Autophagy Regulates Expression of Argonaute 2, a Critical Regulator of the MicroRNA Silencing Pathway

by

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Abstract

Genome-wide association studies have implicated autophagy in Crohn’s Disease (CD) pathogenesis. The functional relevance of autophagy in CD remains unknown. I hypothesized that autophagy is involved in microRNA silencing, another process implicated in CD pathogenesis. MicroRNAs are short non-coding RNAs that are loaded onto RNA-induced silencing complex (RISC) and promote degradation and/or repress translation of target mRNAs. RISC formation and turnover occurs on endosomal membranes. Since autophagosomes and endosomes are closely related and RISC components are downstream effectors of microRNA silencing, I hypothesized that autophagy affects RISC, hence modulates microRNA expression. Using immunoblotting and immunofluorescence, I showed that Ago2, a critical component of RISC, is increased in cells with defective autophagy. Using microarray technology, I discovered 5 microRNAs that are differentially expressed in these cells. Taken together, my results propose a compelling mechanism by which autophagy regulates Ago2, thereby affects miRNA expression, which is implicated in the development of CD.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Ago</td>
<td>Argonaute</td>
</tr>
<tr>
<td>AIEC</td>
<td>adherent invasive <em>E. coli</em></td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATG-/-</td>
<td>ATG5 knockout</td>
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<tr>
<td>CagA</td>
<td>Helicobacter pylori cytotoxin-associated gene A</td>
</tr>
<tr>
<td>CagE</td>
<td>Helicobacter pylori cytotoxin-associated gene E</td>
</tr>
<tr>
<td>CCMS</td>
<td>conditioned culture media supernatants</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
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<td>DAMP</td>
<td>danger-associated molecular patterns</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modification of Eagle's Medium</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulphate</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EBM-2</td>
<td>endothelial cell basal medium-2</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's balanced salts solution media</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF4G</td>
<td>eukaryotic initiation factor 4G</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FDR</td>
<td>false-discovery rate multiple hypothesis testing correction</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GWAS</td>
<td>Genome-wide association studies</td>
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<td><em>H. pylori</em></td>
<td><em>Helicobacter pylori</em></td>
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<tr>
<td>HCL</td>
<td>Hierarchical Clustering</td>
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<tr>
<td>hEGF</td>
<td>Human Epidermal Growth Factor</td>
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<td>HeLa</td>
<td>human cervical carcinoma epithelial cells</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>IFN-γ</td>
<td>interferon gamma</td>
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<td>IL-18</td>
<td>interleukin-18</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
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<tr>
<td>IRGM</td>
<td>Immunity related guanosine triphosphatase M</td>
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<tr>
<td>LC3</td>
<td>microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MDAMC</td>
<td>human breast epithelial cells</td>
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<td>MDP</td>
<td>muramyl dipeptide</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MHC</td>
<td><em>major histocompatibility complex</em></td>
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<td>MIP</td>
<td>Macrophage inflammatory peptide</td>
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<td>miRNA</td>
<td><em>microRNA</em></td>
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<tr>
<td>MNV</td>
<td>murine norovirus</td>
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<tr>
<td>Mode-K</td>
<td>mouse intestinal epithelial cells</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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mTOR mammalian target of rapamycin
MVB multi-vesicular body
NFκB nuclear factor kappa-light-chain-enhancer of activated B cells
Nod2 nucleotide-binding oligomerization-domain-containing protein 2
Nod2fs Nod2 frameshift mutation
PABPC1 Poly(A)-binding protein 1
PAMP pathogen-associated molecular patterns
PBMC peripheral blood monocytes
PBS Phosphate-Buffered Saline
PCA Principal Component Analysis
PE Phosphatidylethanolamine
PI3K Phosphoinositide 3-kinase
RISC RNA-induced silencing complex
ROS reactive oxygen species
RT-PCR Reverse transcription polymerase chain reaction
*S. typhimurium* *Salmonella enterica serovar Typhimurium*
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA Small interfering RNA
SNP single nucleotide polymorphisms
T300A rs2241880 SNP nonsynonymous mutation encodes for a threonine instead of an alanine at the 300 position
TBST Tris-buffered saline with tween
TCF4 Transcription factor 4
TGF-β Transforming growth factor beta
TLR4 Toll-like receptor 4
UC  ulcerative colitis
UTR  untranslated region
VacA  *H. pylori* vacuolating cytotoxin
V-ATPase  vacuolar type H(+)-ATPase
VEGF  Vascular endothelial growth factor
WT  Wildtype
Chapter 1
Introduction

1.1. Crohn’s Disease

Crohn’s disease (CD) is a chronic, idiopathic inflammatory bowel disease (IBD) which can affect any area of the gastrointestinal (GI) tract. The hallmark of CD is segmental transmural inflammation with sparing of intermediary areas. Over time, the majority of patients progress to develop more severe complications, such as strictures, fistulas and abscesses (Solberg et al., 2007).

The disease has considerable morbidity, shortened life expectancy and currently no cure (Cosnes et al., 2002; Louis et al., 2001). Patients face lifelong illness, as they undergo periods of relapse and remission. The current course of treatment focuses on alleviating symptoms and minimizing recurrence. Patients are dependent on medication, require surgery and lose their quality of life and productivity.

CD is found extensively within families, as first-degree relatives of CD patients are ten times more likely to develop the disease, as compared to the general population (Orholm et al., 1991). There is a concordance rate of 35% within monozygotic twins with CD (Spehlmann et al., 2008), suggesting that environmental triggers may play a key role in disease development. Indeed, it is known that the individual’s genetic background determines about 50 percent of the risk to develop CD (Van Limbergen et al., 2007), however little is known about the remaining 50 percent, the environmental factors that trigger CD development.

The current hypothesis suggests that in the genetically susceptible individual, there is an aberrant and prolonged immune response triggered by environmental factors such as commensal microbiota, which leads to persistent inflammation (Baumgart and Carding, 2007; Khor et al., 2011; Xavier and Podolsky, 2007). However, extensive research is needed to reveal the functional role of susceptibility genes, as well as the exact environmental factors involved in CD.
1.1.1. Genome-wide association studies

Genome-wide association studies (GWAS) in chronic inflammatory diseases in general and CD in particular, have been highly successful in identifying numerous definitive gene associations (Wellcome-Trust-Case-Control-Consortium, 2007). GWAS involve the genotyping of hundreds of markers throughout the genome in very large cohorts. Some markers demonstrate significantly different allele frequencies between disease cases and controls. These markers suggest that their surrounding genetic loci, regions ~150 kb long that contain about ~4 genes, encompass functional alleles that increase disease susceptibility (Hardy and Singleton, 2009).

The associations found by GWAS have provided enormous insight into the mechanisms of CD. A sizeable meta-analysis combining the results of several GWAS in CD has increased the number of confirmed and replicated susceptibility loci to 71 (Franke et al., 2010). GWAS have implicated completely novel and unexpected pathways in the development of the disease. These include nucleotide-binding oligomerization-domain-containing protein 2 (Nod2) signalling (Hugot et al., 2001; Ogura et al., 2001) and the autophagy pathway (Hampe et al., 2007; McCarroll et al., 2008; Parkes et al., 2007; Rioux et al., 2007), important for recognition and defense against microbial components. Other implicated immune-related pathways include mucosal barrier function, antigen presentation, immune cell recruitment, innate/adaptive immune regulation, and epithelial restitution, all of which are critical for maintaining intestinal homeostasis. Cellular pathways highlighted by association to CD include apoptosis, endoplasmic reticulum (ER) stress, and reactive oxygen species (ROS) production.

These various pathways underscore the pathophysiologic heterogeneity underlying this devastating disease. However, the functional correlations between these single nucleotide polymorphisms (SNP) and gastrointestinal inflammation remain unclear. Additional studies are necessary to elucidate the biological role of these variants and to eventually lead to new therapeutic approaches.

Despite the vast progress of GWAS in CD, the susceptibility alleles are believed to account for only a fraction (~20%) of the predicted heritability in CD (Franke et al., 2010). This conception is based on the assumption that susceptibility genes are independent, and their additive combined
effect contributes to pathogenesis. However, a recent study undermines this conception, and claims that susceptibility genes are not independent, but actually interrelated, influenced by one another via epistasis and are part of the same functional pathways. Thus, the missing heritability is far less than expected and the susceptibility alleles account for 80% of heritability (Zuk et al., 2012).

The new hypothesis proposed by Zuk et al. is strengthened by several studies. A recent study has utilized next generation sequencing methods to deep-sequence CD association loci in order to identify rare variants. Subsequently, these variants were genotyped in a large cohort of patients and controls. Very few additional associations were discovered, supporting the notion that these variants are indeed rare and cannot account for a large percentage of missing heritability (Rivas et al., 2011). Another study from our group has demonstrated a direct and functional interaction between Nod2 and Atg16L1. We have shown that Nod2 ligands trigger autophagy, lending further support to the new hypothesis by showing that these two significant CD susceptibility genes interact in a shared pathway (Travassos et al., 2010). These recent findings further emphasize the need to focus CD research on the functional roles of identified genetic loci.
1.2. Autophagy

Autophagy is an evolutionary conserved mechanism for lysosomal degradation of cytosolic components, such as intracellular organelles and macromolecules. There are three types of autophagy: microautophagy, the engulfment of cytosolic components directly by lysosomes through invagination; chaperon-mediated autophagy, the translocation of unfolded proteins across the lysosomal membrane by chaperone molecules; and macroautophagy, hereafter referred to as autophagy (Klionsky and Emr, 2000).

During autophagy, cytoplasmic material is sequestered by a cup-shaped double membrane. This initial isolation membrane matures into a vacuole termed the autophagosome. The autophagosome then fuses with lysosomes, a compartment termed the autolysosome. The autolysosome acquires lysosomal enzymes and acid phosphatases, thereby resulting in degradation of the inner membrane and the sequestered cargo (Figure 1). At the termination step, the autolysosome becomes fragmented. The broken down substrates are subsequently used as building blocks for amino acids and to maintain energy homeostasis in the cell.

Once thought to be a nonselective bulk recycling mechanism triggered by nutrient deprivation and energy depletion, autophagy is now known to target specific cargo selectively. It can target damaged organelles and insoluble protein aggregates for degradation, as well as controlling the clearance of intracellular pathogens in a process termed xenophagy (Kraft et al., 2010). Xenophagy is triggered by infection or bacterial toxins, as well as by a variety of immune signals such as pathogen-associated molecular patterns (PAMP), danger-associated molecular patterns (DAMP) and cytokines (Levine et al., 2011).

The origin of the autophagy membrane is unclear. Current evidence proposes the endoplasmic reticulum (ER) (Hayashi-Nishino et al., 2009), mitochondria (Hailey et al., 2010), plasma membrane (Ravikumar et al., 2010), and the nuclear membrane (English et al., 2009) as potential sources. The autophagosome is capable of engulfing considerably large cargo and plays a key role in pathogen elimination due to its ability to form gradually around its cargo, as opposed to budding off a source organelle.
For autophagosome maturation, at least two ubiquitin like conjugation systems are required. The Atg5-Atg12-Atg16L1 complex is required for the elongation of the isolation membrane (Klionsky and Emr, 2000). The complex is bound to the outer membrane of the isolation compartment, and disassociates as the isolation membrane matures into a complete autophagosome. In addition, the microtubule-associated protein light chain 3 (LC3)-phosphatidylethanolamine (PE) complex is also required for elongation and maturation of the autophagosome. LC3 in its non-lipidated form in the cytosol is termed LC3-I. When bound to PE and the autophagic membrane, it is termed LC3-II. LC3-II is present on the outer and inner membranes (Figure 1). At the inner membrane, it binds to adaptor proteins such as p62 that target polyubiquitylated cargo or intracellular bacteria for selective degradation. LC3-II remains bound to the membrane until degradation at the autolysosomal stage.

Autophagy proteins can also function in immune signalling through autophagy-independent mechanisms. Studies show that in response to pathogen invasion autophagy proteins are recruited to phagosomes, pathogen-containing vacuoles created by invagination of the plasma membrane. These vacuoles later fuse with lysosomes to create a degradation compartment that eliminates the pathogen (Berger et al., 2010; Huang et al., 2009; Lee et al., 2010; Sanjuan et al., 2007). Autophagy proteins are also recruited to parasite-containing vacuoles, which acquire immunity related GTPases to eliminate the parasite, in a process dependant on interferon gamma (IFN-γ) (Khaminets et al., 2010; Zhao et al., 2009; Zhao et al., 2008). Furthermore, autophagy proteins regulate various immune signaling molecules that control type I IFN and cytokine production (Jounai et al., 2007; Saitoh and Akira, 2010; Saitoh et al., 2009). In addition to playing diverse roles in immunity, autophagy proteins also play an important role in inflammation, as discussed in the next section.
Figure 1: Overview of the autophagic process in mammalian cells. During autophagy, cytoplasmic material is engulfed by a double membrane structure, the autophagosome, which fuses with lysosomes to generate a compartment where sequestered materials are degraded. The formation of the autophagosome requires a series of Atg proteins. The Atg5-Atg12-Atg16 complex, depicted in red, is conjugated to the autophagosome membrane and is required for its maturation. Next LC3-I, depicted in yellow, is linked to PE yielding the LC3-II form. LC3-II is located on both the inner and outer membrane of the autophagosome and remains associated with it until destruction at the termination step. Autophagosomes fuse with lysosomes, become acidified and acquire cathepsins, thereby the contents of the compartment are degraded. (This information was originally published in The Canadian Journal of Gastroenterology December 2011, Volume 25 Issue 12: 667-674).
1.2.1. **Atg16L1 in Crohn’s Disease**

A SNP in Atg16L1 is consistently associated with CD in numerous GWAS. The rs2241880 SNP encodes for a threonine instead of an alanine (T300A) at the c-terminal of a WD-repeat region, a region which usually mediates protein-protein interactions. The frequency of T300A risk allele is 50% in healthy individuals and 60% in CD patients of Caucasian descent. It confers an increased odds ratio of 1.4-1.9 of having the disease (Zhang et al., 2009).

Several research studies have focused on delineating the functional role of Atg16L1-CD variant in CD pathogenesis. Although Atg16L1 is required for autophagy and, together with Atg5 and Atg12, serves as the nucleating factor for the growing autophagosome, the WD repeat region does not seem to be essential for its function. In comparison with control cells, cells devoid of the Atg16L1 WD-repeat region or containing the T300A variation exhibit similar levels of canonical autophagy and xenophagy in response to *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) (Fujita et al., 2009). On the contrary, other studies have shown reduced bacterial clearance of *Escherichia coli* (*E. coli*) (Lapaquette et al., 2010) and *S. typhimurium* through xenophagy (Kuballa et al., 2008). Hence the functional relevance of Atg16L1-CD variant remains controversial, which suggests that the Atg16L1-CD variant might be implicated in CD pathogenesis through another, unique pathway.

Additional studies have focused on the role of Atg16L1 protein in inflammatory signalling. Mice hypomorphic for Atg16L1 display increased transcription of pro-inflammatory cytokines in Paneth cells. Paneth cells are intestinal immune epithelial cells in the crypts of the small intestine that secrete granules containing antimicrobial peptides, such as defensins and lysozyme, thereby controlling the microbial environment of the gut lumen and protecting against pathogens. Paneth cells from Atg16L1 hypomorphic mice display significant abnormalities in packaging and secretion of these granules through exocytosis, as well as damaged organelles, which may indicate a defective autophagic clearance of organelles (Cadwell et al., 2008; Cadwell et al., 2010). Despite the Paneth cell abnormalities, the Atg16L1 hypomorphic mice did not exhibit reduced clearance of *Listeria monocytogenes*. Interestingly, Paneth cells from CD patients carrying the Atg16L1-CD allele displayed similar abnormalities to the hypomorphic mice.
(Cadwell et al., 2010). In follow up to this study, the authors showed that Paneth cell abnormalities, increased pro-inflammatory cytokine expression, and increased susceptibility to DSS-induced colitis all depended on a viral infection by murine norovirus (MNV), frequently found in mice facilities. Of note, Atg16L1 hypomorphic mice did not develop DSS-induced colitis in a pathogen-free environment, lending further support to the notion that the intestinal microbiota is essential for the development of inflammation (Cadwell et al., 2010).

Atg16L1 also affects the inflammasome, a multi-protein complex activated by infection or stress, which is required for maturation of pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18). Recent studies show that autophagy is induced following inflammasome activation, and acts to limit inflammasome activity and regulate IL-1β levels (Shi et al., 2012). Murine macrophages deficient of Atg16L1 display enhanced secretion of IL-1β, following stimulation of Toll-like receptor 4 (TLR4) by endotoxin, which suggests an aberrant autophagic response. Furthermore, Mice deficient of Atg16L1 in haematopoietic cells display increased susceptibility to intestinal inflammation induced by treatment with dextran sodium sulphate (DSS) (Saitoh et al., 2008).

As noted earlier, our group has recently shown a link between Atg16L1 and Nod2. Nod2 is an intracellular pattern recognition receptor that binds to muramyl dipeptide (MDP), a component of peptidoglycan from the bacterial cell wall, and initiates an inflammatory response. Nod2 is expressed in intestinal epithelial cells such as Paneth cells, as well as antigen-presenting cells. Nod2 was the first susceptibility gene discovered in CD, and encompasses 3 major SNPs associated with the disease. One of these SNPs is a frameshift mutation (Nod2fs). It was unclear how this loss of function mutation leads to an increased inflammatory response. However, our work has shown that Nod2 directly interacts with Atg16L1 and recruits it to the site of bacterial entry in order to induce xenophagy. As opposed to the protective Nod2 variant localized to the plasma membrane, Nod2fs is present mainly in the cytosol, and displays impaired recruitment of Atg16L1, thereby substantially altering xenophagy (Travassos et al., 2010). Furthermore, human peripheral blood monocytes (PBMC) carrying the Atg16L1-CD variant display reduced autophagy in response to Helicobacter pylori (H. pylori) vacuolating cytotoxin A (VacA) (Raju et al., 2012). Failure to recruit autophagy via stimulation with Nod2 ligands also results in
increased IL-1β and IL-6 production (Plantinga et al., 2011). In addition, human dendritic cells carrying Nod2 mutations or Atg16L1-CD also displayed impaired xenophagy and major histocompatibility complex (MHC) class II antigen presentation (Cooney et al., 2010).

These studies emphasize the significance of Atg16L1 in the development of inflammation, and suggest Atg16L1 as a key player in regulation of inflammatory signalling in response to environmental triggers. However, the exact functional relevance of the CD variant remains controversial. Therefore I hypothesized that a novel pathway involving miRNA might explain the role of Atg16L1 in CD.
1.3. MicroRNA

While the functional relevance of pathways associated with CD is not fully understood, the microRNA (miRNA) pathway emerge as an alternate mechanism involved in gene expression dysregulation in CD (Iborra et al., 2010). MiRNAs are single stranded, non-coding RNA molecules that are 19-25 nucleotides long. They contain a seed region of 7 or 8 nucleotides through which they bind imperfectly to a complementary region on the 3' untranslated region (UTR) of a target mRNA, thus repressing translation and/or promoting its degradation. Over a 1000 miRNAs are either functionally confirmed or predicted (Griffiths-Jones, 2004, 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011), and regulate thousands of genes (Friedman, 2009; Krol et al., 2010). Each miRNA can regulate the expression of hundreds of target mRNAs, and each mRNA can be regulated by several miRNAs. Therefore, miRNAs comprise a significant posttranscriptional regulation mechanism, and have profound effects on gene regulatory networks.

Therefore, it is not surprising that miRNAs are involved in numerous cellular processes including growth, proliferation, differentiation, apoptosis, migration, and metabolism (Esquela-Kerscher and Slack, 2006). Differential expression profiles of miRNAs have been associated with numerous diseases such as various cancers, heart, liver, neurological, and autoimmune diseases, as well as CD (Jiang et al., 2009; Lu et al., 2008; Wu et al., 2010; Wu et al., 2008a). Furthermore, miRNA expression profiles have been shown to define the different stages of cancerous tumors more precisely than mRNA expression profiles. These characteristics deem miRNAs as novel biomarkers for disease diagnosis, prognosis and therapeutic response (Ferracin, 2010; Lu et al., 2005; Volinia, 2006).

MiRNAs are also found circulating in peripheral blood as extracellular entities highly resistant to nucleases. Although the origin of circulating miRNA is not fully understood, the current hypothesis is that these miRNAs are secreted via exocytosed vesicles called exosomes into the blood stream (Turchinovich et al., 2011). These miRNA containing exosomes have also been shown to transport into another cell to release their contents there, hence affecting cellular function in a neighboring cell (Mittelbrunn et al., 2011; Skog et al., 2008; Valadi et al., 2007).
Due to their resistant nature, circulating miRNAs in the bloodstream can be utilized as non-invasive biomarkers for several diseases, including CD (Chen et al., 2008; Mitchell et al., 2008; Paraskevi et al., 2012; Rabinowits et al., 2009; Simpson et al., 2009; Taylor and Gercel-Taylor, 2008).
1.3.1. MiRNA biogenesis

MiRNA are initially transcribed by RNA polymerase II as a pri-miRNA, a primary transcript that contains a stem-loop. The pri-miRNA is cleaved by the RNase enzyme Drosha to form a shorter stem-loop, termed pre-miRNA. Exportin-5 exports the pre-miRNA into the cytoplasm, where the hairpin structure is cleaved off by Dicer, another RNase enzyme, to form a short double-stranded miRNA duplex. One of the strands is then degraded, while the other becomes the mature miRNA and is loaded onto an Argonaute (Ago) protein. Ago binds to GW182, another protein required for gene silencing, and together they form the RNA-induced silencing complex (RISC).

Typically, mRNA is stabilized by forming a circular structure. A cytoplasmic Poly(A)-binding protein 1 (PABPC1) binds to the mRNA 3’ tail, as well as to a eukaryotic initiation factor 4G (eIF4G), which binds the 5’ cap, which stabilizes the mRNA in a circular structure. During mRNA silencing, RISC binds to the mRNA 3’ UTR region, causing GW182 to occupy PABPC1 binding site. Therefore, the mRNA is exposed to decapping and deadenylation enzymes that facilitate mRNA degradation and diminish translation efficiency (Tritschler et al., 2010).

The Ago proteins are key components of RISC, as they bind mRNA as well as miRNA, thereby directing the miRNA to its target mRNA (Ender and Meister, 2010). There are 4 homologous Ago proteins in humans, Ago1 to Ago4, all are highly specialized to bind small silencing RNA molecules and inhibit mRNA translation. Moreover, Ago2 also has a unique capability to directly cleave complementary pre-miRNA targets, creating a pool of miRNA processed independently of Dicer. For example, Ago2 has been shown to generate mature miR-451, which cannot be generated in Ago2 deficient cells (Cheloufi et al., 2010; Cifuentes et al., 2010).

Ago proteins also regulate miRNA silencing downstream of miRNA biogenesis. Mature miRNA differ in their affinity for the different Ago proteins. Furthermore, miRNA abundance is sensitive to Ago levels (Kai and Pasquinelli, 2010). Overexpression of Ago proteins increases mature miRNA abundance, while knockdown of Ago proteins leads to a decrease in miRNA levels. In addition, miRNA-bound Ago proteins vary in their ability to silence target mRNA, with an order of Ago4>Ago3>Ago2>Ago1 (Wu et al., 2008b). Ago expression also varies across tissue types,
suggesting specific effects depending on cellular context. As changes in Ago levels have a profound effect on miRNA expression and mRNA levels, it is not surprising that altered Ago2 expression has been implicated in disease processes such as cancer (Kim et al., 2010; Zhou et al., 2010).

GW182 colocalizes with mRNA-processing bodies, called p-bodies (Eystathioy et al., 2003). These cytoplasmic granules contain proteins required for mRNA decay and translation repression along with mRNAs destined for degradation (Eulalio et al., 2007). The role of these p-bodies is unclear, as gene silencing can occur in their absence (Chekulaeva et al., 2009; Chu and Rana, 2006; Eulalio et al., 2007). However, GW182 is necessary for silencing, as cells depleted of GW182 show compromised silencing (Behm-Ansman et al., 2006; Eulalio et al., 2009; Eulalio et al., 2008; Rehwinkel et al., 2005) while Ago and miRNA levels remain unaffected (Eulalio et al., 2009), suggesting GW182 as an effector of miRNA silencing downstream of Ago.

Thus, the regulation of miRNA expression and function occurs at three points: transcription, processing and subcellular localization (Krol et al., 2010). Various cellular and extracellular signalling events regulate the expression of miRNA (Neilson et al., 2007; Smirnova et al., 2005; van Rooij et al., 2007), which are then able to make fine adjustments to cell functions in response to the signalling stimuli (Bartel and Chen, 2004). At processing, miRNAs are regulated by the various proteins discussed above. Subsequently, miRNAs can be localized to p-bodies, to stress granules, cytoplasmic aggregates of mRNA that form in response to global translation repression under stress conditions, or multi-vesicular bodies (MVBs), discussed further below.
1.3.2. MiRNA in Crohn’s disease

Recent studies have implicated aberrant miRNA expression in IBD pathogenesis (Dalal and Kwon, 2010; Iborra et al., 2010). A number of studies show that intestinal tissue from patients with IBD display altered miRNA expression compared with normal controls. For example, colonic tissues from patients with active ulcerative colitis (UC), another form of IBD, displayed aberrant expression of 11 miRNAs, as compared with healthy controls. Furthermore, a decrease in miR-192 in active UC was inversely correlated with an increase in its predicted target, Macrophage inflammatory peptide (MIP)-2, a chemokine expressed by epithelial cells (Wu et al., 2008a). Expression levels of miRNAs also depend on intestinal location. For example, intestinal tissue obtained from patients with ileal CD or colonic CD patients displayed altered expression of 5 and 4 miRNAs respectively as compared with healthy controls (Wu et al., 2010).

In studies of circulating miRNAs from peripheral blood samples, miRNA were differentially expressed in both CD and UC in comparison to healthy controls (Paraskevi et al., 2012; Wu et al., 2011; Zahm et al., 2011). These newly identified aberrant circulating miRNA confirmed some of the known CD-associated miRNAs in tissue samples, as well as identified novel aberrant miRNAs. Interestingly, distinct miRNA profiles emerged between CD and UC patients, suggesting unique pathways are involved in the pathogenesis of CD and UC (Wu et al., 2011).

Additionally, alterations in miRNA expression were also identified in non-inflamed quiescent tissues obtained from CD and UC patients compared to normal controls (Fasseu et al., 2010). Unique miRNA profiles were discernible in quiescent CD, as compared with quiescent UC, though some of the miRNAs overlap between the diseases. Overall, these results suggest a key role for miRNA dysregulation in susceptibility to CD by showing that aberrant miRNA profiles exist prior to development of inflammation.

Intriguingly, intestinal-specific Dicer knockout mice displayed dramatically altered miRNA and mRNA profiles, especially in gene pathways involved in immunity (McKenna et al., 2010). These mice presented with abnormal intestinal development caused by an increase in cell migration and apoptosis in the crypts of the jejunum and colon, as well as an increase in goblet
cells, epithelial cells that function in production of mucus. These mice also displayed abnormal barrier function, leading to intestinal inflammation. This elegant study underscores the significance of miRNA in normal intestinal development and function.

Interestingly, some of the CD associated SNPs appear in intron regions, 3'/5' UTR regions or are synonymous SNPs that do not affect protein sequence. These SNPs have been regarded as ‘silent’ mutations. However, these ‘silent’ mutations suggest a regulatory effect rather than a structural effect on proteins. Their specific role in disease remains unclear. Yet, since miRNAs can form from intron regions and the 3’ UTR constitutes a potential binding site for miRNAs, SNPs in these regions might be especially relevant in miRNA biogenesis and function. Indeed, I have compiled a list of several miRNAs that are transcribed from CD associated loci using a Microsoft visual basic program I wrote (Table 1). Moreover, some of these miRNA are predicted to target genes implicated in CD pathogenesis, including Atg16L1 (Table 2) and Nod2.

Indeed, a recent study showed that a ‘silent’ mutation, a synonymous SNP in Immunity related guanosine triphosphatase M (IRGM) gene associated with CD, alters a miRNA binding site, which leads to loss of regulation under inflammatory conditions (Brest et al., 2011). A critical threshold of IRGM is needed for xenophagy, the autophagic clearance of intracellular pathogens. However the exact mode of regulation of IRGM is unknown. The SNP in IRGM 3’ UTR is in perfect linkage disequilibrium with a deletion polymorphism in the gene’s promoter region, close to a transcription factor binding site. Interestingly, this deletion polymorphism leads to reduced IRGM expression levels in certain cell types, and to increased IRGM levels in other cell types (McCarroll et al., 2008). Consistent with this finding, Brest et al. showed that the effect of the 3’ UTR SNP depends on context. An inflammatory signal caused by CD–associated adherent invasive E. coli (AIEC) leads to an increase in expression of miR-196 family. In individuals harbouring the protective 3’ UTR SNP allele, miR-196 targets IRGM and leads to a decrease in its expression, a decrease which is necessary for bacterial clearance. In patients with IRGM-CD 3’ UTR SNP, miR-196 binding site is abolished, leading to increased expression of IRGM and defective bacterial clearance by xenophagy. Thus, this study demonstrated a clear functional link between the miR-196 family and a ‘silent’ mutation in IRGM, as well as a function role in intestinal inflammation.
Taken together, the findings from these various studies emphasize miRNAs as key modulators of gene expression and intestinal inflammation, and suggest a mechanism by which miRNA play an important role in CD pathogenesis. Further research is needed to delineate the mechanisms that regulate miRNAs, as well as the functional role of miRNA regulation in gene dysregulation in CD.
<table>
<thead>
<tr>
<th>CD SNP</th>
<th>Genes highlighted by genomic location</th>
<th>MiRNA transcribed from region</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12521868</td>
<td>SLC22A4, SLC22A5</td>
<td>hsa-mir-3936</td>
</tr>
<tr>
<td>rs1799964</td>
<td>MCCD1, LTA, HLA-DQA2</td>
<td>hsa-mir-1236, hsa-mir-3135b, hsa-mir-4646</td>
</tr>
<tr>
<td>rs415890</td>
<td>CCR6</td>
<td>hsa-mir-3939</td>
</tr>
<tr>
<td>rs12242110</td>
<td>CREM</td>
<td>hsa-mir-3611</td>
</tr>
<tr>
<td>rs2076756</td>
<td>NOD2</td>
<td>hsa-mir-3181</td>
</tr>
<tr>
<td>rs2872507</td>
<td>GSMDL, ZPB2, ORM DL3</td>
<td>hsa-mir-4728</td>
</tr>
<tr>
<td>rs11871801</td>
<td>MLX, STAT3</td>
<td>hsa-mir-548at, hsa-mir-5010</td>
</tr>
<tr>
<td>rs3180018</td>
<td>SCAMP3, MUC1</td>
<td>hsa-mir-92b, hsa-mir-555</td>
</tr>
<tr>
<td>rs13428812</td>
<td>DNMT3A</td>
<td>hsa-mir-1301</td>
</tr>
<tr>
<td>rs2058660</td>
<td>IL18RAP</td>
<td>hsa-mir-4772</td>
</tr>
<tr>
<td>rs1847472</td>
<td>BACH2</td>
<td>hsa-mir-4464</td>
</tr>
<tr>
<td>rs102275</td>
<td>FADS1</td>
<td>hsa-mir-611, hsa-mir-1908</td>
</tr>
<tr>
<td>rs694739</td>
<td>PRDX5, ESRRA</td>
<td>hsa-mir-1237</td>
</tr>
<tr>
<td>rs151181</td>
<td>APOB48R, IL27, SULT1A2, SULT1A1, SH2B1, EIF3C</td>
<td>hsa-mir-4517, hsa-mir-4721</td>
</tr>
<tr>
<td>rs12720356</td>
<td>TYK2, ICAM1, ICAM3</td>
<td>hsa-mir-1181</td>
</tr>
<tr>
<td>rs181359</td>
<td>YDJC</td>
<td>hsa-mir-130b, hsa-mir-301b</td>
</tr>
</tbody>
</table>

**Table 1: MiRNAs expressed from CD associated loci.** MiRNAs transcribed from CD loci as previously published (Franke et al., 2010) were retrieved from miRBase version 18 (Griffiths-Jones, 2004, 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011).
<table>
<thead>
<tr>
<th>MiRNA</th>
<th>Predicted by number of algorithms</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-130b</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-301b</td>
<td>4</td>
</tr>
<tr>
<td>hsa-miR-1228</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-191</td>
<td>2</td>
</tr>
<tr>
<td>hsa-miR-92b</td>
<td>2</td>
</tr>
<tr>
<td>hsa-miR-555</td>
<td>2</td>
</tr>
<tr>
<td>hsa-miR-566</td>
<td>1</td>
</tr>
<tr>
<td>hsa-miR-1236</td>
<td>1</td>
</tr>
<tr>
<td>hsa-miR-1237</td>
<td>1</td>
</tr>
<tr>
<td>hsa-miR-425</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2: MiRNAs predicted to target Atg16L1, expressed from CD associated loci.** MiRNAs predicted to target Atg16L1 were found using miRecords (Xiao et al., 2009), a database that integrates the results of 11 leading miRNA target prediction algorithms. MiRNAs expressed from CD genomic loci and the number of algorithms that predicted their target as Atg16L1 is depicted in the table.
1.3.3. MiRNA regulation by intracellular membranes

This process of driving the miRNA away from the transcription and translational machinery, into bodies potentially involved in mRNA decay might be a profound form of control on miRNA function. However the players involved in this mechanism are mostly unknown. Two studies have suggested endosomal membranes as the platforms where RISC assembly and turnover occurs in Drosophila (Lee et al., 2009) and mammalian cells (Gibbings et al., 2009). These studies shed light on RISC function in miRNA silencing, as well as suggest a novel link between miRNA gene silencing and intracellular membranes.

During endocytosis, plasma-membrane proteins are internalized into the cell via small vesicles. Endosomal sorting complex required for transport (ESCRT) proteins control the formation of inward budding vesicles and the sorting of plasma-membrane proteins into these vesicles. After entering the cell, the vesicles fuse with an early endosome, which gradually acquires more and more vesicles, thus maturing into a late endosome, termed a multi-vesicular body (MVB). MVBs subsequently fuse with lysosomes to degrade their cargo, or fuse with the plasma membrane to release their content into the extracellular space. The exocytosed vesicles are termed exosomes.

Gibbings et al. and Lee et al. showed that RISC components Ago and GW182 colocalize to endosomal membranes. Furthermore, they suggest a mechanism by which GW182 disassociates from RISC and is subsequently degraded by lysosomes or secreted in exosomes. This step is crucial for RISC turnover, as GW182 acts as a temporal lock, competing for the same binding site with Dicer, hence preventing it for loading a new miRNA onto Ago. Blocking ESCRT protein impaired MVB formation and blocked GW182 clearance, thereby altering RISC turnover and impairing miRNA silencing. Together, these studies propose a mechanism by which MVBs modulate gene silencing by small RNAs.

However, ESCRT proteins also control autophagy, as blocking ESCRT has also been shown to cause impaired autophagy (Rusten and Stenmark, 2009). Furthermore, the subcellular compartments identified as MVBs are closely related to autophagosomes, as they share similar characteristics as autophagosomes. Indeed, current evidence suggests that autophagosomes can
interact directly with MVBs to form a compartment termed an amphysome, which later fuses with lysosome to degrade its cargo (Fader and Colombo, 2009). Therefore, I hypothesized that autophagy may affect miRNA silencing by specifically targeting and degrading RISC components, resulting in alterations in RISC component expression levels, thereby impairing gene silencing.
1.4. Summary and hypothesis

GWAS have implicated autophagy in the pathogenesis of CD via a non-synonymous SNP in Atg16L1, a protein necessary for autophagic function. Atg16L1-CD modulation of autophagy remains unclear, as some studies show that the CD variant impairs autophagic clearance of pathogens, while other studies show that it has no effect. This controversy suggested that the Atg16L1 and autophagy pathway are involved in CD via another, distinctive mechanism. MiRNAs have arisen as key modulators of pro- and anti-inflammatory cytokine expression, as well as have been implicated in CD pathogenesis, intestinal development and mucosal barrier function. A direct connection between miRNA gene silencing and endolysosomal trafficking has arisen, as MVBs were suggest as modulators of RISC formation and turnover. Yet, autophagosomes and MVBs are closely related and share similar characteristics, which led me to hypothesize that autophagy plays a novel role in regulating and degrading RISC components AGO and GW182, thereby altering miRNA gene silencing, a process implicated in CD etiology.
Chapter 2
Methods

2.1. Cell growth conditions

Atg5 knockout and wild-type mouse embryonic fibroblast cells (MEF) (kindly provided by Dr. Tamotsu Yoshimori, Osaka University, Osaka, Japan), human cervix epithelial cells (HeLa) (provided by Dr. Philip Sherman, University of Toronto, Toronto, Ontario, Canada), stably LC3-GFP transfected human breast epithelial cells (MDAMC) and mouse intestinal epithelial cells (Mode-K) (both provided by Dr. Dana Philpott, University of Toronto, Toronto, Ontario, Canada) were grown in Dulbecco's Modification of Eagle's Medium (DMEM) (Wisent St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Gaithersburg, Maryland, USA) and incubated at 37°C in 5% CO2 environment.

Human umbilical vein endothelial cells (HUVEC) were grown in endothelial cell basal medium-2 (EBM-2) (Lonza Walkersville, Maryland, USA) supplemented with 10% FBS and the following growth supplements: 0.04% hydrocortisone; 0.4% Heparin-Binding Growth Factor 2 (hFGF-B); 0.1% Vascular endothelial growth factor (VEGF); 0.1% human recombinant analog of insulin-like growth factor-I with the substitution of arginine for glutamine at position 3 (R3-IGF-1); 0.1% ascorbic acid; 0.1% heparin; 0.1% Human Epidermal Growth Factor (hEGF); 0.1% Gentamicin-Amphotericin mixture (GA-1000) (all from Lonza) and incubated at 37°C in 5% CO2 environment.

2.2. Preparation of H. pylori concentrated culture supernatants

Frozen stock of H. pylori strain 60190 (cagA+ cagE+ VacA+ strain, number 49503, American Type Culture Collection (ATTC), Manassas, Virginia, USA) was inoculated on blood agar plates with 5% sheep blood (Oxoid Biotech, Ottawa, Ontario, Canada). The culture was incubated at 37°C in a microaerophilic environment for 72 hours. Cultured H. pylori strains were then grown at 37°C overnight in Brucella broth (Sigma-Aldrich, Oakville, Ontario, Canada) under microaerophilic conditions with shaking at 120 rpm.
After reaching log phase, *H. pylori* cultures were centrifuged at 4000 rpm for 20 minutes to separate the bacterial pellet from the culture supernatants. The culture supernatants were filtered through a 0.22 μm filter and concentrated 10 times in Ham’s F-12 medium (Wisent, St. Bruno, Quebec, Canada) supplemented with 10% FBS using a 30 kDa cut-off Amicon Ultra centrifugal filter (EMD Millipore, Billerica, Maryland, USA). The culture supernatants were diluted five times in Ham’s F-12 prior to addition to cells for intoxication assays.

### 2.3. Isolation of Peripheral Blood Monocytes

Venous blood was drawn from healthy genotyped volunteers who carry the ATG16L1 CD-protective allele of rs2241880 in sterile Ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Vacutainer; BD Biosciences, Franklin Lakes, New Jersey, USA) diluted 1:1 with pyrogen-free saline and layered over Ficoll-Paque (GE Healthcare Sciences, Baie d’Urfe, Quebec, Canada). Approval for the study was provided by the local ethics board (approval number MSH REB #02-0234-E). Cells were spun at 400 g for 30 minutes to obtain the mononuclear cell fractionation via density centrifugation, washed, and suspended in culture medium. Cells were counted and plated in 6-well culture plates where they were treated with various autophagy stimuli for 4 hours of incubation. Cells were collected, lysed and used for immunoblots.

### 2.4. Induction of autophagy

Autophagy was induced by incubating cells in Earle's balanced salts solution media (EBSS) (Life Technologies, Burlington, Ontario, Canada) at 37°C for 4-6 hours. Alternatively, autophagy was chemically induced by incubating cells in the presence of 100 ng/mL of Rapamycin (Enzo Life Sciences International, Farmingdale, New York, USA) in appropriate culture medium at 37°C for 4-6 hours.

### 2.5. Disruption of autophagy

In order to disrupt autophagy, cells were incubated with 200 μL/mL concentrated culture supernatants of *H. pylori* strain 60190 for 4 hours or with 50 μg/mL bafilomycin (Sigma-Aldrich, Oakville, Ontario, Canada).
2.6. Inhibition of autophagy by Atg12 siRNA

Autophagy was inhibited by transfecting HUVEC cells with siRNA directed to the human autophagic protein Atg12 (NM_004707). A sense/antisense siRNA pair targeting nucleotides 356 to 375 of Atg12 sequence, GUG GGC AGU AGA GCG AAC Adt/ UGU UCG CUC UAC UGC CCA Cdt (Paludan et al., 2005) was used (Dharmacon Inc., Chicago, Illinois, USA). HUVEC cells that were 30% confluent grown in serum free Opti-MEM culture media (Invitrogen Life Technologies, Gaithersburg, Maryland, USA), were treated for 6-8 hours with 200 pmol siRNA and 5 μL of Oligofectamine (Invitrogen Life Technologies, Gaithersburg, Maryland, USA) per well of a 6-well culture plate. Thirty-six hours later, siRNA treated cells were transfected utilizing FuGENE-HD (Roche Diagnostics Indianapolis, Indiana, USA) reagent and further incubated for 72 hours before being assessed for autophagy inhibition by immunoblotting and immunofluorescence.

2.7. Immunofluorescence

Cells were grown on 12-well plates and transiently transfected using FuGENE-HD (Roche Diagnostics Indianapolis, Indiana, USA) for plasmids LC3-GFP (kindly provided by Drs. Noboru Mizushima and Tamotsu Yoshimori, Tokyo Medical and Dental University and Osaka University, Japan), LC3-RFP (produced as previously described (Gutierrez et al., 2005)) and Ago2-HA (kindly provided by Dr. Stephen Girardin, University of Toronto, Toronto, Ontario, Canada). Following transfection and treatments with various stimuli, cells were washed with Phosphate-Buffered Saline (PBS) (Wisent St. Bruno, Quebec, Canada), fixed for 20 min in 4% paraformaldehyde (Sigma-Aldrich, Oakville, Ontario, Canada) in PBS, permeabilized by incubation in 0.1% Triton X-100 (vol/vol) (Sigma-Aldrich, Oakville, Ontario, Canada) in PBS for 20 min, and blocked for 1 hour with 5% milk in PBS (vol/vol). Permeabilized cells were incubated overnight at 4°C with primary antibody diluted in 5% milk in PBS (vol/vol). Primary antibodies dilutions used: Rabbit polyclonal AGO2 antibody (Abcam, Cambridge, Maryland, USA) 1:200; mouse monoclonal HA antibody (Abcam, Cambridge, Maryland, USA) 1:1000; Rabbit polyclonal p62 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) 1:1000. Following incubation, cells were washed extensively with PBS, and incubated for 1 hour
at room temperature with secondary Cy5 and Cy3-conjugated antibodies (Jackson ImmunoResearch Laboratories West Grove, Pennsylvania, USA).

Confocal image acquisition was performed in a Quorum Spinning Disk Confocal Microscope consisting of a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head, 4 laser lines (Spectral Applied Research: 405 nm, 491 nm, 561 nm, 638 nm), an ASI motorized XY stage, and an Improvision Piezo Focus Drive. The equipment is controlled by Volocity acquisition software (Improvision) and powered by an Apple Power Mac G5.

2.8. Immunoblotting

After incubation with various stimuli, cells were put on ice, washed with PBS, and scraped with 100 µl of ice cold RIPA buffer containing phosphatase and protease inhibitors (all from Sigma-Aldrich, Oakville, Ontario, Canada). Cell suspensions were centrifuged and supernatants either stored at -80°C or immediately boiled at 100°C with 1x Laemmli buffer for 5 minutes. Equal amounts of protein were run on 7-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 110V for 1.5 hours at room temperature. Proteins were then transferred onto a nitrocellulose membrane (Pall Corporation, Ann Arbor, Michigan, USA) at 30V for 8 hours at 4°C. Membranes were blocked with 5% milk in Tris-buffered saline with tween (TBST) (Invitrogen Life Technologies, Gaithersburg, Maryland, USA) and probed with 1:500 dilution of AGO2 rabbit polyclonal antibody (Abcam, Cambridge, Maryland, USA), 1:2000 dilution of LC3 rabbit polyclonal antibody (Novus Biologicals, Littleton, Colorado, USA) or 1:1000 dilution of GFP Rabbit polyclonal antibody (Invitrogen Life Technologies, Gaithersburg, Maryland, USA) and developed with the appropriate HRP-conjugated secondary antibody.

2.8.1 Densitometric analysis

Immunoblotting images were captured and quantitated using FluorChem FCII software (Alpha Innotech, San Leandro, California, USA). Densities of Ago2 and β-actin were measured for each treatment and expressed as ratio of Ago2 to β-actin (Ago2/β-actin). For graphic representation,
the ratio of Ago2/β-actin for each treatment was expressed relative to the measured Ago2/β-actin ratio for control cells and identified as fold increase on the graphs.

2.8.2 Statistical analysis

All experiments were performed at least 3 times (n≥3). Student’s t-test or ANOVA and Dunnet’s post-hoc tests were performed to compare the means ± standard error (SE) for treatment groups using GraphPad Prism 6 (GraphPad Inc., La Jolla, California, USA). A p-value of less than 0.05 was determined to be statistically significant.

2.9. MiRNA expression microarray

2.9.1 MiRNA expressed from CD SNP regions

A program in Microsoft Visual Basic for Applications 7.0 (Microsoft, Redmond, Washington, USA) was written to predict miRNA genes transcribed from CD-associated genomic locations previously published (Franke et al., 2010). Genomic locations of miRNA genes were derived from miRBase version 18, and were compared with the regions encompassing CD SNPs. The results are listed in Table 1.

2.9.2 MiRNA extraction

After incubation with various stimuli, MEF cells were washed with PBS and 200 µl of trypsin (Wisent St. Bruno, Quebec, Canada) was added until the cells detached from the plate. Trypsin was neutralized with 500 µl of DMEM. Cells were then spun at 300 g for 10 minutes, supernatants were aspirated and 500 µl of PBS was added. Cells were spun again at 300 g for 5 minutes, and frozen at -80°C for a period of less than 2 months. Total RNA was extracted using with miRNeasy mini kit (Qiagen, Toronto, Ontario, Canada).

2.9.3 NanoString nCounter miRNA expression assay

Total RNA from n=3 experiments was processed at university Health Network (UHN) microarray centre using the NanoString nCounter mouse miRNA expression assay (NanoString, Seattle, Washington, USA), which contains over 600 mouse and mouse-associated viral miRNAs
derived from miRBase (Release 15.0). The assay utilizes 14 probes against short RNA sequences foreign to any known organism. Probes matching 8 such sequences are used as negative controls, since they bind to targets which are absent in the samples and can be used to assess assay background. 6 such RNA sequences are added to the samples in predetermined concentrations. These sequences are processed in an identical manner to the endogenous miRNA, thereby can be used as positive controls and account for experimental platform variation in ligation and hybridization of samples. Raw data obtained was normalized to positive spike-in RNA hybridization controls. Then, the data was normalized to the top 100 expressed miRNAs in order to account for sample input variation. A background threshold of mean plus two standard deviations of the negative control counts was subtracted from miRNA counts. A one sided, heteroscedastic t-test was performed in order to statistically determine whether a miRNA is detected in the sample by assessing the likelihood of the miRNA counts being significantly different than the negative controls counts. Percent coefficient of variation (CV) was calculated in order to detect miRNA counts that do not display similar pattern to their other biological replicates. MiRNAs with positive counts after background subtraction significantly different than negative control counts, and have less than 45% variation across triplicates were considered as detected.

2.9.4 Data analysis

Batch effects caused by experiment and processing date were estimated with a mixed model ANOVA using Partek Genomics Suite (PGS) version 6.6 (Partek, St Louis, Missouri, USA). Each of the batches contained a combination of control and treated cells in a balanced proportion. The batch effects were removed from the data and the expression values were adjusted to remove batch-to-batch differences. Unsupervised methods of data analysis, including Hierarchical Clustering (HCL) and Principal Component Analysis (PCA), were performed using PGS. HCL clustering was performed using Pearson dissimilarity and Complete Linkage method. Kolmogorov-Smirnov and Fligner-Killeen tests to assess whether the distribution of miRNA expression data is normal and whether variances are homogeneous in the data were performed using R (http://www.r-project.org/) (R-Development-Core-Team, 2011). ANOVA was
performed using R and verified using PGS. Raw p-values were then corrected for multiple hypothesis testing using the FDR method (Benjamini and Hochberg, 1995).

2.9.5 Pathway enrichment analysis

Target identification for differentially expressed miRNA was performed using the miRNA target filter in Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) (Ingenuity Systems, Redwood City, California, USA). A list containing the aberrant miRNA and the target mRNA gene identifiers was uploaded into the application. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured by a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed, and a Fisher’s exact test to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the molecules in the network. The network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher’s exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.
3.1. Disrupted autophagy Increases Ago2 in multiple cell lines

In order to evaluate how autophagy regulates the degradation of the components necessary for miRNA processing, I assessed Ago2 levels in multiple cell lines that displayed normal autophagy, increased induction of autophagy and disrupted autophagy. I focused on Ago2 since it is a critical component of the RISC complex, a downstream effector of miRNA silencing, and the only Ago protein with Slicer activity which results in the generation of unique mature miRNA independent of Dicer processing. Ago2 mRNA and protein levels are also the most abundant out of all Ago family members in many cell lines (Petri et al., 2011).

In order to induce autophagy, cells were starved or treated with rapamycin, a pharmacological inhibitor of mammalian target of rapamycin (mTOR) which leads to autophagy induction. To disrupt autophagy, cells were treated with bafilomycin, which affects the fusion of autophagosomes with lysosomes and prevents acidification of lysosomes by inhibiting the vacuolar type H(+-)ATPase (V-ATPase), thereby blocking degradation of cargo (Yamamoto et al., 1998). I also used conditioned culture media supernatants (CCMS) from an *H. pylori* strain expressing the vacuolating cytotoxin A (VacA). VacA is secreted by the bacteria and has been shown to induce autophagy, as well disrupt the degradation capacity of autolysosomes by affecting lysosomal hydrolases such as cathepsin D (Raju et al., 2012; Raju and Jones, 2010; Terebiznik et al., 2006). In addition to using pharmacological inhibitors of autophagy, I utilized autophagy deficient ATG5 knockout (ATG5-/−) mouse embryonic fibroblast (MEF) cells, as well as ATG12 siRNA. As the Atg5-Atg12-Atg16 complex is essential for the maturation of the autophagosome, both methods block autophagy at the initiation step (Figure 1).

First, I assessed Ago2 expression in wildtype (WT) and autophagy disrupted ATG5-/− MEF cells. To confirm autophagy was disrupted in ATG5-/− MEFs, I transfected cells with LC3-GFP and assessed by immunofluorescence using confocal microscopy. WT cells transfected with LC3-GFP displayed basal autophagy exhibiting green puncta indicative of autophagosomes,
while ATG5-/- MEFs lacked these puncta (Figure 2). Western blotting for LC3 in WT and ATG5-/- MEFs showed an increase in the cytosolic form LC3-I in ATG5-/- MEFs in comparison with control MEFs, indicating that autophagy was inhibited at the initiation step (Figure 2). Furthermore, ATG5-/- MEFs displayed an increase in Ago2 expression compared to WT MEFs as assessed by immunofluorescence microscopy (Figure 2). Similarly, ATG5-/- cells displayed an increase in Ago2 in comparison to WT MEFs when assessed by western blotting (Figure 2, 3). Densitometric analysis of at least 3 repeated experiments revealed a statistically significant increase in Ago2 in ATG5-/- cells compared to WT cells (Figure 3).

In addition, bafilomycin was used to disrupt autophagy in the WT MEF cells and a statistically significant increase in Ago2 was detected. As expected, since autophagy is already disrupted in ATG5-/- MEFs, bafilomycin treatment did not result in a further increase in Ago2 levels in these cells (Figure 3). Moreover, cells were treated with autophagy stimuli rapamycin and starvation, followed by treatment with bafilomycin to disrupt autophagy. Under each of these conditions, an increase in Ago2 was detected in WT cells but not ATG5-/- cells (Figure 3). WT and ATG5-/- cells treated with autophagy inducers alone did not display a significant change in Ago2 levels, except for cells treated with rapamycin (Figure 3).

I next determined whether the effect of autophagy inhibition on Ago2 levels was unique to MEFs or could be generalised to other cell types derived from different tissues. I selected cell types reflective of different tissues including breast, intestinal and cervical epithelial cells, endothelial cells and immune cells. A variety of complementary methods were utilized to disrupt autophagy to confirm that any observed effects were dependent on autophagy disruption.

MDAMC human breast carcinoma epithelial cells were treated with ATG12 siRNA to disrupt autophagy at the induction stage or VacA toxin which we have shown induces autophagy that is disrupted at the autolysosome stage (Raju et al., 2012). Ago2 levels and conversion of LC3-I to LC3-II were compared between cell lysates from control and autophagy disrupted cells to verify the effect of the disrupters on autophagy. In comparison with control cells, ATG12 siRNA treated cells demonstrated an increase in the cytosolic free form LC3-I, indicating autophagy is indeed inhibited at the induction step due to knockdown of Atg12. An increase in LC3-II was detected in VacA treated cells compared to control cells confirming disrupted autophagy at the
autolysosome stage. Under conditions of autophagy disruption such as ATG12 siRNA knockdown or VacA-toxin treatment, a significant increase in Ago2 was detected by western blotting (Figure 4A, 4B).

Immunofluorescent confocal microscopy was also used to assess Ago2 levels in MDAMC cells. VacA treated cells showed an increase in green puncta indicative of autophagosomes, as well as Ago2 and p62, an adaptor protein that directs ubiquitylated cargo to the autophagosome for degradation. Increased levels of p62 are indicative of disrupted autophagy in the cells. ATG12 siRNA treated cells do not display green puncta since autophagy is disrupted at the induction step. The cells also demonstrate an increase in Ago2 and p62. ATG12 siRNA treated cells incubated with VacA showed vacuolation, consistent with VacA-toxin treatment. However, autophagosomes were not detected, as expected by ATG12 siRNA disruption of autophagy. In the ATG12 siRNA and VacA treated cells, an increase in Ago2 and an accumulation of p62 were detected (Figure 4C).

HeLa cells, human cervical adenocarcinoma epithelial cells, showed a significant increase in Ago2 when autophagy was inhibited by VacA, bafilomycin, or both (Figure 5A, 5B). Starvation media did not lead to a statistical significant increase in Ago2 levels. To detect Ago2 by immunofluorescence, cells were transfected with AGO2-HA. An increase in Ago2 is detected in VacA treated cells, however less pronounced due to the higher baseline level of Ago2 as a result of the AGO2-HA transfection. HeLa cells treated with VacA displayed a notable increase in autophagosomes, verifying that autophagy was inhibited at the autolysosomal fusion step (Figure 5C).

In order to assess Ago2 in intestinal epithelial cells, murine derived Mode-K cells were used. Cells were treated with VacA, bafilomycin and starvation media. Compared to control cells, VacA treated cells, bafilomycin treated cells, and cells incubated in starvation media with bafilomycin all displayed significantly elevated Ago2 levels (Figure 6). Similarly to the other cell lines, starvation media did not lead to a significant elevation of Ago2. As assessed by immunofluorescence, an increase in autophagosomes in the VacA treated cells indicated that the toxin indeed induced autophagy and disrupted autolysosomal degradation within the cells. These cells also displayed an increase in Ago2.
In addition, the effect of autophagy inhibition on Human umbilical vein endothelial cells (HUVEC) treated with ATG12 siRNA to inhibit autophagy was examined. Cells treated with VacA, bafilomycin, or both, however not with starvation media, displayed a significant increase in Ago2 as assessed by western blotting (Figure 7A). ATG12 siRNA treated cells displayed an increase in Ago2, as detected by immunofluorescence (Figure 7B). Treated cells did not show any autophagosomes, and displayed an accumulation of p62, confirming autophagy inhibition in these cells.

Finally, I assessed Ago2 levels in peripheral blood monocytes (PBMCs) obtained from healthy volunteers who carry the protective ATG16L1-CD allele. PBMCs were isolated, cultured, and treated with autophagy modulators VacA and bafilomycin. Ago2 levels were significantly increased in cells treated with autophagy inhibitors VacA, bafilomycin or both (Figure 8).
Figure 2: Increased AGO2 in ATG5 deficient MEF cells. A: Western blotting of cell lysates from WT or ATG5-/- MEF cells. ATG5-/- cells show an increase in AGO2, as well as an increase in LC3-I, indicative of disrupted autophagy. B: Immunofluorescence microscopy of the same cells showing an increase in AGO2, depicted in red, in ATG5-/- compared to WT cells. The green puncta in WT cells represent autophagosomes, marked by LC3-GFP constructs. WT cells display basal levels of autophagy, while ATG5-/- cells do not show LC3-GFP puncta or a diffuse LC3-I pattern, potentially due to transfection efficiency.
**Figure 3: Increased AGO2 in MEF cells with disrupted autophagy.**

**A:** Western blotting of lysates from ATG5-/- or WT cell treated with bafilomycin to disrupt autophagy, and rapamycin and starvation media to induce autophagy. Cells with disrupted autophagy show an increase in Ago2. **B:** Quantification of Ago2 relative to β-actin levels was performed using blots from n=5 experiments. An unpaired t-test was performed to compare the ATG5-/- control and the WT treated groups to the control untreated WT cells. The results demonstrate a significant increase in Ago2 in ATG5-/- cells as well as WT cells treated with bafilomycin, rapamycin with bafilomycin, as well as starvation media with bafilomycin. Starvation media did not have a significant effect on Ago2 levels. Cells treated with rapamycin, a pharmacological inducer of autophagy, did display a significant increase in Ago2. * p-value < 0.05, ** p-value < 0.01.
**Figure 4: Disruption of autophagy increases AGO2 in MDAMC cells.**

**A:** Western blotting of MDAMC cells treated with control siRNA, as well as autophagy disrupters ATG12 siRNA and VacA. Ago2 is increased in cells with disrupted autophagy. LC3-I increase in ATG12 siRNA treated cells indicates autophagy is indeed disrupted at induction. LC3-II increase in cells treated with VacA alone indicates disrupted autophagy at the autolysosomal degradation step. **B:** Quantification of Ago2 relative to β-actin levels was performed using blots from n=3 experiments. ANOVA and Dunnett’s post-hoc tests were performed to compare the various groups to the control group. The results demonstrate a significant increase in Ago2 in MDAMC cells treated with ATG12 siRNA, or treated with VacA toxin. **p-value < 0.01.** **C:** Immunofluorescence microscopy showing an increase in Ago2, depicted in red, and p62, depicted in blue, in cells treated with ATG12 siRNA and VacA. P62 is an adaptor protein whose accumulation is indicative of disrupted autophagy.
Figure 5: Disruption of autophagy increases AGO2 in HeLa cells. A: Western blotting of lysates from control HeLa cells or treated with VacA which disrupts autophagy. AGO2 is increased in cells with disrupted autophagy. B: Quantification of Ago2 relative to β-actin levels was performed using blots from n=3 experiments. An unpaired t-test was performed to compare the various groups to the control untreated cells. The results demonstrates a significant increase in Ago2 in cells treated with VacA (n=4). * p-value < 0.05. C: IF microscopy showing an increase in AGO2 (red) in cells treated with VacA. LC3-GFP puncta is increased in VacA treated cells indicative of induction and disruption of autophagy.
Figure 6: Disruption of autophagy increases Ago2 in Mode-K cells. A: Western blotting of lysates from Mode-K cells treated with VacA and bafilomycin to disrupt autophagy, as well as MDP and starvation media to induce autophagy. AGO2 is increased in cells with disrupted autophagy. B: Quantification of Ago2 relative to β-actin levels was performed using blots from n=3 experiments. An unpaired t-test was performed to compare the various groups to the control untreated cells. The results demonstrate a significant increase in Ago2 in cells treated with autophagy disrupters VacA and starvation media with bafilomycin. * p-value < 0.05. C: Immunofluorescence imaging of Mode-K cells revealed an increase in Ago2 when cells are treated with VacA which induces and disrupts autophagy. An increase in LC3-GFP puncta is indicative of increased levels of autophagy and suggests that VacA indeed blocked autolysosomal degradation in the cells.
Figure 7: Disruption of autophagy increases AGO2 in HUVEC cells. A: Western blotting of lysates from HUVEC cells treated with VacA and bafilomycin to disrupt autophagy, as well as starvation media to induce autophagy. AGO2 is increased in cells with disrupted autophagy. B: Quantification of Ago2 relative to β-actin levels was performed using blots from n=3 experiments. An unpaired t-test was performed to compare the various groups to the control untreated cells. The results demonstrate a significant increase in Ago2 in cells treated with autophagy disrupters VacA, Bafilomycin, and VacA with bafilomycin. * p-value < 0.05, ** p-value < 0.01. C: Immunofluorescence microscopy indicates an increase in Ago2 in HUVEC cells treated with ATG12 siRNA. Absence of LC3-GFP puncta and an increase in p62 are indicative of disrupted autophagy.
Figure 8: Disruption of autophagy increases AGO2 in human PBMC. A: Lysates from peripheral blood monocytes cells were isolated from healthy volunteers and treated with autophagy disrupters VacA and bafilomycin. AGO2 is increased in cells with disrupted autophagy. B: Quantification of Ago2 relative to β-actin levels was performed using blots from n=3 experiments. ANOVA and Dunnett’s post-hoc tests were performed to compare the various groups to the control group. The results demonstrate a significant increase in Ago2 in peripheral blood monocytes cells treated with autophagy disrupters VacA and bafilomycin. * p-value < 0.05, *** p-value < 0.001.
3.2. Disrupted autophagy leads to differential expression of miRNA

Since my results indicated that autophagy modulates Ago2, a critical effector of miRNA silencing, I hypothesized that miRNA expression profiles would be altered in cells with defective autophagy compared to cells with normal autophagy. I assessed whether disrupted autophagy affects miRNA expression using the NanoString nCounter system and compared miRNA profiles between 4 experimental conditions: WT MEF cells as control cells, WT MEF treated with bafilomycin, ATG5-/- MEF untreated cells, and ATG5-/- MEF treated with bafilomycin. Each miRNA microarray assay was performed on 3 biological replicates of each condition, with a total of 12 samples.

Raw miRNA expression values were first normalized to positive controls and to the top 100 most expressed miRNAs. A background threshold was determined as the mean plus two standard deviations of the negative control counts. A t-test was performed to determine whether the mean miRNA counts for the biological triplicates are significantly different than the mean negative control counts. Furthermore, percent coefficient of variation (CV) was calculated for the biological triplicates in order to identify outliers in the data when the triplicates do not follow the same trend. A total of 43 miRNAs had counts greater than the background threshold, with counts significantly different than the negative control counts (p-value < 0.05), and a percent CV less than 45%.

The microarray assay contained three biological replicates for each condition, each obtained and processed on a different date, therefore there were 3 sample processing batches, which account for some of the variation in the data. I estimated the effect of these sample processing batches with a mixed model ANOVA and adjusted the expression values to what they would be if there were no batch-to-batch variations. I then classified the samples into subgroups using several unsupervised analyses, methods that take into account only expression data and are used in an exploratory fashion to find patterns of differential expression in the data. Principal component analysis (PCA) demonstrated a distinct subgrouping of the samples according to cell type.
(Figure 9). Unsupervised hierarchical clustering (HCL) also distinguished between the two subgroups of WT cells and autophagy deficient ATG5-/- cells (Figure 10).

I next performed a supervised analysis, a method that takes into account expression data as well as any a priori hypothesis of which samples are expected to group together, namely the four experimental conditions: WT, WT with bafilomycin, ATG5-/-, and ATG5-/- cells treated with bafilomycin. Prior to performing an analysis of variance (ANOVA) test, I verified ANOVA test’s assumptions of normal distribution and homogeneity of variances using the Kolmogorov-Smirnov normality test and Fligner-Killeen homogeneity of variance test. ANOVA test was performed to determine differential expression between the different experimental groups with contrasts between: normal autophagy and disrupted autophagy; WT control and bafilomycin-treated WT cells; WT cells and ATG5-/- cells; WT and bafilomycin-treated ATG5-/- cells; ATG5-/- and bafilomycin-treated ATG5-/- cells; and WT cells, treated and untreated, vs. ATG5-/- cells, treated and untreated. Raw p-values were corrected for multiple hypothesis testing using the false-discovery rate (FDR) method (Benjamini and Hochberg, 1995). The results highlighted 5 miRNAs with fold-change equal or greater than ±1.49 and an FDR-adjusted p-value smaller than 0.05 (Table 3) between WT cells, untreated and treated with bafilomycin, and ATG5-/- cells, untreated and treated with bafilomycin. Indeed, this trend was indicated in the unsupervised analysis as well. An HCL analysis of the supervised gene set is presented in Figure 11.

Pathway enrichment analysis was done in order to discover genes and canonical pathways that are regulated by the aberrant miRNAs. Since it is unclear whether the putative miR-1944 gene is indeed processed in vivo (Chiang et al., 2010), 4 miRNAs were included in the analysis. MiR-148a, miR-15b, miR-23a, and let-7c have been experimentally observed to target 315 mRNA. The top canonical pathways with significant changes under disrupted autophagy conditions are listed in figure 12. Several CD associated pathways are implicated by the pathway enrichment analysis, such as the mTOR, PI3K/AKT and MAPK signalling involved in autophagy, IL-8 and NFκB signalling involved in inflammation Wnt/β-catenin and actin cytoskeleton signalling involved in mucosal barrier. Notably, many of the most significantly associated pathways are associated with cancer. Indeed, examining interconnected genes from the dataset and their
function highlights several top biological networks with a Fisher's Exact Test p-value $< 1 \times 10^{-30}$, such as gastrointestinal disease, gene expression mechanisms, cellular function and maintenance, and cancer.
Figure 9: Principal Component Analysis of miRNA expression demonstrates two distinct subgroupings according to cell type. Principal component analysis was done using Partek Genomics suites. Samples are colored according to experimental conditions: WT cells untreated are colored purple, WT cell treated with bafilomycin are colored green, ATG5-/- cells untreated are colored blue, and ATG5-/- cells treated with bafilomycin are colored red. A pink ellipse circles all WT cells, while a yellow ellipse circles all ATG5-/- cells. A distinct subgrouping of the samples according to cell type emerges from this unsupervised analysis.
Figure 10: Unsupervised hierarchical clustering analysis of miRNA expression demonstrates two distinct sub-groupings according to cell type. HCL was done using Partek Genomics Suite, with the Pearson dissimilarity and complete clustering method. The cluster on the left shows clustering of the samples. It indicates a clear subgrouping according to cell type, with the top sub-cluster for autophagy deficient cells and the bottom sub-cluster for wildtype cells. The letter ‘c’ next to the sample indicates control untreated cells, while the letter ‘b’ indicates bafilomycin-treated cells. The cluster on the top shows clustering of the miRNAs detected in the samples and indicates subgrouping of miRNA expression patterns. The scales next to the top and left clusters indicate the Pearson dissimilarity values. The color scale at the bottom of the figure designates color to fold changes, with blue indicating a downregulation and red indicating an upregulation. HCL analysis shows a clear pattern of distinction between WT samples and ATG5 knockout samples.
### Table 3: 5 miRNA are differentially expressed in WT vs. ATG5-/- MEFs.

MiRNAs with a fold change of over ±1.49 and FDR adjusted p-value < 0.05 were considered to be differentially expressed between samples. A positive fold change indicates that a miRNAs is upregulated in WT cells compared to ATG5 deficient cells. A negative fold change indicated that a miRNA is downregulated in WT cells compared to ATG5 deficient cells. MiR-1944 is considered a putative miRNA locus, and it is unclear if it is indeed generated in vivo.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>unadjusted p-value</th>
<th>FDR adjusted p-value</th>
<th>fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-148a</td>
<td>0.0011</td>
<td>0.0392</td>
<td>-2.06</td>
</tr>
<tr>
<td>mmu-miR-1944</td>
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<td>0.0392</td>
<td>-2.61</td>
</tr>
<tr>
<td>mmu-miR-15b</td>
<td>0.0035</td>
<td>0.0392</td>
<td>1.49</td>
</tr>
<tr>
<td>mmu-miR-23a</td>
<td>0.0038</td>
<td>0.0392</td>
<td>1.63</td>
</tr>
<tr>
<td>mmu-let-7c</td>
<td>0.0046</td>
<td>0.0392</td>
<td>1.78</td>
</tr>
</tbody>
</table>
Figure 11: Hierarchical clustering analysis of the differentially expressed miRNAs. HCL was done using Partek Genomics Suite, with the Pearson dissimilarity and complete clustering method. The cluster on the left shows clustering of the samples. It indicates a subgrouping according to cell type, with the top sub-cluster for autophagy deficient cells and the bottom sub-cluster for wildtype cells. The letter ‘c’ next to the sample indicates control untreated cells, while the letter ‘b’ indicates bafilomycin-treated cells. The cluster on the top shows clustering of the significant miRNAs according to expression patterns and indicates 2 subgroups: miR-148a and miR-1944 that are downregulated in wildtype cells, and let-7c, miR-15b, and miR-23a that are upregulated in wildtype cells. The scales next to the top and left clusters indicate the Pearson dissimilarity values. The color scale at the bottom of the figure designates color to fold changes, with blue indicating a downregulation and red indicating an upregulation.
Figure 12: Top canonical pathways predicted to be regulated by the differentially expressed miRNAs. Ingenuity Pathway Analysis (IPA) software was used to identify experimentally validated mRNA targets of the 4 aberrant miRNAs and to identify the canonical pathways that were most significant to this data set. The significance of the association between the data set and the canonical pathway is presented here as the result of a Fisher’s exact test to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. Pathways with enrichment FDR adjusted p-value smaller than 0.05 are presented. The negative logarithm of the FDR adjusted p-value is listed next to each bar. Several pathways implicated have been associated with CD, such as the mTOR, PI3K/AKT and MAPK signalling involved in autophagy; inflammatory pathways such as IL-8 and NFκB signalling; and pathways involved in mucosal barrier, including Wnt/β-catenin and actin cytoskeleton signalling. Interestingly, several of the highly associated pathways are involved in various cancers.
Chapter 4
Discussion

Current evidence shows that MVBs affect Ago2 turnover and RISC formation (Gibbings et al., 2009; Lee et al., 2009). Ago2 is enriched in endosomal membrane fractions obtained using cellular ultracentrifugation (Gibbings et al., 2009), thus Ago2 could also be enriched in autophagosomes. I hypothesized that Ago2 is degraded by autophagy. Since MVBs fuse with autophagosomes, disruption of autophagy might also lead to aberrant turnover of Ago2. My results demonstrate a consistent increase in Ago2 levels, a critical RISC component, in cells with altered autophagy. I have verified that Ago2 is increased in multiple cell lines, including epithelial, endothelial, and immune cells of myeloid origin following a variety of complementary means to inhibit autophagy. This increase across numerous cell types proposes a universal mechanism and a central role for autophagy in regulation of Ago2, a crucial miRNA modulator.

In WT MEF cells, rapamycin, an autophagy inducer also led to a significant increase in Ago2. Similarly, an increase in Ago2 was detected in ATG5 knockout cells in which autophagy is defective. Since rapamycin leads to an increase in Ago2 in both normal and disruptive autophagy cell lines, this effect by rapamycin may represent an autophagy independent mechanism that causes an increase in Ago2 levels.

However the exact mechanisms by which Ago2 increases upon disrupted autophagy remains to be determined. I did not detect colocalization of Ago2 with LC3 as assessed by confocal microscopy. However, this lack of colocalization might be due to the inability to capture the autophagic flux at the right exact moment when Ago2 is degraded. Transfection is also a challenge in this context as several cell lines present low transfection efficiency, hence the percentage of cells exhibiting autophagic puncta is low. Alternatively changes in Ago2 expression could be through an indirect mechanism as a result of disrupting autophagy. For example, RISC components might be degraded by the proteasome, which can also be affected by autophagy disruption. Autophagy and the proteasome have a functional compensatory relationship as proteasome inhibition triggers autophagy (Ding et al., 2007) and autophagy inhibition will alter degradation of substrates by the ubiquitin-proteasome pathway (Korolchuk et
Cytoplasmic levels of RISC components might also be affected by secretion in exosomes that are targeted out of the cells. Whether or not exosomal secretion of Ago2 is regulated by autophagy is unknown. Another option is that AGO2 transcription rises as an indirect result of autophagy disruption which leads to increased levels of Ago2 protein. Studies of Ago2 mRNA levels using RT-PCR can determine whether this mechanism is involved in Ago2 increase.

In future studies, it will be of interest to examine the effects of disrupted autophagy on other members of the Ago protein family in humans, Ago1, Ago3, and Ago4, as well as their subsequent effect on miRNA silencing. Gw182 is another critical regulator of miRNA gene regulation, and examining its levels and function in response to disrupted autophagy might also shed light on miRNA involvement in disease.

To determine if changes in Ago2 would result in corresponding changes in miRNA expression I compared miRNA expression profiles in control cells and cells with disrupted autophagy. In order to explore for patterns of miRNA expression, I used unsupervised analysis methods on the microarray dataset, which indicated a distinct division between WT and ATG5 knockout cells. Since autophagy is already disrupted in the ATG5 knockout cells, bafilomycin treatment is not expected to affect autophagy in these cells. Indeed, the unsupervised analyses did not show a sub-division of ATG5-/- samples according to untreated cells and bafilomycin-treated cells. In addition, the unsupervised analyses did not show subgrouping of WT cells according to untreated cells and bafilomycin-treated cells. A total number of n=6 WT samples in which it is possible to examine bafilomycin’s effect is a limitation of this study design. Six samples might not be enough to detect a different pattern of expression between WT cells with normal autophagy and bafilomycin-treated WT cells with defective autophagy.

Using a supervised analysis, a significant change in expression of 5 miRNAs was detected between all WT cells and all ATG5 knockout cells (total n=12), but not when comparing the experimental subgroups WT cells versus bafilomycin-treated WT cells (total n=6). Again, since bafilomycin is not expected to affect autophagy in ATG5-/- cells in which autophagy is readily disrupted, the sample size for comparing bafilomycin’s effect on miRNA expression in an autophagy dependant manner was limited only to 6 WT samples. A low sample size may account
for the FDR corrected p-value for differentially expressed miRNAs not passing the significance threshold. Comparing ATG5-/- cells with bafilomycin-treated ATG5-/- cells in order to detect expression changes caused by bafilomycin in an autophagy independent manner also did not yield significant expression changes, potentially due to low sample size.

In order to determine which miRNAs are expressed in the samples, raw microarray data is normalized and non-specific background in the expression values is assessed. In doing so, a stringency level is determined, as setting the background threshold at a low value, for example mean of negative control counts, will yield a long list of detected miRNAs. Setting the FDR adjusted p-value at a higher threshold of 0.1 may also be done in order to avoid false negatives and achieve a long list of significant miRNAs. On the contrary, setting a low stringency method might lead to more false positives, hence setting the stringency is a trade-off between false positives and false negatives which effects the ability to validate the aberrant miRNA expression data. In this study, I picked a high stringency background of mean negative control counts plus 2 standard variations, as well as setting the FDR adjusted p-value threshold at 0.05. This method may explain the resulting relatively short list of 5 aberrant miRNAs. Alternatively, a physiological explanation might account for the short list of miRNAs, as miRNAs are variably expressed among various cell types.

Indeed, miRNA levels in our samples did not exhibit a global change across all miRNAs as a result of disruptive autophagy. Since miRNA differ in their affinity to the different Ago proteins, and miRNA levels are sensitive to Ago2 levels, this list of aberrant miRNA might represent a subset of miRNAs that have increased affinity to Ago2.

This study highlighted 4 experimentally validated miRNAs: miR-148a, miR-15b, miR-23a, and let-7c. The let-7 miRNA family plays a key role in regulation of inflammation (Schulte et al., 2011). It also regulates IL-6 and IL-10 cytokine expression, and is downregulated in response to bacteria in order to increase the production of these major cytokines. Let-7 miRNA also regulates TLR4 signalling, as microbial infection downregulates let-7 expression, which in turn increases TLR signalling and bacterial sensing (Androulidaki et al., 2009). Moreover, let-7c and miR-23a have been associated with active ulcerative colitis (Takagi et al., 2010; Wu et al.,
2008a). All the identified miRNAs have been linked to various cancers including gastric and colorectal cancer (Chen et al., 2010; Slaby et al., 2009).

Interestingly, pathway enrichment analysis has highlighted several canonical pathways which have been implicated in CD previously (Figure 12). The Wnt/β-catenin and actin cytoskeleton signalling pathways are regulators of mucosal barrier (Banan et al., 2000), which is linked to pathogenesis of CD (Xavier and Podolsky, 2007). Actin cytoskeleton signalling was also shown to regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation by Nod2 (Legrand-Poels et al., 2007). NFκB, also implicated in this study by pathway analysis, is a transcription factor that is activated in response to various stimuli such as cellular stress, cytokines, and bacterial products. Its activation is involved in the immune response to infection, and thus has a central role in inflammation. It has been associated with CD through Nod2, which mediates NFκB activation (Girardin et al., 2003).

Transcription factor 4 (TCF4) is involved in Wnt signalling and regulates Paneth cell function. Its reduced expression is linked to CD and the characteristic Paneth cell abnormalities and dysfunction (Wehkamp et al., 2007). Vascular endothelial growth factor (VEGF) and Transforming growth factor beta (TGF-β) altered expression are also associated with CD (Del Zotto et al., 2003; Kader et al., 2005; Kanazawa et al., 2001). TGF-β plays a role in intestinal homeostasis and inflammatory bowel disease, which depends on the inflammatory cytokine context (Xavier and Podolsky, 2007).

Finally, mitogen-activated protein kinase (MAPK) signalling regulates various processes such as proliferation, differentiation, inflammation and apoptosis, and mTOR signalling. Indeed mTOR is a nutrient sensor and a master regulator of the autophagy pathway. Phosphoinositide 3-kinase (PI3K)/AKT signalling also regulates autophagy through mTOR signalling.

Notably, many of the canonical pathways emphasized by this study are related to cancer mechanisms. Autophagy has a context dependant role in cancer and acts as the fulcrum balancing tumor suppression and tumorigenesis. Autophagy can act to stimulate oncogenesis by promoting cellular survival via enhanced metabolism under hypoxic conditions, nutrient deprivation, and during chemotherapy. On the other hand, autophagy can act to contain cell
growth and tissue damage that accompany carcinogenesis. Since apoptosis mechanisms are often disrupted in cancerous cells, autophagy can function as a form of programmed cell death. Furthermore, autophagy has the ability to clear damaged organelles such as mitochondria and control levels of ROS production. Since ROS levels increase the rate of genomic mutations that promote oncogenesis, controlling ROS has beneficial consequences in the context of tumor suppression. However, what causes the balance to change from tumorigenesis to tumor suppression is currently unknown (White, 2012).

In the future, gene expression analysis as well as miRNA expression analysis should be performed. Gene expression data will complement the miRNA profiling by confirming that putative miRNA targets are indeed regulated in the samples. Expression values for mRNA can also lend further support to the pathway enrichment analysis, by highlighting specific predicted pathways, as well as implicating additional unexpected pathways, that might be involved in the pathogenesis of CD.

In future studies, RT-PCR should be used to confirm the expression levels of the aberrantly expressed miRNA discovered by microarray. Another informative validation method is high-throughput sequencing of cross-linked immunoprecipitation (HITS-CLIP), a method which has been used successfully to immunoprecipitate Ago2 along with bound miRNA and mRNA identifiable by high-throughput sequencing (McKenna et al., 2010). Furthermore, In order to study the function of the aberrant miRNAs in terms of potentially regulating RISC or autophagy components, a luciferase reporter assay may be utilized. In this method, 3’UTR of target mRNA is cloned into luciferase reporter vector is transfected into cells. Suppression of luciferase activity reflects the regulation of the 3’UTR by miRNAs present in the samples.

An Interesting aim for further research is to determine how the Atg16L1-CD variant affects RISC components and miRNA silencing. Studying the effects of the Atg16L1-CD variant on Ago2 in cells expressing this allele, as well as profiling miRNA and mRNA in cell lines and in tissue samples from patients carrying the CD variant, can tremendously contribute to the understanding of the functional relevance of Atg16L1 CD variant.
Taken together, the findings brought here suggest a novel mechanism by which autophagy regulates RISC components, hence altering miRNA expression. As aberrant expression of miRNA has been implicated in CD, these results provide a novel potential mechanism by which altered autophagy is involved in CD pathogenesis (Figure 13).
Figure 13: Model for altered autophagy involvement in CD pathogenesis. This study has shown that altered autophagy leads to increase in Ago2 levels. To alter autophagy I used pharmacological agents and microbial products which were shown to subvert autophagy. In nature, this might occur through host mutations or defective microbial sensing. Ago2 is a critical regulator of miRNA silencing, and miRNA levels are sensitive to its levels, which leads to aberrant miRNA profiles in cells with disrupted autophagy. Important miRNAs emerge from the data, including the let-7 family that mediates inflammation and several other miRNAs which are involved in autophagy and mucosal barrier, implicated in CD pathogenesis. Differential miRNA profiles have already been recognized in CD. However this study suggests a novel model for the regulation of miRNA. Altered miRNA profiles affect inflammatory and metabolic processes that have been associated to the persistent inflammation characteristic to CD. Interestingly, altered tumor suppression is also implicated by the data.
References


