Fall 12-15-2017

Identifying the Role of Janus Kinase 1 in Mammary Gland Development and Breast Cancer

Barbara Swenson
University of Nebraska Medical Center

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IDENTIFYING THE ROLE OF JANUS KINASE 1 IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

By

Barbara Louise Swenson

A DISSERTATION

Presented to the Faculty of
The University of Nebraska Graduate College
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy

Eppley Institute for Research in Cancer & Allied Diseases
Cancer Research Graduate Program
Under the Supervision of Professor Kay-Uwe Wagner
University of Nebraska Medical Center
Omaha, NE

November 2017
The development of the postnatal mammary gland is tightly controlled by peptide hormones and cytokines. The signaling of these extracellular ligands through their corresponding receptors rely on Janus Kinases (JAKs) that activate downstream Signal Transducers and Activators of Transcription (STATs). The JAK/STAT signaling pathway is crucial for processes such as growth, proliferation, and cell survival of the epithelial tissue, but also for the breakdown and remodeling of the mammary gland via IL-6 class inflammatory cytokines (e.g. LIF and OSM). JAK1 and JAK2, which are expressed in the mammary gland, are thought to have redundant functions. However, our previous studies demonstrated that JAK2 is exclusively required for the activation of STAT5 as well as the growth and functional differentiation of the gland. In contrast to the general paradigm, JAK2 might be dispensable for the activation of STAT3 in response to IL-6 class inflammatory cytokines. Utilizing the recently generated JAK1 conditional knockout mouse model, we demonstrate that Janus kinase 1 is the essential kinase responsible for mediating inflammatory cytokine signaling in the mammary gland, specifically during postlactational remodeling events where the absence of JAK1 delayed involution.
Ablation of JAK1 in the mammary epithelium of mice dramatically prevented the activation of STAT3 and uncoupled signaling of IL-6 class ligands such as OSM and LIF from their downstream effectors. Completing the profile of STATs, JAK1 was also found to play a non-redundant role in the activation of STAT1 and STAT6 but not STAT5.

Building on the evidence that JAK1 contributes to the activation of STAT3 in non-transformed mammary epithelial cells, we next tested if JAK1 maintained discretion in the activation of STAT3 in neoplastic tissue. A notorious transcription factor, active STAT3 has been tightly linked to aggressive disease, promoting a metastatic phenotype in breast cancers, notably, a subset of which that overexpress the human epidermal growth factor ErbB2/Her2. Using the mammary-specific deletion of JAK1 in the context of ErbB2 oncogenesis, we found that while JAK1 does not affect the tumor onset, it dramatically decreases STAT3 activity and occurrence of metastasis. Interestingly, we found that JAK1 is also required for the tumor-initiating cell population of established disease.

Collectively, the results of these studies describe the novel role of Janus kinase 1 specifically in mammary gland development and the contribution of JAK1 to disease progression.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activating Protein-1</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BLG</td>
<td>Beta-lactoglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>Chromatin immunoprecipitation sequencing</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled H2O</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Floxed (fl)</td>
<td>Flanked by loxP sites</td>
</tr>
<tr>
<td>FPKM</td>
<td>Fragments Per Kilobase of transcript per Million mapped reads</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma interferon Activation Site</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>Jak Homology</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>Neu</td>
<td>Rat homolg to the human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin m</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylemethane Sulfonyl Fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline with Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TRK</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine Kinase 2</td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Acknowledgements

First, I would like to thank Dr. Kay-Uwe Wagner for accepting me to join your lab the summer of 2012. My first week into the initial rotation, I was certain that the lab was the best fit, and I am beyond grateful that you allowed me to join the team. You have trained some of the best students, and it’s because of your passion for science combined with your critical eye in experimental design that they are able to cultivate successful careers. Thank you for maintaining a collaborative team. From my early experience, I can say that not many labs are like yours, and that working together is by far the most efficient method to move science forward. I am so grateful for the development of presenting data: you put time and effort into helping clarify ideas and underscore importance, because you know the impact that a well-delivered message can have. Your support for leadership and teaching experience throughout the years has been important to me not only as a developing scientist but also as contributing member of society. Thank you for the opportunity to work on the mammary gland project; without your experienced guidance, the lab and data analysis could not be possible. Overall, being a part of adding knowledge to the field of mammary gland development and breast cancer is irreplaceable.

I would also like to thank express my thanks to Dr. Vimla Band, Dr. Xu Luo, Dr. Rakesh Singh, and Dr. Hallgeir Rui for serving on my committee and providing critical critique throughout my graduate career. From the first committee meeting when we talked about the (then) potential implications of a conditional JAK1-knockout mouse model, to presenting data on how a conditional deletion affects mammary tumor cells, I cannot thank you enough for sharing enthusiasm for data and providing constructive feedback.
I would not be very far had it not been for the entire Wagner laboratory. From the first members that I interacted with—Dr, Kazuhito Sakamoto—for teaching the basics of tail PCR, maintaining mouse colonies, and allowing me to observe the construct development of the JAK1 conditional knockout mouse. These studies would not have been possible without your dedication and scientific detail. I would also like to thank Dr. Karoline Manthey for being a positive personality around in the lab while I was new and integrating into the group. I would like to thank Dr. Jeffrey Schmidt; your data presentation, scientific ideas, and writing abilities were nothing short of incredible. Thank you for teaching me the basics of lab technique along with being an exemplary student. To Dr. Nirakar Rajbhandari, thank you for all your contributions throughout the years with your calm presence that was nearly unshakable; you were a true role model as a student, developing scientist, and artist. A very big thank you to Aleata Triplett; your consistency and organization were factors that drew me to the lab but also propelled me over the course of repeated experiments. You not only know where every reagent is put in the lab, but also have the steadiest hand in PCR and RNA-Seq preparation. Thank you as well for being reliable and sharing the joy of athletics, especially triathlon. And to Patrick Raedler, thank you for being patient as I taught experimental techniques when you joined the lab, and now also for your patience as you reciprocate the teaching and explain introductory bioinformatics. You are highly committed, persistent, and critical in your scientific work, which will no doubt lead to success. Thank you for being a dependable fellow student and friend. I can’t thank Karen Dulany enough for your time as you have been instrumental with your experience in preparation of the histological sections that are integral to our studies.

To Dr. Joyce Solheim, thank you for being a student advocate and director for the Cancer Research Graduate Program, responding to emails quickly, and also looking
out for the students as a whole. Thank you for your guidance as I navigated the world of a student in science; and sincerely, thank you for welcoming me into the program.

I would also like to thank the opportunities to teach while at UNMC, as a teaching assistant for BRTP 822, I developed in presenting abstract concepts to students and I thank the students for giving me feedback on how to be a better instructor. Along with the teaching opportunities, I would like to thank the funding sources that have supported me over the years. The Cancer Biology Training Grant (CA009476) has supported me financially, but also in the development as a scientist by the rigorous presentation training, organized by Dr. Jennifer Black, who has encouraged students to continue to hone their communication style. Also, the UNMC Fellowship/Assistantship for critiquing the funding application for the JAK1 project and awarding the stipend based on scientific merit and career progression. An award that I respect very much and is truly an honor to receive is the Shawn Jessen Memorial Graduate Research Award. It was a privilege to be recognized by fellow students based on seminar presentations and active involvement in Eppley seminars, I hope to continue to uphold the enthusiasm for science and sharing data with others. I would also like to thank the International Student Research Forum for sponsoring my travel and presentations to China; it has made my world bigger and promoted culture as well as collaborations worldwide.

To Dr. Aaron Harmon at Novartis Animal Health for encouraging me to pursue graduate school while I worked as an intern and technician. I owe the phrase “there’s always one question” to you, for seeming to always having a question to ask at a seminar.

I also want to thank Dr. Sue Kane, for taking me under your wing while you were a PhD student at South Dakota State University and at the time also battling breast cancer. You taught me how to do immunohistochemistry, but more importantly showed me persistence. You made a great impression on me, from presenting information so
clearly as a teaching assistant and also your brimming passion and curiosity for science which was contagious. I hope that I can repay what you taught me in values and science to future students.

To Lindsey Muraskin, it was serendipitous that you were able to join the lab during your high school summer internship. Thank you for allowing me to teach you, and for being a receptive student with a drive to understand and learn. I truly appreciated watching you grow in experimental processes, confidence, and communication; it added great value to my graduate career.

I owe an overwhelming thank you to my parents, Jim and Deb Wehde, for allowing me to be independent, pursue creative outlets through art, grow in determination and perseverance through sport, and for instilling authentic work ethic by raising me to appreciate honest work and effort. Thank you for your love and support through the years; I can never thank you enough.

And to Nathan, my husband; you have encouraged me throughout my entire graduate career, helping me through disappointments and celebrating successes. Your heart and personality are uplifting every day, and I am beyond words for your continued love and motivation. This has been a journey. I thank you for helping me develop my scientific communication, talking over ideas and better avenues for explaining abstract science to others. From one adventure to another, thank you for your support.
Chapter 1
Introduction
1.1. **Structure and Function of Janus Kinases and STATs**

1.1.1. **Activation of JAKs and STATs**

Mammalian systems have an abundance of growth factors, peptide hormones, and cytokines, and rely on these ligands to initiate and promote physiological changes such as growth, proliferation, or conversely, apoptosis. Signaling of these extracellular ligands through their corresponding receptors rely on Janus Kinases (JAKs) to activate downstream Signal Transducers and Activators of Transcription (STATs). Upon ligand binding, the corresponding receptor will recruit membrane receptor-associated Janus kinases which then autophosphorylate at tyrosine residues and become activated. Activated JAKs will provide phosphorylation on tyrosine sites of their associated receptor. These activated tyrosine sites provide a docking site for intracellular signal transducers and activators of transcription (STATs). Following recruitment to the receptor, STATs will be activated by JAKs through phosphorylation at tyrosine residues. On the STAT protein, the modification promotes binding sites of Src Homology 2 domains of subsequent STAT proteins. Binding produces a stable homo- or heterodimerization of the STATs, allowing the transcription factors to undergo translocation to the nucleus, where they bind DNA and affect transcription activity.

While there are four members of the JAK family are JAK1, JAK2, JAK3, and TYK2, each are able to phosphorylate select STATs of the seven-member family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). The crucial determinate to exploring which JAK that will activate a STAT protein is likely context-dependent, first taking into consideration the tissue and initiating ligand.
Fig. 1.1.

1. Unbound receptor

2. Activated JAKs

3. Active, Dimerized STATs

4. DNA binding, Activating transcription

Ligand

JAK

JAK

JAK

JAK

PIAS

PTPs

SOCS

STATs

Nucleus
Figure 1.1. The JAK/STAT signaling pathway. The activation of the JAK/STAT signal transduction pathway begins with 1) the unbound receptor that binds with its corresponding ligand. 2) The binding induces a conformation change, recruits a Janus kinase that will auto-phosphorylate and create docking sites for the unbound STAT proteins. 3) The phosphorylated, and now active STATs dimerize and translocate to the nucleus where they recognize and bind GAS consensus sequences, initiating transcription of target genes. Among the regulators within the pathway are suppressors of cytokine signaling (SOCS) which inhibit active JAKs, protein tyrosine phosphatases (PTPs) that function to dephosphorylate JAKs as well as STATs, and protein inhibitors of activated STATs (PIAS), which can inhibit the translocation of STATs to the nucleus.
1.1.2 Structure and function of Janus kinases

Janus kinases are comprised of seven domains and are described by homology between the Janus kinases, referred to as Jak homology (JH) domains. Their hallmark characteristics are the tandem kinase domains, discovered in 1989 by Wilks et al., during the initial cloning [1, 2]. When it was first discovered, the kinase was named JAK, for ‘Just Another Kinase’, until it was found to have the two kinase domains, with one lacking enzymatic activity. It was aptly renamed ‘Janus kinase’, modeling the Roman two-faced god of the gates and doorways [3-6]. The first domain at the C-terminus is JH1 and is the catalytically active site. The second domain, JH2 is the catalytically inactive, pseudokinase domain, which could serve as a negative regulator for the catalytic domain, substrate localization, or supply a structural role in supporting the catalytic JH1 domain [7, 8]. The family also shares five other homology domains, directed from C to N-terminus. JH3 holds the src-homolgy 2 (SH2) domain, and little is known about its function. The remaining region, JH4-7, contains the four-point-one, ezrin, radixin, moesin (FERM) domain. The FERM domain is thought to coordinate with the cytoplasmic portion of the cell receptor, as a study explored the truncated amino-terminus of JAK2 and observed the kinase lost its ability to associate with the growth hormone receptor [9] (Fig. 1.2. Top). The resulting protein ranges from 120-140 kDa in size.

1.1.3 Developmental Analysis: Knockout models of the Janus kinases

To understand the biological implications in development of the Janus kinase family, knockout models have been generated. Conventional deletion refers to the target gene being inactivated in all cells of the organism. Conditional models are an elegant tool used to not only bypass embryonic lethality that is commonly seen in conventional models, but also an effective way to excise genes in a temporal and spatial manner.
Knockout models have been generated to address the role of several Janus kinases in development. Janus kinase 2 was found to have the most severe phenotype, embryos died in utero near embryonic day 12.5, due to inadequate erythropoiesis [10, 11]. To circumvent the lethality during gestation, a conditional \textit{Jak2} mouse model was generated, and in concordance with observations made from the germ line knockout, \textit{JAK2} has been shown to mediate the signaling of growth hormone (GH), erythropoietin, as well as prolactin [12-15]. Additionally, conditional knockout models describe integral function of \textit{JAK2} in mediating signaling in gonadotropin-releasing neurons [16]. The commonality of the ligands is that they are associated with single-chain receptors.

\textit{Janus kinase 3} is mainly restricted to the hematopoietic compartment, associating with the common gamma chain of interleukin (IL) receptor-2, IL-4, IL-7, IL-9, and IL-15 [17, 18]. Deletion of \textit{Jak3} was not lethal, and mice were viable and fertile, although when \textit{Jak3}-deficient mice were analyzed, the role of \textit{JAK3} and gamma chain receptors was confirmed, as mutant mice were found to have a thymocyte phenotype as ablation of \textit{Jak3} resulted in severe lymphopenia, a decrease of the B and T cells [19].

\textit{Janus kinase 1} knockout mice have been generated, and mutant mice lacking the tyrosine kinase die shortly after birth due to neurologic defects and its effects had not been addressed in adult tissue. In the 1998 study, analysis of collected cardiomyocytes, macrophages, and embryonic fibroblasts demonstrate that \textit{Jak1} may be responsible for cytokines such as IL-2, IL-6, LIF, and IFN [20]. In order to address the response of cells from these ligands in postnatal development, a conditional knockout would need to be generated.

The fourth member of the \textit{JAK} family, and first to be identified during a search for tyrosine kinases, is \textit{Tyrosine kinase 2} (\textit{TYK2}) [21, 22]. The knockout revealed a partial dependency of this \textit{JAK} family member for the response of IFN α/β and gamma, as well as IL-12. The phenotypic role was described by Karaghiosoff et al. to have a
predominate effect in the immune response, specifically in select antiviral mechanisms [23]. In contrast to the other JAK family members, TYK2 had redundant role in mediating receptor complex activation, although it had a crucial role in innate and adaptive immune responses.

1.1.4 Structure of STATs

The STAT proteins carry two roles in the cascade of signaling events, the first being signal transduction, carrying the signal after becoming activated by a Janus kinase and the second is activating transcription. STATs perform the later by translocating to the nucleus as a dimer and bind to recognition sequences on the DNA, the gamma interferon activation sites (GAS), recognizing consensus sequences, TTCNNNGAA. STATs are composed of five domains: the amino-terminus, DNA-binding, SH3-like, SH-2, and the carboxy-terminus transactivating domain (Fig. 1.2. Lower). Both the amino-terminus and the DNA-binding domains are highly conserved between the STAT members. The DNA-binding domain will interact with the DNA. The C-terminal activating domain is also conserved among the STAT proteins. The tyrosine in this domain, once phosphorylated allows for the subsequent dimerization at the SH2 domain, mediating the binding partners (other STAT proteins). Dimerization occurs as homo dimers between STATs or as heterodimers. Whether a one dimerization occurs over another is context and STAT-dependent, as some STATs are exclusive for only forming one or the other and with specific STATs. Such as, STAT1 and STAT3 can heterodimerize with one another, and STAT2 will only form a heterodimer with STAT1, and but will not form a homodimer [24]. Serine phosphorylation has been reported in the case of STAT1 and STAT3, at S727 at the C-terminus (Wen 1995) Zhang 1995), and may enhance the tyrosine phosphorylation, although the two activation events may be independent of each other.
Fig. 1.2.

Structure of the Janus kinase

Structure of the STAT
Figure 1.2. Structure of the Janus kinase and STAT protein. The Janus kinase (top) is comprised of seven JAK homology domains (JH), regions that share similarity between the four family members. JH1 serves as the catalytically active kinase domain, followed by the JH2, pseudokinase domain. The amino-terminal FERM domain interacts with the cytoplasmic portion of the receptor. The STAT protein (lower) contains the domains necessary for protein-protein interaction, the amino-terminus domain and the coiled-coil domain. The DNA-binding domain recognizes consensus sequences in the DNA. The SH2 domain is responsible for the tyrosine phosphorylation at the receptor and recruitment with other STAT proteins.
1.1.5 Developmental Analysis: Knockout models of the STAT proteins

STAT1 deficiency is most apparent in IFN signaling, where STAT1 null mutants have decreased signaling efficiency in response to type1 and II IFNs [25, 26]. These knockout models produced a truncated protein with no activity (Mera, et al.) or an elimination of STAT1 protein (Durbin et al.). STAT1 knockout mice were viable, although extremely susceptible to viral infections. Conditional knockout models were later developed in 2010 [27], and later in 2011, which targeted exons 6-10, the DNA-binding domain, for deletion [28]. Using the myloid-specific promoter driving Cre recombinase, LysM-Cre, macrophages and neutrophils were analyzed and confirmed the unresponsiveness of cells to IFN signaling.

Park et al, generated the STAT2-deficient mouse and while they were viable, they were found to be deficient in IFN signaling and like the STAT1 knockout mice, also susceptible to viral infections [29].

A whole-body knockout of STAT3, generated by deletion of the SH2 domain and tyrosine residue, was reported to be embryonic lethal [30]. The 1997 by Takeda et al., saw degeneration of embryos as gestation day 6. Since then, tissue-specific STAT3 knockouts in T-cell, macrophages and neutrophils, keratinocytes, and the mammary gland have been analyzed [31]. STAT3-deficient T cells displayed impaired proliferation due to an impaired response to IL-6 signaling [32]. Macrophages and neutrophils lacking STAT3 had decreased IL-10 production, and experienced inflammatory response mediated by high levels of TNF-alpha, IFN-gamma, and IL-1 [33, 34]. Skin tissue was analyzed for the deletion of STAT3 [35], using the Keratin5-Cre promoter and was found to have decrease wound healing thought to be from the deceased motility of cells during epidermal regeneration, such as neovascularization and response to inflammation. On a closer look, the in vitro studies demonstrated that while proliferation of STAT3-deficient epidermal cells was unaffected, a migration assay of cells compared to wild type, a
knockout of STAT3 severely impaired the motility of cells. The function of STAT3 was addressed in the mammary gland by specifically deleting the transcription factor using the beta-lactoglobulin (BLG) promoter driving Cre recombinase expression [36]. The study was the first to describe that STAT3 played a prominent role in the remodeling of the gland after offspring weaning, by suppressing apoptosis.

STAT4 knockout mice had no observable phenotype and were viable, although studies have shown STAT4 to be highest in the spleen and testes [37]. STAT4 was demonstrated to mediate IL-12 signaling in both organs, specifically in the spleen STAT4 decreased the cytolytic activity of cytotoxic T cells, and a STAT4 deficiency phenocopied an IL-12 knockout [38, 39].

STAT5 has two isoforms, STAT5a and STAT5b and conventional models generated are viable [40, 41]. STAT5a knockout mice display a phenotype during pregnancy, where mammary gland development is impaired. STAT5b mice experienced growth-retardation caused by a decreased response to growth hormone (GH) [40]. Conditional models have addressed the role of STAT5 in the hematopoietic system and mammary gland development. The role of STAT5 in the context of stem cell transplants provided evidence that total STAT5 inhibition could promote tissue engraftment [42].

Similar to the STAT4 model, mice deficient in STAT6 did not display a noticeable phenotype, but were responsible for the select ligand signaling of IL-4 [4, 43]. Mice lacking STAT6 lacked efficient class switching and full upregulation of B cells.

The use of in vivo models opens the possibility to depict the full biological process and roles of select genes and their proteins. Genetically engineered mouse models allow for describing the role of the gene in development using conventional whole-body knockouts as well as its function in select tissue using tissue-specific gene deletions.
Table 1.1. Phenotypes: Murine models of *jak* knockouts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Knockout: Conventional/Conditional</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyk2</td>
<td>Conventional</td>
<td>Viable, impaired antiviral immune response.</td>
<td>Karaghiosoff et al</td>
</tr>
<tr>
<td></td>
<td>Conditional, mammary gland</td>
<td>Impaired alveologenesis</td>
<td>Wagner, et al. 2004</td>
</tr>
<tr>
<td><em>Jak2</em></td>
<td>Conditional, neuroendocrine</td>
<td>Reduced fertility, impaired signaling involving gonadotropin releasing hormone</td>
<td>Wu, et al. 2012</td>
</tr>
<tr>
<td></td>
<td>Conditional, adipocytes</td>
<td>Reduced lipolysis</td>
<td>Nordstrom, et al. 2013</td>
</tr>
<tr>
<td><em>Jak3</em></td>
<td>Conventional</td>
<td>Viable, severe lymphopenia</td>
<td>Nosaka, et al. 1995</td>
</tr>
</tbody>
</table>

Table 1.1. Knockout murine models for Janus kinase family members. Knockout models have been generated and phenotypes observed in whole body knockouts (conventional) or in a tissue specific manner (conditional).
Table 1.2. Phenotypes: Murine models of Stat knockouts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Knockout: Conditional/Conventional</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conditional, Mammary gland</td>
<td>Viable, increased tumor burden</td>
<td>Kloever, et al. 2010</td>
</tr>
<tr>
<td>Stat2</td>
<td>Conventional</td>
<td>Viable, impaired IFN signaling</td>
<td>Park, et al.</td>
</tr>
<tr>
<td></td>
<td>Conditional, T-cells</td>
<td>Viable, impaired IL-6 signaling</td>
<td>Takeda, et al. 1998</td>
</tr>
<tr>
<td></td>
<td>Conditional, macrophages, neutrophils</td>
<td>Increased Th1 response</td>
<td>Takeda, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Conditional, keratinocytes</td>
<td>Viable, impaired wound healing</td>
<td>Sano, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Conditional, mammary gland</td>
<td>Viable, delayed mammary gland remodeling</td>
<td>Chapman, et al. 1999</td>
</tr>
<tr>
<td>Stat4</td>
<td>Conventional</td>
<td>Viable, fertile, impaired IL-12 signaling</td>
<td>Thierfelder, et al. 1996</td>
</tr>
<tr>
<td></td>
<td>Conventional, STAT5a</td>
<td>Viable, impaired alveologenesis and failure to lactate in female mice</td>
<td>Liu, et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Conditional, mammary gland</td>
<td>Impaired development. Loss of differentiated alveolar cells</td>
<td>Cui, et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Conventional, STAT5b</td>
<td>Dwarfism, deregulated liver dimorphism</td>
<td>Udy, et al. 1997</td>
</tr>
</tbody>
</table>

Table 1.2. Knockout murine models for STAT family members. Deletion of individual STATs during development as a whole-body knockout (conventional) or targeted to specific tissues (conditional) demonstrate the critical function the proteins have in signal transduction.
1.2. JAKs and STATs in Mammary Gland Biology

1.2.1 Overview of Mammary Gland Development

The postnatal mammary gland undergoes dramatic structural changes throughout its development cycle. The majority of growth is seen in the nascent gland at the time of birth. The most prolific growth occurs throughout puberty followed by changes associated with pregnancy, and also addresses the remodeling of the gland following offspring weaning. Throughout development, the organ experiences robust controlled growth, cellular proliferation, as well as massive orchestrated cell death.

This development cycle is tightly regulated by peptide hormones and cytokines, which elicit specific responses in the cells of the gland. Signaling of these extracellular ligands through their corresponding receptors rely on Janus Kinases (JAKs) to activate downstream Signal Transducers and Activators of Transcription (STATs). The JAK/STAT signaling pathway is crucial for processes such as growth, proliferation, and cell survival, but also for the breakdown and remodeling of the mammary gland after the cessation of lactation. Of the four JAK family members, (JAK1, JAK2, JAK3, and Tyk2) the predominately expressed Janus kinases in the mammary gland are JAK1 and JAK2. Currently, both kinases are thought to have redundant functions in the activation of selected STAT proteins. However, studies have shown that JAK2 is exclusively required for the activation of STAT5. JAK2/STAT5 then drives growth and functional differentiation of the gland in response to growth hormone and prolactin. In contrast to the general paradigm of redundancy between the two kinases, JAK2 may be dispensable during the remodeling process. The mammary gland experiences rapid decrease in circulating growth hormones and a rise in IL-6 class inflammatory cytokines (e.g. LIF and OSM) in the transition from lactation to involution. These inflammatory
cytokines are mediated by a Janus kinase to activate STAT3, which promotes genes involved in matrix remodeling and apoptosis.

### 1.2.2 Postnatal Mammary Gland Development

Development of the murine mammary gland begins as early as embryonic day 10.5, when two milk lines begin to appear. Derived from the ectoderm, five symmetrical placodes form along each milk line at embryonic day 11.5 [44]. These placodes grow into the epidermis near day 14 of development to initiate the characteristic epithelial buds. This establishes the epithelial mesenchyme and elongation of the bud protrudes into the underlying fat pad, producing a small ductal tree[45]. The continued development occurs after birth.

In females, the architecture of the ductal tree remains constant until approximately 4 weeks of age, estrogen rises during puberty and growth in the mammary gland is again stimulated. Proliferation occurs by ductal branching from the terminal end buds. The repeated estrous cycles promote branched network of ducts and will continue growth to fill the mammary fat pad until 10 weeks of age. At this stage the mature virgin mammary gland will stop growing, until prompted by a surge of growth hormones induced by pregnancy.

The mammary gland is composed of many cell types. Adipose, immune cells, and fibroblasts are found in the stroma, while a bilayer of cells composes the alveolar and ductal epithelium. The bilayer consists of myoepithelial and luminal cells. Connecting to the basement membrane, myoepithelial cells are basally located while luminal cells are oriented apically. The luminal cells will function to secrete milk during pregnancy.

Pregnancy induces a large, regulated proliferation in the ductal network, this time as tertiary structures that terminate in alveolar buds. The alveolar buds give rise to the
robust growth of the differentiated luminal epithelial cells to form alveoli. These cells will secrete milk during lactation, which will empty into the ducts to feed offspring. The alveolar cells are maintained throughout lactation, but when offspring are weaned the terminally differentiated luminal cells are no longer needed. The differentiated alveolar cells undergo cell death as the entire gland shifts into the next phase of development, known as postlactational remodeling.

The remodeling event, also termed involution, is a dramatic example of orchestrated cell death. The apoptosis is necessary for the clearance of differentiated alveolar cells. The process of involution proceeds in two steps. The first reversible stage being massive programmed cell death of the differentiated alveolar cells. The second is irreversible, as organ undergoes architectural restructuring as the gland returns to its pre-pregnancy state. This stage involves breakdown of the basement membrane and clearance of the apoptotic cells. The once-maintained epithelium regresses and is replaced by adipose tissue. This reset prepares the gland for subsequent pregnancies.

1.2.3 Mammary Gland Development is Orchestrated by Hormones and Locally Produced Cytokines

The postnatal development of the mammary gland is regulated by growth factors, hormones, and locally derived cytokines. Initially, the gland responds to long-range endocrine hormones produced by the ovaries for the formation and elongation of ducts. Estrogen and progesterone mediate the ductal branching at the onset of puberty and during estrous cycles for the development into mature virgin glands. Peptide factors such as FGF and IGF have also been shown to promote ductal outgrowth and lobuloaveolar development[46, 47]. The growth factor amphiregulin, transcriptionally regulated by ERα acts through EGF receptors to also promote ductal growth [48]. The known pregnancy hormone, prolactin, is the prominent driver for cell proliferation and
survival of the secretory alveolar cells during pregnancy and the maintenance of the epithelium during lactation.

When offspring are weaned, the secretory alveolar cells need to be removed, shifting survival signals to apoptosis. Locally derived growth factors facilitate the involution process. As circulating levels of prolactin decline, IL-6 class inflammatory cytokines rise. These inflammatory cytokines include, Oncostatin M (OSM) and leukemia inhibitory factor (LIF), although, IL-6 itself does not play a role in signaling during involution [49]. In addition, soluble factors and members of the interleukin family (IL-1α, IL-1β, and IL-13) [50, 51], and TGF-β3 are present [52]. The secretion of TNFα also signals for the recruitment of macrophages, aiding in the clearance of cell debris.

Although they are contrasting molecular events, the signals of lactation and involution, together with development of the organ as a whole, share the general signal transduction pathway.

1.2.4 Peptide hormones and locally synthesized cytokines that are essential for MG development utilize particular JAK/STAT pathways for intracellular signaling

Circulating hormones and growth factors initiate their effects in the mammary gland by binding to their corresponding receptors, and as most cytokine receptors lack intrinsic kinase activity, they recruit a Janus kinase to become active. The active JAK will autophosphorylate and create binding site for Signal Transducers and Activators of Transcription (STATs). The STAT proteins will become activated by phosphorylation, dimerize, and translocate to the nucleus to bind to the DNA, promoting gene transcription. STATs may promote cell survival or cell death signals, depending on the upstream hormone or cytokine signal and activating Janus kinase.

The JAK family is comprised of four members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). Primarily restricted to the hematopoietic system, JAK3 and TYK2 are
necessary for immune function, while JAK1 and 2 are more ubiquitously expressed[53]. In the mammary gland, JAK1 and JAK2 are recognized as being the prominent Janus kinases, mediating signaling of cytokines to activate downstream STATs by tyrosine phosphorylation. There are seven members in the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. In the mammary gland, all are expressed with the exception of STAT2, which is found in the immune system. The activation of the STAT proteins varies according to the stage of mammary gland development. Prompted by growth and proliferation signals during pregnancy and subsequent lactation, STAT5 is the main driver for genes associated with cellular differentiation and survival. STAT1 has been demonstrated to be active in the stroma to promote ductal branching [54]. IL-4 initiates activation of STAT6 and phenotypically, the early pregnant mammary glands of STAT6-deficient mice are shown to have impaired alveolar growth, although this was less apparent during late pregnancy [55]. The non-deleterious effects of STAT1 or STAT6 in the mammary gland suggest these transcription factors play subordinate roles in development. In contrast to cell-sustaining events, following lactation and the induction of mammary gland remodeling, STAT3 becomes the prominent transcription factor, inducing transcription of genes necessary to breakdown epithelial cells. The activity of the appropriate STAT protein in stages of mammary gland development is determined by specific growth hormones, cytokines, and a distinct Janus kinase.

In the case of prolactin (PRL), the pregnancy-associated hormone binds to the prolactin receptor (PRLR). The dependence of alveolar cell growth on this pathway is defined by PRLR-null mice, upon pregnancy, alveolar buds failed to proliferate in response to PRL [56, 57]. The receptor recruits Jak2 then activating STAT5 to promote the AKT/PI3K pathway, upregulating genes associated with proliferation, differentiation, and cell survival [58]. The association of JAK2/STAT5 and mammary epithelial cell differentiation is well documented by in vitro and animal studies. Mice deficient in STAT5
specifically in the mammary epithelium failed to produce alveolar buds and undergo proliferation in response to prolactin stimulation[59]. A conditional deletion of Jak2 [60] in the mammary gland parallels the STAT5 phenotype, as ligand binding fails to activate the receptor and subsequent lack of STAT5 activation. Both studies phenocopy a prolactin receptor-deficient model, such as the mammary glands that lack any of the previous demonstrate loss of alveolar bud formation[56].

1.2.5 Postlactational Remodeling and Inflammation

The remodeling event is a necessary phase of mammary gland development and is the most dramatic series of organized cell death in the organism. The purpose is to terminate the functionally differentiated cells utilized during pregnancy and lactation, allowing the gland to reorganize into a virgin-like state before another pregnancy takes place. Beginning shortly after the offspring are weaned, the remodeling proceeds in two phases. The first phase is characterized by the apoptosis of epithelial cells no longer needed for milk secretion. The second phase includes cell death and also the breakdown and remodeling of the gland, replacing alveolar cells with adipocytes[61].

Inflammatory cytokines are necessary to initiate each phase. As prolactin levels decrease, inflammatory cytokines, leukemia inhibitory factor (LIF) and oncostatin M (OSM) are locally produced. Common to the family members of IL-6 class inflammatory cytokines, OSM receptor (OSMR) and LIF receptor (LIFR) require a GP130 subunit to be activated by a Janus kinase[62]. The phosphorylation by the Janus kinase allows the signal to be relayed from the plasma membrane to the nucleus via activated STAT proteins. These transcription factors will to bind to or complex with other transcription factors to drive transcription of genes necessary for cell death machinery and matrix remodeling. Given the commonality in the IL-6 signal transduction, the key convergence
at which a specific Janus kinase activates the receptor may represent a mechanism to specifically target the inflammatory pathway.

The prominent signal transducer and activator of transcription necessary for mammary gland remodeling is STAT3. Conventional knockout mice identified that a STAT3 deficiency proved embryonic lethal [30]. Bypassing embryonic lethality, conditional deletion of STAT3 within the mammary gland verified STAT3 activity in remodeling [36]. Proceeding studies identified that loss of STAT3 and its tyrosine phosphorylation domain inhibited remodeling [63]. In fact, a loss of STAT3 is two-fold, as it extends the functionality of the gland and delays involution [64]. Previous work has described the integral function of LIF for the initial activation of STAT3, in the first stage of remodeling, as LIF-deficient mice have an absence of phosphorylated STAT3 and delayed mammary gland remodeling [65, 66]. Delayed remodeling is also seen in gp130-deficient mice [67]. This initial activation of STAT3 transcriptionally upregulates LIF receptor (LIFR), along with STAT3, and oncostatin m receptor (OSMR) creating a feed-forward loop, perpetuating the activation of STAT3 by OSM, as LIF levels diminish leading into the second phase of remodeling [36]. Extensive tissue remodeling coupled with removal of the apoptotic cells in the second, irreversible phase concludes involution and the gland is prepared for a subsequent pregnancy.
1.3. Jak and Stat in Disease

1.3.1 Roles of Inflammatory Cytokine Signaling in Cancer

As one of the hallmarks of cancer, inflammation has long been associated with cancer progression and aggressive disease [68, 69]. Categorically separated into acute and chronic, inflammation attributing to disease is typically the later, as perpetual exposure of the inflammatory milieu may induce plasticity in epithelial cells. At the site of chronic inflammation, increases in immune cells, primarily macrophages, and cytokines are common. As chemical messengers, cytokines associated with inflammation can induce tissue repair and remodeling. Prevalent cytokines include tumor necrosis factor (TNF) and interleukin family members such as IL-6, IL-10, and IL-21. Cytokine signals from the environment as well as from cancer cells themselves illicit the activation primary cytokines and transcription factors such as nuclear factor kappaB and STAT3. Important for cell growth, STAT3 has been implicated in mediating the transformation of both human and murine cells, contributing to cancer, particularly when constitutively active [70]. Whereas normal cells have tight regulation on STAT3 activation, cancer cells have unrestricted STAT3 activity, fueling cell growth and metastatic potential. In a model of human prostate cells expressing a constitutively active STAT3, it was shown that STAT3 had the capability to transform cells and modify the actin cytoskeleton thereby promoting migration [71]. Epithelial-Mesenchymal Transition STAT3 has also found a role in stem cells, as studies have linked its activity in regulating tumor-initiating cells [72-74].

The cytokine interleukin 6 (IL-6) has been described to have many functions throughout the body. Functioning from hematopoiesis to the endocrine system the cell types that respond to IL-6 range from macrophages to cells of the heart, liver, and ovary [75]. In inflammatory hepatocellular adenoma, it was demonstrated that Representative
of the many roles for IL-6 within the body, it's apparent in a disease setting that the presence of IL-6 is not the issue but rather the persistent production of the cytokine. Likewise, the increased or sustained inflammatory signaling perpetuates STAT3 activity, such as the case in inflammatory hepatocellular adenoma and hepatocellular carcinoma [76, 77]. Cheng et al., demonstrated IL-6 promoted tumor growth and metastasis in a STAT3-dependant manner in a murine model of breast cancer [78].

The importance of knowing the biological function of Janus kinases is crucial when targeting these kinases in disease. Mouse models of breast cancer clearly demonstrate that inhibiting JAK2 prior to disease onset results in substantial decrease of tumor formation[79]. This implies that JAK2 is a rational preventive therapy in human malignancy. However, JAK2 is dispensable not only for tumor growth in established disease but also for STAT3 tyrosine phosphorylation, clearly indicating that inhibiting STAT3 requires an alternative target [80]. Persistent activation of STAT3 downstream of IL-6 signaling is well documented in disease progression. While there are attempts at inhibiting STAT3, by targeting the SH2 domain or DNA-binding domain, directly inhibiting STAT3 remains difficult, as it is a latent transcription factor until activated [81]. Perhaps an efficient strategy to limit the DNA-binding activity of the transcription factor is distinguishing its activator in specific tissues and malignancies.

1.4. Generation of a Jak-1 Conditional knockout Model

1.4.1. Rationale for Pursuing the Generation of a Conditional Knockout

Since 1998, no published attempts have been made to analyze Janus kinase 1 in postnatal development via knockout. In the seminal study by Schreiber, the first two coding exons of Jak1 were replaced by a neomycin cassette, rendering a disrupted allele [20]. This disruption produced a null protein in both a heterozygous cross as well
as a homozygous breeding. This conventional, whole-body knockout resulted in offspring that experienced perinatal lethality. Thus, the functional role of Janus kinase in adult tissue was unable to be performed. What was observed was momentous, in a way, as the study pointed to a non-redundant biological role for Janus kinase 1, in mediating the signaling of several subfamilies of cytokine receptors (class II cytokine receptors: IFNα/β, IFNγ, and IL-10, the yc receptor: IL-2, IL-4, IL-9, and the gp130 receptor subunit class: IL-6, OSM, LIF). The observations and conclusions were made from assessing Jak1 wild type and Jak1-deficient macrophages, embryonic fibroblasts, and cardiomyocytes.

As JAK1 had been implicated to be central in the signaling node for several cytokine receptors, it was left to question whether the same signaling cascades would be mediated in a similar way in vivo in postnatal development. To address the biological function of JAK1 in postnatal development, a spatial and temporal model to delete Jak1 would need to be designed.

1.4.2. Construct Design

Detailed accounts of generating the conditional knockout mouse model can be found in the 2016 publication by Sakamoto, et al [82]. The targeting vector was generated using bacterial artificial chromosome (BAC) recombineering as described previously by Sakamoto, et al, 2014 [83]. Director of the UNMC Mouse Genome Engineering Core Facility, Dr. Channabasavaiah Gurumurthy provided valuable assistance to Dr. Sakamoto in the generation of the targeting vector. To construct a conditional knockout allele of Jak1 gene, homologous recombination was used to insert loxP sites around exon 2 (floxed allele), and was generated in mouse embryonic stem (ES) cells at the UNMC Mouse Genome Engineering Core Facility. Thirteen correctly targeted ES cell clones were identified and two were injected into C57Bl/6 blastocysts.
Chimeric animals were produced and germ line transmission of the \( \text{Jak}1^{\text{fl/fl}} \) allele was achieved. Further breeding of heterozygous mice identified offspring with both \( \text{Jak}1 \) floxed alleles (homozygous \( \text{Jak}1^{\text{fl/fl}} \) mice). Mice carrying both mutant alleles developed normally, regardless of gender and were fertile.

1.4.3. Breeding Scheme and Phenotypic Characteristics

Recombination occurs when Cre recombinase is present. The recombinase will specifically recognize the loxP sites and will excise the DNA fragment between two directly oriented loxP sites. From the \( \text{Jak}1^{\text{fl/fl}} \) construct design, recombination would delete exon 2, causing a frameshift of the coding sequence, resulting in an absence of protein. To analyze whether our recently generated \( \text{Jak}1 \) conditional knockout mouse would phenocopy that of the previously published conventional knockout, the floxed allele of \( \text{Jak}1 \) was transmitted in the germline of MMTV-Cre mice (line A) [84], as the developing oocytes express active Cre recombinase [85]. Transgenic female mice carrying the MMTV-Cre and \( \text{Jak}1^{\text{fl/wt}} \) genes were bred, activating the Cre recombinase in the oocyte and thereby deleting one allele of \( \text{Jak}1 \). The progeny were screened by PCR and observed that \( \text{Jak}1^{\text{wt/}} \) mice developed normally and were fertile. These observations support the 1998 study of the conventional \( \text{Jak}1 \) knockout, where one allele deficiency did not promote a dominant-negative phenotype. To generate \( \text{Jak}1 \)-null offspring, \( \text{Jak}1 \) heterozygous mice (\( \text{Jak}1^{\text{+/h}} \)) were crossed. To rule out early embryonic lethality, fetuses at embryonic day 12.5 through 18.5 were collected. In total, 43 embryos were collected and were near an expected Mendelian ratio, as 9 (21%) of the embryos were \( \text{Jak}1 \)-deficient, 22 (51%) were heterozygous, and 12 (28%) were wild type. In our observation of the embryos at day 12.5, homozygous null, heterozygous, and wild type were indistinguishable from one another. Although, at embryonic day 15.5, 17.5, and 18.5, the \( \text{Jak}1 \)-deficient fetuses were noticeably smaller in size compared to wild type embryos.
While observing Jak1 knockout offspring, pups survived birth and were able to move similar to wild type pups, but had noticeably more difficult time breathing, with longer pauses or gasps similar to apnea. Knockout mice perished 30 minutes to 2 hours after birth. Similar to Rodig et al., the newly generated Jak1 knockout phenotypically mimics the reported conventional deletion.

1.4.4. Molecular Characteristics of a Jak1-deficiency

To confirm the deletion of Jak1 on the molecular level, PCR was used to identify wild-type or null alleles in developing embryos, and murine embryonic fibroblasts were derived from embryonic day 12.5 for primary and immortalized cell cultures. Cells were derived from Jak1 wild-type, heterozygous and knockout embryos for biochemical analysis (see Materials and Methods: Generation of Murine Embryonic Fibroblasts). The MEFs deficient in Jak1 were not exclusively dependent on the kinase for survival in 2D culture, as they grew and proliferated at the same rate as the wild type. Immunoblotting was performed on the MEF cells, and the Jak1 deletion was confirmed in the homozygous null fibroblasts, confirming that the deletion of exon 2 resulted in the absence of protein [86]. As well, a minor reduction in protein was seen in the heterozygous cells. The total levels of JAK2 remained constant, although interestingly, at the steady-state level, the cells exhibited a loss of activated STAT3, as detected by Western blot. The loss of tyrosine (T705) phosphorylation was evident, although serine phosphorylation (S737) was unchanged, suggesting that a Jak1-independent mechanism is needed for serine activation. The predominant modification is tyrosine phosphorylation for STATs to localize to the nucleus, and it has been proposed that serine and tyrosine activation are not interdependent. In analyzing other members of the STAT family, the activation of STAT1 and STAT6 were also ablated in the absence of Jak1.
1.4.5 Conclusions from Generation of the Janus kinase Knockout model

In conclusion, the generation of the conditional deletion of Jak1 mirrors that of the previously reported conventional, whole body knockout of Jak1 [20]. The recently generated data from Sakamoto et al., 2016, suggests that this kinase is necessary for the tyrosine activation of three members of the STAT family, as analyzed by in vitro data.
1.5. Conclusion and Dissertation Hypothesis

The JAK/STAT pathway has many functions in development and tissue homeostasis, yet also contributes to disease progression and maintenance, and thus has been a target in cancer. Specifically in breast cancer, the premise that STATs can contribute to cancer initiation and survival is well-studied, but the concise identification and the biological function of their upstream activators has been less explored.

Through prior exploration of JAK/STAT signaling in the mammary gland, it is evident that the pathway is crucial not only for processes such as growth, proliferation, and cell survival, but also for the breakdown and remodeling of the gland via IL-6 class inflammatory cytokines (e.g. LIF and OSM). The two members of the Janus kinase family that are expressed within the mammary epithelium, JAK1 and JAK2, have generally been thought to have redundant functions in STAT activation. This is due, in part, to the emphasis on JAK2 in mammary studies and also the lack of data and appropriate genetic tool to investigate the postnatal role of JAK1 in development. We have demonstrated that JAK2 is exclusively required for the activation of STAT5, promoting growth of the gland and also contributing to alveolar proliferation [60]. The involution events have characterized STAT3 as the prominent STAT protein driving expression of genes necessary for remodeling the gland [36]. Still, the upstream kinase responsible for STAT3 activation is unclear, as both JAK1 and JAK2 are thought to mediate the inflammatory cytokines. In contrast to the general paradigm, JAK2 may be dispensable for involution events and mediating IL-6 class inflammatory cytokines, and resulting activation of STAT3. Supporting this premise, we have observed a persistent activation of STAT3 in mammary tumor cell lines that are genetically deficient in Jak2 [80], indicating that another Janus kinase may be responsible for STAT3 signaling. Therefore, we hypothesize that the Jak1 signaling cascade is an important factor in inflammatory cytokine signaling and has a role in postlactational remodeling of the
normal mammary gland and as inflammatory cytokines play a significant role in cancer development, Jak1 may contribute to neoplastic transformation and malignant progression. To approach these questions, we will first determine whether ablation of Jak1 affects normal mammary gland remodeling. Second, we will analyze the importance of Jak1 signaling in mammary carcinogenesis.

The purpose of this work is to identify the molecular contribution of JAK1 to STAT activation, characterize the function and biological relevance of JAK1 in adult mammary tissue, and identify its implications in breast cancer.
Chapter 2: Materials & Methods

Materials & Methods
Mouse Colonies and Strains Used

The use of rodents for studies in this body of work is approved by the Institute for Animal Care and Use Committee (IACUC). Mouse colonies were maintained according to IACUC protocol and standards, and monitored by UNMC Comparative Medicine. Animals were treated fairly and humanely. Premature euthanasia was employed when any signs of distress appeared in mice during the course of the studies such as impaired movement, pain, and lack or eating or drinking. Mice were first euthanized by applying anesthesia by CO2 followed by cervical dislocation, as recommended by American Veterinary Medical Association.

The MMTV-Cre strain was used to target recombination events specifically to the mammary gland. The Jak1\textsuperscript{fl/fl} strain was recently generated and used as a littlemate control to double transgenic experimental mice MMTV-Cre Jak1\textsuperscript{fl/fl}.

Tumor studies utilized the MMTV-neu strain, amplifying the neu oncogene in a mammary-specific manner. The CAG-lox-CAT-lox-GFP model was utilized as a visual aid for recombination events. Athymic nude, FoxN1\textsuperscript{nu/nu}, from National Cancer Institute were used for orthotopic transplant studies.

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Background</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>MMTV-Cre (A line)</td>
<td>FVB</td>
<td>Wagner et al., 1997</td>
</tr>
<tr>
<td>MMTV-neu</td>
<td>FVB</td>
<td>Muller et al., 1988</td>
</tr>
<tr>
<td>Jak1\textsuperscript{fl/fl}</td>
<td>FVB</td>
<td>Sakamoto et al., 2016</td>
</tr>
<tr>
<td>CAG-lox-CAT-lox-GFP</td>
<td>FVB</td>
<td>Kawamoto et al., 2000</td>
</tr>
<tr>
<td>NCr-Foxn1\textsuperscript{nu}</td>
<td>NCr</td>
<td>National Cancer Institute</td>
</tr>
</tbody>
</table>

Whole mount analysis

The thoracic (referred to as number three mammary glands) or the abdominal mammary (referred to a number four mammary glands) glands were dissected out and
spread onto a glass microscope slide (3" x 1" x 1mm, Fisher Scientific) and placed in Carnoy’s fixative solution for at least 1 hour or up to three days. Carnoy’s fixative: 6 parts 100% EtOH, 3 parts CHCL3, and 1 part glacial acetic acid. Fixative was kept in 50mL Falcon tubes, where the tissue on glass slide was placed. Fixative reused. The mammary gland on glass slide was then washed in 70% EtOH for 15 minutes and gradually changed to distilled water. Gland was rinsed in distilled water for 5 minutes. The gland was then placed in Carmine Alum Stain in 4°C overnight or for up to one week (depending on stain penetration). Carmine Alum Stain: 1 g Carmine (Sigma C1022) and 2.5 g aluminum potassium sulfate (Sigma A7167) in 500 mL distilled water and boiled for 20 minutes. Final volume was adjusted to 500 mL with distilled water. Carmine was filtered and a crystal of thymol was added as a preservative. Completed stain was stored at 4°C and used for several months. After Carmine Alum stain, gland was washed in gradients of EtOH for 15 minutes each (70%, 95%, and 100%). Glands were transferred to xylene for 6 hours or up to three days and then mounted with Permount mounting medium (Fischer Scientific, SP15-100). Whole mounts were visualized using the Zeiss Stereo microscope (Carl Zeiss).

**Extracting DNA**

Tissue was gathered for genotyping by snipping 0.5cm from end of mouse tail (typically 4 weeks of age) and digested overnight at 56°C in a Digestion Buffer solution. Digestion buffer was comprised of Tail Buffer: (for 500mL buffer) 1% SDS (50mL of 10% solution), 50nM TrisHCl pH8 (25mL of 1M solution), 100nM NaCl (10mL of 5M solution), and 50 mM EdTA (50mL of 0.5M solution) and Proteinase K (PK), 10mg/ml. For each sample, 500 uL of Tail buffer and 20 uL of PK were used. DNA was extracted by mechanical means, using the AutoGenprep2000 (all reagents used proprietary to AutoGen and contain phenol, ethanol, I-Butanol 9%, Iso Amyl Alcohol 0.01%, and
isopropanol 40%) and maintained high levels of DNA yield and quality. In the event that the mechanical extraction was used, DNA was extracted by hand using the following protocol: Each sample (in 1.5mL tube) was suspended in Digestion Buffer (700 uL of Tail Buffer and 30 uL of PK) and digested overnight at 56°C. The samples were then briefly vortexed and spun down at 13,000 RPM for 20 minutes at room temperature. The supernatant was then poured off into new 1.5 mL tube and 600 uL Phenol added (Invitrogen, 15513-047). The tube was gently inverted 7 times to mix and centrifuged at 13,000 RPM for 20 minutes. The top layer (containing water and DNA) was pipetted off and placed into new 1.5 mL tube and 600 uL chloroform (Fisher BP1145-1) added, inverted 7 times to mix and centrifuged at 13,000 RPM for 20 minutes. The top layer was carefully pipetted off and placed into a new 1.5 mL tube and 1 mL of 100% EtOH and was added and the tube gently inverted 7 times to precipitate the DNA, and spun at 13,000 RPM for 20 minutes, The supernatant was poured off and the DNA pellet was dried by laying the tube on its side for 15 minutes. 100 uL TE (Fischer Scientific, BP2473-1) was added to resuspend the DNA.

**Genotyping**

Extracted genomic DNA was then analyzed by polymerase chain reaction (PCR) to verify the presence or absence of specific genetic modifications. A single PCR reaction was comprised as follows: A final reaction volume of 50uL was made of 32.5uL of ddH2O, 10uL of 5x PCR reaction buffer (Promega, M890A), 3uL MgCl2 (25mM, Promega, A351H), 1uL of dNTPs (10mM, Invitrogen), 0.5uL each of forward and reverse primer (made through IDT, 100pM), 0.5uL of 5U/uL Taq DNA polymerase (Promega, M829B), and 1uL of genomic DNA template. Primer sets utilized and PCR conditions in Table 2.2. PCR programs were run on a T3 Thermocycler (Biometra). PCR product run on a 0.8% agarose gel (BioExpress, E-3119-500), dissolved in 1xTAE buffer (Tris-
Acetate-EDTA) (Fisher Scientific, BP1332-20) containing ethidium bromide (Ameresco, X328-10ML). Gel was visualized using BioDoc-It Imaging System (UVP).

**Preparation of murine tissue for analysis**

**Histology**

Tissue was removed, cut several times to allow increased buffer penetration, and placed in 10% buffered formalin (Fisher Healthcare, 245-684) in a 20 mL scintillation vial for 24 hours. After 24 hours, the formalin was poured off and discarded in appropriate waste container and the tissue was resuspended in 70% EtOH for long-term storage. Tissue was prepared for paraffin embedding by placing tissue in tissue cassette and processing and embedding according to manufacturers directions using the Thermo Scientific Excelsior AS (an automated system that processes tissue starting from 70% EtOH, water, and progressing to dehydrant solution, Clearant solution, and wax). Tissue was embedded with the Thermo Scientific Histostar. The embedded tissue was sectioned using a microtome, cutting tissue 4 μM thick onto microscope slides (Fisher Scientific, 12-550-15). The slides were baked at 56°C overnight and stored in slide boxes.

**Hematoxylin & Eosin**

The staining protocol has been optimized to produce strong nuclear stain by hematoxylin and resilient cytoplasmic stain by eosin. Beginning with paraffin embedded tissue sections, deparaffinize in three changes of 100% Xylene (Histo-Clear II, National Diagnostics, HS-202) for 5 minutes each, followed by two 5 minute changes in 100% EtOH, and two changes each in 95% EtOH, 3 minutes each. Rinse briefly in distilled H2O and incubate slides in Hematoxylin (Fisher Healthcare, 220-100) for 20 minutes. Rinse slides in glass slide box briefly in running tap water, avoiding direct running water.
contact with the slides, until water is clear. Also be sure to use cold water, as warm water will fade staining. Dip slides into acid alcohol solution (1% HCl in 70% EtOH) followed by a brief rinse in tap water. Next, slowly dip slides into ammonia water solution (1 mL NH₄OH in 1L H₂O), here the sections should darken slightly. Rinse again in tap water, briefly, and then incubate in Eosin Y (Statlab, SL98-32) for 1-2 minutes dependent on desired intensity. Rinse briefly under tap water. Place slides into 95% EtOH for several seconds, here eosin will come off slightly. Move slides to 100% EtOH and incubate in two changes for 5 minutes each, to fully dehydrate slides before placing in 100% Xylene. Incubate in Xylene for 10 minutes to 1 hour and mount using Permount and coverslip. Slides were viewed and using a Zeiss Axio Imager microscope (Carl Zeiss) and images captured using SPOT Flex camera and SPOT 5.1 software (Diagnostic Instruments).

Immunofluorescence and Immunohistochemistry

Immunostaining on fixed-formalin paraffin embedded tissue

The protocol is designed for formalin-fixed paraffin embedded tissue, addressing immunofluorescence (IF). Tissues were deparaffinized in 100% Xylene (3 incubations for 10 minutes each) and rehydrated in decreasing concentration of EtOH (2 incubations in 100% for 3 minutes each, and one incubation of 3 minutes each of 95%, 90%, 70%, 50%, and 30%) finishing with a final 5 minute wash in 1xPBS (Fisher Scientific BP399-1, diluted 1:10 in ddH₂O) for 3 minutes. Slides went through antigen retrieval, using 938 uL of Antigen Unmasking Solution (Vectastain, H-3300) into 100 uL ddH₂O. Solution was pre-warmed first before adding to slides. 45mL of prepared Antigen Unmasking Solution were added to slides in 50 mL conical tubes, and heated (avoiding boil) for 20 minutes at a power level of 1. Slides were left to cool to room temperature in solution before rinsing in 1xPBS for 5 minutes. Slides were marked with hydrophobic pen, and tissue area
blocked with 3% BSA (1.5% BSA, Sigma, A9418-100G, powder, dissolved in 50mL of 1x PBS) for 1 hour. Slides were rinsed for five minutes in 1xPBS. Primary antibody added, diluted to desired concentration in blocking buffer. Only one section of the two on the slide received primary antibody, the other received only blocking buffer for background staining control. Primary incubation carried out overnight in a moist chamber at 4°C. The following day slides were rinsed three times in 1xPBS for 5 minutes each. Secondary antibody was applied to all tissue, diluted in blocking buffer, appropriate to primary antibody host and reporter fluorophore. Care was taken to perform tasks in low light to avoid diminishing secondary antibody fluorescence. Secondary antibody incubated in moist chamber for 1 hour, away from light, at room temperature. Slides washed twice in 1xPBS for 5 minutes each followed by one wash in ddH2O for 5 minutes to remove salts that may increase background staining. One drop of Vectasheild DAPI mounting media (Vector, H-1200) was added per tissue and coverslipped. Sealed with clear nail polish, and stored away from light at 4°C. Primary and secondary antibodies used for both immunofluorescence and IHC listed in Table 2.3. Slides were then visualized using Zeiss Axio Imager A1 (Carl Zeiss) and images taken using SPOT Imaging Software 5.1 2014.

To perform immunohistochemistry (IHC), all steps are similar to the IF protocol, from deparaffinization and through rehydration. After rehydration, background peroxidase was treated with a 30 minute incubation in a 3% H2O2 (Fisher, H325-500) solution in methanol. (EMD Millipore, MX0475-1). Slides were rinsed and then treated by antigen retrieval and blocked as in the IF protocol. Primary antibodies for IHC are listed in Table 2.3 and diluted in blocking buffer. Slides were incubated overnight in moist chamber in 4°C. The next day, biotinylated secondary antibody was prepared using the Vector ABC staining kit (Vector PK-6101, PK-6102), diluted 1:200 in blocking buffer. ABC reagent was prepared by adding 1 drop of each reagent A and B into 5 mL of
1xPBS, vortexed, and incubated at room temperature for 30 minutes. Slides were rinsed of secondary antibody in 1xPBS for 5 minutes and ABC reagent added and incubated for 30 minutes in a damp chamber at room temperature. Slides were rinsed in 1xPBS for 5 minutes while DAB reagent (3,3-diaminobenzadine, a peroxidase substrate) was prepared (Vector labs, SK-4100). Slides were dried slightly and DAB was added to slides and watched as staining intensity developed under light microscope. Reaction was terminated by placing slides in tap water. Slides were counterstained in hematoxylin for 10 seconds and rinsed with cold tap water. Slides were dehydrated in reverse process as the rehydration steps and mounted.

**IHC for pSTAT1**

The following protocol was used for the identification of pSTAT1 (Origene pY-STAT1, TA309955) in mouse tissue. Used IDetect™ Universal Mouse Kit-HRP (IDSTM003). IHC protocol was followed through from deparaffinization to rehydration. Next, slides were treated with detergent permeabilization, 0.02% Tween 20 in 1xPBS for 20 minutes, then washed in 1xPBS. Antigen retrieval is same as previously described IHC then cooled to room temperature for 1 hour. Slides were then treated for endogenous peroxidase with 3% H2O2 in methanol for 20 minutes, then washed 3 times in 1xPBS for 3 minutes each. Super Block (part of kit) added for 7 minutes, incubated room temperature after circling tissue with hydrophobic pen. Slides washed 3 times in 1xPBS 3 minutes each. Universal Mouse Block (part of kit) added to tissue, incubated for 1 hour, then washed 3 times in 1xPBS. Primary antibody was diluted 1:100 in 1% BSA in 1xPBS and incubated overnight at 4°C in moist chamber. Next day slides washed 3 times in 1xPBS and biotinylated secondary antibody, Anti-Polyvalent, added for 20 minutes (part of kit), incubated at room temperature. Washed 3 times in 1x PBS, then HRP (part of kit) added, incubated 20 minutes at room temperature. Washed 3
times in 1xPBS, then stained with DAB reagent (prepared as described previously) until reaction developed. Counterstain, dehydration, and mounted as described previously.

**Immunoblotting Protocol**

Tissue and cells were prepared for Western blot (WB) by resuspending in prepared complete lysis buffer. Complete lysis buffer, a mixture of RIPA buffer and proteinase inhibitors, is prepared as follows: RIPA buffer: (radioimmunoprecipitation lysis buffer, which will lyse cells and solubilize proteins, used for whole cell extracts and membrane bound proteins) contains: 0.1% SDS, a denaturing detergent, 0.5% sodium deoxycholate for protein/protein disruption, 1% Nonidet P-40, in 1xPBS, stored at 4°C. Proteinase inhibitors are as follows: Sodium orthovanadate (Sigma, S6508), PMSF (Stock solution of 10mg/mL in isopropanol): Leupepin (Midwest Scientific, J580-2, stock at 10mg/mL in ddH2O), Aprotinin (Sigma #A6279), and Sodium fluoride (Fisher Scientific, S299-100, stock solution 1M in ddH2O). Quantities of reagents to add for complete lysis buffer are as follows: For final volume of 500 uL, 471 uL of RIPA buffer, 9 uL of PMSF, and 5 uL each of aprotinin, leupeptin, sodium orthovanadate, and sodium fluoride were combined and vortexed.

Cell pellets were prepared for immunoblot by resuspending in 50-150 uL of complete lysis buffer in 1.5 mL tubes, on ice, and lysed using the Fisher Scientific Model 500 Sonic Dismembrator, amplitude set to 13 and pulsed for 3 seconds, twice, with resting 30 seconds on ice in between. Cells were placed on ice for 20 minutes followed by centrifugation at 13,000 RPM for 30 minutes at 4°C. The resulting supernatant was moved to a new 1.5 mL tube and quantified by Bradford reaction using BSA protein standard in Coomassie Plus Protein Assay Reagent (Thermo Scientific, 1856210) for standard curve. 25-75 ug of protein was prepared for analysis by adding 25 uL of Tris-Glycine SDS sample buffer (Novex, LC2676) and 1 uL of beta-mercaptoethanol (Fisher
Scientific, BP176-100) to each sample and boiled for 10 minutes at 100°C. Samples were placed immediately on ice before loaded onto 4-20% SDS-PAGE gel (Invitrogen, XP04200BOX). Gels were run for 1 hour at 80 volts followed by 120 volts for 1.5 hours. Gel was transferred onto solid support PVDF membrane filter paper, 0.2um pore size (Novex, LC2002). Transfer was run at 17mAmps for 1 hour followed by 30mAmps for 1 hour. Membrane blocking was in 5% dry milk in 1xTBST (Tris-buffered saline with 0.05% Tween-20) buffer or 3% BSA in 1xTBST for phosphorylation-detecting antibodies, for 1 hour at room temperature, rocking. Membranes were washed in 1xTBST and primary antibody applied in appropriate concentration for overnight incubation, rocking, at 4°C. Next day membranes washed 3 times 5 minutes each before HRP-conjugated secondary antibody added in appropriate concentration in blocking buffer for 1 hour, room temperature. Protein was detected using the ECL chemiluminescence kit (GE, Amersham, RPN2106). For further probing on same membrane, membranes stripped using 0.2M sodium hydroxide solution for 15 minutes. Film used for exposure (BioExpress, F-9029-8X10) List of antibodies used, Table 2.3. The Janus kinase 1 antibody was generated in the lab of Dr. Hallgeir Rui and kindly shared. Sodium azide (Sigma, S8032) was used to extend primary antibody use in buffer (several crystals added per 10mL antibody cocktail prepared), this aided in reusing antibody several times before discarding.

Tissue was prepared for WB by suspending in complete lysis buffer, 200-500uL, dependent on tissue size and homogenized twice, 5 seconds each, to dissociate cells, samples incubated on ice for 20 minutes. The remaining steps follow protocol written above.

Generating Expression Vectors
To facilitate expression of selection markers or target genes, the following vector constructs were used: pHIV-dTomato (Gift of Bryan Welm, Addgene plasmid #21374), pHIV-ZsGreen (Gift of Bryan Welm and Zena Werb, Addgene plasmid #1812) [87]. The pHIV-Luciferase-ZsGreen was generated using the pHIV-ZsGreen construct was digested with BamHI and Not1 overnight, the next day calf intestinal phosphatase (CIP) was added for one hour to limit self-ligation. The T7-IRES-Luciferase (cloned by Kay-Uwe Wagner, PhD) luciferase insert was PCR amplified and digested with BamHI and Not1. The backbone and insert were ligated overnight and transformed in Top10 cells. Clone "g" was used for virus production.

The shJAK1 was generated using shJAK1 (TRCN0000003102 and TRCN0000003105) and cloning into the Tet-pLKO-puro (Gift from Dmitri Wiederschain, Addgene #21915) [88], with AgeI and EcoRI.

The pHIV-FOS-dTomato was generated using the pHIV dTomato construct, digested with HbaI and XbaI overnight. The pcDNA3-FLAG-Fos WT was a gift from John Blenis (Addgene #8966) [89], and was digested with Asp718, then subsequently digested with Xba1. The backbone and insert were ligated overnight and transformed in DH5α cells. Virus was made from DNA isolated from clone “A”. The pHIV-caSTAT3-dTomato was generated using the pHIV dTomato backbone and STAT3-C (pMXs-Stat3-C was a gift from Shinya Yamanaka [Addgene plasmid # 13373] [90]). Not1 site was added to 5’ end of STAT3-C construct by PCR, as well as BamHI cloned into 3’ terminus, following amplification the DNA was digested with BamHI and Not1. dTomato was digested with BamHI and Not1, followed by CIP treatment. Insert and backbone were ligated overnight and transformed in DH5α cells. Proceeded with clone 9 to make virus after sequencing. The pWZL Neo Myr Flag MAP3K8 was a gift from William Hahn & Jean Zhao (Addgene plasmid # 20521) [91], and was cloned into the pHIV-dTomato
by PCR amplifying the Map3k8 with Eco1 and Xba1, ligating and transforming in Stbl3 competent cells.

**Virus production**

Virus was produced by transfecting low-passage 293T cells (ATCC) using Lipofectamine (Invitrogen 2000,11668-019) and collecting supernatant over the course of three days. Collected virus was filtered using 0.45uM filter by syringe and aliquoted in 4mL increments and frozen at -80C. To infect cells, virus was thawed prior to used and Polybrene was added. Virus was added to cells plated in 6-well plates and centrifuged at 1200RPM for 90 minutes at room temperature. 48 hours later, successful infection could be identified in cells expressing fluorescent markers and sorted at the UNMC Flow Cytometry Research Facility. Antibiotic selectable markers could also be treated for at the 48 hour time point, post infection. Puromycin would begin at 2-5ug concentration added to media.

**In vivo Bioluminescence Imaging**

Expression of luciferase reporters was analyzed using the in vivo bioluminescence imaging system (IVIS200). Animals were injected intraperitoneally 5 minutes prior to imaging with luciferin. 1mg D-Luciferin mixed with 1mL of 2xPBS, [92], and (Xenogen, XR-1001) and dosed at 150mg/kg of body weight. Equipped with the IVIS200 is an XGI-8 gas anesthesia system, which was used at a 2.5% Isoflurane flow rate (Butler Schein, 029404). Mice were anesthetized immediately prior to and during imaging. Images were taken at 10 second to 2 minute exposure length.

**Orthotopic Transplantation**
Mammary tumors were excised in sterile environment from tumor-bearing mice. Tumors were either minced and transplanted immediately or frozen. Tissue was minced and immediately put into Recovery Cell Culture freezing media (Gibco, 12648-010) and stored at -80°C until later transplantation. Frozen tissue used for transplant was quickly thawed in 42°C water bath and resuspended in DMEM growth media, spun down, resuspended again in DMEM growth media and taken for transplant. For transplantation, 0.1cm fragment was transplanted into both number four mammary glands of Athymic nude mice, under anesthesia. Incisions were closed by staples, which were removed after 7 days. Tumors were monitored for growth weekly using calipers.

Murine mammary tumor cell lines were harvested by trypsinization, counted, resuspended in sterile 1xPBS and 1x10⁵-1x10⁶ tumor cells were injected into both number four mammary glands of Athymic nude mice. Tumor size was monitored for growth weekly using calipers.

Mammary gland transplants were achieved using viable frozen fragments of target epithelium (example, BIM/BMF double knockout glands). Tissue was minced into 1 mm³ pieces and transplanted into the cleared mammary fat pad of both thoracic glands of 21-day-old female Athymic nude mice. Tissue was permitted to engraft, forming ductal structures, for 12 weeks. Females were bred and glands collected immediately after birth or time points during involution.

**Whole Genome RNA-Sequencing**

The genome wide survey of the transcriptome was achieved by first extracting RNA from mammary tissue or collected cell lines using the RNeasy Mini Kit (Qiagen, 74134). RNA was quantified using NanoDrop, and run on a gel to assess RNA quality. Samples were processed using TruSeq kit (RS-122-9001DOC). Generated cDNA was quantified on a Qubit Fluorometer and assayed for quality control using the BioAnalyzer.
Sequencing was performed using the HiSeq2000 (Illumina). In silico analysis was performed on the Tusker Supercomputer (Lincoln, NE) using the Unix commands. FASTQC (computes and provides quality profile of sequencing reads) demonstrated a Phred score of over 32 for all samples. Tuxedo Tools (index of samtools, open source bioconductor) were used in order to analyze the single-end reads: TopHat (uses Bowtie for read alignment, mm10 for mouse and hg38 for human, and splice junction mapping. Output as bam files), Cufflinks (assemble transcriptomes from RNA-Seq data and quantifies their expression) followed by Cuffmerge (merges individual transcriptomes into master transcriptomes, required for differential expression analysis). Cuffquant (the output is cxb file) followed by Cuffdiff (compares expression levels of genes and transcripts) and Cuffnorm (creates a table of normalized FPKM values). FPKM (Fragments Per Kilbase of transcript per Million mapped reads) values were used to analyze the differential transcript expression between control and experimental groups. Gene Set Enrichment Analysis (GSEA) was performed from output FPKM valued, comparing whole gene sets or pathways. For the analysis of the control tumor cell lines versus the Jak1 knockout cell lines GSEA permutation type parameters were set to utilize the phenotype permutation. Analysis of the paired isogenic mammary tumor cell lines required use of the gene_set permutation, as the program does not allow for the phenotype permutation in a sample size less than 3 per group. Individual genes were viewed using the Broad Institute’s Integrative Genomic Viewer (IGV).

Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

ChIP seq analysis was performed for the binding of STAT3 and STAT5, using mammary tissue from mid-pregnant females. In brief, snap-frozen tissue was homogenized and DNA was cross-linked using 1% formaldehyde. Nuclei were isolated in Farnham lysis buffer, samples were sonicated, centrifuged, and suspended in RIPA
buffer. Lysate was quantified and 600-1000ug lysate were added to Dynabead antibody complexes. Antibodies were ChIP-certified against STAT3 and STAT5. Samples were reversed-crosslinked and was DNA purified before libraries were generated using TruSeq ChIP sample kit (Illumina).

**ChIP and Quantitative Real-time PCR (RT-PCR)**

Following RNA extraction using the RNeasy Mini Kit (Qiagen, 74134), complementary DNA (cDNA) was synthesized using Superscript cDNA synthesis kit (protocol). ChIP was performed using primers against STAT3, STAT5, and isotype-matched control IgG as described previously [58, 93]. STAT binding was assessed for Bim and Bcl2l11 using gene-specific primers. Background and normalization was tested using non-promoter sites as described previously [93]. Quantitative RT-PCR was carried out using the C1000 Thermal Cycler (BioRad System, CFX96-Real Time). Conditions and primers used listed in Table 2.2.

**Statistical Analysis**

Graphs and accompanying statistics were prepared using Prism 6 software (GraphPad Software). Data computed as the mean ±SD, unless otherwise noted. The unpaired Student t test was used to compare data sets and verifying significance. A P value of < 0.05 was ruled significant.

**Cell culture**

Murine epithelial and tumor cells were maintained in the following growth media: DMEM/F12 powder (12400-024), 10 ug/mL of Insulin (Gibco, 12585-014), 10 ng/mL EGF (Life Technologies, E3476), 1 mg/mL BSA (Sigma, A9576), 5 ug/ML linoleic acid complex, 50ug/mL gentamicin (Gibco, 15750-060), 0.25 units/mL fungizone (Gibco,##),
20mg/mL FBS (Atlas, F-0500-A), 100U/mL Pen/Strep (Penicillin Streptomycin) (Gibco, 12140-122), 1.5g sodium bicarbonate (Mallinckrodt Chemicals, 7412-12), beta-mercaptoethanol (Gibco, 21985-023). Media was adjusted to a pH of 7.6 with NaOH and filter sterilized using 0.22 uM filter (Millipore, SGCPS05RE). 0.05% Trypsin used for cell passage (Gibco, 25300-054).

**Generation of Primary Tumor Cell Lines**

Preparation of digestion buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
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</tr>
<tr>
<td>100 units/mL pen-strep</td>
<td></td>
<td>200 uL</td>
</tr>
<tr>
<td>100 ug/mL gentamicin</td>
<td></td>
<td>40 uL</td>
</tr>
<tr>
<td>0.75 units/mL Fungizone</td>
<td></td>
<td>60 uL</td>
</tr>
<tr>
<td>2 mg/mL collagenase</td>
<td></td>
<td>1 mL</td>
</tr>
<tr>
<td>100 units/mL hyaluronidase</td>
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<td>300 uL</td>
</tr>
</tbody>
</table>

Tissue was removed by sterile means and was minced, placed in 20mL of digestion buffer in 50mL Falcon tube, and incubated in 37C hybridization oven, rotating speed 11 for 1-3 hours, or until dissociation was evident. Cells were centrifuged 1200RPM for 5 minutes at room temperature and supernatant removed by suction. Cells were washed by resuspension in growth media for a total of 5 washes. Following final wash, cells resuspended in 6mL of growth media and strained into 3 wells of 6-well plate (2 mL each). The filter was then flipped over and washed off three times with 2mL each growth media into the remaining 3 wells, as this captured the organoid cell clusters for growth. Cells were maintained in 10% FBS for 6-8 weeks followed by a decrease to 5% FBS-containing growth media. During this time, fibroblast cells were removed by partial trypsin (1mL trypsin added to a well of 6-well plate, monitored at room temperature under light microscope, light agitation was applied to lift fibroblast-like cells. Once lifted, trypsin removed by suction and growth media applied). Cells selected for growth were epithelial in appearance (cobblestone structure versus spindle cells).

**Generation of Murine Embryonic Fibroblasts**
Mouse embryonic fibroblasts (MEFs) were generated from Jak1-deficient embryos at embryonic day 12.5, following the initial primary cell generation technique described above. MEFs were cultured in DMEM, 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, 10 ug mL\(^{-1}\) streptomycin. MEFs were immortalized by serial passaging.

**Treatment of cells**

Cells were serum-starved (Growth media without FBS) for 16 hours prior to cytokine stimulation. Treatment included: ovine GH (20nM), recombinant mouse IL-4 (50ng/mL, BD Phamingen), recombinant mouse OSM (25ng/mL, R&D Systems), LIF (103 U/mL, Millipore), for 15 minutes at 37C, followed by cell collection. Ovine GH was a gift by Dr. A.F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) under sponsorship of the National Hormone and Pituitary Program, NIDD (National Institutes of Health).

**shRNA induced by Doxycyline (Dox)**

Treating cells with Doxycyline hyclate (Sigma D9891-25G): stock solution was kept at 1 mg/mL and then used at 1-2 ug/mL in cells in appropriate growth media for 48 hours.

**Migration assay**

Cells were trypsinized, resuspended in serum-free media, and counted using hemacytometer. Using a 24-well plate, 1mL of serum-rich media (20% FBS) was added. An 8 micron, insert was placed in well (8um, Corning, 353097) and desired amount of cells resuspended in 200uL of serum-free media added (2.5x10\(^4\) cells/insert) and incubated cells for 18-24 hours. The following day migrated cells were collected and
stained by taking the insert, washing 3 times in cold 1xPBS, and consecutively dipping the insert 15 times each in fixative, cytoplasmic stain, and nuclear stain. The staining was achieved using Hema 3 Stat Pack (Fisher Scientific, 123-869). The back of the insert was wiped with a cotton swab to remove cells that did not migrate. Permount was added to a microscope slide and the membrane insert was cut out with a razor blade and placed in the Permount and coverslip added. Migrated cells were counted under 200x magnification the average of three field views was calculated and recorded as cells per 1x1mm².

Flow Cytometry Analysis

Cells were trypsinized, and washed twice in buffer (1% FBS in 1x PBS) and counted. A total of 1x10⁵ cells were stained for surface markers. Cells were spun down and resuspended in 150 uL of buffer, antibody added, vortexed gently and incubated for 30 minutes on ice, protected from light. Following antibody incubated, cells were centrifuged at 4K RPM, for 5 minutes. Cells were resuspended in 1 mL of buffer to wash, and centrifuged, repeated twice. After the final wash, cells were strained into FACS tubes and analyzed. Appropriate controls were used, such as unstained samples and fluorescence controls. Flow cytometry was performed using the LSR II and analyzed using BD FACSDiva 8.0.1 software (BD Biosciences). Antibodies used were confirmed for flow analysis: CD61 APC Conjugate (Life technologies). OneComp eBeads (eBioscience) used for antibody staining control.
<table>
<thead>
<tr>
<th>Target</th>
<th>Lab ID</th>
<th>Direction</th>
<th>Sequence: 5'-&gt;3'</th>
<th>PCR Conditions</th>
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<tr>
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<td>TTTCTGCAGCAGCCTAGGC</td>
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<td>1785</td>
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<td>Step 5: 72C, 5 min</td>
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<td>Step 6: 4C, pause</td>
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<tr>
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<td>Step 3: 60C, 30 sec (to Step 2, 39x)</td>
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<td>Step 1: 95C, 3 min.</td>
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Table 2.2. Primer Sets for Genotyping and qRT-PCR
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Chapter 3: Janus kinase 1 mediates inflammatory cytokine signaling during mammary gland involution


*Contributed equally
Introduction

The postnatal growth and development of the mammary gland is dependent on hormones, growth factors, and locally derived cytokines to induce ductal elongation and branching, functional differentiation of the epithelium, and the postlactational remodeling of the gland. Initially, estrogen serves to induce ductal elongation after puberty. Meanwhile, during pregnancy and nursing offspring progesterone and prolactin (PRL) promote the functional differentiation, proliferation, and maintain cell-sustaining signals. Following weaning of the offspring, a decrease in the circulating levels of prolactin along with milk stasis initiate the local production of interleukin-6 (IL-6)-class cytokines. Notably, Oncostatin M (OSM) and leukemia inhibitory factor (LIF) are the prominent members of the IL-6 class that initiate the cascade of intracellular events that culminate in the programmed cell death and removal of select terminally differentiated epithelium.

The ligands such as PRL and OSM rely on nonreceptor associated Janus kinases to activate their corresponding receptor, mediating the signal and phosphorylating subsequent signal transducers and activators of transcription (STATs). Activated STATs function to bind to the DNA, altering the transcriptional profile and molecular events of the cells. In the family of seven STAT members (STAT1, 2, 3, 4, 5a, 5b, and 6), five are expressed within the mammary epithelium (STATs 1, 3, 5a/b, and 6), although they are activated at defined stages of mammary gland development. While STAT1 and STAT6 play subordinate role in development, the effects of STAT5 and STAT3 are robust. During pregnancy and lactation, STAT5 responds to the increase of PRL, and through its activation by JAK2, drives the transcription of genes associated with proliferation and differentiation. Contrasting the cell-sustaining signals, the drastic physiological event of involution is characterized by a decrease in STAT5 activation and surge in the phosphorylation of STAT3. In response to the cytokines LIF and OSM,
STAT3 is driven to the nucleus and upregulates genes associated with matrix remodeling and apoptosis.

The contribution of STATs to mammary gland development has been well studied, although curiously, little has been investigated on their upstream Janus kinase activators. In the family of four Janus kinases, two are expressed within the mammary epithelium as JAK3 and Tyk2 are primarily restricted to the hematopoietic system while JAK1 and JAK2 have increased ubiquitous expression. The functional role of JAK2 in the mammary gland has been established through the development of conditional knockouts, demonstrating its crucial role in mediating signaling of PRL to activate STAT5 for alveologenesis as well as differentiation of the mammary epithelium. Conversely, the role of JAK1 has not been addressed in adult organ development, and specifically, the STATs activated during mammary gland development and sequential STAT activation is unknown.

We demonstrate, for the first time, the analysis of a conditional deletion of JAK1 in a mammary –specific manner. The results support a nonredundant role for JAK1 in activating STAT1, 3, and 6, and demonstrate the biologically relevant role for JAK1 in coupling extracellular cytokines to cell death machinery the differentiated mammary epithelium.

Results

JAK1 expression levels in the virgin and pregnant female mammary gland parallel with phosphorylation of STAT1 and STAT6 as well as STAT3 activation during involution.

Previous studies have explored the biological significance of five of the seven STAT proteins (STAT1, STAT3, STAT5a, STAT5b, and STAT6) during mammary gland development. To identify changes in STAT activation in the context of JAK1 expression
in the mammary gland, we followed protein expression and activation status of the five STATs along with JAK1 throughout a complete postnatal development cycle. Mammary tissues from virgin glands, several time points during pregnancy, lactation, and involution (i.e., day 2 post-weaning) were analyzed by immunoblot (Fig. 3.1A). While all of the STATs are expressed at nearly consistent levels throughout the development cycle, as indicated by total protein levels, their various windows of activation may suggest differing upstream activators. STAT1 phosphorylation is prominent during virgin state and early to mid-pregnancy before declining during lactation and remaining low during involution. Active STAT6 displayed low levels throughout the whole development cycle, declining during involution. STAT5 was robustly phosphorylated during mid-pregnancy and lactation, and decline sharply at the onset of involution. In near-contrast, active STAT3 was observed during pregnancy, decreased during late pregnancy and lactation, but prominently increased during involution. The expression of JAK1 was seen during virgin and stages of pregnancy, and mirroring STAT3 activation, also fell during late pregnancy and lactation yet increased during involution. The STATs demonstrate activity during mid-pregnancy, although the activation of STAT1 and STAT6 follow the expression of JAK1 during the virgin stage and pregnancy, as well as STAT3 activation coinciding with JAK1 expression during involution event.

**JAK1 mediates cytokine signaling to activate STAT1, STAT3, and STAT6 but not STAT5 in embryonic fibroblasts in the mammary gland epithelium.**

In order to investigate the biologically relevant functions of JAK1 during postnatal and adult development, we used the previously described conditional knockout model for Jak1 [82]. In summary, the gene-targeted Cre/lox-based system inserted loxP sites upstream and downstream of the second coding exon of Jak1, and employed a neomycin selection marker in intron 3 which did not cause phenotypic abnormalities in
homozygous mice. Resulting $^{Jak1^{fl/fl}}$ mice were comparable and indistinguishable to wild type controls ($^{Jak1^{+/+}}$). To validate the null allele during embryogenesis, the $^{Jak1^{fl/fl}}$ was recombined in the female germ line. The resulting deletion of $^{Jak1}$ led to perinatal lethality in newborn homozygous mice. Embryonic murine fibroblasts were derived from homozygous $^{JAK1}$-knockout embryos at embryonic day 12.5 as well as from heterozygous embryos and wild type controls, to assess STAT activation. The deletion of the $^{Jak1}$ second coding exon causes a frameshift of the coding sequence and results in the absence of the $^{JAK1}$ protein without a compensatory upregulation by $^{JAK2}$ (Fig. 3.1B). In the absence of $^{JAK1}$, cells displayed a drastic decrease in the tyrosine activation of $^{STAT1}$ and $^{STAT3}$, although $^{STAT1}$ serine phosphorylation on S727 was preserved, indicating that the two events may occur independently of each other. Stimulation of cells by $^{IL-4}$ and $^{GH}$ demonstrated that $^{STAT6}$ relies on $^{JAK1}$ for $^{IL-4}$-induced signaling, conversely $^{STAT5}$ activation by growth hormone stimulation was unperturbed by a loss of $^{JAK1}$ (Fig. 3.1C).

For the analysis of $^{JAK1}$ specifically in the mammary gland, a cohort of female mice were generated carrying the $^{MMTV-Cre}$ transgene in a $^{Jak1}$-floxed homozygous background. Littermate controls carried the homozygous $^{Jak1}$, but lacked Cre recombinase. Mammary glands from the experimental and control group were compared at gestation day 14.5, a point where all STAT proteins exhibit various activity (Fig. 3.1A). The architecture of the glands were similar and a $^{Jak1}$ deficiency appeared to have little effect on alveoli differentiation. Although, analysis by immunohistochemistry indicated a loss of p$^{STAT3}$ activity in the epithelial compartment, meanwhile active tyrosine-phosphorylated $^{STAT5}$ was unaffected (Fig. 3.1D). To understand the activation of $^{STAT1}$, $^{STAT3}$, and $^{STAT6}$, mammary epithelial organoids were freshly prepared and treated for 20 minutes with OSM and $^{IL-4}$ (Fig. 3.1E). Similar to the initial studies in
fibroblasts, an absence of Jak1 uncoupled ligands from downstream mediators, STAT1, STAT3, and STAT6.

A deficiency in JAK1 leads to impaired postlactational mammary gland remodeling.

To reveal the biological functions of JAK1 throughout mammogenesis, we examined the composition and morphology of mammary glands from tissue of JAK1 conditional knockout females and controls during a complete gestation cycle. A GFP-based reporter transgene for Cre recombinase demonstrated that deletion of Jak1 by MMTV-Cre occurred throughout the ductal system (Fig. 3.2A) and indicated no negative selection prior to pregnancy. Deficiency of JAK1 also had no effect on ductal elongation or branching. Even though JAK1 contributes to the activation of STAT6, which is implicated in alveologenesis, no defects were observed during pregnancy. Experimental females were also able to lactate effectively compared to controls, supporting offspring until weaning. Histologically, the glands during lactation were similar between the two groups (Fig. 3.2B, left) and there was no remarkable difference at 24 hours post-weaning (Fig. 3.2B, middle). Strikingly, JAK1-deficient females displayed a pronounced delay in postlactational remodeling at 3 days post-weaning (Fig. 3.2B, right) and extended into day 5 of the remodeling process (Fig. 3.2C, left). The impairment of effective remodeling was also confirmed in a second mammary-specific model, WAP-Cre, for the deletion of Jak1 (Fig. 3.2C, right), where recombination of the target gene occurs in differentiated alveolar cells during late pregnancy and lactation.

Deletion of JAK1 uncouples gp130-mediated signaling through IL-6-class cytokine receptor complexes from its downstream effector, STAT3.

The most dramatic switch in tyrosine phosphorylation of STAT3 and STAT5 occurs between the physiology events of lactation and involution (Fig. 3.1A). To confirm,
immunostaining was performed demonstrating the high levels of nuclear STAT5 in the lactating glands, present in both control and JAK1-knockout female mice, which then decreased 24 hours into the remodeling phase (FIG. 3.3A). Although few, noticeable pSTAT5 staining was observed in the nucleus of JAK1-deficient glands within the first 24 hours of involution. In contrast to the strong nuclear STAT5 staining, nuclear accumulation of tyrosine-phosphorylated STAT3 was dramatically decreased in the JAK1-knockout female glands in the initial stages of involution. This striking molecular phenotype along with our previous observations that the expression of JAK1 is increased during the onset of involution, suggests that JAK1 plays a crucial role in mediating the signaling events necessary for remodeling and activation of STAT3 in the mammary gland.

The two IL-6 class inflammatory cytokines that initiate the signaling of remodeling events in the mammary gland signal through specific ligand-receptor complexes that share the glycoprotein 130 (gp130) signal transduction unit, culminating in the activation of STAT3 for the involution of the mammary gland. To assess the importance of JAK1 as a mediator of signaling through gp130, wild type embryonic fibroblasts along with those hetero or homozygous Jak1-deficient were treated with OSM and LIF. The activation of STAT1 and STAT3 were tested for activation and the results by immunoblot demonstrate the prominent role of JAK1 for mediating the IL-6-class ligands, as a lack of JAK1 uncouples the activation of STAT1 and STAT3 from the inflammatory cytokines (Fig. 3.3B). To translate the in vitro data to the mammary gland, virgin female mice were injected with both OSM and LIF before gland were collected and analyzed by immunofluorescent staining of pSTAT3 (Fig. 3.3C). The strong staining in the wild type glands of OSM and LIF indicate active STAT3 being driven to the nucleus, as well as a presence in the surround stroma (Fig. 3.3C, left). Conversely, JAK1-deficient females treated with cytokines have a failure of active and nuclear STAT3, although
phosphorylated STAT3 is evident in the stroma of the knockout female glands (Fig. 3.3C, right). The active STAT3 in the surrounding stroma serves as an internal positive control, wild type in JAK1, as the Cre recombinase is only active within the mammary epithelium. Both the studies in JAK1-deficient fibroblasts and mammary gland-specific deletion indicate that Janus kinase 1 is the dominant tyrosine kinase to mediate signaling through gp130-receptor complexes in response to inflammatory cytokines.

**Ablation of Jak1 produced deregulated expression of downstream target genes associated with the acute-phase response, inflammation, wound healing, and programmed cell death.**

To further explore the downstream effect and mechanism that a Jak1-deficiency causes in mammary gland remodeling, we performed genome-wide RNA-Seq analysis comparing tissue from lactating glands to involuting mammary tissue lacking JAK1 and wild-type controls. First, we focused on comparing wild-type lactating mammary gland tissue to the involuting mammary gland, observing the genes and pathways are upregulated during the remodeling phase. Next, we concentrated on identifying genes that demonstrated a lack of normal expression during involution with an absence of JAK1. The initial comparison between three wild-type lactating and involuting mice revealed that 1.131 genes had a 2-fold or higher upregulation during involution and 1,378 genes were downregulated. Supporting the phenotype and stage of mammogenesis, a distinct reduction in expression of genes linked to proliferation and cell survival signaling driven by JAK2/STAT5 coincided with an increased expression of genes associated with inflammatory signaling. The known targets of PRL and JAK2/STAT5 signaling such Socs2, Ccnd1, Akt1 were downregulated in the involution tissue, while an increased expression of Lif, Osmr, Stat3, and Socs3 was seen. The cytokine receptor switch between the two phases of lactation and involution, as well as a
synchronous exchange of the JAK/STAT pathway are illustrated by gene set enrichment analyses (GSEAs) of the RNA-Seq datasets (Fig. 3.4A). We also observed an increased expression of transforming growth factor B signaling and apoptosis during involution, as well as deregulated expression of NF-kB signaling and upregulated expression of the tumor necrosis factor receptor superfamily, particularly Tnfrsf1A, Tnfrsf10B, and Tnfrsf21.

To identify the genes deregulated during involution in a JAK1-dependant manner, the gene expression profiles for four conditional knockout mice were compared with three wild-type controls at the second day of involution. Only 135 genes displayed a 2-fold or more deregulation expression, while 203 genes resulted in an increased expression. Consistent with what we observe at the protein level, genes that demonstrated deregulation in JAK1-deficient glands include Stat3 itself, as well as known STAT3 targets such as Socs3, Osmr, and Bcl3 (Fig. 3.4B). Also consistent with the STAT3-deficient mice [63], we observe a reduced expression in genes associated with inflammation, acute-phase response (Cd14, Saa1/2, and Lrg1) and wound healing (Chi3L1). Together with the known targets of STAT3, we found Runx1 and c-Fos to be deregulated, as a lack of increased expression was seen compared to their dramatic rise observed in the involution tissue. A new JAK1-dependant milk protein was also found, the WAP four-disulfide core domain protein (Wfdc5), which was found upregulated during involution and is unlike the milk protein WAP that decreases over the same time, Interestingly we also discovered several JAK1-dependant genes that regulate cell survival and apoptosis. Their downregulation could be responsible for the lack of remodeling cell death in the mammary epithelium. These candidate genes include the death receptor 6 gene (Tnfsf21) and the tumor susceptibility gene Tpl2 (Map3k8). Although, the most consistently deregulated proapoptotic factor was, Bmf, a gene
encoding for the Bcl2-modifying factor and BH3-only protein. Related to BMF, the gene Bim (Bcl2l11) and Bid were also found downregulated.

The RNA-Seq analysis was complete with the addition of three expression libraries of lactating JAK1-knockout mammary glands. The remarkable precision of RNA-Seq allowed us to view the expression of individual exons of genes, notably, Jak1. In all sets of tissue, in the sample comparison of experimental and control groups during lactation and involution, we were able to precisely identify the excision of the second coding exon in the experimental tissue (Fig. 3.5, top). We noted an increase in Jak1 expression levels throughout the involution tissues, which correlates to the increased JAK1 protein levels tested at the same developmental window (Fig. 3.1A). JAK1 had no effect on JAK2/STAT5-responsive genes, such as beta-casein (Csn2) (Fig.3.5, middle). Observing the STAT3 target genes such as Cd14 and c-Fos, their transcriptional levels were remarkable decreased, as were the newly identified proapoptotic genes, Bmf and Bim. Although both genes displayed over 2-fold deregulated expression, Bmf had 4-fold higher expression profile in wild-type tissue overall (Fig. 3.5, lower). Similar to the previous report of the upregulation of Pik3r1, the p50/55 isoforms of the phosphatidylinositol 3-kinase (PI3 kinase), we found that Runx1 was selectively expressed from an intronic promoter that encodes the shorter isoform 3. This finding exemplifies the fidelity of RNA-Seq technology but also demonstrates the importance of using appropriate antibodies that recognize various isoforms at the protein level.

Confirmation of the newly identified JAK1 targets was done by immunoblot, Supporting the earlier findings that JAK1 had no effect on the activation of STAT5 during lactation, phosphorylated STAT5 was consistent in wild-type and knockout lactation tissue (Fig. 3.6A). Analysis of STAT3 confirmed upregulation of active STAT3 at the onset of involution throughout control samples, but was significantly reduced in the involution tissue of the knockout. The targets identified by RNA-Seq, RUNX1, BMF, BIM,
and WFDC5 also demonstrated reduced protein expression. Both RUNX1 and c-FOS have known expression in the hematopoietic system and immunostaining of these targets on histological sections of control and JAK1-deficient tissues demonstrate that they are indeed upregulated during involution, but their expression depends on JAK1 (Fig. 3.6B and C).

**BMF and BIM are downstream targets of JAK1, and their combined deficiency phenocopies an impairment of mammary gland remodeling similar to JAK1-deficient mice.**

The analysis of the mRNA profiles of lactating and involuting mammary tissues show that several genes of the Bcl2 family members display notable changes in their expression. We observed not only more than a 2-fold upregulation of Bax, Bcl-x (Bcl2l1), Bmf, Bim (Bcl2l11), and Bid, but a 3 and 7-fold upregulation of Bbc3 (Puma) and Bik (Fig. 3.4). A more than 2-fold downregulation of prosurvival factor Bcl-w (Bcl2l2) was also seen during involution (not shown). The most significantly downregulated Bcl2 family member was Bmf, along with Bim. In order to identify whether these two genes were preferentially bound by either STAT5 or STAT3, we performed preliminary analysis of the ChIP-Seq data sets from female glands during mid-pregnancy, a time point that represents when all STAT proteins that are expressed in the gland are active at various levels (Fig. 3.7). Socs2 and Socs3 served as preferential binding controls for STAT5 and STAT3, respectively. We observed a strong preference for STAT3 binding sites near Osmr, Bcl3, and Stat3, which is depicted in the histograms (Fig. 3.7). STAT3 was also showed enrichment in binding near Bmf and Bim. To quantify the binding of STAT3 of both Bmf and Bim, during the development phases of lactation and involution, we performed ChIP followed by quantitative reverse transcription-PCR (qRT-PCR) for canonical gamma interferon activation sites (GAS). We found that STAT3 occupied GAS
sequences near transcriptional start sites of both genes, and that the binding of DNA was greatly increased during involution (Fig. 3.8). The combined results of the ChIP-Seq and qRT-PCR data further suggest that STAT3 promotes the transcription of these pro-apoptotic family members during involution.

As Bmf and Bim appear to be downstream targets of JAK1/STAT3 signaling we anticipated that a functional loss of Bmf alone or in combination with Bim may phenocopy the delayed involution signature of the JAK1 conditional knockout mice. To answer this question we examined the histology of lactating and involuting glands of BMF- and BIM-single-knockout mice. Similar to the JAK1-knockout phenotype, the functional loss of either protein did not affect lactating tissue, although, we saw a delay in the remodeling process in both mutant tissue, with the observation being more pronounced in the BMF-deficient glands (Fig. 3.9). These phenotypic observations of the BH3-only family members provide evidence for their functional role for initiation apoptosis in differentiated mammary epithelial cells. Based on our data comparing lactation tissue to involution, where the two proteins are upregulated during involution in tandem, we hypothesized that they might act synergistically during the remodeling phase. Experimentally, we addressed this question by transplanting BMF/BIM-double-knockout tissue into the cleared mammary fat pad of wild-type recipient female mice. Tissue engraftment was successful and following ductal outgrowth after 12 weeks post-transplantation, recipient mice were bred and mammary glands were collected immediately following birth of offspring (Fig. 3.10, postpartum). Similar to a deficiency in a single gene, the double knockout tissue was indistinguishable from that of wild-type glands during lactation. However, we saw a striking delay in the remodeling process of the double-knout tissue (Fig. 3.10, involution). Histological analysis of active STAT3 in BIM- and BMF-single and double knockout mice demonstrate a clear and strong nuclear stain for the transcription factor, and was comparable to the wild-type involution tissue
Because the recipient mice are wild-type for JAK1, it is evident that these proteins act in a cell-autonomous manner. These results suggest that both proapoptotic family members have a significant role in the remodeling process and biologically function downstream of JAK1 and STAT3.
Fig. 3.1. JAK1 is essential for the activation of STAT1, STAT3, and STAT6 in embryonic fibroblasts and mammary epithelial cells. (A) Immunoblot analysis of the expression of JAK1 and tyrosine phosphorylation of STAT1, STAT3, STAT5 and STAT6 during mammary gland development. (P numbers indicate day of pregnancy; L7, lactation day 7; I2, involution day 2). The individual panels represent immunoblots of tissues from different mice. (B) Expression of JAK1 and JAK2 as well as steady-state activation of STAT1 and STAT3 in primary mouse embryonic fibroblasts (MEFs) from heterozygous and homozygous JAK1-knockout embryos which were generated through germ line deletion of the floxed locus (Jak1<sup>+/−</sup> and Jak1<sup>−/−</sup>, respectively) as well as MEFs from wild-type controls (Jak1<sup>+/+</sup>). (C) IP/Western blot analysis of STAT5 and STAT6 in GH- and IL-4-treated wild-type and JAK1-knockout MEFs. (D) Immunohistochemical staining of active STAT5 and STAT3 on mammary tissue sections from a JAK1 conditional knockout mouse (MMTV-Cre Jak1<sup>floxed.floxed</sup>[MCre Jak1<sup>fl/fl</sup>] and a littermate wild-type control (Jak1<sup>fl/fl</sup>) at day 14.5 of gestation. The slides were counterstained with hematoxylin. Bar, 50 μm. (E) Western blot analysis of phosphorylated STAT1, STAT3, and STAT6 in explanted mammary epithelial organoids from mice with mammary gland-specific JAK1 knockout and their wild-type controls with and without treatment with OSM and IL-4.
Fig. 3.2.
Fig. 3.2. The biologically relevant function of JAK1 is restricted to the remodeling phase. (A) (Top) Fluorescence images using the GFP-based reporter for recombination in wild-type (left) (MMTV-Cre Jak1<sup>lox</sup>/ CAG-LSL-GFP) and JAK1-knockout (right) (MMTV-Cre Jak1<sup>lox/lox</sup> CAG-LSL-GFP) nulliparous mammary glands. Whole mount images of control and knockout mammary glands fixed and stained by carmine alum, display similar ductal architecture (Lower). (B) H&E-staining of sectioned mammary glands from control and JAK1-deficient experimental females at the timepoints of lactation (left) and two involution timepoints (right). Bars, 100 μm. (C) Whole mount analysis comparing the MMTV-Cre deletion of Jak1, and the WAP-Cre-mediated excision of Jak1, along with wild-type controls, each as involution day 5. Bars, 2 mm. LN, lymph node.
Fig. 3.3.
Fig. 3.3. Analysis of STAT activation in the mammary gland and embryonic fibroblasts in response to cytokines OSM and LIF. (A) Immunostaining of tyrosine phosphorylated STAT3 and STAT5 in the wild-type and conditional JAK1-knockout mouse mammary gland during lactation and involution. Bar, 50 μm. (B) Western blot analysis of tyrosine-phosphorylated STAT1 and STAT3 in embryonic fibroblasts wild-type, heterozygous, or homozygous-deficient in Jak1, in response in LIF and OSM treatment. (C) Nulliparous mammary glands from wild-type and JAK1-knockout females after treatment with exogenous OSM and LIF. Bars, 50 μm.
Fig. 3.4.
Fig. 3.4. Comparison of the gene expression between normal lactation and involuting glands, and identification of genes deregulated in a JAK1-dependent manner during remodeling. (A) Gene set enrichment plots with heat maps of genes that are selectively upregulated during the second day of involution compared to their status in lactation. (B) Heat maps of selected genes that are upregulated during remodeling but experience a 2-fold or more downregulation in the JAK1-deficient gland. Both control and JAK1-knockout tissue were from the second day of involution.
Fig. 3.5.
Fig. 3.5. Janus kinase 1 is necessary for the expression of STAT3 target genes and newly-identified JAK1-dependent genes. The individual histograms depicting the RNA-Seq data from wild-type and JAK1-knockout glands during lactation and involution, depict the expression levels of the identified genes as well as their exon usage. (Upper) the box highlighting the exon 2 of Jak1 indicates the deletion of the exon in the knockout samples. (Middle) the milk protein gene Csn2, serves a control for a STAT5-responsive target that is downregulated during involution. Cd14 and Pik3r1 provide controls for STAT3 targets known to increase during involution under normal conditions, and whose expression is reverted in the knockout glands. C-Fos, Bmf, Bim, and Runx1 are newly identified targets of JAK1 signaling.
Fig. 3.6.
Fig. 3.6. Confirmation of target gene protein expression in wild type tissue and JAK1-deficient tissue by Western blot and immunofluorescent staining. (A) Western blot analysis of control and knockout tissue during lactation and involution depict a decrease in the target proteins RUNX1, BMF, BIM, WFDC5. (B and C) Immunofluorescence of the target genes RUNX1 and c-FOS on lactation day 9 tissue and involution day 2, with E-cadherin (CDH1) and DAPI served as counterstain. Bars, 50 um. (C) Inset panel demonstrates closer view of tissue lacking c-FOS expression
Fig. 3.7.
Fig. 3.7. STAT3 and STAT5 demonstrate various binding affinities for downstream target genes. The individual panels display the histogram of ChIP-Seq data sets for select target genes, and portrays the transcription factor binding in midpregnant glands. Socs2 and Socs3 serve as binding controls for STAT5 and STAT3, respectively. STAT3 has prominent binding near sites of Osmr, Bcl3, and Stat3 itself. The newly identified targets, Bmf and Bim display higher affinity for STAT3 binding in proximity to these loci (boxes indicate canonical STAT recognition sites [TTCN3GAA] referred to in Fig. 3.8.)
Fig. 3.8.

A  

Bmf  

ATG  

1  

2  

3  

4  

5  

TCAATTCCAAGAACTCA  

GAS 1  

GGGTTCTAGGAATGGA  

GAS 2  

GAS 1  

GAS 2  

Normalized Enrichment  

(arbitrary units)  

Lactation  

Involution  

IgG  

STAT5 P  

STAT3 P  

**  

B  

Bcl2l11 (Bim)  

ATG  

1  

2  

3  

4  

GGAATTCTAGGAAGG  

GAS 1  

GATCTTCATCTGAACTAT  

GAS 2  

GAS 1  

GAS 2  

Normalized Enrichment  

(arbitrary units)  

Lactation  

Involution  

IgG  

STAT5 P  

STAT3 P  

**
Fig. 3.8. qRT-PCR from ChIP by STAT3 and STAT5 demonstrates the binding of STAT3 and STAT5 to target genes during lactation and involution. Schematic outline of the location of canonical STAT binding sites near or within the genes encoding for BH3-only proteins BMF (A) and BIM (B). Boxes indicate the location of exons, and numbers refer to exon number. Bar graphs demonstrate results of quantitative real-time PCR from DNA isolated by ChIP in lactation day 7 and involution day 2 mammary tissue with antibodies directed against STAT3, STAT5, and IgG control. Primer sets used to amplify select regions surrounding STAT-binding sites are specific for sequences located within the 5' intragenic regions of BMF and Bim. Values were normalized against primers sets for nonconsensous binding sites. Bars represent means and SDs, **, P<0.01.
Fig. 3.9.
Fig. 3.9. The remodeling events are delayed in mice that genetically lack BMF or BIM. The H&E staining was performed on tissue collected from mice during lactation and involution from mice wild-type for both genes (BMF<sup>+/+</sup> Bim<sup>+/+</sup>), lacking only BMF (Bmf<sup>−/−</sup> Bim<sup>+/+</sup>) or deficient in only BIM (Bmf<sup>+/−</sup> Bim<sup>−/−</sup>). Bars, 100 μm.
Fig. 3.10.
Fig. 3.10. Double knockout mice for BMF and BIM display impaired remodeling.

Whole mount analysis reveals that while wild-type mice progress through normal involution (left panel) the recipient mice of the double knockout tissue have a notable delay in the involution process (middle, right). Tissue was collected after birth of offspring and four days after weaning. Immunohistochemical staining of the wild-type and double knockout tissue for phosphor-tyrosine STAT3 indicate strong nuclear localization. Bars, 50 μm.
Discussion

The biologically essential functions of JAK1 are restricted to the events of postlactational remodeling of the mammary gland.

Through the extensive characterization of STAT proteins and their activity in the JAK1 conditional knockout mouse model, it was shown that JAK1 contributed to the cytokine-induced tyrosine phosphorylation of STAT1, STAT3, and STAT6, not only by cell culture in embryonic fibroblasts but in the developing female mammary gland. Although JAK1 was responsible for the activation of three STAT family members, the phenotypic consequence for the lack of JAK1 in a developing gland was largely restricted to the postlactational remodeling phase where there was a failure of the gland to respond to the acute rise in inflammatory cytokines. This delay in remodeling resembles the phenotype of STAT3-deficient glands, as well as models that lack gp130 or IL-6 [49, 67]. The loss in activation of STAT1 and STAT6 within the mammary epithelium did not impede growth or development of the gland. Previous reports are indicate that STAT1 is dispensable for ductal elongation and alveologenesis and that the role of STAT1 in mediating optimal ductal branching is dependent on active STAT1 in the stroma [54]. A STAT6 deficiency has been shown to impair mammary alveolar development during early pregnancy, a phenotype that was observed to be repaired during later stages of gestation with no defects noted during lactation. The rebound of the gland during stages in pregnancy could be due to a paracrine effect of STAT6 in the stroma inducing mammary epithelial proliferation [55]. This may explain why a JAK1-deficiency in the mammary gland does not phenotypically compare with a STAT6-knockout model.

The Janus kinase 1 and 2 may have nonredundant roles in the activation of STAT proteins during mammary gland development.
Previous work investigating JAK2 in the mammary gland describes the kinase to specifically activate STAT5 in the mammary gland compartment, in response to PRL signaling. The mammary-specific deletion of JAK2 resulted in the impaired development and growth of alveolar cells, and no compensation by another kinase or downstream mediators of the JAK2/STAT5 pathway was observed to rescue the phenotype [60]. Strikingly similar, the loss of JAK1 in the developing mammary gland had no effect on the activity of STAT5, and in the involution events, and the loss of response to inflammatory cytokine signaling, no other kinase was able to effectively compensate for the genetic lack of JAK1 and subsequent STAT3 activation. The collective findings of each in each of these studies indicate that JAK1 or JAK2 is not able to compensate for the loss of the other to activate STAT5 or STAT3 in the same cell type, in response to ligand-receptor signaling (Fig. 3.11). Observations built from the biological and molecular phenotype support the idea that there may be no functional overlap between the kinases during normal gland development. The impact of this premise could be far-reaching as there are man reports that state JAK1 and JAK2 are capable of phosphorylating STAT3, in response to inflammatory cytokine signaling in vitro. It has been observed in initial studies that JAK1 mediates IL-6 signaling [94-97], yet current work has displayed JAK2 as the key mediator for STAT3 activation in the disease setting [98-101]. This could largely be due to the use of pharmacological inhibitors that are labeled as “JAK2 inhibitors”, when in they actually may inhibit both JAK1 and JAK2 with near similar kinetics [102]. A strong argument again the role of JAK2 activating STAT3 is displayed in recent work, where a genetic deletion of JAK2 in mammary cancers still display constitutive STAT3 activation [80]. Furthermore, STAT3 signaling in malignancies has been shown to depend on IL-6 autocrine signaling [103-106], initiated by receptor tyrosine kinases. We have demonstrated that JAK1 is essential for gp130-mediated
signaling, and that this kinase and not JAK2 may be crucial for targeting the persistent activation of STAT3 in disease.

**JAK1 is a crucial mediator of IL-6-class inflammatory cytokine signaling and activating downstream cell death complexes in differentiated mammary epithelium.**

The collective findings provide rational that JAK1 maintains a crucial function during the involution phase of mammary gland development. The expression levels of JAK1 were observed to rise during the involution time points by protein analysis and validated at the transcriptional level by RNA-Seq. The deletion of this kinase closely mirrored the biological consequences seen in STAT3-, gp130-, or IL-6-deficient mice [36, 49, 67]. The functional loss of JAK1 uncoupled inflammatory cytokine signals from their downstream effector STAT3, which led to a deregulated expression of STAT3 target genes during this biology time point. The genome-wide investigation of genes selectively upregulated during involution in a JAK1-dependant manner revealed known STAT3 targets such as Osmr, Cd14, and STAT3 itself, as well as targets such as c-Fos, a proto-oncogene known for its regulation in tissue remodeling by regulation of metalloproteinases [107-109]. Other targets included Tpl2 (Map3k8), and death receptor 6 (Tnfsf21), although their functions have not been explored. The function of JAK1 in coupling cytokine signaling to crucial mediators of mitochondria-associated cell death machinery was found in its regulation of the BH3-only protein BMF and BIM. Single knockouts of each BMF and BIM confirm their role in aiding in remodeling, and synergistically, a double knockout of both proteins dramatically delays remodeling [110]. This phenotype corresponds to the knockout JAK1, where upending JAK1 deregulated both BMF and BIM in the mammary epithelium. Corroborating data from the RNA-Seq analysis and qRT-PCR demonstrate that STAT3 binds in close proximity to these genes.
Although, the STAT3-deficient knockout did not show similar deregulation, suggesting that other transcription factors whose expression level depend on JAK1 also contribute to the regulation of Bmf and Bim, or that STAT5 may function a suppressor or Bim [111]. Although, the nuclear localization of active STAT3 is unchanged from wild-type mice to knockouts of BMF and BIM, which supports that these two factors function downstream of STAT3. While the lysosome-mediated pathway, driven by STAT3, an alternative model for remodeling in the mammary gland [111], its activity was not able to offset the inhibited canonical apoptotic pathway. The conditional knockout model of JAK1 in the mammary gland led to impaired remodeling and downstream targets of JAK1/STAT3 signaling were verified on a genome-wide scale and confirmed at the protein level and validated in genetic in vivo knockouts, demonstrating that this kinase is essential for driving involution in response to inflammatory cytokine signaling.
Fig. 3.11. JAK1 and JAK2 display nonredundant functions and activate specific STAT proteins in mammary epithelial cells. JAK2 mediates the signal of PRL to activate STAT5 during lactation. The switch to involution requires JAK1 to activate STAT3 downstream of inflammatory cytokines OSM and LIF during involution.
Chapter 4: JAK1 is crucial for persistent STAT3 activation in mammary tumors
Introduction

The results of the previous chapter clearly illustrate that JAK1 is the essential mediator for the inflammatory signaling in the mammary gland [86]. The Cre/lox system, under control of the MMTV promoter, specified the deletion of Jak1 to the mammary gland and avoided perinatal lethality [20]. As the MMTV promoter is active during development, the mice were monitored from birth to pregnancy and through involution. Most striking was the phenotype of female mice undergoing remodeling. While a mammary-specific Jak1 deletion had no effect on mammary gland development prior to or during pregnancy, involution was strongly delayed, evidenced in the sustained maintenance of the mammary epithelium, failure of matrix remodeling, and absence of activated STAT3. This phenocopies the STAT3 and LIF-deficient models, and highlights that JAK1 is crucial for mediating the effects of IL-6 class cytokines.

Surprisingly, JAK1 not only contributed to the activation of STAT3, but also to the phosphorylation of STAT1 and STAT6. While these two transcription factors are active in the developing mammary gland and early stages of pregnancy, development of JAK1-deficient mammary glands were indistinguishable from wildtype. This may be attributed to knockout models of STAT1 and STAT6 demonstrating their effects in the stroma [54, 55].

The previous studies of JAK2 along with the results in Chapter 3 indicate that the Janus kinases may have non-redundant roles in STAT activation. Evidence is seen in data of JAK2 deletion leading to inactivation of STAT5 in the mammary gland, with no compensation by another kinase [60, 112]. JAK1-deficient mammary epithelial cells had no effect on STAT5 activation downstream of PRL signaling but failed to phosphorylate STAT3. This signifies that the two kinases may not have functional overlap during mammary gland development. Understanding the role of the Janus kinases exemplifies the importance of targeting kinases according to function and STAT activation.
Across several types of cancer, such as head and neck squamous cell carcinoma, melanoma, and breast cancer; STAT3 is demonstrated to be a contributor to malignant disease [70, 104, 113]. The function of STAT3 appears to support migration [35]. In breast cancer, 20-30% of cases exhibit amplification of HER2/Neu, which contributes to a growth regulatory network that sees a high prevalence of active STAT3 driving aggressive disease and contributing to metastasis [114-116].

Comparing normal mammary gland development to breast cancer, STAT3 appears to have opposing functions. During postlactational remodeling, STAT3 is essential for cellular breakdown and apoptosis, compared to its described role in breast cancer where it drives growth and aggressive disease. Although in development STAT3 was described as necessary for initiating apoptosis during the remodeling process, this is one facet of the involution phase. Several other important components are occurring as well, such as upregulation of matrix metalloproteinases to degrade the underlying matrix and allow the cells to detach from the basement membrane. In this way, STAT3 may be preferentially active in established disease to promote aggressive phenotypes.

We proposed to address the contribution of JAK1 to activating STAT3 in the context of tumor formation and disease progression by generating in vivo cohorts that express the neu oncogene in a mammary-specific manner, while the experimental group also had Jak1<sup>fl/fl</sup> locus deleted prior to disease formation using the mammary-directed Cre recombinase. If JAK1 maintains nonredundant activation for STAT3, as shown in normal development profile, it may display biological relevance in mediating the migration properties associated with STAT3.

Results

JAK1-deficient MMTV-neu tumor model displays absence of metastatic disease while tumor onset remains unaffected
Janus kinase 1 is necessary for the activation of STAT3 in immortalized embryonic fibroblasts and is the key mediator of inflammatory cytokine signaling in the mammary gland during the postlactational remodeling events during development, coupling the IL-6-class ligands to downstream effectors through STAT3 [86]. The high prevalence of tyrosine phosphorylated STAT3 in aggressive cancers, and STAT3's association with driving proliferative disease, led us to test if a JAK1-deficiency in the mammary gland-specific ErbB2-driven tumor model would experience differential tumor onset or malignant progression. In order to study the tumorigenesis profile, we generated a mouse model that overexpressed the ErbB2 gene specifically in the mammary gland concurrent with a mammary-specific deletion of Jak1 in the same epithelium, and also tracked with a GFP reporter (MMTV-neu MMT-Cre Jak1 \(^{fl/fl}\) CAG-LSL-GFP). As the MMTV promoter is expressed within the luminal and myoepithelial compartments during development, both oncogene expression and the deletion of the floxed alleles occur within the same cell. Latency of MMTV-neu-driven mammary tumors is approximately 220 days, and MMTV expression occurs well before puberty, thus producing Jak1-deficient cells prior to tumor onset. The littermate controls lacked Cre recombinase, preserving the Jak1\(^{fl/fl}\), rendering it wild-type (MMTV-neu Jak1\(^{fl/fl}\) CAG-LSL-GFP). Both experimental and control female mice were bred twice to induce proliferation and expansion of cells in the mammary gland, and monitored for mammary lesions by palpation biweekly for up to 12 months. Histological observation of control and knockout glands depicted normal patterning of ductal branching and alveologenesis. Also the absence of negative selection in Jak1-deficient cells was noted by observing the Cre-mediated expression of the GFP reporter in the mammary glands (Fig. 4.1A). Analysis of 39 tumor-bearing control females and 14 JAK1-deficient mice revealed that the tumor onset did not vary statistically between the two groups (Fig. 4.1B). At the time of necropsy, the resected tumors were analyzed for GFP, if induced by Cre recombinase,
and analyzed as well for evidence of metastatic lesions in the lung, as the MMTV-neu model is well-documented to readily metastasize to the lung [114]. Consistent with these reports, the control females demonstrated lesions to the lung, identified by GFP, interestingly, the JAK1-deficient females had no comparable lesions in pulmonary tissue (Fig. 4.1C). This observation raises a critical question on the need for JAK1 during tumor initiation versus disease progression.

**Janus kinase 1 is necessary for the activation of STAT1, STAT3, and STAT6 in mammary tumor epithelium in vivo and tumor cells in vitro.**

To assess if JAK1 maintained regulation and activation of STAT3 in tumors, Western blot was performed on control MMTV-neu mammary tumor lysate alongside tumors from mice deficient JAK1. The results demonstrate that even in the disease setting, an absence of JAK1 correlated with a deceased activation of STAT3, along with tyrosine-activation of STAT1 (Fig. 4.2A). The activation of STAT6 was not evident in the control tumors, when compared with cells stimulated with IL-4 (Fig. 4.2A). Immunohistochemistry was performed and staining displayed strong nuclear staining of activated STAT3 within control tumors; interestingly, the strongest signal was produced along the leading edge of the tumors and near blood vessels. In comparison tumors deficient in JAK1 had a marked decrease of active STAT3 within the tumor tissue, yet retained activity in the stroma (Fig.4.2B). When immunostaining was performed for localizing phosphorylated STAT1, low levels were seen within the tumor, consistent with the decrease in protein expression by Western blot (Fig.4.2B).

In order to verify the tumor intrinsic characteristics with a lack of JAK1, isogenic cell lines were derived from control mice harboring the Jak1^fl/fl^ alleles, deleting Jak1 by viral mediated Cre recombinase. The confirmed excision of JAK1 by immunoblot was followed by an inhibition of STAT3 activity (Fig. 4.2C). The deletion of JAK1 also led to a
significant decrease in active STAT1. STAT6 demonstrated the same lack of phosphorylation in the knockout cell lines when stimulated with IL-4, as the cells did not express active STAT6 at steady state levels (Fig. 4.2C). The Jak1-deficient cells had no growth deficiencies while in culture, consistent with reports from STAT3-deficient epithelial cells [35]. Although curiously, their phenotype changed from a stretched cobblestone, spindle appearance, to a compact epithelial morphology in the absence of JAK1 (Fig.4.2D).

A lack of JAK1 drastically decreases tumor growth of transformed cells in vivo and reduces incidence of metastatic disease.

While tumor initiation and tumor progression are commonly associated as the same events, they are two very different biological processes, requiring distinct mechanisms for initial transformation of cells to sustained proliferation and migration capacity. To address the function of JAK1 in mediating progression of disease, the isogenic cell lines were treated by lentiviral infection, incorporating a luciferase reporter construct for in vivo bioluminescence to monitor for growth and distant disease. Cells were transplanted into the number 4 mammary glands of recipient female mice. 15 mice per group were transplanted with 1x10^6 tumor cells per gland (total of 30 transplants per cell line were performed) and monitored weekly for growth using calipers and bioluminescence. Over the course of 8 weeks, the wild-type cells engrafted and proliferated robustly, indicating that they maintained their neoplastic properties, strikingly the isogenic-matched JAK1-deficient cells appeared to have engrafted, as indicated by the continued bioluminescence assays, but demonstrated a lack of proliferation as compared to the wild-type cells within the same amount of time (Fig. 4.3A, B). At necropsy, the mice transplanted with the MMTV-neu tumor cells carried metastasis within the lung, along with two mice presenting with metastasis in the liver and pancreas.
Assessment of the lungs and pancreas of the mice transplanted with the JAK1-deficient cells found no evidence of metastatic disease (Fig. 4.3C). Verifying that transplant had no effect on the activation of STAT3, the tumors were subjected to immunoblot, confirming the sustained STAT3 phosphorylation in wild-type cells and a maintained decrease in JAK1-deficient transplanted cells (Fig. 4.3D). Histological analysis of the transplanted tumors of both wild type and knockout tumors demonstrated an invasive phenotype seen in the wild-type tumors, as they lacked defined borders, while the JAK1-deficient tumors maintained a compact phenotype. When the lungs were surveyed for metastatic disease, distant disease was evident, and at time multifocal, while lungs of the mice transplanted with JAK1-null cells remained free of disease (Fig. 4.3E). To ensure that this was not an artifact of cell culture, minced tumor fragments from control and JAK1-deficient tumors were transplanted into the number 4 glands of recipient female mice (mice each cohort, total of 16 transplants performed) and monitored for growth. The growth patterns mirrored that of the cell line transplants, although the knockout tissue exhibited growth several weeks earlier, possible due to a selection of wild type or unrecycled Jak1 floxed alleles. When the tumors were analyzed for active STAT3, the control tumors as well as the resulting lung metastasis (Fig. 4.3G) were positive for phosphorylated STAT3, indicating that active STAT3 may be important not only in the primary tumor but also disease progression, and critical for the metastatic process and propagation at the secondary site. The JAK1-deficient tumors continued to display very little activation of STAT3 within the tumor, although the stroma contained persistent STAT3 phosphorylation (Fig. 4.3G). While the signaling events necessary for tumor formation seem independent of JAK1, these results evidence JAK1 as an integral mediator of disease progression.

Signaling through JAK1 is required for cancer cell migration
Prompted by the behavior of the transplanted cells, we performed in vitro assays to verify migration characteristics. The trans-well migration assay demonstrated that the migration patterns of the tumor cells were inhibited by a JAK1 deletion, reducing the number of cells that migrated by nearly fifty percent and paralleling the same behavior seen in vivo (Fig.4.4A). The results are consistent with the previous reports in STAT3-deficient cells, that while the cell growth in vitro remained unchanged, migration was impaired, suggesting that STAT3 acts in mechanisms associated with motility [35]. The extent to which the JAK1-deficient cells lacked growth compared to the wild-type tumor cells is reminiscent of data and phenotypes that depict the loss of tumor-initiating cells [117, 118]. Performing a tumorsphere assay, we identified whether cells have the required biological properties to generate tumors under low-attachment conditions. Permitting the cells to grow for a duration of seven days, the wild-type cells readily formed multiple spheres, meanwhile the tumor cells lacking JAK1 failed to proliferate under the same conditions, synergizing with their behavior in vivo (Fig.4.4B). A marker of tumor-initiating capability, specifically within the ErbB2-driven tumor population was found to be CD61 [119], when we analyzed our control cells against the JAK1-deficient cells, we observed a shift in the expression of the surface marker (Fig. 4.4C). The data suggest that more than STAT3 is deregulated with the deficiency of JAK1.

Identifying target genes downstream of JAK1 signaling mediating tumor progression.

To identify potential JAK1-dependent mechanisms that contribute to tumor progression, we prepared cell lines for whole genome RNA-Seq analysis followed by Gene Set Enrichment Analysis. Initially, five control tumor cell lines were compared against four JAK1-deficient cell lines, validating the loss of JAK1 in a larger sample size. 241 genes displayed a significant deregulation by 2.5-fold change or greater, 185 genes
were downregulated in the absence of JAK1 and 56 had increased expression in JAK1-deficient cells, represented by a heat map of the top deregulated genes (Fig. 4.5A). Deriving genes directly targeted by the JAK1/STAT3 signaling axis, analysis was performed comparing the two isogenic cell lines. The top genes expressing the greatest fold change by deregulation in the knockout cell lines are illustrated in Fig. 4.5B, the focus for the following experiments expound on the genes with lower expression in the JAK1-deficient cells, capitalizing on the function of STAT3 as a transcription factor. The reversal of expression (lower Fig. 4.5B) may be a secondary result of JAK1 deletion. Genes set enrichment analysis was performed on the isogenic cell lines, to identify genes and functional pathways that may apply to biological processes. The deletion of JAK1 led to the deregulation of downstream target genes associated with JAK/STAT signaling, focal adhesion, and toll like receptor signaling (Fig. 4.5C). Specifically, potential targets, such as Fos, Map3k8, and MyD88 were found downregulated in a JAK1-dependent manner. The tumor susceptibility gene, Tpl2 (Map3k8) activates pathways such as the MAP kinase and NF-κB, and has demonstrated transforming capabilities [120], along with MyD88, whose expression integrates into the toll-like receptor pathway to promote metastasis and proliferation [121]. Reinstating expression of these targets in a JAK1-deficient background would identify their contribution to disease progression.

Next, we validated the expression of putative JAK1 targets. A sizeable downregulation was seen in the JAK1-deficient cells in the expression of the oncogene Fos, as well as a known STAT3 binding target, Socs3. The Jak1 locus depicted the loss of exon 2, supporting the fidelity of the sequencing and the identification of downstream targets (Fig. 4.6A). Testing the expression and localization of select target genes on tumor sections demonstrated a loss of platelet-derived growth factor alpha (PDGFA), the STAT3 target OSMR, a reduction in CD14, large reduction in expression of BCL3 and c-
FOS (Fig. 4.6B). Immunoblot analysis confirmed the downregulation of the select targets in the Jak1-knockout cell lines (Fig. 4.6C).

Re-expression of downstream JAK1-signaling target FOS partially rescues growth-deficient phenotype

Expression of Fos was dramatically deregulated in the JAK1-deficient cells, as identified by the RNA-Seq results (Fig. 4.6A), and perpetuated in the analysis by immunostaining tumors wild type and deficient in JAK1 (Fig. 4.7A). Protein expression was also concurrent in the cell line analysis by Western blot (Fig. 4.6C). Curious, we postulated if this protein could rescue the growth-deficiency seen in the Jak1-knockout tumors. To test this hypothesis, we generated a lentiviral construct to reinstate expression of FOS in JAK1-deficient tumor cells. The cells were established and 1x10⁶ cells transplanted into the number 4 mammary glands of recipient Athymic nude mice and monitored for growth weekly. Tumors began to proliferate noticeably at week 6, where the tumor size was considerably larger than the JAK1-knockout cells (Fig. 4.7). The partial rescue in growth of the FOS-expressing JAK1-deficient cells did not completely mirror the growth patterns of the Jak1⁺/⁻ cells suggesting that complimentary factors must be associated with growth in a JAK1-dependent manner. Further rescue studies will describe additional proteins that can be tested in the same design, identifying and validating critical effectors of JAK1 signaling. These studies may identify potent JAK1-dependent targets necessary for in vivo tumor growth along with identifying genes necessary for tumor-initiating capability.
Fig. 4.1.
Fig. 4.1. Characterizing a deletion of Jak1 during mammary tumor initiation and progression. The GFP-based reporter identifies Cre recombinase activity. The deletion of Jak1 (knockout, right) did not affect growth of the duct or the alveolar branching as viewed by Carmine alum staining (A). Tumor onset was showed no statistical difference between the control and experimental groups (B). Mammary tumors displayed GFP-fluorescence, with lung metastasis seen in the lung of control mice, bar, 0.5 cm (C).
Fig. 4.2.

A

B

C

D
Fig. 4.2. JAK1-deficient tumors display a lack of active STAT3 and STAT1 (A). The whole tumors lysates also have active STAT1, which is absent in the knockout tumors. STAT6 is not active within the tumors, when compared to cells stimulated with IL-4. Immunohistochemistry demonstrates the localization of active STAT3 to the nucleus of tumor cells, with staining evident tumors (T) as well as the surrounding stroma (S), compared to a notable decrease to near absence of STAT3 staining in the tumor, but still seen in the stroma. Bar, 100 μm (B). Tyrosine-phosphorylated STAT1 is evident within the control tumors, while decreased within JAK1-knockout tissue. Isogenic cell lines were generated and verified for the presence of JAK1 in wild type cells (WT) and deletion for Jak1 in the knockout cell lines (KO). JAK1 is shown to contribute to the activation of STAT6 in the presence of IL-4 stimulation, as well as STAT1 at stead-state conditions (C). Morphologically, the Jak1 wild type cells appear stretched, while a knockout of Jak1 seems to drive them to cobblestone, epithelial appearance (D).
Fig. 4.3.

A

+Luc -Luc

Counts

Counts

B

Jak1^{+/+} vs. Jak1^{--} Tumor Cell Growth

Weeks

JAK1

counts

C

Incidence of Metastasis

Lung

Liver

Pancreas

D

JAK1

E

pY-STAT3

STAT3

GAPDH

F

Jak1^{+/+} vs. Jak1^{--} Tumor Fragment Growth

Weeks

G

Mammary Tumor

Lung

Mammary Tumor

M

T

S
Fig. 4.3. **In vivo growth of transplanted JAK1-deficient cells results in a severe decrease in growth and occurrence of metastasis.** To address the implications of a JAK1-deficiency in established tumor cells, the isogenic cell lines were infected with a luciferase construct to allow for bioluminescence tracking (A). The transplanted JAK1-deficient cells displayed a large inhibition in growth compared to the JAK1 wild type cells (A). The growth curve demonstrates the rapid growth of the control cells versus the JAK1-deficient cells (B). The wild type mice displayed multiple lung lesions, as well as metastasis to the liver and pancreas, while metastatic disease was absent in the Jak1-knockout recipient mice (C). The transplanted cell line tumors were analyzed for STAT3 activity, and the knockout retained the muted activation of tyrosine-phosphorylated STAT3 (D). Histology of the control mammary tumors and lung tissue presenting metastasis (M), compared to the JAK1-knockout cell transplant mice display the engrafted and formed tumor and lung tissue, bar 100 μm (E). Verifying that the previous results are not an artifact of cell culture, whole tumors, JAK1 wild type, and JAK1-deficient were minced and transplanted into recipient Athymic nude female mice. The growth curve resulted in the similar trajectory of the transplanted cell lines (F). Analysis of the transplanted tumor fragments depict the morphology by H&E of the wild type tumors and lung tissue displaying metastatic disease (M), as well a tumor tissue from the Jak1-deficient tumors. Control and knockout tissue was stained for phosphorylated STAT3 (red), and luminal cell marker, CK* (green), demonstrating high activity with the control tumor as well as the lung metastasis, while this was absent in the transplanted knockout tissue, although active STAT3 was retained in the stroma (S) bar 100 μm.
Fig. 4.4.

A

Cell Line 1

Cell Line 2

B

Tumorsphere Assay

1mm

C

Unstained Count

CD61-BV21 Count

Violet 450/50-A
Fig. 4.4. The deletion of Jak1 results in decreased migration, tumorsphere formation, and reduction in tumor-initiating cell marker CD61. The migratory capacity of the wild-type cells (Jak1+/+) and JAK1-deficient cells (Jak1−/−) was tested by trans-well assay (bar= 1 mm), decreasing migration nearly 50%, \( P = <0.05 \) (A). The cells were then analyzed for tumor-forming capability by tumorsphere-forming assay. Wild type cells (Jak1+/+) readily formed tumorspheres, while in the knockout cells (Jak1−/−), this morphology was greatly reduced (B). Cells were tested for the cell surface marker expression of CD61, a marker implicated in tumor-initiating cells and was found to be decreased in the knockout cells (C).
Fig. 4.5.
Fig. 4.5. Whole genome RNA-Sequencing revealed the JAK/STAT, toll-like receptor, focal adhesion, and ECM receptor pathways to be deregulated in a JAK1-dependent manner. Five JAK1 wild type cell lines (Jak1^{+/+}), and four JAK1-deficient cell lines (Jak1^-^-) were compared for differentially expressed genes (A). To compare the JAK1-specific effects in a homogeneous population, the isogenic cell lines were compared and the resulting heat maps display the top fold change of genes regulated in a JAK1-dependent manner (B). Gene set enrichment analysis (GSEA) was performed on the isogenic cell line data, and significant deregulated pathways are listed (C).
Fig. 4.6. Expression of JAK1 target genes confirmed by immunostaining and Western blot analysis. The expression of Jak1 demonstrates the loss of exon 2 in the knockout cell line samples (KO), highlighted by the blue-shaded area. Example targets deregulated by decreased JAK1 expression are Fos and Socs3 (A). Targets identified by RNA-Seq results were analyzed by immunostaining in wild type tumors (Jak1+/+) and JAK1-deficient mammary tumors (Jak1−/−) and included PDGFRA, OSMR, CD14, BCL3, c-FOS, SOCS3, and SOC1, bar, 100 μm (B). Select targets verified by Western blot for protein expression, and displayed differential expression between the JAK1 wild type (WT) and knockout (KO) cell lines (C).
Fig. 4.7.

A) FOS (red), DAPI

B) Western blot analysis
- Jak1 WT
- Jak1 cKO
- Jak1 cKO + FOS
- pY-STAT3
- FOS
- GAPDH

C) Jak1 WT, Jak1 cKO, and Jak1 cKO + cFOS Cell Transplant
- Control (n=10)
- KO (n=10)
- KO+cFOS (n=10)

D) Immunostaining
- Jak1 WT
- Jak1 cKO
- Jak1 cKO + cFOS
- DAPI
- FOS (red) 
- CB8 (green)
**Fig. 4.7.** The re-expression of FOS partially rescues the growth-deficient phenotype observed in the JAK1-deficient tumor cells. The expression of FOS in JAK1 wild type tumors compared to JAK1-deficient mammary tumors, bar, 100 μm (A). Re-expression of FOS in a Jak1 knockout cell line (Jak1<sup>−/−</sup>) restores levels of FOS at the protein level (B). Transplanted FOS-expressing JAK1-deficient cells partially restores growth, and can be tracked over the course of the study by bioluminescence, NC, negative control for luciferase activity (C). cFOS expression by immunostaining demonstrates positive staining in tumors wild-type for JAK1 (Jak1 WT) and JAK1-deficient, but expressing c-FOS (Jak1 cKO + cFOS), while JAK1-deficient (Jak1 cKO) tissues are negative. Bar, 100 μm (D).
Discussion

JAK1 plays a critical role in ErbB2-induced mammary cancer progression

The deletion of Jak1 in mammary epithelial cells prior to tumor onset did not affect the tumor initiation; however, the JAK1-deficient tumors displayed a decrease in active STAT3 and STAT1, as well as a decreased propensity to metastasize. The results suggest that JAK1 is not crucial for molecular events involved in the initial transformation of tumor cells, even though previous work has identified STAT3 in driving transformation events [70]. Turkson, et al. demonstrated that STAT3, downstream of Src signaling, could produce transformation in NIH3T3 fibroblasts [122]. A similar study, also using NIH3T3 cells, showed a role for STAT3 in transformation as well as cell growth downstream of tyrosine kinase receptors (TRK) [123]. It should be pointed out, that these studies identified a role for STAT3-induced transformation in vitro, and while STAT3 may promote transformation in 2D culture, it is either not activated in a JAK1-dependent manner or necessary in vivo for ErbB2,neu transformation. The Neu protooncogene has been shown to induce tumor formation through intrinsic kinase activity along with somatic mutations within the transgene, increasing the transforming capabilities [114, 124]. Muthuswamy, et al. identified the Neu transforming capability to also include recruitment of c-Src proto-oncogene (Src homology 2) for signal transduction events [125]. Due to the unchanged tumor onset in the Jak1-deficient mice, it is possible that these transforming events act independently of JAK1. Conversely, early events initiate Akt for cell survival and proliferation, which is modulated by JAK2, before becoming independent of this Janus kinase for fully transformed and established cancer [79], underscoring the importance of signaling events for the various phases of tumor progression.

Addressing tumor progression, our results synergize with ErbB2-driven tumor models deficient in STAT3 [126]. Ranger, et al. reported that while there was no
difference comparing in vivo tumor initiation of ErbB2 tumors that are Stat3<sup>+/+</sup> versus Stat3<sup>−/−</sup>, there was a significant decrease in the metastasis to the lung and concurrent reduction in angiogenic response. We observed a similar phenotype of an absence of lung metastasis in the transplant of isogenic cell lines and tumor fragments deficient in Jak1, both of which displayed a lack of tyrosine-phosphorylated STAT3 in JAK1-deficient samples compared to wildtype. The transplant data show that when placed back in vivo, there was no indication of reactivation of STAT3, suggesting that another Janus kinase family member cannot contribute to STAT3 activation in established disease in the absence of JAK1. First, this potentiates the findings from Chapter 3, describing the role of JAK1 in normal mammary gland development, where the deletion of the Janus kinase 1 produced a decrease in activated STATs 1, 3, 6. Also, decreased activation of these transcription factors demonstrates that JAK2 could not compensate for their lack of activation. In the mammary tumorigensis model, the observation that JAK1 contributes to the activation of a similar STAT profile, which also cannot be rescued by another JAK family member, supports the critical role for JAK1 in activating specific targets, namely STAT3. These results demonstrate that inhibition of this kinase directly may provide effective therapy for reducing the activity of STAT3 in established tumor cells, while concurrently inhibiting their growth properties in Erbb2-driven human malignancy.

JAK1 contributes to the tumor-initiating cell population

The striking observation of the impaired growth of JAK1-deficient tumor cells upon transplantation indicated a depletion of a tumor-initiating population of cells. The in vivo results were replicated in vitro by tumorsphere analysis, as the ErbB2-driven Jak1<sup>+/+</sup> cells displayed neoplastic characteristics and formed spheres readily in nonadherent conditions, whereas Jak1<sup>−/−</sup> cells had a reduction in sphere formation. The results indicate that the ErbB2 model is enriched in tumor-initiating cells, the minor subset of
cells that are capable of propagating the bulk of tumors. The ErbB2/Neu tumorigenesis drives luminal disease that has been shown to express elevated levels of integrin-beta3 (CD61) as well as increased amounts of CD49 [118, 127]. While the study by Vaillant, et al. described the variation of tumor-initiating cells by CD61 expression in three mammary tumor models, MMTV-wnt-1, MMTV-neu, and p53+/− they concluded that the MMT-neu was a homogenous population with little variation in a tumor initiating capability [127]. They arrived at this conclusion because they were not able to subdivide additional populations of cells from the MMTV-neu tumors, such as Sca-1, CD18, and CD14. Our data correlate with their findings, that MMTV-neu tumor cells are comprised of a luminal population with high levels of CD61, although the level of CD61 expression is dramatically decreased in the JAK1-deficient tumor cells. Interestingly, the expression of CD61 correlates with capacity of cells to form spheres, supporting the hypothesis that ErbB2 tumors are highly enriched for tumor-initiating cells. This finding implies that JAK1 may be implicated in the tumor-initiating cell population of established disease. The tumor initiating cells have been suggested to be responsible for metastatic disease [128], and may explain the reduced propensity of JAK1-deficient cells to metastasize.

Downstream targets of JAK1 signaling contribute to tumor growth

We found that the deletion of Jak1 in the Erbb2 murine tumor model establishes JAK1 as the critical activator for STAT3 in a tumor cell-intrinsic mechanism. To identify downstream targets of JAK1-mediated signaling, we performed whole genome RNA-Seqencing and GSEA, identifying genes and pathways deregulated in a JAK1-dependent manner. Among the targets identified were proto-oncogenes, Fos, and Map3k8. Interestingly, these genes were also found to be deregulated in a JAK1-dependent manner during remodeling events of the mammary gland as identified by RNA-Seq and verified by immunostaining (Chapter 3, FOS data shown, Map38 data not
shown). These data support that JAK1 maintains contribution to similar downstream targets from normal development to established disease, pointing to a possible conserved mechanism for their activation. We hypothesized whether either of these targets would able to reinstate a growth, migratory, or tumorsphere-forming behavior, on their own in the absence of JAK1.

In the first line of rescue experiments, we targeted FOS for re-expression, as the proto-oncogene is recognized in cancer progression. FOS family members dimerize with Jun to form the Activating Protein-1 transcription factor complex (AP-1), which affects target gene expression. In the breast, oncogenic functions of FOS are described to contribute to proliferation, loss of polarity, and implicated in aggressive ErbB2-positive breast cancer [129, 130]. Re-expression of FOS partially rescued the in vivo growth properties of the JAK1-deficient cells, although not to the degree of wild-type JAK1 tumor cells. Past studies have demonstrated that a knockdown of FOS inhibits MCF7 breast cancer cell proliferation [131] and correlated with our paired observations of significant Fos deregulation in the JAK1-deficient cells combined reduced growth in vivo. However, the replaced expression could not recapitulate a full growth profile compared to JAK1 wild-type tumor cells. These results indicate that re-expression of FOS in a JAK1-deficient background is not sufficient to drive cancer cell growth. Further studies will identify if FOS contributes to migration and the tumor-initiating cell population. Our observations indicate that JAK1 contributes to the expression and activation of a network of genes that are necessary for driving growth in transformed cells. Further studies that replace expression of deregulated targets will depict if there are direct targets of JAK1 signaling that promote the tumor-initiating cells and growth of the bulk tumor.
Chapter 5: Summary
Summary

The primary focus of our work explores the molecular regulation of the JAK/STAT pathway and its relevance in signal transduction, promoting proliferation and cell maintenance. Extensive work has described the role of STAT proteins in development, along with their presence and activation in malignancy, much less has been explored in relation to their upstream activators, the Janus kinases. Although JAK2 had been highly characterized in the developing organ, little was known of the function of JAK1, the other predominantly expressed Janus kinase in the mammary epithelium. The mechanism of JAK2 is described to mediate PRL and GH signaling, driving the differentiation of the alveolar cells through STAT5 [12, 13]. JAK2 was also thought to contribute to the involution events, mediating the inflammatory cytokines and activating STAT3 to initiate and promote the remodeling of the differentiated gland. However, JAK2 appeared to be dispensable for activating STAT3 due to the observation of persistent STAT3 activation in Jak2-deficient tumor cells [80]. Due to these observations, we questioned whether JAK1 would play a crucial role in the postlactational remodeling events, mediating inflammatory cytokine signaling. Furthermore, does JAK1 contribute to inflammatory cytokine signaling in mammary tumorigenesis and disease progression?

We first began studies by developing a conditional knockout of Jak1 to bypass embryonic lethality. We confirmed from the previously generated Jak1 conventional knockout by Rodig et al. 1998, that JAK1 contributes to the activation of STAT3, and additionally, the activation of two other transcription factors expressed in the mammary epithelial tissue, STAT1 and STAT6. Followed by a targeted deletion in the mammary gland. We saw no evidence of negative selection against Jak1-deficient cells during development, although importantly, we saw a striking phenotype by a massive delay in involution. Further studies confirmed this delay was due to impaired mediation of the inflammatory cytokines OSM and LIF, and also the absence in phosphorylation of
STAT3. Using RNA-Seq, we identified an array of genes selectively regulated in a JAK1-dependant fashion, and by ChIP-Seq verified binding targets of STAT3 versus STAT5. Two of these targets were BMF and Bcl2l11 (BIM), both proapoptotic proteins that are responsible for the initial breakdown of the mammary epithelial cells during involution. This was verified first in silico, confirmed at the protein expression level in involution samples of wild-type and JAK1-deficient tissue, and demonstrated biologically with Bim/Bmf double knockout tissue during involution. These experiments confirmed the integral function of JAK1 and its necessity for mediating STAT3 activation in response to inflammatory cytokine signaling in mammary gland remodeling.

Next, we extended our findings to disease initiation and malignant progression. There is a plethora of previous publications describing the association of STAT3 and aggressive disease [70, 104, 132], and although efforts have been made to target this transcription factor directly, success has been limited due to the inherent nature of STAT3 existing as a latent factor in the cytoplasm. We targeted the deletion of JAK1 prior to tumor onset in ErbB2-driven mammary cancer, though we did not observe a significant effect on tumor initiation, there was a defined reduction in the occurrence of metastatic disease. To compare the effects on tumor progression, we established cell lines from tumor mice bearing the Jak1^fl/fl alleles and deleted Jak1 via viral-mediated Cre recombinase. The cells that were deficient in JAK1 displayed a drastic decrease in STAT3 activation. Also, when transplanted into recipient wild-type mice, the tumor cells lacking JAK1 had a significant reduction in growth rate and no evidence of metastasis; whereas tumor cells harboring wild-type JAK1 grew prolifically and metastasized to the lung and in some cases the liver and pancreas. The substantial differences in the growth rates indicated an effect on the tumor initiating cell population. Using tumor sphere assay in vitro, we observed a striking decrease in the amount and size of tumor-forming cells. RNA-Seq analysis identified deregulated genes that mirrored what we observed
phenotypically, that the tumor cells had deregulation of adhesion molecules and oncogenes (such as c-FOS) in a JAK1-dependant manner. Initial rescue studies showed a partial rescue of the delayed growth of tumor cells. Further experiments will demonstrate the genes and subsequent proteins necessary for tumor growth downstream of JAK1/STAT3 signaling. Interestingly, when human breast cancer cells lines which overexpress HER2 are depleted of JAK1, the same transcription factors seen in the murine tissue—STAT1, STAT3, and STAT6—demonstrated a lack of activation. Functional studies will illustrate whether JAK1 depletion has the same effect in human malignancy as observed in our murine studies. If this is the case, then efforts made to develop a JAK1-specific inhibitor would be advantageous for targeting active STAT3 and preventing metastatic dissemination of breast cancers. The aspect of JAK1 affecting established cancer is pertinent, as the majority of clinically addressing malignancies is for the treatment of established cancers.
Chapter 6: Future Directions
Future Directions

The findings from the biological impact of deleting Jak1 in neoplastic tumors were surprising and left many questions to be answered. Namely, what are the tumor-initiating roles of JAK1 in the primary murine tumor cells, and does the absence of JAK1 in human cancer cells impart similar behavior observed in the murine cells? The following experiments address JAK1, but also the downstream targets of JAK1 signaling, as well as the effect of impacting human health by targeting JAK1 specifically by inhibitors.

Verifying the biological significance of JAK1-dependant targets

In continuation of identifying JAK1-dependant target genes responsible for growth and proliferation, the target Tpl2 (MAP3K8) will be re-expressed. MAP3K8 has been demonstrated to be a potent transforming factor in lung cancers as well as in hepatocellular carcinomas [120, 133]. Little is known of its role in mammary tumor progression. Results of re-expression in murine mammary tumor cells will verify its transformation capabilities and potential to influence growth. Preliminary results indicate that MAP3K8 is indeed an oncogene capable of reinstating neoplastic characteristics to JAK1-deficient cells (Fig. 6.1).

To further verify the expression and JAK/STAT molecular pathways influencing expression of these target genes, STAT3 activity will be re-instated in JAK1-deficient cells by constitutive mutation of STAT3. The expression of STAT3 and the correlation with increased expression of FOS and MAK3K8 would solidify the dependence of STAT3 for transcriptional activation, which is regulated in a JAK1-dependant manner. Verification by Western blot, qPCR, and immunostaining is expected to confirm expression of the targets.
Addressing the role of JAK1 specifically in ErbB2-driven cancer cells and breast cancer tissue

In the previous chapter, we described the role of JAK1 contributing to the activation of STAT3 along with STAT1 and STAT6 in murine Erbb2-driven tumor cells. Extending the role of JAK1 into human disease, we generated a doxycycline-inducible shRNA knockdown of human JAK1, and tested the knockdown on MDA-453 breast cancer cells, which overexpress the ErbB2 oncoprotein. Preliminary results demonstrate similar findings, that JAK1 is necessary for the activation of STAT1, STAT3 and STAT6, in steady-state cell culture conditions (Fig. 6.2A). This contradicts previous reports that promote the activation of JAK2 phosphorylating these downstream transcription factors [134]. After verifying the knockdown of JAK1 in the human breast cancer cell lines, RNA-Seq was performed and verified the knockdown of JAK1 in a Dox-inducible manner (Fig. 6.2B). In conclusion these initial results are encouraging, as perhaps the JAK1-knockdown in human cells may show similar behavior of the murine counterparts. Additionally, we performed a JAK1-knockdown in the triple-negative cell line, MDA 231, and the activation of STAT3 was also decreased, suggesting that JAK1 may have a role in regulating STAT3 expression across multiple breast cancer subtypes. These results are encouraging, as triple negative tumors lack targeted therapy. The inhibition of JAK1 could influence the growth and metastatic potential observed in the previously described ErbB2 studies (Fig. 6.2C) (Chapter 4). To address the role of JAK1 in human breast cancer tissue, we are able to collaborate with Dr. Hallgeir Rui at the Medical College of Wisconsin for staining human tumor samples. First, ERBB2-driven cancer tissue can be stained for the presence of active STAT3 in human breast cancer tissue. Protein expression could be analyzed by quantitative imaging for expression of
STAT3 in various stages of breast cancer progression, such as pre-malignant tissue, pre-treatment biopsies, post-treatment tissue, and recurrent disease. Next, the activation of STAT3 would then be compared with the expression of targets identified in the previous chapter such as FOS and the previously undescribed oncogene in the mammary gland, Tpl2 (MAP3K8). The validation in conjunction with STAT3 levels would support the method of using the expression of these targets as biomarkers for JAK1 inhibition.

**Novel avenues to test JAK1 inhibitors for efficacy both in vitro and in vivo**

We have the tools to directly assess the effects of current and developing drugs/compounds targeting either Jak1 or STAT3 activity in cells deficient in Jak1. Genetic knockouts biologically simulated the use of an inhibitor, by directly ablating the function of a target. Using a novel inhibitor INCB039110, developed by Incyte, targeting JAK1 could reveal the specificity of the compound and delineate off-target effects. The compound, which has not been validated in an array of normal and transformed cells, has been demonstrated to be well tolerated in topical application studies [135]. The study could be constructed in a way to test the compound on epithelial cells, exemplifying non-tumor samples, tumor cells wild-type for JAK1, and tumor cells genetically deficient in Jak1. First describing the effects on non-tumor epithelial cells versus immortalized and oncogenically-transformed epithelial cultures, comparing the phenotypes and molecular downstream targets of the JAK/STAT pathway such as the STATs themselves. This data could then be expanded, comparing the effects of the inhibitor on tumor cells lacking JAK1 alongside matching wild-type tumor cells, assessing the results for synergy of a JAK inhibitor on wild-type cells versus genetically ablated kinase. Concise verification of the effects of drugs in vitro could be analyzed by whole genome RNA-Seq, verifying differential gene expression. Building from the initial
cell culture analysis, the in vivo application could be demonstrated by transplanting MMTV-neu-driven cancer cells wild-type for JAK1, which have shown to engraft, proliferate, and produce distant metastasis, or the Jak1-null tumor cells, into the mammary gland of Athymic nude mice. This would provide several lines of evidence describing the efficacy of the inhibitor. First, it would provide insight to the targeted inhibition of JAK1 reproducing the same effects displayed in the previous study described in Chapter 4, where a deletion in Jak1 inhibited tumor growth and metastasis in transformed cells. This could be done in several stages to address the role of JAK1 extensively in tumor progression. The transplanted wild-type cells could be allowed engraftment and inhibitor could be administered several days post-engraftment, when the tumor is 0.5 cm in size, or the tumor could also be allowed to engraft, grow to a select size, then biopsied with drug administration shortly after, observing the effects of treatment on residual disease concurrently with its effect on metastasis. This assessment in drug performance would mimic the clinical administration in human disease, where a HER2+ breast cancer may be treated upon diagnosis, during advanced disease, or adjuvant/post-operative therapy for refractory disease of the primary tumor as well as controlling for present or potential metastatic disease. Moving the studies to human breast cancer, HER2+ breast cancer cell lines that have been stably infected with an inducible JAK1-knockout by shRNA in a doxycycline-responsive manner, can also be used in vitro and in vivo (transplanted into Rag2−/−Il2rg−/− recipient mice), using the same inhibitor treatment strategy. Comparison of the transplanted mice with the JAK1-knockdown via Dox alongside the inhibitor treatment would begin with tumor measurement by calipers, followed by collection of tumors for molecular analysis. Initial studies using Western blot would test for the deregulation of STAT proteins in a JAK1-dependent manner. Analysis of previously identified targets of JAK1/STAT3
signaling would verify that the same targets are deregulated by the inhibitor similar to a JAK1 knockdown or deficiency.

These future studies will provide valuable insight on the direct contribution of JAK1 to breast cancer progression. Understanding the role of JAK1 and its downstream effectors in established cancer will demonstrate the significance of clinically targeting JAK1.
Fig. 6.1.

A

\[ \text{Jak1KO} \quad \text{Jak1 KO + Map3K8} \]

B

\[ \text{Jak1 WT} \quad \text{Jak1KO} \quad \text{Jak1 KO + Map3K8} \]
Fig. 6.1. Expression of MAP3K8 in JAK1-deficient cells demonstrates rescue of neoplastic capabilities. Expression of MAP3K8 in cell lacking JAK1 activity demonstrates loss of polarity in 2D cell culture (A) (view 10x magnification). Reinstating MAP3K8 rescues the phenotype of JAK1-deficient cells (Jak1 KO) by producing tumorspheres in low-attachment conditions.
Fig. 6.2.

A

MDA 453 shJAK1A

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B

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MDA 231 shJAK1

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Fig. 6.2. Expression of JAK1 in human breast cancer cell lines corresponds to the activity of STAT3. Using the ErbB2-driven human breast cancer cell line, JAK1 was knocked down by inducible shRNA using Doxycycline. Knockdown of JAK1 demonstrates reduced activation of STAT1, STAT3, and STAT6, by Western blot, similar to Erbb2-driven murine disease (A). Initial studies of RNA-Seq depict the reduced expression of JAK1 in a Dox-induced manner (B). Preliminary Western blot results of a knockout of JAK1 in the triple negative cell line correlates with a decrease in phospho-tyrosine STAT3, indicating a broad application of JAK1 regulating STAT3 in breast cancer (C).


98. Abubaker, K., et al., Targeted Disruption of the JAK2/STAT3 Pathway in Combination with Systemic Administration of Paclitaxel Inhibits the Priming of Ovarian Cancer Stem Cells Leading to a Reduced Tumor Burden. Front Oncol, 2014. **4**: p. 75.


