

THESIS AND ABSTRACT

Autophagy in Hematopoiesis and Acute Myeloid Leukemia

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Abstract

Autophagy in Hematopoiesis and Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) develops following oncogenic alterations to hematopoietic stem (HSC) and progenitor cells (HSPCs) in the bone marrow, resulting in dysregulated proliferation of immature myeloid progenitors that interferes with normal hematopoiesis. Understanding the mechanisms of HSPC protection against damage and excessive division, and how these pathways are altered during leukemic progression, is vital for establishing effective therapies. Here, we show that autophagy, a lysosomal degradation pathway, is increased in HSPCs using a novel imaging flow cytometry autophagy assay. Loss of hematopoietic autophagy following deletion of key gene *Atg5* resulted in increased HSC proliferation, leading to HSC exhaustion and bone marrow failure. Although erythrocyte and lymphocyte populations were negatively impacted by autophagy loss, myeloid cells showing immature characteristics were expanded. Deletion of *Atg5* in an AML model resulted in increased proliferation under metabolic stress, dependent on the glycolytic pathway, and aberrant upstream mTOR signaling. Moreover, modulation of *Atg5* altered leukemic response to culture with stromal cells. Finally, primary AML cells displayed multiple markers of decreased autophagy. These data suggest a role for autophagy in preserving HSC function, partially through suppression of HSPC proliferation, and indicate that decreased autophagy may benefit AML cells. We postulate that modulation of autophagy could help maintain stem cell function, for example during transplantation, and aid AML therapy in a setting-specific manner.

Author's Publications

Watson, A. S., A. Stranks, O. Williams, J. De Boer, K. Cain, M. McFarlane, J. McGouran, B. Kessler, S. Khandwala, M. Lutterop, P. Woll, O. Chowdhury, D. Puleston, K. Phadwal, M. Mortensen, G. Djordjevic, T. Riffelmacher, D. Ferguson, E. Soilleux, S. E. Jacobsen, and A. K. Simon. Deficient autophagy drives hematopoietic stem cell dysfunction, myeloid leukemic proliferation and glycolysis. *Cell Stem Cell*, under revision.

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Abbreviations

(e)GFP	(Enhanced) green fluorescence protein
+I	+ Inhibitors (E64d/Pepstatin A)
4E-BP1	eIF4E-binding protein 1 inhibition
7AAD	7-amino-actinomycin-D
AF4/9	ALL1-fused gene from chromosome 4/9
AKT	AKT8 virus oncogene cellular homolog
ALFY	Autophagy-linked FYVE protein
AMBRA1	Activating molecule in Beclin1-regulated autophagy 1
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
Ara-C	Cytosine arabinoside
ARF	Alternate reading frame family
ATG or Atg	Autophagy-related gene
ATP	Adenosine triphosphate
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2 associated X-protein
BCA	Bicinchoninic
BCL	B-cell lymphoma 2
BCR-ABL	Breakpoint cluster region-ableson leukemia oncogene cellular homolog
BDS	Bright detail similarly
BM	Bone marrow
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMNCs	Bone marrow mononuclear cells
BMP4	Bone morphogenetic protein 4
BNIP	BCL2/adenovirus E1B 19kDa protein-interacting protein 3
BrdU	Bromodeoxyuridine
C/EBP	Ccaat-enhancer binding proteins
C57BL/6	C57 Black 6 (mouse strain)
cAMP	Cyclic adenosine monophosphate
Cbl	Casitas b-lineage lymphoma
Cbp	CREB-binding protein
CD	Cluster of differentiation
CDK	Cycling-dependent kinase
CDKI	CDK inhibitor
CDKN1	Cyclin-dependent kinase inhibitor 1
cDNA	Complementary DNA
CFC	Colony forming cell
CIP1	CDK-interacting protein 1
CLP	Common lymphoid progenitor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO ₂	Carbon dioxide

CSF-1	Colony stimulating factor 1
Ct	Cycle threshold
CXCL12	Chemokine (C-X-C motif) ligand 2
DAPI	4',6-diamidino-2-phenylindole
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated autophagy modulator
DSC	Dermal stem cells
EDTA	Ethylenediaminetetraacetic acid
eIF2a	Eukaryotic translation initiation factor 2a
EM	Electron microscopy
ENL	Eleven-nineteen-leukemia protein
EPO	Erythropoietin
ER	Endoplasmic reticulum
Er	Erythropoietin receptor
Erk	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
ESCRT	Endosomal sorting complexes required for transport
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FCCP	Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone
FCS	Fetal calf serum
FIP200	Focal-adhesion kinase-family interaction protein of 200kDa
FITC	Fluorescein isothiocyanate
Fl/fl	Flox
Floxed	LoxP-flanked
Flt-3	FMS-related kinase 3
FOXO	Forkhead box O
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2
FS	Forward scatter
Fwd	Forward (primer)
g	Grams/G-Force (context dependent)
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gfi1	Growth factor independent 1
GM	Granulocyte-macrophage
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMFI	Geometric mean fluorescence intensity
GMP	Granulocyte macrophage progenitor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GβL	G protein beta subunit-like
H&E	Haematoxylin and eosin
HCL	Hydrochloric acid
HET	Heterozygote (for <i>Atg</i>)
HIF	Hypoxia inducible factor
HMGB1	High-mobility group 1
Hox	Homeobox

hrs	hours
Hrs	Hours
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
iBMK	Immortalized baby mouse kidney cells
IC	Intracellular
ICAM-1	Intracellular adhesion molecule 1
iCre	Codon improved Cre recombinase
IFCAA	Imaging flow cytometry autophagy assay
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMDM	Iscoe's Modified Dulbecco's Medium
INK4	Inhibitor of cyclin-dependent kinase 4)
iPS	Induced pluripotent stem cells
IRE1	Inositol-requiring protein 1
Kb	Kilobase
KDa	Kilo Daltons
KEAP1	Kelch-like ECH-associated protein 1
Ki67	Kiel 67 protein
KO	Knockout (for <i>Atg5</i>)
LAMP1/2	Lysosomal-associated membrane protein 1/2
LC3	MAP1LC3B
Lck	Lymphocyte-specific protein tyrosine kinase
LK	Lin-Sca1-cKit ⁺ (progenitors)
LKB1	Liver kinase B1
LMPP	Lymphoid-primed multipotent progenitor
LSC	Leukemic stem cell (leukemic initiating cell)
LSK	Lin-Sca1+cKit ⁺ (MPPs)
LT-HSC	Long-term HSC
Lys-M	M-lysozyme
M	Molar
MACS	Magnetic-activated cell sorting
MAP1LC3	Microtubule-associated protein 1 light chain 3 beta
MCL-1	Myeloid cell leukemia
MDS	Myelodysplastic syndrome
ME	MLL-ENL
MEF	Mouse embryonic fibroblast
Meis1	Myeloid ecotropic viral integration site
MEK	Mitogen activated protein kinase Erk kinase
MEM	Minimal Essential Media
MEP	Megakaryocyte-erythroid progenitor
MFI	Mean florescence intensity
Min	Minute
Mitophagy	Mitochondrial autophagy
MLL	Mixed-lineage leukemia
Mpl	Myeloproliferative leukemic virus oncogene
MPP	Multipotent progenitor
mRNA	Messenger RNA

MSC	mesenchymal stem cell/stromal cell (Chapter 6)
mTOR(C1/C2)	Mammalian target of rapamycin (complex 1/complex 2)
mtROS	Mitochondrial ROS
Mx	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78
myc	Avian myelocytomatosis virus oncogene cellular homolog
n.s.	Not significant
NAC	N-acetyl cysteine
NaCl	Sodium Chloride
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NAO	Nonyl-acridine orange
NaOH	Sodium hydroxide
NBR1	Neighbor Of BRCA1 Gene 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NHS	National Health Service
NIX	NIP3-Like Protein X
NK cell	Natural killer cell
NRF2	Nuclear factor-like 2
OCR	Oxygen consumption rate
P/p	Phosphorylated
PAS	Pre-autophagosomal structure
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK	3-phosphoinositide-dependent protein kinase 1
PE	Phosphatidylethanolamine/R-Phycoerythrin (context dependent)
PERK	Double-stranded RNA activated protein kinase (PKR)-like ER kinase
Pi	Phosphate
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A
PML-RARalpha	Promyelocytic leukemia protein - retinoic acid receptor alpha
PreMegE	Pre megakaryocyte erythroid progenitor
PTEN	Phosphatase and tensin homolog
PVDF	Polvinylidene fluoride
qRT-PCR	Quantitative reverse transcription PCR
Rab	Ras-related protein
Raf	Rapidly accelerated fibrosarcoma
Rag	Ras-related GTPase
RAPTOR	Regulatory-associated protein of mTOR
RAS	Rat sarcoma (oncogene)
RB	Retinoblastoma protein
RBC	Red blood cell
Rev	Reverse (primer)
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of mTOR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid

RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RPKM	Reads per kilobase per million mapped reads
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcription
S6K	p70S6 kinase
SAHA	Suberoylanilide hydroxamic acid
SCF	Stem cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Sec	Seconds
SEM	Standard error of the mean
Ser	Serine
Sox2	SRY-box-2
SQSTM1	Sequestosome 1 (also p62)
Src	Rous sarcoma oncogene cellular homolog
SSC	Side scatter
TCA	Tricarboxylic acid
TE	Tris-EDTA (buffer)
TEM	Transmission electron microscopy
TFEB	Transcription factor EB
TGF	Transforming growth factor
TGF	Transforming growth factor
Thr	Threonine
Tie2	Tunica interna endothelial cell kinase
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TSC1/2	tuberous sclerosis 1/2 complex
UCP	Uncoupling proteins
ULK	Unc-51 like autophagy activating kinase 1
UV	Ultraviolet
UVRAG	UV-irradiation resistance-associated gene
V	Volts
VPS	Vacuolar protein sorting (family)
WIPI	WD repeat domain, phosphoinositide interacting 1
WT/wt	Wildtype
XIAP	X-linked inhibitor of apoptosis protein
B-Gal	Beta-galactosidase
O ₂	Oxygen

Chapter 1: General Introduction

Autophagy

1.1 An introduction to autophagy

Cellular homeostasis requires that anabolic (synthetic) activities be balanced by catabolic turnover, which in eukaryotic cells involves either the ubiquitin-proteasome system or the main degradative organelle, the lysosome. Autophagy is a catabolic cellular pathway responsible for the sequestration of cytoplasmic constituents and their delivery to lysosomes for degradation, hence the name ‘auto-phagy’ which comes from the Greek for ‘self-eating’. The process, from morphology to the main protein players involved, is conserved among eukaryotes, from yeast to higher organisms (1); a parallel pathway has been found in plants (2, 3).

Within the cell, autophagic activity serves to both remove superfluous items or damaged organelles from the cytoplasm and to provide a recycled supply of macromolecules for metabolic activities, something particularly vital under stressful conditions. Indeed, although previously considered a cell death mechanism (4, 5), autophagy is now accepted as a pathway primarily promoting cell survival. Autophagy has been implicated in a wide range of cellular processes, including roles in cell development and differentiation (6) (7), growth (8), aging, (9) (10), senescence (11, 12) and cell death (13). Its dysfunction is associated with a variety of pathological conditions, including neurodegeneration (14, 15) and cancer (16, 17).

The autophagic process can be subdivided by the method of cargo delivery to the lysosome. In chaperone-mediated autophagy, proteins containing a recognition sequence are specifically targeted to lysosomes by chaperone proteins (18, 19) whereas during microautophagy, cargo is taken up by vacuoles/lysosomes non-selectively or selectively

via membrane invagination (20). The third variant, macroautophagy (hereafter referred to as autophagy) is distinct in that it involves the de-novo formation of a double membraned vesicle, the autophagosome, which sequesters cargo and then fuses with a lysosome for degradation of its contents (21); it is the latter which is the subject of this thesis.

1.2 Autophagy regulation - signaling

Autophagy has been found to occur at low basal levels in most cell types for the purposes of homeostatic maintenance (22), but it is induced to much higher levels in response to developmental control (6) and a variety of cellular stresses (22, 23), including nutrient deprivation/starvation, hypoxia and oxidative stress, as well as diverse external stimuli such as growth factors and inflammatory cytokines (22, 24, 25). The preeminent signals involved in autophagy regulation are summarized in Figure 1.1.

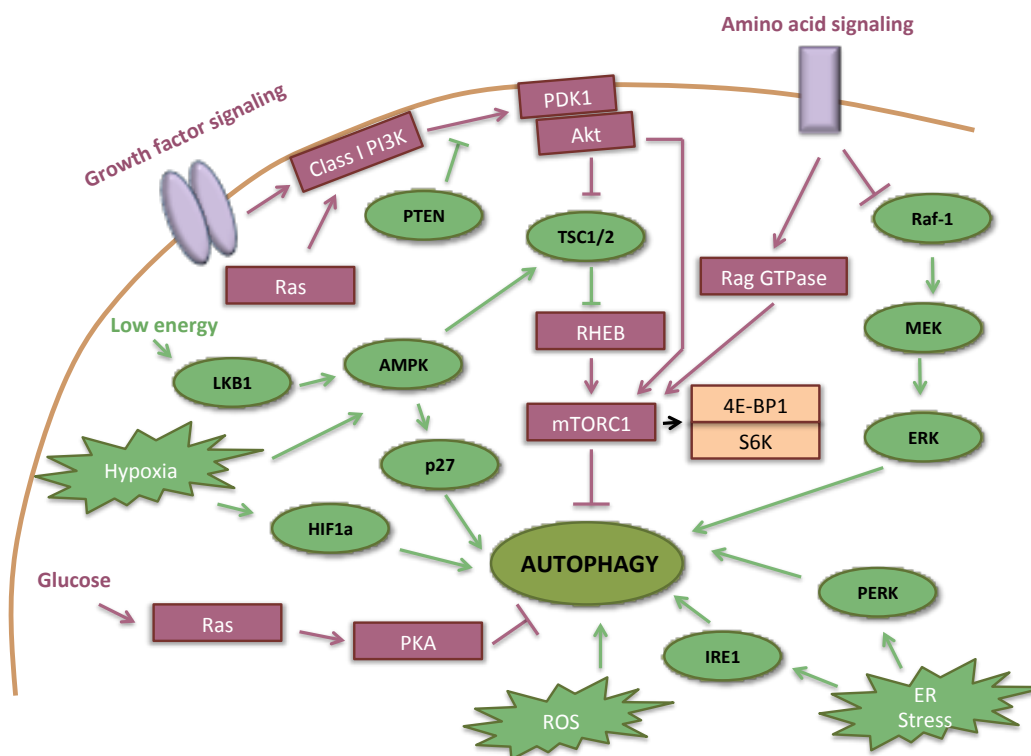


Figure 1.1: Major signaling pathways regulating mammalian autophagy. Components, activating or inhibiting arrows in green indicate positive autophagy regulators; red indicates negatively regulating proteins/steps. Growth factor activation of class I phosphoinositide 3-kinase (PI3K) results in $PI(3,4,5)P_3$ synthesis, recruiting and activating phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/Akt) in turn. Phosphatase and tensin homolog (PTEN) negatively regulates PIP_3 levels. When activated, Akt signaling both inhibits tuberous sclerosis complex 1 and 2 (TSC1/2), which relieves mammalian target of rapamycin (mTOR) activator RHEB from its GTPase, and directly phosphorylates mTOR. mTOR signaling, when in the mTOR complex 1 (mTORC1) association with RAPTOR, drives protein synthesis through activation of p70 S6 kinase (S6K) and eIF4E-binding protein 1 inhibition, while directly inhibiting autophagy initiation. Autophagy is negatively regulated by amino acid transporter signaling, through active Rag-GTPase or inhibited Erk, and by increased glucose levels, through protein kinase A (PKA). By contrast, cellular stresses, including low energy levels (through AMP-activated protein kinase, AMPK, signaling), hypoxia, oxidative stress (through reactive oxygen species, ROS, production) and endoplasmic reticulum stress, all activate autophagy. See text for reference details, figure adapted from (22).

Mediating these changes is a substantial signaling network, converging on a conserved set of Autophagy Related Genes (Atg's); over 30 Atg's (36 at the time of writing) (26) have been identified in yeast, and the mammalian homologs for many have also been described (1). A central upstream regulator of the Atg's is the serine/threonine kinase TOR (target of rapamycin), mTOR (mammalian TOR) in mammals. In the presence of growth factor signaling via the class I Phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway, mTOR is directly phosphorylated by Akt (27) and activated by positive regulator RHEB (28, 29). Active mTOR, in the mTOR-complex 1(mTORC1) with RAPTOR, in turn drives protein synthesis and cell growth through S6K and 4E-BP1 phosphorylation (30) while inhibiting catabolism and autophagy through the inactivation of the phosphorylation of initiation complex member ULK1, the mammalian homolog of Atg1 (31, 32). There is also recent evidence that Akt regulates autophagy proteins directly, phosphorylating and sequestering autophagy protein Beclin1 (33), while mTOR can also inhibit Beclin1 binding partner AMBRA1 (34). As such, negative regulators of Akt signaling, such as PTEN (35), or mTOR inhibitors, such as rapamycin (36), activate autophagy.

mTOR serves as an effector for other environmental and nutrient sensory networks; it is positively regulated by increased amino acid levels through Rag GTPases (37) and by increased cellular energy levels through AMP/ATP ratio sensing liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signaling (38). The latter can alternatively *increase* autophagy under low-energy conditions by activating the Rheb-regulating tuberous sclerosis 1/2 complex (TSC1/2) (39), leading to decreased mTOR signaling, as well as through the cyclin-dependent kinase inhibitor p27, which also mediates cell cycle arrest (23). Outside the mTOR complex, elevated cAMP and Ras-glucose sensing both activate PKA (40) which negatively regulates Atg1.

Finally, autophagy is under the control of various stress-response pathways, including hypoxia through HIF1 α (41) and AMPK regulation, oxidative stress, due to reactive oxygen species (ROS) dependent inactivation of autophagy protein ATG4 (42) and LKB1/AMPK activation by hydrogen peroxide (43), and ER stress, through multiple pathways including PERK and IRE1 (44). There is extensive cross-talk between the autophagy and the apoptotic pathways (45), which mediate cell death typically characterized by caspase activation, DNA/nuclear fragmentation, membrane blebbing and corresponding loss of cell size (46). Apoptosis is triggered by autophagy inhibition during cell stress (13).

1.3 Autophagy regulation - transcription

Accompanying autophagy signaling changes in response to cellular stress, several Atg's are regulated at a transcriptional level following extended cellular strain (22).

The Forkhead box O family of transcription factors are key stress regulators, activating anti-oxidant response along with proteasomal degradation, and in keeping with this

FOXO3 in particular has been identified as a key autophagy modulator, responding to decreased Akt activity by upregulating multiple autophagy genes, including Bnip3 and Atg8 homologs *Map1lc3b* and *Gabarp11*, in a rapamycin (and therefore mTOR) independent manner (47) (48) (49). Further down the Akt pathway, decreased nutrients and amino acids in the lysosomal lumen results in mTORC1 inactivity and therefore allows nuclear translocation of transcription factor EB (TFEB), which controls a largely different set of autophagy and lysosomal genes to FOXO3, including *Atg9*, *Sqstm1*, *Uvrag* and *Lamp-1* (50) (51).

The above transcription factors primarily respond to environmental stimuli, but internal factors are also integrated into autophagy transcriptional regulation. The key DNA damage and stress response protein p53 is known to regulate autophagy directly, as well as indirectly through the effector DRAM (52). Although p53-autophagy relationship is context dependent (53), nuclear p53 has recently been confirmed to directly bind to and upregulate several core autophagy genes, including Atg1 homologs *Ulk1/2*, *Atg7*, *Atg10* and *UVRAG*, along with *FOXO3* (54). The retinoblastoma protein (RB), another tumor suppressor, and associated CDKI's have been shown to induce autophagy (55) in a complex relationship with binding partner E2F1 (56). Similarly, ER stress results in eIF2a dependent transcription of *Atg12* (57). Interestingly, these three pathways are all associated with cell cycle arrest (22). Separately, there is recent exciting evidence of autophagy gene regulation by developmental transcription factors, including GATA-1 (58), which will be discussed further in Chapters 3 and 4. Epigenetic factors also influence autophagy, as autophagy genes have also been found to be upregulated by histone deacetylase inhibition (59).

1.4 Autophagosome formation

Following activation by the factors described above, Atg genes act in sequential complexes to mediate autophagosome formation (Figure 1.2); although the autophagy network includes over 60 genes (60) the products of a group of 18 Atgs (and mammalian homologs where known) in particular are vital for this process and are shared across the autophagy sub-types, thus these are referred to as ‘core’ Atg’s (1).

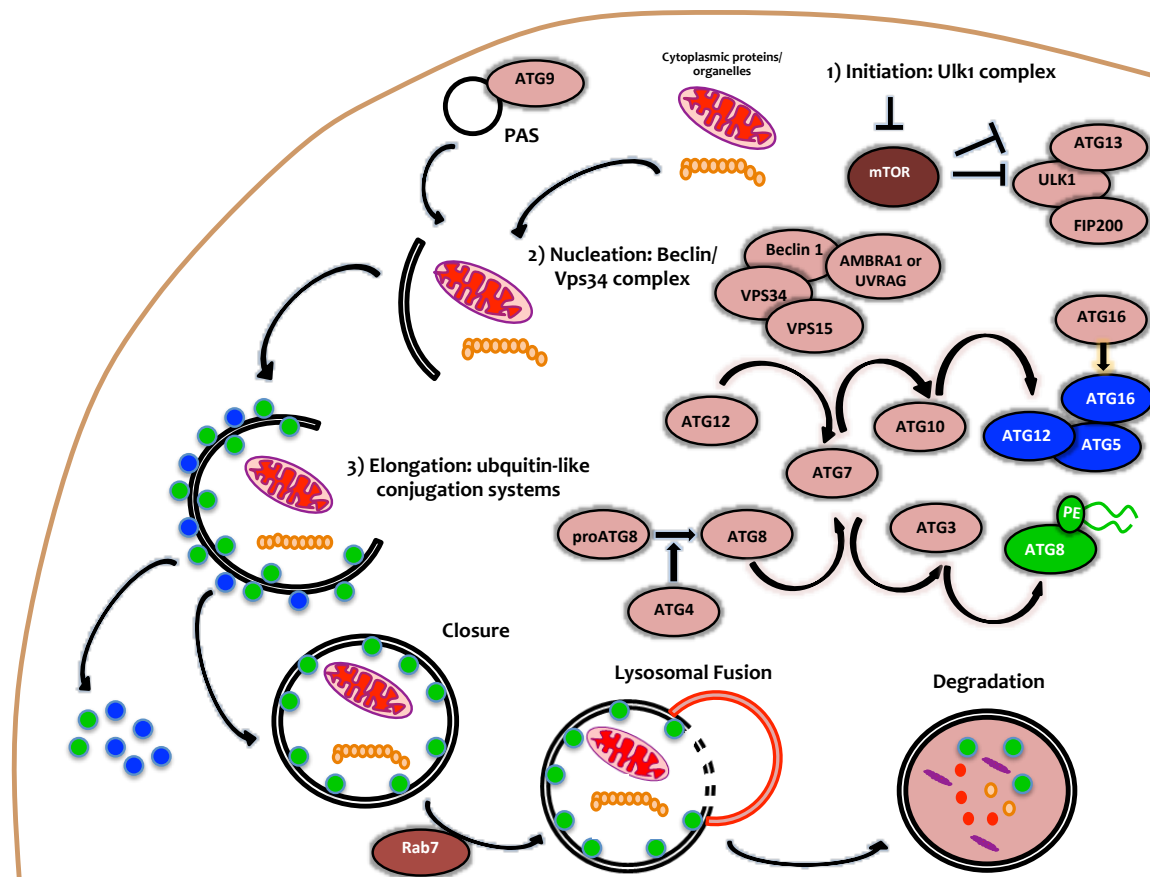


Figure 1.2: Outline of mammalian autophagosome formation. Autophagy is initiated by the ULK1 (mammalian Atg1 homolog) complex following loss of mTOR inhibitory phosphorylation of ULK1 and ATG13. The ULK1 complex combines with the Beclin 1 (mammalian Atg6 homolog) and class III PI3K VPS34 complex, which has several mutually exclusive binding partners including AMBRA1 and UVRAG, to control formation of the phagophore (also called the isolation membrane), aided by membrane transporting ATG9 complex, which includes WIP1/2 (mammalian Atg18 homologs, not shown). The site of autophagosome membrane formation, the pre-autophagosomal structure (PAS), is debated. The elongation of the isolation membrane is mediated by two ubiquitin-like conjugation systems. In the first, ATG7 (E1-like enzyme) activates the C-terminus of ATG12, which is transferred to ATG10 (E2-like enzyme) before being

conjugated to ATG5. ATG5-12 conjugate binds ATG16, which self associate to form a tetramer (in blue) that aids the second ubiquitin-like cascade and autophagosome formation. In the second ubiquitin-like cascade, ATG8, having had a C-terminal glycine exposed by cysteine protease ATG4 cleavage, is passed from ATG7 and ATG3 to bind phosphatidylethanolamine (PE) via an amine bond (forms lipidated ATG8, shown in green); ATG8-PE acts as a scaffold in the autophagosome membrane. Following autophagosome formation, ATG5-12/16 dissociates while exterior ATG8-PE is cleaved by ATG4 for recycling, interior ATG8 is degraded following autophagosome-lysosome fusion, a process requiring Rab7. Adapted from (61) (1).

The primary initiation complex in mammals includes ULK1 (mammalian Atg1 homolog) (62) and ATG13, both directly negatively regulated by mTOR (32), and FIP200 (mammalian Atg17 functional homolog) (63). Also involved in autophagosome formation are ATG9A/B (64), which transports membrane to the growing PAS (pre-autophagosomal structure), and binding partners ATG2 and WIPI1/2 (mammalian Atg18), which are thought to aid ATG9 recycling (65). A third set of complexes, involved in nucleation of the growing vesicle, contain the type III PI3K VPS34, VPS15 and Beclin1 (mammalian Atg6) as core complex members, with a network of binding partners including ATG14L and AMBRA1 (66) or UVRAG (67) (68).

Together, these three associations cooperate to form the initial autophagosome membrane, or phagophore (1). Interestingly, the precise origin of the membrane components used is a matter of debate, with the plasma membrane (69), Golgi (70) and mitochondria/ER contact points (71) (72) being suggested; on balance the evidence points towards ER contact with other membranous compartments being a dogma (26).

Once initiated, the elongation of the autophagosome membrane (called the isolation membrane) requires two ubiquitin-like conjugation systems, first defined in yeast; in the one, Atg7 (E1-like) and Atg10 (an E2-like protein) act to conjugate Atg5 to Atg12 (73) (74), which then associates with Atg16. The Atg5-Atg12/16 complex is able to bind and tether autophagosome membranes directly (75) and functions as E3-like protein (76). It

acts in concert with Atg7 and Atg3 (an E2-like protein) to conjugate Atg8 homologs, when first prepared by C-terminal cleavage mediated by Atg4 or its four mammalian homologs (77, 78), to phosphatidylethanolamine (PE) and locate the conjugate in the membrane of the growing autophagosome (79, 80). Atg8-PE is proposed to play a scaffolding role in membrane growth (21).

Following autophagosome completion, the Atg5-12/16 complex dissociates from the membrane (81) while exterior Atg8 is cleaved from PE by Atg4 and recycled (77).

Interestingly, mammalian cells have six functional Atg8 homologs, including MAP1LC3A, MAP1LC3B, MAP1LC3C, GABARAP, GABARAPL1, GABARAPL2 (GATE-16) and while the precise functions of each is still to be determined, it does appear that they may preferentially be processed by different Atg4 homologs (82). Meanwhile, the formed autophagosome undergoes fusion events, either with late endosomes (to form amphisomes), which then in turn fuse with lysosomes, or with lysosomes directly, in a process which requires ESCRT family proteins (83) along with Rab7 (84) and allows degradation of autophagosome contents. As would be expected for a vesicular process, cytoskeletal transport (85) and resulting localization (86) of these compartments is an important factor in determining autophagic rate ('flux') and targets.

1.5 Targets of autophagy

As mentioned above, the inclusion of cargo within the autophagosome can be non-selective or selective in a stimulus-dependent manner. While starvation induced autophagy is largely considered a bulk engulfment of cytoplasmic regions for the purpose of metabolite liberation, homeostatic surveillance/repair activities and responses to particular stressors are now known to be highly targeted (87). This includes autophagic degradation of damaged or surplus organelles, such as peroxisomes (pexophagy) (88), ribosomes

(ribophagy) (89) and lipid droplets (90). Another well studied form is mitophagy, the degradation of mitochondria in response to damage (ex. loss of membrane potential), developmental cues, oxidative stress, and hypoxia (91). Each of these substrates involves the core autophagy machinery and organelle-specific adaptors (56).

Autophagy has a significant role in degradation of accumulated cytosolic proteins, whether superfluous, damaged or misfolded, in particular helping to clear aggregates of such proteins. As with organelle targeting, autophagic protein degradation is mediated by adaptor proteins: p62 (product of *SQSTM1*) (92), NBR1 (93), and ALFY (94) all bind ubiquitinated proteins and, in the case of the first two, also bind Atg8 homologs to facilitate autophagosome inclusion. Indeed, p62 has been shown to also target ubiquitinated proteins on organelles and facilitate their autophagic clearance (95). The E3 ligase c-Cbl can act as a cargo receptor independent of its ligase activity (96). Finally, as mentioned above, there is evidence selective receptors exist for autophagic organelle targeting, for example the adaptor NIX (BNIP3L) has been found to bind Atg8 and mitochondrial membrane proteins through a C-terminal domain (97), which may interact with BNIP3 on the membrane surface (98).

1.6 Autophagy products and outcomes

One of the primary roles of autophagic degradation is to liberate products vital for supporting cell metabolism and cell survival under stressful conditions. Glucose and lipids produced by the breakdown of cellular macromolecular stores can be fed into energy metabolism, via glycolysis and/or mitochondrial oxidative phosphorylation, or converted into biosynthetic intermediates, while nucleosides can be used for nucleic acid synthesis. (99). Proteins are a key target of autophagy, and the resulting amino acids can be used for

novel protein synthesis, ATP production via the tricarboxylic acid (TCA) cycle, or the generation of other key metabolites, such as citrate for lipid synthesis (99).

The other side of autophagy is the removal of potentially harmful excess components (such as otherwise healthy mitochondria in hypoxic conditions) or waste (for example, protein aggregates) and autophagic malfunction is associated with the accumulation of autophagic targets with toxic consequences. For example, without mitophagic pruning, damaged mitochondria build up in the cell, resulting in altered metabolism, increased reactive oxygen species, and inhibited maturation of hematopoietic cells (100, 101). Similarly, deletion of key Atg's or p62 itself causes protein aggregates to collect in the cytoplasm (102), which are implicated in aging, neurological disease (103), and tumorigenesis (104).

Hematopoiesis

1.7 The process of hematopoiesis

Hematopoiesis is the generation of the cells of the blood, the main components of which are erythrocytes (red blood cells), platelets and leukocytes (white blood cells).

Erythrocytes are the most common cell type, serving to deliver oxygen to tissues and to transport carbon dioxide to the lungs for expulsion. Megakaryocytes fragment to produce platelets, responsible for creating aggregate plugs in vascular injury response. Leukocytes, which can be divided into myeloid and lymphoid cells, mediate the body's immune system (105).

1.8 Location of hematopoiesis

Giving rise to these cell types are hematopoietic stem cells (HSCs), a rare population of pluripotent cells which top the hematopoietic hierarchy and replenish the blood through differentiation and gradual lineage commitment (106). Prior to birth, hematopoiesis progresses from the yolk sac to aorta-gonad mesonephros, the placenta and fetal liver of developing mammals, before HSCs move to the bone marrow (BM) following birth and continue blood cell production throughout adult life (106). In the BM, HSCs reside in a specialized niche, created by multiple cell types including endothelial cells, mesenchymal stromal cells and osteoblasts, which regulate and support HSC activity (107); the former two in particular produce factors vital for HSC maintenance, such as stem-cell factor (SCF) (108). These niches, often located near trabecular bone (bone with many small projections and spongy appearance, increasing marrow contact) and sinusoid blood vessels, were described as particularly hypoxic, although this evidence has recently been called into question (107). Movement between particular niche locations may regulate HSC quiescence and self-renewal, as a recent study found quiescent HSCs associated with periarteriolar sites in an osteoblastic niche within the endosteal bone marrow, whereas actively cycling HSCs localized to perisinusoidal niches (109).

1.9 The hematopoietic cascade

In contrast to fetal HSCs, which divide actively, a significant proportion of adult HSCs are in a quiescent, non-dividing state, which is important for retaining the HSC reservoir as well as resisting damage/stress and therefore for lifelong HSC health (110). HSC quiescence has been found to be partially controlled by BM niche-provided factors, including thrombopoietin signaling (111) and TGF- β (112). The actively cycling HSC

population either undergo self-renewal, that is form a new HSC to maintain the population, or else begin the process of differentiation towards lineage commitment (106).

HSC differentiation begins with a loss of that self-renewal potential, subdividing long-term HSCs (LT-HSCs), which are capable of life-long self-renewal, from short-term HSCs and multi-potent progenitors (MPPs), both of which retain multi-lineage potential but have either limited (~6 week) or no long-term hematopoietic reconstitution ability respectively (113-115). Downstream of these populations, progenitors become increasingly lineage biased. Although cytokines were previously thought to simply drive survival and expansion of progenitors based on receptor expression (116), therefore supporting already committed progenitors, it is now clear that they can also instruct lineage choice in progenitor and stem cells, including myeloid (117, 118) and erythroid (119) fates. External stimulus/cytokines combine with transcriptional programs (120) affecting the balance between key transcription factors in progenitors, which are the major drivers of cell fate decisions. Such transcription factor relationships are complex and often antagonistic, for example GATA-1 and PU.1 in early erythroid versus myeloid lineage choices in common progenitors (106, 121, 122). Evidence suggests that progenitor populations, including HSCs, are heterogeneous in both function (123, 124) and gene expression (125) resulting in differential lineage potential and signatures within those population prior to fate commitment (120) (126).

The extent and manner of lineage bifurcation in the hematopoietic cascade has recently been revisited. Classical models outlined an early division downstream of HSCs to either a common myeloid progenitor (CMP), with myelo-erythroid potential, or a common lymphoid progenitor (CLP), with sole lymphoid potential (127). However, recent observations cannot be explained by that model, for example the first step in lymphoid

commitment is instead a lymphoid-primed multipotent progenitor (LMPP), with downregulated megakaryocyte-erythroid potential yet retained granulocyte-monocyte (GM) potential (128, 129). Similarly, early T-cell progenitors in the thymus retain GM potential (130).

As a result, revised models of the hematopoietic hierarchy emphasize that plasticity of choice and include multiple myeloid-lymphoid branching points. One such model for murine hematopoiesis is represented in Figure 1.3.

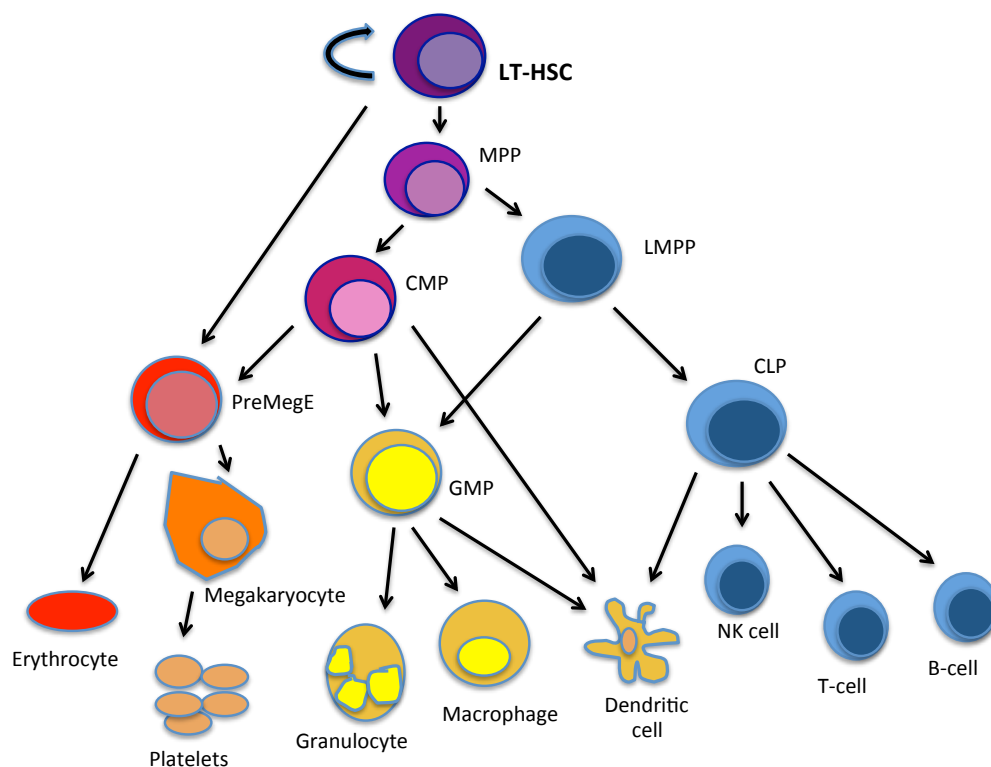


Figure 1.3: Adult murine hematopoietic cascade. Long-term hematopoietic stem cell (LT-HSC), multipotent progenitor (MPP), lymphoid-primed multipotent progenitor (LMPP), common myeloid progenitor (CMP), common lymphoid progenitor (CLP), granulocyte macrophage progenitor (GMP), pre megakaryocyte erythroid progenitor (PreMegE), natural killer cell (NK cell), adapted from (120).

1.10 Thesis Summary: The role of autophagy in hematopoiesis and acute myeloid leukemia

As outlined in the following chapters, previous work in our lab had found that the deletion of the autophagy gene *Atg7* leads to multiple hematopoietic defects, including HSC loss and a significant myeloproliferation, progressing to eventual bone marrow failure (131). The objective of the research presented herein was twofold: (i) to investigate the involvement of autophagy in healthy hematopoiesis, in particular focusing on autophagy's impact on HSCs and MPPs, and (ii) to examine the role autophagy plays under the aberrant hematopoietic conditions of acute myeloid leukemia (AML). Quantification of autophagy in rare populations, such as HSCs, is extremely difficult with existing techniques, and this work uses a novel autophagy assay based on imaging flow cytometry to demonstrate a positive correlation between hematopoietic progenitor immaturity and autophagy levels in *ex vivo* murine and human systems. These observations support a general role for autophagy in HSCs, therefore a murine model with hematopoietic deletion of the essential autophagy gene *Atg5* was examined in order to expand on our lab's previous findings in the *Atg7* deletion model and rule out gene specific phenotypes. Finally, given an accumulation of myeloid cells under autophagy deficient conditions, this work combines measurement of autophagy in primary AML samples with autophagy modulation in murine and human AML models to explore autophagy's impact on this myeloid neoplasia. The data described here show an autophagy requirement for maintaining a healthy HSC reservoir, and connects autophagy regulation with growth and proliferation of transformed myeloid cells. These findings may inform application of autophagy modulating treatments in cancer and suggests further research into autophagy's connections to the cell cycle.

Chapter 2: Materials and Methods

2.1 Mice and breeding

Mice were bred and housed in the Department of Biomedical Services, University of Oxford in individually ventilated, filter top cages and fed *ad libitum*. *Atg7^{fl/fl}* mice were obtained from M. Komatsu, *Atg5^{fl/fl}* kindly gifted by N. Mizushima, *Vav-iCre* mice were kindly given to the lab by D. Kioussis (London). *Vav-Atg7* breeding and genotyping has been described previously (131). *Atg5^{fl/fl}* (132) mice were crossed to *Vav-iCre* mice to obtain *Vav-Atg5^{-/-}* (*Atg5^{fl/fl};Vav-iCre⁺*) and genotyped as below. Male and female mice were used equally in all experiments; *Atg5^{fl/fl};Vav-iCre⁻* and *Atg5^{fl/wt};Vav-iCre⁺* littermates were used equally as wildtype littermate controls, as no phenotype was observed in heterozygous mice. All mice were bred on a C57BL/6 background. C57BL/6 CD45.1+ mice were bred in, and obtained from, Department of Biomedical Services, University of Oxford. All animal experiments were approved by the local ethical review committee and performed under UK home office personal (PIL: 30/9996) and project licenses (PPL 39/2809).

2.2 Genotyping

Genotyping was performed as previously described (133, 134) (132) using tissue taken via ear punch. The tissue was digested for a minimum of two hours (maximum overnight) at 55°C in a lysis buffer (5mM EDTA, 0.1M Tris-HCl, 0.2% SDS, 0.2M NaCl, 7.8 mg/ml proteinase K from Sigma Aldrich), spun down (16000 x g for 7 min) then the supernatant transferred to a new 1.5ml tube. DNA in the supernatant was precipitated by addition of an equal volume of isopropanol, mixing via inversion, pelleting (16000 x g 7 min), washing in 70% ethanol then air-drying. Dried DNA was dissolved in 50µl distilled water (incubated 65°C for 30 minutes) and stored at -20°C

PCR reactions were performed in 20µl total volume with specific procedures as follows:

iCre PCR

iCre PCR reactions were performed using Ampli-TaqGold DNA polymerase kits (Applied Biosystems) using the following conditions:

1. 94°C for 3 min
2. 94°C for 40 sec
3. 62°C for 40 sec
4. 72°C for 30 sec
5. 30 cycles repeat above from step 2
6. 72°C for 5 min
7. Hold at 4°C

Primers were (iCre-Fwd 5'-AGATGCCAGGACATCAGGAACCTG-3' and iCre-Rev 5'-ATCAGCCACACCAGACACAGAGATC-3').

Atg7 fl and wt PCR

Atg7 PCR reactions were performed using MyTaq Red Mix combined PCR mix (Applied Biosystems) using the following conditions:

1. 95°C for 1 min
2. 95°C for 15 sec
3. 55°C for 15 sec
4. 72°C for 10 sec
5. 30 cycles repeat above from step 2
6. Hold at 4°C

Primers were (WT fwd Primer) 752: 5'-CCATGCTGATGGCTAATGTCTC-3', (Flox fwd Primer) 753: 5'-CTGCAGGAATTCGATATCATAACTTCG-3', (Reverse Primer) Atg7-14R: 5'-GTCCAGAGTCCGGTCTCTGGTTG-3'.

Atg5 fl and wt PCRs

Atg5 fl and wt PCR reactions were performed using Roche Taq DNA polymerase and under the following conditions:

1. 95°C for 3 min
2. 95°C for 30 sec
3. 64.8°C for 30 sec
4. 72°C for 1 min
5. 34 cycles repeat above from step 2
6. 72°C for 10 min
7. Hold at 4°C

WT and Fl genotyping performed separately: WT PCR used primers 1&2, Fl PCR used 2&3 (1: 5'-GAATATGAAGGCACACCCCTGAAATG-3', 2: 5'-GTACTGCAT-AATGGTTTAACTCTTGC-3', 3: 5'-ACAACGTCGAGCACAGCTGCGCAAGG-3')

The resulting PCR products were run on 1% agarose gels (Invitrogen) prepared with 50ml TBE buffer (45mM Tris-Borate, 1mM EDTA) and stained with 5µl SYBR Safe DNA Stain (Invitrogen). Samples were run at 70V according to expected band size.

2.3 Primary cell sources and preparation

Mouse bone marrow

Hind limb bones of 6-9 week old mice were collected in aseptic conditions, cleaned of tissue and fur and rinsed in 70% ethanol before being transferred into PBS/5% FCS. The bones were crushed in a sterile mortar and pestle in PBS/5% FCS, passed through a 70µm cell strainer (BD Falcon) and enumerated by Trypan Blue (Sigma) counts.

Mouse splenocytes

Spleens collected from 6-9 week old mice in aseptic conditions were transferred to PBS/5%FCS and mashed with regular washing through a 70µm strainer (BD Falcon). The resulting suspension was passed through a second strainer, pelleted, incubated for 5 min in 1x RBC Lysis Buffer (eBioscience), washed in PBS/5% FCS and enumerated by Trypan Blue (Sigma) counts.

Human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were separated from healthy donor buffy cones obtained from NHS Blood Services (John Radcliffe Hospital, Oxford). 30 ml buffy cones (1:5 diluted in sterile RPMI [Life Technologies]) was layered onto 10 ml of Lymphocyte Separation Medium (PAA), pelleted (1200 x g 20 min with brake off) and washed twice before suspension in RPMI/10% FCS and enumeration.

Human bone marrow

Healthy human bone marrow mononuclear cells (BMNCs) were obtained from Lonza (2M-125C). AML and MDS BMNCs were obtained from MDSBio (experiments approved by local REC, REC# 11/H0605/4, samples taken with informed consent by named biobank). Frozen samples were maintained in liquid nitrogen storage before thawing into IMDM (Life Technologies)/20% FCS; cells were rested for a minimum of 4 hours prior to analysis.

2.4 *In vitro* culture

Cells were maintained at 37°C and 5% CO₂. NB4 and KG1a cells were bought from DMSZ and ATCC respectively. HEL, K562, HL-60, MV4-11, OCI-AML, MCF7 were gifts from the labs of collaborators Prof S.E. Jacobsen or Prof. A. Harris and recent passages. The lab of Prof. F. Powrie gifted HCT116 cells. All lines were mycoplasma tested. Generation of ATG5-overexpressing and vector control NB4 and HEL lines, and that of the murine MLL-ENL lines, is described below.

For cell lines actively maintained in the lab, medias used were as listed in Table 2.1 unless indicated otherwise in figure legends. Human leukemic cell lines were kept at a density of 5×10^5 - 1×10^6 cells/ml through passage 2-3 times per week. Adherent lines were split 1:5 on reaching 90% confluence. MLL-ENL lines in liquid culture were counted and plated at 0.5×10^5 cells per ml every 48 hours.

Table 2.1: Cell Lines and Growth Medias

Cell Line	Media (from Life Technologies unless otherwise stated)
MCF-7, HCT116	DMEM + 10% FCS + 2mM L-glutamine + penicillin/streptomycin
HL-60, NB4, HEL	RMPI + 10% FCS + 2mM L-glutamine + penicillin/streptomycin
KG1a, K562	IMDM + 10% FCS + 2mM L-glutamine + penicillin/streptomycin
MLL-ENL lines	RMPI + 10% FCS + 2mM L-glutamine + penicillin/ streptomycin + 50 μ M 2-mercaptoethanol, 100 ng/ml mSCF, 10 ng/ml mIL-3, 10 ng/ml mIL-6 (all recombinant cytokines from Peprotech)
MS-5	α MEM (nucleosides, no ascorbic acid) + 10% FCS + 2mM L-glutamine + penicillin/streptomycin + 2mM sodium pyruvate

2.5 ATG5 overexpression line generation

HEL and NB4 cells were transfected using the Neon Transfection System (Invitrogen) as per kit instructions with pEGFP-C1 (Clontech; empty vector) or pEGFP-Atg5, plasmids were gifts from Dr. Yoshimori and generated as described (78). Neon transfection settings were:

HEL: 1300V (pulse voltage), 20ms (pulse width), 3 (pulse number), 1 μ g DNA/5x10⁵ cells per transfection

NB4: 1350 (pulse voltage), 35ms (pulse width), 1 (pulse number), 1 μ g DNA/5x10⁵ cells per transfection

Transfected lines were grown for 4 weeks with 500 μ g/ml G418 (Life Technologies) from 48hrs post-transfection, then bulk FACS sorted for GFP expression and grown under selection for another 4 weeks prior to immunoblot assessment.

2.6 MLL-ENL line generation

Bone marrow from *Atg5^{fl/fl}*, *Atg5^{fl/wt}* and *Atg5^{wt/wt}* mice (3 per genotype) was extracted as above and pooled by genotype. Lineage cells were depleted using Lineage Cell Depletion Kit and LS columns (both Miltenyi Biotec) as directed by the manufacturer, and lineage negative cells were transduced with constitutive MLL-ENL as previously described (135, 136). Transduced cells were grown in complete methylcellulose without EPO (R&D systems) for 7 days, the resulting colonies pooled, transduced with pMSCV-IRES-CD2, containing tailless human (h) CD2 cDNA (137) either alone (empty vector) or with additional iCre (133) then FACS sorted on hCD2 expression. Resulting cell lines were grown in suspension, but were passaged through methylcellulose pre- and post-thaw. Lines were generated with the assistance of Dr. O. Williams, University College London.

2.7 Flow cytometry and Image Stream

Surface marker and membrane-permeable dye staining

Primary or cultured cells were pelleted (1500 rpm, 5 minutes, 4°C unless indicated otherwise) and transferred to round-bottomed 96 well plates for staining. If mitochondrial, autophagosome (CytoID) or lysosome (LysoID) stains (listed below) were used, pelleted cells were immediately resuspended in staining solutions and incubated (in dark/wrapped in foil; all staining steps likewise unless otherwise stated) at 37°C for 10-30 min as instructed by the manufacturer. Otherwise, cells were washed (for this section, wash indicates sample resuspended, spun down and supernatant removed) with cold FACS buffer (PBS, 1% FCS, 0.2% sodium azide) and incubated with 50µl of Fc blocking reagent (as below) for 10 min at 4°C where appropriate prior to addition of 50µl 2x surface marker mix (including fixable live/dead stain if used), for 100µl staining volume, and incubation

(covered in foil) for 10 minutes at room temperature or 20 minutes at 4°C on shaker.

Samples were then washed with cold FACS buffer and analyzed or sorted immediately, resuspended in FACS buffer or viability stain (DAPI/7AAD) where appropriate, or else fixed (IC Fixation Buffer, eBioscience) for storage or intracellular analysis. Antibodies, dyes and reagents used in flow cytometry or imaging flow cytometry are listed in Tables 2.2, 2.3 and 2.4.

Table 2.2: Reagents used in mouse flow cytometry or imaging flow cytometry (antibodies are all anti-mouse, some may cross react)

Name (clone)	Fluorochrome (working dilution)	Company
CD4 (RM-4.5)	Unconjugated (1:1600), APC (1:800) or PE-Cy5 (1:800)	eBioscience
CD5 (53-7.3)	Unconjugated (1:800), APC (1:700) or PE-Cy5 (1:600)	eBioscience
CD8a (53-6.7)	Unconjugated (1:800), APC (1:800 in lineage stain, 1:300 alone), FITC (1:300) or PE-Cy5 (1:800)	eBioscience
CD11b (M1/70)	Unconjugated (1:200), APC (1:1600 in lineage, 1:500 alone), PE-Cy5 (1:400), PE (1:400), eFluor 450 (1:400)	eBioscience
B220 (RA3-6B2)	Unconjugated (1:200), APC (1:200), PE-Cy5 (1:200), eFluor 450 (1:300)	eBioscience
Ter119 (TER-119)	Unconjugated (1:50), APC (1:200), PE-Cy5 (1:300), PE (1:400)	eBioscience
Gr1/Ly6g (RB6-8C5)	Unconjugated (1:100), APC (1:400), PE-Cy5 (1:400), FITC (1:300)	eBioscience
F4/80	APC (1:400)	eBioscience
CD45.1 (A20)	PE-Cy7 (1:500)	eBioscience
CD45.2 (104)	eFluor 450 (1:250)	eBioscience
Goat anti-rat IgG (Poly 4054)	Dylight 405 (1:200)	Biolegend
Goat anti-rat IgG	Cy5-R-PE (1:100)	Invitrogen
CD34 (MEC14.7)	PE (1:100)	eBioscience
CD71 (R17217)	Biotinylated (1:100), PE (1:300)	eBioscience
CD19 (MB19-1)	APC (1:100)	eBioscience
CD3 (145-2C11)	FITC (1:150)	eBioscience
CD117/c-Kit (2B8)	APC-eFluor 780 (1:1200); APC (1:400)	eBioscience
Sca-1 (E13-161.7)	Pacific Blue (1:200)	Biolegend
Sca-1 (D7)	Biotinylated (1:100)	eBioscience
Streptavidin	PE (1:200) APC (1:150) PE-TexasRed (1:800)	BD eBioscience BD
CD150 (TC15-12F12.2)	PE-Cy7 (1:100)	Biolegend
CD105 (MJ7/18)	Biotinylated (1:400)	eBioscience
CD48 (HM48-1)	PE (1:400) APC (1:600)	eBioscience Biolegend

CD16/32 (93)	PE (1:150)	eBioscience
CD31 (390)	Pacific Blue (1:500)	Biolegend
CD45 (30-F11)	Alexa Fluor 546 (1:300)	Biolegend
ICAM (YN1/1.7.4)	Alexa Fluor 647 (1:300)	Biolegend
Fc blocking reagent (anti-mouse CD16/32) (93)	N/A (1:400)	eBioscience
Annexin V	APC (1:50)	Biolegend
Ki67	FITC (1:300)	eBioscience
Phospho-mTOR (Ser2448)(D9C2)	Unconjugated (1:50)	Cell signaling
Phospho-Akt (Ser473)(D9E)	Alexa Fluor 647 (1:25)	Cell signaling
CD2 (RPA-2.10) (anti-human, for ME lines)	PE (1:500)	eBioscience

Table 2.3: Reagents used in human flow cytometry or imaging flow cytometry (antibodies are all anti-human)

Name (clone)	Fluorochrome	Company
CD34 (581)	PE (1:100) FITC (1:100)	Biolegend BD
CD33 (P67.6), CD13 (WM15)	eFluor 450 (1:100)	eBioscience
CD14 (TuK4)	PE (1:100)	BD
CD117 (104D2)	Pacific Blue (1:100)	Invitrogen
CD19 (HIB19)	PE (1:100)	Biolegend
CD3 (UCHT1)	eFluor 450 (1:100)	BD
LAMP1 (H4A3)	FITC (1:100)	BD
WIPI1 (38-W)	PE (1:300)	Biolegend
Human Fc Blocking Reagent	Unconjugated	Santa Cruz
	N/A (1:10)	Miltenyi Biotec

Table 2.4: General reagents used in both human and mouse flow cytometry or imaging flow cytometry

Reagent	Fluorochrome (working dilution/concentration)	Company
Goat anti-rabbit IgG	Alexa Fluor 488 (1:500 image stream; 1:300 phosflow) or 546 (1:500)	Invitrogen
Rabbit anti-mouse LC3 (cross reacts with human)	Unconjugated (1:1000)	Gift from Dr. C. Munz
Rabbit anti-mouse LC3 (cross reacts with human)	Unconjugated (1:1000)	MBL
CytoID	1:1000	Enzo Life Sciences
LysoID	1:666	Enzo Life Sciences
7AAD	(2:1; cell cycle)(1:200; viability)	eBioscience
DAPI	1:50	Invitrogen
Aqua Fluorescent reactive dye	1:2000	Invitrogen
Violet Fluorescent reactive dye	1:2000	Invitrogen
nonyl Acridine Orange	100nM	Invitrogen

MitoID	1:2500	Enzo Life Sciences
MitoSOX Red	5 μ M	Invitrogen
MitoTracker Red	100nM	Invitrogen
MitoTracker Green	100nM	Invitrogen
Total ROS Detection Kit	1 μ M	Enzo Life Sciences
BrdU Flow Kit	APC (1:50)	BD

Intracellular staining

For routine intracellular stainings (including LC3), following cell surface marker staining and/or organelle staining, cells were resuspended in 100 μ l IC Fixation Buffer (eBioscience) and incubated 20 min at room temperature. Samples were permeabilized through two washes in 1x Permeabilization buffer (eBioscience, perm buffer) then resuspended in intracellular antibody mix (made up in 1x perm buffer) and incubated for 20 min at room temperature. Cells were then washed twice in perm buffer and resuspended in secondary antibody mixes (in 1x perm buffer) for 20min at room temperature where appropriate. Following final staining layer, samples were washed twice with FACS buffer prior to analysis.

BrdU incorporation and cell cycle analysis

For BrdU cell cycle analysis, 10 μ M BrdU was either added to culture media 48 hours (long-term incorporation) or 45 minutes (pulse experiments) prior to analysis. Samples were stained using the BrdU Flow Kit (BD, APC-conjugate) as per manufacturers instructions, buffers being provided with the kit. For Ki67 staining, samples were incubated in 1x Fc γ 3 Fixation/Permeabilization Buffer (1 part concentrate, 3 parts diluent, eBioscience) for 1 hour at room temperature, before washing with 1 x perm buffer (eBioscience) and intracellular staining as above (absenting fixation). Samples were

resuspended in 20µl of a 2:1 mix of 7AAD(eBioscience):FACS buffer for 5 minutes at room temperature prior to dilution in 300µl FACS buffer and analysis.

Phosflow experiments

After transfer to 96 well V-bottom plate, cells were resuspended in violet fixable live/dead (Invitrogen) in PBS/FCS (FCS content matched to complete/starvation media culture conditions) for 5 minutes at room temperature before centrifugation (1500 rpm for 5 min) and rapid resuspension in Cytofix buffer (BD, prewarmed to 37°C). Samples were incubated 15 min at 37°C, then washed twice with cold FACS buffer, resuspended in Phosflow Perm Buffer III (BD, chilled to -20°C) and incubated 30 min at 4°C. Following two washes in cold FACS buffer, phosflow antibody mixes were added (in FACS buffer) and cells incubated 60 min at room temperature. FACS washes and incubation (30 min) was repeated for secondary stains; stained cells were washed twice more with FACS buffer and rapidly analyzed.

Absolute cell counts

Absolute cell counts were performed on peripheral blood taken from the tail vein of live animals, collected in heparin-coated tubes (Microvette 300, Sarstedt) to avoid coagulation. 75µl of blood was transferred to TruCount tubes (BD) with 30µl of antibody mix. Tubes were incubated 15 min at 4°C before addition of 450µl 1x RBC Lysis Buffer (eBioscience) for lymphocyte/myeloid cell counts, followed by 10 min incubation at room temperature, or else 450µl FACS buffer for erythrocyte counts. Samples (555µl total volume) were analyzed immediately by flow cytometry and absolute counts calculated as per manufacturer instructions. Absolute count analysis performed by S. Khandwala or D. Puleston.

Instrumentation

Flow cytometry experiments were performed on LSRII or Fortessa (both BD) or Cyan (Beckman Coulter) instruments unless otherwise stated, FACS sorts were performed on Aria II (BD) with aid of Dr. H. Ferry and Dr. K. Alford. Data were analyzed with FlowJo 9.1 for Mac (Tree Star, Inc).

Image Stream and imaging flow cytometry autophagy assay (IFCAA)

Primary or cultured samples were suspended $<1 \times 10^6$ cells/ml in normal growth media (IMDM 10% FCS for primary cells and as above for cell lines) with 10 μ g/ml E64D/Pepstatin A (Enzo Life Sciences), 1 μ M Rapamycin (Sigma), 20 μ M Chloroquine (Sigma) or 100 μ M Wortmannin (Sigma) for two hours. Samples were stained for LysoID (Enzo), fixable violet live-dead (Invitrogen) and surface markers then intracellular LC3 as outlined above.

Image stream measurement of autophagy performed as previously described (12), samples were acquired on the Image Stream 100 or Image Stream X (Amnis), laser strengths were set off single color controls and samples were run with a minimum brightfield cell classifier of 25. Data obtained were analyzed with IDEAS 4.0.735 software (Amnis) for LC3/LysoID Bright Detail Similarity (BDS). BDS quantifies the degree of overlapping pixel intensities between fluorescent imagery channels (138), calculated as a non-mean normalized log-transformed Pearson's correlation coefficient applied to the open residue image. Cells from LC3-GFP transgenic mouse (139) or stained for endogenous LC3 were acquired on the IS100 and analyzed for spot count on a LC3 peak mask with a spot-to-cell background ratio of 2.5 or 3.5. *Note on BDS/Ratio Readout usage:* As described in Phadwal *et al.*, the frequency of cells with a BDS higher than a control defined cut off,

normally ~ 2 , provided optimal detection for known autophagy modulating treatments. For very rare populations, median BDS was employed as lower population numbers made frequency measurements unstable. In situations of complete genetic autophagy loss, increased level of endogenous LC3 meant BDS alone did not always accurately report autophagy levels, therefore the ratio of % of cells LC3/LysoID BDS_{hi} following lysosomal inhibitor treatment divided by the untreated, or basal level (Basal+I/Basal) is used in this thesis as a measure of basal autophagic function, or flux, and to control for autophagic/lysosomal defects (140). Note however this is distinct from LC3II/I turnover measurements of flux, although the trend would be similar. Thanks to Dr. K. Phadwal for assistance with the development of this assay.

2.8 qPCR and Fluidigm

Quantitative PCR

Cells ($1-2 \times 10^6$) were transferred to 1.5ml microcentrifuge tubes and spun for 5 min at 2000 x g. Supernatant was removed and the pellet was washed with PBS then either frozen at -80°C or used immediately. RNA was extracted using RNeasy Kit (Qiagen) as per manufacturers instructions and quantified using Nanodrop spectrophotometer (Thermo Scientific). RNA was reverse transcribed (RT) using High Capacity RNA to cDNA kit (Applied Biosystems, AB), with 20 μl reaction volume consisting of 9 μl RNA, 1 μl 20x RT Enzyme Mix and 10 μl 2x RT Buffer. RT settings were: 1 hour 37°C , 5 min 95°C , 10 min 4°C . Resulting cDNA was stored at -20°C .

Real-time quantitative PCR (qPCR) using comparative Ct method ($\Delta\Delta\text{Ct}$) was employed to evaluate gene expression, using validated Taqman probes (AB) as listed in Table 2.5. Reactions were performed in 20 μl (9 μl RT RNA, 10 μl TaqMan Gene Expression Mix

(AB), 1µl 20x probe) in 96 well MicroAmp Fast Optical reaction plates (AB), sealed with Optical Adhesive Film (AB) and run in standard mode on a 7500 Fast Real-time PCR machine (AB). Conditions:

1. 50°C 2min
2. 95°C 10min
3. 95°C 15sec
4. 60°C 1min
5. 40 cycles 3+4

Fluidigm

Prior to sorting, 0.2x Taqman assay mix (probe list below, Table 2.5) was prepared (1µl of each 20x probe used, TE buffer to 100µl total). AML BMNCs were stained and FACS sorted on selected surface marker expression (blast immunophenotyping as per Figure 5.4) into marker negative and positive populations; 3x200 cells/population per donor. Cells were sorted directly into 0.2ml tubes containing 10µl reverse transcription mix (5µl Cells direct 2x Reaction mix [Invitrogen], 0.1µl SUPERase-In RNase Inhibitor [Ambion], 1.2µl TE buffer [Invitrogen], 2.5µl 0.2x Assay mix [AB], 1.2µl SuperScript III RT/Platinum Taq Mix [Invitrogen]; Invitrogen components all part of Cells Direct One-Step qRT-PCR Kit). Tubes were vortexed, spun down (1800rpm for 30 seconds), and pre-amplification RT-PCR reaction performed. Conditions:

1. 50°C 15min
2. 95°C 2min
3. 95°C 15sec
4. 60°C 4min
5. 20 cycles 3+4

Samples were then diluted with 40µl TE buffer and stored at -20°C prior to Fluidigm analysis. Assay mixes (3.0 µl 20x Taqman probe [AB], 3.0 µl 2x Assay Loading Reagent [Biomark Fluidigm] - loaded 5µl) and sample pre-mixes (3.0µl TaqMan Universal PCR Master Mix [AB], 0.3µl 20x GE Sample Loading Reagent [Biomark Fluidigm], 2.7µl cDNA – loaded 5µl) were prepared separately on ice. Sample pre-mixes with cDNA from each sort (3/donor) were prepared and loaded in triplicate onto a dynamic array (Biomark Fluidigm) along with assay mixes and analyzed per manufacturers instructions as previously described (141). Relative gene expression was calculated using comparative Ct method ($\Delta\Delta C_t$); array loading and analysis performed in collaboration with Dr. P. Woll.

Table 2.5: *qRT-PCR Taqman probes (from Applied Biosystems)*

Species	Target Gene	Probe ID
Human	GAPDH	Hs02758991_g1
Human	Atg5	Hs00169468_m1
Human	Atg7	Hs00883770_m1
Human	Atg10	Hs00919721_m1
Human	Atg12	Hs00740818_m1
Human	Becn1	Hs00186838_m1
Human	GABARAP	Hs009258991_g1
Human	GABARAPL1	Hs00740588_mH
Human	GABARAPL2	Hs00371854_m1
Human	MAP1LC3B	Hs00917682_m1
Human	Atg9A	Hs00225393_m1
Mouse	GAPDH	Mm99999915_g1
Mouse	Atg9A	Mm01264429_m1
Mouse	Atg7	Mm00512209_m1
Mouse	Becn1	Mm00517174_m1
Mouse	Atg5	Mm00504340_m1
Mouse	Atg12	Mm00503201_m1
Mouse	Atg10	Mm00470550_m1
Mouse	HPRT	Mm01545399_m1
Mouse	B2M	Mm00437762_m1

2.9 RNA-seq

RNA-sequencing was performed by collaborator Dr. P. Woll, who provided the data for analysis of autophagy pathway genes. Briefly, human bone marrow was sorted for HSC (Lineage⁻CD34⁺CD38⁻CD90⁺CD45RA⁻), GMP (Lineage⁻CD34⁺CD38⁺CD123⁺CD45RA⁺) and MEP (Lineage⁻CD34⁺CD38⁺CD123⁻CD45RA⁻) populations, RNA was extracted and RNA-seq analysis was performed, reads per kilobase per million mapped reads (RPKM) for autophagy genes was calculated using standard techniques (142).

2.10 Colony formation assays

Whole bone marrow and myeloid cell line CFC assays

Murine bone marrow was extracted as above. Cells were suspended at 1×10^6 /ml in RPMI and 400µl was added to 4ml complete methylcellulose (M3434, Stem Cell Technologies). The mixture was homogenized by vortexing and, after bubbles had dissipated, 1.1ml (1×10^5 cells) was plated onto three 35mm dishes (Stem Cell Technologies) using a blunt end needle. Dishes corresponding to each mouse were incubated together in a 100mm culture dish (with uncovered 35mm dish of sterile water providing humidity) for 7 days before lineage colonies were identified and scored by inverted microscope.

For MLL-ENL line colony formation assays, cells were harvested from liquid culture, enumerated and 1.65×10^4 cells were resuspended in 3ml complete media. Suspension was mixed and 300µl (1.65×10^3 cells) was added to 3ml methylcellulose (R&D Systems), which was vortexed, plated 1.0ml/plate (500 cells) in triplicate and cultured as above either in normal conditions (37°C, 5% CO₂, normoxia) or in a hypoxic chamber (37°C, 0.1% O₂) for 3 days prior to colony counts.

Myeloid single-cell clonogenic (Terasaki) assay

Murine bone marrow was extracted from four 6-week-old mice per genotype. Cells were pooled by genotype, 2.0×10^7 cells were resuspended in MACS buffer (PBS, 5% FCS, 2mM EDTA) and enriched for c-kit by bead separation using CD117 Microbeads mouse (Miltenyi) and LS columns (Miltenyi) as per manufacturers instructions. Enriched cells were stained for surface markers as above. 150 HSCs (Lineage-, Sca1+, c-Kit+, CD150+, CD48-) or GMPs (Lineage-, Sca1-, c-Kit+, FcγRII/III+) of each genotype were FACS sorted into 3ml of myeloid growth media (X-vivo 15 media [Lonza], supplemented with 10% FCS, 20mM HEPES, 2mM L-glutamine, penicillin/streptomycin/gentamicin, 100μM 2-mercaptoethanol [all Invitrogen], 2ng/ml ratSCF, 10ng/ml GM-CSF, 5ng/ml hTPO, 10ng/ml hG-CSF, 5ng/ml hFlt3L, 5ng/ml mIL-3 [from collaborator, all available from Peprotech]), which was kept on ice at all times. Media was vortexed to homogenize population and immediately plated 20μl/well in 60-well Terasaki plates (Greiner), mixing prior to each aspiration. Wells were scored for clonal growth by inverted microscope after 10 days culture (plates cultured together in culture boxes with water dishes to protect against evaporation; boxes were placed into incubators and closed after 15 min equilibration), cloning potential assessed against expected number of wells containing 1 cell (37.8 wells by Poisson distribution). GM potential assay as previously published (143) (144), planning, culture and assessment performed with aid of Dr. M. Lutteropp.

2.11 Competitive chimera

Bone marrow cells were extracted as above from an 8-week-old *Vav-Atg5*^{-/-} mouse, wildtype littermate (both CD45.2+) and C57BL/6 mouse expressing CD45.1. Cells were enumerated by trypan blue exclusion and resuspended at 10×10^6 /ml in PBS/5% FCS. 800μl (8×10^6 cells) from either *Vav-Atg5*^{-/-} mouse or littermate control was added to

800µl CD45.1+ cells (1:1 CD45.2+:CD45.1+), resulting mixes were washed three times (resuspended then spun down 500 x g for 5 minutes) in PBS. Mixes were kept on ice and 200µl of each (2×10^6 cells total, 1×10^6 from each donor) was injected intravenously into lethally irradiated C57BL/6 CD45.1+ recipients (split dose irradiation 4 hrs apart, 2 x 450cGy, injections 2 hours after last dose) as rapidly as possible, three hosts per genotype mix. Injections performed with aid of Oxford Biomedical Services technicians. Mice were bled from tail vein at 4-week intervals post-injections to assess engraftment and taken down for bone marrow analysis after 24 weeks.

2.12 Seahorse and metabolic analysis

Cell Tak (BD) plate coating

One day prior to assay, 51.9µl Cell Tak (BD) added to 26.0µl 1M NaOH and 1122.2µl 0.1M NaHCO₃, mixed well and aliquoted 43µl per well to a XF 24-well cell culture microplate (Seahorse Bioscience) under sterile conditions. The plate was let stand 20 min at room temperature, gently washed with 200µl PBS (no Ca⁺ or Mg⁺) (adding then removing 200µl to avoid touching the Cell Tak layer) then carefully aspirated dry. After 1 hour open at room temperature, the plate was closed and stored at 4°C until use.

Seahorse assay

MLL-ENL lines grown in complete media were harvested and enumerated by trypan blue exclusion. 1.5×10^6 cells were line were washed with 3ml unbuffered DMEM (supplemented with 200mM Glutamax [Life Technologies] and 100nM sodium pyruvate [Sigma]) then resuspended in 535µl of DMEM. 100µl (2.8×10^5 cells) of the cell mixes was added to 5 wells of the Cell Tak-coated (as above) microplate. The plate was spun (550rpm for 2 min) and transferred to a non-CO₂ 37°C incubator for 30 min. Following

incubation, 900µl of un-buffered DMEM was added to each experimental well, then exchanged (900µl removed, 900µl added) twice more to fully wash away old media. After the last 900µl wash was removed, 575µl of DMEM was added (to 100µl dead volume still present) for 675µl DMEM/per well. The plate was equilibrated for 30 min at 37°C in a CO₂ free incubator then loaded onto a XF24 Seahorse Analyzer (Seahorse Bioscience), where oxygen consumption rate (OCR) was analyzed with sequential addition of oligomycin (400nM), FCCP (400nM) and rotenone (1µM) as previously described (145). Following analysis, cell protein was extracted with RIPA buffer (Sigma) and quantified as below for well normalization. Seahorse analysis assistance and reagents provided by Prof. M. MacFarlane and Prof. K. Cain (Leicester University).

Enzymatic lactate determination

Enzymatic lactate assessment used the Instruchemie kit (2864) in a 96-well plate. Standards curve was created through 2-fold dilution series (50µl previous standard to 50µl dH₂O) from 4.44mM kit-provided lactate standard to 0.069375mM. MLL-ENL cell cultures (seeded at 0.5×10^5 cells) were counted after 48 hours culture and spun down (2000 x g for 5 min) to harvest cell-free supernatants (supernatants were sometimes frozen at -80°C for a week prior to analysis). Aliquots of culture media (no cells) were also obtained for control purposes. Supernatants were diluted 1:3 in dH₂O and added in duplicate 10µl per well to a 96 well plate flat-bottom plate, standards (created as above) were similarly added 10µl per well. 290µl Lactate-NAD mixture (2ml Lactate Buffer, 0.1ml Lactate LDH reagent, 4ml dH₂O all added to one Lactate NAD reagent bottle [powder], all but dH₂O from kit; made fresh for each to measurement) was added to each well (containing standard, supernatant or media), plate was gently mixed then incubated 15 min at 37°C prior to absorbance measurement at 340nm. Lactate concentration was

calculated from standard curve, normalized to media alone and divided by final cell count ($\times 10^5$ cells) to give lactate production.

2.13 Immunoblotting

Protein extraction

Protein from cell pellets ($1-3 \times 10^5$ cells per condition) was extracted using RIPA buffer (Sigma) as per instructions. Briefly, pellets were washed with 1ml PBS, transferred to 1.5 microcentrifuge tubes and spun down (2000 rpm, 5 min, 4°C). Cells were resuspended in 100µl of RIPA buffer (Sigma), supplemented with 1µl 100x Protease/Phosphatase Inhibitor Cocktail (Cell Signaling), vortexed and incubated on ice for 30 min. Lysates were spun at 13,000rpm for 15 min to pellet cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube via small gauge needle to shear DNA. Protein content was assayed using BCA Protein Assay Kit (Thermo Scientific) as per manufacturers instructions, diluted in RIPA buffer to lowest sample content and stored at -20°C until analysis.

Polyacrylamide gel electrophoresis

20µl samples were mixed with 10µl NuPage buffer (7.5µl NuPage LDS Sample buffer, 3µl NuPage Reducing Reagent) and boiled at 95°C for 10min before 20µl was loaded onto NuPAGE 4-12% BIS-Tris gel (Invitrogen), along with 15µl of PageRuler Prestained NIR Ladder (Thermo Scientific) and run at 200V in 1x NuPage MES SDS Running Buffer (Invitrogen).

Western/Immunoblotting

Proteins separated by electrophoresis were transferred onto PVDF membranes (GE Healthcare; prepared prior to transfer by 1 min immersion in methanol, wash in dH₂O then soaking in transfer buffer) using XCell SureLock wet transfer module (Invitrogen) in 1x NuPage transfer buffer (Invitrogen) for 40 min at 30V. Membranes were then blocked with Licor Odyssey Blocking buffer (Licor) for 2 hours at room temperature in the dark. Following blocking, membranes were washed in 0.2%Tween/PBS (Sigma) then incubated with primary antibody (made up in 50% blocking buffer, 50% 0.2%Tween/PBS) overnight at 4°C on a shaker (in dark). The membrane was washed 3 times in 0.2%Tween/PBS before 2-hour room temperature (dark on shaker) incubation with secondary antibodies (1:20,000 goat anti-rabbit IRDye 800 [Licor] or goat anti-mouse IRDye 680LT [Licor] in 20ml 0.2%Tween/PBS with 40µl 20% SDS solution). Membranes were washed twice more in 0.2%Tween/PBS, once in PBS then rinsed with dH₂O prior to detection on Odyssey CLx Infrared Imaging System [Licor]. Primary antibodies were rabbit anti-Atg5 (Novus), mouse anti-tubulin (Cell signaling), rabbit anti-LC3 (Sigma) and mouse-anti-GAPDH (Millipore).

2.14 Immunohistochemistry and Histology*p62 immunohistochemistry*

Paraffin embedded, mounted AML bone marrow trephine sections and healthy controls were obtained with the help of Dr. Elizabeth Soilleux (NDCLS). Slides were deparaffinised and rehydrated via incubation in (5 min each) citrocLEAR I, citrocLEAR II (both TCS Biosciences), 100% ethanol I, 100% ethanol II, 50% ethanol and dH₂O, before being transferred to boiling 1x Dako Target Retrieval Solution (Dako UK) and microwaved (15

minutes medium/low). The slides were left to cool for 10 min in PBS then stained for p62 as per the Dako EnVision+ System (Dako EnVision+ Dual Link System HRP DAB+ml) kit instructions. For negative control, primary rabbit anti-human p62 (Enzo Life Sciences; 1:800 in PBS/2% horse serum) was incubated one hour on ice with 400x molar excess blocking peptide (a.a 387-436 of human p62, sequence H-CPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSKH-OH, gift from Enzo Life Sciences). Following staining, slides were washed with dH₂O (gently poured/squirted above section to run over specimen), counterstained with 100µl hemotoxylin (Sigma) for 30 seconds before washing again with dH₂O. Slides were then washed five times with deionized water (in vertical holder so fully submerged, adding fresh water then dumping out) to 'blue' samples, before being mounted with cover slip using Immu-Mount (Thermo Scientific). Slides were dried overnight at 4°C in dark before imaging. Technique now in press (146), thanks to Dr. E. Soilleux for analysis and image provision.

Histology

Major organs from 6 week old *Vav-Atg5*^{-/-} mice and wildtype littermates (two per genotype) were examined macroscopically, harvested and whole organs fixed in 4% w/v paraformaldehyde before delivery to Nuffield Department of Pathology (John Radcliffe Hospital, Oxford, OX3 9DS) who performed paraffin-embedding, sectioning and H&E staining. Thanks to thanks to Dr. E. Soilleux for analysis and image provision.

2.15 Electron microscopy

MLL-ENL lines grown in complete media were pelleted and directly resuspended in 4% glutaraldehyde for fixation. Pellets were processed for, and analyzed by, transmission

electron microscopy by Prof. D. Ferguson of the Nuffield Department of Pathology as previously described (147).

2.16 Bone marrow cytospins

Filter cards were moistened with PBS prior to spinning. 5×10^3 BM cells from 6-week-old *VavAtg5*^{-/-} mice or wildtype littermates were resuspended in 200 μ l of PBS/20% FCS and pipetted onto the filter cards then centrifuged with low acceleration, 1000 rpm for 10 min before drying overnight. Slides were soaked in May-Grünwald (Sigma) for 5 minutes, washed with deionized water, before being transferred to Giemsa (Sigma) for 20 minutes and washed with deionized water again. Slides were then left to dry prior to mounting with Pertex. Slides were inspected with an un-inverted microscope and 100 cells per slide were morphologically scored as: neutrophil, immature myeloid, lymphocyte, erythrocyte.

Thanks to L. Stenson for help performing the cytospin and Dr. O. Chowdhury for assistance with cytospin assessment.

2.17 Statistics

Statistics were calculated using GraphPad Prism 6 for Macintosh, version 6.0a (GraphPad Software Inc.). Specific test and significance values for each experiment are provided in figure legends.

Chapter 3: Increased Autophagy in Immature Hematopoietic Progenitors

Introduction

As a pathway important in cell development, survival and damage prevention, autophagy has multiple potential roles in stem cells. Studies examining mammalian autophagy have been conducted in immortalized cell lines *in-vitro*, conditions which work well for traditional methods of quantifying autophagy, such as electron microscopy (EM), western blot and fluorescent microscopy. However, hematopoietic studies and models are predominantly *in-vivo* based and involve rare progenitor populations defined by surface phenotypes, factors that have complicated investigation of autophagy in HSCs. The evidence for autophagy's importance to stem cells, in particular HSCs, will be presented and the existing techniques for measuring autophagy briefly reviewed.

3.1 Stem cell properties

Stem cells are immature, non-specialized cells that can differentiate to form the many mature cells of the body. Although stem cells vary with developmental stage and tissue type (148), they are characterized by certain general properties, in particular: i) multipotency, the capacity to form multiple cell types and morphologies, ii) self-renewal, the ability to undergo long-term self-replication, in some cases life-long, and related to that, iii) quiescence, meaning periods of reversible cell-cycle arrest in G0 (149).

Autophagy's functions are hypothesized to be highly important for these stem cell characteristics, and indeed there exists evidence, direct and indirect, of such a role.

3.2 Autophagy in differentiation and development

In the change from a non-specialized stem cell to a mature, functionally specialized cell, significant changes must be made to cellular structure and function, requiring degradation of existing cellular bodies and availability of metabolites for biosynthesis (150).

Autophagy plays an important part in controlling the turnover of proteins and the degradation of existing cellular structures (151), for example during cytoskeletal remodeling (152). It might therefore be expected that loss of autophagy would delay or arrest full differentiation, and indeed maturation of erythrocytes has been found by the host lab and others to be severely inhibited following removal of core autophagy genes *Atg7* (101) (153) and *Ulk1* (154) or mitophagy adaptor *Nix/Bnip3l* (155), due to delayed mitochondrial clearance leading to accumulation of damaged mitochondria, cell death and severe anemia. Similar lineage specific defects in differentiation and survival have been found in other hematopoietic cell types, including T- and B-lymphocytes (156) (157) along with myeloid cells (158), as well as a variety of other cell types including adipocytes (159), osteoclasts (160), keratinocytes (161) and nervous tissue (162, 163) (102) (for an excellent review of autophagy in differentiation and development, see (6)). Moreover, there is evidence autophagy is specifically upregulated by transcriptional regulators of differentiation, for example the key hematopoietic transcription factor GATA-1 directly activates the transcription of Atg8 homologs *Map1lc3b*, *Map1lc3a*, *Gabarap*, *Gabarapl1* and *Gabarapl2*, as well as indirectly driving several others through FOXO3 activation, during erythroid maturation (58).

Such differentiation steps are often temporally or spatially removed from stem cells, but autophagy is also regulated during the initial steps of stem cell differentiation. In embryogenesis, embryonic stem cells (ES cells) lacking ATG5 arrest at the 4 to 8 cell stage, a stage where autophagy was observed to be transiently upregulated in wild type counterparts (164), and autophagy genes have been separately reported as required for embryo cavitation in-vitro (165). Similarly, autophagy is upregulated and required at early stages of neuronal development (166). Interestingly however, mouse models lacking key autophagy genes, including *Atg7* (167) and *Atg5* (168), are still viable at birth due to

maternally inherited oocyte ATG protein (164), although they typically display lower birth weight and die soon after in the neonatal starvation period (169). An exception is the *Becn1* knockout, which displays defective ESC autophagy and a severe embryonic defect (170), suggesting that the BECN1 protein may be involved in ESC *in vivo* development events independent of the core autophagy machinery. However, other early acting autophagy genes such as BECN1 binding partner AMBRA1 (66) and initiation complex member FIP200 (also known as RB1CC1) (171) also display early ESC developmental defects, therefore it may be that the early, autophagosome-initiating autophagy protein complexes are linked to phenotype severity (6).

3.3 Autophagy in self-renewal and maintenance of multi/pluri-potency

The studies outlined above suggest autophagy's importance to stem cells during maturation, but it also appears to play an important role in the maintenance of the stem cell population itself. Long-term stem cell self-renewal is vital for tissue homeostasis, balancing out loss via differentiation or death. Although the pathways mediating self-renewal in HSCs are relatively unknown, as an opposing force to stem cell maturation it is no surprise that controlling levels of stem cell maintenance- (ex: Hox genes (172) (173)) or lineage-driving gene transcription is vital (123, 174) (120) (more fully reviewed in (174)). As such, transcription factors, for example Gfi1 (175) and transcriptional activators, such as Cbp (176), impact HSC fate decisions. Similarly, chromatin remodeler BMI1 is known to be involved through controlling expression of cell cycle modulators p16INK4a and p19ARF (177) along with mitochondrial function (178).

What is also known is that self-renewal requires the ability to control and/or respond to chronic stress; failure leads to tissue damage and potential transformation (179).

Moreover, cellular damage triggers checkpoints that limit self-renewal and force

differentiation (180). Therefore external insults, for example cytotoxic agents or irradiation (181) (182) or internal stressors, including metabolic byproducts such as unfolded proteins and reactive oxygen species (ROS), have been shown to initiate differentiation and loss of self-renewal in stem cells (183) (184) (185). Indeed, self-renewal ability in hematopoietic stem cells segregates on ROS levels even under steady-state conditions (186) and replicative stress following serial transplantation triggers oxidative DNA damage, HSC specific expression of cell cycle inhibitors p16INK4a, p14ARF (the human p19arf homolog) and p21CIP1, premature senescence and loss of HSC self-renewal (187). In a similar fashion, insults such as irradiation lead to loss of HSC function through chronic oxidative stress, mediated by NADPH oxidase activity, and senescence, visualized via senescence associated B-gal and p16 expression (188-190).

To reduce these deleterious impacts of stress on function, stem cells display a metabolic state that limits potentially damaging waste. Mitochondria are a primary producer of cellular ROS (9) as a byproduct of their efficient ATP-generating electron transport chain (191), and excess ROS can lead to multiple deleterious effects on the cell, including macromolecular and genomic damage such as point mutations (192) and double strand breaks (193), particularly dangerous to HSCs as they preferentially employ the error prone non-homologous end-joining DNA repair mechanism when quiescent (194). To counterbalance/avoid this risk, stem cells have reduced mitochondrial activity, either through reduced mitochondrial content (195), as in ESCs and iPS cells (196), or through active uncoupling of mitochondrial potential via the UCP proteins, seen in both ESC (197) and HSCs, where re-coupling is required for later differentiation (198). Instead, both ESCs (199) and HSCs (200) rely on aerobic glycolysis, that is conversion of glucose to lactate in the presence of oxygen, to meet energy requirements. In the past, the quiescent and glycolytic metabolic state of HSCs was hypothesized to be supported by a hypoxic stem

cell niche. This is underpinned by LT-HSC staining for the hypoxic marker pimonidazole and the importance of key hypoxia (and autophagy regulating) response factor hypoxia-inducible factor 1 α (HIF1 α) for HSC maintenance (201). However, while recent evidence suggests bone marrow space is generally hypoxic (202), the hypoxic profile of HSCs does not appear to segregate with vascular proximity, therefore this evidence has been argued to reflect cell autonomous metabolism, rather than the unique microenvironment (107, 203).

Autophagy provides a primary cellular response to many forms of cellular stress, removing damaged macromolecules along with potential sources of toxic byproducts (204), and may form another metabolic adaption in stem cells. Indeed, autophagic removal of damaged mitochondria is vital for maintaining ESC self-renewal (195) while autophagy is required for the irradiation resistance of mesenchymal stem cell (MSC) (205) and HSC response to cytotoxic agents (181). Moreover, increased constitutive autophagy has been observed in certain stem cell types, including MSCs (206), and, as discussed in more detail in Chapter 4 of this thesis, deletion of autophagy genes *FIP200* (207) or *Atg7* (131) in HSCs results in loss of HSC self-renewal, accompanied by increased numbers of damaged mitochondria, ROS and DNA damage. Accumulating damage and loss of self-renewal in stem cells, including HSCs (208), is an important part of organismal aging. Intriguingly, levels of autophagy have been shown to fall with age in multiple cell types, including hematopoietic cells (140). Moreover, old HSCs have increased levels of active mTOR. mTOR over activation in young HSCs increased CDK inhibitors p16INK4a, p19ARF and p21CIP1 (209), while mTOR pathway inhibiting, therefore potentially autophagy inducing, treatments have been demonstrated to extend lifespan (210) and rejuvenate aged HSCs (209).

In a complementary fashion, the acquisition of self-renewal and pluripotency, as seen in iPS cell generation, has been found to be supported by mTOR pathway inhibition (211) and importantly this has been linked to a requirement for autophagy activation, mediated by Sox2 downregulation of mTOR itself (212). However, contradictory reports state that autophagic removal of mid-bodies, organelles formed during cell division, may reduce iPS reprogramming (213), therefore autophagy's role may be setting specific.

3.4 Autophagy in quiescence

A final important, and related, property of adult stem cells is the ability to undergo lengthy periods of reversible cell cycle exit, or quiescence, in order to preserve long-term proliferative capacity and health (110). In murine HSCs, there is a transition from close to 100% cycling cells in the fetal liver until 3 weeks of age to 50% or more of the population quiescent from 4 weeks and after (214, 215), with analogous changes in other species (148). Moreover, normal HSCs in the actively cycling cell cycle phases (S/G2/M) displayed engraftment defects, demonstrating cell cycle regulation is important for short term HSC function as well as long-term maintenance (214). Quiescent HSCs form a reserve to repopulate MPPs following their proliferation and depletion in response to blood loss, infection or other stressor stimuli (216).

Control of cellular quiescence is not fully understood but appears multifactorial: external factors provided by the stem cell niche and microenvironment are important in multiple stem cell types, including (receptor/ligand) Notch/Jagged-1 signaling in muscle stem cells (217) and HSCs (218), Mpl/Thrombopoietin (111, 215), Tie2/Angiopoietin-1 (219), c-Kit/SCF (108), Wnt (220) and TGF- β (112) signaling for HSCs, therefore the particular niche (endosteal/osteoblastic or vascular) location of HSCs may regulate cell cycle status (109). Internally, it comes as no surprise that cell cycle regulators are crucial, with

knockout of multiple cyclin-cdk complex proteins, such as D-cyclins (221), or inhibitors, including p57CDKN1c, (222) through cooperation with p27CDKN1b (223), p21CIP1 (224), p18INK4c (225), p16INK4a (226), p14ARF (murine equivalent being p19ARF) (187) and the RB family proteins (227), along with cell cycle regulator p53 (228) causing HSC engraftment defects and altered proliferation (229) (230).

As in self-renewal, metabolic factors appear crucial, with deletion of glycolysis regulator *HIF1A* promoting decreased cell cycle quiescence while HIF1 α accumulation induced quiescence (201). Loss of liver kinase B1 (LKB1), an important regulator of AMPK and mTOR pathways, has similarly been proven to critically impair HSC quiescence in a cell autonomous and oxidative stress independent manner (231-233). However, as with self-renewal, oxidative stress signaling is indeed important for quiescence, as deletion of oxidative stress response regulators *FOXO1*, *FOXO3* and *FOXO4* caused increased HSC cycling (234). Finally, the cell growth, cell cycle (235) and glycolytic metabolism (236) regulating Akt-mTOR pathway has an important role in HSC quiescence regulation: constitutive Akt signaling (237), therefore increased mTOR activity, or loss of negative pathway regulators TSC1 (238) or PTEN (239), which also lead to activated mTOR, all increase HSC cycling. By contrast, loss of mTORC1 did not impact HSCs but impaired later lineage differentiation of myeloid cells and lymphocytes (240). Importantly, these pathways, from HIF1 α to mTOR, all regulate autophagy and, although not directly implicated by the authors, their quiescence-maintaining signaling states would upregulate autophagy. Indeed, a survey by Cheung and Rando compared quiescent stem cell gene expression across multiple stem cell types and, of 49 upregulated genes in a ‘quiescent signature’, they identified five genes in the autophagy pathway, namely *Ulk2*, mitophagy factor *Pink1*, Atg8 homologs *Gabarapl1* and *Map1lc3a* as well as the transcription factor *Foxo3* (110).

A summary of the important factors controlling HSC/stem cell quiescence, self-renewal and differentiation is outlined in Figure 3.1, along with current links to autophagy.

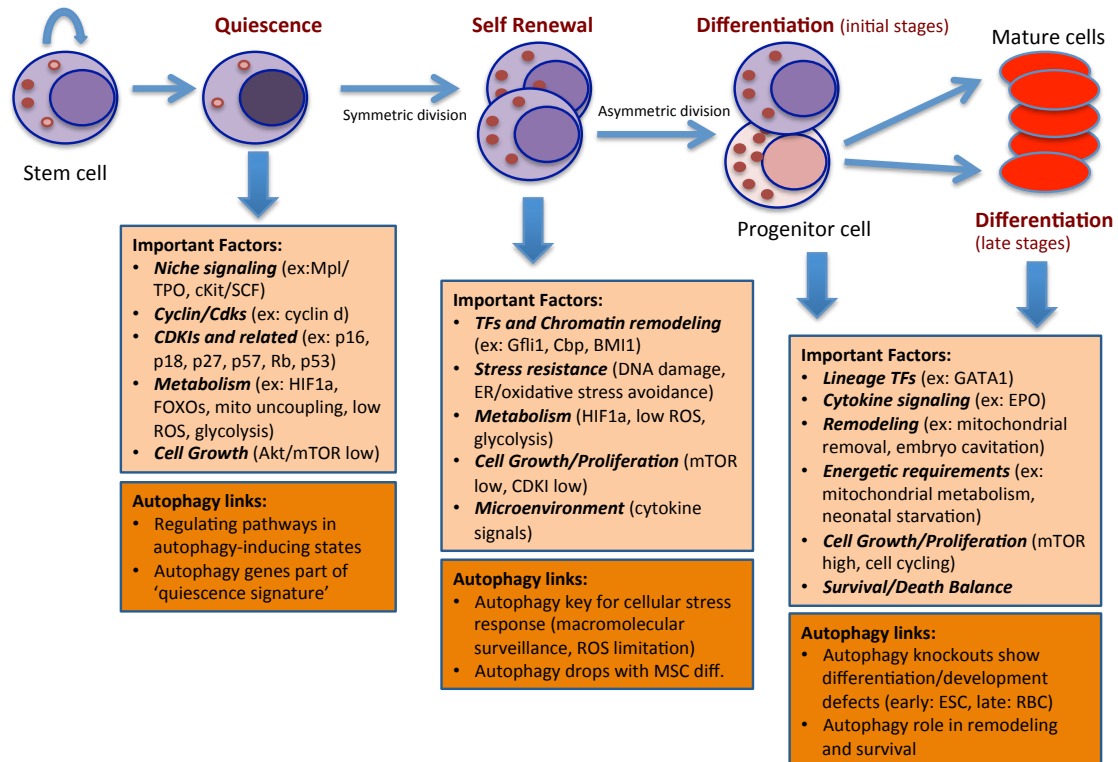


Figure 3.1: Stem cell properties and autophagy. Abbreviated summary of important factors controlling stem cell quiescence, self-renewal and differentiation, with a focus on HSCs, see text for details.

3.5 Autophagy in HSCs – status of knowledge

These data connecting autophagy itself, or regulating pathways, to stem cell differentiation, self-renewal and quiescence is suggestive of a requirement for upregulated autophagy in HSCs, however when this thesis project began there were extremely few studies, and no robust measurements, of autophagy in HSCs (169). This is surprising for perhaps the best studied body stem cell, benefiting from ease of access to primary tissues (blood and bone marrow) and defined functional in-vitro and in-vivo assays (241). Recent years have seen multiple reports of stem cell markers in several different tissue types (242-

244), including surface phenotypes of hematopoietic progenitor populations (245) and HSCs themselves (246) allowing identification of these populations in tissue samples/sections and leading to leaps in understanding. However, classical methods of autophagy quantification have not allowed optimal use of these advances (140).

3.6 Classical techniques for autophagy detection

There now exist a myriad of techniques for detection of the presence and level of autophagy within cells, each with their own advantages and potential pitfalls (247).

Autophagy was originally identified in the late 1950s by transmission electron microscopy (TEM), where autophagosomes appear as double membraned bodies encircling cytoplasmic contents, and quantification of autophagosomes within cells via TEM remains a respected assay within the field (248, 249). Similarly, autophagosomes can be visualized by fluorescence or confocal microscopy, most often via staining for lipid-conjugated Atg8 (most often the MAP1LC3B, or simply LC3, homolog in mammals) or LC3-II associated with the autophagosome membrane, where it acquires a punctate appearance to differentiate it from cytosolic, or non-conjugated, LC3-I. A related technique utilizes systems where LC3 is GFP conjugated, allowing autophagosome identification in cell lines, even human ESCs (250), and in-vivo systems (139), although such systems carry a risk of GFP aggregates misrepresenting LC3-puncta, particularly if overexpressed (251). In microscopy, numbers of autophagosomes or LC3-puncta per cell is used as a readout of increased autophagy, however a challenge when using such autophagosome-quantifying techniques is differentiating between autophagy induction, and therefore increased autophagosomes and autolysosomes, and late-stage inhibition of autophagy (ex: lysosomal defects or shortage), where autophagosomes are unable to fuse with lysosomes and can accumulate (249). To better measure autophagic activity, autophagosomes can be counted

with or without addition of pharmacological lysosome inhibitors, with the resulting accumulation of autophagosomes representing the rate of autophagy, or autophagic flux (249).

Using LC3 lipidation as a marker of autophagosome formation is also common by biochemical means. A standard assay for autophagy is immuno-, or Western-, blotting for LC3 in protein lysates run on SDS-PAGE gels, where LC3II migrates faster than LC3I due to its lipid-acquired hydrophobicity (despite the additional weight of the phosphatidylethanolamine tag) and is largely representative of the amount of autophagic membrane (or autophagosomes) formed (252). However, it seems that some Atg8 lipidation is possible even when key autophagy components are missing (253), and similar concerns with regards to late autophagic defect-related autophagosome accumulation exist with LC3-II as with microscopic LC3-puncta detection. Figure 3.2 shows how late stage inhibition of can result in deceptive autophagy readouts. The latter concern can be addressed by ‘autophagic flux’ assays, for example pharmacological lysosomal inhibition, where the ratio of LC3II after treatment over control levels represents LC3-II turnover, and therefore the level of ‘flow’, or flux, through the autophagy ‘pipeline’ (254) (247). An alternative biochemical method involves measurement of autophagic substrate degradation, and the autophagy adaptor p62 is the best-used example. p62 selectively targets cargo to autophagosomes via LC3 binding and is degraded along with its target, meaning cellular levels of p62 inversely correlate to autophagy levels (255). However, a confounding factor with this assay can be transcriptional changes to p62 (22). Indeed care must be taken in the interpretation of all the above assays and, where possible, the comparison of multiple readouts is best. (247).

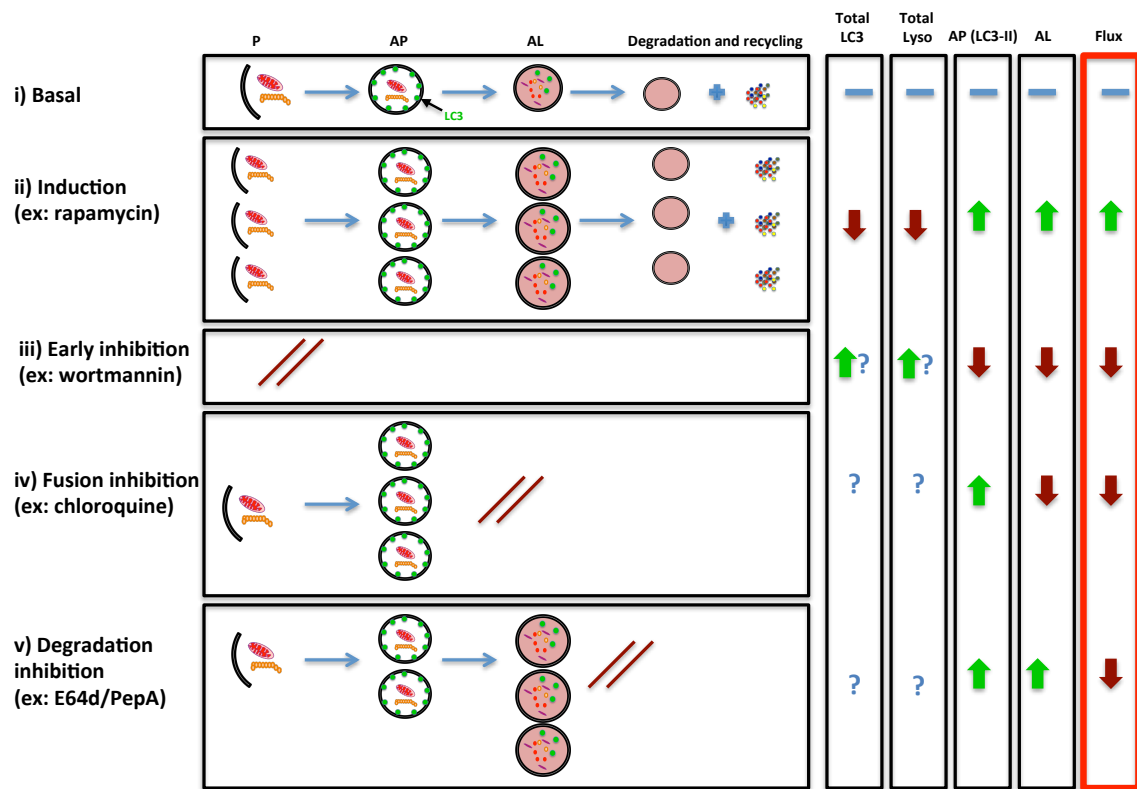


Figure 3.2: Impacts of autophagy modulation on marker abundance. Diagram indicates the impact of different autophagy modulating treatments on the amount of phagophore (P) formation, autophagosome formation (AP, marked by lipid-conjugated LC3-II or punctate LC3), autolysosome numbers (AL, where LC3 and lysosomal markers will colocalize early on), and completion of autophagic degradation, with metabolite liberation and recycling of lysosomal enzymes, relative to basal conditions. Impacts on total cellular LC3 and lysosome levels are also shown where consistent, a question mark (?) indicates lack of LC3/lysosome use/consumption, where cellular synthesis rate will determine whether marker builds up. Flux indicates actual amount of autophagic degradation ('flow' of cargo from phagophore to degradation). Adapted from (249).

3.7 The HSC challenge and flow based assays

The major challenge in the application of classical autophagy techniques to the study of HSCs is the rarity of the population. Thanks to the identification of surface markers, HSC or progenitor isolation via FACS or bead-based cell-sorting is possible, but accumulating the homogenous cell numbers necessary for microscopic or biochemical methods is extremely challenging without *in vitro* culture or xenografting, both of which, along with

sorting itself, potentially alter physiological autophagy levels through substantial changes in stress and growth factor signaling (140, 149, 247). As a result, the recent development of FACS based autophagy readouts provides promise of high-throughput methods that can utilize the hematopoietic system's best assets of availability and surface phenotypes. As of the time of this thesis however, these assays have been applied using single-channel intensity alone, for example using lysosomal dyes, which is not recognized as a reliable indicator within the field (249), or else have used an LC3-GFP transgenic cell line (256) (257), whereas detection of endogenous protein is needed for examination of primary human samples.

Results

3.8 Outline of a novel high throughput Imaging Flow Cytometry

Autophagy Assay (IFCAA)

In order to overcome the challenges facing autophagic detection in hematopoietic stem cells (HSCs) and early progenitor populations when using classical techniques such as microscopy or immunoblotting, an assay would need to (i) incorporate surface marker definitions of HSCs and progenitor populations (246) (245), ideally by flow cytometry to take advantage of suspension populations, allow for large samples sizes and adequate HSC numbers for unbiased assessment without sorting or manipulation (247) (149) (ii) utilize endogenous protein rather than transgenic constructs, allowing application to both varied murine settings and human samples (iii) be able to distinguish between late-stage autophagic impairment and induced autophagic degradation, through measurement of functional autophagy at a late stage or by using lysosomal inhibitors for a ‘flux’ based assay (249) (247). The latter is particularly important in flow cytometry based assays because, as above, previous single-readout intensity-based assays detecting autophagosomes (via fluorophore-labeled antibody staining for endogenous LC3, note that there is currently no antibody which detects LC3-II alone and classical flow cytometry cannot detect puncta) or lysosomes (using antibodies against lysosomal proteins or acidotropic lysosomal dyes), where a decrease in LC3 or lysosomal volume is interpreted as increased autophagic degradation (ie. inversely correlative) (257) (249), have been found unreliable in isolation (254) (247) (249) (also in unpublished data from host lab).

Imaging flow cytometry provides a means of combining the benefits of flow cytometry for hematopoietic study with the better-characterized fluorescence microscopy readouts for autophagic activity (247). Image Stream is a multi-spectral imaging flow cytometer capable of detecting either 6 channels (Image Stream 100) or 12 channels (Image Stream X) of per-cell fluorescence imagery (two of which are brightfield and darkfield images), analyzed on the custom image analysis IDEAS software to permit objective assessment of immunofluorescence and morphological patterns within cells. As the localization of LC3 to autophagosome membranes, the re-positioning of lysosomes to common sub-cellular areas (86) and the delivery of autophagosomal LC3 to lysosomes (ie. vesicle fusion to create autolysosomes) are three hallmarks of autophagic degradation (249), the colocalization of LC3 and lysosomal fluorescence signals in single cells was determined to be a prospective method of autophagic detection (140), able to reflect autophagic degradation (flux) under most situations (Figure 3.2).

Therefore, the Image Stream was employed to measure LC3/Lysosomal colocalization within a surface-marker defined cell population; this novel assay is termed hereafter the Imaging Flow Cytometry Autophagy Assay (IFCAA) (Figure 3.3). Endogenous LC3 was detected by anti-LC3 polyclonal antibody/fluorophore-conjugated secondary labeling and lysosomal content detected using the dye LysoID, which is characterized to localize to vesicles positive for late-endosome/lysosome markers (258). LysoID does not display the photoconversion or emission artifacts found in other lysosomal dyes (259). Samples were stained with LysoID, a viability dye (note that all IFCAA autophagy measurements represent live-cells) and any antibodies against surface markers before being rapidly fixed and stained for intracellular LC3 before being acquired on the Image Stream. Following population selection using surface markers (not shown), cells positive for both LysoID and LC3, a population found to generally increase in frequency following autophagy induction

(140), were gated on in order to avoid inaccurate colocalization values due to minimal intensity of one or both signals within certain cells (Figure 3.3). LC3/LysolID BDS (Bright Detail Similarity, or colocalization) values calculated for individual cells are expressed as a histogram for a selected population (Figure 3.3). To compare between populations, either the percentage of cells with a BDS higher than a control-defined cutoff (ie. % cell gated BDS hi), which was more sensitive to autophagy modulation in large populations, or the median BDS of the population were employed, with the latter required where population rarity meant a frequency-based measurement was vulnerable to outliers.

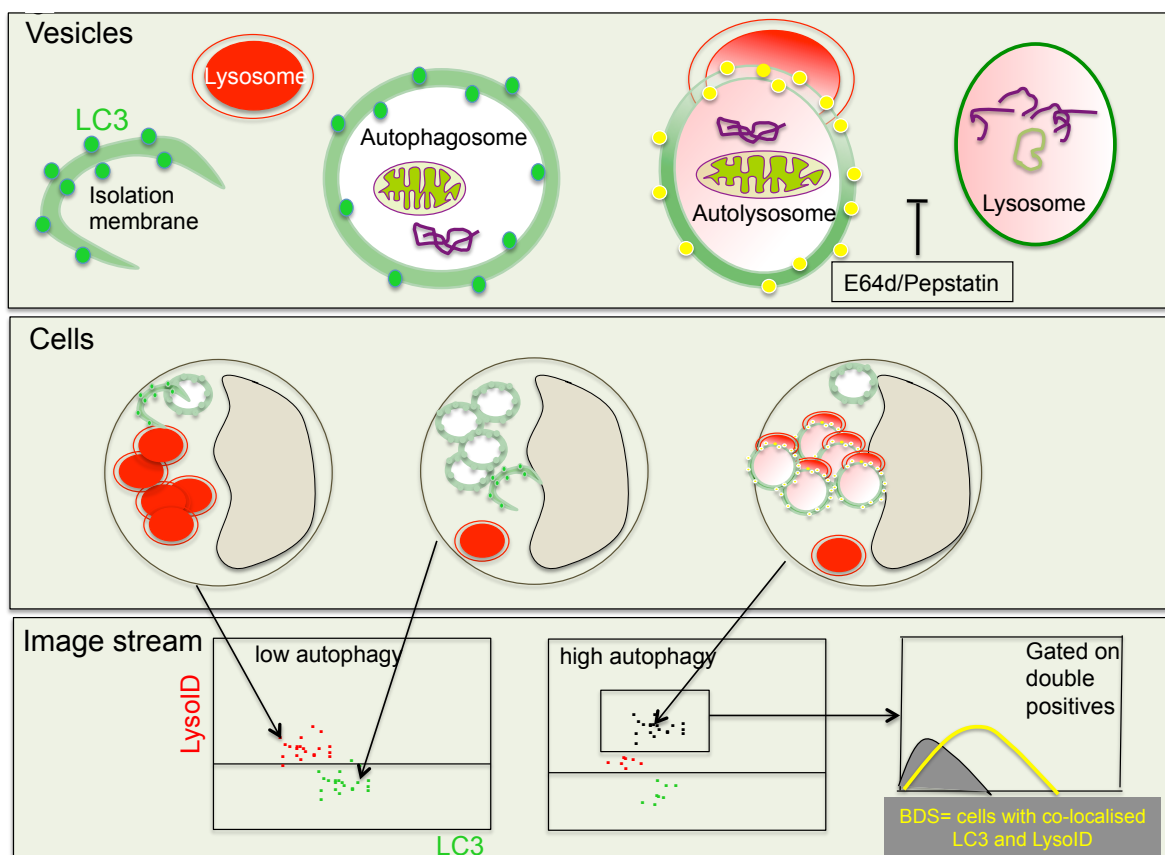


Figure 3.3: Design of the Imaging Flow Cytometry Autophagy Assay (IFCAA). Upper panel: outline of the vesicles involved in autophagic degradation, yellow shows LC3 and lysosome signal colocalization, stage where lysosomal inhibitors E64d/Pepstatin A arrest autophagic flux indicated. Middle panel: cells representing three staining populations based on vesicle abundance. Lower panel: schematic of LC3/LysolID double positive gating and histogram BDS (Bright Detail Similarity; LC3 and LysolID colocalization) readout for the population.

This readout was combined, where possible, with the culture of target cells for two hours with the membrane permeable cathepsin inhibitors E64d (inhibits cysteine proteases cathepsin B, H, L and calpain) (260) (261) (262) and Pepstatin A (inhibits aspartic proteases cathepsin D and E) (263) (264) (265) (E64d/Pepstatin A treatment indicated on figures in later chapters as ‘+I’). These compounds impair lysosomal degradation, meaning degradation of LC3-II following autophagosome-lysosome fusion is slowed (254) (247), resulting in increased LC3/LysoID colocalization as a function of the amount of LC3 susceptible to lysosomal degradation (ie. autophagosome/lysosome fusion) within the treatment period, therefore controlling for autophagic/lysosomal defects. Direct protease inhibitors were used for this purpose, rather than the lysosomal-condition altering inhibitors Bafilomycin A1 and Chloroquine, as the impact of the latter compounds on autophagosome/lysosome fusion is under debate (266) (267). Full details of analysis and design are in Materials and Methods and published here (140).

3.9 LC3/LysoID colocalization responds more robustly than intensity alone to pharmacological autophagy inducers and inhibitors in KG1a myeloid leukemic cells

To test the LC3/LysoID BDS response to characterized pharmacological autophagy modulators that impact the autophagy pathway at various stages, KG1a hematopoietic cells were cultured for two hours with either early acting autophagy inducer rapamycin, early inhibitor wortmannin, autophagosome-lysosome fusion inhibitor chloroquine or lysosomal enzyme inhibitors E64d/Pepstatin A. Rapamycin acts at beginning of the autophagy pathway, inhibiting mTOR activity (268) (269) and therefore relieving mTOR-mediated negative regulation of the autophagy initiation complexes, resulting in increased autophagic activity (247). By contrast, autophagy inhibitor wortmannin interferes with the

initiation complexes, inhibiting the activity of the class III PI3K required for autophagosome nucleation and progression (270). Lysosomal inhibitors act at a late stage of autophagy, and while all ultimately interfere with autophagic degradation their precise impact on vesicle dynamics varies. Chloroquine interferes with the fusion of autophagosomes to lysosomes, therefore preventing the delivery of autophagic cargo for degradation (271) (267). This differs from E64d and Pepstatin A which, as described above, permit autophagosome/lysosome fusion but inhibit the activity of proteases within lysosomes (260) (261) (262) (263) (264) (265) and therefore create a buildup of fused, but undegraded, autolysosomes, (and lipidated LC3) by acting at a late stage in the autophagy process (254).

KG1a showed strong staining of LC3 and LysoID on the Image Stream; representative images are shown in Figure 3.4. Note that increased colocalization between the LC3 and LysoID channels was often accompanied by the collection of these signals in a common sub-cellular location, particularly in larger cell lines studied, and indeed larger cells with larger cytoplasm were in general more amenable to the IFCAA. Chloroquine treatment resulted in decreased overall LC3/LysoID BDS per cell compared to untreated (basal) levels (Figure 3.4). Rapamycin treatment resulted in increased population BDS, either by median BDS for the population or using the frequency of cells with a BDS higher than a cutoff gate (% gated BDS_{hi}) defined by the negative control (chloroquine) histogram (Figure 3.4). When quantified for multiple independent cultures, rapamycin treatment caused a significant increase in the % cells BDS_{hi}, as did inhibiting autolysosome progression with E64d/Pepstatin A treatment. Inhibiting autophagy initiation with wortmannin caused a significant decrease in % cell BDS_{hi}, as did treatment with chloroquine, supporting the ability of BDS to distinguish alterations in the fusion of the autophagosome and lysosome populations (Figure 3.5).

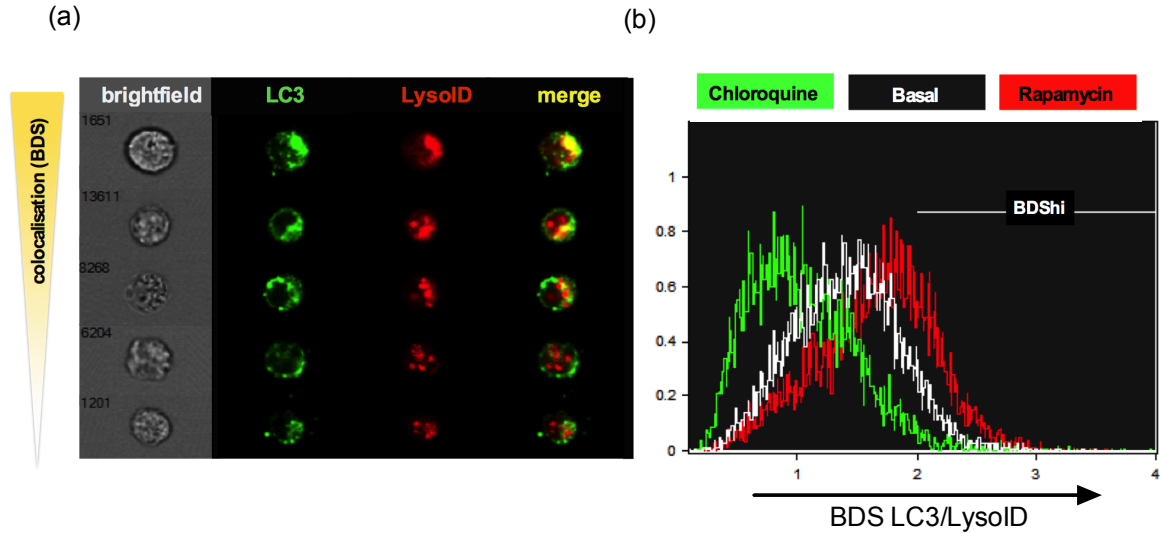


Figure 3.4: LC3 and LysoID colocalization in KG1a cells. (a) Representative images of KG1a cells from Image Stream (10,000 analyzed per sample), individual scaling was used for example display images. (b) Representative histograms of LC3 and LysoID colocalization (Bright Detail Similarity, BDS) in KG1a cells cultured in growth medium alone (basal) or treated with 1 μ M rapamycin or 20 μ M chloroquine for 2 hours, gated on LC3/LysoID double positive cells (n=3 cultures/condition, representative of two independent experiments).

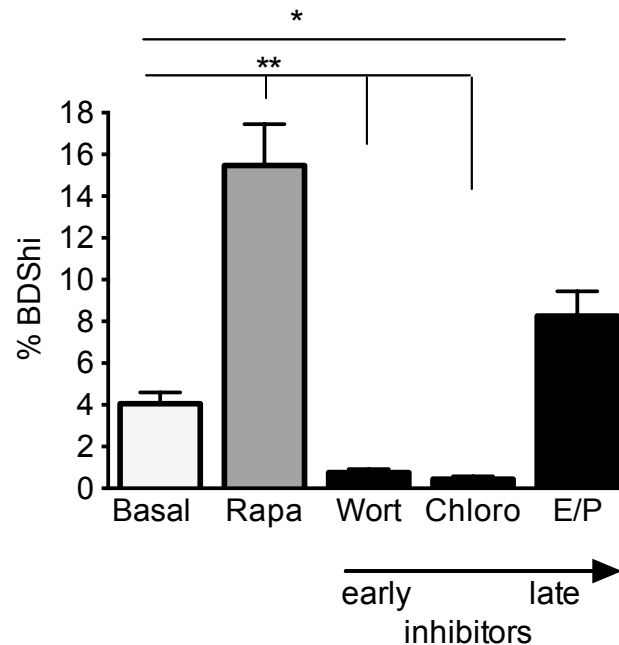


Figure 3.5: IFCAA responds to pharmacological modulators of autophagy in a hematopoietic cell line. Frequency of KG1a cells with high LC3/LysoID BDS under basal conditions or following 2 hour treatment with 1 μ M rapamycin (Rapa), 100nM wortmannin, 20 μ M chloroquine or 10 μ g/ml E64d/Pepstatin A, gated on LC3/LysoID double positive cells. Error bars represent SEM (n=3), * p <0.05, ** p <0.01, unpaired t test.

Previous flow cytometry assays have employed intensity of LC3 or LysoID alone as a readout for autophagy, measuring the consumption of the labeled compartments by autophagic degradation, although such approaches have been questioned in the field (247) (249). The Image Stream also collects mean intensity per cell data for each fluorescence channel, and the changes in KG1a LC3 and LysoID intensity was compared to the BDS readout following the above treatments. Rapamycin treatment caused a significant decrease in LC3 intensity (note not LC3II but overall LC3) after two hours, possibly through increased autophagic flux and therefore degradation (Figure 3.6). However, wortmannin and chloroquine treatment demonstrated only mild increases in total LC3, and no inhibitor significantly altered cellular LC3 in this context (Figure 3.6).

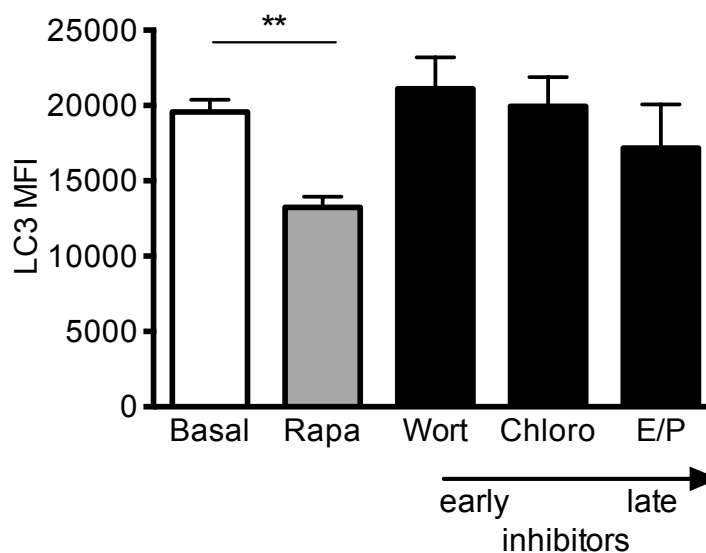


Figure 3.6: LC3 intensity response to pharmacological modulators of autophagy in a hematopoietic cell line. Mean fluorescence intensity of LC3 in KG1a cells under basal conditions or following 2 hour treatment with 1 μ M rapamycin (Rapa), 100nM wortmannin, 20 μ M chloroquine or 10 μ g/ml E64d/Pepstatin A, gated on LC3/LysoID double positive cells. Error bars represent SEM (n=3), ** p <0.01, unpaired t test.

LysoID intensity was significantly decreased by rapamycin treatment, while wortmannin created a significant buildup of LysoID intensity. However, E64d/Pepstatin A caused a decreased LysoID intensity; the reason behind this is uncertain but it was also found in other cell types and may reflect altered lysosomal recycling when fused autophagosomes are unable to be degraded (Figure 3.7). These results demonstrate use of LC3 or LysoID intensity alone, although modulated by some autophagy inhibitors and inducers as previously reported, does not reliably reflect autophagy status and cannot distinguish certain late autophagy impairments. Using IDEAS software to quantify the number of LC3 spots was found to be highly similar to LC3 intensity in this context (not shown). The lack of a decrease in LysoID intensity following chloroquine treatment indicates chloroquine-mediated alterations to intra-lysosomal conditions does not appear to impede LysoID staining (Figure 3.7).

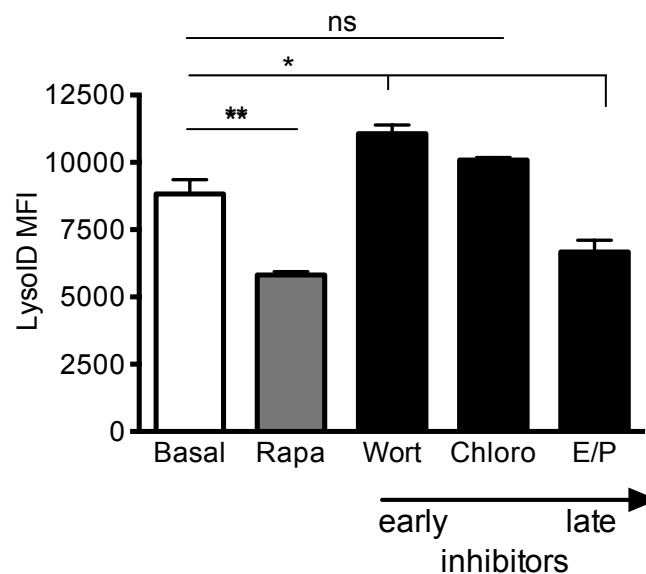


Figure 3.7: LysoID intensity response to pharmacological modulators of autophagy in a hematopoietic cell line. Mean fluorescence intensity of LysoID under basal conditions or following 2 hour treatment with 1 μ M rapamycin (Rapa), 100nM wortmannin, 20 μ M chloroquine or 10 μ g/ml E64d/Pepstatin A, gated on LC3/LysoID double positive cells. Error bars represent SEM (n=3), ns=not significant, *p<0.05, **p<0.01, unpaired t test.

3.10 LysoID correlates with LAMP1 on lysosomes

The above intensity data suggests LysoID largely behaves as expected following modulator treatment, however it is possible that the fixation step in the IFCAA protocol causes cytoplasmic leakage of staining or alterations to intensity. To exclude this, human peripheral blood mononuclear cells (PBMCs) were stained with LysoID and antibodies detecting LAMP1 (lysosomal associated membrane protein 1), the latter, along with related protein LAMP2, known to be major protein components of the lysosomal membrane (272). The two markers displayed a high level of colocalization and intensity correlation (Figure 3.8).

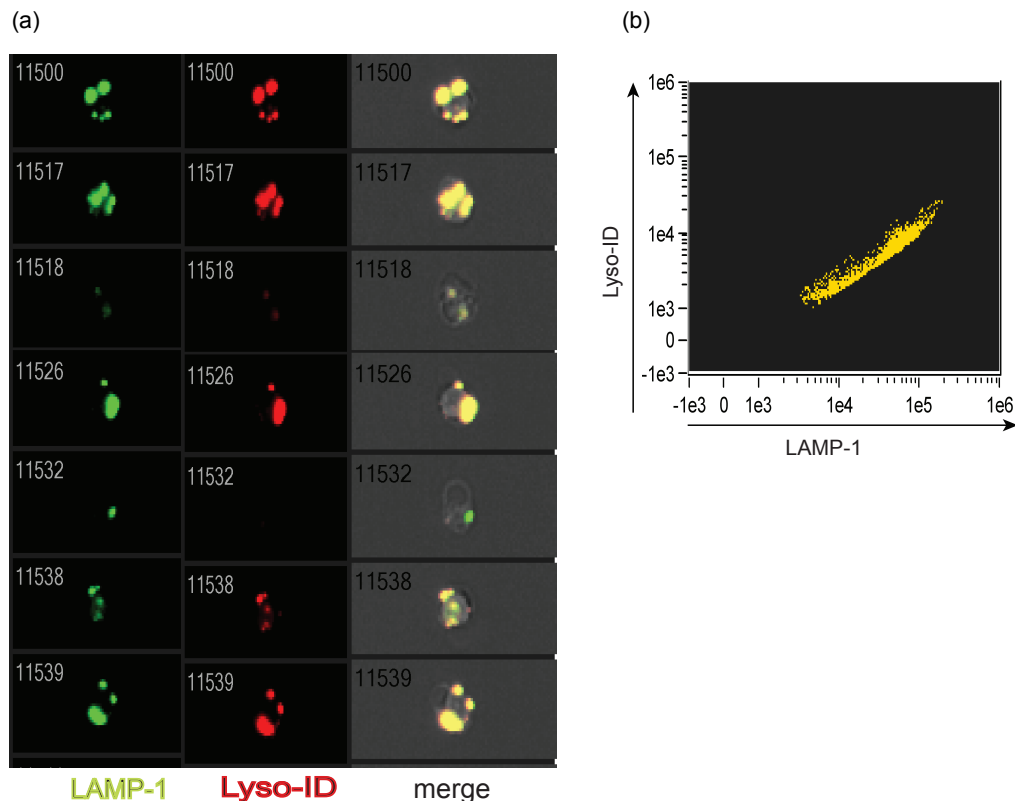


Figure 3.8: Lysosomal markers correlate on Image Stream. (a) Example merge (yellow) images of human PBMCs stained for LAMP1 (green) and LysoID (red), individual scaling was used for example display images. (b) Dot plot showing the mean fluorescence intensity of LAMP1 and LysoID in human PBMCs. Grateful acknowledgements to Dr Kanchan Phadwal for assisting with this experiment.

3.11 LysoID colocalization is higher with LC3 than with early autophagy pathway member WIPI1

Autophagosome formation and cargo incorporation is a multi-stage process, which may begin in several cellular compartments (26) and culminates in trafficking to, and fusion with, lysosomes (85) (86). To determine whether Image Stream resolution permitted the distinction of autophagic vesicles at different stages of maturation, human PBMCs were stained for LysoID, LC3 and WIPI1 (performed by K. Phadwal). WIPI1, a human paralogue of Atg18, is a phosphatidylinositol-3-phosphate scaffold thought to act with ATG9A/B and ATG2 in the delivery of membrane to a newly forming autophagosomes (65), therefore functions upstream of LC3 (273) and is not extensively associated with autophagosomal membrane at the point of lysosome fusion (274) (275); puncta of WIPI1 have been utilized as a readout for early autophagosome/phagophore levels in fluorescence microscopy (276). Acquired on the Image Stream, and although WIPI1 staining was overall quite low in PBMCs, PBMCs showed a higher degree of LC3/LysoID BDS than WIPI1-LysoID BDS (Figure 3.9), indicating a certain degree of resolution between highly related vesicle compartments.

3.12 LC3/LysoID colocalization is increased in stable ATG5 overexpression myeloid leukemic lines

In order to validate the IFCAA on a genetic model of autophagy modulation, myeloid leukemic lines HEL and NB4 were engineered to stably overexpress an Atg5-GFP fusion protein. ATG5 overexpression has been previously shown to enhance autophagy both in vitro (277) (278) and recently in vivo (279), where intriguingly overexpression is linked to oxidative stress resistance and extended lifespan. Early passages of the lines were transfected with either an eGFP-Atg5 expression construct or an eGFP-alone empty vector

by Neon electroporation (plasmids were kind gifts from Dr. Yoshimori as previously published (78)) and grown under 500ug/ml G418 (geneticin) selection for 4 weeks, before being FACS sorted for GFP⁺ cells and grown under G418 selection for a further 4 weeks.

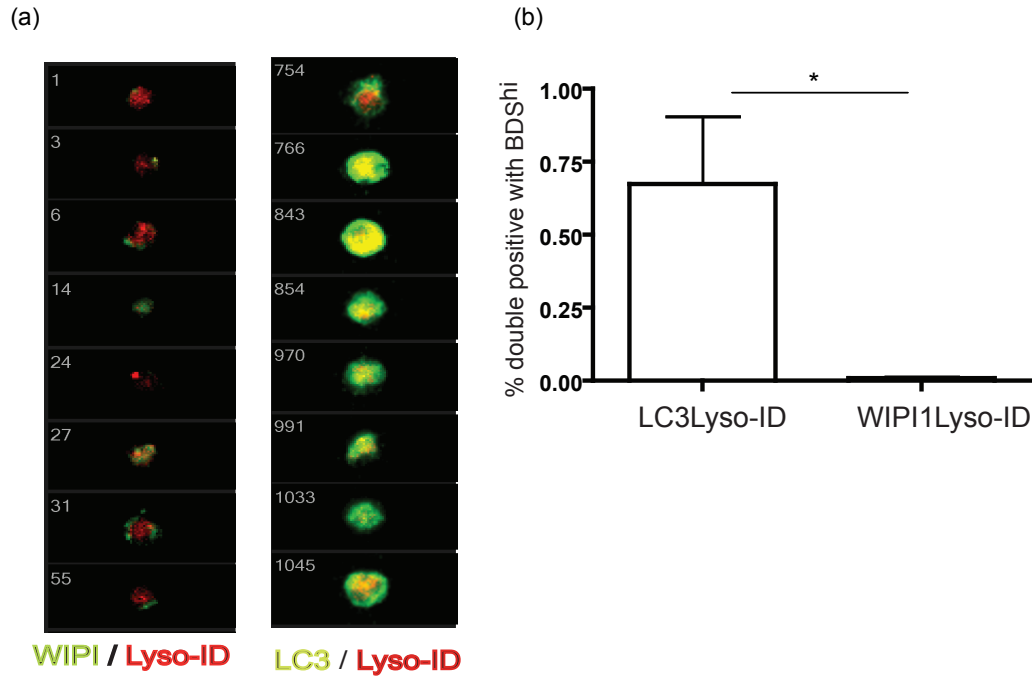


Figure 3.9: Colocalization of stage-specific autophagosome markers with LysoID on the Image Stream. (a) Example merge (yellow) images of either WIPI1 (green, left column) or LC3 (green, right column) and LysoID (red, both columns) staining in human PBMCs, individual scaling was used for example display images. (b) Frequency of human PBMCs with high LC3/LysoID BDS or WIPI1/LysoID BDS, gated on double positive cells, identical cutoffs used for both features. Error bars represent SEM (n=4), * $p < 0.05$, unpaired t test. Grateful acknowledgements to Dr Kanchan Phadwal for performing this experiment.

Western blotting for ATG5 in the lysates of the lines thus generated showed moderate overexpression of Atg5-GFP in the NB4-Atg5eGFP line (NB4-Atg5) and the HEL-Atg5eGFP line (HEL-Atg5), absent as expected in NB4-eGFP and HEL-eGFP vector control lines and the untransfected original lines (Figure 3.10).

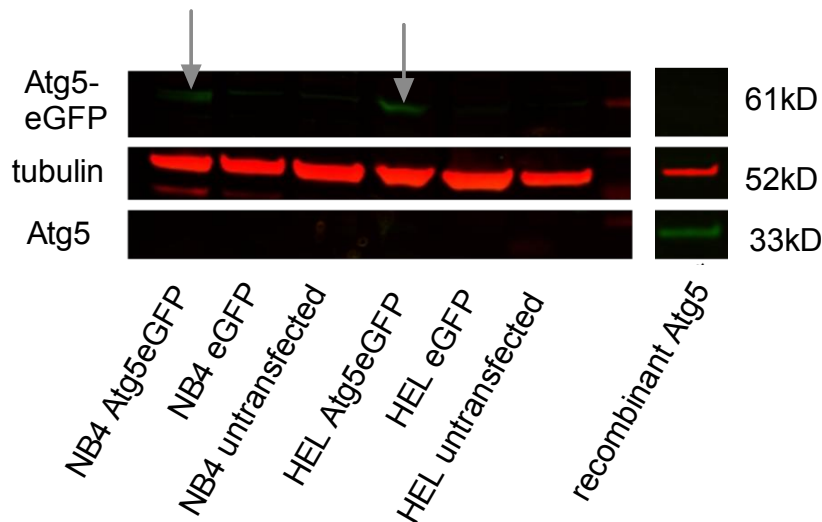


Figure 3.10: Generation of NB4 and HEL lines stably overexpressing Atg5-eGFP. Immunoblot against ATG5 and tubulin in lysates of NB4 and HEL cells transfected with either Atg5-eGFP or eGFP (empty vector) constructs, grown for 4 weeks under G418 selection (500 μ g/ml), FACS sorted on GFP expression and grown another 4 weeks under G418 selection, or untransfected parent lines, recombinant human ATG5 (Novus) used as a positive control.

To confirm the GFP tag does not hamper Atg5 function within the autophagy pathway, as previously reported (78), the transfected lines were stained with LysoID and run on the Image Stream. Within the autophagy pathway, ATG5 functions in the Atg5-12-16L complex to mediate autophagosome formation and LC3-conjugation, therefore, although it is partly recycled from the surface of closed autophagosomes, it has been reported on both autophagosome and autolysosomes (78) (273) (280). In both NB4 and HEL lines, Atg5-eGFP was found to significantly colocalize with LysoID, whereas un-tagged eGFP did not (Figure 3.11), and was similarly colocalized with LC3 (not shown); eGFP also exhibited a more diffuse fluorescence pattern in general. Finally, the levels of autophagy in the ATG5 overexpression lines were quantified with the IFCAA. The Atg5-eGFP expression lines showed a significant increase in the frequency of cells with a high LC3/LysoID BDS relative to their vector control lines (Figure 3.12). Interestingly, the EGFP-expressing vector control lines in turn showed increased BDS compared to the original untransfected

lines (not shown), suggesting that plasmid-overexpression of a protein alone may increase autophagy levels to a limited degree, possibly due to increased ER stress or recognition of foreign DNA (281). These findings show that the IFCAA detects increased basal autophagy in a genetic Atg overexpression model.

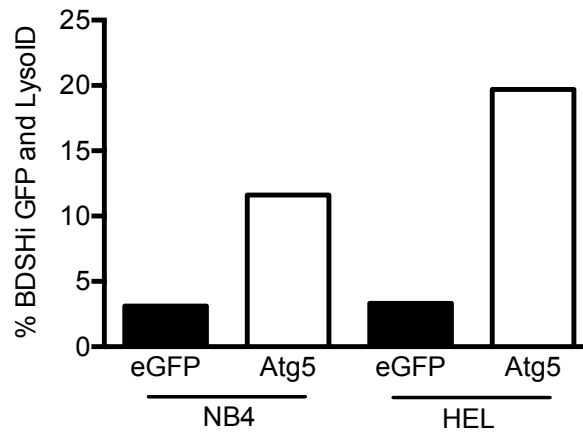


Figure 3.11: Atg5-eGFP colocalizes with lysosomal markers. Frequency of NB4 and HEL cells with high GFP/LysoID BDS after stable transfection with either Atg5-eGFP or eGFP empty vector constructs, gated on double positive cells, representative of two independent experiments.

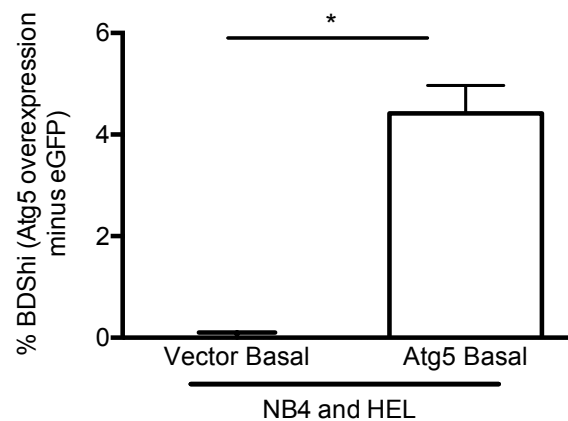


Figure 3.12: NB4-Atg5 and HEL-Atg5 show increased LC3/LysoID BDS on IFCAA. Difference between the frequency of NB4 and HEL cells stably overexpressing ATG5 with high LC3/LysoID BDS and frequency of relevant empty vector control, gated on double positive cells. Error bars represent SEM (n=2), * $p < 0.05$, unpaired t test.

3.13 LC3/LysoID colocalization is decreased in *Atg7*^{-/-} CD8⁺ T-cells

To test the IFCAA readout on the converse setting, that is genetic loss of an Atg, the level of LC3/LysoID BDS *ex-vivo* in CD8⁺ T-cells from the spleen of *Vav-Atg7*^{-/-} mice or wild-type littermates were measured. The removal of *Atg7* by Cre under the Vav promoter, and subsequent loss of autophagy in the hematopoietic cells of these mice, has been previously confirmed (7) (131). Splenic *Atg7*^{-/-} CD8⁺ T-cells showed significantly decreased % cells BDS hi compared to wild-type counterparts (Figure 3.13).

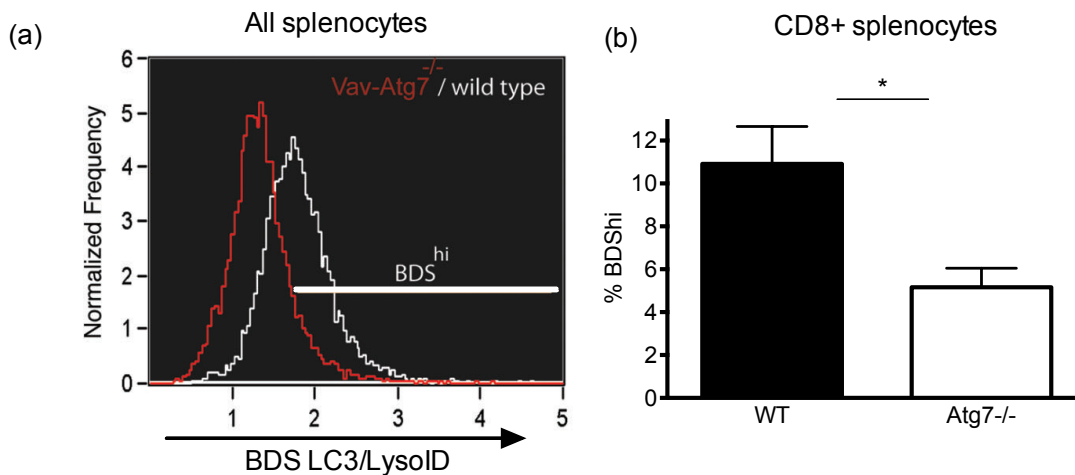


Figure 3.13: IFCAA shows decreased LC3/LysoID BDS in *Vav-Atg7*^{-/-} splenocytes. (a) Representative LC3/LysoID BDS histograms of splenocytes from *Vav-Atg7*^{-/-} mice (red) or wildtype littermate (white), gated on double positive cells. (b) Frequency of CD8⁺ splenocytes from *Vav-Atg7*^{-/-} mice or wildtype littermates with high LC3/LysoID BDS, gated on double positive cells. Error bars represent SEM (n=3), **p*<0.05, unpaired *t* test.

Together, validation performed with pharmacological modulators, genetic models of increased and dysfunctional autophagy as well as comparisons of pathway markers suggest the IFCAA detects relative autophagy levels in a variety of hematopoietic cell types.

3.14 IFCAA on primary human PBMCs – Peripheral T-cells display higher basal autophagy than B-cells

One of the primary advantages of the IFCAA over previous flow cytometric techniques is that endogenous LC3, rather than transgenic LC3-GFP is detected, allowing application to primary human hematopoietic tissue samples, including the blood and bone marrow often obtained from patients for diagnostic purposes (140). To test this capacity, the autophagy levels of human lymphocyte populations were explored. PBMCs taken from healthy donors were run on the IFCAA, gating on CD3+ T-cell and CD19+ B-cell lymphocyte populations. CD3+ T-cells showed substantially increased autophagy, via frequency of BDS-hi cells, while application of E64D/Pepstatin A suggested that peripheral CD19+ B-cells have little active basal autophagic activity (Figure 3.14). I contributed this set of data (Figure 3.13-14) to a publication describing this assay, authored by a postdoc, Kanchan Phadwal, in the host lab (140). This observation may reflect differential requirements of lymphocyte populations for ongoing autophagy in the periphery, evidenced by the degree of survival impairment following *Atg5* deletion (156) (282).

3.15 IFCAA on primary murine bone marrow – HSCs show increased basal autophagy

Another goal behind the development of the IFCAA technique was the quantification of basal autophagy in murine HSCs and early progenitors *ex vivo* using surface phenotype definitions and endogenous markers, yet to be accomplished in the literature. Murine HSCs and multipotent progenitors (MPPs) have been found to express different combinations of the SLAM (signaling lymphocyte activation molecule) family of surface markers CD150 and CD48 (246), which have been subsequently combined with the older Lin-Sca1+cKit+ (LSK) definition of a heterogeneous murine multi-potent progenitor

population (283) (284) (285) to delineate largely quiescent and self-renewing HSCs as LSK CD150⁺ CD48⁻ in young (286) (287) as well as older mice (288). These definitions can be improved by addition of CD229 and CD244 (287) or CD34. Similarly, the Lin⁻ Sca1-cKit⁺ (LK) population, which contains the oligopotent murine myeloid and erythroid progenitor subsets, can be further broken down using CD150, CD105, CD41 and the Fc receptor CD16/32 (II/III) (245) (131) to delineate progenitor subtypes, including the LK FcγRII/III⁺ CD150^{lo} granulocyte-macrophage progenitor (GMP) population.

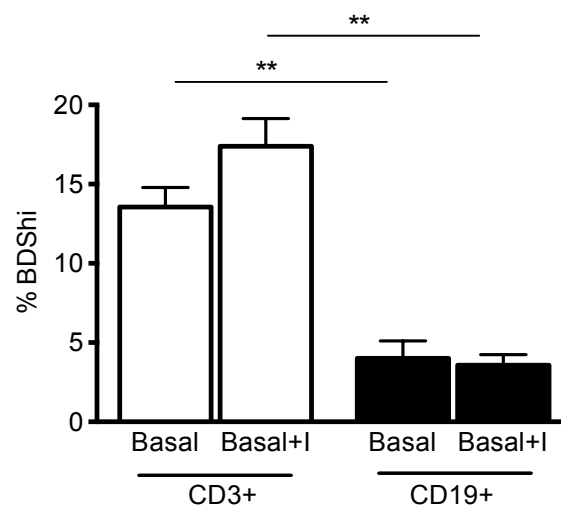


Figure 3.14: Autophagy measurement in human peripheral blood lymphocyte populations. Frequency of CD3⁺ T-cells or CD19⁺ B-cells in human healthy donor PBMCs with high LC3/LysoID BDS after 2 hours in RPMI with 10% FCS alone (basal) or following treatment with 10µg/ml E64d/Pepstatin A (basal+I), gated on double positive cells. Error bars represent SEM (n=3), **p<0.01, unpaired t test.

Using the HSC and GMP populations surface phenotypes, along with their un-enriched LSK (MPP) and LK (largely oligopotent) progenitor parent populations, a gating strategy for IFCAA analysis of HSC and progenitor populations within wild type primary murine bone marrow was developed (Figure 3.15), with the newer Image Stream X imaging flow cytometer required due to its expanded 12 channel capacity. The first three gatings shown were representative of all Image Stream analysis, with selection of focused cells by Gradient RMS, the exclusion of doublets via Aspect Ratio against Area on the Brightfield

image, both as guided by the manufacturer, and finally selection of live cells using a fixable live-dead marker before application of surface marker grating (Figure 3.15).

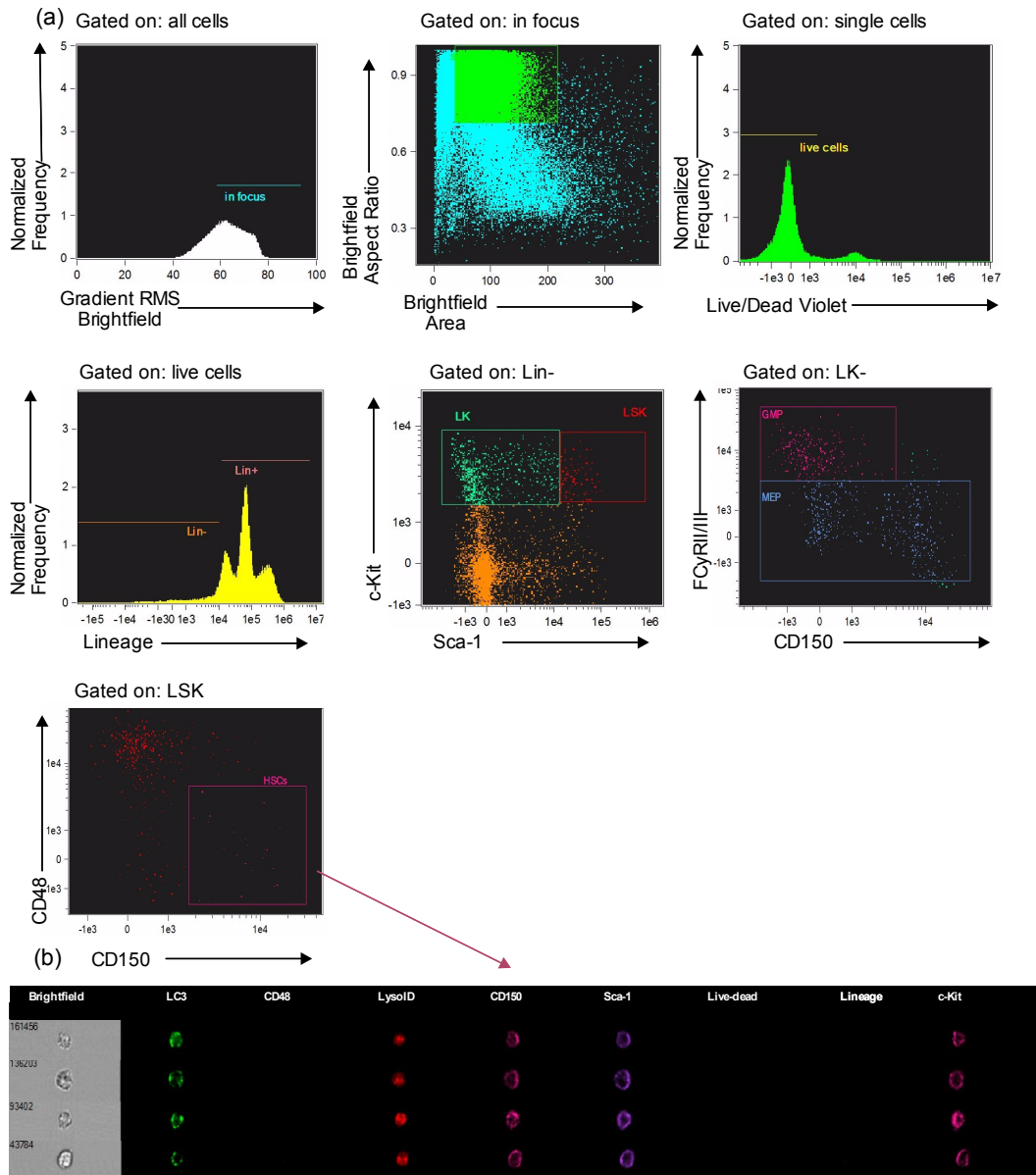


Figure 3.15: Image Stream surface marker gating strategy for HSC and GMP populations. (a) Gating on wild type mouse bone marrow cells stained for indicated surface markers, with live-dead aqua and LysoID and for intracellular LC3 then acquired on ImageStream X, IDEAS plots shown; cells were selected for being in focus, single, live cells then gated on lineage marker expression (Lin-: Lineage negative), c-Kit and Sca-1 expression (for LK: Lin-cKit-Sca1+, LSK: Lin-cKit+Sca1+), CD150 and CD48 expression (for HSC: LSK CD150+CD48-) or CD150 and FcγRII/III expression (for GMP: LK FcγRII/III+, MEP: megakaryocyte-erythroid progenitor) (b) Representative images of HSCs for all used fluorescence channels (Lin-cKit+Sca1+CD150+CD48-), optimal individual scaling was used for example display images.

Note that certain aspects of the progenitor definitions, using alternate viewing modes in flow cytometry analysis software (ex: contour plots) are not possible on IDEAS. Also, fluorescence compensation was a significant challenge on the Image Stream X due to the number of channels used, and final matrices required careful confirmation via manual image and plot verification. The LC3 and LysoID staining patterns of the resulting populations could then be visualized, with representative images of LSK CD150+ CD48+ HSCs shown (Figure 3.15).

The median LC3/LysoID BDS for each population was determined and HSCs were found to display significantly increased basal colocalization *ex vivo* compared to the bulk of MPP progenitors (LSK cells), which in turn had higher BDS than the more mature progenitor populations (Figure 3.16). As outlined above, median BDS was chosen as opposed to cutoff gate readouts due to HSC population rarity and file size limits on acquisition (also addressed in Methods chapter). These results suggest that relative basal autophagy in murine hematopoietic progenitors may correlate with multipotency and quiescence.

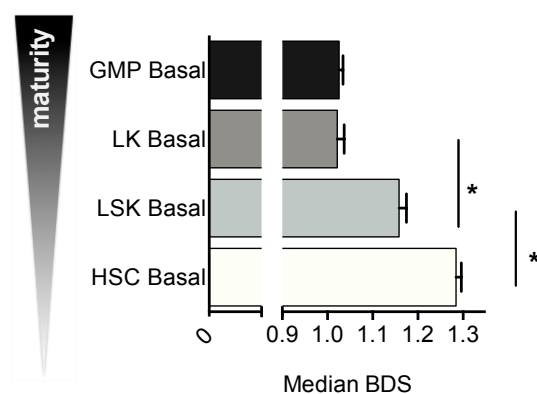


Figure 3.16: Elevated autophagy in HSC and LSK populations via IFCAA. Median LC3/LysoID BDS of murine HSC (*Lin-Sca1+cKit+CD150+CD48-*), LSK (*Lin-Sca1+cKit+*), LK progenitor (*Lin-Sca1-cKit+*) and GMP (*Lin-Sca1-cKit+FcgRII/III+*) populations in wild type murine bone marrow (150,000 cells analyzed per sample), gated as Figure 3.15, note that median BDS is employed rather than %BDS_{hi} due to HSC population rarity. Error bars represent SEM (n=3), **p*<0.05, unpaired *t* test.

3.16 Murine HSCs have higher levels of GFP-LC3 puncta via imaging flow cytometry

While IFCAA determination of LC3 and lysosome marker colocalization represents a novel autophagy readout, the quantification of GFP-LC3 puncta by fluorescence microscopy is a recognized assay within the field, where increased puncta counts and/or accumulation of puncta following lysosomal inhibition indicates the level of autophagic activity (139) (249). The advent of GFP-LC3 mice has permitted the *in-vivo* and *ex-vivo* analysis of autophagy's homeostatic role in several tissues (139), however at the time of this thesis this tool had not been applied to wild type hematopoietic progenitors. The Image Stream allows for the objective quantification of fluorescent puncta per cell in surface marker gated populations, therefore the GFP-LC3 model was combined with imaging flow cytometry to confirm the *ex-vivo* IFCAA analysis of hematopoietic progenitors. As the Image Stream 100 was employed, the joint MPP and HSC population of LSK cells was the most immature population assessed. LSK cells showed highest frequency of cells with 4 or more LC3 puncta (spot to background ratio 2.5), both at basal levels and post-lysosomal inhibitor treatment (Figure 3.17). The frequency of puncta-high LSK cells was over 1.5x the frequency in the more mature LK progenitor population, which in turn displayed higher autophagy levels than various mature lineage populations, including CD11b⁺ Gr1⁻ myeloid cells and B220⁺ B-cells. Identical results were found using puncta count alone (not shown). Considered with the IFCAA measurement, this demonstrates further evidence for increased basal autophagy in the most immature hematopoietic cascade populations.

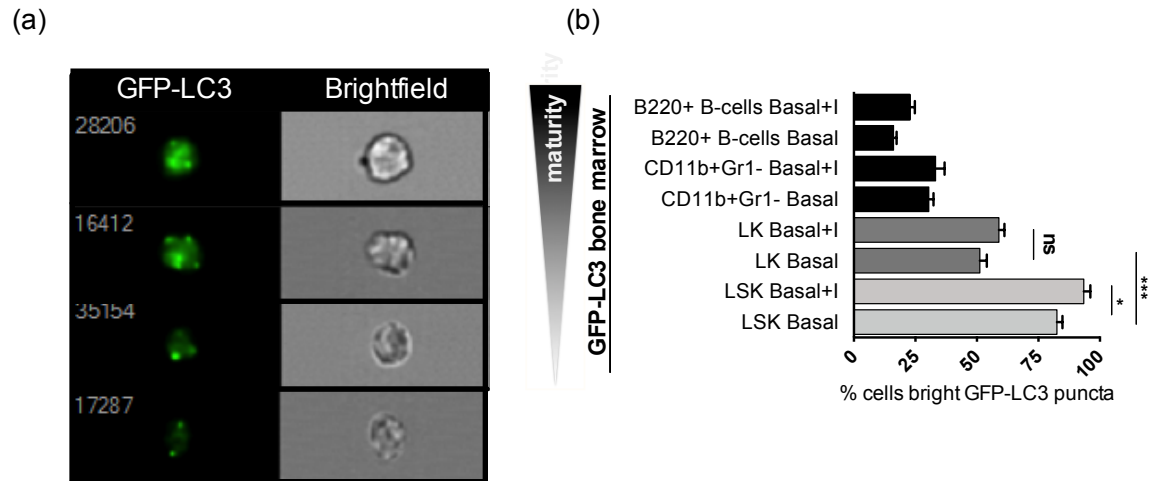


Figure 3.17: Increased GFP-LC3 puncta in LSK population. (a) Representative images of LSK cells showing decreasing (top to bottom) numbers of GFP-LC3 puncta and corresponding brightfield image, optimal individual scaling was used for example display images. (b) Quantification of LC3-GFP puncta in LSK, LK, CD11b+Gr1- (neutrophils) or B220+ (B cells) bone marrow populations from transgenic GFP-LC3 mice (40,000 cells analyzed per sample) after 2 hours in RPMI with 10% FCS alone (basal) or following treatment with 10µg/ml E64d/Pepstatin A (basal+I), quantification using Image Stream assessment of % cells with 4 or more bright puncta. Error bars represent SEM (n=4), ns=not significant, *p<0.05, ***p<0.001, unpaired t test.

3.17 IFCAA on human bone marrow - CD34+ progenitors show higher levels of basal autophagy, correlating with decreased oxidative stress

To confirm the observed murine progenitor autophagy trends in human systems, the IFCAA was employed on healthy human bone marrow mononuclear cells (BMNCs). As the Image Stream 100 was utilized for these measurements, the number of available surface markers was severely restricted. CD34 is a phosphoglycoprotein originally identified on human hematopoietic stem and progenitor cells (HSPCs) (289), and although it has since been found in other tissues as a marker of general progenitor-like traits (290) and is more restrictive in combination with other markers (ex: CD38, CD90, CD45RA), among human hematopoietic cells it is strongly expressed from HSC to oligopotent progenitors and can be used as a single marker to enrich for self-renewal and progenitor capacity (290) (291). Therefore, LC3/LysoID BDS was investigated in the CD34+ and CD34- BMNC

populations. Frozen BMNCs from healthy donors were obtained from Lonza; freezing was found to not significantly alter BDS profiles in human primary cells in control experiments (not shown). Donor to donor variation in LC3/LysoID staining was substantial and gating on cells highly double positive for both markers was required for population BDS comparisons; despite this, CD34⁺ cells were found to display significantly more basal autophagy than CD34⁻ from the same donor (Figure 3.18).

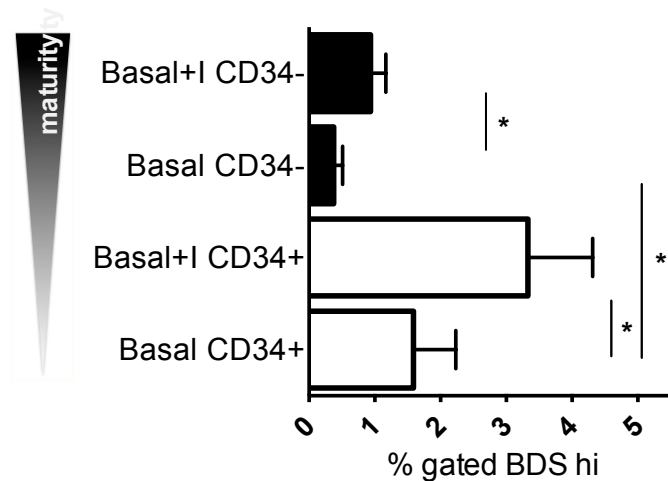


Figure 3.18: Human hematopoietic progenitors show increased basal autophagy via IFCAA. Frequency of CD34⁺ and CD34⁻ cells from healthy donor human bone marrow mononuclear cells with high LC3/LysoID BDS (20,000 cells analyzed per sample) after 2 hours in RPMI with 10% FCS alone (basal) or following treatment with 10 μ g/ml E64d/Pepstatin A (basal+I), gated on double positive high cells. Error bars represent SEM (n=6), * p <0.05, paired t test.

Suppression of oxidative stress is an important physiological role of the autophagy pathway (292) (293), particularly through policing damaged mitochondria and their potential reactive oxygen species (ROS) production (294). Oxidative stress has been found to severely inhibit HSPC function (185) (186) (234) and increased ROS have been observed in autophagy deficient stem cells (131). To confirm whether the CD34⁺ cells displayed reduced oxidative stress, BMNCs were stained with nonyl-acridine orange (NAO), a florescent dye which stains cardiolipins in the mitochondria membrane but is excluded when cardiolipins are oxidized by excess ROS production (295) (296), and with

MitoSOX, which fluoresces in the presence of the mitochondrial superoxide ROS species. CD34⁺ cells showed decreased superoxide generation by MitoSOX and increased mitochondrial health by NAO compared to the CD34⁻ compartment in the same donor (Figure 3.19), indicating reduced mitochondria-generated oxidative stress in the autophagy high population.

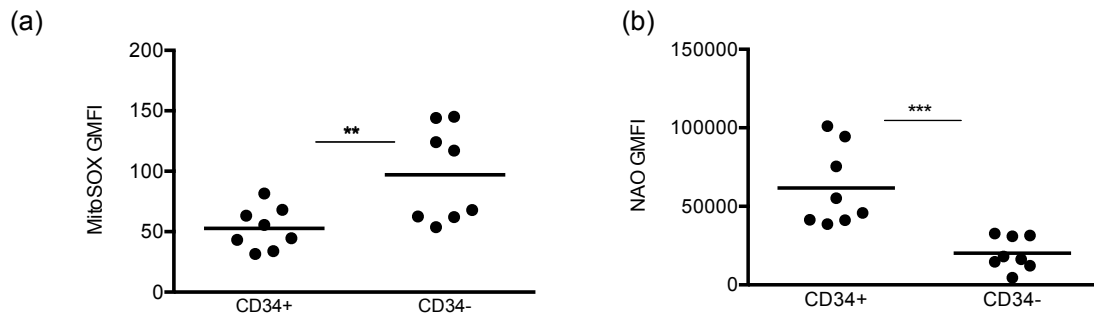


Figure 3.19: Human hematopoietic progenitors show decreased oxidative stress. (a) GMFI of MitoSOX Red staining in CD34⁺ and CD34⁻ populations from healthy donor human bone marrow mononuclear cells. (b) GMFI of NAO (nonyl acridine orange) staining in CD34⁺ and CD34⁻ populations from healthy donor human bone marrow mononuclear cells. ** $p < 0.01$, *** $p < 0.001$, paired t test.

3.18 Human hematopoietic stem cells show higher expression of autophagy genes

In order to assess the autophagy status within the human CD34⁺ compartment in more depth, the expression of autophagy genes in human HSCs (defined as Lineage⁻CD34⁺CD38⁻CD90⁺CD45RA⁻), GMPs (defined as Lineage⁻CD34⁺CD38⁺CD123⁺CD45RA⁺) and megakaryocyte-erythroid progenitors (MEPs, defined as Lineage⁻CD34⁺CD38⁺CD123⁻CD45RA⁻) populations was investigated. RNA-seq was performed on FACS sorted healthy donor HSC, GMP and MEP populations in the lab of collaborator Professor S.E Jacobsen by Dr. Woll, and reads per kilobase per million reads (RPKM) were calculated by Dr. Woll as previously published (142). Analysis of autophagy pathway genes, as defined by Behrends et al. (297) within this data set showed that, despite a limited sample size, expression of autophagy modulating transcription factor

FOXO3 was increased in HSCs compared to GMPs and MEPs, along with mammalian Atg8 homolog *GABARAPL2* (Figure 3.21). Atg8 homolog *GABARAP* was unaltered between HSC and GMP populations, but both showed relatively increased expression in both compared to MEPs (Figure 3.20). Importantly, no autophagy pathway genes showed significantly increased expression in downstream progenitors compared to HSCs.

Although additional donors would strengthen conclusions, this analysis suggests transcript levels of important autophagic pathway components are higher in the most immature CD34+ compartment, HSCs, agreeing with the murine autophagy measurements in correlating increased autophagy signature to multipotency, self-renewal and quiescence.

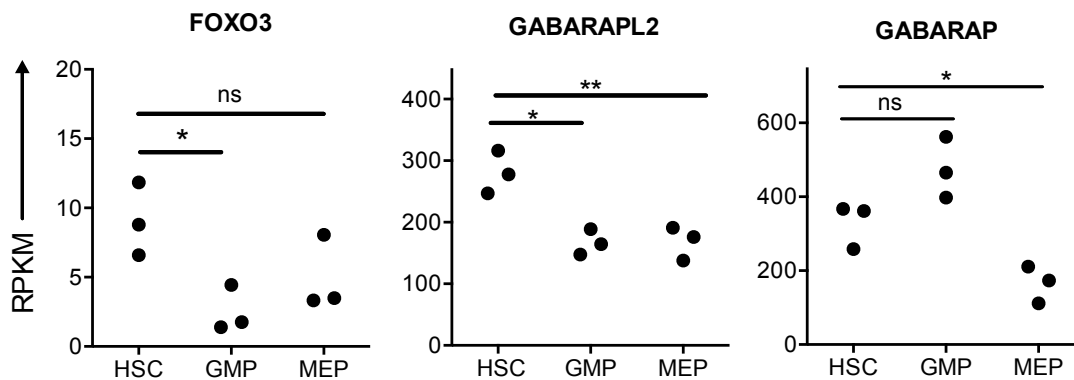


Figure 3.20: Human HSCs show increased *FOXO3* and *GABARAPL2* expression. RNA-seq on human HSC (Lineage-CD34+CD38-CD90+CD45RA-), GMP (Lineage-CD34+CD38+CD123+CD45RA+) and MEP (MEP: Lineage-CD34+CD38+CD123-CD45RA-) populations, reads per kilobase per million mapped reads (RPKM) for autophagy genes with significant changes are shown. ns=not significant, * $p < 0.05$, ** $p < 0.01$, unpaired *t* test. Grateful acknowledgements to Dr Petter Woll for performing the RNA-seq experiment and assisting with the analysis.

Discussion

3.19 A novel imaging flow cytometry autophagy assay detects hematopoietic autophagy using endogenous markers

The field of autophagy is rapidly expanding, with prospective roles in a myriad of biological process covering cell development, disorder and death (6) (15). The study of autophagy in healthy and diseased biological systems requires assays that detect autophagy, an extremely dynamic and sensitive process that is intrinsically tied to cellular stress, with minimal manipulation and subjectivity (247). Robust assays that capture the autophagic process in mammals, such as electron microscopy, florescence microscopy and western blotting, were optimized on cell lines and are difficult to apply to complex primary or clinical samples without prior purification and/or culture (140, 247).

The use of this imaging flow cytometry autophagy assay (IFCAA) to detect *physiological* autophagy had several prospective advantages over classical assays in such circumstances. As a flow based platform, it allowed for the use of multiple surface markers (as opposed to much more limited options in florescence microscopy) and their application to define subsets of a large (20,000-150,000 cells) sample that would be unfeasible in microscopy, even with population purification. Moreover, the associated image analysis software (IDEAS for Image Stream) means populations of thousands of cells can be objectively and efficiently analyzed for morphological characteristics and vesicular sub-cellular locations, either for LC3 and LysoID colocalization (140) or for more classical LC3-puncta quantification (247). The end result, when employed correctly, is a reduction in the subjectivity of cell sample selection inherent in the majority of microscopic analysis. That being said, confocal results and western analysis were found to generally correlate well with IFCAA readout (published in (140)).

These benefits are of course shared by recently published assays that use traditional flow cytometry, most of which have employed GFP-LC3 transgenic cell lines and assessed a decrease in LC3 intensity as increased autophagic activity (256, 257). However, such intensity based readouts, using either LC3 or lysosomal markers (298) without an imaging component, have been found to harbor several disadvantages, including the dangers of foreign protein expression for physiological measurement of an ER stress sensitive pathway (247), the potential impacts of larger protein tags on smaller protein (LC3) function, the confounding factor of GFP-LC3 aggregates (251) and the inability to distinguish certain impairments from induction by monitoring a single vesicular compartment (249)(shown in Figure 3.2). Indeed, stable overexpression of GFP alone was observed to mildly increase autophagy in vector controls for the Atg5-eGFP lines. It is worth noting that LC3 single-intensity flow cytometric assays would be vastly improved if lipidated LC3, that is LC3-II, could be distinguished by antibody from un-conjugated LC3-I. Approaches like that of Eng et al. (256) which use detergents such as saponin to extract LC3-I from the cytoplasm prior to staining, are therefore superior, but detergents have been found to induce autophagy independent LC3 aggregates when employed prior to fixation (299) and it is not clear how detergent use prior to fixation impacts surface marker staining. Samples in the IFCAA were fixed as rapidly as possible, post-surface labeling and prior to permeabilization by detergents.

The IFCAA overcomes single-intensity complications by combining staining of both autophagosomes (endogenous LC3) and lysosomes (LysoID), using the trafficking of these vesicles into a common sub-cellular area (86) and subsequent fusion as a dynamic stage of autophagy to monitor within the pathway. Examining the fusion step and subsequent

autolysosome numbers has been previously proposed for microscopy (298), and reflects autophagic flux direction in most cases. The application of different autophagy inhibitors demonstrated the flexibility of this approach; upstream induction via rapamycin or inhibition via wortmannin led to increased and decreased population BDS respectively. Moreover, downstream inhibition with autophagosome-lysosome fusion inhibiting chloroquine (267) also decreased BDS, importantly suggesting that the IFCAA resolution allows for differentiation of fusion activity and can distinguish such autophagy blockages in a way which LC3 or lysosome marker intensity cannot. By contrast, in line with previous reports (256) (249), LC3 or LysoID intensity could reflect increased degradation after autophagy stimulation by rapamycin but was unable to capture all forms of autophagy inhibition effectively (depicted in Figure 3.2).

Assessing LC3/LysoID colocalization alone showed robust directional responses to most pharmacological and genetic autophagy modulation, including the overexpression of *ATG5*, previously reported to increase autophagy (277) (278, 279), and deletion of *Atg7*. However, E64-D/Pepstatin A treatment demonstrated that inhibition of lysosomal activity in a way which does not impact vesicle fusion can still cause a BDS increase. LC3 in the autophagosome membrane is normally either degraded after lysosomal fusion, if on the inner membrane, or cleaved by Atg4 and recycled (78), if on the outer membrane, rendering the LC3/lysosomal marker-positive autolysosome population a dynamic one under normal conditions (298). Therefore, E64D/Pepstatin A inhibition of lysosomal proteases can assay for LC3 immediately susceptible to lysosomal degradation, with the accumulation of LC3/LysoID colocalization post-treatment reflecting inhibition of normal autolysosomal LC3 clearance (140). E64D and Pepstatin A was employed in parallel with basal BDS/autophagy assessment wherever possible, as is recommended for all autophagy assays (254), both to control for lysosomal function and, when used as a ratio of BDS post-

inhibitor treatment over basal BDS (Basal+I/Basal), as a readout for impaired autophagy. This readout is considerably different from LC3-II turnover ‘flux’ assays via Western blot, although the trend may be largely similar. Also, E64-D and Pepstatin A were found to be sometimes ineffective on primary murine samples compared to human samples; whether this reflected sample preparation or biological differences is unknown.

Another worthwhile consideration for this assay was that application to cell lines provided more reproducible results than in primary samples. This could be due to natural variation between donors, possible even within one donor over time (300), or the limitations of the current Image Stream resolution and magnification (40x) in dealing with smaller cell types. Regarding the latter, improved results were obtained with the newer Image Stream X, and future technological advances are anticipated to further improve this readout. As with all autophagy readouts (247), it was best combined with a second confirmatory assay where possible. Despite these caveats, the IFCAA allowed high throughput assessment of autophagy levels using endogenous markers and minimal manipulation.

3.20 Increased autophagy in hematopoietic stem and progenitor cells

The IFCAA was designed to be particularly applicable in two hematopoietic situations: to assess autophagy in heterogeneous clinical samples, such as patient blood, and to quantify physiological autophagy of a very rare population within a suspension sample (140) (149). Exemplifying the former case, autophagy levels of the two major lymphocyte cell types, CD3⁺ T-cells and CD19⁺ B-cells, were compared in healthy donor human PBMC samples, revealing increased BDS in the CD3⁺ population. Autophagosomes and autolysosomes have been previously described in T-cells (156) (301), and peripheral T-cells have been found to require ongoing autophagy for survival in *Atg5*^{-/-} chimeric mouse models (156), where it mediates mitochondrial homeostasis and metabolism (302)

(303). By contrast, most mature B-cells survive in the periphery without *Atg5* (282) and naïve B-cells have been reported to display little autophagy (304). The meaning behind these observations requires further investigation, but demonstrates the use of the IFCAA on populations in primary human blood.

Employing the ability of imaging flow cytometry to monitor LC3 and LysoID in combination with surface marker panels, images of LC3 and LysoID staining in individual murine LSK CD150⁺ CD48⁻ HSCs were obtained *ex vivo* without sorting or culture. Comparison of LC3/LysoID BDS between progenitor compartments suggested that multipotent LSK cells display higher levels of basal autophagy compared to more mature, committed progenitors, and autophagy was further increased in self-renewing HSC population. The general correlation between hematopoietic population immaturity and autophagy levels was confirmed using quantification of GFP-LC3 puncta with and without lysosomal inhibitors. Also, puncta count demonstrated an alternate imaging flow cytometry based autophagy detection method which may be highly useful for study of *in vivo* and *ex vivo* hematopoietic autophagy with the LC3-GFP mouse model (139); however, as published and in the above cell line experiments, endogenous LC3 puncta provided a less reliable readout (249, 305).

At the time of these assays, the only autophagic quantification in HSCs examined human CD34⁺ CD133⁺ cells following bead sorting and culture, along with epidermal (ESC) and dermal (DSC) stem cells, reporting increased autophagosome number via EM, confocal and LC3 Western blot as compared to NB4 myeloid leukemic cells or mature neutrophils (181). These findings were restricted by their manipulations, experimental conditions (which the authors acknowledge) and chosen controls, however the IFCAA data supports these observations. The authors reported loss of stem cell properties, including stem cell

colony formation, ability to differentiate and drug (etoposide) resistance, following *Atg5* knockdown or autophagy inhibitor (3-Methyladenine) treatment (181). Moreover, the decrease in autophagy and autophagy gene expression during their differentiation assays agrees with the finding presented in this chapter, and with previous observations in MSCs. (206).

Very recently, a study investigated murine HSC autophagy more completely (49). Using the LC3-GFP model, they described little basal GFP-LC3 puncta (via fluorescence microscopy) or flux (defined as the increase in GFP-LC3 fluorescence on Bafilomycin A treatment) in *ex vivo* HSCs (LSK CD150⁺ 48⁻) from young mice as compared to GMPs, however they observed a substantial increase in HSC autophagy via both readouts following cytokine withdrawal (49). Their conclusion was that HSCs undergo only limited basal autophagy but are primed for autophagic activation following metabolic stress, which is required for their survival, with the transcription factor FOXO3 suggested to be a key factor controlling this switch. Supporting this, they found high levels of ‘upstream’ autophagy gene transcripts, yet low expression of ‘core’ Atg’s, at basal level, which was lost on *FOXO3* deletion. Finally, increased autophagy was reported in older murine HSCs, where it was found to be vital for function (49). Together, these pioneering observations were highly important in furthering the knowledge of HSC autophagy, implying a significant HSC autophagy capacity and underlining the role of FOXO3 in HSC autophagy, which is supported by the RNA sequencing data from human HSCs and GMPs as presented above. However, these data and the findings presented in this chapter differ in their assessment of basal HSC autophagy. Reasons for this difference may be that the control (basal) condition for several key assays presented by Warr et al. involved culture in cytokine-rich conditions for 6 hours, and cytokine/growth factor signaling is a key negative regulator of ongoing autophagy through PI3K/mTOR (22). Indeed, the basal

GFP-LC3 level intensity in HSCs appeared much higher than in GMP populations by flow cytometry, despite selected fluorescence microscopic images, in agreement with increased expression of multiple Atg8 homologs (Map1lc3b/a and Gabarapl1/l2) they reported in HSCs relative to GMPs, which were not included as ‘core’ Atg’s in their analysis, and the RNA seq data. Increased expression of Atg8 homologs has been reported as part of a general signature of stem cell quiescence (110).

On the other hand, my analysis by IFCAA was preceded by a brief rest period in IMDM medium supplemented with FCS after extraction from BM, and it is possible that these conditions resulted in stressed HSCs and impacted the observed autophagy levels. However, a requirement for ongoing basal autophagy in maintaining the basic HSC properties of self-renewal, multipotency and quiescence is in keeping with indirect evidence, presented in the introduction to this chapter, for a connection between autophagy and these characteristics, including a requirement for low levels of negative autophagy regulators such as mTOR signaling in HSCs (238) (239) (240), the vital role of autophagy inducing transcription factors FOXO3 (234) and HIF1 α (201) in HSC homeostasis, along with HSC metabolic requirements for ROS repression (184) (185) (186) and decreased mitochondrial oxidative stress in human CD34⁺ BMNCs. Indeed, a recent oral communication, again employing GFP-LC3 mice, provides additional support for ongoing basal autophagy in HSCs relative to myeloid progenitors (306). Confirmation of HSC autophagy levels and inducibility is therefore required and will be vital for understanding the impact of autophagy inhibitors (such as chloroquine) or inducers for therapeutic purposes and in the generation of iPS cells.

3.21 Conclusions

This chapter outlines the development of a novel method of autophagy detection using imaging flow cytometry, permitting high throughput and statistically robust detection of autophagy in surface marker-defined hematopoietic populations *ex vivo* based on endogenous proteins. Its application to human peripheral lymphocyte populations showed increased autophagy in T-cells relative to B-cells. Moreover, this assay enabled autophagic quantification in the murine hematopoietic progenitor populations, revealing a positive correlation between basal autophagy and progenitor immaturity. This agrees with an autophagy requirement for maintenance of HSCs, and suggests the pathway may be actively upregulated in immature populations.

Chapter 4: Autophagy is required for HSC quiescence and maintenance

Introduction

While work performed in yeast and cell lines has been invaluable in unraveling the machinery of autophagy, discerning the precise role of mammalian autophagy genes within normal tissues has required the more recent application of murine knockout models. The importance of *in-vivo* studies is particularly true for HSCs and progenitors, as many gold standard assays and outputs for function are *in-vivo* or *ex-vivo*, whereas culture based assays are potentially unreliable (307). The results presented in Chapter 3 of this thesis suggest increased autophagy in immature HSCs and early progenitors; previous work in the host lab on a hematopoietic knockout of autophagy gene *Atg7* show that it is required for maintaining the HSC population. However subsequent papers have shown *Atg7*, as with several *Atgs*, may have important roles outside the strict autophagy pathway. Here, the phenotypic highlights of existing murine autophagy knockout models will be reviewed, culminating in a description of the *Atg7* hematopoietic knockout model, along with the questions surrounding the autophagy specificity of that phenotype.

4.1 Systemic knockout of *Atg* or related genes – autophagy is vital for development

Ablation of autophagy genes during early development has revealed several points of *Atg*-requirement, but the variety in phenotypes demonstrates the importance of examining multiple pathway genes (summarized in Table 3.1). The first systemic autophagy knockout targeted *Beclin1*, the mammalian *Atg6* homolog which acts early in the autophagy pathway in the nucleation complex (22). *Beclin1* is directly phosphorylated by Akt (33), leading to cytoskeletal sequestration and downregulated autophagy. The *Becn1*^{-/-} mouse was lethal at an early stage of embryogenesis, with embryos at day 7.5 (E7.5) demonstrating abnormally small size, widespread apoptosis and pro-amniotic canal defects

(170). *Becn1*^{+/-} heterozygotes were viable but showed reduced cellular autophagy, increased proliferation and suffered from increased tumor development (308). Similarly, mice deficient for Beclin1 interacting protein AMBRA1 via gene-trap mutagenesis arrested at E14, displaying increased cellular proliferation and altered neural tube development (66). Knockout of *FIP200*, a member of the ULK1/2 initiation complex, is also lethal from E13.5-16.5 with failures in heart and liver development (171). Together, these phenotypes suggest that autophagy initiation complexes may be required during embryonic development. However, complicating this picture, Beclin1 also plays a role in apoptotic regulation through interaction with anti-apoptotic protein Bcl-2 (309), although *Becn*^{-/-} ESC apoptosis was normal in response to UV light or serum withdrawal (170). Similarly, FIP200 can act in decreased proliferation and cell cycle arrest through p53 (310) and retinalblastoma-1 (Rb1) (311). Therefore, caution in interpretation is warranted. Nor is the phenotype of all early-complex genes the same; removal of *Ulk1* (154), a key member of the initiation complex with FIP200, leads to defective erythrocyte development but is viable, likely due to general functional redundancy with fellow Atg1 homolog ULK2 (312). Supporting this, *Ulk2*^{-/-} knockout mice are also viable, with no overt phenotype (313), but recently created *Ulk1/2*^{-/-} double knockout mice show neonatal mortality and a defect in lung development (314).

The phenotypes of later autophagy genes, specifically those involved in the two ubiquitin-like cascades required for autophagosome membrane elongation (21), are largely similar to the *Ulk1/2*^{-/-} mice, with individual systemic deletion of *Atg3* (315), *Atg5* (168), *Atg7* (134), *Atg9a* (316) and *Atg16L1* (317) all displaying neonatal lethality combined with reduced birth weight and lower levels of tissue amino acids. These data confirm autophagy's role in survival during the first hours post-birth, a period associated with increased autophagy as observed in LC3-GFP mice (168). The exact cause of death in

knockout mice is debated, with suckling defects due to neurological impairment suggested (169). Also, autophagy's role in early development is left in balance, as mice missing members of autophagy's ubiquitin-like cascades appear able to survive embryogenesis, likely supported through initial ESC division by maternal Atg protein (164). However unique and subtle defects have been reported in individual knockouts. Examples include decreased adipose mass in *Atg5*^{-/-} mice, impaired innate immune response in *Atg9a*^{-/-} mice and tissue specific autophagy defects along with tumorigenesis in *Atg4C*^{-/-} mice (318). As a result, a general autophagy role in tissue development cannot be ruled out (6). Interestingly, full body loss of the Atg8 homologs *Map1Lc3b* (319) or *Gabarap* (320) did not result in an observable phenotype, suggesting redundancy among Atg8 homologs.

Table 4.1: Mouse models with systemic autophagy gene deletion

Gene	Autophagy Stage	Cre/Tissue	Phenotype	Lethal	Ref
<i>Beclin1</i> ^{-/-}	Nucleation	Systemic	E7.5 arrest, embryonic apoptosis, proamniotic canal defects	Yes	(170)
<i>Beclin1</i> ^{+/-}	Nucleation	Systemic	Increased proliferation, tumor development	No	(308)
<i>Ambra</i> ^{-/-}	Nucleation	Systemic	E14 arrest, increased proliferation, neural tube defects	Yes	(66)
<i>FIP200</i> ^{-/-}	Initiation	Systemic	E13.5-16.5 lethal, heart and liver defects	Yes	(171)
<i>Ulk1</i> ^{-/-}	Initiation	Systemic	Anemia, impaired erythrocyte mitochondrial clearance	No	(154)
<i>Ulk2</i> ^{-/-}	Initiation	Systemic	No overt phenotype	No	(313)
<i>Ulk1/2</i> ^{-/-} <i>Atg3</i> <i>Atg5</i> <i>Atg7</i> <i>Atg9a</i> <i>Atg16L1</i>	Initiation (Ulk1/2), Elongation (Atg3,5,7, 16L1)	Systemic	Neonatal lethal, reduced birth weight, lower tissue amino acid levels (all), certain gene/model specific defects reported (ex. <i>Atg5</i> ^{-/-} decreased adipose mass; <i>Atg9a</i> ^{-/-} innate immune defects; <i>Atg16L1</i> ^{-/-} inflammation)	Yes*	(314), (134, 168, 315-317)
<i>Atg4C</i> ^{-/-}	Elongation	Systemic	Increased tumorigenesis	No	(318)
<i>Nix</i> ^{-/-}	Mitophagy	Systemic	Anemia, impaired erythrocyte mitochondrial clearance	No	(97)
<i>Map1lc3b</i> ^{-/-} <i>Gabarap</i> ^{-/-}	Ubiquitin-like cascades	Systemic	No overt phenotype	No	(319, 320)

*Mice survive embryogenesis due to maternal Atg protein (164) but die soon after birth, due to suggested neurological impairment resulting in suckling defects.

4.2 Tissue-restricted knockout of Atg or related genes

Due to the early lethality in systemic knockouts of Atg's and related genes, there exist many tissue-specific Atg deletion models, used to study the role of autophagy in adult tissues *in-vivo*. Most commonly employing the Cre-LoxP system (321) (322) (323), these cover neurons, hepatocytes, cardiomyocytes, skeletal muscle, adipose tissue as well as multiple hematopoietic cell types, to name a few (6). These models have revealed the importance of autophagy for homeostasis of organelles and proteins in non-dividing, mature cells, as common features of Atg knockouts across cell types include abnormal organelle accumulation (particularly mitochondria) and aggregates positive for ubiquitin and p62, with associated toxic effects on the tissue (6). Of particular note are the neuronal knockouts; loss of function in the gene encoding ubiquitin ligase PARKIN is associated with early-onset neurodegeneration in Parkinson's disease, and PARKIN has been found to play a vital role in targeting mitochondria for selective degradation via autophagy in a manner dependent on the PINK1 kinase and p62 (324) (325) (326). The delineation of this mechanism, combined with the severe neurodegenerative phenotypes seen in central nervous system (nestin-Cre driven) knockouts of *Atg5* and *Atg7* (162, 163) (102), with an even more severe phenotype observed in a nestin-driven *FIP200* knockout (327), have created intense interest in autophagy's involvement in neuronal pathologies (328). Similarly, defects in organelle clearance and survival in the absence of autophagy have been noted in several hematopoietic lineages.

4.3 Atg knockout in hematopoietic lineages

The first hematopoietic tissue-specific Atg knockout examined *Atg5*^{-/-} chimeras, generated via transplant of *Atg5*^{-/-} E14 fetal hematopoietic progenitors to lethally irradiated hosts (156). These progenitors were able to successfully reconstitute the host hematopoietic

compartment. However chimeric mice showed reduced thymic cellularity 6-10 weeks after rescue, despite a normal CD4/CD8 subset developmental profile and no enhanced apoptosis in *Atg5*^{-/-} thymocytes. Peripheral survival of T and B cells appeared impaired, with CD8⁺ T-cells in particular undergoing increased apoptosis. T-cells also showed impaired proliferation in response to CD3 stimulation. In contrast, Gr1⁺ cells were able to survive normally (156). These results suggested a particular lymphocyte requirement for autophagy, supported by subsequent studies of *Atg7* deletion (100, 303) (7) and linked to metabolism, with altered mitochondrial transcription and increased mitochondrial mass in *Atg5*^{-/-} peripheral T-cells (303), while *Atg7*^{-/-} T-cells showed defective ATP generation following activation (329). Interestingly, differences were reported between lymphocytes in *Atg5*^{-/-} and *Atg7*^{-/-} models using *Lck-Cre* to drive excision in early thymocytes. For example, *Atg7*^{fl/fl};*Lck-Cre*⁺ mice showed mitochondrial retention in CD4⁺ and CD8⁺ single positive populations in the thymus, not replicated in the equivalent *Atg5*^{-/-} model (100).

The B-cell phenotype of *Atg5*^{-/-} chimeric mice was expanded on by a subsequent study, which employed in parallel *CD19-Cre* to drive excision of *Atg5* in B-cells specifically. The authors concluded ATG5 was required both during B-cell development in the bone marrow at the pro- to pre- B-cell stage as well as for maintenance of B-1 a B-cells in the periphery, with both subsets associated with increased apoptosis in autophagy's absence (282). Separately, a recent study has defined a role for autophagy in the function of plasma cells. Differentiation of plasma cells from B-cells in *Atg5*^{fl/fl};*CD19-Cre* mice was largely unimpeded, despite increased autophagy during the process, but *Atg5* deficient plasma cells displayed increased immunoglobulin synthesis, leading to higher ER stress and decreased viability over time, ultimately impairing immune response *in vivo* (330). Indeed,

it appears that, unlike most developing and circulating B-cells, the maintenance of longer lived memory B-cells requires autophagy (331).

By contrast, there have been relatively few investigations published of myeloid-specific Atg knockout mice, compared to several studies using Atg ablation in transformed myeloid cell line models (158, 332, 333). A study of mice with CD11c-Cre driven deletion of *Atg5* found *Atg5*^{-/-} dendritic cells suffered from impaired antigen presentation, leading to decreased survival following viral infection (334). More recently, mice with Cre under the control of the M lysozyme (Lys-M) promoter, and therefore specific for myeloid cells (335), were crossed to *Atg5*^{fl/fl} mice and assayed for their response to *Mycobacterium tuberculosis* infection. *Atg5*^{fl/fl};*LysM-Cre* mice showed increased bacterial burden and increased pulmonary neutrophil infiltration, while *Atg5*^{-/-} macrophages secreted excess IL-1a even under steady-state conditions (336). Therefore autophagy appears dispensable for the survival of myeloid cells in-vivo, but may play a role in functional maturation. There is evidence that autophagy is required for full development of macrophages ex-vivo using *Atg7*^{-/-} monocytes (337).

As noted above, mice with systemic knockout of autophagy initiation complex member *Ulk1* were viable, likely due to redundancy with ULK2, but developed a moderate anemia (154). The generation of red blood cells, or erythropoiesis, starts in erythroblastic islands, specialized structures in the fetal liver, spleen and bone marrow that consist of a macrophage surrounded by erythroid cells developing from a common progenitor, the pronormoblast (338) (105). Pronormoblast division creates normoblasts, which extrude their nuclei then leave the island as reticulocytes, which mature into erythrocytes in the peripheral blood by degradation of remaining organelles, including ribosomes, golgi and, importantly, mitochondria (339, 340). Systemic knockout of the mitochondrial protein and

Bcl-2 family member NIX (or BNIP3L) resulted in anemia with reduced mature erythrocytes in circulation (341). Remaining erythrocytes showed reduced lifespan associated with retention of mitochondria, and NIX has subsequently been shown to be an adaptor protein for mitophagy (97) (98). Similarly, mitochondrial clearance is impaired, although not completely abrogated, in *Atg7*^{-/-} fetal liver chimeras (153). Autophagy was found to increase with erythrocyte differentiation stage in-vitro, corresponding with mitochondrial removal, although such measurements have not been confirmed in-vivo (154). Thus autophagy convincingly plays a vital role in the internal remodeling required during the transition from progenitor to functional erythrocyte. Interestingly, no erythroid phenotype or lethality was reported in *Atg5*^{-/-} fetal liver chimeric mice described above (156), contrasting the *Atg7*^{-/-} chimera (153), although viability defects in *Atg5*^{-/-} chimeras have subsequently been suggested (303). Also, a lineage specific knockout of *Atg7* driven by the erythropoietin receptor (ErGFP-Cre) showed a relatively mild anemia (342), and indeed in all the models above a subset of erythrocytes were still able to clear mitochondria (342). This suggests an alternative method of organelle degradation may act at this stage to partially compensate for autophagy's loss, and in fact an *Atg5/7* independent autophagy pathway has been proposed (343). Therefore some questions of stage and gene-specificity regarding autophagy in erythrocyte development remain.

4.4 Knockout of Atgs in HSCs – *FIP200* and *Atg7*

The first study to outline the importance of autophagy for maintenance of HSCs used a Tie2 driven Cre recombinase to drive excision of *FIP200* (344). *Tie2* encodes an angiopoietin receptor expressed in both hematopoietic and endothelial cells during embryogenesis (345) as well as in a quiescent, osteoblast-associating side population of adult HSCs (219). *Tie2-Cre* is reported to drive expression in 80-85% of all lineages of

blood cells from the embryonic yolk sac to adult bone marrow (346). The Tie2 mediated excision of *FIP200* led to perinatal lethality, with reduced knockout frequency at birth and all *FIP200^{fl/fl};Tie2-Cre⁺* mice dying within 7 days (344). Analysis revealed a progressive depletion of *FIP200*^{-/-} fetal HSCs from E14.5, along with a severe HSC functional defect in competitive repopulation assays, which was reproduced using a conditional *Mx-Cre* chimera to demonstrate the cell-autonomous nature of the phenotype. In keeping with defects observed in other autophagy depleted cell types, E14.5 *FIP200*^{-/-} fetal HSCs showed increased mitochondrial mass and ROS levels along with increased proliferation, yet unaltered apoptosis. The model also displayed defects in hematopoietic lineages, as *FIP200*^{-/-} embryos were anemic, associated with an erythrocyte maturation block, whereas cells of the myeloid lineage were observed to be greatly expanded by E18.5. Thus, FIP200, and potentially autophagy, were concluded to be indispensable for fetal hematopoiesis (344).

However, subsequent studies by our lab have suggested the HSC autophagy requirement arises later. *Atg7^{fl/fl}* mice were crossed to *Vav-iCre* (133) mice to drive excision of *Atg7* in hematopoietic cells (7). *Vav-iCre* has been shown to drive efficient Lox-P excision shortly after HSC emergence in the embryo (347), with expression in fetal hematopoietic progenitors (348) as well as adult HSCs (349) and therefore in all hematopoietic lineages (348), while it is inactive in non-hematopoietic cell types including endothelial cells (348) (133) (350). Unlike *FIP200^{fl/fl};Tie2-Cre⁺* mice, *Atg7^{fl/fl};Vav-iCre⁺* (hereafter *Vav-Atg7*^{-/-}) mice are able to survive post-natally, however they died within 12 weeks with bone marrow failure. Despite an early expansion of multipotent progenitors, which displayed increased ROS and mitochondrial content, *Atg7*^{-/-} HSCs were gradually lost, and *Atg7*^{-/-} bone marrow was unable to reconstitute irradiated hosts (131). These data suggest *Atg7* is required for adult HSC maintenance but perhaps not in fetal progenitors. *Vav-Atg7*^{-/-} mice

also had lineage defects, including progressive anemia, with an erythroid developmental block (development defined by surface phenotype as (351)) and mitochondrial accumulation, along with lymphopenia, supporting results from *Atg7*^{-/-} chimeric mice. Moreover, similar to the FIP200 model, *Vav-Atg7*^{-/-} mice showed an accumulation of CD11b⁺Gr1⁺ myeloid cells in the blood, bone marrow and infiltrating into several non-hematopoietic organs (131). Table 3.2 contains a summary of hematopoietic-system Atg knockout models.

Table 4.2: Mouse models with hematopoietic autophagy gene deletion

Gene	Cre/Tissue	Phenotype	Lethal	Ref
Atg5 ^{-/-}	Fetal liver chimera	Reduced thymic cellularity, impaired lymphocyte peripheral survival, altered T-cell metabolism, increased T-cell peripheral mitochondrial mass	No*	(156)
Atg5 ^{-/-}	Lck-Cre/Thymocytes	Increased peripheral T-cell mitochondrial mass, increased ROS, peripheral survival defect	No	(100)
Atg7 ^{-/-}	Lck-Cre/Thymocytes	Increased peripheral T-cell mitochondrial mass, increased ROS, increased CD4 ⁺ /CD8 ⁺ single positive thymocyte mitochondrial mass, peripheral survival defect	No	(100)
Atg5 ^{-/-}	CD19-Cre/B-cells	Defect at pro- to pre- B-cell development stage, peripheral B-1 a B-cell survival decreased, defective long-term plasma cell and memory B-cell maintenance	No	(282) (330). (331)
Atg5 ^{-/-}	CD11c-Cre/Dendritic cells	Impaired antigen processing	No	(334)
Atg5 ^{-/-}	LysM-Cre/myeloid cells	Increased steady state macrophage IL-1a secretion, impaired response to bacterial infection	No	(336)
Atg7 ^{-/-}	Fetal liver chimera	Anemia, impaired erythrocyte mitochondrial clearance	No**	(153)
Atg7 ^{-/-}	ErGFP-Cre/erythrocytes	Mild anemia, moderate impairment to reticulocyte mitochondrial clearance	No	(7)
FIP200 ^{-/-}	Tie2-Cre/HSCs and hematopoietic system	Perinatal lethal, HSCs decreased from E14.5, display repopulation defects, increased mitochondrial mass and ROS, anemia, myeloid expansion	Yes	(344)
Atg7 ^{-/-}	Vav-iCre/HSCs and hematopoietic system	Lethal 10-12 weeks, progressive anemia, lymphopenia, myeloid expansion, HSC population depleted, LSK increased mitochondrial mass and ROS	Yes	(131)

*No reported lethality (156), contrasting subsequent reports (303) **50% of *Atg7*^{-/-} fetal liver recipient mice died, surviving mice generally healthy

4.5 The gene-specificity of Atg hematopoietic phenotypes

The data generated thus far by murine Atg knockout models has provided convincing evidence of a role for these genes at several stages of mammalian development, including in the hematopoietic system. However, as observed in the phenotypes of the systemic Atg knockouts, the severity and timing of the defects differ between models. In lymphoid cells, differences were observed in Lck-Cre driven *Atg5*^{-/-} and *Atg7*^{-/-} models, with additional abnormalities detected in the latter (100). Similarly, no erythroid defects were reported in *Atg5*^{-/-} fetal liver chimeric mice, whereas *Atg7*^{-/-} chimeric mice (153) and *Vav-Atg7*^{-/-} mice (7) both develop severe anemias. Examining HSCs, *Vav-Atg7*^{-/-} mice show a progressive loss of adult HSCs (131), while in *FIP200^{fl/fl};Tie-2⁺* mice, the negative impacts seem to arise much earlier during fetal hematopoiesis (344), despite both recombinases being active at that stage.

Differences between Atg knockout phenotypes in similar or identical contexts suggest Atg gene-specific effects. As in HSCs, the systemic (171) or neuronal removal (327) of *FIP200* seems particularly detrimental, which may be due to FIP200's role in the early initiation complexes of autophagy, or possibly autophagy independent mechanisms. FIP200 has been reported to regulate cell cycle progression and G1 arrest by interacting with key cell cycle protein p53 (310) and driving transcription of cell cycle inhibitors RB1, p16 and p21 (311). Similarly, Beclin1 impacts autophagy and apoptosis through its interactions with anti-apoptotic protein B-cell lymphoma 2 (BCL-2) (45).

ATG7 and ATG5, both required at a similar stage of autophagy during autophagosome membrane elongation by the ubiquitin-like cascades, have separate roles outside the canonical autophagy pathway. Subsequent to our lab's publications on the *Vav-Atg7* model, ATG7 was reported to directly bind p53 in mouse embryonic fibroblasts following

metabolic stress and, independent of E1-like activity in the elongation cascades, induce cell cycle inhibition through *p21cdkn1a* transcription (352). Loss of the ATG7-p53 interaction was implicated in the increased mitochondrial ROS, DNA damage response and apoptosis observed following *Atg7* deletion. Rescue of the pro-apoptotic response and, interestingly, partial rescue of the neonatal survival defect in systemic *Atg7*^{-/-} mice, could be achieved by simultaneous deletion of p53 apoptosis-regulating kinase *Chk2* (353), attributing that phenotype to upregulated p53 programs rather than autophagy loss (352). Although the autophagy-independent nature of these ATG7 effects have been questioned by field experts, given general similarity between *Atg* knockout models (354), these observations do cast doubt on whether autophagy is solely to blame for HSC DNA damage, ROS and loss in *Vav-Atg7*^{-/-} mice. Separately, Radoshevich *et al.* have suggested that, distinct from starvation induced autophagy/mitophagy, ATG12 may be linked to ATG3, rather than its normal final autophagy cascade partner ATG5, in an ATG7 and ATG3 activity dependent manner. The ATG12-ATG3 complex may regulate mitochondrial homeostasis by controlling mitochondrial fusion/fission and may play a role in mitochondrial cell death pathways (355). Therefore, *Atg7* loss (but not *Atg5* deletion) could impact mitochondrial dynamics and cell cycle arrest outside the canonical autophagy pathway.

Like ATG7, ATG5 can affect apoptosis directly; however its impacts appear to be largely pro-apoptotic. ATG5 can bind to extrinsic apoptosis effector FADD (fas-associated protein with death domain) via its C-terminal region to drive interferon- γ associated cell death (277). Separately, ATG5 has been reported to be cleaved by the proteases calpain 1 and 2 following apoptotic stimuli (278). The resulting ATG5 fragment was translocated to the mitochondria where it bound anti-apoptotic protein BCL-XL to enable mitochondrial destabilization, cytochrome-c release and apoptosis (278) (356). Although overexpression

of ATG5 was associated with increased autophagy and apoptosis sensitivity, truncated ATG5 induced only the latter (278). These pro-apoptotic ATG5 functions have thus been proposed as dissociated from its role in the autophagy pathway (45).

Importantly, none of these autophagy-independent, gene-specific roles for *Atg7* or *Atg5* have been reported to involve both genes. Therefore comparisons of *Atg5* and *Atg7* deficient phenotypes under similar circumstances would assist in the exclusion of gene-specific artifacts from the roles of autophagic degradation.

Figure 4.1: Genotyping of *Vav-Atg5^{flox/flox};Vav-iCre* mice from ear biopsy DNA. (a) Example PCR amplification of *Atg5* Fl (Flox) and WT (wildtype) alleles from extracted ear biopsy DNA, PCRs performed separately and run in separate wells on 1% agarose/TBE for 30min at 76V. (b) Example PCR Amplification of *iCre* from extracted ear biopsy DNA, run on 1% agarose/TBE for 30min at 76V. Genotypes: Mouse 1 *Atg5^{fl/wt};Vav-iCre⁻*, Mouse 2 *Atg5^{fl/fl};Vav-iCre⁺*, Mouse 3 *Atg5^{wt/wt};Vav-iCre⁻*.

To confirm that the presence of *Vav-iCre* and floxed *Atg5* alleles in *Atg5^{fl/fl};Vav-iCre⁺* mice (hereafter called *Vav-Atg5^{-/-}* mice) led to loss of *Atg5* expression in hematopoietic cells, bone marrow was extracted from these mice or wildtype littermates (those lacking *Vav-iCre*). The level of *Atg5* mRNA, along with the expression of several other autophagy genes, was quantified by real-time quantitative PCR (qPCR). The comparative Ct ($\Delta\Delta C_t$) method was used to determine autophagy gene expression. Ct values for each sample were normalized to the average Ct of three endogenous control genes in that sample (*Gapdh*, $\beta 2M$ and *Hprt*) and the resulting ΔC_t for autophagy genes in *Vav-Atg5^{-/-}* samples was compared to a wildtype reference to obtain a $\Delta\Delta C_t$ value. This was converted to a measurement of relative abundance by a $2^{-\Delta\Delta C_t}$ calculation. The resulting data showed that *Atg5* expression was almost completely ablated in *Vav-Atg5^{-/-}* bone marrow (Figure 4.2).

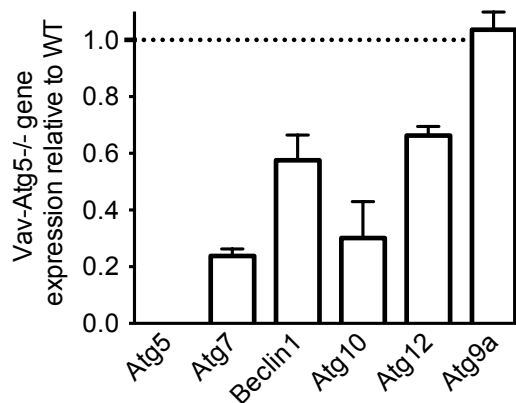


Figure 4.2: Loss of *Atg5* expression in *Vav-Atg5^{-/-}* bone marrow. qRT-PCR of key autophagy genes in bone marrow of *Vav-Atg5^{-/-}* mice, values determined by $\Delta\Delta C_t$ method, normalized to an average of *GAPDH*, *HPRT* and $\beta 2M$ expression and compared to wildtype littermate values for relative expression. Error bars represent SD ($n=3$ pooled).

Interestingly, the expression of several other autophagy genes, including *Atg7*, *Beclin1* and *Atg10*, was also decreased in these cells, although not to the same extent (Figure 4.2).

Similar patterns were observed in other autophagy knockout situations, and it is possible that the dysfunctional pathway, fatally hampered by *Atg5* deletion, is generally down-regulated by cells over time as compensation for its loss.

Cre driven loss of *Atg5* has been previously shown to result in the absence of autophagic activity (162). To confirm this in *Vav-Atg5*^{-/-} hematopoietic progenitors, the imaging flow cytometry autophagy assay (IFCAA) was employed to examine progenitor autophagic function, using the IFCAA ‘flux’ measurement. This approach, described in Chapter 3.8, assesses the colocalization between LC3 and lysosome populations after inhibition of lysosomal proteases compared to basal levels (140). Due to limitations on surface marker channels on the Image Stream 100, the progenitor marker CD34 was combined with a lineage (Lin) marker staining to identify Lin-CD34⁺ hematopoietic progenitors; in murine bone marrow, CD34⁺ is expressed on Lin⁻ multipotent progenitors and early oligopotent progenitors (291). While Lin-CD34⁺ cells in wildtype littermates showed a net increase in LC3/lysosomal colocalization after autolysosome clearance was artificially impaired (BDS Basal+I/Basal > 1), the *Vav-Atg5*^{-/-} progenitors showed no such accumulation, indicating no ongoing flow through the autophagic pathway and therefore loss of autophagic function (Figure 4.3). Overall LC3 levels in murine cells missing *Atg5* or *Atg7* were often found to be higher than wildtype equivalents under basal conditions, likely attributable to increased unconjugated LC3-I in the cytoplasm. Also, it is worth noting that, as has been previously reported for *Vav-Atg7*^{-/-} mice (7) (131) and in other murine *Atg5* deletion models (162), mice with heterozygote loss of *Atg5* in the hematopoietic system (*Atg5*^{fl/wt}; *Vav-iCre*⁺) were found to have functional autophagy and displayed no overt observable phenotype

(not shown). Therefore heterozygotes were used equally along with *Atg5^{fl/wt};Vav-iCre-* and *Atg5^{fl/fl};Vav-iCre-* mice in littermate controls for *Vav-Atg5^{-/-}* mice.

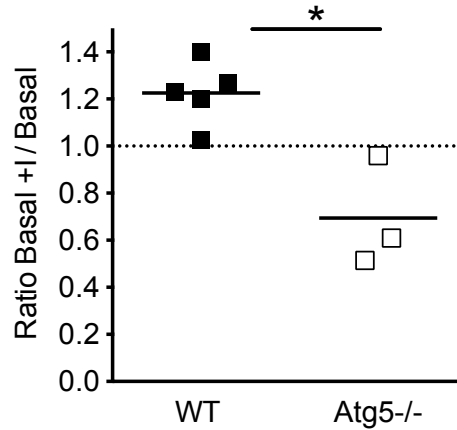


Figure 4.3: Loss of autophagy in *Vav-Atg5^{-/-}* hematopoietic progenitors. Ratio of LC3/LysolID BDS of Lin-CD34⁺ cells from *Vav-Atg5^{-/-}* or wildtype littermate bone marrow after 2hrs treatment with 10µg/ml E64d/Pepstatin A (basal+I) or medium alone (basal). **p*<0.05, unpaired *t* test.

4.7 Erythrocytes in *Vav-Atg5^{-/-}* mice have a developmental block and accumulate damaged mitochondria

Loss of *Atg7* has been previously reported to result in erythrocyte mitochondrial accumulation, damage and anemia *in vivo* (153) (7). However, anemia was not reported in *Atg5^{-/-}* fetal liver chimeric mice (156) (342). To investigate the state of erythropoiesis in *Vav-Atg5^{-/-}* mice, blood taken from *Vav-Atg5^{-/-}* or wildtype littermates was stained for the transferrin receptor (CD71) and glycophorin A (Ter119). These markers subdivide erythroid developmental stages in the bone marrow into four stages: Ter119⁻/CD71^{high} (Stage I, proerythroblasts), Ter119⁺CD71^{high} (Stage II, basophilic erythroblasts), Ter119⁺CD71^{med} (Stage III, polychromatophilic erythroblasts) and Ter119⁺CD71⁻ (Stage IV, orthochromatophilic erythroblasts) (351). In wildtype mice at 6 weeks of age, the more immature erythroid stages accounted for only a small proportion of the total population. In *Vav-Atg5^{-/-}* the mature population (stage IV) was significantly decreased,

with the frequency of the immature stages abnormally high (Figure 4.4). This loss of mature erythroid cells, or anemia, was confirmed via absolute numbers quantification with TruCount beads (Figure 4.4).

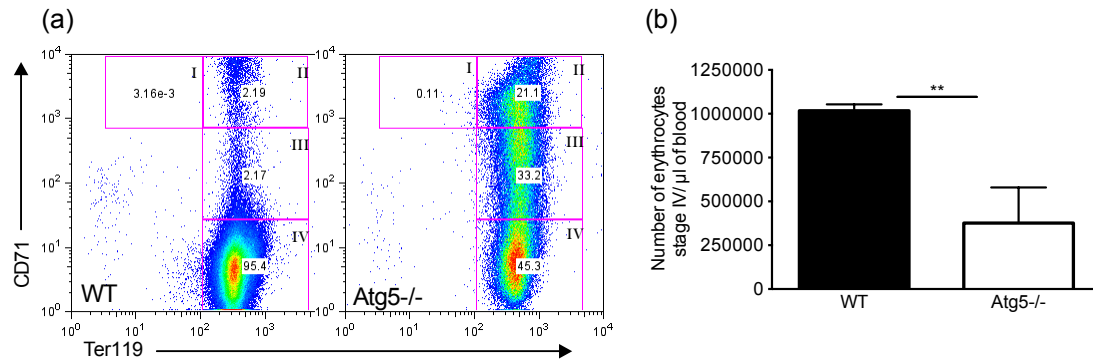


Figure 4.4: *Vav-Atg5*^{-/-} mice display erythroid developmental block and anemia. (a) Representative dot plots from *Vav-Atg5*^{-/-} (right) and wildtype littermate (WT) (left) blood at 6 weeks of age showing CD71/Ter119 surface marker defined erythroid development stages I-IV. (b) Absolute blood count in *Vav-Atg5*^{-/-} mice and WT littermates of stage IV erythroid cells at 9 weeks of age. Error bars represent SEM (n=3), **p<0.01, unpaired t test. Grateful acknowledgement of Shivani Khandwala for the absolute blood counts.

The removal of organelles, including mitochondria, is a vital stage of erythroid development in the periphery (339) (340), and loss of other autophagy genes has been shown to impair mitochondrial removal (155) (154). In particular, *Atg7*^{-/-} erythrocytes have been found to retain significant mitochondrial volume, associated with increased mitochondrial ROS (7). However, mitochondrial retention by thymocytes (as measured by mitochondrial volume-indicating fluorescent dye Mitotracker Green) has previously reported in absence of *Atg7* but not *Atg5* (100). To determine if ATG5 deficiency altered mitochondrial dynamics in *Vav-Atg5*^{-/-} erythrocytes, the staining levels of Mitotracker Green and mitochondrial superoxide indicator MitoSOX were measured in the abnormally increased erythrocyte developmental compartments. These stainings showed heightened levels of mitochondrial content and ROS production in the Ter119⁺CD71^{med} blood cells of *Vav-Atg5*^{-/-} mice (Figure 4.5), the stage when mitochondria are degraded during

erythroid development. This indicates that ATG5 is indeed required for proper mitochondrial removal during peripheral erythrocyte development. Absolute quantifications and mitochondrial stainings were performed with lab member Shivani Khandwala.

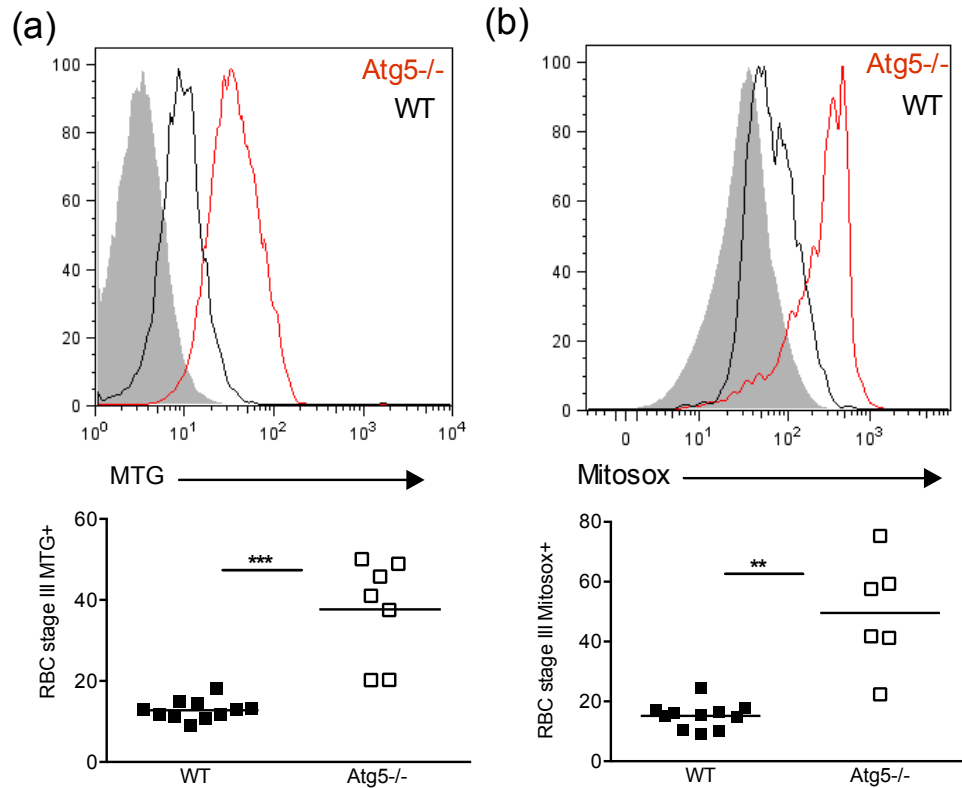


Figure 4.5: Abnormal mitochondrial retention in Vav-Atg5^{-/-} erythroid cells. (a) MitoTracker Green intensity in stage III Vav-Atg5^{-/-} and wildtype littermate (WT) erythroid cells (defined as Figure 4.4), with representative overlaid histograms for each genotype (top, tinted histogram indicates unstained control) and frequency of MitoTracker Green positive cells (bottom). (b) MitoSOX intensity in Stage III Vav-Atg5^{-/-} and WT littermate erythroid cells, with representative overlaid histograms for each genotype (top, tinted histogram indicates unstained control) and frequency of MitoSOX positive cells (bottom). ** $p < 0.01$, *** $p < 0.001$, unpaired t test. Grateful acknowledgement of Shivani Khandwala for assistance with this experiment.

Loss of mitochondrial integrity may be a natural step in the development of erythrocytes, signaling to autophagy for mitochondrial removal, but failure to clear such damaged mitochondrial is a likely cause of mitochondrial ROS accumulation and cell death (7). Increased levels of autophagy during erythroid development has been previously

correlated with the stage of mitochondrial removal *in vitro* (154). To assess autophagy during the above erythroid developmental populations *in vivo*, Ter119/CD71 expression stages were identified in the bone marrow of GFP-LC3 mice and the number of GFP-LC3 puncta quantified by Image Stream as in Chapter 3.17. The number of erythroid cells expressing 4 or more GFP-LC3 puncta, initially absent in early developmental stages, was significantly increased in Stage III (Ter119+CD71med) and Stage IV (Ter119+CD71-) populations (Figure 4.6), correlating well with the stages reported to show progressive mitochondrial removal *in vivo* (7) and with the developmental block seen in *Vav-Atg5*^{-/-} or *Vav-Atg7*^{-/-} mice.

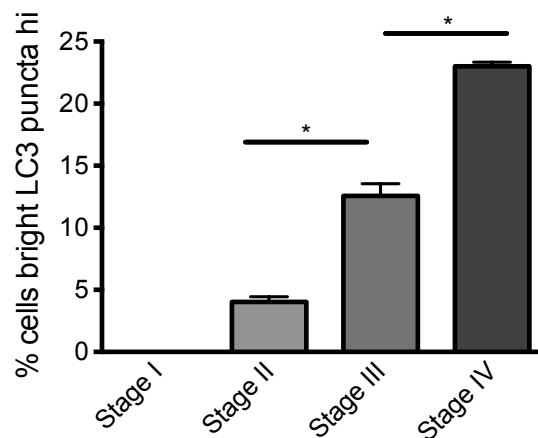


Figure 4.6: Autophagy in wildtype erythroid cells increases during development. Frequency erythroid cells stage I-IV (defined as Figure 4.4) with four or more bright GFP-LC3 puncta in GFP-LC3 transgenic mouse bone marrow, quantified using Image Stream (40,000 cells analyzed per sample). Error bars represent SEM ($n=4$), $*p<0.05$, unpaired t test.

4.8 Peripheral lymphocyte populations are reduced in *Vav-Atg5*^{-/-} mice

As alluded to above, similar to erythrocytes, peripheral survival of lymphocytes was found severely hampered in *Vav-Atg7*^{-/-} mice (7), and ATG5 has been reported as vital for T-cell peripheral survival in *Atg5*^{-/-} chimeric mice (156). The impact of hematopoietic-specific

Atg5 deletion on lymphocyte survival was investigated in *Vav-Atg5*^{-/-} mice, with severe decreases in the frequency of both CD8⁺ and CD4⁺ cells detected in the spleen of *Vav-Atg5*^{-/-} mice at 6 weeks of age as compared to wildtype littermates (Figure 4.7). The magnitude of the CD8⁺ and CD4⁺ loss was comparable to that found in *Vav-Atg7*^{-/-} mice of a similar age (7). Lymphopenia was confirmed by quantification of absolute CD3⁺ cell counts in the blood of *Vav-Atg5*^{-/-} (Figure 4.7). These results confirm a role for autophagy and ATG5 in the maintenance of T-lymphocytes.

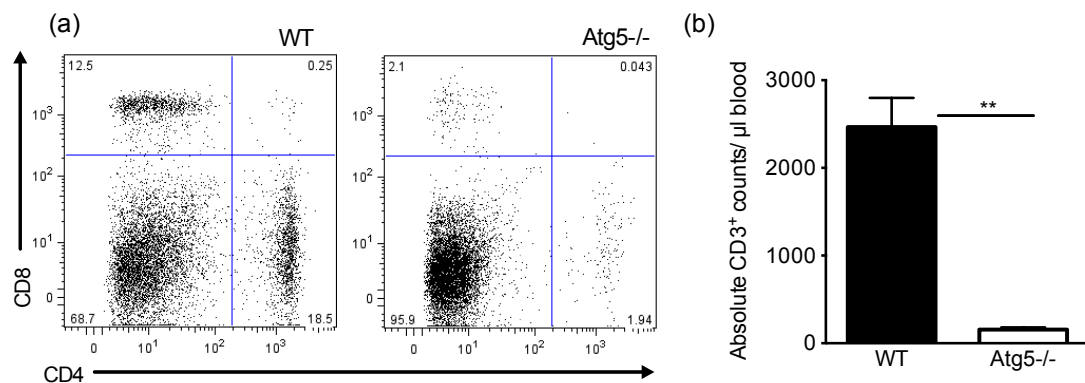


Figure 4.7: T-cell lymphopenia in *Vav-Atg5*^{-/-} mice. (a) Representative dot plots showing CD4 and CD8 expression in *Vav-Atg5*^{-/-} (right) and wildtype (WT) littermate (left) spleen at 6 weeks of age. (b) Absolute blood counts in *Vav-Atg5*^{-/-} and WT mice of CD3⁺ T-cells at 6 weeks of age. Error bars represent SEM ($n=3$), ** $p<0.01$, unpaired t test. Grateful acknowledgement of Daniel Puleston for assistance with absolute blood counts.

The importance of autophagy for the peripheral survival of B-lymphocytes has been reported less consistently than T-cell counterparts, with the peripheral survival defect in a CD19-Cre driven B-cell knockout of *Atg5* restricted to B-1a cells (282). However, investigation of *Vav-Atg5*^{-/-} showed that there was a significant loss of CD19⁺ B-cells in the blood by 6 weeks of age (Figure 4.8). A similar decrease in B-cells was found in *Vav-Atg7*^{-/-} mice (7), and may represent cumulative damage stemming from the earlier expression of *Vav-iCre* in the development of hematopoietic lineages. Lymphoid absolute counts were aided by lab member Dan Puleston.

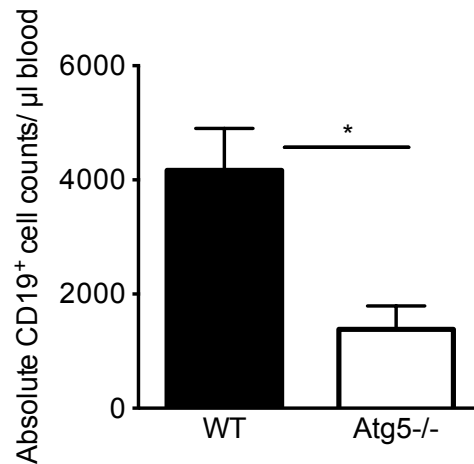


Figure 4.8: B-cell lymphopenia in *Vav-Atg5*^{-/-} mice. Absolute blood counts in *Vav-Atg5*^{-/-} and WT mice of CD19⁺ B-cells at 6 weeks of age. Error bars represent SEM (n=3), ***p*<0.01, unpaired *t* test. Grateful acknowledgement of Daniel Puleston for assistance with absolute blood counts.

4.9 *Vav-Atg5*^{-/-} mice show early expansion of multi-potent progenitors followed by progenitor loss and lethal bone marrow failure

Although the lineage phenotypes of the *Vav-Atg7*^{-/-} mouse were significant, a dominant contributor to the lethality of the genotype, with a median survival of 12 weeks (7), was a progressive stem cell defect leading to bone marrow failure (131). Similar to *Vav-Atg7*^{-/-} mice, *Vav-Atg5*^{-/-} mice became symptomatic (lethargy, piloerection, weight loss, pale paws) at approximately 9 weeks of age. Penetrance was 100%, although precise onset of symptoms varied between individuals. Culling was required by 10-12 weeks (not shown). To ascertain whether this reflected alterations to the stem cell compartment, the relative abundance of the Lin-Sca1+ckit⁺ (LSK) multipotent progenitors was assessed in the bone marrow of 6 week old (asymptomatic) and 9 week old (symptomatic) *Vav-Atg5*^{-/-} mice by flow cytometry, as compared to wildtype littermates (therefore aged-matched controls).

At 6 weeks, the LSK compartment of *Vav-Atg5*^{-/-} was significantly increased (Figure 4.9), indicating a possible expansion of surface-phenotypic multipotent progenitors at this stage.

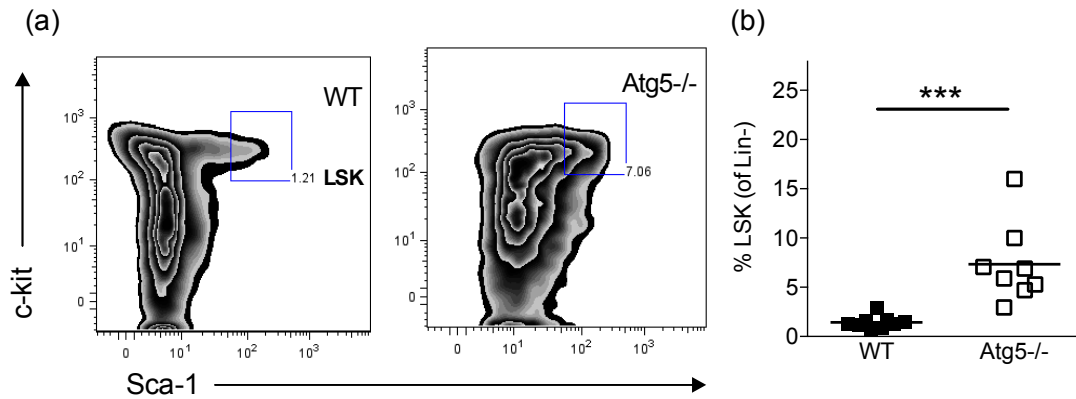


Figure 4.9: LSK population is expanded in *Vav-Atg5*^{-/-} mice at 6 weeks of age. (a) Representative contour plots of cKit and Sca1 expression in lineage negative (Lin⁻) bone marrow of *Vav-Atg5*^{-/-} mouse (right) and wildtype (WT) littermate (left) at 6 weeks of age. (b) Frequency of Lin⁻Sca1⁺cKit⁺ cells (gated as in a) among Lin⁻ parent population in *Vav-Atg5*^{-/-} or WT littermate bone marrow at 6 weeks of age. ****p* < 0.001, unpaired *t* test.

However this expansion was only temporary, as LSK frequency dropped to significantly below wildtype levels by 9 weeks of age (Figure 4.10), correlating with the appearance of clinical symptoms.

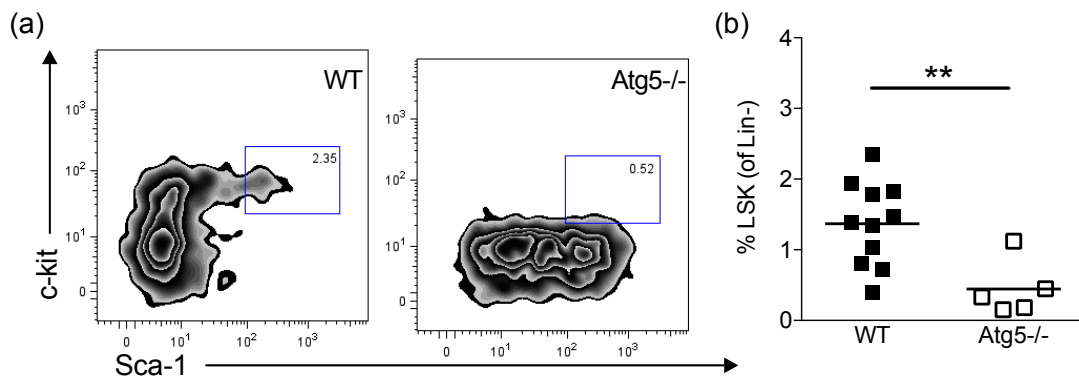


Figure 4.10: LSK population is contracted in *Vav-Atg5*^{-/-} mice at 9 weeks of age. (a) Representative contour plots of cKit and Sca1 expression in lineage negative (Lin⁻) bone marrow of *Vav-Atg5*^{-/-} mouse (right) and wildtype (WT) littermate (left) at 9 weeks of age, showing Lin⁻Sca1⁺cKit⁺ (LSK) population gating. (b) Frequency of LSK cells (gated as in a) among Lin⁻ parent population in *Vav-Atg5*^{-/-} or WT littermate bone marrow at 9 weeks of age. ***p* < 0.01, unpaired *t* test.

The loss of upstream progenitors would be expected to severely hamper bone marrow function. To assay the lineage production potential of *Vav-Atg5*^{-/-} bone marrow at 9 weeks, 1×10^5 cells from *Vav-Atg5*^{-/-} or wildtype littermates was suspended in complete methylcellulose media with recombinant cytokines and erythropoietin (M3434). *Vav-Atg5*^{-/-} bone marrow produced significantly fewer colonies of all lineages (myeloid, erythroid, megakaryocyte, mixed)(Figure 4.11). The majority of knockout colonies produced were lost on replating (not shown), indicating the loss of early hematopoietic progenitors in *Vav-Atg5*^{-/-} mice mediates failure of bone marrow colony production.

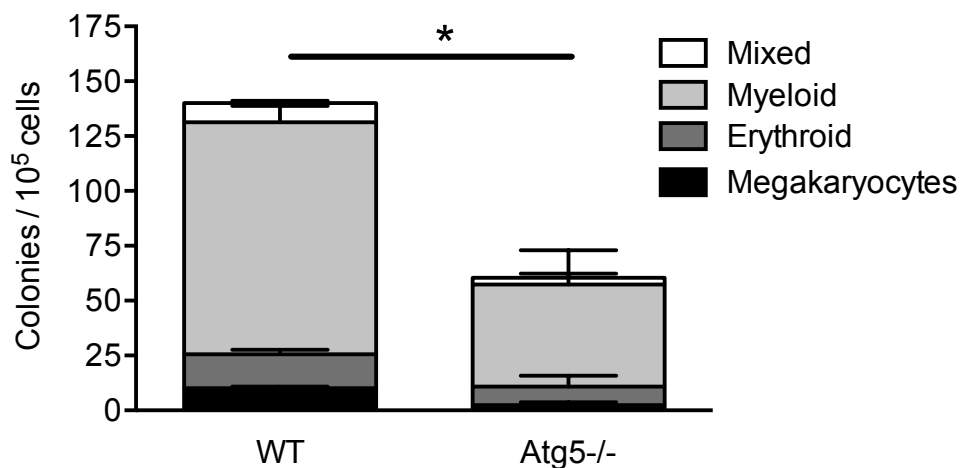


Figure 4.11: Multi-lineage colony formation is impaired in 9 week old *Vav-Atg5*^{-/-} bone marrow.

Quantification of lineage colonies formed by 10,000 bone marrow cells from *Vav-Atg5*^{-/-} or WT mice plated in triplicate in complete methylcellulose (M3434, STEMCELL Technologies) and analyzed at 7 days. Error bars represent SEM ($n=4$), * $p<0.05$, unpaired t test.

4.10 HSCs and multipotent progenitors lose quiescence and are overproliferative in *Vav-Atg5*^{-/-} mice

The expansion of the *Vav-Atg5*^{-/-} LSK multipotent progenitor compartment at 6 weeks of age, followed by LSK contraction and bone marrow failure, suggests a possible loss of HSC division avoidance, or quiescence. Such a loss would be manifested in HSCs (a rare subset of LSK cells) becoming more proliferative and differentiating into multi-potent

progenitors, leading to a loss of true HSCs along with a brief increase in downstream progenitors. Eventual exhaustion of the HSC compartment would lead to cascading loss of downstream progenitors and bone marrow failure. To investigate this hypothesis, the frequency of HSCs (LSK CD150⁺CD48⁻) in 6-week-old *Vav-Atg5*^{-/-} mice was investigated by flow cytometry, revealing a substantial proportional decrease in HSCs (Figure 4.12).

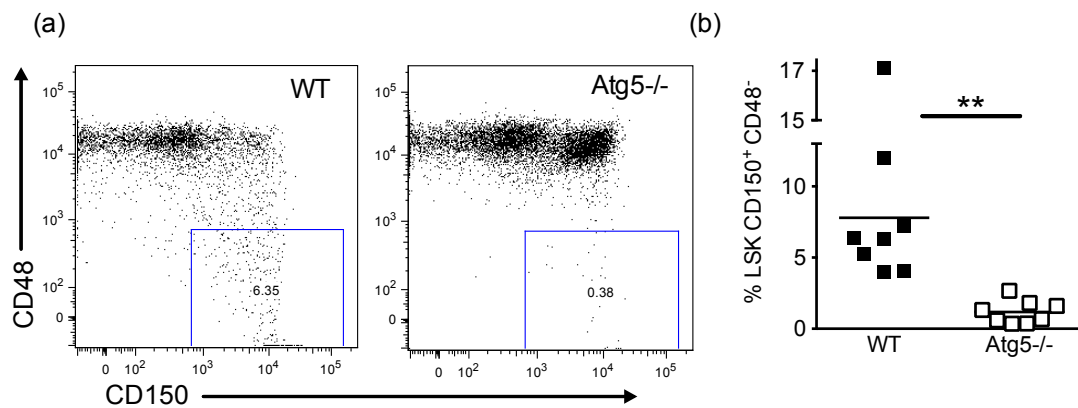


Figure 4.12: Hematopoietic stem cell (HSC) frequency is decreased in *Vav-Atg5*^{-/-} mice by 6 weeks of age. (a) Representative dot plots of CD150 and CD48 expression within the LSK population (gated as in Figure 4.9) in bone marrow of *Vav-Atg5*^{-/-} mouse (right) and wildtype (WT) littermate (left) at 6 weeks of age, showing CD150⁺CD48⁻ (HSC) population gating. (b) Frequency of HSC (gated as in a) among LSK parent population in *Vav-Atg5*^{-/-} or WT littermate bone marrow at 6 weeks of age. ***p* < 0.01, unpaired *t* test.

A similar phenotype of HSC loss preceding progenitor compartment failure was observed in *Vav-Atg7*^{-/-} (131). To expand on the knowledge obtained from both models, the cell cycle status of the *Vav-Atg5*^{-/-} HSC and LSK compartment at 6 weeks was assessed by flow cytometry using the proliferative marker Ki67 and a dye for cellular DNA content, 7-aminoactinomycin D (7AAD). Although its function within the cell remains uncertain, the expression of the Ki67 nuclear protein is characterized to be present during the ‘active’ stages of the cell cycle, namely G1, S, G2 and mitosis (M), yet absent from cells undergoing cell cycle arrest in G0 (357). As such, when plotted against DNA content, it can be used to differentiate those cells in G0 (no Ki67), cells in G1 (Ki67⁺, 2n DNA

content) and cells in S-G2-M (Ki67+, 4n DNA content); this technique has been applied to multiple cell types, including hematopoietic stem cells (215). Applied to *Vav-Atg5*^{-/-} HSCs and LSKs, this delineation revealed a significant increase in the proportion of both populations in S-G2-M phases of the cell cycle, therefore actively proliferating, particularly evident in HSCs (mean of 2.98% S-G2-M in wildtype HSCs to 20.45% in *Vav-Atg5*^{-/-})(Figure 4.13). The proportion of quiescent HSCs was correspondingly decreased to roughly half that found in wildtype counterparts (Figure 4.13).

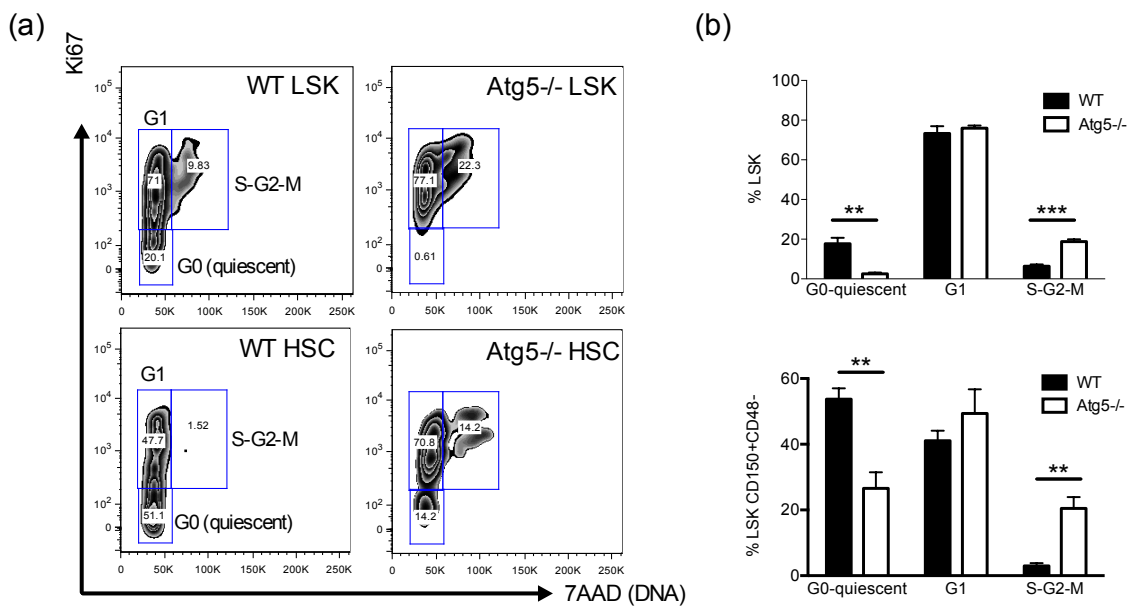


Figure 4.13: Increased proliferation in *Vav-Atg5*^{-/-} HSC and LSK populations by 6 weeks of age. (a) Representative contour plots of LSK or HSC populations (defined as Figure 4.9 and Figure 4.12 respectively) from 6 week old *Vav-Atg5*^{-/-} mice (right) or wildtype (WT) littermates (left) in G0 (quiescence), G1 or S-G2-M cell cycle stages defined by Ki67 staining against DNA content (7AAD intensity) as indicated. (b) Frequency of LSKs and HSCs from *Vav-Atg5*^{-/-} or WT littermate bone marrow in indicated cell cycle stage (populations gated as in a) at 6 weeks of age. Error bars represent SEM (n=4), **p<0.01, ***p<0.001, unpaired t test.

To ascertain if excess proliferation was a general feature of *Vav-Atg5*^{-/-} progenitors or restricted to multipotent progenitors, the status of the more mature oligopotent granulocyte macrophage progenitors (GMPs, Lin-Sca1-cKit+FcyRII/III+) was also probed. At 6 weeks, the relative frequency of GMPs was unaltered in *Vav-Atg5*^{-/-} mice (Figure 4.14)

and their cell cycle phase status was unchanged from wildtype littermates (Figure 4.15). Together, these data indicate ATG5, and autophagy, may be vital for maintaining quiescence in HSCs and thereby preventing excess proliferation. Additionally, the alterations to HSC and LSK cell cycle profiles suggest that deficient autophagy could promote the G1/S transition among proliferating early progenitor cells, as the relative proportion of Ki67+ (proliferating) cells in S-G2-M as compared to G1 was specifically increased in both *Vav-Atg5*^{-/-} populations. However, it is important to note that lineage defects in the *Vav-Atg5*^{-/-} and *Vav-Atg7*^{-/-} models could result in systemic stress signaling and HSC proliferation. As a result, conclusions on the intrinsic nature of the in vivo HSC phenotype require confirmation in an ongoing inducible competitive bone marrow chimera.

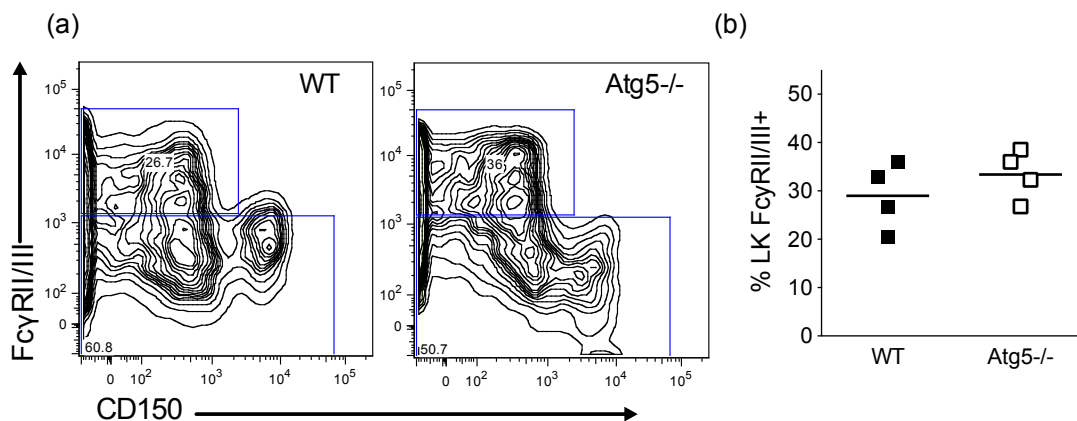


Figure 4.14: Granulocyte macrophage progenitor (GMP) frequency is unaltered in 6 week old *Vav-Atg5*^{-/-} mice. (a) Representative dot plots of CD16/32 (FcγRII/III) and CD150 expression within the Lin-Sca1-cKit⁺ (LK) population in the bone marrow of *Vav-Atg5*^{-/-} mouse (right) and wildtype (WT) littermate (left) at 6 weeks of age, showing FcγRII/III⁺ (GMP) population gating. (b) Frequency of GMP (gated as in a) among LK parent population in *Vav-Atg5*^{-/-} or WT littermate bone marrow at 6 weeks of age.

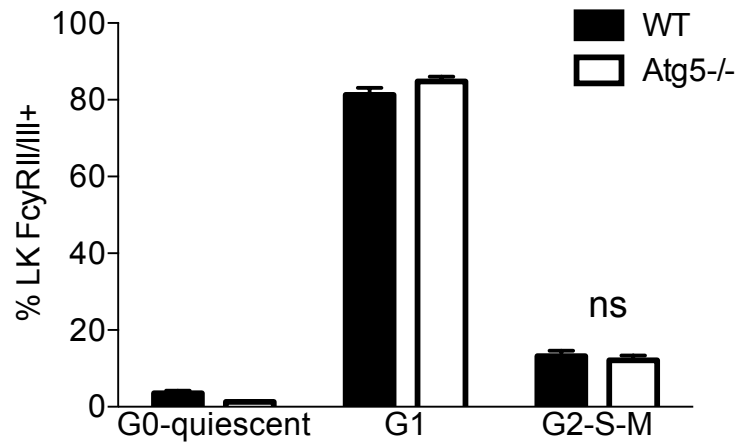


Figure 4.15: *Vav-Atg5*^{-/-} GMP population does not show increased proliferation. Frequency of GMPs (defined as Figure 4.14) from *Vav-Atg5*^{-/-} or WT littermate bone marrow in indicated cell cycle stage (populations gated as in Figure 4.13). Error bars represent SEM (n=4), ns=not significant, unpaired t test.

4.11 *Vav-Atg7*^{-/-} HSCs have reduced myeloid clonogenic capacity in vitro

As outlined in the introduction to this chapter, the absence of autophagy genes, including *Atg5* and *Atg7*, has been characterized to result in damage and loss of function in multiple cell types (6), including hematopoietic stem cells in the *Vav-Atg7*^{-/-} model (131). The evidence presented thus far suggests a significant part of the hematopoietic failure in *Vav-Atg5*^{-/-} and *Vav-Atg7*^{-/-} mice is due to a loss of HSC quiescence and excessive differentiation into multipotent progenitors. However, it is not clear if the hematopoietic loss of autophagy has *also* resulted in reduced function and differentiation potential in individual adult HSCs, as previously suggested (181), or whether HSCs function normally and are simply being driven to excess division, or indeed whether their ability to differentiate exceeds that of wildtype counterparts.

To help address this question *ex vivo* for the myeloid lineage, HSC and GMP populations were sorted from the bone marrow of *Vav-Atg7*^{-/-} mice or that of wildtype littermates, and single cells were transferred to individual wells of terasaki plates supplemented with myeloid growth media as previously published (143). After 10 days, the percentage of wells containing colonies was assessed and compared to expected frequencies, resulting in a measurement of the average ‘myeloid clonogenic potential’ of single HSCs and GMPs. As an additional readout, the size (well coverage) of the colonies that formed provided information on the proliferative ability of the progenitors. Cell plating and colony scoring were performed with the assistance of Dr. Michael Lutteropp. As expected, nearly all HSCs from wildtype mice showed myeloid potential under these conditions, whereas the clonogenic frequency of *Atg7*^{-/-} HSCs was reduced (Figure 4.16). Moreover, while colonies formed by wildtype HSCs were dominantly large (>50% well coverage), the average size of *Atg7*^{-/-} colonies was lower (Figure 4.16), indicating either delayed colony induction, retarded division or the failure (through death or arrest) of some downstream progenitors formed. Similarly, most GMP progenitors from wildtype mice were able to form myeloid colonies, although with a reduced colony size compared to HSCs, expected from a downstream progenitor with fewer potential divisions before reaching maturity. *Atg7*^{-/-} GMPs produced similar overall colony numbers, but these were uniformly of a smaller size (<50% well coverage), indicating reduced single progenitor production (Figure 4.16). Together, these data suggest that individual autophagy deficient HSCs and, to a more limited extent, GMPs, have reduced myeloid differentiation ability. Therefore, impaired progenitor function may contribute to bone marrow defects in these mice prior to upstream progenitor loss.

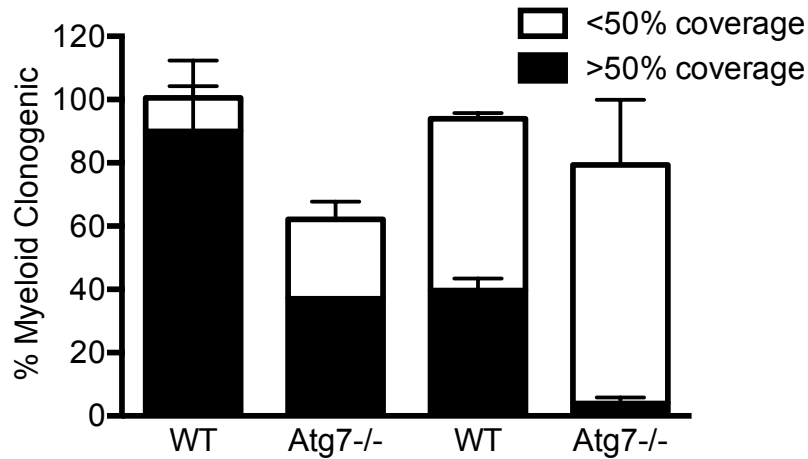


Figure 4.16: Reduced myeloid colony formation ability in *Vav-Atg7^{-/-}* HSCs *ex vivo*. Myeloid cloning potential (percentage colonies formed against Terasaki wells plated) by single HSC and GMP progenitors (defined as Figure 4.12 and Figure 4.14) sorted from 6 week old *Vav-Atg7^{-/-}* and wildtype (WT) littermate bone marrow, divided into number of colonies covering <50% or >50% of the well after 10 days. 4 mice pooled per genotype, 2 plates per progenitor type. Error bars represent SD (n=2). Grateful acknowledgement of Dr. Michael Lutteropp for assistance with setup and assessment.

4.12 *Vav-Atg5^{-/-}* HSCs show severe defect in in-vivo reconstitution ability

The monitoring of the *Vav-Atg5^{-/-}* progenitor compartments, combined with the *ex-vivo* data on autophagy deficient HSCs, strongly suggests that the HSC population in *Vav-Atg5^{-/-}* is compromised by 8-9 weeks of age. To confirm this functionally *in vivo* via competitive repopulation, bone marrow taken from either *Vav-Atg5^{-/-}* mice or wildtype (WT) littermates (CD45.2+) was mixed 1:1 with CD45.1+ competitor bone marrow cells and 2×10^6 cells of the resulting mixes (WT:WT and *Vav-Atg5^{-/-}*:WT) were injected into lethally irradiated CD45.1+ hosts. By 4 weeks, analysis of WT:WT bone marrow chimeras showed wildtype littermate CD45.2 cells had contributed just under 50% of the total blood hematopoietic cells in hosts, a proportion which gradually increased throughout the 24 week assay. By contrast, in *Vav-Atg5^{-/-}*:WT chimeric mice the CD45.2+ population

represented under 5% of the blood at 4 weeks, and even this contribution failed with time and was lost by 24 weeks (Figure 4.17).

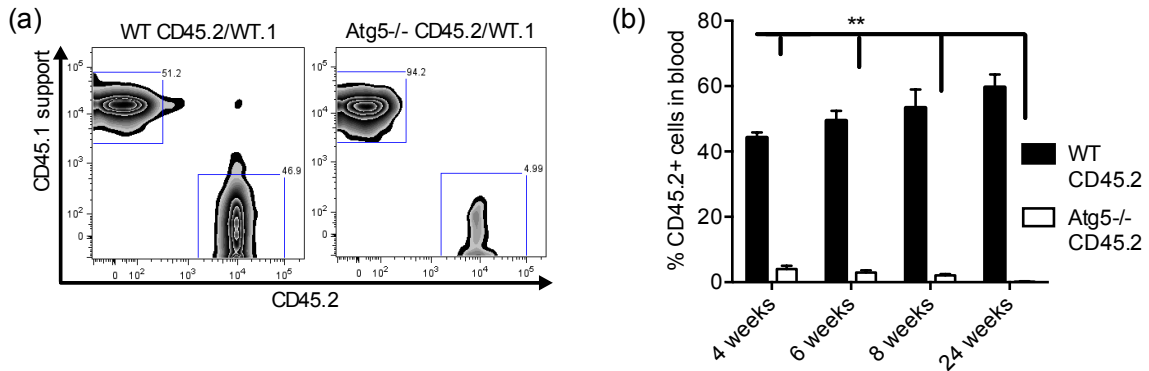


Figure 4.17: *Vav-Atg5*^{-/-} HSCs have defective in-vivo reconstitution ability in competitive chimera. Lethally irradiated CD45.1 hosts (WT) were transplanted with CD45.1 competitor BM cells (8 week old) mixed 1:1 with CD45.2 BM cells from either 8 week old *Vav-Atg5*^{-/-} mice or wildtype (WT) littermates to make WT CD45.2/WT.1 or *Atg5*^{-/-} CD45.2/WT.1 chimeric mice (a) Representative contour plots showing CD45.2 and CD45.1 expression in the blood of indicated chimeras 4 weeks post-transplant. (b) Frequency of CD45.2+ cells in blood of indicated chimeras over time post-transplant. Error bars represent SEM (n=3), ***p* < 0.01, unpaired *t* test.

To confirm that this represented a failure of *Vav-Atg5*^{-/-} HSPCs to engraft and contribute to the multipotent progenitor population, the LSK cells in the bone marrow of the chimeric mice was examined after 24 weeks. While WT:WT chimeras showed a strong CD45.2+ presence in their LSK compartment, *Vav-Atg5*^{-/-}:WT chimera LSKs were all CD45.2- (Figure 4.18). These results demonstrate that *Vav-Atg5*^{-/-} HSPCs are severely functionally deficient in a competitive engraftment setting, unable to contribute to long-term hematopoiesis, and combined with the quiescence and cloning data, support a possible autophagy requirement of HSCs.

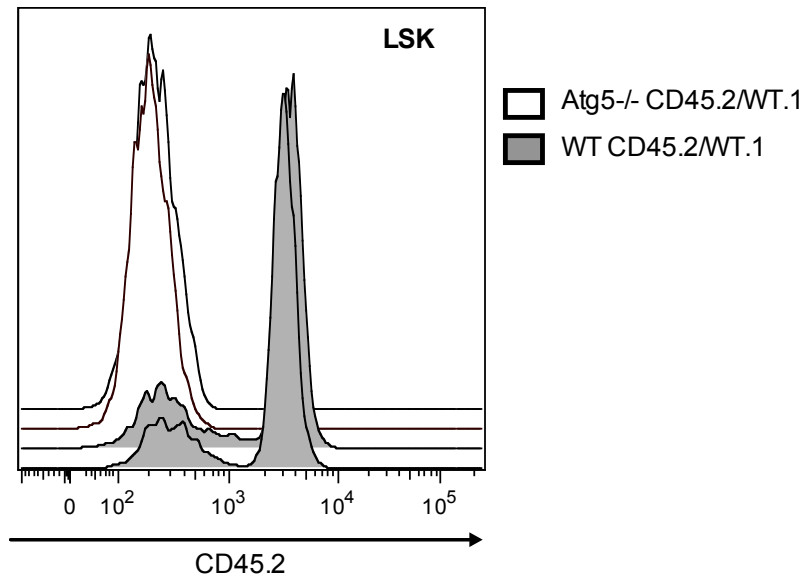


Figure 4.18: *Vav-Atg5^{-/-}* HSCs show no long-term engraftment in competitive chimeras. Histograms showing CD45.2 expression in the LSK compartment (defined as Figure 4.9) in the bone marrow of indicated chimeric mice, generated as described in Figure 4.17, 24 weeks post-transplant (representative of 3 WT mice, one KO chimera died of unrelated causes prior to takedown).

4.13 CD11b⁺Gr1⁺ myeloid cell are expanded in the blood, bone marrow and spleen of *Vav-Atg5^{-/-}* mice

The observation that HSC, erythrocyte and lymphocyte populations progressively decrease with time in *Vav-Atg5^{-/-}* mice suggests that Vav-driven *Atg5* loss is generally detrimental to the survival of affected cell types, resulting in multi-lineage cytopenias. However, as previously observed in *Vav-Atg7^{-/-}* mice (131), investigation of the *Vav-Atg5^{-/-}* myeloid lineage revealed kinetics distinct from those of other lineages. Myeloid cells were defined by expression of Ly6g/Gr1, a neutrophil-specific marker (358) with a role in neutrophil recruitment (359), and CD11b, a pan-myeloid marker expressed on granulocytes, monocytes, macrophages with roles in adhesion (360) and as a receptor in activation (361); together they mark mature neutrophils. In contrast to erythroid and lymphoid populations, the relative abundance of the CD11b⁺Gr1⁺ myeloid subset was observed to be increased in the blood of *Vav-Atg5^{-/-}* at 6 weeks of age relative to wildtype frequencies (Figure

4.19). Other hematopoietic organs were interrogated; the CD11b+Gr1+ population was also expanded in the bone marrow (Figure 4.20) as well as the spleen (Figure 4.21) of *Vav-Atg5*^{-/-} mice, as compared to wildtype counterparts. Note that increased numbers of Gr1med cells in the bone marrow, creating a broader Gr1 expression peak, was characteristics of *Vav-Atg5*^{-/-} (Figure 4.20) and *Vav-Atg7*^{-/-} models.

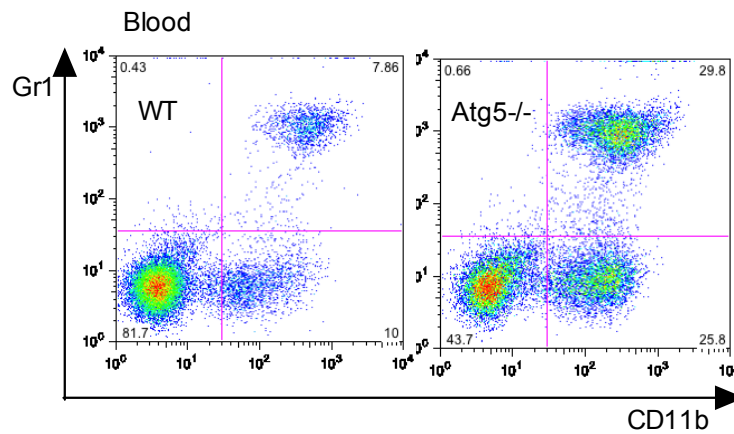


Figure 4.19: Increased frequency of CD11b+Gr1+ cells in the blood of *Vav-Atg5*^{-/-} mice. Representative dot plots of Gr1 and CD11b expression in the blood of *Vav-Atg5*^{-/-} mice and wildtype (WT) littermates at 6 weeks of age. Findings typical of 3 or more independent experiments.

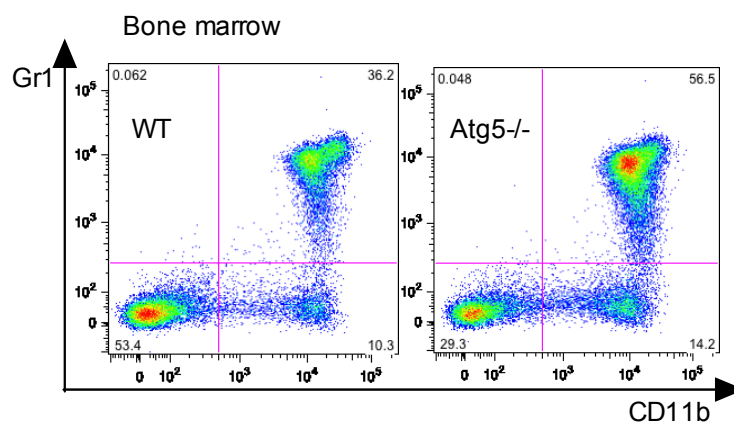


Figure 4.20: Increased frequency of CD11b+Gr1+ cells in the bone marrow of *Vav-Atg5*^{-/-} mice. Representative dot plots of Gr1 and CD11b expression in the bone marrow of *Vav-Atg5*^{-/-} mice and wildtype (WT) littermates at 6 weeks of age. Findings typical of 3 or more independent experiments.

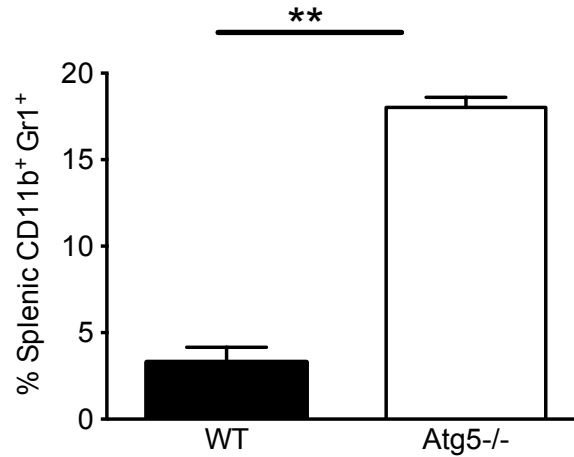


Figure 4.21: Increased frequency of CD11b+Gr1⁺ cells in the spleen of *Vav-Atg5*^{-/-} mice. Frequency of CD11b+Gr1⁺ cells among splenocytes from *Vav-Atg5*^{-/-} mice and wildtype (WT) littermates at 6 weeks of age. Error bars represent SEM (n=3), **p<0.01, unpaired t test.

As the blood and bone marrow of *Vav-Atg5*^{-/-} mice are generally hypocellular at this stage, TruCount quantitative counts of the myeloid populations were performed to determine whether the myeloid alterations were relative or absolute (performed with lab member Shivani Khandwala). CD11b+Gr1⁻ cells, also increased by frequency in the blood, were moderately decreased by absolute numbers along with the CD11b-Gr1⁺ subset, but CD11b+Gr1⁺ numbers were indeed elevated in *Vav-Atg5*^{-/-} mice (Figure 4.22). Therefore, it appears that the survival of myeloid lineages is not dependent on autophagy.

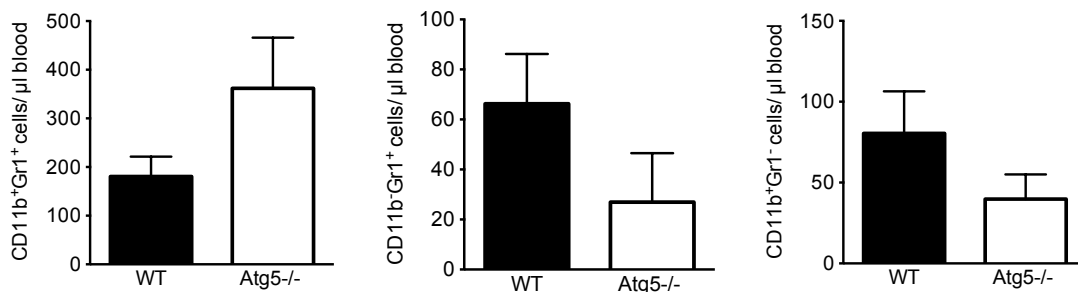


Figure 4.22: Quantification of CD11b and Gr1 expression subsets in the blood of *Vav-Atg5*^{-/-} mice. Absolute counts of CD11b+Gr1⁺, CD11b-Gr1⁺ and CD11b+Gr1⁻ cells in the blood of *Vav-Atg5*^{-/-} mice and wildtype (WT) littermates at 6 weeks of age. Error bars represent SEM (n=3). Grateful acknowledgement of Shivani Khandwala for the absolute blood counts.

In light of the differential impact of ATG5 loss on the myeloid subsets in *Vav-Atg5*^{-/-}, the levels of basal autophagy in wildtype myeloid cells was measured using GFP-LC3 transgenic mice and related to CD11b and Gr1 expression. Intriguingly, although the number of GFP-LC3 puncta quantified via the Image Stream was overall low in mature myeloid cells, the mean puncta per cell in the CD11b+Gr1⁺ subset was significantly lower than that of either single-positive myeloid cell type (Figure 4.23), and in fact was the lowest mean of any mature lineage cell type studied (not shown). These data require further confirmation, in particular given the low puncta threshold overall, but could indicate a correlation between basal autophagy level and the impact of autophagy loss among myeloid subsets.

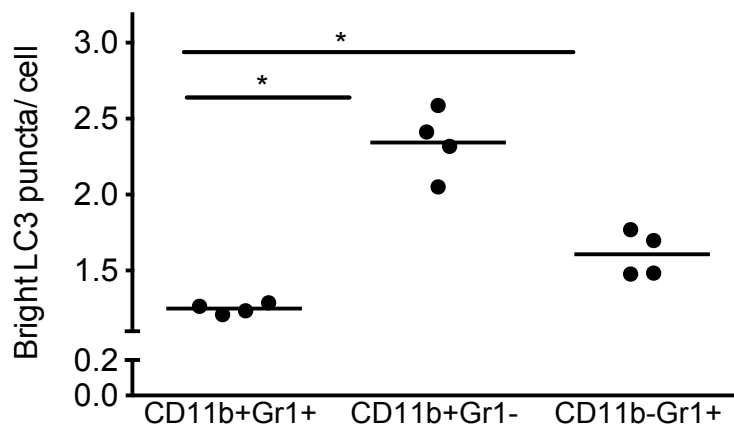


Figure 4.23: *CD11b+Gr1⁺ have the lowest GFP-LC3 puncta count of CD11b/Gr1 myeloid subsets. Quantification of number of bright GFP-LC3 puncta/cell in indicated CD11b/Gr1 defined myeloid subsets in GFP-LC3 transgenic mouse bone marrow, quantified using Image Stream (40,000 cells analyzed per sample). Error bars represent SEM (n=4), *p<0.05, unpaired t test.*

4.14 Expanded, autophagy deficient myeloid cells are infiltrative and show immature features by histology, cytospin and electron microscopy

To augment the flow cytometric data on the expanded myeloid population with distributive information, organs were taken from phenotypic *Vav-Atg5*^{-/-} mice for histological examination. Consistent with the relative myeloid expansion in the spleen and bone marrow of *Vav-Atg5*^{-/-} mice, histology performed on these organs to investigate the distribution of cell types revealed atypical myeloid infiltrates in these hematopoietic organs, along with lymph nodes, of all mice examined (Figure 4.24).

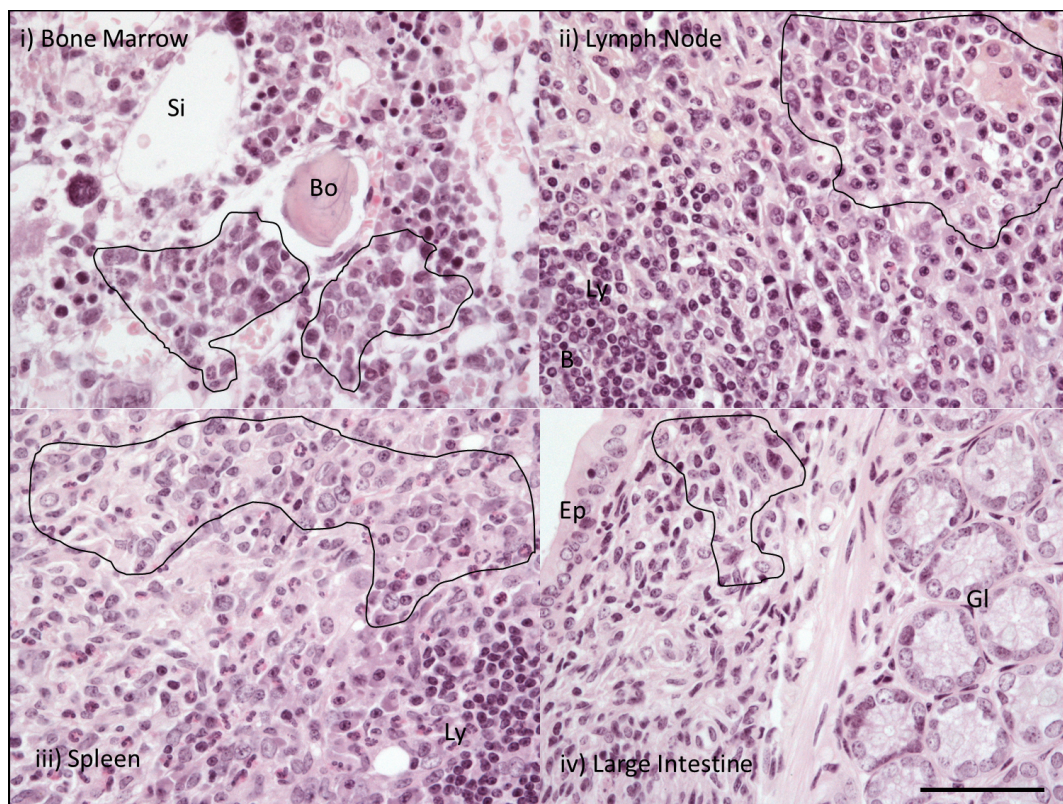


Figure 4.24: *Vav-Atg5*^{-/-} mice develop myeloid infiltrations in hematopoietic and non-hematopoietic organs. H&E staining of 4- μ m paraffin sections taken from i) bone marrow (Bo-bone, Si-sinus), ii) lymph node (Ly-residual lymphocytes), iii) spleen, iv) large intestine (Gl-glands, Ep-luminal epithelium) of *VavAtg5*^{-/-} mice at 6 weeks of age, dysplastic myeloid cells encircled (scale bar is 50 μ m for all panels). Grateful acknowledgement of Dr. Elizabeth Soilleux for assistance with histology.

Moreover, infiltrates of myeloid cells were found in non-hematopoietic organs of *Vav-Atg5*^{-/-} mice, including the large intestine (Figure 4.24). Histological interpretation of sections, performed by histopathologist Dr. Elizabeth Soilleux, also noted the dysplastic features of some infiltrating cells, including irregular nuclei and high nuclear/cytoplasmic ratios.

Cell cytopins from blood or bone marrow are a common diagnostic tool when hematological irregularities are suspected. To help assess the hematological phenotype of 6-week-old mice, cytopins of *Vav-Atg5*^{-/-} and wildtype littermate bone marrow were performed. Quantification of cell types present (performed by Dr. Onima Chowdhury) similarly revealed an increased myeloid frequency relative to littermate counts, including a significant number of myeloid cells displaying immature features (Figure 4.25). Cytospin analysis also confirmed the *Vav-Atg5*^{-/-} lymphopenic phenotype in the bone marrow (Figure 4.25). However, the phenotype was not judged to represent leukemic features.

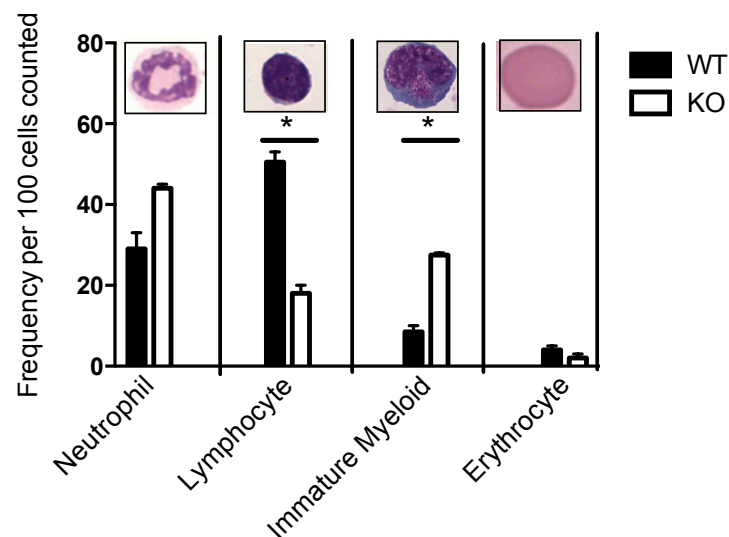


Figure 4.25: Increased frequency of immature myeloid cells in *Vav-Atg5*^{-/-} bone marrow cytospin. Frequency per 100 cells of indicated cell types (hematologist assessed) in cytospin of 6 week old *Vav-Atg5*^{-/-} mouse bone marrow or that of a wildtype littermate, along with representative morphological images of each cell type (representative experiment of two time points). Grateful acknowledgement of Laura Stenson for aid performing cytospin and Dr. Onima Chowdhury for assistance with cytospin assessment.

The presence of increased CD11b+Gr1+ cells prior to progenitor failure, myeloid infiltrates in hematopoietic and non-hematopoietic organs, including myeloid cells displaying immature morphological features, in *Vav-Atg5*^{-/-} mice matches the *Vav-Atg7*^{-/-} model, suggesting the autophagy pathway may have a role in regulation of myeloid proliferation/accumulation and maturation (131).

Work performed by lab member Amanda Stranks helped to clarify the nature of the CD11b+Gr1+ population: cells expressing CD11b and Gr1 were sorted from the bone marrow of *Vav-Atg7*^{-/-} mice or wildtype littermates by flow cytometry and examined by transmission electron microscopy. Both *Vav-Atg7*^{-/-} and wildtype populations generally displayed features consistent with neutrophils, including cytoplasmic granules and multi-lobed (>3) nuclei (Figure 4.26). However, these features were reduced in the *Vav-Atg7*^{-/-} neutrophils; they displayed smaller, less electron-dense granules along with a reduced overall granule count per cell (Figure 4.26), while many more cells containing non- or bi-lobed nuclei (as opposed to multi-lobed nuclei) were also observed among the *Vav-Atg7*^{-/-} population (Figure 4.26). Therefore, histology, cytospin and TEM analysis indicate increased immaturity in the expanded autophagy deficient myeloid populations.

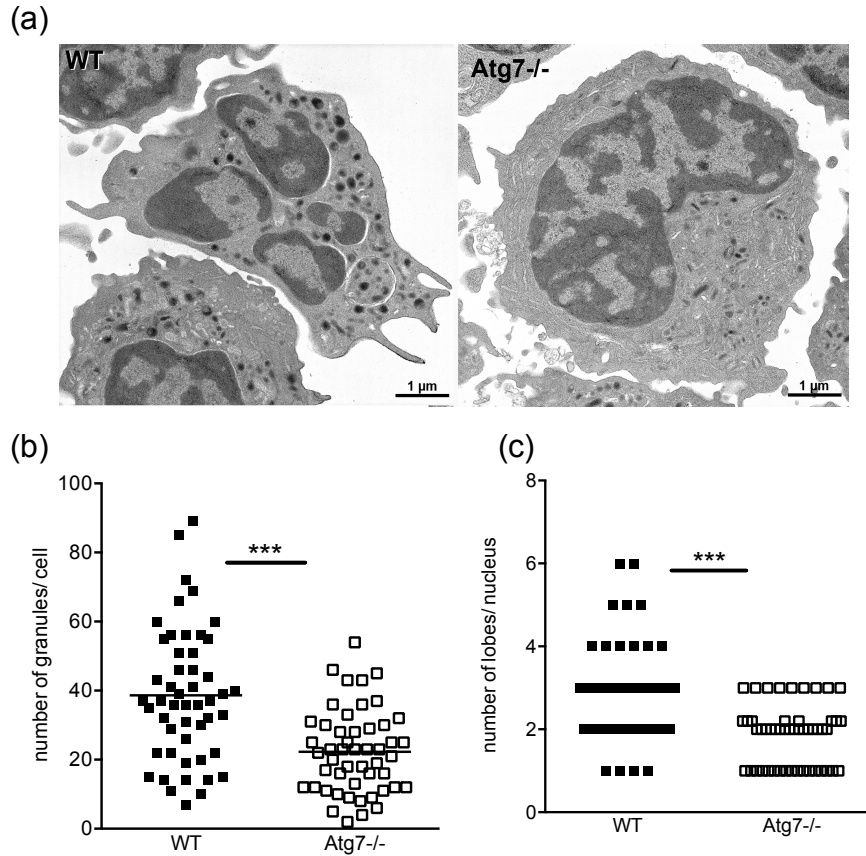


Figure 4.26: *CD11b+Gr1+* cells from *Vav-Atg7^{-/-}* mice display immature features. *CD11b+Gr1+* cells sorted from pooled bone marrow of 4 *Vav-Atg7^{-/-}* or wildtype (WT) littermates, fixed and analyzed by transmission electron microscopy. (a) Representative micrographs from either WT (left) or *Vav-Atg7^{-/-}* (right). (b) Enumeration of cytoplasmic granules from 50 neutrophils per indicated genotype. (c) Enumeration of visible nuclear segments (lobules) from 50 neutrophils per indicated genotype. *** $p < 0.001$, unpaired *t* test. Data courtesy Amanda Stranks, grateful acknowledgement also of Professor David Ferguson for TEM image acquisition.

Discussion

4.15 *Atg5* is required for maintenance of erythroid and lymphoid lineages

Given the phenotypic variation between systemic autophagy gene deletion models (6), and the gene specific functions recently defined for *Atg7* and *Atg5* (352) (278) (277), the stark correlation between all aspects of the *Vav-Atg5*^{-/-} and *Vav-Atg7*^{-/-} models is an important confirmation that their phenotypes stem from the autophagy pathway. *Vav-Atg5/7*^{-/-} will be used to refer to shared phenotypes of the single knockout models in this discussion.

The role of ATG5 in T-cell peripheral survival has been demonstrated previously (156) (100), and the lineage-intrinsic nature of the T-cell phenotype has been shown using a CD4-Cre driven model in our lab (D. Puleston, unpublished data). However the B-cell impact in the *Vav-Atg5*^{-/-} model, shared by *Vav-Atg7*^{-/-} mice (7), appears less subset-restricted than defects following B-cell lineage deletion of *Atg5* mediated by CD19-Cre (282) (330) (331). This difference may be due to the earlier excision mediated by the *Vav* promoter and resulting progenitor abnormalities prior to CD19 expression, supported by the exacerbated B-cell defect reported in *Atg5*^{-/-} chimeras (156).

In erythroid cells, previous work examining autophagy pathway initiator ULK1 (154) and mitochondrial adaptor NIX (341) defined an important role for elements of the autophagy pathway in red blood cell development, in particular during reticulocyte mitochondrial clearance. Subsequent work using *Atg7*^{-/-} models (7, 153) extended the autophagy gene network involved, however, due to the lack of reported anemia in an *Atg5*^{-/-} fetal liver chimeric model (156) and successful mitochondrial clearance observed in *Atg5*^{-/-} neonates (362) the role of ATG5 and the canonical autophagic ubiquitin cascades was questioned

(342). Indeed, ATG5 and classical-cascade independent roles for ATG7 in mitochondrial dynamics and death have been demonstrated (355). However, that *Vav-Atg5*^{-/-} mice develop anemia equivalent to *Vav-Atg7*^{-/-} mice, including an erythrocyte maturation block at the same stage (stage III to stage IV transition) and abnormal accumulation of damaged mitochondria, argues that the ubiquitin-like cascades are indeed required players in this developmental organelle clearance. The stage III/IV block corresponded with a significant increase in LC3 puncta in developing wildtype erythrocytes *ex vivo*, delineating what may be a point of particular autophagic requirement.

Differences in anemic severity have been reported between Atg knockout models, including among those investigating *Atg7* deletion, where *Vav-Atg7*^{-/-} mice were the most severely impacted (342). That *Vav-Atg5*^{-/-} erythrocytes, with an alternate Atg but identical Cre, phenocopy *Vav-Atg7*^{-/-} supports i) the timing of autophagic impairment (fetal vs. adult, progenitor stage), known to impact gene requirements in hematopoietic contexts (363) (364), and ii) the method of model generation (irradiated transplant vs. physiological) as major contributors to anemia variation among Atg knockout models. It cannot be excluded that damage to early HSPCs, which also suffer following Vav-mediated *Atg5* and *Atg7* deletion prior to lineage commitment, exacerbates the erythroid-specific phenotype, as hypothesized for the B-cell phenotype. That being said, autophagic regulation appears a vital and regulated part of erythrocyte development: recent evidence shows that the key hematopoiesis and erythroid lineage transcription factor GATA-1 upregulates autophagy both directly, driving transcription of Atg8 homologs *Gabrapl1*, *Gabarapl2*, *Gabarap*, *Maplc3b* and *Maplc3a*, and indirectly through FOXO3 (58). This is an important example of specific developmental upregulation of autophagy at a stage of morphological remodeling during lineage differentiation, agreeing with the autophagic

upregulation observed *ex vivo* and previously *in vitro* (154), and helping to confirm a role for ubiquitin cascade members in erythrocyte development.

A common feature among Atg-deficient erythrocyte models, including *Vav-Atg5*^{-/-}, is that the developmental and mitochondria clearance blockades are not complete (342).

Alternative degradation via 15-lipoxygenase mediated proteolysis has been shown to aid reticulocyte organelle degradation (365), but plays only a limited role in mitochondrial breakdown (366). Similarly, *Atg5*/*7* independent macroautophagy, which precedes without MAP1LC3 lipidation, has been shown to aid reticulocyte mitochondrial clearance (343) and could also assist in the clearance of other organelles during erythroid maturation. As this process is described as ULK1 dependent (343), while the erythroid phenotype in *Ulk1*^{-/-} erythrocytes is not more severe than other models, further study is required to clarify the overlapping organelle clearance mechanisms operating during red blood cell development.

4.16 *Atg5* is required for the *in vivo* maintenance of hematopoietic stem and progenitor cell populations

As with lineage defects, the use of different Cre constructs and autophagy genes, namely *FIP200* (344) and *Atg7* (7), in the two published *in vivo* studies outlining an HSC phenotype in the absence of Atg's has strongly suggested the importance of pathway genes in HSC maintenance, but has not refined timing (169) or pathway specificity. Both *FIP200* and *Atg7* have proposed autophagy-independent roles in the cell cycle (310, 311, 352), with ATG7's reported function in mediating cell cycle arrest in combination with p53 following metabolic stress in particular potentially augmenting the knockout phenotype and the role of the autophagy pathway in HSCs (179) (49). However, the implications of that study, already questioned in relation to Atg systemic knockout phenotypes (354), appear limited in HSCs, as the impact of Vav-driven deletion of *Atg5*, which has no

reported autophagy independent cell cycle roles, on HSCs in *Vav-Atg5*^{-/-} mice phenocopies *Vav-Atg7*^{-/-} mice. In both models, LSK multipotent progenitors were expanded in the asymptomatic stage. However, this was accompanied by a reduction in the HSC population. Interestingly, relative GMP abundance was similar, indicating initial progenitor impacts may be harsher in immature populations. Subsequent loss of LSK cells heralded the onset of clinical symptoms, bone marrow failure, as visualized by lineage colony formation, and death. The HSC and progenitor defect was confirmed by competitive repopulation assay, where 8-week-old *Vav-Atg5*^{-/-} bone marrow showed only very limited short-term and no long-term reconstitution ability in irradiated hosts. Therefore the kinetics and severity of the *Vav-Atg5*^{-/-} HSC phenotype match that of *Vav-Atg7*^{-/-} mice, suggesting the autophagy ubiquitin-like cascades, and likely the autophagy pathway itself, are a required element for HSC preservation *in vivo*. The loss of the HSC population with LSK expansion may indicate defective HSC self-renewal, in that HSCs are no longer balancing out differentiation and death through formation of new HSCs, however this would need to be confirmed through serial transplantation of younger (<8 week old) *Vav-Atg5*^{-/-} HSCs.

4.17 Autophagy regulates HSC quiescence and function

With the models judged equivalent, investigation into the mechanism of HSC loss delineates the role of autophagy in vital HSC properties. Previously, *Vav-Atg7*^{-/-} LSKs were found to be damaged, with increased ROS, caspase-3 activity and higher expression of proliferation marker Ki67 (131). However, true cell cycle status and the state of HSCs were not investigated. In this study, the quiescent HSC population was revealed to be greatly reduced in 6-week-old *Vav-Atg5*^{-/-} mice, with a corresponding increased frequency of HSCs in the S-G2-M phases. Such increased proliferation could lead to the loss of stem

cells observed at this stage, along with the transient LSK expansion through HSC differentiation and the final BM failure through HSC exhaustion.

That HSCs and multipotent progenitors, but not GMPs, show alterations to proliferation in *Vav-Atg5*^{-/-} mice suggests autophagy's impact may be highest in early progenitors. The loss of autophagy-promoting transcription factors HIF1a (201) and the FOXOs causes decreased HSC quiescence, with the latter in particular showing similar HSC-specific (non-GMP) cell cycle alterations, (234). Increased HSC proliferation is also a mark of multiple forms of activating mutations within the Akt-mTOR pathway (237-239), which is a negative autophagy regulator. The impact on HSCs in *Vav-Atg5/7*^{-/-} mice indicates autophagy loss may be an important contributor to these phenotypes. This correlates with the increased autophagy observed in HSCs and LSKs in Chapter 3, and autophagy genes have been previously identified as part of a general multi-tissue 'quiescent stem cell' signature (110).

Should it prove intrinsic, the mechanism of autophagy's cell cycle regulation requires future clarification, with three primary possibilities hypothesized (Figure 4.27). Firstly, stress resistance is a primary autophagy function, and oxidative (234) or ER stress (183) have been implicated in excessive stem cell cycling.

Secondly, the mTOR is known to regulate the cell cycle (8, 367, 368), in particular impacting the G1/S transition which appeared most significantly altered in *Vav-Atg5*^{-/-} HSCs (367), and HSC quiescence (238); the endolysosomal trafficking of the mTORC1 complex has been shown to regulate kinase activity and cell growth (369) (370) (32, 371, 372), and autophagy adaptor p62 has recently been identified as a binding partner of mTORC1 complex member RAPTOR, required for its lysosomal localization (373), therefore autophagy loss has been hypothesized to feedback to increased mTOR activity

(8). Such feedback may be supported by the observations in *Atg5* deficient leukemic cells (presented in Chapter 5), and could explain partly why cell cycle inhibitors including p14ARF (374) and p27KIP1 (375) induce autophagy. However, this must be reconciled with increased cell cycle inhibitor expression following mTOR overactivation in HSCs (209), possibly due to compensatory mechanisms.

The third possibility is autophagic degradation of cell cycle regulators. Cell cycle progression is controlled by the cyclin-dependent kinase (CDK) family and the cyclin binding partners required for their activity (376). Regulation of cyclin levels via degradation and renewal is an important component of that control; cyclins have traditionally been thought to be degraded by ubiquitination and subsequent proteasomal targeting (376). Autophagy can also target ubiquitinated proteins via p62 (92). Previous work suggests cyclin D1, a member of the cyclin D family which functions at the G1-S transition and is linked to HSC quiescence (221), suppresses autophagy (377), conceivably to prevent degradation. Moreover, a new study has implicated autophagy in the degradation of cyclin A2; the authors found a subset of ubiquitinated cyclin A2 colocalized with lysosomes and could be immunoprecipitated with LC3 and p62, while autophagy modulation could alter cyclin A2 levels (378). Cyclin A2 can bind CDK's 1, 2 and 3 (376). While its activity is mainly associated with S and mitotic phases (378), both CDK2 and 3 activity have been associated with quiescence exit (379, 380). Autophagy is known to be negatively regulated by CDK1 via inhibition of VPS34-Beclin1 binding during mitosis (381). Elucidating the relevance of autophagic cyclin regulation in HSCs, the state of mTOR activation in *Vav-Atg5/7-/-* HSCs, and indeed the balance between the three hypotheses above, will be important goals for future studies.

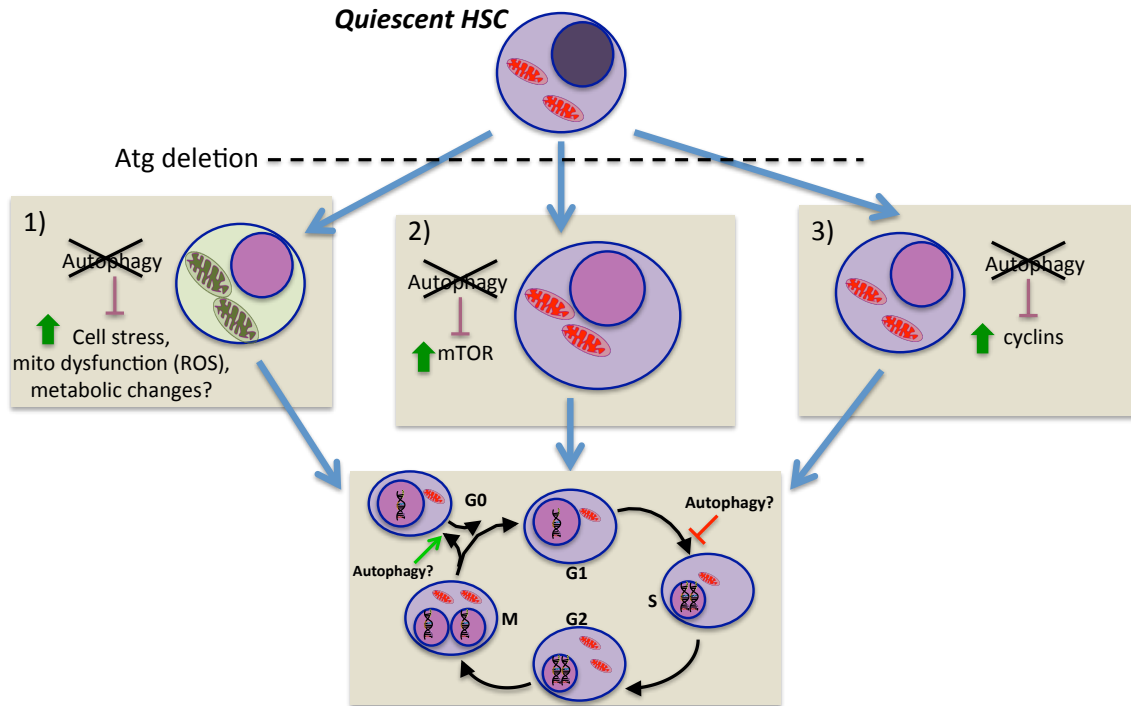


Figure 4.27: Three hypothesized mechanisms for HSC-intrinsic increased proliferation in the absence of autophagy, see text for details.

Similarly, the precise stage of autophagy's impact on HSC proliferation requires further investigation. Murine HSCs are known to undergo a sharp shift in cell cycle status around 4-5 weeks of age, transitioning from a near 100% cycling fraction in fetal and young HSCs to less than 5% found in S-G2-M phases (214). Considering the timing of the *Vav-Atg5/7-/-* HSC phenotype, where Vav excision operates from a fetal stage (347), and the successful generation of *Atg5/7-/-* fetal chimeras (156) (153), it is tempting to suggest that autophagy may be particularly important during, or after, this shift in adult hematopoiesis (ie: when HSCs become less proliferative). Indeed, as noted above when considering erythrocyte models, differential requirements between fetal and adult hematopoiesis have been found in multiple pathways (363) (364), and analogously autophagy has been found to be vital in longer lived, memory lymphocyte populations (330) (331)(D. Puleston, unpublished data). However *FIP200^{fl/fl};Tie2-Cre* mice display increased HSC

proliferation at a much earlier fetal stage and die perinatally (344). This could indicate gene specific impacts but, as there is evidence of less severe survival defects in *Atg5*^{-/-} (303) and *Atg7*^{-/-} (153) fetal chimeras as well, a likely factor is the stress of chimera generation, previously observed to alter hematopoietic requirements (382).

It is important to note that the deficiency in myeloid clonogenic potential observed in single *Vav-Atg7*^{-/-} HSCs suggests that autophagy deficient HSCs are not simply cycling more but also may have altered function, assessed as *in vitro* myeloid differentiation, compared to activated wildtype counterparts at an individual level. This may contribute to the reduced colony formation of *Vav-Atg5/7*^{-/-} bone marrow, and is an important indicator that autophagy deficient HSCs are not only proliferating more but may be also intrinsically damaged. The drop in colony formation was stronger in HSCs than more mature GMPs, but both *Vav-Atg7*^{-/-} progenitor types produced smaller colonies *in vitro*, which could be a product of the lack of increased proliferation of downstream progenitors combined with the loss of some progenitor clones within colonies. As it has not been directly assayed, it is conceivable that *Vav-Atg5/7*^{-/-} HSCs are actually damaged from an earlier age, as seen in *FIP200*^{-/-} HSCs, in a cumulative fashion before a threshold results in visible symptoms. An inducible *Atg5^{fl/fl};Mx-Cre* competitive chimera will help to elucidate whether autophagy is indeed still required at an adult stage, and to confirm whether the *Vav-Atg5/7*^{-/-} HSC defect is cell intrinsic instead of a product of a damage microenvironment. Preliminary investigations in this model indicate a cell intrinsic ATG5 requirement for adult HSCs (data not shown). Intriguingly, mTOR overactivation is a feature of aged HSCs and restricts their function (209), while *Atg5* overexpression in mice has recently been shown to extend lifespan (279). Therefore, the question of whether increasing autophagy can benefit HSCs will be a promising avenue of investigation.

4.18 Autophagy deficient mice accumulate immature and infiltrative myeloid cells

Contrasting the lineage and HSC loss in the *Vav-Atg5/7-/-* models, the myeloid phenotype of CD11b+Gr1+ subset expansion and infiltration into hematopoietic and non-hematopoietic organs indicates autophagy is not required for their survival, confirmed by previous lab investigations of a *Atg7^{fl/fl};LysM-Cre* model ((131); unpublished data) and the limited murine myeloid knockout models reported (334) (336). The differential impact on CD11b/Gr1 defined subsets in *Vav-Atg5/7-/-* mice, namely CD11b+Gr1+ expansion yet decreased CD11b+Gr1- cells, is the opposite to that observed in mice with mTORC1 deficiency through *Raptor* deletion (240), which, although not examined in that study, would likely cause an increase in autophagy induction. Wildtype CD11b+Gr1+ cells also displayed the lowest basal level of GFP-LC3 puncta among myeloid subsets, and could conceivably be favored in an autophagy-low setting and vice-versa. This evidence supports a putative role for autophagy levels in balancing myeloid subsets, although this is purely speculative without further study.

An autophagy requirement for the proper maturation and function of myeloid cells is better supported. The expanded myeloid cells in *Vav-Atg5/7-/-* mice displayed an immature phenotype, while both *Vav-Atg7-/-* macrophages and neutrophils have been demonstrated to have altered function *ex vivo* (A. Stranks, unpublished work). Myeloid-specific autophagy deficiency has been previously shown to result in cell-intrinsic antigen processing defects in dendritic cells (334) and an inflammatory phenotype in macrophages (336). Autophagy has been shown to be required for *ex-vivo* macrophage differentiation driven by CSF-1 (337) in addition to the maturation of myeloid leukemic cell lines (158)

(332) (333). It may be that, similar to erythroid cells, certain stages of myeloid development require autophagic remodeling, or that autophagy deficiency creates intra/extracellular conditions that favor immaturity.

The expansion, infiltration and immaturity of the accumulated myeloid cells in *Vav-Atg5/7-/-* mice, combined with multi-lineage cytopenias, led to an initial diagnosis of a myelodysplastic/pre-leukemic like phenotype in these models. Supporting this, their phenotype fulfills the majority of the Bethesda criteria for murine myeloid malignancy, including infiltration of dysplastic myeloid cells into hematopoietic and non-hematopoietic organs and greater than 20% dysplastic myeloid cells in the bone marrow of affected mice (383). However the expanded *Vav-Atg7-/-* myeloid population was not transplantable, although it was replicated on transplantation of bone marrow or LSK cells, and lacks clonal genetic gains or losses via genomic hybridization (131). Furthermore, as GMP progenitors were neither increased in number, more proliferative or able to form larger myeloid colonies *ex vivo*, the progenitor source of the expanded myeloid cells is uncertain. A markedly similar phenotype following FOXO family deletion, including HSC proliferation, loss of myeloid colony formation yet expansion of CD11b+Gr1+ cells, was blamed on ROS levels (234), and this remains a distinct possibility. Alternatively, a block late in maturation might result in myeloid accumulation, and it is possible the *Vav-Atg5/7-/-* *in vivo* environment promotes the myeloid cells survival in an extrinsic manner. In either case, while the autophagy deficient HSC and progenitor environment is likely to be one promoting damage (384), the HSC phenotype lethality and cytotoxicity seem to prevent the true leukemic transformation that is observed following Akt/mTOR overactivation (385).

4.19 Conclusions

These results demonstrate that the loss of autophagy gene *Atg5* in the murine hematopoietic system results in lineage defects, including anemia, lymphopenia and abnormal myeloproliferation, along with a progressive stem cell defect, extending previous findings beyond gene-specific roles. Specifically, autophagy loss results in loss of stem cell quiescence, overproliferation and exhaustion; HSC colony formation was also impaired *in vitro*. Ongoing experiments in an inducible supported chimera will reveal whether the impacts of autophagy loss are HSC intrinsic.

Chapter 5: Decreased Autophagy is Associated with Proliferation in Acute Myeloid Leukemia

Introduction

As presented in Chapter 4, knockout of autophagy leads to excess cell cycling and damage in HSCs, while immature myeloid cells accumulate in tissues. This picture resembles a pre-cancerous phenotype (383) (384), raising the question of autophagy's role in myeloid leukemias. As a process intrinsically tied to cell growth, maintenance and damage response, it is no surprise that autophagy has been linked to the development and progression of malignancies. However, as has been well reviewed within the field (16) (386) (387), the precise nature of that effect remains complex: autophagy appears to be a 'two edged sword' in cancer development, exerting tumor suppressive or tumor promoting at different stages and in different tumor types. Here, evidence for autophagy's role on both sides of the process will be briefly presented, including autophagy's dual role in metabolism, before focusing on autophagy in acute myeloid leukemia.

5.1 Autophagy in tumor suppression

Data gathered in mouse models has shown a defined link between autophagy and tumor suppression, with deletions of autophagy genes including *Atg4C* (318), *Atg5*, *Atg7* (388) and *Beclin1* (308) resulting in increased tumor development in mouse models. Work over the last several decades has revealed the development of cancer to be a multifaceted and complex process, with marked heterogeneity both between and within tumors complicating diagnosis and treatment (389). However, broad hallmarks of cancer, meaning common properties or abilities that tumors of different types must acquire in order to cause disease, have been proposed (390). Selected characteristics include sustained proliferation, genomic instability and evading death or immune recognition; recent work has illustrated how autophagy's cellular actions resist these oncogenic alterations (summarized in Figure 5.1).

Impaired autophagy promotes tumorigenesis

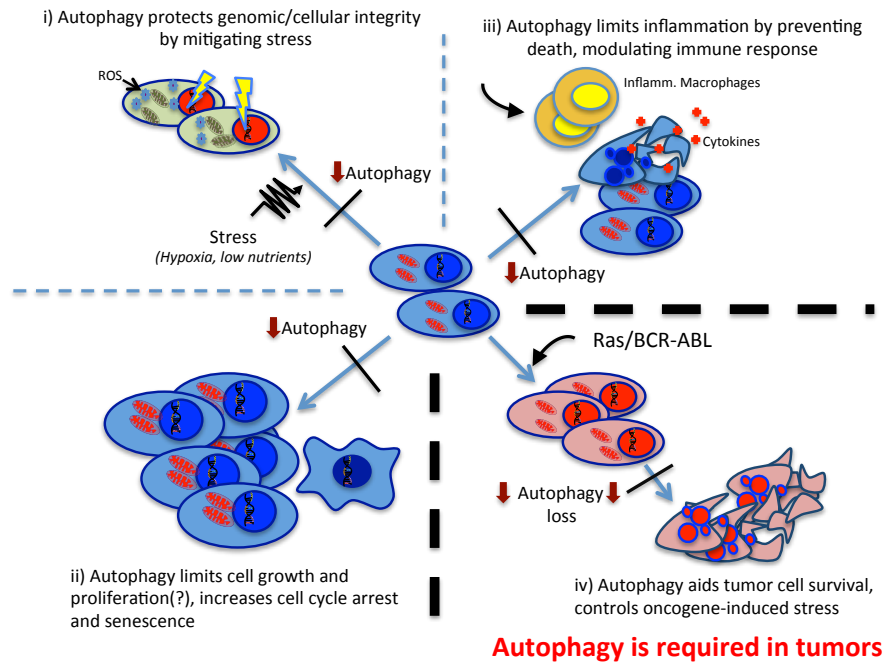


Figure 5.1: Autophagy's 'two edged sword' role in cancer. A summary of the major ways autophagy protects against tumor development (i-iii), and therefore impaired autophagy may aid tumorigenesis (diagrams i-iii), and its contrasting reported requirement for tumor cell survival in certain cancer models (iv). See text for details.

5.2 Autophagy maintains macromolecular and genomic integrity through stress response

At its simplest, oncogenesis involving acquisition of 'hallmark' capabilities requires cellular damage leading to genomic or epigenetic changes (390). As discussed in the previous two chapters, autophagic degradation plays a vital role in cellular quality control, removing damaged macromolecules and organelles. Disabled autophagy in murine models leads to the accumulation of old, misfolded and aggregated proteins in cells (134) (162) (102), damaged organelles (7) and toxic byproducts such as reactive oxygen species (131), causing oxidative and ER stress (391). The latter two processes are well characterized to

contribute to mutations, transformation and further cancer evolution over time (179) (392) (193) (393) (192).

Indeed under conditions of metabolic stress, and despite impaired survival via increased necrosis, deficient autophagy following allelic loss of *Beclin1* or *Atg5* deletion in immortalized epithelial cells has been directly tied to increased tumorigenicity (394) and several other phenomena. These include nuclear and centrosomal abnormalities, aneuploidy, DNA double-strand breaks and chromosomal copy number changes, in particular when accompanied by apoptotic deficiency (395) (394). The ‘metabolic stress’ conditions used comprised 1% oxygen and nutrient restriction believed to mimic the typical ischemic tumor microenvironment (396) (395). Under these conditions, the combination of inflammation induced by necrosis and increased mutagenesis accelerated the selection for growth-promoting mutations. This in turn was purported to create a net tumor benefit. One of the mechanisms behind autophagy’s protective effects against oncogenesis was the elimination of autophagy adaptor p62. This adaptor otherwise collects with un-degraded protein aggregates, organelles and ER chaperone molecules in the absence of autophagy *in vitro* and *in vivo*, associated with both ER and oxidative stress (397). ROS suppression and p62 elimination helped mitigate cellular damage, with p62’s role also being linked to suppression of pro-apoptotic signaling via NF-κB activation in response to ROS (397) (398). In fact, p62 appears to have multiple signaling roles outside its adaptor function, as it binds to ubiquitin ligase KEAP1 to stabilize the oxidative stress transcription factor NRF2 (399) which in turn further upregulates p62 (400). Deletion of p62 helped to reduce the size of liver tumors formed in mice already lacking *Atg5* or *Atg7* (388).

5.3 Autophagy limits cell growth, proliferation, and is linked to cell cycle arrest

Integral to many cancers is a state of uncontrolled proliferation, driven by aberrations in oncogene or tumor suppressor gene signaling. This is accompanied by suppression of cell mechanisms designed to counteract such growth, such as senescence or programmed cell death (390). In order to divide, tumor cells uniformly require increased biosynthetic/anabolic activities to increase cell size in preparation for division, as well as to overcome negative cell cycle regulation, while catabolic activities are suppressed (401) (8). In healthy cells, the mTORC1 complex serves at a hub of growth signaling, integrating growth factor (PI3K), amino acid (Rag GTPase, tRNA), energy levels (LBK/AMPK) and stress sensors (DNA damage, oxidative and ER stress) (22) to either drive protein translation, cell growth and cell cycle progression in response to favorable signals (368) (367) (8) or, in poor conditions, allow cell arrest and survival through autophagic catabolism (8). Over activation of the Akt/mTOR pathway is a common feature of cancers, including leukemias (402) (403), and indeed mTORC1 signaling has been found to be required for proliferation in acute myeloid leukemias (403) (404) (405) while excessive mTOR activation mediates leukemogenesis (385). Multiple such transformative Akt/mTOR pathway alterations, including PI3K constitutive signaling (406) or loss of the tumor suppressor and negative Akt-regulator PTEN (35), have been shown to downregulate autophagy in cancer models.

In order to maximize cell mass, suppression of self-catabolism and autophagy is vital for growth activities, as induction of autophagy by metabolic cellular stresses, common in the tumor microenvironment, prolongs survival at the cost of cell size and growth (407). Overexpression of autophagy genes has been found to inhibit proliferation of epithelial

cancer cells (408). By contrast, knockout of autophagy genes in mice is associated with hyperproliferation in tissues (66, 308). The mechanism of hyperproliferation in the absence of autophagy is not clear (8); possibilities are outlined further on in the chapter discussion.

What is better characterized is the link between mediators of cell cycle arrest and autophagy induction. The p53 protein is a central tumor suppressor in cells, reacting to cellular stress by inhibiting cell cycle progression to await cellular repair and/or activating apoptosis. Following DNA damage in mouse cells, p53 has been found to activate AMPK, inhibit mTOR and thereby induce autophagy (409). Nuclear p53 directly drives transcription of several core autophagy genes (54) and upregulates positive autophagy regulator DRAM1 (52). Additionally, as discussed last chapter, autophagy gene *Atg7* may directly bind p53 to direct its activities towards cell cycle arrest, rather than apoptosis, following metabolic stress (352). Thus autophagy seems to be positively connected to p53-induced proliferation impairment; complicating this picture however, are reports that cytoplasmic p53 may suppress autophagy in tumor cells, as its removal was associated with autophagic induction and increased survival (410). This negative regulatory activity was subsequently connected to p53 mutants located preferentially in the cytoplasm (411), therefore it seems that a balance between cytoplasmic and nuclear p53 is vital for autophagy regulation: stress-induced nuclear p53 activity may drive autophagy in healthy cells, while in tumor cells autophagy may be suppressed by cytoplasmic p53 (16). Nor is p53 the only cell cycle inhibitor to influence autophagy. RB1 and pathway members p16INK4a and p27KIP1 induce autophagy (55), as do eIF2a (57) and p19ARF (412), with the latter acting both through p53-dependent mechanisms or independently through BCL-XL (374, 413).

Given that mediators of cell cycle arrest induce autophagy, it is not surprising that autophagy has also been linked to permanent arrest of cell division or senescence, induced by oncogenic signaling. Induction of autophagy was reported during the senescent transition (414) and ablation of *Atg7* and *Atg5* impairs onset of senescence (414) (415). By contrast, senescence associated with a long-term decrease of autophagy has been observed by our lab in primary cell populations including senescent T-cells (140). This matches similar reports in primary human fibroblasts (11). However these observations are not incompatible with a role for autophagy in oncogenic senescence transitions. In primary cell culture contexts, deficient autophagy may lead to long term damage and proliferation to initiate population senescence over time (11), as seen following chronic stress and DNA damage in HSCs (187) (188) (189), yet still suppress other acute senescence pathways.

Finally, autophagy is tightly linked to the ultimate protection against transformation, cell death. The primary death pathway initiated by several tumor suppressors is apoptosis (416), and the autophagy pathway has extensive crossover with apoptotic signaling (417). Autophagy proteins including Beclin1 and ATG5 have been shown to play roles in apoptotic pathways (45), with ATG5 mediating apoptosis following cellular stress (277) (278) and Beclin1 interacting with anti-apoptotic proteins BCL-2 (418) and family-member BCL-XL (419) to co-regulate both processes. Similarly, both autophagy and apoptosis can be induced by tumor suppressors like p53 and its effector DRAM1 (52) (420). Autophagy impairment in apoptosis-competent cells is well characterized to result in increased damage and apoptosis *in vivo* and *in vitro* (7, 395). In the context of cancer, in transformed cells where apoptosis has been impaired or disabled, for example by knockout of pro-apoptotic molecules BAX/BAK or BCL-2 inhibition (395), autophagy initially aids survival following metabolic stress by limiting metabolism and cellular damage (5). Therefore inhibition of autophagy in transformed cells (leaving them unable to undergo

apoptosis or autophagy) reduces viability (increased necrosis) but aids genomic instability and oncogenesis (421). Even when transformed cells are autophagy-competent, extended autophagic self-digestion following long-term metabolic stress can eventually lead to cell death independent of apoptosis (394) (422). This process was initially termed ‘autophagic cell death’ despite the fact that it likely results from attempts at survival. Indeed, an autophagy inducing Beclin1 peptide has recently been shown to cause a form of cell death that is dependent on key autophagy components, a process termed ‘autosis’ by the authors (423). As discussed below, the induction of autophagy-dependent cell death under stress has been observed to be associated with many cancer therapies (424).

5.4 Autophagy modulates inflammation and anti-tumor immunity

An important contributor to cancer development is an inflammatory microenvironment, which is induced by oncogenic oxidative stress and aids a transformative cellular phenotype by promoting proliferation, survival and angiogenesis (425). In transformed, apoptotic-deficient cells, autophagy protects against necrotic cell death that otherwise causes inflammation through pro-inflammatory cytokine secretion and release of inflammation-driving protein high-mobility group 1 (HMGB1), thereby promoting tumor growth (394) (395) (426). Similarly, deletion or mutation of *Atg16l1* and *Atg5* was found to contribute to inflammation in Crohn’s disease (427). As described above, impaired autophagy can lead to p62 accumulation, which can activate pro-inflammatory NF- κ B as well as oxidative response regulator NRF2 (399), mediating tissue damage (397). Defects in autophagic degradation have been linked to mechanisms underlying acute pancreatitis (428) and hepatitis (429) (430).

If autophagy helps prevent pro-tumorigenic inflammatory conditions, it also plays a vital role in mediating the immune response when cancer does develop. As discussed in the

previous chapter, autophagy plays an important role in differentiation, survival and proper function of immune cells (156) (282) (334), including their ability to mediate anti-tumor activities in the tumor microenvironment (431). Moreover, active autophagy in tumor cells may promote anti-tumor immune responses following chemotherapy by aiding ATP release (432), while deficient autophagy in tumor cells has been found to suppress cross-presentation of tumor antigens by dendritic cells (433), potentially limiting anti-tumor immune activity.

5.5 Other tumor suppressive effects - differentiation and metastasis

While the above categories represent autophagy's most commonly attributed anti-cancer abilities, other mechanisms of tumor suppression by autophagy have also been proposed. For example, as discussed in the previous chapter, we have found that low autophagy results in immature characteristics in myeloid cells, and indeed upregulated autophagy has been associated with the differentiation of leukemic cells (332) (333), often via degradation of leukemic oncoproteins (434) (158). Also, while autophagy's role in metastasis is stage dependent, it has been found to inhibit metastasis in some conditions (435).

5.6 Autophagy inducers in therapy

Functional autophagy therefore contributes to biological prevention of initiation of tumorigenesis or suppression of developing tumors, and its induction also appears required for the anti-cancer activity of certain therapeutic agents, in particular in cancers resistant to conventional apoptosis-inducing therapies (436). For example, histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) has been reported to induce autophagy-

mediated cell death in breast cancer cells resistant to tamoxifen treatment (437). Similarly, while the fact that autophagy accompanies the action of mTOR inhibitors such as rapamycin is no surprise, it seems that the toxicity of some mTOR inhibitors may be enhanced by autophagic activity and death (438). For example the action of RAD001 when used as a single agent or when used in combination with radiation to treat glioblastoma (439) or prostate cancer (440). Another mTOR inhibitor NVP-BEZ235 induced both autophagy and cell cycle arrest, consistent with the anti-proliferative effects of autophagy (441). Therapies that appear to mediate tumor cell death through autophagy also exist for hematological cancers. For example arsenic trioxide, an agent used to treat acute promyelocytic leukemia, induces autophagy in target cells and this induction is required for anti-leukemic effects (442) (443). The mechanism may be through degradation of oncoproteins in these oncogene-addicted leukemias (444), although ‘autophagic cell death’ in arsenic trioxide treated tumor cells has also been attributed to general self-digestion following upregulation of Beclin1 (445) or BNIP3 (446). Similarly, metformin, a mitochondrial inhibitor which induces AMPK activation, impairs lymphoma via mTOR inhibition and autophagy activation (447), and a similar impact has been found in T-cell acute lymphoblastic leukemia (448). A summary of autophagy inducing therapies can be found here (449).

5.7 Autophagy aids tumor cell survival under stress

As discussed above, autophagy can suppress cancer by maintaining cell integrity under metabolic stress, therefore downregulation of autophagy can aid tumorigenesis through promoting cell damage, genomic instability, proliferation and cancer evolution (395) (394) (387). However, once a tumor develops, the pro-integrity activity of autophagy may actually be required for transformed cells to survive stressful conditions (5), as excessive

damage and death which outbalances division may begin to inhibit tumor growth (shown in Figure 5.1). In such contexts, autophagy would actually promote cancer, and indeed autophagy appears to be required in transformed cells of certain cancer models (16). For example, autophagy gene knockout in immortalized baby mouse kidney cells (iBMK) expressing Ras family oncogenes (H-Ras and K-Ras) leads to an energetic crisis during starvation, with depleted metabolites and impaired growth (450); here, the pro-tumorigenic effects of impaired autophagy seem to be outweighed by loss of viability. Similarly, in leukemias driven by the BCR-ABL fusion protein, cells displayed low levels of autophagy. However this activity was still required as deletion of *Atg3* during cell stress resulted in cell cycle arrest and apoptotic cell death, reflected in *in-vivo* leukemic inhibition (451). Here, cell death was attributed to p53 mediated pro-apoptotic activation rather than metabolic failure (451), an alternate mechanism to the Ras autophagy requirement in iBMK cells. However a subsequent study by the same group in Ras-expressing non-small-cell lung cancer found the tumor impairment following *Atg7* deletion was indeed p53-dependent (452).

Thus it may be that specific oncogenes create cellular conditions requiring autophagic activity for growth during stress, whereas others can tolerate its loss (394). Autophagy requirement by tumors also appears stage dependent, for example autophagy induced by the tumor suppressor ARHI aided the initiation of tumor dormancy and maintained tumor cell health in that state, however active autophagy also restricted subsequent re-expansion of tumor cells (453). Additionally, active autophagy may aid cancer in non-cell intrinsic ways. For example hypoxia induced autophagy in cancer cells aids resistance to cytotoxic T-cell killing (454).

5.8 Autophagy inhibition in therapy

In keeping with a survival role for autophagy during stress, several studies have focused on a role for upregulated autophagy in mediating therapy resistance. Protective autophagy induction has been observed following treatment with diverse chemotherapeutic agents (455) (456) (457), including tyrosine kinase inhibitor imatinib in leukemia and also in the setting of radiation therapy (458). As a result, inhibition of autophagy in combination with therapy is a tempting option to consider where resistance to standard apoptosis-targeting or radiation therapies complicates treatment, and indeed synergistic effects have been reported. (5) (458) (459). Autophagy may be required for viability in dormant tumor cells (453), and in keeping with the previous chapter's connection of stem cells and autophagy, there is evidence that autophagy inhibition can sensitize cancer stem cells, notoriously less proliferative and therapy resistant (460) (461) (462).

The most common autophagy inhibitor used in several clinical trials, is hydroxychloroquine, a derivative of chloroquine. This is a lysosomotropic agent which is well characterized to inhibit lysosomal degradation and autophagy, possibly through inhibiting lysosome-autophagosome fusion (267). Most trials are still at preliminary stages, but data from cancer models has found chloroquine mediated autophagy inhibition to be effective in complementing therapy. For example in a myc-induced lymphoma model in the setting of treatment with cyclophosphamide to stimulate p53-mediated apoptotic cell death (463) and in drug-resistant BCR-ABL driven leukemia when chloroquine was combined with histone deacetylase inhibitor SAHA (464).

5.9 Cancer metabolism – both sides of the coin

As a pathway mediating turnover of cellular macromolecular stores, autophagy has a key role in cancer, where metabolic building blocks are typically in short supply (421). In differentiated tissues, most cells utilize mitochondrial metabolism under oxygenated conditions: here, glucose is converted into pyruvate through glycolysis, and pyruvate is shuttled into mitochondria, where it is oxidized to produce carbon dioxide and NADH (nicotinamide adenine dinucleotide, NAD⁺ when reduced) in the tricarboxylic acid (TCA) cycle. NADH then acts as electron donor for the electron transport chain (ETC) mediated oxidative phosphorylation of ATP. Only under oxygen-limited conditions do cells switch to converting glycolytic pyruvate to lactate. This anaerobic glycolytic metabolism produces only 2 molecules of ATP per glucose, as compared to 36 for oxidative phosphorylation (465) (466). In multicellular organisms, glycolytic lactate can be recycled to glucose using the Cori cycle in the liver (466).

However, as observed by Otto Warburg (467), the majority of cancer cells appear to utilize aerobic glycolysis, producing lactate even in the presence of oxygen (467) despite functional mitochondria (468) (469) (470). This preference, since termed the ‘Warburg Effect’, has been subsequently attributed to the requirement of dividing cells to produce sufficient biomass, including nucleotides, amino acids and lipids, to allow for rapid division (465). As a result, neoplastic cells shuttle the majority of carbon from glucose and glutamine uptake (also increased in cancers), in the form of TCA cycle as well as glycolytic intermediates, into biosynthetic pathways (465); glycolytic metabolism provides stoichiometric ATP production per carbon donor, whereas oxidative phosphorylation’s efficient use of oxidative potential from a carbon source provides excess ATP while reducing biosynthetic capacity (465). Therefore, oncogenes including *myc* (471) (472),

Ras (473), *Src* (474) and genes such as *Hif1 α* (475, 476) have been demonstrated to upregulate aerobic glycolysis, while loss of tumor suppressors such as p53 similarly increases glycolytic flux (477, 478). Interestingly, although the Warburg effect was envisaged as being accompanied by impaired oxidative phosphorylation, in many cancers the switch to glycolysis is reversible, glucose-dependent and occurs in the presence of a still functional mitochondrial metabolism. This variant of the original concept is termed the ‘Crabtree effect’ (479). AKT/mTOR pathway activation in cancers has been linked in several studies to initiation of the Warburg/Crabtree effect (simplified to ‘Warburg effect’ hereafter). This occurs through upregulation of glycolytic metabolism, including mTOR-mediated upregulation of rate limiting enzyme pyruvate kinase M2, and increased lactate generation (480) (481) (482) (483) (484) (485). Also, multiple studies have implicated the Warburg effect and its mediators in leukemias (486, 487) (488) (489) (484) (485).

Autophagy plays a vital role in cellular metabolic homeostasis. It is extensively tied into energy state signaling networks, including AMPK, Ras and mTOR. When stimulated, autophagic breakdown of cellular stores can free glucose, lipids, nucleotides and amino acids to maintain energy generation and biosynthetic activities when nutrients or growth factors are lacking (99) (5). Relating to the Warburg effect, the key glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase has been reported to positively regulate autophagy through mTORC1 inhibition under low glucose conditions (490) and *Atg12* transcription following mitochondrial damage (491), helping to promote survival under those conditions (491). Similarly, tumor hypoxia is one hypothesized promoter of Warburg-effect glycolysis; hypoxia-inducible factor HIF exists as a dimer of HIF1 α or HIF2 α with HIF1 β , and under hypoxic or oxidative stress conditions HIF-1/2 α stabilization allows HIF formation and upregulation of response pathways (492). Those pathways include glycolysis (492) and mitophagy, mediated by BNIP3 (41). Similarly,

hypoxia causes reduced translation and increased autophagy via ER-stress and the unfolded protein response (493). Finally, the switch to glycolytic metabolism of glucose means mitochondrial TCA cycle biosynthetic pathways, vital for the Warburg effect-mediated growth promotion (465), must be aided by other carbon sources such as the amino acid glutamine, through glutaminolysis (494). Glutaminolysis is upregulated by several glycolysis-promoting oncogenes (495) (496) and is important for cell cycle progression (496); ammonia generated as a byproduct of glutaminolysis has been shown to positively regulate autophagy (497).

These points above suggest autophagy may be important for metabolic support and glycolysis under certain conditions, for example in tumors driven by oncogenes like *myc* and *Ras* which create extreme metabolic stress (421), and indeed Ras-driven glycolysis has been found to be facilitated by functional autophagy (473). However, this contrasts with the vital roles of negative autophagy regulators PI3K and mTOR in mediating the Warburg effect and glycolysis (480) (481) (484). Autophagy defects also produce mitochondrial dysfunction (7, 292) which would favor a switch to non-mitochondrial, glycolytic metabolism in tumors (421). Recent reports have made a direct link between autophagy inhibition and increased glycolysis in pancreatic tumors *in vivo*, therefore the connection between autophagy and metabolism is complex (498) and further understanding requires investigations that take into account particular cancer settings (421), including leukemia. A conundrum is that there appears to be a requirement for autophagy liberated metabolic substrates to support the metabolism of certain cancers, and yet there is a benefit from lower autophagy in tumor cells; this may be partially explained by the two-compartment cancer metabolism model (499) (294) (500) (408), as outlined in Chapter 6.

5.10 Alterations to autophagy genes and protein expression

The above sections outline sometimes conflicting evidence concerning the role of autophagy in cancer pathogenesis and the effect of key cancer-related changes, including oncogene gain and tumor suppressor loss, on autophagy regulation. Another clue to resolve the overall impact autophagy has on cancer is to look at how autophagy genes (Atg's) and proteins are altered in human tumors. Multiple autophagy genes have been found to be lost in a hemizygous manner in cancer, and therefore have been characterized as tumor suppressors. The most prominent example is *BECN1* (encodes Beclin1), which is mono-allelically lost in almost half of human breast, ovarian and prostate cancers (61). Beclin1 has a proven link to tumor suppression (501) through its VPS34 binding domain, which mediates Beclin1's functions in the autophagy pathway (502). Similarly, the Beclin1 binding partner and positive autophagy regulator UVRAG has been found to be deleted and tumor suppressive in colon cancers (503). Downstream of the Beclin1 complex, frameshift mutations in the core autophagy genes *ATG2*, *ATG5*, *ATG9B* and *ATG12* have been found in colorectal and gastric cancers (504). In hematological cancers, the data is far more limited, however *ATG16L2* was identified as hypermethylated in chronic myelogenous leukemia and associated with poor prognosis (505).

An excellent review by Gorski *et al.* recently summarized published data regarding alterations to autophagy gene transcription and protein expression levels in cancer (506). A general trend towards decreased mRNA and protein expression for *BECN1*, decreased mRNA but either increased or decreased protein for Atg8 homologs LC3 and GABARAP, increased p62 protein and increased mTOR protein expression levels across multiple tumor types suggests a low or dysfunctional autophagy phenotype (506). LC3 protein levels in particular are worth highlighting; increased LC3 has been reported and

interpreted by some as an indicator of increased autophagy in certain solid tumors (507) (508). However strong LC3 staining in the absence of key autophagy genes like *Atg7* (498) demonstrates how LC3 protein accumulation can result from autophagic defects instead. Decreased expression of *Atg5* and *Atg7*, along with several other autophagy genes, has been observed in hepatocellular carcinoma (438). Separately, down regulation of ATG5 levels has recently been characterized in a large subset of melanoma patients, associated with reduced progression-free survival for patients and increased proliferation in a melanoma model (415).

5.11 Myelodysplastic syndrome and acute myeloid leukemia

Acute myeloid leukemia is a cancer of the myeloid lineage, it is thought to occur due to the transformation of an HSC or early hematopoietic progenitor cell (509); recent evidence points to leukemic stem cells most resembling hematopoietic progenitors at the LMPP or GMP stage, with the more immature LMPP-like population giving rise to the GMP-like cells (510). The dysregulated production and proliferation of myeloid cells in the bone marrow (BM) interferes with normal hematopoiesis, resulting in a reduction in red blood cell (RBC), platelet and leukocyte numbers. AML is often associated with common mutations and chromosomal abnormalities; as with many cancers, a multiple genetic-hit model for its initiation has been proposed (511). The WHO classifies AML into: (1) AML with characteristic genetic abnormalities (translocations and inversions), (2) AML with multi-lineage dysplasia (progressed from MDS), (3) therapy-related AML and (4) AML not otherwise categorized (512). AML is the most common myeloid leukemia, with a prevalence of 3-8 cases per 100,000, which rises to 17-19 per 100,000 in those 65 and older. Despite recent advances in AML treatment, remission is still not achieved in 20-

40% of patients with de novo AML, while 50-70% of cases with successful remission relapse within 3 years, therefore novel therapeutic strategies are urgently required (513).

AML can be preceded by bone marrow dysfunction pathologies (514). One such condition is myelodysplastic syndrome, which comprises a group of anemic disorders of uncertain etiology characterized by abnormal cell morphology in the BM, peripheral blood cytopenias and a roughly 30% overall transformation rate to leukemia (384). Increased caspase-dependent apoptosis, higher levels of reactive oxygen species (ROS) and mitochondrial damage are observed in BM cells from MDS patients (515). Although classification issues make prevalence hard to determine accurately (516), recent studies have estimated MDS to be more common than previously thought, with a prevalence of 2/1000 above the age of 65 in the US and Canada (517).

While there are many links between autophagy and cancer pathogenesis, there is very little data regarding autophagy's modulation in AML (384), with the vast majority existing reports focusing on the impact of treatments which alter autophagy rather than cancer development (518) (477). As a result, in this chapter, the levels of autophagy in AML patients and the impact of autophagy gene loss on an AML model are explored.

Results

5.12 Autophagy genes are deleted in human AML

AML is characterized by clonal chromosomal abnormalities, genetic mutations and changes in gene expression, some of which are known to impact disease progression and prognosis (519, 520) (521). Chromosome alterations include defined translocations and inversions, but also more complex karyotypes, often appearing in older patients and associated with poor outcome (522) (523). To assess the status of the autophagy pathway in human AML patients, the locations of copy number alterations (CNA) in recent studies of AML genomes (524) (525) (526) were cross-referenced with the locations of autophagy network genes, as defined by Behrends *et al.* (297). In the larger study of Walter *et al.* covering 86 patients, out of 8 deleted regions, those on 5q, 7q, 16q and 17p contained core autophagy genes, including *ATG10* and *ATG12* (5q) and Atg8 homologs *GABARAP* (17p), *GABARAPL2* and *MAP1LC3B* (16q), as summarized in Table 1 (524). Another Atg8 homolog, *GABARAPL1*, was located in a region of genetic loss on 12p identified by Akagi *et al.* (526). It is important to note these chromosomal regions encompass many genes, and autophagy gene locations are often outside narrow consensus regions defined by other studies; for example 5q31.1 (527) on chromosome 5, with *ATG10* and *ATG12* falling within a region more variably lost (527). However no core autophagy genes were found within amplified regions, and basic statistical analysis points to the inclusion of core autophagy genes within deleted regions being significant (performed with the help of Dr. L. Jostins). Rucker *et al.* defined losses at the following approximate regions occurring at frequencies of 77% for 5q, 55% for 17p, 45% for 7q and 32% for 16q among 60 patients with complex karyotype AML (525).

Table 5.1: Autophagy genes are hemizygotously deleted in human Acute Myeloid Leukemia (AML). Correlation between the locations of autophagy pathway genes in the human genome, as compiled by Behrends et al. (Behrends et al., 2010), and chromosomal regions commonly deleted in AML patients (Akagi et al., 2009; Rucker et al., 2006; Walter et al., 2009) (* indicates Rucker et al., 2006 only); ** indicates the statistical probability ($p=0.008$) of this number of core autophagy genes being deleted by chance, assuming independent gene locations, Fisher's exact tests. Grateful acknowledgement of Dr. L. Jostins for assistance with statistical analysis.

Chromosome arm lost (normally heterozygous)	Deleted Regions (from Walter et al., 2009, except where indicated)	Autophagy genes	Autophagy gene locations (human genome, taken from Ensembl Genome Browser)	Frequency of Chromosome Loss in Complex Karyotype AML (n=60, Rucker et al. 2006)
5q	Chr5: 79,741,823-159,483,514	Atg10 Atg12	Chr 5: <u>81,267,844-81,572,241</u> Chr 5: <u>115,163,897-115,177,555</u>	77%
7q	Chr7: 107,570,049-158,821,424	KLHDC10 PRKAG2 Atg9B	Chr 7: <u>129,710,350-129,773,596</u> Chr 7: <u>151,253,203-151,574,316</u> Chr 7: <u>150,709,302-150,721,586</u>	45%
12p	Chr12: 100,000-32,700,000*	GABARAPL1	Chr 12: <u>10,365,489-10,375,722</u>	18%
16q	Chr16: 59,672,953-88,827,254	GABARAPL2 MAP1LC3B	Chr 16: <u>75,600,249-75,611,779</u> Chr 16: <u>87,425,406-87,438,385</u>	32%
17p	Chr17: 1-8,353,903	GABARAP	Chr 17: <u>7,143,333-7,145,772</u>	55%

Leukemic cell lines are widely used to investigate aspects of disease and have been found to be largely faithful models for the cytogenetic abnormalities they carry (528). To examine the impact of genetic loss on autophagy gene expression, focusing on the 5q locus, three AML cell lines reported to carry hemizygous loss of 5q, namely HL60, NB4 and KG1a (528, 529), and three leukemic lines with normal 5q, namely HEL, THP1 and K562 (528), were chosen for study. The expression of *ATG10*, *ATG12* and selected other autophagy genes was compared by qPCR, referenced to the Atg-normal HEL cell line. HL60 cells in particular demonstrated reduced expression of *ATG10* and *ATG12*, while KG1a cells demonstrated reduced expression of all autophagy genes assayed and *ATG12* in particular (Figure 5.2). Decreased *ATG12* expression has also been correlated with 5q loss in patients (524). By contrast, NB4 cells showed similar expression of the autophagy

genes assayed relative to the HEL cell line, while K562 and THP1 showed significantly increased expression levels (Figure 5.2). These results demonstrate heterogeneity in Atg expression among AML cell lines, which carry alterations to many chromosomes and pathways (528), but suggest *ATG12* expression in particular could be altered by hemizygous loss of 5q.

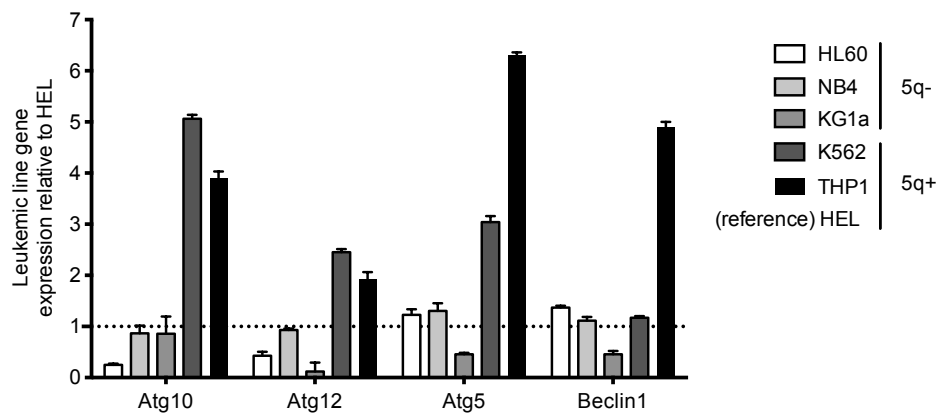


Figure 5.2: Leukemic cell lines with reported 5q deletions show reduced *Atg12* expression. *qRT-PCR* analysis of selected autophagy genes in six leukemic cell lines, values determined by $\Delta\Delta C_t$ method, normalized to *GAPDH* expression and compared to expression in HEL cell line for relative expression. Error bars represent SD.

To assess the impact of heterogeneity of autophagy gene expression on autophagy levels in leukemic cells, the degree of LC3/LysoID colocalization in HL60, NB4, KG1a, HEL and K562 cells, in addition to the MV4-11 and OCI-AML lines, was quantified via the Imaging Flow Cytometry Autophagy Assay (IFCAA) as described in Chapter 3.8. Relatively low levels of basal LC3/LysoID BDS were detected in all leukemic lines studied (Figure 5.3). These observations may reflect the impact of common autophagy regulating signaling changes in AML, including overactivation of the Akt/mTOR pathway (402) (403), and emphasize that genetic changes are only one means of an altered autophagy phenotype (16). Interestingly, the levels of basal autophagy in AML cell lines were generally much lower than those in exemplar epithelial breast and colon cancer lines,

MCF7 and HCT116 respectively (Figure 5.3). Moreover, attempted generation of ATG10, ATG12, ATG5 or empty vector (GFP) overexpressing NB4, HL60, KG1a, K562 and HEL cell lines showed that NB4, HL60 and KG1a (all with chromosome 5 deletions) were generally resistant to autophagy gene overexpression (compared to tolerance of vector alone), and of ATG10 and ATG12 in particular (data not shown), with no ATG10/12 positive cells surviving for those lines after 4 weeks. Measurement of autophagy levels using other methods and in more epithelial models would be required to confirm these findings, and excessive autophagy induction has previously been shown to be potentially toxic (423), but these results could suggest a particular addiction to lower autophagy levels among leukemic lines.

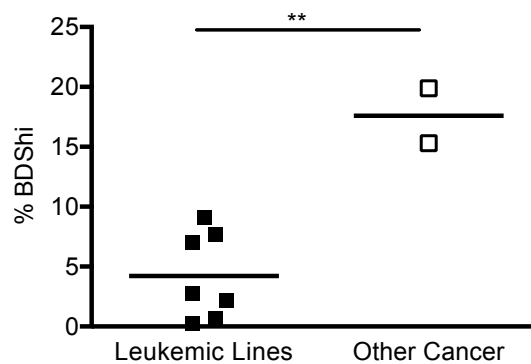


Figure 5.3: Reduced basal autophagy in leukemic cell lines compared to epithelial cell lines. Frequency of leukemic cell lines (HL60, KG1a, K562, HEL, NB4, MV4-11, OCIAML3) and epithelial cell lines (HCT116, MCF7) with high LC3/LysoID BDS (bright detail similarly, colocalization) under normal culture conditions, assay outlined in Chapter 3.8. ** $p < 0.01$, unpaired t test.

5.13 Human primary AML blasts display decreased autophagy gene expression

The loss of autophagy genes in AML is in keeping with Atg deletions in other cancer types (61) and suggests that the autophagy pathway may be functionally altered in AML patients. The BM of AML patients is a heterogeneous mixture of residual normal BM and

different AML subclones (530) (531) (532), which are organized in a hierarchical structure from leukemic stem cells to the bulk proliferating ‘blast’ population (510). The abnormal expression of surface markers (‘blast markers’) on leukemic cells is commonly used to characterize the blast populations within and between AML samples (531) (533). In order to investigate the autophagy phenotype of the bulk blast population in AML via the IFCAA, BM mononuclear cells (BMNCs) taken from a cohort of AML patients at diagnosis (obtained through *MDSbio* biobank) were first stained for a panel of blast markers (CD34, CD33, CD13, CD14 and CD117). Due to the limited available surface marker channels on Image Stream 100 for the IFCAA, the single or two-marker (combined in one channel) combination which represented the highest proportion of the blast population was chosen; although five markers were assessed, either CD34 or CD13 and CD33 (alone or in combination) was found to provide the best division, and therefore these were used on a donor-specific basis for subsequent experiments. Example blast marker gatings are shown in Figure 5.4.

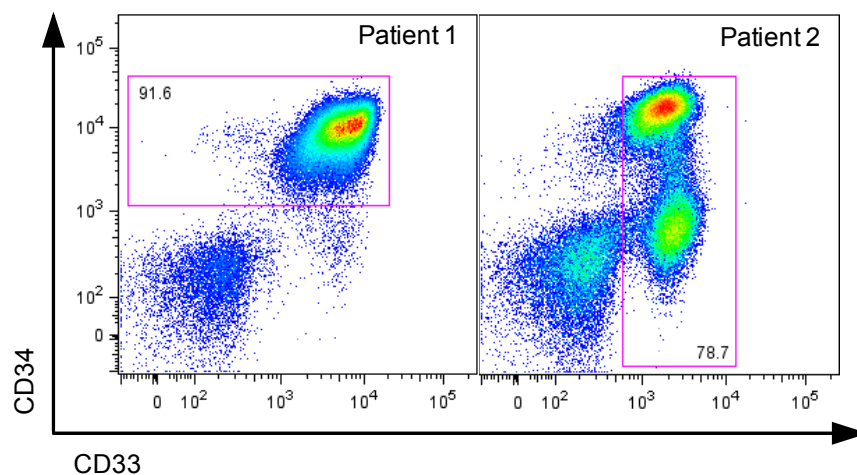


Figure 5.4: Acute myeloid leukemia patient immunophenotyping and blast marker gating. Example dot plots for CD33 and CD34 expression of bone marrow mononuclear cells (BMNCs) from two acute myeloid leukemia patients (five different five blast markers were assessed for each donor), showing donors where CD34 (left) or CD33 (right) best defines the blast population. A single (majority) or two-marker combination that represented the highest proportion of the blast population was chosen for Image Stream and Fluidigm experiments.

To assess the autophagy gene expression status within AML BM using those markers, cells from the blast marker positive population, chosen as above to represent the dominant AML blast population, and the blast marker negative population, representing residual hematopoietic cells and less proliferative minor clones (531), were sorted three times independently for each donor, and each sort was analyzed for expression of key autophagy genes via Fluidigm dynamic array as previously published (534). The blast marker positive cells were found to express relatively lower levels of several autophagy genes (Figure 5.5), particularly the Atg8 homologs *GABARAPL1*, *GABARAPL2* and *MAP1LC3B*, which were previously found to be located in complex karyotype AML deleted regions. Lower transcript levels of tumor suppressor *BECN1* as well as *ATG5* were also noteworthy. The donor cohort possessed only limited genetic/prognostic information, and no clear correlation between expression levels and those variables was found.

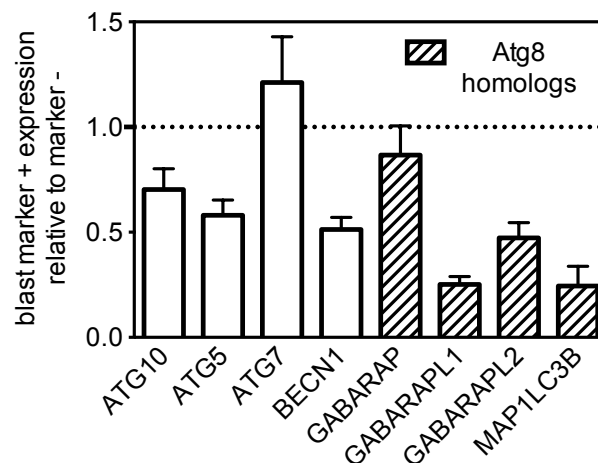


Figure 5.5: Expression of autophagy genes is reduced in AML blast marker positive cells. AML BMNCs were sorted on selected blast surface marker expression (as per Figure 5.4) into marker negative and positive populations, 3x200 cells/population per donor. RNA was extracted, selected autophagy genes amplified and analyzed in triplicate for expression using a dynamic array (Biomark Fluidigm) as previously described (141), see Methods for additional details. Values determined by $\Delta\Delta C_t$ method, normalized to GAPDH expression, with blast marker positive cell values compared to blast marker negative values for relative expression. Error bars represent SEM ($n=7$ donors). Grateful acknowledgement of Dr. Petter Woll for assistance with this experiment.

5.14 Human primary AML shows decreased autophagy and increased oxidative stress

To investigate the autophagy levels of AML blasts, patient BMNCs were stained for blast markers as described above and run on the IFCAA. Analysis revealed a low level of basal autophagy, via LC3/LysoID colocalization (BDS), in the blast positive cells (Figure 5.6), and although in most donors BDS did increase on application of lysosomal inhibitors (E64d and Pepstatin A, as described in Chapter 3.8), those levels were still markedly reduced compared to those observed in the blast marker negative population of each patient (Figure 5.6).

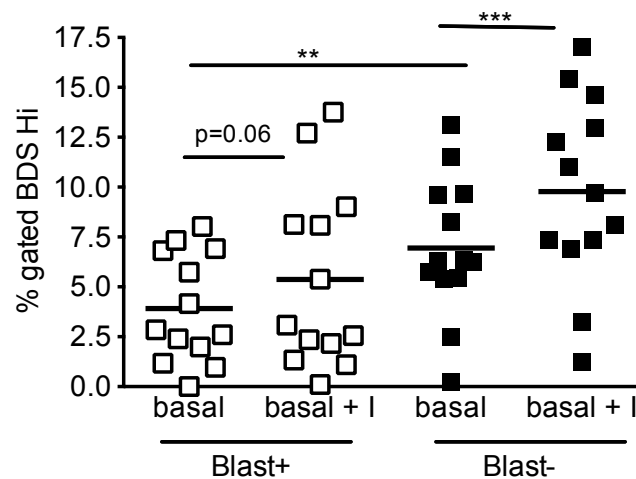


Figure 5.6: AML blast marker positive cells show decreased autophagy. Frequency blast marker positive or negative cells from AML BMNCs with high LC3/LysoID BDS after 2hrs treatment with 10µg/ml E64d/Pepstatin A (basal+I) or medium (IMDM 10% FCS) alone (basal), assay outlined in Chapter 3.8. Error bars represent SEM (n=13 donors), ** $p < 0.01$, *** $p < 0.001$, paired t test.

Moreover, when the relative level of BDS increase on lysosomal inhibitor treatment (IFCAA ‘flux’) in blast positive cells was compared to that found in healthy donor CD34+ progenitors, or indeed those from patients with the AML precursor MDS, it was found to

be significantly reduced (Figure 5.7). These data suggest basal autophagy levels are suppressed in the dominant blast population of AML patients relative to the remainder of the BM.

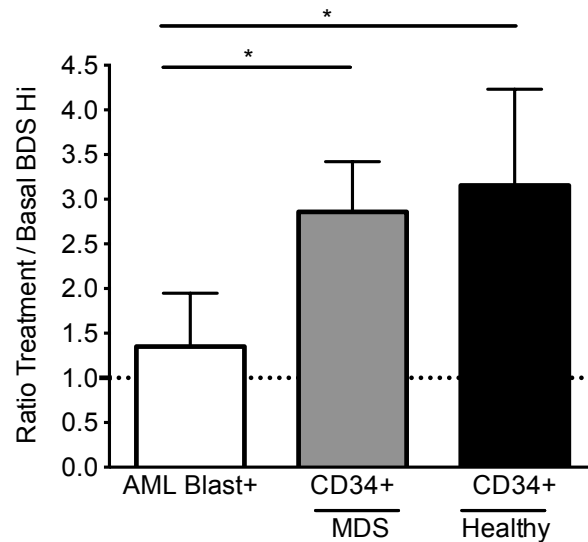


Figure 5.7: AML blast marker positive cells have reduced autophagic flux. Ratio of frequency cells LC3/LysoID BDS high in blast marker positive cells among AML BMNCs, CD34+ cells from healthy donor BMNCs or CD34+ cells from myelodysplastic syndrome (MDS) patient BMNCs after 2hrs treatment with 10µg/ml E64d/Pepstatin A (basal+I) versus medium alone (basal) (ratio basal+I/basal), assay outlined in Chapter 3.8. Error bars represent SEM (n=13 AML donors, 6 healthy donors, 8 MDS donors), *p<0.05, unpaired t test.

To assess the autophagy status of AML BM using an alternative readout, we examined the levels of p62 in BM trephine samples from AML patients and healthy controls by immunohistochemistry (IHC) (technique in press, (146)). As described in the introduction to Chapter 3, p62 is an adaptor molecule with both LC3 and ubiquitin binding domains which helps target cargo for autophagic degradation and is itself degraded in the process (92) (255). As such, p62 accumulation can be used as a marker of decreased autophagy (255) (498) and has been assessed via IHC in a variety of tumor types (506); increased p62 levels correlated with poor prognosis in lung cancer (535). To control for non-specific

background, each sample was also stained with antibody pre-incubated with saturating amounts of p62 peptide, providing a negative control. 7 out of 8 AML samples showed widespread positive p62 staining, compared to 0 out of 4 healthy controls; three representatives of each group are shown in Figure 5.8 along with negative controls (assessed with the help of Dr. E. Soilleux). Increased transcription of p62 cannot be excluded, but increased p62 staining indicates a potential low autophagy phenotype and matches the observation of generally increased p62 staining that is found across tumor types (506).

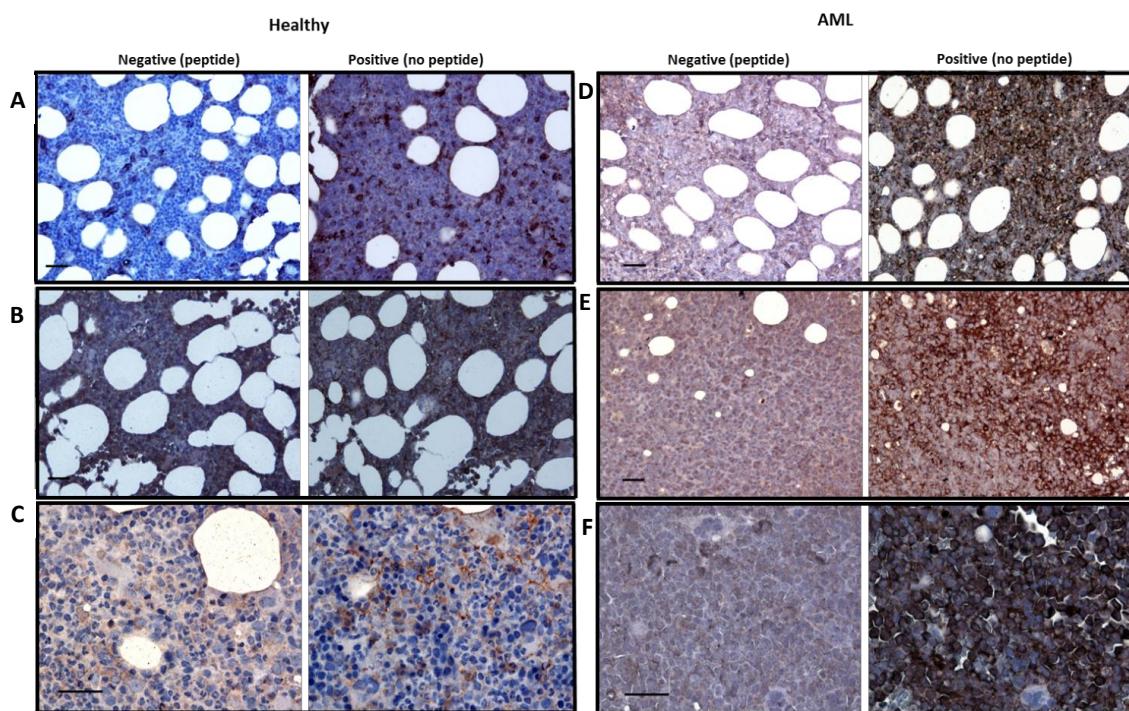


Figure 5.8: AML bone marrow trephine samples show increased p62 staining. Representative immunohistochemistry of p62 in bone marrow trephine samples from AML patients (A-C above, of n=8 donors total) or healthy controls (D-F above, of n=4 donors total) stained with anti-p62 antibody alone (positive) or with antibody pre-incubated with 400x molar excess of synthetic human p62 peptide (negative) to block specific staining. Scale bar 50µm. Grateful acknowledgement of Dr. Elizabeth Soilleux for assistance with setup and assessment.

Autophagy functions to dampen oxidative stress, primarily through mitophagy and removal of damaged cytoplasmic components (294). We analyzed the levels of ROS and oxidative stress in AML bone marrow, specifically assessing mitochondrial superoxides (using MitoSOX) and mitochondrial health (using NAO, which stains non-oxidized cardiolipins in healthy mitochondrial membranes (296) (295) as outlined in Chapter 3.18). AML blasts showed evidence of higher levels of superoxides and lower levels of healthy mitochondria compared to CD34⁺ cells from healthy donors and MDS patients (Figure 5.9), possibly indicating increased oxidative stress, although the latter may also relate to mitochondrial content alterations (536) (537). Oncogene-induced increases in oxidative stress, including excessive Akt pathway activation (538), have been previously characterized to drive proliferation and genomic instability in AML models (537) (539) (540) and targeting oxidative stress responses may be therapeutically beneficial (541).

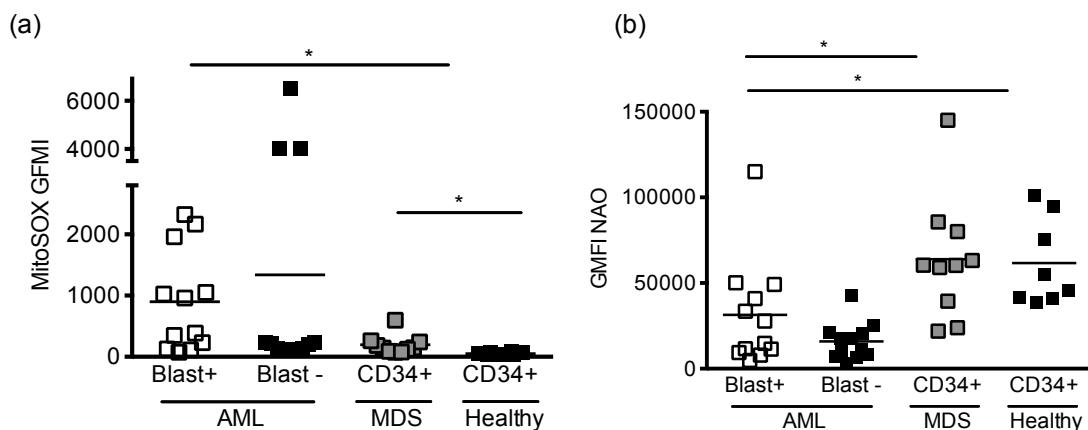


Figure 5.9: AML BMNCs display high oxidative stress. GMFI of MitoSOX (left) or nonyl-acridine orange (right) staining in blast marker positive and negative population among AML patient BMNCs, CD34⁺ cells from myelodysplastic syndrome (MDS) patient BMNCs or CD34⁺ cells from healthy donor BMNCs ($n=12$ AML donors, 10 MDS donors, 8 healthy donors). $*p=0.05$, unpaired t test.

MDS CD34⁺ cells also showed a mild increase in mitochondrial superoxides relative to healthy counterparts (Figure 5.9), in agreement with mitochondrial abnormalities reported in MDS patients (542) (384). Intriguingly, blast negative cells in AML BM also showed

evidence of high ROS levels (Figure 5.9), an observation suggesting general BM microenvironmental oxidative stress, the potential impacts of which are explored in Chapter 6 (294).

5.15 Generation of a murine MLL-ENL (ME) AML model with *Atg5* deletion

These data presented above indicate that decreased autophagy may be an acquired feature in a proportion of AML patients, however the impact of reduced autophagy on the biology of leukemic pathogenesis is uncertain (384). To investigate this, a murine model of autophagy-deficient AML driven by the MLL-ENL fusion oncoprotein was developed (Figure 5.10). Translocations that involve the mixed lineage leukemia (MLL) gene, active during normal embryonic development and hematopoiesis (543-545), can result in both myeloid and lymphoid leukemias (546) (547), including 60% of infant AMLs (548). MLL can fuse to over 40 different partner genes, the most prevalent being ENL, AF9 and AF4 (549), which aberrantly activate transcriptional pathways (546). AML leukemogenesis mediated by MLL fusion to chromatin modification and transcription elongation complex member ENL (550) has been shown to require multiple Hox family genes (136), FRAT2 (135), C/EBP α (551) and anti-apoptotic factor MCL-1 (552). To study autophagy in MLL-ENL AML pathogenesis, enriched lineage-negative cells from mice with *Atg5^{fl/fl}*, *Atg5^{fl/wt}* and *Atg5^{wt/wt}* genotypes (3 pooled per genotype) were transduced with constitutive MLL-ENL as previously described (136) (135). Transformation of progenitors via MLL-ENL transduction has been shown to be sufficient to cause leukemia *in vivo* (553). The transformed lines were subsequently transduced with either retrovirus containing a human CD2 reporter alone (empty vector) or one also carrying iCre (133) (137) and sorted for purity (Figure 5.10). The addition of Cre post-transformation was

required due to the high potential for cytotoxic damage to be already present in *Atg5*^{-/-} progenitors. The generation of these new cell lines from primary murine progenitors, rather than established cell lines, permitted study of the impacts of *Atg5* deletion with minimal background mutations.

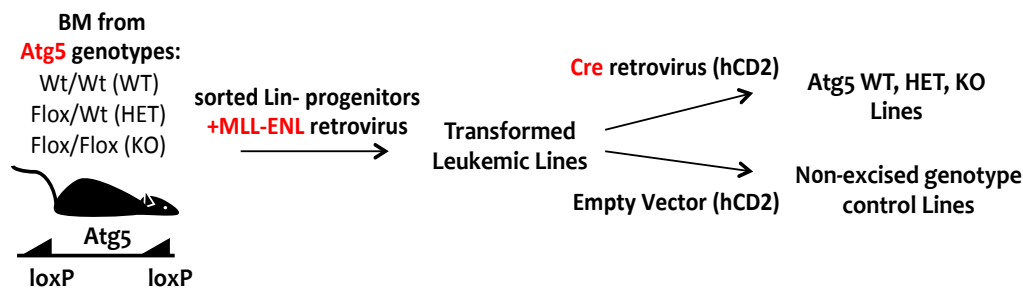
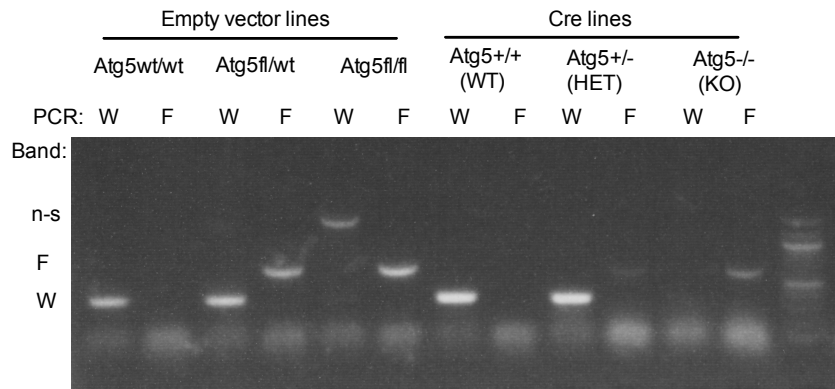


Figure 5.10: Schematic showing generation of *Atg5*^{-/-}, *Atg5*^{+/-} and control murine MLL-ENL (ME) cell lines. Bone marrow from *Atg5*^{fl/fl}, *Atg5*^{fl/wt} and *Atg5*^{wt/wt} (B16) mice (3 per genotype) was extracted, crushed and pooled by genotype. Lineage cells were depleted and lineage negative cells transduced with constitutive MLL-ENL as previously described (135, 136). The resulting lines were transduced with pMSCV-IRES-CD2, containing the tailless human CD2 cDNA (137) either alone (empty vector) or with additional *iCre* (133), sorted on hCD2 expression for purity than grown in RPMI, 10% FCS, 2mM L-Glutamine, penicillin/streptomycin, 50μM 2-mercaptoethanol, 100 ng/ml mSCF, 10 ng/ml mIL-3, 10 ng/ml mIL-6 except when indicated. Final lines Cre transduced lines are WT (*Atg5*^{wt/wt}; *iCre*⁺), HET (*Atg5*^{fl/wt}; *iCre*⁺), and KO (*Atg5*^{fl/fl}; *iCre*⁺), and corresponding empty vector controls (*Atg5*^{wt/wt}; *Atg5*^{fl/wt}; *Atg5*^{fl/fl}) with no Cre expression.

The resulting *iCre* carrying MLL-ENL transformed lines (ME lines) with *Atg5*^{+/+} (WT), *Atg5*^{+/-} (HET) or *Atg5*^{-/-} (KO) genotypes, along with associated Cre-negative empty vector control lines (*Atg5*^{wt/wt}; *Atg5*^{fl/wt}; *Atg5*^{fl/fl}), were genotyped for the presence of the wildtype *Atg5* allele, *Atg5* flox allele and *iCre* as described in Chapter 4.6, confirming their expected genotypes (Figure 5.11). This method of transformation is published to give rise to myeloid cell lines (136) (553) (135). To confirm this, the lines were stained for expression of myeloid markers CD11b and Gr1 and the progenitor marker c-Kit, all showing similar positive expression (Figure 5.12).

(a) Atg5 Flox/WT PCR



(b) iCre PCR

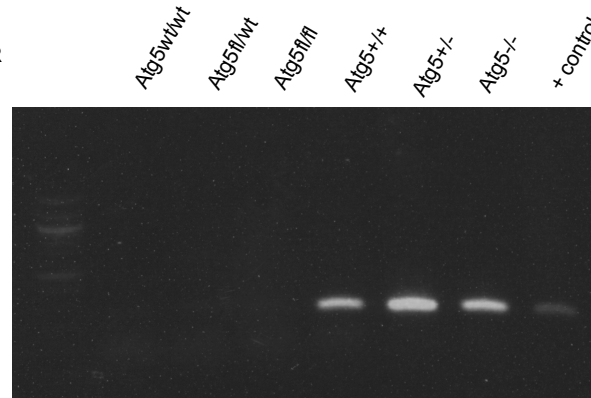


Figure 5.11: Genotyping of ME leukemic lines. (a) PCR Amplification of Atg5 F (Flox) and W (wildtype) alleles from DNA extracted from MLL-ENL immortalized cell lines of the indicated genotype, PCRs performed separately and run in separate wells on on 1% agarose/TBE for 30min at 76V, n-s indicates non-specific band sometimes observed in W PCR of Atg5fl/fl murine cells. (b) PCR Amplification of iCre from indicated cell line DNA or Vav-iCre containing mouse ear biopsy (control), run on 1% agarose/TBE for 30min at 76V.

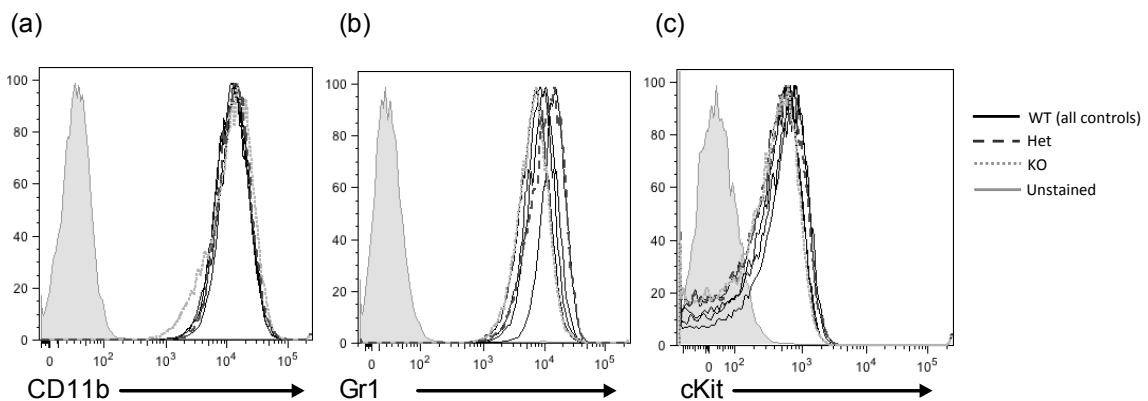


Figure 5.12: ME leukemic lines express equivalent levels of myeloid surface markers. Histograms of (a) CD11b, (b) Gr1 and (c) c-Kit surface marker staining intensity of MLL-ENL lines, genotypes as indicated.

To confirm the autophagy phenotype of the Cre lines, cells were cultured with or without lysosomal inhibitor bafilomycin A1 before lysates were extracted for LC3 immunoblot. The KO cells showed increased basal levels of LC3-I, but lysosomal inhibition resulted in no accumulation of lipid-conjugated LC3-II, indicating an arrest in autophagosome formation and autophagy pathway impairment (Figure 5.13). Both WT and HET lines showed robust LC3-II buildup, but on band quantification the normalized LC3-II/I ratio on bafilomycin A1 treatment (249) (247) was slightly lower in the HET line (Figure 5.13).

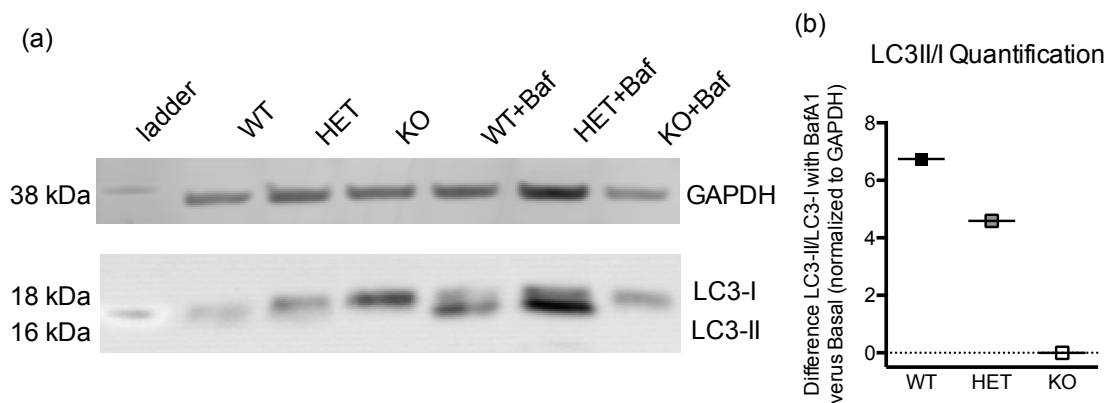


Figure 5.13: Autophagy is lost in KO ME line by LC3 immunoblot. (a) Immunoblot against LC3 and GAPDH in protein lysates of iCre-transduced MLL-ENL cell lines, WT, HET and KO after two hours treatment with 100nM bafilomycin A1 (Baf) or in normal media. (b) Quantification of LC3I and LC3II band intensity (of immunoblot shown in a), normalized to GAPDH loading control intensity and showing change in LC3-II/LC3-I intensity ratio in bafilomycin treated lanes minus non-treated controls (note that all controls showed no LC3-II band, therefore a ratio of zero). Thanks to Hanlin Zhang for help performing this blot.

Mild decreases in autophagy on heterozygous deletion of *Atg5* has been previously reported in transformed epithelial cells (395), while *Atg5* has been shown to be quantitatively important for autophagy in overexpression models (277) (278) (279). However no phenotype was observed in *Atg5^{flox/wt};Vav-Cre⁺* mice (data not shown) or *Atg7^{flox/wt};Vav-Cre⁺* mice (7). To assess the autophagy levels using alternative readouts, the mean number of LC3 puncta per cell was quantified via Image Stream, and the lines were separately stained with putative autophagosome dye CytoID (Figure 5.14). The four

wild-type lines showed similar levels of both LC3 puncta and CytoID intensity, with the existing variation strikingly similar between the different readouts (Figure 5.14). Similarly, the KO line possessed significant reductions in both basal LC3 puncta and CytoID intensity (Figure 5.14). Interestingly, the HET line displayed significantly reduced numbers of LC3 puncta, however CytoID intensity was within wild-type variation (Figure 5.14); without knowing the precise mechanism of CytoID it is hard to assess this difference. Therefore the KO line displays a clear autophagy defect, while the HET line phenotype is less clear.

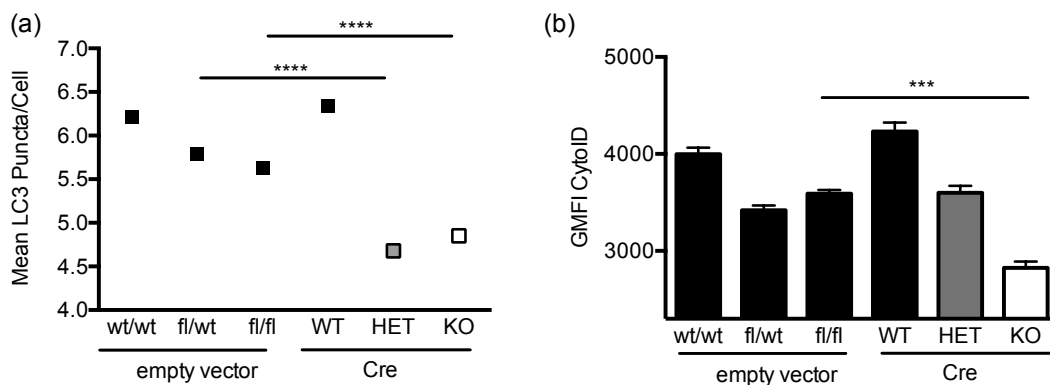


Figure 5.14: Variable autophagosome content in HET ME line by flow cytometry. (a) Quantification of mean number of LC3-puncta per cell using Image Stream on endogenous LC3 staining in ME lines under normal (basal) growth conditions (spot to background ratio 3.25, minimum 1500 live, single cells quantified per line). (b) GMFI of CytoID staining in ME lines as measured by standard flow cytometry under normal (basal) growth conditions ($n=3$ cultures per line). Error bars represent SEM, *** $p<0.001$, **** $p<0.0001$, unpaired t -test.

5.16 *Atg5* loss results in increased ME leukemic proliferation under metabolic stress

As outlined in the introduction to this chapter, links have been reported between autophagy and cell proliferation (66) (8), while autophagic activity can limit cell growth and division under conditions that induce metabolic stress (554), which are commonly found in the tumor microenvironment (395) (555). The proliferation of the ME lines was investigated

via short-term BrdU pulse and subsequent co-staining for BrdU incorporation against DNA content as shown in Figure 5.15, where the proportion of cells in S-phase is a well-characterized readout for proliferation.

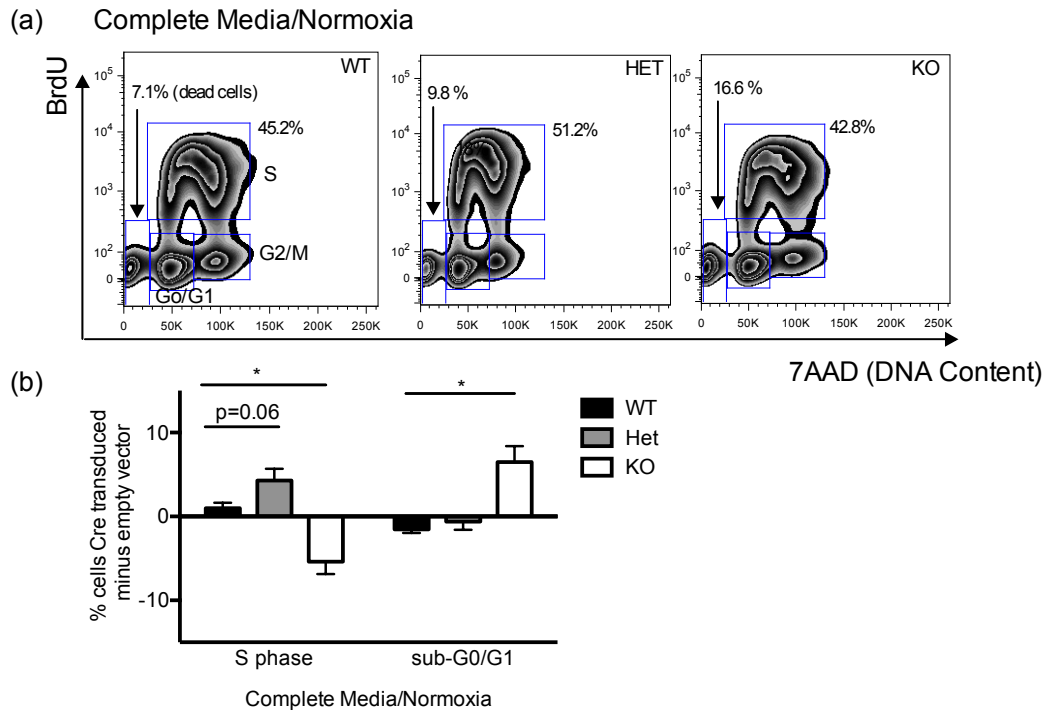


Figure 5.15: Cell cycle profiling of ME leukemic lines shows mild alterations following *Atg5* loss in normal culture conditions. Bromodeoxyuridine (BrdU) was added to ME line cultures (final concentration 10 μ M) 45 minutes prior staining (a) Representative contour plots showing BrdU intensity (incorporation) against 7AAD intensity (DNA content) for iCre-transduced ME lines (WT, HET, KO as Figure 5.10), with gateings representing cell cycle stages and dead cells (sub-Go/G1) as indicated. (b) Difference in frequency of cells in S-phase or sub-G0/G1 (Cre transduced subtract relevant genotype empty vector) after culture in normal growth conditions (complete media with 10% and cytokines/normoxia), gating as in (a). Error bars represent SEM (n=6 cultures over three experiments), *p<0.05, unpaired t test.

Dead cells were characterized by their sub-G0/G1 DNA levels, a traditional readout for apoptosis (556) (557, 558). However recent studies have highlighted that necrotic cells can also display DNA fragmentation and sub-G0/G1 staining (559). Therefore this population was simply judged to indicate dead/dying cells from either pathway (apoptosis/necrosis). For cell cycle analysis, the frequency of cells in S-phase or sub-G0/G1 in the Cre transduced lines was routinely subtracted from the relevant empty vector line as an internal

control, as shown in Figure 5.15. The ME lines were grown in complete media containing 10% FCS supplemented with SCF, IL-3 and IL-6 (136). Under these growth conditions, the WT and HET lines showed comparable cell cycle profiles (Figure 5.15), with the KO cells displaying moderately decreased S-phase fraction and increased in cell death (Figure 5.15). However, under conditions of metabolic stress following serum withdrawal (18 hours 2% FCS no cytokines), both HET and KO line showed significantly increased continued proliferation relative to wildtype lines, while the KO line also showed increased cell death (Figure 5.16). The role of autophagy in resisting cell death following metabolic stress has been previously found in immortalized models (560), whereas autophagic loss was reported to cause not only increased death but also genomic instability (395) (397).

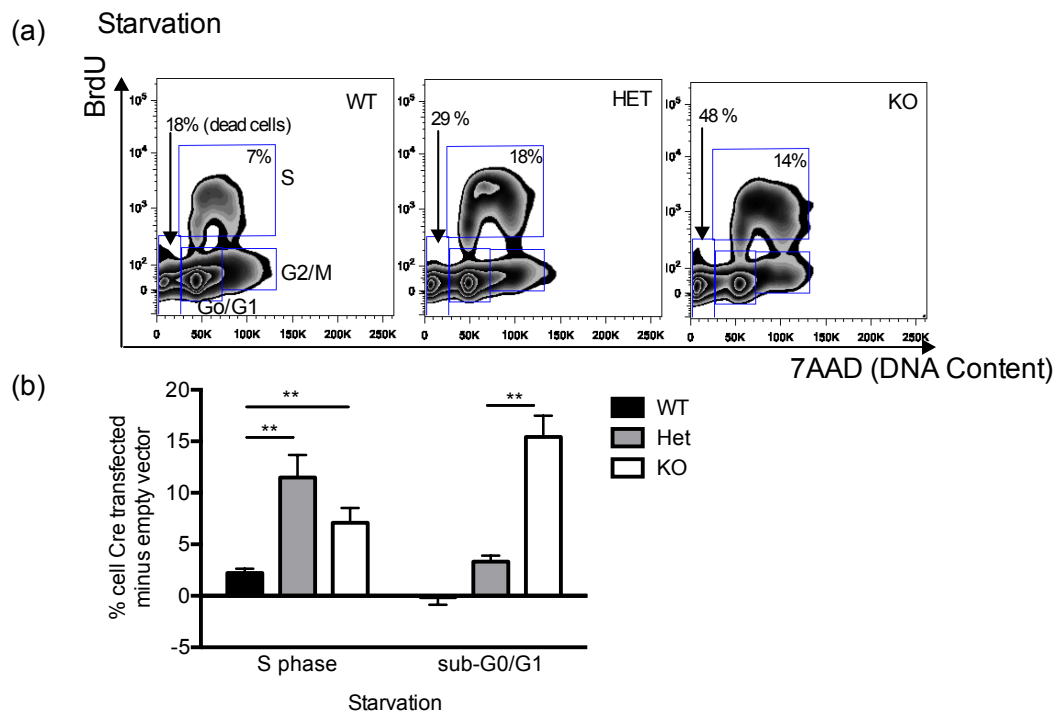


Figure 5.16: KO and HET ME lines show increased proliferation under serum starvation. (a) Representative contour plots showing BrdU intensity (incorporation following pulse as Figure 5.15) against 7AAD intensity for iCre-transduced ME lines (WT, HET, KO as Figure 5.10) cultured under starvation (2% FCS, no cytokines) conditions for 18 hours, with gatings representing cell cycle stages and dead cells (sub-G0/G1) as indicated. (b) Difference in frequency of cells in S-phase or sub-G0/G1 (Cre transduced subtract relevant genotype empty vector) after culture in starvation conditions for 18 hours as (a). Error bars represent SEM (n=5), **p<0.01, unpaired t test.

A form of metabolic stress particularly common in the bone marrow is hypoxia (561) (202), which is exacerbated by leukemic development (562) (563). Therefore AML cells require, and commonly show, adaptive ability to proliferate under low oxygen conditions (564) (565). The proliferation of the ME lines was therefore investigated under this alternate stressor, and the KO line in particular was found to display increased proliferation following 40hrs in 0.1% oxygen (Figure 5.17), and although KO cells underwent increased cell death (apoptosis/necrosis) in hypoxic conditions (Figure 5.17), the difference was not as dramatic as that following nutrient withdrawal, and indeed was similar to levels in complete media.

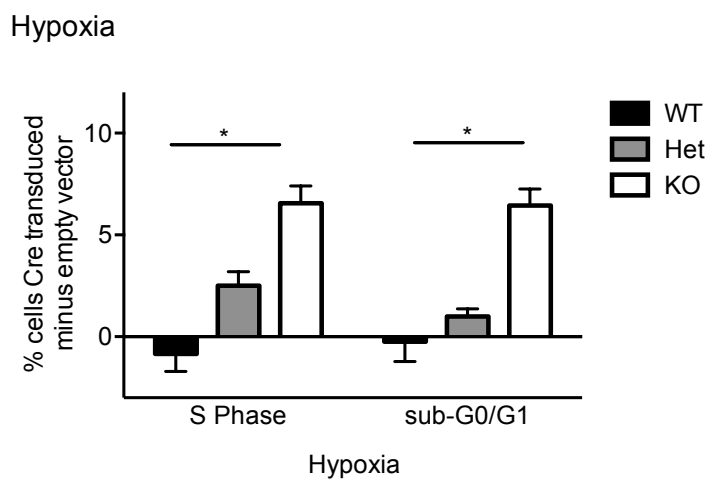


Figure 5.17: KO ME cells are more proliferative in hypoxic conditions. Difference in frequency of cells in S-phase or sub-G0/G1 (dead) (Cre transduced subtract relevant genotype empty vector) after culture in complete media (10% FCS and cytokines), 0.1% oxygen for 40 hours and BrdU pulse as Figure 5.15. Error bars represent SEM (n=5), * $p < 0.05$, unpaired t test.

To provide a different measure of proliferative ability in the ME lines, they were plated in complete methylcellulose without erythropoietin and grown for 72hrs under either normoxic conditions or 0.1% oxygen. Relative to control lines, the HET cells were able to form more colonies under normoxic conditions, however the KO colony formation was significantly favored under hypoxic conditions (Figure 5.18). Together, these results

suggest that *Atg5* deficiency enables continued proliferation under conditions of microenvironmental metabolic stress that are known inducers of autophagy.

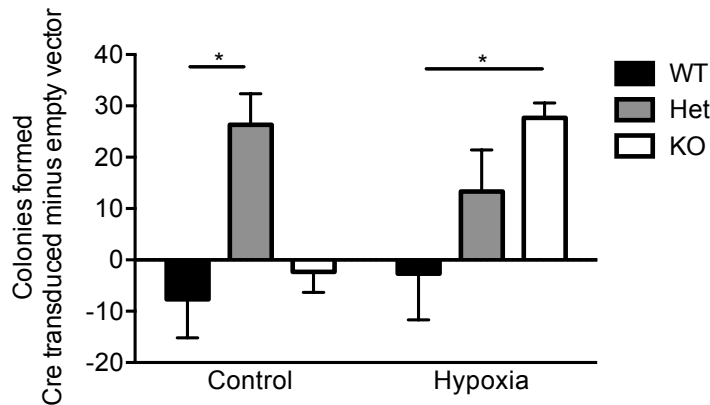


Figure 5.18: KO ME cells form more colonies under hypoxic conditions. Different numbers (#) of colonies formed in complete methylcellulose without EPO by 500 cells from ME lines (Cre transduced subtract relevant genotype empty vector) following 72 hrs culture in 0.1% oxygen. Error bars represent SEM ($n=3$ per line, representative of two experiments), $*p<0.05$, unpaired t test.

5.17 NAC treatment does not decrease *Atg5*-deficient proliferation gains in ME leukemia

A common feature following homozygous deletion of autophagy genes in mouse models is accumulation of damaged mitochondria (6) (205) and associated increases in reactive oxygen species (131), particularly under conditions of metabolic stress (397). As reactive oxygen species (ROS) are known to promote proliferation of cancer cells at certain concentrations (540) (566) (294), oxidative stress was considered as a potential mediator of the increased proliferation following *Atg5* allelic loss. However addition of ROS-scavenger N-acetyl cysteine (567) (568) to serum-starved cultures left WT and HET cell cycle profiles unaffected, while in fact increasing the proportion of KO cells in S-phase and partially rescuing KO apoptosis/necrosis (Figure 5.19). These results require confirmation with other anti-oxidants, but could indicate that, while the excess cell death

in the KO line is NAC sensitive, the excess proliferation in the HET and KO lines under metabolic stress may not be ROS-mediated.

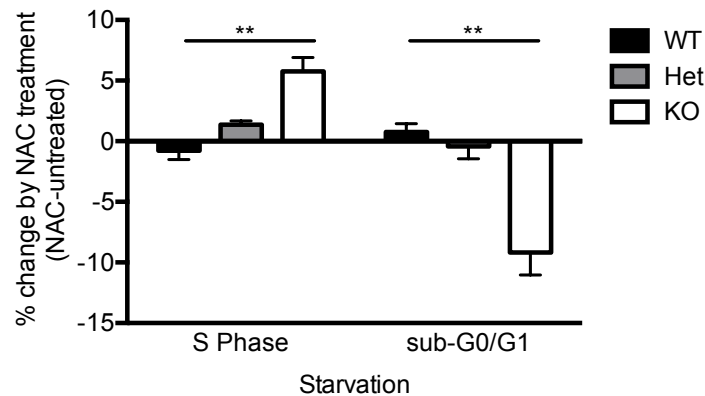


Figure 5.19: NAC does not hamper HET and KO proliferation. Difference in frequency of cells in S-phase or sub-Go/G1 (dead, apoptotic/necrotic) in Cre-transfected ME lines after culture under starvation (2% FCS, no cytokines) conditions for 18 hours with or without 5mM N-acetyl cysteine (NAC), BrdU pulse as Figure 5.15. Error bars represent SEM (n=5), * $p < 0.05$, unpaired t test.

Supporting this, wildtype lines showed substantially increased whole cell ROS following serum-withdrawal mediated metabolic stress as assessed via flow cytometry, while the HET and KO lines actually displayed reduced ROS build up (Figure 5.20). Mitochondria are a primary source of intracellular ROS, with components of the electron transport chain accounting for up to 90% of ROS generation (9), therefore mitochondrial content of the ME lines was assessed to see whether reduced ROS generation under stress reflected lower numbers of mitochondria in HET and KO lines. However, staining for healthy mitochondrial content via NAO showed similar levels across lines (Figure 5.21). As an alternative readout, the number of mitochondria in representative cells of each genotype was quantified by transmission electron microscopy. Counts showed some variation among the lines, possibly reflecting lower sample size, and although the data from the HET and KO cells were towards the lower end of the count ranges they were not significantly lower than the Cre containing WT line, which itself trended towards lower

mitochondrial counts (Figure 5.21). Similarly, mitochondrial ultrastructure assessment revealed no observed morphological defects in HET and KO mitochondria (data not shown). Therefore, *Atg5* deficient ME lines showed no evidence of mitochondrial accumulation and associated ROS build-up above that found in wildtype cells.

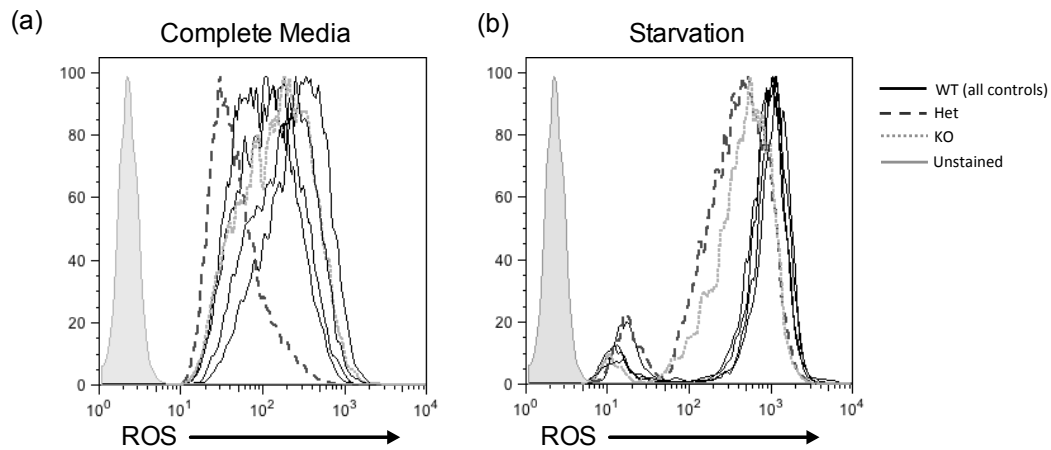


Figure 5.20: KO and HET ME lines show decreased ROS under starvation. Histograms showing intensity of oxidative stress detection reagent (Enzo Life Sciences) in ME lines after 18 hours in (a) complete media (10% FCS and cytokines) or (b) under starvation (2% FCS, no cytokines).

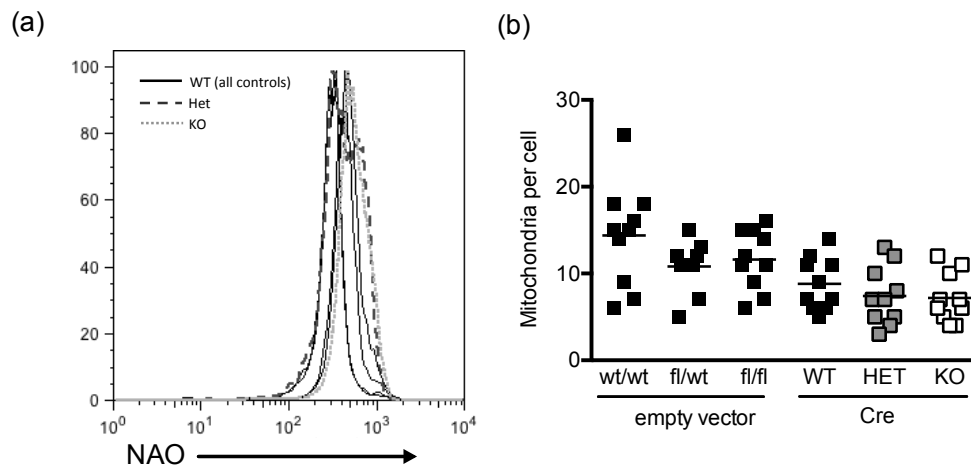


Figure 5.21: Equivalent mitochondrial content across ME line genotypes. (a) Histograms of nonyl-acridine orange (NAO) intensity in ME lines, representative experiment of three (b) Enumeration of visible mitochondria in transmission electron microscopy (TEM) micrographs of 10 cells per indicated genotype, ME lines were grown in complete media conditions prior to fixation and analysis. Grateful acknowledgement of Professor David Ferguson for TEM image acquisition and assistance with analysis.

5.18 Metabolic reprogramming aids ME leukemia proliferation in the absence of *Atg5*

Proliferation is intimately tied to cell metabolism and, as outlined in the chapter introduction, tumor cells commonly display altered metabolisms to support sustained growth and division (465). Autophagy plays an important role in metabolic homeostasis (99) (421), with autophagy loss in the context of Ras-driven tumors reported to inhibit the TCA cycle and oxidative phosphorylation (450) (569). The reduced ROS generation exhibited by the HET and KO lines was a potential indicator of metabolic changes; increased use of mitochondrial metabolism by certain types of cancer is associated with more abundant cellular ROS (570), while upregulated aerobic glycolysis, preferred for energy generation in a majority of tumor types (465), helps to control excess generation of ROS, which can inhibit growth at higher concentrations (571) (565) (572) (573).

To investigate the metabolic state of the ME leukemia lines, their mitochondrial function was probed via measurement of oxygen consumption rate (OCR) over time using the Seahorse XF24 Bioanalyzer, with the step-wise addition of pharmacological modulators of the electron transport chain (ETC) (Figure 5.22). Unlike reports from studies of Ras associated tumors, the KO cells showed no reduction in basal oxygen consumption, in fact showing a mild increase in initial OCR relative to WT cells (Figure 5.22). Treatment with oligomycin, an inhibitor of ATP synthase at the end of the ETC that arrests oxidative phosphorylation-dependent oxygen consumption to estimate mitochondrial ATP generation (574), showed all lines utilized low levels of mitochondrial ATP synthesis (defined as the drop in OCR on ATP synthase inhibition), with the WT line showing the lowest mitochondrial-dependent oxygen consumption (Figure 5.22). To assess for mitochondrial dysfunction, the cells were treated with ETC uncoupler FCCP to estimate

the maximum respiratory capacity (oxygen consumption after FCCP treatment) and the mitochondrial spare capacity (difference between oxygen consumption post-FCCP treatment and basal) (574). KO cells showed a significantly reduced maximal respiratory capacity (Figure 5.22), an indication of potential mitochondrial dysfunction (574). Intriguingly, the HET line showed a quite different phenotype, with an increased, yet unused, spare capacity (Figure 5.22). Complete ETC inhibition using complex I-targeting rotenone showed little non-mitochondrial oxygen consumption across all lines (Figure 5.22).

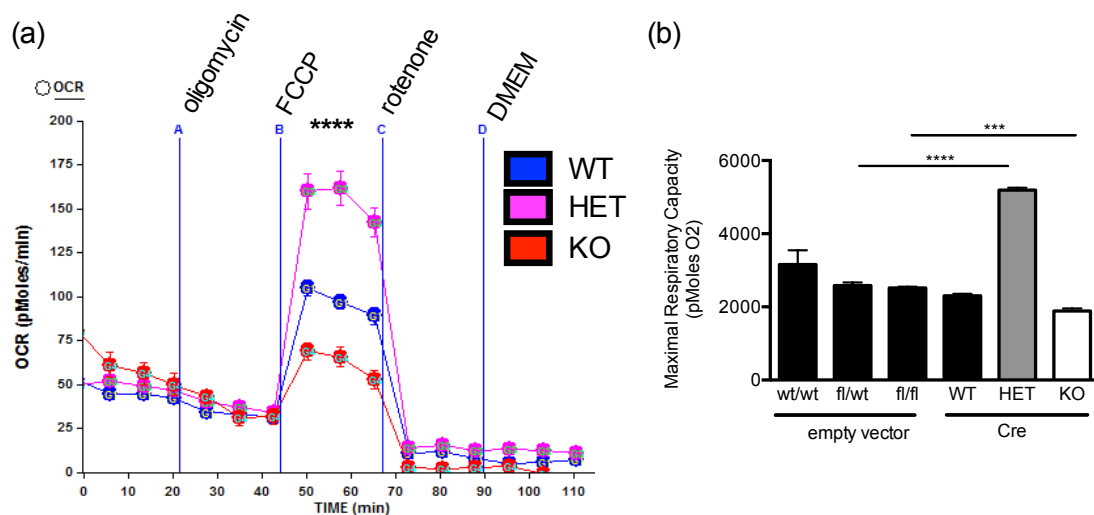


Figure 5.22: KO and HET ME lines have altered mitochondrial respiratory capacity. ME lines grown in complete media were plated 2.8×10^5 cells/well on Cell Tak (BD) coated microplates, washed and rested for 1 hour in low-buffered bicarbonate-free DMEM (pH 7.4) prior to analysis on XF24 XF (Seahorse Bioscience) as previously described (145). (a) Oxygen consumption rate (OCR, pMoles/min) was measured over time, with addition of oligomycin (400nM), FCCP (400nM), rotenone (1 μ M) or DMEM media control at indicated time points. (b) Quantification of maximal respiratory capacity (OCR multiplied by time during FCCP treatment, prior to rotenone addition) from analysis in (a) for indicated genotypes. Error bars represent SD ($n=5$ replicates per ME genotype, over two experiments), *** $p < 0.001$, **** $p < 0.0001$, unpaired t test.

Combined, this assay reveals that all lines generated only low levels of ATP using mitochondrial pathways, and that both KO and HET basal mitochondrial use was not impaired, in fact being slightly elevated in KO cells. The reduction in maximal and spare

respiratory capacity in *Atg5* KO cells could point to mitochondrial dysfunction and impaired mitochondrial potential generation (574), consistent with reports in *Atg7*^{-/-} mouse models (292). By contrast, the HET OCR phenotype suggests increased, but unused, mitochondrial potential. It is worth noting that all ME lines suffered in the serum free conditions required for Seahorse analysis, and as the KO cells were particularly prone to serum-starvation induced death, it is possible the respiratory capacity drop is an artifact.

As ME lines displayed low mitochondrial ATP generation, it seems likely that they use alternative mechanisms for fulfilling energy requirements. This is in keeping with previous reports that MLL-ENL myeloid immortalization upregulates aerobic glycolytic metabolism (575). Lactate production by the ME lines was assayed to judge glycolysis levels, and although all lines showed strong lactate generation, the HET cells in particular were found to have increased lactate generation compared to wildtype controls, while the KO line displayed a more moderate rise (Figure 5.23).

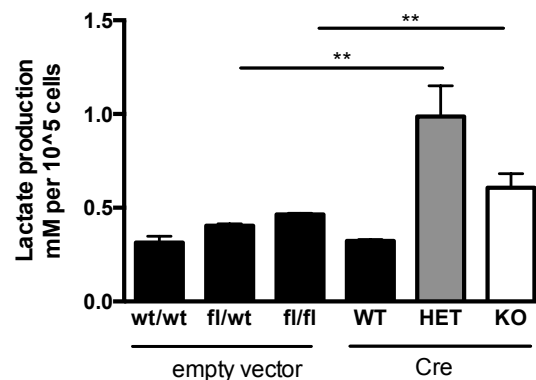


Figure 5.23: Increased lactate production following *Atg5* loss in ME leukemic lines. Enzymatic determination of lactate production over 48 hours under normal growth conditions by ME lines of indicated genotype, 0.5×10^5 cells initially plated per well, cells counted by trypan blue after 48 hours before supernatant collection, lactate concentration in supernatant normalized to media alone control and divided by final cell count ($\times 10^5$ cells). Error bars represent SD ($n=4$, two duplicates over two independent Cre transductions), $**p < 0.01$, unpaired *t* test.

Upregulated glycolysis is known to support oncogenic growth in a variety of cancer models (465). To assess whether HET and KO glycolysis provided a proliferative advantage, the ME lines were subjected to the metabolic stress of serum starvation as before, but with the sugar galactose exchanged for the normal glucose in their media. Due to ATP requirements for galactose processing, conversion of galactose to pyruvate via glycolysis generates no net ATP, therefore cells must switch to oxidative phosphorylation to meet energy needs (576, 577). Supporting this, culture in galactose resulted in a sharp drop in lactate production across all cell lines (Figure 5.24). ME lines were grown in complete media supplemented with either galactose or glucose for 24 hours and then underwent serum starvation in the presence of the same sugar type. Apoptosis levels were not impacted by galactose in any line under either condition, indicating that the metabolic switch was well tolerated, although the proliferation of all lines was mildly reduced under complete media conditions (data not shown). However, under starvation the proliferative advantage of all lines were reduced but in particular that of the HET and KO cells (Figure 5.24). This implies that the increased growth observed following *Atg5* allelic deletion is supported in part by upregulated glycolysis.

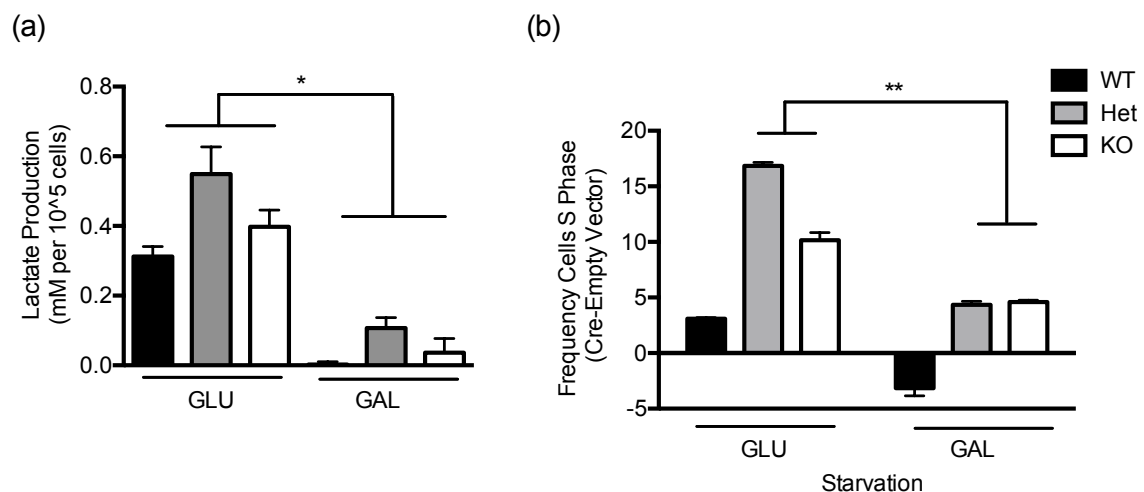


Figure 5.24: KO and HET proliferation under stress is glycolysis dependent. (a) Enzymatic determination of lactate production over 48 hours in Cre transduced lines (calculated as in Figure 5.23) after culture in growth media (10% FCS and cytokines) containing either 20mM glucose (GLU) or 20mM galactose (GAL).

(b) Difference in frequency of cells in S-phase (Cre transduced subtract relevant genotype empty vector) after culture in RPMI containing either 20mM glucose (GLU) or 20mM galactose (GAL), first for 24 hours in complete media (10% FCS and cytokines) than under starvation conditions for 18 hours (2% FCS, no cytokines) (as other starvation treatments, both medias contained 2mM L-Glutamine, penicillin/streptomycin, 50uM 2-mercaptoethanol). Error bars represent SEM (n=2), * $p < 0.05$, ** $p < 0.01$, unpaired t test.

5.19 *Atg5*-deficient ME leukemic cells exhibit Akt/mTOR phosphorylation under starvation

The growth regulating Akt/mTOR pathway is an important mediator of the Warburg effect and glycolysis in cancer (480) (481) (482) (483) (484) (578). As discussed at the end of Chapter 4, a connection between the downregulation of autophagy and altered mTOR signaling has been hypothesized but not demonstrated (393). The activation of Akt and mTOR in the ME lines was assessed by flow cytometric staining for their phosphorylated forms (phosflow), as previously performed on hematopoietic progenitors (209).

Phosphorylation of Akt at Ser473 following growth factor signaling is required for its full activity (579-581), and once activated Akt directly phosphorylates mTOR at both Thr2446 and Ser2448 to promote its activity (27, 582-584). As expected, serum withdrawal resulted in a uniform loss of phospho-Ser473-Akt (p-Akt) intensity for all wildtype ME lines (Figure 5.25). However, both HET and KO lines retained significant p-Akt staining under starvation conditions, retaining 54% and 63% of their complete medium level respectively (where the average wildtype line intensity difference from starvation to complete media is taken as 100%). Similarly, serum starvation evenly decreased phospho-Ser2448 mTOR (p-mTOR) in wildtype ME lines, but HET cells retained 30% of activated wildtype intensity, while KO cells retained 74% (Figure 5.26). KO cells also displayed a trend towards increased intensity under complete media conditions, although this was not significant. Together, these data suggest that Akt and mTOR may be aberrantly activated under metabolic stress following *Atg5* allelic loss in the ME lines.

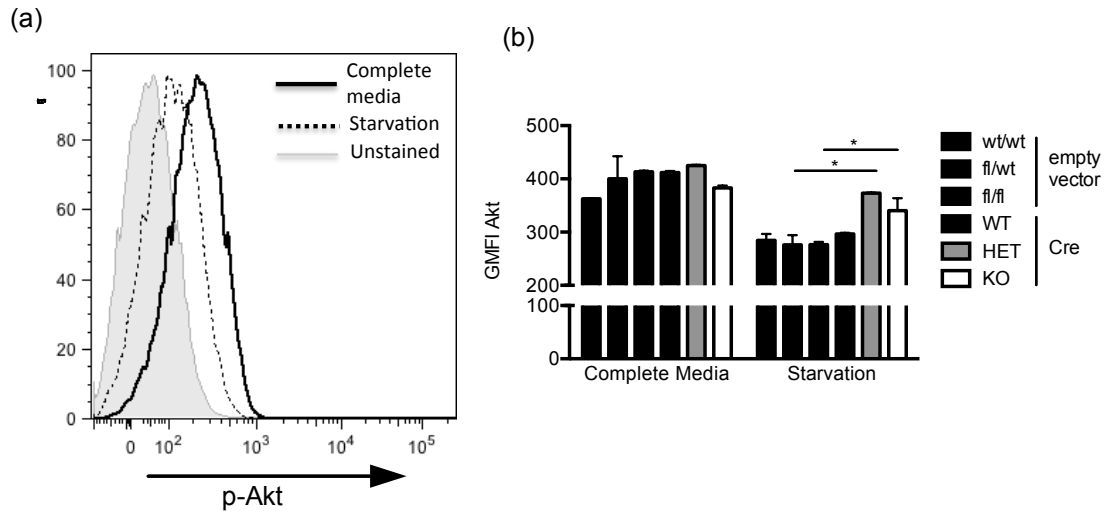


Figure 5.25: KO and HET cells show Akt activation under starvation. ME leukemic cells were cultured in complete media (10% FCS, cytokines) or starvation conditions (2% FCS, no cytokines) for 18 hours than rapidly fixed, permeabilized and stained for phospho-Akt (Ser473). (a) Representative wildtype histogram showing intensity of p-Akt staining under complete media and following starvation. (b) GMFI of p-Akt staining under complete media conditions and following serum starvation for indicated genotypes. Error bars represent SD (n=2, representative of three independent experiments), *p<0.05, unpaired t test.

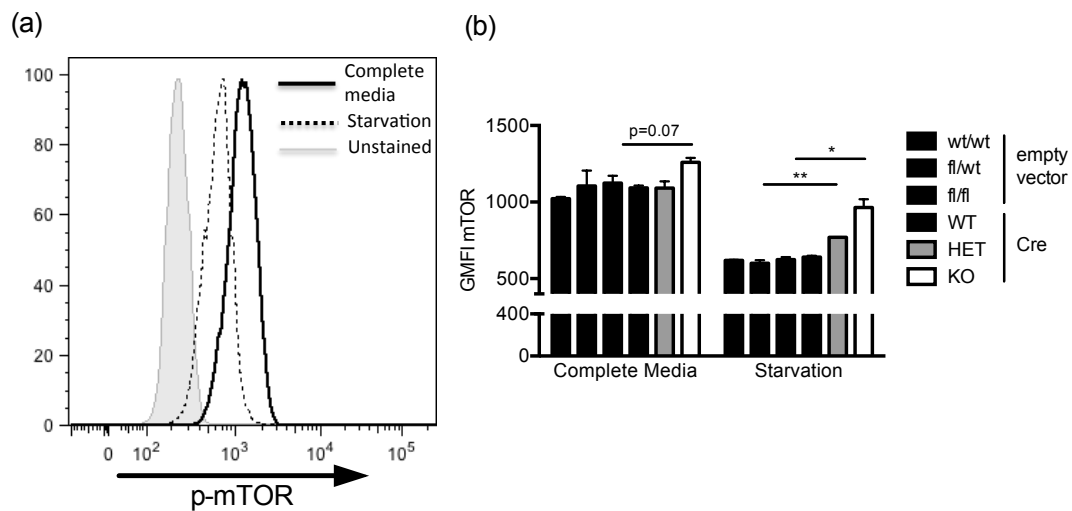


Figure 5.26: KO and HET cells show mTOR activation under starvation. ME leukemic cells were cultured in complete media (10% FCS, cytokines) or starvation conditions (2% FCS, no cytokines) for 18 hours than rapidly fixed, permeabilized and stained for phospho-mTOR (Ser2448). (a) Representative wildtype histogram showing intensity of p-mTOR staining under complete media and following starvation. (b) GMFI of p-mTOR staining under complete media conditions and following serum starvation for indicated genotypes. Error bars represent SD (n=2, representative of three independent experiments), *p<0.05, **p<0.01, unpaired t test.

mTOR kinase has a well described role in promoting cell growth and regulating cell size (585). Loss of mTOR signaling following nutrient starvation results in a reduction in cell size, while mTOR hyperactivation limits the loss of cell area (585, 586). Using the Image Stream brightfield images to calculate the mean cell area of each ME line under complete media and serum starvation conditions (>3000 cells counted each), HET and KO cells retained 5% and 4% more cell area than WT cells on growth factor loss (Figure 5.27). This agrees with the observations that mean area, quantified by Image Stream, of *Vav-Atg5*^{-/-} and *Vav-Atg7*^{-/-} hematopoietic cells, including bone marrow cells and lymphocytes, was consistently greater than wildtype equivalents (data not shown). Although further tests with rapamycin (which will reduce mTOR activity without inducing autophagy in the KO line) might help ensure that loss of catabolism does not contribute to size retention, these data help support deregulated cell size and hyperactivated mTOR signaling in HET and KO cells on metabolic stress.

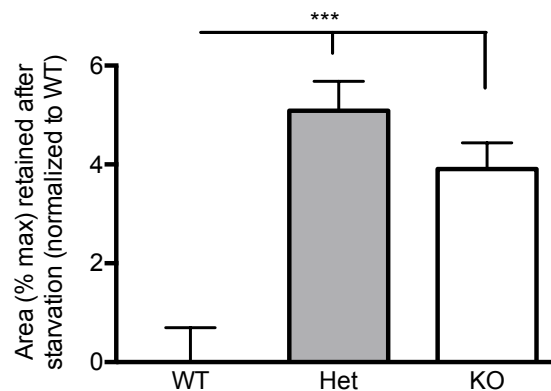


Figure 5.27: KO and HET cells retain cell size on starvation. Percentage of maximal (complete media) cell area retained (Cre transduced subtract relevant genotype empty vector) on starvation (18 hours, 2% FCS, no cytokines), as quantified from Image Stream brightfield images, normalized to WT. Error bars represent SEM (minimum 3000 cells assessed per ME line), *** $p < 0.001$, unpaired t test.

ROS-dependent activation of p-Akt and p-mTOR has been reported in mice with deletion of oxidative stress and autophagy regulating transcription factor FOXO3, leading to myeloproliferation which was rescued by treatment with the ROS-scavenger NAC. (587). The lack of differential impact on proliferation of the ME lines following treatment with NAC suggests this is not the mechanism involved in the HET/KO lines. To confirm this p-Akt and p-mTOR levels were examined under serum starvation combined with NAC addition. In agreement with previous observations (587), culture with NAC further suppressed p-Akt and p-mTOR levels, however the magnitude of reduction was similar across all lines (Figure 5.28). As before, these results require confirmation with alternative antioxidants, but may indicate increased ROS is not the mechanism behind Akt/mTOR signaling changes in HET and KO lines.

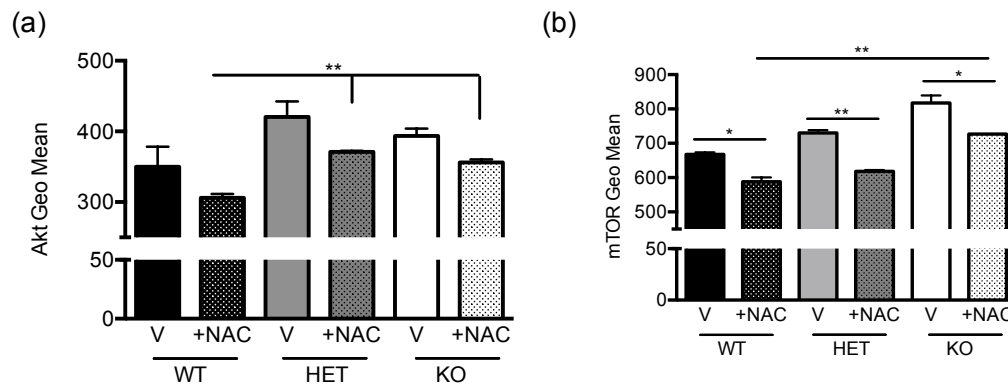


Figure 5.28: NAC results in equivalent decreases in p-Akt and p-mTOR across ME lines. ME leukemic cells were cultured in starvation conditions (2% FCS, no cytokines) treated with vehicle (V, dH2O) or 5mM N-acetyl cysteine (NAC) for 18 hours then rapidly fixed, permeabilized and stained for phospho-Akt (Ser473) and phospho-mTOR (Ser2448). (a) GMFI of p-Akt staining in Cre-transduced ME lines of indicated genotype under starvation with or without NAC treatment. (b) GMFI of p-mTOR staining in Cre-transduced ME lines of indicated genotype under starvation with or without NAC treatment. Error bars represent SD (n=2), *p<0.05, **p<0.01, unpaired t test.

5.20 mTORC1 activity promotes ME line glycolysis

As increased Akt/mTOR signaling is recognized to drive glycolysis upregulation and dependence in a variety of cancer types, including leukemias (480) (481) (482) (483) (484) (578), the effect of mTOR inhibition on the ME leukemia line lactate production was investigated. mTOR exists in two distinct complexes: mTOR complex 1 (mTORC1) with GβL and RAPTOR, which regulates autophagy and cell growth through S6K and 4E-BP1 (585), or mTOR complex 2 (mTORC2) with GβL and RICTOR, the targets of which are still being identified but include cytoskeletal proteins (588). Both complexes have been proposed to have impacts on glycolysis (480) (481).

The classical mTOR inhibitor rapamycin inhibits mTORC1 alone at nM doses, whereas the recently developed inhibitor Torin1 inhibits both mTORC1 and mTORC2 (589) (590). Incubation of the ME lines with Torin1 reduced lactate production in all lines in a dose dependent manner and was able to reduce the excess KO lactate production towards WT levels (Figure 5.29). Rapamycin treatment had a similar effect on WT and KO lines, and it also reduced HET lactate production towards WT levels, not seen with Torin1 inhibition, although HET lactate generation remained slightly elevated in all conditions (Figure 5.29). As Torin1 is reported to be the more potent inhibitor (590), the reasons for the differential impact on HET glycolysis require more investigation. What this experiment does confirm is the role of mTOR signaling in promoting glycolysis in ME leukemia lines, and suggests this is mediated by the mTORC1 complex.

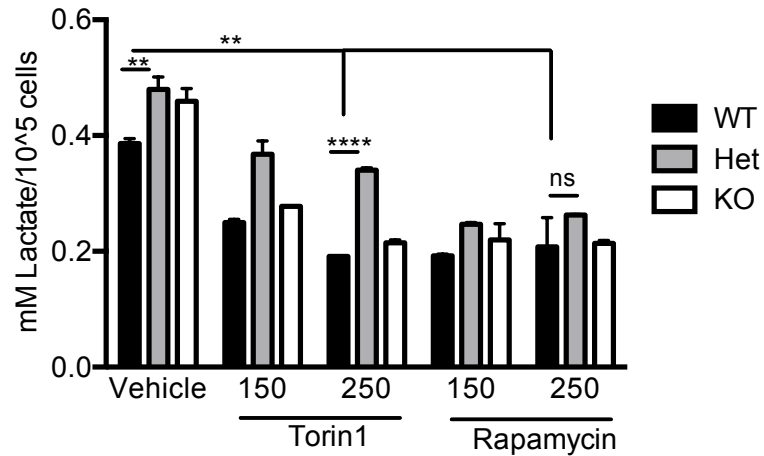


Figure 5.29: *mTORC1 controls glycolysis in ME leukemic lines. Enzymatic determination of lactate production over 48 hours in complete media by Cre-transduced ME lines of indicated genotype treated with vehicle (DMSO), Torin 1 (150nM or 250nM) or rapamycin (150nM or 250nM). 0.5x10⁵ cells initially plated per well, cells counted by trypan blue after 48 hours before supernatant collection, lactate concentration in supernatant normalized to media alone control and divided by final cell count (x10⁵ cells). Error bars represent SD (n=4), **p<0.01, ****p<0.0001, unpaired t test.*

Discussion

5.21 Alterations to Akt/mTOR pathway signaling in the absence of autophagy

The integration of external and internal environmental signals is vital for the regulation of cellular activities related to growth, function and division in both normal and cancer cells (8). The mTOR pathway and autophagy receive input on environmental suitability for proliferation from nutrient (growth factor-PI3K), amino acid (Rag GTPase) and energy level (LBK/AMPK) sensing pathways. These pathways then integrate this with information on cell suitability for growth, including factors such as DNA damage (591), oxidative and ER stress (22) (582) to influence growth regulation. With mTOR driving anabolic metabolite usage (99), mass accumulation through protein translation, and division through cell cycle regulation, while autophagy mediates catabolism, cellular breakdown and metabolite liberation (99), the two pathways are fundamentally complementary (585). However their described relationship has thus far been hierarchical, with autophagy suppressed by mTOR activity (8). Recent evidence has pointed to a more complex, bidirectional interaction, where just as mTOR suppresses autophagy when conditions are favorable, so autophagy and lysosomal degradation may help suppress mTOR signaling when conditions are negative (8, 585).

The data from the murine MLL-ENL leukemia model suggest loss of autophagic function, through *Atg5* deletion, can lead to continued activation of the Akt/mTOR pathway in unfavorable conditions, when wildtype (autophagy competent) lines have largely lost kinase phosphorylation. This was observed for both the *Atg5*^{+/-} HET line and the *Atg5*^{-/-} KO line, however while the HET displayed increased phospho-Akt, the retention of p-mTOR was a largely KO phenomenon. Examining first the activation of mTOR, both

indirect and direct feedback effects on mTOR signaling have been proposed from decreased or disabled autophagy. Indirect mechanisms include alterations to mTOR trafficking; the mTORC1 complex is found on the lysosomal membrane (29), where it receives information on the nutrient content of the lysosomal lumen and regulates autophagy through TFEB and ULK1 suppression (86, 371). As such, endolysosomal trafficking regulates mTOR activity, with peripheral localization bringing mTOR in contact with the upstream PI3K/Akt complexes, while central lysosomal trafficking following starvation inhibits mTOR activation (86, 371). Indeed, vesicle movements and fusion profoundly influence mTOR signaling (370), with activation of the intracellular trafficking-regulating Rab and Arf GTPases, which play a role in autophagosome maturation, inhibiting mTOR (369). Alterations to the fate of mTORC1-carrying endolysosomal compartments in the absence of autophagosome formation could therefore influence mTORC1 trafficking and signaling (8, 592). Supporting this, overexpression of Atg1 and Atg13 was sufficient to inhibit mTOR kinase activity via its localization to perinuclear vesicular structures (32); autophagy inhibition could have an alternative, liberating effect.

Other possible indirect mechanisms include changes to the intracellular environment. Metabolite levels may be altered following autophagy loss (99) and this can impact mTOR activation (593), although under stress metabolite shortages without autophagy would be predicted to inhibit mTOR. Similarly, ROS have been shown to activate both Akt and mTOR to cause myeloproliferation (587), and while neither acute HET and KO proliferation nor Akt/mTOR activation was reduced by NAC treatment, sensitivity to oxidative stress could potentially be an early survival pressure following Cre transduction in our model.

Direct mechanisms of mTOR activation following autophagy inhibition center around alterations to the abundance, location and activity of autophagy proteins at either end of the autophagy pathway following the deletion of key Atg's. Overexpression of ULK1, an autophagy initiator negatively regulated by mTOR, results in decreased phosphorylation of mTORC1 substrates and strongly impairs cell growth (594, 595), likely via phosphorylation of mTORC1 complex member RAPTOR and subsequent loss of complex activity (372). Under wildtype conditions this may help to amplify a loss of mTOR signaling to induce autophagy via feedback, and it is possible chronic autophagy impairment could result in pathway downregulation and relieve this interaction. Similarly, WIPI1, which functions in autophagosome formation, can inhibit mTORC1, although the impact of autophagy inhibition on this interaction is unclear (596). The most direct role for autophagy inhibition on mTOR signaling comes from the multi-functional adaptor p62, which has been identified as a member of the mTORC1 complex and is required for mTORC1 lysosomal localization and activation (373). p62 is characterized to be degraded by autophagy and is present at low levels in cells where autophagy has been stimulated (92), but autophagy inhibition could promote p62 accumulation and increased mTORC1 lysosomal localization. It should be noted that primary explanations for altered mTOR signaling favor complete autophagy loss, rather than heterozygous Atg deletion, agreeing with experimental observations. Further investigation of these possible mechanisms, including p62 levels and location in the HET and KO cells along with the sub-cellular location of mTORC1 itself, will be important for separating the contribution of these factors.

Akt signaling too is increased following heterozygous and homozygous deletion of *Atg5*. Less is known regarding autophagy's impact on Akt signaling, but Akt can directly regulate autophagy through Beclin1 phosphorylation and resulting sequestration on

vimentin cytoskeletal elements (33), therefore not all Akt/mTOR pathway-autophagy interactions involve mTOR. Notably however, the mTORC2 complex has been found to phosphorylate and activate Akt at Ser473, which appears important for subsequent Akt phosphorylation by PI3K activated PDK at Thr308 (580). Therefore investigating the status of Akt Thr308 phosphorylation and the impact of mTOR inhibition on both will be necessary to establish whether mTOR activation can explain the p-Akt increase as well.

5.22 Metabolic reprogramming in the absence of *Atg5*

To promote cell growth, mTOR plays a coordinating role in cellular metabolism, positively regulating synthesis of proteins, through translation, nucleotides, through the pentose phosphate pathway, and lipids (597). In addition to these anabolic activities, and despite distinct roles in different cellular processes, both mTOR complexes have been separately demonstrated to upregulate aerobic glycolytic energy generation. Specifically, mTORC1 has been shown to drive an aerobic glycolysis program through HIF1 α and c-Myc, targeting the key pyruvate kinase M2 enzyme in particular (480). mTORC1 also drives increased lactate production through Stat-3 mediated lactate dehydrogenase upregulation (482), and these impacts have been confirmed through unbiased metabolic and proteomic approaches following mTORC1 inhibition with rapamycin (598). Similarly, c-Myc is also regulated by mTORC2, through FOXO inhibition, to upregulate tumor glycolysis (481). Moreover, upstream of mTOR, Akt has been shown to strongly upregulate aerobic glycolysis without impacting oxidative phosphorylation to promote glucose dependent growth (483-485). Together, the Akt/mTOR pathway is understood to be an important mediator of Warburg effect metabolism (578).

The combination of increased glycolysis and aberrant p-Akt/p-mTOR activation observed in HET and KO leukemic cells makes that pathway a potential candidate for certain aspects of their metabolic phenotype. Torin1 and rapamycin treatment confirmed mTORC1 as a regulator of lactate production in all ME leukemic lines, with both inhibitors appearing to negate the KO glycolytic advantage. However, only rapamycin was able to reduce a continued HET glycolytic advantage; as rapamycin specificity is known to vary with concentration (589), further experiments are required to understand the inhibitor phenotypes. Moreover, neither inhibitor equalized lactate production entirely. Continued Akt activation, observed to be stronger in the HET line, independent of mTOR could be one explanation, to be confirmed via co-Akt inhibition. However, as increased lactate production by *Atg5* deficient lines, in particular HET cells, was strong in complete media, where only the KO line had observed differences in Akt/mTOR activation, the Akt/mTOR pathway is likely to be only one contributing factor. The mitochondrial phenotype is a reminder that the impact of autophagy loss goes beyond the mTOR pathway.

A very similar phenotype of upregulated glycolysis, accompanied by mitochondrial dysfunction (indicated by decreased maximal respiratory capacity) has been observed in *Atg7*^{-/-} MEFs and was attributed to increased oxidative stress (292). Myeloid cells from *Vav-Atg7*^{-/-} mice also showed upregulation of glycolysis proteins when studied by proteomic analysis, and correspondingly increased lactate production, although intriguingly here it was accompanied by *increased* unused respiratory capacity (A. Stranks, unpublished data); these contrasting results mean conclusions on the KO line respiratory capacity will require further experiments to exclude decreased viability impacting the Seahorse assay. Finally, a recent study has reported increased glycolysis in the absence of decreased mitochondrial oxygen consumption (maximal capacity was not

assessed) in *Atg7*^{-/-} pancreatic tumor cells with a *p53* null background (498); indeed, here a mild increase in oxygen consumption rate was observed, similar to the KO ME line.

Therefore, a link between autophagy loss and glycolytic upregulation appears well supported, however the accompanying mitochondrial phenotype may be cell type and status specific. Loss of mitochondrial function and increased oxidative stress necessitating a switch from mitochondrial use is one explanation for the metabolic changes (292).

While, somewhat surprisingly, increased oxidative stress was not found following *Atg5* deletion, it could conceivably have had a role during the initial survival-selection of cells immediately after Cre excision, with the end phenotype an adaptation to avoid damaging ROS production. Indeed, despite equal or lower levels of ROS measured in the KO line, treatment with NAC significantly rescued KO apoptosis, indicating, dependent on confirmation with other antioxidants, that mitochondrial dysfunction and ROS sensitivity may still be important factors in the KO phenotype. However, a loss of mitochondrial metabolism might be expected to hamper cancer development (599), and the KO and HET lines showed no decrease in mitochondrial oxygen consumption or decreased survival following galactose culture (only decreased proliferation), indicating their glycolytic switch is likely reversible. Alternatively, alterations to the internal cell environment and metabolite levels following the loss of autophagic homeostatic activities, in particular the loss of amino acids (99), might necessitate a shift to increased glucose consumption, the shuttling of TCA cycle intermediates into biosynthetic pathway and therefore use of glycolysis for energy generation (99, 465). Indeed, although autophagy has been seen as a vital contributor in cellular responses to glucose levels through AMPK, and has been reported to be required for glycolysis and survival in some Ras-driven tumors (473) (421), recent studies have shown that autophagy is *not* robustly induced on glucose starvation of immortalized cells (600). Autophagy loss did not sensitize cells to glucose withdrawal, but

populations of autophagy deficient cells exhibited significantly higher rates of cell death when glycolysis was inhibited by glucose analog 2-deoxyglucose (600). The lack of mitochondrial buildup following *Atg5* deletion in the ME lines may indicate little ongoing mitophagy across all lines due to a shift away from mitochondrial use (and therefore potential for damage) towards glycolysis, as previously reported in MLL-ENL transformed cells (575), which is further accelerated by *Atg5* deletion. Thus autophagy loss may create a metabolic state in the ME lines which favors glycolytic activities.

The HET metabolic phenotype was relatively strong, while measured autophagy level alterations appeared comparatively mild. Although no phenotype was observed in *Vav-Atg5/7+/-* heterozygote mice, phenotypes following incomplete Atg loss have been reported (66, 308), including in *Atg5+/-* transformed cells (395). The HET/KO differences may simply stem from decreased viability in the KO cells impacting metabolic readouts, and further investigation will be required to understand the metabolic impacts of allelic *Atg5* loss.

5.23 *Atg5* allelic loss promotes ME leukemia proliferation

Our study of the MLL-ENL leukemic model revealed consistently increased proliferation in the HET and KO lines. Although the HET line showed some proliferative advantage in normal growth conditions, both lines showed increased cell division relative to wildtype cells following serum reduction, while the KO line in particular showed an advantage under hypoxic conditions. These stressors are both well characterized to induce autophagy (22), suggesting that under conditions of metabolic stress protective autophagic induction limits cell growth in an *Atg5*-dependent manner (585). Combined serum withdrawal and oxygen reduction was previously shown important to the genomic damage mediated by

Atg5 homozygous deletion or *Beclin1* heterozygous loss in immortalized lines (395, 397), indicating that the autophagy pathway is little utilized under optimal *in vitro* conditions (ie. complete media, normoxia). However such nutrient high conditions are unlikely in physiological environments, in particular in the bone marrow and tumor microenvironment (395) (555), and allelic loss of *Beclin1* or *Ambra1* results in hyperproliferation *in vivo* (66, 308), similar to *Atg5/7* deficient multipotent progenitors and HSCs (Chapter 4). In cancer, reduced *Atg5* expression has recently been shown to drive proliferation in melanoma cells (415) and correlate with poor prognosis. Indeed, preliminary results from an ongoing experiment suggest that *Atg5* allelic loss, and in particular full (homozygous) deletion, accelerates *in vivo* leukemic formation in the MLL-ENL model (Figure 5.30, ongoing).

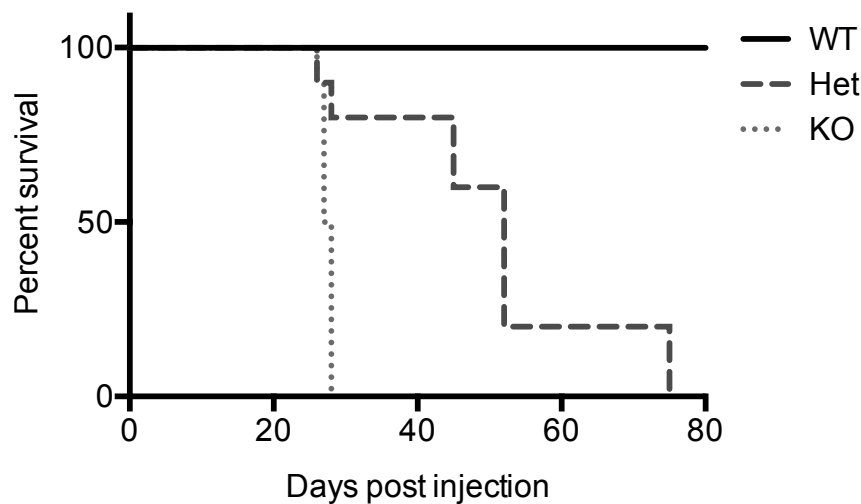


Figure 5.30: Preliminary results from ongoing experiment; *Atg5*^{-/-} ME cells form more aggressive leukemias *in vivo*. Survival of sublethally irradiated C57BL/6 mice (2x3Gy) intravenously injected with 1×10^6 primary ME leukemic cells with *Atg5*^{wt/wt}, *Atg5*^{fl/wt} or *Atg5*^{fl/fl} genotypes transduced with Cre/GFP (CRE-IRES-GFP retrovirus, purchased from Allele Biotech), sorted on GFP expression; mice were scored regularly and killed on development of clinical symptoms past a pre-determined score threshold, approved by UK Home Office. *n*=10 mice per genotype.

The possible drivers of that proliferation are largely similar to those impacting HSC quiescence. Oxidative stress and ROS can either promote (537) (539) (540) or inhibit

(571) (565) (572) (573) proliferation in a concentration and setting dependent manner, however on current experimental evidence ROS appear to not be a major factor in ME line proliferation. mTOR activation, on the other hand, is known to promote both growth and division through combined metabolic regulation (described above) (597), cell cycle signaling, and autophagy regulation. Achieving sufficient cell size and mass (including organelle numbers) through PI3K/Akt/mTOR regulated anabolism is a requirement prior to cell division (601), while autophagy loss prevents the pathway hindering mass accumulation via catabolic breakdown of cytoplasmic structures (585). Increased cell size has been repeatedly observed following autophagy loss, both in the ME lines and in *Vav-Atg5/7-/-* mice, and there is evidence that excessive cell size itself can promote division in stem cells (602). In most cases however, once sufficient mass is achieved, cell cycle checkpoints must be bypassed to achieve cell division, and mTOR has been shown to promote cell cycle progression, in particular driving expression of the key G1-regulating pair cyclin D1/Cdk4 (603) and promoting G1/S progression (367, 368). Again, autophagy loss could aid this role through impaired degradation of cyclins (377, 378, 381). Therefore mTOR activation and autophagy loss could synergize to drive proliferation, and testing the impact of Akt/mTOR inhibitors on the Atg5 HET and KO lines will help clarify this. Interestingly, preliminary experiments have shown metformin, which inhibits mTOR through AMPK activation (447) (448), may cause KO/HET proliferative arrest and cell death (data not shown).

What was suggested by the experiments reported here is the importance of the metabolic reprogramming described above for the proliferation of *Atg5* deficient MLL-ENL leukemic lines, as a galactose-enforced switch away from glycolysis greatly reduced the excess HET and KO proliferation under stress. Increased glycolytic requirements have been noted in autophagy deficient lines (600) as well as in leukemias driven by Akt

overactivation (484). Recent studies have demonstrated direct cross talk between glycolytic enzymes and cell cycle regulators (604) (605), therefore whether increased glycolysis is sufficient to drive increased proliferation in the HET and KO lines, or simply permits a proliferative advantage as part of a program of cellular changes following autophagy loss, remains to be seen.

5.24 Decreased autophagy in AML and cancer

As described in the introduction to this chapter, the precise role of autophagy in cancer pathogenesis and treatment remains controversial, with differential impacts depending on tumor type and stage (16, 386). The caveat to autophagy's role in suppressing proliferation is its importance in cell survival under stress (5, 395), and indeed the KO line displayed significantly increased cell death following both serum withdrawal and hypoxia. In tumor models driven solely through hyperactivation of tyrosine kinase proliferation pathways, specifically certain Ras models (450, 452, 569) and BCR-ABL (451), autophagy appears required for metabolic support, with Atg deletion inhibiting tumorigenesis through excessive cell death and metabolic failure (473). By contrast, transformation through other means, such as apoptotic inhibition via BCL-2 overexpression or BAX/BAK deletion, is aided by autophagy loss. This is despite increased cell damage, and in fact occurs through promotion of genomic damage and inflammation (395, 397). Moreover, deletion of autophagy genes including *Beclin1* (heterozygous only) (308), *Atg4C* (318), *Atg5* and *Atg7* cause cell damage and tumor development (388), although in the latter cases these tumors appeared to be benign. Deletion of autophagy genes (16, 61) and downregulation of autophagy proteins (506) is the common phenotype in human cancers, while *Atg* overexpression was poorly tolerated in leukemic cell lines; these data indicate an overall tumor suppressive role for autophagy as more likely prevalent.

Thus an emerging model indicates autophagy deficient cells are more prone to dysplastic growth and initial tumor formation because of excess proliferation, metabolic changes, genomic instability and inflammation. However malignant development may depend on the status of the apoptotic pathway (Figure 5.31). In tumor models where primary drivers are kinase oncogenes (without supporting mutations) which cause excessive metabolic stress, the survival defect following autophagic loss is likely to outweigh the benefits in tumor evolution. Conversely, in cells with a handicapped apoptotic pathway, autophagic loss is permissible, with more limited cell death driving inflammation (452) and growth/mutation benefits accelerating tumorigenesis.

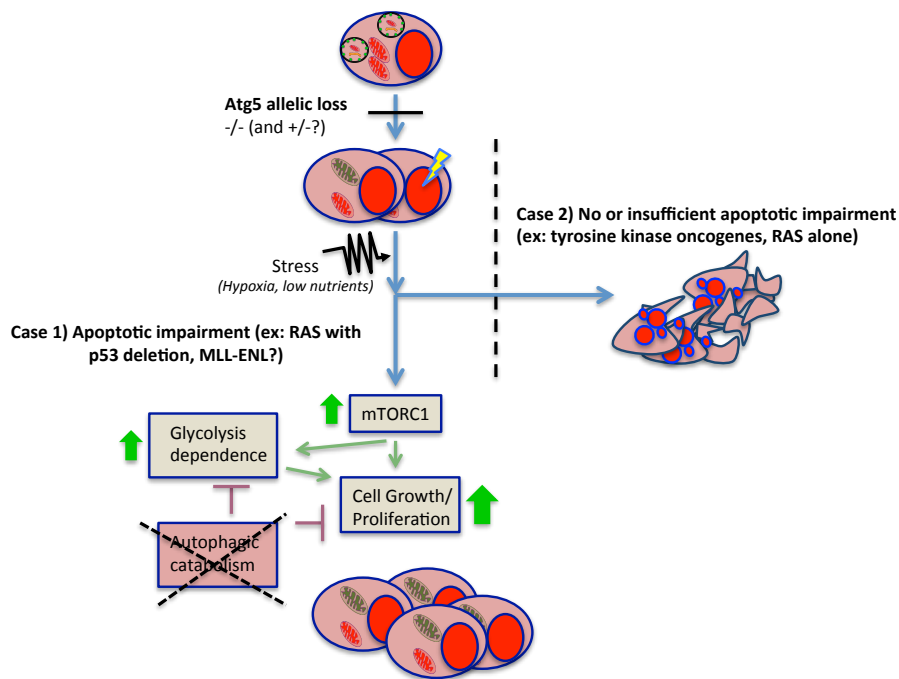


Figure 5.31: Hypothesis of apoptotic pathway involvement in the impact of autophagy loss on cancer models. In case 1, apoptotic impairment allows autophagy-deficient cells to survive metabolic stress, with autophagy loss promoting mTOR activation, dependence on glycolysis and increased cell growth as observed in MLL-ENL driven leukemic lines. In case 2, oncogene-driven stress without apoptotic impairment results in an autophagy-requirement for survival. See text for details.

This hypothesis is supported by new findings in Ras driven tumors. First, in a K-Ras driven model of lung cancer, *Atg5* deletion accelerated initial tumor development, however co-deletion of *p53* was required for malignant progression (606). Similarly, in a K-Ras driven pancreatic cancer model, *Atg7* null mice developed lesions that did not progress to cancer; however, additional deletion of *p53* resulted in accelerated *Atg7* null cancer development, and those tumors demonstrated increased glycolysis (498). Indeed, in BCR-ABL associated leukemias, cell death following autophagy loss is mediated through upregulation of pro-apoptotic proteins and p53 activation (451). This may explain why deletion of *Atg4C*, *Atg5* and *Atg7* results in largely pre-malignant tumors (318, 388), and indeed why *Vav-Atg5/7*^{-/-} mice display pre-leukemic symptoms but no transformation, whereas Beclin1 deletion, which frees the anti-apoptotic BCL-2 protein from its interaction, can result in malignancies (318).

The studies in K-Ras and p53 models are highly important steps for understanding autophagy in tumors, and testing the extension of these findings to other tumors, oncogenes and apoptosis proteins will be vital to clarify the above hypothesis. The MLL-ENL model provides one such look; apoptosis in MLL-ENL driven tumors has been previously demonstrated to be handicapped through the key regulated genes *Hoxa9* and *Meis1* (575, 607) and control of the anti-apoptotic protein MCL-1 (552). Autophagy deficient KO ME cells were still able to form increased colony numbers in a hypoxic setting, while ongoing *in vivo* experiments show strongly decreased survival in mice receiving *Atg5*^{-/-} cells compared to both *Atg5*^{+/-} and *Atg5*^{+/+} genotypes (Figure 5.30), therefore, pending the full completion and confirmation of the *in vivo* experiments, the balance between benefits and costs to autophagic loss in this model indeed appears to

favor the KO condition. More precisely investigating the mechanism of KO cell death under stress will clarify whether increased necrosis might aid the *in vivo* phenotype. Therefore the ability of tumor models to tolerate the increased damage and metabolic costs of deficient autophagy may predict whether autophagy is beneficial or harmful to tumor development.

A final point is the application of these murine model findings to observations in primary human leukemia. Importantly, AML blasts from patient BMNCs displayed hallmarks of decreased autophagy, including lower LC3/LysoID colocalization, increased p62 levels and decreased expression of autophagy genes. Regarding the latter, a recent study reported decreased transcript levels of Atg8 homologs *GABARAPL1* and *GABARAPL2* compared to healthy controls, yet similar levels of *GABARAP* (333), an identical pattern to the Fluidigm results which helps validate the blast marker choices and findings. The Atg8 homologs appear particularly tightly regulated in AML and HSCs (110), suggesting further inquiry into the precise roles of the different homologs in hematopoiesis. The heterozygous deletion of Atg's observed in AML cases may be one method of autophagy downregulation, in particular for *ATG12* and the Atg8 homologs, with knockdown of *GABARAPL2* shown to be sufficient to impair autophagic flux in leukemic cells (333). However, signaling changes, in particular overactivation of the Akt/mTOR pathway, which mediates leukemogenesis (385) and is commonly observed in AML (402) (403) and required for leukemic proliferation (403) (405), is another possible contributing factor. A minority of donors (4/13) displayed full loss of autophagic flux on the IFCAA, while a majority of donors showed evidence of lower but continued autophagic function. As the MLL-ENL HET line showed pro-leukemic benefits both *in vitro* and *in vivo*, and autophagy genes are observed to be heterozygously deleted in AML along with many other cancer types (although mutation and/or epigenetic silencing of other copies is

possible), these findings could indicate intermediate degrees of autophagy loss (ex. downregulation) as a compromise in some AML patients between the benefits and costs of autophagy loss (16). Decreased autophagy may be relevant to mediate damage during initial transformative events, as downregulated expression of *ATG12* and lysosomal gene *LAMP1*, accompanied by increased mitochondrial oxidative stress, was identified in t-MDS/AML patients at the time of transformation (537).

Moreover, although these data suggest decreased autophagy as the dominant phenotype in the fast-proliferating AML blast population, the evidence from *Vav-Atg5/7-/-* mice suggests autophagy is vital for the quiescence of stem-cell like populations, which includes leukemic stem cells (169, 510). Supporting this, deletion of mTORC1 component *Raptor* in an AML-AF9 AML model resulted acute impairment and loss of blasts in the blood and bone marrow, yet an undifferentiated initiating-cell population was able to survive long term with sustained self-renewal (403). An initiating-cell autophagy requirement, possibly avoided in the MLL-ENL model due to *in vitro* selective pressures, would argue for autophagy inhibiting therapies in combination with chemotherapy/radiation, despite the benefit of autophagy loss for the majority of the AML blasts, and must be carefully investigated prior to decisions on implementation of treatment targeting autophagy.

5.25 Conclusions

In conclusion, autophagy was found to be decreased in primary human AML patients, including downregulated expression of multiple *Atgs*, agreeing with the presence of *Atgs* in chromosomal regions found heterozygously deleted in AML patients. In a murine model of MLL-ENL driven AML, deletion of one or both copies of autophagy gene *Atg5* lead to increased proliferation under microenvironmental stress, while *Atg5* knockout cells were also more susceptible to cell death under those conditions. The increased growth following

Atg5 loss was supported by glycolysis and accompanied by activation of the Akt/mTOR pathway under stress conditions, which controlled glycolysis in the leukemic cells. Together, these results point to decreased autophagy promoting proliferation in AML, while suggesting a possible feedback between the autophagy pathway and the Akt/mTOR pathway which may also be relevant in other tumor types. Understanding whether these data translate to leukemic initiating cells will be important for future decisions regarding autophagy modulation in therapy.

Chapter 6: Autophagy in AML - stroma interactions

Introduction

The data presented in Chapter 5 demonstrated that modulation of autophagy could play a cell-intrinsic role in the growth of some forms of acute myeloid leukemia (AML). Like the early hematopoietic stem and progenitor cells (HSCs and HSPCs) it develops from (509) (510), leukemia does not progress in isolation but rather within the bone marrow microenvironment, which shapes and aids leukemic pathogenesis (608). Autophagy-mediated alterations to both tumor cells and stromal cells have been suggested to help shape cancer-stroma interactions (609), but its connection in AML remains virtually unexplored. Here, the leukemic microenvironment is very briefly introduced before a model for autophagy's non-cell autonomous importance to stromal interactions in solid tumors is explored, along with possible applications to AML.

6.1 The leukemic microenvironment

Chapters 1 and 3 introduced the importance of the bone marrow niche in hematopoiesis and HSC regulation. Briefly, HSPCs reside in specialized microenvironments, shaped by multiple bone marrow 'stromal' cell types, such as endothelial cells, mesenchymal stromal cells and osteoblasts (107). Through contact or secretion, these cell types provide various factors vital to proper HSC regulation, such as SCF (108), TPO (111, 215), Jagged-1 (218), Ang-1 (219) and CXCL12 (610) (611). This includes metabolic factors, the most characterized of which is a hypoxic environment; although hypoxia as a characteristic of the niche alone has recently been questioned (107), the bone marrow in general is known to be hypoxic at the endosteum (202) (561), and this may help to keep quiescent HSCs from excessive ROS-generating mitochondrial activity (200, 201, 219, 612).

Leukemic stem cells develop from early HSPCs within the stromal support of the bone marrow niche, and features of both the osteoblastic and vascular niches contribute to their growth (218, 219, 608, 613). Examples include CXCL12 homing (614), provision of ligands for adhesion molecules like CD44 (such as hyaluronan or other extracellular matrix components (615)), and SCF signaling (530). Leukemic cell use of niches can both directly impair function of nearby normal HSC or else force them to less optimal locations (530). Furthermore, leukemic stem cells have been shown to alter stromal cells to produce a microenvironment more favorable to their growth, and less favorable for normal hematopoiesis (608, 616). Leukemic-derived exosomes are able to induce cell cycling in normal HSCs while altering stromal surface adhesion molecule and cytokine expression, including that of pro-inflammatory IL1 β . Culture of healthy HSCs on such leukemia-altered stromal cells resulted in an engraftment defect compared to culture with normal stromal populations (617). A separate study similarly implicated pro-inflammatory cytokine secretion by stromal cells as consequence of leukemic co-culture (618). Stromal cell growth is altered in the presence of leukemic cells, with MSCs from acute lymphoblastic leukemia patients showing decreased proliferation, correlating with increased bone morphogenetic protein 4 (BMP4) levels, and viability of healthy CD34⁺ cells cultured with ALL stroma was reduced (619). Interestingly, BMP4 has previously been shown to induce autophagy (620). Leukemias can also mediate physical changes to the bone marrow structure, for example increased angiogenesis (621) (622)

Genetic changes to stromal compartments have been shown to promote leukemogenic growth: deletion of *Dicer* in mesenchymal osteoprogenitors results in myelodysplasia and AML (613), overactivation of the NF- κ B pathway in stromal cells drives a myeloproliferative disorder (623), and deregulated Wnt-B-catenin signaling provided by stroma helps maintain LCS self renewal (624). Similarly, leukemic development following

deletion of key tumor suppressors has been found to be non-intrinsic to hematopoietic progenitors. For example, loss of retinoblastoma 1 (*RBI*) (625) or *PTEN* (385) promoted full leukemic development only when also lost in the microenvironment. Intriguingly, different leukemias may benefit from particular microenvironmental factors, as osteoblastic activation of the parathyroid hormone receptor was found to impair BCR-ABL expressing leukemias but promote MLL-AF9 AML (626).

Stromal cells have also been shown to contribute to metabolism in the leukemic microenvironment. Mesenchymal stem cell and stromal cell provision of amino acids, including asparagine (627) and cysteine, via cystine conversation (628), has been shown to have protective effects for leukemic cells *in vitro* and *in vivo*. In the latter study, stromal-provided cysteine supported leukemic metabolism and reduced potentially toxic reactive oxygen species (ROS) levels by allowing increased synthesis of the anti-oxidant glutathione (629). Meanwhile, AML cells alter their metabolic microenvironment through arginase II release, which helps impair anti-leukemic immune response (630). Evidence suggests the leukemic microenvironment is even more hypoxic than normal bone marrow (562, 631) and that LSCs display HIF1 α -dependent increased glycolysis that aids drug resistance (631-633). Indeed, metabolomics of acute leukemic bone marrows, as compared to blood, before and after remission found lactate and lipids relatively enriched in untreated bone marrow microenvironments, whereas amino acid metabolites were increased on remission (634).

6.2 Leukemic impact on stromal cells *in vitro*

In keeping with the import of stromal cells and microenvironmental changes to leukemia *in vivo*, bone marrow stromal cell lines, including the murine stromal line MS-5 (635), are widely used in *in vitro* culture systems for AML and other leukemias, where they have

been demonstrated to promote long term growth, survival and resistance to metabolic stress (636, 637) (638, 639) (640) (641). For example, culture of AML cell lines with MS-5 stromal layers protected the leukemic cells from serum deprivation and ara-C mediated apoptosis (642). The proposed mechanism was stromal-induced, partly non-contact dependent, upregulation of anti-apoptotic BCL-2 and BCL-XL levels, which was replicated in primary patient samples. Of note is that BCL-2 upregulation is associated with decreased autophagy via Beclin-1/BCL-2 interactions (643, 644). Similarly, culture with primary bone marrow stromal cells has been shown to activate the pro-survival PI3K/Akt pathway and upregulate the X-linked inhibitor of apoptosis protein (XIAP) in leukemic cells (645). Microenvironment signaling *in vitro* has been shown to reduce the efficacy of certain treatments, such as Flt-3 inhibition (646). The benefits of stromal cells to leukemic cultures have been linked to stromal provision of cytokines, both secreted and non-secreted, as well as direct contact with stromal surface adhesion molecules, as positive impacts are often only partially replicated in non-contact culture conditions (635) (641, 642, 647-650).

Although the role of autophagy in leukemic-stromal interactions has not been examined, models for the interactions between proliferating tumor cells and stromal cells in solid tumors indicates an important role for autophagy in the tumor microenvironment (609).

6.3 Autophagy in tumor-stromal interactions – the parasitic metabolism model

The importance of non-tumor stromal cells in driving and supporting oncogenic growth in the heterogeneous tumor environment has been well characterized, and autophagy has several proposed roles *in vitro* and *in vivo* (609). Communication between the tumor and non-tumor compartments can create feedback and co-evolution, altering the ‘tumor

secretome' in an autophagy-dependent manner to impact on disease progress (651) (652) (653) and immune reaction (426) (609, 654).

A predominant model in epithelial cancers proposed by Lisanti and colleagues, called either the 'autophagic tumor stromal model' or 'two compartment tumor metabolism' (408), suggests a key role for autophagy in both the tumor and stromal compartments, shaping a tumor-supportive microenvironment to create parasitic cancer metabolism (294, 499, 500, 655-657). As summarized in Figure 6.1, lower autophagy and high proliferation rates in tumour cells promote oxidative stress in surrounding stromal fibroblasts, which is itself a direct activator of autophagy (658), causes cell and organelle damage, and also results in the activation of NF- κ B and HIF1 α , in turn inducers of autophagy. NF- κ B activation is proposed to drive an inflammatory 'cytokine storm' which induces autophagy in nearby fibroblasts (659), while HIF1 α induces stromal aerobic glycolysis; this increase in aerobic glycolysis in non-tumor stromal cells, as opposed to the increase in cancer cells originally observed by Otto Warburg (467), has been termed the 'reverse-Warburg' effect. Glycolysis, along with autophagy, liberates high-energy metabolites from stromal cells; these include lactate, ketones and amino acids like glutamine. They are secreted into the tumour microenvironment and become available to tumour cells (656, 657, 660), where they allow efficient mitochondrial metabolism and TCA biosynthesis while promoting the biogenesis of healthy mitochondria (294, 499). In stromal cells, damage caused by oxidative stress and high autophagy results in mitochondrial removal (655), thus further reliance on glycolysis. Meanwhile, microenvironmental oxidative stress increases due to ROS production from increasingly damaged stromal tissue. In co-culture this production has been shown to outstrip that of tumours after 5 days, promoting genetic instability and cancer evolution in a 'bystander', or field, effect (294). Tumor cells are protected against ROS-induced apoptosis by a robust antioxidant defense (294), aided by stromal-provided

amino acids and transcriptional changes. This produces a final ‘two compartment’ picture where tumour cells, with low autophagy yet healthier mitochondria, anti-oxidant defense and efficient stromal-supported metabolism, are driven to evolve and supported parasitically by surrounding stromal cells, which are burdened by high autophagy, glycolysis and ROS production along with unhealthy mitochondria (Figure 6.1). Supporting their elucidation of this model, overexpression of autophagy genes *ATG16L1* and *BNIP3* in cancer-associated fibroblasts resulted in mitochondrial dysfunction, increased metabolite production and enhanced tumor support, while *ATG16L1* overexpression in cancer cells inhibited tumor growth (408).

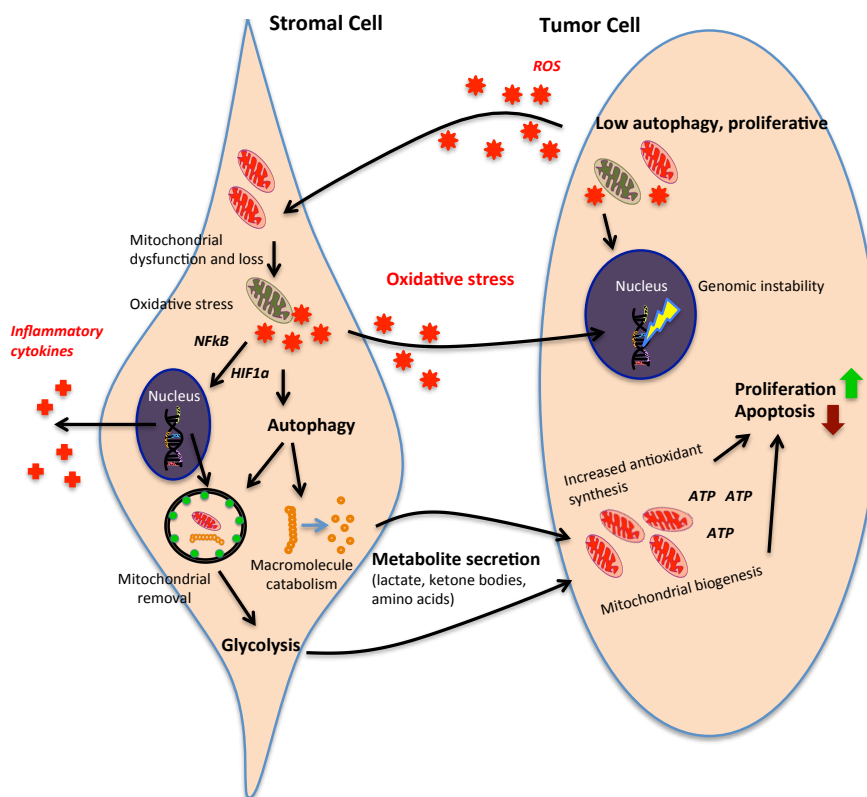


Figure 6.1: The ‘autophagic tumor stromal’ or ‘two compartment tumor metabolism’ model for cancer-stromal cell interactions, see text for details.

6.4 Autophagy mediated tumor-stroma coevolution in acute myeloid leukemia

Lab data suggests that hematopoietic progenitors lacking either *Atg5* or *Atg7*, and therefore with deficient autophagy levels, become damaged, accumulate mutations and produce excess ROS, as do autophagy-deficient myeloid cells (Chapters 4 and (131)). Similarly, as presented in Chapter 5, the proliferating blast population in the bone marrow of AML patients showed evidence of decreased autophagy and increased oxidative stress.

Interestingly however, the bone marrow environment of these patients in general was under high oxidative stress; despite increased autophagy levels, the non-blast marker positive population (which will contain stromal cells and non-transformed hematopoietic progenitors, although likely diluted by minor blast populations) showed equivalent mitochondrial ROS generation and decreased healthy mitochondrial content (NAO staining) compared to blast marker positive cells. Together, these observations fit with the initiating tenants of the tumor-stroma coevolution hypothesis: differential autophagy, an oxidatively stressed microenvironment and non-tumor cells displaying mitochondrial dysfunction. Therefore, elements of the model presented by Lisanti *et al.* were tested in the context of *in vitro* culture of AML cells with MS-5 stromal layers, focusing on the impact of AML autophagy levels on stromal interactions, and in the context of *in vivo* mouse models.

Results

6.5 Stromal co-culture improves mitochondrial health and reduces apoptosis of human AML cell lines following serum withdrawal

In order to study the effects of autophagy modulation in AML on stromal support, the benefits of *in vitro* stromal co-culture first had to be established. An important feature of the ‘autophagic tumor stromal model’ in epithelial cancers is increased tumor cell mitochondrial health and metabolic support in co-cultures (294, 499, 655). Similarly, culture with the MS-5 murine bone marrow stromal cell line has been shown to protect leukemic cell lines from dysfunction and death under serum starvation (642); microenvironmental stress (in the form of reduced nutrients and growth factors) is a common property of the tumor microenvironment (395) (555). To assess the mitochondrial health of leukemic cells under serum starvation with and without stromal support, the NB4 leukemic line, an acute promyelocytic leukemic model with t(15:17) PML-RARalpha fusion (661), was cultured either alone or with a 90% confluent monolayer of MS-5 cells for 72 hours in both complete media and serum-free conditions before being stained with the mitochondrial superoxide (mtROS) sensitive dye MitoSOX and viability dye DAPI. Detached stromal cells could be excluded from leukemic cells by size, and even under starvation very few were observed without trypsinization. The elevated mtROS observed in live (DAPI negative), serum starved NB4 cells grown alone (ie: no stromal support) was reduced by stromal co-culture, and indeed mtROS levels were even reduced in complete media cultures following co-culture (Figure 6.2). Moreover, NB4 cell death (DAPI+) following serum starvation was largely mitigated by co-culture (Figure 6.2). Similar results were obtained for the HEL AML cell line, which as a type M6 erythroleukemia is genetically distinct from NB4 cells (662) (data not shown).

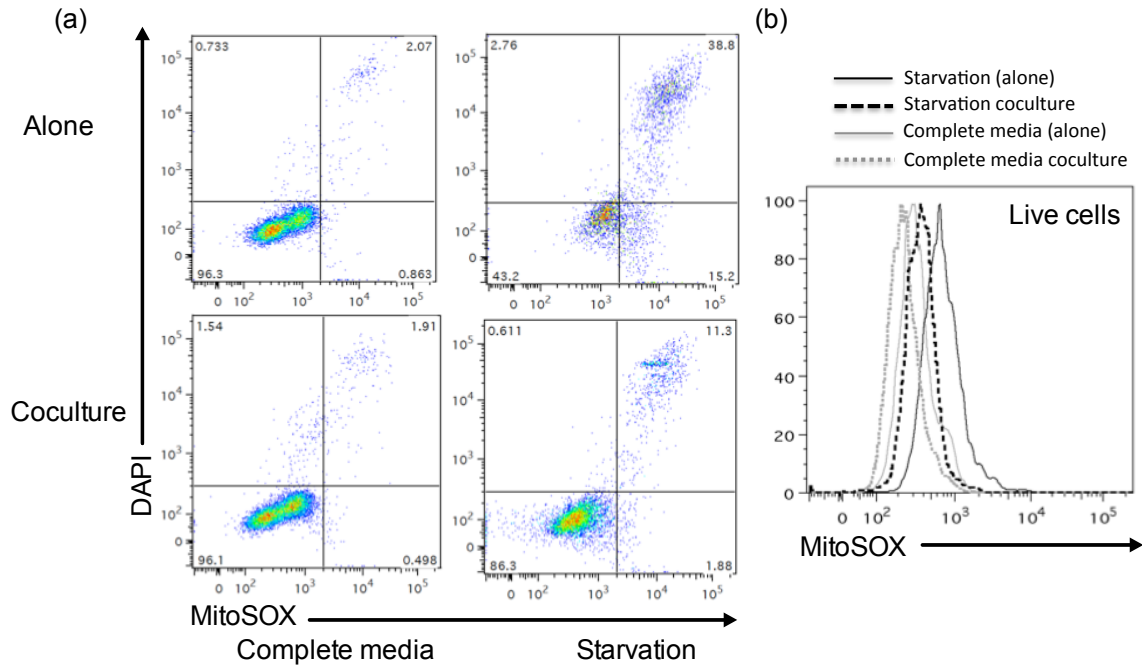


Figure 6.2: Co-culture with MS-5 stromal layers reduces NB4 mitochondrial ROS production and death following serum starvation. 3×10^5 NB4 cells were cultured with (co-culture) or without (alone) a 90% confluent MS-5 stromal layer in complete media (10% FCS) or starvation media (no FCS) for 72 hours before flow cytometry analysis. (a) Representative dot plots showing intensity of DAPI against MitoSOX staining intensity in NB4 cells cultured as indicated. (b) Representative histograms of NB4 MitoSOX intensity, gated on live (DAPI negative) cells in the indicated condition. Results representative of three independent experiments.

Cell death with high mitochondrial oxidative stress, as observed in DAPI positive NB4 and HEL cells, is an indicator of apoptosis (295) (296). To confirm this, apoptosis levels in NB4 and HEL cell lines were measured using Annexin V, which stains apoptotic marker phosphatidylserine on the cell surface, after 72 hours serum starvation. As expected, significant Annexin V intensity was observed in AML cells following serum withdrawal (HEL results shown), which was reduced in co-culture with stromal cells (Figure 6.3), as reported in MCF-7 breast cancer cells (663) and other leukemic lines (642). Together, these results point to stromal maintenance of mitochondrial integrity in nearby AML cells reducing the induction of the apoptotic pathway.

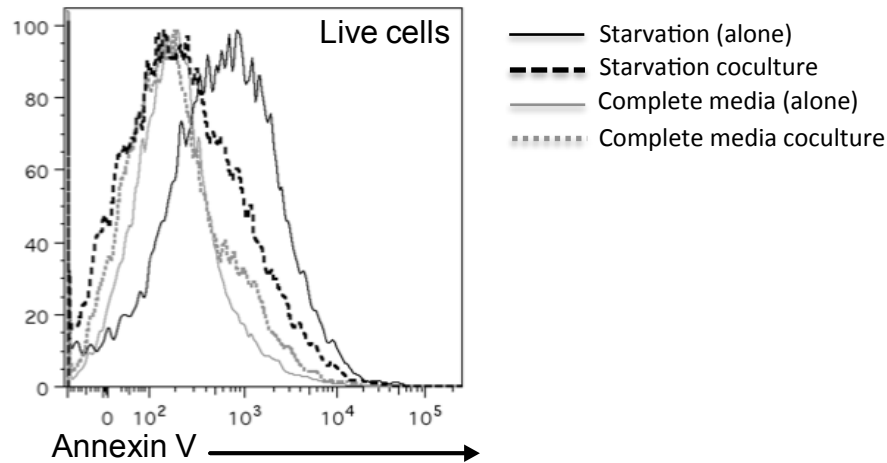


Figure 6.3: Co-culture rescues serum starvation induced apoptosis in HEL leukemic cells. Representative histograms showing Annexin V staining intensity of HEL cells cultured with or without a MS-5 stromal layer in starvation or complete media conditions as in Figure 6.2, gated on live (DAPI negative) cells.

6.6 Stromal support increases proliferation of human AML cells under stress

Tumor associated stromal cells have been reported to increase tumor growth *in vitro* and *in vivo* (663) (655) (408). In breast cancer models, this was proposed to be largely through increased survival (655), however increased proliferation under serum withdrawal has been reported in leukemic lines with stromal support (642). To validate this, AML/MS-5 co-cultures were set up as before and thymidine analog BrdU was added after 24hrs. Cells were stained for BrdU incorporation, reflecting DNA synthesis and therefore a readout for S-phase activity, after a further 48hrs (HEL data shown, representative of NB4 data). This revealed that, although co-culture had little effect on proliferation in complete media, it increased BrdU intensity in serum-starved AML cells, in particular reducing the number of AML cells showing no incorporation (Figure 6.4).

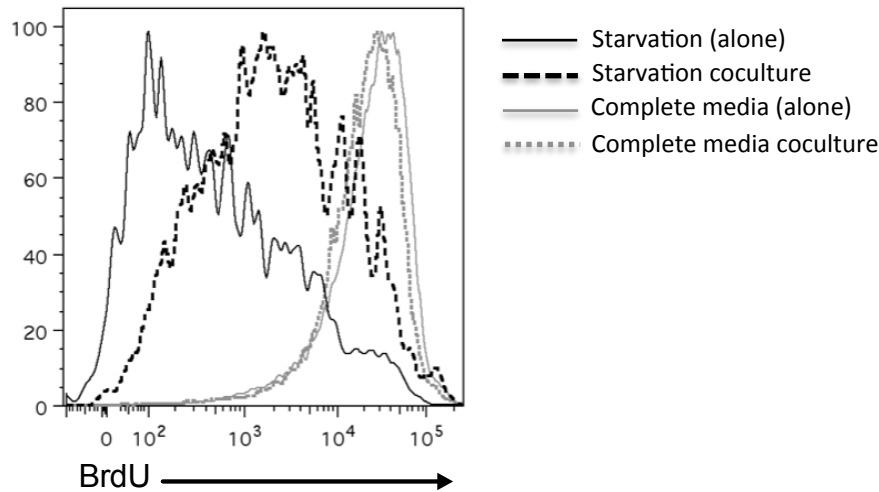


Figure 6.4: Stromal cells maintain HEL leukemic cell proliferation during serum starvation. HEL cells were grown with or without a MS-5 stromal layer in starvation or complete media conditions as in Figure 6.2, BrdU (10 μ M) was added after 24 hours and cells stained for incorporation after a further 48 hours, representative histograms show BrdU intensity in the indicated culture condition.

To confirm the BrdU readout for proliferation, the number of NB4 cells in the co-cultures (3x10⁵ cells/ml starting concentration) was quantified after 72 hours, showing that cell numbers in serum-starved conditions were modestly but significantly increased with MS-5 co-culture (Figure 6.5). Combined with the benefits to mitochondrial health and reductions in apoptosis, these results demonstrate that MS-5 cells are able to support nearby AML cells under stressful conditions.

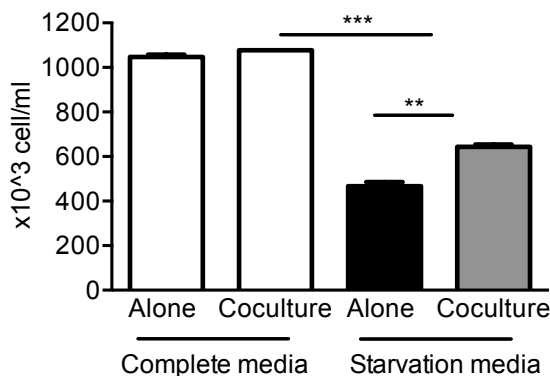


Figure 6.5: Stromal cells maintain NB4 leukemic cell proliferation during serum starvation. 3x10⁵ NB4 cells were grown with or without a MS-5 stromal layer in starvation or complete media conditions as in Figure 6.2, final cell counts were taken after 72 hours. Error bars represent SEM (n=3), **p<0.01, ***p<0.001, unpaired t test. Thanks to Tom Riffelmacher for performing cell counts.

6.7 ATG5 overexpression alters BrdU incorporation under starvation and hypoxia

Low levels of autophagy in tumor cells is an important factor in the epithelial model of tumor-stroma coevolution, allowing increased tumor growth (408) (499, 664) and exacerbating microenvironmental oxidative stress which drives pro-tumor metabolic changes and cytokine release in associated stromal cells (294) (659). With the benefits of the co-culture model for the HEL and NB4 AML lines established, the role of AML cell autophagy was investigated. Both HEL and NB4 cells, along with all other AML cell lines investigated, displayed low basal autophagy levels relative to epithelial cells types (Chapter 5.12). To alter this, HEL and NB4 cells stably overexpressing either eGFP-Atg5 or the eGFP empty vector alone were generated as described in Chapter 3.12, with the eGFP-Atg5 transfected lines both showing increased levels of basal autophagy. These lines were grown with stromal support in complete or serum-free media as before, with BrdU added after 24 hours and incorporation assayed after 72hrs (with gating on GFP signal ensuring no stromal contamination). HEL cells overexpressing eGFP-Atg5 (HEL-Atg5) showed no difference in proliferation in complete media conditions relative to empty vector (HEL-eGFP) controls (Figure 6.6, grey overlay) but under serum-starvation they showed reduced BrdU incorporation in co-culture (Figure 6.6). This difference was not observed when the lines cultured without stromal support (not shown). Repeating the experiment with NB4 cells overexpressing eGFP-Atg5 (NB4-Atg5) similarly showed a reduction in BrdU incorporation under serum-starved, co-culture conditions, although this difference was not significant (Figure 6.7). The reason for the less severe impact in NB4 cells requires further investigation, however these results indicate that Atg5 overexpression in AML lines may reduce the proliferative benefit from MS-5 co-culture under serum starvation.

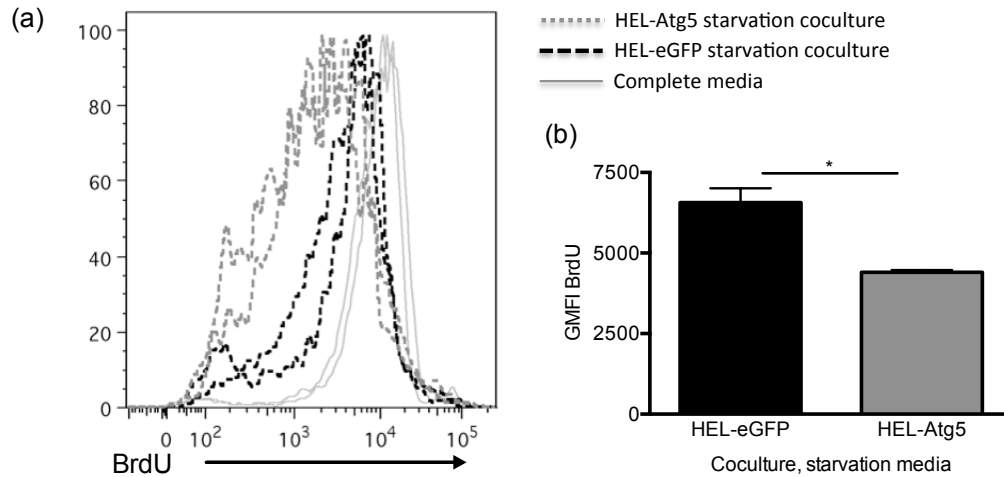


Figure 6.6: HEL cells overexpressing Atg5 (HEL-Atg5) show reduced BrdU incorporation in stromal co-cultures under starvation. HEL-Atg5 and empty vector control (HEL-eGFP) lines, generated as outlined in Chapter 3.12, were grown with or without a MS-5 stromal layer in starvation or complete media conditions, with 10 μ M BrdU added for 48 hours as in Figure 6.4. (a) Histograms of BrdU intensity in HEL-Atg5 and HEL-eGFP lines when co-cultured with MS-5 cells under serum starvation conditions, representative complete media control histograms also shown from both lines, gated on GFP⁺ positive cells. (b) Quantification of BrdU MFI from histograms shown in (a). Error bars represent SEM (n=2), * p <0.05, unpaired t test.

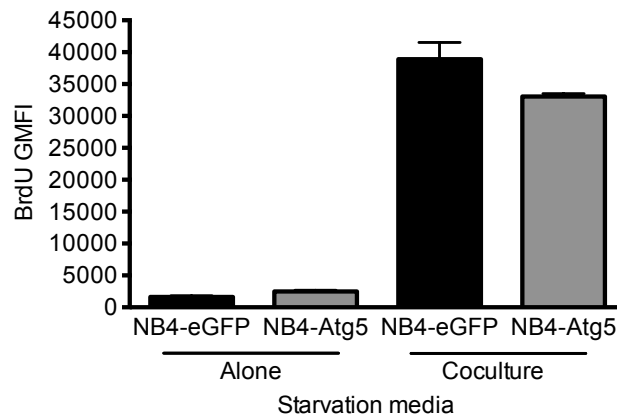


Figure 6.7: Proliferation of Atg5 overexpressing NB4 cells (NB4-Atg5) in stromal co-cultures. GMFI of BrdU staining in NB4-Atg5 and empty vector control (NB4-eGFP) lines, generated as outlined in Chapter 3.12, grown with or without a MS-5 stromal layer in starvation or complete media conditions, with 10 μ M BrdU added for 48 hours as in Figure 6.4. Error bars represent SEM (n=2).

Hypoxia is an alternate metabolic stress which is commonly observed in the microenvironment of both the tumors and normal bone marrow (561) (202), where oxygen levels are further decreased during AML pathogenesis (562) (563). The ability of AML

cells to proliferate under these conditions is a significant selective pressure (564) (565). To extend the investigation of stromal support of AML cells, wild-type NB4 cells were first cultured in the presence of BrdU for 48hrs in 0.1% oxygen or normal growth conditions (normoxia). As expected, hypoxic NB4 cells proliferated well both with and without stromal support, however a minority population showed little BrdU incorporation under hypoxic conditions, and this population showed marginally increased BrdU intensity with stromal support (Figure 6.8).

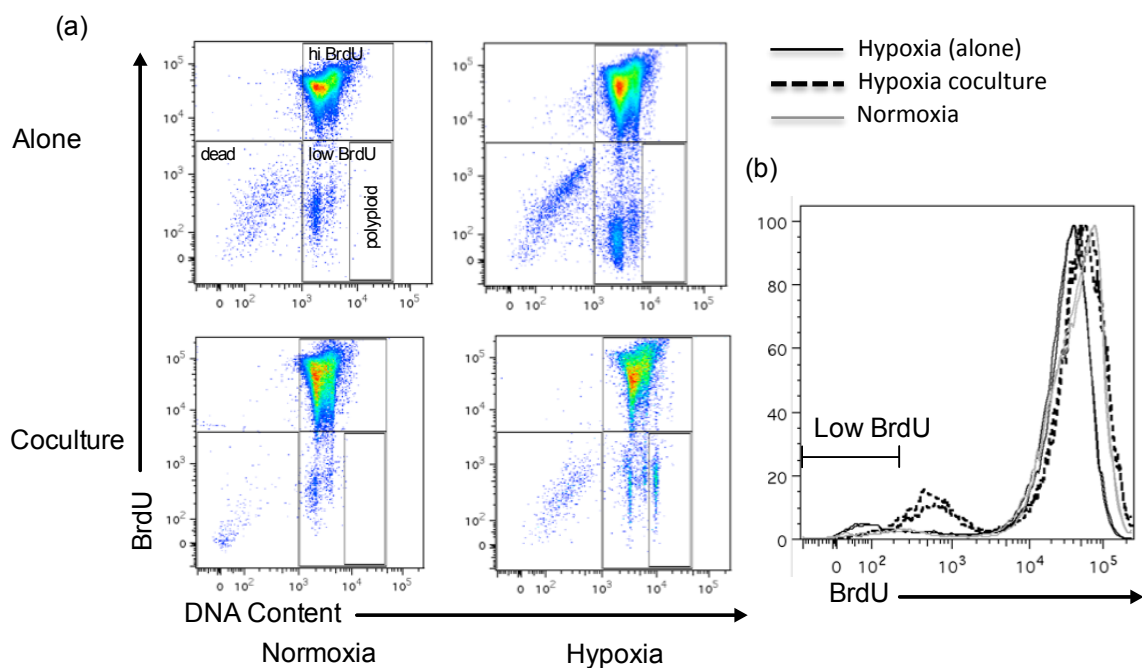


Figure 6.8: Stromal cells alter NB4 BrdU intensity in hypoxic conditions. 3×10^5 NB4 cells were grown in complete media (10% FCS) under normoxic (21% O₂, normal culture conditions) or hypoxic (0.1% O₂) conditions for 48 hours with 10 μ M BrdU with or without MS-5 stromal support. (a) Representative dot plots of BrdU intensity against 7AAD intensity (DNA content) in NB4 cells in indicated culture conditions, gates divide dead, high BrdU, low BrdU and abnormal DNA content cells in the latter. (b) Histograms of BrdU intensity in co-cultured NB4 cells under normoxia, hypoxia and grown alone as indicated.

Whether this shift truly indicates any alleviation of proliferative arrest would require further confirmation. It should also be noted that abnormal polyploidy in DNA content was observed in hypoxic, co-cultured AML cells, not seen in either condition alone (Figure 6.8). Alterations to DNA content could potentially change BrdU staining, however

populations with increased DNA content did not display increased BrdU intensity.

Similarly, whether the increased BrdU intensity in the high expression peak under co-culture (Figure 6.8) reflects proliferative differences is unclear.

To assess the impact of ATG5 overexpression on hypoxic stromal support, NB4-Atg5 cells and the empty vector control line (NB4-eGFP) were similarly assayed. As under serum starvation, NB4-Atg5 cells did not display substantially altered BrdU incorporation under hypoxia when grown alone (not shown), however in co-culture the population of cells with low BrdU intensity showed further reductions on Atg5 overexpression (Figure 6.9). This could indicate the stromal benefit to growth-arrested NB4 cells under hypoxia is being reduced by increased autophagy, however the implications of the single peak intensity shift need to be clarified via direct cell counts.

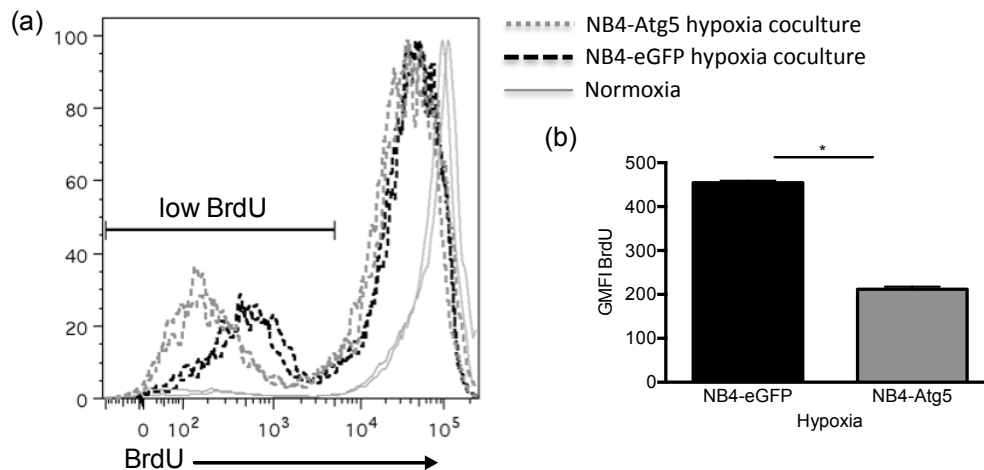


Figure 6.9: Decreased BrdU-intensity of NB4-Atg5 cells in hypoxic co-cultures. NB4-Atg5 or empty vector (NB4-eGFP) cells were grown in complete media (10% FCS) under normoxic (21% O₂, normal culture conditions) or hypoxic (0.1% O₂) conditions for 48 hours with 10 μ M BrdU with or without MS-5 stromal support. (a) Histograms showing representative BrdU intensity of NB4-Atg5 and NB4-eGFP cells in hypoxic co-culture, representative normoxic histograms also shown, gating shows low BrdU population. (b) GMFI of gated low BrdU population from (a) in NB4-Atg5 and NB4-eGFP cells in MS-5 co-cultures under hypoxic conditions. Error bars represent SEM (n=3), *p<0.05, unpaired t test.

6.8 Increased mitochondrial content in co-cultured NB4 cells is reduced by ATG5 overexpression

An important contribution of stromal cells to tumor cell metabolism in the ‘autophagic tumor-stromal model’ is their stimulation of tumor cell mitochondrial biogenesis (499) (655). This is hypothesized to be mediated through tumor cell use of secreted stromal-produced metabolites, including lactate, amino acids and ketones (408, 499), which permit increased tumor cell TCA-cycle biosynthesis and growth (655).

As the advantage to cell proliferation is reduced in ATG5-overexpressing leukemic cells, the mitochondrial content of NB4-Atg5 and vector control lines was assessed to see if this stromal-provided benefit was similarly reduced. Using the mitochondrial potential-sensitive dye Mitotracker Red to measure healthy mitochondrial content (used in place of NAO due to GFP emission overlap), no difference between the two cell types was detected in solitary cultures. However, culture with stromal cells was sufficient to significantly increase mitochondrial content by 1.4 fold in the vector control line, whereas the upregulation in NB4-Atg5 cells was significantly less (Figure 6.10). Whether this represents an intrinsic block to increased mitochondrial content in the NB4-Atg5 cells, for example through mitophagic mitochondrial turnover, or represents a reduction in stimulated stromal cell support, is not certain. Reduced mitochondrial content could impact proliferation.

6.9 NB4 cell differentiation is rescued by stromal co-culture

The NB4 cell line has been frequently employed as a model of neutrophilic differentiation, induced through treatment with all-trans retinoic acid. Autophagy has repeatedly been

found vital for NB4 cell maturation, with knockdown of autophagy genes and modulators attenuating the process (332, 333), possibly as autophagy degrades the PML-RARalpha

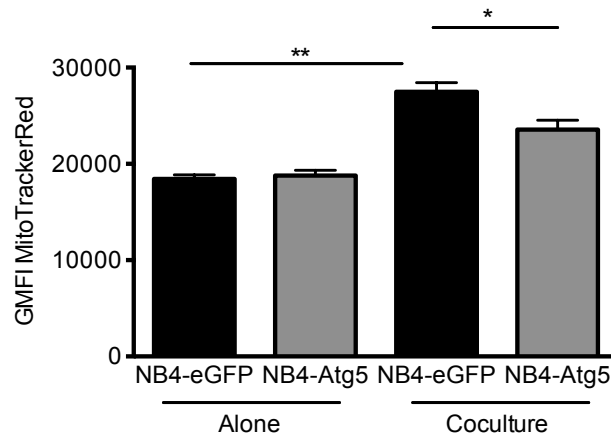


Figure 6.10: Stromal co-culture-induced mitochondrial content increases are Atg5-status dependent. GMFI of MitoTrackerRed staining of NB4-Atg5 and NB4-eGFP empty vector lines grown in complete media (10% FCS) with or without MS-5 stromal layers. Error bars represent SEM (n=3), * $p < 0.05$, ** $p < 0.01$, unpaired *t* test.

oncoprotein (158, 434). Serum starvation is an autophagy stimulus, and stress can induce differentiation of immature cell types (183). Therefore expression of CD11b on the cell surface, a marker of NB4 differentiation (332, 333, 665), was quantified during culture of NB4-Atg5 and vector control (eGFP) lines. While both lines showed minimal CD11b expression in complete media, a stark upregulation of CD11b expression was seen following extended serum deprivation, particularly in NB4-Atg5 cells where twice as many cells became CD11b positive (Figure 6.11). This upregulation provides indications that increased autophagic capacity may mediate NB4 cell differentiation under inducing conditions, although additional differentiation parameters, such as morphology, would be to be assessed. Interestingly, co-culture with stromal cells was able to significantly rescue NB4-Atg5 CD11b expression, suggesting stromal reduction of cell stress may mitigate the differentiation induced by ATG5 overexpression.

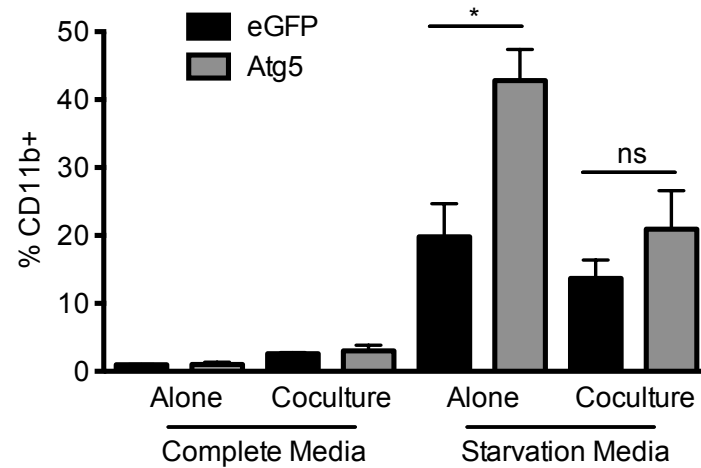


Figure 6.11: Stromal co-culture and *Atg5* overexpression modulate NB4 CD11b expression under starvation. Frequency of CD11b positive cells in NB4-*Atg5* and empty vector control (NB4-eGFP) lines grown with or without a MS-5 stromal layer in starvation or complete media conditions as in Figure 6.2. Error bars represent SEM (n=2), * $p < 0.05$, unpaired *t* test.

6.10 Stromal co-culture increases proliferation and reduces cell death in a murine AML model in a contact-independent manner

Different cancer models, including AML cells, have ranging growth requirements, yet a feature of the ‘autophagic tumor stromal model’ is the relatively broad applicability of metabolic benefits tumor-stroma coevolution provides (294). Also, although stromal cells have been widely used to support primary healthy hematopoietic cells and patient AML cells in culture (636, 637) (638, 639) (640) (641), mechanistic work has often been performed on established cell lines (642, 645) which grow well in solitary culture, are often growth factor independent and carry substantial genetic abnormalities (528). Chapter 5.15 outlined the generation of MLL-ENL immortalized murine cell lines (ME lines) (135, 136), and the impact of MS-5 stromal co-culture on proliferation was investigated using this more primary, genetically-defined AML model.

As with the human AML cell lines, the ME lines were cultured with or without a 90% confluent layer of MS-5 cells, either in complete growth media (10% FCS, supplemented

with SCF, IL-3 and IL-6, as published (136)) or in serum starvation conditions (2% FCS, no cytokines; note that 2% serum was retained as full serum withdrawal was poorly tolerated in preliminary experiments) for 18 hours before their cell cycle status was analyzed. Cell cycle phase and dead cell (sub-G0/G1, indicates apoptosis or necrosis) gateings were performed as in Chapter 5.16. Wildtype ME lines in complete media showed a mild but consistent decrease in cell death when co-cultured with stromal cells, with a corresponding slight increase in the S-phase population (Figure 6.12). Those differences were heightened under serum starvation, where stromal co-culture doubled the proliferating fraction incorporating BrdU (Figure 6.12, white bars). These differences observed on stromal co-culture are relatively small, but help corroborate the human cell line data on the benefits stromal cells provide *in vitro* to AML cells under stress.

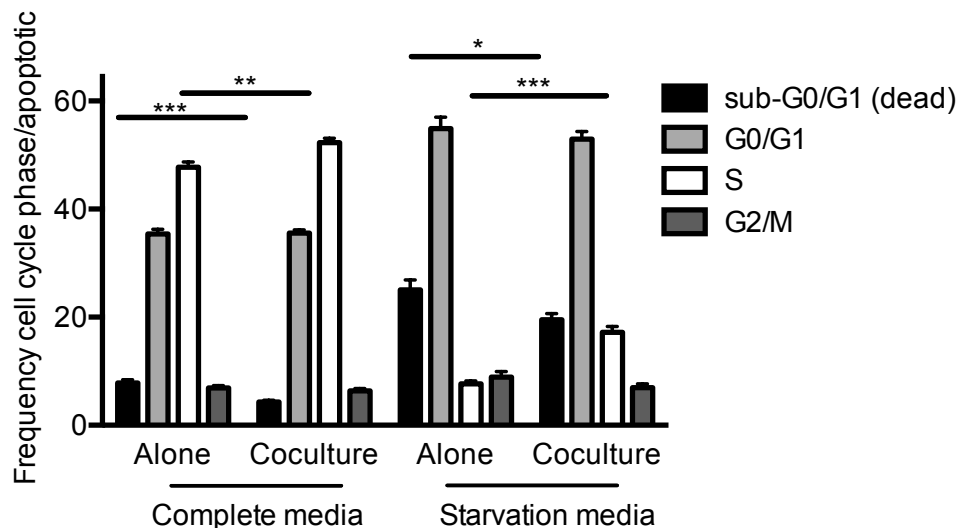


Figure 6.12: Co-culture with MS-5 stromal cells increases proliferation and decreases cell death under starvation in a murine MLL-ENL (ME) AML model. 1.5×10^5 wildtype MLL-ENL murine cell lines (generated as described in Chapter 5.15) were grown in complete media (10% FCS and cytokines as Chapter 5.15) or in starvation media (2% FCS, no cytokines) with or without a 90% confluent MS-5 stromal layer for 18 hours, $10 \mu\text{M}$ BrdU was added for final 45 minutes before fixation, staining for BrdU incorporation and flow cytometric assessment against 7AAD intensity (DNA content), cell cycle stages and dead (sub-G0/G1) cells were gated as Figure 5.15. Error bars represent SEM ($n=12$ cultures over 4 experiments), $*p<0.05$, $**p<0.01$, $***p<0.001$, unpaired *t* test.

Another key factor in the ‘autophagic tumor stromal model’ is the contact-independence of the tumor-stromal interaction: oxidative stress from tumor cells stimulates stromal metabolic changes, leading to stromal secretion of pro-growth metabolites (294, 499). In primary hematopoietic cell and leukemic *in vitro* co-cultures, stromal cell (including MS-5 specifically) provision of soluble/diffusible factors has been implicated in the benefits provided by stromal culture. Examples include cytokines such as SCF, GM-CSF, IL-6 and TPO (641, 647-650), which likely synergize with contact-dependent factors, including extracellular matrix and adhesion molecules or non-secreted cytokines (642, 645, 648, 666-668). Indeed, a combination of these factors is often cited as transwell separation of the stromal and leukemic cells, or addition of conditioned media, is reported to provide only partial benefits (642, 648, 666, 667, 669).

To assay the influence of cell-to-cell contact on the stromal support of the ME lines, the above experiments were repeated with the cell populations separated by a transwell filter (0.4µm pore size). As before, MS-5 cells were able to moderately reduce ME apoptosis and improve the S-phase fraction without direct cell contact (Figure 6.13), and the magnitude of these impacts was not altered from the direct contact experiment. This experiment indicates that the benefits to ME line health are indeed provided by soluble factors.

6.11 *Atg5* KO leukemic cells may particularly benefit from stromal co-culture

As noted above, oxidative stress plays an important role in the ‘autophagic tumor stromal model’. Tumor cell-produced ROS stimulates stromal oxidative stress and production of

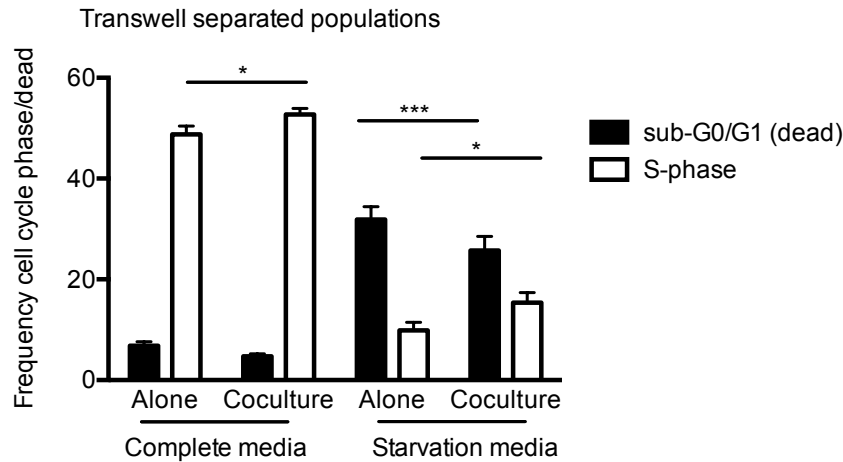


Figure 6.13: Proliferative benefits of stromal co-culture for ME leukemic cells are contact-independent. Frequency of cells in S-phase or sub-G0/G1 (dead) in ME leukemic lines grown in complete media or starvation media (as in Figure 6.12) in the top section of a transwell permeable insert (Costar, 0.4 μ m pore size, 6.5mm insert) with or without a MS-5 stromal layer in the other transwell-separated compartment, for 18 hours then BrdU pulsed and assayed as Figure 6.12. Error bars represent SEM (n=6), * p <0.05, *** p <0.001, unpaired t test.

pro-tumor factors (499), while co-culture with stromal cells stimulates antioxidant defense in tumor cells (294). This defense includes expression of TIGAR (TP53-induced glycolysis and apoptosis regulator) (663), which dampens ROS, and inhibits apoptosis as well as autophagy (478, 670). Therefore, despite epithelial cancer cells initially displaying the highest ROS, the majority of ROS was observed in stromal cells after extended culture (294). The levels of ROS in ME leukemic lines was investigated after 18 hours of co-culture with MS-5 stromal cells under serum starvation conditions, and reduced levels of ROS were found in co-cultured leukemic cells relative to those cultured alone (Figure 6.14; ROS reduced for all genotypes, representative lines shown), even at this early time point. Reduced levels of ROS were also observed in ME cells cultured with stromal cells in complete media, although to a lesser extent (not shown), matching mitochondrial ROS measurements in HEL and NB4 cells.

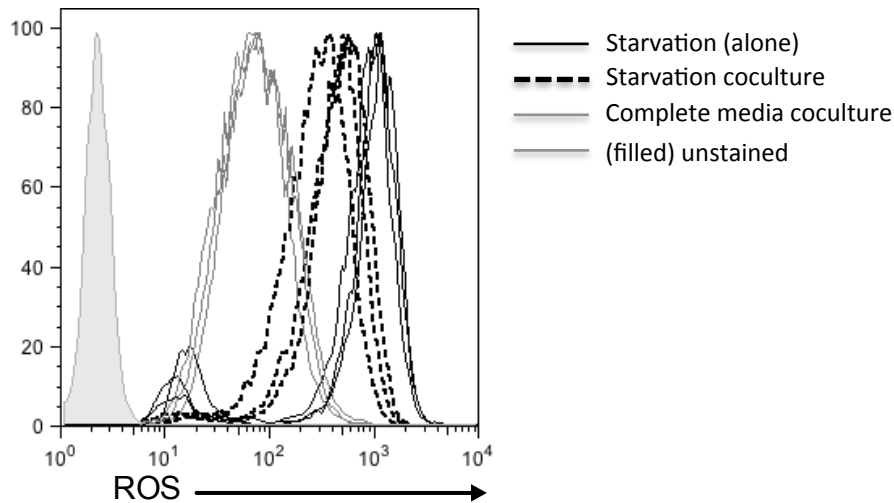


Figure 6.14: Co-culture reduced total ROS in starved ME leukemic cells. Histograms showing intensity of oxidative stress detection reagent (Enzo Life Sciences) in three representative wildtype ME lines after 18 hours in starvation media (2% FCS, no cytokines) with or without an MS-5 stromal layer, as compared to cells grown in complete media with stromal layers.

Chapter 5.15 outlines the generation and characterization of ME leukemic lines with heterozygous (HET) and homozygous (KO) deletion of *Atg5*. HET and KO cells demonstrated increased proliferation following serum starvation, while KO cells also showed increased cell death, which could be partially rescued by treatment with antioxidant N-acetyl cysteine (NAC). As ME cell ROS are actually reduced through MS-5 co-culture, the impact of NAC treatment on the benefits of stromal co-culture was investigated, including the effect across all lines and by genotype. Examining first the proportion of cells in S-phase (cells actively incorporating BrdU following a short pulse), culture with NAC significantly tempered the stromal proliferation benefit observed in both complete media and serum starvation co-culture conditions, while not impacting proliferation significantly in leukemic cells cultured alone (Figure 6.15). When the effect of NAC treatment under serum starvation was broken down by genotype, the KO line received the most substantial benefit (increased S-phase fraction) from co-culture and a corresponding reduction on NAC treatment. However, these trends were not significant due to variation and experimental numbers (Figure 6.15). As noted in the previous chapter,

serum-starved KO cells alone showed significantly increased proliferation following NAC treatment in solitary culture, and this was not further increased by co-culture with NAC treatment. This could suggest that while ROS sensitivity may impact KO proliferation specifically in a cell-intrinsic manner, certain co-culture effects (although not all) are non-cell autonomously ROS-dependent (Figure 6.15). Conclusions though require confirmation with alternative antioxidants, and absolute effects seen are relatively small. Finally, although all genotypes showed increased proliferation following co-culture, the proportion of WT cells in S-phase was still below that of HET and KO cells, demonstrating the increased proliferation seen in the latter two genotypes under metabolic stress is not culture-type dependent (Figure 6.15).

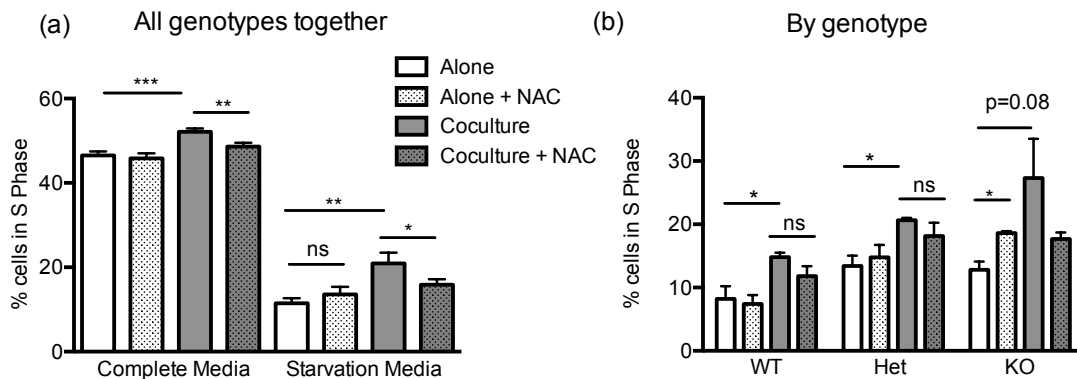


Figure 6.15: Proliferative benefit to ME leukemic cells in MS-5 co-culture is partially NAC-sensitive and is *Atg5* independent. Frequency of ME leukemic cells in S-phase in complete media or starvation co-cultures setup and assayed as Figure 6.15, either with or without 5mM N-acetyl cysteine (NAC) addition to each culture conditions, showing either (a) Cre-transduced ME *Atg5*-genotypes (WT, HET, KO) pooled together ($n=9$, three cultures each of three lines), therefore common impact of treatment or (b) divided by *Atg5* genotype under starvation conditions into WT (*Atg5*^{+/+}), HET (*Atg5*^{+/-}) or KO (*Atg5*^{-/-}) lines ($n=3$ cultures per line). Error bars represent SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, unpaired *t* test.

Interestingly, NAC treatment was able to very mildly but consistently reduce the proportion of apoptotic/necrotic (sub-G0/G1) ME leukemic cells under complete media conditions across all genotypes in solitary cultures, whereas the already further reduced apoptosis levels in complete media co-cultures were unaltered (Figure 6.16). In contrast,

only KO cell death in solitary cultures were effected under serum starvation conditions, bringing the frequency of dying cells to a similar level as those observed in WT cells (in these experiments, HET cells showed lower apoptosis than WT cells following starvation, however this was not consistently observed across all experiments). Combined with the S-phase data, these results suggest the S-phase and apoptotic frequency changes on NAC treatments are differential effects (ie. altered S-phase frequencies are not simply due to changes in apoptotic fraction), as alterations were seen under co-culture and solitary culture conditions respectively. Furthermore, in the WT and HET lines in particular, NAC treatment could not replicate co-culture benefits under starvation. Although NAC treatment could alter co-culture S-phase frequencies, the magnitude of changes are slight, and further study is required to fully understand NAC-effects (671). Finally, S-phase and cell death data suggest KO ME cells in particular could benefit from the stromal provided microenvironment, as co-culture was able to reduce otherwise elevated apoptosis levels to equivalent low levels across all three genotypes (Figure 6.16).

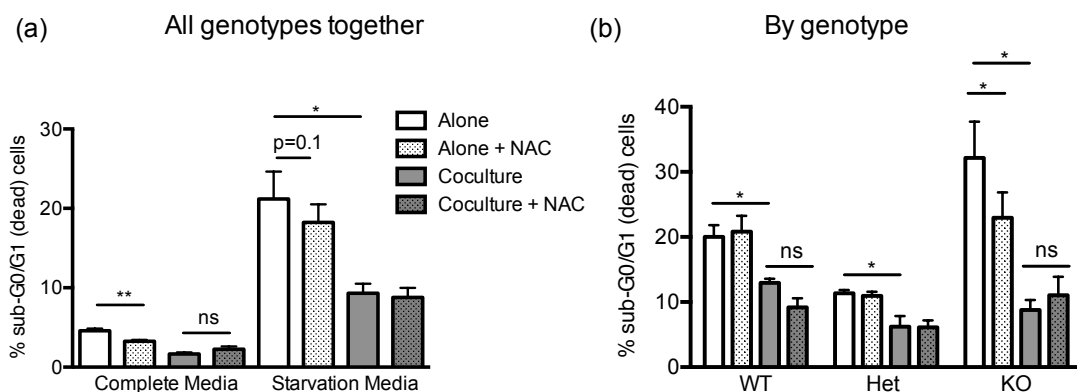


Figure 6.16: Excess starvation-induced cell death of *Atg5* KO ME leukemic cells is rescued by stromal co-culture. Frequency of apoptotic/necrotic (sub-G0/G1) ME leukemic cells in complete media or starvation co-cultures setup and assayed as Figure 6.15, either with or without 5mM N-acetyl cysteine (NAC) addition to each culture conditions, showing either (a) *Cre*-transduced ME *Atg5*-genotypes (WT, HET, KO) pooled together ($n=9$, three cultures each of three lines), therefore common impact of treatment or (b) divided by *Atg5* genotype under starvation conditions into WT (*Atg5*^{+/+}), HET (*Atg5*^{+/-}) or KO (*Atg5*^{-/-}) lines ($n=3$ cultures per line). Error bars represent SEM, * $p<0.05$, ** $p<0.01$, unpaired *t* test.

6.12 Co-culture reduces stromal healthy mitochondrial content *in vitro*

Coevolution of the tumor and stromal populations is an important point in the model proposed by Lisanti *et al.*, where the presence of tumor cells results in loss of mitochondria in the stromal population, accompanying a stromal glycolytic shift which benefits the tumor population (‘reverse Warburg effect’) (294, 499). Although metabolic alterations have not been previously examined in leukemic culture systems, the ability of leukemic cells to alter the phenotype of co-cultured stromal cells has been reported (608, 616) with leukemic-altered stromal cells promoting increased proliferation of normal healthy progenitors (617). The mitochondrial content of the MS-5 stromal layer was assessed by NAO staining and, although 3 days of serum starvation did not significantly alter MS-5 NAO intensity, 3 days of growth with NB4 leukemic cells resulted in a loss of healthy mitochondrial content in MS-5 cells (Figure 6.17).

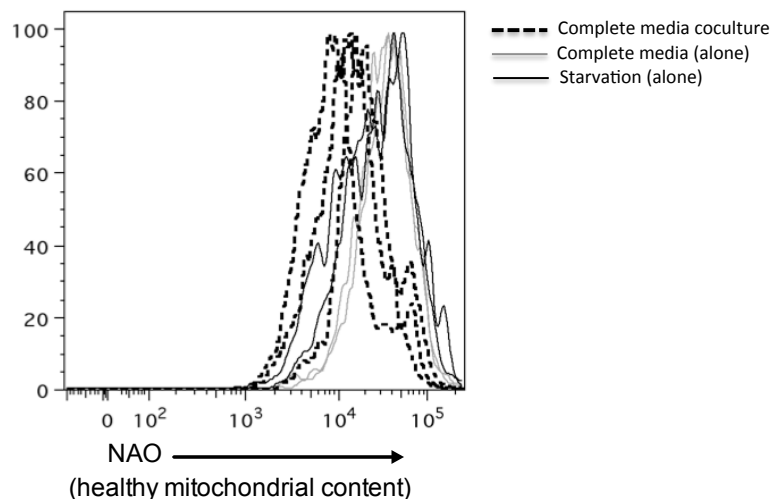


Figure 6.17: Leukemic co-culture reduces healthy mitochondrial content of MS-5 stromal cells by NAO staining. Histograms showing intensity of nonyl-acridine orange (NAO) staining in live (DAPI negative) MS-5 cells grown in complete media (10% FCS) alone or with NB4 leukemic cells for 72 hours as in Figure 6.2, or in starvation media (no FCS) alone. Leukemic cells were washed off with PBS prior to MS-5 trypsinization and remaining leukemic cells were excluded by size (FCS/SSC) gating.

NAO stains non-oxidized cardiolipins in the mitochondrial membrane, so it is responsive to mitochondrial oxidative health in addition to content (ie. stains ‘healthy mitochondrial content’). Therefore, the non-potential or ROS sensitive dye MitoID was used to ensure decreased NAO staining represented content changes, rather than solely decreased health, and indeed leukemic co-culture resulted in the reduction of MS-5 MitoID intensity as well (Figure 6.18).

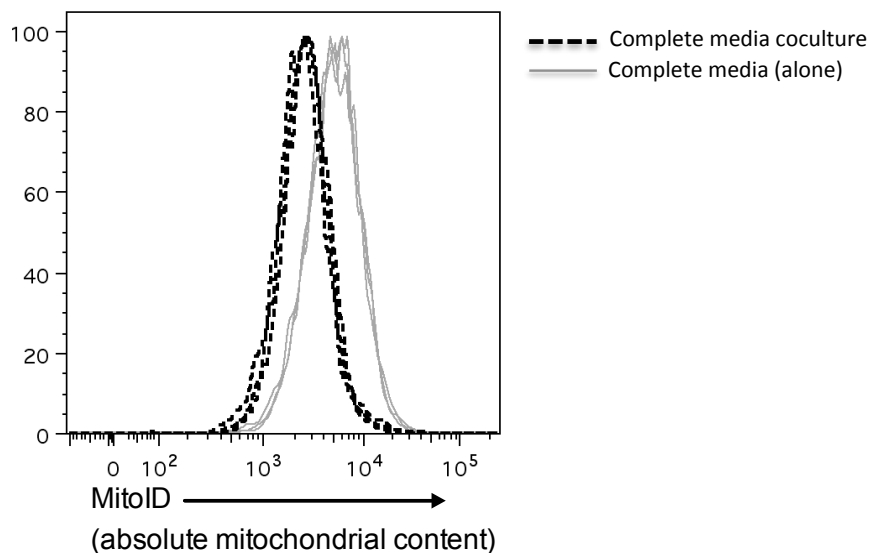


Figure 6.18: Leukemic co-culture reduces mitochondrial content of MS-5 stromal cells by MitoID staining. Histograms showing intensity of MitoID staining in live (DAPI negative) MS-5 cells grown in complete media (10% FCS) alone or with NB4-eGFP (empty vector) leukemic cells for 48 hours. Leukemic cells were washed off with PBS prior to MS-5 trypsinization and remaining leukemic cells were excluded by size (FCS/SSC) and GFP expression.

6.13 Bone marrow stromal cells have reduced mitochondrial health in *Vav-Atg7*^{-/-} mice

The presence of leukemic cells, which displayed reduced basal levels of autophagy as assayed in Chapter 5, is therefore sufficient to reduce stromal mitochondrial content *in*

vitro. Although *Vav-Atg7*^{-/-} mice do not develop leukemia prior to death, their bone marrow was characterized to display a pre-leukemic phenotype around 6 weeks of age, including bone marrow infiltration by dysplastic *Atg7*-null myeloid cells, increased multipotent progenitor proliferation, increased progenitor mitochondrial content, and increased oxidative stress (131). HSCs and progenitors are known to develop in vascular niches in close contact with endothelial stromal cells (107). As *Vav-iCre* excision is limited to the hematopoietic compartment (348) (133) (350), these mice provide a prospective *in vivo* model where wildtype stromal cells are in close proximity to stressed, autophagy-deficient myeloid and progenitor cells in the bone marrow.

Therefore, the mitochondrial content, via NAO intensity, was investigated in endothelial stromal cells in the *Vav-Atg7*^{-/-} bone marrow at 6 weeks of age. Endothelial cells, defined by lack of surface expression of CD45 and presence of CD31 (672), were divided by expression of ICAM1(673) as shown (Figure 6.14). ICAM1 is an endothelial activation marker which is upregulated during inflammation (673) (674) and aids leukocyte adhesion to vessel walls (675). Stromal cells from *Vav-Atg7*^{-/-} mice showed reduced healthy mitochondrial content as compared to stromal cells in wildtype mice, indicating an *in vivo* autophagy-low, oxidative stress-high environment is able to impact the mitochondrial health of nearby stromal cells (Figure 6.19). This was true for both ICAM1⁺ and ICAM1⁻ populations.

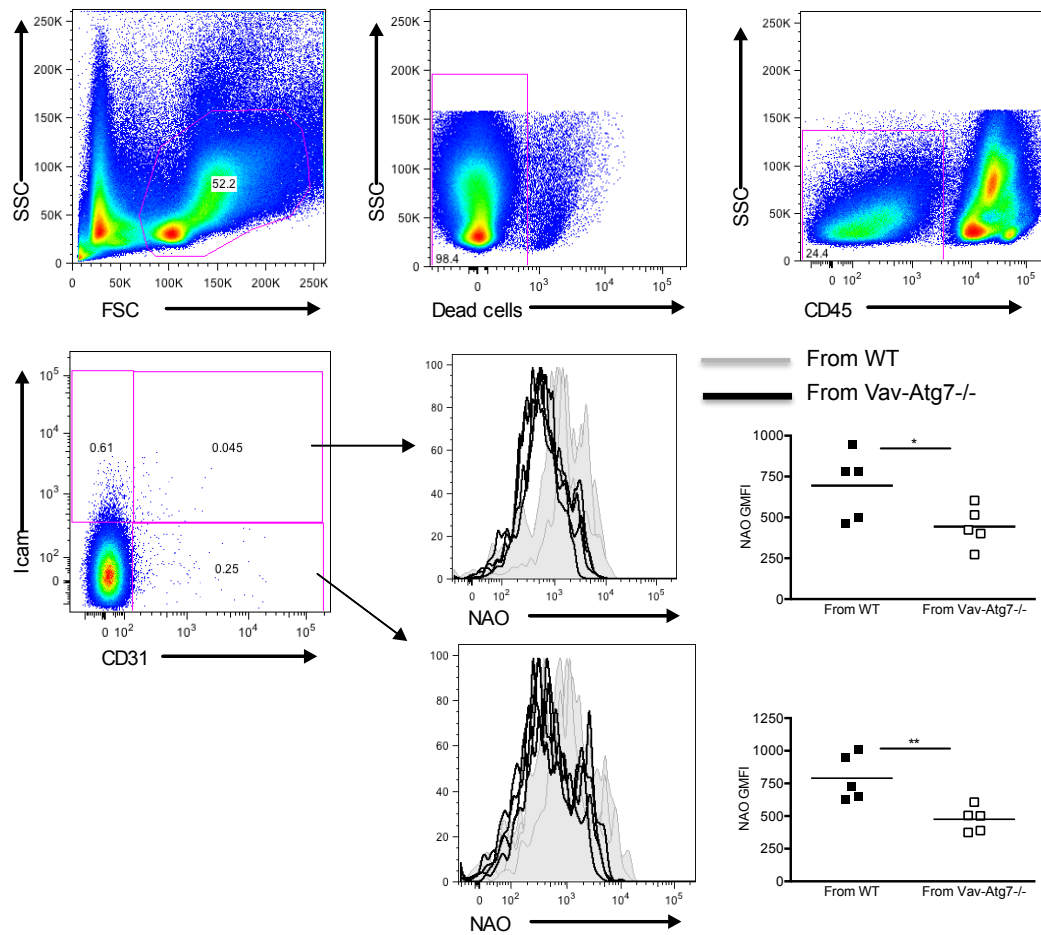


Figure 6.19: Endothelial stromal cells in *Vav-Atg7*^{-/-} bone marrow have reduced healthy mitochondrial content. Gating strategy for stromal cells in crushed bone marrow from 6 week old *Vav-Atg7*^{-/-} mice or wildtype littermates, gating (left to right) size (FSC/SSC), live cells, CD45⁻ than selecting for Icam1⁺ CD31⁺ (activated, top histogram showing three representatives per genotype and graph) or Icam1⁻ CD31⁺ cells (bottom histogram showing three representatives per genotype and graph) and quantifying nonyl-acridine orange staining (NAO) intensity. **p*<0.05, ***p*<0.01, unpaired *t* test.

Discussion

6.14 AML cells benefit from stromal co-culture

As a system frequently employed to both study the relationship leukemic cells have with their microenvironment and to culture AML cells from patients for investigative and diagnostic purposes (636, 637) (638, 639) (640) (641), understanding the interactions between AML cells and stromal cell layers *in vitro* is highly important. MS-5 cells, a murine bone marrow stromal cell line, are often used to provide support to mouse and human hematopoietic cells, including HSCs (for up to 5 weeks)(669), and leukemic cells (636), with their advantages over primary stroma being homologous and consistent support across experimental conditions (635) (647) (669) (649) (642) (645). In particular, MS-5 cells and related lines have been described to protect human AML cells from both therapy- and metabolic stress- (serum starvation) induced apoptosis (648) (642) (666). These conditions are relevant to the *in vivo* leukemic bone marrow microenvironment (395, 564) (555) (608, 609). In our hands, MS-5 stromal cells were found to increase BrdU intensity and reduce cell death following serum starvation of two human leukemic cell lines, with more mild impacts on a murine AML model with different growth requirements (136, 661, 662). As the hypoxic impact on NB4 BrdU incorporation was slight (agreeing with the intrinsic resistance of leukemic cells to hypoxia (564)), repeating these experiments with the other models and using additional readouts such as cell counts will be important for confirmation. Increased healthy mitochondrial content in co-cultures is one potential internal mediator of the growth-promotion observed in these lines under stress, as is hypothesized in other tumor types (656). Increased mitochondrial ROS from unhealthy mitochondrial following therapy have been frequently implicated in leukemic apoptosis and growth arrest (676, 677).

Although past reports have indicated stromal support of AML cells is through a combination of contact and non-contact dependent mechanisms (642, 648, 666), transwell experiments with the MLL-ENL cells suggested stromal pro-leukemic effects are *not* contact-dependent in this model. A frequently cited potential primary mode of action for diffusible stromal support of AML *in vitro* is the provision of growth promoting cytokines in serum-starvation conditions (642, 647, 648). MS-5 cells are known to produce SCF and GM-CSF but not IL-3 (641), although studies have suggested MS-5 promotion of hematopoiesis is not necessarily tied to these or several other hematopoietic cytokines (669). From the AML perspective, MS-5 cells have been reported to upregulate anti-apoptotic proteins BCL-2 (642) and XIAP, related to activation of PI3K/Akt signaling pathway (645) that can be mediated by cytokines such as SCF (678). In our experiments, MLL-ENL cells would no doubt benefit from SCF under starvation conditions. However, this does not explain the mild stromal benefits in complete media where SCF is provided. Similarly, provision of soluble SCF did not reproduce MS-5 benefits on a human cytokine-dependent AML line (647), nor did SCF, GM-CSF, IL-3 or IL-6 supplementation in culture of AML cells with primary bone marrow monolayers (648). Moreover, HEL and NB4 are largely growth factor independent cell lines. Re-addition of individual cytokines to starved ME co-cultures, as well as cytokine blockade experiments, would help clarify their role in the co-culture setting.

6.15 Application of ‘two-compartment metabolism model’ to AML and stromal *in vitro* cultures

Another contributing factor could be metabolic support of leukemic cells by the stroma, as in the ‘two compartment metabolism’ or ‘autophagic tumor stroma model’ (499) (656)

(294) (408). As outlined in above, this model represents a primary hypothesis for autophagy-dependent solid tumor interactions with stromal populations which has not previously been tested in hematopoietic contexts. Some observations from leukemic cell-stromal cell interactions appear to partially follow model predictions, but several key experiments remain before conclusions can be drawn on model applicability to the leukemic setting.

First, oxidative stress produced by tumor cell proliferation is hypothesized to create microenvironmental oxidative stress and leads to loss of stromal mitochondrial health and mitochondrial removal, potentially through autophagy/mitophagy activation (499) (294). Consistent with this, co-culture with leukemic cells reduced MS-5 cell mitochondrial volume, while the *Vav-Atg7*^{-/-} bone marrow microenvironment (which contains accumulated myeloid cells and increased oxidative stress (131)) produced a similar effect in endothelial stromal cells measured *ex vivo*. Treatment with NAC reduced the ME line proliferation benefit seen in co-culture. However, as changes were small, and considering NAC has been reported to have effects beyond ROS reduction (671), repeating these experiments with other antioxidants as well as examining their impact on stromal mitochondrial loss in co-culture will be vital prior to interpreting these results.

Secondly, in the model proposed by Lisanti *et al.*, altered metabolism in stromal cells following mitochondrial loss results in increased secretion of metabolites, including lactate and ketones (408), utilized by tumor cells to drive increased mitochondrial biogenesis and promote mitochondrial biosynthetic pathways (499) (656). MS-5 co-culture increased healthy mitochondrial content in co-cultured leukemic cells, accompanied by decreased ROS. However, confirming the mechanism behind these changes requires much investigation; preliminary experiments revealed elevated lactate levels in the supernatant

of stromal/AML co-cultures (data not shown). This elevation has also been reported in the bone marrow of AML patients (634), but the cell origin of the lactate would need confirmation as it could simply represent increased proliferation. Metabolomics on media from co-culture cells could assess levels of specific contributors, such as amino acids. Glutamine metabolism has been shown vital for AML growth via mTORC1 activation (679), while stromal-provided cysteine has been previously shown to help reduce leukemic ROS levels through glutathione synthesis (629). Future experiments will focus on such examinations of stromal secretome, and if metabolites are found to be increased, their effects on leukemic cell mitochondrial levels would require independent confirmation via culture supplementation.

Third, the solid tumor model suggests a role for autophagy in tumor-stromal interactions (499) (408). Decreased levels of autophagy in tumor cells may lead to increased stromal-provided benefit, while overexpression of autophagy gene *ATG16L1* in epithelial cancer cells reduced their growth (408). In primary AML BMNCs, blast marker positive cells were observed to have decreased autophagy and increased NAO staining (healthy mitochondria) relative to blast marker negative cells (Chapter 5, Figures 5.6 and 5.9), generally agreeing with the above model. *ATG5* overexpression in leukemic cells reduced their growth in serum-starved co-cultures, while decreasing NB4 cell mitochondrial content and increasing CD11b expression (661). NB4 cell CD11b expression has previously been shown to be autophagy-dependent, with autophagy degrading PML-RARalpha to induce differentiation (158, 434), and further tests could ascertain whether oncoprotein levels decrease in NB4-Atg5 cells on starvation. However, the mechanisms behind the other observations remain uncertain, as no differential impact on MS-5 was observed (not shown), indicating overexpression impacts may be cell intrinsic.

In the MLL-ENL lines, although most stromal-benefits were of a small magnitude, *Atg5*^{-/-} (KO) cell death under serum starvation appeared substantially rescued by MS-5 co-culture. Repeating this result with alternative cell death assays (ex. Annexin V staining for apoptosis) will be required to clarify the cell death pathway involved (ie. apoptosis versus necrosis), as the mechanisms of cell death (ie. ROS dependent, as suggested by the NAC result, due to metabolic crisis or DNA damage via p53 ect.) will be important in unraveling how MS-5 cells rescue that phenotype.

Finally, increased stromal autophagy is an important part of the ‘autophagic tumor-stroma model’ (499), with stromal cells overexpressing the autophagy- related genes *ATG16L1* or *BNIP3* reportedly showing increased lactate and ketone secretion while promoting increased tumor growth (408). This leads the authors to suggest stromal autophagy as a potential therapeutic target (294). MS-5 autophagy has yet to be fully probed in the leukemic model, but a proper understanding of the implications of autophagy modulation on both leukemic cells and their interactions with stroma is vital for the systemic application of autophagy modulating therapies like hydroxychloroquine (477).

6.16 Conclusions

This chapter has presented an investigation into the *in vitro* interactions between AML cells and the bone marrow stromal line MS-5, specifically testing the application of a prominent model for autophagy’s impacts in epithelial tumor-stroma interactions. MS-5 cells were able to reduce AML cell death while promoting AML cell proliferation under metabolic stress in a contact independent manner, and *Atg5* status impacted on co-culture benefits. Moreover, stromal cell mitochondrial content was reduced *in vivo* following autophagy loss in the hematopoietic system. However, these results are still preliminary and much work remains to define autophagy’s role in the AML microenvironment.

Chapter 7: Discussion and Conclusions

7.1 Autophagy in hematopoiesis and HSCs

That the severity and timing of lineage and stem cell defects in *Vav-Atg5*^{-/-} mice phenocopied *Vav-Atg7*^{-/-} mice (7, 131) argues strongly for the role of the canonical autophagy pathway in hematopoiesis. This is particularly true for the ubiquitin-like conjugation systems, potentially at both early and late stages of the hematopoietic cascade.

In the differentiation of the erythroid lineage, previous studies have outlined a developmental block following the defective removal of mitochondria in the absence of autophagy genes (341) (154) (7, 153). The loss of *Atg5* reproduces this, dismissing suggestions that aspects of autophagic mitochondrial maintenance occurring in the absence of *Atg5* could compensate. Indeed no mitochondrial phenotype was relieved by the substitution of *Atg5* deletion for *Atg7*. The ability of *Atg5* and *Atg7* null erythroid cells to partially remove mitochondria, and to fully remove other organelles, indicates erythroid cells do not rely on canonical autophagy alone for cytoplasmic clearance during maturation (342, 343). However the progressive anemia *Vav-Atg5*^{-/-} mice show suggests autophagy is an indispensable part of that process. The stage-specific block in erythroid maturation corresponds to an observed spike in autophagic activity, as well as the action of key lineage transcription factor GATA1 in upregulating the autophagy pathway (58). Erythroid differentiation, therefore, represents an important example of a non-homeostatic role for autophagy, rather the developmental regulation of the autophagy pathway for the purposes of cellular remodeling (150). By contrast, the lymphoid requirement for autophagy and *Atg5* appears more homeostatic, with the strongest defect in survival of mature populations in the periphery rather than developmental organs.

Experiments with *Vav-Atg5*^{-/-} HSCs show hematopoietic autophagy is required for maintaining the balance between HSC quiescence, self-renewal and differentiation. This is

underpinned by severe defects in steady-state maintenance of the HSC population, *in vitro* myeloid differentiation and *in vivo* reconstitution capacity. The increased basal autophagy observed in the HSC and multipotent progenitor compartment suggests the requirement for autophagy may be HSC intrinsic, and that upregulated autophagy, or a high autophagy capacity (49) is a general feature of long-lived hematopoietic populations, and indeed many stem cell types (206) (212) (110). Potential contributors to constitutive HSC autophagy may be microenvironmental conditions in the niche (107), HIF1 α (201), enforced low mTORC1 signaling (238, 403) and, in particular, FOXO3 (49), which was found upregulated in human HSCs. Combining the novel imaging flow cytometry autophagy assay with analysis of transcription factor translocation could aid the separation of these factors, as could application of LC3-GFP transgenic mice, which will be a powerful tool for *in vivo* investigation of hematopoietic autophagy going forward.

Still to be determined are the precise impacts of autophagy loss which mediate loss of quiescence and exhaustion. Possibilities include a (i) damaged microenvironment due to lineage defects (extrinsic), or intrinsic mechanisms such as (ii) oxidative stress due to mitochondrial retention (185, 538), (iii) accumulation of macromolecular damage without turnover activities (protein and genomic), (iv) aberrant Akt/mTOR signaling, as observed in autophagy deficient myeloid leukemic cells, or (v) altered levels of autophagy-regulated cell cycle proteins. Extrinsic factors will be largely addressed in an ongoing Mx-Cre inducible competitive chimera experiment, where it appears so far that autophagy-deficient HSCs may still be defective in a healthy microenvironment. An intrinsic autophagy requirement in HSCs and early progenitors may explain the increased severity of lineage defects, including in erythroid and B-cell populations, in the Vav models compared to lineage specific Cre models (282, 342). mTOR pathway phosflow on *Vav-Atg5*^{-/-} HSCs, rapamycin, metformin and antioxidant treatment experiments would all be intriguing

options for follow-up investigation of intrinsic factors. Similarly, the differences in timing between the HSC defect in the Vav driven models and *FIP200*^{-/-} fetal liver chimeras (344), which indicate autophagy requirement at early adult and fetal stages respectively, will require further clarification. Vav-deletion is more physiological, and the preliminary results from the inducible model would indicate autophagy is still required in adult HSCs, therefore it may be that the stress of chimera generation intensifies autophagy requirement, as previously observed for cell cycle regulator RB1 (382), or that FIP200 phenotype is exacerbated by ubiquitin-like cascade independent roles.

A model newly arrived in the host lab which may help define the quantitative importance of autophagy in HSCs is a Vav-driven overexpression model of transcription factor EB (TFEB), a positive regulator of autophagy and lysosomal biogenesis (50, 51). If TFEB overexpression generates a phenotype of HSC quiescence or altered differentiation, it would suggest regulatory roles in growth pathways as opposed to oxidative stress suppression. The comparison of the *Vav-TFEB* and *Vav-Atg5/7* models may also help clarify the importance of autophagy levels in HSC metabolism. The combined hypoxic bone marrow (if not niche) environment and the dangers of ROS would advocate for a minimal, glycolytic metabolism in the majority of HSCs, which appears supported by recent direct measurements (200). Autophagy loss could hamper HSC mitochondrial function (131), and both autophagy deficient myeloid leukemic cells and macrophages (A. Stranks, unpublished) upregulate glycolysis, therefore a metabolic switch may not be behind the exhaustion phenotype of autophagy deficient HSCs. However, as autophagy is important for metabolite recycling (99), and the activation of HSC mitochondrial function is important for differentiation (198), metabolic alterations may yet impact the *Vav-Atg5/7*^{-/-} HSC phenotype.

A final hematopoietic phenotype in the *Vav-Atg5* and *Vav-Atg7* models is the myeloproliferation observed prior to bone marrow failure. Increased frequency, immaturity, decreased function and organ infiltration by myeloid cells all support a pre-leukemic phenotype. However the lack of transplantability and clonal genetic lesions (131), or clear progenitor origins, all argue for either a late maturation block, delayed clearance/death or microenvironmental factors in a damaged, inflammatory hematopoietic system. The fact that the few myeloid lineage-specific autophagy deletions investigations have revealed altered function (336) and differentiation abnormalities (337, 680), albeit of a milder nature, support the existence of cell intrinsic autophagy requirements, and study of myeloid populations in the Mx-Cre inducible chimera will further clarify the role of microenvironment in the autophagy-deficient myeloid expansion. It has been suggested that the myeloid lineage is particularly tolerant to ROS (234, 681), which may explain their persistence in the *Vav-Atg5/7*^{-/-} environment, as may their relatively quick turnover compared to long-lived lineages such as lymphocytes. Whatever the cause of the *Vav-Atg5/7*^{-/-} myeloproliferation, the ability of myeloid cells to survive with deficient autophagy, combined with the damage and proliferation in the early HSPC compartment of *Vav-Atg5/7*^{-/-} mice, lead to the hypothesis that decreased autophagy could be favored in the pathogenesis of myeloid neoplasias.

7.2 Autophagy in Acute Myeloid Leukemia

Indeed, decreased autophagy, as observed in primary AML patients blasts relative to the remainder of the bone marrow, may mediate several interrelated advantageous phenotypes in AML cells, including (i) increased proliferation (ii) increased activation of the pro-growth Akt/mTOR pathway under stress and (iii) upregulation of glycolytic metabolism. Although certain aspects of these alterations were observed in optimal growth conditions,

they were strongest under metabolic stress, conditions where autophagy's importance and activity are heightened. As increased growth and proliferation were also observed in the *Vav-Atg5/7-/-* mice, along with other Atg deletion mouse models (66, 308), it will be intriguing to see if the mechanisms behind the proliferation of autophagy-deficient HSCs are also at work in AML.

These results add to a growing body of evidence that may help clarify the contradiction between autophagy's broadly tumor suppressive role, supported by heterozygous Atg deletions in multiple cancer types along with the pro-tumor impacts observed on Atg gene deletion in mouse models, and the autophagy requirement observed in certain tumor types (16). A new potential model, as seen in other cancer phenotypes, appears background specific: certain cell types, such as myeloid cells, or cancer backgrounds, such as those with significant apoptosis pathway impairment (ex: *p53* mutation) (498), may tolerate autophagy deficiency to reap competitive advantages in tumor microenvironmental conditions, whereas in others where oncogene stress exceeds apoptosis inhibition (ex: Ras models), concomitant sudden loss of the autophagy survival pathway is not tolerated. Validation of this model will be required, for example it would predict that *p53* mutation in BCR-ABL transfected cells may rescue the negative impacts of autophagy loss (451). Microenvironmental factors may also impact autophagy requirements; in the MLL-ENL model, *Atg5* loss resulted in excess cell death following serum withdrawal, yet this was rescued *in vitro* by bone marrow stromal cell support. This requires further confirmation, but could explain why, despite excess death compared to transformed cells lacking only one copy of *Atg5*, the knockout phenotype appears to strongly accelerate *in vivo* leukemia growth. Alternatively, a limited amount of cancer cell damage and death may benefit tumors overall through increased inflammation and genomic instability (394) (395). Finally, there is evidence that tumor cells can adapt over time to better tolerate autophagy

loss (682), which is both an important potential factor during clonal selection and a caveat to be considered following extended in vitro culture of cancer models.

The importance of autophagy in leukemic development must still be addressed. The lack of transformation in the shortened lifetime of *Vav-Atg5/7-/-* mice and benign nature of most tumors developing in autophagy deletion models (388) indicate apoptotic protein mutation may precede (if not accompany) modulation of the autophagy pathway. In other words, the development of spontaneous tumors in most Atg knockouts may be greatly aided by a previous ‘hit’. To investigate this, the *Vav-Atg5* model has been crossed to mice with an internal tandem duplication of Flt-3 (Flt3-ITD) (683), a common mutation in AML and one where autophagy genes including *FOXO* and *GABARAPL1* are found downregulated (684). An initial hit may also be required to observe a phenotype following heterozygous Atg deletion, as heterozygous Vav-driven deletion of *Atg5* or *Atg7* did not result in a observed phenotype, yet MLL-ENL immortalized cells with allelic *Atg5* deletion presented with a proliferative advantage, something also seen in Ras-driven pancreatic cancer (K. Ryan, personal communication). Indeed, the phenotype of the heterozygote line, observable following two separate generations, appears stronger than the measurable autophagic reduction in the line, leading to the possibility that feedback into other regulatory pathways or alternate *Atg5* functions (278) play a role.

Downregulation of *ATG5* has been recently linked to increased proliferation and progression of melanomas (415).

The mechanism of autophagy modulation in tumors may be through signaling alterations (ex: mTOR pathway activation), genetic or epigenetic changes (506), or indeed a combination of these factors. In AML, the correlation of deleted chromosomal regions with autophagy gene locations must be taken further, to assess whether deletions correlate

with expression changes in a cohort of patients; a collaboration to partly address this question is ongoing.

A final point is the potential for autophagy heterogeneity within a tumor; autophagy was observed to be still functional, although significantly decreased, in most AML patients, and indeed the autophagy gene deletions observed in many tumor types, including AML, are heterozygous. Mutation or silencing (685) of the remaining allele is possible, as predicted in the Knudson two-hit model for tumor suppressor loss (686), however the lack of complete Atg loss-of-function observed thus far in tumors could also indicate differential requirements of expanding tumor/blast cell populations and the cancer initiating ‘stem cells’ they arise from. If autophagy is required for the more quiescent, stress-resistant ‘cancer stem cell’ population, autophagy levels within tumors could resemble those measured in the hematopoietic cascade, with downregulation of autophagy to support proliferation as opposed to clonal Atg ablation (which would uniformly remove autophagy, therefore in initiating cells too). This is purely conjecture at this point, however any application of autophagy modulating therapies would need to consider the differential requirements of tumor populations.

7.3 Conclusions

Analysis of the healthy and diseased hematopoietic systems has revealed the multi-faceted roles of autophagy across hematopoietic cell types and states.

Application of imaging flow cytometry techniques to measurement of hematopoietic autophagy was able to reveal substantially increased autophagy in early hematopoietic progenitors, particularly HSCs. Similarly, expression of autophagy pathway genes FOXO3 and Atg8 homologs were observed to be upregulated in HSCs. Supporting an HSC autophagy requirement, the deletion of *Atg5* in HSCs and cellular descendants under the

Vav promoter lead to defective HSC maintenance, characterized by increased HSC proliferation and impaired myeloid colony formation, leading to HSC exhaustion and bone marrow failure. The HSC phenotype, along with significantly impaired erythroid differentiation and lymphocyte survival, in the absence of *Atg5* supports previous observations in *Vav-Atg7* mice to confirm these autophagy-pathway roles in the hematopoietic system. Furthermore, these findings argue against differential importance of *Atg5* and *Atg7* for autophagic functions in hematopoietic cell types, including mitochondrial removal.

On the other hand, autophagy was not required for the persistence of CD11b+Gr1+ myeloid cells *in vivo*, which were expanded at 6 weeks in *Vav-Atg5*^{-/-} mice, infiltrative and displayed immature characteristics. *Atg5* deletion in MLL-ENL immortalized myeloid cell lines mediated increased *in vitro* proliferation under metabolic stress, supported by glycolysis, and increased phosphorylation of Akt and mTOR under serum withdrawal. Conversely, in human AML cell lines, overexpressing *Atg5* limited MS-5 stromal cell-provided support of proliferation, while cells missing *Atg5* significantly benefited from stromal presence. As the dominant blast population in primary AML BMNCs showed decreased autophagy levels and decreased autophagy gene expression relative to other bone marrow cells, while low autophagy marker p62 was increased in leukemic bone marrow sections by immunohistochemistry, these results suggest lower autophagy as a beneficial phenotype in AML. New therapeutic strategies are urgently required in AML (513), and with autophagy modulating therapies already in trial (518) (449) (477), understanding the biological roles autophagy plays in hematopoietic cells will inform therapy application and development.

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