# INTERROGATING THE REGULATION AND FUNCTION OF *ZFP30* IN AIRWAY INFLAMMATION

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## ABSTRACT

Lucas Tanner Laudermilk: Interrogating the Regulation and Function of *Zfp30* in Airway Inflammation
(Under the direction of Samir Kelada)

Neutrophils play an integral role in the innate immune system, but robust recruitment of these cells can cause collateral host tissue damage. Neutrophils have been linked to the severity of a number of pulmonary diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and acute respiratory distress syndrome. These diseases represent a significant economic and health burden, so increased understanding of neutrophil chemotaxis is of vital importance. In my dissertation, I have utilized molecular biology and mouse models to characterize *Zfp30*, a candidate regulator of the hallmark neutrophil chemokine CXCL1. I identified a specific SNP, rs51434084, that underlies a previously identified *Zfp30* eQTL, and I demonstrated the mechanism through which this SNP impacts transcription: differential binding of the transcriptional repressor ZFP148. ZFP148 has a known regulatory role on the neutrophil chemokine CXCL5, and our results suggest an additional impact on CXCL1 via *Zfp30*.

Additionally, I utilized an *ex vivo* model of airway epithelial cells generated from a *Zfp30* knockout mouse strain on a C57BL/6J background to demonstrate that *Zfp30* significantly modulates CXCL1 and IL-6 secretion in airway epithelial cells. *In vivo* models utilizing this knockout mouse strain revealed additional effects of *Zfp30*

knockout on IL-6 secretion in BALF, liver and spleen development, and blood glucose level. These studies explored and confirmed an impact of *Zfp30* on the secretion of cytokines that modulate airway neutrophilia, and this body of work lays a foundation off of which future studies of *Zfp30*'s impact on development and diabetes may extend.

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## LIST OF ABBREVIATIONS

ARDS: acute respiratory distress syndrome

BALF: bronchoalveolar lavage fluid

bp: basepair

CBC: complete blood count

CC: collaborative cross

COPD: chronic obstructive pulmonary disease

EMSA: electrophoretic mobility shift assay

ENCODE: Encyclopedia of DNA Elements

eQTL: expression quantitative trait locus

FBS: fetal bovine serum

$FEV_1$: forced expiratory volume in 1 second

H&E: hematoxylin and eosin stain

HCG: human chorionic gonadotropin

FLA-ST: flagellin protein isolated from *Salmonella typhimurium*

HDM: house dust mite

KRAB: Krüppel associated box

KO: knockout

LPS: lipopolysaccharide

mRNA: messenger RNA

MTEC: mouse tracheal epithelial cell

NET: neutrophil extracellular trap

NLR: neutrophil-to-lymphocyte ratio

| | |
|---|---|
| PCR: | polymerase chain reaction |
| PMSG: | pregnant mare’s serum gonadotropin |
| qPCR: | quantitative polymerase chain reaction |
| QTL: | quantitative trait locus |
| RNA-seq: | RNA sequencing |
| SDM: | site-directed mutagenesis |
| siRNA: | small interfering RNA |
| SNP: | single nucleotide polymorphism |
| TRD: | transmission ratio distortion |
| WT: | wildtype |
| ZFP: | zinc finger protein |

# CHAPTER 1: INTRODUCTION

## Overview and aims

Neutrophils are an abundant white blood cell population that plays an essential role in the innate immune system. They respond to infections with an arsenal of pathogen-killing mechanisms, but the activities of these mechanisms are largely not pathogen-specific (Henson and Johnston 1987; Weiss 1989). This means that poorly regulated or over-abundant neutrophil activity can lead to host tissue damage. This regulation is particularly important in the lungs and airways, because these tissues are constantly exposed to airborne pathogens, pollutants, and allergens that represent potential innate immune stimuli.

*Zfp30* emerged from a quantitative trait locus (QTL) mapping study of airway inflammation in mice as a candidate gene of interest in modulating neutrophil recruitment to the lung through regulation of the chemokine CXCL1 (Rutledge *et al.* 2014). This study identified an expression quantitative trait locus (eQTL) that regulated *Zfp30* expression and suggested that ZFP30 down-regulates *Cxcl1* through transcriptional repression (Rutledge *et al.* 2014). The aims of my research were to identify the causal variant(s) underlying the *Zfp30* eQTL and to further characterize the role that *Zfp30* plays in neutrophil recruitment to the lungs utilizing *in vivo*, *ex vivo*, and *in vitro* approaches.

In Chapter 2, I utilize molecular biology techniques, including cloning, luciferase assays, western blotting, and electrophoretic mobility shift assays to probe

the causal variants underlying the *Zfp30* eQTL. These experiments identify rs51434084 as a causal variant in the regulation of *Zfp30* expression in mice via altered binding of the transcription factor ZFP148. In Chapter 3, I explore the impact of ZFP30 loss in mice on multiple baseline phenotypes and on the inflammatory response to multiple immune stimuli: house dust mite (HDM) allergen, lipopolysaccharide (LPS), and ozone exposure. I additionally make use of *ex vivo* models generated from this mouse strain to probe cytokine responses of LPS exposure and find a significant impact on CXCL1 and IL-6 production. In Chapter 4, I reflect on the implications and impact of my results and discuss some of the nuances of my findings.

**Overview of neutrophils**

An innate immune response is typically initiated by soluble signals associated with pathogens, host damage, or both (Nathan 2002). These initial signals to mount a response are sensed by resident mast cells and macrophages of a tissue, and these cells secrete pro-inflammatory signals to recruit additional leukocytes. Neutrophils are a major population among these recruited cells.

Neutrophil migration to sites of damage or infection typically proceeds through a stepwise process that begins with rolling and adhesion of circulating neutrophils to activated endothelial tissue (von Andrian *et al.* 1991; Butcher 1991; Springer 1994). From this point, neutrophils polarize to achieve a leading edge with concentrated chemokine receptors, and gradients of chemotactic molecules guide neutrophils into the tissue through haptotaxis (Middleton *et al.* 2002; Handel *et al.* 2005; Wang *et al.* 2005). Chief among the chemokines that direct neutrophil migration are the CXCR2

ligands (Charo and Ransohoff 2006). CXCR2 is a G-protein-coupled rector expressed on a number of cell types, including leukocytes, endothelial cells, and epithelial cells in the lung (Stadtmann and Zarbock 2012). CXCL8 (IL-8) (Baggiolini *et al.* 1989), CXCL1 (KC (mice)/Gro-α (human)) (Anisowicz *et al.* 1987; Richmond *et al.* 1988), CXCL2 (MIP-2 (mice)/Gro-β (human))(Haskill *et al.* 1990), and CXCL5 (LIX (mice)/ENA-78 (human)) (Walz *et al.* 1991) are among the hallmark neutrophil recruitment cytokines in humans and mice, though there is no known murine CXCL8 homolog.

Once recruited to the site of inflammation, the phagocytic activity of neutrophils increases (Sørensen *et al.* 2001), and a combination of integrin engagement and chemokine stimulation leads to neutrophil respiratory burst (Nathan 1987). The generation of reactive oxygen intermediates during respiratory burst is a key process in the innate immune system's antimicrobial response (Nauseef 2007; Robinson 2008; Flannagan *et al.* 2009). A second major antimicrobial mechanism is the release of toxic proteins and enzymes, such as myeloperoxidase, neutrophil elastase, and defensins via degranulation (Cortjens *et al.* 2017). The antimicrobial activity of neutrophils can even extend beyond the life of the cell itself via neutrophil extracellular trap (NET) formation. NETosis is a mechanism of cell death in which the nuclear envelope and granule membranes disintegrate, and strands of de-condensed DNA coated in antimicrobial proteins are extruded from the cell (Fuchs *et al.* 2007). The formation of NETs is thought to be a last line of defense via which neutrophils can catch and destroy nearby pathogens (Brinkmann *et al.* 2004).

Careful balance of neutrophil recruitment is important, as impaired neutrophil recruitment to sites of inflammation can lead to unchecked infections, as seen in leukocyte adhesion deficiency (Abram and Lowell 2009), and an overzealous recruitment of neutrophils can lead to host tissue damage. Much of the neutrophil's armory of antimicrobial mechanisms lacks specificity to pathogens and represents a potential source of collateral host tissue damage during a robust response (Nathan 2006). In addition to direct impacts on host tissue, neutrophils secrete a broad repertoire of cytokines to recruit additional leukocytes to sites of infection, and they participate in complex cross-talk with multiple immune cell types (Mantovani *et al.* 2011). To better understand how a neutrophilic response may minimize collateral damage without compromising response to invading microbes, further study of the tight regulation of neutrophil recruitment is vital.

**Neutrophils in pulmonary disease**

The literature points to a broad importance of airway neutrophilia in pulmonary disease severity, so the regulation of neutrophil recruitment is a vital area for study. Acute respiratory distress syndrome (ARDS) is a disease of lung damage that can include diffuse alveolar damage, neutrophil alveolitis, edema, and hemorrhage (Reilly 2017). Clinical risk factors alone are poor predictors of patients that will develop or survive ARDS, and it is thought to be a complex disease in which many genetic variants each confer a small amount of risk (Reilly 2017). Neutrophils play a clear role in the pathogenesis of ARDS, with neutrophil-associated gene expression levels and neutrophil chemotaxis levels corresponding to increased epithelial permeability, edema, and disease progression (Chollet-Martin *et al.* 1992;

Grommes and Soehnlein 2011; Williams and Chambers 2014; Kangelaris *et al.* 2015).

Chronic obstructive pulmonary disease (COPD) is a progressive disease of the small airways characterized by persistent airflow limitation and a chronic inflammatory response (Vestbo *et al.* 2013). Smoking is the major risk factor for development of COPD, but non-smokers may develop COPD and it is believed that genetics may play a role in disease development (Vestbo *et al.* 2013). Chronic lower respiratory diseases, primarily COPD, were the third leading cause of death in the United States in 2014 (National Center for Health Statistics 2016), and COPD impacts an estimated 6.5 percent of people in the United States (Ford *et al.* 2015). In 2010, medical costs associated with COPD were estimated at $32.1 billion, and this was projected to increase to $49 billion in 2020 (Ford *et al.* 2015). A neutrophilic infiltrate in the airways and elevated CXCR2 chemokine levels are associated with the pathology of COPD and disease severity, and the activity of neutrophil proteases and NETs are associated with tissue damage and declining lung function (Qiu *et al.* 2003; Quint and Wedzicha 2007; Hoenderdos and Condliffe 2013; Günay *et al.* 2014; Duman *et al.* 2015; Grabcanovic-Musija *et al.* 2015).

Cystic Fibrosis (CF) is an often fatal autosomal recessive genetic disorder that impacts a chloride conducting transmembrane channel (Elborn 2016). In the lungs, this leads to progressive respiratory impairment caused by mucus retention, chronic infection, and inflammation (Elborn 2016). In CF, patient sputum has considerable neutrophil-stimulating activity, and neutrophil elastase serves as a biomarker of disease progression (Mackerness *et al.* 2008; Gifford and Chalmers

2014). Additionally, aberrant expression of *CXCL8* is thought to play a role in CF disease severity and is being pursued as a potential drug target (Jundi and Greene 2015). Recent evidence suggests that the role of neutrophils in CF may even extend beyond their direct mechanisms of antimicrobial toxicity (Laval *et al.* 2016).

Asthma is an allergic disease of the lungs and airways with both genetic and environmental underpinnings (Whitehead *et al.* 2003; Kelada *et al.* 2011; Ober and Vercelli 2011; Thomsen 2015). The disease has an increasingly appreciated heterogeneity, but the clinical presentation is typically characterized by reversible limitation of expiratory airflow, bronchial hyperresponsiveness, and airway inflammation (Wenzel 2012). In the United States, asthma impacts 8.3 percent of adults and children and costs the nation an estimated $81.9 billion per year (Centers for Disease Control 2016; Nurmagambetov *et al.* 2018). In 2016, asthma lead to 1.7 million emergency department visits and 3,518 deaths in the United States (Centers for Disease Control 2016). Asthma is typically thought of as an eosinophil-dominated disease, but in the 1990s, studies of severe asthma spurred interest in the heterogeneity of the disease. Many patients with severe asthma exhibit appreciable neutrophilia with varying presence of eosinophils, and subsets of inflammatory presentation are associated with differing clinical outcomes (Wenzel 2012). Neutrophil levels in the lung are associated with asthma disease severity and progression, and severe asthmatics show elevated levels of CXCL8 and neutrophil myeloperoxidase in sputum (Stănescu *et al.* 1996; Jatakanon *et al.* 1999; Wenzel *et al.* 1999; Little *et al.* 2002). Severe asthmatics also experience prolonged neutrophil survival not solely attributable to inhaled corticosteroid use and display poorer

response to glucocorticoids (Green *et al.* 2002; Uddin *et al.* 2010), and neutrophil levels scale with healthcare utilization among severe asthmatics (Moore *et al.* 2010). There is also accumulating evidence that NETosis contributes to airway obstruction in chronic pulmonary diseases, including asthma, COPD, and CF (Cortjens *et al.* 2017).

**The importance of CXCR2 signaling in pulmonary disease**

In recognition of the role that neutrophils play in the pathology of pulmonary diseases, CXCR2 is being heavily explored as a potential drug target (Jones *et al.* 1997; Walters *et al.* 2008; Chapman *et al.* 2009; Austin *et al.* 2015; Ha *et al.* 2015). Promising results from a number of studies suggest tolerability of CXCR2 antagonists and demonstrate a promising impact on neutrophil recruitment in multiple disease contexts (Matzer *et al.* 2004; Zarbock *et al.* 2008; Russo *et al.* 2009; Lazaar *et al.* 2011; Virtala *et al.* 2011; Konrad and Reutershan 2012; Nair *et al.* 2012; Leaker *et al.* 2013; Moss *et al.* 2013; Kirsten *et al.* 2015; Planagumà *et al.* 2015; Rennard *et al.* 2015; Todd *et al.* 2016; Watz *et al.* 2017). Upon CXCR2 antagonism, a reduction in airway neutrophils is noted in both mild atopic asthmatics and patients with severe or persistent neutrophilic asthma (Nair *et al.* 2012; Todd *et al.* 2016; Watz *et al.* 2017). Patients with COPD tolerated CXCR2 antagonism well with no significant increase in infections and showed fewer neutrophils in sputum along with improved forced expiratory volume ($FEV_1$) (Kirsten *et al.* 2015; Rennard *et al.* 2015), and patients with CF showed improvements in neutrophil counts and neutrophil elastase levels in sputum upon CXCR2 antagonism (Moss *et al.* 2013). These results highlight the importance of CXCR2 signaling regulation in contexts

where robust neutrophil recruitment may be detrimental and point to the need for increased understanding of this pathway's regulation.

**Overview of the collaborative cross**

The Collaborative Cross (CC) is a multiparental genetics reference population of recombinant inbred strains derived from eight founder strains of inbred mice (Collaborative Cross Consortium 2012). The eight founder strains consist of five classical inbred strains of mice (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, and NZO/HlLtJ) and three wild-derived strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ), and these strains represent genetic contributions from three mouse subspecies (*M. m. musculus*, *M. m. domesticus*, and *M. m. castaneum*). These founders represent at least 36,155,524 single nucleotide polymorphisms (SNPs) between them (Collaborative Cross Consortium 2012). The autosomes of the resulting inbred lines initially appeared to display similar levels of contribution from each of the CC founder strains (Collaborative Cross Consortium 2012), but more recent findings reveal a significant depletion in genome-wide haplotype frequencies of CAST/EiJ and PWK/PhJ (Srivastava *et al.* 2017).

Genetic reference populations exist for a number of model organisms, and they are popular for the study of complex traits (Collaborative Cross Consortium 2012). These populations are particularly valuable for the ability to design treatment vs. control studies or gene/environment interactions across a large number of recombinant inbred lines. The CC advances mouse genetic reference populations by increasing the level of genetic diversity above that of previous populations, and it displays more uniformly distributed genetic variants across the genome

(Collaborative Cross Consortium 2012). This increased genetic diversity makes the CC an ideal population for mapping studies and for the identification of novel mouse models of disease.

**Overview of Zinc Finger Proteins**

Zinc finger proteins (ZFPs) with $C_2H_2$ domains are the largest family of transcription factors in mice and humans (Gray *et al.* 2004; Vaquerizas *et al.* 2009). C2H2 ZFPs were the first class of ZFPs characterized and are named as such due to the Cys(2)His(2) residues that hold a single zinc atom in their structure (Razin *et al.* 2012). These proteins can bind to DNA in a sequence-specific fashion via their $C_2H_2$ domains, and a large percentage of these ZFPs additionally contain a KRAB domain that allows for recruitment of the known transcriptional repressor KAP1 (Friedman *et al.* 1996). KAP1 interacts with HP1, SETDB1, and histone deacetylases to induce heterochromatin formation (Ryan *et al.* 1999; Lechner *et al.* 2000; Schultz *et al.* 2002; Medugno *et al.* 2005), and these heterochromatin domains can spread across tens of kilobases to silence nearby genes (Groner *et al.* 2010). The sequence specificity conferred by $C_2H_2$ domains combined with the local heterochromatin-mediated silencing mechanism of KAP1 makes $C_2H_2$ zinc finger proteins potent regulators of gene expression.

Many ZFPs remain uncharacterized, but there is precedent among known ZFPs for regulation of immune response. ZFP36 (TTP) is an important immune regulator that impacts the turnover rate of TNFα and GM-CSF messenger RNAs (mRNAs) (Patial and Blackshear 2016). ZBTB20 impact the induction of inflammatory gene expression in macrophages (Liu *et al.* 2013). ZFP64 binds to the

promoters of TNFα, IL-6, and IFN-β and modulates NF-κB activity (Wang *et al.* 2013), and ZFAT is an important transcriptional regulator of immune genes in B and T cells (Koyanagi *et al.* 2008). ZNF160 is a zinc finger protein that down-regulates TLR4 in intestinal epithelia via KAP1-mediated heterochromatin domains (Takahashi *et al.* 2009), and *Zfp30* is hypothesized to down-regulate *Cxcl1* expression via a similar mechanism (Rutledge *et al.* 2014). The overall aim of this dissertation is to shed additional light on the regulation and function of *Zfp30*.

## CHAPTER 2: DIFFERENTIAL REGULATION OF *ZFP30* EXPRESSION IN MURINE AIRWAY EPITHELIA THROUGH ALTERED BINDING OF ZFP148 TO RS51434084[1]

### Introduction

Recruitment of neutrophils to the lung is a hallmark of innate immune responses to inhaled pathogens and air pollutants, and is associated with decreased lung function and disease susceptibility (Nathan 2006; Borregaard 2010; Mantovani *et al.* 2011). Neutrophils play an integral role in immune response to external stimuli by migrating toward resulting chemotactic signals and responding to pathogens through phagocytosis and secretion of reactive oxygen species, proteases, and cytokines. These reactive oxygen species and proteases, however, do not distinguish between host and pathogen, therefore a robust recruitment of neutrophils can adversely impact host tissue. This makes tight regulation of neutrophil recruitment and their activity crucial. A neutrophilic infiltrate in the airways is seen in a range of pulmonary diseases, including asthma (Jatakanon *et al.* 1999; Wenzel *et al.* 1999), acute respiratory distress syndrome (ARDS) (Chollet-Martin *et al.* 1992; Grommes and Soehnlein 2011; Williams and Chambers 2014; Kangelaris *et al.* 2015), chronic obstructive pulmonary disease (COPD) (Qiu *et al.* 2003; Quint and Wedzicha 2007; Hoenderdos and Condliffe 2013; Günay *et al.* 2014; Duman *et al.*

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[1]Chapter 2 is featured as an article, "Differential Regulation of *Zfp30* Expression in Murine Airway Epithelia Through Altered Binding of ZFP148 to rs51434084" in *G3: Genes, Genomes, Genetics*, Volume 8, No. 2, Pages 687-693.

2015; Grabcanovic-Musija *et al.* 2015), and cystic fibrosis (CF) (Mackerness *et al.* 2008; Gifford and Chalmers 2014; Laval *et al.* 2016). In asthma, neutrophil levels in sputum have been shown to correlate with disease severity and lung function, as well as a lack of responsiveness to glucocorticoids (Stănescu *et al.* 1996; Green *et al.* 2002; Uddin *et al.* 2010).

Previously, we applied a house dust mite model of asthma to incipient lines of the Collaborative Cross (CC) population to identify novel genes and pathways associated with neutrophil recruitment responses in the context of allergic inflammation. The CC is a new mouse genetics reference population composed of recombinant inbred lines that are derived from eight founder strains (Collaborative Cross Consortium 2012). We measured neutrophil recruitment responses and levels of the hallmark neutrophil chemokine CXCL1 (aka KC) in bronchoalveolar lavage fluid and found that both were regulated by a locus on chromosome 7 (25.6–29.7 Mb) that we called *Dpc1* (Rutledge *et al.* 2014). Likewise, the expression of the gene Zinc finger protein 30 (*Zfp30*) was also regulated by this locus [*i.e.*, is an expression quantitative trait locus (eQTL)], and *Zfp30* expression was strongly correlated with CXCL1 and neutrophil counts (Figure 2.S1). We also found that *Zfp30* is expressed in airway epithelia and that knockdown of *Zfp30* in an epithelial cell line resulted in increased CXCL1 production in response to endotoxin. These results led to development of a model in which ZFP30 negatively regulates CXCL1, and CXCL1 then affects neutrophil recruitment, all under the control of *Dpc1*.

Relatively little is known about *Zfp30* except that it encodes an as yet uncharacterized C2H2 zinc finger protein that contains a Krüppel-associated box

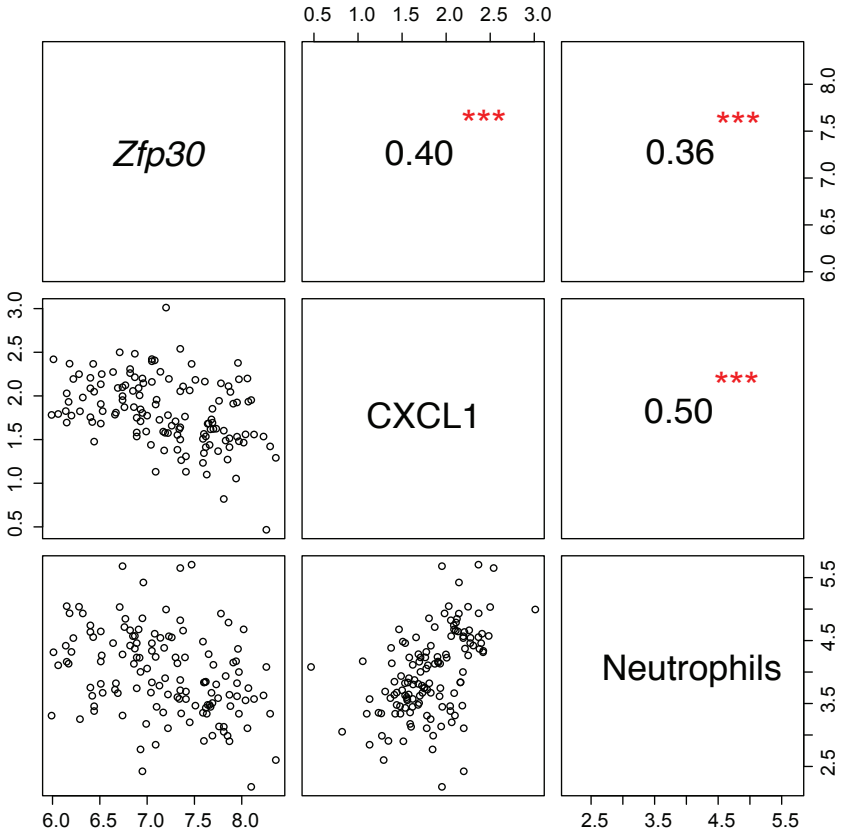


**Figure 2.S1: Pairwise correlations between *Zfp30* expression, CXCL1, and neutrophils in incipient CC lines**. Plot is derived from previously published data (Rutledge et al., 2014). *Zfp30* expression is expressed in normalized (log2) units, CXCL1 units are log(pg/ml), and neutrophil counts are log10(cell count). Numbers above diagonal denote Pearson correlation values, and asterisks denote p-value threshold of <0.001.

domain, signifying a role in gene repression via heterochromatin formation (Friedman *et al.* 1996). However, two features of the *Zfp30* eQTL provided important insights about *Zfp30* regulation. First, allele-specific gene expression data provided convincing evidence that the eQTL acts in *cis*. Second, haplotypes spanning the 5′ region of the gene, but not the 3′ region, were strongly correlated with *Zfp30* expression. More specifically, we found that expression levels in CC mice correlated strongly with the strain distribution pattern of 5′ region haplotypes: mice with haplotypes from NOD/ShiLtJ, NZO/H1LtJ, 129S1/SvImJ, or A/J founder strains had high expression; mice with haplotypes from C57BL/6J or WSB/EiJ founder strains had moderate expression; and mice with haplotypes from CAST/EiJ or PWK/PhJ founder strains had low gene expression.

These findings prompted us to seek to identify the specific variant(s) that regulate *Zfp30* expression. In this study, we utilized sequence data, epigenomic data, reporter gene assays, and electrophoretic mobility shift assays (EMSA) to interrogate the effect that specific variants have on *Zfp30* expression. In aggregate, our data point to a causal variant responsible for the modulation of *Zfp30* expression, and show that this variant alters transcription factor binding.

## Materials and Methods

### Cell culture

The MLE12 mouse lung epithelia cell line (generated from FVB/N mouse strain) was cultured in a 1:1 mix of Dulbecco's Modified Eagle Medium and Ham's F12 supplemented with 5% fetal bovine serum (FBS). The LA4 mouse lung airway cell line (generated from the A/He mouse strain) was cultured in Ham's F12K

medium supplemented with 15% FBS. Both cell lines were grown at 37° with 5% $CO_2$. Twenty-four-well plates were seeded at a density of 120,000 cells per well for MLE12 or 125,000 cells per well for LA4 24 hr prior to transfection for luciferase assays.

**Dual luciferase reporter assay**

A 500 basepair (bp) genomic region of the *Zfp30* promoter surrounding a euchromatic region containing a variant of interest was amplified from five of the eight CC founder strains. Amplified candidate promoters were subcloned into the multiple cloning site upstream of the firefly luciferase gene in the promoterless pGL4.10 vector (Promega, Madison, WI) using a Gateway subcloning approach, and then verified by sequencing. MLE12 or LA4 cell lines were cotransfected in 24-well cell culture plates with 500 ng of candidate promoter constructs and 28 ng of *Renilla* control vector pRL-SV40 (Promega), using Lipofectamine 3000 (Life Technologies, Carlsbad, CA), according to manufacturer's protocol. Transfected cells were cultured 48 hr before cell lysates were collected. Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity as a control for transfection efficiency. Data are reported as the ratio of firefly to *Renilla* luciferase activity, and these data are provided in Table S1[2]. Assays include four replicates for each candidate promoter and two technical replicates for each lysate collected. Luciferase assays were performed in each cell line at least two times, and results were

[2]Table S1 is available online at:
http://www.g3journal.org/content/8/2/687.supplemental

consistent across experiments. Welch's two-sided *t*-tests were performed to compare luciferase activity between promoter constructs.

**Quantitative Polymerase Chain Reaction (qPCR) of luc2 and Renilla luciferase**

mRNA expression of firefly and *Renilla* luciferase was assayed in MLE12 RNA samples collected 48 hr post-transfection with candidate promoters and pRL-SV40. iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) was used in conjunction with *luc2* primers (Fwd: 5′-GTGGTGTGCAGCGAGAATAG-3′; Rev: 5′-CGCTCGTTGTAGATGTCGTTAG-3′) or *Renilla* luciferase primers (Fwd: TCACTATAGGCTAGCCACCAT-3′; Rev: 5′-CACTGCGGACCAGTTATCATC-3′). We validated the qPCR primers for efficiency by performing assays with serial dilutions of template and verifying the expected changes in Cq values. In subsequent assays, *Renilla* luciferase levels were used to normalize *luc2* Cq values, and fold change in expression of A/J and 129S1/SvImJ over C57BL/6J was calculated.

**Identification of genetic variants among CC founder strains**

We utilized the Sanger Mouse Genomes Project variant database (Keane *et al.* 2011) (http://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1505) to identify variants within our cloned promoter region, which spans 29783622–29784093 bp on chromosome 7. These variants are shown in Table 2.1.

**Site-directed mutagenesis**

Site-directed mutagenesis to change the rs51434084 allele in pGL4.10 constructs was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) according to manufacturer's protocol. Primers (Fwd:

5′-CCTCCAACCCCCTTCCCGTGACCAAGAGCTAGGGGCC-3′; Rev: 5′-GGCCCCTAGCTCTTGGTCACGGGAAGGGGGTTGGAGG -3′) were designed to mutate the position corresponding to rs51434084 from C to G.

**EMSA**

Complementary 17 bp oligonucleotide probes centered on rs51434084 were purchased from (Integrated DNA Technologies, Coralville, IA) for use in EMSA experiments. Sequences for the moderate (C57BL/6J) and high (A/J) expression haplotypes, respectively, were as follows: Fwd: 5′-CCCTTCCCCTGACCAAGAG-3′; Rev: 5′-CTCTTGGTCAGGGGAAGGG-3′; and Fwd: 5′-CCCTTCCCGTGACCAAGAG-3′; Rev: 5′-CTCTTGGTCACGGGAAGGG-3′. Sets of complementary probes were ordered with and without biotin end-labeling (Integrated DNA Technologies). Complementary oligos were annealed into double-stranded DNA probes at a concentration of 2 pmol/µl by heating complimentary oligos to 95° in an annealing buffer (10 mM Tris, 50 mM NaCl; 1 mM EDTA pH 8.0) and cooling them to 25° in a thermocycler at a rate of 1° per minute. NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA) were used to collect nuclear protein lysates from MLE12 cells. Protein concentrations were quantified using the Pierce BCA Protein Assay (Thermo Scientific).

EMSA experiments were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's protocol. DNA–protein binding reactions contained 1× binding buffer, 1 µg poly(dIdC), 4 µg nuclear protein lysate, 200 fmol biotinylated DNA probe, and water, for a total reaction volume of 20 µl. Reactions were incubated at room temperature for 30 min prior to gel loading.

Reactions including nonbiotinylated probes for DNA competition were incubated with 45-fold excess unlabeled probe for 25 min prior to addition of biotinylated probe. ZFP148 supershift reactions contained either 10 µg (Figure 2.4) or 20 µg (Figure 2.S3) antibody incubated for 30 min prior to addition of biotinylated probe. Antibodies for ZFP148 (Fisher Scientific, Hampton, NH) and KLF4 (Millipore, Burlington, MA) were ordered for use in EMSA supershifts. DNA–protein complexes were separated on 6% DNA retardation gels (Life Technologies) using 0.5× TBE buffer (Life Technologies). Complexes were transferred to Biodyne B Pre-Cut Modified Nylon Membranes (Thermo Scientific) and UV cross-linked before chemiluminescent detection.

**Transcription factor binding site prediction**

To identify transcription factor binding sites potentially affected by rs51434084, we used TRANSFAC (http://generegulation.com/pub/programs.html#match) and HOCOMOCO (http://opera.autosome.ru/perfectosape/scan/new) prediction tools. Based on EMSA results indicating enhanced binding of nuclear proteins to the C57BL/6J allele (*vs.* 129S1/SvImJ), we limited our search to putative transcription factor binding sites in which the C57BL/6J allele is predicted to bind the transcription factor better than the 129S1/SvImJ allele. We then performed literature searches on the initial list of candidates to prioritize any transcription factors with a known connection to regulation of neutrophil recruitment.

## Results

### Candidate regulatory region for Zfp30

We sought to identify the causal variant (or variants) responsible for the *Zfp30* *cis*-eQTL on chromosome 7 we previously identified in whole lung tissue from incipient CC lines, which we showed was associated with variation in CXCL1 and neutrophil counts in response to allergen challenge (Rutledge *et al.* 2014). In that study, we found that incipient CC lines with chromosome 7 QTL region haplotypes from NOD/ShiLtJ, NZO/H1LtJ, 129S1/SvImJ, or A/J strains had high *Zfp30* expression, mice with haplotypes from C57BL/6J or WSB/EiJ founder strains had moderate expression, and mice with haplotypes from CAST/EiJ or PWK/PhJ founder strains had low gene expression. This same pattern of expression was observed among CC founder lines (Table 2.1), indicating that the *cis*-eQTL is the major determinant of *Zfp30* expression.

To identify regulatory regions that may contribute to variation in *Zfp30* expression, we examined Encyclopedia of DNA Elements (ENCODE) data for euchromatic regions marked by DNAse I hypersensitivity near the 5′ region of *Zfp30* in mouse lung tissue, because our previous data indicated a strong correlation between 5′ region haplotypes and gene expression (Rutledge *et al.* 2014). We examined a region spanning 10 kb upstream through intron 1 and identified a ~ 500 bp region of open chromatin near the putative *Zfp30* promoter and transcription start site (Figure 2.S2), which contains 10 SNPs. An examination of the strain distribution patterns within the identified region of open chromatin at the *Zfp30* promoter

| Strain[a] | *Zfp30* Expression[b] | Relative Expression | Expression Group | rs32436680 | rs258741331 | rs31291744 | rs211739374 | rs230547036 | rs252419650 | rs578896492 | rs581321876 | rs239071968 | rs51434084 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A/J | 7.72 | 1.86 | High | C | T | C | C | G | C | T | C | G | G |
| NOD/ShiLtJ | 7.65 | 1.77 | High | C | T | C | C | G | C | T | C | G | G |
| NZO/H1LtJ | 7.56 | 1.67 | High | C | T | C | C | G | C | T | C | G | G |
| 129S1/SvImJ | 7.57 | 1.68 | High | T | T | T | C | G | C | T | C | G | G |
| C57BL/6J | 6.82 | 1.00 (Ref.) | Moderate | T | T | T | C | G | C | T | C | G | C |
| WSB/EiJ | 6.69 | 0.91 | Moderate | T | T | T | C | G | C | T | C | G | C |
| CAST/EiJ | 6.48 | 0.79 | Low | C | C | C | A | C | T | C | T | A | C |
| PWK/PhJ | 6.42 | 0.76 | Low | C | T | C | A | C | T | C | T | A | C |

**Table 2.1: *Zfp30* expression levels among CC founder strains and strain distribution patterns (SDPs) for 5' region SNPs.** a: The Sanger Mouse Genomes Project variant database was utilized for SNP data. b: Expression data (log2 units) based on data from Rutledge *et al.* (2014).

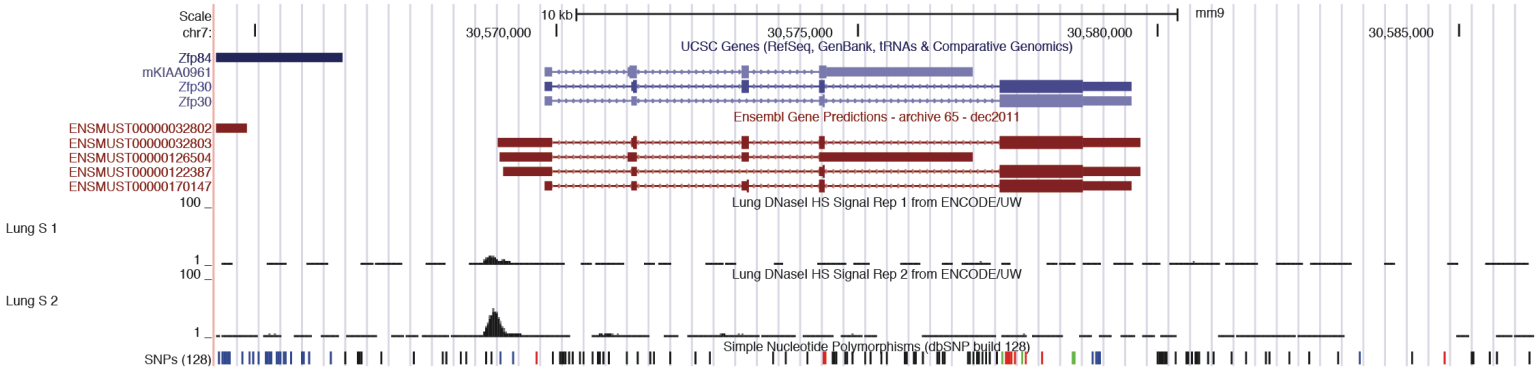


**Figure 2.S2: ENCODE Data reveals a candidate regulatory region.** DNase I hypersensitivity peaks in mouse lung tissue reveal an open region of chromatin and candidate regulatory region. The region cloned for luciferase assays spans Chr7:29783622-29784093 bp (mm10).

revealed a single shared SNP (rs51434084) among the high expression strains and six shared SNPs among the low-expressing strains (Table 2.1). These variants were thus considered priority candidates that might affect regulation of *Zfp30* in a manner consistent with the three expression groups.

**Candidate regulatory region significantly modulates reporter gene activity in mouse airway epithelia cell lines**

To interrogate the effect of this candidate regulatory region on *Zfp30* expression, we cloned the 500 bp putative promoter region from representative strains of each *Zfp30* expression group into a promoterless luciferase vector for use in luciferase reporter assays. More than one strain was chosen from the high and low expression groups to account for potential differences in gene expression due to variants in the region. Specifically, promoter regions were cloned from A/J and 129S1/SvImJ to represent the high expression group, from C57BL/6J to represent the intermediate expression group, and from CAST/EiJ and PWK/PhJ to represent the low expression group.

We then tested whether these promoter constructs recapitulate relative patterns of expression seen *in vivo*, using luciferase reporter assays in two mouse lung epithelia cell lines, MLE12 and LA4. Mouse lung epithelia cell lines were chosen because *Zfp30* expression was previously reported in this tissue. Additionally, we previously reported that knockdown of *Zfp30* using small interfering RNAs (siRNAs) in MLE12 cells led to increased CXCL1 secretion in response to LPS exposure, which is consistent with predictions based on *in vivo* data (Rutledge *et al.* 2014). Concordant with previously measured *in vivo* expression data (see

Table 2.1), initial luciferase experiments in the MLE12 mouse lung epithelia cell line (Figure 2.1A) revealed a significant twofold difference in expression driven by promoters from high-expressing strains (A/J and 129S1/SvImJ) compared with lower-expressing strains (C57BL/6J, CAST/EiJ, and PWK/PhJ). No difference in luciferase activity was observed between the moderate-expressing (C57BL/6J) and low-expressing (CAST/EiJ and PWK/PhJ) promoter constructs in this cell line. This assay was also performed in the LA4 mouse airway cell line to compare results across differing genetic backgrounds. In LA4 cells, the expression difference between high and moderate expression groups was also evident, and at a similar magnitude. Additionally, CAST/EiJ and PWK/PhJ promoters produced significantly lower expression than C57BL/6J promoter (Figure 2.1B), at a magnitude roughly equivalent to the *in vivo* differences shown in Table 1. To verify that the differences we observed exist at both the protein and RNA levels, we measured *luc2* and *Renilla* luciferase mRNA levels in transfected MLE12 cells using a custom qPCR assay. The results, shown in Table 2.S2[3], confirmed that the difference we observed in protein activity was also present at the RNA level.

We then focused our attention on the identification of SNPs that could explain the observed twofold expression difference between moderate and high *Zfp30* expressing promoters, for two reasons. First, the difference between moderate and high expression strains is much larger than the expression difference between moderate and low expression strains (Table 2.1). Second, in addition to our previous

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[3]Table S2 is available online at:
http://www.g3journal.org/content/8/2/687.supplemental

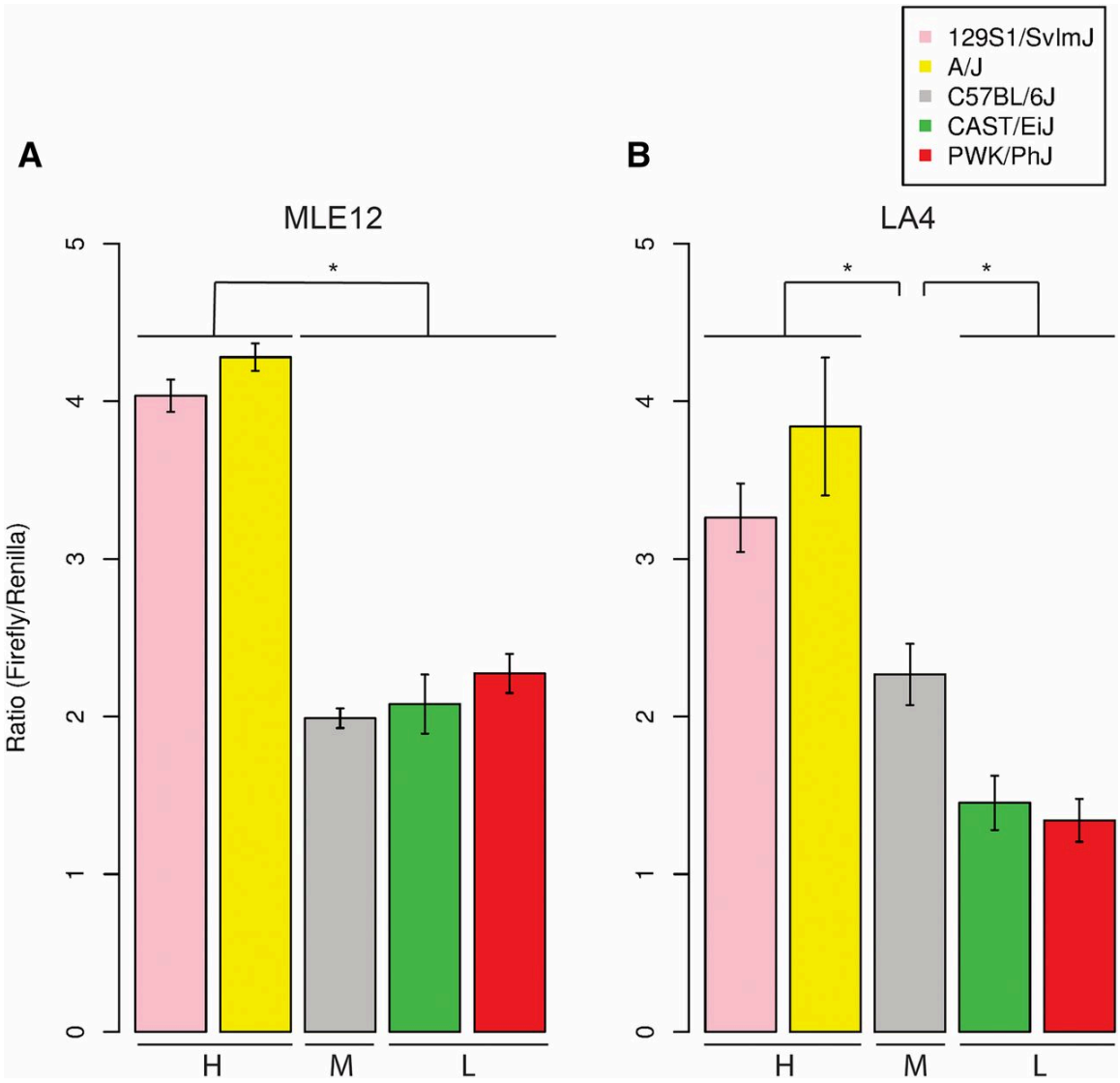



**Figure 2.1: *Zfp30* promoter region haplotypes recapitulate *in vivo* patterns of gene expression in reporter assays.** Dual luciferase reporter assays were performed, and firefly/*Renilla* ratios are shown. Reporter clones are grouped by *in vivo Zfp30* expression level: high (H), moderate (M), and low (L). (A) Results from assays in MLE12 cells recapitulate *in vivo* differences between moderate-expressing (C57BL/6J) and high-expressing (A/J and 129S1/SvImJ) strains but not low-expressing (CAST/EiJ) and (PWK/PhJ) strains. (B) Results from assays in LA4 cells recapitulate *in vivo* differences between all three expression groups. * P<0.05.

finding regarding the link between *Zfp30* expression and immune response in the lung (Rutledge *et al.* 2014), a previous report from a different research group indicates that the aforementioned expression difference is likely also the cause of contrasting response to *Streptococcus pneumonia* infection (Denny *et al.* 2003).

Within the cloned regulatory region, A/J and 129S1/SvImJ share only one variant, rs51434084. To test the regulatory effect of this SNP, we used a site-directed mutagenesis approach to modify a C57BL/6J promoter at this locus to match the allele of the high-expressing strains. The resulting promoter contains a single bp modification that results in a doubling of expression in both MLE12 ($P < 0.001$) and LA4 ($P < 0.05$) cell lines (Figure 2.2), matching the expression levels of A/J and 129S1/SvImJ.

**Variation at rs51434084 results in differential binding of ZFP148**

We then performed EMSA experiments to detect differential binding of transcription factors from MLE12 nuclear lysates to 17 bp probes mimicking either the C57BL/6J or 129S1/SvImJ allele at rs51434084 (Figure 2.3). Multiple DNA–protein complexes were observed for each probe, and each complex showed higher affinity for the C57BL/6J probe. Competition reactions with unlabeled probes revealed that the unlabeled C57BL/6J probe competed these interactions away with higher affinity than the unlabeled 129S1/SvImJ probe. Given that *in vivo* data and *in vitro* reporter assays both indicated that the C57BL/6J haplotype is associated with lower gene expression than the 129S1/SvImJ haplotype, we reasoned that the observed nuclear binding to the probe sequence containing rs51434084 is repressive.

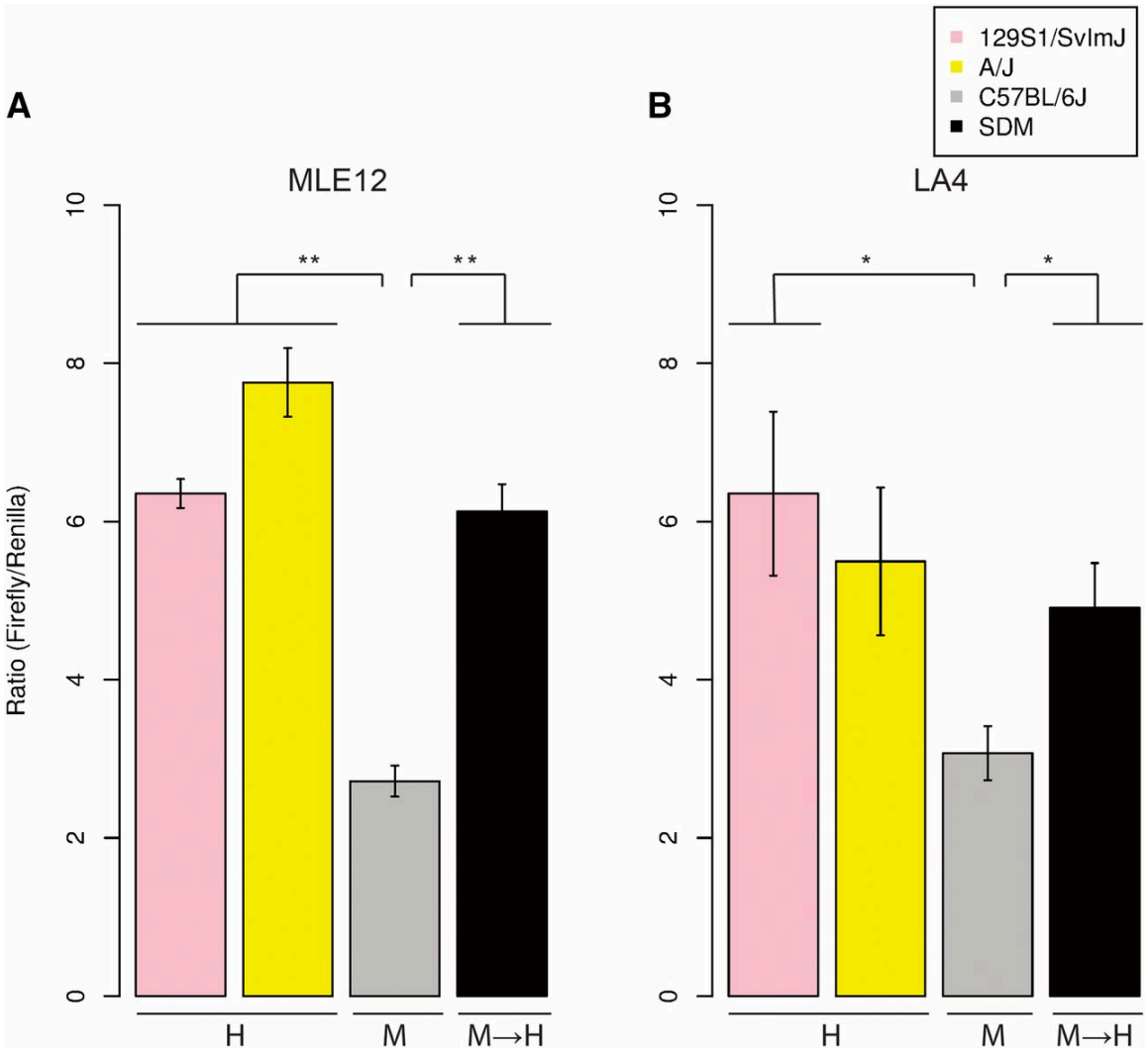
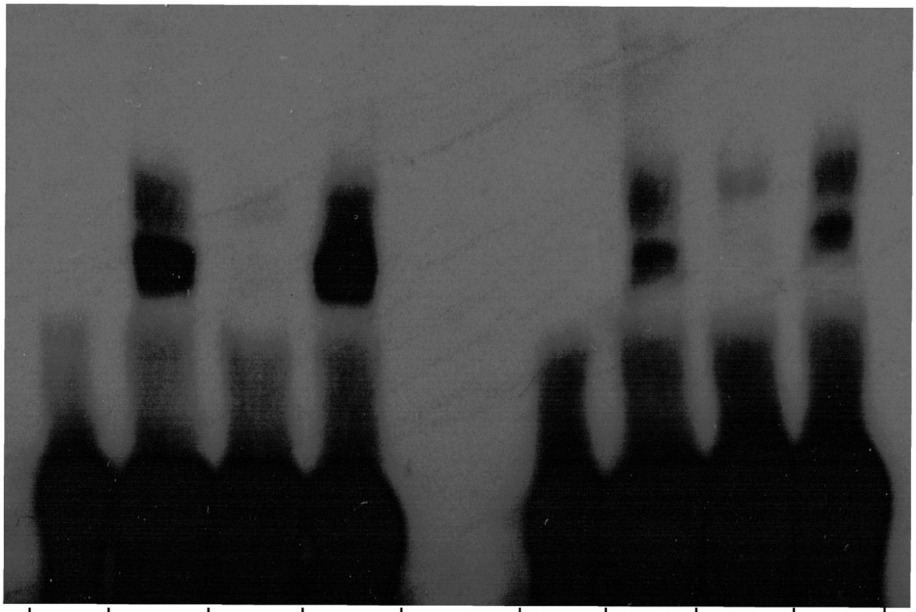



**Figure 2.2: rs51434084 significantly modulates reporter gene expression.** Dual luciferase reporter assays with haplotypes from A/J, 129S1/SvImJ, and C57BL/6J strains, as well as C57BL/6J haplotype with the rs51434084 C > G site-directed mutant (SDM) allele, were performed, and firefly/*Renilla* ratios are shown. Reporter clones are grouped by *in vivo Zfp30* expression as in Figure 1, with the addition of a haplotype generated by SDM of rs51434084 (M→H). (A) Results from assays in MLE12 cells show a significant increase in SDM construct compared with the C57BL/6J construct. (B) Results from assays in LA4 cells show a significant increase in SDM construct compared with the C57BL/6J construct. ** $P < 0.001$, * $P < 0.05$.



| Biotinylated Probe | C | C | C | C | | G | G | G | G |
|---|---|---|---|---|---|---|---|---|---|
| Nuclear Lysate (MLE12) | | + | + | + | | | + | + | + |
| Unlabeled Competitor Probe | | | C | G | | | | C | G |

**Figure 2.3: Electrophoretic mobility shift assays reveal differential binding of a transcription factor at the rs51434084 locus.** Biotin-labeled 17-bp double-stranded DNA oligonucleotides containing contrasting rs51434084 alleles were incubated with 4 µg of MLE12 nuclear lysates. Unlabeled probes were incubated at 45x concentration of the labeled probes. The unmodified image is shown in Figure S4[4].

[4]Figure S4 is available online at: http://www.g3journal.org/content/ggg/suppl/2017/12/13/g3.117.300507.DC1/FigureS4.jpg

To identify candidate proteins that participate in these DNA–protein complexes, we used the transcription factor binding site prediction tools TRANSFAC and HOCOMOCO to search for known mouse DNA-binding proteins predicted to differentially bind to the C57BL/6J and 129S1/SvImJ probe sequences. Multiple candidates emerged from this analysis (Table S3[5]), and literature searches revealed that two candidates have established roles in immune regulation and have repressive transcriptional activity, rendering them priority candidates. ZFP148 (also known as ZBP-89, BERF-1, and BFCOL1) has been shown to regulate CXCL5, a chemokine similar to CXCL1 in function (Keates *et al.* 2001). KLF4 (also known as GKLF) was associated with endothelial barrier integrity and severity of lipopolysaccharide-induced lung injury (Cowan *et al.* 2010).

EMSA experiments with a supershift condition did not reveal a supershift with KLF4 antibody (data not shown). In contrast, addition of ZFP148 antibody produced a supershift (Figure 2.S3), indicating that ZFP148 is among the DNA–protein complexes identified in Figure 2.3. We detected differential intensity of the supershifted band when probing with oligos representing the rs51434084 C or G alleles (Figure 2.4, lanes 3 and 6), accompanied by differential intensity of the lower band that is reduced by ZFP148 antibody addition (Figure 2.4, lanes 2 and 5). These results indicate that ZFP148 is among the nuclear proteins with higher affinity to the C57BL/6J probe.

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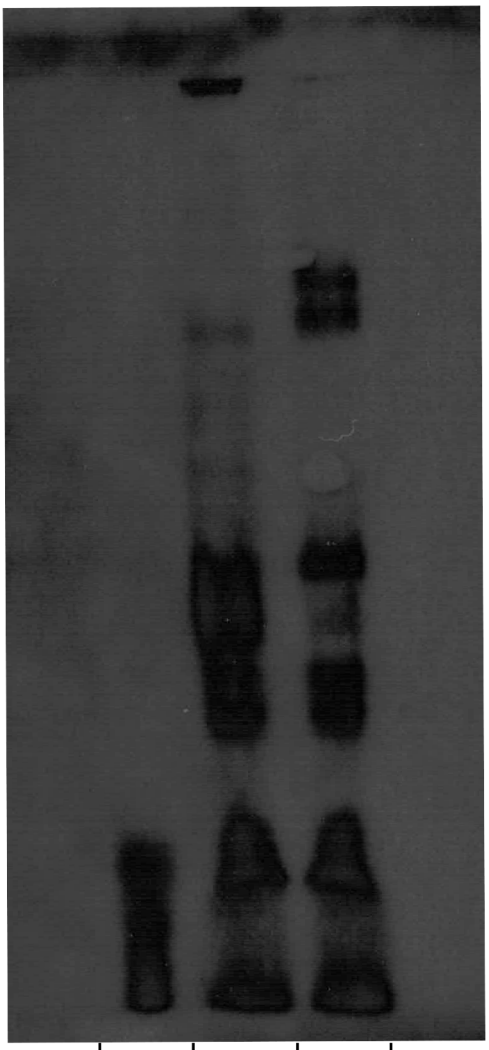
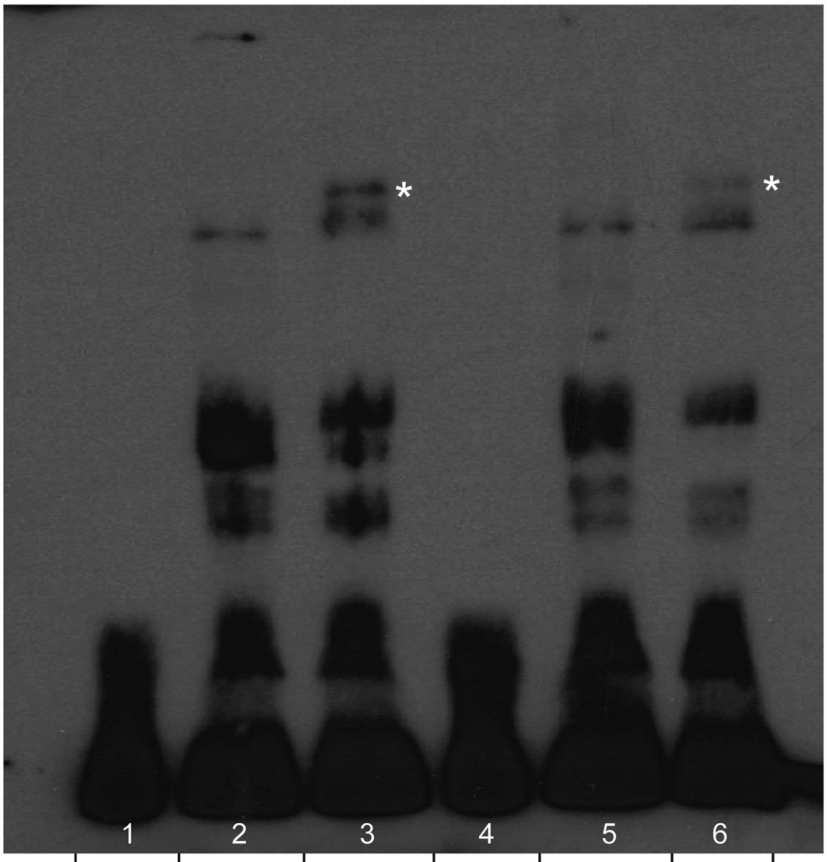
[5]Table S4 is available online at:
http://www.g3journal.org/content/8/2/687.supplemental



| | | | |
|---|---|---|---|
| Biotinylated Probe | + | + | + |
| Nuclear Lysate (MLE12) | | + | + |
| ZNF148 Ab | | | + |

**Figure 2.S3: Electrophoretic mobility shift assay super-shift reveals ZFP148 binding.** Biotin-labeled 17-bp double-stranded DNA oligonucleotides containing the C57BL/6J rs51434084 allele was incubated with 4 µg of MLE12 nuclear lysates. Super-shift condition incubated with 20 µg ZFP148 antibody. Adobe Photoshop was used to adjust image brightness (+88) and contrast (-49). Adobe Illustrator was used to crop image down to relevant gel lanes.




| | | | | | | |
|---|---|---|---|---|---|---|
| Biotinylated Probe | C | C | C | G | G | G |
| Nuclear Lysate (MLE12) | | + | + | | + | + |
| ZFP148 Antibody | | | + | | | + |

**Figure 2.4: Electrophoretic mobility shift assay supershift reveals differential ZFP148 binding.** Biotin-labeled 17-bp double-stranded DNA oligonucleotides containing contrasting rs51434084 alleles were incubated with 4 $\mu$ g of MLE12 nuclear lysates. The supershift condition was incubated with 10 $\mu$ g ZFP148 antibody. White asterisks denote the supershift band. The unmodified image is shown in Figure S5[6].

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[6]Figure S5 is available online at: http://www.g3journal.org/content/ggg/suppl/2017/12/13/g3.117.300507.DC1/FigureS5.jpg

## Discussion

Neutrophil levels in human airways correlate with asthma disease severity (Wenzel *et al.* 2003; Moore *et al.* 2014), poor response to inhaled corticosteroids (Green *et al.* 2002), and sudden-onset fatal asthma attacks (Sur *et al.* 1993). In COPD, neutrophil-to-lymphocyte ratio (NLR) is associated with disease state, and increased NLR is associated with longer hospital stays and higher readmission rates (Günay *et al.* 2014; Duman *et al.* 2015). Additionally, neutrophil extracellular traps are associated with increased airflow limitation among patients with COPD, and neutrophilic inflammation leads to decreased lung function (Quint and Wedzicha 2007; Hoenderdos and Condliffe 2013; Grabcanovic-Musija *et al.* 2015). In cystic fibrosis, neutrophil secretory proteins drive disease states and increase mucus production (Mackerness *et al.* 2008; Gifford and Chalmers 2014; Laval *et al.* 2016). In acute lung injury, neutrophils are a key component to lung damage, and their recruitment is controlled by complex chemokine networks (Grommes and Soehnlein 2011; Williams and Chambers 2014). Genes related to neutrophils and their recruitment are also upregulated in early sepsis-induced ARDS (Kangelaris *et al.* 2015). These findings indicate that increased understanding of neutrophil recruitment to the lung would have broad importance.

To gain insight into the regulation of neutrophil recruitment, we exploited a mouse model of asthma featuring neutrophilic inflammation. In the context of this model system, we identified *Zfp30* as a candidate regulator of CXCL1 levels and neutrophil recruitment (Rutledge *et al.* 2014). Here, we follow up this finding by

identifying a causal variant for the variation in *Zfp30* expression, namely rs51434084.

Our reporter assays with *Zfp30* promoter constructs provided two main findings. First, this 500 bp region successfully recapitulated the twofold range of variation in *Zfp30* expression we previously characterized *in vivo* (Rutledge *et al.* 2014). Second, allele-swapping of rs5143408 using site-directed mutagenesis shows that this variant is the primary driver of the twofold expression difference between these strain groups. We also note that in our *in vitro* reporter assays, the expression pattern for CAST/EiJ and PWK/PhJ differed between the two cell lines we used (MLE12 and LA4). The expression pattern seen in the LA4 cell line (generated from the A/He mouse strain) matched mRNA expression results seen *in vivo*, but the pattern seen in MLE12 (generated from FVB/N mouse strain) did not. This suggests that these cell lines differentially express a repressor protein that downregulates expression of *Zfp30* in CAST/EiJ and PWK/PhJ. Future experiments will be directed toward identifying the causal variant(s) and protein(s) responsible for this modulation of *Zfp30* expression.

Our EMSA results indicate that ZFP148 is among the transcription factors that bind to the rs51434084 locus in the *Zfp30* promoter region and thereby regulate *Zfp30* expression. Thus, we propose that our initial model, in which *Zfp30* expression regulates CXCL1 and neutrophil recruitment (Rutledge *et al.* 2014), be expanded to include ZFP148. ZFP148 has a known role in the negative regulation of *Cxcl5*, a CXCR2 ligand similar to CXCL1, in human colonic epithelial cells (Keates *et al.* 2001). In combination with previous findings, our results suggest that ZFP148

downregulates both *Cxcl5* and *Zfp30*. These functions may seem contradictory at first, as *Zfp30* is proposed to downregulate *Cxcl1*, but decreased expression of *Cxcl5* has actually been linked to increased neutrophil recruitment into the lungs in an *Escherichia coli* pneumonia mouse model (Mei *et al.* 2010) through a mechanism involving Duffy antigen receptor for chemokines (DARC)–mediated sequestration of CXC chemokines. This mechanism results in a steeper gradient of CXCL1 and CXCL2 chemokines between vasculature and lungs. Thus, the function of ZFP148 proposed here could be cooperative with its known effect on CXCL5 levels.

ZFP30 is a C2H2 zinc finger protein with domains that suggest DNA-binding and KAP-1–binding functions. Wherever ZFP30 binds in the genome, a complex of proteins recruited by KAP-1 are predicted to induce heterochromatin domains and silence nearby genes (Friedman *et al.* 1996; Ryan *et al.* 1999; Schultz *et al.* 2002; Medugno *et al.* 2005). Specific binding sites for ZFP30 have not yet been identified, and additional work is needed to characterize the mechanism of ZFP30-mediated control of neutrophil recruitment. *Cxcl1* is a priority locus of interest; however, KAP-1/zinc finger protein complexes could have multiple binding sites and can act over tens of kilobases (Groner *et al.* 2010), so multiple genes may be affected.

This work contributes to an increased understanding of how *Zfp30* is regulated as a function of an allelic variant, which we previously showed was linked to neutrophils in the airways in a mouse model. Future work examining the relevance of the genes/proteins studied here, namely ZFP148 and ZFP30, in neutrophil chemotaxis in other model systems and in human patients is a critical next step to evaluating the biomedical importance of our findings. Additionally,

identification and characterization of additional functional variants that impact neutrophil recruitment will further develop our understanding of the complex regulatory networks that drive disease severity phenotypes in both model systems and human patients with asthma, acute lung injury, cystic fibrosis, or COPD.

# CHAPTER 3: BASELINE AND IMMUNE RESPONSE CHARACTERIZATION OF A ZFP30 KNOCKOUT MOUSE STRAIN

## Introduction

Neutrophils are a vital participant in the innate immune system's response to pathogens, but the mechanisms through which these cells respond to immune challenge are prone to collateral host tissue damage (Nathan 2006). Production of reactive oxygen intermediates, degranulation of cytotoxic molecules, and NETosis are each effective at killing invading microbes, but they can all damage host tissue and drive disease if left unchecked. Neutrophils are also potent signaling cells that recruit additional leukocytes to sites of inflammation and participate in complex cross talk with other immune cell types (Mantovani *et al.* 2011). For these reasons, neutrophils drive pulmonary disease severity in acute respiratory distress syndrome (ARDS) (Chollet-Martin *et al.* 1992; Grommes and Soehnlein 2011; Williams and Chambers 2014; Kangelaris *et al.* 2015), chronic obstructive pulmonary disease (COPD)(Qiu *et al.* 2003; Quint and Wedzicha 2007; Hoenderdos and Condliffe 2013; Günay *et al.* 2014; Duman *et al.* 2015; Grabconovic-Musija 2015), and cystic fibrosis (CF) (Mackerness *et al.* 2008; Gifford and Chalmers 2014). A growing body of evidence also suggests a role for neutrophils in asthma disease severity (Stănescu *et al.* 1996; Jatakanon *et al.* 1999; Wenzel *et al.* 1999; Green *et al.* 2002; Little *et al.* 2002; Uddin *et al.* 2010; Moore *et al.* 2010).

Dysregulation of the chemokines that attract neutrophils into tissues may tip the scale from appropriate innate immune response to unwanted damage. The CXC chemokine family members CXCL8 (IL-8), CXCL1 (KC/Gro-α), CXCL2 (MIP-2/Gro-β), and CXCL5 (LIX/ENA-78) are all hallmark neutrophil recruitment molecules that signal through the chemokine receptor CXCR2 (Charo and Ransohoff 2006). Pharmaceutical targeting of CXCR2 has proven effective in reducing airway neutrophilia in early chronic pulmonary disease trials (Nair *et al.* 2012; Moss *et al.* 2013; Kirsten *et al.* 2015; Rennard *et al.* 2015; Todd *et al.* 2016; Wats 2017). After CXCR2 antagonism, patients with COPD present with fewer neutrophils in sputum alongside improved forced expiratory volume (Kirsten *et al.* 2015; Rennard *et al.* 2015). Likewise, cystic fibrosis patients treated with CXCR2 antagonists also showed fewer neutrophils and lower amounts of neutrophil elastase in sputum (Moss *et al.* 2013). CXCR2 antagonism also reduced airway neutrophils in mild atopic asthmatics and patients with severe or persistent neutrophilic asthma (Nair *et al.* 2012; Todd *et al.* 2016; Watz *et al.* 2017).

To further probe the pathways underlying pulmonary neutrophil recruitment, we applied a house dust mite model of asthma to incipient line of the Collaborative Cross, a genetics reference population ideal for the study of complex traits (Rutledge *et al.* 2014). This study pointed to *Zfp30* as a candidate repressor of CXCL1 and neutrophil recruitment to the lungs. *Zfp30* is a C2H2 zinc finger protein with a KRAB domain. The C2H2 domains allow ZFP30 to bind to DNA in a sequence specific manner, and the KRAB domain recruits the KAP1 transcriptional repressor to these binding sites (Friedman *et al.* 1996). This transcriptional repression proceeds

through recruitment of HP1, SETDB1, and histone deacetylases that induce heterochromatin formation and silence nearby genes (Ryan *et al.* 1999; Schultz *et al.* 2002; Medugno *et al.* 2005; Groner *et al.* 2010). In this study, we further characterize *Zfp30* and its role in neutrophil recruitment through generation of a CRISPR-Cas9 knockout mouse model for use in *ex vivo* and *in vivo* experiments. Our results confirm a regulatory effect of *Zfp30* on CXCL1 secretion and undercover novel connections between *Zfp30* and IL-6, blood glucose levels, and multiple organ weight phenotypes.

## Materials and Methods

### Generation of *Zfp30* Knockout Mice by CRISPR/Cas9 Embryo Microinjection

A CRISPR/Cas9 guide RNA (5'-GAATCCAGATACAGCAGTAA(CGG)-3') was designed to target mouse *Zfp30* gene near the 5' end of exon 5. While targeting earlier exons would have been preferable, exon 5 was the only region that could be targeted with specificity owing to high homology across ZFP family members. Exon 5 encodes the C2H2 zinc finger domains of ZFP30 that are involved in DNA binding. The guide RNA was produced by T7 *in vitro* transcription and validated *in vitro* by incubating guide RNA, Cas9 enzyme, and plasmid harboring the guide RNA target site. This was followed by gel electrophoresis to determine the extent of in vitro cleavage activity. A donor oligonucleotide ("Z30-H1-T": 5'-GTTTTTCTTCTTTTTGCTTTCAGATCTGGAATCCAGATACAGC[TGA][TAG]GATCC[TAG]ACCGGTAACGGGTTACTTCCAGAAAAGAATACTTACGAAATTAATCTATCT-3') was used for homologous recombination to insert stop codons (brackets) and a BamHI restriction site (underlined) at the target site.

C57BL/6J females were superovulated by injection with pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) and then mated with C57BL/6J stud males for zygote production. One-cell embryos were collected from the ampulla oviducts the morning after mating and microinjected with either "low" or "high" mix, containing, respectively, 20 ng/μl or 100 ng/μl in vitro transcribed Cas9 mRNA, 20 ng/μl or 50 ng/μl Zfp30 guide RNA and 100 ng/μl Z30-H1-T donor oligonucleotide. The microinjected embryos were then implanted into pseudopregnant recipients.

Fourteen live pups born from microinjected embryos were screened by polymerase chain reaction (PCR) amplification of the *Zfp30* target site followed by digestion of the PCR product with BamHI restriction enzyme. The BamHI restriction site was detected in nine animals. Two founders with apparent biallelic insertion of the BamHI restriction site were mated to C57BL/6J animals for germline transmission of the targeted allele.

The founder animals harboring *Zfp30* mutations were screened for mutations at 10 potential off-target sites (Supplementary Table 1) predicted by crispr.mit.edu in October 2013. Each potential off-target site was PCR amplified and products were analyzed by T7endo1 assay. Founders chosen for line establishment were further analyzed by Sanger sequencing of PCR products for all 10 off-target sites. A single founder line was subsequently backcrossed to C57BL/6J again to remove detected off-target mutations. The resulting colony was maintained via heterozygous matings.

In the initial stages of breeding, genotyping of *Zfp30* knockout mice was performed using allele-specific PCR. Primer sets were designed to specifically target

either the *Zfp30*$^{+/+}$ (Fwd: GGGCTGCTAAGTCCATTCAG; Rev: GGAAGTAACCCGTTACTGCTG) or *Zfp30*$^{-/-}$ (Fwd: GGGCTGCTAAGTCCATTCAG; Rev: CGTTACCGGTCTAGGATCCT) allele. We later transitioned to a proprietary qPCR-based genotyping protocol through Transnetyx (Cordova, TN).

**qPCR for *Zfp30* Quantification in the Knockout Strain**

To quantify *Zfp30* gene expression level in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice, we designed primer sets that specifically quantify the *Zfp30*$^{+/+}$ allele (Fwd: TGTTGGAACAAGGGAAGGAG; Rev: GTAACCCGTTACTGCTGTAT) or specifically quantify the *Zfp30*$^{-/-}$ allele (Fwd: TGTTGGAACAAGGGAAGGAG; Rev: CGGTCTAGGATCCTATCAGCT). qPCR reactions were carried out using iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA USA).

**Complete Blood Count Assays and Blood Glucose Testing**

For complete blood counts, blood was collected in EDTA tubes and stored on ice for a minimal amount of time before processing via a ProCyte Dx Hematology Analyzer.

For blood glucose measurements, blood was collected, and serum was separated via centrifugation using Microtainer tubes with lithium heparin (BD). Blood glucose measurements were taken using an Alfa Wasserman Vet Axcel Chemistry System.

**Neutrophil Recruitment Models**

LPS challenge: Intratracheal instillation of LPS from *E. coli* (LIST Biologicals) into lungs of *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice was carried out at a dose of 0.3 mg per kg of bodyweight using the method of Limjunyawong, Mock, and Mizner (Limjunyawong *et*

*al.* 2015). Bronchoalveolar lavage fluid was collected between 8 and 48 hours after exposure, and differential cell counts in bronchoalveolar lavage fluid were performed. Aliquots of BALF were saved for cytokine quantification. Oropharyngeal aspiration of LPS from *E. coli* (LIST Biologicals) into lungs of *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice was carried out with 5μg LPS in 40μl PBS.

Sensitization to House dust mite allergen Der p 1: *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice were sensitized with 10 μg Der p1 administered through intra-peritoneal injection (in 100 μl of PBS) on days 0 and 7 of the experiment, and a 50 μg Der p1 challenge was administered on day 15 of the experiment (Kelada *et al.* 2011). Mice were sacrificed 48-72 hours after challenge, and differential cell counts in bronchoalveolar lavage fluid were performed. Aliquots of BALF were saved for cytokine quantification.

Ozone exposure: *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice were exposed to filtered air, 1 ppm ozone, or 2 ppm ozone for three hours (Smith *et al.* 2018). Bronchoalveolar lavage fluid was collected 24 hours after exposure, and differential cell counts in bronchoalveolar lavage fluid were performed.

**MTEC Culture**

Mouse tracheal epithelial cell (MTEC) cultures were generated and cultured according to a previously established protocol (You and Brody 2013). Tissues isolated from 4 week old mice were grown using PluriQ differentiation media and plated in 12 well plates with Transwell inserts. Cells were maintained at air-liquid interface for a minimum of three weeks to allow for differentiation. LPS and FLA-ST

exposures were carried out at 10μg/mL in 100μl of PBS added to the apical surface of MTECs for 24h.

**Luminex Assays**

Cytokines in bronchoalveolar lavage fluid or PBS used in MTEC LPS exposures were measured using Milliplex assays (Millipore, Billerica, MA) according to manufacturer's instructions.

**Histology**

Histological preparation and analysis of lungs was carried out using previously described methods (Donoghue *et al.* 2017). Briefly, left lung lobes were fixed in formalin and cut in cross-section starting at the hilum and 2mm apart along the main stem bronchus. Sections were embedded in paraffin and stained with Hematoxylin and eosin (H&E) stain. Images were captured on an Olympus BX605F microscope with CellSens Standard software.

**Results**

We sought to investigate the function of *Zfp30* through a CRISPR-Cas9 mediated *in vivo* knockout model. Of fourteen pups born from microinjected embryos, nine had successful insertion of our knockout cassette, and two founders with biallelic insertion were mated to C57BL/6J for germline transmission of the targeted allele. These founder animals were screened for ten potential off-target insertions, and a single founder was subsequently backcrossed once more to C57BL/6J to establish our knockout colony. Throughout the maintenance of this colony, transmission ratio distortion (TRD) was a periodic issue. A significant depletion of knockouts was apparent in the early stages of breeding, and this

depletion was initially overcome via selective breeding of mice that produced normal litters. TRD appeared again after several generations of breeding, however. Our total genotype ratios for the history of the colony reveal a significant depletion of *Zfp30*$^{-/-}$ mice (Table 3.1).

| | ***Zfp30*$^{+/+}$** | ***Zfp30*$^{+/-}$** | ***Zfp30*$^{-/-}$** | $x^2$ | ***P*** |
|---|---|---|---|---|---|
| **Observed Genotype Ratio** | 298 (26.6%) | 575 (51.3%) | 247 (22.1%) | 9.47 | .009 |
| **Expected Genotype Ratio** | 298 (25%) | 596 (50%) | 298 (25%) | | |

**Table 3.1: Genotype ratios reveal Transmission Ratio Distortion among the *Zfp30* knockout colony.** Genotypes of all mice born from *Zfp30*$^{+/-}$ x *Zfp30*$^{+/-}$ breeding pairs were tallied, and observed genotype ratios are reported along with the expected Mendelian genotype ratio. A $x^2$ calculation was performed to test the significance of the *Zfp30*$^{-/-}$ depletion.

Because no reliable antibody for mouse ZFP30 exists, we initially queried the effect of *Zfp30* knockout at the RNA level. We designed a qPCR-based approach to specifically quantify expression of either the *Zfp30*$^{+/+}$ allele or the CRISPR-Cas9 modified *Zfp30*$^{-/-}$ allele. We detected expression of only the *Zfp30*$^{+/+}$ allele in WT mice, only the *Zfp30*$^{-/-}$ allele in KO mice, and intermediate expression of the two alleles in heterozygous mice (Figure 3.1A).

**Baseline Phenotyping**

As a family, the KRAB domain-containing C2H2 zinc finger proteins are thought to play an important role in differentiation and development (Urrutia 2003; Lupo *et al.* 2013). To asses the impact that a whole body knockout of *Zfp30* might have on development, we tracked total body weight in a population of *Zfp30*$^{+/+}$, *Zfp30*$^{+/-}$, and *Zfp30*$^{-/-}$ mice (derived from Zfp30$^{+/-}$ x Zfp30$^{+/-}$ matings) between four and eight weeks of age, and this revealed no significant differences by genotype (data not shown). We also assayed the weights of lungs, pancreases, spleens, and livers in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice. We detected no significant differences in lung or pancreas weights, but *Zfp30*$^{-/-}$ mice exhibit significantly smaller spleens and livers relative to total body weight (Figure 3.1 B and C), and we additionally see significantly reduced blood glucose levels among *Zfp30*$^{-/-}$ mice, suggesting an impact on pancreas function (Figure 3.1 D). Complete blood count (CBC) assays did not reveal any significant reproducible differences in red blood cell or circulating leukocyte phenotypes between *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ samples (data not shown). Finally, we also carried out histological analysis of lungs from *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$

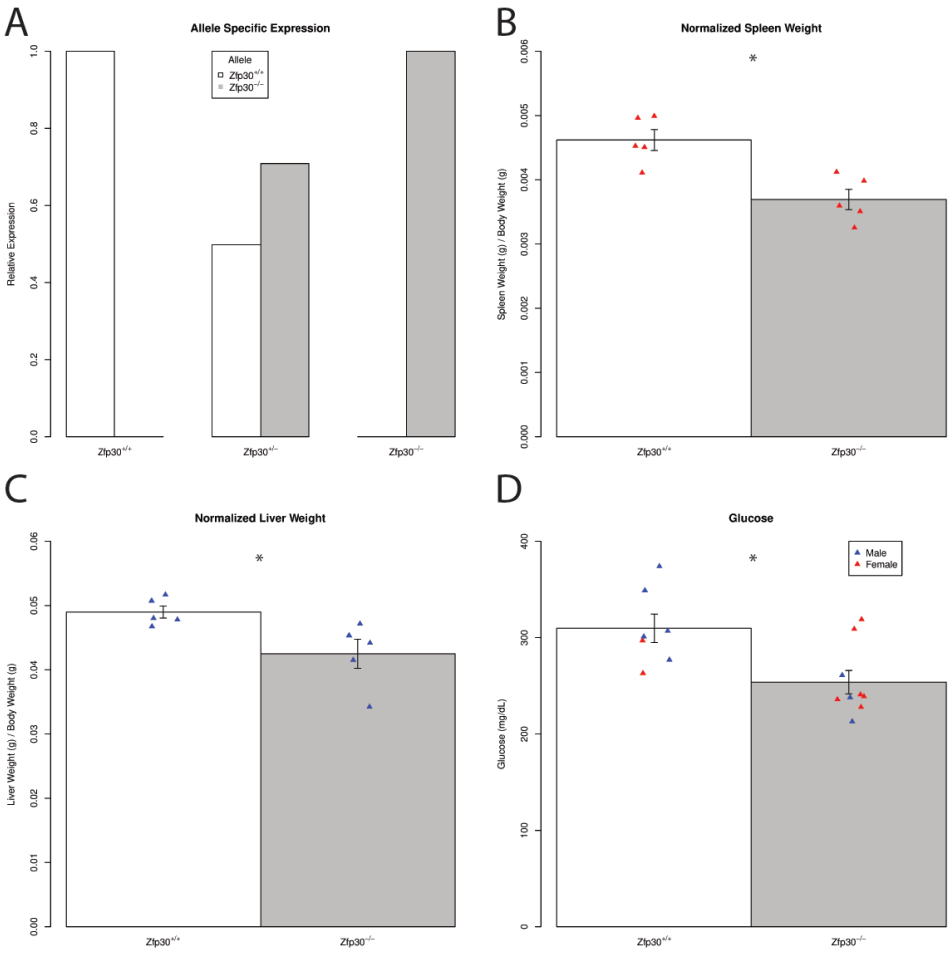


**Figure 3.1: Baseline phenotyping reveals impacts of *Zfp30* on spleen weight, liver weight, and blood glucose levels.** (A) Allele-specific qPCR reveals expression of only the inserted *Zfp30*$^{-/-}$ allele among KO mice and intermediate expression of the two alleles among *Zfp30*$^{+/-}$ mice. Reactions for each allele were carried out on samples from mice of each genotype, and results are normalized to homozygous expression level. (C-D) Weights of excised organs reveal significantly smaller spleens (C) and livers (D) among *Zfp30*$^{-/-}$ mice. (E) Blood glucose measurements reveal significantly lower blood glucose levels among *Zfp30*$^{-/-}$ mice.

lungs to investigate any obvious differences in the airways, alveoli, or vasculature and detected no striking differences (Figure 3.2).

### *Ex vivo* Mouse Tracheal Epithelial Cell Cultures

Given our previous results indicating a correlation between *Zfp30* expression and innate immune response in the lung (Rutledge *et al.* 2014), we tested innate immune responses in mouse tracheal epithelia cultures (MTECs) from *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice. This system was particularly well suited to study the impact of *Zfp30* knockout on neutrophil recruitment, because recent single-cell RNA sequencing (RNA-seq) data suggests expression of *Zfp30* across a broad array of cell types in the airway epithelium (Plasschaert *et al.* 2018). Additionally, we previously showed that MTEC cultures have high expression of *Zfp30* and that perturbation of *Zfp30* expression with siRNAs results in increased CXCL1 production following LPS exposure in a mouse airway epithelia cell line (Rutledge *et al.* 2014). We saw significant differences in CXCL1 secretion in the basolateral media of these cultures following LPS stimulation, indicating that knockout of *Zfp30* produced the expected impact initially observed in the CC and MLE12 airway epithelial cell line (Rutledge *et al.* 2014) (Figure 3.3 A). We also observe differential CXCL1 secretion in response to Flagellin protein (FLA-ST), a TLR5 ligand. These experiments additionally revealed significantly decreased secretion of IL-6 on the apical surface of *Zfp30*$^{-/-}$ MTEC cultures in response to LPS and FLA-ST (Figure 3.3 B). To survey potential differences in airway epithelial cell type ratios among *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ MTEC cultures, we carried out qPCR for cell type-specific markers: *Foxj1* (ciliated cells), *Krt5* (basal cells), *Scgb1a1* (club cells), and *Muc5ac* (goblet cells). Our results reveal

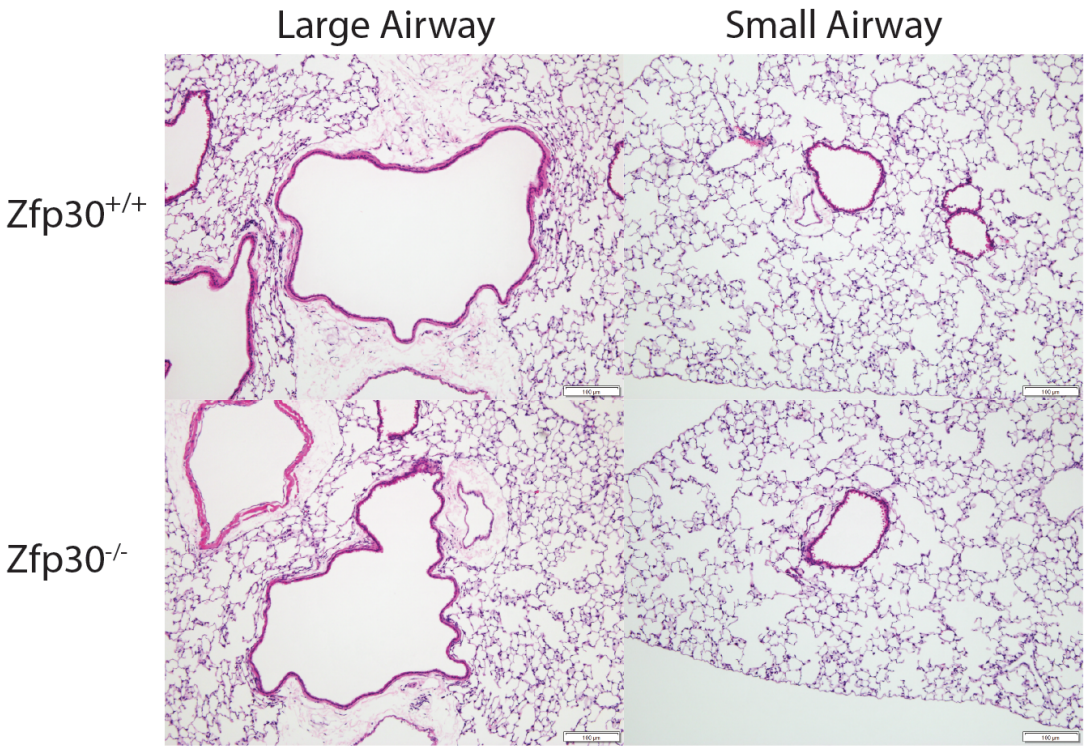


**Figure 3.2: Histological analysis reveals no striking phenotypic differences among the Zfp30 knockout colony.** H&E staining was carried out on lung sections from *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice. No significant differences are seen in large airways (top left, bottom left) or among small airways and alveoli (top right, bottom right). Bar, 100μM.

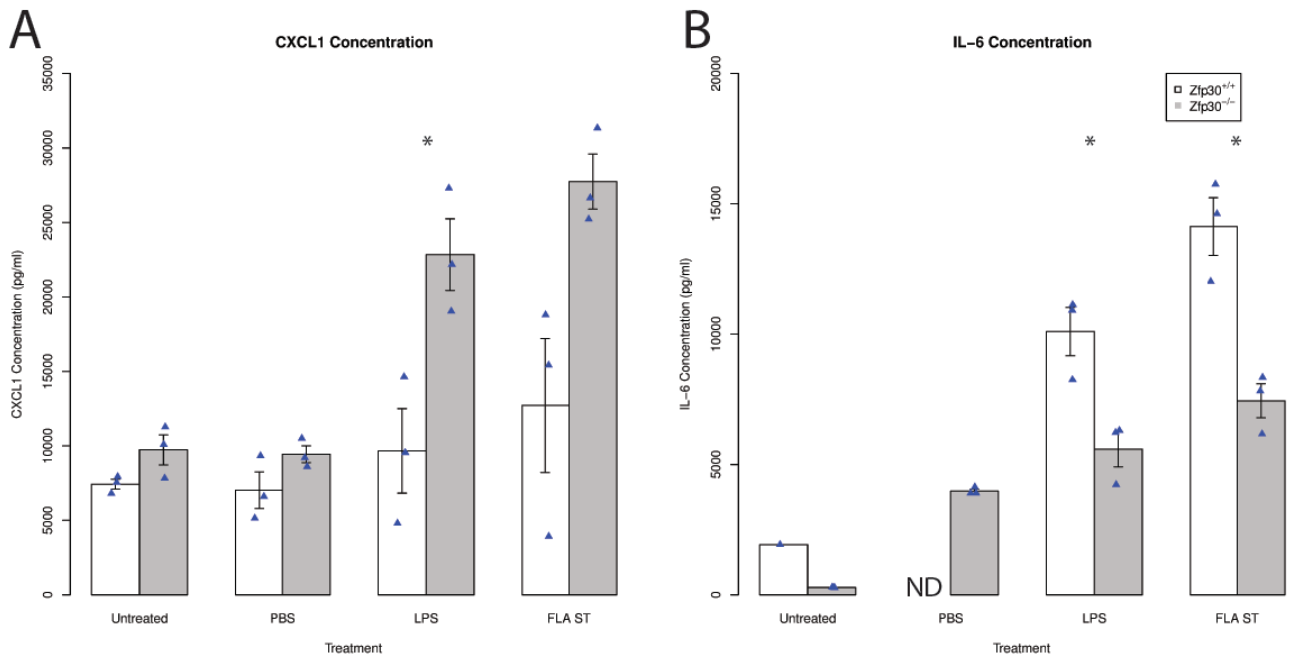


**Figure 3.3: *Zfp30* modulates CXCL1 and IL-6 secretion in cultured MTECs.** MTEC cultures were generated from *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice and maintained at air-liquid interface. LPS and FLA-ST exposures induce significantly more CXCL1 (A) and significantly less IL-6 (B) secretion in response to TLR4 (LPS) and TLR5 (FLA-ST) ligands. Exposures were carried out for 24 hours. ND= not detected.

no differences in cell type ratios between *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ cultures (data not shown).

### *In vivo* Lung Inflammation Phenotypes in *Zfp30*$^{-/-}$ mice

*Zfp30* was identified as a candidate regulator of CXCL1 levels and neutrophils in bronchoalveolar lavage fluid in the context of a model of allergic airway disease (Rutledge *et al.* 2014). As a direct follow-up to these experiments, we utilized the same house dust mite model of allergic airway disease in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice to further probe the connection between *Zfp30* and neutrophil recruitment. We saw no significant differences in neutrophil counts or CXCL1 in bronchoalveolar lavage fluid (BALF) 48 or 72 hours post-challenge (Figure 3.4 A-B).

Our allergic airway disease model is dominated by eosinophilia. To test for differences in CXCL1 secretion or neutrophil recruitment into the lungs in the context of neutrophil-dominated immune responses, we employed LPS and ozone exposure models. These models induce a much more robust neutrophilic airway recruitment than the HDM model, so differences in chemotactic signaling may be more apparent. Intratracheal instillation of LPS does not reveal a significant difference in neutrophil or CXCL1 levels in BALF (Figure 3.5 A-B). We did however note significant impacts on IL-6 in BALF in a subset of our LPS experiments (Figure 3.5 C). We also see no significant differences in neutrophilia utilizing 1ppm or 2ppm ozone exposures (Figure 3.6).

## Discussion

Further study of the underlying genes that preclude or contribute to host tissue damage via CXCR2-mediated neutrophil chemotaxis poses great potential

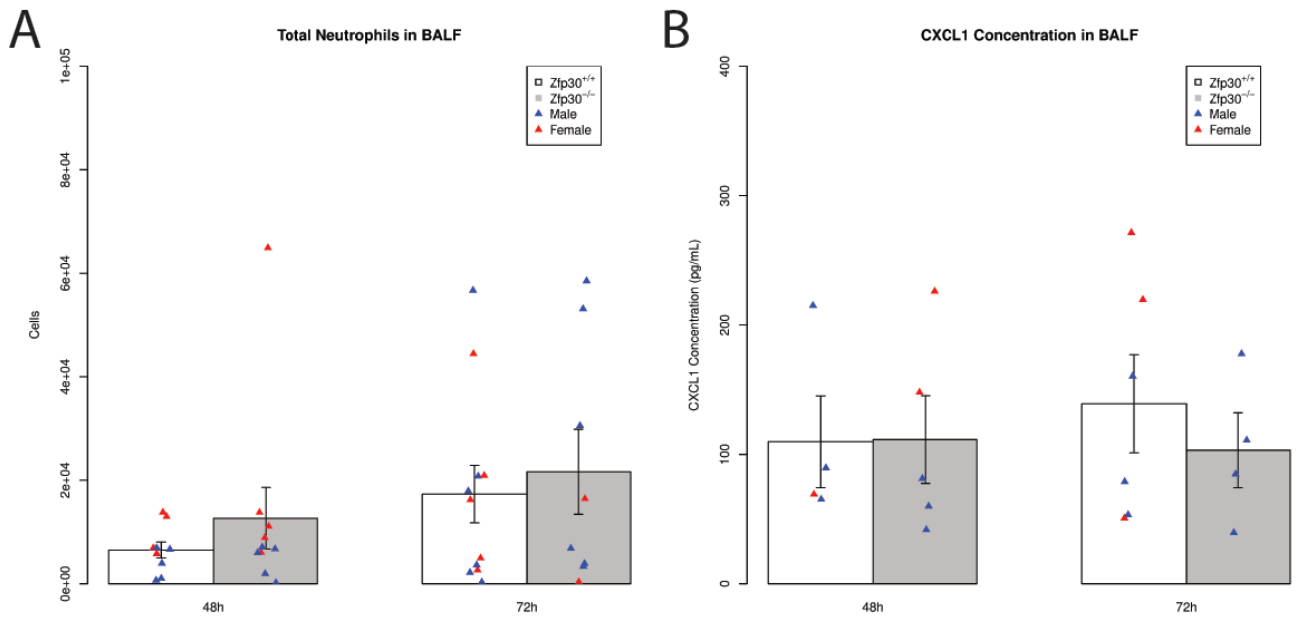


**Figure 3.4: House dust mite allergen challenge in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice reveals no significant difference in neutrophil count or CXCL1 concentration in BALF.** *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice were sensitized to and challenged with HDM allergen, and effects on neutrophil counts (A) and CXCL1 concentration (B) in BALF were monitored 48 and 72 hours after challenge. Cell count results are the combined data of two experiments. Neutrophil levels were assayed via differential cell counts, and cytokine measurements were carried out via multiplex assays.

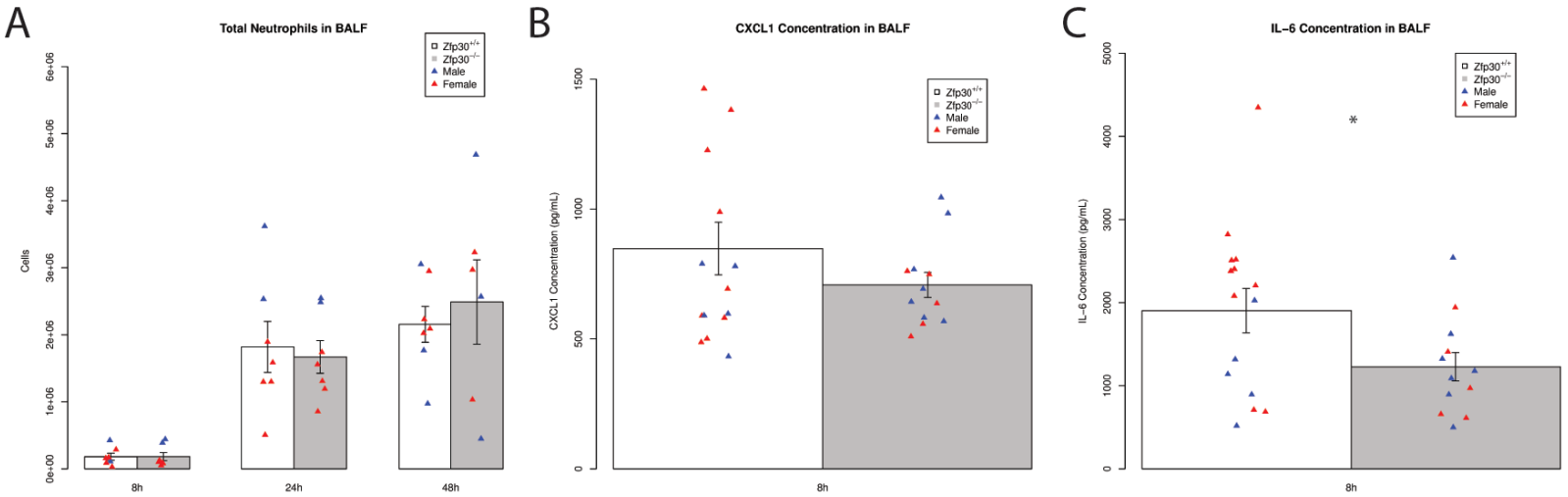




**Figure 3.5: LPS challenge in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice reveals a significant difference in IL-6 concentration but not neutrophil count or CXCL1 concentration in BALF.** Intratracheal LPS administration in *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice reveals no impact on neutrophil counts (A) or CXCL1 concentration (B), but a significant impact on IL-6 (C) concentration is observed. Neutrophil counts reported are from a single intratracheal administration and are representative of 2 OPA administration experiments and 2 intratracheal administration experiments. CXCL1 and IL-6 results are the combined data from two OPA administration experiments. Neutrophil levels were assayed via differential cell counts, and cytokine measurements were carried out via multiplex assays.* $P < 0.05$.

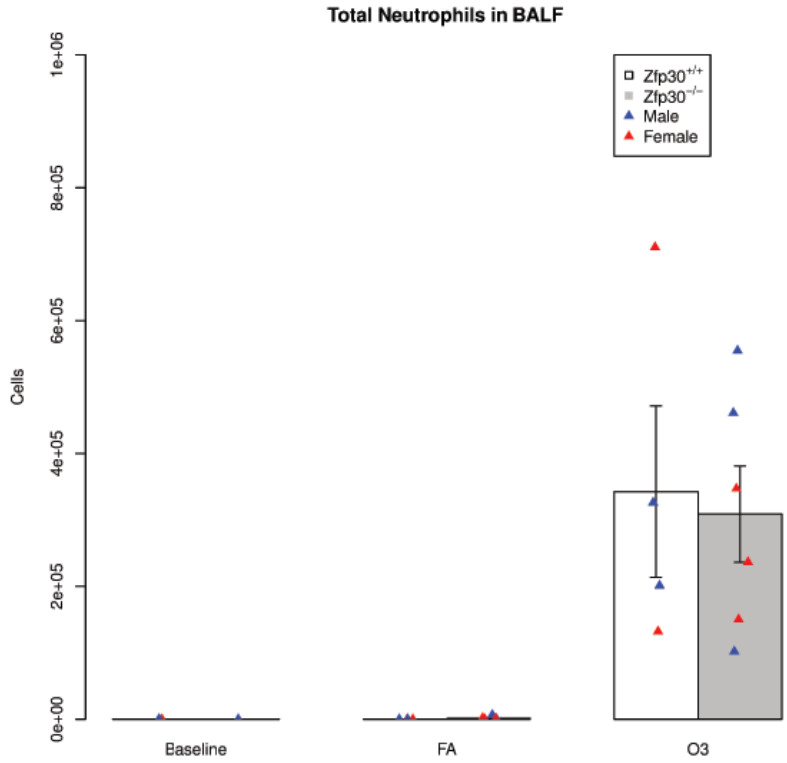


**Figure 3.6: Ozone exposure of *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice reveals no significant impact on neutrophil counts in BALF.** A three-hour exposure of *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ mice to 2 ppm ozone reveals no significant differences in neutrophils in BALF. Results are representative of a single 2ppm exposure and a single 1ppm exposure. Neutrophil levels were assayed via differential cell counts, and cytokine measurements were carried out via multiplex assays.

health and economic benefits, as neutrophil-driven pulmonary diseases are common and lead to increased healthcare utilization. We confirmed in this manuscript a regulatory impact of *Zfp30* on CXCL1 secretion in an *ex vivo* model of airway epithelia and revealed a novel impact of *Zfp30* on epithelia-secreted IL-6. Interestingly, we saw opposite directions of effect in CXCL1 and IL-6. CXCL1, a hallmark neutrophil chemokine, was significantly up-regulated in *Zfp30*$^{-/-}$ epithelia after immune stimulus. IL-6, however, is significantly down-regulated in the same LPS-stimulated MTEC cultures. This may at first seem contradictory, as IL-6 is typically thought of as a pro-inflammatory signaling molecule, but IL-6 has been implicated in the clearance of neutrophils and down-regulation of CXCL1 in a model of acute inflammation (Fielding *et al.* 2008). IL-6 activation of STAT3 is thought to comprise a checkpoint in acute inflammation in which a neutrophil recruitment response transitions to a more monocyte-dominated response (Fielding *et al.* 2008), and our results suggest that *Zfp30* may play some role in this checkpoint.

Our cell type-specific qPCR results with *Foxj1*, *Krt5*, *Scgb1a1*, and *Muc5ac* suggest that the impacts of *Zfp30* on cytokine secretion are not due to differences in the abundance of cytokine-secreting cells. The observation that *Zfp30* leads to differential cytokine secretion in response to both TLR4 and TLR5 ligands may imply that ZFP30 targets some shared member of the downstream signaling machinery of these receptors. Follow-up experiments to probe specific shared members of these signaling pathways or to assay genome-wide expression in *Zfp30*$^{-/-}$ data may give further insights into the targets of ZFP30-mediated repression.

In contrast to our *ex vivo* results, our *in vivo* experiments largely did not reveal significant differences in chemokine secretion or neutrophil recruitment. We observed an impact on IL-6 secretion in a subset of our *in vivo* results, but we did not see reproducible effects on CXCL1 or neutrophil levels. One potential explanation is that *Zfp30* may have a modest effect on CXCL1 secretion *in vivo*. Our *ex vivo* cytokine measurements represent an accumulated difference in CXCL1 and IL-6 secretion in cell culture media over a 24 hour period; at any particular snapshot of time *in vivo*, the difference in CXCL1 or IL-6 levels may not be large enough to produce an observable effect with our BALF analysis methods. Another potential explanation is the genetic background that our knockout was generated on. C57BL/6J has a known mutation in *Nlrp12* that impacts neutrophil recruitment and CXCL1 production by leukocytes (Ulland *et al.* 2016; Hornick 2017). If *Zfp30* impacts CXCL1 secretion by neutrophils, this may have played a role in its original discovery in the CC and may not be apparent in C57BL/6J mice. Introducing a knockout into an already-mutated cytokine secretion pathway may mask the effects of the knockout. As a follow-up to the results presented here, one could consider a *Zfp30* knockout or knockdown in an alternate mouse strain. If *Nlrp12* masks the effect of *Zfp30* on neutrophil recruitment in C57BL/6J, use of another mouse strain could facilitate a more in-depth investigation of ZFP30's role in neutrophil recruitment *in vivo*.

Our results additionally revealed effects of ZFP30 loss on spleen weight, liver weight, and blood glucose levels. *Zfp30* is differentially expressed in the pancreatic beta cells of type-2 diabetes patients (Lawlor 2017), so the results presented here

may warrant a follow-up investigation into the impact of *Zfp30* in a mouse model of diabetes. Moving forward, the role of *Zfp30* on development and pancreas function should be investigated more closely. RNA-seq and ATAC-seq in MTEC cultures, spleens, livers, and pancreases of $Zfp30^{+/+}$ and $Zfp30^{-/-}$ mice would help to identify the specific targets of ZFP30-mediated repression in each of these tissues. RNA-seq will reveal genes that are differentially expressed in the $Zfp30^{-/-}$ mice, and ATAC-seq will provide information on the chromatin states of these tissues. $Zfp30^{-/-}$ mice likely have altered chromatin states near genes that are directly repressed by ZFP30, as the KAP1-mediated heterochromatin domains at those sights will not be maintained via ZFP30 binding.

The cause of our periodic TRD phenotype remains unclear, though there is precedent for zinc finger protein knockout mice to display some degree of embryonic lethality. Knockout of *Zfp57*, a C2H2 ZFP with a KRAB domain, impacts the establishment and maintenance of critical DNA methylation imprints, and disruption of this imprinting leads to death (Li *et al.* 2008). It is possible that we are observing a phenotype with incomplete penetrance or variable expressivity due to *Zfp30*'s impact on the local chromatin landscape of the causal gene(s). If there is epigenetic inheritance of *Zfp30* chromatin effects, this could potentially explain why the TRD disappeared after selective breeding. Additional generations of breeding may have allowed slow erosion of ZFP30-maintained heterochromatin domains among heterozygous mice used for breeding given that heterozygous mice have intermediate expression of the $Zfp30^{+/+}$ allele.

# CHAPTER 4: DISCUSSION

Many polymorphisms contribute to variation in a complex trait, and identification of these variants can be difficult due to intricate genetic architecture (Lander and Schork 1994). Genetics reference populations like the Collaborative Cross make it easier to tease apart the underlying mechanisms of disease-related phenotypes through use of a large number of inbred lines, particularly in the context of studies with both treatment and control groups. In this dissertation, I follow up on a QTL mapping study in the Collaborative Cross (Rutledge *et al.* 2014) that identifies *Zfp30* as a candidate regulator of CXCL1 secretion levels and neutrophil recruitment to the lung. In Chapter 2, I identify a causal variant underlying the bulk of variation in *Zfp30* expression. In Chapter 3, I explore the role of *Zfp30* in cytokine secretion and neutrophil recruitment to the airways using a whole body knockout mouse model, and I uncover a novel role of *Zfp30* in IL-6 regulation.

A large proportion of genetic variants associated with disease are found in regulatory regions of the genome and overlap transcription factor binding sites (Maurano *et al.* 2012). In Chapter 2, I show that this is the case with rs51434084, a SNP underlying the *Zfp30* eQTL identified in the Collaborative Cross study (Rutledge *et al.* 2014). I utilize luciferase assays and site-directed mutagenesis in a candidate regulatory region of *Zfp30* to test the impact that specific SNPs have on expression, and I conduct EMSA experiments to identify the mechanism through which candidate SNPs impact transcription. I find that variation at rs51434084

produces a two-fold difference in expression level of *Zfp30* via differential binding of ZFP148, a known transcriptional repressor.

Identifying the mechanisms that produce differential expression is not always straightforward, however. rs51434084 accounts for the difference in *Zfp30* expression between the high (A/J, NOD/LtJ, NZO/H1LtJ, 129S1/SvImJ) and moderate (C57BL/6J and WSB/EiJ) expressing strains described in Chapter 2, but the mechanism that differentiates the moderate and low (CAST/EiJ and PWK/PhJ) expressing groups remains unclear. Using site directed mutagenesis and luciferase assays, I find that modification of any of the 6 SNPs shared by the low expression group in our cloned promoter region (Table 2.1) produces a shift from moderate to low expression in a C57BL/6J promoter (Figure 4.1). We initially expected a single SNP to explain the full difference between the moderate and low expression groups or for multiple SNPs to each modulate expression by a smaller fraction. Our results, however, suggest that variation at any of these six shared SNPs that span a ~120bp region is sufficient to produce the difference in expression between the moderate and low *Zfp30* expression groups. This suggests that each SNP impacts the same mechanism of *Zfp30* regulation. Future experiments could include combinations of SNPs in site-directed mutagenesis or prediction of upstream binding of transcription factors with mechanisms of action that are impacted by local DNA sequence.

In Chapter 3, I utilize a knockout mouse model to explore the role of *Zfp30* in cytokine secretion and neutrophil recruitment, and *ex vivo* results confirm an

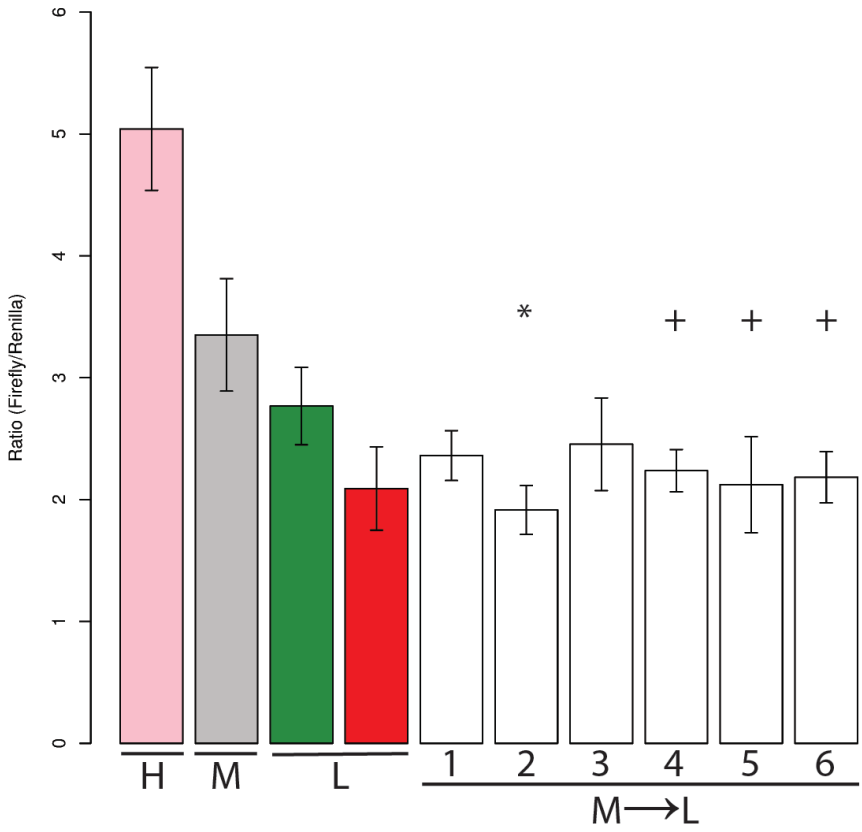


**Figure 4.1: SNPs shared among low-expressing promoters significantly modulate *Zfp30* expression level.** Dual luciferase reporter assays with haplotypes from 129S1/SvImJ, C57BL/6J, CAST/EiJ, and PWK/PhJ strains, as well as C57BL/6J haplotype with site-directed mutagenesis of the rs211739374 (1), rs211739374 (2), rs252419650 (3), rs578896492 (4), rs581321876 (5), and rs239071968 (6) sites to match the low expressing CAST/EiJ and PWK/PhJ strains were performed, and firefly/*Renilla* ratios are shown. Reporter clones are grouped by *in vivo Zfp30* expression, with the addition of haplotypes generated by SDM (M→L). * $P < 0.05$; $^{+}$ $P < 0.1$.

important regulatory role for *Zfp30* in the signaling pathways that modulate neutrophilia in the airways. *Zfp30* impacts CXCL1 secretion, which is a known chemotactic factor for neutrophils. I also uncover a novel effect of *Zfp30* on IL-6 production, which indicates that *Zfp30* may play a role in the eventual clearance of neutrophils. However, our *in vivo* results reveal no significant differences in neutrophil recruitment as assayed by differential cell counts from BALF.

It is not typically clear how many genes contribute to complex phenotypes in disease, and networks of epistasis further complicate this genetic architecture (Shao *et al.* 2008). Thousands of polymorphisms that individually explain less than 1 percent of variation may underlie a complex phenotype (Goddard *et al.* 2016). With this in mind, it may not be surprising that the knockout of *Zfp30* does not produce a striking effect on neutrophil level in the lung. *Zfp30* is likely one of multiple regulators of CXCL1 and IL-6, CXCL1 is only one of several potent CXCR2 ligands, and CXCR2 is only one of multiple receptors that impacts neutrophil chemotaxis. This signaling complexity is compounded by the complications that can arise when choosing a single mouse strain to test a knockout in. C57BL/6J has known mutations that impact CXCL1 expression in leukocytes (Ulland *et al.* 2016, Hornick 2017), and epistasis can have significant buffering effects in complex gene networks (Shao *et al.* 2008). All of these factors, together with normal technical variation in *in vivo* experiments, may mask subtle differences in neutrophil recruitment in our knockout colony. Our *ex vivo* results together with the result from the Collaborative Cross (Rutledge *et al.* 2014) suggest that *Zfp30* does have some effect on neutrophil

levels in the lung, even if *Zfp30* knockout does not cause a statistically significant difference upon immune challenge *in vivo*.

An additional aim of this body of work was to identify targets of ZFP30-mediated repression on a genome-wide scale. A comprehensive exploration of ZFP30 targets would ideally include both RNA-seq and ChIP-seq analyses. RNA-seq analysis when *Zfp30* is overexpressed or knocked down will reveal the downstream transcriptional effects of ZFP30-mediated repression. ChIP-seq will reveal ZFP30 binding sites, and genes near those binding sites are the likely candidates for direct repression by ZFP30 due to the mechanism of KAP-1 heterochromatin domain spreading. Together, these analyses will identify direct functional targets of ZFP30-mediated repression. ChIP-seq alone does not reveal how far the ZFP30-mediated heterochromatin domains spread and does not quantify the effect that these heterochromatin domains have on expression of nearby genes. RNA-seq alone allows for the possible identification of indirect targets if ZFP30 modulates the expression of any transcription factors.

However, this aim of the project has proven difficult to accomplish. ChIP-seq requires the ability to probe the protein of interest with an antibody, and we were unable to validate any commercially available ZFP30 antibodies. To work around this, we attempted to overexpress HA and eGFP-tagged ZFP30 proteins in mouse airway epithelia cell lines. We utilized constructs with each tag at either the N or C terminus of *Zfp30*, and we attempted this overexpression in seven cell lines. We were able to successfully detect expression of tag control vectors at the protein level, but, ultimately, we were unable to achieve tagged overexpression of ZFP30 in

a mouse cell line at the protein level. This prevented us from directly identifying ZFP30 binding sites via ChIP-seq, and this prevented us from quantifying the protein-level effect of *Zfp30* shRNAs for knockdown experiments. Future efforts to identify ZFP30 binding sites could involve ATAC-seq or H3k9me3 ChIP-seq when *Zfp30* is over-expressed or knocked down to assess heterochromatin domains that increase or decrease along with *Zfp30* expression level.

RNA-seq experiments using MTEC cultures from Chapter 3 are underway. *Zfp30*$^{+/+}$ and *Zfp30*$^{-/-}$ samples at baseline and after LPS exposure will be assessed for differentially expressed genes. The protein-level cytokine results in Chapter 3 suggest that we may see a significant impact on *Cxcl1* and *Il-6* expression, but the data may reveal a more complex signaling network than we can currently predict. Genes that are differentially expressed across treatment groups will reveal the overarching signaling pathways that are activated in MTECs upon LPS exposure, and any genes within these pathways that are differentially expressed across genotypes are likely targets of ZFP30-mediated repression that impact neutrophilia. If no genes upstream of *Cxcl1* or *Il-6* are impacted by *Zfp30* knockout, it may suggest that *Cxcl1* is a direct target of KAP-1 mediated repression. The direction of effect seen in IL-6 secretion, however, suggests that is not directly repressed in *Zfp30*$^{-/-}$ mice. If any immune signaling molecules are differentially expressed that we have not tested at the protein level, we may revisit the *in vivo* or *ex vivo* models for follow-up experiments. This RNA-seq analysis may also provide leads on any roles that *Zfp30* plays in development or other disease contexts.

As the field of genetics shifts more and more toward large-scale mapping experiments with genomic data, follow-up studies of candidate genes will become increasingly important for understanding the mechanisms that underlie disease. In aggregate, this dissertation represents the follow-up characterization of a candidate gene identified in QTL mapping studies of immune response phenotypes in the CC. Roadblocks in each chapter of the project highlight the intricacies of complex disease traits and reveal the difficulty in fully characterizing the mechanisms that modulate these traits. Chapter 2 is a journey from large-scale QTL findings to the identification of a specific polymorphism that underlies variation in expression. Chapter 3 is a functional characterization of *Zfp30* after *in vivo* and *ex vivo* immune challenge to assess the impact that variation in *Zfp30* expression might have on disease. Each of these chapters expands the known *Zfp30* signaling network and its downstream impact. The findings presented here have implications for a number of pulmonary diseases, and it is likely that *Zfp30* plays a role in any disease context in which the robustness of neutrophil chemotaxis or clearance is important.

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