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Investigating Gp96 Biology and its Macrophage-Intrinsic Role in Colitis-Associated Colon Tumorigenesis

Crystal Morales

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Investigating gp96 Biology and its Macrophage-Intrinsic Role in Colitis-Associated Colon Tumorigenesis

Crystal Morales, Ph.D.

University of Connecticut, 2013

Gp96, a mammalian endoplasmic reticulum resident chaperone, is required for the proper folding and expression of multiple Toll-Like Receptors (TLRs) and integrins. The expression of this heat shock protein (HSP) is elevated in cancer, and it is known to cross-present tumor antigens. A better understanding of gp96 biology will aid the design of cancer drugs, as gp96 is a target. To study this molecule, we investigated an orthologous system in Drosophila, a fruitful approach used in understanding HSP90 biology. A BLAST search using the mammalian gp96 sequence identified Drosophila gp93, an uncharacterized molecule. With 74% amino acid homology and various conserved elements, we hypothesized that gp93 was a gp96 ortholog. This gene was therefore cloned and transduced into a murine pre-B cell line deficient for gp96. It was next tested whether or not gp93 could functionally compensate for gp96-loss by rescuing gp96 client expression. Despite the long evolutionary gap, gp93 was indeed able to rescue gp96 client expression in mouse cells, albeit to a lower expression level than gp96. It was further demonstrated that CNPYb is a TLR-specific cochaperone of gp93, similar to the gp96 cochaperone CNPY3. Important residues in each molecule were then elucidated.
Therefore, we have not only identified gp93 as the *Drosophila* ortholog of gp96, but we have also established a simpler system by which to further study gp96 biochemistry.

Since gp96, TLRs, and macrophages have all been implicated in cancer, we decided to further explore their role in tumor initiation and progression. We utilized a macrophage-specific gp96 knockout (KO) mouse model to study the role of macrophage derived gp96 in tumorigenesis. Both wild type and KO mice were treated with either Dextran Sulfate Sodium (DSS) alone to induce colitis, or Azoxymethane plus DSS to induce inflammation-associated colonic tumors. KO mice are protected from colitis and colon tumorigenesis, suggesting that macrophage intrinsic gp96 plays a promoting role in both diseases. Mechanistically, gp96 induces IL-17, IL-23, TNFα, Wnt signaling, and β–catenin mutations while reducing CD4+ IFNγ+ cells. Our data thus help to explain the manner in which gp96 promotes cancer, thus identifying possible targets for therapeutic development.
Investigating gp96 Biology and its Macrophage-Intrinsic Role in Colitis-Associated Colon Tumorigenesis

Crystal Morales

B.A., Boston University, 2005

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2013
November 12, 2012

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2013
Doctor of Philosophy Dissertation

Investigating gp96 Biology and its Macrophage-Intrinsic Role in Colitis-Associated Colon Tumorigenesis

Presented by

Crystal Morales, B.A.

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Linda Cauley, Ph.D.

University of Connecticut
2013
DEDICATION

It is with great pleasure that I dedicate this thesis to my mother, Ms. Laudelina Morales. She supported me in every way possible since I was a child; from band, class grades, graduations, to jobs. She instilled morals into me which made me the person I am today. She also passed down to me her passion for school and success, always pushing me to pursue higher education, and become a role model for other Latinos while helping to fade away stereotypes against our race. Just as I am sure she is proud of me as her daughter, I am proud of her as my mother. For if it was not for her, this thesis would have never come into fruition. This is for you, so thank you mom. I love you.
ACKNOWLEDGEMENTS

The past five years of my life have been extremely arduous, and I could have never finished my thesis without the help of many people. First, I would like to express my sincerest gratitude to my PI, Dr. Zihai Li. You not only accepted me into your lab, but also into the University. You trained me to be a technically well rounded scientist, you taught me to be critical, and showed me how to write grants, animal protocols, and publications. You have always supported me as your student, and I know you always will. For these reasons, I am forever grateful. Thank you Zihai.

I would also like to thank my committee members, Dr. Anthony Vella and Dr. Linda Cauley. Tony, before I was even a student, you met with me and gave me advice on my application. And Linda, you had interviewed me. Once I became a student, you both helped me not only in my classes, but then as committee members. You guided me with my experiments and provided so much support during both my preliminary exam and thesis preparation. Thank you so much for your help. During my preliminary exam, Dr. Robert Clark, Dr. Stefan Brocke, and Dr. Carol Wu (RIP) all served on my committee. They guided and supported me throughout the process. I would like to thank all of them for their help as well.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td></td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td></td>
<td>ii</td>
</tr>
<tr>
<td>APPROVAL PAGE</td>
<td></td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td></td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td></td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td></td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>xi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td></td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>I.</td>
<td>Gp96</td>
<td>2</td>
</tr>
<tr>
<td>II.</td>
<td><em>Drosophila</em> homologues</td>
<td>11</td>
</tr>
<tr>
<td>III.</td>
<td>Macrophages and cancer</td>
<td>14</td>
</tr>
<tr>
<td>IV.</td>
<td>NF-κB in colitis and cancer</td>
<td>22</td>
</tr>
<tr>
<td>V.</td>
<td>Molecules and pathways implicated in cancer</td>
<td>31</td>
</tr>
<tr>
<td>VI.</td>
<td>Colitis and colon cancer</td>
<td>40</td>
</tr>
<tr>
<td>VII.</td>
<td>Outline of this thesis dissertation</td>
<td>42</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>MATERIALS AND METHODS</td>
<td>46</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>GP93 IS THE <em>DROSOPHILA</em> ORTHOLOG OF MAMMALIAN GP96.</td>
<td>63</td>
</tr>
<tr>
<td>I.</td>
<td>Rationale</td>
<td>64</td>
</tr>
<tr>
<td>II.</td>
<td>Results</td>
<td>65</td>
</tr>
</tbody>
</table>
CHAPTER 4 MACROPHAGE-SPECIFIC GP96 PROMOTES COLITIS-ASSOCIATED COLON TUMORIGENESIS

I. Rationale................................................................................................. 103
II. Results.................................................................................................... 104
III. Discussion............................................................................................. 110
IV. Figures.................................................................................................... 114

CHAPTER 5 CONCLUDING REMARKS......................................................... 128

I. Conclusions............................................................................................ 129
II. Outstanding questions........................................................................... 131

REFERENCES............................................................................................... 140
List of Tables

Table I  Comparison of mammalian and Drosophila protein families…………….44
Table II Literature review of DSS-colitis and AOM+DSS colon cancer………….45
Table III Gene accession numbers……………………………………………………………57
Table IV List of cloning primer sequences……………………………………………….58
Table V List of Drosophila RNAi primer sequences……………………………………….59
Table VI List of Drosophila qRT-PCR primer sequences………………………………….60
Table VII List of murine qRT-PCR primer sequences……………………………………..61
List of Figures

Figure 1: Sequence alignment and structure conservation between mammalian gp96 and
*Drosophila* gp93.................................................................82

Figure 2: gp93 expressed in murine gp96-mutant pre-B cells can rescue gp96 client
expression.................................................................84

Figure 3: Both gp96 and gp93 interact with TLR9.................................86

Figure 4: gp96 and gp93 heterodimerize........................................87

Figure 5: gp93^{A137C} rescues N-terminal disulfide bond dimerization, yet does not
enhance chaperone function...........................................89

Figure 6: gp93^{96CBD} does not have improved client expression....90

Figure 7: gp93^{658-75D} has decreased client expression....................92

Figure 8: gp93^{2YA} has decreased client expression.........................93

Figure 9: CNPYa and CNPYb improve β2 expression while only CNPYb improves TLR
expression.................................................................94

Figure 10: gp96 and gp93 both interact with CNPY3 and CNPYb...........96

Figure 11: *Drosophila* CNPY structure and sequence alignment........97

Figure 12: Cysteines 36, 39, and 92 of CNPYb are important for folding of TLRs.....98

Figure 13: CNPYb^{C183A} phenocopies CNPYb^{C39A}..........................99

Figure 14: gp93 may not chaperone endogenous *Drosophila* Toll........100

Figure 15: Model of gp93, CNPYa, and CNPYb chaperone function.........101

Figure 16: Macrophage-specific gp96 KO mice are less susceptible to colitis....114

Figure 17: Decreased tumor burden in Macrophage-specific gp96 KO mice......116
Figure 18: KO mice harbor less cytokine expression…………………………………117
Figure 19: Similar macrophage markers between WT and KO mice………………….118
Figure 20: KO mice exhibit diminished lamina propria CD4⁺ IFNγ⁺ cells……………..120
Figure 21: Wnt activation and β-catenin mutations are decreased in KO mice………121
Figure 22: WT and KO mice demonstrate similar DNA repair machinery……………123
Figure 23: BMT verifies a hematopoietic intrinsic role……………………………………124
Figure 24: Model of the promoting roles of macrophage-specific gp96………………126
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium Chloride Kalium-hydrogen-carbonate</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
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<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
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<td>BER</td>
<td>Base Excision Repair</td>
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<td>BM</td>
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<td>Bone Marrow Transfer</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CAC</td>
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<tr>
<td>CBD</td>
<td>Client Binding Domain</td>
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<td>CNPY</td>
<td>Canopy</td>
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<td>Cys</td>
<td>Cysteine</td>
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<td>Day 5</td>
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<td>Day 8</td>
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<tr>
<td>DAMP</td>
<td>Danger Associated Molecular Pattern</td>
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<tr>
<td>DSP</td>
<td>Dithiobis-Succinimidyl Propionate</td>
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<tr>
<td>DSS</td>
<td>Dextran Sulfate Sodium salt</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylene-Diamine-Tetraacetic Acid</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EV</td>
<td>Empty Vector</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>Gp93</td>
<td>Glyco-Protein of 93kDa</td>
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<tr>
<td>Gp96</td>
<td>Glyco-Protein of 96kDa</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>ICS</td>
<td>Intra-Cellular Stain</td>
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<td>Interleukin</td>
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<td>IP</td>
<td>Immuno-Precipitation</td>
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<td>i.p.</td>
<td>Intra-Peritoneal</td>
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<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
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<td>KO</td>
<td>Knock Out</td>
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<tr>
<td>LysM</td>
<td>Lysozyme M</td>
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<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
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<td>ml</td>
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<td>MMR</td>
<td>MisMatch Repair</td>
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<td>MUSC</td>
<td>Medical University of South Carolina</td>
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<tr>
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<td>Poly Acrylamide Gel Electrophoresis</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal Exudate Cells</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time PCR</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SP</td>
<td>Signal Peptide</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<td>University of Connecticut Health Center</td>
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</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
This introduction will start with a focus on gp96 biology, detailing what is known about this molecule. We will then move on to discuss the two major client families of gp96: TLRs and integrins. Next, we will discuss the cochaperone CNPY3 and its family members. Since we elucidate the Drosophila ortholog of gp96, we will then examine the Drosophila Toll, integrin, and CNPY families. As our experiments analyze the role of macrophage-specific gp96 in colitis and colon cancer, we will next review macrophage biology, including differentiation and subtypes, and consider what is already known about the role of macrophages in cancer. Furthermore, since TLRs signal via NF-κB, we next discuss the NF-κB pathway, and the role of NF-κB and TLRs in both colitis and colon cancer. The following section will examine various molecules and pathways associated with both cancer and our mouse model. Finally, we will consider the clinical aspects of colitis and colon cancer, thereby highlighting the significance of our work. The introduction will then end with an outline of the thesis.

I. gp96

The mammalian heat shock protein (HSP) gp96 is located in the endoplasmic reticulum (ER) and is ubiquitously expressed. A member of the HSP90 family, it is also known as HSP90B1, grp94, endoplasmin, TRA1, CaBP4, ERp99, ECGP, and HSPC4 (1, 2). Gp96 is a chaperone with two major client families: Toll-like receptors (TLRs) and integrins. It is required for the folding of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9, but not TLR3 (3-5). It is also required to fold the integrins α1, α2, α4, αD, αE, αL, αM, αV, αX, β2, β5, β6, β7, and β8 (3, 6). Additionally, gp96 is required for expression
of the platelet GPIb-IX-V complex via folding GPIX directly (7). Refer to Table I for a list of gp96 clients.

Besides being a chaperone, gp96 also plays a role in the ER-associated degradation (ERAD) pathway, where it associates with OS-9 which binds ERAD substrates and brings them in proximity to the ubiquitin ligase Hrd1 (8). Gp96 is also postulated to play a role in the unfolded protein response (UPR), as its absence leads to decreased XBP-1 splicing yet increased compensatory expression of calreticulin, calnexin, and GRP78 (9). Furthermore, gp96 contains various low and high affinity calcium binding sites, and may thus play a role in calcium storage (2). Gp96 was also thought to act as a kinase and an aminopeptidase, but this was later proven to be the result of contaminating proteins in the purified fraction. However, gp96 itself can be phosphorylated (10-12).

The structure of gp96 was first solved in 2007 when Dollins et al crystalized the purified protein. Present as a homodimer, the structure reveals a twisted V conformation with dimerization at the C-terminus. The N-terminus remains open and the structure does not change between bound ADP or AMP-PNP (13). The gp96 structure was later analyzed by small angle X-ray scattering and electron microscopy, which further confirmed the V shape of the dimer. Highly homologous, the structure of gp96 mirrors that of cytosolic HSP90 (14).

Various structural elements of gp96 are well documented. The molecule contains an N-terminal signal peptide (SP) for targeting to the ER, while a C-terminal KDEL ER-retention sequence prevents it from continuing down the secretory pathway. Moreover, the N-terminus contains an ATP-binding pocket, followed by a highly charged middle
domain, and then the C-terminal dimerization domain (2). While the ATP and dimerization domains are important for function, the charged middle domain is not required (3). Although at a low rate, gp96 has proven ATPase properties, and this ability is required for function (3, 15-17).

The gp96 protein is 803 amino acids long and the importance of various amino acids has been elucidated. In 1996, a stretch of 44 amino acids in the C-terminus (676-719 in canine gp96) was demonstrated to act as the dimerization domain (18). Gp96 has five potential N-linked glycosylation sites and it is phosphorylated on threonine and serine residues (2, 10). The amino acid D159, which binds ATP, is important for chaperone function. The amino acid E103, important for ATP hydrolysis, is required for folding of TLRs but not integrins (3). Importantly, a potential client-binding domain was postulated from the gp96 crystal, where a methionine pair surrounded by hydrophobic residues in alpha helix C2 protrude outward from the structure (13). This domain, residues 652-678, was later verified to indeed be the client-binding domain for both TLRs and integrins. Moreover, the methionine pair was further proven to be critical for integrins but not TLRs (19).

Gp96 is intimately linked to innate immunity via TLRs. As thus, its expression is important for the function of macrophages which express high levels of TLRs (4). Gp96 is also important for proper tissue compartmentalization of B cells due to integrins, and for optimal antibody responses to TLR agonists (20). These studies were carried out with conditional knockout mice. However, using pan inducible knockout mice, gp96 was further shown to be important for the pro- to pre-B transition, where cells were blocked and prevented from differentiating. Similarly, thymocytes were stopped at the double
negative stage (CD4^- CD8^-), and thus no mature T cells developed. In contrast, myeloid cell development was unimpaired in these mice (6).

Gp96 has various clinical implications. Its expression in macrophages promotes endotoxin shock, yet it is protective against *Listeria* infection (4). Gp96 is also important in vesicular stomatitis virus (VSV) infection, where its absence prevents the envelope glycoprotein VSV-G from binding the cell, and thus prevents cell entry (21). Although primarily located in the ER, gp96 has been found on the cell surface (22). Utilizing a transgenic mouse with enforced surface gp96 expression, it was found that such localization induced lupus like autoimmune disease. Specifically, surface gp96 induced IL-12 production by DCs in a MyD88 dependent manner (23, 24). TLR4 not only caused inflammation in this model, but also induced Treg suppressive function (25). Furthermore, gp96 has proven to be a therapeutic target for this disease, where a chemical gp96-inhibitor relieved lupus symptoms (26).

Although cell surface expression of gp96 causes autoimmunity, its expression in tumor cells promotes anti-tumor immunity via cross-presentation of cellular antigens and the induction of tumor-specific T cells (27, 28). Even without enforced cell surface gp96 expression, gp96 purified from tumors is an effective immunization inducing specific anti-tumor immunity via macrophages and both CD4 and CD8 T cells (29). This tumor-specific immunogenicity of gp96 derives from chaperoned antigenic peptides which are cross-presented via MHC I (30). Moreover, gp96 expression is induced in various cancers, both spontaneous and chemically induced, as well as tumor associated macrophages (31, 32). Clinically, gp96 is the target of various cancer trials. The vaccine Vitespen, for instance, is a HSP-peptide complex modeled off of gp96 (33).
TLRs

TLRs are a family of pattern recognition receptors (PRRs) which recognize various pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, fungi, and parasites. Additionally, a few endogenous ligands have also been found, termed danger-associated molecular patterns (DAMPs), such as HMGB1, HSPs, and degradation products of the extracellular matrix. As an important component of innate immunity, TLRs are a first line of defense which signal immune cells and turn on adaptive immunity. These receptors can be broadly grouped by their location. The majority of TLRs are present on the cell surface: TLR1, TLR2, TLR4, TLR5, and TLR6. Another group of TLRs are located within endosomes: TLR3, TLR7, TLR8, and TLR9. The former group recognizes mainly microbial membrane components such as proteins, lipoproteins, and lipids. The latter group recognizes foreign nucleic acids. Mice express TLR1-13, but TLR10 is nonfunctional. Meanwhile, humans only express TLR1-10 (34).

The cytoplasmic tail of TLRs recruits TIR domain containing adaptor proteins. All TLRs recruit MyD88 except for TLR3, which recruits TRIF. TLR4 is the only member which recruits both MyD88 and TRIF. However, this receptor first interacts with TRAM before recruiting TRIF (34). As well, both TLR2 and TLR4 interact with TIRAP before MyD88 recruitment (34, 35). MyD88 then interacts with various IRAK family members, which in turn activate various TRAF members, which then activate both NF-κB and MAPK pathways. The TRIF cascade induces TRADD, Pellino-1, RIP1, and then both NF-κB and MAPK pathways. The MyD88-dependent pathway primarily stimulates
inflammatory cytokine production, while the TRIF pathway primarily stimulates a type I interferon response (34).

TLR1 and TLR6 can form a heterodimer with TLR2. The TLR1/2 dimer recognizes triacylated lipopeptides from mycoplasma and gram-negative bacteria, while the TLR2/6 dimer recognizes diacylated lipopeptides from mycoplasma and gram-positive bacteria. The specificity of these dimers derives from structural differences between TLR1 and TLR6; specifically, a hydrophobic channel in TLR1 is missing from TLR6 (34, 36). The triacylated lipopeptide Pam3CSK4, for instance, is recognized by the TLR1/2 dimer. TLR2 further functions with the help of coreceptors, such as dectin-1 and CD36, which aid in PAMP recognition (34).

TLR3 recognizes foreign dsRNA, mainly from viruses. A synthetic analog, poly(I:C), is commonly used during experimentation. Receptor ligation results in the production of both proinflammatory cytokines and type I interferon. Since the structure was crystalized, TLR3 was determined to homodimerize at the C-terminus. The horseshoe-like shape allows for ligand binding at both the N and C termini at the convex lateral side (34).

TLR4 recognizes lipopolysaccharide (LPS), a constituent of the gram-negative outer membrane. The soluble LPS-binding protein (LBP) binds LPS, which is then bound by the cell surface CD14, and is then transferred to TLR4 in complex with another molecule, MD2. Receptor ligation can then trigger proinflammatory cytokine and type I interferon production (34). In Alzheimer’s disease and atherosclerosis, TLR4 has been found to heterodimerize with TLR6. In this situation, amyloid-β and oxidized low-
density lipoprotein act as the ligands and produce a sterile inflammation. Furthermore, the CD36 scavenger receptor was found to regulate this pathway (37).

**TLR5** recognizes bacterial flagellin, a protein of flagella. In particular, small intestinal lamina propria DCs express high levels of TLR5. These DCs can promote Th1 and Th17 differentiation, as well as B cell differentiation into IgA producing plasma cells. TLR11 is a relative of TLR5. Although less is known about this TLR, it appears to recognize a profilin-like molecule in *Toxoplasma gondii*, as well as uropathogenic bacterial constituents (34).

**TLR7** recognizes ssRNA from viruses. It can also recognize some small interfering RNAs, synthetic poly (U) RNA, resiquimod (R-848), imiquimod, and guanine analogs like loxoribine. In particular, TLR7 is expressed highly on pDCs where it induces a strong type I interferon response as well as proinflammatory cytokines. Ligand recognition occurs within endolysosomes. TLR8 is most similar to TLR7. In humans, TLR8 recognizes viral ssRNA and R-848. In mice however, the ligand is unknown, as TLR8 KO mice respond normally to such agonists (34).

**TLR9** recognizes unmethylated CpG DNA, common in viruses and bacteria, yet rare in mammals (34). Specifically, it was determined that the sugar backbone of DNA is important for recognition (38). TLR9 is also stimulated by the hemozoin crystal which is a byproduct produced by *Plasmodium falciparum*. Ligation of this receptor induces a strong Th1 response, and it is highly expressed on pDCs (34). More recently, TLR9 was found to be post-translationally cleaved, yielding a functionally mature receptor. This cleavage appears to be carried out by a member of the cathepsin protease family (34, 39,
40). Another level of TLR9 regulation is achieved through granulin, a soluble cofactor which potentiates CpG-TLR9 binding and signaling (41).

TLRs 10-13 have yet to be fully characterized. However, TLR13 is a murine TLR located within the endosomes. Recently, Oldenburg et al discovered that the ligand for TLR13 is a conserved 23S ribosomal RNA sequence (42). Shortly after, Hidmark and colleagues found that TLR13 recognizes whole gram-positive bacteria, and specifically bacterial RNA. TLR13 signaling seems to be MyD88 and UNC93B dependent, and can induce both inflammatory cytokines and type I interferon (43).

Integrins

Integrins are a family of cell surface molecules consisting of an alpha and beta subunit. Mammals have 18 different alpha integrins (α1, α2, αIIb, α3, α4, α5, α6, α7, α8, α9, α10, α11, αD, αE, αL, αM, αV, and αX) and 8 beta integrins (β1, β2, β3, β4, β5, β6, β7, and β8; Table I) which heterodimerize to form 24 distinct dimers. Some integrins are restricted to certain tissues or cell types, such as αIIbβ3 on platelets and β2 integrins on leukocytes. Various structural elements of integrins have been characterized, such as the I domain important for ligand binding, and integrins can be grouped according to their ligand specificities: collagen, laminin, RGD, and leukocyte (44).

Integrins link the extracellular matrix and other cells outside to the cytoskeleton inside the cell. Integrins can modulate various cellular processes such as polarity, shape, motility, adhesion, apoptosis, survival, proliferation, differentiation, gene expression, and haptotaxis. Two types of signaling can occur, either inside-out or outside-in. With inside-out signaling, G-protein-coupled receptors signal a conformational change in the integrin
allowing for higher affinity ligand binding. This occurs with leukocyte extravasation from the blood through the endothelial barrier, causing the cell to stop instead of roll. Ligand binding with outside-in signaling also induces a conformational change in the integrin. This leads to interaction of the cytoplasmic tail with various adaptor proteins and enzymes which assemble into adhesion structures. (44)

Besides being involved in many cellular processes, integrins serve as receptors for various bacteria and viruses. Mutations in β2 can cause leukocyte adhesion deficiency (LAD) (45). As such, they have become potential therapeutical targets for various conditions. Anti-α4 antibodies have been used for Crohn’s disease and other inflammatory bowel diseases, as well as for multiple sclerosis. Anti-β3 antibodies have even been used for thrombosis. Various other integrin antagonists have been designed for clinical use as well, such as for β2 (44, 45).

**CNPYs**

Gp96 requires a co-chaperone for folding TLR9, called CNPY3 (5). The CNPY family is highly conserved across the animal kingdom with four mammalian members: CNPY1, CNPY2, CNPY3, and CNPY4 (Table I). This small family is divided into two subgroups, where the first two members have shorter C-terminal sequences than the last two members. The CNPY family is similar to the Saposin-like proteins, in that they contain a characteristic pattern of six cysteine residues. All members have a signal peptide and ER retention signal. However, CNPY3 also contains a C-terminal basic region high in lysine residues. CNPY1 displays self-binding properties, and it is thus postulated that CNPYs form a Saposin-like dimeric structure (46).
CNPY3 is also known as PRAT4A (protein associated with TLR4 A) for its ability to regulate TLR4 cell surface expression and signaling (47). CNPY3 was later found to also play a role in regulating TLR1, TLR2, TLR7 and TLR9. Moreover, similar to gp96, CNPY3 does not regulate TLR3 (48). Three single-nucleotide polymorphisms (R95L, S231I, and M145K) were characterized in CNPY3, yet only M145K was found to alter function (49). As a cochaperone of gp96, the two molecules are found to physically interact, yet ATP causes complex dissociation. Since CNPY3 seems to bind the ATP-sensitive form of gp96, it is postulated to promote client loading (5).

Other CNPY family members are not as well studied as CNPY3. CNPY4 for instance, also called PRAT4B (protein associated with TLR4 B), appears to regulate TLR4 cell surface expression similar to CNPY3. It is also conserved among various vertebrate species, and it is ubiquitously expressed in different tissues and leukocyte subsets (50). The most dissimilar family member, however, appears to be CNPY1. As opposed to the ubiquitous nature of CNPY3 and CNPY4, CNPY1 is restricted to neural tissues. In zebrafish, CNPY1 regulates FGF signaling along the midbrain-hindbrain boundary via its interaction with FGFR1 (46).

II. Drosophila homologues

Mammalian gp96 chaperones TLRs with the help of its cochaperone CNPY3, and it also chaperones integrins (3-6). An orthologous system would therefore not only contain a paralogue of gp96, but also similar client and cochaperone molecules. Next, we therefore explore these homologous genes in Drosophila, where Toll, integrin, and CNPY families also exist.
**Toll**

Although mammalian TLRs are highly characterized, the original *Drosophila* Toll (Toll-1) was discovered first. The first mammalian TLR was not discovered until 1997, which was TLR4 (51). *Drosophila* Toll, however, is not only important for innate immunity, but also for dorsal-ventral patterning of the embryo (52, 53). *Drosophila* contains nine Toll family members, Toll-1 to Toll-9 (Table I). They all consist of an ectodomain comprised of leucine rich repeats flanked by cysteine-rich domains. They also contain a transmembrane domain and cytosolic tails. Toll-5 is most similar to Toll-1 while Toll-9 is different from the other Tolls, containing only one cysteine-rich domain more characteristic of mammalian TLRs. Only Toll-1, however, has been definitively proven to be involved in innate immunity. Although some evidence exists for a role of Toll-5 and Toll-9 in immunity, this relationship has not been fully characterized (54).

Toll-1, similar to mammalian TLR4, requires accessory proteins in order to be activated. Fungal and yeast derived β-glucan is first recognized by GNBP3, while bacterial peptidoglycan is recognized by either PGRP-SD or PGRP-SA/GNBP1. These complexes then activate a protease cascade, starting with ModSP. This molecule in turn activates Grass, which then activates Spirit, Spheroide, or Sphinx1/2. These proteases then activate SPE, which cleaves Spatzle (Spz). Spz is released as an inactive protein, and when cleaved into the C-terminal 106 amino acids (C-106), serves as the direct ligand of Toll-1. Cell surface dimeric Toll-1 binds two C-106 dimers, initiating the downstream signaling cascade (54).
Comparable to mammalian TLRs, the cytosolic tail of Toll-1 also contains a TIR domain which recruits adaptor molecules. The first adaptor recruited is the *Drosophila* MyD88 homolog, which then binds Tube and then Pelle. Then either Pelle directly phosphorylates Cactus, or it first recruits Cactus Kinase. Once phosphorylated, the inhibitory Cactus is degraded, thus releasing the Dif/Dorsal transcription factor to translocate to the nucleus. Various antimicrobial peptide genes are then transcribed, such as Drosomycin and Defensin, which stimulate an immune response for microbial clearance (54, 55).

**Integrins**

*Drosophila* integrins consists of both alpha and beta subunits which heterodimerize similar to the mammalian integrin family. *Drosophila* has five alpha integrins (\(\alpha_{ps1}\), \(\alpha_{ps2}\), \(\alpha_{ps3}\), \(\alpha_{ps4}\), and \(\alpha_{ps5}\)), and only two beta integrins (\(\beta_{ps}\) and \(\beta_{\nu}\); Table I). \(\beta_{ps}\) (myospheroid) is widely expressed and can heterodimerize with each alpha subunit (44). \(\beta_{\nu}\) expression is very restricted, only being expressed at 12-15 hours of embryogenesis, and selectively in the midgut endoderm (56). It is not essential for viability or fertility, and it can partially rescue loss of \(\beta_{ps}\) (57).

\(\beta_{ps}\) and \(\alpha_{ps1}\) dimerize to form the PS1 (position specific) integrin; \(\beta_{ps}\) and \(\alpha_{ps2}\) form PS2; \(\beta_{ps}\) and \(\alpha_{ps3}\) form PS3; and so on. The ligands for these integrins appear to be components of the extracellular matrix, such as collagen IV, tiggrin, laminins, and tenascin-m. Specifically, PS1 and PS2 are known to bind laminins, and PS2 can further bind tiggrin. PS3 is also thought to bind laminins. Other specific ligands are unknown. However, data suggests that there are multiple ligands for each integrin (58, 59).
Integrins $\alpha_{ps1}$ (multiple edematous wings) and $\alpha_{ps2}$ (inflated) are both expressed in the embryo, larva, and adults. Their expression patterns seem to be complimentary, with $\alpha_{ps1}$ expressed in the ectoderm and $\alpha_{ps2}$ in the mesoderm. Specifically, $\alpha_{ps1}$ is highly expressed in the midgut primordia while $\alpha_{ps2}$ appears to concentrate at muscle attachment sites, and both are expressed in the wing imaginal disc (60, 61). $\alpha_{ps3}$ (scab, volado) is also expressed in all developmental stages: embryo, larva, and adult. It is expressed in the amnioproctodeal invagination, amnioserosa, midline of the ventral nerve cord, dorsal vessel, midgut, trachea, and salivary gland (62). Furthermore, PS1 and PS2 are expressed during early and mid-oogenesis, while PS3, PS4, and PS5 are expressed in late-oogenesis (59).

**CNPYs**

The CNPY family is highly conserved. In *Drosophila*, there are two family members: CNPYa and CNPYb (Table I). These CNPYS appear to also contain a signal peptide and ER retention sequence. The six saposin-like cysteine residues are also conserved in both *Drosophila* CNPYS. Furthermore, CNPYb has a highly basic region similar to CNPY3, although smaller in size. Meanwhile, CNPYa appears to be more similar to CNPY1 and CNPY2 (46). However, the function of the *Drosophila* CNPYS is unknown.

**III. Macrophages in cancer**

Macrophages have been implicated in promoting both colitis and cancer (63-66). However, which molecules and pathways are involved in this process is not completely understood. We believe that macrophage intrinsic gp96, in part via chaperoning TLRs,
promotes colitis and cancer. Therefore, we will next describe macrophages in general, and what is known in the literature about their role in colitis and cancer progression.

**Macrophages**

Macrophages are hematopoietic derived immune cells. Hematopoietic stem cells in the bone marrow give rise to myeloid progenitors which differentiate into monoblasts, promonocytes, and then into monocytes. Monocytes leave the bone marrow to circulate in the blood and migrate to various tissues throughout the body. In the presence of CSF-1, these monocytes differentiate into macrophages. However, they can also give rise to osteoclasts in the presence of RANKL, or to myeloid DCs (67, 68). Multiple macrophage subsets exist in different tissues which differ in various ways: microglia in the brain, alveolar macrophages in the lung, Kupffer cells in the liver, and Langerhans cells in the skin, among others (69, 70).

Although macrophages are thought of as immune cells, they also play trophic roles in development. Macrophages are required for proper ductal branching, such as in the mammary gland and pancreatic islets. Microglia are important for neural networking, specifically within the hypothalamic-pituitary-gonadal axis and both excitatory and inhibitory signals in auditory and visual evoked potentials. Macrophages are also important for angiogenesis after wound healing, and in the eye, heart, and lungs. Macrophages even play roles in adipogenesis, myocyte development and growth, and erythrogenesis (68).

The immune functions of macrophages include phagocytosis/endocytosis and antigen presentation. Macrophages, present in high numbers, are the first line of defense
against an invading pathogen. They are able to phagocytize pathogens either directly or indirectly via PRRs. The phagocytized material is processed and then presented for activation of adaptive immunity via MHC I and II. The engagement of various PRRs results in the production of cytokines and chemokines. Macrophages also have microbicidal properties, and can fuse into giant cells (71).

In order to have diverse functions, macrophages must harbor different receptors and other molecules. For instance, macrophages express various PRRs, such as TLRs, NLRs, and C-type lectin receptors. They also express complement molecules, scavenger receptors, MHCI and II, costimulatory molecules, and integrins. Some common macrophage markers include CD11b, F4/80, CD68, CSF-1R, MAC2, CD163, and Marco (69-71).

Macrophages can further be divided into classically- or alternatively-activated macrophages (M1 versus M2). While M1 cells are typically stimulated with IFNγ, M2 cells are stimulated by IL-4 and IL-13, therefore being associated with either a Th1 or Th2 response, respectively. M1 cells are predominantly inflammatory, expressing IL-1, IL-6, IL-12, IL-23, TNFα, MHCIi, CD86, Nos2, SOCS3, pSTAT1 and/or pSTAT3. M2 cells are more suppressive, expressing IL-10, Dectin-1, Arg1, CCL12, CCL24, CXCL13, SOCS2, and pSTAT6. M2 macrophages are more similar to trophic macrophages (69-71).

Macrophages within the small and large intestines are primarily located within the subepithelial lamina propria (72). These macrophages are unique, being highly phagocytic and bactericidal due to the constant presence of commensal bacteria within the gut. However, they weakly produce proinflammatory cytokines, and this phenotype is
induced by the intestinal stromal cells (67). Besides tolerance to commensals, intestinal macrophages must also be nonresponsive to food antigens (70). While they express various TLRs, they do not express CD14 or various Ig receptors. When stimulated by TLR ligands, however, these macrophages do not produce inflammatory cytokines. Instead, they act to maintain homeostasis in an environment with constant foreign stimuli. When pathogenic organisms invade into the lamina propria, however, blood monocytes are recruited which can induce a needed inflammatory response (72).

Macrophages have been implicated in various diseases: cancer, rheumatoid arthritis, atherosclerosis, multiple sclerosis, Crohn’s disease, autoimmune hepatitis, diet-induced obesity, type II diabetes, allergy, asthma, infection, and fibrosis (68, 70). In cancer, macrophages constitute a large percentage of cells within the tumor microenvironment and have thus been called Tumor Associated Macrophages (TAMs) (73). TAMs are more similar to alternatively activated macrophages (M2), and differ from classically activated macrophages (M1) in terms of secreted molecules, among other factors (74).

Roles in colitis and cancer

Macrophages harbor a promoting role in colitis. Patients with ulcerative colitis have increased numbers of CD14+ macrophages within the intestinal mucosa. These cells are activated by IgG+ plasma cells to produce inflammatory cytokines such as TNFα and IL-1β. These macrophages are also stimulated by commensal bacteria to produce TNF and IL-23, and thus promote colitis progression (64). CD14+ macrophages are also increased in Crohn’s disease patients, and they produce more IL-6, IL-23, and TNFα than
macrophages in either normal controls or ulcerative colitis patients. In Crohn’s disease, these macrophages contribute to IFNγ production by lamina propria mononuclear cells (65). Therefore, macrophages promote both ulcerative colitis and Crohn’s disease.

Jeffrey Pollard and colleagues have performed tremendous work on the tumor-promoting roles of macrophages in vivo, primarily in the context of mammary cancers. Pollard first became interested in macrophage colony-stimulating factor (CSF-1, M-CSF) and its receptor CSF-1R due to the fact that the expression of both of these molecules is increased in breast, uterine, and ovarian cancers and that they correlate with poor prognosis and high grade (66). CSF-1 regulates differentiation, proliferation, and survival of monocytes (macrophages, microglia, and osteoclasts) (66, 75). However, both CSF-1 and CSF-1R expression have been found in tumor cells, suggesting a direct role in tumor promotion (66).

Mice with a recessive null mutation of CSF-1 (Csf1op), which have an almost complete absence of mature tissue macrophages, were crossed to transgenic mice that are susceptible to breast cancer (Polyoma Middle T oncoprotein, PyMT). Although primary tumor incidence and growth were not affected in these mice, the progression to invasive carcinoma and metastasis were delayed. As well, the recruitment of macrophages to the tumor microenvironment was also reduced. Furthermore, overexpression of CSF-1 accelerated tumor progression and metastasis (66). Macrophages thus harbor tumor promoting capabilities.

Within the Csf1op PyMT mouse model, tumor cells only express EGFR while macrophages only express CSF-1R. The two cell types interact in a paracrine manner and migrate via EGF and CSF-1 gradients into microneedles. In this manner, macrophages
promote the migration of carcinoma cells away from the primary tumor, and thus promotes metastasis (76). DeNardo and colleagues expanded upon this notion, discovering that CD4+ IL-4+ T cells directly promote macrophage secretion of EGF, which then signals tumor cells via EGFR (77). Furthermore, multiphoton microscopy demonstrated that tumor cells must associate with perivascular macrophages within the mammary tumor in order to intravasate when local angiogenesis is absent (78).

Using Csf1<sup>op</sup> PyMT mice, CD11b-DTR mice, and L-clodronate to deplete macrophages from mammary tumors by both genetic and chemical means, Pollard demonstrated that macrophages are important for cancer cell extravasation, metastatic seeding and subsequent growth, obtaining similar data from each model. For instance, when tumor cells are injected iv, they accumulate in the lung. However, the number of tumor cells drastically drop by 36 hours. The cells remaining have successfully seeded the lung, and then start to proliferate. L-clodronate treatment significantly decreased the number of seeded tumor cells. Not only do less metastatic nodules form, but the ones which grow are smaller in size. Furthermore, the phenotype of macrophages recruited into the metastatic site differs from resident macrophages. Recruited macrophages have similar F4/80 expression, but increased CD11b, CCR2, CX3CR1, and VEGFR1, yet decreased CD11c (63). Therefore, a distinct macrophage population is recruited which promotes tumor cell metastasis.

Tumors must be in proximity to blood vessels in order to survive, grow, and metastasize. Not only can tumors utilize the host vasculature, but they can also promote angiogenesis within the tumor microenvironment. Moreover, the occurrence of tumor angiogenesis is associated with progression to malignancy. Again using the Csf1<sup>op</sup> PyMT
model, Pollard’s group found that macrophages promote tumor angiogenesis. While Csf1\(^{op}\) PyMT mice had delayed angiogenesis, premature macrophage infiltration into premalignant tumors promoted early vasculature formation (79). In addition, these macrophages were newly recruited right before malignant progression, and were essential for angiogenesis (80).

In order to uncover the role of vascular endothelial growth factor (VEGF-A) produced by TAMs in tumorigenesis, Pollard’s group genetically restored VEGF-A expression in mammary epithelial of PyMT mice, and hence in tumors. Compared to control mice, angiogenesis and tumor progression were restored with VEGF-A. Leukocyte infiltration was also induced, consisting predominantly of macrophages (81). Therefore, TAMs promote tumor angiogenesis via production of VEGF-A.

Similar to Pollard’s data, Kubota used a CSF-1R inhibitor and blocking antibody in a mouse model of osteosarcoma. AX osteosarcoma cells were injected s.c. in the back, followed by daily s.c. injections of either the inhibitor or blocking antibody, creating a local decrease in monocyte differentiation. In this model, CSF1/CSF-1R inhibition resulted in decreased tumor growth, angiogenesis, lymphangiogenesis, metastasis, mortality, and macrophage infiltration into the tumor. Importantly, healthy angiogenesis and lymphangiogenesis were not affected, contrary to that seen with VEGF inhibition. Moreover, \textit{in vitro} studies proved that the inhibitor only affects macrophages, and not AX cells (75).

The use of clodronate encapsulated in liposomes has proven an effective chemical method for depleting macrophages. Although touched upon above in Pollard’s work, many other investigators have taken advantage of this reagent. Zeisberger and colleagues
used clodronate with human A673 rhabdomyosarcoma and murine F9 teratocarcinoma in mouse tumor models. Efficient depletion of TAMs resulted in decreased tumor growth and angiogenesis (82).

From a clinical standpoint, the adverse role of macrophages in cancer can also be seen. For example, patients with classic Hodgkin’s lymphoma have large numbers of macrophages. Higher numbers of macrophages in these patients correlated with shorter progression-free survival, shorter disease-specific survival, and increased likelihood of relapse. Thus, macrophages can be used as a biomarker for risk stratification in Hodgkin’s lymphoma (83).

Although the above mentioned literature defines a tumor promoting role of macrophages, various macrophage mediators have also been demonstrated. Coculture experiments of macrophages with breast cancer cell lines increased tumor cell invasiveness. Macrophages upregulated TNFα, which lead to increased secretion of matrix metalloproteases (MMPs). Inhibition of either TNFα or MMPs reduced invasiveness. Neither invasiveness nor induction of TNFα or MMPs was seen in cocultures of macrophages with a benign mammary epithelial cell line (84). Matsumoto demonstrated another macrophage mediator in colitis-associated premalignant cancer. Macrophages from treated mice were found to produce increased IL-6 and soluble IL6Ra, which signaled on gut epithelial cells inducing pSTAT3. Antagonizing the IL-6 coreceptor (gp130) on epithelial cells significantly reduced disease (85).

Another mediator of macrophage function is nitric oxide (NO), considered to be a M1 marker. In a model of skin cancer, transfer of CD8+ T cells lead to tumor cell killing. These T cells produced IFNγ, which activated macrophages to produce NO. Surprisingly,
NO played a dual role, being required for tumor regression, yet suppressing CD8$^+$ T cell activity (86). In another study, combined IL-1/anti-CD40 immunotherapy had anti-tumor effects in a renal cell carcinoma model. This treatment induced high NOS2/iNOS expression in macrophages. Both macrophage depletion and iNOS inhibition reduced the ability of the immunotherapy treatment to reduce lung metastases. However, NO did not play a role in primary tumor reduction (87). Additionally, higher levels of iNOS are seen in TAMs after irradiation therapy, as well as Arg1 and Cox2. Irradiated prostate cancer cells had quicker growth kinetics than unirradiated tumor cells, suggesting a tumor-promoting role for iNOS, Arg1, and Cox2 in macrophages (88).

Many tumor cells have an ER stress response which actually promotes tumor growth and progression. Curiously, secreted factors from such stressed tumor cells can activate ER stress within macrophages as well. These stressed macrophages then amplify an inflammatory response beneficial for tumor cells. Macrophage-specific TLR4 was identified to play a role in this transmission of ER stress to the macrophages, although TLR2 had no effect (89). Therefore, TLR4 plays a tumor promoting role in macrophages.

IV. NF-κB in colitis and cancer

The NF-κB signaling pathway is the main pathway downstream of TLRs, and it has been implicated in both colitis and cancer (90-97). Since gp96 chaperones TLRs, we next describe the NF-κB signaling pathway in general, and then explore what is known about its role in these two diseases.
**NF-κB pathway**

NF-κB is a family of five similar transcription factors: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). They combine as homo and heterodimers, and are inhibited by the binding of IκB in the cytosol. The IκB family consists of five members: IκBα, IκBβ, IκBγ, IκBε, and BCL3. Upon activation of various pathways, these inhibitors are phosphorylated by IKK kinase and degraded by the proteasome. The IKK family has three family members, IKKα, IKKβ, and IKKγ (NEMO). IKK itself is activated by NIK kinase. Once the IκB inhibitor is degraded, liberated NF-κB translocates to the nucleus where it acts as a transcription factor. Multiple pathways activate NF-κB, such as TLRs, TNFα, IL-1, IL-18, T cell receptor, B cell receptor, BAFF, CD40L, and lymphotixin β. The NF-κB pathways can also regulate multiple events, such as proliferation, apoptosis, differentiation, inflammation, immune response, and even tumorigenesis (90, 91).

**NF-κB in colitis**

Myeloid differentiation factor 88 (MyD88) is an important adaptor molecule necessary for TLR, IL1R, and IL18R activation of NF-κB. MyD88 also engages IFNGR1, where it activates the p38 MAPK pathway (98). Three groups demonstrated the importance of MyD88 in intestinal homeostasis and protection from colitis utilizing the DSS model (92-94). Rakoff-Nahoum showed that MyD88 KO mice dramatically lost weight and died after acute DSS treatment. Compared to WT mice, these KO mice had increased rectal bleeding, ulceration, erosion, and epithelial injury, while red blood cell concentration, hematocrit, and levels of IL-6 and CXCL1 were decreased. Using antibiotic treatments, TLRs were implicated as the potential upstream receptors involved. While WT mice had
100% survival rates for DSS-colitis, antibiotic treatment decreased survival to ~10%, similar to MyD88 KO mice treated with DSS. Furthermore, the addition of LPS or LTA ameliorated survival rates (92). However, this report does not investigate the potential roles of IL1R, IL18R, or IFNGR1.

Araki et al confirmed the protective role of MyD88 in acute DSS-colitis, where MyD88 KO mice exhibited increased weight loss, bleeding, clinical and histological scores, and colonic shortening (93). Kirkland also demonstrated the increased mortality of MyD88 KO mice treated with DSS, but used a chronic model as opposed to an acute model. Contrary to Rakoff-Nahoum, antibiotic treatment ameliorated survival of both WT and MyD88 KO mice (94). DSS directly attacks epithelial barrier integrity, allowing exposure of the underlying lamina propria cells to gut commensal bacteria. It appears that direct sensing of commensals and the ensuing inflammation is protective during acute colitis, presumably promoting wound repair. During chronic colitis however, uncontrolled commensals proliferate and become systemic, where they can be found in the liver and lung, and lead to mortality (94).

The importance of Kirkland’s work is in the investigation of cell specific roles of MyD88 in colitis. Although epithelial cell specific MyD88 KO mice had no phenotype compared to WT mice treated with DSS, all other cell specific KO mice had decreased survival: B cells, T cells, DCs, and macrophages. While the later three cell specific mice had roughly 40-50% survival, only B cell specific MyD88 KO mice had 0% survival, similar to total MyD88 KO mice. It appeared that B cell specific MyD88 controlled lethal dissemination of commensals, where B cell activation of complement played a key role
(94). However, since TLRs are more prevalent in DCs and macrophages, it’s curious to know whether IL1R, IL18R, or IFNGR1 may also play protective roles in B cells.

Besides MyD88, other NF-κB pathway members also play roles in colitis. Nenci demonstrated that intestinal epithelial cell specific IKKγ (NEMO) KO mice developed spontaneous intestinal inflammation. These mice exhibited pathology characteristic of colitis, with shortened colons, bacterial translocation, and immune cell infiltration (CD4+ T cells, DCs and granulocytes). These mice also had increased levels of cytokines and chemokines as early as two weeks of age, which became more pronounced over time. Furthermore, MyD88 and TNFR1 upstream of IKK were crucial for pathogenesis, as disease was ameliorated in KO mice crossed to either MyD88 or TNFR1 KO mice. The phenotype of spontaneous colitis was also recapitulated in IKKα/β double KO mice, but not in single KO mice, presumably due to compensation by the other (99). Although intestinal epithelial cell specific IKKβ KO does not lead to spontaneous colitis, Greten et al demonstrated that these mice are more susceptible to DSS-colitis. KO mice lost more weight, and had increased histological damage, ulceration, and expression of inflammatory cytokines than WT mice (100). Therefore, epithelial specific IKK is important for intestinal homeostasis, as well as amelioration of colitis.

Single immunoglobulin IL-1 receptor related molecule (SIGIRR/TIR8) is a negative regulator of TIR signaling, such as with IL1R and TLR. SIGIRR KO mice therefore have uninhibited signaling through these receptors. Under the DSS regimen these mice are highly susceptible, with 100% mortality by 13 weeks and high induction of inflammatory molecules. SIGIRR KO mice were then crossed to gut epithelial specific SIGIRR transgenic mice and then subjected to DSS-colitis. These mice had an
intermediate phenotype compared to WT or SIGIRR KO mice (101). Therefore, NF-κB induction plays a colitis promoting role in epithelial cells, as well as another cell type(s) not examined in this model. Collectively, these data demonstrate that NF-κB plays a promoting role in colitis (Table II).

NF-κB in cancer

As discussed in more detail below, mice with an autosomal dominant mutation within the adenomatous polyposis coli (APC) gene develop spontaneous multiple intestinal neoplasia (Min). These APC<sup>Min</sup> mice develop numerous tumors in both the small and large intestines (102). Later, this phenotype was found to be mediated via MyD88. Rakoff-Nahoum et al demonstrated that compared to APC<sup>Min/+</sup> mice, APC<sup>Min/+</sup> MyD88 KO mice had increased survival, hematocrit, and apoptotic tumor cells, while polyp number and size were decreased (95). Another group further demonstrated that the APC<sup>Min</sup> phenotype was driven by TLR and EGF activation of ERK and subsequent increase in cMyc, leading to increased proliferation and reduced apoptosis in enterocytes, thereby promoting tumorigenesis (103).

A study by Hagemann and colleagues demonstrated that IKKβ polarized TAMs towards an alternatively activated phenotype with increased IL-10 and ARG1, yet decreased IL-12. When IKKβ was inhibited, macrophages reverted to a classical phenotype with increased iNos, IL-12, and tumoricidal activity, while IL-12 recruited NK cells (104). Although IKK seemed to protect against colitis as previously mentioned, here it promoted cancer.
Swann et al showed the tumor promoting effects of MyD88 in two separate mouse models: DMBA+TPA induced skin papilloma, and MCA induced fibrosarcoma. Tumor incidence was significantly decreased in MyD88 KO mice under both models. The number of lesions was also decreased in the DMBA+TPA model. However, MyD88 was not required for transplanted tumor rejection (96). Therefore, MyD88 appears to be more important for tumor initiation than for tumor progression.

Schiechl and colleagues discovered a colitis promoting role of MyD88 in oxazolone (Oxa)-induced colitis, as well as tumor promoting effects in an inflammation-induced colon cancer model utilizing Oxa with AOM. MyD88 KO mice were more resistant than WT mice to either model, with decreased weight loss and tumor numbers. Tumor formation was found to be dependent on IL-6, and the role of MyD88 appeared to be hematopoietic intrinsic by BM chimeric studies (105).

Although epithelial IKKβ is protective against colitis as previously discussed, it actually harbors tumor promoting capabilities. Under the AOM+DSS model of colitis-induced colon cancer, epithelial specific IKKβ KO mice develop fewer tumors, although tumor size is not affected. This decrease in tumor number was associated with increased apoptosis and pro-apoptotic factors, and decreased anti-apoptotic Bcl-xL. The tumor promoting role of IKKβ was further identified in myeloid cells, such as macrophages and neutrophils, using a different cell-specific conditional KO mouse. When treated with AOM+DSS, these mice had fewer tumors, and a higher percentage of smaller tumors than WT mice. Although levels of apoptosis were not affected in these mice, there were reduced levels of proliferation and inflammatory molecules (100). So while epithelial IKKβ prevents tumor cell apoptosis, myeloid IKKβ promotes tumor cell growth.
As discussed earlier, the TIR negative regulator SIGIRR demonstrates that hyperactive NF-κB signaling promotes colitis. SIGIRR KO mice are also more susceptible to AOM+DSS colon cancer, exhibiting increased tumor incidence, tumor number, and tumor size. These mice had increased tumor cell proliferation and expression of inflammatory molecules (101). Therefore, uncontrolled NF-κB activation via SIGIRR KO causes an exacerbated inflammatory response resulting in death with colitis, and increased tumor burden with colon cancer.

Similar to the protective role of MyD88 in colitis, Salcedo et al demonstrated that MyD88 is also protective from AOM+DSS induced colon cancer. MyD88 KO mice exhibited increased bleeding, diarrhea, inflammation, ulcers, polyps, and even squamous metaplasia, while hematocrit and colon length were decreased. Although KO mice had decreased proliferation and DNA repair molecules, there was also increased apoptosis, angiogenesis, and inflammation. The tumors in MyD88 KO mice even progressed into adenocarcinomas over time. To determine which upstream receptor was involved, IL1R, IL-18, and IL18R KO mice were also treated with AOM+DSS. Although IL1R KO mice were phenotypically similar to WT controls, IL-18 and IL18R KO mice demonstrated a similar phenotype to MyD88 KO mice with increased bleeding and polyp numbers. Additionally, IL-18 KO mice are also more susceptible to DSS-colitis, with increased bleeding, diarrhea, ulceration, and colonic shortening (97). Therefore, MyD88 is protective in both colitis and colon cancer, at least in part due to IL-18 signaling. However, the precise role of TLRs was not investigated in this model.
TLRs in colitis and cancer

TLRs are expressed highly on antigen presenting cells, such as DCs, B cells, and macrophages, with lower expression on epithelial, paneth, and T cells. Within the gut, leukocytes are located in the lamina propria, just under the epithelial lining. Although TLR2 and TLR4 are expressed throughout the small and large intestines, higher expression was found in the colon. TLR2 was more dominant in the proximal colon, while TLR4 was greater in the distal colon. DSS-colitis upregulated both TLRs, but the expression patterns remained similar (106). Patients with ulcerative colitis and Crohn’s disease also have increased levels of intestinal TLR2 and TLR4 (107). Rakoff-Nahoum subjected TLR2 and TLR4 KO mice to DSS-colitis, and found that both KO mice had increased weight loss and mortality (92). TLR2 and 4 may therefore be protective against colitis, and their expression is upregulated in order to ameliorate disease.

In sporadic human colorectal cancer, TLR2 mRNA is increased in tumor tissue, while TLR4 expression does not differ (107). In another study however, TLR4 protein was increased in tumor tissue from colitis-associated cancer samples (108). Fukata and colleagues characterized the expression level of various TLRs in the murine AOM+DSS model. TLR2, 3, 4, 5, and 9 were all increased upon treatment in both tumor tissue and nondysplastic surrounding mucosa. While most TLRs had higher expression in the surrounding mucosa than the tumor, only TLR4 was higher within the tumor. Treating TLR4 KO mice with AOM+DSS demonstrated the tumor promoting role of TLR4. KO mice had decreased tumor incidence, tumor number, tumor size, and area affected. TLR4 was found to induce Cox2 expression, which induced PGE₂, amphiregulin, and EGF, thus leading to increased proliferation of cancer cells (108).
Contrary to Fukata’s work, Lowe et al demonstrated that TLR2 protects against AOM+DSS induced colon cancer. Treated TLR2 KO mice had increased weight loss, mortality, tumor number, tumor size, high grade dysplasia, and aberrant crypt foci. Expression of β-catenin, pSTAT3, and inflammatory molecules were also increased, as well as Th17 responses and proliferation. Apoptosis and NO production, however, were reduced (109). Therefore, TLR2 and TLR4 have opposing roles in colon cancer, which raises the question of the roles of other TLRs in colon cancer. Collectively, these data demonstrate that NF-κB plays both protective and promoting roles in the AOM+DSS model (Table II). While IL18R and TLR2 protect, TLR4 promotes. The role of other upstream receptors must therefore be determined in order to clarify the role of NF-κB.

TLRs have also been directly implicated in other cancer types. For instance, Kim et al demonstrated that Lewis lung carcinoma secretes versican, an extracellular matrix proteoglycan, which is recognized by the TLR2/6 heterodimer on macrophages. TLR ligation leads to macrophage activation with IL-6 and TNFα production which promoted metastatic tumor growth (110). In this setting, TLR2/6 signaling promotes metastasis. Another study by Earl et al demonstrated metastatic promotion by TLR4. MC38 murine colon cancer cells were injected subcapsularly into the spleen of WT and TLR4 KO mice. This method mimics the portal circulation of metastatic colorectal cancer cells. Although host TLR4 expression had no effect, TLR4 expression on MC38 cells promoted metastasis, as evidenced by MC38 cells treated with TLR4 siRNA (111).
V. Molecules and pathways implicated in cancer

Due to the significant findings of our data and the direction of our work, we will now discuss molecules and pathways which will be important for understanding and interpreting our results. We will discuss IL-17 and IL-23, the Wnt/β-catenin pathway, DNA repair, and inflammasomes. We will explain the basics of each of these, and then go into what is known about their role in colitis and cancer.

**IL-17 & IL-23**

IL-17 is a cytokine which is predominately produced by proinflammatory Th17 cells. Wu and colleagues demonstrated that colitis caused by enterotoxigenic *Bacteroides fragilis*, a colonic bacterium, is mediated by IL-23, STAT3, and IL-17 producing Th17 cells (112). Utilizing the AOM+DSS model of colitis-associated colon tumorigenesis in IL-17A KO mice, Hyun et al demonstrated the tumor promoting role of IL-17A. KO mice exhibited decreased tumor number, tumor size, inflammation, proliferation, and proinflammatory cytokine expression (113).

Another group used recombinant and retroviral transduction of IL-17 into tumors to reveal pro-tumor growth and pro-angiogenic characteristics, such as cord formation and vascular endothelial cell migration (114). Additionally, IL-17A promotes growth of the bladder cancer cell line MB49 and the melanoma cell line B16 when injected into mice. IL-17A was found to promote IL-6 expression, leading to STAT3 activation, and stimulating survival and angiogenesis, thus aiding tumor growth. Furthermore, IL-17 is elevated in various cancers as well as patients with ulcerative colitis (115, 116).
IL-23 is a dimeric cytokine consisting of the specific p19 subunit together with the p40 subunit, which is also shared with IL-12. This cytokine, which is required for Th17 cell differentiation, is also linked to cancer. Langowski and colleagues discovered the tumor promoting role of IL-23 in the DMBA+TPA skin papilloma model, demonstrating its role in both tumor growth and incidence (117). This work was later verified by Teng et al, who further describe the tumor promoting and metastatic potentiating effects of IL-23 in MCA-induced fibrosarcoma and B16F10 melanoma transfer models (118). IL-23 has also been shown to play a promoting role in the T cell transfer model of colitis, where it suppresses Tregs while inducing Th17 accumulation (119).

Besides mouse models, a clinical role for IL-23 has also emerged. This cytokine is elevated in various human cancers, such melanoma, stomach, breast, lung, and head and neck cancer, with the highest fold induction found in ovarian and especially colon cancer (117). IL-23, along with its receptor IL23R, is elevated in both ulcerative colitis and Crohn’s Disease patients (116). Additionally, mutations within IL23R have further highlighted the link between this cytokine and Crohn’s disease (120). Therefore, both IL-17 and IL-23 promote tumorigenesis and colitis.

Wnt/β-catenin
The Wnt pathway supports multiple cellular processes, such as proliferation, differentiation, apoptosis, cell motility, and tissue patterning (121, 122). The ligands for this pathway are the secreted Wnt family molecules, composed of 19 members: Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a,
Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt16 (121). These ligands are recognized by the Frizzled (Fzd) cell surface receptors together with the LRP coreceptors (122). The family of Fzd receptors is composed of 10 members, Fzd1-10 (123).

The canonical Wnt pathway is usually turned off. In this state, a cytosolic destruction complex exists, composed of glycogen synthase kinase 3 (GSK3), APC, Axin, and casein kinase Iα (CKIα). This complex phosphorylates β-catenin, causing it to be ubiquitinated by βTrCP and degraded by the proteasome. Upon ligation of the receptors with Wnt, the downstream pathway is activated. Receptor aggregates form which recruit disheveled (Dsh) to phosphorylate LRP. Phospho-LRP then recruits Axin, thus causing dissociation of the destruction complex and liberation of β-catenin. β-catenin accumulates in the cytoplasm, and then translocates to the nucleus where it acts as a coactivator with the transcription factors TCF/LEF (T cell factor/lymphoid enhancer family) (122).

Unlike the canonical pathway, activation of the noncanonical Wnt pathway is independent of β-catenin and LRP. Instead, the release of intracellular calcium activates the pathway, where Wnt associates with a Fzd receptor to activate Dsh, but this time activating the calcium sensitive kinases CamKII and PKC. Dsh can also activate small GTPases (Rho, Rac, Cdc42), which activate JNK, MAPK, ROCK, and AP-1. Importantly, the Wnt pathway is deregulated in various diseases, such as hyperactivation in colorectal cancer (122, 124).

Various Fzd receptors have been associated with colon cancer. One study shows that Fzd1 and Fzd2 were not present in normal tissue, but were expressed at the protein level in poorly differentiated human colon cancer samples at the border of invasion,
suggesting a role for these receptors in tumor invasion (125). Another study shows that among the ten different Fzd receptors, Fzd3, 5, and 8 are moderately expressed, and Fzd4, 6, and 7 are highly expressed in human colon cancer cell lines. In particular, Fzd7 was more consistently highly expressed in all cell lines examined, and it is also expressed in various primary human colon cancer samples. Furthermore, Fzd7 is able to induce Wnt target genes in cancer cell lines, even in the presence of APC and β-catenin mutations (123).

Utilizing ethynitrosourea to induce germline mutations, Moser and colleagues discovered a mouse line which developed spontaneous adenomas in both the small and large intestines. Since mice exhibited multiple intestinal neoplasia, they termed the mutated gene Min (102). Later, this gene was identified as APC, an important molecule in the Wnt signaling pathway, and these mice are now known as APCMin mice. Furthermore, Wnt ligands themselves are also associated with colon cancer. Although the expression of Wnt1, 4, 5b, 7a, 10b, and 11 were unaltered between normal and colon cancer, Wnt2, and to a lesser extent Wnt5a, were both upregulated in human colon cancer samples compared to normal adjacent tissue (125).

Macrophages in particular are important for linking Wnt pathway activation and cancer. Macrophages secrete IL-1β via Stat1 activation, which activates the Wnt pathway in human colon cancer cells, inducing their growth. This pathway can be inhibited with vitamin D3, which attenuates Stat1 activation (126). Activated macrophages can also secrete TNFα, which inhibits GSK3β and thus activates the Wnt/β-catenin pathway in gastric tumor cells (127). Conversely, Ojalvo and colleagues showed that TAMs, which promote murine mammary tumor cell invasion, have increased Wnt pathway molecules.
Multiple Fzd receptors and Wnt ligands were increased, as well as TCF7, Axin, and LRP5 (121). Similarly, Pukrop et al demonstrated that macrophages cocultured with breast cancer cells upregulate Wnt5a, leading to increased MMP7 and enhanced invasiveness of tumor cells (128).

Murine macrophages infected with *Mycobacterium tuberculosis* upregulated Fzd1 expression in a manner dependent on TLRs, MyD88, and NF-κB. When activated with the Wnt3a ligand, these macrophages became anti-inflammatory with reduced TNF release (129). This study therefore links together the TLR and Wnt pathways. Collectively, these data demonstrate the strong link between increased Wnt signaling and cancer, with a promoting role of macrophages in particular.

**DNA Repair**

Many intrinsic and extrinsic insults exist which cause DNA damage. Intrinsic factors include alkylation, oxidation, hydrolysis, and mismatched DNA bases. Extrinsic factors include ultraviolet radiation, ionizing radiation, and chemical agents. Such DNA damage can cause genetic mutations, leading to senescence, apoptosis, and increased susceptibility to cancer, neurological disorders, and immunodeficiency. Organisms have therefore evolved a series of checkpoints to recognize DNA damage, and multiple DNA-damage repair pathways to correct the problems (130).

Six DNA repair pathways exist which correct different types of DNA damage: 1. Mismatch repair (MMR), 2. Base excision repair (BER), 3. Direct reversal, 4. Non-homologous end joining, 5. Homologous recombination, and 6. Nucleotide excision repair (130). However, only the first two pathways are associated with colon cancer (130-
The MMR pathway repairs insertion/deletion loops and base mismatches which usually occur during DNA replication (130, 133). It can also correct base modifications caused by oxidizing, alkylating, and methylating agents, among others. In particular, the MMR pathway repairs O\textsuperscript{6} methylguanine-containing mismatches, which are recognized by the MSH heterodimer (133). The BER pathway consists of two sub pathways, the short- and long-patch BER. These pathways fix both short (1 nucleotide) and long (2-13 nucleotides) base damage, respectively (130).

The MMR pathway has been associated with colon cancer in both mice and humans. MLH1, MLH3, MSH2, MSH3, and MSH6 KO mice all develop spontaneous intestinal tumors (130). Additionally, hereditary non-polyposis colorectal cancer (HNPCC), or Lynch syndrome, has been associated with mutations in MLH1 and MSH2 at high frequencies (70-80%) (130, 131, 134). Mutations in MSH6 have been found in 10% of cases, and to a lesser extent, mutations have also been found in MLH3, PMS1, PMS2, and EXO1 (130, 131). Biallelic MLH1 inactivation has further been associated with sporadic colorectal tumors (130, 133). Microsatellite instability (MSI) also occurs in colon cancer, which is associated highly with hypermethylation of the MLH1 promoter, loss of MLH1 protein, and mutations in various MMR genes (135). Furthermore, mismatch repair-deficiency syndrome, which causes multiple malignancies including colon cancer, is associated with inherited biallelic dysfunction of MMR genes (131).

The BER pathway has also been associated with both human and murine colon cancer. The autosomal recessive disorder, MYH-associated polyposis (MAP), is correlated with biallelic mutations in the MYH gene and is associated with colonic adenomas and carcinomas. Mutations within the adenomatous polyposis coli (APC) gene
are usually found in MAP tumors as a result of loss of MYH function. MYH KO mice even develop spontaneous intestinal tumors (130). Furthermore, although AAG KO mice do not develop spontaneous tumors, they are more susceptible to the AOM+DSS colon tumorigenesis model, harboring more tumors, worse pathology, and more severely shortened colons (132). This work was later extended to show that ALKBH2 and ALKBH3, DNA glycosylases which recognize etheno-base lesions similar to AAG, also confer increased susceptibility to the AOM+DSS model (136).

Inflammasomes

Inflammasomes are multi-protein complexes located within the cytosol. A few inflammasomes have been described, the majority of which contain NOD-like receptors (NLRs): NLRP1, NLRP3, NLRP6, and NLRC4. Another inflammasome has been identified which contains the HIN200 molecule absent in melanoma 2 (AIM2). Murine NLRP1 and NLRC4 contain caspase recruitment domains (CARDs) which bind various caspases, such as Casp1 and Casp11. NLRP3 and NLRP6, however, lack these CARD domains, yet contain pryin (PYD) domains. These NLRs require an adaptor protein, ASC, which itself has both a PYD and CARD domain. After ASC binding, these NLRs can then recruit caspase via the CARD domain of ASC (137).

Caspase recruitment is essential for inflammasome effector mechanisms. Caspase activation leads to the cleavage of proIL-1β and proIL-18 into the mature IL-1β and IL-18 cytokines, which are then secreted. IL-1β promotes antibody production, B cell proliferation, T cell survival and polarization (Th1, Th2, and Th17), leukocyte migration, and fever. IL-18 stimulates IL-4 and IL-5 production which can promote Th2 responses.
It can also synergize with IL-12 to induce T cell and NK production of IFNγ to promote Th1 polarization. IL-18 can also facilitate IL-17 production and promote Th17 responses (137).

Pyroptosis, a method of proinflammatory cell death via caspase 1 activation, is another effector mechanism of inflammasomes. When a host cell is infected by a virus or intracellular bacterium, cell death is a way to prevent pathogen propagation. Inflammasomes can be triggered by both PAMPs and DAMPs, either directly or indirectly. The exact molecular pattern recognized depends on which inflammasome is activated. Hyaluronan, amyloid β fibrils, uric acid crystals, ATP, bacterial PrgJ basal bodies, flagellin, and pathogenic genomic material have all been identified as ligands of inflammasomes. In the case of ATP, a microbial protease cleaves host mitogen-activated protein kinase kinases (MKKs), and the resulting inhibition of p38 then triggers ATP release from the cell. ATP is then recognized by the surface receptor P2X7, inducing proteasome activation and calcium fluxes, which then activate NLRP1 (137).

Various data have proven a protective role for the inflammasomes in colitis and colitis-induced colon cancer (Table II). Casp1 KO mice are extremely susceptible to DSS-induced colitis, demonstrating its protective role. ASC KO mice had a milder phenotype, while Casp12 KO mice showed no phenotype compared to WT controls. However, Casp12 KO mice were more susceptible to AOM+DSS colon cancer. Interestingly, exogenous IL-18 treatment, which bypasses the requirement for caspases, rescues Casp1 KO mice from colitis (138). Besides the caspases, NLRP genes have also proven to play a role. NLRP12 protects against both DSS-colitis and AOM+DSS colon cancer. This was shown to be due to regulation of the noncanonical NF-κB pathway, and
NLRP12 expression was important in both hematopoietic and non-hematopoietic compartments, although the latter was more important for tumorigenesis (139, 140).

Besides NLRP12, many studies have also demonstrated a role for NLRP3 in cancer. Ghiringhelli et al found that tumor cells release ATP, which is recognized by the P2X7 receptor on DCs and triggers the NLRP3/Casp1 inflammasome to produce IL-1β, which activates cytotoxic T lymphocytes to produce IFNγ (141). Zaki et al found that NLRP3, ASC, and Casp1 KO mice are all more susceptible to DSS-colitis than WT mice. This protection was dependent on IL-18 and nonhematopoietic expression of NLRP3 (142). A couple weeks later, another study by Allen et al demonstrated that NLRP3, ASC, and Casp1 KO mice are all susceptible to both acute and chronic DSS-induced colitis, as well as AOM+DSS colon cancer. The colonic tumors were further associated with reduced levels of IL-1β and IL-18, and NLRP3 expression in hematopoietic cells was important for protection against tumors. However, NLRC4 KO mice had similar resistance to colon cancer as WT mice (143).

The previous data suggests that IL-18 in particular, through inflammasome activation, is important for protection against colitis and inflammation-induced colon cancer. A couple of months after the previous papers were published, Zaki et al came out with another article which confirmed Allen’s data on the protective role of NLRP3, ASC, and Casp1 in AOM+DSS colon cancer. Furthermore, although levels of IL-1β were unaltered in these mice, IL-18 was significantly decreased. Utilizing IL-18 KO mice demonstrated the protective role of this cytokine in AOM+DSS colon cancer. IL-18 administration was also able to rescue Casp1 KO mice. Moreover, reduced IL-18 was associated with decreased IFNγ, and thus diminished Stat1 activation in both epithelial
and immune cells (144). However, overproduction of IL-18 can also exacerbate colitis with increased macrophage infiltration (145). Therefore, the dose of IL-18 is extremely important for disease outcome.

VI. Colitis and colon cancer
Since we utilize mouse models of colitis and colon cancer, we will next describe the clinical aspects of these diseases to help convey the translational importance of our work.

Inflammatory Bowel Diseases
Inflammatory Bowel Diseases (IBD) encompass ulcerative colitis and Crohn’s disease. Although the etiology for either disease is unknown, some common themes have been observed. Crohn’s disease is associated with defective intracellular bacterial processing, while ulcerative colitis is associated with epithelial barrier integrity. In Crohn’s disease, 71 susceptibility genes have been discovered, many of which are linked to the immune system, such as the intracellular pathogen recognition receptor NOD2. In ulcerative colitis, 47 genes have been determined, some of which are important for immunity and epithelial barrier function. Furthermore, 28 genes are linked to both diseases, such as various immune genes and the IL-17/IL-23 axis. Moreover, 51 of these IBD susceptibility genes are associated with 23 other diseases, both immune- and nonimmune-mediated: asthma, alopecia, dermatitis, lupus, multiple sclerosis, rheumatoid arthritis, psoriasis, vitiligo, and diabetes (146).

Chronic IBD is associated with abdominal pain, which can be either somatic or visceral, and can even persist during remission. Four categories of pain are seen in IBD
patients: inflammation, bacterial overgrowth, surgical complications, and neurobiological processes. Even common medications used to treat the inflammation can cause pain, which itself can become disabling (147). A more severe concern in IBD patients is the increased risk of colorectal cancer. It presents at a younger age, accounts for 10-15% of IBD deaths, and is due to both genetic and acquired factors. The potential risk factors are severity of inflammation, duration and extent of colitis, gender, age at diagnosis, coexistent primary sclerosing cholangitis, and family history of sporadic colon cancer. Therefore, routine colonoscopy surveillance is performed 8 to 10 years after IBD diagnosis (148). In order to develop IBD therapies, the etiological understanding of these diseases must be elucidated.

Colon cancer

There are two forms of colon cancer, sporadic and inherited, where benign polyps may form throughout the length of the colon. Although the majority of these will remain benign, a few will progress with age to adenomas, or even carcinomas. There are various colon cancer syndromes: familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC), Gardner syndrome, Turcot’s syndrome, attenuated adenomatous polyposis coli, Peutz-Jeghers syndrome, Cowden disease, Juvenile polyposis syndrome, and MYH-associated polyposis. The majority of colon cancers are associated with other cancer types, such as renal, ovarian, hepatobiliary, endometrial, and gastric, among others. Some of the sporadic colon cancers are associated with chronic inflammatory conditions. For instance, patients with ulcerative colitis are 10 times more likely to develop colon cancer (124).
Inherited colon cancer syndromes have facilitated the identification of genes commonly mutated within this disease. Two pathways are highly mutated: the Wnt/β-catenin and DNA repair pathways. APC is the most commonly mutated gene within the Wnt pathway, while β-catenin, Axin1, Axin2, and TCF4 have also been found to have mutations. The various DNA repair genes which are mutated include MLH1, MSH2, MSH6, MYH, and PMS2. Another category of mutations occur within Ras small-G proteins downstream of growth factor receptors, such as KRAS and NRAS. Another common phenomenon in colon cancers is the presence of microsatellite instability, with high and low frequencies being characterized. Unfortunately, 150,000 Americans are diagnosed with colon cancer annually, and a third of these patients will die from the disease (124). As with other cancer types, treatments are heavily sought after.

VII. Outline of this thesis dissertation

The following chapter will detail all experimental methods used. Chapter 3 contains data elucidating gp93 as the Drosophila ortholog of mammalian gp96. We will show that gp93 is capable of rescuing gp96 client protein expression. Gp93 interacts with TLR9 and can form a dimer with gp96. We demonstrate that although gp93 is incapable of forming disulphide bond dependent C terminal dimerization, this ability is not required for chaperone function. We further elucidate various amino acids which are important for function, helping to characterize the gp96 CBD.

The second half of the following chapter focuses on characterization of the Drosophila CNPY family, focusing on CNPYb in particular. We determine that CNPYb is a TLR-specific cochaperone. We also demonstrate that both CNPYa and CNPYb can
rescue β2 expression without gp93. We further reveal that both gp96 and gp93 are able to physically interact with both CNPY3 and CNPYb. Upon scrutiny of the CNPYb amino acid sequence, we determine that disulfide bonds between cysteine residues are important for function. Unfortunately, we are not able to determine whether or not gp93, CNPYa, or CNPYb play a role in chaperoning endogenous Drosophila Toll.

Chapter 4 of this thesis moves to determine the role of macrophage-specific gp96 in colitis and inflammation-associated colon tumorigenesis. We first demonstrate a promoting role in colitis, characterized by differences in pathology, colon length, stool consistency, and cytokine expression. We also show a promoting role in colon tumorigenesis, illustrated by differences in pathology, tumor burden, cytokine expression, CD4+ IFNγ+ cells, and Wnt signaling. In particular, differences in IL-17, IL-23, and TNFα are found. We also describe huge differences in β-catenin mutations, yet no differences in DNA repair between WT and KO mice. Finally, we determine that the phenotype seen is indeed hematopoietic intrinsic.

At the end of each results chapter is a discussion section where we examine the data, make conclusions in relation to the literature, and debate the significance. The final chapter of this thesis starts with a summary of all the data and their conclusions. We then discuss future directions in sight of the gathered data, and bring the thesis to a conclusion.
Table I: Comparison of mammalian and *Drosophila* protein families.

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<th>Mammals</th>
<th>Drosophila</th>
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Underlined genes represent identified gp96 clients. * indicates identified gp96 cochaperone.
Table II: Literature review of DSS-colitis and AOM+DSS colon cancer.

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Chapter 2

Materials and Methods
**Cell lines:** Phoenix Eco cells were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% FBS (Atlas Biologicals), 100U/ml Penicillin and 100μg/ml Streptomycin (Gibco). 14.GFP (WT) and gp96 mutant E4.126 (KO) murine pre-B cells were obtained from Randow and Seed (Harvard University) (3). Cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100U/ml Penicillin and 100μg/ml Streptomycin, and 0.055 mM 2-mercaptoethanol (Life Technologies). The above cells were all cultured at 37°C with 5% CO₂ in a humidified incubator.

*Drosophila* S2 and S2* cells were gifts from Dr. Brenton Graveley (UCHC) and Dr. Neal Silverman (UMASS), respectively. S2 cells were cultured in Express Five SFM medium (Life Technologies) supplemented with 18mM L-Glutamine (Gibco), 100U/ml Penicillin and 100μg/ml Streptomycin. These cells were cultured at 27°C while gently shaking. S2* cells were cultured in Schneider's Drosophila Medium (PromoCell) supplemented with 10% FBS, 20U/ml Penicillin, 20μg/ml Streptomycin, 1% GlutaMax (Gibco), and 12.33mM L-Glutamine. These cells were cultured at 27°C without shaking. All cells were cultured using sterile technique.

**Sequence Alignment & Conservation Mapping:** Murine (NP_035761) and Canine (NP_001003327) gp96 were blasted on the National Center for Biotechnology Information (NCBI) database to search for genes with high sequence homology. We identified the gene gp93 (NP_651601) from *Drosophila melanogaster* with unknown function. ClusterW was used to align all three gene sequences (149). ConSurf and PyMol (http://www/pymol.org/) were then used to map sequence conservation among the genes onto the molecular surface of the structure of canine gp96 (PDB ID:2O1U) (150).
**Cloning Constructs:** mRNA was isolated from S2 cells and reverse transcribed into cDNA in order to amplify gp93, CNPYa, and CNPYb by PCR. Gp93 was cloned into the pGEM-T Easy vector (Promega) and then subcloned into the MigR1 retrovector expressing a GFP reporter. WT gp93 was then used to amplify gp93\textsuperscript{Flag} by PCR using a different reverse primer. Both constructs were engineered with a KDEL instead of a HDEL ER retention signal after the FLAG epitope (DYKDDDDK) to ensure proper localization in a murine system. CNPYa and CNPYb were cloned directly into the MigR1 retrovector. Both constructs were engineered with a C-terminal Myc tag (EQKLISEEDL). Refer to Table III for gene accession numbers and Table IV for cloning primer sequences.

**Site-Directed Mutagenesis:** All gp93 mutants were PCR amplified from gp93\textsuperscript{Flag} with the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) per the manufacturer’s protocol. All CNPYb mutants were PCR amplified from CNPYb myc using the same kit. All primers were designed using Stratagene’s online primer design program to incorporate the mutations. Refer to Table III for gene accession numbers and Table IV for primer sequences.

**Virus Production and Transduction:** Phoenix Eco cells were transfected with ecotropic retrovectors using Lipofectamine 2000 (Invitrogen). Virus was collected 2 days later and used to transduce $2 \times 10^5$ E4.126 cells with the addition of hexadimethrine bromide (Sigma-Aldrich). Cells were then centrifuged at 1900g, 32°C for 1.5hrs to
achieve viral transduction. The GFP-reporter of the retrovector was used to determine transduction efficiency by FACS, and two to three rounds of transduction was performed to obtain high transduction efficiency.

**FACS:** Single cell suspensions were obtained and washed in PBS, and then washed in FACS Buffer (1x PBS, 2% FBS, and 0.9g/L sodium azide). Cells were then blocked with FACS Buffer containing serum and an FcR blocking antibody. Cells were incubated with primary antibody, washed, and then incubated with fluorochrome-labeled secondary antibody. Cells were washed, and then stained with propidium iodide to exclude dead cells. For intracellular staining, cells were first fixed with 4% formaldehyde and then permeabilized with methanol. Cells were then stained as above, except without FcR blocking antibody or propidium iodide. Cells were acquired on a FACS Calibur (BD Biosciences) using Cell Quest software, and data was analyzed using FlowJo software.

**Western Blot:** Single cell suspensions were washed with PBS and then lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 0.01 M sodium phosphate pH 7.2, 1% Nonidet P-40, 2 mM EDTA, 0.1% SDS, and 1% sodium deoxycholate). Protein concentration was quantified by Bradford Protein Assay (BioRad) and read with a microplate reader. Equal amounts of total cell lysate were denatured with the addition of SDS loading buffer and 0.1 M DTT, and boiled for 5 minutes. Denatured protein was then resolved on 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk-PBS/Tween for 2hrs at RT, and then incubated with primary antibody diluted in 5% milk-
PBS/Tween at 4°C overnight. Membranes were washed and then incubated with the appropriate HRP-conjugated secondary antibody diluted in 5% milk-PBS/Tween for up to 2hrs at RT. Membranes were then washed and developed with chemiluminescent substrate (Pierce Chemical) and exposed to film.

**Immunoprecipitation:** Single cell suspensions were washed with PBS, and then treated with or without 0.1 mg/ml dithiobis-succinimidyl propionate (Thermo Scientific) at RT for 30 minutes, and then lysed with RIPA buffer. 1200ug of lysate was precleared with Protein G beads (GenScript) and then removed and incubated with HA, Flag, or isotype control antibodies at 4°C for 1hr. Lysate-Ab mixtures were then incubated with fresh Protein G beads at 4°C overnight while rotating. RIPA lysis buffer was used to vigorously wash the beads, and then protein was eluted by boiling in SDS-loading buffer with 0.1M DTT. Eluted protein was then resolved on 10% SDS-PAGE and immunoblotted as above.

**RNAi and Toll Signaling:** Primers were designed to specifically recognize the gene of interest and span approximately 600-800bp. T7 promoter sites were designed within the primers to flank the gene sequence. The negative control RNAi construct was designed to amplify the MigR1 retrovector without recognizing any DNA sequences in *Drosophila*. Refer to Table V for RNAi primer sequences. CNPYa, CNPYb, and Toll were first amplified via PCR from S2 cDNA using the RNAi primers, and cloned into the pGEM-T Easy vector (Promega) to facilitate future production of RNAi. These vectors were then used as templates, along with the MigR1 and gp93-MigR1 vectors, for PCR amplification.
of RNAi constructs. The PCR products were then purified and in vitro transcribed to RNA using T7 polymerase and a kit from Epicentre. The sense and anti-sense RNA strands were then annealed together at 75°C for 15 minutes, and then allowed to cool to RT. Annealed RNA was treated with DNase at 37°C for 15 minutes to digest remaining DNA, and then purified using Centri Spin-20 columns (Princeton Separations).

S2* cells were cultured at a density of one million cells per ml. The following day, two million cells were seeded in 3ml of medium in a 6-well dish. The next day the cells were treated with 6μg of annealed RNAi mixed with 125mM CaCl₂ in BBS buffer. The mixture was vortexed and incubated for 15 minutes before being added drop-wise to the cells. The plates were gently swirled to mix, and left over night. The following day, cells were split to one million cells per ml to get rid of CaCl₂, and then returned to culture. An aliquot of cells was taken at day four to isolate RNA for determination of knockdown efficiency via qRT-PCR (described below).

RNAi knockdown cells were treated with 1μM 20-hydroxyecdysone (Sigma) for 24hrs on day 6 to amplify Toll signaling as previously published (151). Cells were then treated on day 7 with 2.4nM rSPZ (C106, gift from Dr. Neal Silverman) for 6 hours (152). mRNA was then collected and Drosomycin induction was analyzed via qRT-PCR.

**Mice:** gp96<sup>flox/flox</sup> (WT) and gp96<sup>flox/flox</sup> LysM Cre (KO) mice were described previously by our lab (4). Mice were maintained at the UCHC animal facility, and then later transferred to the MUSC animal facility. Animal protocols were approved at both facilities and strictly followed.
**DSS-Colitis:** Mice were fed 3% DSS (MP Biomedical) dissolved in their drinking water and fed *ad libitum* for 5 days. One cohort of mice was euthanized at day 5, while another cohort received 3 days of normal drinking water and were euthanized on day 8. Weight, stool scores (Stool consistency: 0, well-formed stool; 1, semi-formed stool; 2, semi-formed stool that adheres to the anus; 3, liquid stool/diarrhea. Rectal bleeding: 0, no blood; 1, visible blood in stool; 2, gross rectal bleeding.), and serum were collected daily. After euthanasia, colon length was measured and various organs were harvested. N=5 per group.

**AOM+DSS Colorectal Cancer:** Mice received one i.p. injection of 12.5mg/kg AOM (Sigma-Aldrich) on day 1. Two cycles of 2.5% DSS were given on weeks 2 and 5 for five days each, and a third cycle of 2% DSS was given on week 8 for four days. Body weight was recorded weekly and serum was collected every two weeks. Mice were euthanized at week 20, or earlier if severely moribund. Various organs were harvested after euthanasia for histology, RNA collection, or single cell suspensions. Colons were washed, cut longitudinally, and fixed in 10% paraformaldehyde overnight. Colons were then stained with 0.2% methylene blue (Sigma) to aid tumor visualization. A dissecting microscope was used to count and measure tumors. Colons were then frozen in OCT as Swiss rolls. N=10-17 per group.

**Bone Marrow Transplantation:** C57BL/6 WT mice were lethally irradiated with a cesium irradiator. The mice received two doses of 550 cGy each, four hours apart, for a total dose of 1,100cGy. Mice were then reconstituted 24hrs later with 2 x 10⁶ bone
marrow cells by iv injection in the lateral tail vein. One group of mice received WT BM (WT→WT), while another group received KO BM (KO→WT). After 12 weeks, mice underwent the above AOM+DSS study with slight modification: DSS was administered on weeks 2, 8, and 11; Mice were sacrificed at week 16. To obtain BM cells, the femurs and tibias of mice were flushed with PBS and then lysed of red blood cells with ACK lysis buffer. Cells were washed and resuspended in PBS to a final concentration of 10^7 cells per ml. Cells were maintained under sterile conditions.

**Histology:** Tissue was harvested and fixed overnight in 4% formalin, switched to 30% sucrose-PBS overnight, and then frozen in OCT medium and stored at -80°C. A Shandon Cryotome was used to cut 5μm sections which were mounted on charged slides. H&E staining was performed, and slides were scored blindly by a pathologist. Some tissue was also paraffin embedded for IHC.

**Immunohistochemistry:** Paraffin embedded tissue was cut at 5μm and mounted onto charged slides. Slides were incubated at 55°C for 30 minutes to remove paraffin. Slides were cleared in xylenes, and then rehydrated in a series of ethanol, water, and then PBS. 20μg/ml proteinase K was used for antigen retrieval, and then slides were washed. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ with 0.3% normal goat serum diluted in PBS. After washing, slides were blocked with 10% goat serum 2% BSA diluted in PBS for 2hrs. Samples were stained with rat IgG2b isotype control or F4/80 Ab (Santa Cruz) overnight at 4°C. Tissue was again washed and then incubated with 2% goat serum and biotinylated anti-rat Ab diluted in PBS (Peroxidase rat IgG Vectastain
ABC kit). Slides were washed, and then incubated in streptavidin Ab diluted in PBS. Samples were washed and then developed with DAB substrate kit (Vector Labs). Hematoxylin was used to counterstain the nucleus, which were then washed and dehydrated in a series of ethanol and xylenes. Slides were then mounted and analyzed under a microscope.

**Lamina Propria Isolation:** Colon and small intestine were removed, flushed with PBS, and Peyers Patches were cut out. The intestines were cut longitudinally, and then into 1cm pieces. Tissue was washed trice in cold Hank’s Balanced Salt Solution (HBSS) with 2% FBS and vortexed for 15 seconds to aid removal of the mucus layer. Tissue was incubated in HBSS + 5% FBS + 1μM DTT and agitated at 100rpm at 37°C for 20mins in a bacterial shaker, and then vortexed. Samples were then incubated in 1.3mM EDTA diluted in PBS and spun at again at 37°C for 20-30 minutes, vortexed, and then this was repeated to remove epithelium. HBSS 2% FBS was used to wash tissue twice, and then RPMI for a final wash, vortexing after each. Tissue was cut further into smaller pieces and then spun as before with collagenase IV and 0.1mg/ml DNAse1 + 5% FBS diluted in RPMI for 20 minutes. The samples were then passed through a 19g syringe and a cell strainer to break apart the remaining tissue, and then the solution was washed with RPMI. This preparation was used as total lamina propria isolates to analyze myeloid cells. To enrich for lymphocytes, percoll centrifugation was used with 44% and 66% percoll in RPMI and centrifuged for 20 minutes at 2800rpm with the brake off. The middle layer was removed and washed in RPMI. A hemocytometer and Trypan blue were used to count live cells.
**T Cell Stimulation:** 10μg/ml anti-CD3 and 2μg/ml anti-CD28 Abs were used to coat plates in 0.1M sodium carbonate buffer at 4°C overnight. PBS was used to wash the wells, and then lymphocyte-enriched lamina propria isolates were added to the wells with 1μg/ml anti-CD3 Ab diluted in RMPI. Cells were incubated at 37°C overnight, and then harvested for analysis via FACS.

**qRT-PCR:** mRNA was harvested from both colon tissue and cells using TriZol reagent (Life Technologies) as per the manufacturer’s protocol. RNA was quantified with a nanodrop, and then reverse transcribed into cDNA using Superscript II H. Reverse Transcriptase (Invitrogen). For qRT-PCR, 5ng of RNA was used per reaction. cDNA and the appropriate primers were mixed with SYBR Green supermix and the reactions were run on a BioRad iCycler. Microsoft Excel was used for data analysis using the dCT method. β-actin and Rp49 were used as internal controls for murine and *Drosophila* qRT-PCR reactions, respectively. Plots depict fold change in mRNA. For a list of primer sequences, refer to Tables VI (*Drosophila*) and VII (murine).

**Gene Sequencing:** Colon cDNA was used as a template to amplify exon 3 of the β-catenin gene via PCR. PCR products were purified with Qiagen kits and then sent to Genewiz for sequencing. Vector NTI software (Invitrogen) was used to align sequencing results, and original chromatograms were analyzed to determine mutations. Primers were based off of the literature (100).
**Statistical Analysis:** All results are shown as mean ± standard error (SEM) or standard deviation (SD) as indicated in figure legends. Student’s T test was performed to determine significance. All experiments were performed at least twice to confirm reproducibility of the results. *p<0.05, **p<0.005
Table III: Gene accession numbers.

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F, forward primer. R, reverse primer.
Table V: List of *Drosophila* RNAi primer sequences.

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F, forward primer. R, reverse primer.
Table VI: List of *Drosophila* qRT-PCR primer sequences.

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F, forward primer. R, reverse primer.
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F, forward primer. R, reverse primer.
Chapter 3

Gp93 is the Drosophila Ortholog of Mammalian gp96

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Rationale:

Gp96 is implicated in sepsis, autoimmunity, and cancer (4, 23, 28, 31-33). Therefore, a better understanding of gp96 will prove beneficial in designing therapeutics. In order to gain a better understanding of gp96 biochemistry, we decided to look for a homolog in a simpler system. Establishing such a model will prove easier to probe, and the knowledge gained can then be applied to studying the mammalian system. This method has already proven to be beneficial in the case of HSP90, where the study of the E. coli (HtpG) and yeast (Hsc82) paralogs has aided the study of mammalian HSP90 (153, 154).

A BLAST search probing for possible gp96 homologs identified the Drosophila molecule gp93, which proved interesting for multiple reasons. First, the two molecules were highly conserved despite the large evolutorial gap, suggesting conserved function. Second, while gp96 chaperones TLRs, the original Toll is present in Drosophila (3, 4, 51). Additionally, while mammals have alpha and beta integrins, Drosophila only has five alpha and two beta integrins. Furthermore, the gp96 cochaperone CNPY3 is part of a four member family in mammals, while Drosophila only has two CNPY family members (5, 46). Therefore, it is plausible that gp93 chaperones Toll and the Drosophila integrins with a CNPY cochaperone, similar to gp96 in mammals. Due to decreased Tolls, integrins, and CNPYs in Drosophila, this system will prove to be easier to study than the mammalian system, yet increase our knowledge of gp96 and aid development of therapeutics. The data demonstrating gp93 as an ortholog of gp96 has already been published in the Journal of Immunology (155). The remaining data is being prepared for publication.
**Results:**

*Identification of gp93*

The mammalian gp96 sequence was searched in the NCBI database in order to uncover genes with high sequence homology. Gp93 (NP_651601), a protein with unknown function in *Drosophila melanogaster*, was identified with a high degree of sequence conservation. The fly database was searched for this gene, confirming its identity and lack of known function. Sequence alignment demonstrated 74% homology at the amino acid level among *Drosophila* gp93, and both mouse and canine gp96 (Figure 1A). A breakdown of domain structure identified further similarities. Gp93 also contains an N-terminal signal peptide (SP) for ER targeting which is 19% homologous. After the SP is an ATPase domain with 65% homology, suggesting the importance of ATP hydrolysis in conserved function. A charged middle domain is next with 48% homology, and a C-terminal dimerization domain, important for function, is 61% homologous (3). Finally, the terminal amino acids of gp96 are KDEL, while those in gp93 are HDEL, both known ER retention signals (156). Even important residues for ATP binding and hydrolysis and thus chaperone function in gp96 are conserved in gp93: Glu103, Asn149, and Arg448 (Figure 1A, highlighted yellow) (3, 13). Thus, both the amino acid sequence and domain structure are conserved between gp96 and gp93.

The crystal structure of canine gp96 has been solved in complex with a nonhydrolyzable ATP, 5’-adenylyl-β,γ-imidodiphosphate, demonstrating a twisted V structure of the gp96 dimer (13). It is possible to use this structure to map the sequence conservation among canine gp96, murine gp96, and *Drosophila* gp93. Depicted in Figure 1B is the front and back (180°) of one monomer of gp96, with conserved residues in red.
This figure illustrates areas within the structure which are highly preserved with invariant residues. For instance, the ATP binding pocket near the N-terminus appears to be highly conserved (Figure 1B, black arrows). Collectively, these data demonstrate that gp96 and gp93 are highly homologous, with shared amino acid sequences, important residues, and shared domain structure. Therefore, we decided to determine whether or not gp93 also shares chaperone function with gp96.

**Gp93 rescues gp96 client expression**

Since it is unknown whether gp93 functions as a chaperone, any possible client proteins are also unknown. Therefore, we decided to determine whether or not gp93 can function as a chaperone in a murine system and fold gp96 clients due to its high homology. For these studies, we utilized a murine gp96-mutant pre-B cell line, E4.126 (KO), which lacks functional gp96 (3). Therefore, these cells do not express surface α4, αL, β2, TLR2, or TLR4, but their expression can be rescued with gp96 restoration (3, 4, 20). We therefore tested whether gp93 can functionally replace gp96 and rescue the aforementioned gp96 clients. We utilized retroviral vectors expressing a GFP reporter in a bicistronic fashion and cloned both WT gp96 and gp93 into these vectors. We further cloned both molecules with Flag epitopes inserted just upstream of the ER retention signals for easier detection (No antibodies exist for gp93, and gp96 antibodies do not cross react with gp93: data not shown). The cloned vectors were transfected into Phoenix Eco cells for viral production, at which point viral particles were transduced into E4.126 cells. GFP expression (Figure 2A), as well as Flag expression (Figure 2B) by both
intracellular stain and western blot, demonstrated high level transduction of each construct.

As a control, empty-vector (EV) transduced E4.126 cells failed to express the integrins α4, αL, or β2, nor TLR2 due to lack of gp96 expression (Figure 2C). As expected, both WT gp96 and gp96\textsuperscript{Flag} rescued all clients. Importantly, both WT and Flag tagged gp93 were also able to rescue all gp96 clients. While the MFI for all clients was about 5 in EV cells, the MFI for α4, αL, β2, and TLR2 increased to 313, 19.3, 7.96, and 17.1 (respectively) in gp93\textsuperscript{Flag} cells. Integrin β1 however, a non-gp96 client protein, retained similar expression levels throughout the different cell types. Additionally, the Flag tag of either gp96 or gp93 did not impede chaperone function. Curiously, although gp93 was competent at chaperoning gp96 clients, it did so less efficiently. Comparing Flag expression levels, gp93\textsuperscript{Flag} is expressed higher than gp96\textsuperscript{Flag} (Figure 2B), yet the MFI of client proteins in gp93\textsuperscript{Flag} cells is lower than those in gp96\textsuperscript{Flag} cells (Figure 2C). Therefore, the chaperone function of folding both integrins and TLRs is conserved between gp96 and gp93. Moreover, the fact that gp93 is less efficient at folding than gp96 provides us a method by which to study structural elements necessary for chaperone function by comparing gp96 to gp93.

\textit{Gp93 interacts with TLR9}

Besides cell surface TLRs, we decided to check if gp93 could also chaperone intracellular TLRs by determining whether or not gp93 could physically interact with TLR9. To this end, we further transduced gp96\textsuperscript{Flag} and gp93\textsuperscript{Flag} cells with TLR9\textsuperscript{HA}, tagged at the C-terminus. TLR9 expression was verified by intracellular stain and western blot,
demonstrating similar levels in each cell type (Figure 3A). We then performed immunoprecipitation experiments by pulling down Flag and immunoblotting for HA. Using the thiolcleavable and membrane-permeable cross-linker DSP to stabilize the complex, we show that both gp96 and gp93 are able to interact with TLR9 (Figure 3B). Performing a reverse immunoprecipitation with HA pull down further verified these results (Figure 3C). Therefore, gp93 is thus able to chaperone multiple gp96 clients, located at both the cell surface and intracellularly.

**Gp96 and gp93 heterodimerize**

Since *Drosophila* gp93 is able to fold murine gp96 client proteins and it is highly homologous, we questioned whether or not gp96 and gp93 could heterodimerize. The C-terminal canine gp96 oligomerization domain has been characterized, and the corresponding murine amino acids are 697-740 (18). This region is 55% conserved in gp93 (Figure 4A). For these experiments we utilized 14.GFP WT pre-B cells which express endogenous gp96. We further retrovirally transduced these cells with either gp96Flag or gp93Flag. GFP reporter expression, as well as Flag and gp96 were determined by FACS (Figure 4B). Endogenous gp96 expression, as well as Flag transgene expression, was also verified by WB (Figure 4C). We then used these cells for gp96 IP with or without DSP crosslinker, followed by Flag WB. Interaction between gp96 and gp93 was only seen under crosslinking conditions (Figure 4D). More convincing was the reverse IP, where we pulled down Flag and looked at gp96 expression. We now saw weak gp96-gp93 interaction in the absence of crosslinker, and strong interaction in the presence of crosslinker (Figure 4E). Therefore, important residues within the
dimerization domain of gp96 and gp93 are highly conserved, allowing the two to dimerize.

*N-terminal disulfide bond formation in gp93*

Although gp93 can chaperone gp96 client proteins, it does so less efficiently. This allowed an opportunity to discover structural motifs important for function by examining differences between the two molecules which may account for the observed phenotype. Gp96 contains six cysteine residues (Cys$^{10}$, Cys$^{11}$, Cys$^{138}$, Cys$^{576}$, Cys$^{645}$), which are important for disulfide bond formation. Ignoring the first two, which are located in the SP and cleaved upon entrance into the ER, we focused on the last three residues. The terminal two cysteines are conserved in gp93, but Cys$^{138}$ is not (Figure 1A, asterisks). Our lab demonstrated that this residue is important for N-terminal dimerization in gp96 (as opposed to C-terminal dimerization depicted in the crystal structure) (13, 155). We therefore sought to determine whether lack of this cysteine in gp93 would prevent N-terminal dimerization, and thus make gp93 less efficient at folding client proteins.

The corresponding residue of gp96 Cys$^{138}$ in gp93 is Ala$^{137}$. In order to scrutinize this residue in gp93, we created the mutant gp93$^{A137C}$ expressing the FLAG epitope. Upon retroviral transduction of E4.126 mutant pre-B cells, both the bicistronic GFP and Flag expression were detected indicating transgene expression (Figure 5A). Then we resolved gp96$^{\text{Flag}}$, gp93$^{\text{Flag}}$, and gp93$^{A137C}$ under both reducing and nonreducing SDS-PAGE (Figure 5B). While gp96 exhibited a dimer at 200kDa under nonreducing conditions, WT gp93 did not. However, gp93$^{A137C}$ gained the ability to form a disulfide bonded dimer. So as seen in gp96, this N-terminal cysteine residue is important for
disulfide bond dependent dimerization. While gp93 lacks the ability to dimerize at the N-terminus due to the presence of an alanine at this position, this ability can be restored by mutating the alanine to a cysteine.

Since gp93\textsuperscript{A137C} gains N-terminal dimerization similar to gp96, we next explored whether or not this would improve chaperone efficiency. We therefore probed these cells for gp96 client expression. Surprisingly, gp93\textsuperscript{A137C} did not have increased \(\alpha_4\), \(\alpha_L\), \(\beta_2\), or TLR2 expression (Figure 5C). Client expression levels were similar between WT gp93 and gp93\textsuperscript{A137C}. Analogous to these results, gp96\textsuperscript{C138A} does not have decreased client expression, although this mutant does lose the ability to form N-terminal disulfide bond dependent dimerization (155). Therefore, although gp93\textsuperscript{A137C} gains the ability to form N-terminal dimerization, this cysteine residue is not important for chaperone function.

Exploring the client binding domain of gp93

Resolution of the crystal structure of gp96 identified a C-terminal hydrophobic loop which protruded outward from the structure (13). Our lab recently identified this region (652-678) as the client binding domain (CBD) of gp96 for both integrins and TLRs (19). This region is highly conserved among different species of gp96, and is 63% homologous to HSP90. Comparing this region in gp96 to the corresponding residues in gp93 (649-675) revealed only 44% homology (Figure 6A). We postulated that this difference could account for the decreased chaperone efficiency of gp93. Taking a closer look revealed that the first third of the CBD (652-660 in gp96) was surprisingly 78% homologous between gp96 and gp93, while the remaining two thirds of the region was only 28%
homologous. We therefore decided to test a mutant gp93 construct containing the gp96 CBD instead of the WT gp93 sequence (gp93\textsuperscript{96CBD}).

\textit{gp93}\textsuperscript{96CBD} was given a Flag tag and retrovirally transduced into E4.126 cells. Transgene expression was verified by GFP reporter and Flag expression by both FACS and WB (Figure 6B and C). Cells were then tested for gp96 client expression by FACS. Surprisingly, \textit{gp93}\textsuperscript{96CBD} did not have improved expression of α4, αL, β2, or TLR2 (Figure 6D). Contrary, this mutant expressed similar levels of αL and β2 as \textit{gp93}\textsuperscript{Flag} cells, yet decreased α4 and TLR2. This suggested that other motifs were responsible for the decreased chaperone efficiency seen with gp93.

Although \textit{gp93}\textsuperscript{96CBD} did not have improved chaperone function, we decided to test whether or not residues 649-675 in gp93 constituted a CBD similar to gp96. Furthermore, we took into account the differences in homology between the first third and remaining two thirds of the region. We hypothesized that since the first third was so conserved, it was likely important for function. Likewise, we postulated that the last two thirds were not as important for function since they were not conserved evolutionarily, and this would explain why \textit{gp93}\textsuperscript{96CBD} did not have increased chaperone efficiency. We first focused on the later region by deleting it from \textit{gp93} (\textit{gp93}\textsuperscript{658-75D}). If our hypothesis was correct, then we could further pin point the important residues within the CBD to the first third. Yet if our hypothesis was wrong, then we could assume that one of the only five conserved residues in the last two thirds was important for function.

\textit{gp93}\textsuperscript{658-75D} was given a Flag tag and again retrovirally transduced into E4.126 pre-B cells. These cells expressed high levels of the GFP reporter by FACS (Figure 7A). Transgene expression was also verified by intracellular stain and WB for Flag expression
(Figure 7B). We then analyzed these cells for gp96 client expression via FACS. Surprisingly, we found that gp93^{658-75D} had decreased expression of α4, αL, β2, and TLR2 (Figure 7C). Therefore, this suggested that one of the five conserved residues within this region was important for function.

Of the five amino acids conserved in the last two thirds of the CBD, there are two hydrophobic tyrosine residues. Tyrosines are important because phosphate and sulfate groups can be added to them, which usually modulate enzymatic activity of the protein. Since gp96 is known to be phosphorylated, we therefore decided to mutate these two amino acids to alanines, which are smaller hydrophobic residues (gp93^{2YA}). Similar to our previous constructs, we again added a Flag tag to this mutant and retrovirally transduced it into E4.126 pre-B cells. GFP reporter expression was first verified by FACS (Figure 8A). Transgene expression was then further verified by Flag intracellular stain and WB (Figure 8B). We then proceeded to analyze gp96 client expression levels by FACS. The gp93^{2YA} mutant had decreased expression of α4, αL, β2, and TLR2 (Figure 8C). This proved that at least one of these tyrosines is important for function, and further implicated the role of phosphorylation or sulfation in gp96 chaperone function.

**Analyzing Drosophila CNPYs as possible gp93 cochaperones**

As discovered recently, CNPY3 is a gp96 cochaperone which promotes TLR folding (5). Since cochaperones are important aspects in chaperone biology, we probed whether or not gp93 also had an endogenous cochaperone. While mammals have four CNPY family members, Hirate showed that *Drosophila* possessed two CNPY members, CNPYa and CNPYb (46). Although CNPYb seemed most similar to CNPY3 as being the only family
members with highly basic domains, we decided to test both Drosophila CNPYs for the capacity to act as a cochaperone.

Utilizing the gp93\textsuperscript{Flag} pre-B cell line, we further transduced these cells with either CNPYa or CNPYb, both tagged with the Myc epitope at the C-terminus. E4.126 KO cells were also transduced with both constructs without gp93 as a control. GFP reporter expression showed high expression of both CNPYa and CNPYb in E4.126 cells (Figure 9A). GFP expression was also increased in cells already containing gp93\textsuperscript{Flag}. Western blot analysis of these cells for Myc further verified expression of both CNPY constructs in all cells (Figure 9B). We next analyzed CNPY cells by FACS for client expression. Neither CNPYa nor CNPYb alone expressed α4, αL, TLR2, or TLR4 (Figure 9C). Interestingly, these cells did express β2 at higher levels than gp93\textsuperscript{Flag} cells, and this expression increased slightly with gp93 expression. However, cells containing gp93 together with either CNPY had decreased α4 and αL expression compared to gp93\textsuperscript{Flag} cells. This suggests that both CNPYs may act as a direct chaperone for beta integrins, but not alpha integrins. As expected, however, gp93\textsuperscript{Flag} CNPYb cells had increased TLR2 and TLR4 expression. This suggests that CNPYb acts as a TLR cochaperone. gp93\textsuperscript{Flag} CNPYa cells had decreased TLR expression. Therefore, while both CNPYa and CNPYb seem to aid the expression of β2 integrin, only CNPYb aids TLR expression.

If CNPYb is indeed a cochaperone for gp93, then the two molecules should physically interact. Furthermore, since gp96 and gp93 are so homologous, we further wondered if gp96 could interact with CNPYb. We therefore further transduced CNPYb into gp96\textsuperscript{Flag} cells and verified transgene expression by Myc WB (Figure 10A). We then did an IP for Flag under crosslinking conditions followed by Myc WB. As expected,
gp93 does interact with CNPYb. Furthermore, gp96 was also able to interact with CNPYb (Figure 10B). We thus pondered whether gp93 could also interact with murine CNPY3. We again used gp96\textsuperscript{Flag} and gp93\textsuperscript{Flag} cells and transduced them with CNPY3 containing the Myc epitope. Transgene expression was first verified by Myc WB (Figure 10A). We then used all four cell lines to perform a Myc IP under crosslinking conditions, followed by Flag WB. As stated in the literature, gp96 and CNPY3 interacted (5). gp93 was also able to interact with CNPY3. Furthermore, we again verified that both gp96 and gp93 could interact with CNPYb (Figure 10C). Thus, both gp93 and gp96 can interact with both CNPYb and CNPY3. This further verifies our hypothesis that CNPYb is a cochaperone for gp93, and demonstrates the highly conserved nature of the system.

**Cysteine residues important for CNPYb function**

Since CNPYb appears to be a TLR-specific cochaperone for gp93 much like CNPY3 is for gp96, we decided to investigate CNPYb further. Similar to Saposin, the CNPY family members each have six cysteine residues and are postulated to form shell like dimers (Figure 11A) (46). Besides intermolecular disulfide bonds, CNPYs are also postulated to form intramolecular bonds where the first cysteine binds the last as the protein folds upon itself as depicted in Figure 11B (UniProt CNPY3 Q9DAU1). Although CNPY3 and CNPYb are only 49% homologous at the amino acid level, all six cysteines are conserved, suggesting their importance in function (Figure 11C, yellow). We therefore decided to test the roles of these cysteines.

Since the six cysteine residues are postulated to bind each other, we only need to test the first three (Figure 11B). We therefore separately mutated C36, C39, and C92 to
alanines: CNPYb\textsuperscript{C36A}, CNPYb\textsuperscript{C39A}, and CNPYb\textsuperscript{C92A}. These constructs were tagged with the Myc epitope and retrovirally transduced into E4.126 pre-B cells expressing gp93\textsuperscript{Flag}. FACS showed that GFP expression increased over the parent gp93\textsuperscript{Flag} cells (Figure 12A). Transgene expression was then further verified by Myc WB (Figure 10B). Interestingly, a second band of approximately double the size (26 vs 52kDa) was also seen, indicating that CNPYb can indeed form dimers, although this band was curiously absent in CNPY3 cells (Figure 10A). We then analyzed these cells for TLR levels by FACS. All three mutants had decreased TLR4 expression compared to gp93\textsuperscript{Flag} cells with WT CNPYb (Figure 12C). However, only gp93\textsuperscript{Flag} CNPYb\textsuperscript{C39A} cells had decreased TLR2 expression. C39 therefore appeared to be more important for chaperone function.

If CNPYb does indeed fold upon itself, then mutating the cysteine that binds to C39 should phenocopy it (Figure 11B). We therefore made the mutant CNPYb\textsuperscript{C183A} with a Myc epitope and retrovirally transduced it into gp93\textsuperscript{Flag} cells. GFP reporter expression was increased as compared to gp93\textsuperscript{Flag} cells as seen by FACS (Figure 13A). Transgene expression was further verified by Myc WB (Figure 13B). We then analyzed TLR expression by FACS. As hypothesized, gp93\textsuperscript{Flag} CNPYb\textsuperscript{C183A} cells phenocopied gp93\textsuperscript{Flag} CNPYb\textsuperscript{C39A} cells with decreased TLR2 and TLR4 expression (Figure 13C). This suggested that C39 and C183 may indeed form a disulphide bond with each other, and that this bond is important for chaperone function. Furthermore, all CNPYb mutants except CNPYb\textsuperscript{C92A} displayed a dimeric band by Myc WB (Figure 12B and 13B). However, CNPYb\textsuperscript{C92A} was expressed at a much lower level than the other CNPYb mutants, and is therefore resolved more easily. We thus cannot rule out the possibility
that CNPYb\textsuperscript{C92A} does form dimers. So although breaking apart one disulfide bond affects chaperone function, it does not affect the ability to dimerize.

*Does gp93 chaperone Drosophila Toll?*

The endogenous clients of gp93 in *Drosophila* are unknown. Since gp93 is capable of chaperoning murine TLR2 and TLR4 and interacting with TLR9, it is possible that gp93 chaperones *Drosophila* Toll. To test this, the *Drosophila* cell line S2\textsuperscript{*} was utilized. RNAi was used to knockdown gp93, as well as CNPY\textsubscript{a}, CNPY\textsubscript{b}, and Toll as a control. Two different RNAi constructs were used to knockdown gp93, one which recognized the N terminus (gp93N), and one which recognized the middle (gp93M). An RNAi construct specific for the MigR1 vector, which does not recognize any *Drosophila* gene, was used as a negative RNAi control. Analysis by qRT-PCR reveals effective knockdown of each molecule (Figure 14A).

Since Toll antibodies are lacking, Toll protein expression could not be assayed directly. Therefore, Toll signaling was used to determine the presence or absence of Toll protein. RNAi treated cells were stimulated with recombinant SPZ (rSPZ), the endogenous ligand for Toll. The induction of downstream antimicrobial peptides, such as Drosomycin, was determined by qRT-PCR. Drosomycin induction could be seen in MigR1-RNAi cells treated with rSPZ compared to cells without rSPZ treatment. Cells with Toll-RNAi lacked Drosomycin induction, indicating that Toll knockdown was functionally efficient. However, cells with gp93, CNPY\textsubscript{a}, and CNPY\textsubscript{b} RNAi still had induction of Drosomycin (Figure 14B). Therefore, gp93 may not chaperone Toll, and neither CNPY\textsubscript{a} nor CNPY\textsubscript{b} may be involved in Toll folding as well. However, although
efficient mRNA knockdown of gp93, CNPYa, and CNPYb was seen, the protein expression levels were not known. It is possible that these molecules have long half-lives, and that the protein is not yet knocked down. Therefore, further examination of endogenous gp93 clients must be performed.

Discussion:

Our data unequivocally proves that *Drosophila* gp93 is a conserved ortholog of mammalian gp96, containing a large degree of sequence and structural homology. When expressed in a mammalian system effectively knocked out for gp96, gp93 is able to rescue gp96 client expression and can even physically interact with TLR9. Further demonstrating high homology between gp96 and gp93, the two molecules were capable of dimerizing. Mutational analysis of gp93 proved exceptionally useful. While gp93<sup>A137C</sup> gained the ability to dimerize at the C-terminus similar to gp96, chaperone function was not enhanced. With this knowledge, reciprocal studies were carried out in gp96, where gp96<sup>C138A</sup> lost C-terminal dimerization, while chaperone abilities were unaltered (155).

Although the crystal structure of gp93 only depicts N-terminal dimerization, other data, such as the gp96<sup>C138A</sup> mutant, demonstrate that C-terminal dimerization also occurs (13, 155). This seems probable, given the fact that HSP90, the cytosolic paralog of gp96, can dimerize at both termini. The presence of either ADP or a nonhydrolyzable ATP did not significantly alter gp93 structure (13). It is possible that either client or cochaperone binding may induce C-terminal dimerization. Gp96 remains in an open V shape, allowing either client and/or cochaperone to bind. Once bound, biochemical changes occur that cause C-terminal dimerization which may be necessary for client folding. Upon folding
of substrate, another biochemical change occurs which disrupts C-terminal dimerization and thus releases the client.

In order to further dissect the potential CBD of gp96 postulated from the crystal structure, the CBD of gp93 was swapped for that of gp96 (13). This mutant, however, did not have improved chaperone abilities. α4 and TLR2 expression were actually decreased. This may be due to unforeseen structural changes induced by the substitution of 27 amino acids. Furthermore, although the second half of the CBD is less conserved across species, the gp93<sup>658-75D</sup> mutant had decreased client expression, implicating the importance of both halves of the CBD. Additionally, the terminal two tyrosines in the CBD were also important for function, as evidenced by the gp93<sup>2YA</sup> mutant with decreased client expression. Although gp96 is known to be phosphorylated on serine and threonine residues, this data implicates a possible role for tyrosine phosphorylation in gp96/93 function (10). Furthermore, this investigation aided the identification of the gp96 CBD (19).

In an attempt to characterize the role of either <i>Drosophila</i> CNPY in gp93 activity, CNPYb was determined to be a TLR specific cochaperone similar to CNPY3 with gp96 (5). Surprisingly, both CNPYa and CNPYb rescued β2 expression in cells lacking gp93, and to a level higher than that in gp93 cells. The addition of gp93 to either CNPY only mildly increased its expression. Therefore, both CNPYs appear to play a dominant role in promoting β2 expression. Furthermore, gp93 cells with CNPYa had decreased levels of TLRs, and both CNPYs had decreased alpha integrin expression compared to gp93 cells. Therefore, the possibility remains that these CNPYs may negatively regulate certain gp93 clients while promoting others, perhaps by inhibiting ATPase activity. This is plausible,
as various cochaperones of HSP90 have been found to either activate or inhibit ATP hydrolysis, thereby affecting chaperone function (153).

A deeper investigation of CNPYb demonstrated that this molecule is able to physically interact with gp93, which is a characteristic of a cochaperone. Additionally, gp96 was also able to interact with CNPYb, while gp93 was also able to interact with CNPY3, identifying further similarities between the mammalian and Drosophila systems. Since not much is known about mammalian CNPY3, we decided to investigate CNPYb further in order to learn more about this molecule using a simpler system. Analyzing the six characteristic saposin-like cysteines by mutational analysis demonstrated that although C36 and C92 were important for TLR4 expression, only C39 and C183 were required for both TLR2 and TLR4 expression. It is possible, therefore, that C39 and C183 form a disulfide bridge important for general cochaperone function. However, CNPYb seems to differentially interact with different TLRs. This is likely, given that such differences have been seen with CNPY3 (49).

The elucidation of the endogenous gp93 clients will enable better utilization of the Drosophila system for understanding gp96 biochemistry. Unfortunately, preliminary data suggests that gp93 does not chaperone Toll. However, although gp93 mRNA levels were efficiently knocked down, the protein level is unknown. If gp93 has a long half-life similar to gp96, then it is possible that we did not achieve knockdown of gp93 protein. Therefore, further studies must be performed in order to conclude whether or not gp93 chaperones Toll. Furthermore, it is possible that gp93 chaperones the other Toll-related proteins (Toll-2 to Toll-9). However, being that TLR4 was discovered due to its
homology to Toll, and that gp93 was able to rescue TLR4 expression, we remain firm on our hypothesis that gp93 chaperones Toll.

After publication of our work identifying gp93 as the *Drosophila* ortholog of gp96, another group published a paper characterizing gp93 (155, 157). Maynard and colleagues demonstrated that gp93 KO flies harbor a growth defect, and die at the third instar larval stage, never developing into adult flies. Closer examination demonstrated multiple defects in the larval midgut. KO larva had decreased nutrient uptake, reduced gut motility, abnormal septate junction structure, decreased gut acidification, abnormal copper cell structure, reduced insulin signaling, and atypical amino acid and triglyceride mobilization. KO flies thus display a starvation-like metabolic disease (157). In *Drosophila*, integrins $\alpha_{PS1}$, $\alpha_{PS3}$, $\beta_{PS}$, and $\beta_{\nu}$ are all expressed in the midgut (56, 57, 60, 62). In $\beta_{PS}$ KO flies, there is a defect in migration of the primordial midgut cells. Furthermore, when both $\beta_{PS}$ and $\beta_{\nu}$ are absent, the phenotype is exacerbated, and the migration of these cells is blocked completely (57). Therefore, gp93 KO flies seem to phenocopy $\beta_{PS}/\beta_{\nu}$ double KO flies. Since gp96 chaperones select integrins, this data highly suggests that gp93 can chaperone *Drosophila* integrins.

In conclusion, gp93 is the *Drosophila* ortholog of mammalian gp96, being able to chaperone both integrins and TLRs. A137 of gp93 fails to support C-terminal dimerization, yet the ability to dimerize at the C terminus did not affect chaperone function. Residues 658-75 of the CBD, and tyrosines 74-75 in particular were important for chaperone function, further implicating the role of phosphorylation in gp93 regulation. CNPYb is a TLR specific cochaperone for gp93, while CNPYa and CNPYb are possible negative regulators of $\alpha4$ and $\alphaL$. Furthermore, these CNPYs are a dominant
requirement for β2 integrin (Figure 15). So not only do these CNPYs differentially modulate TLRs versus integrins, but there appears to also be differences in alpha versus beta integrin regulation. Not only does the study of the Drosophila gp93 system create an avenue to better study the gp96 system, but insights gained from gp93 have already aided the discovery of certain aspects of gp96 biochemistry, with hopes of uncovering more.
Figure 1: Sequence alignment and structure conservation between mammalian gp96 and Drosophila gp93. (A) Amino acid sequence alignment among murine (m) gp96, canine (c) gp96, and Drosophila (d) gp93. Conserved residues among all three are highlighted red. Residues conserved between two are blue. Similar amino acids are highlighted green. Weakly similar residues are grey. Yellow highlighted residues are those important for ATPase activity. Numbers denote amino acid position. Asterisks
indicate cysteine residues. (B) Conservation mapping using the canine gp96 structure. Conserved residues in ‘A’ are highlighted in red. Nonconserved residues are grey. The front and back (180° turn) of one monomer of gp96 is depicted. The N-terminus is at the top, and the C-terminus is at the bottom. Black arrows indicate the ATP binding pocket.
Figure 2: gp93 expressed in murine gp96-mutant pre-B cells can rescue gp96 client expression. E4.126 murine pre-B cells, deficient for gp96, were retrovirally transduced
with the indicated constructs and then analyzed. (A) GFP reporter expression of the indicated cells was determined by FACS. (B) Flag intracellular stain of gp96$^{\text{Flag}}$ and gp93$^{\text{Flag}}$ cells (top). Flag western blot of the indicated cells (bottom). β-actin was used as a loading control. (C) FACS staining of gp96 clients (α4, αL, β2, and TLR2) as well as one non-client protein (β1). Shaded histograms indicate isotype staining. Numbers denote MFI. Data shown is one of many experiments with similar results.
**Figure 3: Both gp96 and gp93 interact with TLR9.** E4.126 pre-B cells already transduced with either gp96\textsuperscript{Flag} or gp93\textsuperscript{Flag} were then further transduced with TLR9\textsuperscript{HA} and then analyzed. (A) HA intracellular stain of the indicated cells (left). Shaded histogram indicates isotype control stain. Numbers denote MFI. HA western blot with β-actin loading control (right). (B) Flag (F) IP of gp96\textsuperscript{Flag} and gp93\textsuperscript{Flag} cells with TLR9\textsuperscript{HA} followed by WB for HA and Flag. Isotype control antibody was used (iso). Cells were treated with (+) or without (-) DSP crosslinker. (C) Same as in ‘B’, but IP for HA (H) or the appropriate isotype control antibody. Data are representative of two separate experiments.
Figure 4: gp96 and gp93 heterodimerize. (A) Amino acid sequence alignment of the dimerization domain between murine (m) gp96 and Drosophila (d) gp93. Conserved amino acids are highlighted red, and similar residues are highlighted blue. Numbers denote amino acid position. (B) 14.GFP (WT) murine pre-B cells were retrovirally
transduced with either gp96\textsuperscript{Flag} or gp93\textsuperscript{Flag} and then analyzed for GFP reporter, Flag, and gp96 expression by intracellular stain. Shaded histograms denote isotype controls. (C) gp96, Flag, and β-actin WB of the indicated cells. (D) Gp96 (96) IP with (+) or without (-) DSP crosslinker, followed by Flag and gp96 WB. (E) Reverse IP of Flag (F) with or without DSP, followed by gp96 WB. Isotype (Iso) control antibody was used. Data represent one of two experiments with similar results.
Figure 5: gp93^{A137C} rescues N-terminal disulfide bond dimerization, yet does not enhance chaperone function. E4.126 murine pre-B cells were transduced with the indicated constructs and then analyzed. (A) GFP reporter expression of gp93^{A137C} cells by FACS (left). Flag intracellular stain (right). (B) gp96^{Flag}, gp93^{Flag}, and gp93^{A137C} cells were resolved by SDS-PAGE with (+DTT) or without (-DTT) denaturing conditions. The membranes were blotted with Flag and β-actin loading control antibodies. Numbers denote molecule size in kDa. (C) FACS for gp96 client proteins (α4, αL, β2, and TLR2). Shaded histogram denotes isotype control stain. Data represent one of two experiments with similar results.
**Figure 6:** gp93<sup>96CBD</sup> does not have improved client expression. (A) Amino acid sequence alignment of the CBD among HSP90, gp93, and different species of gp96. Conserved residues are highlighted red. Amino acids conserved among the majority are highlighted blue, while those conserved by a minority are highlighted green. Residues in green font are similar amino acids. Numbers denote amino acid length of the CBD. (B) E4.126 murine pre-B cells were transduced with gp93<sup>96CBD</sup> and then analyzed by FACS for GFP reporter expression. (C) Flag intracellular stain of gp93<sup>96CBD</sup> cells (left). Flag
WB of the indicated cells with β-actin loading control (right). (D) FACS of gp96 client proteins (α4, αL, β2, and TLR2). Shaded histogram denotes isotype control stain. Data represent one of two experiments with similar results.
Figure 7: gp93\textsuperscript{658-75D} has decreased client expression. E4.126 murine pre-B cells were retrovirally transduced with the indicated constructs and then analyzed. (A) GFP reporter expression of gp93\textsuperscript{658-75D} cells by FACS. (B) Flag intracellular stain of gp93\textsuperscript{658-75D} cells (left). Flag WB of the indicated cells with β-actin loading control (right). (C) FACS of gp96 client proteins (α4, αL, β2, and TLR2). Shaded histogram denotes isotype control stain. Data represent one of two experiments with similar results.
**Figure 8: gp93^{2YA} has decreased client expression.** E4.126 murine pre-B cells were transduced with the indicated constructs and then analyzed. (A) GFP reporter expression of gp93^{2YA} cells by FACS. (B) Flag intracellular stain of gp93^{2YA} cells (left). Flag WB with β-actin loading control of the indicated cells (right). (C) FACS of gp96 client proteins (α4, αL, β2, and TLR2). Shaded histogram denotes isotype control stain. Data represent one of two experiments with similar results.
Figure 9: CNPYa and CNPYb improve β2 expression while only CNPYb improves TLR expression. E4.126 murine pre-B cells retrovirally transduced with the indicated constructs were then analyzed. (A) GFP reporter expression of the indicated cells by
FACS. (B) WB for Flag (gp93), myc (CNPYα and CNPYβ), and β-actin loading control. (C) FACS of the indicated cells for gp96 client proteins (α4, αL, β2, TLR2, and TLR4). Shaded histogram denotes isotype control stain. Numbers indicate MFI. Data represent one of two experiments with similar results.
Figure 10: Gp96 and gp93 both interact with CNPY3 and CNPYb. E4.126 murine pre-B cells already transduced with either gp96\textsuperscript{Flag} or gp93\textsuperscript{Flag} were further transduced with either CNPY3 or CNPYb and then analyzed. (A) Myc WB of the indicated cells. \(\beta\)-actin was used as a loading control. (B) Flag (F) IP under DSP crosslinking conditions, followed by Myc WB. (C) Reverse IP of Myc (M) with DSP, followed by Flag WB. Isotype (Iso) control antibody was used. Data represent one of two experiments with similar results.
Figure 11: *Drosophila* CNPY structure and sequence alignment. (A) Schematic of *Drosophila* CNPYa and CNPYb. Signal peptide, SP, green. Cysteine residues, C, yellow. Basic region, BR, grey. ER retention signal, ER, purple. (B) Model of CNPYb folded clam structure. Lines represent disulfide bonds between Cysteine/C residues. Colors are as in ‘A’. (C) Amino acid sequence alignment between murine (m) CNPY3 and *Drosophila* (d) CNPYb. Conserved residues are highlighted red. Similar residues are blue. Cysteines are highlighted yellow. Numbers indicate amino acid position.
Figure 12: Cysteines 36, 39, and 92 of CNPYb are important for folding of TLRs.

E4.126 murine pre-B cells already transduced with gp93^Flag were further transduced with the indicated CNPYb construct and then analyzed. (A) GFP reporter expression of the indicated cells by FACS. (B) Myc (all CNPYs) and β-actin control WB. Numbers denote molecule size in kDa. (C) FACS of the indicated cells for TLR2 and TLR4 expression. Shaded histograms denote isotype control stains. Data represent one of two experiments with similar results.
Figure 13: CNPYb\textsuperscript{C183A} phenocopies CNPYb\textsuperscript{C39A}. E4.126 murine pre-B cells already transduced with gp93\textsuperscript{Flag} were further transduced with the indicated CNPYb construct and then analyzed. (A) FACS for GFP reporter expression of the indicated cells. (B) WB for Flag (gp93), Myc (all CNPYs), and β-actin loading control. Numbers denote molecule size in kDa. (C) FACS for TLR2 and TLR4 of the indicated cells. Shaded histograms are isotype control stains. Data represent one of two experiments with similar results.
Figure 14: gp93 may not chaperone endogenous Drosophila Toll. (A) Gp93, Toll, CNPYa, and CNPYb qRT-PCR of S2* cells treated with the indicated RNAi constructs for four days. The MigR1 RNAi construct was used as a negative control. (B) RNAi treated S2* cells were given 1μM 20-hydroxyecdysone for 24 hours on day six. On day seven, cells were treated with or without (+/-) 2.4nM rSPZ for 6 hours. Cells were then harvested and assayed for Dromosycin RNA levels by qRT-PCR. Rp49 was used as an internal control. Data represent one of two experiments with similar results.
Figure 15: Model of gp93, CNPYa, and CNPYb chaperone function. Our data indicates that CNPYb aids gp93 in chaperoning TLRs. Gp93 chaperones integrins α4, αL, and β2 independently of any CNPY. CNPYa and CNPYb are able to improve β2 expression on their own.
Chapter 4

Macrophage-Specific gp96 Promotes Colitis-Associated Colon Tumorigenesis
Rationale:

Although both gp96 and macrophages have been implicated in promoting cancer, the majority of work done on macrophages has involved the cell’s deletion (66, 82). In order to further this area of research and design better therapeutics targeting macrophages in cancer, the mechanisms which activate protumor properties of macrophages need to be better delineated. PRRs are a large class of molecules which are able to activate macrophages. TLRs are a type of PRR which are chaperoned by gp96, and signal through MyD88 to induce NF-κB signaling. Since TLRs induce inflammation and inflammation has been shown to promote cancer, TLRs are an attractive molecule to study in the role of cancer. In particular, our interest was focused on colitis and colitis-associated colon cancer.

The literature revealed a protective role for NF-κB in general, and TLR2 and TLR4 specifically in colitis. However, this picture became more obscured in colon cancer, where NF-κB appeared to be both protective and promoting. This identifies conflicting roles for receptors upstream of NF-κB. MyD88 proved to be protective in colon cancer, and a look at upstream receptors demonstrated that IL1R had no effect, while IL18R was also protective. The direct role of TLRs, however, was not investigated. As well, other receptors upstream of NF-κB must be studied in order to determine which receptors are responsible for cancer promotion.

Since the TLR family is so large, a pan-TLR KO mouse is hard to develop. Although many investigations have used the downstream adaptor MyD88, deficiency of this gene also interferes with IL1R and IL18R. The knockdown of gp96, however, provides a pan-TLR KO mouse (except TLR3) without affecting IL1R or IL18R. We
therefore decided to use macrophage-selective gp96 KO mice as a means to study macrophage activation via TLRs in colitis-associated colon tumorigenesis.

**Results:**

*KO mice are less susceptible to DSS colitis*

LysM Cre gp96<sup>Flox/Flox</sup> (KO) mice were described previously. Yang demonstrated that there were different levels of gp96 knock down depending on the macrophage tissue of origin, with more mature macrophages having greater knock down (4). In order to study colitis-associated colon tumorigenesis, we decided to first analyze colitis. We treated WT and KO mice with 3% DSS for five days, and then euthanized the mice at either day five or day eight. DSS is a chemical irritant which breaks apart epithelial tight junctions, both destroying the architecture and allowing commensal bacteria to encounter lamina propria-resident leukocytes. Histological examination of the colons by H&E staining demonstrated normal architecture basally in both WT and KO mice. Upon DSS exposure, WT mice had worse pathology than KO mice at both day 5 and day 8, characterized by greater erosion, loss of structure, and immune infiltrate (Figure 16A). When scored blindly by a pathologist, the worst pathology was seen with day 8 colons. Furthermore, WT mice had significantly worse pathology at day 8 than KO mice (Figure 16B).

Colonic shortening, a characteristic of colitis, was examined at time of sacrifice. At day 5 of DSS exposure, colonic shortening was seen in both WT and KO mice, but it was significantly more drastic in WT mice. At day 8, both mice started to recover, but no significant difference was seen (Figure 16C). During treatment, mice were also analyzed
daily for stool consistency. WT mice had significantly worse stool consistency scores than KO mice (Figure 16D).

Commensal bacteria can trigger leukocytes via TLRs, and since gp96 chaperones TLRs, we decided to look at differences in TLR activation. We analyzed cytokine expression downstream of TLR activation by qRT-PCR of distal colon samples. The following cytokines were analyzed: IL-1β, IL-6, IL-17A, IL-17F, IL-12 p35, IL-12/23 p40, IL-23 p19, IL-18, IL-22, IFNγ, and TNFα. A trend developed whereby WT mice expressed higher cytokine levels, although insignificant. The macrophage marker F4/80 was also analyzed. Although its expression significantly increased on day 8 upon inflammation, similar levels were seen between WT and KO mice, suggesting similar macrophage recruitment (Figure 16E). Collectively, these data demonstrate that KO mice are less susceptible to DSS-induced colitis.

KO mice are less susceptible to colitis-associated colon tumorigenesis

Since KO mice were protected from colitis, we expected to see an exacerbated phenotype with colitis-associated colon tumorigenesis. Mice were administered with the carcinogen AOM via ip injection on day one. Mice were then treated with three cycles of DSS as described in the methods, and then sacrificed on week 20. Nearly 100% tumor incidence was seen in WT and KO mice (data not shown). Upon necroscopy, macroscopic tumors could be seen in both WT and KO mice when the colons were cleaned and opened longitudinally. Distal and medial localization of the tumors was similar between WT and KO mice, while the proximal colon was void of tumors (Figure 17A). Closer pathological examination of tumors via H&E staining revealed differences between WT and KO mice.
WT mice had higher grade adenomas with greater immune infiltration than KO mice (Figure 17B). When scored blindly by a pathologist, WT mice indeed had higher scores (Figure 17C). When adenomas were enumerated, WT mice had significantly more tumors than KO mice (Figure 17D). Upon closer scrutiny, WT mice had significantly more large tumors than KO mice, being more than 2mm in diameter (Figure 17E). When scored, WT mice also had worse tumor burden than KO mice (Figure 17F). Therefore, KO mice seem to be more resistant to the AOM+DSS treatment.

Similar to our colitis experiments, we again sought to determine differences in cytokine levels by qRT-PCR of distal colon samples. As in Figure 16E, we analyzed the same cytokines and again found the trend whereby WT mice exhibited greater expression levels (Figure 18). Importantly, IL-17A, IL-17F, TNFα, and IL-23 (but not IL-12) were significantly decreased in KO mice. This data is consistent with the literature which shows a role for IL-17 and IL-23 in tumor promotion (113-115, 117, 120).

**Similar macrophage markers in WT and KO mice**

Since gp96 is only knocked out of macrophages, it is important to investigate different macrophage populations within our mice. We therefore decided to look at different macrophage markers in distal colon samples from our mice by qRT-PCR. The general macrophage markers CD11b and F4/80 were similar between WT and KO mice in both untreated and AOM+DSS treated samples. Both M1 (iNOS) and M2 (ARG1, Cox2, and IDO1) markers were also similar between WT and KO mice (Figure 19A). We also looked at F4/80 expression by immunohistochemistry, which demonstrated similar localization and number of macrophages in both WT and KO untreated colons and
AOM+DSS-induced tumors (Figure 19B). Therefore, differences seen between WT and KO mice are not due to differences in macrophage recruitment, but rather to differences in macrophage activation.

*Less CD4⁺ IFNγ⁺ cells in KO mice*

We next sought to uncover differences in immune activation by looking at different T cell subsets. Importantly, no differences were seen in overall CD4⁺ or CD8⁺ lamina propria T cells from either untreated or AOM+DSS treated WT and KO mice when analyzed by FACS (Figure 20A and C). The CD4 to CD8 ratio was also unaltered between genotypes (Figure 20D). However, when stimulated *ex vivo*, we did see a significant decrease in CD4⁺ IFNγ⁺ cells in AOM+DSS treated KO mice (Figure 20B and E), but not in other T cell subsets (data not shown). Therefore, KO mice exhibit less CD4⁺ IFNγ⁺ cells, such as Th1 cells, contributing to a decreased immune response compared to WT mice.

*KO mice are protected from Wnt activation and β-catenin mutation*

We next decided to look into the Wnt pathway, as its activation has been associated with various cancers (124). We first performed qRT-PCR on distal colon samples for various Wnt pathway molecules and downstream targets: Axin, β-catenin, cMyc, CyclinD1, Fzd1, and p53. As expected, all molecules were significantly increased upon AOM+DSS exposure. Furthermore, a trend again emerged whereby WT mice harbored greater expression levels than KO mice. Notably, only the Wnt receptor Fzd1 and the downstream target p53 were significantly greater in WT mice than in KO mice (Figure 21A).
Mutations within exon 3 of β-catenin occur under the AOM+DSS model, as previously reported (100). Exon 3 in particular is important for protein regulation, harboring GSK3β phosphorylation sites. We therefore sequenced β-catenin in our colon samples. Neither WT nor KO untreated mice had any mutations. As expected, more than 60% of AOM+DSS treated WT mice exhibited mutations within exon 3. Strikingly however, not a single KO mouse treated with AOM+DSS had any mutations (Figure 21B). Upon closer scrutiny, individual WT mice had anywhere from zero to six mutations within exon 3 (Figure 21C). Mutations found in treated WT mice were not only located in exon 3, but were also clustered around the GSK3β phosphorylation sites (Figure 21D). Therefore, the β-catenin mutation rate in KO mice is drastically reduced.

Since disruption of GSK3β phosphorylation sites can activate β-catenin, we next assayed β-catenin nuclear localization by immunohistochemistry. Both cytoplasmic and nuclear expression patterns were similar between WT and KO mice in areas of normal epithelium and within the tumor (Figure 21E). Collectively however, these data indicate that WT mice exhibit altered Wnt pathway activation and β-catenin mutation rates than KO mice.

**DNA repair is activated with inflammation-associated colon tumorigenesis**

Due to striking differences in β-catenin mutation rate between WT and KO mice, we wondered if DNA repair pathways would be altered. We focused on molecules involved in the MMR and BER pathways of DNA repair, as they have been implicated in colon cancer and repair of O6-methylguanine containing mismatches caused by AOM. We first checked DSS samples for expression of the MMR genes MLH1, MSH2, and MSH6, as
well as the BER gene AAG by qRT-PCR. No significant differences were seen (Figure 22A). When we analyzed the same genes in AOM+DSS treated samples, we saw significant increases upon treatment. However, there were again no differences between WT and KO mice (Figure 22B). Therefore, the reason underlying decreased β-catenin mutation rate in KO mice remains unknown.

**Bone marrow transplantation confirms the role of hematopoietic cells**

Utilizing LysM Cre-mediated gp96 deletion in our system allows a remote possibility that the recombinant Cre could be ectopically expressed in parenchymal cells as well as macrophages, thus complicating our interpretation of the results. To rule out this possibility, we performed bone marrow transplantation studies. Congenic WT mice were lethally irradiated, and then reconstituted with either WT (WT→WT) or KO (KO→WT) bone marrow. We first verified over 90% donor chimerism by FACS of peritoneal exudate cells, analyzing gp96 expression on gated F4/80 cells (Figure 23A). These mice were then subjected to the AOM+DSS regimen. We found that treated KO→WT mice gained more weight than WT→WT mice (Figure 23B). Similar to our previous data, KO→WT mice again exhibited decreased tumor burden compared to WT→WT mice (Figure 23C). F4/80 immunohistochemistry also showed similar macrophage localization and number between WT→WT and KO→WT mice (Figure 23D). These data collectively show that parenchymal cells are not affected, and that the phenotype of decreased susceptibility seen in KO mice is indeed hematopoietic intrinsic.
Discussion:

The focus of our work was to determine the role of macrophage-specific gp96 in cancer. We used gp96 as a means to produce a pan-TLR knockout in order to investigate the role of macrophage activation. Up to now, the majority of studies on the role of macrophages in cancer have used macrophage depletion strategies to answer this question. However, we were interested in how macrophages mediated these effects, and in particular, which PRRs were responsible for activation of their tumor promoting roles. Since TLRs are integral in promoting inflammation, we decided to look at a model of inflammation-induced colon tumorigenesis: AOM+DSS. In addition, DSS-colitis was checked separately in order to better characterize this model.

Our data demonstrate colitis and colon cancer promoting roles of macrophage-specific gp96. Upon DSS treatment, KO mice were protected from colitis with decreased pathology, colonic shortening, stool consistency, and inflammatory cytokine expression. This role was further amplified in colitis-associated colon tumorigenesis, where gp96 again played a promoting role. KO mice displayed decreased pathology, tumor number, tumor size, and tumor burden. This was characterized with decreased inflammatory cytokine expression, particularly IL-17, IL-23, and TNFα. These three cytokines, which have previously been implicated in cancer, are therefore possible mediators of gp96 cancer promoting functions. The use of bone marrow transplantation coupled to AOM+DSS treatment demonstrated the hematopoietic intrinsic role of the phenotype seen in KO mice, where KO→WT mice harbored decreased tumor burden compared to WT→WT mice.
Scrubinizing macrophage subsets, no differences were found between WT and KO mice in either M1 or M2 markers. Likewise, macrophage infiltration into the tumor microenvironment was also comparable, demonstrating that integrin loss due to gp96 absence did not affect macrophage migration. Upon AOM+DSS treatment, CD4+ IFNγ+ cells were decreased in KO mice, suggesting that macrophage specific gp96 may promote maintenance of Th1 cells in colon tumorigenesis. Wnt signaling also appeared to be down regulated in KO mice, yet there was no difference in nuclear β-catenin translocation within the tumor. Furthermore, mutations within the β-catenin gene characteristic of the AOM+DSS model were absent in KO mice.

In order to determine the apparent difference in mutation rates within these mice, DNA repair pathways were investigated. No differences were seen in colitis. With colon cancer, however, there was a marked increase of DNA repair genes with AOM+DSS treatment. However, there were no differences between WT and KO mice. It is possible that differences exist in other DNA repair genes not studied in this model. As well, there may be epigenetic differences affecting mutation rates independent of DNA repair. Most likely however, TLR signaling in WT mice leads to increased levels of reactive oxygen and nitrogen species (RONS) which can directly insult DNA. With more DNA insult in WT mice than KO mice, the DNA repair machinery is not able to keep up with correcting these changes, and mutations are thus able to persist and be passed down to progeny cells.

Macrophage-specific gp96 may exert its affects through TLRs to promote colitis. This is complimentary to the literature where NK-κB displays colitis promoting roles, yet the upstream receptors involved are not clearly delineated. However, a pan-TLR specific
KO mouse does not exist, and only a few TLR KO mice have been used for colitis experiments. Therefore, our data for the first time shows that the culminative effect of knocking out all TLRs (except TLR3) exhibits a colitis promoting role. However, the role of other NK-κB signaling receptors needs to be investigated in order to ascertain a comprehensive understanding of the role of NK-κB in colitis.

Although macrophages have pro-tumor properties, the activating mechanism is unknown. In inflammation induced cancers, especially within the intestines where commensals are prevalent, PRRs are likely to be the culprit. Macrophage-specific gp96 KO creates macrophages which lack all TLRs except TLR3. The literature demonstrated that NK-κB harbors both tumor promoting and protective roles in the AOM+DSS model. However, the role of individual upstream receptors was unclear. Our data therefore demonstrates that the upstream TLR receptor family plays a promoting role. This is in contrast to IL18R which plays a protective role, also implicating the role of the inflammasomes. Furthermore, MyD88 also plays a protective role, demonstrating that the role of IL18R is dominant over that of TLRs. As well, TLR2 is protective while TLR4 promotes colon cancer. Therefore, although specific TLRs may have different effects, the collective effect of all TLRs is tumor promoting.

Under homeostatic conditions within the colon, the epithelial cells create a physical barrier separating the intestinal lumen full of commensal bacteria from the lamina propria. Immune cells such as macrophages within the lamina propria produce IL-10 to maintain an immunosuppressive environment (Figure 24A). In the case of DSS-colitis, epithelial tight junctions are directly antagonized by DSS, creating space in between enterocytes by which commensal bacteria can pass into the lamina propria.
Bacterial-derived ligands then stimulate macrophages via cell surface TLRs to secrete proinflammatory cytokines. Macrophages promote inflammation which causes colitis (Figure 24B). When AOM is further added, IL-17, IL-23, and TNFα in particular are induced by macrophages. As a carcinogen, AOM can directly cause DNA mutations. As well, proinflammatory macrophages are known to secrete RONS which also cause mutations. Since KO mice are devoid of β-catenin mutations, we believe that this is the direct result of RONS, which we assume to be drastically reduced in KO mice if macrophages are not activated. As well, the inflammatory environment induced the Wnt pathway within enterocytes. This, coupled with activating β-catenin mutations as well as the induction of other oncogenes, led to increased proliferation and adenoma formation (Figure 24C). Whether other genes harbor mutations, and if differences exist between WT and KO mice, is unknown.

In conclusion, macrophage-specific gp96 promotes colitis and colitis-associated colon cancer. Therefore, TLR expression on macrophages may activate protumor properties of the cells, and TLRs are at least one receptor upstream of NF-κB which promotes colon cancer. This data pinpoints macrophage-derived gp96 and TLRs as therapeutic targets against colitis and colon cancer.
Figure 16: Macrophage-specific gp96 KO mice are less susceptible to colitis. WT and KO mice were treated with 3% DSS for 5 days, and then sacrificed on either day five (D5) or day eight (D8). Three experiments were performed with similar results. (A) H&E staining of transverse colon sections from untreated (UT) or DSS treated mice. (B) Pathology scores based on ‘A’. N=3 (C) Colon length measured from cecum to anus at time of sacrifice. N=5 (D) Stool was scored daily for consistency as follows: 0, well-
formed stool; 1, semi-formed stool; 2, semi-formed stool that adheres to the anus; 3, liquid stool/diarrhea. N=10 for days 0-5, n=5 for days 6-8. (E) qRT-PCR of distal colon samples for various cytokines. β-actin was used as an internal control, and data was analyzed using the dCT method. N=5 Error bars depict standard deviation in ‘B-D’, and standard error in ‘E’. p<0.05 *, p<0.005 **
Figure 17: Decreased tumor burden in Macrophage-specific gp96 KO mice. Mice were injected ip with 12.5mg/kg AOM on day 1, followed by two cycles of 2.5% DSS for 5 days on weeks 2 and 5, and then one cycle of 2% DSS for 4 days on week 8. Mice were monitored weekly and then sacrificed on week 19-21. Two experiments were performed with similar results. (A) Gross photographs of colons ex vivo which were washed and opened longitudinally after AOM+DSS treatment. (B) H&E staining of colonic tumors. (C) Pathology score based on ‘B’. N=6 WT and 7 KO. (D) Colons were fixed in paraformaldehyde, and then stained with methylene blue for adenoma visualization. The number of tumors per mouse was counted using a dissecting microscope. (E) Distribution of tumors based on size. The diameter of each tumor was measured in two perpendicular directions, and then the average diameter was used for tumor size. (F) Tumor burden was calculated for each mouse as the number of tumors times the average tumor size. N=9 WT and 16 KO for D-F. Error bars depict standard deviation. p<0.05 *, p<0.005 **
Figure 18: KO mice harbor less cytokine expression. Upon sacrifice of mice treated with AOM+DSS (AOM), the distal colon was harvested for RNA. After reverse transcription into cDNA, qRT-PCR was performed for various cytokines. β-actin was used as an internal control, and data was analyzed using the dCT method. Error bars depict standard error. UT, untreated. N=7 WT UT and KO UT, 9 WT AOM, and 14 KO AOM. p<0.05 *, p<0.005 **
Figure 19: Similar macrophage markers between WT and KO mice. (A) Upon sacrifice of mice treated with AOM+DSS (AOM), the distal colon was harvested for RNA. After reverse transcription into cDNA, qRT-PCR was performed for macrophage markers. β-actin was used as an internal control, and data was analyzed using the dCT method. Error bars depict standard error. N=7 WT UT and KO UT, 9 WT AOM, and 14 KO AOM. p<0.05 *, p<0.005 ** (B) After tumors were counted and measured, colons were distained in ethanol, and then incubated in 30% sucrose overnight. Whole colons were then frozen in OCT as Swiss Rolls, and 5µm sections were cut using a cryostat and
mounted onto charged slides. F4/80 (brown) IHC of untreated colons or AOM+DSS
treated colonic tumors was then performed. Tissue was counterstained with hematoxylin
(blue). UT, untreated.
**Figure 20: KO mice exhibit diminished lamina propria CD4$^+$ IFN$\gamma^+$ cells.** Upon sacrifice of AOM+DSS mice, lamina propria lymphocytes were isolated. (A) CD4 and CD8 FACS staining of lamina propria cells from either untreated (UT) or AOM+DSS treated mice. (B) Lamina propria lymphocytes were stimulated *ex vivo* with anti-CD3 and anti-CD28 antibodies overnight, and then analyzed by FACS for CD4 and IFN$\gamma$ expression. Plots are gated on CD4$^+$ cells. Numbers in ‘A’ and ‘B’ denote percentages. (C) CD4$^+$ cell percentages from ‘A’ are quantified. (D) The ratio of CD4$^+$ to CD8$^+$ cells from ‘A’ are quantified. (E) CD4$^+$ IFN$\gamma^+$ cell percentages from ‘B’ are quantified. Error bars depict standard deviation. N=3 WT UT and KO UT in ‘C’ and ‘D’, and 2 in ‘E’. N=9 WT AOM and KO AOM in ‘C-E’. $p<0.05$ *
Figure 21: Wnt activation and \( \beta \)-catenin mutations are decreased in KO mice. (A) RNA was isolated \textit{ex vivo} from distal colon samples and reverse transcribed into cDNA.
qRT-PCR of Wnt pathway molecules from untreated (UT) or AOM+DSS treated distal colon samples was performed. β-actin was used as an internal control, and data was analyzed using the dCT method. Error bars depict standard error. N=7 WT UT and KO UT, 9 WT AOM, and 14 KO AOM. (B) Exon three of β-catenin was sequenced after PCR amplification from distal colon cDNA samples. Graph depicts percent of mice with mutations. N=3 WT UT and KO UT, 9 WT AOM, and 14 KO AOM. (C) Number of β-catenin mutations in individual WT mice treated with AOM+DSS. (D) β-catenin exon 3 sequence. Underlined codons are sites of GSK3β phosphorylation. Capitalized and red residues signify mutated bases. (E) β-catenin (brown) IHC of a normal section of colon, and of a colonic tumor from Swiss Rolls of AOM+DSS treated mice. Slides were counterstained with hematoxylin (blue). p<0.05 *, p<0.005 **
Figure 22: **WT and KO mice demonstrate similar DNA repair machinery.** cDNA from distal colon samples was reverse transcribed from RNA. (A) qRT-PCR was performed for various DNA repair molecules from either untreated (UT) or DSS treated samples at day 8. N=5. (B) The same as ‘A’, but from UT or AOM+DSS treated samples. N=7 WT UT and KO UT, 9 WT AOM, and 14 KO AOM. β-actin was used as an internal control, and data was analyzed using the dCT method. Error bars depict standard error. p<0.05 *, p<0.005 **
Figure 23: BMT verifies a hematopoietic intrinsic role. C57BL/6 mice were lethally irradiated with two doses of 550cGy, and then reconstituted 24hrs later with either 2x10^6 WT or KO BM cells injected iv into the lateral tail vein. After 12 weeks, mice were either left untreated (UT) or were treated with the AOM+DSS regimen, and then sacrificed 16 weeks later. (A) FACS staining of PEC cells from BMT mice. Top: F4/80 vs SSC. Numbers denote percentages. Bottom: gp96 intracellular stain of gated F4/80^+ cells from above. Isotype antibody was used as a control. (B) Percent weight change over time of UT or AOM+DSS treated BMT mice. N=4 WT UT, 6 KO UT, 15 WT AOM, and 16 KO AOM. (C) Quantification of tumor burden as the number of tumors times the average tumor size. Error bars depict standard deviation. N=12 WT and 11 KO. p<0.05 * (D)
F4/80 (brown) IHC of colonic tumors from Swiss Rolls of BMT mice treated with AOM+DSS. WT, WT→WT. KO, KO→WT.
Figure 24: Model of the promoting roles of macrophage-specific gp96. (A) Under basal conditions, gut epithelial cells create a physical barrier so that commensal bacteria cannot penetrate. Within the lamina propria, macrophages secrete IL-10, maintaining a tolerant environment. (B) DSS breaks apart tight junctions holding together epithelial cells, creating gaps between the cells and deteriorating the physical barrier. Commensal
bacteria are now able to cross into the lamina propria, where gp96+ macrophages recognize bacterial constituents via cell surface TLRs. This activation leads to production of proinflammatory cytokines, causing inflammation and colitis. (C) In the AOM+DSS model, DSS causes colitis in the same manner as described in ‘B’. Besides cytokines, proinflammatory macrophages are known to secrete reactive oxygen and nitrogen species (RONS) which can directly insult DNA. The carcinogen AOM is also able to cause DNA mutations. In particular, β-catenin is frequently mutated (stars) in this model. As well, the inflammatory environment leads to increased Wnt signaling in enterocytes. This leads to increased proliferation of epithelial cells, and in conjunction with mutations in oncogenes, leads to the formation of adenomas.
Chapter 5

Concluding Remarks
Conclusions:

Our study of the biology of gp96 has led to the discovery of the *Drosophila* ortholog gp93, identifying a simpler system by which to further examine gp96 structure and function. The following are our conclusions:

1. *Drosophila* Gp93 is the ortholog of mammalian gp96.
   a. Gp93 rescues gp96 client expression.
   b. Gp93 physically interacts with TLR9.
   c. Gp93 and gp96 can dimerize.

2. Gp96/Gp93 undergoes N-terminal dimerization.
   a. A137 of gp93/C138 of gp96 is important for N-terminal dimerization.
   b. N-terminal dimerization does not affect chaperone function of gp93.

3. Gp96/Gp93 contains an N-terminal CBD.
   a. Residues 658-75 in gp93 are important for chaperone function.
   b. Tyrosines 674 and 675 are important for gp93 function.

4. CNPYa and CNPYb rescue β2 expression.

5. *Drosophila* CNPYb is the ortholog of mammalian CNPY3.
   a. CNPYb and CNPY3 bind both gp93 and gp96.
   b. CNPYb is a TLR-specific cochaperone for gp93.

6. Disulphide bond formation is important for CNPYb function.
   a. Cysteines 36, 39, 92, and 183 of CNPYb are important for TLR4 expression.
   b. Cysteines 39 and 183 of CNPYb are important for TLR2 expression.
The study of macrophage-specific gp96 has demonstrated a promoting role in both colitis and colitis-associated colon cancer. This work thus identifies gp96 in macrophages as a potential therapeutic target. The following are our conclusions:

1. KO mice are less susceptible to DSS-induced colitis, exhibiting decreased:
   a. Pathology score
   b. Colonic shortening
   c. Stool consistency
   d. Inflammatory cytokine expression

2. KO mice are less susceptible to AOM+DSS colitis-associated colon cancer, demonstrating decreased:
   a. Pathology score
   b. Tumor burden (size and number)
   c. IL-17, IL-23, and TNFα expression
   d. CD4^+ IFNγ^+ Th1 cells
   e. Wnt signaling

3. Macrophage-specific gp96 does not affect:
   a. Macrophage polarization
   b. Macrophage infiltration
   c. DNA repair pathways


5. Decreased tumor burden in KO mice is a hematopoietic effect.
Outstanding Questions:

Drosophila gp93

1. What are the endogenous clients of gp93 in Drosophila?

As previously mentioned, the following molecules are present in Drosophila: Toll, Toll-related proteins, $\alpha_{PS1}$, $\alpha_{PS2}$, $\alpha_{PS3}$, $\alpha_{PS4}$, $\alpha_{PS5}$, $\beta_{PS}$, and $\beta_{v}$. Since gp96 chaperones TLRs and various integrins, it is quite possible that all or some of these molecules may be endogenous clients of gp93. To explore this question, either S2 cells or flies can be used. S2 cells are a Drosophila hemocyte (similar to a macrophage) cell line (158). RNAi techniques can be used to knockdown gp93 expression, and then the expression of the potential client proteins can be analyzed. Immunoprecipitation experiments can be performed to determine whether gp93 and the various ‘clients’ can physically interact. As well, if loss of gp93 results in loss of Toll, then knockdown cells will be resistant to Toll ligation. Cells can either be transfected with a constitutively activated form of SPZ (C106, the Toll ligand), or they can be treated with recombinant C106. Then the activation of downstream targets can be analyzed, such as Drosomycin. If gp93 knockdown results in loss of expression of any of these molecules (by FACS or WB), and if gp93 physically interacts with them, then we can conclude that these molecules are clients of gp96.

A gp93 knockdown fly can also be used. Such a fly was already characterized, but they do not survive past the third instar larval stage (157). However, other strains of flies with mutations within the gp93 gene exist which are available from the Bloomington Drosophila Stock Center and are awaiting characterization. Whole fly lysates can be prepared from WT and KO flies for WB and IP to determine expression of the potential
client proteins, and whether or not they physically interact with gp93. Furthermore, flies can be easily infected with fungus and then probed for Toll downstream target genes to determine if Toll remains intact in KO flies. Therefore, either S2 cells or flies can be used to determine the endogenous clients of gp93.

2. Is CNPYb the endogenous cochaperone for gp93?

Although CNPYb appears to act as a cochaperone for gp93 in relation to TLR2 and TLR4, we would like to investigate whether or not CNPYb is also an endogenous cochaperone for gp93. However, this question relies on the answer to the former question. If Toll or the Toll-related proteins prove to be endogenous clients for gp93, then it would be interesting to find out if CNPYb aids gp93 in their folding. S2 cells can be used for immunoprecipitation experiments to determine not only if gp93 binds CNPYb, but if tri-molecular complexes can be found among chaperone, cochaperone, and client. RNAi can also be used to knockdown CNPYb expression in WT S2 cells, to determine if client expression is decreased. Toll signaling can also be analyzed in these cells to determine if it’s affected. Such experiments will definitively prove whether or not CNPYb is an endogenous gp93 cochaperone.

3. What is the endogenous role of CNPYa in gp93 biology?

Once again, this question relies upon the elucidation of the endogenous clients of gp93. Similar experiments as the ones proposed in ‘question 2’ can be used to determine whether or not CNPYa is a cochaperone for gp93. Immunoprecipitation experiments with S2 lysate can determine whether or not CNPYa physically interacts with gp93 or any of
its clients. RNAi specific for CNPYa will demonstrate if the expression of any of these client proteins is affected. Since our results demonstrate increased β2 expression in cells containing CNPYa, it is possible that CNPYa may chaperone the *Drosophila* beta integrins. Collectively, these results will demonstrate whether or not CNPYa is a cochaperone of gp93, either positively or negatively regulating various clients.

4. What else can we learn from gp93 and CNPYb mutations?

Mutational analysis of both gp93 and CNPYb has already gained insight into the model of action of both of these molecules. Again, the purpose of characterizing the *Drosophila* system was to gain knowledge which could be applied to the mammalian gp96 system. In this respect, we have already proven successful demonstrating the role of a C-terminal cysteine in dimerization and characterizing the CBD (19, 155). We wish to further this study to learn more. For instance, gp93\(^{2YA}\) demonstrates the importance of these two tyrosine residues in gp93 function. The corresponding mutation in gp96 might uncover a similar defect. This data implies the role of tyrosine phosphorylation in chaperone function. Therefore, identification of the tyrosine kinase involved may prove beneficial in designing therapeutic targets which regulate gp96 activity.

CNPY3 M145K has defects in TLR interaction, trafficking, and signaling, with differences among varying TLRs (49). The reason for these differences is unknown. The corresponding residue in CNPYb is L133. Both methionine and leucine are hydrophobic residues, while lysine is basic. Therefore, L133 in CNPYb may play a similar role to M145 in CNPY3. Since the Toll-like family in *Drosophila* is smaller than the mammalian...
TLR family, the mutant CNPYb L133K may therefore prove to be a useful tool in understanding the differential regulation of various TLRs/Toll by this residue.

5. Does gp93 play a role in the UPR of *Drosophila*?

As a major chaperone inside the ER, gp96 plays a role in the UPR. While general protein translation is stopped, ER chaperones take part in chaperoning unfolded proteins to thereby restore homeostasis. The UPR response is conserved from mammals down to *Drosophila*, with key molecules such as BiP/grp78 and XBP1 being highly conserved. Since gp93 is the ortholog of gp96, it is therefore possible that gp93 also plays a role in the Drosophila UPR. S2 cells treated with gp93-specific RNAi can be treated with tunacamycin to induce a UPR response. If gp93 knock down cells are more sensitive to UPR induction, this will imply that gp93 is required for a proper UPR response; if a UPR response cannot restore homeostasis, then the cell will undergo apoptosis. Furthermore, WT S2 cells can also be treated with tunacamycin and probed for gp93 levels. Although general protein translation is halted, the translation of UPR specific genes is increased, such as the ER chaperones. Therefore, if gp93 expression is increased after tunacamycin treatment, then this will imply that gp93 plays a role in the *Drosophila* UPR.

*Macrophage specific gp96*

1. Are reactive oxygen & nitrogen species (RONS) reduced in KO mice?

The mutation rate of β-catenin was drastically different between WT and KO mice, yet no differences in DNA repair were seen. Since both mice were treated with AOM, we wondered if β-catenin mutations in particular were the result of another toxic
agent. It is known that proinflammatory macrophages produce RONS which can directly cause mutations. Since KO macrophages lack TLRs and are thus not as highly activated as WT macrophages, we hypothesized that there must be drastically reduced levels of RONS in KO mice. If so, this could explain the differences seen in β-catenin mutation.

2. Does the role of macrophage-specific gp96 differ in other cancer types?

   Our data demonstrate that macrophage-specific gp96 promotes colitis associated colon cancer. We have not yet tested other models of cancer with our mice. However, the literature demonstrates that some molecules may play promoting roles in one cancer type, and protective roles in another. One case in point is MyD88. Although MyD88 is protective in the AOM+DSS model, it has proven promoting in many other models (97). In a very similar model of colitis-associated colon cancer using AOM+Oxa, MyD88 actually played a promoting role (105). Yet in the absence of IL-10 in a model that uses six injections of AOM without DSS, MyD88 promotes cancer (159). MyD88 is also tumor-promoting in the MCA-induced fibrosarcoma model, the DMBA+TPA skin papilloma model, and in the spontaneous intestinal tumor model of APC\textsuperscript{Min} mice (95, 96). Therefore, the role of macrophage-specific gp96 must be elucidated in other cancer models to determine its effects. It will be interesting to compare tumors in different tissues, as well as spontaneous versus inflammation-induced models. A better understanding will determine the applicability of therapeutics targeting gp96 in macrophages for different cancer types.
3. What is the role of integrins?

Gp96 chaperones select integrins (6). It is possible that the loss of some integrins can be compensated by others. In the AOM+DSS model, gp96 KO macrophages can still migrate to the lamina propria and to the tumor microenvironment, demonstrating little overall effect by loss of integrins. The gp96-dependent integrin αv, however, leads to spontaneous colitis when knocked out of myeloid cells, including macrophages, monocytes, neutrophils, and some DCs (6, 160). In particular, αvβ1 on intestinal macrophages is important for production of IL-10, and thus the maintenance of an immunosuppressive environment (161). Since our KO mice do not develop spontaneous colitis, it is likely that this phenotype was caused by a different myeloid cell population. However, this demonstrates that a single integrin subunit on a restricted cellular population can drastically impact colitis. Therefore, it will prove interesting to determine if any of the integrin subunits knocked down in our KO macrophages plays a role in the protective phenotype seen with our mice. One way to deduce the role of integrins is to create a transgenic knock-in mouse harboring the gp96<sup>E103A</sup> mutation, and then treating it with AOM+DSS. This mutant gp96 is able to chaperone integrins, but appears devoid of TLR chaperone function (3). Therefore, if the phenotype is recapitulated, integrins must not play a role in our model.

4. Are there differences in the gut microbiota?

The gut microbiota plays an immense role in the induction of DSS-colitis and colon cancer, and inflammation alone can alter the composition of the gut microbiota (162). Within the intestine, IL-10 expression is required for Treg development which
dampens T cell driven inflammation caused by commensals, thereby maintaining homeostasis (163, 164). Therefore, IL-10 KO mice get spontaneous colitis. However, IL-10 KO mice do not develop colitis when housed in germ free conditions, and mono-association with the *B. vulgatus* bacterium causes an intermediate phenotype. Furthermore, although IL-10 KO mice treated with AOM develop colon cancer, polyp formation is absent when housed in germ free conditions, and mono-association again produced an intermediate phenotype. Therefore, the development of both colitis and colon cancer is dependent on the presence of commensals (159).

Another group demonstrated that mono-colonization of IL-10 KO mice with *E. coli* NC101 together with AOM led to invasive carcinomas. However, deletion of the polyketide synthase (pks) genotoxic island from *E. coli* NC101 resulted in decreased tumorigenesis. The presence of Pks+ *E. coli* is even associated with a large percent of IBD and colon cancer patients (162). Furthermore, different commensal species can produce varying effects. For instance, segmented filamentous bacteria (Clostridium) are a potent inducer of T cell maturation within the gut (165). Therefore, we are interested in analyzing the microbiota composition in WT and KO mice, both untreated and under DSS and AOM+DSS treatments. It is possible that the composition of the microbiota in KO mice is altered, being associated with less disease.

5. Does gp96 promote EMT conversion and the appearance of stemness genes?

Epithelial to Mesenchymal transition (EMT) is a normal process occurring in embryogenesis, organogenesis, and tissue regeneration. However, it abnormally occurs in cancer, where the dedifferentiation of epithelial cells promotes tumor progression and
metastasis (166). This phenomenon is associated with the loss of epithelial markers, such as E-cadherin, cytokeratins, and CD326, with a concomitant increase in mesenchymal markers such as vimentin and CD90. This transition has also been associated with the appearance of stemness genes, such as CD133, Sox2, Nanog, and Oct4 (167). Furthermore, the notion of a cancer stem cell postulates that within a tumor, a few cells are able to self-renew and thus contribute to tumor growth. Being stem cell-like, these cells will also express various stemness genes (168). Therefore, we would like to determine whether macrophage-specific gp96 can promote EMT conversion and the appearance of stemness genes as a means to promote tumorigenesis.

WT and KO macrophages can be stimulated ex vivo with TLR ligands and AOM to simulate our in vivo model. Either the cells directly, or culture supernatant can then be used to activate an enterocyte cell line, such as YAMC (169). As well, these cells can be treated directly with IL-17, IL-23, or TNFα, the three cytokines which were significantly reduced in KO mice. The YAMC cells will then be analyzed for Wnt pathway activation, β-catenin mutation, EMT transition, and stemness genes. This data may further prove mechanistically how macrophage-derived gp96 promotes tumorigenesis.

6. What is the role of gp96 in microglia and seizures?

Kinetic scrutiny of our KO mice demonstrated the most prominent phenotype of these mice: age-related spontaneous seizures characterized by abnormal behavior and hyperactivation of multiple muscles at once. Although detailed observations have not been made, the onset is around 6 months of age and incidence is roughly 90-95%. Furthermore, the severity of episodes also seems to increase with age, lasting roughly 30-
90 seconds. The incidence in WT littermate controls was 0%. A neurological disorder, better characterization of brain macrophages, microglia, is warranted. Furthermore, the trigger for such episodes needs to be determined. However, anxiety, fear, increased heart rate, and/or adrenalin may be possible causes, as episodes frequently occur upon transferring the cage into the hood and opening the lid, an action which may startle the mice. Therefore, the study of macrophage-specific gp96 may gain valuable insight into seizure biology.
REFERENCES

14. Krukenberg KA, Böttcher UMK, Southworth DR, Agard DA. 2009. Grp94, the endoplasmic reticulum Hsp90, has a similar solution conformation to cytosolic Hsp90 in the absence of nucleotide Protein Science 18: 1815-27
cellular antigens and the generation of tumor-specific T cell memory Cancer Immunity 3: 1
34. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors Nature Immunology 11: 373-84

43. Hidmark A, von Saint Paul A, Dalpke AH. 2012. TLR13 Is a Receptor for Bacterial RNA. Journal of Immunology 189: 2717-21


61. Bogaert T, Brown N, Wilcox M. 1987. The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments Cell 51: 929-40


73. Erreni M, Mantovani A, Allavena P. 2011. Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer *Cancer Microenvironment* 4: 141-54


86. Vicetti Miguel RD, Cherpes TL, Watson LJ, McKenna KC. 2010. CTL induction of tumoricidal nitric oxide production by intratumoral macrophages is critical for tumor elimination Journal of Immunology 185: 6706-18
91. Dixit V, Mak TW. 2002. NF-kappaB signaling. Many roads lead to madrid Cell 111: 615-9
immunoediting during primary tumorigenesis Proceedings of the National Academy of Sciences 105: 652-6


121. Ojalvo LS, Whittaker CA, Condeelis JS, Pollard JW. 2010. Gene Expression Analysis of Macrophages That Facilitate Tumor Invasion Supports a Role
for Wnt-Signaling in Mediating Their Activity in Primary Mammary Tumors Journal of Immunology 184: 702-12
130. Hakem R. 2008. DNA-damage repair; the good, the bad, and the ugly. EMBO Journal 27: 589-605


the development of colitis with markedly infiltrated macrophages in interleukin-18 transgenic mice. *Journal of Gastroenterology and Hepatology* 18: 960-9


163. Li MO, Flavell RA. 2008. Contextual Regulation of Inflammation: A Duet by Transforming Growth Factor-b and Interleukin-10. Immunity 28: 468-76


