old wild-type FVB mice. One fat pad received control Py2T cells and the other in the same mouse received CIRP knockdown Py2T cells. Tumors were measured using digital callipers twice per week for 7 weeks and mice were euthanized, and tumor harvested at 14 weeks of age. Tumors were weighed at necropsy (7 weeks post-injection). Final tumor weight was averaged and plotted for each genotype (n=5). Digital calliper measurements for length and width were used to calculate tumor volume using the tumor ellipsoid formula ($\pi/6 \times$ length x width) [105]. Tumor volume was plotted over time as age in weeks.

CIRP Knockdown

Stable CIRP knockdown was achieved using Alt-R CRISPR/Cas9 system (IDT). Guide RNAs (Alt-R crRNAs) were designed using the design tool by IDT to target the coding sequence of the murine CIRP gene. The sequences of each Alt-R crRNA are shown in Table S3. crRNA- trans-activating crRNA (tracrRNA) duplexes were prepared by mixing equimolar concentrations of each crRNA and tracrRNA (10µl Alt-R crRNA and 10µl Alt-R tracrRNA) in nuclease-free duplex butter (IDT catalog #

11010301) and annealing at 95°C for 5 minutes in a thermocycler. Equal amounts (working concentration of 1μM) of each Alt-R crRNA-tracrRNA duplex (3 total) were then used to prepare crRNA-tracrRNA-Cas9 RNP complexes. Alt-R S.p. HiFi Cas9 nuclease (IDT catalog # 1081060) was mixed at a recommended working concentration of 1μM along with 1μM of mixed crRNA-tracrRNA duplexes into Opti-MEM media (Thermo Fisher Catalog #51985091). RNP complexes were mixed with gentle pipetting or gentle vortexing and incubated at room temperature for 20-30 minutes. RNP complexes were used to prepare transfection complexes by mixing 750μl of RNP complex with 36μl of CRISPRMAX Cas9 lipofectamine reagent (Thermo Fisher Catalog # CMAX00001) and 714μl of Opti-MEM media. Transfection complexes were mixed by gentle pipetting and incubating at room temperature for 20-30 minutes. Transfection complexes were then gently mixed with Py2T cells at concentration of 400,000 cells/mL and plated in 6-well plates. Cells were mixed with either positive control (targeting the HPRT gene) crRNA RNP complex, negative control crRNA RNP complex, CIRP crRNA RNP complex, or lipofectamine only (untreated). Cells were allowed to grow for 48 hours before splitting and harvesting some for protein and preserving the rest for subsequent transfections. A total of three transfections were performed to achieve desired knockout/knockdown efficiency of ~85% at the protein level.

CIRP knockdown efficiency was measured at the protein level using an ELISA specific for murine CIRP protein (CUSABIO Wuhan, China). Cells were trypsinized and collected from 6-well plates after 48-hours of incubation with RNP complexes. Half of the cells were kept for subsequent transfections and half were

harvested for protein for use in the ELISA. Results CIRP knockdown efficiency shown in figure S1.

Luminex Arrays

Tissue supernatants and serum were used to assess 25 different cytokine protein levels (G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF-A, Eotaxin/CCL11). Cytokine production in mammary tissue was assessed using Milliplex mouse cytokine array assays (EMD Millipore, Billerica, MA). Multiplex arrays were used for these analyses in accordance with the manufacturer's protocols.

CIRP ELISA

Human CIRP (hCIRP) and mouse CIRP (mCIRP) specific enzyme-linked immunosorbent assay (ELISA) kit were from CUSABIO (Wuhan, China). Serum samples were diluted 10-fold, in accordance with the manufacturer's instructions. Tissue homogenates were prepared from 100mg of mammary tumor tissues using 2 freeze-thaw cycles followed by 5 minutes of centrifugation at 5000 x *g* at 2-8°C. The resulting supernatants were collected and assayed in the ELISA protein assays in accordance with the manufacturer's instructions. These colorimetric assays were read on a Tecan Infinite F500 (Tecan, Switzerland) microplate reader at 450nm.

Murine Breast Tumor Digestion

Mammary tumors developed in PyMT/+ and CIRP/PyMT mice were isolated from mice at necropsy (14 weeks of age). Tumors were washed in PBS, cut into 6-8 roughly equal pieces and placed in a 10ml digestion solution of DMEM/F12 + 5% Fetal Bovine Serum with 1x Collagenase/Hyaluronidase in DMEM (Stemcell Technologies Cambridge, Massachusetts), 0.05 U/mL Dispase (Stemcell Technologies Cambridge, Massachusetts), and DNAse I Solution (Stemcell Technologies Cambridge, Massachusetts). Tumors were incubated at 37°C for 100 minutes and briefly vortexed at 60% power every 10 minutes. The resulting cell suspension was then vortexed at full power for 30-45 seconds and then strained through a 70-micron cell strainer. Cells were then pelleted and washed in PBS twice. Cells were then resuspended in flow cytometry staining buffer containing PBS with 2mM EDTA and 5% FBS.

Cell Staining and Flow Cytometry

Prior to the addition of antibodies, cells were suspended in staining buffer containing phosphate buffered saline, 2mM EDTA with 5% fetal bovine serum. Cells were then incubated with 8 antibodies specific for F4/80, CD11b, Ly6C, Ly6G, CD19, CD3, CD4 and CD8 (See table S3.2 for working concentrations) for 30 minutes at 4°C. Two wash steps were then completed where cells pelleted by spinning at 500x*g* for 5 minutes at 4°C and washed in PBS. Cells were resuspended in staining buffer prior to assaying on the Attune NxT flow cytometer (Thermofisher Scientific Waltham, Massachusetts). Cells were first gated to eliminate dead cells and then to identify only singlets before gating for specific

immune cells markers. Flow cytometry panel was designed using Fluorofinder Design Tool (Broomfield, Colorado) to minimize spill over from fluorochromes.

Statistics

The average tumor weight, tumor burden, average tumor volume, levels of individual cytokines, average number of metastatic foci and average percentage gated were compared with 2 sample t-tests. Correlation analysis was done in Excel and Google Sheets to obtain the R^2 value. Experiments were repeated a minimum of 3 times.

Results

Human CIRP expression impeded early tumorigenesis in MMTV-PyMT Mice

To assess the effects of CIRP on early mammary tumor development, mammary gland whole mounts were prepared from the number 4 mammary gland from 7-week old MMTV-PyMT and MMTV-CIRP/PyMT mice. ImageJ [106], was used to assess the total area of mammary intraepithelial neoplasias (MIN) relative to the total epithelial area. Quantitation revealed that PyMT mice had significantly more MINs than CIRP/PyMT mice (not shown). Since MINs develop in a proximal to distal direction, as the epithelial ducts proliferate and extend into the fat pad during mammary gland development [86, 107, 108], MIN burden distal to the midline of the lymph node was also assessed. Figure 1A shows a representative whole-mount mammary gland image for a CIRP/PyMT mouse overlaid with a counting grid. The area counted is indicated by a red line drawn vertically through the middle of the lymph node and extending horizontally in the

direction of the arrow. Figure 1B shows that CIRP/PyMT mice had significantly fewer MINs distal to the lymph node when compared to PyMT mice (23% vs 39%, respectively).

Histopathological analysis of the mammary glands of 7-week-old mice confirmed that not only were there fewer MINs in CIRP/PyMT mammary glands, but that the lesions were less advanced. Mammary lesions in both mice were similar in character, with a primary lesion containing multiple MINs and occasional foci suggestive of early carcinoma. However, the mammary lesions in CIRP/PyMT mice were less advanced than those found in PyMT mice, with PyMT mice having larger primary lesions, greater secondary duct involvement and more advanced secondary lesions (Table 1). Mammary lesions from PyMT mice also had more mitotic figures and fewer apoptotic figures on average when compared to the lesions in CIRP/PyMT mice (Table 1). These results suggested that an altered balance between proliferation and apoptosis could be responsible for the less advanced lesions and reduced number of MINs in CIRP/PyMT mice.

Table 3.1. Histopathology of Mammary Glands from 7-week CIRP/PyMT and PyMT mice (n=5)		
7-week old Mice	PyMT	CIRP/PyMT
Primary Lesions	Larger, less clearly demarcated	Smaller, more clearly demarcated
Secondary Duct Involvement	Considerably greater	Less secondary duct involvement
Secondary Lesions	More Advanced	Less Advanced
Atypia	No Difference	
Vascularity		
Inflammatory Cell Infiltrate		
Mean Mitotic Figures	2.2 figures per field	0.7 figures per field
Mean Apoptotic Figures	1.15 figures per field	4.1 figures per field

Based on the results shown in Figure 1B and Table 1, the effect of CIRP overexpression on epithelial proliferation was examined. Proliferation was assessed by immunostaining for proliferation marker Ki67. Figure 1C shows representative Ki67 staining (brown) in sections of PyMT and CIRP/PyMT mammary glands. Figure 1D shows that there was significantly less proliferation in CIRP/PyMT mammary glands (24% of epithelial cells were Ki67 positive) compared to mammary glands from PyMT mice (38% of epithelial cells were Ki67 positive). This finding indicated that CIRP reduced epithelial proliferation, which may account, in part, for the reduced MIN burden and less advanced lesions in CIRP/PyMT mice. Figure 1E shows the visual difference in MINs between mammary gland whole mounts taken from PyMT and CIRP/PyMT mice at 7 weeks of age.

CIRP impeded late tumorigenesis and pulmonary metastasis

Tumor progression was tracked in mice from 8 to 14 weeks of age in order to assess effects on later tumorigenesis. Analysis of tumor volume revealed that tumors in CIRP/PyMT mice were smaller and grew more slowly when compared to PyMT mice at the same time point, with significant differences in total tumor volume at 11, 12 and 13 weeks of age (Figure 2A). At 14 weeks of age, mice were weighed at necropsy before tumors were removed and weighed to determine tumor burden. Figure 2B shows that CIRP/PyMT mice had significantly lower tumor burden when compared to PyMT mice. The weights of the heaviest four tumors were also measured (Figure 2C), with CIRP/PyMT mice having smaller tumors when compared to tumors from PyMT mice. A large percentage of PyMT mice have developed not only carcinomas by 14 weeks of age, but also pulmonary metastases [104]. To assess CIRP's effect on pulmonary metastases, lungs from PyMT and CIRP/PyMT mice were sectioned and stained with haematoxylin and eosin and imaged to quantify the number of metastatic foci. Figure 2D shows the average number of metastatic foci in lungs from CIRP/PyMT and PyMT mice. Lungs from CIRP/PyMT mice had greatly reduced pulmonary lesions when compared to PyMT mice; an average of 0.3 compared to 58. Taken together, these results suggested that CIRP impeded late stage tumor growth and metastasis.

In order to more directly assess the relationship between CIRP expression and tumor development, ELISAs were used to specifically quantitate human CIRP protein in mammary tumors from 14-week CIRP/PyMT mice. Correlation analysis was then used to assess the relationship between CIRP level and tumor weight. Figure 2E shows the results of this analysis. As would be expected if CIRP were

inhibiting tumor growth, CIRP expression negatively correlated with tumor size; tumor size decreased with increased CIRP protein. These results showed that CIRP impedes breast tumorigenesis.

CIRP knockdown increased tumor growth in Py2T tumor cell grafts

The effects of CIRP loss on tumor growth was also assessed. Py2T cells were generated by passaging primary mammary tumor cells from a transgenic PyMT mouse and characterized as described [104]. CIRP was stably knocked down in these cells using CRISPR-Cas9. Control treated Py2T or CIRP knockdown (CIRP KD) Py2T cells were injected into the number 4 mammary gland of wild type FVB mice and tumor growth monitored. Figure 3A shows that CIRP KD cell grafts resulted in larger tumors when compared to control Py2T cell grafts at 14 weeks (weighed at necropsy). Figure 3B shows that although there was no significant difference in tumor growth rate overall, tumor latency was decreased with CIRP KD cells when compared to control Py2T cells. Two weeks after injection of cells, CIRP KD Py2T cells produced a tumor of an average volume of 36 mm^3, while negative control treated Py2T cells produced a just detectable tumor of an average volume of 3 mm^3. The overall increase in tumor size coupled with decreased latency with CIRP KD cells reinforce overexpression data showing that CIRP suppressed tumorigenesis.

CIRP decreases pro-tumorigenic cytokines in mammary tumors

CIRP has been shown to modulate inflammation in various types of cancer [15, 16, 52]. In addition, cytokine levels in both serum (systemic inflammation) and mammary glands (local inflammation) are known to influence tumor growth in

both human breast cancer [109, 110] and in the PyMT mouse model [107, 111]. To ask if the effects seen on tumorigenesis were secondary to changes in the inflammatory environment, cytokines produced by mammary glands (7-weeks) and mammary tumors (14 weeks) were assessed using tissue supernatants [112] . Tissue supernatants were obtained by incubating mammary tissue from 7-week or mammary tumors from 14-week old PyMT and CIRP/PyMT mice overnight in complete cell culture media. Cytokines released into the media were assayed using multiplex Luminex cytokine arrays, as were cytokines in serum these mice.

Of the 25 cytokines assessed, 7-week old CIRP/PyMT mammary glands produced significantly lower levels of chemokine ligand 1 (KC), monocyte chemoattractant protein (MCP-1), leukaemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF-A) compared to PyMT mammary glands (Figure 4). LIF promotes tumorigenesis and metastasis in breast cancer [113] and was shown to inhibit p53 in colorectal cancer [114]. KC (CXCl1) promotes inflammation and metastasis by recruiting myeloid cells in breast cancer, including neutrophils [115]. MCP-1 (monocyte chemoattractant or CCL2) targets monocyte migration into the tumor microenvironment, facilitating metastatic seeding by VEGF-A-mediated angiogenesis [116]. Assessment of serum cytokines in 7-week old mice revealed no significant differences in cytokine levels between CIRP/PyMT and PyMT mice (not shown). These results suggested that CIRP impeded early mammary tumor growth by decreasing pro-tumorigenic cytokine production.

Consistent with results in 7-week mammary glands, several pro-tumorigenic cytokines were also significantly lower in supernatants from

CIRP/PyMT mice during late stage tumorigenesis (14 weeks) when compared to PyMT mice. There were significantly lower levels of interferon-γ (IFNγ), Interleukin-13 (IL13), chemokine ligand 3 (MIP-1α), Interleukin-10 (IL10), and Interleukin 6 (IL6) (Figure 5A). In addition, serum from 14-week CIRP/PyMT mice had significantly less Interleukin-1α (IL1α), Interleukin-1β (IL1β), Interleukin-5 (IL5), KC, MCP-1 and chemokine ligand 2 (MIP-2) (Figure 5B). IFNγ is typically associated with an tumor rejection immune response, however high expression is not always beneficial in breast cancer [117, 118]. MIP-1α has been linked to metastasis and chemoresistance in breast cancer [119]. IL-10 and IL-6 are both known to promote breast tumor growth and are often central to breast tumorigenesis and metastasis [120-122]. IL13 is upregulated in breast cancer [123]. These results suggested that CIRP is decreasing a number of cytokines that are known to promote tumor growth in both mice at both 7 and 14 weeks of age.

CIRP decreases $CD4^+$ helper T-cells and increases $CD8^+$ cytotoxic T-cells in mammary tumors during late stage tumorigenesis

In order to ask if the effects of CIRP on several protumorigenic cytokine levels was either affecting or due to the immune cell cohort within the tumors, we performed flow cytometry to assess the levels of B-cells ($CD19^+$), $CD4^+$ helper T-cells ($CD3^+CD4^+$), $CD8^+$ cytotoxic T-cells ($CD3^+CD8^+$), macrophages ($F4/80^+$), Neutrophils ($CD11b^+$,$Ly6G^+$,$Ly6C^+$) and Monocytes ($CD11b^+$, $Ly6G^+$). Analysis of these immune cells were chosen based on our results from assessment of cytokine levels. Mammary tumors were taken from PyMT/+ and CIRP/PyMT mice at 14 weeks of age and digested into a single-cell suspension. Of the five immune cell

types analyzed, the percentage gated of macrophages, monocytes, neutrophils and B-cells were not found significantly different between PyMT/+ mice and CIRP/PyMT mice (Data not shown). However, CIRP/PyMT mice had significantly lower $CD4^+$ helper T-cells and significantly higher $CD8^+$ cytotoxic T-cells when compared to PyMT/+ mice (Figure 3.6). Higher levels of $CD8^+$ cytotoxic T-cells and lower levels of $CD4^+$ helper T-cells are typically associated with better prognosis and outcomes in human breast cancer [110, 111]. These results suggest that the effects of CIRP on tumor growth and protumorigenic cytokines is potentially due to differences in the immune cell cohort.

Discussion

Cold-inducible RBP (CIRP) is a stress-induced RBP that has recently been shown to modulate inflammation in response to cellular stress. CIRP expression is altered in several cancers, including breast cancer. While the normal physiological role of CIRP as a stress-induced RBP has been well characterized, more recent studies have detailed roles for CIRP in several diseases including different forms of cancer and in inflammation [15, 16, 20, 21, 30, 44, 52]. Our previous studies identified a role for CIRP in suppressing epithelial proliferation during mammary gland development, specifically at the transition from lactation to involution [23]. The current study assessed the potential effects of CIRP on the development and progression of mammary tumorigenesis in the MMTV-PyMT mouse model of breast cancer that recapitulates many key features of human disease.

To our knowledge, this is the first study to explore the role of CIRP in breast cancer and inflammation *in vivo*. Our results show that overexpression of human

CIRP in the mammary epithelium impedes tumor growth and metastasis in the MMTV-PyMT model. Early tumorigenesis was inhibited, with fewer, less advanced lesions in CIRP/PyMT mammary glands when compared to PyMT glands, due in part to decreased proliferation Inhibition of tumorigenesis extended into late stages, with increased tumor latency, decreased tumor size and burden, and a remarkable decrease of pulmonary metastases. Final tumor size negatively correlated with CIRP protein level, suggesting a direct influence of CIRP on tumor growth. These findings were reinforced by CIRP knockdown experiments. Py2T cells in which CIRP was stably knocked down had decreased tumor latency and larger tumor mass compared to control Py2T cells when grafted into wild type FVB mammary glands. Consistent with these findings, other studies have demonstrated a role of CIRP in decreasing proliferation [6, 20, 21, 50]. CIRP expression negatively correlated with malignancy in both ovarian tumors, endometrial cancers and nasal cancers [20, 21, 103]. Altogether, our observations support a role for CIRP as a tumor suppressor that reduced the progression and invasive potential of mammary gland tumors and encourage further delineation of the effects and benefits of CIRP on breast (and potentially ovarian and endometrial) cancer development and progression.

Previous studies on the effects of CIRP on inflammation support an investigation of how CIRP may affect the inflammatory environment within our models. Generally, our results showed that pro-tumorigenic cytokines were reduced in CIRP/PyMT mice when compared to PyMT mice at both early and late time points. Interestingly, assessment of the cytokines produced by mammary

glands with early lesions showed significantly lower levels of VEGF-A, whose role in angiogenesis is well-established [124]. However, there was no discernible difference in vascularity between the MINs examined by histopathology in CIRP/PyMT and PyMT mice. This discrepancy may be due to the early stage of the disease. The lower levels of cytokines LIF, MCP-1 and KC in CIRP/PyMT supernatants likely play a role in the decreased tumor growth observed, as these cytokines are all known to play a role in maintaining the tumor microenvironment in breast cancer [107, 111, 125].

In late stage tumorigenesis, CIRP decreases the levels of pro-tumorigenic cytokines both locally and systemically. CIRP/PyMT mammary gland tumors showed decreased levels of IL13, MIP-1α, IL10, IL1β, IL5, IL6, KC, MCP-1, MIP-2, LIF and VEGF-A compared to PyMT tumors. In breast cancer, MIP-1α, MIP-2, KC and MCP-1 are chemokines that recruit macrophages, neutrophils and monocytes to maintain the tumor microenvironment [111]. MIP-1α and MIP-2 have also been linked to metastasis and chemoresistance in breast cancer [119]. IL10 downregulates the expression of Th1 type cytokines and increases immune tolerance of cancer in the tumor microenvironment [111, 126], while IL1β is associated with increased risk for bone metastasis in human breast cancer [127, 128]. LIF was shown to promote migration and metastasis in breast cancer through the AKT-mTOR pathway [111, 125], and elevation of VEGF-A levels in breast cancer are typically associated with poor prognosis and higher rates of pulmonary metastasis [129]. IL-6 is known to promote invasiveness through epithelial to mesenchymal transition (EMT) and is associated with the formation and

maintenance of breast cancer stem cells [130, 131]. IL-5 and IL-13 have been reported as elevated in some breast cancers, but their potential roles in human breast cancer are not known [132, 133]. In addition, CIRP decreased the number of $CD4^+$ helper T-cell and increased the number $CD8^+$ cytotoxic T-cells in tumors from CIRP/PyMT mice when compared to PyMT mice, potentially due to the aforementioned changed in cytokines. This difference in immune cell cohort was somewhat expected, given that CIRP decreased a number of cytokines associated with tumor progression in breast cancer. These results suggest that CIRP overexpression may decrease breast tumor growth and metastases by decreasing these cytokines and changing the immune cell cohort present within these tumors. In particular, the decreased levels of IL6, KC, MCP-1, VEGF-A and $CD4^+$ T-cells in the tumors taken from 14-week-old CIRP/PyMT mice compared to PyMT mice could explain the rather large difference in the number of pulmonary metastases observed between these two genotypes. No difference in the number of macrophages was observed between CIRP/PyMT mice and PyMT mice, however, it is possible that macrophage polarity may be different, given the observed differences in cytokines.

Although a direct mechanism of how CIRP regulates inflammation has not been explored in our model, many studies have described how CIRP functions as regulator of inflammation in both cancer and other diseases. Previous studies on CIRP as a modulator of inflammation in cancer have revealed that CIRP can act as a damage-associated molecular pattern (DAMP) and stimulate MyD88-NFκB and subsequent cytokine release via toll-like receptor 4 (TLR4) binding [16, 52]. This

mechanism of CIRP was initially described in sepsis in both humans and murine models [14]. This function of CIRP was also described as a pro-tumorigenic event in both colorectal and liver cancer[16, 52]. However, recent findings have shown that the ability of CIRP to function as a TLR4 ligand can induce an anti-tumorigenic inflammatory response in mice with E.G7-OVA tumors, showing that this aspect of CIRP function can potentially be applied clinically to drive a tumor rejection response [134].

Our results, taken in context with previous findings, suggest that the role CIRP plays in inflammation in cancer is context dependent, explaining why CIRP increases pro-tumorigenic inflammation in colorectal and liver cancers [16, 52], while decreasing pro-tumorigenic inflammation in breast cancer [27] as well as in wound healing [50]. Future studies will focus on the mechanistic role CIRP plays in modulating cytokine levels, to provide clarity on how CIRP variably modulates inflammation. Further exploring how immune cell cohorts are affected, particularly regulatory T-cells and macrophages polarization, within the tumor tissue and in blood will be key to determine if CIRP is a clinically relevant marker for prognosis in human breast cancer.

Acknowledgments

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P30CA118100 and the Flow Cytometry shared resource for usage of Luminex plate reader and analysis software.

Figures

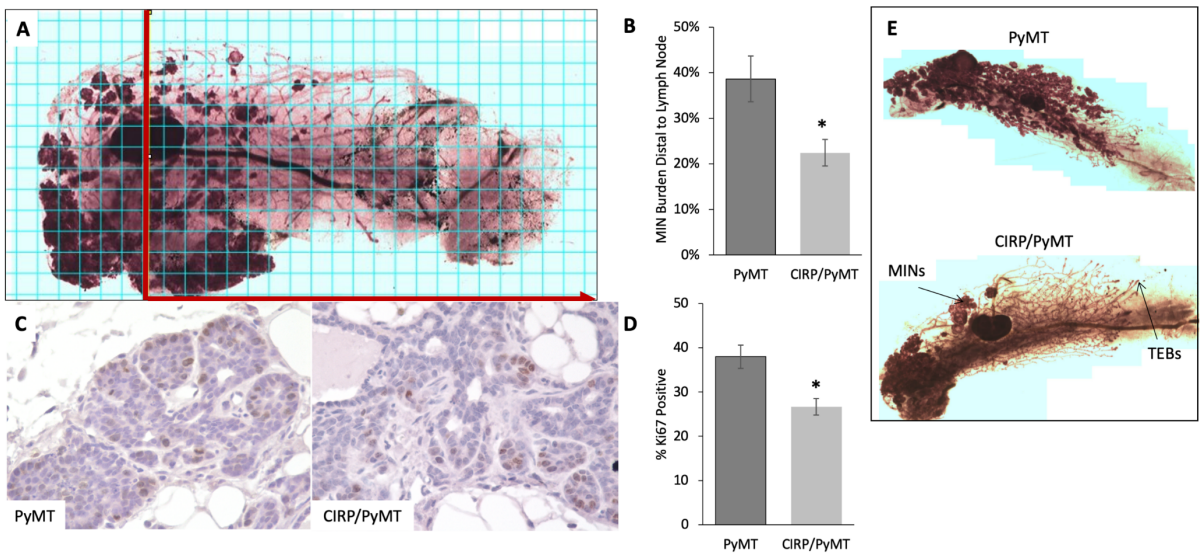


Figure 3.1. CIRP Decreases Early Tumorigenesis and Proliferation in 7-week PyMT Mice. A) Representative image of a mammary gland whole mount from a 7-week PyMT mouse with the grid overlay and division of lymph node (red line) used for quantitation in panel B. B) Quantitation of mammary intraepithelial neoplasias (MIN). MIN burden is percentage of gland containing MINs distal to the lymph node (n =8). C) Representative Ki67 immunostaining (brown) of PyMT/+ and CIRP/PyMT mammary glands. Five-micron sections were counterstained with hematoxylin (purple). D) Quantitation of Ki67 positive cells relative to total cells (n=4). *p<0.05, two-sample t-test. E) Visual comparison of PyMT and CIRP/PyMT mammary gland whole mounts. MINs and Terminal end buds (TEBs) are labeled.

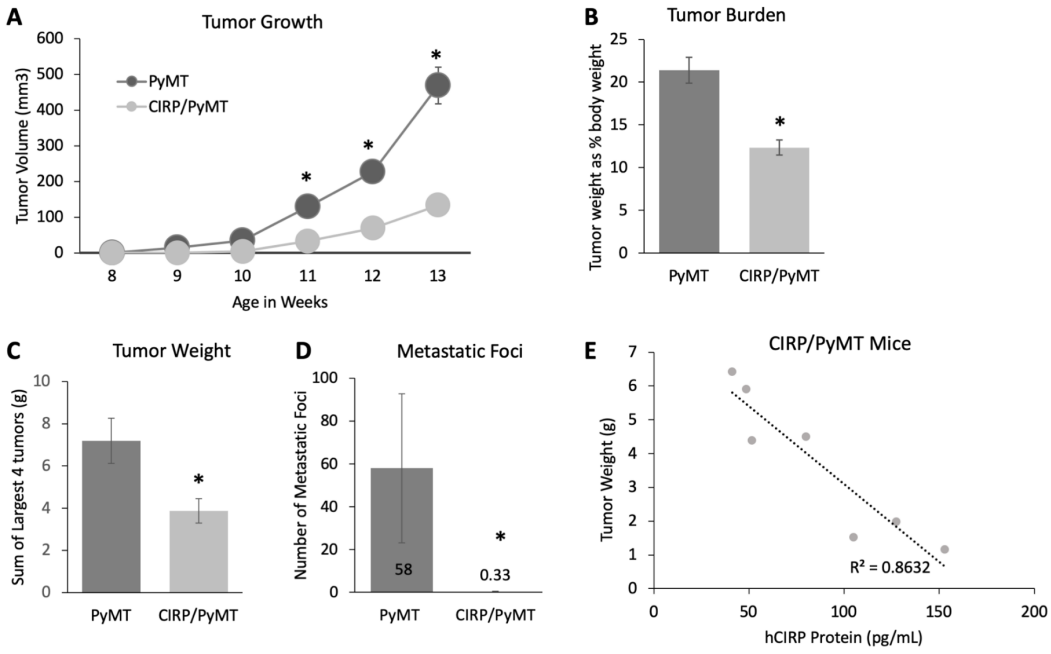


Figure 3.2. CIRP Decreases Late Tumorigenesis and Pulmonary Metastasis in 14-week PyMT mice. A) Tumor volume as calculated from the ellipsoid area formula: $V = \frac{\pi}{6} \times length \times width$ (n=13). B) Tumor burden is tumor weight as a percentage of total body weight (n=13). C) Sum of the largest four tumors averaged from mice of each genotype (n=13). D) Average number of pulmonary metastatic foci from each genotype (n=5). E) Correlation analysis between hCIRP protein level (measured by ELISA) and tumor size (sum of 4 largest tumors); R^2 = 0.8632. *p value <0.05 (two sample t-test).

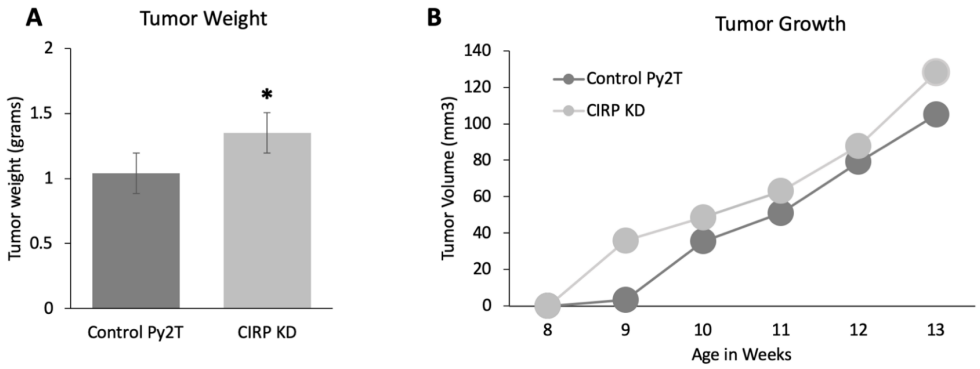


Figure 3.3. CIRP knockdown increases tumor growth in Py2T cell grafts. A) Tumor weight shown at necropsy (n=5). B) Tumor volume in mm^3 from the ellipsoid area formula $V = \frac{\pi}{6} \times length \times width$ (n=5) as measured at the indicated times. *p value <0.05 (two sample t-test).

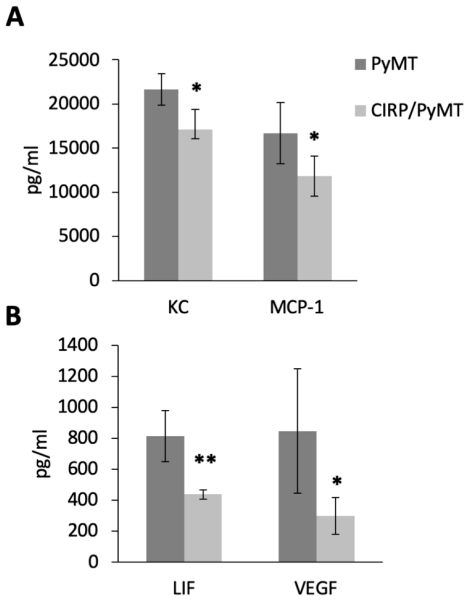


Figure 3.4. CIRP decreases pro-tumorigenic cytokines in early tumorigenesis (7-week mice). Quantitation of cytokines in supernatants from overnight cultures of PyMT/+ and CIRP/PyMT mammary glands; measured by multiplex ELISA (n=3). A) Levels of KC and MCP-1. B) Levels of LIF and VEGF-A. *p<0.05, **p<0.005.

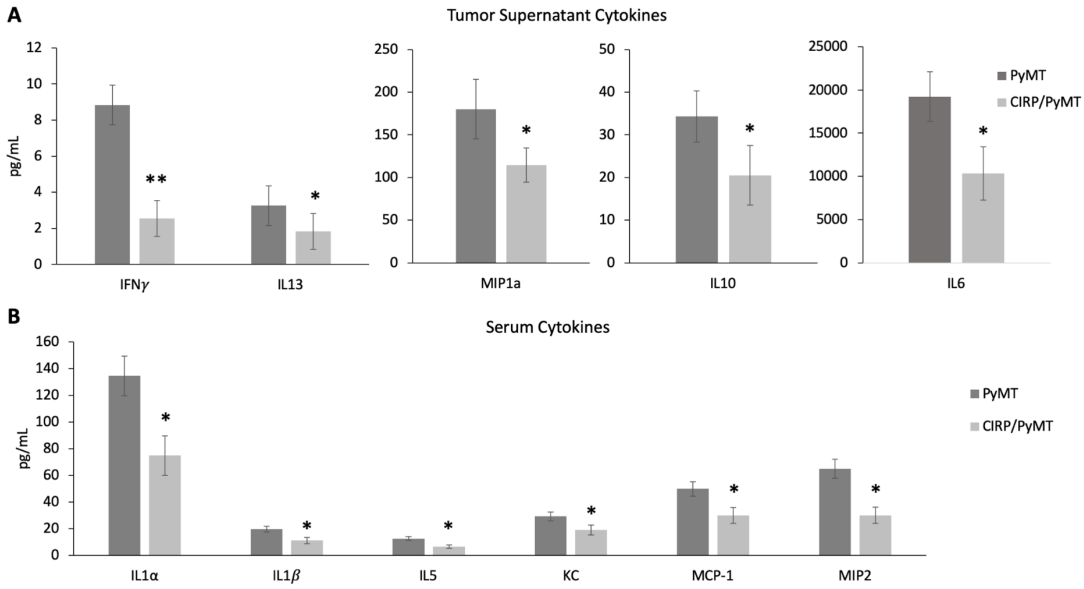


Figure 3.5. CIRP decreases pro-metastatic and pro-tumorigenic cytokines in late tumorigenesis (14-week mice). A) Quantitation of cytokines in supernatants from overnight cultures of PyMT/+ and CIRP/PyMT mammary gland tumors; measured by multiplex ELISA (n=6). B) Quantitation of cytokines in serum from 14-week PyMT/+ mice and CIRP/PyMT mice (n=6). *p<0.05 **p<0.005 (two sample t-test).

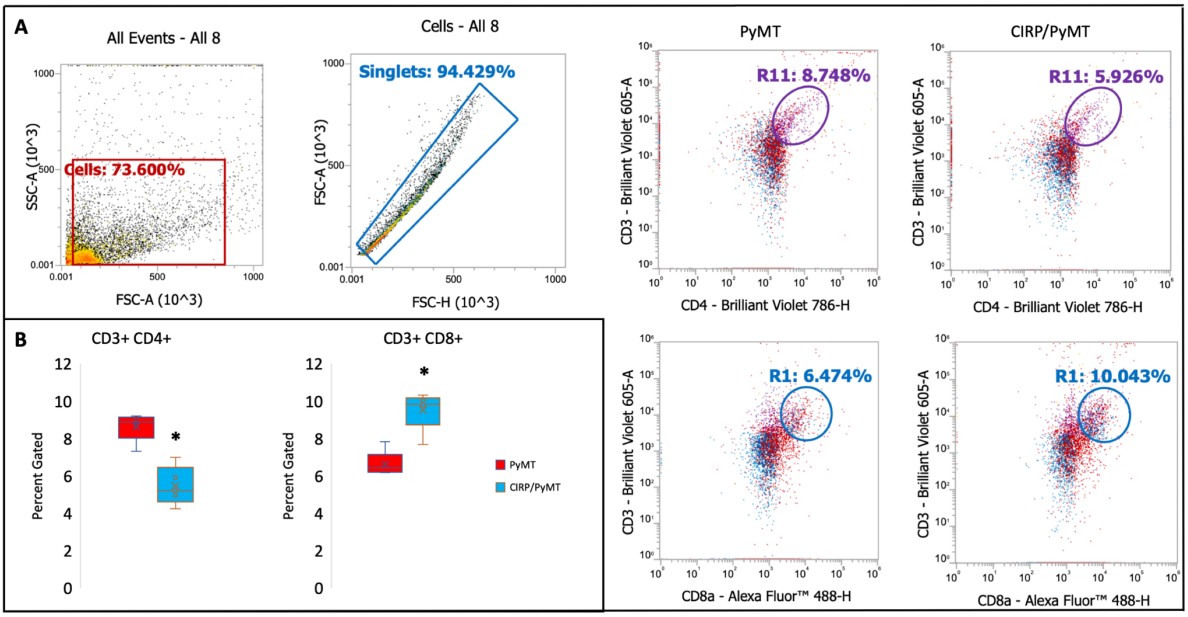


Figure 3.6. CIRP decreases CD4⁺ helper T-cells and increases CD8⁺ Cytotoxic T-cells during late stage tumorigenesis (14 weeks of age) A) Representative dot plots showing gating strategy for all events, singlets and for quantitating CD4⁺ ($CD3^+CD4^+$) and CD8⁺ ($CD3^+CD8^+$) T-cells. Representative plots shown for PyMT/+ and CIRP/PyMT mice when gated for CD3 (y-axis) and either CD4 or CD8 (x-axis). B.) Box plots of average percent gated cells from total cells in PyMT/+ and CIRP/PyMT mice. (n=5) *p<0.05

Supplemental Figures and Tables

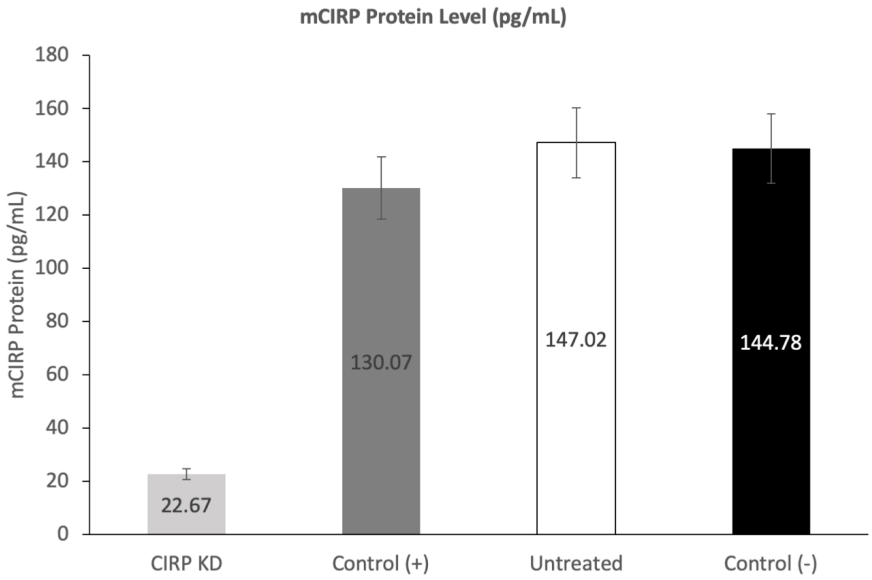


Figure S3.1. CIRP Knockdown Efficiency. Measured by ELISA and shown in picograms per milliliter. Cells were mixed with either positive (+) control (targeting the HPRT gene) crRNA RNP complex, negative (-) control crRNA RNP complex, CIRP crRNA RNP complex (CIRP Knockdown or KD), or lipofectamine only (untreated). n=4.

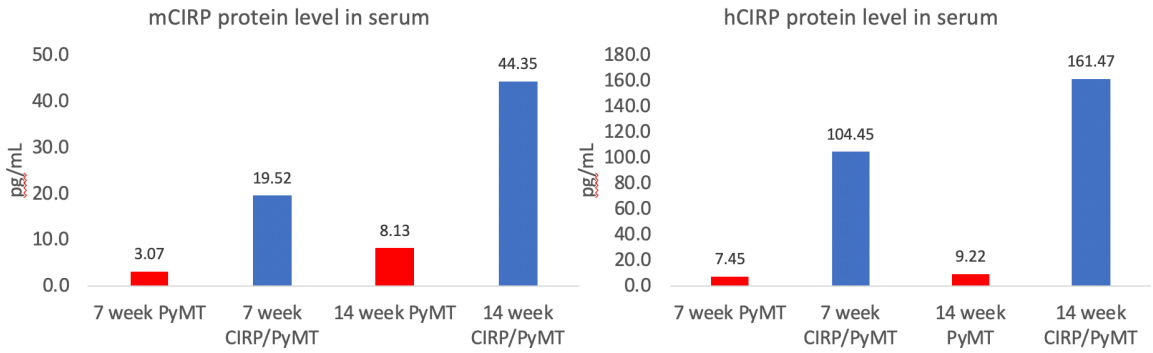


Figure S3.2 mCIRP and hCIRP protein levels in Serum from CIRP/PyMT and PyMT Mice. CIRP serum levels were measured using an ELISA specific for either mouse (mCIRP) or human CIRP (hCIRP). Levels below 15 pg/ml are considered baseline. n=3.

Table S3.1: Genotyping Primers	
Target Gene Name	**Primer Sequence**
PyMT Forward	5'- CGG CGG AGC GAG GAA CTG AC 3'
PyMT Reverse	5'- TCA GAA GAC TCG GCA GTC TTA -3'
CIRP Forward	5'- TAC TAT AGC AGC CGG AGT CAG AGT -3'
CIRP Reverse	5'- AAC AGA TGG CTG GCA ACT AGA AGG -3'

Table S3.2: Description of Antibodies				
Antibody	**Source**	**Catalog Number**	**Dilution or Working Concentration**	**Stock Concentration**
Ki67 (SP6)	NeoMarkers	RM-9106-S0	1:300 Dilution	Not Provided
CD11b	Life Technologies	25-0112-82	0.1μg/10^6 cell per 100μl	0.2mg/ml
CD19	Life Technologies	47-0193-82	0.1μg/10^6 cell per 100μl	0.2mg/ml
F4/80	Life Technologies	61-4801-82	0.8μg/10^6 cell per 100μl	0.2mg/ml
CD8A	Life Technologies	53-0081-82	0.4μg/10^6 cell per 100μl	0.5mg/ml
Ly6G	Biolegend	127616	0.2μg/10^6 cell per 100μl	0.2mg/ml
Ly6C	Biolegend	128014	0.2μg/10^6 cell per 100μl	0.5mg/ml
CD3	Biolegend	100351	0.4μg/10^6 cell per 100μl	0.2mg/ml
CD4	Biolegend	100552	0.2μg/10^6 cell per 100μl	0.2mg/ml

Table S3.3: Sequences of Alt-R crRNAs (Guide RNAs)			
Product	**Sequence Name**	**Sequence**	**Number of Bases**
Alt-R CRISPR/Cas9 crRNA 10nmol	Mm.Cas9.CIRBP.1.AA	Alt-R1/UCU UCU GAC AAC CGG UCC CGG UUU UAG AGC UAU GCU/Alt-R2	36
Alt-R CRISPR/Cas9 crRNA 10nmol	Mm.Cas9.CIRBP.1.AB	Alt-R1/CAG CUU CGA CAC CAA CGA GCG UUU UAG AGC UAU GCU/Alt-R2	36
Alt-R CRISPR/Cas9 crRNA 10nmol	Mm.Cas9.CIRBP.1.AC	Alt-R1/AGC GUC AUC GAU AUU UUC AAG UUU UAG AGC UAU GCU/Alt-R2	36

Chapter 4: General Discussion

CIRP was initially described as both a DNA-damage inducible transcript and a transcript inducible by mild-hypothermia [3, 7]. Several initial studies of CIRP outlined a role as a tumor suppressor in various cell lines and tissue types, where it slowed proliferation and cellular growth [3, 18, 19]. Some of the first studies of CIRP in human tissues described that CIRP inversely correlated with proliferation and malignancy in endometrial carcinoma and ovarian tumors, respectively [20, 21]. These studies, taken as a whole, outlined an intriguing role for CIRP as a tumor suppressor in several contexts and cellular models for disease. Conversely, many studies have shown that CIRP can also function as an oncogene, where it increases the survival of prostate cancer cells [26], allows cells to bypass replicative senescence [25], and increases telomerase activity in several cancer cell lines [33]. Prior to the studies presented in this dissertation, our lab and others showed that CIRP positively regulates proliferation in breast cancer cells, as well as in other cell lines in culture [27, 135].

Intrigued by our results in breast cancer cell lines, as well as the clear dichotomy of CIRP in the context of cancer, we aimed at studying the physiological role of CIRP in the breast and in breast cancer. In order to investigate this, we created a transgenic mouse model that overexpresses CIRP in the mammary epithelium. This mouse model served as a valuable tool for exploring the role that CIRP plays in mammary gland development as well as its role in breast cancer. The data presented in this dissertation provide new information for how CIRP

functions in mammary gland development, breast cancer and inflammation in the context of breast cancer. These findings fit into the recent studies on CIRP in the context of inflammation and cancer, where CIRP not only functions to promote or decrease tumor growth, but modulates inflammation in various contexts.

Aim 1: Determine the role of CIRP in normal mammary gland development

Since CIRP is expressed in mammary epithelial cells, Chapter 2 was aimed at determining what role CIRP plays in normal mammary gland development. Our primary finding was that CIRP decreases proliferation during the lactational switch when proliferation is no longer need during development, but not during puberty or gestation, when proliferation is normally high as this are the periods when functional epithelial ducts are being formed or expanded, respectively. This result suggests that CIRP functions to decrease proliferation when it is no longer needed. A role for CIRP in shutting down proliferation is consistent with other studies that outline a role for CIRP as a tumor suppressor *in vivo*. For example, CIRP expression inversely correlates with proliferation in normal endometrium, implicating CIRP in decreasing proliferation during the normal menstrual cycle. CIRP also inhibits proliferation through its function as an RBP, binding DNA damage response genes. CIRP is also part of the transcriptomic signature that decreases the lifetime risk for breast cancer in multiparous women [18-21, 24]. Thus, one role for CIRP may be in tissue homeostasis, maintaining normal levels of proliferation in certain tissues. CIRP expression also inversely correlates with malignancy in ovarian cancers, with low levels of CIRP seen in malignancy, suggesting that CIRP loss disrupts homeostasis by releasing inhibition of proliferation. In cells in culture, CIRP is also known to decrease proliferation through upregulation of $p27^{kip1}$

[136], a negative regulator of the cell cycle. This provides another possible mechanism for CIRP to decrease proliferation at the lactational switch during development.

Our findings also highlight the significance of studying biomolecules *in vivo*, as previous findings in breast cancer cell lines found CIRP to be a positive regulator of cyclin E1, a positive regulator of the cell cycle [27]. Future studies should focus on the specific mechanism of how CIRP influences proliferation during mammary gland development *in vivo*. One potential mechanism for this could be CIRP's function in binding and stabilizing or increasing translation of mRNAs encoding known tumor suppressor genes such as ATR, TRX-1 and RPA2 [18, 19]. These genes are not currently known to play a role in mammary gland development; however these mRNAs are known targets for CIRP under various conditions of cell stress and could be downstream effectors of CIRP's normal physiological role during mammary gland development.

In contrast to its effects on proliferation, we found that CIRP overexpression did not affect apoptosis at involution day 3, which is the peak of apoptosis during mammary gland development. This result was somewhat expected, due to our previous findings of how CIRP is a positive regulator of Cyclin E1 *in vitro* which is a positive regulator of proliferation. It is also possible that the initial onset of apoptosis or its duration was affected [137], as other time points during involution were not assessed. Evidence against this is the normal morphology of the mammary gland following involution (data not shown). Future studies examining the full time course of involution, from its onset through its completion would be necessary to rule out a role for CIRP in apoptosis during mammary gland development.

CIRP's relatively novel role as a mediator of inflammation has yet to be explored in the context of organ development, but inflammation has been shown to play a role in mammary gland development [86, 137, 138]. Given our results in breast cancer (Aim 2), in which we showed that CIRP decreases several cytokines known to be protumorigenic in human breast cancer, it is possible that CIRP could be influencing inflammatory cytokines or cells that may also influence proliferation during involution as there is some evidence that inflammation is actively suppressed during mammary gland development to allow for remodeling [139].

Aim 2: Determine the effects of CIRP overexpression on breast tumorigenesis and inflammation

Chapter 3 shows that CIRP overexpression decreases both early and late stage tumorigenesis in the MMTV-PyMT mouse model for breast cancer. The mechanisms by which CIRP decreases tumor growth in this model could be cell intrinsic, cell extrinsic or both. CIRP could affect tumorigenesis in a cell extrinsic fashion through its effects on cytokines that are known to be protumorigenic in breast cancer, its effects on T-cell populations, and/or through its function as a DAMP. CIRP may also be affecting tumor growth in a cell intrinsic fashion, via its function as a classic RNA-binding protein. As a classic RBP, CIRP is known to decrease proliferation through binding and increasing translation of stress response proteins such as TRX-1, RPA2 and ATR [3, 6, 25]. In addition, CIRP is also known to bind and upregulate $p27^{Kip1}$, a known cell-cycle inhibitor [136]. CIRP may also be affecting cytokines through a cell intrinsic mechanism, as CIRP is known to affect cytokine levels by affecting Iκκ phosphorylation through an unknown mechanism [11].

We showed that inhibition of late stage tumorigenesis was associated with a higher level of $CD8^+$ cytotoxic T-cells and a lower level of $CD4^+$ helper T-cells, along with lower levels of IL-6, LIF, IL-10 and IL-13 are associated with tumor rejection in human breast cancer [111]. Since CIRP/PyMT mice also have significantly lower levels of cytokines that play key roles in metastasis in human breast cancer, such as MIP1, MIP2 and LIF [119], the decreases in these cytokines likely contributed to the lower levels of metastatic foci found in CIRP/PyMT mice when compared to PyMT/+ mice. Interestingly, CIRP/PyMT mice also had significantly lower levels of IFN$\boldsymbol{\gamma}$, which is typically associated with tumor rejection in human breast cancer [111]. However, there is evidence that elevated levels of IFN$\boldsymbol{\gamma}$ can induce immune escape, mediated by programmed cell death ligand 1 (PD-L1) [118, 140, 141]. Future investigations in our mouse models could focus on this mechanism of potential immune escape, as this could reveal why tumorigenesis is consistently decreased in CIRP/PyMT mice despite lower levels of IFN$\boldsymbol{\gamma}$. Also, since $CD4^+$ T-cells are significantly lower in CIRP/PyMT cells, looking at specific sub-sets of $CD4^+$ T-cells could shed further light on the potential prognosis, as the presence of significant levels of $CD4^+$ Th17 cells and regulatory T-cells are typically associated with poor prognosis in human breast cancer [111, 142].

Due to the observed differences in myeloid chemoattractants (chemokines) such as KC and MCP-1, we expected to see differences in the number of macrophages present in tumors from CIRP/PyMT mice and PyMT mice. However, no difference in overall macrophage levels were detected between CIRP/PyMT and PyMT/+ tumors. Despite this result, it is possible that differences in macrophage polarization are present in our mouse models. M1 macrophage polarity is associated with tumor rejection, while M2

macrophage polarity is associated with tumor promotion in human breast cancer [43, 47, 143, 144]. CIRP mediated alteration of the levels of cytokines, starting in early tumorigenesis, could affect the subsequent immune cell cohort and macrophage polarity. Future experiments could assess macrophage polarity in CIRP/PyMT as compared to PyMT mice at both early and late stages of tumorigenesis to test this possibility.

CIRP is known to trigger changes in inflammation by acting as a DAMP [16, 43, 47, 52] and is also known to increase Iκκ phosphorylation through an unknown mechanism. Further exploration of the mechanistic details of CIRP's role in regulation of the NF-κB pathway will be critical to understanding how CIRP causes changes in immune cell cohorts and associated cytokines. The relevance of CIRP's role as a DAMP in human breast cancer could be investigated in human breast cancer cell lines, since it is known that CIRP alters inflammation in this way in colorectal and liver cancers [16, 52]. Specifically, assays in human breast cancer cell lines could investigate whether or not CIRP triggers cytokine release in manner similar to known TLR4 agonists, such as LPS. We also have some evidence showing that CIRP protein is present in human breast cancer patient serum (Figure AP1.1), which is significant because CIRP is known to affect inflammation in a cell extrinsic manner as a DAMP when found in detectable levels in serum [14, 16, 52].

Overall, results from this aim show that CIRP overexpression impedes breast tumorigenesis, likely by acting as a negative modulator of protumorigenic inflammation. The mechanistic details of how CIRP functions in this regard have yet to be explored and provides a clear avenue for future experiments. Our findings also provide rationale for

investigating CIRP protein levels in human cancer patient serum and tumors and how they may correlate with protumorigenic inflammatory cytokines and immune cell cohorts in those patients. There is precedent for measurement of CIRP protein levels in patients with other cancers and diseases. For example, CIRP increases tumor promoting inflammation in colorectal and liver cancers. CIRP also increases tissue damaging inflammation in patients with sepsis [14, 16, 44, 52]. Our preliminary results in human breast cancer patient sera are shown below, where we begin to investigate how CIRP protein levels correlate with inflammatory cytokines (See Appendix). These unpublished preliminary results show that CIRP levels negatively correlate with RANTES, and positively correlate with TNFα and IL1β. RANTES is a chemokine that attracts T-cells to the tumor microenvironment and is associated with more advanced disease in breast cancer [145]. TNFα and IL1β are both associated with better prognosis and tumor clearance in human breast cancer [111]. These results suggest that CIRP could be functioning as a tumor suppressor by increasing cytokines that promote tumor rejection in human breast cancer. These preliminary results fall in line with our results in the PyMT mouse model for breast cancer and provide rationale for further examination of how CIRP function in human breast cancer and inflammation.

While the PyMT mouse model mirrors the formation and progression of human breast cancer in several key facets [105], it is critical to validate these findings in human disease in order to identify potential avenues for clinical application or early detection. If CIRP levels correlate with higher levels of cytokines associated with tumor rejection, it can be a potential biomarker for better prognosis in human breast cancer. There is precedent for using CIRP as a biomarker for reduced risk of breast cancer in multiparous

women [24]. It may also be possible to upregulate CIRP or its targets, once definitively identified, for therapy or prevention.

The most intriguing unknowns include the mechanism for CIRP's extracellular localization [14-16] and CIRP's binding of the TLR4-MD2 complex when found extracellularly, which provokes the question of whether it has the ability to bind other toll-like receptors, and how this binding alters the overall cytokine environment. Similarly, determining how CIRP increases IκκB and IκBα phosphorylation, is both intriguing and necessary for understanding CIRP's role in the NFκB pathway, which is a pathway that plays important roles in cytokine production and regulation [146]. CIRP is pro-inflammatory via its function as a stabilizing RBP, but also decreases inflammation by unknown mechanisms. Future studies should be aimed at elucidating the mechanistic roles CIRP plays in these various contexts, including human breast cancer.

Aim 3: Determine the effects of CIRP loss in a PyMT graft model

Chapter 3 also explores the effects of CIRP loss on tumor growth *in vivo* using a PyMT graft model. A cell line (Py2T cell line) was established from a mammary tumor taken from an MMTV-PyMT FVB mouse. These cells were then treated with CRISPR-Cas9 ribonucleocomplexes designed to target the coding sequence of CIRP. Py2T cells with CIRP knocked down were then injected/grafted into the mammary fat pads of wild-type FVB mice, with control treated Py2T cells injected into the contralateral mammary fat pad. CIRP knockdown resulted in greater tumor burden when compared to control Py2T cells where CIRP levels were unaltered. Interestingly, CIRP loss did not result in significant differences in the overall growth rate of tumors when compared to control Py2T cells. The

significant differences found in overall tumor burden are likely due to decreased tumor latency in CIRP knockdown tumor cell grafts; CIRP knockdown Py2T tumors appeared a full week earlier on average compared to control Py2T tumors.

We achieved an 84% knockdown of CIRP at the protein level prior to Py2T graft injections; it is possible that results would be more dramatic with a complete knockout. Many RBPs positively regulate their own expression by increasing the translational efficiency of their own mRNA via binding regulatory regions in the untranslated regions, leading to increased protein levels [147]. Since CIRP was not knocked out in all of the Py2T cells in the graft, it is possible that the cells still expressing CIRP had a growth advantage over those with CIRP knocked out and led to the observed similar rates of tumor growth after a full week of decreased tumor latency. Future analysis of CIRP protein level in tumors taken at necropsy will explore this possibility, as will *in vitro* experiments examining CIRP potential auto-regulation. Despite this caveat, CIRP knockdown led to a significant increase in tumor latency and final tumor weight. Taken as a whole, our results with CIRP knockdown substantiate evidence from CIRP overexpression experiments for a role in decreaing breast tumor growth in the PyMT mouse model for breast cancer. In addition, CIRP appears to act in a dose dependent manner as shown in Figure 3.2E where higher CIRP protein correlates with lower final tumor weight.

Overall Role of CIRP in Mammary Gland Development and Tumorigenesis

Our results show that CIRP acts as tumor suppressor by decreasing proliferation as well as by decreasing levels of several protumorigenic cytokines. Overall, we believe these results reinforce the current literature on CIRP, which

shows that the various roles of CIRP in inflammation and cancer are tissue and cell-type specific. Based on our results and the work of others, the tumor suppressive role that CIRP plays in breast cancer may be cell intrinsic, through its function as a stabilizing RBP. CIRP is known to bind and upregulate the stress response proteins TRX-1, RPA2 and ATR as well as $p27^{kip1}$. TRX-1, RPA2 and ATR upregulation causes cell-cycle arrest, while 27^{kip1} is a cell-cycle inhibitor [6, 136]. Since our data show that proliferation is decreased in development and during tumorigenesis, it is possible that the observed decreases in proliferation in both are being affected via these known actions of CIRP.

Evidence also shows that CIRP can affect cytokines levels in a cell intrinsic manner, where it affects Iκκ phosphorylation and subsequent cytokine production through an unknown mechanism. We show that CIRP decreases cytokine levels during tumorigenesis, but we did not examine Iκκ phosphorylation and activation, or its known downstream cytokine targets. CIRP-associated cytokine changes were associated with increased $CD8^+$ T-cells and decreased $CD4^+$ T-cells, which could influence the immune system towards breast tumor rejection. These results, along with the known cell intrinsic function of CIRP in Iκκ phosphorylation, provide a potential mechanism and clear direction for future experiments. Assessment of Iκκ phosphorylation status in our mouse model may provide more insight into the mechanistic details of how CIRP influences inflammation in breast cancer.

It is also important to note that the role of CIRP in inflammation may not be limited to disease processes, since inflammation is also known to play a role in mammary gland development [138, 148]. Of note, inflammation is known to be

actively suppressed during mammary gland development. This allows for extensive remodelling while avoiding the potentially damaging effects of cellular inflammatory responses and cytokine storms during these processes [148]. While our findings show that CIRP decreases several inflammatory cytokines during tumorigenesis, our studies leave CIRP's potential role in suppressing inflammation during mammary gland development unexplored. Given that our results show that CIRP has a role in mammary gland development and suppresses inflammation during mammary tumorigenesis, further investigation of a potential role for CIRP suppressing inflammation during development is an intriguing future direction. Future analyses should also include assessment of CIRP's potential roles in binding cytokine mRNAs in mammary gland development and tumorigenesis, as several cytokine mRNAs contain U-rich elements that CIRP preferentially binds as an RBP.

Several studies also show that CIRP has a role in affecting inflammation in a cell extrinsic fashion, acting as a DAMP via TLR4 binding [14, 16, 44, 52]. This function of CIRP typically occurs when detectable levels of CIRP are present in the serum of humans and animals. While our studies do not investigate CIRP-TLR4 binding, our data show that CIRP is detectable in the serum of our mouse model as well as in human sera from breast cancer patients and in normal human sera. This suggests the potential for CIRP to affect cytokine levels through a cell extrinsic mechanism. It is also important to note that TLR4 signalling typically leads to increases in cytokine production and release in cells, while we see consistent decreases in several cytokines when CIRP is overexpressed during tumorigenesis. We believe this discrepancy highlights not only the multiple mechanisms CIRP may

use to influence tumorigenesis, proliferation and inflammation, but also the numerous ways in which cytokine profiles and ultimately immune cell cohorts can be affected.

As a whole, when viewed in the larger context of other studies on CIRP, our data is shows that the overall functions of CIRP in mammary gland development and tumorigenesis are likely a combination of cell intrinsic and cell extrinsic functions that affect both proliferation and inflammation in the mammary gland.

Scientific Impact

These studies provide a solid rationale for further exploring the role of CIRP in modulating inflammation associated with human breast cancer. To our knowledge, this is the first attempt to assess the role of CIRP in breast tumorigenesis *in vivo*. It is also the first to explore the effects of CIRP on inflammation in breast cancer. These studies add new insights for how CIRP functions in not only in breast tumorigenesis, but to the newer and rapidly growing list of studies showing that CIRP is a mediator of inflammation in different contexts. Ideally, our findings in the CIRP mouse and MMTV-PyMT and mouse model for breast cancer will translate to human breast cancer, where CIRP could potentially be used as a biomarker to assess outcomes in human breast cancer, or to design novel therapies for prevention or treatment, as indicated by our prelimary human sera studies.

Appendix: CIRP and Cytokines in Patient Serum

Introduction

The goal of this appendix is to show our unpublished data on CIRP in normal and breast cancer patient serum samples. As referenced above, CIRP can modulate inflammation in a variety of contexts. In some cases, such as colorectal and liver cancers, CIRP increases cytokines that are known to be protumorigenic in human breast cancer. In other cases, such as in liver ischemia and in sepsis, CIRP increases cytokines that are known to promote tumor rejection. Our long-term goal is to understand how CIRP functions in human breast cancer and associated inflammation. Based on our studies in a mouse model of breast cancer, which show that CIRP inhibits tumorigenesis and decreases protumorigenic cytokines both locally and in serum, we hypothesize that higher CIRP level may correlate with lower protumorigenic cytokines in serum from breast cancer patients.

Materials and Methods

Patient Serum Samples

Ten serum samples were obtained through the Expanded Breast Cancer Registry (EBCR) at the University of New Mexico Human Tissue Repository. Five serum samples were from female patients diagnosed with ductal carcinoma in-situ (DCIS) and five serum samples were from female patients diagnosed with invasive breast cancer. Ten “normal” serum samples were obtained from BIOIVT (Hicksville, New York). These samples were taken from ten individual female donors, approximately age matched.

Luminex Arrays

Patient serum samples were used to assess 25 different cytokine protein levels (G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF-A, Eotaxin/CCL11). Cytokines in sera were assessed using Milliplex human cytokine array assays (EMD Millipore, Billerica, MA). Multiplex arrays were used for these analyses in accordance with the manufacturer's protocols. Sera were diluted ten-fold for analysis.

CIRP ELISA

Human CIRP (hCIRP) specific enzyme-linked immunosorbent assay (ELISA) kits were from CUSABIO (Wuhan, China). Serum samples were diluted ten-fold, in accordance with the manufacturer's instructions. These colorimetric assays were read on a Tecan Infinite F500 (Tecan, Switzerland) microplate reader at 450nm. Samples were run a minimum three times.

Statistics

Correlation analysis was done using Excel data analysis package to obtain the R^2 value for correlations between average CIRP level and levels of each cytokine assessed in the multiplex cytokine array. R^2 values greater than 0.7 were considered strong correlations for our analyses. R^2 values less than 0.7 were considered weak correlations.

Results

Serum levels of CIRP protein were assessed using ELISA and compared between breast cancer patients and non-patients. Figure AP1.1 A shows that CIRP protein level was not significantly different between breast cancer patient sera and normal patient sera. The average CIRP level in normal sera was 217.8 pg/ml, with the highest measured sample 292.5 pg/ml and the lowest 120.7 pg/ml. CIRP level in breast cancer patient sera averaged 287.5 pg/ml, with the highest measured sample 804.7 pg/ml and the lowest 101.4 pg/ml. Correlation analyses of CIRP protein level and cytokine levels in breast cancer patient sera, showed that CIRP protein level had strong positive correlations with IL-1β and TNFα (Figure AN1.1 B). A strong negative correlation was also found between CIRP and RANTES. Several other weak positive correlations were found between CIRP and IL2, Mip1, GMCSF, IL-12p40, IL-15, IFNα2, IP10, IL-12p70, IL9, IL4, VEGF, IL6 and IL8.

Discussion

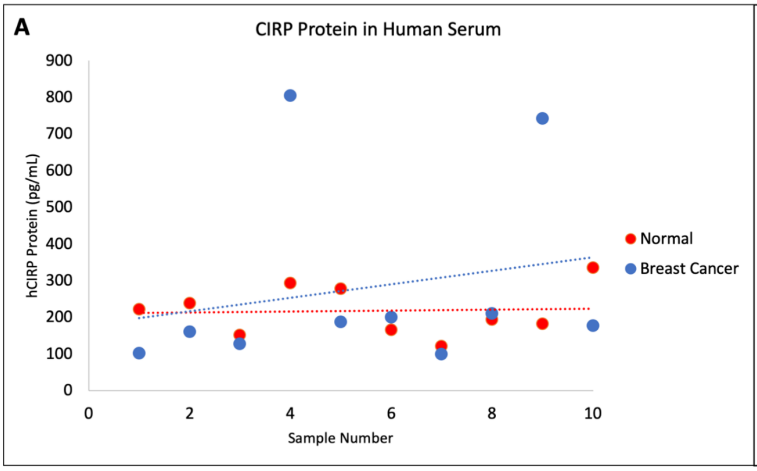
Although CIRP level was lower in normal sera compared to breast cancer patient sera, the difference was not significant, most likely due to the variation of CIRP level in patient sera. CIRP in breast cancer sera ranged from 101 to 805 pg/ml, while in normal sera CIRP ranged from 121 to 293 pg/ml. If the two patients with the highest CIRP levels are excluded, breast cancer sera has significantly lower levels of CIRP compared to normal sera (150 vs 225 pg/ml, $p< 0.03$, not shown). It is clear that more samples are needed for more definitive results.

Future analyses could assess CIRP level in breast cancer patient and matched normal tissues, since CIRP protein is known to be elevated in at least a subset of human breast cancers [25, 30], but hasn't been compared to matched normal tissue. It is important to note that if CIRP is elevated in both breast cancer tissues as well as serum, then there is potential for CIRP to affect proliferation and inflammation through both

cell intrinsic and cell extrinsic mechanisms. For this pilot study, we focused our analyses on human serum based on studies showing that detectable levels of CIRP in serum typically leads to CIRP affecting inflammation in a cell extrinsic fashion, by acting as a DAMP. Patient serum was also easier to obtain as compared to tumor and matched normal tissue.

Strong correlations between CIRP level and RANTES, IL1β, and TNFα were found. CIRP levels negatively correlated with RANTES, which is a chemokine that positively correlates with lymph node metastasis in human breast cancer [149]. CIRP levels positively correlated with IL1β and TNFα, both of which are known to promote tumor rejection in human breast cancer [111]. These results suggest that CIRP may inhibit cytokines involved in metastasis and promote cytokines involved in tumor rejection. These results are also consistent with our studies in the PyMT mouse model.

Although preliminary and limited, these results provide a proof of principle, showing detectable levels of CIRP in breast cancer patient sera. In addition, since CIRP levels correlated with cytokines that promote tumor rejection, and inversely correlated with a cytokine that promotes metastasis, a potential role for CIRP in modulating systemic inflammation in human breast cancer is implicated.



B CIRP Protein Correlation With Cytokines (R^2 values)

RANTES	**-0.80373**
IL1B	**0.765141**
TNFa	**0.706035**
IL2	0.667643
Mip1B	0.667138
GMCSF	0.658784
IL-12p40	0.629336
IL15	0.599889
IFNa2	0.588068
IP10	0.573497
IL-12p70	0.548903
IL9	0.50914
IL4	0.483051
VEGF	0.452619
IL6	0.452256
IL8	0.420859

Figure AP1.1 CIRP in Human Patient Serum A) CIRP protein level in breast cancer and normal patient sera as measured by ELISA each dot represents the results from one patient. Dashed lines are lines of best fit. B) Correlation analyses between CIRP protein levels and cytokine levels in breast cancer patient sera (R^2 values).

References

1. Lukong KE, Chang K-w, Khandjian EW, Richard S. RNA-binding proteins in human genetic disease. Trends in Genetics. 2008;24(8):416-25.
2. Gerstberger S, Hafner M, Tuschl T. A census of human RNA-binding proteins. Nature Reviews Genetics. 2014;15(12):829-45.
3. Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J. A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. The Journal of cell biology. 1997;137(4):899-908.
4. De Leeuw F, Zhang T, Wauquier C, Huez G, Kruys V, Gueydan C. The cold-inducible RNA-binding protein migrates from the nucleus to cytoplasmic stress granules by a methylation-dependent mechanism and acts as a translational repressor. Experimental cell research. 2007;313(20):4130-44.
5. Yang C, Carrier F. The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. Journal of Biological Chemistry. 2001;276(50):47277-84.
6. Fornace AJ, Alamo I, Hollander MC. DNA damage-inducible transcripts in mammalian cells. Proceedings of the National Academy of Sciences. 1988;85(23):8800-4.
7. Sheikh MS, Carrier F, Papathanasiou MA, Hollander MC, Zhan Q, Yu K, et al. Identification of several human homologs of hamster DNA damage-inducible transcripts cloning and characterization of a novel uv-inducible cDNA that codes for a putative RNA-binding protein. Journal of Biological Chemistry. 1997;272(42):26720-6.
8. Wilusz J, Feig DI, Shenk T. The C proteins of heterogeneous nuclear ribonucleoprotein complexes interact with RNA sequences downstream of polyadenylation cleavage sites. Molecular and cellular biology. 1988;8(10):4477-83.
9. Lleonart M. A new generation of proto-oncogenes: cold-inducible RNA binding proteins. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2010;1805(1):43-52.
10. Zhu X, Bührer C, Wellmann S. Cold-inducible proteins CIRP and RBM3, a unique couple with activities far beyond the cold. Cellular and Molecular Life Sciences. 2016:1-21.
11. Brochu C, Cabrita MA, Melanson BD, Hamill JD, Lau R, Pratt MC, et al. NF-κB-dependent role for cold-inducible RNA binding protein in regulating interleukin 1β. PloS one. 2013;8(2):e57426.
12. Chen L, Ran D, Xie W, Xu Q, Zhou X. Cold-inducible RNA-binding protein mediates cold air inducible airway mucin production through TLR4/NF-κB signaling pathway. International Immunopharmacology. 2016;39:48-56.
13. Juan Y, Haiqiao W, Xie W, Huaping H, Zhong H, Xiangdong Z, et al. Cold-inducible RNA-binding protein mediates airway inflammation and mucus hypersecretion through a post-transcriptional regulatory mechanism under cold stress. The International Journal of Biochemistry & Cell Biology. 2016;78:335-48.
14. Qiang X, Yang W-L, Wu R, Zhou M, Jacob A, Dong W, et al. Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nature medicine. 2013;19(11):1489-95.
15. Ren WH, Zhang LM, Liu HQ, Gao L, Chen C, Qiang C, et al. Protein overexpression of CIRP and TLR4 in oral squamous cell carcinoma: an immunohistochemical and clinical correlation analysis. Medical Oncology. 2014;31(8):1-9.

16. Sakurai T, Kashida H, Watanabe T, Hagiwara S, Mizushima T, Iijima H, et al. Stress response protein cirp links inflammation and tumorigenesis in colitis-associated cancer. Cancer research. 2014;74(21):6119-28.
17. Yoo IS, Lee SY, Park CK, Lee JC, Kim Y, Yoo SJ, et al. Serum and synovial fluid concentrations of cold-inducible RNA-binding protein in patients with rheumatoid arthritis. International Journal of Rheumatic Diseases. 2016.
18. Yang R, Weber DJ, Carrier F. Post-transcriptional regulation of thioredoxin by the stress inducible heterogenous ribonucleoprotein A18. Nucleic acids research. 2006;34(4):1224-36.
19. Yang R, Zhan M, Nalabothula NR, Yang Q, Indig FE, Carrier F. Functional significance for a heterogenous ribonucleoprotein A18 signature RNA motif in the 3'-untranslated region of ataxia telangiectasia mutated and Rad3-related (ATR) transcript. Journal of Biological Chemistry. 2010;285(12):8887-93.
20. Hamid AA, Mandai M, Fujita J, Nanbu K, Kariya M, Kusakari T, et al. Expression of cold-inducible RNA-binding protein in the normal endometrium, endometrial hyperplasia, and endometrial carcinoma. International journal of gynecological pathology. 2003;22(3):240-7.
21. Biade S, Marinucci M, Schick J, Roberts D, Workman G, Sage E, et al. Gene expression profiling of human ovarian tumours. British journal of cancer. 2006;95(8):1092-100.
22. Peri S, de Cicco RL, Santucci-Pereira J, Slifker M, Ross EA, Russo IH, et al. Defining the genomic signature of the parous breast. BMC Med Genomics. 2012;5:46. doi: 10.1186/1755-8794-5-46. PubMed PMID: 23057841; PubMed Central PMCID: PMCPMC3487939.
23. Lujan DA, Garcia S, Vanderhoof J, Sifuentes J, Brandt Y, Wu Y, et al. Cold-inducible RNA binding protein in mouse mammary gland development. Tissue and Cell. 2016;48(6):577-87.
24. Peri S, de Cicco RL, Santucci-Pereira J, Slifker M, Ross EA, Russo IH, et al. Defining the genomic signature of the parous breast. BMC medical genomics. 2012;5(1):46.
25. Artero-Castro A, Callejas FB, Castellvi J, Kondoh H, Carnero A, Fernández-Marcos PJ, et al. Cold-inducible RNA-binding protein bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation. Molecular and cellular biology. 2009;29(7):1855-68.
26. Zeng Y, Kulkarni P, Inoue T, Getzenberg RH. Down-regulating cold shock protein genes impairs cancer cell survival and enhances chemosensitivity. Journal of cellular biochemistry. 2009;107(1):179-88.
27. Guo X, Wu Y, Hartley RS. Cold-inducible RNA-binding protein contributes to human antigen R and cyclin E1 deregulation in breast cancer. Molecular carcinogenesis. 2010;49(2):130-40.
28. Gao S, Ma J-J, Lu C. Prognostic value of cyclin E expression in breast cancer: a meta-analysis. Tumor Biology. 2013;34(6):3423-30.
29. Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, et al. Cyclin E and survival in patients with breast cancer. New England Journal of Medicine. 2002;347(20):1566-75.
30. Chang ET, Parekh PR, Yang Q, Nguyen DM, Carrier F. Heterogenous ribonucleoprotein A18 (hnRNP A18) promotes tumor growth by increasing protein translation of selected transcripts in cancer cells. Oncotarget. 2016;7(9):10578.

31. Mangé A, Lacombe J, Bascoul-Mollevi C, Jarlier M, Lamy P-J, Rouanet P, et al. Serum autoantibody signature of ductal carcinoma in situ progression to invasive breast cancer. Clinical Cancer Research. 2012;18(7):1992-2000.
32. Jian F, Chen Y, Ning G, Fu W, Tang H, Chen X, et al. Cold inducible RNA binding protein upregulation in pituitary corticotroph adenoma induces corticotroph cell proliferation via Erk signaling pathway. Oncotarget. 2016;7(8):9175-87.
33. Zhang Y, Wu Y, Mao P, Li F, Han X, Zhang Y, et al. Cold-inducible RNA-binding protein CIRP/hnRNP A18 regulates telomerase activity in a temperature-dependent manner. Nucleic acids research. 2015:gkv1465.
34. Akincilar SC, Unal B, Tergaonkar V. Reactivation of telomerase in cancer. Cellular and Molecular Life Sciences. 2016;73(8):1659-70.
35. Lee HN, Ahn S-M, Jang HH. Cold-inducible RNA-binding protein promotes epithelial-mesenchymal transition by activating ERK and p38 pathways. Biochemical and Biophysical Research Communications. 2016;477(4):1038-44.
36. Lee HN, Ahn S-M, Jang HH. Cold-inducible RNA-binding protein, CIRP, inhibits DNA damage-induced apoptosis by regulating p53. Biochemical and biophysical research communications. 2015;464(3):916-21.
37. Sakurai T, Itoh K, Higashitsuji H, Nonoguchi K, Liu Y, Watanabe H, et al. Cirp protects against tumor necrosis factor-α-induced apoptosis via activation of extracellular signal-regulated kinase. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2006;1763(3):290-5.
38. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011;144(5):646-74.
39. Chin L, Artandi SE, Shen Q, Tam A, Lee S-L, Gottlieb GJ, et al. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell. 1999;97(4):527-38.
40. Beauséjour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. The EMBO journal. 2003;22(16):4212-22.
41. Chen Z, Trotman LC, Shaffer D, Lin H-K, Dotan ZA, Niki M, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature. 2005;436(7051):725-30.
42. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature. 2007;445(7128):656-60.
43. Zhou Y, Dong H, Zhong Y, Huang J, Lv J, Li J. The Cold-Inducible RNA-Binding Protein (CIRP) Level in peripheral blood predicts sepsis outcome. PloS one. 2015;10(9):e0137721.
44. Bolognese AC, Sharma A, Yang W-L, Nicastro J, Coppa GF, Wang P. Cold-inducible RNA-binding protein activates splenic T cells during sepsis in a TLR4-dependent manner. Cellular & Molecular Immunology. 2016.
45. Yang W-L, Sharma A, Wang Z, Li Z, Fan J, Wang P. Cold-inducible RNA-binding protein causes endothelial dysfunction via activation of Nlrp3 inflammasome. Scientific reports. 2016;6.
46. Fein AM, Calalang-Colucci MG. Acute lung injury and acute respiratory distress syndrome in sepsis and septic shock. Critical care clinics. 2000;16(2):289-317.

47. Zhou M, Yang W-L, Ji Y, Qiang X, Wang P. Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia. Biochimica et Biophysica Acta (BBA)-General Subjects. 2014;1840(7):2253-61.
48. Lopez M, Meier D, Müller A, Franken P, Fujita J, Fontana A. Tumor necrosis factor and transforming growth factor β regulate clock genes by controlling the expression of the cold inducible RNA-binding protein (CIRBP). Journal of Biological Chemistry. 2014;289(5):2736-44.
49. Lopez MA, Meier D, Wong WW-L, Fontana A. TNF induced inhibition of Cirbp expression depends on RelB NF-κB signalling pathway. Biochemistry and Biophysics Reports. 2016;5:22-6.
50. Idrovo JP, Jacob A, Yang WL, Wang Z, Yen HT, Nicastro J, et al. A deficiency in cold-inducible RNA-binding protein accelerates the inflammation phase and improves wound healing. International journal of molecular medicine. 2016;37(2):423-8.
51. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nature Reviews Cancer. 2013;13(11):759-71.
52. Sakurai T, Yada N, Watanabe T, Arizumi T, Hagiwara S, Ueshima K, et al. Cold-inducible RNA-binding protein promotes the development of liver cancer. Cancer science. 2015;106(4):352-8.
53. Godwin A, Yang W-L, Sharma A, Khader A, Wang Z, Zhang F, et al. Blocking Cold-Inducible RNA-Binding Protein (CIRP) Protects Liver from Ischemia/Reperfusion Injury. Shock (Augusta, Ga). 2015;43(1):24.
54. Doi K, Horiuchi T, Uchinami M, Tabo T, Kimura N, Yokomachi J, et al. Hepatic ischemia–reperfusion promotes liver metastasis of colon cancer. Journal of Surgical Research. 2002;105(2):243-7.
55. Kim M-Y, Hur J, Jeong S-J. Emerging roles of RNA and RNA-binding protein network in cancer cells. BMB reports. 2009;42(3):125-30.
56. Katsanou V, Papadaki O, Milatos S, Blackshear PJ, Anderson P, Kollias G, et al. HuR as a negative posttranscriptional modulator in inflammation. Molecular cell. 2005;19(6):777-89.
57. Neelamraju Y, Hashemikhabir S, Janga SC. The human RBPome: from genes and proteins to human disease. Journal of proteomics. 2015;127:61-70.
58. Guo X, Hartley RS. HuR contributes to cyclin E1 deregulation in MCF-7 breast cancer cells. Cancer research. 2006;66(16):7948-56.
59. Wu Y, Guo X, Brandt Y, Hathaway HJ, Hartley RS. Three-dimensional collagen represses cyclin E1 via β1 integrin in invasive breast cancer cells. Breast cancer research and treatment. 2011;127(2):397-406.
60. Lopez de Silanes I, Lal A, Gorospe M. HuR: post-transcriptional paths to malignancy. RNA Biol. 2005;2(1):11-3. Epub 2006/11/30. PubMed PMID: 17132932.
61. Lukong KE, Chang KW, Khandjian EW, Richard S. RNA-binding proteins in human genetic disease. Trends Genet. 2008;24(8):416-25. Epub 2008/07/04. doi: 10.1016/j.tig.2008.05.004. PubMed PMID: 18597886.
62. Kim MY, Hur J, Jeong S. Emerging roles of RNA and RNA-binding protein network in cancer cells. BMB Rep. 2009;42(3):125-30. Epub 2009/04/02. PubMed PMID: 19335997.

63. Yang C, Carrier F. The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. J Biol Chem. 2001;276(50):47277-84. Epub 2001/09/28. doi: 10.1074/jbc.M105396200. PubMed PMID: 11574538.
64. Nishiyama H, Danno S, Kaneko Y, Itoh K, Yokoi H, Fukumoto M, et al. Decreased expression of cold-inducible RNA-binding protein (CIRP) in male germ cells at elevated temperature. American Journal of Pathology. 1998;152(1):289-96. PubMed PMID: WOS:000071439200031.
65. Yang R, Zhan M, Nalabothula NR, Yang Q, Indig FE, Carrier F. Functional significance for a heterogenous ribonucleoprotein A18 signature RNA motif in the 3'-untranslated region of ataxia telangiectasia mutated and Rad3-related (ATR) transcript. J Biol Chem. 2010;285(12):8887-93. Epub 2010/01/28. doi: 10.1074/jbc.M109.013128. PubMed PMID: 20103595; PubMed Central PMCID: PMC2838310.
66. Yang R, Weber DJ, Carrier F. Post-transcriptional regulation of thioredoxin by the stress inducible heterogenous ribonucleoprotein A18. Nucleic Acids Res. 2006;34(4):1224-36. Epub 2006/03/04. doi: 10.1093/nar/gkj519. PubMed PMID: 16513844; PubMed Central PMCID: PMC1388095.
67. Hamid AA, Mandai M, Fujita J, Nanbu K, Kariya M, Kusakari T, et al. Expression of cold-inducible RNA-binding protein in the normal endometrium, endometrial hyperplasia, and endometrial carcinoma. International Journal of Gynecologic Pathology. 2003;22(3):240-7.
68. Peri S, de Cicco RL, Santucci-Pereira J, Slifker M, Ross EA, Russo IH, et al. Defining the genomic signature of the parous breast. BMC medical genomics. 2012;5(1):1.
69. Artero-Castro A, Callejas FB, Castellvi J, Kondoh H, Carnero A, Fernandez-Marcos PJ, et al. Cold-inducible RNA-binding protein bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation. Mol Cell Biol. 2009;29(7):1855-68. Epub 2009/01/23. doi: 10.1128/MCB.01386-08. PubMed PMID: 19158277; PubMed Central PMCID: PMC2655622.
70. Sakurai T, Itoh K, Higashitsuji H, Nonoguchi K, Liu Y, Watanabe H, et al. Cirp protects against tumor necrosis factor-alpha-induced apoptosis via activation of extracellular signal-regulated kinase. Biochim Biophys Acta. 2006;1763(3):290-5. Epub 2006/03/30. doi: 10.1016/j.bbamcr.2006.02.007. PubMed PMID: 16569452.
71. Wellmann S, Bührer C, Moderegger E, Zelmer A, Kirschner R, Koehne P, et al. Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. Journal of cell science. 2004;117(9):1785-94.
72. Tang C, Wang Y, Lan D, Feng X, Zhu X, Nie P, et al. Analysis of gene expression profiles reveals the regulatory network of cold-inducible RNA-binding protein mediating the growth of BHK-21 cells. Cell biology international. 2015. Epub 2015/01/20. doi: 10.1002/cbin.10438. PubMed PMID: 25597958.
73. Rajayer SR, Jacob A, Yang W-L, Zhou M, Chaung W, Wang P. Cold-inducible RNA-binding protein is an important mediator of alcohol-induced brain inflammation. PloS one. 2013;8(11):e79430.
74. Guo X, Hartley RS. HuR contributes to cyclin E1 deregulation in MCF-7 breast cancer cells. Cancer Res. 2006;66(16):7948-56. Epub 2006/08/17. doi: 10.1158/0008-5472.CAN-05-4362. PubMed PMID: 16912169.
75. Guo X, Wu Y, Hartley RS. Cold-inducible RNA-binding protein contributes to human antigen R and cyclin E1 deregulation in breast cancer. Mol Carcinog.

2010;49(2):130-40. Epub 2009/09/25. doi: 10.1002/mc.20582. PubMed PMID: 19777567; PubMed Central PMCID: PMC2813375.
76. Blaxall BC, Dwyer-Nield LD, Bauer AK, Bohlmeyer TJ, Malkinson AM, Port JD. Differential Expression and Localization of the mRNA Binding Proteins, AU-Rich Element mRNA Binding Protein (AUF1) and Hu Antigen R (HuR), in Neoplastic Lung Tissue. Mol Carcinog. 2000;28(2):76-83.
77. Denkert C, Weichert W, Pest S, Koch I, Licht D, Köbel M, et al. Overexpression of the embryonic-lethal abnormal vision-like protein HuR in ovarian carcinoma is a prognostic factor and is associated with increased cyclooxygenase 2 expression. Cancer Res. 2004;64(1):189-95.
78. Denkert C, Weichert W, Winzer K-J, Müller B-M, Noske A, Niesporek S, et al. Expression of the ELAV-like protein HuR is associated with higher tumor grade and increased cyclooxygenase-2 expression in human breast carcinoma. Clinical Cancer Research. 2004;10(16):5580-6.
79. de Silanes IL, Fan J, Yang X, Zonderman AB, Potapova O, Pizer ES, et al. Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene. 2003;22(46):7146-54.
80. Heinonen M, Bono P, Narko K, Chang S-H, Lundin J, Joensuu H, et al. Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. Cancer Res. 2005;65(6):2157-61.
81. Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, et al. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proceedings of the National Academy of Sciences. 2003;100(26):15853-8.
82. Rudolph MC, McManaman JL, Phang T, Russell T, Kominsky DJ, Serkova NJ, et al. Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. Physiological genomics. 2007;28(3):323-36.
83. Ip MM, Asch BB. Methods in mammary gland biology and breast cancer research: Springer Science & Business Media; 2000.
84. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol. 1992;12(3):954-61. Epub 1992/03/01. PubMed PMID: 1312220; PubMed Central PMCID: PMC369527.
85. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. The Journal of Immunology. 1984;133(4):1710-5.
86. Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. An atlas of mouse mammary gland development. Journal of mammary gland biology and neoplasia. 2000;5(2):227-41.
87. Kaneko T, Kibayashi K. Mild hypothermia facilitates the expression of cold-inducible RNA-binding protein and heat shock protein 70.1 in mouse brain. Brain research. 2012;1466:128-36.
88. Li S, Zhang Z, Xue J, Liu A, Zhang H. Cold-inducible RNA binding protein inhibits H(2)O(2)-induced apoptosis in rat cortical neurons. Brain research. 2012;1441:47-52. Epub 2012/02/03. doi: 10.1016/j.brainres.2011.12.053. PubMed PMID: 22297174.
89. Saito K, Fukuda N, Matsumoto T, Iribe Y, Tsunemi A, Kazama T, et al. Moderate low temperature preserves the stemness of neural stem cells and suppresses apoptosis of the cells via activation of the cold-inducible RNA binding protein. Brain research.

2010;1358:20-9. Epub 2010/08/26. doi: 10.1016/j.brainres.2010.08.048. PubMed PMID: 20735994.
90. Marti A, Ritter PM, Jäger R, Lazar H, Baltzer A, Schenkel J, et al. Mouse mammary gland involution is associated with cytochrome c release and caspase activation. Mechanisms of development. 2001;104(1):89-98.
91. Lockshin RA, Zakeri Z. Caspase-independent cell death? Oncogene. 2004;23(16):2766-73.
92. Förster C, Mäkela S, Wärri A, Kietz S, Becker D, Hultenby K, et al. Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium. Proceedings of the National Academy of Sciences. 2002;99(24):15578-83.
93. Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng C-X, et al. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol Cell Biol. 2004;24(18):8037-47.
94. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. Journal of the National Cancer Institute. 2011.
95. Masuda T, Itoh K, Higashitsuji H, Higashitsuji H, Nakazawa N, Sakurai T, et al. Cold-inducible RNA-binding protein (Cirp) interacts with Dyrk1b/Mirk and promotes proliferation of immature male germ cells in mice. Proceedings of the National Academy of Sciences. 2012;109(27):10885-90.
96. Peng Y, Yang P-H, Tanner JA, Huang J-D, Li M, Lee HF, et al. Cold-inducible RNA binding protein is required for the expression of adhesion molecules and embryonic cell movement in Xenopus laevis. Biochemical and biophysical research communications. 2006;344(1):416-24.
97. Sakurai T, Kashida H, Watanabe T, Hagiwara S, Mizushima T, Iijima H, et al. Stress response protein cirp links inflammation and tumorigenesis in colitis-associated cancer. Cancer Res. 2014;74(21):6119-28. Epub 2014/09/05. doi: 10.1158/0008-5472.CAN-14-0471. PubMed PMID: 25187386.
98. Coussens LM, Pollard JW. Leukocytes in mammary development and cancer. Cold Spring Harbor perspectives in biology. 2011;3(3). Epub 2010/12/03. doi: 10.1101/cshperspect.a003285. PubMed PMID: 21123394; PubMed Central PMCID: PMC3039933.
99. Zhou M, Yang WL, Ji Y, Qiang X, Wang P. Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia. Biochim Biophys Acta. 2014;1840(7):2253-61. Epub 2014/03/13. doi: 10.1016/j.bbagen.2014.02.027. PubMed PMID: 24613680.
100. Rajayer SR, Jacob A, Yang WL, Zhou M, Chaung W, Wang P. Cold-inducible RNA-binding protein is an important mediator of alcohol-induced brain inflammation. PLoS One. 2013;8(11):e79430. Epub 2013/11/14. doi: 10.1371/journal.pone.0079430. PubMed PMID: 24223948; PubMed Central PMCID: PMC3815202.
101. Qiang X, Yang WL, Wu R, Zhou M, Jacob A, Dong W, et al. Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nature medicine. 2013;19(11):1489-95. Epub 2013/10/08. doi: 10.1038/nm.3368. PubMed PMID: 24097189; PubMed Central PMCID: PMC3826915.
102. Lujan DA, Ochoa JL, Hartley RSJWIRR. Cold-inducible RNA binding protein in cancer and inflammation. 2018;9(2):e1462.

103. Lin T-Y, Chen Y, Jia J-S, Zhou C, Lian M, Wen Y-T, et al. Loss of Cirbp expression is correlated with the malignant progression and poor prognosis in nasopharyngeal carcinoma. Cancer Management and Research. 2019;11:6959.
104. Waldmeier L, Meyer-Schaller N, Diepenbruck M, Christofori G. Py2T murine breast cancer cells, a versatile model of TGFβ-induced EMT in vitro and in vivo. PLoS One. 2012;7(11):e48651.
105. Franci C, Zhou J, Jiang Z, Modrusan Z, Good Z, Jackson E, et al. Biomarkers of residual disease, disseminated tumor cells, and metastases in the MMTV-PyMT breast cancer model. PLoS One. 2013;8(3):e58183.
106. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012;9(7):671.
107. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. The American journal of pathology. 2003;163(5):2113-26.
108. Marjon NA, Hu C, Hathaway HJ, Prossnitz ER. G protein–coupled estrogen receptor regulates mammary tumorigenesis and metastasis. Molecular Cancer Research. 2014;12(11):1644-54.
109. Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. Cytokine & growth factor reviews. 2006;17(5):325-37.
110. Ben-Baruch A. Host microenvironment in breast cancer development: inflammatory cells, cytokines and chemokines in breast cancer progression: reciprocal tumor–microenvironment interactions. Breast cancer research. 2002;5(1):31.
111. DeNardo DG, Coussens LMJBcr. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. 2007;9(4):212.
112. Morris KT, Castillo EF, Ray AL, Weston LL, Nofchissey RA, Hanson JA, et al. Anti-G-CSF treatment induces protective tumor immunity in mouse colon cancer by promoting NK cell, macrophage and T cell responses. Oncotarget. 2015;6(26):22338.
113. Li X, Yang Q, Yu H, Wu L, Zhao Y, Zhang C, et al. LIF promotes tumorigenesis and metastasis of breast cancer through the AKT-mTOR pathway. Oncotarget. 2014;5(3):788.
114. Yu H, Yue X, Zhao Y, Li X, Wu L, Zhang C, et al. LIF negatively regulates tumor suppressor p53 through Stat3/ID1/MDM2 in colorectal cancers. Nature communications. 2014;5:5218.
115. Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell. 2012;150(1):165-78.
116. Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011;475(7355):222.
117. García-Tuñón I, Ricote M, Ruiz A, Fraile B, Paniagua R, Royuela M. Influence of IFN-gamma and its receptors in human breast cancer. BMC cancer. 2007;7(1):158.
118. Mandai M, Hamanishi J, Abiko K, Matsumura N, Baba T, Konishi I. Dual faces of IFNγ in cancer progression: a role of PD-L1 induction in the determination of pro-and antitumor immunity. Clinical cancer research. 2016;22(10):2329-34.
119. Feliciano PJNG. CXCL1 and CXCL2 link metastasis and chemoresistance. 2012;44(8):840.

120. Yang C, He L, He P, Liu Y, Wang W, He Y, et al. Increased drug resistance in breast cancer by tumor-associated macrophages through IL-10/STAT3/bcl-2 signaling pathway. Medical oncology. 2015;32(2):14.
121. Llanes-Fernández L, Álvarez-Goyanes RI, del Carmen Arango-Prado M, Alcocer-González JM, Mojarrieta JC, Pérez XE, et al. Relationship between IL-10 and tumor markers in breast cancer patients. The Breast. 2006;15(4):482-9.
122. Knüpfer H, Preiß R. Significance of interleukin-6 (IL-6) in breast cancer. Breast cancer research and treatment. 2007;102(2):129-35.
123. Srabovic N, Mujagic Z, Mujanovic-Mustedanagic J, Muminovic Z, Softic A, Begic L. Interleukin 13 expression in the primary breast cancer tumor tissue. Biochemia medica: Biochemia medica. 2011;21(2):131-8.
124. Toi M, Hoshina S, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. Japanese Journal of Cancer Research. 1994;85(10):1045-9.
125. Li X, Yang Q, Yu H, Wu L, Zhao Y, Zhang C, et al. LIF promotes tumorigenesis and metastasis of breast cancer through the AKT-mTOR pathway. 2014;5(3):788.
126. Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ, Selvan SRJIr. Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy. 2011;51(2-3):170-82.
127. Nutter F, Holen I, Brown HK, Cross SS, Evans CA, Walker M, et al. Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line. 2014;21(2):327-41.
128. Tulotta C, Ottewell PJE-rc. The role of IL-1B in breast cancer bone metastasis. 2018;25(7):R421-R34.
129. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. 2001;7(2):192.
130. Sanguinetti A, Santini D, Bonafè M, Taffurelli M, Avenia N. Interleukin-6 and pro inflammatory status in the breast tumor microenvironment. World journal of surgical oncology. 2015;13(1):129.
131. Xie G, Yao Q, Liu Y, Du S, Liu A, Guo Z, et al. IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. International journal of oncology. 2012;40(4):1171-9.
132. Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissière F, Laune D, et al. Oestrogen receptor negative breast cancers exhibit high cytokine content. 2007;9(1):R15.
133. Srabovic N, Mujagic Z, Mujanovic-Mustedanagic J, Muminovic Z, Softic A, Begic LJBmBm. Interleukin 13 expression in the primary breast cancer tumor tissue. 2011;21(2):131-8.
134. Villanueva L, Silva L, Llopiz D, Ruiz M, Iglesias T, Lozano T, et al. The Toll like receptor 4 ligand cold-inducible RNA-binding protein as vaccination platform against cancer. 2018;7(4):e1409321.
135. Tang C, Wang Y, Lan D, Feng X, Zhu X, Nie P, et al. Analysis of gene expression profiles reveals the regulatory network of cold-inducible RNA-binding protein mediating the growth of BHK-21 cells. Cell biology international. 2015;39(6):678-89.

136. Roilo M, Kullmann MK, Hengst L. Cold-inducible RNA-binding protein (CIRP) induces translation of the cell-cycle inhibitor p27Kip1. Nucleic acids research. 2018;46(6):3198-210.
137. Strange R, Li F, Saurer S, Burkhardt A, Friis R. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development. 1992;115(1):49-58.
138. Brady NJ, Chuntova P, Schwertfeger KL. Macrophages: regulators of the inflammatory microenvironment during mammary gland development and breast cancer. Mediators of inflammation. 2016;2016.
139. Monks J, Geske FJ, Lehman L, Fadok VA. Do inflammatory cells participate in mammary gland involution? Journal of mammary gland biology and neoplasia. 2002;7(2):163-76.
140. Abiko K, Matsumura N, Hamanishi J, Horikawa N, Murakami R, Yamaguchi K, et al. IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. British journal of cancer. 2015;112(9):1501-9.
141. Hamanishi J, Mandai M, Matsumura N, Abiko K, Baba T, Konishi I. PD-1/PD-L1 blockade in cancer treatment: perspectives and issues. International journal of clinical oncology. 2016;21(3):462-73.
142. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. Cancer research. 2015;75(18):3788-99.
143. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. International Journal of Developmental Biology. 2011;55(7-8-9):861-7.
144. Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Mönkkönen J, et al. Human breast cancer cells educate macrophages toward the M2 activation status. Breast Cancer Research. 2015;17(1):101.
145. Soria G, Yaal-Hahoshen N, Azenshtein E, Shina S, Leider-Trejo L, Ryvo L, et al. Concomitant expression of the chemokines RANTES and MCP-1 in human breast cancer: a basis for tumor-promoting interactions. Cytokine. 2008;44(1):191-200.
146. Serasanambati M, Chilakapati SR. Function of nuclear factor kappa B (NF-kB) in human diseases-a review. South Ind J Biol Sci. 2016;2:368-87.
147. Pullmann R, Kim HH, Abdelmohsen K, Lal A, Martindale JL, Yang X, et al. Analysis of turnover and translation regulatory RNA-binding protein expression through binding to cognate mRNAs. Molecular and cellular biology. 2007;27(18):6265-78.
148. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Research. 2004;6(2):R92.
149. Smeets A, Brouwers B, Hatse S, Laenen A, Paridaens R, Floris G, et al. Circulating CCL5 levels in patients with breast cancer: is there a correlation with lymph node metastasis? ISRN Immunology. 2013;2013.