Epigenetic regulation of autophagy genes in stem cells in steady-state and stress conditions

A Thesis

Submitted to

Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

Renu Raveendran

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, India

April, 2020

Supervisor: Dr. Deepa Subramanyam

Name: Renu Raveendran

All rights reserved

CERTIFICATE

This is to certify that this dissertation entitled '**The epigenetic regulation of autophagy genes in stem cells in steady-state and stress conditions**' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Renu Raveendran at National Centre for Cell Science (NCCS) Pune under the supervision of Dr.Deepa Subramanyam, Scientist 'E', during the academic year 2019-2020.

Dr Deepa Subramanyam

Committee:

Dr Deepa Subramanyam

Dr Girish Ratnaparkhi

Declaration

I hereby declare that the matter embodied in the report entitled '**The epigenetic regulation of autophagy genes in stem cells in steady-state and stress conditions**' are the results of the work carried out by me at the National Centre for Cell Science(NCCS) Pune under the supervision of Dr.Deepa Subramanyam and the same has not been submitted elsewhere for any other degree.

Renu Raveendran

02.04.2020

Abstract

Autophagy is a process by which the components of a cell are recycled via a lysosomal degradation pathway to ensure the maintenance of cellular homeostasis and viability. The autophagic pathway is largely referred to as a cytosolic pathway and is found to be actively upregulated during cellular differentiation. But not much is known about the nuclear components of this pathway. Recent reports from studies suggest that epigenetic modifications, by regulating signalling pathways act as a major player in cellular fate decisions during development and differentiation. However, the contribution of epigenetic mechanisms in autophagy regulation is relatively unclear. Our study was aimed at understanding how different epigenetic modifications regulate the expression of autophagy genes and in turn the process of autophagy itself during different cell states. It was found that the epigenetic inhibition of chromatin domains led to changes in the expression of autophagy genes and the process of autophagy itself.

Table of contents

Abstract……………………………………………………………………………............03

Acknowledgements…………………………………………………………………..08

Chapter:Introduction……...……………………………………………………………09

1.1 Autophagy…………………………………………………………….…………09

1.2 Types of autophagy…………………………………………………………...09

1.3 Major steps in an autophagic pathway………………………………….……………..10

1.4 Epigenetic regulation of autophagy……………………………….………....11

1.5 Autophagy in development……………………………………………….……..15

1.6 Mouse ES cells as a model organism to study autophagy……………….…………16

Chapter 2: Materials and methods………………………………………..………...20

2.1 Materials……………………………………...…………………...……. 20

2.2 List of genes analysed by qRT-PCR using primer array *MATPL-I* obtained from RealTime Primers…………………………………………..…………………………20

2.3 Epigenetic inhibitors from SGC used for screening……………………...………. 24

2.4 CYTO ID test………………………………………………….……………..... 25

2.5 Fluorescent construct…………………………………………………........26

2.6 V6.5 mESC line maintenance…………………………………………...….26

2.7 FUW-mCherry-GFP-LC3 infected V6.5 mESC line maintenance ………..….........26

2.8 Viral Infection for stable autophagy reporter cell line generation…………………...26

2.9 Epigenetic Inhibitor Screen……………………………..………………..……....28

2.10 Confocal Imaging………...…………………………………………………......29

2.11 corticosterone standardisation…….…………………………………………..29

2.12 qRT-PCR……………………………………………………………………….29

Chapter 3: Results…………………………………………………………………...31

3.1 Stable reporter cell line generated using fluorescent construct FUW-mCherry-GFP-LC3 visualized autophagy………………………...………..…....31

3.2 Epigenetic inhibitor screen on mESCs identified epigenetic modulators that can regulate autophagy…………………………………………………………….………..32

3.3 Confocal Imaging of reporter cell line generated after epigenetic inhibitor treatment validated data obtained from FACS………………………………………………..……….34
3.4 Epigenetic modification of G9a/GLP, EZH2 and BET domain regulates autophagy in mESCs……………………………………………………………………...……………..39
3.6 EED as a novel regulator of autophagy in mESCs…………………….…………..40
3.7 Autophagy is upregulated in cells under stress induced by corticosterone………………………………………………………………………………43
Chapter 4: Discussion……………………………………………………………..…..48

Chapter 5: References……..…………………………………………………………...53

List of Figures

1.1 Representation of different types of autophagy in a mammalian cell…………….10
1.2 Representation of the major steps in the process of autophagy………………….11
1.3 Histone modifications leading to the regulation of autophagic flux……………….15
1.4 Epigenetic modification as a result of histone tail modification…………………....17
1.5 Derivation of mESCs from mouse embryo……………………………………..18

2.1 FUW-mCherry-GFP-LC3 construct from Addgene……………………………….27
2.2 GFP fluorescence is quenched as a result of the fusion of the autophagosome with the autolysosome……………………………………………………………………...28

3.1 Confocal images of FUW-mCherry-GFP-LC3 infected V6.5 mES cells…………..32
3.2 Autophagy was upregulated on inhibition of G9a/Glp, UTX, BET, EZH2, EED and LSD domain in the chromatin…………………………………………………………...33
3.3 Increased autophagic flux on inhibition of G9a/GLP……………………………….35
3.4 Increased autophagic flux on inhibition of BET domain…………………………....37
3.5 Increased autophagic flux on treatment with the EZH2 inhibitor…………………..38

3.6 Candidate epigenetic modulators affect the expression of crucial autophagy genes………………………………………………………………………………………...40
3.7 Inhibition of EED upregulates autophagy………………………………………..….42
3.8 EED regulates expression of crucial autophagy genes…………………………...…43
3.9 Upregulation of autophagy in mESCs under conditions of stress induced by corticosterone treatment……………………………………………………………...…46

4.1 Different histone modifications and their effect on autophagy…………………..…48
4.2 Regulation of the process of autophagy by regulation of expression of autophagy associated genes by protein domains in the chromatin………………………………...50

List of Tables

1.1 List of known epigenetic modulators……………………………………………..…14
2.1 List of epigenetic inhibitor drugs from SGC…………………………....………..…25

List of Abbreviations

mESCs: mouse Embryonic Stem Cells

AMPK: 5' AMP-activated protein kinase/5' adenosine monophosphate-activated protein kinase

ATG1/ULK1: Autophagy related 1/Unc-51 like autophagy activating kinase

PI3K: phosphoinositide 3-kinase

ATG5: Autophagy related 5

ATG12: Autophagy related 12

ATG16L1: Autophagy related 16 like 1

LC3: microtubule-associated light chain 3

LAMP: Lysosome-associated membrane protein

RAB7: Ras-related protein

corticosterone: corticosterone

EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit

ChIP: chromatin immunoprecipitation

Acknowledgement

I express my heartfelt gratitude to Dr Deepa Subramanyam for believing in me and supporting me throughout this one year with everything related to the project and also in trying to understand and help me with my chaotic self. I would also like to thank Dr Deepika Puri for guiding me during this project and also for being a good friend to whom I could talk whenever I was stuck. Next, I would like to thank all the members of Dr Deepa Subramanyam's lab for helping me with whatever little help I needed.

This acknowledgement would be incomplete without the mention of my family and friends. I would like to thank my mother, for instilling in me the love for science, my father, for providing me with everything I needed and my sister for being there always. Feba, Manjari and Shivani, thank you for being the family here at IISER. I do not think I would have survived IISER without you guys.

I would also like to acknowledge Dr Girish Ratnaparkhi for his valuable suggestions in this project. Finally, I would like to acknowledge the microscopy facility at IISER, the FACS facility at NCCS, the fifth year committee, NCCS Pune and IISER Pune for all the help provided related to the project.

Chapter One: Introduction

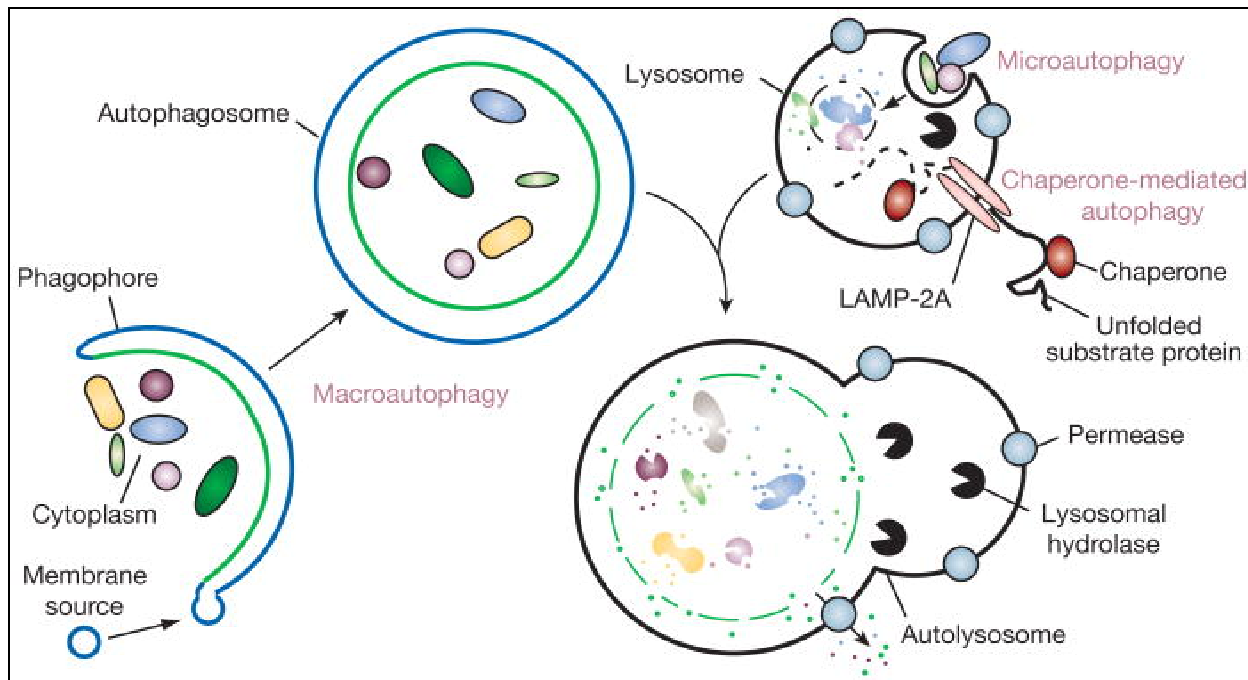
1.1 Autophagy

Autophagy means to eat oneself (‘auto’=self, ‘phagy’=eat) and is a process that the cell utilises to degrade and recycle the waste generated in a cell. These waste materials could either be a dying organelle or a protein aggregate or other cellular components. Christian de Duve first discovered the process in rat liver and coined the term autophagy. He found that cellular organelles like mitochondria in the liver of a rat underwent degradation on the passage of glucagon to the liver tissues. However, the molecular mechanism behind this remained a mystery until autophagy was studied in yeast. Most of the details that we know about the molecular mechanism and the significance of autophagy were identified based on studies done on yeast. It was reported that the genes associated with autophagy in yeast were also conserved in mammals suggesting conservation of autophagy genes through the phylogeny (Glick et al., 2010).

1.2 Types of autophagy

Autophagy functions to maintain homeostasis in the cell, and based on the key players involved in the process of degradation, autophagy is classified into three kinds (Figure 1.1) (Kohli et al.,2010). In all the three different kinds of autophagy, the components of the cell that are to be degraded, (the cargo), is finally degraded by proteolytic cleavage by the action of enzymes present in the lysosome. Macroautophagy, one of the three defined types of autophagy is a process by which the cargo is engulfed by a membranous structure that gets pinched off from the endoplasmic reticulum called the autophagosome. The autophagosome transports the cargo to the lysosome. Proteolytic cleavage of the cargo gets initiated once the autophagosome gets fused with lysosome forming an autophagolysosome/autolysosome inside which the degradation occurs. In microautophagy, the cargo is engulfed into the lysosome and gets degraded inside. In chaperone-mediated autophagy (CMA), the cargo gets transported to the lysosome in a

protein complex along with Hsc-70, a well-known chaperone protein (Mizushima et al.,2008).



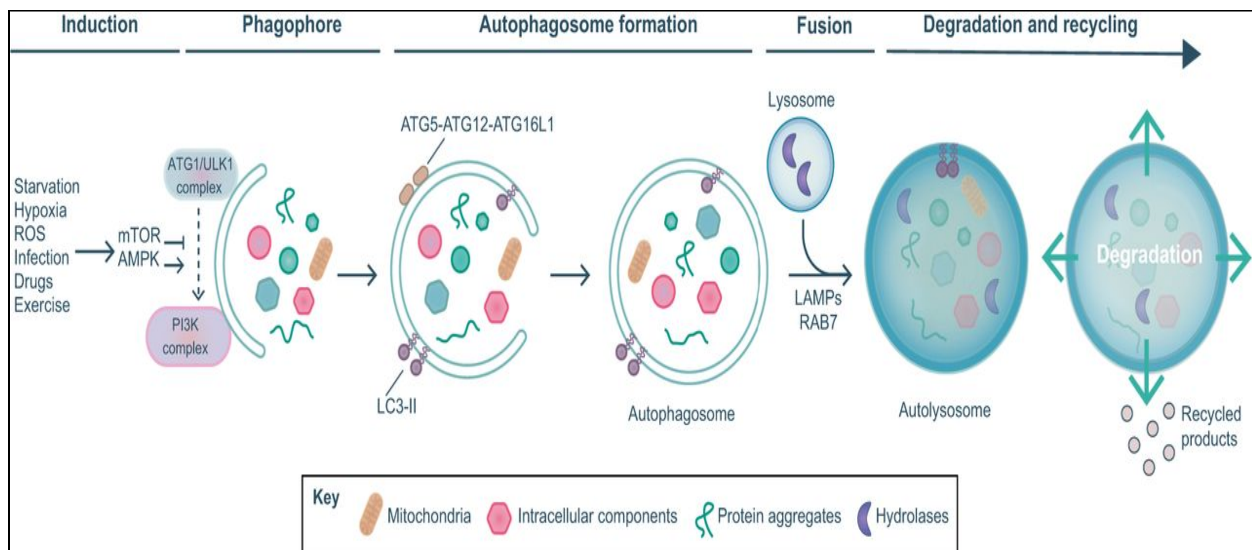
Figure(1.1): Diagrammatic representation of the three different types of autophagy seen in a mammalian cell

Macroautophagy, mediated by autophagosomes; microautophagy, in which cells are degraded by engulfment followed by proteolytic cleavage by lysosomes; and chaperone-mediated autophagy, where cargo is transported into the lysosome by the help of chaperone proteins. (Mizushima et al.,2008).

1.3 Major steps in an autophagic pathway

The process of macroautophagy henceforth referred to as autophagy, has five significant steps viz, induction, phagophore formation, autophagosome formation, fusion and degradation (Kohli et al.,2010) (Figure1.2). This induction of autophagy is the cell's way of maintaining homeostasis under conditions of stress. The cells induce autophagy under conditions such as starvation, exercise, hypoxia, infection, drug treatment or even during exposure to reactive oxygen species (ROS). The induction of autophagy is made possible by either the inhibition of the mTOR pathway or activation of the AMPK

pathway. This inhibition/activation is dependent on the formation of the ATG1/ULK1 complex and the PI3K complex. Further, a part of the endoplasmic reticulum is pinched off to form an immature structure called the phagophore. The phagophore later matures to form the autophagosome, the vesicle into which the cargo to be degraded is recruited. The maturation of the phagophore to form the autophagosome is mediated by the conjugation of various autophagy proteins such as ATG5, ATG12, ATG16L1 and LC3. LC3II, which is formed as a result of the conjugation of LC3 and lipid phosphatidylethanolamine (PE) is anchored on to the membrane of the autophagosome. The fusion of the autophagosome with the lysosome occurs, leading to the formation of the autophagolysosome/autolysosome. The lysosome is a cellular sac consisting of hydrolases that are capable of degrading the cargo inside the autophagolysosome/autolysosome (Boya et al.,2018).



Figure(1.2): Diagrammatic representation of the significant steps in the process of autophagy
Autophagy is induced under conditions of stress. A phagophore is formed and gets matured to form the autophagosome. The autophagosome is fused with the lysosome. The cargo gets degraded inside the autolysosome, and the broken-down cargo is released into the cell cytoplasm to be recycled by the cell. (Boya et al.,2018)

LAMPs and RAB7 are two essential proteins that play a crucial role in the fusion of autophagosome to the lysosome. The cargo is degraded by the hydrolases that are activated on the fusion between the autophagosome and the lysosome. The degradation of the cargo results in the release of amino acids, lipids and nucleotides, which further signals the mTOR/AMPK pathway to terminate autophagy (Boya et al.,2018).

1.4 Epigenetic regulation of autophagy

Recent studies have brought to light many of the cytosolic components of autophagy, but the focus on the nuclear components of autophagy remains less understood. Studies have also shown that the autophagic flux can be regulated at the level of chromatin by epigenetic modifications (Figure 1.3). The chromatin is composed of DNA, RNA and histone proteins. The process of transcription, which is what decides the expression levels of a gene, is regulated by modulating the accessibility of the DNA to the transcription machinery. This regulation of accessibility of the chromatin is a result of chromatin modifiers which in turn regulate gene expression (Li et al.,2002; de et al., 2013 and Reik et al., 2007). Histone Acetyltransferases (HATs), Deacetylases (HDACs), Methyltransferases (HMTases) and Kinases are all enzymes known for the addition or deletion of molecular flags from the DNA-histone complex, making a gene transcriptionally active or repressed depending on the epigenetic mark. The discovery of small-molecule inhibitors specific to particular chromatin modifiers has facilitated the inhibitor-based screens that help in elucidating their role in response to various signals. Among the well known epigenetic modulators are A-366, UNC 0642 that are known to inhibit the G9a/GLP complex. Table 1.1 enlists a few of the known epigenetic modulators and their target domains.

Name of Epigenetic Inhibitor	Target Domain
GSK 2801	BAZ 2A/2B

BAZ2-ICR	
JQ1 -JQ1	BET
CBP 112 CBP 30 A-485 A-486	CREBBP/EP300
SGC 0946	DOT1L
A-395 A-395N	EED
UNC 1999 UNC 2400 GSK 343	EZH2
GSK 366 UNC 0642	G9A/GLP
GSK LSD1	LSD1
MS023	PRMT1
SGC 707 SGC XY	PRMT3
TP 064 TP 064N	PRMT4
R(PFI)2 S(PFI)2	SETD7

BAY 598 BAY 369 PFI 5	SMYD2
A-196	SUV420H1/H2
OICR 9429 OICR 547	WDR5
GSK J4 GSK J5	UTX

Table (1.1): List of known epigenetic modulators

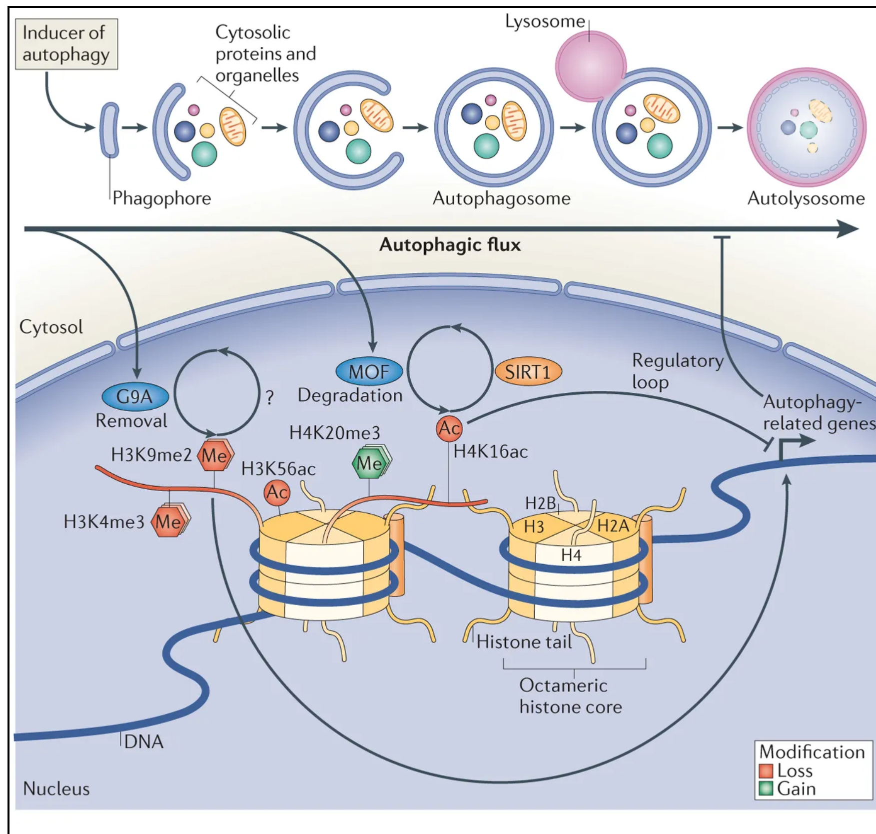
The table contains a list of known small-molecule epigenetic inhibitors and the domains that each of them would inhibit

The processes resulting in a change in the activity of a gene without actually altering the gene itself are known as an epigenetic modification (Weinhold et al.,2006). Over the years, there have been multiple reports suggesting that the process of autophagy can be epigenetically modified (Vellai et al.,2009). The polyamine, spermidine can increase the levels of autophagy by deacetylation of the HATs (Eisenberg et al.,2009). Similarly, on starvation, an upregulation in autophagy was seen in drosophila when G9a was modified (Ding et al.,2013). As in the case of mouse fibroblast cells, upregulation in autophagy as a result of starvation showed a decrease in the acetylation of H4K16 (de et al.,2013). The following figure (Figure 1.4) depicts different histone modifiers leading to epigenetic regulation.

1.5 Autophagy in development

There are multiple reports suggesting the role of autophagy in development. The degeneration of intersegmental muscles is a typical scenario observed during insect metamorphosis, and it was found that autophagic vesicles played a crucial role in the

degeneration of these muscles in *Lepidoptera* (Beaulaton et al.,1977). The involvement of the lysosomal machinery and the autophagosomes were seen in a large number of insects during development (Baehrecke et al., 2003; Berry and Baehrecke, 2007). Embryonic development is a process during which the cell undergoes a lot of structural changes for which the cells undergo cycles between a quiescent state and a state of



Figure(1.3): Histone modifications leading to the regulation of autophagic flux

The figure depicts how different histone modifications influence different steps of the autophagic process.H3K4me regulates the process of autophagy by influencing the autophagy-related genes. H4K16ac inhibits the autophagy-related genes and hence regulate the autophagic flux (Füllgrabe et al.,2014)

high metabolism to be functional during embryogenesis. This requires the cellular machinery to have a mechanism by which a significant turnover of proteins can be

obtained, which cannot be met by just proteasomal degradation. This requirement can be met by the autophagic degradative pathway that will help the cell change structurally within a short time (Cecconi et al.,2008). The manipulation of Ulk1 gene, a gene that is crucial in the process of autophagy resulted in defects in neuronal development in mice (Tomoda et al., 1999). In mice, embryos in which the Beclin1 gene was knocked out, the embryos displayed a defect in size (Yue et al., 2003). These findings indicate that autophagy is a process that is required during the time of embryogenesis, and it is vital to understand the molecular mechanisms through which autophagy regulates the process of development and differentiation. To understand the changes happening in a tissue undergoing development and differentiation, a model system such as mESCs can be used (Guasch et al., 2005).

1.6 Mouse embryonic stem cells (mESCs) as a model s to study the epigenetic regulation of autophagy

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of the pre-implantation embryo, the blastocyst (Figure 1.5). These cells have the potential to differentiate into the three germ layers, namely, ectoderm, endoderm and mesoderm (Czechanski et al.,2014). Hence mESCs have the distinguishing property of pluripotency and self-renewal, that is the cells can divide and also give rise to new stem cells (Czechanski et al.,2014, Guasch et al., 2005). It is from these stem cells, various tissues of an organism form, and according to reports, in order for the cells to develop into cells/tissues that are functional and morphologically precise, the process of autophagy is required as it can regulate the turnover of different proteins in a cell (Lum et al.,2005).

While earlier reports have studied autophagy in a handful of cell types, there is a gap in our understanding of autophagy regulation in stem cells. Reports suggest that well-regulated autophagy machinery is required for maintaining pluripotency in stem cells (Phadwal et al., 2012; Vessoni et al., 2018 and Chen et al.,2018) but the regulation

remains to be understood. mESCs present an excellent model system to understand autophagy regulation in stem cells and during differentiation as these can give an insight into autophagy regulation during development.

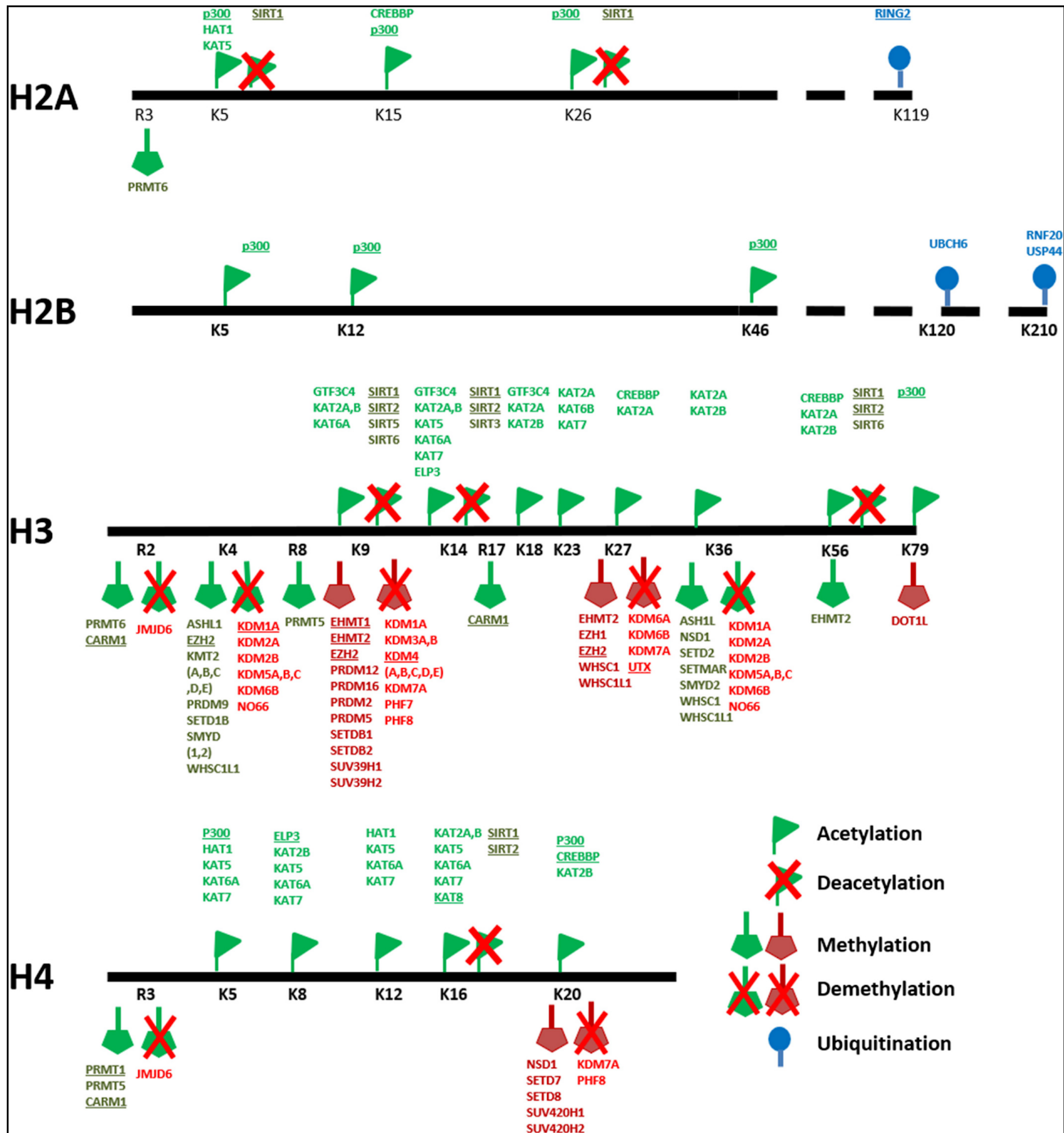
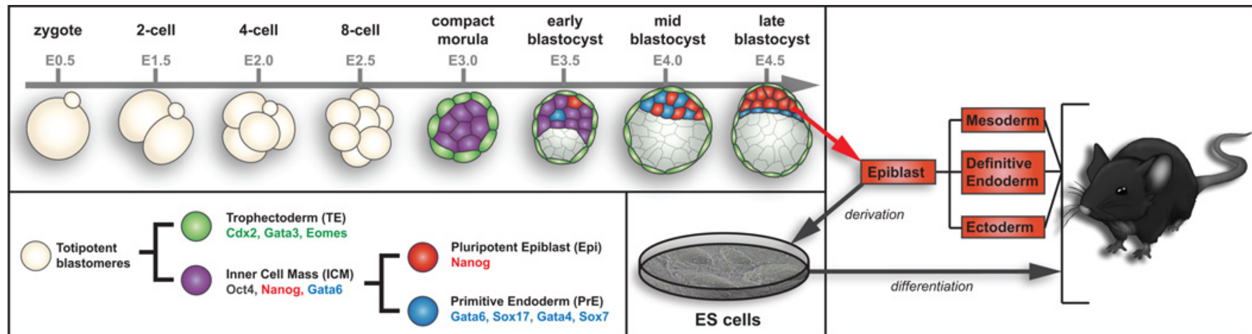


Figure (1.4): Epigenetic modification as a result of histone tail modification

There are two kinds of epigenetic modification resulting in either the repressive or activated transcriptional

state of the chromatin. In this figure, green flags/green pentagons represent the activating marks and red flags/red pentagon indicate repressive marks. A crossed flag represents deacetylation while a crossed pentagon represents demethylation((Puri and Subramanyam, 2019).



Figure(1.5): Derivation of mESCs from a mouse embryo

The inner cell mass from a blastocyst is extracted and grown in culture and is called mESCs. These cells on differentiation can develop into either the mesoderm or the endoderm or the ectoderm (Czechanski et al.,2014).

Objectives

This project aims at understanding the epigenetic regulation of autophagy genes at the level of chromatin using mouse embryonic stem (mES) cells as a model system. The major aims of this project are as follows:

1) Generating a stable autophagy reporter cell line using a fluorescent construct.The fluorescent construct FUW-mCherry-GFP-LC3 (Addgene_110060) labels the autophagosome (GFP,mCherry) and autolysosome (mCherry). The construct is designed in such a way that there is a quenching of GFP fluorescence due to the acidic pH as the autophagosome fuses with the lysosome, forming the autolysosome. This allows us to visualise the change in levels of autophagy occurring in a cell when treated with the different epigenetic inhibitors on the basis of fluorescence.

2) Conducting epigenetic inhibitor screens to identify chromatin modulators of autophagy genes in different cell states

Reports have shown that there is a regulation of autophagic flux as a result of epigenetic modifications, but the role of these modifiers in autophagy in mESCs is

unclear. Using different epigenetic inhibitors from Structural Genomics Consortium (SGC), the change in levels of autophagy upon treatment with each epigenetic inhibitor will be studied to narrow down the epigenetic modulators resulting in a significant regulation of autophagy in mESCs.

3) Studying the mechanism of regulation of autophagy genes in the context of the chromatin by

(i) Determining the effect of epigenetic inhibitors on the expression levels of autophagy genes using qPCR

Potential epigenetic modulators of autophagy identified from the epigenetic screen may act by upregulating or downregulating autophagy genes. To study this, the mRNA levels of different autophagy genes will be quantified using qRT-PCR after treatment of cells with the selected epigenetic modulators. An autophagy qRTPCR primer array will be used, which consists of primers for 88 autophagy genes. The list of autophagy genes analysed in mentioned in materials and methods

(ii) Identifying the mechanism of autophagy regulation by candidate autophagy modulators by ChIP

Once the regulation of different autophagy genes due to the identified epigenetic modulators is quantified, the mechanism by which these epigenetic modulators regulate the expression of autophagy genes will be understood using ChIP.

Chapter 2: Materials and Methods

2.1 Materials

0.2% gelatin, Corning Costar Cell Culture Plates 24 well (Sigma), Knockout DMEM (Invitrogen), FBS(Invitrogen), MEM Non-Essential Amino Acids Solution 100X(Invitrogen), Penstrip (Invitrogen), 0.25% Trypsin-EDTA (1X), L-Glutamine, 200mM, UltraPure™ DNase/RNase-Free Distilled Water, Knockout DMEM, L-Glutamine, Dulbecco's Modified Eagle's Medium-high glucose, 2-Mercaptoethanol, FBS certified USA origin, Paraformaldehyde, Dimethyl sulfoxide, Tween20, Corning Costar Cell culture 6w flat-bottomed, Corning Costar Cell culture 12w flat bottomed, Vectashield slide mounting medium, Power SYBR Green PCR master mix, Optical adhesive cover, Stemolecule CHIR99021, Stemolecule™ PD0325901 2mg, RIPA buffer, 4% PFA, 37% formaldehyde, 33 epigenetic inhibitors from structural genomics consortium, Tris-HCl (pH 8.0),1M Tris-HCl (pH 9), 0.5M EDTA (pH 8.0), Sodium Dodecyl Sulphate(SDS), 5M NaCl, 1M NaHCO3 (pH 8), 1M Glycine, PMSF, Protease Inhibitor, Proteinase K, BSA, Triton X-100, LiCl, NP-40, deoxycholic acid, glacial acetic acid, Glycogen, Isopropanol, Sodium acetate, dynabeads(Protein A), CYTO ID test (ENZO).

2.2 List of genes analysed by qRT-PCR using primer array *MATPL-I* obtained from RealTime Primers

AKT1S1	AKT1 substrate 1 (proline-rich)
AMBRA1	Autophagy/beclin-1 regulator 1
APOL1	Apolipoprotein L, 1
ATF4	Activating transcription factor 4
ATG10	ATG10 autophagy related 10 homolog (S. cerevisiae)
ATG12	ATG12 autophagy related 12 homolog (S. cerevisiae)
ATG16L1	ATG16 autophagy-related 16-like 1 (S. cerevisiae)

ATG16L2	ATG16 autophagy-related 16-like 2 (S. cerevisiae)
ATG2A	ATG2 autophagy related 2 homolog A (S. cerevisiae)
ATG2B	ATG2 autophagy related 2 homolog B (S. cerevisiae)
ATG3	ATG3 autophagy related 3 homolog (S. cerevisiae)
ATG4A	ATG4 autophagy related 4 homolog A (S. cerevisiae)
ATG4B	ATG4 autophagy related 4 homolog B (S. cerevisiae)
ATG4C	ATG4 autophagy related 4 homolog C (S. cerevisiae)
ATG4D	ATG4 autophagy related 4 homolog D (S. cerevisiae)
ATG5	ATG5 autophagy related 5 homolog (S. cerevisiae)
ATG7	ATG7 autophagy related 7 homolog (S. cerevisiae)
ATG9A	ATG9 autophagy related 9 homolog A (S. cerevisiae)
BARKOR	BARKOR (KIAA0831)
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BECLIN1	Beclin1
BECN1L1	Becn1L1
BIRC5	Effector cell peptidase receptor 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
DDIT3	DNA-damage-inducible transcript 3
DRAM	Damage-regulated autophagy modulator
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
EIF4EBP2	Eukaryotic translation initiation factor 4E binding protein 2

EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1
EPS15L1	Epidermal growth factor receptor pathway substrate 15-like 1
FKBP15	FK506 binding protein 15, 133kDa
FRAP1	FK506 binding protein 12-rapamycin associated protein 1
FRS2	Fibroblast growth factor receptor substrate 2
FRS3	Fibroblast growth factor receptor substrate 3
GABARAP	GABA(A) receptor-associated protein
GABARAPL1	GABA(A) receptor-associated protein-like 1
GABARAPL2	GABA(A) receptor-associated protein-like 2
GBL	G protein beta subunit-like
GFI1B	Growth factor independent 1B transcription repressor
GNAI3	Guanine nucleotide-binding protein (G protein), alpha inhibiting activity polypeptide 3
GPSM1	G-protein signalling modulator 1 (AGS3-like, C. Elegans)
GPSM2	G-protein signalling modulator 2 (AGS3-like, C. Elegans)
GPSM3	G-protein signalling modulator 3 (AGS3-like, C. Elegans)
HIF1A	Hypoxia-inducible factor 1, alpha
HSPA5	Heat shock 70kDa protein 5
LAMP1	Lysosomal-associated membrane protein 1
LAMP2	Lysosomal-associated membrane protein 2
LAMP3	Lysosomal-associated membrane protein 3
LETM1	Leucine zipper-EF-hand containing transmembrane protein 1

LETM2	Leucine zipper-EF-hand containing transmembrane protein 2
MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha
MAP1LC3B	Microtubule-associated protein 1 light chain 3 beta
MAP1LC3B2	Microtubule-associated protein 1 light chain 3 beta 2
MAP1LC3C	Microtubule-associated protein 1 light chain 3 gamma
MCL1	Myeloid cell leukaemia sequence 1 (BCL2-related)
PIK3C3	Phosphoinositide-3-kinase, class 3
PIK3R4	Phosphoinositide-3-kinase, regulatory subunit 4
PPM1K	Protein phosphatase 1K (PP2C domain containing)
RAPTOR	Raptor
RASD1	RAS, dexamethasone-induced 1
RB1CC1	RB1-inducible coiled-coil 1
RGS19	Regulator of G-protein signalling 19
RICTOR	Rapamycin-insensitive companion of mTOR
SEC16A	SEC16 homolog A (S. cerevisiae)
SEC16B	SEC16 homolog B (S. cerevisiae)
SEC23A	Sec23 homolog A (S. cerevisiae)
SEC23B	Sec23 homolog B (S. cerevisiae)
SEC24A	SEC24 related gene family, member A (S. cerevisiae)
SEC24B	SEC24 related gene family, member B (S. cerevisiae)
SEC24C	SEC24 related gene family, member C (S. cerevisiae)
SEC24D	SEC24 related gene family, member D (S. cerevisiae)
SH3GLB1	SH3-domain GRB2-like endophilin B1

SH3GLB2	SH3-domain GRB2-like endophilin B2
SNX30	Sorting nexin family member 30
SQSTM1	Sequestosome 1
TP53	Tumour protein p53
TP73	Tumour protein p73
TPR	Translocated promoter region (to activated MET oncogene)
ULK1	Unc-51-like kinase 1 (C. Elegans)
ULK2	Unc-51-like kinase 2 (C. Elegans)
ULK3	Unc-51-like kinase 3 (C. Elegans)
ULK4	Unc-51-like kinase 4 (C. Elegans)
UVRAG	UV radiation resistance-associated gene
WDR45L	WDR45-like
WIPI1	WD repeat domain, phosphoinositide interacting 1
WIPI2	WD repeat domain, phosphoinositide interacting 2

2.3 Epigenetic inhibitors from SGC used for screening

For the epigenetic screening, 33 epigenetic inhibitors (including negative controls) from Structural Genomics Consortium (SGC) were used. These epigenetic inhibitors targeted different domains in the chromatin. The period of treatment and the concentration of the epigenetic inhibitors were decided after a toxicity screen that was done by Dr Deepika Puri.

The epigenetic inhibitors were grouped into three different groups on the basis of their treatment periods. The different groups into which these were grouped and the respective concentrations are as follows.

4 Day Treatment		1 Day Treatment		Overnight Treatment	
Name of epigenetic inhibitor	concentration (μM)	Name of epigenetic inhibitor	Concentration (μM)	Name of epigenetic inhibitor	Concentration (μM)
UNC 1999	3	SGC-707	1	SGC-CBP30	1
UNC 2400	3	XY	1	I-CBP-112	3
A 395 N	1	R-PFI-2	1	A-486	0.8
UNC 0642	1	S-PFI-2	1	GSK-2801	3
GSK 343	3	BAY-598	1	BAZ2-ICR	1
TP 064	1	PFI-5	3	A-485	0.8
TP 064N	1	OICR-9429	3	JQ1	0.2
A 395	1	OICR-0547	3	(-)JQ1	0.2
A 366	1	GSK J4	5	MS023	0.1
SGC 0946	1	GSK J5	5	SGC 0649	0.8
A 196	1	GSK LSD1	1	SGC 2043	0.8
		BAY-369	1		

Table (2.1): List of epigenetic inhibitor drugs from SGC

The different epigenetic drugs from SGC were categorised into three groups - 4 days, 1 day and overnight and concentration were decided by toxicity screen.

2.4 CYTO ID test from ENZO

The trypsinized cells were resuspended in 0.5μl cyto ID test dye diluted in 500μl 1x PBS + 5% FBS and incubated at 37℃ for 30 minutes. After incubation, washed with PBS+5% FBS and resuspended in the same and filtered and subjected to flow cytometry. Fluorescence intensity was acquired using the FITC channel as suggested

by manufacturers. The mean fluorescence intensity was quantified after plotting a graph between FITC and cell count.

2.5 Fluorescent construct

FUW-mCherry-GFP-LC3 plasmid is a transgenic plasmid in which the LC3B gene is tagged to the fluorescent proteins EGFP and mCherry. The autophagosomes and autolysosomes are labelled using this fluorescent construct. Construct id :Addgene_110060

2.6 V6.5 mESC Line Maintenance

V6.5 mESCs were cultured in tissue culture plastic dishes that were coated with 0.2% gelatin. The cells were maintained in standard cell culture conditions that are mESC medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM) with 15% FBS, 0.1 mM nonessential amino acids, two mM glutamine, 1000U LIF, 100 µg/ml pen strep, and 0.1 mM 2-mercaptoethanol and incubated at 37°C, 5% CO_2. Cells were passaged every third day to maintain the undifferentiated state. To passage cells, a single-cell suspension was generated by treatment with 0.25% trypsin. Trypsin activity was then quenched with an equal volume of mESC media. The cells were centrifuged at 1000 rpm for 5 minutes and resuspended in mESC media. Viable cells were plated.

2.7 FUW-mCherry-GFP-LC3 infected V6.5 mESC line maintenance

The stable cell line that was generated using transfection was maintained under standard cell culture conditions.

2.8 Viral Infection for stable autophagy reporter cell line generation

The construct FUW-mCherry-GFP-LC3 was packaged into lentiviral particles using pLKO, psPAX2 and pMD2.G. Plasmids at the appropriate concentration were incubated in DMEM for 20 minutes. After 20 minutes, FuGENE HD was added and incubated further for 30 minutes. The transfection mix was added onto a 10 cm plate containing

HEK293T cells at 60% confluency in 10ml complete media. 8ml of fresh media was added the next day, and after 48 hours, media was filtered to obtain the viral supernatant.

V6.5 mESCs were infected with the viral supernatant to generate a stable cell line. The lentiviral construct used is called FUW-mCherry-GFP-LC3 and is a mCherry, and GFP tagged to either side of the LC3 gene. After 72 hours of infection, expression levels of GFP and mCherry fluorescence were observed under an epifluorescence microscope, and double-positive cells were sorted using flow cytometry.

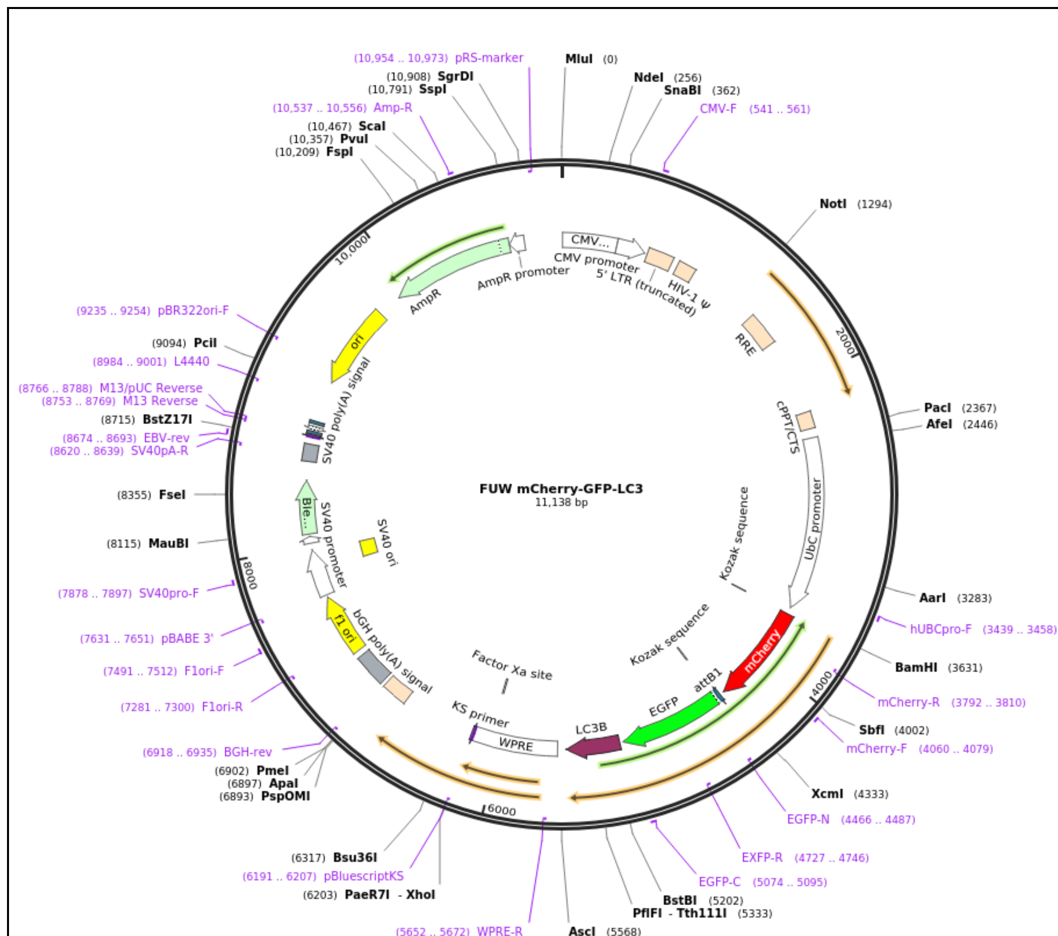


Figure (2.2): FUW-mCherry-GFP-LC3 construct from Addgene

The plasmid has a GFP and mCherry gene tagged to the LC3 gene allowing the visualization of the autophagic flux.

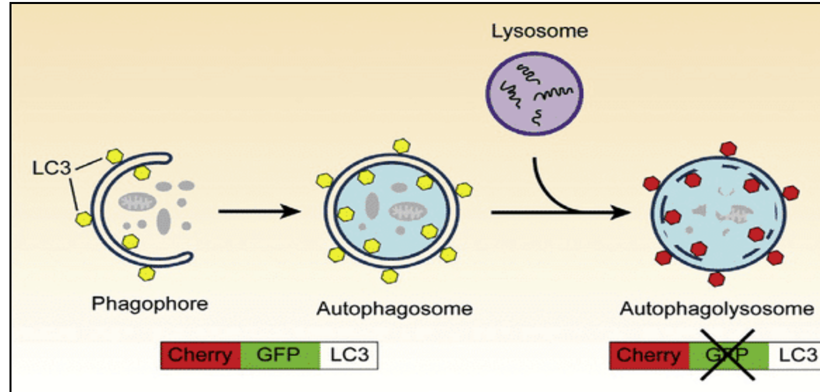


Figure (2.3): GFP fluorescence is quenched as a result of the fusion of the autophagosome with the autolysosome (Castillo et al., 2017)

The EGFP and mCherry fluorescence are tagged to the LC3 gene now labelling the autophagosome yellowish-green.On the fusion of the autophagosome with the lysosome to form the autolysosome, the GFP fluorescence is quenched as a result of of the low pH from hydrolases in the lysosome.

2.9 Epigenetic Inhibitor Screen

2.9.1 V6.5 mES cells

Cells were plated on tissue culture plastic dishes that were coated with 0.2% gelatin and maintained in ES+LIF media. The cell number per well was kept 3000 cells per well for 4-day treatment and 5000 cells per well for the rest of the treatment. After 24h of plating, the cells were treated with the epigenetic inhibitors from SGC. The media was changed with the appropriate drug every other day, and the cells were harvested at the end of the specified treatment period. The harvested cells were then subjected to an autophagy assay using a CYTO ID test from ENZO and mean GFP fluorescence was quantified using flow cytometry.

2.9.2 FUW-mCherry-GFP-LC3-expressing mESCs

Cells were plated onto tissue culture plastic dishes that were coated with 0.2% gelatin and maintained in ES+LIF media. The cell number per well was kept 3000 cells per well for 4-day treatment and 5000 cells per well for the rest of the treatment. After 24h of plating, the cells were treated with the epigenetic inhibitors from SGC. The media was changed with the appropriate drug every other day, and the cells were harvested in PBS

+ 5% FBS at the end of the specified treatment period. The cells were then analysed using flow cytometry to quantify mean GFP and mCherry fluorescence.

2.10 Confocal Imaging

The stable cell line that was generated using the fluorescent construct from Addgene was plated on to sterile coverslips in a cell culture dish. The coverslips were fixed using 4% PFA after the epigenetic drug screening period was over. The cells were then stained using DAPI, mounted onto slides using Vectashield and imaged.

All the images were taken on Nikon A1 confocal laser microscope, and the lasers that were used were EGFP, mCherry and DAPI and the respective wavelengths were 488nm, 568nm and 408nm. All images were imaged at 100X magnification and with oil immersion.

2.11 corticosterone standardisation

FUW-mCherry-GFP-LC3 cells were plated onto coverslips in tissue culture plastic dishes that were coated with 0.2% gelatin and maintained in ES+LIF media. The cell number per well was kept 3000 cells per well. Individual wells were then treated with different concentrations of corticosterone ranging from 0.1 μM to 10 μM and observed for a period of 4 to 10 days. The media was changed every other day with the respective concentration of corticosterone and was maintained under standard cell culture conditions. The coverslips were harvested and fixed using 4% PFA on the second, fourth, seventh and tenth day. The fixed coverslips were mounted on a glass slide using vectashield and imaged using a Nikon confocal microscope.

2.12 qRT-PCR

The cells after the epigenetic inhibitor assay were treated with 500μl TRIzol (Invitrogen), and the total RNA from the cell was isolated. The amount of RNA was quantified using NanoDrop and cDNA was synthesised using Verso cDNA synthesis kit. The expression

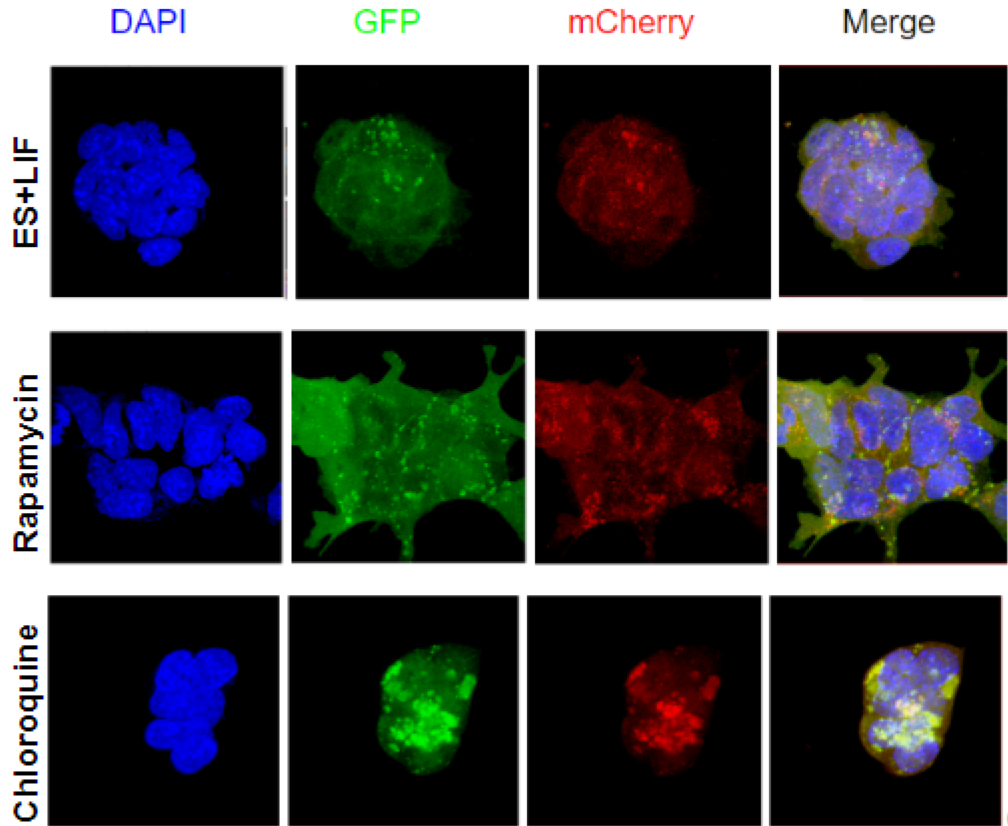
levels of various autophagy genes after treatment with the epigenetic inhibitors were quantified using SYBR green PCR master mix from Invitrogen.

Chapter Three: Results

3.1. Stable reporter cell line generated using fluorescent construct FUW-mCherry-GFP-LC3 to visualize autophagy

A stable cell line was generated by infecting V6.5 mESCs with the fluorescent construct FUW-mCherry-GFP-LC3 to visualize autophagy under a microscope in live /fixed cells. The fluorescent construct FUW-mCherry-GFP-LC3 is designed in such a way that the cells exhibit both mCherry and GFP fluorescence while the LC3 (microtubule-associated light chain) protein is associated with the autophagosome and exhibits only mCherry fluorescence as the autophagosome fuses with the lysosome, resulting in the degradation of the GFP protein and hence quenching of GFP fluorescence. After infection, the cells were sorted using flow cytometry to obtain a population that was both GFP and RFP positive.

The generated cell line exhibited increased levels of GFP and mCherry fluorescence when treated with rapamycin, which is a known inducer of autophagy (Figure 3.1). When treated with chloroquine a chemical known to inhibit the fusion of the autophagosome with the lysosome, fewer isolated mCherry puncta were observed and most mCherry puncta observed overlapped with the GFP puncta suggesting that these puncta (yellowish-green in merge) represent autophagosome (Figure 3.1). This suggests an accumulation of autophagosomes and its inability to be fused with the lysosome, which is what is expected on treatment with chloroquine. These observations validate that the construct follows the pattern of fluorescence expression as expected and can be used for further experiments.



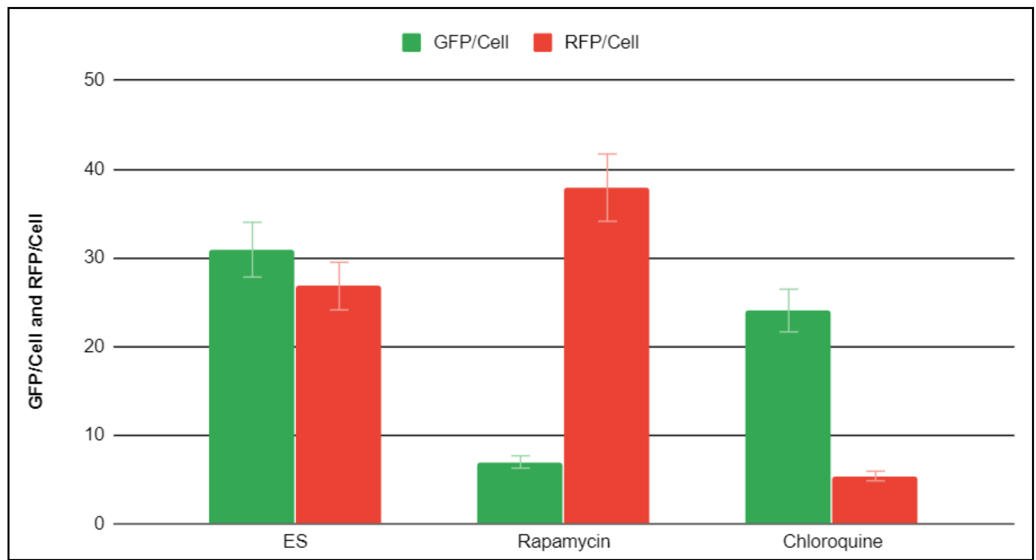


Figure (3.1): Confocal images of FUW-mCherry-GFP-LC3 infected V6.5 mES cells

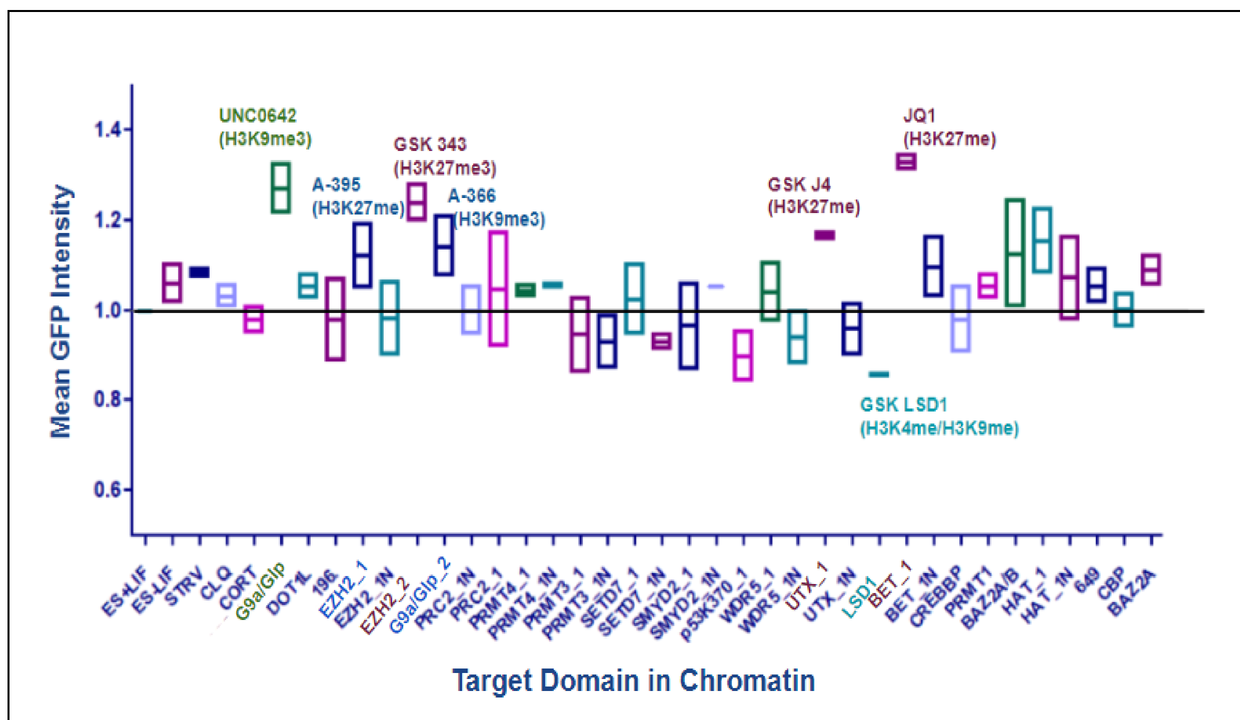
The figure represents the change in the levels of autophagic flux in V6.5 mES cells when treated with chemical regulators of autophagic flux. ES+LIF, the untreated sample shows basal levels of autophagy which is represented by almost equal levels of GFP and mCherry expression. Rapamycin, an activator of autophagy led to an increase in mCherry expression alluding to an increased autophagic flux. The cells

when treated with the repressor of autophagy, chloroquine, expressed higher green fluorescence as compared to mCherry fluorescence depicting a decrease in autophagic flux. All images were analysed using the software Imaris and analysis was done for 2 data points.

3.2 Epigenetic inhibitor screen on mESCs identified epigenetic modulators that can regulate autophagy

The regulation of autophagy in the context of chromatin was studied using 33 epigenetic inhibitors from the Structural Genomics Consortium (SGC). These are epigenetic modulators capable of inhibiting certain protein domains in the chromatin, thus altering the transcriptional status of various genes.

The epigenetic screen revealed a change in the levels of autophagy in mESCs when treated with the epigenetic modulators. Out of the 33 epigenetic modulators, 7 showed a significant change in the levels of autophagy based on mean fluorescence readout after flow cytometry analysis (Figure 3.2). The more the mean intensity readout obtained from flow cytometry analysis, more is the autophagy happening in a cell as the autophagosomes and autolysosomes in the cell express green fluorescence due to treatment with ENZO cyto id dye during flow cytometry. These epigenetic modulators that showed an upregulation in autophagy were GSK 343 (EZH2), UNC 0642 (G9A/GLP), A-395 (EED), A-366 (G9A/GLP), GSK J4 (UTX) and JQ1 (BET) while the epigenetic inhibitor drug GSK LSD1 (LSD) showed significant downregulation in autophagy.



Figure(3.2): Autophagy was upregulated on inhibition of G9a/Glp, UTX, BET, EZH2, EED and LSD domain in the chromatin

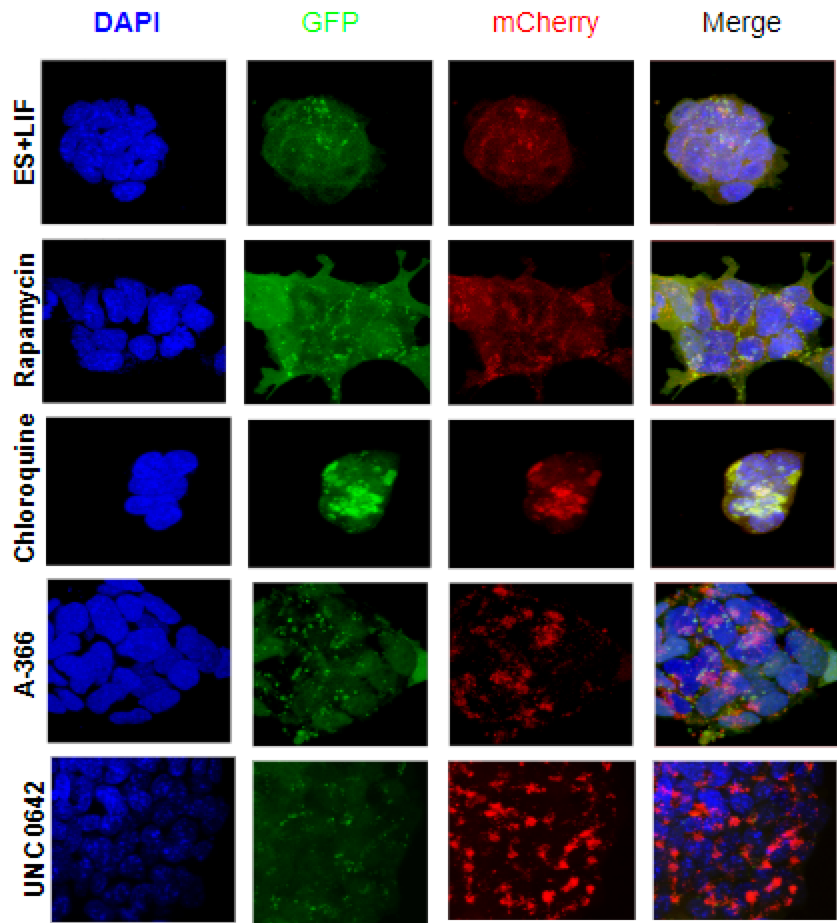
The graph depicts the mean intensity readout from flow cytometry analysis after autophagy assay of the epigenetic inhibitor-treated V6.5 mES cells. Chloroquine(CLQ), inhibitor of autophagy was used as a negative control and Starvation(STRV), a known activator of autophagy was used as positive control. All samples were normalised to the untreated sample represented as ES+LIF.The graph was plotted for 2 data points(n=2). The bars indicate the range of mean fluorescence intensity readouts (2 data points) from flow cytometry analysis and their mean. Demarcated samples represent the epigenetic inhibitor drugs that showed a significant change in autophagic flux after flow cytometry analysis, henceforth will be referred to as candidate epigenetic modulators.

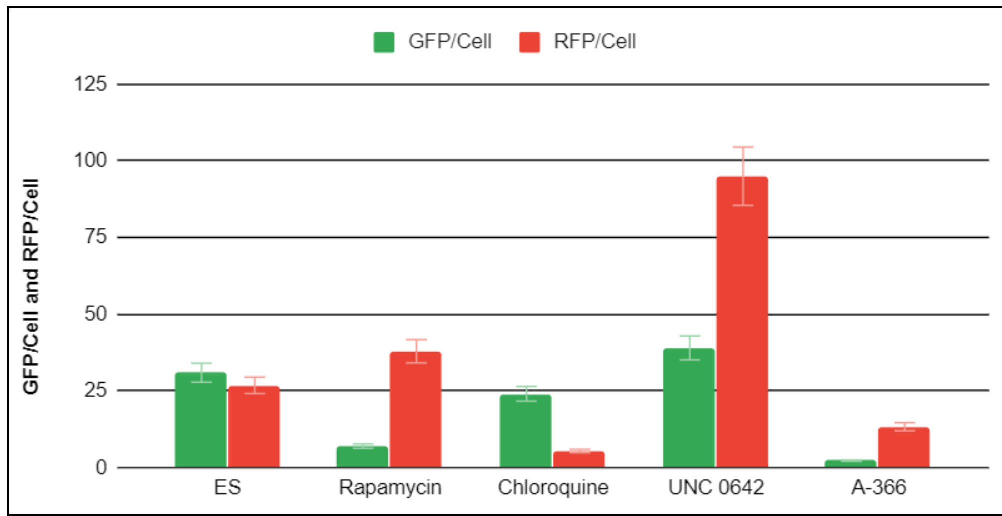
Hence these 7 epigenetic inhibitor drugs that showed a change in levels of autophagy and their respective negative controls A-395N, -JQ1 and GSK J5 were chosen as candidate epigenetic regulators of autophagy for further experiments quantifying the fold changes in the levels autophagy gene expression and the mechanism of regulation of autophagy.

3.3 Confocal Imaging of reporter cell line generated after epigenetic inhibitor treatment validated data obtained from FACS

The autophagy reporter cell line that was generated exhibited changes in the levels of autophagy taking place in the cell that is represented by the respective changes in GFP and mCherry fluorescence (Figure 3.3, 3.4 and 3.5). The change in the levels of autophagy was similar to that observed after the epigenetic screen of V6.5 mESCs that were stained using the ENZO CYTO id dye and analysed after flow cytometry. It was found that the seven epigenetic inhibitor drugs that were chosen as the candidate epigenetic regulators of autophagy showed increased mCherry fluorescence proposing an increased autophagic flux in the cells as a result of treatment with the same.

It was observed that after treatment with A-366 and UNC 0642, the epigenetic inhibitor drugs from SGC that targets the G9a/GLP domain that recruits methyltransferases and thus lead to the repressed state of a gene, the confocal images of mESCs showed mCherry expression levels to be higher as compared to the GFP levels in the cells (Figure 3.3). The treatment with the epigenetic inhibitor drugs was given for a duration of four days. When merged, very few yellowish-green puncta were seen suggesting a lower number of autophagosomes and a higher number of autolysosomes (red). This increase in the levels of mCherry expression implies an increase in the number of autolysosomes pointing to an increased autophagic flux as the rate of autophagosomes (green) being converted into autolysosomes (red) has now increased. The conversion of autophagosomes to autolysosomes is indicated by the decreased levels of GFP expression as GFP fluorescence is quenched on the fusion with lysosomes.





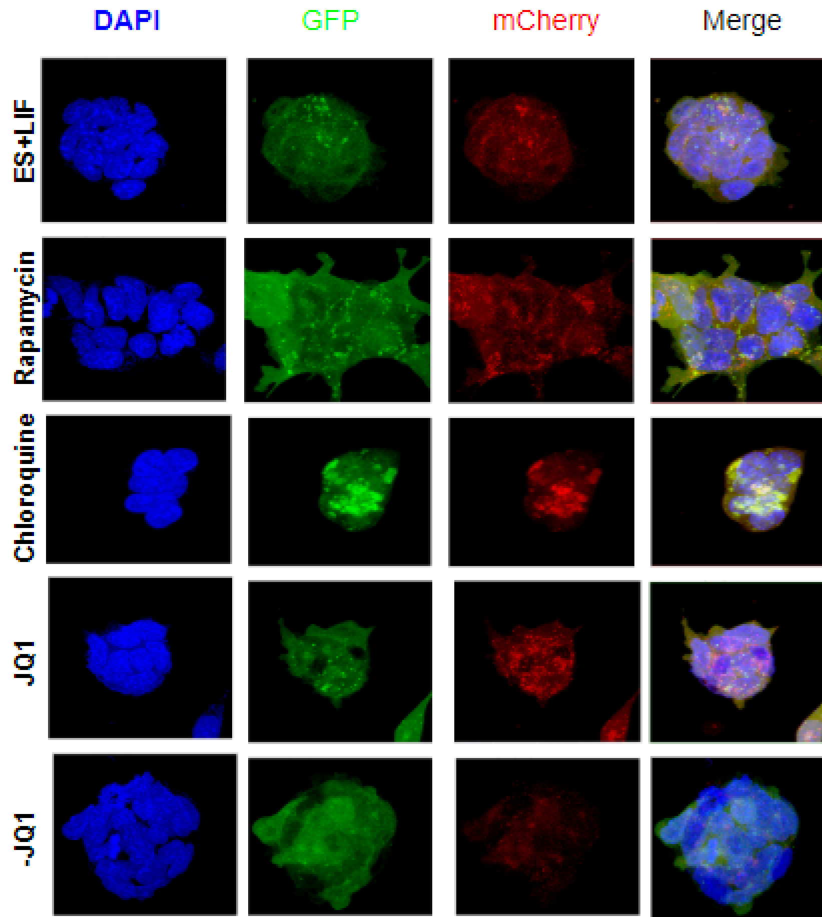
Figure(3.3): Increased autophagic flux on inhibition of G9a/GLP

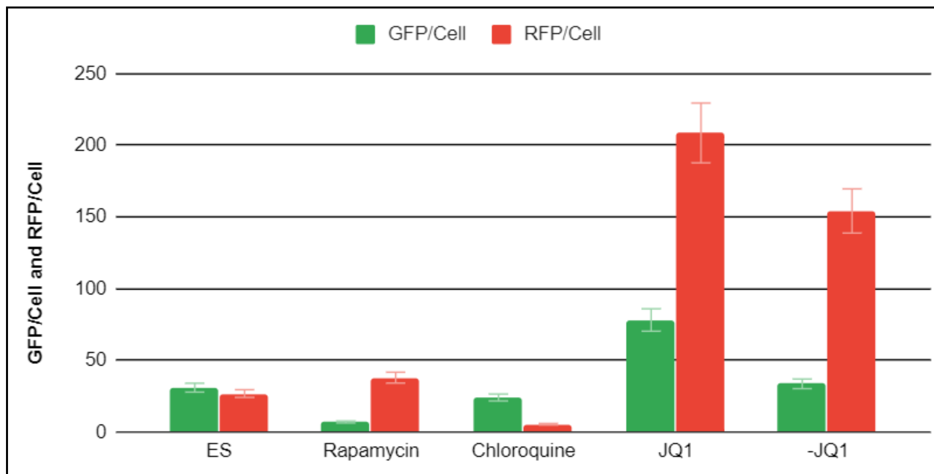
Basal levels of autophagy were seen in mESCs that were untreated (ES+LIF) and mCherry expression levels were found to be increased as compared to GFP levels indicating increased autophagic flux. Rapamycin and Chloroquine were used as positive and negative control for autophagy respectively.

Quantification of images confirmed an increase in mCherry expression levels as compared to GFP expression levels and hence increased autophagic flux for mESCs treated with the epigenetic inhibitor drugs A-366 and UNC 0642, targeting G9a/GLP complex in chromatin . All images were analysed using the software Imaris and analysis was done for 2 data points.

Similarly, autophagy was found to be upregulated on treatment with the BET domain inhibitor JQ1 (Figure 3.4). Confocal images after treatment with epigenetic inhibitor drug JQ1 showed an increase in mCherry puncta numbers. When merged, very few yellowish-green puncta were seen suggesting a lower number of autophagosomes and a higher number of autolysosomes (red). This increase in the levels of mCherry expression implies an increase in the number of autolysosomes being formed proposing an increased autophagic flux as the rate of autophagosomes (green) being converted into autolysosomes (red) has increased. The conversion of autophagosomes to autolysosomes is indicated by the decreased levels of GFP expression as GFP fluorescence is quenched on the fusion with lysosomes. The cells on treatment with the negative control for JQ1 that is, -JQ1 showed fluorescence levels similar to that of an untreated cell where the mCherry and GFP expression levels are comparable.

The epigenetic inhibitor drugs UNC 1999 and GSK 343 inhibit the EZH2 domain in the PRC2 complex (Figure 3.5). After treatment of mESCs with these epigenetic inhibitor drugs, the cell population showed an increase in mCherry expression, implying an increased autophagic flux. When merged, very few yellowish-green puncta were seen suggesting a lower number of autophagosomes and a higher number of autolysosomes (red). This increase in the levels of mCherry expression implies an increase in the number of autolysosomes proposing an increased autophagic flux as the rate of autophagosomes (green) being converted into autolysosomes (red) has increased.



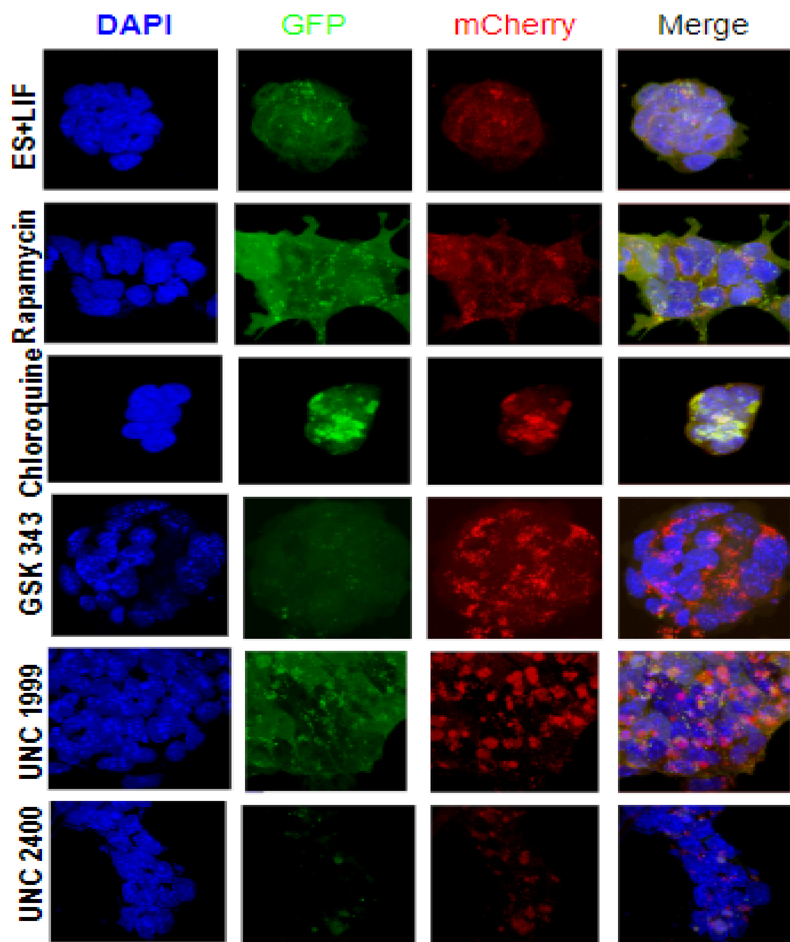


Figure(3.4): Increased autophagic flux on inhibition of BET domain

Basal levels of autophagy seen in untreated cells (ES+LIF). Increased mCherry expression in cells treated with JQ1, the inhibitor of BET domain in the chromatin suggesting an increased conversion of autophagosomes to autolysosomes. -JQ1, the negative control for JQ1 shows similar levels of autophagy as that in untreated (ES+LIF) mESCs. Rapamycin and Chloroquine were used as positive and negative

control for autophagy respectively. Quantification of images confirmed an increase in mCherry expression levels as compared to GFP expression levels and hence increased autophagic flux . All images were analysed using the software Imaris and analysis was done for 2 data points.

The conversion of autophagosomes to autolysosomes is indicated by the decreased levels of GFP expression as GFP fluorescence is quenched on the fusion with lysosomes. The negative control for the UNC1999, UNC 2400 showed GFP and mCherry fluorescence expression levels similar to that of an untreated cell population.



Figure(3.5): Increased autophagic flux on treatment with the EZH2 inhibitor

Basal levels of autophagy observed in untreated cells (ES+LIF). Increased mCherry expression on treatment with EZH2 inhibitor UNC 1999. Basal levels of autophagy similar to that as in untreated (ES+LIF) population when treated with negative control for UNC 1900 that is UNC 2400. Increase in mCherry expression on treatment with GSK 343, epigenetic inhibitor targeting the EZH2 domain.

Rapamycin and Chloroquine were used as positive and negative control for autophagy respectively.

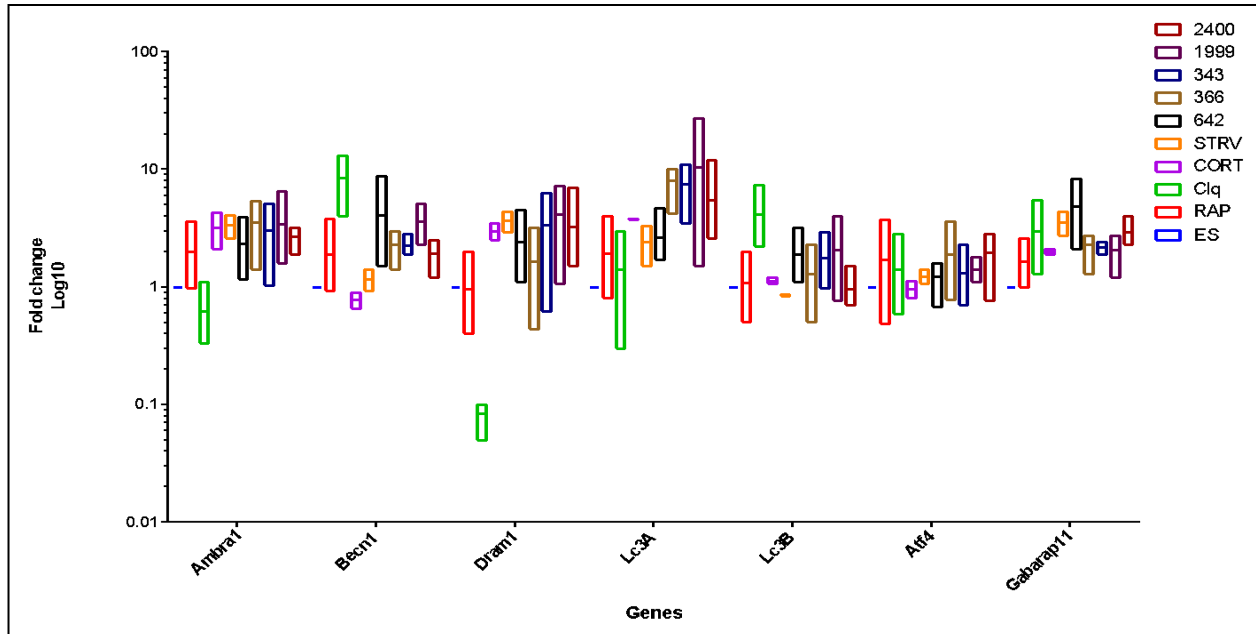
3.4 Epigenetic modification of G9a/GLP, EZH2 and BET domain regulates autophagy in mESCs

G9a/GLP, one of the lysine methyltransferases, is known to regulate autophagy by the inhibition of the autophagy genes. It is known to inhibit autophagy by methylating ATG12 when under no stress. But under conditions of starvation, studies in Drosophila suggest an increase in the expression of Atg8a gene as a result of demethylation by G9a/GLP (Ding et al., 2013; de et al., 2013).

One of the subunits of the PRC2 complex, EZH2 can regulate autophagy by inhibiting the expression of the following genes; SC2, RHOA, DEPTOR, FKBP11, RGS16, and GPI (Wei et al.,2015). These genes are known suppressors of the mTOR pathway, and the inhibition of the mTOR pathway is crucial for the induction of autophagy (Wei et al.,2015).

The epigenetic modulator JQ1, which targets the BET domain has shown to induce autophagy by activating the LKB1/AMPK pathway (Li et al., 2019).
The expression levels of some of the crucial autophagy genes based on qRT-PCR suggest that there is a regulation of these genes by the candidate epigenetic modulators targeting G9a/GLP, EZH2 and BET domain, validating the effect of these modulators on autophagy genes in mESCs.

The effect of the candidate epigenetic modulators on different autophagy genes are represented in the following graph (Figure 3.7).



Figure(3.6): Candidate epigenetic modulators affect the expression of crucial autophagy genes
The graph represents the expression levels of the autophagy genes Ambra1, Becn1, Dram1, LC3A, LC3B, Alf4 and Gabarap1 obtained from RT-qPCR screen after treatment with the epigenetic inhibitors UNC 2400, UNC 1999, GSK 343, A 366, UNC 0642 and corticosterone,a stress mimic. It was normalised to the expression levels of these genes with untreated (ES). Starvation (STRV), Chloroquine (Clq) and Rapamycin (Rap) were set as controls.The data represents results from 2 biological replicates.

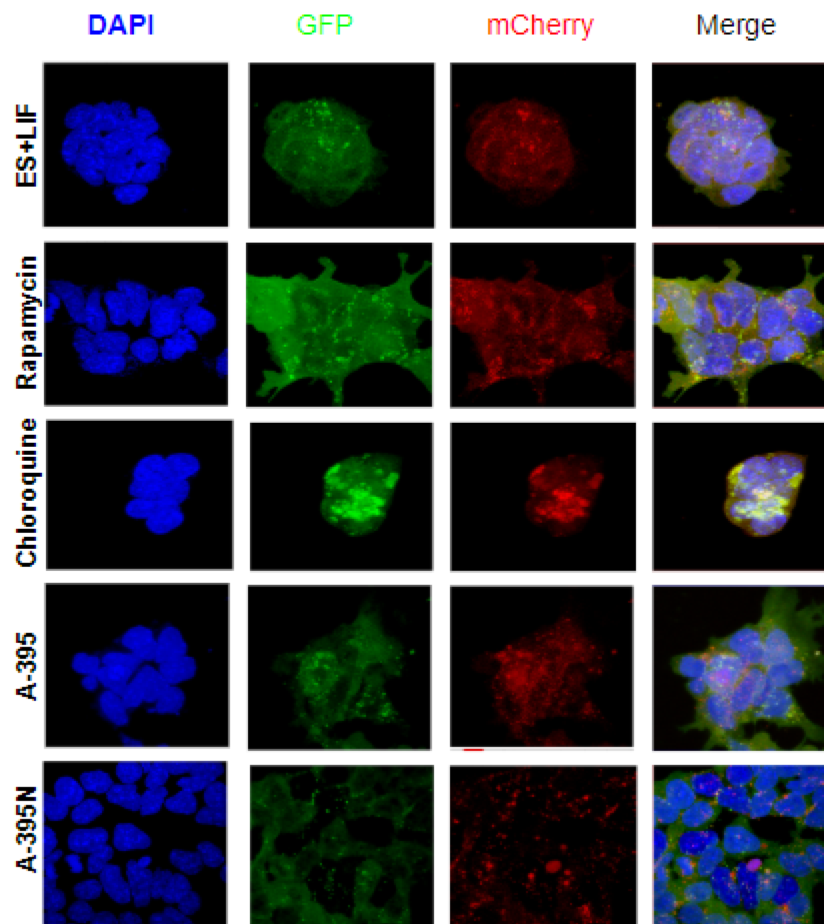
3.5 EED as a novel regulator of autophagy in mESCs

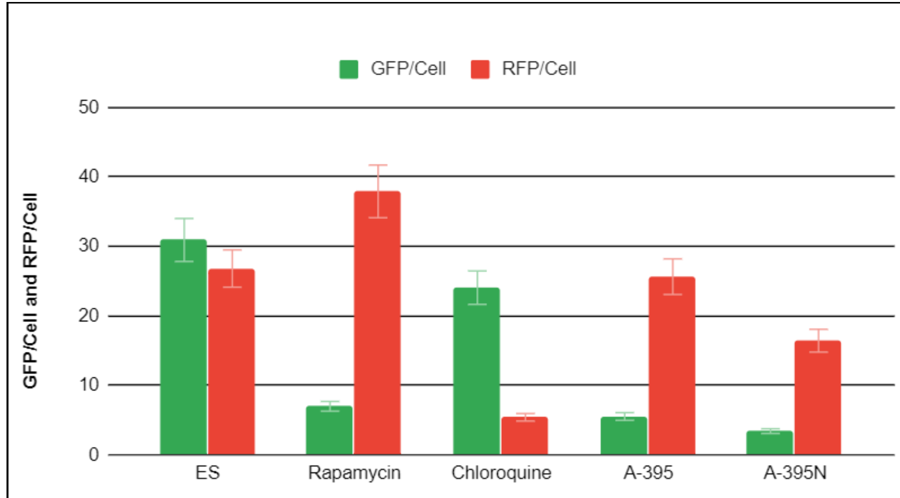
Embryonic ectoderm development (EED) protein is a subunit of the polycomb repressive (PRC2) group. EED group of proteins interact with the EZH2 group and bring about the repression of genes by histone deacetylation. Studies have also shown the association of EED with repressive methyl-lysine marks leading to the activation of the methyltransferase activity (Wei et al.,2015).

Confocal images of mESCs after treatment with the epigenetic inhibitor drug A-395, targeting the EED complex, showed an increase in the levels of autophagy, the same was quantified using image analysis. When merged, very few yellowish-green puncta were seen suggesting a lower number of autophagosomes and a higher number of autolysosomes (red). This increase in the levels of mCherry expression implies an

increase in the number of autolysosomes proposing an increased autophagic flux as the rate of autophagosomes (green) being converted into autolysosomes (red) has now increased. The conversion of autophagosomes to autolysosomes is indicated by the decreased levels of GFP expression as GFP fluorescence is quenched on the fusion with lysosomes.

It was observed that the mESCs after treatment with A-395N showed fluorescence expression levels similar to that of an untreated cell population. This increase in expression of mCherry fluorescence after treatment with A 395 implies an increase in autophagy as a result of inhibition of the EED complex by the epigenetic modulator A-395.

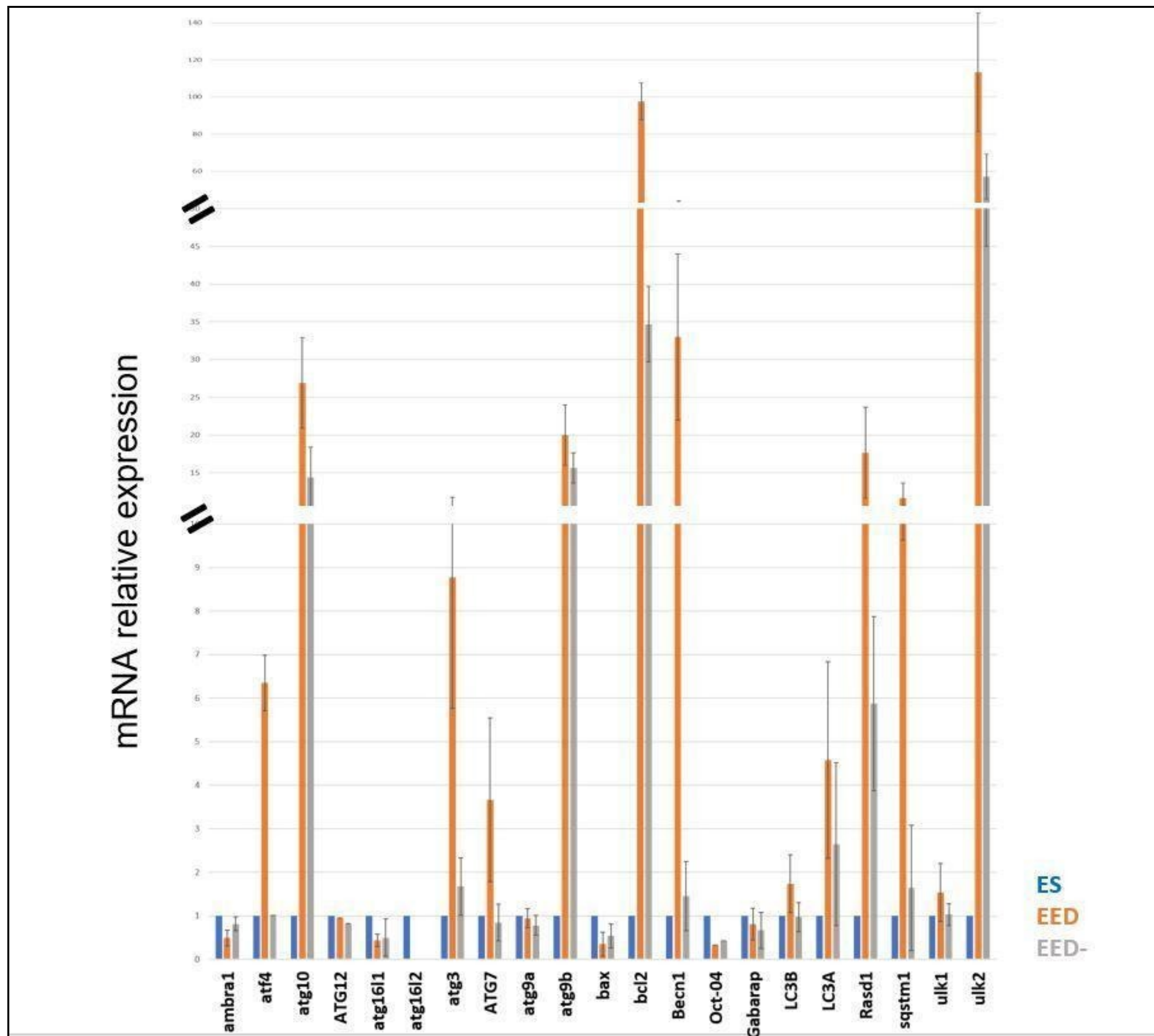




Figure(3.7): Inhibition of EED upregulates autophagy

Basal levels of autophagy observed in untreated (ES+LIF) cells. Increased autophagic flux represented by an increase in mCherry fluorescence on inhibition of EED. Basal levels of autophagy similar to that of an untreated(ES+LIF) cell population. Rapamycin and Chloroquine were used as positive and negative control for autophagy respectively. Quantification of images confirmed an increase in mCherry expression levels as compared to GFP expression levels and hence increased autophagic flux . All images were analysed using the software Imaris and analysis was done for 2 data points.

qRT-PCR of cDNA that was synthesised using RNA isolated from mESCs after treatment with A-395, that inhibits the EED domain showed an increase in the expression of genes that are crucial in the autophagy pathway (Figure 3.9). The genes that showed an upregulation include ATG10, ATG3, ATG9, BCL2, BECN1, LC3A, RASD1, SQSTm1 and ULK2.



Figure(3.8): EED regulates expression of crucial autophagy genes

The graph represents the mRNA levels of different autophagy genes after treatment with EED inhibitor A395. The autophagy associated genes ATG3, ATG4, ATG7,BCL2, Becn1, LC3, RASD1 and SQSTM1 showed an increase in their mRNA expression levels post treatment with A-395, the inhibitor of EED domain in chromatin. The graph was plotted for 2 data points.

3.6 Autophagy is upregulated in cells under stress induced by corticosterone

In 1956, Selye coined the term “stress” to define anything that interrupts homeostasis (Schneiderman et al.,2005). Studies have revealed that the regulation of autophagy has an effect on the regulation of depressive behaviour and antidepressant activity, that is seen as a result of stress (Abelaira et al.,2014). According to some studies, epigenetic

regulation of autophagy and stress have shown that in a number of cell lines, there is an accumulation of repeat elements as a result of misexpression and autophagy regulation when a cell is under stress (Xiao et al.,2019). There are studies on rats suggesting an increase in the levels of a repressive epigenetic mark, H3K27 as a response to acute stress in the hippocampal regions of the brain. When chronic stress was induced in the hippocampus of the brain, this epigenetic mark was no longer seen (Hunter et al.,2012). These findings suggest a possible involvement of autophagy in clearing the repeat elements in a cell under conditions of stress. Also, now that we know that there is epigenetic regulation of autophagy, it is crucial to look at how the epigenetic regulation of autophagy would vary under conditions of stress.

Corticosterone is a glucocorticoid hormone that the body produces as one of the first responses of stress (Mark et al.,2015). It is this glucocorticoid hormone that helps the body during stress to utilise stored energy, initiate escape and maintenance behaviours and immune component enhancement to cope with the stress that was induced. Reports from studies on rats have shown that corticosterone can be used as a mimic for chronic stress (Mark et al.,2015).

The concentration and period of treatment with corticosterone were standardised to 3μM for four days after treating the cells with different concentrations of corticosterone and treating the cells for different time periods. The concentrations used ranged from 0.1μM to 10μM, and the treatment period ranged between 2 days to 10 days. At higher concentrations and higher treatment period, the cells started dying and detaching from the coverslips on to which they were plated. At day four and 3μM concentration, the cells showed an increase in the expression level of mCherry fluorescence, suggesting an increase in the conversion of autophagosomes to autolysosomes (Figure 3.10). This means that the autophagic flux in the cells has now been increased. When merged, very few yellowish-green puncta were seen suggesting a lower number of autophagosomes and a higher number of autolysosomes (red). This increase in the

levels of mCherry expression implies an increase in the number of autolysosomes pointing to an increased autophagic flux as the rate of autophagosomes (green) being converted into autolysosomes (red) has increased. The conversion of autophagosomes to autolysosomes is indicated by the decreased levels of GFP expression as GFP fluorescence is quenched on the fusion with lysosomes.

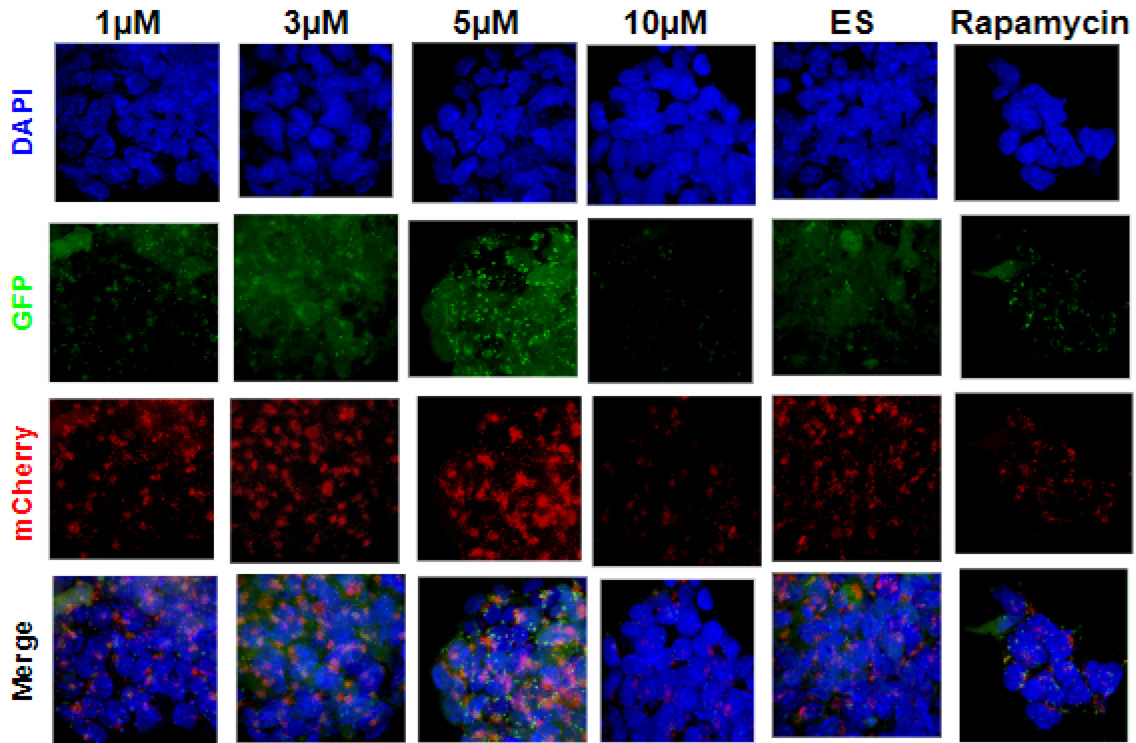


Figure (3.9): Upregulation of autophagy in mESCs under conditions of stress induced by corticosterone treatment

Increased mCherry fluorescence as a result of upregulation in autophagy in mESCs after treatment with corticosterone of concentrations 3μM,1μM and 5μM for 4 days .Cell death due to treatment with a higher concentration of corticosterone. mESCs expressing basal levels of autophagy under untreated condition (ES+LIF). Increased mCherry fluorescence on treatment with rapamycin, a known inducer of autophagy.

The change in fluorescence expression levels after treatment with different concentrations of corticosterone that was used as a mimic for stress indicated that the autophagic flux in a cell could be regulated depending on the levels of corticosterone in the cell. Nevertheless, the regulation in the expression levels of autophagy genes and

the mechanism by which corticosterone regulates the expression of autophagy genes are still to be understood.

The above results conclude that the process of autophagy can be regulated at the level of chromatin via epigenetic modifications. This study showed that the epigenetic inhibitor drugs from SGC, A-395 (EED), A-366 (G9A/GLP), GSK 343 (EZH2), GSK J4 (UTX), GSK LSD1, JQ1 (BET), and UNC 0642 (G9A/GLP).
This regulation of the process of autophagy is a result of the change in transcriptional states of the autophagy genes, which was the result of inhibition of the respective domains in the chromatin by the epigenetic inhibitor drugs.

Chapter 4: Discussion

Autophagy is still believed to be a cytosolic event though recent studies have shown that there are other players contributing to the regulation of autophagy. This includes regulation of autophagy genes by nuclear components like transcription factors, histone modifiers, microRNAs and others. Among these, the regulation of autophagy by epigenetic modifications like DNA methylation and histone modifications are now being studied actively (Zhou et al.,2005). The table below enlists a few of the histone modifications and the effect of these modifications on autophagy (Hargarten et al.,2018).

Histone modification	Regulator	Effect on autophagy
H3K9Ac	SIRT6	↑ATG5
H4K16Ac (H1.2 variant)	SIRT1/HDAC1	↑Autophagy
H3K9me	HIF-1α, KDMs	↑BNIP3
H3R17me2	TFEB/co-activator-associated arginine methyltransferase 1	↑ATG14
H4R3me2	C/EBPβ/PRMT5	Unknown
Multiple	HDAC6	SQSTM1 autophagic clearance

Figure (4.1): Different histone modifications and their effect on autophagy

The figure depicts a table containing the effect on the process of autophagy based on a particular modification of the histone and the regulator responsible for this modification.(Hargarten et al.,2018)

Though in recent years, there have been many reports implying the role of epigenetics in the regulation of autophagy in different systems, there has been no systematic study on how epigenetics regulates autophagy in embryonic stem cells.

From this study, we found that autophagy is regulated by epigenetic regulators in mES cells validating results in other model systems. The epigenetic screens followed by qRT-PCR showed an increase in expression of autophagy genes when treated with epigenetic inhibitors that targeted the PRC2 complex proteins EED and EZH2, UTX domain, BET domain, and the G9a/GLP. Earlier observations from studies on epigenetic regulation of autophagy have confirmed the role of EZH2, BET and UTX domain in the regulation of autophagy in other model systems. This study has succeeded in validating the same in mESCs.

Our results also identify EED as a novel regulator of autophagy in mESCs. It was observed that when the cells were treated with A-395, which inhibits the EED domain, autophagy was upregulated in the cell. The expression levels of essential autophagy genes also went up as a result of treatment with A-395. The genes that showed a significant deviation in their expression pattern include ATG3, ATG4, ATG7,BCL2, Becn1, LC3, RASD1 and SQSTM1.Basal levels of autophagy and autophagy gene expression were seen in the case of A-395N, which is the negative control for A-395, suggesting that the effect of A-395 on autophagy is direct.

EED works by deacetylating the lysine 27 and lysine 9 of histone three and this results in the transcriptional repression of the respective gene. A-395, an epigenetic inhibitor drug targeting the EED domain of the PRC2 complex resulted in an upregulation of the autophagy associated genes . This upregulation could be a result of the removal of the H3K27 mark. EED is also known to be involved in gene silencing by binding to sites of histone methylation. Figure 4.2 depicts the summary of the findings of this project. It was found that the autophagy associated genes ATG4 and LC3A are regulated by EED,

EZH2 and the G9a/Glp complex, whereas the genes Ambra 1 and Dram 1 are regulated by EZH2 and G9a/Glp complex and EED alone regulates the expression of ATG3, ATG7, BCL2, BECN1, RASD1 and SQSTM1. Since EED and EZH2 are components of the PRC2 complex, we can say that the PRC2 complex regulates the process of autophagy by regulating the gene expression of ATG3, AtG4, ATG7, BCL2, BECN1, LC3A, RASD1 and SQSTM1. More targeted and detailed studies are required to find the role of these complexes in the transcriptional pathway of these genes.

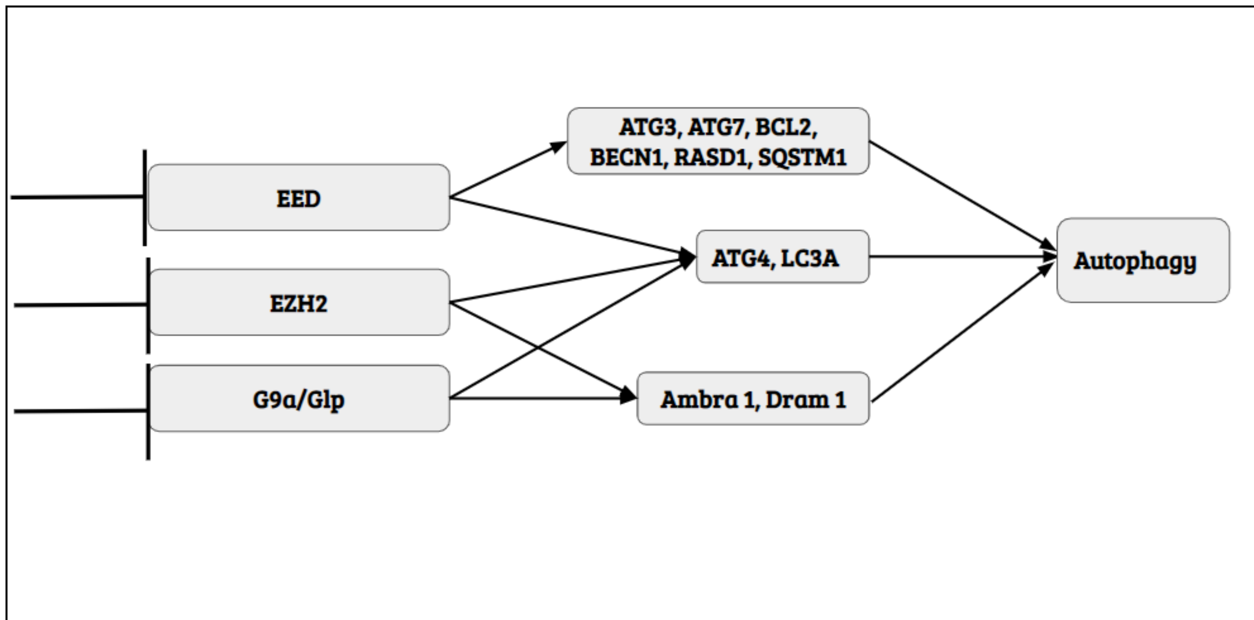


Figure (4.2): Regulation of the process of autophagy by regulation of expression of autophagy associated genes by protein domains in the chromatin

Figure depicts a constructed model of regulation of autophagy genes by chromatin complexes EED,EZH2 and G9a/Glp. '⟶' indicates induction and '⊢' indicates repression.

In conclusion, we can say that autophagy, which is a process that the cell depends on to renew its components, can be regulated at the level of chromatin in mESCs. From our results, the regulation of autophagy by epigenetic modulators targeting different domains of the chromatin in mESCs in steady-state was identified. It was found that EZH2, G9a/GLP, BET, LSD1 and UTX domain in the chromatin plays a role in the regulation of autophagy by regulating the expression of various genes crucial during

various steps of autophagy. However, the exact mechanism by which these epigenetic modifications regulate the autophagy genes are still to be understood. Also, how these epigenetic modifications would affect the autophagy regulation in a cell under conditions of stress also remains to be unravelled. For this, the glucocorticoid hormone corticosterone, the concentration and period of treatment that was standardised using a toxicity screen will be used as a mimic for stress.

The epigenetic inhibitor assay screen was used to identify the epigenetic modulators regulating autophagy and the pathway by which the process of autophagy is regulated. It is imperative to have a targeted approach by generating gene knockdowns of the identified autophagy associated genes that showed a deviation in their normal expression levels upon epigenetic inhibitor treatment.

It is also important to elucidate how these epigenetic modifications would regulate autophagy during the process of differentiation and development. To understand this, these studies will be done on the neuronal cell line as it differentiates from its neuronal progenitor cells.

Future work

1. Confocal Imaging for cells after treatment with GSK LSD1 and GSK J4 that are inhibitors of LSD domain and UTX domain. The epigenetic screen suggested that GSK LSD1 and GSK J4 shows upregulation in autophagy. Same will be validated with confocal imaging if the expression of mCherry increases in the cells treated with GSk LSD1 and GSK J4.
2. Western Blotting to quantify the levels of LC3 protein in mESCS after treatment with all candidate epigenetic modulators to validate FACS data.
3. qRT-PCR for all the autophagy genes, as mentioned in chapter 2, section 4(2.4) after treatment with candidate epigenetic modulators.
4. A ChIP of cmESCs after treatment with candidate epigenetic inhibitors will be

done against the epigenetic marks of these to understand the mechanism by which they regulate the expression of autophagy genes.

5. The regulation of autophagy in cells under stress(corticosterone) will be looked at using autophagy assay, confocal imaging, the expression levels of different autophagy genes will be studied using RT-qPCR, and the mechanism of regulation of these genes will be understood using ChIP.

The results from this study show that the process of autophagy in mESCs is regulated at the level of chromatin. Changing the transcriptional states of the autophagy genes using epigenetic modulators can bring about a change in the expression levels of the autophagy genes and the process of autophagy itself under steady-state.

Results from the treatment of mESCs with corticosterone also hint to the conclusion that the process of autophagy in mESCs are regulated by the levels of corticosterone in a cell and that under conditions of stress, the levels of autophagy occurring in a cell can vary in order for the cell to maintain its homeostasis and viability. The molecular mechanism behind this regulation in the context of chromatin is yet to be understood and could provide insights into how epigenetic regulation of the process of autophagy can influence the process of development and differentiation.

Chapter 5: References

1. Abelaira HM, Réus GZ, Neotti MV & Quevedo J (2014) The role of mTOR in depression and antidepressant responses. *Life Sci* 101, 10– 14.

2. An PNT, Shimaji K, Tanaka R, Yoshida H, Kimura H, Fukusaki E & Yamaguchi M (2017) Epigenetic regulation of starvation-induced autophagy in Drosophila by histone methyltransferase G9a. *Sci Rep* 7, 7343.

3. Baehrecke, E. (2003). Autophagic programmed cell death in Drosophila. Cell Death & Differentiation *10*, 940-945.

4. Beaulaton, J., and Lockshin, R. (1977). Ultrastructural study of the normal degeneration of the intersegmental muscles of Antheraea polyphemus and Manduca sexta (Insecta, Lepidoptera) with particular reference to cellular autophagy. Journal Of Morphology *154*, 39-57.

5. Berry, D., and Baehrecke, E. (2007). Growth Arrest and Autophagy Are Required for Salivary Gland Cell Degradation in Drosophila. Cell *131*, 1137-1148.

6. Boya, P., Codogno, P., and Rodriguez-Muela, N. (2018). Autophagy in stem cells: repair, remodelling and metabolic reprogramming. Development *145*, dev146506.

7. Castillo, K., Valenzuela, V., Oñate, M., and Hetz, C. (2017). A Molecular Reporter for Monitoring Autophagic Flux in Nervous System In Vivo. Methods In Enzymology 109-131.

8. Cecconi, F., and Levine, B. (2008). The Role of Autophagy in Mammalian Development: Cell Makeover Rather than Cell Death. Developmental Cell *15*, 344-357.

9. Chen, X., He, Y., and Lu, F. (2018). Autophagy in Stem Cell Biology: A Perspective on Stem Cell Self-Renewal and Differentiation. Stem Cells International *2018*, 1-12.

10. Czechanski, A., Byers, C., Greenstein, I., Schrode, N., Donahue, L., Hadjantonakis, A., and Reinholdt, L. (2014). Derivation and characterization of mouse embryonic stem cells from permissive and nonpermissive strains. Nature Protocols *9*, 559-574.

11. de Narvajas AA-M, Gomez TS, Zhang J-S, Mann AO, Taoda Y, Gorman JA, Herreros-Villanueva M, Gress TM, Ellenrieder V, Bujanda L *et al*. (2013) Epigenetic regulation of autophagy by the methyltransferase G9a. *Mol Cell Biol* 33, 3983– 3993.

12. Ding J, Li T, Wang X, Zhao E, Choi J-H, Yang L, Zha Y, Dong Z, Huang S, Asara JM *et al*. (2013) The histone H3 methyltransferase G9A epigenetically activates the serine-glycine synthesis pathway to sustain cancer cell survival and proliferation. *Cell Metab* 18, 896– 907.

13. Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L *et al*. (2009) Induction of autophagy by spermidine promotes longevity. *Nat Cell Biol* 11, 1305–1314.

14. Füllgrabe, J., Klionsky, D. and Joseph, B. (2014).The return of the nucleus: transcriptional and epigenetic control of autophagy. Nat Rev Mol Cell Biol 15, 65–74.

15. Guasch, G., and Fuchs, E. (2005). Mice in the world of stem cell biology. Nature Genetics *37*, 1201-1206.

16. Glick D, Barth S, Macleod KF.(2010). Autophagy: cellular and molecular mechanisms. J Pathol. 221(1):3-12.Medicine *8*, 4792-4805.

17. Hunter RG, Murakami G, Dewell S, Seligsohn M, Baker MER, Datson NA, McEwen BS & Pfaff DW (2012) Acute stress and hippocampal histone H3 lysine 9 trimethylation, a retrotransposon silencing response. *Proc Natl Acad Sci USA* 109, 17657– 17662.

18. Kohli, L., and Roth, K. (2010). Autophagy. The American Journal Of Pathology *176*, 1065-1071.

19. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 3, 662.

20. Li, F., Yang, C., Zhang, H., Ma, J., Jia, J., Tang, X., Zeng, J., Chong, T., Wang, X., and He, D. et al. (2019). BET inhibitor JQ1 suppresses cell proliferation via inducing autophagy and activating LKB1/AMPK in bladder cancer cells. Cancer

21. Lum, J., DeBerardinis, R., and Thompson, C. (2005). Autophagy in metazoans: cell survival in the land of plenty. Nature Reviews Molecular Cell Biology *6*, 439-448.

22. Mark D. Kvarta, Keighley E. Bradbrook, Hannah M. Dantrassy, Aileen M. Bailey, and Scott M.Thompson.(2015).Corticosterone mediates the synaptic and behavioural effects of chronic stress at rat hippocampal temporoammonic synapses.

23. Meshorer E & Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. *Nat Rev Mol Cell Biol* 7, 540.

24. Mizushima, N., Levine, B., Cuervo, A., and Klionsky, D. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

25. Phadwal, K., Watson, A., and Simon, A. (2012). Tightrope act: autophagy in stem cell renewal, differentiation, proliferation, and ageing. Cellular And Molecular Life Sciences *70*, 89-103.

26. Puri, D., and Subramanyam, D. (2019). Stress – (self) eating: Epigenetic regulation of autophagy in response to psychological stress. The FEBS Journal *286*, 2447-2460.

27. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 447, 425.

28. Schneiderman, N., Ironson, G., and Siegel, S. (2005). Stress and Health: Psychological, Behavioral, and Biological Determinants. Annual Review Of Clinical Psychology *1*, 607-628.

29. Selye H (1955) Stress and disease. *Science* 122, 625– 631.

30. Tomoda, T., Bhatt, R., Kuroyanagi, H., Shirasawa, T., and Hatten, M. (1999). A Mouse Serine/Threonine Kinase Homologous to C. Elegans UNC51 Functions in Parallel Fiber Formation of Cerebellar Granule Neurons. Neuron *24*, 833-846.

31. Vellai T (2009) Autophagy genes and ageing. *Cell Death Differ* 16, 94– 102.

32. Vessoni, A., Muotri, A., and Okamoto, O. (2012). Autophagy in Stem Cell Maintenance and Differentiation. Stem Cells And Development *21*, 513-520.

33. Wei, F., Cao, Z., Wang, X., Wang, H., Cai, M., Li, T., Hattori, N., Wang, D., Du, Y., and Song, B. et al. (2015). Epigenetic regulation of autophagy by the methyltransferase EZH2 through an MTOR-dependent pathway. Autophagy *11*, 2309-2322.

34. Weinhold, B. (2006). Epigenetics: The Science of Change. Environmental Health Perspectives *114*.

35. Xiao X, Shang X, Zhai B, Zhang H & Zhang T (2018) Nicotine alleviates chronic stress-induced anxiety and depressive-like behaviour and hippocampal neuropathology via regulating autophagy signalling. *Neurochem Int* 114, 58– 70.

36. Yue, Z., Jin, S., Yang, C., Levine, A., and Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumour suppressor. Proceedings Of The National Academy Of Sciences *100*, 15077-15082.

37. Zhou Y & Grummt I (2005) The PHD finger/bromodomain of NoRC interacts with acetylated histone H4K16 and is sufficient for rDNA silencing. *Curr Biol* 15, 1434– 1438.