Regulatory Role of miR-93 in Autophagy Induction

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To My Family

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SUMMARY

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression. miRNAs play multiple functions in many essential cellular processes such as proliferation, apoptosis and differentiation.

Autophagy is a conserved cellular process in which cell digests spent components and organelles to regenerate energy and "building blocks" to cope with various kinds of stress.

To date, the effect of autophagy on miRNA expression and the corresponding mechanisms through which miRNAs regulate autophagy are still not clear. Therefore, the main objective of this study is to investigate the effect of autophagy induction on endogenous miRNA expression and the corresponding mechanisms through which miRNAs regulate autophagy.

Here, we first screened the differential expression of miRNAs by microarray when autophagy was induced by starvation in Atg5 wide type mouse embryonic fibroblasts (WT MEF) and Atg5 knock out mouse embryonic fibroblasts (KO MEF). Starvation and Rapamycin treatments are established common inducers for autophagy. The MEF cells were chosen as they were among the most popular cell line models for the current autophagy research, knock out of Atg5 can efficiently block autophagy induced by starvation or Rapamycin. Among these miRNAs, miR-93 and miR-181a were up-regulated while miR-221 was down-regulated when autophagy was induced. Such changes were confirmed using real time qRT-PCR in cells undergoing autophagy induced by either starvation or Rapamycin. Those miRNAs deregulated in the period of autophagy induction were predicted to target a variety of autophagy related genes, indicating the important regulatory role of miRNAs in autophagy.

miR-93 was selected for further examination. Through screening of the predicted autophagy related target genes, we successfully identified Ulk1 as a novel target gene of miR-93, based on the following evidence: (1) Compared with MEF cells transfected with negative control miRNA, the expression levels of Ulk1 protein and mRNA in MEF cells transfected with pre-miR-93 were decreased whereas they were relatively more abundant in MEF cells transfected with anti-miR-93. (2) Through ß-Gal reporter gene assay, miR-93 was proved to interact with the predicted binding sites on the 3'UTR of Ulk1 mRNA. Since Ulk1 is an essential Atg gene which forms a complex with mammalian orthologue of Atg13 and FIP200 during the initiation of autophagosome formation, miR-93 places its negative regulatory effect on autophagy initiation through suppression of Ulk1 expression.

In summary, this study reveals a new negative feedback loop mechanism between autophagy and miR-93. Data from this study provided new evidence that certain miRNAs could play important roles in autophagy via regulation of key Atg genes such as Ulk1. Understanding such a novel mechanism is beneficial for the development of miRNA-based therapeutics against various autophagy related diseases through modulation of autophagy.

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ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E binding protein 1
AMPK	AMP-activated protein kinase
ATG	AuTophaGy related
BAD	Bcl-xL/Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	basal cell lymphoma-extra large
Beclin 1	coiled-coil, myosin-like Bcl-2 interacting protein
BH3	Bcl-2 homology domain
BNIP3	Bcl-2/adenovirus E1B 19 kDa-interacting protein 3
cDNA	complementary DNA
CQ	chloroquine
DTT	dithiothreitol
EBSS	earle's balanced salt solution
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FIP200	focal adhesion kinase (FAK) family interacting protein of 200 kDa
FoxO	Forkhead box O
HIF	hypoxia inducible factor

hVps34	homolog of yeast Vps34
IGF1	insulin-like growth factor
JNK	c-Jun N-terminal kinase
KO	knockout
Lamp-2	lysosomal-associated membrane protein 2
LC3	microtubule-associated protein 1 light chain 3, the mammalian homolog of yeast Atg8
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence-1
MEF	mouse embryonic fibroblasts
miRNA	microRNA
mTOR	mammalian target of Rapamycin
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAS	the pre-autophagosomal structure
PARP	poly(ADP-ribose) polymerase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PtdIns(3)P	phosphatidylinositol 3-phosphate
PtdIns(3,4)P2	phosphatidylinositol 3,4-biphosphate
PTEN	phosphatase and tensin homolog deleted on chromosome 10

RISC	RNA induced siliencing complex
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
S6	ribosomal protein S6
S6K	70 kDa S6 kinase
SDS	sodium dodecyl sulfate
SQSTM1	sequestosome 1
STAT	signal transducers and activators of transcription
TNFα	tumor necrosis factor α
TORC1	target of Rapamycin complex 1
TORC2	target of Rapamycin complex 2
Tris	tris(hydromethy) aminomethane
TSC	tuberous sclerosis complex
ULK	UNC-51-like kinase
UTR	untranslated region
UV	ultraviolate
UVRAG	UV radiation resistance-associated gene
Vps	vacuolar protein sorting
WT	wild type
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

1.1 Autophagy:

In order to achieve cellular homeostasis which favors cell survival and normal growth, besides the regular synthesis of macromolecule and organelles, cells must also adopt several degradation mechanisms. The major turnover pathway for cytosolic protein is the proteasome pathway while the major degradation pathway for bulk cellular components is Autophagy - the "self-eating" pathway.

In mamalian cells, there are mainly three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Chaperone-mediated autophagy is a secondary response to starvation which involves direct translocation of the protein cargoes across the lysosome membrane (Massey et al., 2006). Microautophagy sequesters cytoplasm by invagination or septation of the lysosomal/vacuolar membrane (Wang and Klionsky, 2004). Macroautophagy (hereafter refer to as autophagy), the most prevalent form, is morphologically featured by the formation of autophagosomes. Autophagy, is a highly conserved cellular process in which double-membrane autophagosomes sequester spent organelles or unwanted cellular components and fuse with lysosomes for degradation and recycling (Klionsky and Emr, 2000).

Autophagy is a highly dynamic process which includes several discrete steps: (1). induction; (2). cargo selection and packaging; (3). nucleation of vesicle formation; (4). vesicle expansion and completion; (5). retrieval; (6). fusion with endosome and

lysosome/vacuole; (7). breakdown of intralumenal cargoes and recycling (Klionsky, 2005). The dynamic autophagy process is briefly illustrated in Figure 1.1.

Autophagy is induced by various cellular cues such as amino acid deprivation, hypoxia and growth factor withdrawal. After receiving autophagy-inducing signals, the autophagosomes will be de novo generated (Yorimitsu and Klionsky, 2005). After the Atg1 complex triggered initiation, the phagophore (PAS) will be formed via the nucleation machinery (Xie et al., 2008). Through elongation machinery, the phagophore develops into autophagosome. Then the matured autophagosome, by fusing with endosome and lysosome/vacuole sequentially, becomes autolysosome in which the contents are degraded and recycled.

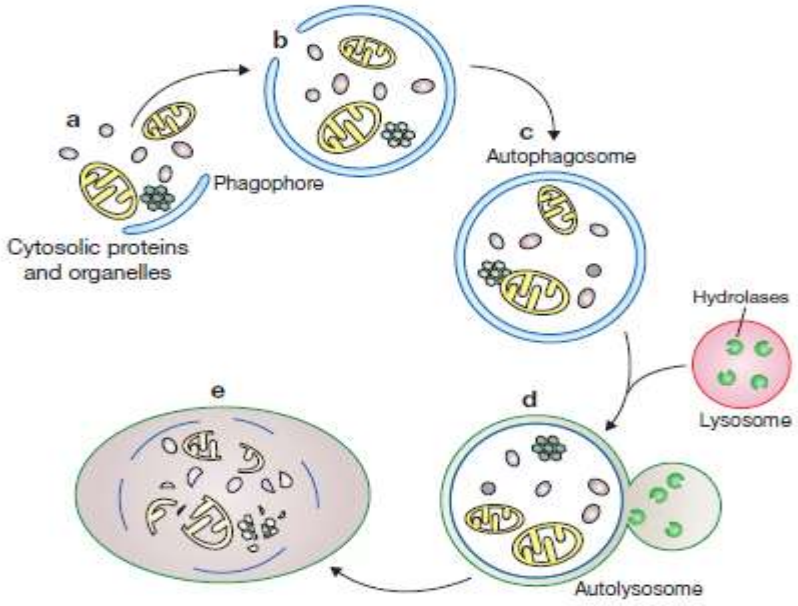


Figure 1 .1 Schematic depiction of the dynamic process of autophagy

(adapted from Xie and Klionsky, Nature Cell Biology, 2007)

1.1.1 Atgs: the autophagy machinery

Autophagy is a highly evolutionarily conserved process and autophagy-related (Atg) genes are found in almost all organisms from monocellular yeasts to fungi, drosophila up to vertebrates. Since the first discovery of Atg1 gene in yeast (Matsuura et al., 1997), to date, 32 Atg genes have been identified in yeast and there are 15 known counterparts in mammalian cells (He and Klionsky, 2009; Kanki et al., 2009; Tanida et al., 2011).

Among these Atg genes, 18 of Atg genes (Atg1–10, Atg12–14, Atg16–18, Atg29, and Atg31) are involved in autophagosome formation (Mizushima, 2007). As this study is conducted using MEF cells, the functions of mammalian Atg genes for the autophagy core machinery is summarized in Table 1.1.

Table 1.1 Fuctions of mammalian Atg genes in the autophagy core machinery

Mammals	Yeast	Functions	References
Ulk1/2	Atg1	protein kinase, target of mTOR kinase and AMPK, form a complex with Atg13, Atg17,and Atg101, trigger autophagosome biogenesis	(Chan et al., 2007; Hara et al., 2008; Young et al., 2006)
Atg3	Atg3	E2-like enzyme in Atg12 and Atg8/LC3 conjugation	(Ichimura et al., 2000; Tanida et al., 2006)
Atg4A-D	Atg4	cytosolic cysteine protease for processing of Atg8/LC3, deubiquitinating-like enzyme for removing PE from Atg8	(Marino et al., 2003; Scherz-Shouval et al., 2003; Tanida et al., 2004)
Atg5	Atg5	target of Atg12 localizing to isolated membranes,	(Mizushima et al.,

		leading to oligomerization with Atg16 to form a complex acting as a E3-like enzyme involved in Atg8 lipidation	1998a; Mizushima et al., 2001)
BECN1	Atg6	interact with PI3K class III required for membrane nucleation, Bcl2-interacting protein	(Liang et al., 1999; Kametaka et al., 1998)
Atg7	Atg7	E1-like enzyme for Atg12-Atg5 conjugation and Atg8 lipidation	(Tanida et al., 2001)
MAP1LC3A-C GABARAP GATE16	Atg8	conjugating with PE and localizing to autophagosomes	(Kabeya et al., 2000; Tanida et al., 2006)
Atg9A/B	Atg9	transmembrane protein involved in autophagosome formation	(Yamada et al., 2005; Young et al., 2006)
Atg10	Atg10	E2-like enzyme for Atg5-Atg12 conjugation	(Mizushima et al., 2002)
Atg12	Atg12	conjugates with Atg5, leading to oligomerization with Atg16 to form a complex act as E3-like enzyme that involved in Atg8 lipidation	(Mizushima et al., 1998b)
Atg13	Atg13	phosphorylated protein in the Ulk1 complex	(Hosokawa et al., 2009; Meijer et al., 2007)
Atg14/Barkor	Atg14	bind with Beclin 1-PI3K class III (Vps34) complex involved in membrane nucleation, enhance autophagosome formation	(Itakura et al., 2008)

Atg16L1/L2	Atg16	interact with Atg5-Atg12 to form complex acting as a E3-like enzyme,determines the site of LC3 conjugation	(Mizushima et al., 2003)
FIP200	Atg17	component of the Ulk1 complex, essential for both stability and phosphorylation of Ulk1	(Ganley et al., 2009; Hara et al., 2008; Jung et al., 2009)
WIPI-1,2,3,4	Atg18	PI(3)P binding protein, involved in nucleation	(Proikas-Cezanne et al., 2004)
Atg101		important for stability and basal phosphorylation of Atg13 and Ulk1	(Hosokawa et al., 2009; Mercer et al., 2009)

The existence of Atg5/ Atg7-independent alternative macroautophagy shows that autophagy is a very complex and finely controlled process (Nishida et al., 2009).

1.1.1.1 The Atg1 (Ulk1) complex in autophagy induction

Atg1, a serine/threonine kinase, has been proved to play an indispensable role in this process. In yeast, the Atg1 complex, consisting of Atg1, Atg13, and Atg17, is the downstream of the target of Rapamycin (TOR) and cAMP-dependent kinase (PKA) pathways (Matsuura et al., 1997). Upon TOR inhibition, both the Atg1 kinase activity and its binding affinity to Atg13 and Atg17 increase, leading to formation of an Atg1-Atg13-Atg17 scaffold and recruitment of multiple Atg proteins to the Pre-autophagosome Structure (PAS) to initiate autophagosome formation (Kamada et al., 2000). In addition, Atg1 kinase activity is suggested to be primarily required for the Cvt pathway (Abeliovich

et al., 2003). Moreover, in *Drosophila*, during nutrient starvation, Atg1 can inhibit the phosphorylation and activation of S6K, a downstream effector of TOR (Lee et al., 2007).

There are two mammalian Atg1 homologs - unc-51-like kinase 1 and 2 (Ulk1 and Ulk2), which have been found to form a complex with mammalian orthologue of Atg13 and Atg17 (FIP200) and localize to the phagophore upon starvation (Hara et al., 2008; Jung et al., 2009). Different from the Atg1 complex components in yeast, Ulks, Atg13 & FIP200 form a stable complex in a nutrient-independent manner in mammalian cells and Atg13 and FIP200 are required for maximizing Ulk1 kinase activity (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009).

Under nutrient-rich conditions, mTOR complex 1 (mTORC1) interacts with the Ulk1-Atg13-FIP200 complex through raptor, one of the mTORC1 components, and sequentially phosphorylates and inactivates Ulks and Atg13 (Ganley et al., 2009; Jung et al., 2009). Upon mTORC1 inhibition, Ulk1 and Ulk2 are freed and phosphorylate Atg13 and FIP200, which are essential for promoting autophagy (Hara et al., 2008; Hosokawa et al., 2009). Atg101, a newly identified component in the Ulk1 complex in mammals, binds and stabilizes Atg13 (Mercer et al, 2009).

Besides their phospohorylation effect on Atg13 and FIP200, Ulks also undergo autophosphorylation which results in conformational changes and autophagy induction (Chan et al., 2009).

Recently, Ulk1 and Ulk2 were found to be substrates of AMPK and similar to mTORC1, AMPK also associated with Ulk1 in a nutrient-dependent manner (Egan et al., 2011a; Lee et al., 2010; Shang et al., 2011). However, AMPK may have dual role in autophagy induction as on one hand, during starvation, besides the classic AMPK-TSC1/2-

mTOR pathway, it can also phosphorylate raptor, the key adaptor in mTORC1, thus lifting the inhibitory effect of mTOR on Ulk1 (Lee et al., 2010; Egan et al, 2011b); on the other hand, when cells are fed, it can form a complex with and confine portions of Ulk1 to suppress autophagy (Shang et al., 2011). Interestingly, Ulk1 and Ulk2 were also shown to be able to in turn phosphorylate AMPK and thereby form a negative feedback circuit (Löffler et al., 2011). These studies show the fact that Ulks are tightly under the control of multiple signaling pathways in mammalian cells.

1.1.1.2 The class III phosphatidylinositol 3-kinase (PtdIns3K) complex in PAS nucleation

Instead of being generated by budding from a preexisting organelle's surface or sealing of a piece of continuous membrane, autophagosome is assembled at the PAS by adding new membranes. In the early stage of autophagosome formation - the nucleation and initial assembly of the phagophore membrane require the participation of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex which produces PI3P from PI. It is also involved in PAS targeting of Atg18 (WIPI-1α), Atg20, Atg21, and Atg24 that bind PtdIns3P (Nice et al., 2002; Obara et al., 2008; Stromhaug et al., 2004; Axe et al., 2008; Proikas-Cezanne et al., 2004) and thus further recruits two ubiquitin-like (Ubl) conjugation systems, Atg12–Atg5-Atg16 and Atg8–PE, to the phagophore, contributes to the membrane elongation (Suzuki et al., 2001; Suzuki et al., 2007).

In yeast, the class III PtdIns3K complex is composed of the PtdIns3K Vps34 (vacuolar protein sorting 34), a myristoylate serine/threonine kinase Vps15, Atg14 and Atg6 (Kihara et al., 2001). In mammals, the class III PtdIns3K complex includes Beclin 1,

hVps34, hVps15 (formerly called p150), and Ambra1 (Fimia et al., 2007). Similar to its yeast counterpart, this complex also binds to Atg14 (Barkor) (Sun et al., 2008) and participates predominantly in autophagy regulation (Itakura et al., 2008). There exists another Beclin 1-hVps34 complex which comprises Beclin 1, hVps34, hVps15, Ambra1 and additionally, the mammalian homolog of Vps38 and the UV irradiation resistance-associated gene (UVRAG) protein whose role in autophagy is still not clear. UVRAG was observed to be able to activate Beclin 1-hVps34 complex and facilitate autophagosome formation (Liang et al., 2006; Takahashi et al., 2007), while other studies found that the UVRAG-incorporated Beclin 1-hVps34 complex was not involved in autophagosome formation (Itakura et al., 2008; Sun et al., 2008) but regulation of autophagosome maturation and endocytotic trafficking (Liang et al., 2008).

Beclin 1 is the mammalian homologue of the yeast Atg6, which was found to be indispensible for autophagy in mammals (Liang et al., 1999). Under nutrient-rich conditions, Beclin 1 is bound by the anti-apoptotic protein B-cell lymphoma/leukemia-2 (Bcl2) and thus autophagy is suppressed. Dissociation of Bcl2 from Beclin 1 is required for the autophagy induction. The activity of Vps34 (hVps34) is tightly controlled by Vps15 (hVps15) (Stack et al., 1995; Yan et al., 2009). Beclin 1 was reported to strengthen the interaction between hVps34 and hVps15 and thus activate hVps34 (Yan et al., 2009).

1.1.1.3 Two ubiquitin-like systems in membrane elongation

Two ubiquitin-like systems, the Atg5-Atg12 system and the Atg8 (LC3) system, are evolutionarily conserved from yeast to mammals and play essential roles in autophagosome

biogenesis. Like ubquitination in which ubquitin is first activated and transferred by an E1 ubiquitin-activating enzyme to an E2 ubiquitin-conjugating enzyme, and then conjugated with the target protein by an E3 ubiquitin-protein ligase (Pickart, 2004), the two ubiquitin-like system also undergo conjugation in a similar manner.

In the Atg5-Atg12 system, Atg12 is activated by Atg7 (E1), transferred to Atg10 (E2) and attached to the substrate protein Atg5. In contrast to ubiquitination, Atg12-Atg5 conjugation does not require E3 ligase counterpart (Geng and Klionsky, 2008) and the conjugate further interacts with Atg16, a coiled-coil protein which links the Atg12-Atg5-Atg16 complex into a tetramer through self-oligomerization and localizes to the phagophore (Mizushima et al., 2003).

In the LC3 conjugation system, the newly synthesized LC3 is first processed by a cysteine protease, Atg4, exposing a C-terminal glycine residue to generate LC3-I. Atg7 activates LC3-I and transfers it to Atg3 (E2). LC3-I is finally conjugated to the phosphatidylethanolamine (PE), catalyzed by the E3-like Atg12-Atg5 conjugate which is the product of the other Ubl system described above (Ichimura et al., 2000). PE conjugated LC3-II is requried for the elongation of autophagic membrane. Upon autophagy induction, LC3 will rapidly change from the cytosolic form to the lipid-conjugated form and translocate to both sides of the PAS (Kabeya et al., 2000). After autophagosome formation, LC3-II on the outter membrane will be released and recycled while the inner membrane-anchored LC3-II will be degraded in the autolysosome (Mizushima et al., 2003).

1.1.1.4 Atg9 in membrane traffic and phagopore expansion

Atg9 is the only identified integral membrane protein during the autophagosome formation. It may function as a vesicle in supplying membrane and may also exert its effect on phagophore expansion. Atg9 is found to be localized to the PAS and non-PAS membranes (peripheral structures in yeast, trans-Golgi network and endosomes in mammal). Researchers have found that Atg9 can move bidirectionally between these two sites (Young et al., 2006) and this membrane cycling machinery is believed to deliver membranes to PAS thus facilitates the autophagosome formation (Reggiori et al., 2005; Reggiori et al., 2004a). In yeast, Atg11, Atg23, and Atg27 assist the anterograde transport of Atg9 to phagophore (Chang et al., 2007a; He et al., 2006; Legakis et al., 2007; Yen et al., 2007); while the Atg1 complex, Atg2, Atg18, and the PtdIns3K complex are essential in its retrograde transport (Reggiori et al., 2004a). In mammals, similarly, when autophagy is induced, Atg9 is transported from the trans-Golgi network (TGN) to late endosomes and Ulk1 and Atg13 are essential in the redistribution of Atg9 from the TGN to late endosomes (Young et al., 2006). Also, dynamic self-multimerization of Atg9 was suggested to facilitate membrane tethering or fusion during phagophore expansion (He et al., 2008). In addition, the interaction between Atg9 and Atg18 which links the membrane cycling machinery to the nucleation machinery was also reported (Reggiori et al., 2004b).

1.1.1.5 The Beclin 1-hVps34 - Rubicon complex in the late stages of autophagy

After formation, autophagosomes will fuse with early or late endosomes to form the amphisomes, and subsequently fuse with the lysosomes to form the autolysosomes (Liou et al., 1997; Razi et al., 2009; Stromhaug et al., 2004). As a result, the acidification of the

autophagosome and the degradation and recycling of cellular components were also observed.

Multiple signals mediate the late stages of autophagy. In addition to the autophagosome formation function of the Beclin 1-hVps34 - Rubicon complex mentioned previously, this complex also regulates the maturation of autophagosome. The Beclin 1-hVps34 - Rubicon complex was proposed to upregulate the endocytic trafficking and the maturation while its association with Rubicon would downregulate these trafficking events (Matsunaga et al., 2009; Zhong et al., 2009). Besides this, the maturation of autophagosome is regulated by Rab proteins, DRAM as well as the endosomal and lysosomal membrane proteins while the kinase activity of ATPases is essential for the acidification and degradation stages (Mehrpour et al., 2010).

1.1.2 Upstream signaling pathways for regulation of autophagy

Since autophagy is such a crucial process in controlling cell death and survival in response to environmental cues during stress periods, it is not a surprise that there are multiple pathways involved in the regulation of intracellular autophagic activity including nutrient & energy signaling pathways, various stress response pathways, and pathogen infection related signaling pathways (He and Klionsky, 2009). As mTOR is in the central point of various pathways that sensing nutrient status, growth factors, energy balance and environmental stresses, it plays a crucial role in the upstream signaling network of autophagy (Schmelzle and Hall, 2000). The upstream signaling pathways regulating autophagy are briefly illustrated in Figure 1.2.

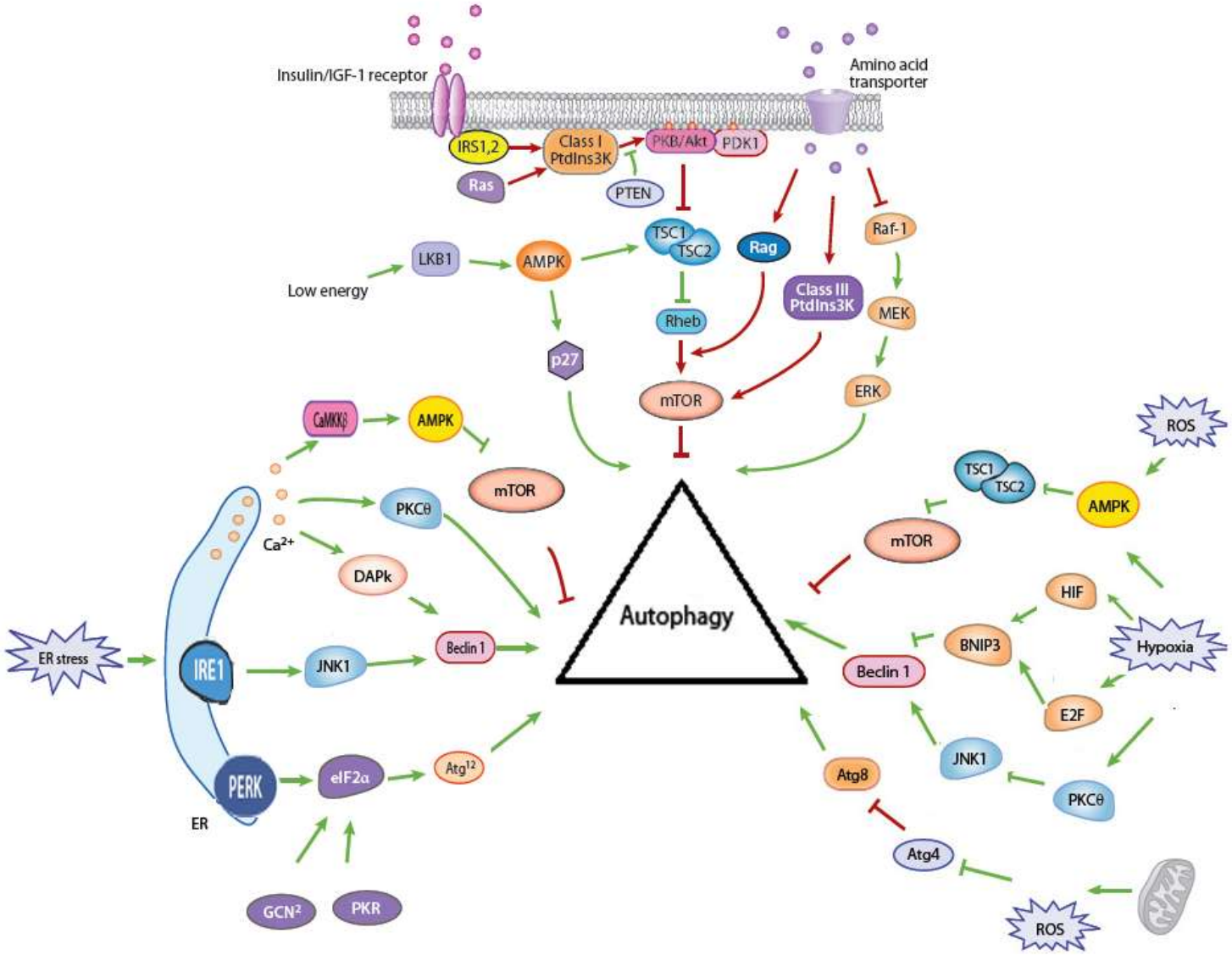


Figure 1.2 Upstream signaling pathways of autophagy

(adapted from He et al., Annu Rev Genet, 2009)

The major pathways are discussed below.

1.1.2.1 mTOR complex 1 pathways

There are two mTOR complexes in mammals. mTOR complex 1 (mTORC1), comprising of mTOR, raptor, mLST8 (also known as GβL), is sensitive to inhibition by Rapamycin. Rapamycin stimulates autophagy by inhibiting mTORC1 even in the presence of nutrients (Noda and Ohsumi, 1998). In contrast, mTORC2 which consists of mTOR, sin1, rictor and mLST8, is not known to be resistant to Rapamycin and its involvement in autophagy is largely unknown.

Activation of mTORC1 leads to phosphorylation of Ulk1 and Atg13 at the inhibitory sites, blocks the Ulk1 complex's role in autophagosome induction and thus suppresses autophagy. Besides this, mTORC1 also suppresses autophagy through phosphorylation of Tap42, an activator of the catalytic subunits of PP2A (the serine/threonine protein phosphatase 2A) which is a negative regulator of autophagy (Yorimitsu et al., 2009). Activation of mTORC1 also leads to phosphorylation of S6K1 and 4E-BP1 which promotes protein translation and transcription of many genes via modulation of URI (unconventional prefoldin RPB5 interactor) and transcription factors, e.g. STAT1 and STAT3 (Wullschleger et al., 2006).

Withdraw of growth factors alone is sufficient to induce autophagy (Lum et al., 2005a). Growth factors such as insulin and insulin-like growth factors exert their

regulatory fuction via modulating mTOR activity. Ligation between insulin and its receptor leads to activation of Class I PI3K and generation of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) which result in the full activation of Akt by PDK1 and mTORC2 (Jacinto et al., 2006; Sarbassov et al., 2005). Activated Akt in turn phosphorylates TSC2 and leads to TSC1-TSC2 complex's dissociation, maintaining the GTP-Rheb (Inoki et al., 2002; Tee et al., 2002; Tee et al., 2003) which activates mTORC1 subsequently (Inoki et al., 2003; Li et al., 2004; Long et al., 2005). Additionally, activated Akt also phosphorylates PRAS40 and thus dissociates the inhibitory 14-3-3 from mTORC1 (Vander Haar et al., 2007). PTEN reverses PIP3 production and sequentially decreases the downstream PKB/Akt signaling that negatively regulates autophagy (Arico et al., 2001). Akt inactivation also leads to activation of the forkhead box O (FoxO) transcription factors 3 (FoxO3) which amplifies a variety of autophagy-related genes including LC3, BNIP3, Atg4, and hVps34 (Mammucari et al., 2007; Zhao et al., 2007).

Besides this, Ras is also involved in the growth factor pathway. In NIH3T3 mouse embryonic fibroblasts (MEFs), growth factor receptor (tyrosine kinases) activated Ras decreases autophagy via the Class I PtdIns3K, but not through Raf-1 (Furuta et al., 2004).

Recent studies are elucidating the mechanisms how nutrients, especially essential amino acids, activate mTORC1. Rag proteins, the Ras-related small GTPases, activate TORC1 in response to amino acids in *Drosophila* and mammals (Kim et al., 2008a; Sancak et al., 2008) through translocation of mTORC1 which facilitates the interaction between TORC1 and its activator Rheb (Ras homolog enriched in brain) (Sancak et al., 2008). The observation that single amino acid fails to activate mTORC1 (Hara et al., 1998) can be explained by the fact that in the upstream of Rag protein, SLC1A5 and SLC7A5, the

bidirectional transporter, simultaneously promote the efflux of L-glutamine and the influx of L-leucine/EAA across cell membranes (Nicklin et al., 2009). Sancak et al. recently identified that Ragulator - a complex including the MAPKSP1, ROBLD3, and c11 or f59 genes, associates with the Rag GTPases and it is essential for the translocation of mTORC1 to lysosomal membranes in response to amino acids (Sancak et al., 2010). In addition, p62 was discovered to be interacting with raptor thus it is an integral part of the mTORC1 complex. p62 was proved to be responsible for the activation of S6K1 and 4EBP1 and its interaction with mTOR and raptor is amino-acid dependent. The interaction and colocalization of p62 and Rags at the lysosomal compartment is essential for the interaction of mTOR with Rag GTPases and subsequent translocation of the mTORC1 complex to the lysosome (Duran et al., 2011).

On the other hand, other studies found that amino acid also could activate mTOR and subsequent autophagy inhibition through the effect of class III PtdIns3K (Byfield et al., 2005; Nobukuni et al., 2005) which makes class III PtdIns3K's effect on autophagy a bit confusing.

1.1.2. 2 The Ras-cAMP-PKA pathway

In glucose sensing, the Ras-cAMP-PKA pathway plays a crucial role. Without starvation, the active small GTPases Ras1 and Ras2 enhance generation of cAMP which binds to Bcy1 and releases its inhibitory effect on PKA. In yeast, constitutively activated Ras-cAMP-PKA pathway suppresses autophagy induced by TOR inhibition though

phosphorylation of PKA's substrate Atg1 (Budovskaya et al., 2004; Budovskaya et al., 2005; Schmelzle et al., 2004).

1.1.2.3 AMPK signaling pathway

AMP-activated protein kinase (AMPK) is activated by an increased ratio of AMP/ATP via its upstream kinase LKB1 (Corradetti et al., 2004; Hardie et al., 1998). Activated AMPK leads to direct phosphorylation of TSC2 which stabilizes the TSC1-TSC2 complex, thereby maintains GDP-Rheb and eventually suppresses mTORC1 (Inoki et al., 2003b). Alternatively, AMPK also phosphorylates raptor, facilitates sequestration of raptor by 14-3-3, thus suppresses mTORC1 activity (Gwinn et al., 2008). In addition, the LKB1-AMPK pathway phosphorylates and activates p27kip1, which is required for autophagy induction in response to bioenergetic stress during growth factor withdrawal and nutrient deprivation (Liang et al., 2007). The yeast homolog of mammalian AMPK, Snf1, also positively monitors autophagy, but through an independent mechanism involving Atg1 (Wang et al., 2001). As previously mentioned, Ulk1 is also a substrate of AMPK in mammal. When cells are fed, it can form a complex with and confine portions of Ulk1 to suppress autophagy (Shang et al., 2011) whereas upon starvation, AMPK can phosphorylate raptor to lift the inhibitory effect of mTOR on Ulk1 (Lee et al., 2010; Egan et al, 2011). Also, AMPK was shown to be negatively feedbacked by Ulk1 and Ulk2 (Löffler et al., 2011).

1.1.2.4 Stress response pathways

1.1.2.4.1 Hypoxia

Hypoxia, an atmosphere in which levels of oxygen is at or below 1% (Warnecke et al., 2003; Persson et al., 2011), induces autophagy in mammalian cells. Hypoxia-inducible factor-1 (HIF-1) is the primary transcription factor acutely induced by hypoxic conditions which helps to minimize deleterious effects caused by O_2 deficiency. In MEFs, hypoxia induced mitophagy is dependent on HIF-1 and its downstream target BNIP3 and BNIP3L (Zhang et al., 2008a; Sandoval et al., 2008; Schweers et al., 2007). BNIP3 competes with Beclin 1 for Bcl-2 binding and thus decreases Bcl-2's inhibitory effect on autophagy. Interestingly, in tumor cells, hypoxia induced bulk autophagy is not dependent on the HIF-1 pathway but the AMPK-mTOR and PKCδ -JNK1 pathway (Papandreou et al., 2008; Vasseur et al., 2009; Chen et al., 2008a). Also, TOR, the eIF4F complex and mRNA translation are inhibited by hypoxia (Brugarolas et al., 2004; Reiling and Hafen, 2004). However, autophagy seems to have dual functions in hypoxia, enhanced mitophagy reduces reactive oxygen species (ROS) and protects cell integrity, while in several cancer cell lines, prolonged hypoxia mediates autophagic cell death (Azad et al., 2008).

1.1.2.4.2 ER stress

ER stress is a common inducer of autophagy which can be accumulated by glucose deprivation, hypoxia, oxidative stress and Ca^{2+} efflux. In yeast, ER stress-induced autophagy is mediated by Ire1 (inositol-requiring kinase 1), an ER transmembrane protein

with a cytosolic endoribonuclease domain. When unfolded proteins accumulates in the ER, an ER-specific member of the heat shock protein 70 family, Grp78/BiP, activates the cytosolic endonuclease activity of Ire1, triggers the splicing of its substrate Hac1 and then subsequently activates transcription of Hac1's target genes involved in protein modification/folding (Ma and Hendershot, 2001). Similarly, in mammals, siRNA downregulation of Grp78/BiP inhibits autophagosome formation induced by ER stress (Li et al., 2008). The mammalian signaling network in response to ER stress is more complex including at least three distinct downstream pathways: IRE1-JNK pathway (Ogata et al., 2006), ATF6 (activating transcription factor 6) pathway, and PERK (RNA-dependent protein kinase-like ER kinase) - eIF2α pathway (Kouroku et al., 2007). ER stress also increases the intracellular Ca^{2+} level and activates Calcium-activated calmodulin-dependent kinase kinase-β (CaMKKβ) which activates AMPK (Høyer-Hansen et al., 2007). Also, increased Ca^{2+} levels leads to phosphorylation of PKCθ that induces LC3 conversion and autophagy (Sakaki et al., 2008).

1.1.2.4.3 ROS and oxidative stress

ROS, the destroyer of organelles such as mitochondria, is another common inducer of autophagy, especially, mitophagy (Narendra et al., 2008; Priault et al., 2005). ROS can cause oxidation of a conserved Cys81 on Atg4 and thereby inhibit Atg4 protease activity and promote lipidation of Atg8/LC3 which contributes to autophagy (Scherz-Shouval et al., 2007).

1.1.3 Biological functions of autophagy

As a cellular degradative pathway in eukaryotes which relieves cells from various types of stresses, autophagy has a variety of physiological and pathological roles such as starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression and antigen presentation (Mizushima et al., 2005).

Among them, the pro-survival or pro-death function of autophagy has been studied extensively and the conclusion remains highly controversial. So far, there is still not a general conclusion about autophagy's role in PCD, it can be pro-survival or pro-death, or even just a bystander as the cell death mechanisms when autophagy is also present are cell line specific and highly dependent on stimulus (Kourtis and Tavernarakis, 2009; Kroemer and Levine, 2008; Levine and Yuan, 2005).

For example, the relationship between autophagy and apoptosis is complex as a number of inducers such as etoposide (Feng et al., 2005) and ceramide (Pattingre et al., 2009) can activate apoptosis and autophagy simultaneously and the fact that there is a lot of crosstalk between these two processes that maintain cell integrity and homeostasis by eliminating damaged and aged cells or spent cellular components.

1.1.3.1 The pro-survival function of autophagy

1.1.3.1.1 Autophagy suppresses apoptosis

In spite of its pro-death function, autophagy also has been proved to be a protective mechanism in various cellular settings to help cell survive through stress times, especially under nutrient deprivation. For example, autophagy can decrease apoptosis induced by ER Stress (Bernales et al., 2006; Ding et al., 2007a; Ogata et al., 2006) and DNA damage (Ito et al., 2005; Paglin et al., 2001).

It is simple to understand that autophagy helps cell cope with tough conditions by providing recycled energy and nutrients. Similar to starvation induced autophagy which digests bulk cytosolic components to provide energy and metabolite supply, DNA-damage induced autophagy also maintains the intracellular ATP level and thus promotes cell survival (Lum et al., 2005b; Katayama et al., 2007). This notion is also supported by in vivo models, the lack of survival of Atg5/Atg7 defective mouse through the neonatal period again demonstrates the crucial pro-survival function of autophagy (Komatsu et al., 2005; Kuma et al., 2004).

Autophagy also acts as a housekeeper to help cell specifically eliminate spent harmful organelles such as damaged mitochondria by ROS which can serve as triggers for apoptosis (Lemasters, 2005; Ravikumar et al., 2006). Misfolded protein aggregates which cause ER stress are also cleaned by autophagy (Ding et al., 2007b).

1.1.3.1.2 Autophagy suppresses necrosis

Numerous studies have reported that autophagy acts as a protective mechanism in necrosis and unlike apoptosis, it does not require energy supply at its late stage. For example, knockdown of Atg5 in the Bax-/- & Bak-/- MEF cells when growth factor is

withdrawn causes the cells to undergo rapid necrosis (Lum et al., 2005a). Upon the signal of metabolic stress, suppression of autophagy by activated Akt or disrupted Beclin 1 sensitizes cancer cells to necrosis (Degenhardt et al., 2006) as autophagy maintains genome integrity by eliminating the harmful effect of DNA damage in such cells context (Karantza-Wadsworth et al., 2007). Colell et al. also found that autophagy and upregulation of glycolysis induced by GAPDH protected cells from necrosis (Colell et al., 2007).

1.1.3.2 The pro-death function of autophagy

1.1.3.2.1 Autophagy promotes apoptosis

Apoptosis is attenuated when autophagy is suppressed by 3-methyladenine (3-MA) or knockdown of Atg7 and Beclin 1 in CD4+ T lymphocytes and leukemia cell lines (Espert et al., 2006). Autophagy is induced by cannabinoid via ER-stress response which is also the upstream signal of cannabinoid-induced apoptosis in cancer cell lines (Salazar et al., 2009). The pro-death function of autophagy may be executed through various pathways such as Akt and ERK, Atg5, Bcl-2 family members. Akt is able to inhibit apoptosis via phosphorylation of Bad and suppression of transcription factor FoxO (Brunet et al., 1999; Datta et al., 1997). Likewise, ERK also can phosphorylate Bad and activate another transcription factor CREB that amplifies anti-apoptosis proteins (Ballif and Blenis, 2001). Thus, during autophagy, the suppression of Akt and ERK may lead to promotion of apoptosis. The calpains-cleaved form of Atg5, one key member of the core autophagy

machinery, has been found to translocate from cytosol to mitochondria to promote cytochrome c release and intrinic apoptosis pathway (Yousefi et al., 2006).

It was proposed that the anti-apoptosis Bcl-2 proteins such as Bcl-2, Bcl-xL, and Mcl-1 could bridge the pro-death function of autophagy in apoptosis as Beclin 1 may sequester anti-apoptotic Bcl-2 proteins. However, the Beclin 1-bound Bcl-2 family proteins are still fully anti-apoptotic (Ciechomska et al., 2009). Qu et al. found that in the embryoid bodies defective in autophagy, the induction of apoptosis did not require autophagy but in the late stage of apoptosis, the energy synthesised from autophagy was crucial for the completion of the apoptosis machinery (Qu et al., 2007; Kroemer and Levine, 2008).

1.1.3.2.2 Autophagy itself serves as a death mechanism: autophagic cell death

Paradoxically, although autophagy plays primarily a pro-survival function, it can also accelerate cell death during necrosis. Autophagic cell death describes a cell death mechanism that is dependent on autophagy but independent on caspases, in which autophagy is the executioner rather than just a bystander (Kroemer et al., 2009). And it was evidenced in several in vitro models, Shimizu et al. found that in response to DNA damage caused by etoposide and staurosporine, the Bax-/- & Bak-/- MEF cells underwent a non-apoptotic cell death dependent on autophagy as decreasing Atg5 or Beclin 1 could preserve cell viability (Shimizu et al., 2004). Another group identified in several cell lines such as fibrosarcoma L929 cells, the non-apoptotic cell death induced by zVAD, a pan-

caspase inhibitor, was autophagy dependent as silencing Beclin 1 and Atg7 ablated cell death, but futher examination of the detailed mechanisms are needed (Yu et al., 2004).

Yet, the evidences for this kind of cell death in vivo systems are still not convincing, for example, in D. discoideum lacking Bcl-2 and caspases, under combinational treatment of starvation and differentiation-inducing factor (DIF), cell death was observed with autophagic structures (Levraud et al., 2003; Roisin-Bouffay et al., 2004), however, autophagy might not be the exact death mechanism as necrosis still existed when autophagy was abrogated by disrupting Atg1 (Kosta et al., 2004). Similarly, in Drosophila, although autophagy induction accelerated developmental degradation in salivary glands, inhibition of autophagy still caused a delayed histolysis (Berry and Baehrecke, 2007). Thus it is still not clear whether autophagy was the cell death executioner.

1.1.4 Autophagy in health and disease

Besides its function in normal physiological conditions, autophagy has been shown to possess diverse functions under pathological conditions and contribute to the pathogenesis of many diseases including cancer, neurodegenerative diseases, metabolic diseases, immunodeficiency diseases, and infectious disease. Figure 1.3 is a brief summary of the relationship between autophagy and human disease (Mizushima et al., 2008).

For example, upregulation of autophagy has been proposed to be a useful therapeutic strategy for neurodegenerative disease treatment, based on the evidence that autophagy is the major clearance pathway for many neurodegenerative disease-associated

mutant proteins and the discovery that neurodegenerative diseases are promoted when autophagy genes are knock out in neural tissues (Rubinsztein, 2006; Martinez-Vicente and Cuervo, 2007).

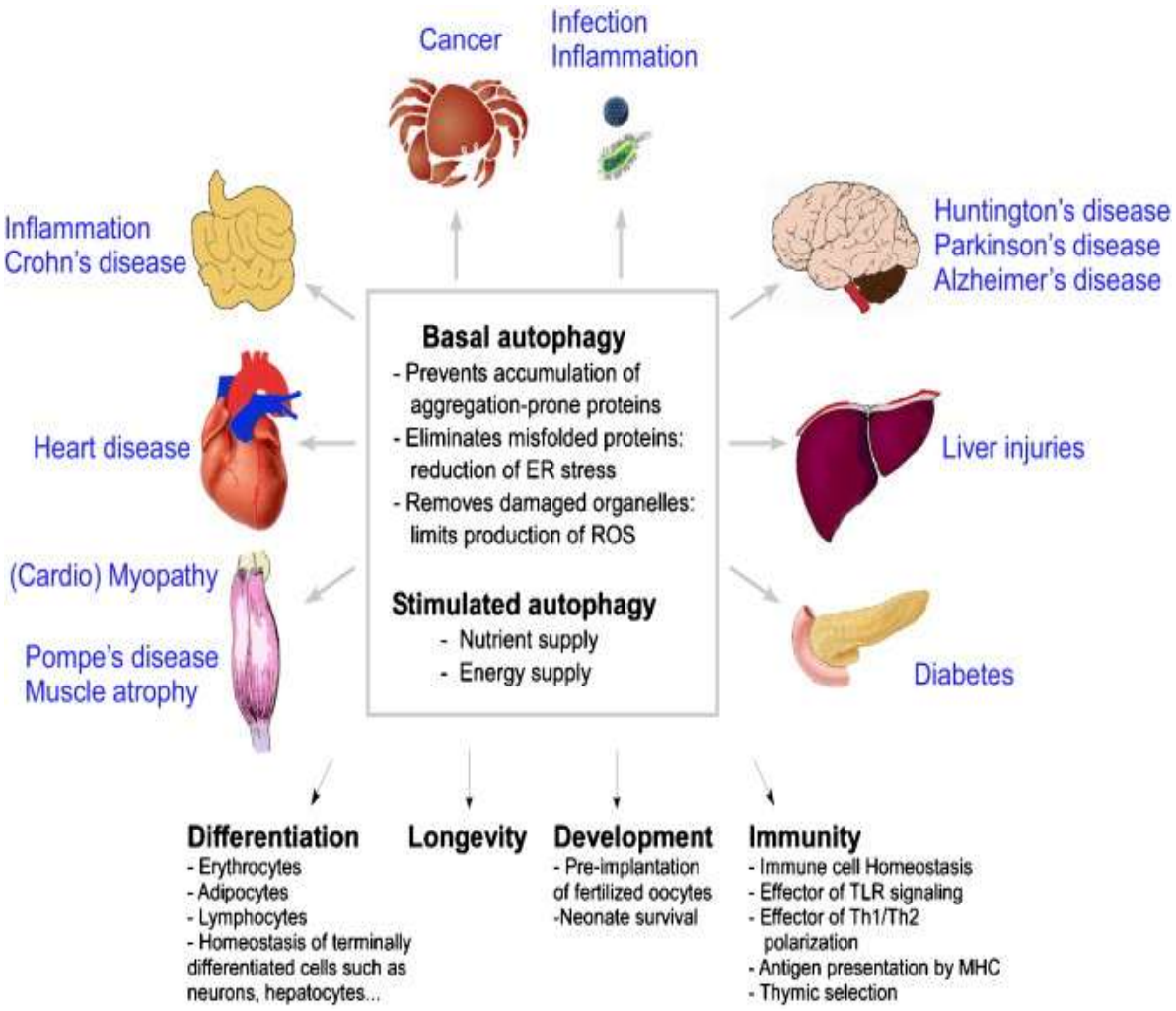


Figure 1.3 The role of autophagy in human diseases

(adapted from Beau et al., Int J Biochem Cell Biol, 2011)

1.1.4.1 Autophagy in cancer

Being a multifaceleted process, the deregulation of autophagy is observed in a series of pathological conditions such as aging, infectious diseases, neurodegenerative diseases, metabolic diseases and most importantly, cancer.

However, autophagy's role in cancer is a little complicated, a fundamental paradox is that it has both positive and negative effects on tumorigenesis as autophagy can act to promote either cell survival or cell death depending on the specific conditions.

1.1.4.1.1 The anti-cancer function of autophagy

1.1.4.1.1.1 Evidence supporting the anti-cancer function of autophagy

Although autophagy might have dual roles in tumorigenesis, the balance has tilted more to the notion that autophagy is a tumor suppressive mechanism. Evidence includes:

(1).Several tumor cells have relative weak autophagic activity (Schwarze et al., 1985; Knecht et al., 1984).

(2). Atg genes are frequently observed to be deleted in various kinds of human malignancies (Levine, 2007a).

(3). Several Atg genes act as tumor suppressors. For example, loss of Beclin 1, which is frequently deleted in human breast, ovarian, and prostate cancer, increases tumorigenesis in mice and tumor growth is inhibited during Beclin 1 overexpression

(Liang et al., 1999; Qu et al., 2003; Yue et al., 2003); Atg4c has also been proved to be a tumor suppressor (Marino et al., 2007). Overexpression of Atg5 inhibits tumor growth and stimulates apoptosis (Yousefi et al., 2006).

(4). The upstream signaling pathways of autophagy include many oncogenes and tumor suppressor genes. Oncogenes like Class I PI3K, Akt or mTOR that inhibit autophagy are activated, while tumour suppressor genes like p53, PTEN, DAPK1, TSC1/TSC2, LKB1/STK11 that stimulate autophagy are mutated or epigenetically silenced in tumors (Botti et al., 2006).

(5). Autophagy contributes to the integrity of genome as immortalized epithelial cells with loss of Beclin 1 or Atg5, display increased DNA damage, gene amplification, and tumorigenicity (Mathew et al., 2007).

1.1.4.1.1.2 Possible mechanisms of the anti-cancer function of autophagy

Autophagy, as a homeostatic process, removes toxic cellular compounds such as spent organelles, misfolded and aggregated proteins which can contributed to various stresses, can help to eliminate the cytotoxic microenvironment which will favor tumorigenesis (Wang et al., 2010). In addition, autophagy helps to prevent the genome mutation and chronic tissue damage which are common causes for cancer development (Chen et al., 2011).

Recent studies have revealed that p62, the selective autophagy substrate protein, might be a possible bridge linking autophagy to tumorigenesis. Defective autophagy will leads to accumulation of p62 which will cause liver damage and facilitate the tumor

development in mice (Komatsu et al., 2007; Mathew et al., 2009a). Indeed, p62 accumulation was proved to be able to abrogate NF-kB activation which will casue cell death and inflammation which will trigger tumorigenesis (Maeda et al., 2005). Additionally, p62 accumulation also activates the expression of transcription factor NF-E2–related factor 2 (Nrf2) and its target genes whose constitutive activation are associated with increased cancer incidence (Komatsu et al., 2010; Lau et al., 2010; Kensler and Wakabayashi, 2010).

1.1.4.1.2 The pro-cancer function of autophagy

On the other hand, autophagy can also favor tumorigenesis by preventing cells from cell death under nutrient deprivation and hypoxia. The protective effects of autophagy on tumor cell's resistance to chemotherapy were also reported (Abedin et al., 2007; Carew et al., 2007). For example, overexpression of the oncogenic Ras gene will result in tumorigenesis, interestingly, upregulation of Ras also dramatically upregulates basal autophagy which is esstential for cell survival under various stress conditions. In human cancer cells in which Ras is activated, genetic suppression of autophagy results in cell death or growth arrest (Guo et al., 2011; Lock et al., 2010; Yang et al., 2011).

1.1.4.1.3 Targeting autophagy as a novel cancer therapeutic strategy

As basal autophagy is employed by many cancer cells as a protective mechanism against hostile microenvironment, once tumorigenesis is initiated, inhibiting autophagy

might be a novel therapeutic strategy to treat cancers (Chen et al., 2011). As mentioned above, suppression of autophagy in Ras-driven cancers decreases tumorigenesis, pharmacological inhibition of autophagy also sensitizes tumor cells to cell death. For instance, CDP, the autophagy late stage inhibitor, can kill Myc-expressing mouse cells in a p53-dependent manner (Maclean et al., 2008; Amaravadi et al., 2007). It was proposed that cancer cells employ autophagy not only for releasing metabolic stress but also therapeutic stress. In this regard, many ongoing clinical trials use HCQ as an autophagy inhibitor for the treatment of a series of advanced diseases (Amaravadi et al., 2011; White and Dipaola, 2009).

1.1.5 Methods for monitoring autophagy

Being a dynamic, multi-stage process, autophagy can be monitored at various steps. During the past decade which witnessed the dramatic increase of autophagy related research projects, a series of related techiniques are established and well recorded in some reviews (Mizushima et al., 2010; Klionsky et al., 2007).

Especially, regarding to the increased number of autophagosome – the most common marker of autophagy, the most critical issue is to distinguish the enhanced autophagic activity from blockage of late stage of autophagy. “Autophagic flux” assay which employs lysosome inhibitor to block the late stages of autophagy was proposed to solve this problem (Mizushima et al., 2010). For example, in the LC3 turnover assay, with introduction of lysosomal inhibitor CDP, the LC3-II degradation will be blocked, thus the difference in the amount of LC3-II between groups treated with or without CDP

represents the genuine amount of LC3-II that delivered to autolysosome for degradation. The starvation-induced difference between groups treated with or without CDP is larger than the difference between control groups (cultured in full medium) treated with or without CDP, indicating that autophagic flux is increased under starvation treatment (Mizushima et al., 2010).

So, a combination of approaches is suggested to be a better way to access the autophagic activity. Table 1.2 is the summary of the classical methods used for monitoring mammalian autophagy.

Table 1.2 Methods for monitoring mammalian autophagic activity

Methods	Technique	Principle	Application
Diagnose the autophago-some and autolysosome	electron microscopy	organelles for execution of autophagy, both autophagosomes and autolyosomes are increased in numbers indicates autophagy activation and an increased autophagosome numbers without a change in autolysosome numbers indicates a block in autophagosome maturation	monitoring autophagy in vitro
Observing the number and distribution of GFP-LC3 puncta	fluorescence microscopy	LC3-Is a specific marker for autophagosome, upon lipidation, the cytosolic LC3-I will become LC3-II and translocate to the membranes of autophagosome. The differences in the amount of GFP-LC3-II puncta between samples in the presence and absence of lysosomal inhibitors reflect the autophagic activity.	monitoring autophagy in vitro and in vivo
Observing the conversion from LC3-I to LC3-II, GFP-	western blot	LC3-Is a specific marker for autophagosome, upon lipidation, the cytosolic LC3-I will become LC3-II and translocate to the membranes of autophagosome.The differences in the amount of	monitoring autophagy in vitro

LC3-I to GFP-LC3-II		LC3-II or free GFP fragment between samples in the presence and absence of lysosomal inhibitors reflect the autophagic activity.	
Observing the degradation of p62	western blot	p62 direct binds to LC3,thus selectively incorporate into the autophagosome and efficiently degraded by autophagy	monitoring autophagy in vitro
Observing quenching of GFP in GFP-labeled autophagic substrates such as LC3	fluorescence microscopy	in systems with both GFP-LC3 and RFP-LC3, GFP-LC3 will be degraded in autolysosome while RFP-LC3 is more stable, autophagosomes and autolysosomes are thus labeled with yellow (RFP and GFP) and red (RFP only), respectively. Both yellow and red puncta are increased indicates increased autophagic flux; only yellow puncta are increased without a concomitant increase in red puncta indicates autophagic flux blockage at late stage.	monitoring autophagy in vitro and in vivo
Measuring bulk degradation of long -lived proteins	radio-activity assay	cells are cultured with isotope-labeled amino acids to label long-lived proteins, followed by a short incubation period without isotope-labeled amino acids to labeled short-lived proteins. the tricholoracetic acid-soluble radioactivity in the culture supernatant is from the degraded long-lived protein and is quantified as an indicator of autophagy.	monitoring autophagy in vitro

1.1.6 MicroRNAs

In the past decades, interest in the detection and function of small RNA molecules has rapidly expanded, resulting into two hot research fields: small interfering RNAs (siRNAs), which can silence the expression of specific genes at the post-transcriptional level; and microRNAs (miRNAs), which have been shown to regulate various target gene

expression in many organisms. Both miRNAs and siRNAs are 18–30 nucleotides in length and their processing pathways have a lot in common (Zeng et al., 2003).

miRNAs are a class of evolutionary conserved, small (18 to 25 nucleotides in length), endogenous non-coding RNA molecules which negatively regulate gene expression either by transcript degradation or translation repression at the transcriptional and post-transcriptional level (Ambros, 2004). miRNAs play important roles in many processes such as development, cell proliferation, differentiation and death. They accomplish their roles by binding to the 3' untranslated region (3'-UTR) of target messenger RNA (mRNA), the regulatory results are depending on the degree of complementarity in base pairing. First discovered by Ambros and colleagues in 1993 in C.elegans (Lee et al., 1993; Wightman et al., 1993), miRNAs are shown to be abundantly expressed in viruses (Sullivan et al., 2006), plants (Mallory and Vaucheret, 2006) and animals (Stefani and Slack, 2008). To date, 16772 miRNAs have been found in 153 species (miRBase Release 17, http://www.mirbase.org/). The sequence and function conservation of many miRNAs between distantly related organisms indicates that this class of small RNAs is an integral part of essential cellular processes (Pasquinelli et al., 2000). Human beings are able to express about 1424 miRNAs (http://www.mirbase.org). These miRNAs are expected to be able to regulate more than 30% of all protein–coding genes (Filipowicz et al., 2008).

1.2.1 miRNA biogenesis

Encoded in the genome, miRNAs are transcribed by RNA polymerase II as primary transcripts (pri-miRNAs) that are typically 3 to 4 kilobases long single-stranded RNAs

with 5'cap, 3' poly(A) tail and complicated secondary structures (Lee et al., 2004; Saini et al., 2007). A microprocessor complex comprising the nuclear RNase III, Drosha, and the double-stranded RNA binding protein, Pasha/DGCR8 (Gregory et al., 2004; Han et al., 2004) then processes the pri-miRNAs into one or more precursor-miRNAs (pre-miRNAs) of ~70-nucleotides. Pre-miRNAs are then actively exported to the cytoplasm through exportin-5 together with RAN-GTPase (Bohnsack et al., 2004; Yi et al., 2003), then another RNase III, Dicer, further processes them into ~22-nucleotide mature miRNAs,which are double stranded (miR duplex). The miRNA strand in the miRNA duplex is incorporated into the multi-protein RNA-induced silencing complex (miRISC) whereas the other stand, the complementary strand (miR* strand) is degraded (Hutvagner et al., 2001; Ketting et al., 2001). Thermodynamic stability of the strand probably determines the choice of strands to be incorporated into miRISC (Khvorova et al., 2003). The general processing pathway of miRNAs is shown in Figure 1.4.

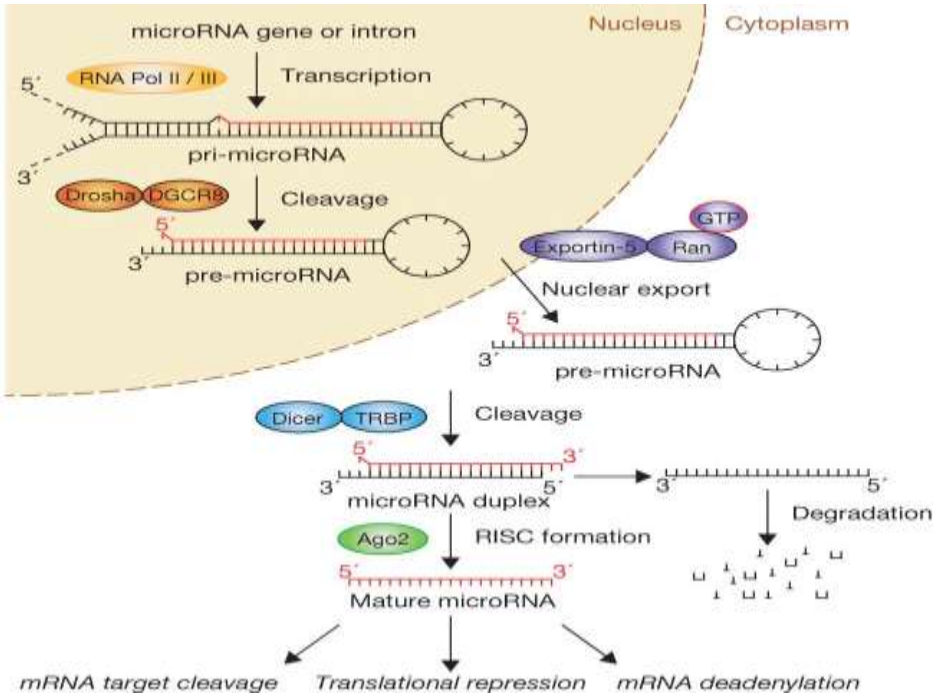


Figure 1.4 miRNA biogenesis pathway

(adapted from Winter et al., Nature Cell Biology, 2009)

1.2.2 Regulatory mechanisms of miRNAs

miRNA and their target genes form a very complex regulatory network since each individual miRNA can potentially regulates hundreads of target genes (Lim et al., 2005), and each target gene can be cooperatively controled by a series of miRNAs (Lewis, 2003).

For each specific interaction, the seed region, an area on the mature miRNA which typically includes nucleotides 2-7 in the 5' end that binds to the 3' untranslated region (3' UTR) of the mRNA, plays a crucial role in determining the regulatory effect. miRNAs binding with imperfect complementarity to the 3'UTR of target mRNA usually repress the

translation of the target mRNAs while miRNAs with perfect complementarity can degrade the corresponding target mRNAs (Meister and Tuschl, 2004).

To date, the mechanism by which miRNAs cause blockage of target gene translation is still not clear. However, a number of pathways that contribute to this process have been revealed. Wu et al. have found that most target mRNAs underwent deadenylation and subsequent destabilization (Wu et al., 2006). And specific miRNAs together with their target mRNAs have been found to be stored in the processing bodies (P bodies) which usually are the cytoplasmic storage sites for proteins involved in mRNA decay, translational repression and non-translating mRNAs (Chan and Slack, 2006). In addition, the argonaute family proteins, Ago2 has been detected in P bodies in a miRNA-dependent manner (Sen and Blau, 2005). Future investigations are required to elucidate this improtant gene regulation mechanism.

1.2.3 Biological functions of miRNAs

Cross-species comparisons demonstrate that miRNAs are evolutionarily conserved and play important roles in a wide array of physiological and pathological processes such as proliferation, apoptosis, differentiation, immunity and angiogenesis. Thus miRNAs are involved in a series of diseases including cancer, cardiac diseases, gastrointestinal diseases and diabetes (Huang et al., 2011).

Evidences for the important roles of miRNAs in tumourigenesis including the following several aspects. About 50% of miRNA genes are in cancer-associated genomic regions (Calin et al., 2004; Sevignani et al., 2006). Deregulated miRNA expression

profiles are associated with many cancer types; more than one-quarter of all known human miRNAs are reported to be deregulated in at least one cancer type (Wang et al., 2009). miRNA expression profiling of specific cancer can be used to identify new biomarkers of tumor subtype. For example, miR-103/107 is important diagnostic and prognostic markers of esophageal cancer (Guo et al., 2008a). The expression of a subset of miRNAs (miR-21, miR-155, miR-181b, miR-221, miR-222) was found to be consistently up-regulated, whereas another subset of miRNAs (miR-143, miR-145, let-7/miR-98 cluster) was consistently down-regulated across different cancer types, suggesting their involvement in common cellular pathways whose deregulation may lead to tumourigenesis (Wang et al., 2009). Emerging evidences have demonstrated that these deregulations indeed implicate miRNAs' roles in tumourigenesis, with the fact that those miRNAs commonly down-regulated in cancers are often tumor suppressors while those commonly up-regulated are often oncogenes. What is more, overexpression or knockdown of some deregulated miRNAs would result in abnormal cell development.

Of several miRNAs that are implicated in cell proliferation and apoptosis, some miRNAs, such as miR-29b and miR-15-16, influence only the apoptotic pathway, whereas others including let-7/miR-98 and miR-17-92 may play roles in both the apoptotic and cell-proliferation pathways (Secombe et al., 2004; Adhikary et al., 2005), indicating the complexity of the miRNA regulatory mechanism. Noticeably, even very similar, closely related miRNAs may have different impacts on oncogenesis. For example, miR-199a, was shown to target several oncogenes such as IKKbeta (Chen et al., 2008b), MET and ERK (Kim et al., 2008b); while miR-199b may play a house-keeping role and do not contribute to oncogenesis as it is seldom reported to be deregulated in cancer (Wang et al., 2009).

Although functional studies with relevance to cancer and cell death are still limited compared to the large amounts of miRNAs, dozens of cellular targets of miRNAs have been validated. In the tumor-suppressive miRNA group, for instance, let-7/miR-98 family negatively regulate HMGA2 (Peng et al., 2008), Ras (Johnson et al., 2005), IGF2BP1 (Lu et al., 2007), c-Myc (Sampson et al., 2007), NF2 (Meng et al., 2007a), LIN28B (Guo et al., 2006) ,CDC25a, CDK6, Cyclin D, CCND2 (Johnson et al., 2005), and caspase-3 (Tsang and Kwok, 2008). miR-34a and miR-34b/c enhance p53 function by targeting signal molecules such as CDK4, E2F3, Bcl2, SIRT, N-Myc, c-Met, CDK6, and c-Myc (Hermeking, 2009). The miR-15-16 cluster target Bcl-2 (Cimmino et al., 2005) and NGN3 (Joglekar et al., 2007); and miR-29b also negatively regulates Mcl-1 (Mott et al., 2007), a member of the Bcl-2 family. In contrast, oncogenic miRNAs also form their complex signal network, for example, miR-21 targets PTEN (Meng et al., 2007b), TPM1 (Zhu et al., 2007), PDCD4 (Lu et al., 2008), MASPIN/SERPINB5 (Zhu et al., 2008), MARCKS (Li et al., 2009a), HNRPK (Papagiannakopoulos et al., 2008), and RECK (Zhang et al., 2008); miR-210 decreases caspase activation which leads to apoptosis (Kulshreshtha et al., 2007) and regulates EPHRIN-A3/EFNA3 (Fasanaro et al., 2008); while miR-155 inhibits TP53INP1 expression and thus reduces apoptosis (Gironella et al., 2007). In addition, E2F1 (Sylvestre et al., 2007), TSP1, CTGF (Dews et al., 2006), PTEN (Xiao et al., 2008), BIM, Rb2 (Wang et al., 2008a), p21(Fontana et al., 2008) , AIB1 (Hossain et al., 2006), AML1, M-CSFR (Fontana et al., 2007) are regulated by miR-17-92 cluster. Interestingly, some miRNAs have dual roles in regulating both cell proliferation and apoptosis during cancer development. For instance, miR-224 was reported to sensitize cells to apoptosis

through the apoptosis inhibitor API-5, yet, it also increased cell proliferation (Wang et al., 2008b).

With the potential of being used as therapeutic drugs that prevent cancer by directly targeting oncogene transcripts, miRNAs are receiving more and more attention these years.

1.2.4 Involvement of miRNAs in autophagy

Despite the large amount of publication elucidating autophagy regulatory mechanisms, so far, research focusing on the relationship between autophagy regulatory elements and miRNAs, espeically Atg genes and miRNAs are still limited. And there is no publication directly elucidating the miRNA expression pattern during autophagy induction and execution. Hopefully, this area will gain more and more attention in the following years.

1.2.4.1 miRNAs directly regulate Atg genes

1.2.4.1.1 miRNAs target Beclin 1

Zhu et al. demonstrated that Beclin 1, the mammalian homologue of yeast Atg6 was negatively regulated by miRNA-30a in human breast and lung cancer cells in which the Beclin 1 expression was usually deregulated. Tumor cells treated with the miR-30a mimic showed a decreased expression of Beclin 1 mRNA and protein, whereas tumor cells treated with the miR-30a antagomiR showed the opposite result. Through dual luciferase

reporter assay, they confirmed that the miR-30a binding sequence is in the 3'-UTR of Beclin 1. Inhibition of Beclin 1 expression by miR-30a mimic blunted activation of autophagy induced by Rapamycin (Zhu et al., 2009).

1.2.4.1.2 miRNAs target LC3-II

Recently, Xiao et al. found that during ischemia-reperfusion (IR) injury induced autophagy, miR-204 was downregulated while its target gene LC3-II was upregulated. Upon transfection of miR-204 mimic, LC3-II protein was attenuated while during AMO-204 (miR-204 inhibitor) transfection, LC3-II protein was up-regulated. Interestingly, They found miR-204 only targeted LC3-II under such condition, LC3-I was not a target of miR-204 (Xiao et al., 2011).

1.2.4.1.3 miRNAs target Atg4d

Frankel et al. has proposed that miR-101, a tumor suppressive miRNA, is a potent inhibitor of basal, etoposide- and Rapamycin-induced autophagy, and this miRNA may execute its inhibitory function via suppressing multiple autophagy related target genes (Frankel et al., 2011). They conducted a functional screen for the miRNA regulators of the autophagic flux in breast cancer cells. Through transcriptome profiling analysis, they identified three autophagy related genes STMN1, Rab5a and Atg4d are targeted by miR-101 under autophagic conditions. And this miR-101 mediated inhibition of autophagy can

sensitize cancer cells to 4-hydroxytamoxifen (4-OHT)-mediated cell death (Frankel et al., 2011).

1.2.4.2 miRNAs target upstream signal molecules that regulate autophagy

Besides the autophagic core machinery, various components in the upstream signaling pathways of autophagy have also been proved to be tightly regulated by a series of miRNAs. However, most of these studies haven't further their functional studies into the autophagic field, so far it is still not very clear whether these miRNAs can affect autophagy in specific settings via corresponding suppression of their target genes.

1.2.4.2.1 Potential positive miRNA regulators of autophagy that target autophagy upstream signaling network

miRNAs have been proved to target a series of autophagy upstream signaling molecules which place unfavorable effects on autophagy induction and execution including mTOR, PI3K/Akt, Ras, ERK, Rb, and Bcl2. Table 1.3 summarize these miRNAs which have potential positive impact on autophagy.

Recently, several groups have discovered that mTOR, the key regulator of autophagy pathway is also under dynamic control of various miRNAs. Fornari et al. has discovered miR-199a-3p which is downregulated in several human cancers including HCC, targeted mTOR and c-Met in HCC cells (Fornari et al, 2010). Attenuating miR-199a-3p will enhance susceptibility of cell to hypoxia treatment, indicating autophagy's

involvement. Similarly, Nagaraja et al. found that miR-100 which is the most downregulated miRNA in their clear cell ovarian cancer cell lines, targets FRAP1/mTOR (Nagaraja et al., 2009). Oneyama et al. has found that mTOR is a target of miR-99a which is downregulated upon c-Src activation. Overexpression of miR-99a downregulates mTOR and leads to suppression of tumor growth (Oneyama et al., 2011).

Growth factors activate receptor tyrosine kinases (RTKs) which stimulate the phosphatidylinositol 3-kinases (PI3K), PI3K then activates mTOR for stimulating cell growth and inhibiting autophagy. miR-126 inhibits PI3K signaling as detected by reduced levels of phosphorylated Akt. The PI3K regulatory subunit p85β is a potential target of miR-126 (Guo et al., 2008b). Blockage of miR-205 by using a synthetic antagomiR, or by the ectopic expression of miR-184, leads to decreased phosphorylated Akt, phosphorylated BAD together with increased apoptosis (Yu et al., 2008).

The mammalian Ras proteins, H-Ras, K-Ras and N-Ras, modulate many proliferation pathways. Ras family also activate mTOR for inhibiting autophagy. Let-7 family, composed of more than nine members, negatively regulates Ras proto-oncogene through multiple complementary sites in the 3'-UTRs of all three human Ras genes (Johnson et al, 2005). miR-143 also negatively regulates Ras (Lin et al., 2009).

ERK, a downstream signal of Ras, can inhibit autophagy by activating mTOR as well. ERK1/2 is reported to be a target of miR-199a (Kim et al., 2008b). miR-143 also targets ERK5 and thus suppresses cell growth and enhances CH-11-induced apoptosis (Akao et al., 2009).

The retinoblastoma (Rb) tumor suppressor functions as a negative regulator of the cell cycle through inhibition of E2F transcription factors (Trimarchi and Lees, 2002). Loss

of pRb has been shown to sensitize tumor cells to apoptosis (Chau and Wang, 2003); and pRb attenuates the induction of BNIP3, a Bcl-2 superfamily member, by hypoxia-inducible factor to prevent autophagic cell death (Tracy et al., 2007). miR-106-363 cluster (Landais et al., 2007) and miR-290 (Benetti et al., 2008) also target Rb. Epigenetic loss of miR-124a is correlated with activation of CDK6 and phosphorylation of Rb (Lujambio et al., 2007).

Table 1.3 Potential positive miRNA regulators of autophagy

miRNA	Favorable Effect on Autophagy	Reference
miR-199a	targets mTOR and c-Met as well as mTOR's activator ERK1/2	(Fornari et al., 2010; Kim et al., 2008b)
miR-100	targets mTOR/FRAP1	(Nagaraja et al., 2009)
miR-99a	downregulates mTOR	(Oneyama et al., 2011)
miR-126	inhibits PI3K signaling and decreasing phosphorylated Akt	(Guo et al., 2008b)
miR-184	decreases phosphorylated Akt	(Yu et al., 2008)
let-7 family	targets mTOR activator - Ras proto-oncogenes	(Johnson et al, 2005)
miR-143	targets Ras and ERK5	(Lin et al., 2009; Akao et al., 2009)
miR-106-363 cluster	targets Rb	(Landais et al., 2007)
miR-290	targets Rb	(Benetti et al., 2008)
miR-124a	deactives phosphorylation of Rb	(Lujambio et al., 2007)
miR-15-16 cluster	targets Bcl-2	(Cimmino et al., 2005)
miR-1	targets Bcl-2	(Tang et al., 2009)
miR-29b	targets Mcl-1	(Mott et al., 2007)
miR-512	downregulates Mcl-1	(Saito et al., 2009)
miR-101	targets Mcl-1	(Su et al., 2009)
miR-153	downregulates Bcl-2 and Mcl-1	(Xu et al., 2010)
miR-133b	targets Mcl-1 and Bcl-W	(Crawford et al., 2009)
miR-122	targets Bcl-W	(Lin et al., 2008)

Bcl-2 family contain both antiapoptotic and proapoptotic proteins. Bcl-2, Mcl-1, Bcl-xL, and Bcl-W are antiapoptotic proteins which can inhibit autophagy by binding to Beclin 1(Atg6) (Maiuri et al., 2007; Pattingre et al.,2005; Erlich et al., 2007). Several tumor suppressive miRNAs such as miR-15-16 cluster (Cimmino et al., 2005) regulate Bcl-2. miR-1 regulates cardiomyocyte apoptosis by targeting Bcl-2 (Tang et al., 2009). miR-29b increases cell apoptosis sensitivity to TRAIL by targeting Mcl-1, a member of the Bcl-2 family (Mott et al., 2007), but whether it can also lead to autophagy still needs to be examined. Epigenetic activation of miR-512-5p induces suppression of Mcl-1, leading to apoptosis in gastric cancer cells (Saito et al., 2009). miR-101 also exerts its proapoptotic function via targeting Mcl-1 (Su et al.,2009). miR-153 downregulates both Bcl-2 and Mcl-1 (Xu et al., 2010), while miR-133b targets Mcl-1 and Bcl-W (Crawford et al., 2009). miR-122 also targets Bcl-W (Lin et al., 2008).

1.2.4.2.2 Potential negative miRNA regulators of autophagy that target autophagy upstream signaling network

Besides those potential positive miRNA regulators of autophagy, there are also miRNA counterparts which place unfavorable effect on autophagy via suppressing those autophagy-inducing upstream signaling molecules such as PTEN, E2F, FoxO1/3 and IRGM. These miRNAs are summarized in Table 1.4.

The PTEN phosphatase is a dual-specificity protein and lipid phosphatase that counteracts PI3K activity and functions as a major tumor suppressor. miR-214 (Yang et al.,

2008) and miR-21 (Meng et al., 2007b) were proved to inhibit PTEN expression in human cancer, resulting in tumor cell proliferation, migration and invasion.

The E2F family play a crucial role in controlling cell cycle and actions of tumor suppressor proteins. In autophagy, the E2F family upregulate four autophagy genes: LC3, Atg1, Atg5 and DRAM (Polager et al., 2008). Several miRNAs such as miR-210 (Giannakakis et al., 2008), miR-106b and miR-93 (Petrocca et al., 2008) are able to regulate E2F1-3. In prostate cancer cells, E2F1 is negatively regulated by miR-330 which induces apoptosis through E2F1-mediated suppression of Akt phosphorylation (Lee et al., 2009).

IRGM1 is essential to promote autophagy that kills bacteria. Recently, in the inflammatory intestinal epithelia of individuals with Crohn's disease, Brest et al. found the overexpressed miR-196 downregulated the IRGM protective variant (c.313C) but not the risk-associated allele (c.313T). This effect affected autophagy's efficiency and subsequently compromised autophagy's control of replication of Crohn's disease-associated adherent invasive Escherichia coli (Brest et al., 2011).

The FoxO1 transcription factor, a putative tumor suppressor, regulates genes involved in the apoptotic response, cell cycle checkpoints, and other cellular metabolisms. Overexpression of either FoxO1 or FoxO3 results in decreased cell viability and enhanced expression of autophagy pathway genes. miR-27a, miR-96, and miR-182 were reported to cooperately down regulate FoxO1 in breast cancer cells (Guttilla et al., 2009). Overexpression of miR-182 promotes melanoma metastasis by repressing FoxO3 and microphthalmia-associated transcription factor (Segura et al., 2009).

Additionaly, the miR-146a mimic upregulates Bcl-xL and STAT3 phosphorylation (Liu et al., 2009). miRNAs also regulate proapoptotic Bcl-2 family members such as BIM. For example, miR-32 (Ambs et al., 2008) targets BIM.

Table 1.4 Potential negative miRNA regulators of autophagy

miRNA	Unfavorable Effect on Autophagy	Reference
miR-214	inhibits PTEN	(Yang et al., 2008)
miR-21	inhibits PTEN	(Meng et al., 2007b)
miR-210	targets E2F1-3	(Giannakakis et al., 2008)
miR-25-106b cluster	targets E2F1-3	(Petrocca et al., 2008)
miR-330	downregulates E2F1	(Lee et al., 2009)
miR-27a	downregulates FoxO1	(Guttilla et al., 2009)
miR-96	downregulates FoxO1	(Guttilla et al., 2009)
miR-182	downregulates FoxO1 and FoxO3	(Guttilla et al., 2009, Segura et al., 2009)
miR-196	targets IRGM1(c.313C)	(Brest et al., 2011)
miR-146a	upregulates Bcl-xL and STAT3 phosphorylation	(Liu et al., 2009)
miR-32	targets BIM	(Ambs et al., 2008)

1.2.4.2.3 Potential miRNA regulators of autophagy that have dual function

p53, a transcription factor encoded by the TP53 gene, regulates cell cycle and thus functions as a tumor suppressor. p53 can transactivate an autophagy-inducing gene, DRAM, which codes for a lysosomal protein (Crighton et al., 2006). p53-dependent induction of autophagy has been identified in response to DNA damage (Feng et al., 2005), Arf activation (Abida and Gu, 2008), or reexpression of p53 in p53-deficient tumor cells (Amaravadi et al., 2007). Inactivation of p53 by deletion, depletion or inhibition also triggers autophagy (TasdemiR et al., 2008). miR-34a, miR-34b/c, miR-26a, miR-182, are

consistently induced after p53 activation, indicating they may serve as direct p53 targets. miR-34 family also increase p53-mediated apoptotic sensitivity (Chang et al., 2007b). miR-192 and miR-215, which can impact cell proliferation, are directly regulated by p53 (Song et al., 2008). miR-372 and miR-373 inhibit p53 directed CDK signaling, promoting tumorigenesis in testicular germ cells by targeting LATS2 (Voorhoeve et al., 2006). In addition, loss of miR-122 is suggested to facilitate genomic instability due to the lack of translational repression of the p53 inducible CCNG1 (Gramantieri et al., 2007). Recently, miR-125b and miR-504 are identified as novel negative regulators of p53 (Le et al., 2009; Hu et al., 2010). miR-127 may increase TP53-dependent apoptosis by disrupting the negative regulatory feedback loop between Bcl-6 and TP53 (Saito et al., 2006), whereas miR-155 inhibits TP53INP1-mediated apoptosis by directly targets TP53INP1 (Gironella et al., 2007).

miR-17-92 cluster also have dual functions in regulating autophagy upstream signaling network. On one hand, miR-17-92 place favorable effect on autophagy. miR-17-92 cluster was reported to activate the cyclinD1/CDK4 complex to release Rb's inhibition on E2F and downregulate Rb directly to drive cell proliferation (Wang et al., 2008a). Meenhuis et al. also identified sequestosome 1 (SQSTM1), a regulator of autophagy-mediated protein degradation, as a major target of miR-17 family in myeloid progenitors and mature neutrophils (Meenhuis et al., 2011). On the other hand, miR-17-92 cluster was proved to inhibit PTEN (Xiao et al., 2008), E2F1-3 (Sylvestre et al., 2007) as well as the proapoptotic Bcl-2 family member BIM (Xiao et al., 2008).

Similarly, miR-34 also has ability to influence both signals that favor autophagy and disfavor autophagy. Bcl-2, the inhibitor of Beclin-1, was proved to be a target of miR-34a

(Bommer et al., 2007). However, miR-34 can also directly target E2F1-3 (Tazawa et al., 2007).

1.3 Objectives of the study

Generally, the regulatory role of miRNAs in autophagy has not been systematically studied. At the time when this project started, Beclin 1 is the only Atg gene that was reported to be regulated by miRNA, although a series of research found that miRNA target genes involved in the upstream regulatory network of autophagy, most of these projects extend their functional studies into the autophagy field. Thus, we want to establish the role of miRNAs in autophagy regulation and function, the hypothesis is that miRNAs are capable of modulating autophagy via targeting the autophagy machinery (Atg genes), or the upstream signaling molecules, and subsequently affect the efficiency of autophagy.

We aim to:

1. Identify and confirm candidate miRNAs that are differentially expressed during autophagy induction.

2. Characterize the target genes of the deregulated miRNAs that involved either in the autophagy machinery or the autophagy upstream regulatory pathways.

Results from this study will advance the current understanding of the regulatory mechanisms of autophagy as well as expand the functional scope of miRNAs. We also hope the findings from this study will contribute to the development of novel therapeutic strategies for autophagy-related human diseases such as cancer.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 Materials and Methods

2.1 Materials and reagents

Taqman miRNA individual assays were purchased from Applied Biosystems, The negative control miRNA, mimic and antagomir of mmu-miR-93 were purchased from Ambion. Rapamycin, earles's balanced salt solution (EBSS), and chloroquine were purchased from Sigma-Aldrich. Primers were purchased from 1st BASE Pte Ltd. SacII restriction endonucleases was purchase from New England Biolab (NEB).The pAPI5-3UTR-WT plasmid was a kind gift from Dr. Wang Yu and A/Prof. Caroline Lee Guat Lay.

2.2 Cell culture

Atg5 WT, Atg5-/- MEF and GFP-LC3 MEF(Tet-off Atg5 MEFs) cell lines were obtained from Prof. Noboru Mizushima, these cell lines are maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum (FBS, from HyClone, Logan,UT, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5 % CO_2 atmosphere at 37 ℃. For starvation, cells are cultured in EBSS (Sigma-Aldrich, St. Louis, USA).

2.3 Microarray

miRNA expression analysis was conducted using the miRXplore™ Microarrays in the Lab of Miltenyi Biotec microarry servicing facility in Germany.

Total RNA Samples including low molecular weight RNAs were first checked for quality using the Agilent 2100 Bioanalyzer platform (Agilent Technologies). RNA Intergrity Number (RIN) was calculated for each sample. All samples revealed RIN values between 9.5 and 10 which indicate the samples are highly processable. The treatment groups were labeled with Hy5 (Red) and the control groups were labeled with Hy3 (Green) according to the undisclosed miRXplore™ user manual. Then the fluorescently labeled samples were hybridized overnight to miRXplore™ Microarrays using a-Hyb™ hybridization station. Fluorescence signals were detected using an Agilent laser scanner. Mean signal and mean local background intensities were obtained for each spot of the microarray images using the ImaGenea software (Biodiscovery). Low quality spots were flagged and excluded from data analysis. Unflagged spots were analysed with the PIQOR™ Analyzer software. After normalization and another quality control step which only took into account those spots with the Hy5/Hy3 ratio from signal that was equal or higher than 50% of the background signal intensities, the Significance Analysis of microarrays (SAM) was then used to identify miRNAs that displayed significant expression difference between the treatment groups and the control groups. The miRNAs were identified at a false discovery rate of less than 0.05 and an absolute SAM score of more than 2.5.

2.4 Target prediction

Generally, prediction for target genes of a specific miRNA or prediction for miRNA regulator of a particular gene was first carried out using a combination of 11 prediction programs including DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget. Some of the 11 prediction programs were further checked for more specific purposes such as binding site conserveness among different species.

2.5 MicroRNA isolation

Total RNA or RNA enriched for small species was extracted with the mirVana™ miRNA Isolation Kit (Ambion) following manufacturer's instructions. 5x 10^5 cells were freshly prepared, first lysed in 500 ul lysis buffer and 50 ul miRNA homogenate addictive, incubated on ice for 10 minutes. Then 500 ul Acid-Phenol: Chloroform was added to the lysate, after centrifugation at 10,000 x g for 5 minutes, the upper phase was removed and added with 625 ul 100% ethanol, and then the lysate was passed through a collection column. The column was washed once with 600 ul wash buffer 1, twice with 500 ul wash buffer 2/3 and once with 700 ul 80% ethanol and centrifuged briefly at 10,000 x g. After that, the columns were centrifuged at the maximum speed for another minute to remove the residue, the total RNA or RNA enriched for small species was then eluted by 50 ul nuclease free water preheated at 99 ℃.

2.6 Real Time quantitative Reverse Transcription PCR

For the analysis of miRNA differential expression, Taqman MicroRNA Individual Assays (Applied Biosystems) were employed for validation. Each sample was analyzed in triplicates. Reverse transcription was carried out with 75 ng template RNA enriched for small species using Iscript TM cDNA Synthesis Kit (Bio-Rad) and miRNA specific reverse transcription primers. Real-Time PCR was performed in a 10 ul reaction mix comprising 2ul 2X diluted reverse transcription product, 5 ul Taqman 2X Universal PCR Master Mix without UNG Amperase (Applied Biosystems), 2ul miRNA specific probes and 1ul nuclease free water, on a Bio-Rad CFX96™ Real-Time PCR Detection System, with an initial denaturation step at 95 ℃ for 10 minutes, followed by 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 1 minute. Fluorescence signal was measured at each extension step. The Taqman microRNA individual assays used in this study are listed in Table 2.1

Table 2.1 List of Taqman microRNA individual assay used in Real Time qRT-PCR

miRNA ID	Mature Sequence(5'- 3')	Assay No.
mmu-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	001090
mmu-miR-221	AGCUACAUUGUCUGCUGGGUUU	001134
mmu-miR-181a	AACAUUCAACGCUGUCGGUGAGU	000480
mmu-let-7b	UGAGGUAGUAGGUUGUGUGGUU	002619
mmu-miR-21	UAGCUUAUCAGACUGAUGUUGA	000397
mmu-miR-30c	UGUAAACAUCCUACACUCUCAGC	000419
mmu-miR-99a	AACCCGUAGAUCCGAUCUUGUG	000435
mmu-miR-34a	UGGCAGUGUCUUAGCUGGUUGU	000426
mmu-miR-17	CAAAGUGCUUACAGUGCAGGUAG	002308
mmu-miR-199a	CCCAGUGUUCAGACUACCUGUUC	000498

For the analysis of autophagy transcripts levels, total RNA was extracted with the RNeasy kit (Qiagen). One microgram of total RNA from each sample was used as a template for cDNA synthesis using Iscript™ cDNA Synthesis Kit (Bio-Rad) according to

the manufacturer's protocol. Real Time PCR was performed on the Bio-Rad CFX96™ Real-Time PCR Detection System using Bio-Rad Ssofast^{TM} EvaGreen Supermix. Amplification reaction mix included cDNA template corresponding to 50 ng total RNA, target gene primers (0.25 um), and 2X PCR Master Mix in a total volume of 20 ul. Amplification conditions included an initial denaturation step at 95 ℃ for 1 minute, followed by 40 cycles of denaturation at 95 ℃ for 5 seconds, annealing at specific annealing temperature for 5 second and extension at 60 ℃ for 5 seconds. The green fluorescence was measured at each extension step. The specific primer sequences for each target gene examined are listed in Table 2.2.

Table 2.2 List of primers for target genes of mmu-miR-93 used in Real Time qRT-PCR

Target Gene	Primer	Sequence(5'- 3')
STAT3 (NM_213660)	Forward	CAATACCATTGACCTGCCGAT
	Reverse	GAGCGACTCAAACTGCCCT
Atg7 (NM_028835)	Forward	CCTGTGAGCTTGGATCAAAGGC
	Reverse	GAGCAAGGAGACCAGAACAGTG
Ulk1 (NM_009469)	Forward	GCAGCAAAGACTCCTGTGACAC
	Reverse	CCACTACACAGCAGGCTATCAG
Atg16l1 (NM_029846)	Forward	GGACACTCATCCTGCTTCTGGT
	Reverse	GCTTCCCAAAGTTTCACCCTGC
Rb1cc1 (NM_009826)	Forward	GGAATCTCTGGTCAGGAAGTGC
	Reverse	GTCCAAGGCATACAGCCGATCT
Atg2a (NM_194348)	Forward	ACCTTCTCCACACTGGTGACAG
	Reverse	CTTGCTCCACATCTAGTACCAGC
Akt1 (NM_009652)	Forward	GGACTACTTGCACTCCGAGAAG
	Reverse	CATAGTGGCACCGTCCTTGATC

The transcript expression was recorded as threshold cycle (CT) - the fractional cycle No. at which the fluorescence signal exceeded a standard threshold, and relative

quantitation of gene expression was calculated by the ΔΔCT method (Livak et al, 2001). The fold change was calculated using the equation $2^{-\Delta\Delta CT}$.

2.7 Transfection

Atg5 WT MEF and GFP-LC3 MEF cells were seeded 12-24 hour before the transient transfection in antibiotics-free medium and 20~30% confluency was achieved at the point of transfection. The transient transfection of miRNA negative control, mimic and antagomir were performed using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's protocol. All transfected cells were harvested within 72 hours post transfection for further analysis. The short oligos used in transfection are listed in Table 2.3.

Table 2.3 List of short oligos used in transient transfection

Oligo Name	Commercial Name	Catalogue No.
Pre-mmu-miR-93	Ambion Pre-miR miRNA Precursors	PM10951
Anti-mmu-miR-93	Ambion Anti-miR miRNA Inhibitors	AM10951
Negative control miR	Pre-miR miRNA Precursor Molecules Negative Control #1	AM17110

2.8 Western blot

After the designated treatments, whole cell lysates was prepared in M2 lysis buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM glycerol phosphate, 1 mM sodium vanadate and 1X proteinase inhibitor cocktail). Insoluble

fractions were discarded after centrifugation at 15,000 g for 15 min. Protein was quantified using UV-2401PC. An equal amount of protein was resolved and loaded into 10~12% SDS-PAGE gel in the Mini-PROTEAN II system (Bio-Rad) and transferred onto PVDF membrane (Bio-Rad). After blocking with starting block (Thermo Scientific) for half an hour, the membrane was probed with designated first followed by corresponding second antibodies, developed with the enhanced chemiluminescence method (Pierce) and visualized with the Kodak Image Station 4000R (Kodak). The major antibodies used in this study are listed in Table 2.4.

Table 2.4 List of major antibodies used in western blot

Primary Antibody	Source	Company	Catalogue No.	Dilution Factor	Size
LC3B	Rabbit polyclonal	Sigma	L7543	1:1000	16, 18kDa
p62	Mouse monoclonal	Abnova	H00008878-2C11	1:2000	62kDa
Atg5	Rabbit polyclonal	Cosmo Bio	CAC-TMD-PH-AT5	1:2000	55kDa
Atg7	Rabbit polyclonal	ProSci	3617	1:1000	80kDa
Ulk1	Rabbit polyclonal	Sigma	A7481	1:1000	150kDa
STAT3	Rabbit polyclonal	Cell signaling	#9132	1:1000	79, 86kDa
p4EBP1	Rabbit monoclonal	Cell signaling	#2855	1:1000	15 to 20 kDa
pS6	Rabbit polyclonal	Cell signaling	#2211	1:1000	32kDa
GFP	Mouse monoclonal	Covance	MMS-118R	1:1000	27kDa
α-tubulin	Mouse monoclonal	Sigma	T6199	1:2000	50kDa
β-actin	Mouse monoclonal	Sigma	A5316	1:2000	43kDa

Secondary Antibody	Source	Company	Catalogue No.	Dilution Factor
anti-Mouse	Goat	Thermo Scientific	#31430	3.513889
anti-Rabbit	Goat	Thermo Scientific	#31460	3.513889

9 Confocal microscopy

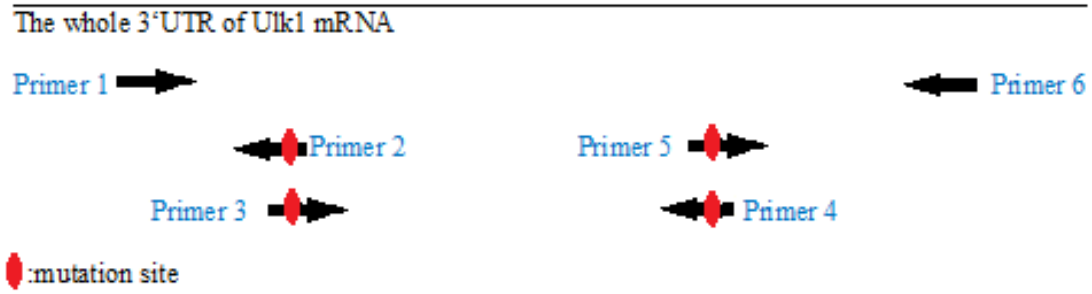
The GFP-LC3 MEF cells were seeded to a 4 well or 8 well coverglass slide chamber (Lab-Tek®, NUNC), maintained in full medium for 16 hours before treatment. After designated treatments, the cells were then examined under confocal microscope Olympus Fluoview FV1000 (Olympus).

2.10 Generation of 3' UTR reporter constructs

The 1683 base pair of wild type Ulk1 3' UTR was amplified from mouse genomic DNA and cloned downstream of a β-Galactosidase reporter gene driven by multidrug resistance associated gene (MRP1) promoter at the SacII restriction site. The human MRP1 promoter was chosen over the human cytomegalovirus (CMV) promoter, because the MRP1 promoter is around 30 times weaker than the CMV promoter which will facilitate the measurement of subtle changes in the reporter gene activity. The reporter construct also carried a CMV promoter driven enhanced green fluorescence protein (EGFP) to normalize for differences in transfection efficiencies. A mutant reporter vector which include the two putative mutated miR-93 binding sites was also generated by PCR mutagenesis. The recombinant reporter vector carrying the widetype Ulk1 3' UTR was termed as pUlk1-

3UTR-WT while the mutant one was termed as pUlk1-3UTR-MUT. The mutant site was verified in silico using PITA. Both constructs were verified by sequencing. Figure 2.1 shows the cloning strategies and results. Primers used for PCR mutagenesis and sequencing are listed in Table 2.5 and Table 2.6.

A



B

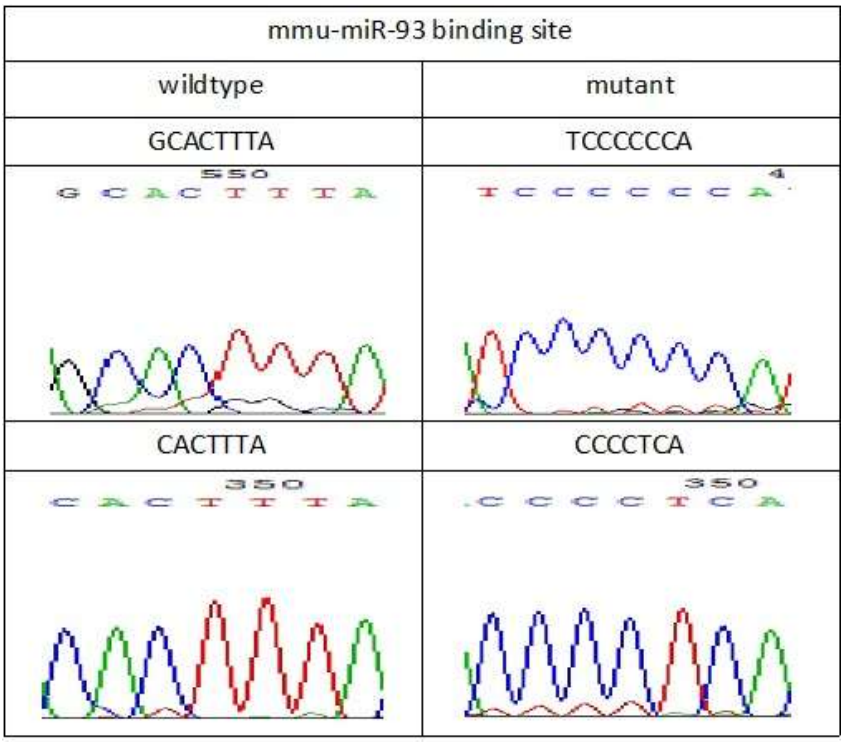


Figure 2.1 Generation of Ulk 1 3'UTR reporter constructs. (A) Strategy for PCR mutagenesis at the two miR-93 binding sites of Ulk1 3'UTR. **(B)** Product of the PCR mutagenesis and the binding site sequences confirmed.

Table 2.5 List of primers for PCR mutagenesis

Primer	Sequence (5'~3')
Primer 1	GCCTGACCGCGGCTACCTGCTGCCAACCTGCAGGG
Primer 2	TCTGT ATGCA T**GGG**G**G**G**A** TCCGG TGACT TACAA AGCTG
Primer 3	AGTCA CCGGA **T**C**C**C**CC**A TGCAT ACAGA GACTG GAGAA
Primer 4	ATCTT AGGAG T**G**A**G**G**G**G TGGAA GTTGA GGAGT TTTAA
Primer 5	TCAAC TTCCA C**C**C**C**T**C**A CTCCT AAGAT GTGTC TCCAC
Primer 6	CACATACCGCGGCAATAATTCCAATTTTTATTTTCTTAA

Table 2.6 List of primers for sequencing

Primer	Sequence (5'~3')
POSITION 400 FORWARD	GAAAAAAGGGTATGGTCTTCTGGCCCC
POSITION 850 FORWARD	GAAAGGGCGGAGGCAGGGCAGGCC
POSITION 1300 REVERSE	TTCAAGTATTGCAAAGAAAAACG

2.11 ß-gal reporter gene assay

Atg5 WT MEF cells cultured in 6 well plates were transfected with either 1.0 ug of the pUlk1-3UTR-WT or the corresponding pUlk1-3UTR-MUT and co-transfected with either 50 nM of control oligos or miRNA precursors (Ambion Pre-miR^{TM} mmu-miR-93) by using siPORT^{TM} Amine Transfection Agent (Ambion) according to the manufacturer's instruction. β-Galactosidase reporter gene activity was assayed kinetically using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate and measured at 1 minute intervals over 60 minute at 570 nm in a SpectraMAX PLUS microplate reader (Molecular Devices, Sunnyvale, California, USA), with crude lysate from the transfected cells harvested 24 hours post transfection.

2.12 Statistical analysis

All numerical data were presented as mean ± Standard Error of at least three independent experiments. Statistical significance was estimated with student's t-test (two-tailed distribution, two-sample equal variance). A p-value less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

Chapter 3 Results

3.1 Identification of potential miRNA regulators in starvation-induced autophagy

3.1.1 miRNA expression profiling of starvation induced autophagy

As mentioned in section 1.1.2, autophagy can be induced under a series of stress conditions in various cell types. To identify deregulated miRNAs in autophagy induction, we examined the expression of 634 mouse mature miRNAs in Atg5 WT MEF cells versus Atg5 KO MEF cells with or without starvation treatment for 6 hours using miRXplore^{TM} miRNA microarrays (Figure 3.1 A, B). Starvation (EBSS) was selected as the initial cellular stress since amino acid deprivation is a well-established inducer of autophagy (Mizushima et al., 2010), EBSS treatment for 6 hours can efficiently induce autophagy in autophagy efficient MEF cell lines as evident from the conversion of LC3-I to LC3-II (Figure 3.1 C). The Atg5 WT and KO MEF cells were chosen as they were among the most popular cell line models for the current autophagy research.

Of these 634 mouse miRNAs, 541 miRNAs were not expressed in these four samples. The remaining 93 miRNAs were included in the subsequent analysis, and only 36 of them were significantly deregulated in at least one sample. The selection criteria was that in comparison with the control sample, only miRNAs that were upregulated or downregulated over 1.5 fold were considered to be significantly deregulated. However, as the microarray was done only once for each comparison, the results could not be statistically significant. Table 3.1 summarized the experiment design and results.

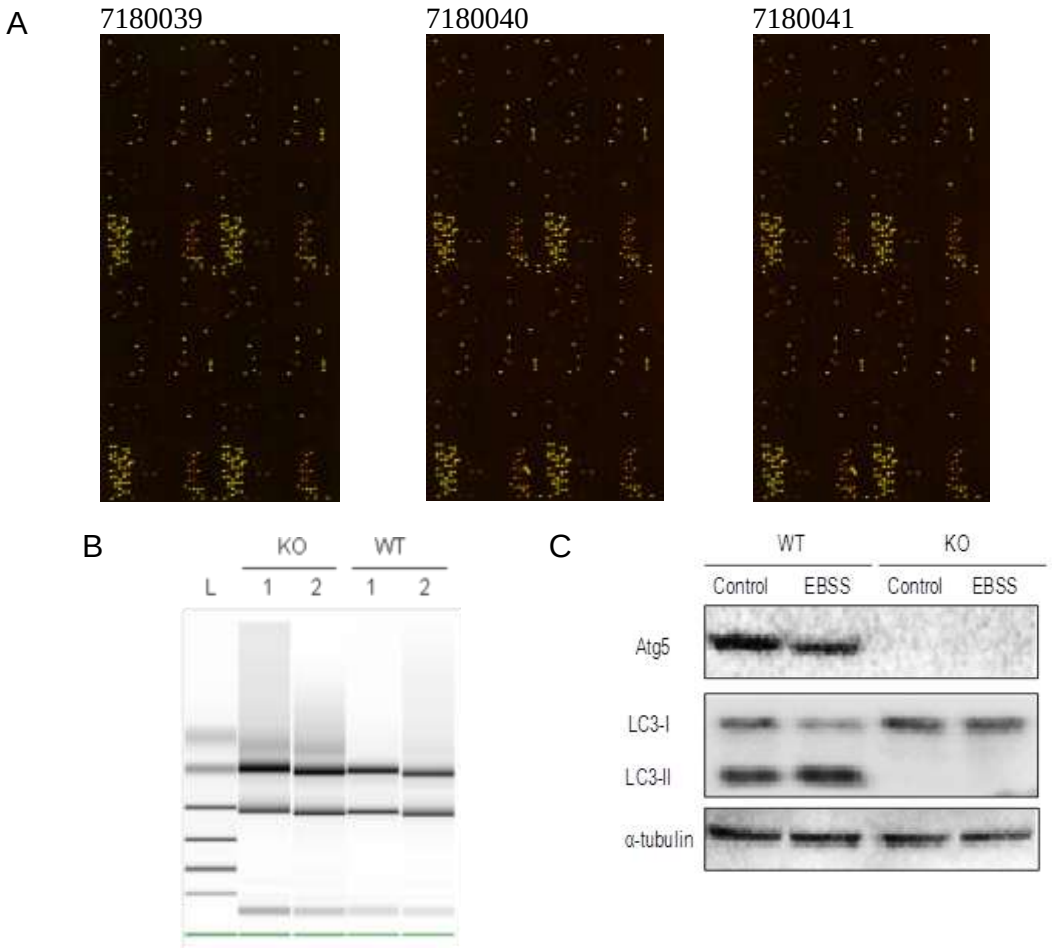


Figure 3.1 The miRNA microarray for miRNA expression profiling of starvation induced autophagy. (A) Hy5/Hy3 false-color image after scanning of the three microarrays. Red spot indicates the corresponding miRNA is overexpressed in the treatment group, green spot indicates the corresponding miRNA is downregulated, yellow spot indicates no deregulation. **(B)** Gel image indicates RNA qualities of the samples. The lowest green band represents an internal standard, the two prominent bands are 18S RNA and 28S RNA respectively. **(C)** Autophagy is induced in Atg5 WT MEF cell following 6 h EBSS treatment while there is no autophagy induction in Atg5 KO MEF cell which received the same treatment as evident by the conversion of LC3-I to LC3-II.

Table 3.1 Experiment design and result of the microRNA microarray *

miRNA Microarray No.	Treatment		Control Samples	Experiment Samples	Upregulated miRNA	Down-regualted miRNA
	Control Samples	Experiment Samples				
7180039	full medium	full medium	Atg5 WT1	Atg5 KO1	miR-152, miR-15a, miR-181a, miR-18a, miR-21, miR-25, miR-30a, miR-30b, miR-30c, miR-30d, Let-7f, miR-20a, miR-34a, miR-467a-467b, miR-503, miR-115-5p, miR-351, miR-m23-1-5p, miR-1274a, miR-181a	Let-7b, Let-7c
7180040	full medium	EBSS	Atg5 WT1	Atg5 WT2	miR-1902, miR-762, miR-30e, miR-711, miR-689, miR-93, miR-26b, miR-m23-1-5p, miR-1274a, miR-181a	miR-221
7180041	EBSS	EBSS	Atg5 WT2	Atg5 KO2	miR-26b, miR-17, miR-1902, miR-34a, miR-30e, miR-222, miR-689, miR-350, miR-487b, miR-28-5p, miR-19a, Let-7f, miR-322-miR-424, miR-m23-1-5p, miR-1274a, miR-181a	Let-7b, Let-7c, miR-99a

*****Generally, four total RNA samples were prepared accordingly and three comparisons were conducted, Atg5 WT1 and KO1 were samples cultured in full medium while Atg5 WT2 and KO2 received a EBSS treatment for 6 h.

The three microarray experiments revealed the clues of a general miRNA expression pattern when MEF cell were under EBSS condition for 6 hours: (1). miRNA induced by starvation (autophagy inducer) in both Atg5 WT and Atg5 KO MEFs: miR-30e, miR-689, miR-26b, miR-1902; (2). miRNA induced by starvation only in Atg5 WT MEFs: miR-93, miR-711, miR-762; (3). miRNA dramatically induced by starvation only in Atg5 KO

MEFs: miR-322-miR-424; (4). miRNA reduced by starvation only in Atg5 WT MEFs: miR-221; (5). miRNA reduced by starvation only in Atg5 KO MEFs: miR-99a; (6). miRNA enhanced by Atg5 deletion (regardless whether starvation or non-starvation): miR-34a, miR-467a-miR-467b, let-7f; (7). miRNA decreased by Atg5 deletion: let-7b, let-7c.

Table 3.2 summarized these potential significantly deregulated miRNAs revealed by the microarray experiments. As shown in Table 3.2, we observed many of those deregulated miRNAs were clustered and associated either with functional family (let-7 family, miR-17 family, miR-221 family, miR-30 family) or genomic loci (let-7 family on chromosome 15, miR-17 cluster on chromosome 14). And these miRNAs were found to be slightly enriched in chromosome 1, 14, 15, and X. Interestingly, when compared with Atg5 WT and Atg5 KO cells, let-7f was significantly upregulated while another two members of the let-7 family, let-7b and let-7c were downregulated. This seems to be chromosome dependent, yet may also indicates that even being in the same functional family, these three miRNAs might have different major targets or there exists a homeostatsis for let-7 family.

Table 3.2 Significantly deregulated miRNAs in the microarray

miRNA	Genomic Loci	Genomic Cluster	Functional Family	Expression Fold Change		
				7180039*	7180040*	7180041*
MMU-LET-7F	chromosome: 13	Let-7a/Let-7f/Let-7d	let-7 family	2.96	1.18	1.61
MMU-LET-7B	chromosome: 15	Let-7c/Let-7b		0.61	1.10	0.62
MMU-LET-7C	chromosome: 15	Let-7c/Let-7b		0.60	0.97	0.62
MMU-MIR-152	chromosome: 11		miR-148 family	2.58	1.10	1.36
MMU-MIR-15A	chromosome: 14	miR-15a/16	miR-15 family	1.68	1.24	1.21
MMU-MIR-487B	chromosome: 12	miR-495/667/376c/654/376b/376a/300/381/487b/539/544/382/134/668/485/453	miR-154 family		1.01	1.63
MMU-MIR-25	chromosome: 5	miR-93/25/106b	miR-17 family	2.22	0.96	0.85
MMU-MIR-18A	chromosome: 14	miR-17/18a/19a/20a/19b/92a		1.56		1.06
MMU-MIR-20A	chromosome: 14	miR-17/18a/19a/20a/19b/92a		1.53	0.73	1.31
MMU-MIR-17	chromosome: 14	miR-17/18a/19a/20a/19b/92a		1.19	1.13	1.53
MMU-MIR-93	chromosome: 5	miR-93/25/106b		1.01	1.89	1.10
MMU-MIR-181A	chromosome: 1	miR-181a/181b	miR-181 family	1.86	2.03	2.14
MMU-MIR-19A	chromosome: 14	miR-17/18a/19a/20a/19b/92a	miR-19 family		0.82	2.41
MMU-MIR-1902	chromosome: 2		miR-1902 family		1.55	2.05
MMU-MIR-21	chromosome: 11		miR-21 family	1.72	1.05	1.04
MMU-MIR-221	chromosome: X	miR-221/222	miR-221 family	0.98	0.65	0.91
MMU-MIR-222	chromosome: X	miR-221/222		0.97	1.18	2.46
MMU-MIR-26B	chromosome: 1		miR-26 family		2.05	1.97
MMU-MIR-151	chromosome: 15		miR-28 family	1.54		1.35
MMU-MIR-28	chromosome 16			1.04		1.54
MMU-MIR-30C	chromosome: 4	miR-30c/30e	miR-30 family	2.53	1.15	1.13
MMU-MIR-30A	chromosome: 1			2.25	1.20	1.24
MMU-MIR-30B	chromosome: 15	miR-30b/30d		1.74	1.05	0.85
MMU-MIR-30D	chromosome: 15	miR-30b/30d		1.61	1.03	1.06

MMU-MIR-30E	chromosome: 4	miR-30e/30c		1.20	2.13	3.58
MMU-MIR-322	chromosome: X	miR-322/503/351/542/450a/450b	miR-322 family			4.19
MMU-MIR-34A	chromosome: 4		miR-34 family	4.67		2.04
MMU-MIR-350	chromosome: 1		miR-350 family	1.11	1.01	1.56
MMU-MIR-351	chromosome: X	miR-322/503/351/542/450a/450b	miR-351 family	1.62		1.24
MMU-MIR-467	chromosome: 2	miR-466m/466f/669f/669e/669b/669d/669l/297a/466o/467c/466b/669a/669k/467a/466b-8/669g/669j/466e/467b/466c/669a	miR-467 family	1.68	1.03	1.75
MMU-MIR-503	chromosome: X	miR-322/503/351/542/450a-2/450a-1/450b	miR-503 family	2.02		1.39
MMU-MIR-711	chromosome: 9		miR-711 family		1.66	0.91
MMU-MIR-762	chromosome: 7		miR-762 family		1.89	1.33
MMU-MIR-99A	chromosome: 16	miR-99a/Let-7c	miR-99 family	0.88	0.89	0.63
MMU-MIR-689			a fragment of rRNA		1.90	1.99
MMU-MIR-1274A			a fragment of a Lys tRNA	1.59	1.53	1.87

*. 7180039, 7180040, 7180041 are the miRNA microarray No. (Table 3.1).

3.1.2 Target prediction of deregulated miRNAs

In order to investigate the potential regulatory effect miRNAs may have on autophagy, a screen for the potential miRNA regulators was first conducted in silico with the expectation that those commonly up-regulated miRNAs in autophagy-deficient cell are often autophagy suppressors and those commonly down-regulated miRNAs are often autophagy inducers, using a combination of 11 miRNA-target prediction programs

(DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan /TargertScanS). Most of these programs predict the target relationship based on the complementarity between the miRNA sequence and mRNA 3'UTR sequence, with additional selection criteria such as complete pairing at seed region, optimal thermodynamic stability, evolutionary conservation, and multiple target sites for the specific mRNA.

As depicted in Figure 3.2, the strategy used was that, on one hand, a search was started from the deregulated miRNAs, aiming to find their predicted targeted autophagy related genes and autophagy upstream regulators; on the other hand, we also conducted a search for the potential miRNA regulators of the genes involved in the autophagy core machinery (a prediction was considered valid only if the target relationship was predicted by at least 3 programs). We gathered prediction results from both directions and selected the overlapping miRNAs and mRNAs as the interested candidates.

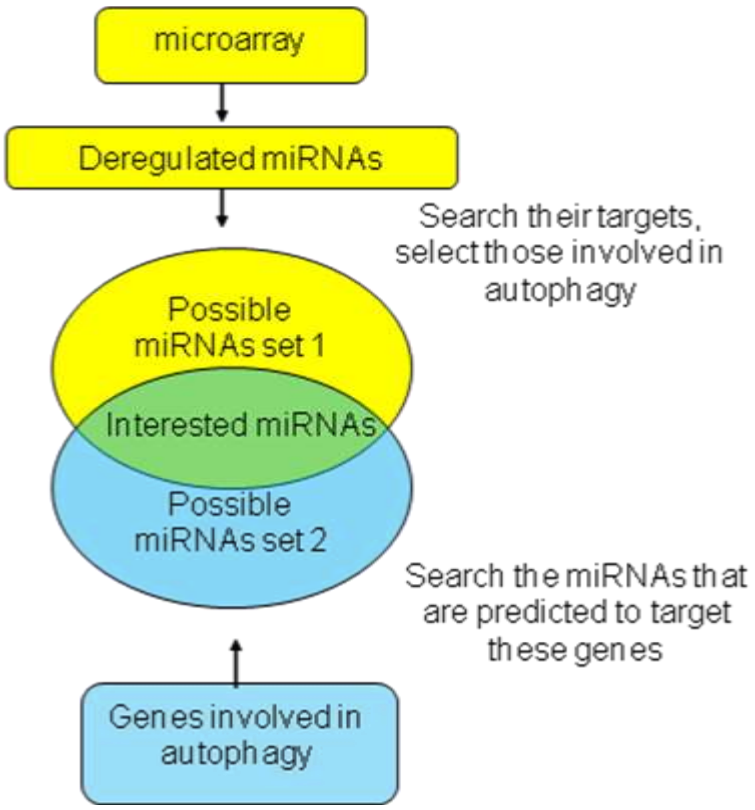


Figure 3.2 Strategy of screening possible miRNAs.

After searching, the interested miRNAs were reselected based on the number of autophagy related genes they target and the importance of their target genes in autophagy. The top 10 interested miRNA are listed below: miR-93, miR-221, miR-181a, let-7b, miR-21, miR-30c, miR-99a, miR-34a, miR-17, miR-199a. Table 3.3 summarizes the prediction results of these interested 10 miRNAs. The 10 miRNAs were also selected for further validation of their expression.

Table 3.3 Prediction result of the top 10 interested miRNAs

miRNA	Reasons for Choosing	Proved Target	Predicted Target	Possible Effect on Autophagy	Hypothesis
miR-34a	upregulated by starvation	SIRT1, E2F1, E2F3, BCL2	Rab43, Casp2, E2F5, Notch1, Bcl2, Akt1, Caspase3, IGF1, IGF1R, EIF1, TRAF3, Map3k3, Mapk1, IRF4, Atg9b, Atg9a	may block due to expression pattern	miR-34a has been demonstrated to be a target of p53, and function as a tumor suppressor. It targets E2F family and SIRT1, and is predicted to target Atg9b, Atg9a; however, it may also target Bcl2, Akt1 and Ras family member. When it downregulates E2F family members, it also upregulates p53 signaling pathway in human colon cancer cell lines, thus it may also have favorable effect on autophagy.
miR-181a	upregulated in all microarrays, consistently predicted to target Atg5	Bcl2, CD69, Tcra	E2F5, Mapk1, Eif4a2, SIRT1, E2F7, IGF2bp2, Bcl2l11, Pak4,Atg5, PTEN, Bcl2, Akt3, CD4, TNF	may block due to expression pattern	miR-181a is predicted to target E2F, SIRT1,PTEN and ATG5, so it may block autophagy, however, it may also target BCL2 and Akt3. And it is upregulated in all experiments, may be this is cell line specific.
miR-30c	upregulated in Atg5 KO cells, miR-30 family is consistently predicted to target Atg5, miR-30a is proved to target Beclin1	UBE2I	Rasd1, Atg5, Rab15, SIRT1, E2F7, Bcl2l11, Atg12, Rhebl1, Arf4, Map2k4, Apaf1	may block	miR-30c may block autophagy by targeting Atg genes such as Atg5, Atg12. It is also predicted to target the upstream signaling molecules such as SIRT1, Arf4. miR-30c may also block apoptosis by targeting Apaf1.

miR-21	upregulated in Atg5 KO cells, related to PI3K/pAkt upregulation	PTEN,TPM1, PDCD4, MASPIN /SERPINB5, MARCKS, HNRPK, RECK	STAT3, EIF2, Mapk10, Eif2ak4, Rab9b, Lamp3, E2F2, Rab18	uncertain, may block due to expression pattern	miR-21 may block autophagy as it targets PTEN,Eif2, eif2ak4,E2F2 and leds to upregulation of PI3K/pAkt, but it may also induce autophagy by targeting STAT3.
miR-221	down-regulated in starvation treated Atg 5 WT cells	p27(Kip1)	Arf4,Rab1, IGF2bp2, STAT1, Bcl2l11, Cd4, Mapk6, Map3k7, Rho, TRAF7, Pak7	may mainly block autophagy due to expression pattern	miR-221 may block autophagy by downregulating p27, Bcl2l11(Bim), but it may also have the opposite effect.
Let-7b	down-regulated in Atg5 KO cells, but the Let-7 family expression pattern in this microarray is different	HMGA2, RAS, IGF2BP1, c-Myc, NF2, LIN28B, CDC25a, CDK6, Cyclin D, CCND2, lin-41, hbl-1, MPTN	IGF2bp3, GF2bp2, TSC1, Rasl10b, Map4k3, E2F5, Bcl2l1, Casp3, Eif2c4, Rab8b, Rab5b, IGF1R, Map3k1, Mapk6, FADD, FASL IGF2, IGF2R	uncertain	Let 7 family is a famous tumor suppressive miRNA family. It may induce autophagy, because it inhibits Ras and its expression pattern. But it may also decrease autophagy by inhibitting TSC1 and E2F family.
miR-17	upregulated in Atg5 KO cells, miR-17* is predicted to target Atg5	Rbl2	Rbl2, Map3k12, STAT3, Map3k2, Rab10, Map3k8, E2F5,Mcl1 ,PTEN, Ulk1,Atg7, Atg16l1, Vps26a, Pak7, STAT3,	may block	miR-17 may block autophagy by targeting Ulk1, Atg7, Atg16l1 as well as FOXO1, however, it may also place favorable effect on autophagy by targeting molecules such as Mcl1 and Akt3.

			Cdk2, Mapk9, FOXO1, Rasl11b, Casp12, FADD, Eif2ak2, Akt3, Bcl2l2, E2F1, TRAF4		
miR-99a	down-regulated in Atg5 KO cells, is consistently predicted to target mTOR	none	Frap1(mTOR),E2F2, Vps37c, Bid,IGF1R, IGF2, Map2k6, TNF	may induce due to the prediction and expression pattern	miR-99a may induce autophagy by directly targeting mTOR, although it may also have some inhibitory effect on autophagy.
miR-199a	expression level relatively high but no obvious change among 3 microarrays, led to ERK down-regulation	ERK1/2, Hif1a, SIRT1	Vps26a, Rab9b, Arfrp1, Map3k12, TRAF3, SIRT1, Map3k11, E2F6,Atg5, Rheb	may block	miR-199a may inhibit autophagy by targeting ERK, SIRT1, Vps26a, even directly targeting Atg5.
miR-93	upregulated in Atg5 WT cell treated with starvation	E2F, p21, Vesicular stomatitis Indiana virus P protein, STAT3	Ulk1, Hif1a, STAT3, Eif4g2, Bnip2, Bcl2l2, E2F1, Atg16l1, Rb1cc1, XIAP, Bcl2, Bcl2l11, Bnip3, Mapk1, Atg2a, Atg2b	may block	miR-93 may inhibit autophagy by targeting Atg genes as well as its upstream signals.

3.2 Validation of differentially expressed miRNAs in autophagy induction

To confirm the deregulated miRNAs' expression is indeed due to autophagy induction, Taqman miRNA individual assays were employed to validate the miRNA expression level in Atg5 WT MEF and GFP-LC3 MEF cells.

Since the Atg5 KO cell line was not generated from the paired Atg5 WT MEF, and compared with Atg5 WT MEF, it responses differently to a series of stresses even cell death stimuli which may not be attributed to the lackage of autophagy (data not shown), We suspected that the genetic backgrounds of these two cell lines are quite different. So in order to identify the real deregulated miRNAs during autophagy induction, we mainly focused on the deregulated miRNAs in the samples of Atg5 WT MEF cells with or without 6h EBSS treatment (microarray 7180040).

Through Taqman Reverse Transcription quantitative PCR, we first examined the 10 interested miRNAs' expression in MEF cell treated with EBSS for 6 hour. As shown in Figure 3.3, among the 10 interested miRNAs, 7 miRNAs (miR-181a, miR-221, miR-30c, miR-93, miR-99, miR-199a, let-7b) showed expression patterns consistent with microarray 7180040. miR-34a whose signal was not readable in the microarray showed no deregulation. And we also found two miRNAs, miR-17 and miR-99a, showed a downregulation trend to some degree while miR-30c showed a slight upregulation. The upregulation of miR-181a & miR-93 and the downregulation of miR-221 were statistically significant (p-value<0.05). Thus the deregulation of the three significantly deregulated miRNAs (miR-181a, miR-93 and miR-221) revealed by microarray were validated by the Real Time qRT-PCR.

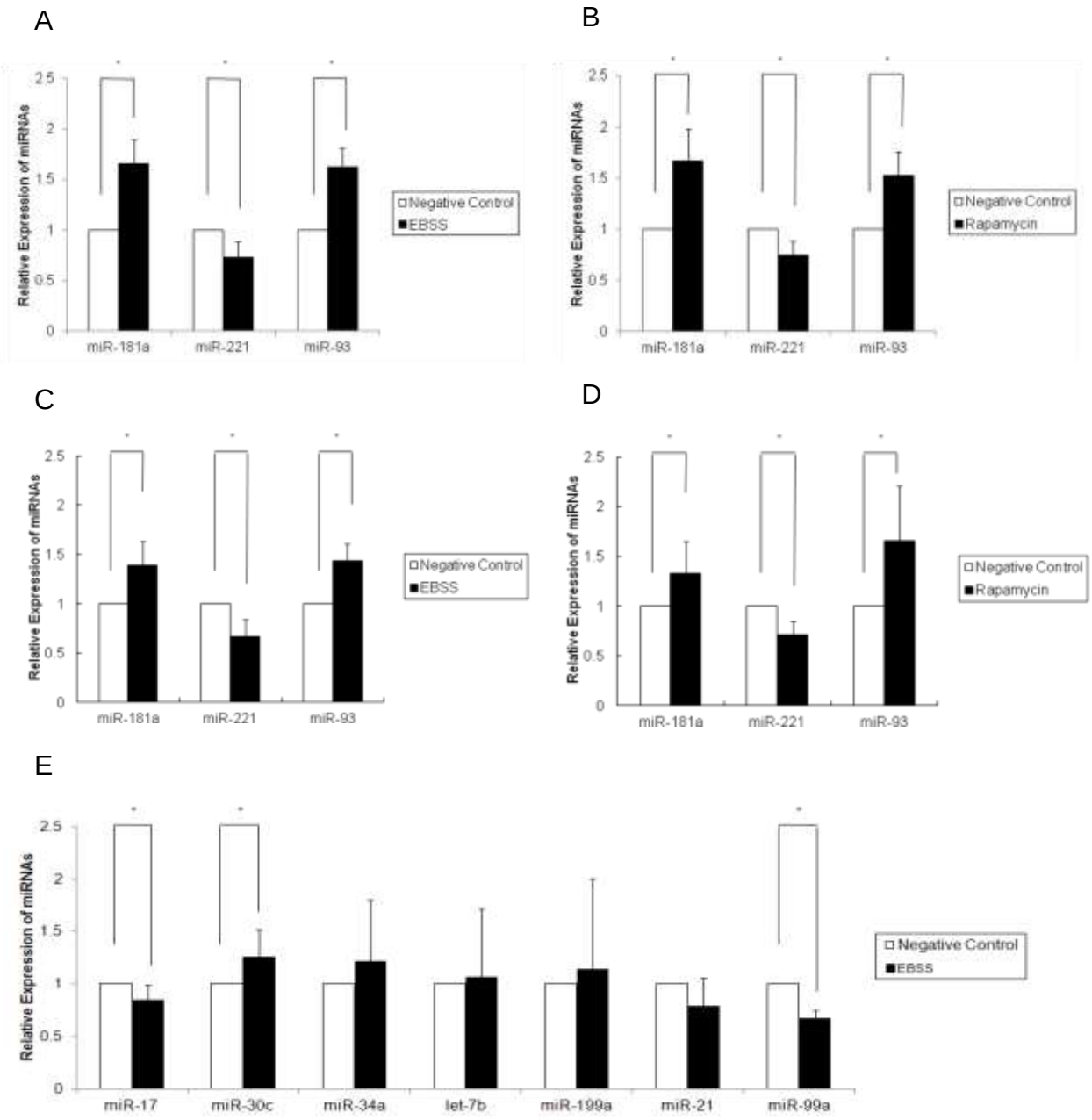
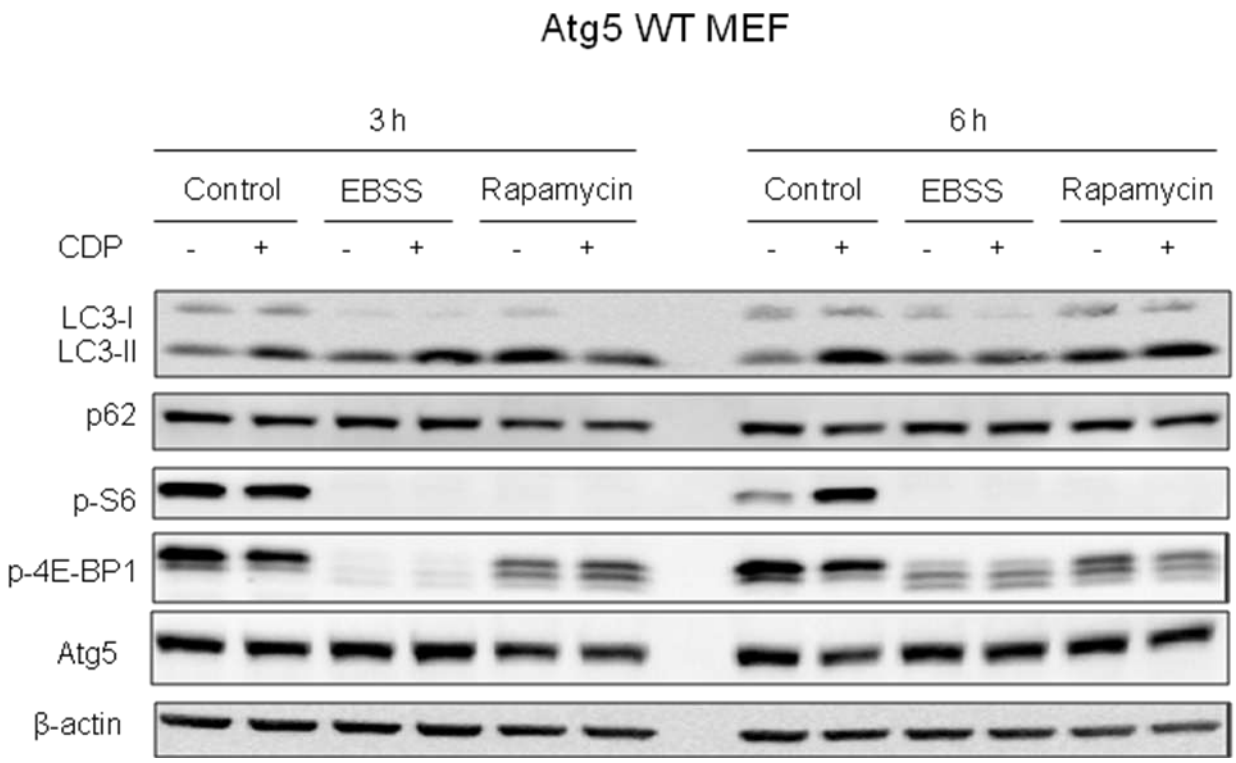


Figure 3.3 Validation of the interested miRNAs' deregulation in Atg5 WT and GFP-LC3 MEF in starvation and Rapamycin induced autophagy. (A) Normalized expression level of miR-181a, miR-221 and miR-93 in Atg5 WT MEF when autophagy

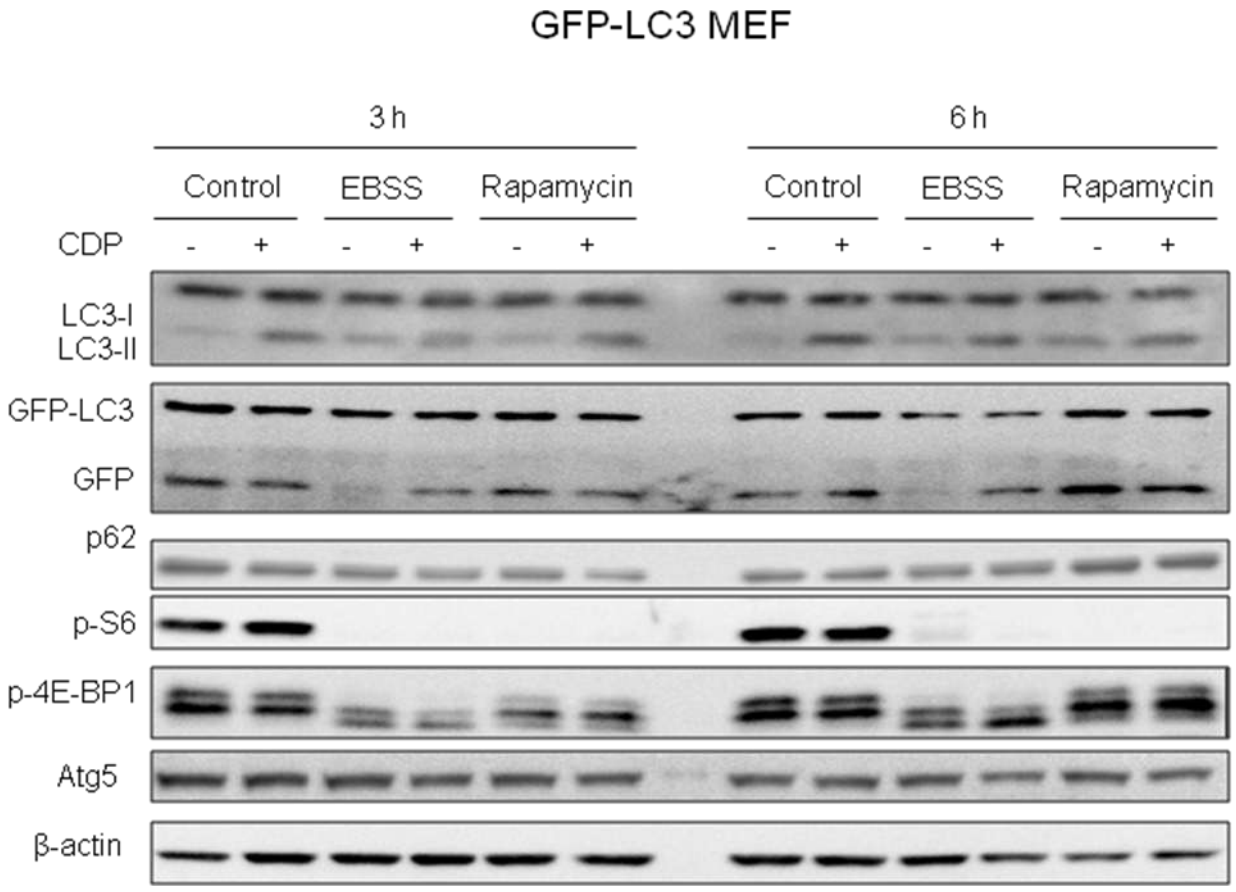
was induced following 6h EBSS treatment. **(B)** Normalized expression level of miR-181a, miR-221 and miR-93 in Atg5 WT MEF when autophagy was induced following 3h Rapamycin (100nm) treatment. **(C)** Normalized expression level of miR-181a, miR-221 and miR-93 in GFP-LC3 MEF when autophagy was induced following 6h EBSS treatment. **(D)** Normalized expression level of miR-181a, miR-221 and miR-93 in GFP-LC3 MEF when autophagy was induced following 3h Rapamycin (100nm) treatment. **(E)** The other 7 interested miRNA expression level in Atg5 WT MEF when autophagy was induced by 6h EBSS treatment. The miRNA levels were measured by Real Time qRT-PCR and normalized against the internal control snoRNA202 or snoRNA234. Error bar presents standard error of at least 3 independent experiments. * means p-value <0.05.

We proceeded to examine whether the deregulation of these three miRNA in Atg5 WT MEF following 6h starvation treatment was indeed due to autophagy induction, by employing another autophagy inducing agent Rapamycin and another MEF cell line GFP-LC3 MEF. Rapamycin has been shown by a lot of studies to be able to efficiently block mTORC1 activation and thus lead to autophagy induction even in the nutrient rich condition (Noda and Ohsumi, 1998). Figure 3.4 shows autophagy is induced in both Atg5 WT MEF and GFP-LC3 MEF following EBSS treatment (starvation) or 100 nm Rapamycin treatment. During autophagy, LC3-I is converted to LC3-II; p62 level is decreased, especially in starvation induced autophagy. EBSS and Rapamycin treatments lead to deactivation of mTOR signaling pathway as evident from the dephosphorylation of two mTOR downstream targets - p-S6 and p-4E-BP1. In GFP-LC3 MEF cells, the release pattern of free GFP is also a marker for autophagy induction.

A


Atg5 WT MEF
3 h
6 h
Control
EBSS
Rapamycin
Control
EBSS
Rapamycin
CDP
- + - + - + - + - + - +
LC3-I
LC3-II
p62
p-S6
p-4E-BP1
Atg5
β-actin

B


GFP-LC3 MEF
3 h
6 h
Control
EBSS
Rapamycin
Control
EBSS
Rapamycin
CDP
- + - + - + - + - + - +
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GFP-LC3
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Atg5
β-actin

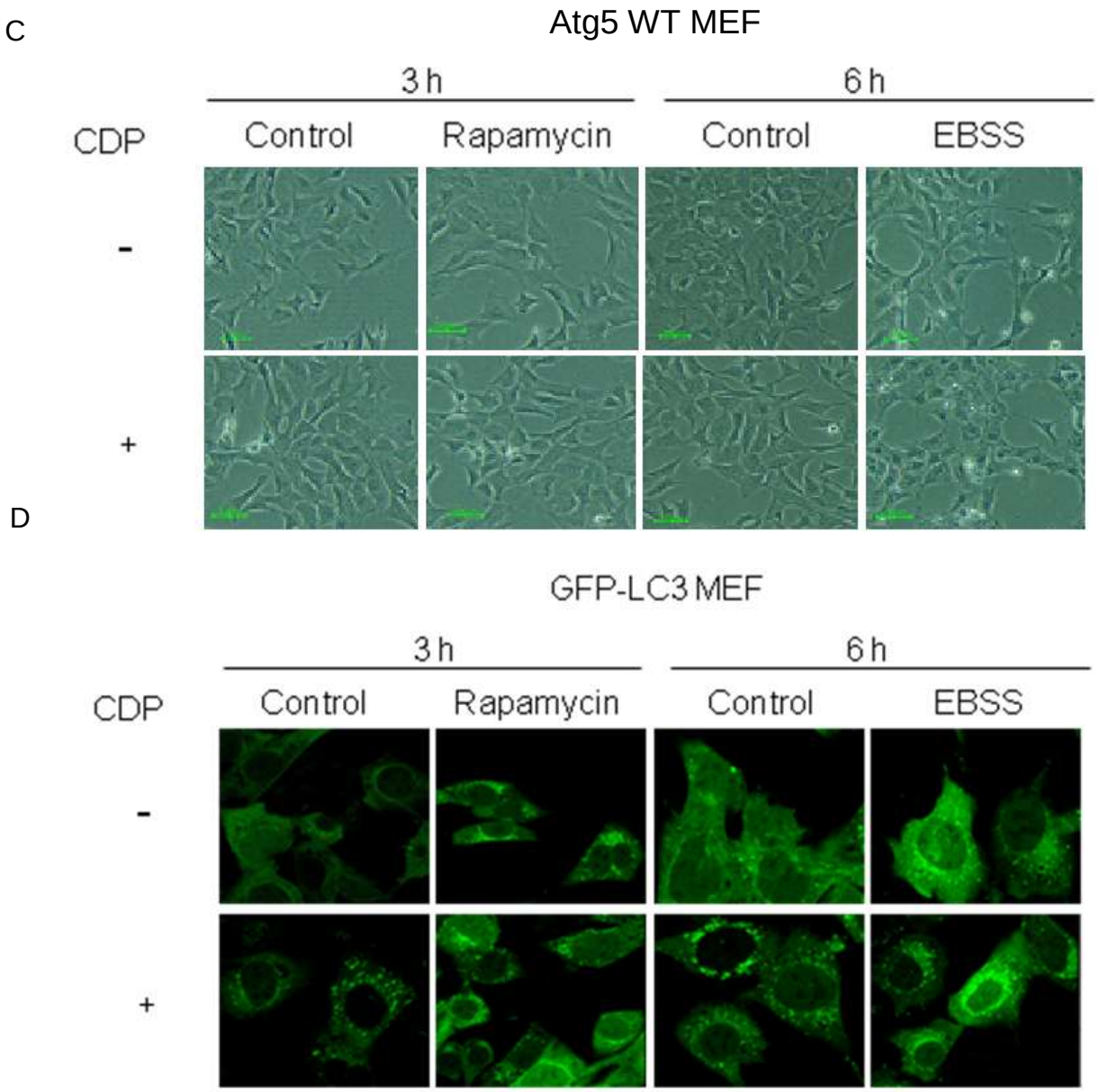


Figure 3.4 Autophagy induction in Atg5 WT MEF and GFP-LC3 MEF cells. Western blot shows that autophagy is induced after EBSS treatment and Rapamycin treatment for 3 hour and 6 hour in Atg5 WT MEF cell **(A)** and GFP-LC3 MEF cell **(B)**. **(C)** morphological character of starvation induced autophagy and Rapamycin induced autophagy in Atg5 WT MEF cell; **(D)** Autophagy is induced in GFP-LC3 MEF cell following 6h EBSS treatment or 3h 100 nm Rapamycin treatment, as evidenced by the increase of observable GFP-LC3

punctas. CDP is the lysosomal inhibitor which can block the LC3-II degradation and thus be used in detecting the intracellular autophagic activity.

Using the same set of cells, we then focused on the endogenous expression of miR-93, miR-181a & miR-221 in these two cell lines after 6h EBSS treatment or 3h 100nm Rapamycin treatment which has already been proved to successfully induce autophagy. As shown in Figure 3.3, consistent with the microarray and the previous validation, miR-93 and miR-181a were upregulated in starvation induced autophagy while miR-221 showed a consistent downregulation in starvation induced autophagy. In addition, compared with the miRNA expression patterns of starvation induced autophagy, all three miRNAs showed similar expression patterns in Rapamycin induced autophagy in both Atg5 WT MEF and GFP-LC3 MEF cells. Interestingly, the degree of deregulation for these three miRNAs in starvation induced autophagy and Rapamycin induced autophagy were also similar. These demonstrated that the deregulation of these three miRNAs was indeed due to autophagy induction, indicating the potential important regulatory role these three miRNAs might have on autophagy. Thus a miRNA signature in autophagy induction was revealed.

Since there exists distinct differences between effects of Rapamycin treatment and effects of amino acid starvation (Peng et al. 2002), the common effect of Rapamycin treatment and EBSS treatment placed on the expression of these three miRNAs implyed that target genes of these three miRNAs regarding to autophagy regulation should be more downstream, possiblely, genes involved in the autophagy core machinery.

3.3 miR-93 negatively regulates cellular autophagic activity

We then proceeded to screen the potential autophagy related target genes of these miRNAs. miR-93 was selected as the first miRNA to be functionally investigated because in MEF cells, it showed consistently steady upregulation in both EBSS and Rapamycin induced autophagy and has more predicted Atg target genes compared with the other two miRNAs (Table 3.3).

Pre-miR™ miRNA Precursors, small chemically modified double-stranded RNAs which includes one strand that is identical to the particular mature miRNA, are designed to mimic endogenous precursor miRNAs. Upon transfection into the cells, miRNA precursors are being processed into mature miRNA using the miRNA processing pathway of the host cell, thus the specific miRNA is overexpressed which will cause an increase in target mRNA suppression. Likewise, negative control miRNA is being processed into a miRNA that is validated not to bind any target genes. In contrast, Anti-miR™ miRNA Inhibitors, small chemically modified single-stranded RNAs, are designed to bind to and subsequently inhibit the activity of specific endogenous miRNAs.

In order to examine the possible regulatory role of miR-93 on autophagy, Pre-miR-93, Anti-miR-93 and negative control miRNA were also transfected transiently into Atg5 WT MEF and GFP-LC3 MEF cells.

As shown in Figure 3.5, upon transfection of miR-93 mimic into the Atg5 WT MEF or GFP-LC3 MEF cells, the amount of mature miR-93 was greatly enhanced while it was also dramatically decreased when antagomir of miR-93 was introduced into the cells. The intracellular basal autophagic activity was reduced in the pre-miR-93 transfected GFP-LC3 MEF cells while it was increased in the anti-miR-93 transfected GFP-LC3 MEF cells,

comparing with that of GFP-LC3 MEF cells transfected with negative control miRNA. When the transfected cells were treated with EBSS or 100nm Rapamycin for 3 hour, there was an obvious autophagy induction in all three populations of cells, similarly, the pre-miR-93 transfected cells again showed a relatively weak autophagy induction while the anti-miR-93 transfected cell showed a stronger autophagy induction compared to the control group of cells (Figure 3.6 A, B). And the pattern of endogenous autophagy activity in these three populations of cells correlated well with the expression pattern of Ulk1 and STAT3. As STAT3 was already a proven target of miR-93, this result supported the hypothesis that miR-93 placed a negative regulatory effect on autophagy induction via targeting Ulk1, one of the core autophagy machinery genes involved in the autophagy initiation process. Figure 3.6 C showed the confocal microscopy images of the three transfected groups of GFP-LC3 MEF, as evidenced by the change of area and numbers of GFP-LC3 punctas, this result confirmed miR-93's negative effect on starvation and Rapamycin induced autophagy.

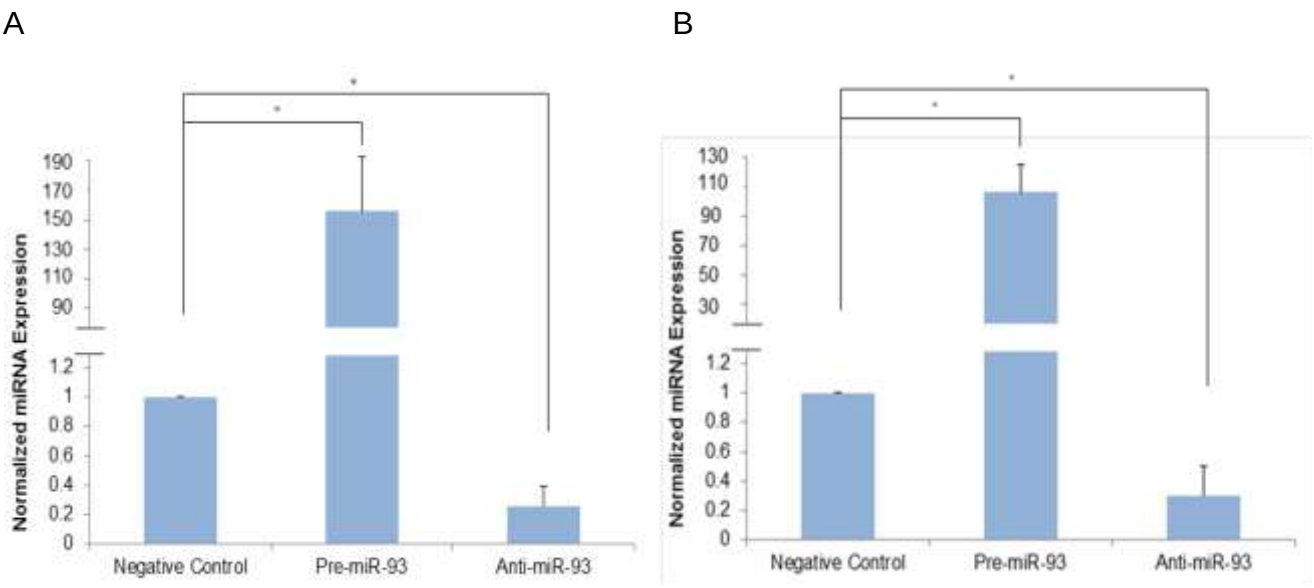
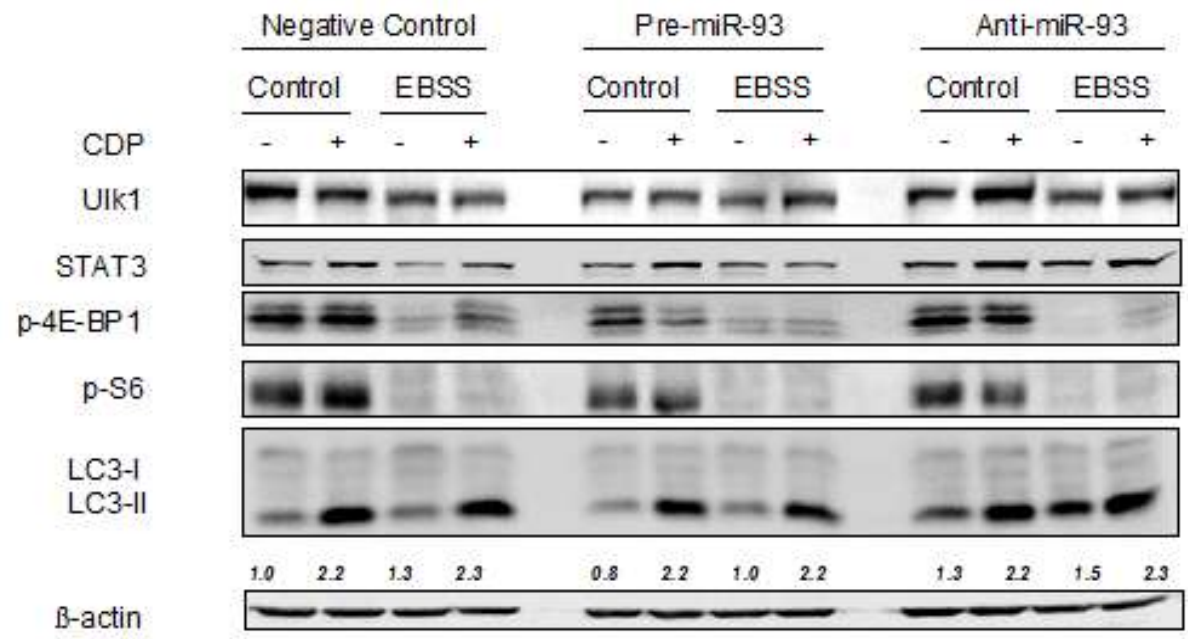


Figure 3.5 The efficiency of transient transfection in MEF cells. (A) Mature miR-93 expression level in Atg5 WT MEF cells after transfected with negative control, miR-93 precursor or miR-93 inhibitor for 24h. **(B)** Mature miR-93 expression level in GFP-LC3 MEF cells after transfected with negative control, miR-93 precursor or miR-93 inhibitor for 24h. Data presented are mean ± S.E. * denotes a p-value less than 0.05.

A


Negative Control
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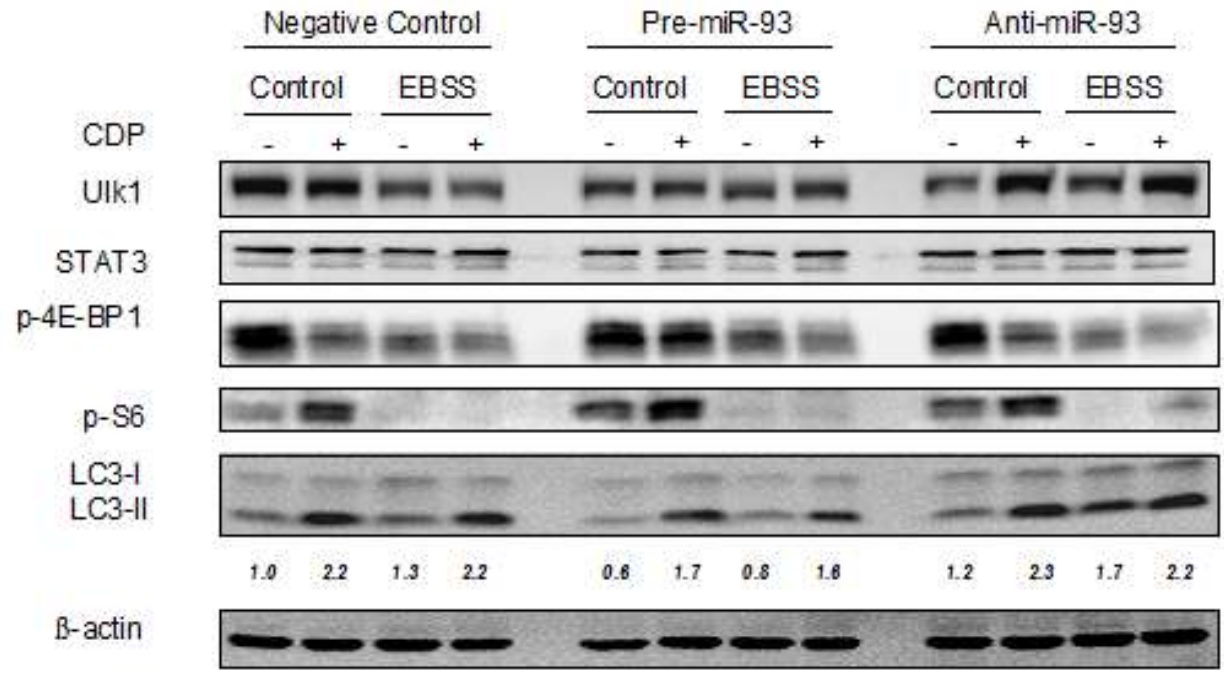
Anti-miR-93
Control
EBSS
Control
EBSS
Control
EBSS
CDP
- + - + - + - + - + - +
Ulk1
STAT3
p-4E-BP1
p-S6
LC3-I
LC3-II
1.0 2.2 1.3 2.3 0.8 2.2 1.0 2.2 1.3 2.2 1.5 2.3
ß-actin

B


Negative Control
Pre-miR-93
Anti-miR-93
Control
EBSS
Control
EBSS
Control
EBSS
CDP
- + - + - + - + - + - +
Ulk1
STAT3
p-4E-BP1
p-S6
LC3-I
LC3-II
1.0 2.2 1.3 2.2 0.6 1.7 0.8 1.8 1.2 2.3 1.7 2.2
ß-actin

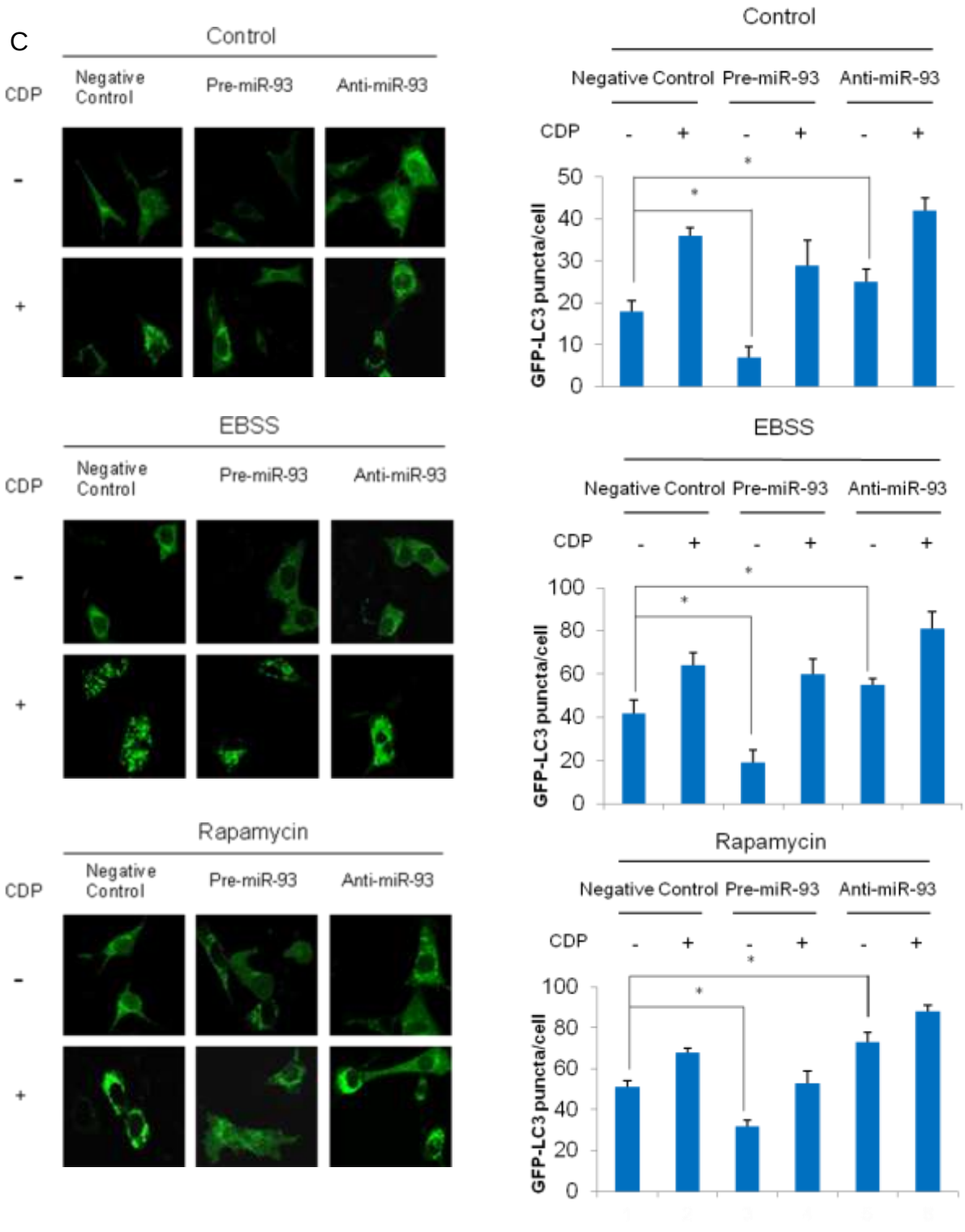


Figure 3.6 miR-93 negatively regulates endogenous autophagic activity in starvation

and Rapamycin induced autophagy. (A) Western blot image showing miR-93 negatively regulates cellular autophagic activity in starvation-induced autophagy. GFP-LC3 MEF cells were transfected with 100 nm negative control miRNA, pre-miR-93, anti-miR-93 respectively. 24h post transfection, cells were treated with EBSS for 3h. The control groups were cultured in full medium. 10 um CDP was added as an inhibitor of autophagic process at late stage to show the real autophagic flux. (B) Western blot image showing miR-93 negatively regulates cellular autophagic activity in rapamycin-induced autophagy. The same set of three transfected GFP-LC3 MEF cells were treated with 100 nm Rapamycin for 3h. Image J software was used to quantify LC3-II expression and the values which are listed above the row of actin were the relative fold change normalized to the control group. (C) Confocal microscopy image of starvation or Rapamycin-induced autophagy in GFP-LC3 MEF cells transfected with 100 nm negative control miRNA, miR-93 mimic and miR-93 antagomir, respectively. GFP-LC3 puncta/cell was counted manually for each group. Data presented are mean ± S.E. * denotes a p-value less than 0.05.

3.4 Screening of possible autophagy related target genes of miR-93

Employing miRecords (http://mirecords.biolead.org) which combines the results of 11 microRNA-target prediction programs including miRanda, PicTar & TargetScan (Xiao et al., 2009), an in silico screen for the autophagy related target gene of miR-93 was first conducted. A prediction was considered valid only if the target relationship was predicted by at least 3 programs.

As briefly summarized in Table 3.4, miR-93 was predicted to target a series of autophagy related genes (both in the autophagy upstream regulatory network and the autophagy core machinery) including Ulk1, Hif1a, STAT3, Eif4g2, BNIP2, Bcl2l2, E2F1, Atg16l1, Rb1cc1, XIAP, Bcl2, Bcl2l11, BNIP3, Mapk1, Atg2a, Atg2b.

Table 3.4 Predicted autophagy related target genes for mmu-miR-93

Target Gene	Base Pairing Complementarity	Conserveness	No. of Programs Predicting the Relationship
Ulk1	partial	Mouse,Human	5
Hif1a	partial	Mouse	5
STAT3	partial	Mouse,Human,Rat	5
Eif4g2	partial	Mouse,Human	5
Rb1cc1	partial	Mouse,Human	4
Bnip2	partial	Mouse,Human	4
Bcl2l2	partial	Mouse,Human	4
E2F1	partial	Mouse,Human	4
Atg16l1	partial	Mouse,Human	4
Atg7	partial	Mouse,Human	3
XIAP	partial	Mouse	3
Bcl2	partial	Mouse	3
Bcl2l11	partial	Mouse	3
Bnip3	partial	Mouse	3
Mapk1	partial	Mouse	3
Atg2a	partial	Mouse,Human	3
Atg2b	partial	Mouse,Human	3
Akt1	partial	Mouse, Human	3

3.4.1 miR-93 negatively regulates Ulk1 expression

To identify the possible target genes, we selected 7 predicted target genes for further examination based on the number of programs predicting the relationship, the importance

of target gene in the autophagy network and their conserveness among species. Target gene expressions were examined at both protein level and mRNA level.

GFP-LC3 MEF were transfected with negative control miRNA, mimic and antagomir of mmu-miR-93 (100nm) and the level of selected target gene transcripts were measured using Real Time qRT-PCR. The results are shown in Figure 3.7 A.

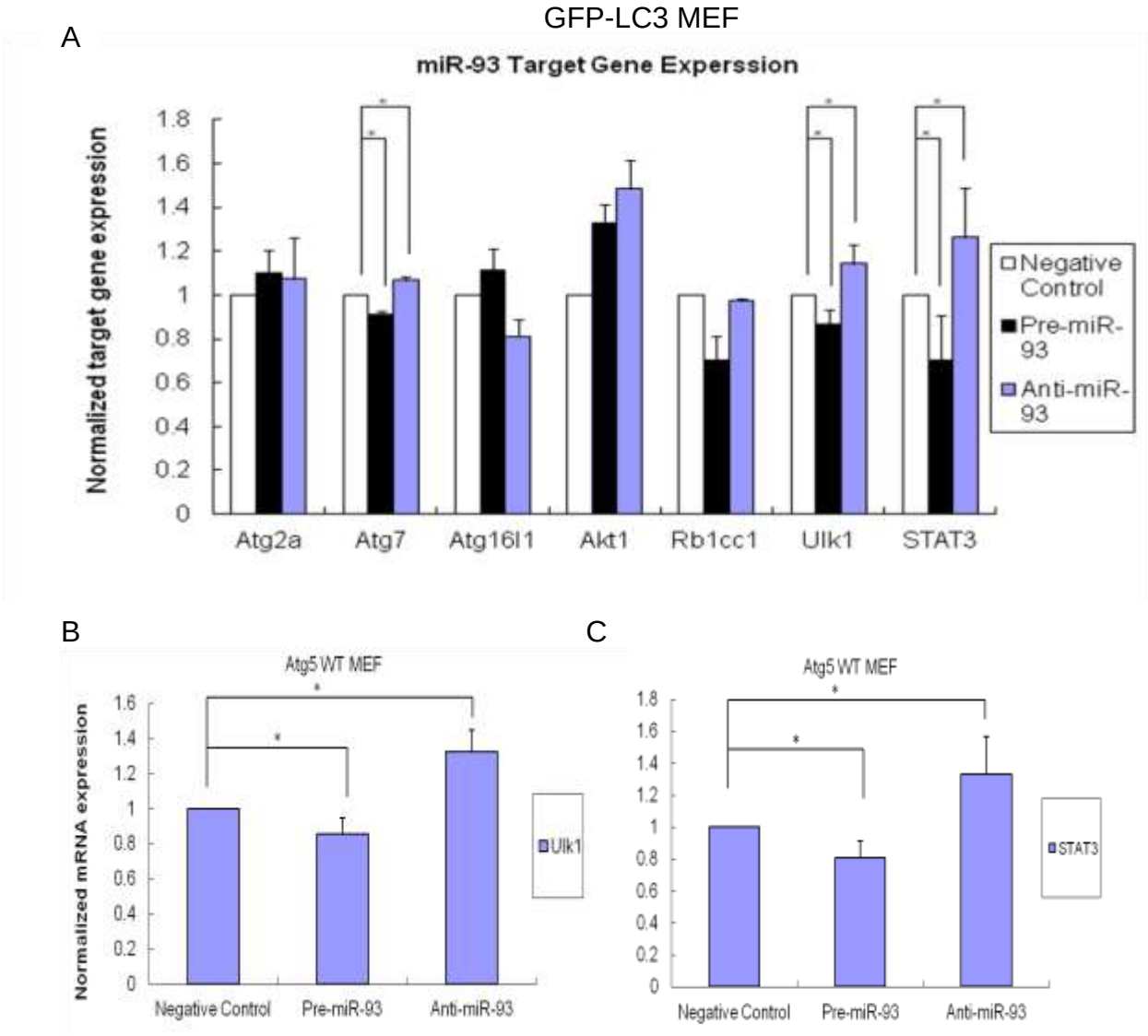


Figure 3.7 The mRNA expression level of miR-93 predicted target genes after transfected with 100nm negative control miRNA, mimic and antagomir of miR-93. **(A)** The mRNA levels of predicted target genes in transfected GFP-LC3 MEF. **(B)** Ulk1 mRNA level in transfected Atg5 WT MEF. **(C)** STAT3 mRNA level in transfected Atg5 WT MEF. 24 hours post-transfection, cells were harvested and the target gene transcript level were measured by Real time qRT-PCR and normalized to b-actin. Data were presented as mean ± S.E., * denotes a p-value less than 0.05.

Among those predicted target genes, mRNAs of STAT3 and Ulk1 were downregulated upon addition of pre-miR-93 into the GFP-LC3 MEF cells while they were relatively more abundant when anti-miR-93 was introduced into the GFP-LC3 MEF cells. To confirm this, Atg5 WT MEF was also transiently transfected with mimic and antagomir of miR-93, indeed, the mRNA levels of Ulk1 and STAT3 were decreased upon pre-miR-93 transfection (Figure 3.7 B, C). Based on this result, STAT3, Ulk1 seemed to be the real target of miR-93 in Atg5 WT MEF and GFP-LC3 MEF cells.

After observing miR-93's negative regulatory effect on autophagy induction, we then focused on elucidating the relationship between miR-93 and its predicted target gene, Ulk1. STAT3 has also been proved as a miR-93 target in mouse (Foshay et al., 2009).

As shown in Figure 3.8, the protein expression of Ulk1 was suppressed when miR-93 precursor was introduced into the Atg5 WT MEF and GFP-LC3 MEF cell. Also, transfection of miR-93 antagomir weakened miR-93's suppression on Ulk1 as evidenced by an increase in the protein expression. Similarly, STAT3 protein expression level was also decreased when miR-93 precursor was transfected into the cells while it was relatively higher when cells were transfected with miR-93 antagomir, compared with that of cells transfected with negative control miRNA (Figure 3.8).

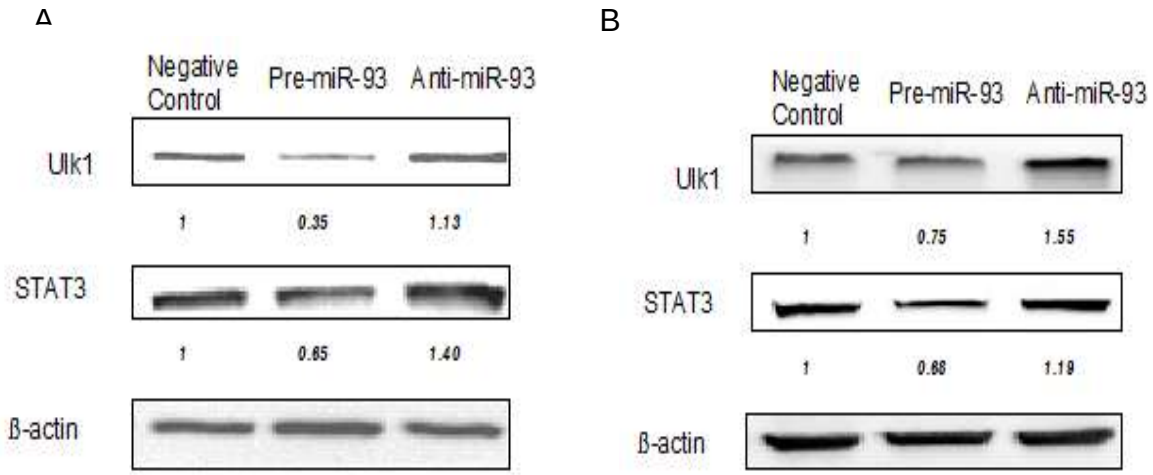


Figure 3.8 miR-93 negatively regulates Ulk1 protein expression. (A) Protein expression of Ulk1 in Atg5 WT MEF cells transfected with100nm negative control miRNA, miR-93 precursor or miR-93 inhibitor. **(B)** Protein expression of Ulk1 in GFP-LC3 MEF cells transfected with100nm negative control miRNA, miR-93 precursor or miR-93 inhibitor. Image J software was used to quantify Ulk1 and STAT3 protein expression and the values listed below protein bands were the relative fold changes normalized to the control group.

3.4.2 Ulk1 is downregulated at specific time points when autophagy is induced

At the same time when the screen for miRNA target was conducted in the transfected MEF cells, the protein expression of interested autophagy related gene was also being examined in both Atg5 WT MEF and GFP-LC3 MEF under conditions of EBSS induced autophagy and Rapamycin induced autophagy.

If the target gene was indeed a major endogenous target of the specific miRNA, it might present an opposite expression trend compared to the expression pattern of the miRNA regulator.

Although the autophagy process was still on-going, as evidenced by the accumulation of LC3 II under both EBSS and Rapamycin treatment (Figure 3.9), Ulk1, one of the core Atg genes involved in the initiation step of autophagy, showed a downregulation trend at specific time points in starvation and Rapamycin triggered autophagy induction. Interestingly, miR-93 was previously proved to be upregulated at the same time points (Figure 3.3), indicating that Ulk1 may be a real endogenous target of miR-93.

STAT3 was proved to be an endogeneous target of mmu-miR-93 (Foshay et al., 2009). So we employed it as a positive control to indicate the possible target genes of mmu-miR-93 in this study.

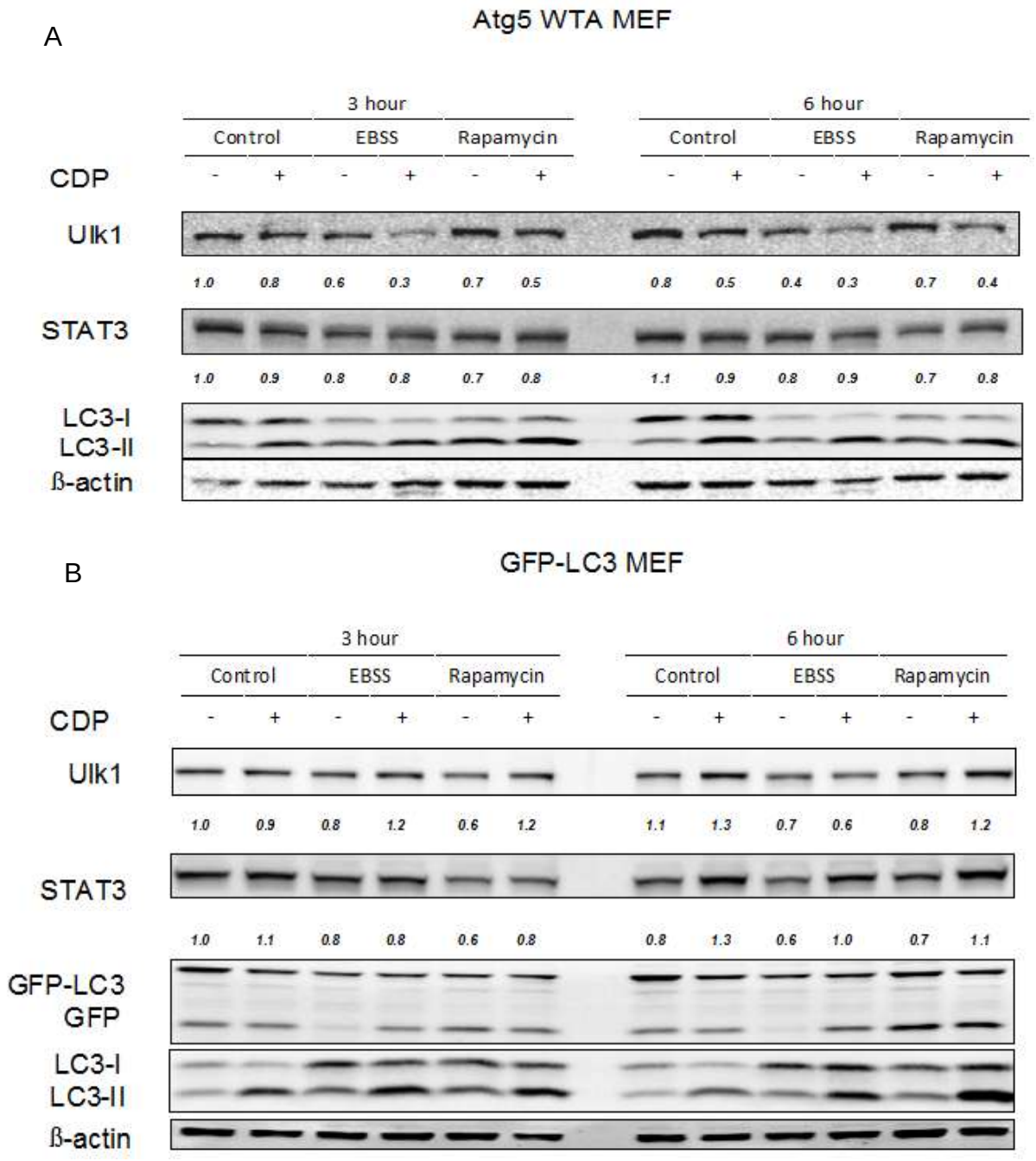


Figure 3.9 Downregulation of Ulk1 in autophagy induction in Atg5 WT and GFP-LC3 MEF cells. (A) Western blot results showing Ulk1 downregulation in Atg5 WT MEF. **(B)** Western blot results showing Ulk1 downregulation in GFP-LC3 MEF. MEF cells were

treated with EBSS or 100nm Rapamycin for 3 hour or 6 hour. Image J software was used to quantify Ulk1 and STAT3 protein expression and the values listed below protein bands were the relative fold changes normalized to the control group.

3.5 Ulk1 is a direct target of miR-93

To answer the question whether miR-93 directly regulatesUlk1 expression by binding to the predicted binding sites located in the 3' UTR of Ulk1 mRNA, we used β-Galactosidase reporter gene assay to elucidate the molecular mechanism. We first identified two putative miR-93 binding sites in the 3' UTR of Ulk1 mRNA using Targetscan prediction program. Then the 1683 bp long wildtype Ulk1 3' UTR as well as the corresponding mutant Ulk1 3' UTR in which the two putative binding sites for miR-93 were mutated were subcloned into the downstream of the β-Galactosidase reporter gene driven by MRP1 promoter. When HCT116 cell were transfected with 50nm miR-93 precursor, a significant reduction of β-Galactosidase activity was observed in cells carrying the wildtype Ulk1 3'UTR reporter construct compared to cells carrying the mutant Ulk1 3'UTR reporter construct. And there was no significant difference in the β-Galactosidase activity between cells carrying the wildtype Ulk1 3'UTR reporter construct and cells carrying the mutant Ulk1 3'UTR reporter construct when negative control miRNAs were introduced into the cells. Figure 3.10 shows that miR-93 can specificaly interact with the two putative binding sites in the 3' UTR of Ulk1 and thus directly target Ulk1.

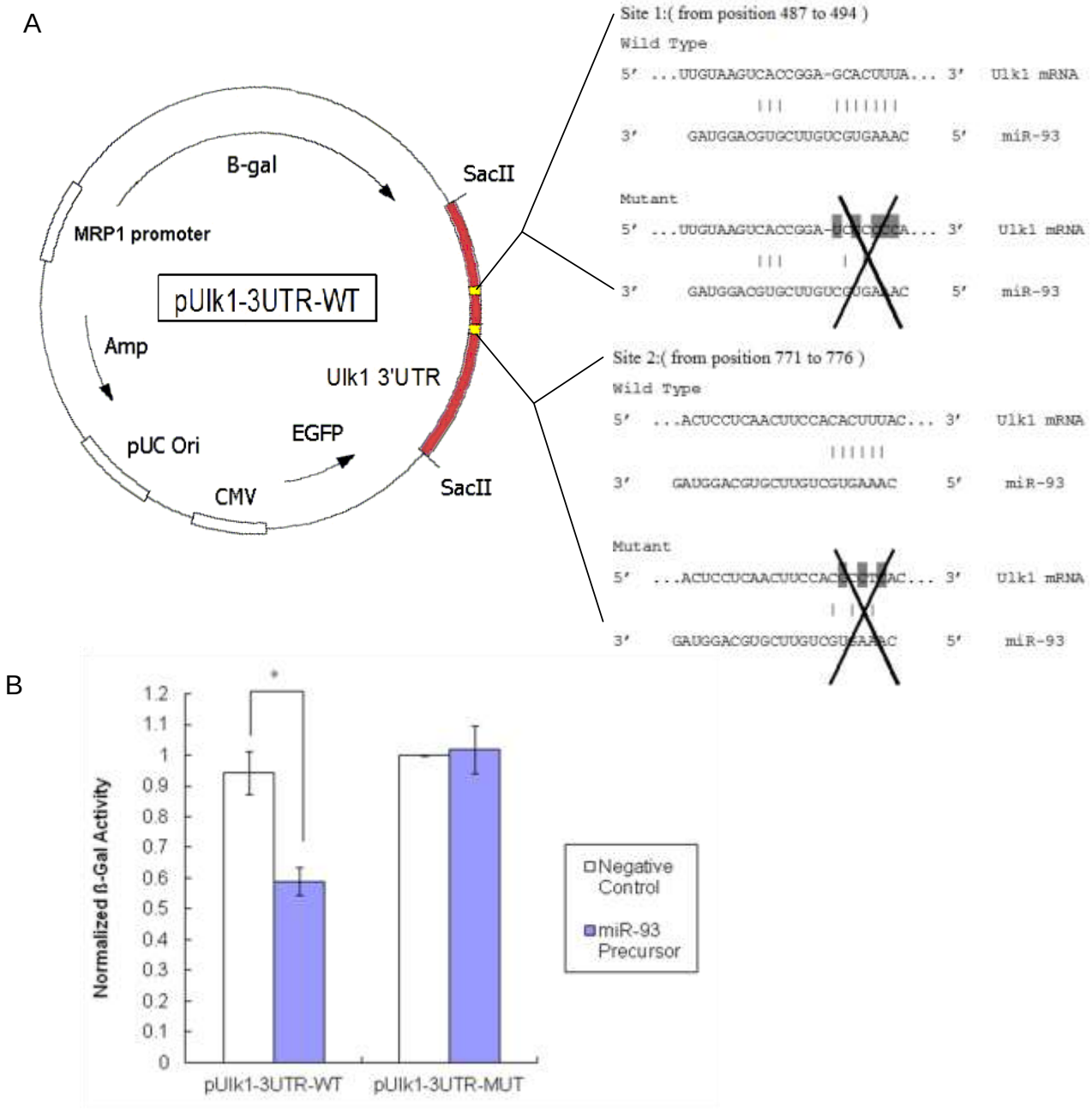


Figure 3.10 miR-93 interacts with the predicted binding sites on Ulk1 3'UTR. (A) Schematic diagram of the constructs pUlk1-3UTR-WT with the two predicted binding sites and corresponding mutant binding sites. "X" means the predicted targeting relationship does not exist when the specific predicted binding site on the 3' UTR of Ulk1 mRNA was mutated. **(B)** β-Galactosidase reporter gene assay result showing that miR-93 binds to the

predicted sites in Ulk1 mRNA 3'UTR. Data presented are mean ± S.E. of three experiments. * denotes a p-value less than 0.05.

CHAPTER 4

DISCUSSION AND CONCLUTIONS

Chapter 4 Discussion and Conclusions

4.1 Autophagy-miR-93-Ulk1 negative feedback loop

One important observation from this study is that, we identified a novel negative regulatory function of miR-93 on autophagy, which is at least, partially, executed by targeting Ulk1 gene expression. Autophagy induced by both starvation or Rapamycin leads to significant up-regulation of miR-93. On the other hand, miR-93 is able to negatively regulate autophagy via suppression of Ulk1 gene expression. Therefore, it is believed that there is a negative feedback loop between autophagy and miR-93 (Figure 4.1).

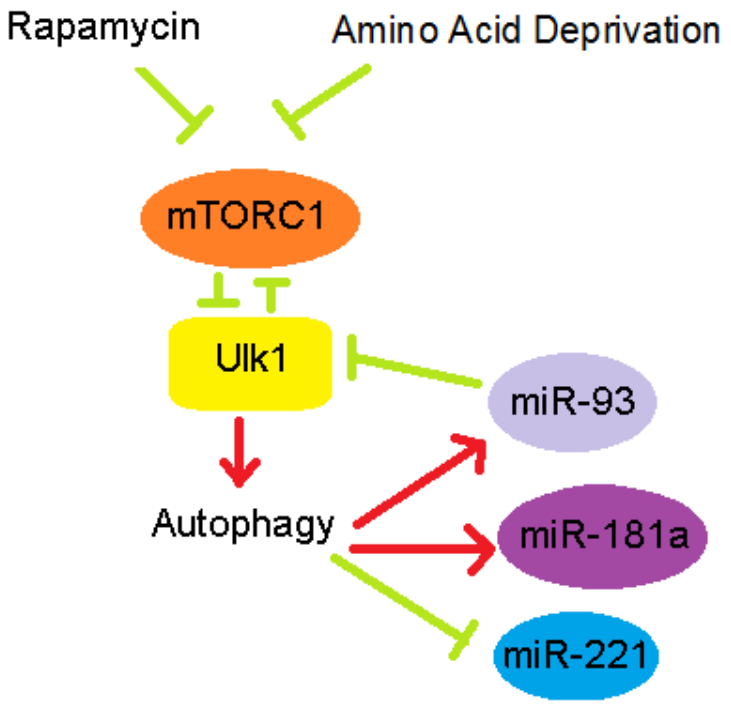


Figure 4.1 The autophagy-miR-93 negative feedback loop

At present, it is still controversial, at least in the mammalian cells, whether autopahgy requires de novo gene transcription and protein synthesis (Martinet et al., 2006; Mizushima et al., 2007). On one hand, it is widely believed that Atg proteins are always sufficiently expressed and their post-translational modifications and/or associations with other members of the autophagic machinery seem more critical to autophagy rather than the regulation on their expression levels (Mizushima et al., 2010); on the other hand, some of the key Atg proteins such as LC3 are found to be significantly up-regulated in the autophagic process (Xiao et al., 2011). Therefore, it is highly possible that there are active regulatory mechanisms at the transcriptional and translational levels in the course of autophagy. Data from this study provided new evidence that certain miRNAs could play important roles in autophagy via regulation of key Atg genes such as Ulk1. Our findings are generally consistent with the existing reports showing that miRNAs could target other Atgs such as Beclin 1, LC3-II (Zhu et al., 2009; Xiao et al., 2011).

A possible explanation of this negative feedback loop involving miR-93 is that when cells are under stress conditions, autophagy is induced to provide cells with essential regenerated energy and "building blocks" such as amino acids, however, the autophagy activity should be tightly limited to a certain degree otherwise the excessive autophagy activity would destroy the normal cellular system and render cells to autophagic cell death (Codogno et al., 2005; Kang et al., 2007). Thus, miR-93 may help cells to survive by limiting the harmful excessive autophagic activity. It is also possible that extra Ulk1, like extra Atg5 (Yousefi et al., 2006), may interact with other signal molecules, lead it to detrimental outcomes such as promoting apoptosis, so cells have to generate some inhibitors to decrease the total expression of Ulk1 to an acceptable level.

However, it should be noted that during autophagy induction, the upregulation of miR-93 does not result in a complete blockage of the Ulk1 expression or the corresponding autophagy induction process. Autophagy is still ongoing under starvation and Rapamycin treatment which might be explained by the fact that even a small amount of Atg1 is often enough for the initiation of autophagy (Kabeya et al, 2009). Therefore, further studies are needed to determine the actual functional impact of miRNAs such as miR-93 on autophagy and whether such regulatory mechanisms could serve as new venue for the intervention of autophagy.

4.2 miR-93 deregulation affects multiple pathways

Autophagy deregulation is a common phenomenon in many human diseases, especially in cancer. So far, autophagy is much more perceived as a tumor suppressive process (Huang et al., 2007), as a lot of previous studies have demonstrated that tumor cells showed reduced autophagic activity (Schweers et al., 1985; Knecht et al.,1984) and several anticancer drugs as well as tumor suppressor genes such as PTEN and p53 exert a favorable effect on autophagy induction (Kondo and Kondo, 2006).

Nevertheless, miR-93 is a component of the miR-106b-25 polycistron which exert potential proliferative, antiapoptotic, cell cycle-promoting effects in vitro and tumorigenic activity in vivo. The miR-106b-25 cluster is a paralog of the miR-17-92 cluster based on the similarity between their nucleotide sequences.

Human miR-93 was observed to be frequently upregulated in a series of tumors including epithelial ovarian cancer (Resnick et al., 2009), T-cell leukemia (Yeung et al.,

2008), hepatocellular carcinoma (Li et al., 2009b), highgrade breast tumors (Blenkiron et al., 2007), gastric cancer (Petrocca et al., 2008; Kim et al., 2009), prostate cancer (Ambs et al., 2008) as well as stress conditions such as hypoxic environment in primary term human trophoblasts (Donker et al., 2007). Its downregulation was also being observed in HCT-8 and HCT-116 colon cancer cells after treatment with 5-fluorouracil (5-FU) and oxaliplatin (L-OHP) (Zou et al., 2010) as well as cells undergo cardiac hypertrophy (Van Rooij et al., 2006).

Generally, miR-93 is considered as an oncomiR and affects a variety of signaling pathways:

The miR-106b-25 cluster including miR-93, was proven to be activated by E2F1 in human gastric cancer in parallel with its host gene, Mcm7 and in turn, negatively regulated E2F1 (Petrocca et al., 2008). As E2F1 is a transcription factor that transactivates a variety of genes involved in chromosomal DNA replication and both oncogenic and tumor suppressive activities, the effects of downregulation of E2F1 by miR-93 may be quite complex. For instance, with regard to autophagy, E2F1 upregulates several autophagy genes including LC3, Atg1, Atg5, DRAM (Polager et al., 2008) and BNIP3 (Tracy et al., 2007), but it also activates PI3K/Akt/mTOR pathway (Ladu et al., 2008). Suppression of E2F1 by miR-93 may subsequently lead to suppression of target genes of E2F1 and subsequent effects.

Indeed, besides directly targeting E2F1, miR-93 was also proved to target several E2F1 upregualted genes including cell cycle regulator p21 (Petrocca et al., 2008) and the tumor suppressor TP53INP1 (Yeung et al., 2008). Overexpression of miR-93 completely abrogated TGFβ-induced cell-cycle arrest, it is consistent with the degree of p21

downregulation. And targeting another tumor suppressor gene FUS1 could also decrease apoptosis (Maes et al., 2008).

Through luciferase reporter assay and western blot, STAT3 was proved to be a target of miR-93 (Foshay et al., 2009). Similar to targeting E2F1, targeting the pluripotent transcription factor STAT3 could also lead to alteration in a lot of downstream processes.

4.3 Autophagy induction induces deregulation of miRNAs

As described in Section 3.1, from the microarray, several clustered miRNAs were found to be deregulated in starvation-induced autophagy. The coordinated deregulation of members of a specific miRNA family or members of different families which shares the common target genes implies that the miRNA families may have a combinational effect on the regulation of autophagy. For example, Bcl-2 is the common predicted target of both upregulated miRNAs – miR-93 and miR-181a, as well as the downregulated miRNA miR-221 (Table 3.3), suggesting that these miRNAs may contribute to the balance of endogenous Bcl-2. Since Bcl-2 familiy members play important roles in both autophagy and apoptosis (Pattingre et al., 2005), the change of intracellular Bcl-2 homeostasis would affect both autophagy and apoptosis.

In addition, from the screening for the possible miRNA regulators of autophagy, we found that miRNAs previously identified to target key Atg genes were rare in the microarray report. For those miRNAs that are predicted to target key Atg genes, usually they are also predicted to target dozens of other genes involved in autophagy upstream signaling network and other types of programed cell death, both proautophagic and

antiautophagic (Table 3.3), so it is hard to estimate the dominant role that these miRNAs would play in the regulation of autophagy induction, completion and function. As miRNA regulation is such a dynamic process, it is very possible that during different stages of autophagy process, a specific miRNA may have different major targets and play distinct functions (Arnold et al., 2011; Ambros and Chen, 2007).

In order to obtain more consistent deregulation of miRNAs in autophagy induction, we employed another autophagy inducing agent Rapamycin and another MEF cell line. Based on our data, there is not much significant difference in the level of expression of the three miRNAs examined following the two different ways of inducing autophagy (Figure 3.3). Nevertheless, further study wil reveal the different expression patterns of other miRNAs following these two treatments. This could be the result of a series of factors such as cell line or treatment specific effect on the expression of miRNAs. It is also possible that this is not associated with induction of autophagy. For example, Rapamycin has different effects on a lot of signaling pathways compared with EBSS treatment (Peng et al. 2002), the altered expression of a specific miRNA may be due to an alteration in a corresponding pathway not related to autophagy.

4.4 Conclusions

Through this study, we identified a new target for mmu-miR-93, and elucidated the negative regulatory effect of miR-93 on autophagy induction was at least, partially, executed by decreasing Ulk1 expression.

It has been suggested that Atg mRNA or protein expression levels are not considered appropriate indicators for monitoring autophagy as Atg proteins are always sufficiently expressed and their post-translational modifications and/or associations with other members of the autophagic machinery seem more critical to autophagy rather than the regulation on their expression levels (Mizushima et al., 2010). This study agrees with the general notion that the expression levels of Atg genes are not proper autophagy markers, however, it does provide an evidence that through regulating the total expression of Atg genes, miRNA does have the ability to affect intracellular autophagic activity.

Through this study, a new negative feedback loop in autophagy induction was revealed, showing that the induction of autophagy is under tight control in the intracellular environment. We can expect there will be more miRNAs being recognized as endogenous regulators of autophagy.

4.5 Directions for future study

4.5.1 miRNA profiling following different autophagy induction stimuli and using different cell lines

One weakness of this study is that the miRNA microarray was conducted once only, using one autophagy induction stimuli - starvation, and the employment of two cell lines with distinct genetic backgrounds further weaken the significance of the comparisons in the three designed experiments. The deregulations of miRNA expression revealed by the microarray may not be consistent and functionally-related as experiment conditions could cause a lot of deviation in the real miRNA expression and thus results in false leads.

More microarrays conducted under different autophagy induction conditions including nutrient deprivation, Rapamycin treatment and hypoxia are expected to reveal the consistent miRNA signature in autophagy induction and thus facilitate future functional studies.

Human cell lines, especially cancer cell lines which showed deregulated basal or induced autophagy activity should also be selected for the miRNA analysis. In addtion, cell lines which always showed the deregulation of the interested miRNAs should also be selected to study their autophagy activity. For example, miR-93 is deregulated in HCC, it is interesting to examine whether miR-93 also suppress autophagy in HCC and thus contributes to tumorigenesis. These will enhance the clinical relevance and prospect of this study.

4.5.2 Characterization of the roles that miRNAs play in the biological functions of autophagy

As autophagy is closely related to cell death and survival, it will be interesting to examine the effect of miRNAs (especially oncomiR and tumor suppressor miRNAs) on cell death, survival and proliferation via monitoring autophagy, thus further revealing miRNA and autophagy's biological function in the various physiological and pathological conditions.

4.5.3 Investigation of miRNAs's role on disease development via modulation of autophagy

As mentioned in 1.1.4, autophagy deregulation is a common phenomenon in many human diseases. The investigation of miRNAs' effect in pathogenesis via monitoring autophagy will contribute to the better understanding of the molecular mechanisms of diseases and bring possible breakthrough points for developing miRNA-based therapies.

For example, regarding the oncogenic miR-93's negative regulatory effect on autophagy, whether or not miR-93 also participates in affecting Ulk1 expression and autophagy in malignant cells remains to be clarified. It would thus be important to further analyze if a direct correlation between the aberrant expression of endogenous miR-93 and disease development such as tumorigenesis via modulation of Ulk1 and corresponding autophagy. Clinical samples of different stages of cancer should be obtained for analyzing the expression level of miR-93, Ulk1 and other classic autophagic markers. In contrast, further investigation is also required to determine in the condition of environmental and metabolic stresses such as nutrient deprivation and hypoxia, whether autophagy also exerts its protective effect via enhanced expression of miR-93 and consequent suppression of target genes such as E2F1 and STAT3.

Besides, miR-93 was also found to have the ability to accelerate the accumulation of toxic agent in aging liver isolated from 33 month old mouse as it targeted several important genes involved in maintaining the intracellular homeostasis and detoxification such Mgst1 & eEF1A-1 (Maes et al., 2008). miR-93 might also downregulate autophagy through decreasing Ulk1 expression in this condition. As autophagy is crucial for maintaining the intracellular homeostasis by providing energy and eliminating spent organelles and other cytotoxic components. The negative effect of miR-93 on autophagy thus may contribute to

the deterioration of cytotoxic environment which can lead to malfunction of aging liver and the pathogenesis of various liver-related diseases.

CHAPTER 5

REFERENCES

REFERENCES

Abedin, M. J., Wang, D., et al. (2007). "Autophagy delays apoptotic death in breast cancer cells following DNA damage." Cell Death Differ 14(3): 500-510

Abeliovich, H., Zhang, C., et al. (2003). "Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy." Mol Biol Cell 14(2): 477-490.

Abida, W. M. and Gu, W. (2008). "p53-Dependent and p53-independent activation of autophagy by ARF." Cancer Res 68(2): 352-357.

Adhikary, S. and Eilers, M. (2005). "Transcriptional regulation and transformation by Myc proteins." Nat Rev Mol Cell Biol 6(8): 635-645.

Akao, Y., Nakagawa, Y., et al. (2009). "Role of microRNA-143 in Fas-mediated apoptosis in human T-cell leukemia Jurkat cells." Leuk Res 33(11): 1530-1538.

Amaravadi, R. K., Yu, D., et al. (2007). "Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma." J Clin Invest 117(2): 326-336.

Amaravadi, R. K., Lippincott-Schwartz, J., et al. (2011). "Principles and current strategies for targeting autophagy for cancer treatment." Clin Cancer Res 17(4): 654-666.

Ambros, V. (2004). "The functions of animal microRNAs." Nature 431(7006): 350-355.

Ambros, V. and Chen, X. (2007). "The regulation of genes and genomes by small RNAs." Development 134(9): 1635-1641.

Ambs, S., Prueitt, R. L., et al. (2008). "Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer." Cancer Res 68(15): 6162-6170.

Arico, S., Petiot, A., et al. (2001). "The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway." J Biol Chem 276(38): 35243-35246.

Arnold, C. P., Tan, R., et al. (2011). "MicroRNA programs in normal and aberrant stem and progenitor cells." Genome Res 21(5): 798-810.

Axe, E. L., Walker, S. A., et al. (2008). "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum." J Cell Biol 182(4): 685-701.

Azad, M. B., Chen, Y., et al. (2008). "Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3." Autophagy 4(2): 195-204.

Ballif, B. A. and Blenis, J. (2001). "Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals." Cell Growth Differ 12(8): 397-408.

Beau, I., Mehrpour, M., et al. (2011). "Autophagosomes and human diseases." Int J Biochem Cell Biol 43(4): 460-464.

Benetti, R., Gonzalo, S., et al. (2008). "A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases." Nat Struct Mol Biol 15(9): 998.

Bernales, S., McDonald, K. L., et al. (2006). "Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response." PLoS Biol 4(12): e423.

Berry, D. L. and Baehrecke, E. H. (2007). "Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila." Cell 131(6): 1137-1148.

Blenkiron, C., Goldstein, L. D., et al. (2007). "MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype." Genome Biol 8(10): R214.

Bohnsack, M. T., Czaplinski, K., et al. (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." RNA 10(2): 185-191.

Bommer, G. T., Gerin, I., et al. (2007). "p53-mediated activation of miRNA34 candidate tumor-suppressor genes." Curr Biol 17(15): 1298-1307.

Botti, J., Djavaheri-Mergny, M., et al. (2006). "Autophagy signaling and the cogwheels of cancer." Autophagy 2(2): 67-73.

Brest, P., Lapaquette, P., et al. (2011). "A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease." Nat Genet 43(3): 242-245.

Brugarolas, J., Lei, K., et al. (2004). "Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex." Genes Dev 18(23): 2893-2904.

Brunet, A., Bonni, A., et al. (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." Cell 96(6): 857-868.

Budovskaya, Y. V., Stephan, J. S., et al. (2004). "The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in Saccharomyces cerevisiae." J Biol Chem 279(20): 20663-20671.

Budovskaya, Y. V., Stephan, J. S., et al. (2005). "An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase." Proc Natl Acad Sci U S A 102(39): 13933-13938.

Byfield, M. P., Murray, J. T., et al. (2005). "hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase." J Biol Chem 280(38): 33076-33082.

Calin, G. A., Sevignani, C., et al. (2004). "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers." Proc Natl Acad Sci U S A 101(9): 2999-3004.

Carew, J. S., Nawrocki, S. T., et al. (2007). "Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance." Blood 110(1): 313-322.

Chan, E. Y., Kir, S., et al. (2007). "siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy." J Biol Chem 282(35): 25464-25474.

Chan, S. P. and Slack, F. J. (2006). "microRNA-mediated silencing inside P-bodies." RNA Biol 3(3): 97-100.

Chan, E. Y., Longatti, A., et al. (2009). "Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism." Mol Cell Biol 29(1): 157-171.

Chang, C. Y. and Huang, W. P. (2007a). "Atg19 mediates a dual interaction cargo sorting mechanism in selective autophagy." Mol Biol Cell 18(3): 919-929.

Chang, T. C., Wentzel, E. A., et al. (2007b). "Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis." Mol Cell 26(5): 745-752.

Chau, B. N. and Wang, J. Y. (2003). "Coordinated regulation of life and death by RB." Nat Rev Cancer 3(2): 130-138.

Chen, J. L., Lin, H. H., et al. (2008a). "Novel roles for protein kinase Cdelta-dependent signaling pathways in acute hypoxic stress-induced autophagy." J Biol Chem 283(49): 34432-34444.

Chen, R., Alvero, A. B., et al. (2008b). "Regulation of IKKbeta by miR-199a affects NF-kappaB activity in ovarian cancer cells." Oncogene 27(34): 4712-4723.

Chen, H. Y. and White, E. (2011). "Role of autophagy in cancer prevention." Cancer Prev Res (Phila) 4(7): 973-983.

Ciechomska, I. A., Goemans, G. C., et al. (2009). "Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function." Oncogene 28(21): 2128-2141.

Cimmino, A., Calin, G. A., et al. (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." Proc Natl Acad Sci U S A 102(39): 13944-13949.

Codogno, P. and Meijer, A. J. (2005). "Autophagy and signaling: their role in cell survival and cell death." Cell Death Differ 12 Suppl 2: 1509-1518.

Colell, A., Ricci, J. E., et al. (2007). "GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation." Cell 129(5): 983-997.

Corradetti, M. N., Inoki, K., et al. (2004). "Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome." Genes Dev 18(13): 1533-1538.

Crawford, M., Batte, K., et al. (2009). "MicroRNA 133B targets pro-survival molecules MCL-1 and BCL2L2 in lung cancer." Biochem Biophys Res Commun 388(3): 483-489.

Crighton, D., Wilkinson, S., et al. (2006). "DRAM, a p53-induced modulator of autophagy, is critical for apoptosis." Cell 126(1): 121-134.

Datta, S. R., Dudek, H., et al. (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." Cell 91(2): 231-241.

Degenhardt, K., Mathew, R., et al. (2006). "Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis." Cancer Cell 10(1): 51-64.

Dews, M., Homayouni, A., et al. (2006). "Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster." Nat Genet 38(9): 1060-1065.

Ding, W. X., Ni, H. M., et al. (2007a). "Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival." J Biol Chem 282(7): 4702-4710.

Ding, W. X., Ni, H. M., et al. (2007b). "Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability." Am J Pathol 171(2): 513-524.

Donker, R. B., Mouillet, J. F., et al. (2007). "The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts." Mol Hum Reprod 13(4): 273-279.

Duran, A., Amanchy, R., et al. (2011). "p62 is a key regulator of nutrient sensing in the mTORC1 pathway." Mol Cell 44(1): 134-146.

Egan, D., Kim, J., et al. (2011a). "The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR." Autophagy 7(6): 643-644.

Egan, D. F., Shackelford, D. B., et al. (2011b). "Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy." Science 331(6016): 456-461.

Erlich, S., Mizrachy, L., et al. (2007). "Differential interactions between Beclin 1 and Bcl-2 family members." Autophagy 3(6): 561-568.

Espert, L., Denizot, M., et al. (2006). "Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4." J Clin Invest 116(8): 2161-2172.

Fasanaro, P., D'Alessandra, Y., et al. (2008). "MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3." J Biol Chem 283(23): 15878-15883.

Feng, Z., Zhang, H., et al. (2005). "The coordinate regulation of the p53 and mTOR pathways in cells." Proc Natl Acad Sci U S A 102(23): 8204-8209.

Filipowicz, W., Bhattacharyya, S. N., et al. (2008). "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" Nat Rev Genet 9(2): 102-114.

Fimia, G. M., Stoykova, A., et al. (2007). "Ambra1 regulates autophagy and development of the nervous system." Nature 447(7148): 1121-1125.

Fontana, L., Pelosi, E., et al. (2007). "MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation." Nat Cell Biol 9(7): 775-787.

Fontana, L., Fiori, M. E., et al. (2008). "Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM." PLoS One 3(5): e2236.

Fornari, F., Milazzo, M., et al. (2010). "MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells." Cancer Res 70(12): 5184-5193.

Foshay, K. M. and Gallicano, G. I. (2009). "miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation." Dev Biol 326(2): 431-443.

Frankel, L. B., Wen, J., et al. (2011). "microRNA-101 is a potent inhibitor of autophagy." EMBO J 30(22): 4628-4641.

Furuta, S., Hidaka, E., et al. (2004). "Ras is involved in the negative control of autophagy through the class I PI3-kinase." Oncogene 23(22): 3898-3904.

Ganley, I. G., Lam du, H., et al. (2009). "ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy." J Biol Chem 284(18): 12297-12305.

Geng, J. and Klionsky, D. J. (2008). "The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series." EMBO Rep 9(9): 859-864.

Giannakakis, A., Sandaltzopoulos, R., et al. (2008). "miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer." Cancer Biol Ther 7(2): 255-264.

Gironella, M., Seux, M., et al. (2007). "Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development." Proc Natl Acad Sci U S A 104(41): 16170-16175.

Gramantieri, L., Ferracin, M., et al. (2007). "Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma." Cancer Res 67(13): 6092-6099.

Gregory, R. I., Yan, K. P., et al. (2004). "The Microprocessor complex mediates the genesis of microRNAs." Nature 432(7014): 235-240.

Guo, Y., Chen, Y., et al. (2006). "Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma." Gene 384: 51-61.

Guo, Y., Chen, Z., et al. (2008a). "Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma." Cancer Res 68(1): 26-33.

Guo, C., Sah, J. F., et al. (2008b). "The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers." Genes Chromosomes Cancer 47(11): 939-946.

Guo, J. Y., Chen, H. Y., et al. (2011). "Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis." Genes Dev 25(5): 460-470.

Guttilla, I. K. and White, B. A. (2009). "Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells." J Biol Chem 284(35): 23204-23216.

Gwinn, D. M., Shackelford, D. B., et al. (2008). "AMPK phosphorylation of raptor mediates a metabolic checkpoint." Mol Cell 30(2): 214-226.

Han, J., Lee, Y., et al. (2004). "The Drosha-DGCR8 complex in primary microRNA processing." Genes Dev 18(24): 3016-3027.

Hara, K., Yonezawa, K., et al. (1998). "Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism." J Biol Chem 273(23): 14484-14494.

Hara, T., Takamura, A., et al. (2008). "FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells." J Cell Biol 181(3): 497-510.

Hardie, D. G., Carling, D., et al. (1998). "The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell?" Annu Rev Biochem 67: 821-855.

He, C., Song, H., et al. (2006). "Recruitment of Atg9 to the preautophagosomal structure by Atg11 is essential for selective autophagy in budding yeast." J Cell Biol 175(6): 925-935.

He, C., Baba, M., et al. (2008). "Self-interaction is critical for Atg9 transport and function at the phagophore assembly site during autophagy." Mol Biol Cell 19(12): 5506-5516.

He, C. and Klionsky, D. J. (2009). "Regulation mechanisms and signaling pathways of autophagy." Annu Rev Genet 43: 67-93.

Hermeking, H. (2010). "The miR-34 family in cancer and apoptosis." Cell Death Differ 17(2): 193-199.

Hosokawa, N., Hara, T., et al. (2009). "Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy." Mol Biol Cell 20(7): 1981-1991.

Hossain, A., Kuo, M. T., et al. (2006). "Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA." Mol Cell Biol 26(21): 8191-8201.

Hoyer-Hansen, M., Bastholm, L., et al. (2007). "Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2." Mol Cell 25(2): 193-205.

Hu, W., C. S. Chan, et al. (2010). "Negative regulation of tumor suppressor p53 by microRNA miR-504." Mol Cell 38(5): 689-699.

Huang, J. and Klionsky, D. J. (2007). "Autophagy and human disease." Cell Cycle 6(15): 1837-1849.

Huang, Y., Shen, X. J., et al. (2011). "Biological functions of microRNAs: a review." J Physiol Biochem 67(1): 129-139.

Hutvagner, G., McLachlan, J., et al. (2001). "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA." Science 293(5531): 834-838.

Ichimura, Y., Kirisako, T., et al. (2000). "A ubiquitin-like system mediates protein lipidation." Nature 408(6811): 488-492.

Inoki, K., Li, Y., et al. (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol 4(9): 648-657.

Inoki, K., Li, Y., et al. (2003a). "Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling." Genes Dev 17(15): 1829-1834.

Inoki, K., Zhu, T., et al. (2003b). "TSC2 mediates cellular energy response to control cell growth and survival." Cell 115(5): 577-590.

Itakura, E., Kishi, C., et al. (2008). "Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG." Mol Biol Cell 19(12): 5360-5372.

Ito, H., Daido, S., et al. (2005). "Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells." Int J Oncol 26(5): 1401-1410.

Jacinto, E., Facchinetti, V., et al. (2006). "SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity." Cell 127(1): 125-137.

Joglekar, M. V., Parekh, V. S., et al. (2007). "MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3." Dev Biol 311(2): 603-612.

Johnson, S. M., Grosshans, H., et al. (2005). "RAS is regulated by the let-7 microRNA family." Cell 120(5): 635-647.

Jung, C. H., Jun, C. B., et al. (2009). "ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery." Mol Biol Cell 20(7): 1992-2003.

Kabeya, Y., Mizushima, N., et al. (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J 19(21): 5720-5728.

Kabeya, Y., Noda, N. N., et al. (2009). "Characterization of the Atg17-Atg29-Atg31 complex specifically required for starvation-induced autophagy in Saccharomyces cerevisiae." Biochem Biophys Res Commun 389(4): 612-615.

Kamada, Y., Funakoshi, T., et al. (2000). "Tor-mediated induction of autophagy via an Apg1 protein kinase complex." J Cell Biol 150(6): 1507-1513.

Kametaka, S., Okano, T., et al. (1998). "Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, Saccharomyces cerevisiae." J Biol Chem 273(35): 22284-22291.

Kang, C., You, Y. J., et al. (2007). "Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation." Genes Dev 21(17): 2161-2171.

Kanki, T., Wang, K., et al. (2009). "Atg32 is a mitochondrial protein that confers selectivity during mitophagy." Dev Cell 17(1): 98-109.

Karantza-Wadsworth, V., Patel, S., et al. (2007). "Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis." Genes Dev 21(13): 1621-1635.

Katayama, M., Kawaguchi, T., et al. (2007). "DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells." Cell Death Differ 14(3): 548-558.

Kensler, T. W. and Wakabayashi, N. (2010). "Nrf2: friend or foe for chemoprevention?" Carcinogenesis 31(1): 90-99.

Ketting, R. F., Fischer, S. E., et al. (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans." Genes Dev 15(20): 2654-2659.

Khvorova, A., Reynolds, A., et al. (2003). "Functional siRNAs and miRNAs exhibit strand bias." Cell 115(2): 209-216.

Kihara, A., Noda, T., et al. (2001). "Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae." J Cell Biol 152(3): 519-530.

Kim, E., Goraksha-Hicks, P., et al. (2008a). "Regulation of TORC1 by Rag GTPases in nutrient response." Nat Cell Biol 10(8): 935-945.

Kim, S., Lee, U. J., et al. (2008b). "MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2)." J Biol Chem 283(26): 18158-18166.

Kim, Y. K., Yu, J., et al. (2009). "Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer." Nucleic Acids Res 37(5): 1672-1681.

Klionsky, D. J. and Emr, S. D. (2000). "Autophagy as a regulated pathway of cellular degradation." Science 290(5497): 1717-1721.

Klionsky, D. J. (2005). "The molecular machinery of autophagy: unanswered questions." J Cell Sci 118(Pt 1): 7-18.

Klionsky, D. J., Cuervo, A. M., et al. (2007). "Methods for monitoring autophagy from yeast to human." Autophagy 3(3): 181-206.

Knecht, E., Hernandez-Yago, J., et al. (1984). "Regulation of lysosomal autophagy in transformed and non-transformed mouse fibroblasts under several growth conditions." Exp Cell Res 154(1): 224-232.

Komatsu, M., Waguri, S., et al. (2005). "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice." J Cell Biol 169(3): 425-434.

Komatsu, M., Waguri, S., et al. (2007). "Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice." Cell 131(6): 1149-1163.

Komatsu, M., Kurokawa, H., et al. (2010). "The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1." Nat Cell Biol 12(3): 213-223.

Kondo, Y. and Kondo, S. (2006). "Autophagy and cancer therapy." Autophagy 2(2): 85-90.

Kosta, A., Roisin-Bouffay, C., et al. (2004). "Autophagy gene disruption reveals a non-vacuolar cell death pathway in Dictyostelium." J Biol Chem 279(46): 48404-48409.

Kouroku, Y., Fujita, E., et al. (2007). "ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation." Cell Death Differ 14(2): 230-239.

Kourtis, N. and Tavernarakis, N. (2009). "Autophagy and cell death in model organisms." Cell Death Differ 16(1): 21-30.

Kroemer, G. and Levine, B. (2008). "Autophagic cell death: the story of a misnomer." Nat Rev Mol Cell Biol 9(12): 1004-1010.

Kroemer, G., Galluzzi, L., et al. (2009). "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009." Cell Death Differ 16(1): 3-11.

Kulshreshtha, R., Ferracin, M., et al. (2007). "A microRNA signature of hypoxia." Mol Cell Biol 27(5): 1859-1867.

Kuma, A., Hatano, M., et al. (2004). "The role of autophagy during the early neonatal starvation period." Nature 432(7020): 1032-1036.

Ladu, S., Calvisi, D. F., et al. (2008). "E2F1 inhibits c-Myc-driven apoptosis via PIK3CA/Akt/mTOR and COX-2 in a mouse model of human liver cancer." Gastroenterology 135(4): 1322-1332.

Landais, S., Landry, S., et al. (2007). "Oncogenic potential of the miR-106-363 cluster and its implication in human T-cell leukemia." Cancer Res 67(12): 5699-5707.

Lau, A., Wang, X. J., et al. (2010). "A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62." Mol Cell Biol 30(13): 3275-3285.

Le, M. T., Teh, C., et al. (2009). "MicroRNA-125b is a novel negative regulator of p53." Genes Dev 23(7): 862-876.

Lee, R. C., Feinbaum, R. L., et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell 75(5): 843-854.

Lee, Y., Kim, M., et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." EMBO J 23(20): 4051-4060.

Lee, S. B., Kim, S., et al. (2007). "ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase." EMBO Rep 8(4): 360-365.

Lee, K. H., Chen, Y. L., et al. (2009). "MicroRNA-330 acts as tumor suppressor and induces apoptosis of prostate cancer cells through E2F1-mediated suppression of Akt phosphorylation." Oncogene 28(38): 3360-3370.

Lee, J. W., Park, S., et al. (2010). "The association of AMPK with ULK1 regulates autophagy." PLoS One 5(11): e15394.

Legakis, J. E., Yen, W. L., et al. (2007). "A cycling protein complex required for selective autophagy." Autophagy 3(5): 422-432.

Lemasters, J. J. (2005). "Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging." Rejuvenation Res 8(1): 3-5.

Levine, B. and Yuan, J. (2005). "Autophagy in cell death: an innocent convict?" J Clin Invest 115(10): 2679-2688.

Levine, B. (2007). "Cell biology: autophagy and cancer." Nature 446(7137): 745-747.

Levraud, J. P., Adam, M., et al. (2003). "Dictyostelium cell death: early emergence and demise of highly polarized paddle cells." J Cell Biol 160(7): 1105-1114.

Lewis, B. P., Shih, I. H., et al. (2003). "Prediction of mammalian microRNA targets." Cell 115(7): 787-798.

Li, Y., Inoki, K., et al. (2004). "Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity." Mol Cell Biol 24(18): 7965-7975.

Li, J., Ni, M., et al. (2008). "The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells." Cell Death Differ 15(9): 1460-1471.

Li, T., Li, D., et al. (2009a). "MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells." Biochem Biophys Res Commun 383(3): 280-285.

Li, Y., Tan, W., et al. (2009b). "Role of the miR-106b-25 microRNA cluster in hepatocellular carcinoma." Cancer Sci 100(7): 1234-1242.

Liang, X. H., Jackson, S., et al. (1999). "Induction of autophagy and inhibition of tumorigenesis by beclin 1." Nature 402(6762): 672-676.

Liang, C., Feng, P., et al. (2006). "Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG." Nat Cell Biol 8(7): 688-699.

Liang, J., Shao, S. H., et al. (2007). "The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis." Nat Cell Biol 9(2): 218-224.

Liang, C., Lee, J. S., et al. (2008). "Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking." Nat Cell Biol 10(7): 776-787.

Lim, L. P., Lau, N. C., et al. (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature 433(7027): 769-773.

Lin, C. J., Gong, H. Y., et al. (2008). "miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines." Biochem Biophys Res Commun 375(3): 315-320.

Lin, T., Dong, W., et al. (2009). "MicroRNA-143 as a tumor suppressor for bladder cancer." J Urol 181(3): 1372-1380.

Liou, W., Geuze, H. J., et al. (1997). "The autophagic and endocytic pathways converge at the nascent autophagic vacuoles." J Cell Biol 136(1): 61-70.

Liu, X., Nelson, A., et al. (2009). "MicroRNA-146a modulates human bronchial epithelial cell survival in response to the cytokine-induced apoptosis." Biochem Biophys Res Commun 380(1): 177-182.

Livak, K. J. and Schmittgen, T. D. (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods 25(4): 402-408.

Lock, R., Roy, S., et al. (2011). "Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation." Mol Biol Cell 22(2): 165-178.

Loffler, A. S., Alers, S., et al. (2011). "Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop." Autophagy 7(7): 696-706.

Long, X., Lin, Y., et al. (2005). "Rheb binds and regulates the mTOR kinase." Curr Biol 15(8): 702-713.

Lu, L., Katsaros, D., et al. (2007). "Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis." Cancer Res 67(21): 10117-10122.

Lu, Z., Liu, M., et al. (2008). "MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene." Oncogene 27(31): 4373-4379.

Lujambio, A., Ropero, S., et al. (2007). "Genetic unmasking of an epigenetically silenced microRNA in human cancer cells." Cancer Res 67(4): 1424-1429.

Lum, J. J., Bauer, D. E., et al. (2005a). "Growth factor regulation of autophagy and cell survival in the absence of apoptosis." Cell 120(2): 237-248.

Lum, J. J., DeBerardinis, R. J., et al. (2005b). "Autophagy in metazoans: cell survival in the land of plenty." Nat Rev Mol Cell Biol 6(6): 439-448.

Ma, Y. and Hendershot, L. M. (2001). "The unfolding tale of the unfolded protein response." Cell 107(7): 827-830.

Maclean, K. H., Dorsey, F. C., et al. (2008). "Targeting lysosomal degradation induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis." J Clin Invest 118(1): 79-88.

Maeda, S., Kamata, H., et al. (2005). "IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis." Cell 121(7): 977-990.

Maes, O. C., An, J., et al. (2008). "Murine microRNAs implicated in liver functions and aging process." Mech Ageing Dev 129(9): 534-541.

Maiuri, M. C., Le Toumelin, G., et al. (2007). "Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1." EMBO J 26(10): 2527-2539.

Mallory, A. C. and Vaucheret, H. (2006). "Functions of microRNAs and related small RNAs in plants." Nat Genet 38 Suppl: S31-36.

Mammucari, C., Milan, G., et al. (2007). "FoxO3 controls autophagy in skeletal muscle in vivo." Cell Metab 6(6): 458-471.

Marino, G., Uria, J. A., et al. (2003). "Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy." J Biol Chem 278(6): 3671-3678.

Marino, G., Salvador-Montoliu, N., et al. (2007). "Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3." J Biol Chem 282(25): 18573-18583.

Martinet, W., De Meyer, G.R., et al. (2006). "In situ detection of starvation-induced autophagy." J Histochem Cytochem 54(1):85-96.

Martinez-Vicente, M. and Cuervo, A. M. (2007). "Autophagy and neurodegeneration: when the cleaning crew goes on strike." Lancet Neurol 6(4): 352-361.

Massey, A. C., Zhang, C., et al. (2006). "Chaperone-mediated autophagy in aging and disease." Curr Top Dev Biol 73: 205-235.

Mathew, R., Karantza-Wadsworth, V., et al. (2007). "Role of autophagy in cancer." Nat Rev Cancer 7(12): 961-967.

Mathew, R., Karp, C. M., et al. (2009). "Autophagy suppresses tumorigenesis through elimination of p62." Cell 137(6): 1062-1075.

Matsunaga, K., Saitoh, T., et al. (2009). "Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages." Nat Cell Biol 11(4): 385-396.

Matsuura, A., Tsukada, M., et al. (1997). "Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae." Gene 192(2): 245-250.

Meenhuis, A., van Veelen, P. A., et al. (2011). "MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice." Blood 118(4): 916-925.

Mehrpour, M., Esclatine, A., et al. (2010). "Overview of macroautophagy regulation in mammalian cells." Cell Res 20(7): 748-762.

Meijer, W. H., van der Klei, I. J., et al. (2007). "ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes." Autophagy 3(2): 106-116.

Meister, G. and Tuschl, T. (2004). "Mechanisms of gene silencing by double-stranded RNA." Nature 431(7006): 343-349.

Meng, F., Henson, R., et al. (2007a). "The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes." J Biol Chem 282(11): 8256-8264.

Meng, F., Henson, R., et al. (2007b). "MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer." Gastroenterology 133(2): 647-658.

Mercer, C. A., Kaliappan, A., et al. (2009). "A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy." Autophagy 5(5): 649-662.

Mizushima, N., Noda, T., et al. (1998a). "A protein conjugation system essential for autophagy." Nature 395(6700): 395-398.

Mizushima, N., Sugita, H., et al. (1998b). "A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy." J Biol Chem 273(51): 33889-33892.

Mizushima, N., Yamamoto, A., et al. (2001). "Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells." J Cell Biol 152(4): 657-668.

Mizushima, N., Yoshimori, T., et al. (2002). "Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method." FEBS Lett 532(3): 450-454.

Mizushima, N., Kuma, A., et al. (2003). "Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate." J Cell Sci 116(Pt 9): 1679-1688.

Mizushima, N. (2005). "The pleiotropic role of autophagy: from protein metabolism to bactericide." Cell Death Differ 12 Suppl 2: 1535-1541.

Mizushima, N. (2007). "Autophagy: process and function." Genes Dev 21(22): 2861-2873.

Mizushima, N., Klionsky, D.J. (2007). "Protein turnover via autophagy: implications for metabolism." Annu Rev Nutr. 27:19-40.

Mizushima, N., Levine, B., et al. (2008). "Autophagy fights disease through cellular self-digestion." Nature 451(7182): 1069-1075.

Mizushima, N., Yoshimori, T., et al. (2010). "Methods in mammalian autophagy research." Cell 140(3): 313-326.

Mott, J. L., Kobayashi, S., et al. (2007). "mir-29 regulates Mcl-1 protein expression and apoptosis." Oncogene 26(42): 6133-6140.

Nagaraja, A. K., Creighton, C. J., et al. (2010). "A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer." Mol Endocrinol 24(2): 447-463.

Narendra, D., Tanaka, A., et al. (2008). "Parkin is recruited selectively to impaired mitochondria and promotes their autophagy." J Cell Biol 183(5): 795-803.

Nice, D. C., Sato, T. K., et al. (2002). "Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy." J Biol Chem 277(33): 30198-30207.

Nicklin, P., Bergman, P., et al. (2009). "Bidirectional transport of amino acids regulates mTOR and autophagy." Cell 136(3): 521-534.

Nishida, Y., Arakawa, S., et al. (2009). "Discovery of Atg5/Atg7-independent alternative macroautophagy." Nature 461(7264): 654-658.

Nobukuni, T., Joaquin, M., et al. (2005). "Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase." Proc Natl Acad Sci U S A 102(40): 14238-14243.

Noda, T. and Ohsumi, Y. (1998). "Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast." J Biol Chem 273(7): 3963-3966.

Obara, K., Sekito, T., et al. (2008). "The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function." J Biol Chem 283(35): 23972-23980.

Ogata, M., Hino, S., et al. (2006). "Autophagy is activated for cell survival after endoplasmic reticulum stress." Mol Cell Biol 26(24): 9220-9231.

Oneyama, C., Ikeda, J., et al. (2011). "MicroRNA-mediated downregulation of mTOR/FGFR3 controls tumor growth induced by Src-related oncogenic pathways." Oncogene 30(32): 3489-3501.

Paglin, S., Hollister, T., et al. (2001). "A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles." Cancer Res 61(2): 439-444.

Papagiannakopoulos, T., Shapiro, A., et al. (2008). "MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells." Cancer Res 68(19): 8164-8172.

Papandreou, I., Lim, A. L., et al. (2008). "Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L." Cell Death Differ 15(10): 1572-1581.

Pasquinelli, A. E., Reinhart, B. J., et al. (2000). "Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA." Nature 408(6808): 86-89.

Pattingre, S., Tassa, A., et al. (2005). "Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy." Cell 122(6): 927-939.

Pattingre, S., Bauvy, C., et al. (2009). "Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy." J Biol Chem 284(5): 2719-2728.

Peng, T., Golub, T. R., et al. (2002). "The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation." Mol Cell Biol 22(15): 5575-5584.

Peng, Y., Laser, J., et al. (2008). "Antiproliferative effects by Let-7 repression of high-mobility group A2 in uterine leiomyoma." Mol Cancer Res 6(4): 663-673.

Persson, M. M., Johansson, M. E., et al. (2011). "HIF-2alpha Expression Is Suppressed in SCLC Cells, Which Survive in Moderate and Severe Hypoxia When HIF-1alpha Is Repressed." Am J Pathol.

Petrocca, F., Visone, R., et al. (2008). "E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer." Cancer Cell 13(3): 272-286.

Pickart, C. M. (2004). "Back to the future with ubiquitin." Cell 116(2): 181-190.

Polager, S., Ofir, M., et al. (2008). "E2F1 regulates autophagy and the transcription of autophagy genes." Oncogene 27(35): 4860-4864.

Priault, M., Salin, B., et al. (2005). "Impairing the bioenergetic status and the biogenesis of mitochondria triggers mitophagy in yeast." Cell Death Differ 12(12): 1613-1621.

Proikas-Cezanne, T., Waddell, S., et al. (2004). "WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy." Oncogene 23(58): 9314-9325.

Qu, X., Yu, J., et al. (2003). "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene." J Clin Invest 112(12): 1809-1820.

Qu, X., Zou, Z., et al. (2007). "Autophagy gene-dependent clearance of apoptotic cells during embryonic development." Cell 128(5): 931-946.

Ravikumar, B., Berger, Z., et al. (2006). "Rapamycin pre-treatment protects against apoptosis." Hum Mol Genet 15(7): 1209-1216.

Razi, M., Chan, E. Y., et al. (2009). "Early endosomes and endosomal coatomer are required for autophagy." J Cell Biol 185(2): 305-321.

Reggiori, F., Tucker, K. A., et al. (2004a). "The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure." Dev Cell 6(1): 79-90.

Reggiori, F., Wang, C. W., et al. (2004b). "Early stages of the secretory pathway, but not endosomes, are required for Cvt vesicle and autophagosome assembly in Saccharomyces cerevisiae." Mol Biol Cell 15(5): 2189-2204.

Reggiori, F., Shintani, T., et al. (2005). "Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts." Autophagy 1(2): 101-109.

Reiling, J. H. and Hafen, E. (2004). "The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila." Genes Dev 18(23): 2879-2892.

Resnick, K. E., Alder, H., et al. (2009). "The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform." Gynecol Oncol 112(1): 55-59.

Roisin-Bouffay, C., Luciani, M. F., et al. (2004). "Developmental cell death in dictyostelium does not require paracaspase." J Biol Chem 279(12): 11489-11494.

Rubinsztein, D. C. (2006). "The roles of intracellular protein-degradation pathways in neurodegeneration." Nature 443(7113): 780-786.

Saini, H. K., Griffiths-Jones, S., et al. (2007). "Genomic analysis of human microRNA transcripts." Proc Natl Acad Sci U S A 104(45): 17719-17724.

Saito, Y., Liang, G., et al. (2006). "Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells." Cancer Cell 9(6): 435-443.

Saito, Y., Suzuki, H., et al. (2009). "Chromatin remodeling at Alu repeats by epigenetic treatment activates silenced microRNA-512-5p with downregulation of Mcl-1 in human gastric cancer cells." Oncogene 28(30): 2738-2744.

Sakaki, K., Wu, J., et al. (2008). "Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum." J Biol Chem 283(22): 15370-15380.

Salazar, M., Carracedo, A., et al. (2009). "Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells." J Clin Invest 119(5): 1359-1372.

Sampson, V. B., Rong, N. H., et al. (2007). "MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells." Cancer Res 67(20): 9762-9770.

Sancak, Y., Peterson, T. R., et al. (2008). "The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1." Science 320(5882): 1496-1501.

Sancak, Y., Bar-Peled, L., et al. (2010). "Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids." Cell 141(2): 290-303.

Sandoval, H., Thiagarajan, P., et al. (2008). "Essential role for Nix in autophagic maturation of erythroid cells." Nature 454(7201): 232-235.

Sarbassov, D. D., Guertin, D. A., et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science 307(5712): 1098-1101.

Scherz-Shouval, R., Sagiv, Y., et al. (2003). "The COOH terminus of GATE-16, an intra-Golgi transport modulator, is cleaved by the human cysteine protease HsApg4A." J Biol Chem 278(16): 14053-14058.

Scherz-Shouval, R., Shvets, E., et al. (2007). "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4." EMBO J 26(7): 1749-1760.

Schmelzle, T. and Hall, M. N. (2000). "TOR, a central controller of cell growth." Cell 103(2): 253-262.

Schmelzle, T., Beck, T., et al. (2004). "Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast." Mol Cell Biol 24(1): 338-351.

Schwarze, P. E. and Seglen, P. O. (1985). "Reduced autophagic activity, improved protein balance and enhanced in vitro survival of hepatocytes isolated from carcinogen-treated rats." Exp Cell Res 157(1): 15-28.

Schweers, R. L., Zhang, J., et al. (2007). "NIX is required for programmed mitochondrial clearance during reticulocyte maturation." Proc Natl Acad Sci U S A 104(49): 19500-19505.

Secombe, J., Pierce, S. B., et al. (2004). "Myc: a weapon of mass destruction." Cell 117(2): 153-156.

Segura, M. F., Hanniford, D., et al. (2009). "Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor." Proc Natl Acad Sci U S A 106(6): 1814-1819.

Sen, G. L. and Blau, H. M. (2005). "Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies." Nat Cell Biol 7(6): 633-636.

Sevignani, C., Calin, G. A., et al. (2006). "Mammalian microRNAs: a small world for fine-tuning gene expression." Mamm Genome 17(3): 189-202.

Shang, L., Chen, S., et al. (2011). "Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK." Proc Natl Acad Sci U S A 108(12): 4788-4793.

Shimizu, S., Kanaseki, T., et al. (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." Nat Cell Biol 6(12): 1221-1228.

Song, B., Wang, Y., et al. (2008). "miR-192 Regulates dihydrofolate reductase and cellular proliferation through the p53-microRNA circuit." Clin Cancer Res 14(24): 8080-8086.

Stack, J. H., DeWald, D. B., et al. (1995). "Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast." J Cell Biol 129(2): 321-334.

Stefani, G. and Slack, F. J. (2008). "Small non-coding RNAs in animal development." Nat Rev Mol Cell Biol 9(3): 219-230.

Stromhaug, P. E., Reggiori, F., et al. (2004). "Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy." Mol Biol Cell 15(8): 3553-3566.

Sullivan, C. S., Grundhoff, A., et al. (2006). "Expression and function of microRNAs in viruses great and small." Cold Spring Harb Symp Quant Biol 71: 351-356.

Sun, Q., Fan, W., et al. (2008). "Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase." Proc Natl Acad Sci U S A 105(49): 19211-19216.

Suzuki, K., Kirisako, T., et al. (2001). "The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation." EMBO J 20(21): 5971-5981.

Suzuki, K., Kubota, Y., et al. (2007). "Hierarchy of Atg proteins in pre-autophagosomal structure organization." Genes Cells 12(2): 209-218.

Sylvestre, Y., De Guire, V., et al. (2007). "An E2F/miR-20a autoregulatory feedback loop." J Biol Chem 282(4): 2135-2143.

Takahashi, Y., Coppola, D., et al. (2007). "Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis." Nat Cell Biol 9(10): 1142-1151.

Tang, Y., Zheng, J., et al. (2009). "MicroRNA-1 regulates cardiomyocyte apoptosis by targeting Bcl-2." Int Heart J 50(3): 377-387.

Tanida, I., Tanida-Miyake, E., et al. (2001). "The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3." J Biol Chem 276(3): 1701-1706.

Tanida, I., Sou, Y. S., et al. (2004). "HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates." J Biol Chem 279(35): 36268-36276.

Tanida, I., Sou, Y. S., et al. (2006). "Atg8L/Apg8L is the fourth mammalian modifier of mammalian Atg8 conjugation mediated by human Atg4B, Atg7 and Atg3." FEBS J 273(11): 2553-2562.

Tanida, I. (2011). "Autophagy basics." Microbiol Immunol 55(1): 1-11.

Tasdemir, E., Maiuri, M. C., et al. (2008). "Regulation of autophagy by cytoplasmic p53." Nat Cell Biol 10(6): 676-687.

Tazawa, H., Tsuchiya, N., et al. (2007). "Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells." Proc Natl Acad Sci U S A 104(39): 15472-15477.

Tee, A. R., Fingar, D. C., et al. (2002). "Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling." Proc Natl Acad Sci U S A 99(21): 13571-13576.

Tee, A. R., Manning, B. D., et al. (2003). "Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb." Curr Biol 13(15): 1259-1268.

Tracy, K., Dibling, B. C., et al. (2007). "BNIP3 is an RB/E2F target gene required for hypoxia-induced autophagy." Mol Cell Biol 27(17): 6229-6242.

Trimarchi, J. M. and Lees, J. A. (2002). "Sibling rivalry in the E2F family." Nat Rev Mol Cell Biol 3(1): 11-20.

Tsang, W. P. and Kwok, T. T. (2008). "Let-7a microRNA suppresses therapeutics-induced cancer cell death by targeting caspase-3." Apoptosis 13(10): 1215-1222.

van Rooij, E., Sutherland, L. B., et al. (2006). "A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure." Proc Natl Acad Sci U S A 103(48): 18255-18260.

Vander Haar, E., Lee, S. I., et al. (2007). "Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40." Nat Cell Biol 9(3): 316-323.

Vasseur, S., Afzal, S., et al. (2009). "DJ-1/PARK7 is an important mediator of hypoxia-induced cellular responses." Proc Natl Acad Sci U S A 106(4): 1111-1116.

Voorhoeve, P. M., le Sage, C., et al. (2006). "A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors." Cell 124(6): 1169-1181.

Wang, Z., Wilson, W. A., et al. (2001). "Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p." Mol Cell Biol 21(17): 5742-5752.

Wang CW, Klionsky DJ. (2004). "Microautophagy. In Autophagy (ed. D.J. Klionsky)", Georgetown, TX: Landes Bioscience. pp. 107-114.

Wang, Q., Li, Y. C., et al. (2008a). "miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130." Proc Natl Acad Sci U S A 105(8): 2889-2894.

Wang, Y., Lee, A. T., et al. (2008b). "Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target." J Biol Chem 283(19): 13205-13215.

Wang, Y. and Lee, C. G. (2009). "MicroRNA and cancer--focus on apoptosis." J Cell Mol Med 13(1): 12-23.

Wang, G., Yang, Z. Q., et al. (2010). "Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential." Am J Transl Res 2(1): 65-74.

Warnecke, C., Griethe, W., et al. (2003). "Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors." FASEB J 17(9): 1186-1188.

White, E. and DiPaola, R. S. (2009). "The double-edged sword of autophagy modulation in cancer." Clin Cancer Res 15(17): 5308-5316.

Wightman, B., Ha, I., et al. (1993). "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans." Cell 75(5): 855-862.

Winter, J., Jung, S., et al. (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nat Cell Biol 11(3): 228-234.

Wu, L., Fan, J., et al. (2006). "MicroRNAs direct rapid deadenylation of mRNA." Proc Natl Acad Sci U S A 103(11): 4034-4039.

Wullschleger, S., Loewith, R., et al. (2006). "TOR signaling in growth and metabolism." Cell 124(3): 471-484.

Xiao, C., Srinivasan, L., et al. (2008). "Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes." Nat Immunol 9(4): 405-414.

Xiao, F., Zuo, Z., et al. (2009). "miRecords: an integrated resource for microRNA-target interactions." Nucleic Acids Res 37(Database issue): D105-110.

Xiao, J., Zhu, X., et al. (2011). "MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II." J Biomed Sci 18: 35.

Xie, Z. and Klionsky, D. J. (2007). "Autophagosome formation: core machinery and adaptations." Nat Cell Biol 9(10): 1102-1109.

Xie, Z., Nair, U., et al. (2008). "Dissecting autophagosome formation: the missing pieces." Autophagy 4(7): 920-922.

Xu, J., Liao, X., et al. (2010). "Downregulations of B-cell lymphoma 2 and myeloid cell leukemia sequence 1 by microRNA 153 induce apoptosis in a glioblastoma cell line DBTRG-05MG." Int J Cancer 126(4): 1029-1035.

Yamada, T., Carson, A. R., et al. (2005). "Endothelial nitric-oxide synthase antisense (NOS3AS) gene encodes an autophagy-related protein (APG9-like2) highly expressed in trophoblast." J Biol Chem 280(18): 18283-18290.

Yan, Y., Flinn, R. J., et al. (2009). "hVps15, but not Ca2+/CaM, is required for the activity and regulation of hVps34 in mammalian cells." Biochem J 417(3): 747-755.

Yang, H., Kong, W., et al. (2008). "MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN." Cancer Res 68(2): 425-433.

Yang, S., Wang, X., et al. (2011). "Pancreatic cancers require autophagy for tumor growth." Genes Dev 25(7): 717-729.

Yen, W. L., Legakis, J. E., et al. (2007). "Atg27 is required for autophagy-dependent cycling of Atg9." Mol Biol Cell 18(2): 581-593.

Yeung, M. L., Yasunaga, J., et al. (2008). "Roles for microRNAs, miR-93 and miR-130b, and tumor protein 53-induced nuclear protein 1 tumor suppressor in cell growth dysregulation by human T-cell lymphotrophic virus 1." Cancer Res 68(21): 8976-8985.

Yi, R., Qin, Y., et al. (2003). "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs." Genes Dev 17(24): 3011-3016.

Yorimitsu, T. and Klionsky, D. J. (2005). "Autophagy: molecular machinery for self-eating." Cell Death Differ 12 Suppl 2: 1542-1552.

Yorimitsu, T., He, C., et al. (2009). "Tap42-associated protein phosphatase type 2A negatively regulates induction of autophagy." Autophagy 5(5): 616-624.

Young, A. R., Chan, E. Y., et al. (2006). "Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes." J Cell Sci 119(Pt 18): 3888-3900.

Yousefi, S., Perozzo, R., et al. (2006). "Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis." Nat Cell Biol 8(10): 1124-1132.

Yu, L., Alva, A., et al. (2004). "Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8." Science 304(5676): 1500-1502.

Yu, J., Ryan, D. G., et al. (2008). "MicroRNA-184 antagonizes microRNA-205 to maintain SHIP2 levels in epithelia." Proc Natl Acad Sci U S A 105(49): 19300-19305.

Yue, Z., Jin, S., et al. (2003). "Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor." Proc Natl Acad Sci U S A 100(25): 15077-15082.

Zeng, Y., Yi, R., et al. (2003). "MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms." Proc Natl Acad Sci U S A 100(17): 9779-9784.

Zhang, H., Bosch-Marce, M., et al. (2008a). "Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia." J Biol Chem 283(16): 10892-10903.

Zhang, Z., Li, Z., et al. (2008b). "miR-21 plays a pivotal role in gastric cancer pathogenesis and progression." Lab Invest 88(12): 1358-1366.

Zhao, J., Brault, J. J., et al. (2007). "FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells." Cell Metab 6(6): 472-483.

Zhong, Y., Wang, Q. J., et al. (2009). "Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex." Nat Cell Biol 11(4): 468-476.

Zhu, S., Si, M. L., et al. (2007). "MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1)." J Biol Chem 282(19): 14328-14336.

Zhu, S., Wu, H., et al. (2008). "MicroRNA-21 targets tumor suppressor genes in invasion and metastasis." Cell Res 18(3): 350-359.

Zhu, H., Wu, H., et al. (2009). "Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells." Autophagy 5(6): 816-823.

Zou, J., Zou, Y., et al. (2010). "5-Fluorouracil and oxaliplatin modify the expression profiles of microRNAs in human colon cancer cells in vitro." Oncol Rep 23(1): 121-128.