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THE RETURN OF THE NUCLEUS - EPIGENETIC REGULATION OF AUTOPHAGY

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The Return of the Nucleus - Epigenetic regulation of Autophagy
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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For Bertrand

In nature's infinite book of secrecy

A little I can read.

-Antony and Cleopatra, William Shakespeare

ABSTRACT

Autophagy is an evolutionary conserved catabolic process activated in response to a variety of cellular stresses, for example nutrient deprivation or chemotherapy. During autophagy, cells engulf parts of their cytplasm in a double-membrane vesicle, where misfolded proteins or damaged organelles are degraded. A plethora of human diseases has been linked to autophagy, including cancer and neurodegenerative disorders.

Previously, autophagy has been considered a purely cytosolic event as even enucleated cells were able to display autophagic vesicles. Here we have shown for the first time that epigenetic changes are a major component of the autophagic process and involve global changes in the level of several histone modifications and local alterations in DNA methylation. Autophagy-related epigenetic modifications are involved during all stages of autophagy and have the potential to influence the autophagic life and death decision.

During the early steps of autophagy, histone modifications are involved in the transcriptional up-regulation of autophagy-related genes. However, global down-regulation of histone H4 lysine 16 acetylation (H4K16ac) occurring at later stages protects cells from an overstimulation of autophagy, which can lead to a lethal level of autophagy. Furthermore, we have uncovered a critical role for the histone H3 lysine 36 (H3K36) demethylase Rph1/KDM4A in suppressing autophagy under baseline conditions.

As we and others have shown that histone modifications are part of the autophagic process, we wondered how long the autophagy induced epigenetic modifications remain. By definition, an epigenetic regulation of autophagy should involve heritable changes that alter the cellular gene expression for a prolonged period of time.

We indeed found that cancer cells in which autophagy has been induced show a lower expression of autophagy-related proteins. Upon renewed stimulation these pre-treated cells show an alteration in autophagic flux compared to untreated cells. This ‘autophagic memory’ involves an early up-regulation of DNA methyl transferase 3A (DNMT3A) which induces a stable down-regulation of autophagy-related genes by DNA methylation.

In the future, development of new drugs for a variety of diseases involving deregulated autophagy may benefit from a widened knowledge about the epigenetic autophagy-regulatory network.

LIST OF SCIENTIFIC PAPERS

I. ***Opposing effects of hMOF and SIRT1 on H4K16 acetylation and the sensitivity to the topoisomerase II inhibitor etoposide.***
Hajji N, Wallenborg K, Vlachos P, **Füllgrabe J**, Hermanson O, Joseph B.
Oncogene, 2010, **29**(15):2192-204.

II. ***The histone H4 lysine 16 acetyltransferase hMOF regulates the outcome of autophagy.***
Füllgrabe J, Lynch-Day MA, Heldring N, Li W, Struijk RB, Ma Q, Hermanson O, Rosenfeld MG, Klionsky DJ, Joseph B.
Nature, 2013, **500**(7463):468-71.

III. ***Rph1/KDM4 mediates nutrient-limitation signaling that leads to the transcriptional induction of autophagy.***
Bernard A, Jin M, González-Rodríguez P, **Füllgrabe J**, Delorme-Axford E, Backues SK, Joseph B & Klionsky DJ.
Current Biology, 2015, **25**(5):546-55.

IV. ***The DNA methyltransferase DNMT3A contributes to autophagy long-term memory.***
Füllgrabe J, González-Rodríguez P, Cheray M, Salli M, Heldring N, Dyczynski M, Li W, Rosenfeld MG and Joseph B.
Manuscript.

RELATED PUBLICATIONS, NOT INCLUDED IN THE THESIS

I. ***Cracking the death code: apoptosis-related histone modifications.***
Füllgrabe J, Hajji N, Joseph B.
Cell Death and Differentiation. 2010,**17**(8):1238-43.

II. ***Histone onco-modifications.***
Füllgrabe J, Kavanagh E, Joseph B.
Oncogene. 2011, **30**(31):3391-403.

III. ***Histone post-translational modifications regulate autophagy flux and outcome.***
Füllgrabe J, Klionsky DJ, Joseph B.
Autophagy. 2013, **9**(10):1621-3.

IV. ***The return of the nucleus: transcriptional and epigenetic control of autophagy.***.
Füllgrabe J, Klionsky DJ, Joseph B.
Nature Reviews Molecular Cell Biology. 2014, **15**(1):65-74.

V. ***Cracking the survival code: autophagy-related histone modifications.***
Füllgrabe J, Heldring N, Hermanson O, Joseph B.
Autophagy. 2014, **10**(4):556-61.

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LIST OF ABBREVIATIONS

3MA	3-Methyladenine
Ac	Acetylation
AcCoA	Acetyl-coenzyme A
ADP	Adenosine diphosphate ribose
AGO2	Argonaute RISC Catalytic Component 2
AMBRA1	Activating molecule in BECN1 regulated autophagy protein1
ATG	Autophagy-related
ATG16L1	ATG16-like 1
Baf	Bafilomycin A1
BCL-2	B-cell lymphoma 2
bp	Base pair
ChIP-seq	Chromatin immunoprecipitation sequencing
CpG	—Cytosine—phosphate—Guanine—
CQ	Chloroquine
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
FOXO3	Forkhead box O3
GFP	Green fluorescent protein
Gis1	GIg1-2 Suppressor
GRO-seq	Global run-on sequencing
H1	Histone H1
H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H3K27me3	Histone H3 lysine 27 trimethylation
H3K36me3	Histone H3 Lysine 36 trimethylation
H3K4me1	Histone H3 lysine 4 monomethylation

H3K4me3	Histone H3 lysine 4 trimethylation
H3K56ac	Histone H3 lysine 56 acetylation
H3K9	Histone H3 lysine 9
H3K9me2	Histone H3 Lysine 9 dimethylation
H4	Histone H4
H4K16ac	Histone H4 Lysine 16 acetylation
H4K20me3	Histone H4 lysine 20 trimethylation
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demthylase
hMOF	Human orthologue of males absent on the first
HMT	Histone methyltransferase
iPS cells	Induced pluripotent stem cells
K	Lysine
KAT8	Lysine acetyltransferase 8
KDM4A	Lysine-specific methylase 4A
KMT2A	Lysine-specific methyltransferase 2A
LC3	Microtubule-associated proteins 1A/1B light chain 3
MAP1LC3	
MBD	Methyl-CpG-binding domain
Me	Methylation
miRNA	MicroRNA
MLL1	Mixed lineage leukemia 1
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NSCLC	Non-small cell lung carcinoma
P	Phosphorylation
PE	Phosphatidylethanolamine
PRC2	Polycomb repressive complex 2
PTM	Posttranslational modification

R	Arginine
Rapa	Rapamycin
RFP	Red fluorescent protein
Rim15	Regulator of IME2
RNA	Ribonucleic acid
Rph1	Regulator of PHR1
S	Serin
SAHA	Suberoylanilide hydroxamic acid
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
SOX2	SRY (sex determining region Y)-box 2
SQSTM1	Sequestosome 1
STAT1	Signal transducer and activator of transcription 1
SUMO	Small ubiquitin-related modifier
T	Threonine
TFEB	Transcription factor EB
TSA	Trichostatin A
TSS	Transcription start site
Ub	Ubiquitination
ULK1	Unc-51-like kinase 1
VP16	Etoposide/Vepeside®
VPA	Valproic acid/Valproate
ZKSCAN3	Zinc finger with KRAB and SCAN domains 3

1 INTRODUCTION

1.1 EPIGENETICS

With only few exceptions, all cells in an organism share exactly the same DNA but no one can deny the major differences seen in their phenotype. A liver cell has to perform very different functions than heart cells or brain cells. The phenotype of these different cell types is assured by different gene expression patterns (Margueron and Reinberg, 2010), which in turn are governed by epigenetic modifications. These epigenetic marks are set during embryonic development and assure cellular identity even after cell division, throughout the lifetime of a cell (Bird, 2007). However, some epigenetic modifications can respond to the environment and change locally or globally within a matter of minutes (Görisch *et al*., 2005). Hence, epigenetic modifications have the potential to modulate gene expression, activating or silencing specific regions of the DNA. By definition, epigenetics is the study of phenotypic changes that are heritable but not caused by changes in the underlying DNA sequence (Berger *et al*., 2009). The main cellular mechanisms that are studied in epigenetics are DNA methylation, histone modifications, nucleosome positioning and RNA silencing. It is worth to note that these different processes are not independent events, but rather their dynamic interplay creates cellular expression patterns (reviewed in Vaissière *et al*., 2008).

1.2 HISTONE MODIFICATIONS

DNA is not floating around freely in the nucleus but is packed in a highly organized and condensed structure called chromatin. The main protein components of the chromatin are the histones which can be imagined as spools winding up the DNA strands (Luger *et al*., 1997). The tight binding between histones and DNA is achieved through a high lysine content in the histone tails which gives the histones an overall positive charge. This opposes the negative charge of the DNA. In the eukaryotic cell, the histone octamer comprises two copies of each of the four core histone proteins H2A, H2B, H3 and H4 (**Figure 1**) (Kornberg and Lorch, 1999). The DNA with the associated histone octamer resemble ‘beads on a string’ when observed by electron microscopy (Luger *et al*., 1997). These 11-nanometre fibres form only the first level of chromatin compaction that eventually allows the packaging of about two metres of DNA into the small size of a nucleus (Margueron and Reinberg, 2010).

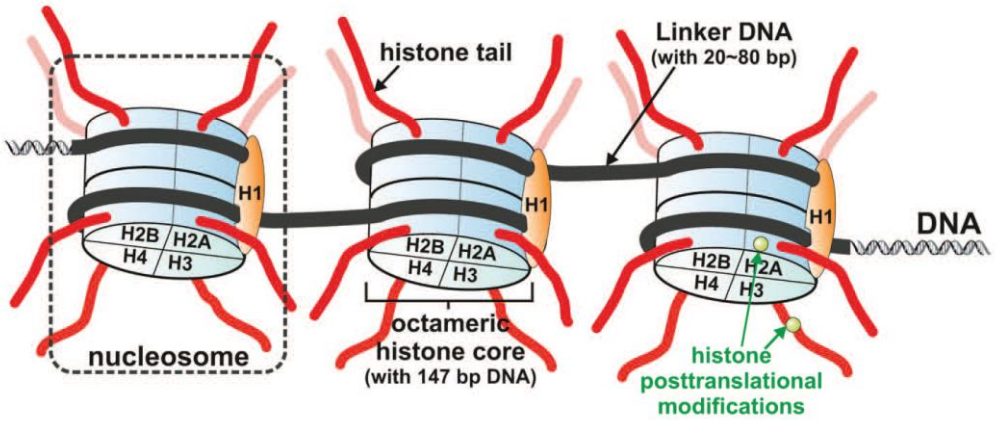

Figure 1. Chromatin is composed of repeating units which are called nucleosomes. The nucleosome consists of ~147 base pairs of DNA which are wrapped around the histone core. The histone core consists of two copies of each of the histones H2A, H2B, H3 and H4. Single nucleosomes are connected by a piece of linker DNA of around 20-80 bp. The linker histone H1 stabilises higher order chromatin structures. Figure was adapted from Füllgrabe *et al*., 2014a.

Interestingly, the histone proteins do not only function as 'DNA packaging' units but are important regulators of chromatin dynamics (reviewed in Cosgrove *et al*., 2004). In this respect, histone post-translational modifications (PTMs) play a major role in the regulation of DNA to histone binding and in the regulation of transcriptional outcome. These PTMs have been described to occur on more than 60 residues, especially on the histone tails which are protruding from the histone core. Moreover, there is a wide range of possible modifications including methylation, acetylation, citrullination, ADP-ribosylation phosphorylation, ubiquitination, and SUMOylation (Khorasanizadeh, 2004; Kouzarides, 2007; Christophorou *et al*., 2014). The functional implication of these modifications are complex and can be influenced by the location (for example promoter *versus* gene body), number of modifications on a single residue (for example H3K4me1 *versus* H3K4me3) or simply by which residue is modified (for example the 'active' H3K4me3 histone mark *versus* the repressive H3K27me3 histone mark) (reviewed in Schneider and Grosschedl, 2007 and Bernstein *et al*., 2007).

1.3 THE HISTONE CODE HYPOTHESIS

The histone code hypothesis states that there are a multitude of layers regulating the interpretation of the genome. In this respect, the combination of specific post-translational histone modifications alters chromatin structures and thus the transcriptional output (Strahl and Allis, 2000; Turner, 2000). These histone modifications are regulated by 'writers' and 'erasers'. For each modification, there are enzymes which are responsible for the specific addition or removal of histone post-translational modifications. Of specific interest for this thesis are acetylation and methylation of histones. Histone residues are methylated by histone methyltransferases (HMTs) and demethylated by histone demethylases (HDMs). Histone

methylation can act as transcriptional activator or repressor. Histones are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs) (Allis *et al*., 2007). Histone acetylation is usually considered an active transcriptional mark (**Figure 2**) (Kurdistani and Grunstein, 2003; Koch *et al*., 2007).

The histone modifications are translated into transcriptional activity by at least two distinct pathways. First, 'reader' molecules use the modified histones as signal platforms to which they can bind in a specific manner, modulating the transcription rate (Vogelauer *et al*., 2000; Yun *et al*., 2011). Second, histone post-translational modifications can change the steric or electrostatic properties of the histone, which adjust the inter-nucleosomal contacts, allowing more or less access to the RNA polymerase machinery (Wolffe Hayes, 1999; Allahverdi *et al*., 2011).

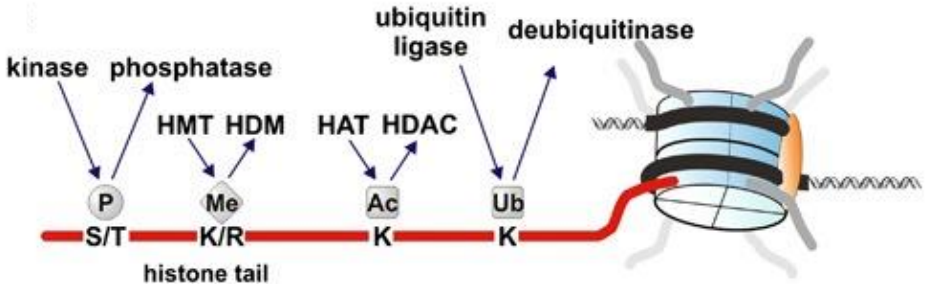

Figure 2. Several residues on the histone tail can be modified by specific histone modifying enzymes. Figure was adapted from Füllgrabe *et al*., 2010.

1.4 HISTONE H4 LYSINE 16 ACETYLATION

Due to its role not only in transcriptional control but also in the regulation of higher order chromatin structures we have focused mainly on histone H4 lysine 16 acetylation (H4K16ac) in this thesis (Kalashnikova *et al*., 2013). H4K16 acetylation can serve as a binding platform for a range of transcription factors or other chromatin modifiers (Ruthenburg *et al*., 2011; Zippo *et al*., 2009). Moreover, the acetylation of H4K16 close to the transcription start site (TSS) may play a role in displacing the nucleosome, which would otherwise hinder the binding of the RNA polymerase complex (Schones *et al*., 2008). However, it should be mentioned that the transcriptional outcome of H4K16 acetylation seems to be dependent on the cell type and environmental stimuli (Horikoshi *et al*., 2013). Deregulation of this particular modification has been linked to aberrant gene expression, DNA repair alterations and genomic instability (Li *et al*., 2010b; Füllgrabe *et al*., 2011). Therefore, changing the H4K16ac level offers a potential target for a range of diseases including cancer, due to its relatively easy modulation (Görisch *et al.*, 2005; Hajji *et al*., 2010).

Apart from its function in maintaining transcriptionally permissive DNA domains and promoting transcription, H4K16ac has a unique role in regulating higher-order chromatin structures. H4K16 distorts compact 30-nanometre-like fibres and cross-fibre interactions (Shogren-Knaak *et al*., 2006; Kalashnikova *et al*., 2013). Work by the group of Karsten

Rippe showed in a very visual approach how histone acetylation, especially H4K16ac, opens up the nucleus for access of high-molecular weight dextran (Görisch *et al*., 2005). H4K16 acetylation is mainly controlled by the HAT KAT8/MYST1/hMOF (human orthologue of the *Drosophila melanogaster* gene males absent on the first) and the NAD-dependent HDAC sirtuin 1 (SIRT1) (Lu *et al*., 2011; Neal *et al*., 2000; Utley and Côté, 2003; Taipale *et al*., 2005). The acetyl-group required for histone acetylation is supplied by acetyl-coenzyme A (AcCoA) (Eisenberg *et al*., 2014)

Remarkably, H4K16 acetylation levels are linked to the level of another histone modification, histone H3 lysine 4 trimethylation (H3K4me3). On a genome-wide scale, H4K16ac was found to accumulate significantly on the same nucleosomes as H3K4me3 (Wang *et al*., 2009; Ruthenburg *et al*., 2011; Katoh *et al.*, 2011). Moreover, Dou *et al.* have shown a physical interaction of hMOF with one of the histone methyltransferases responsible for placing H3K4me3, MLL1/KMT2A (Dou *et al*., 2005).

1.5 DNA METHYLATION

Repressed chromatin is often marked by both, a set of repressive covalent histone modifications and DNA methylation. The interplay between histone modifications and DNA methylation is complex (Tachibana *et al*., 2008 and reviewed in Vaissière *et al*., 2008). It has been suggested that DNA methylation can guide the establishment of histone modifications (Reddington *et al*., 2013; Nan *et al*., 1998); however several studies indicate that DNA methylation is also influenced by the initial presence of specific histone modifications patterns (Ooi *et al*., 2007; Zhao *et al*., 2009; Baubec *et al*., 2015 and reviewed in Cedar and Bergman, 2009). DNA methylation is established and maintained by the activity of a family of DNA methyltransferase enzymes (DNMTs), which catalyse the transfer of a methyl group from *S*-adenosyl-L-methionine to position 5 of cytosines in CpG dinucleotides, generating 5-methylcytosine (5mC) (Bird, 2011). DNA methylation usually acts as a transcriptional repressor (Weber *et al*., 2007). It could perform this task either by physically hindering the binding of transcriptional activators to the DNA or by recruitment of methyl-CpG-binding domain proteins (MBDs) (Klose and Bird, 2006). These can in turn recruit chromatin remodelling proteins like HDACs, leading to heterochromatin formation (Ooi *et al*., 2007).

1.6 DNA METHYL-TRANSFERASES

DNMT1 is mainly responsible for maintaining DNA methylation patterns during mitosis while DNMT3A and DNMT3B are central for de novo methylation (Herman *et al*., 2004; Okano *et al*., 1999). Folic acid/folate acts as the major 1-carbon donor necessary for the DNA methylation pathway (Crider *et al*., 2012; Rogers, 1995). Despite the fact that DNMT3A and DNMT3B are highly similar in their protein sequence, they show different preferences for DNA sequences around the CpG target sites *in vivo* (Wienholz *et al.*, 2010). Moreover,

DNMT3A and DNMT3B seem to methylate different genomic regions. In this respect, DNMT3B has an increased ability to methylate the nucleosome core region, while DNMT3A is better at methylating naked DNA (Takeshima *et al*., 2006). Interestingly, DNMT3A and DNMT3B also show a different expression pattern during embryogenesis, pointing to distinct functions of both enzymes (Watanabe *et al*., 2002).

DNA methylation and histone PTMs are controlled by different chemical reactions and also require different enzymes, however they are not disconnected but are working in concert to modulate gene expression (Vaissière *et al*., 2008). DNA methylation can both influence and be influenced by histone PTMs (Reddington *et al*., 2013; Ooi *et al*., 2007). Recent evidence indicates that histone methylation can help to direct *de novo* DNA methylation patterns (Baubec *et al*., 2015). At the molecular level, this crosstalk can be mediated through direct interactions between HMTs, HDACs and DNMTs (Robertson *et al*., 2000; Probst *et al*., 2009; Rose and Klose, 2014). Promoters with unmethylated CpG islands show elevated levels of methylated H3K4, suggesting that this chromatin mark protects from DNA methylation (Ooi *et al*., 2007; Hu *et al*., 2009). In fact, *de novo* DNA methylation is laid down by DNMT3A or DNMT3B in a complex with DNMT3L, a closely related homologue that lacks methyltransferase activity. DNMT3L recognizes unmethylated H3K4 through its amino terminus and recruits DNMT3A through its carboxyl terminus, hence linking unmethylated H3K4 to the DNA methylation machinery (Ooi *et al*., 2007). Therefore, demethylation of H3K4 appears to be a critical step in the establishment of DNA methylation patterns. Remarkably, DNMT3A was suggested to be a relevant tumour suppressor gene (Peters *et al*., 2014; Raddatz *et al*., 2012; Gao *et al*., 2011).

1.7 TRANSGENERATIONAL EPIGENETIC MEMORY

Calorie restriction has been shown to be able to increase promoter DNA methylation, inducing gene silencing (Chung *et al*., 2013). Interestingly, it has long been described that DNA methylation patterns which are established *in utero* are usually sustained for the rest of life, determining the fate of every cell (Lillycrop *et al*., 2005). Research on starvation in human populations seems to support these findings. A study on individuals conceived during the Dutch Hunger Winter from 1944 to 1945 showed alterations in DNA methylation and an increased risk of schizophrenia and depression (De Rooij *et al*., 2010; Painter *et al*., 2008; Tobi *et al*., 2014; Heijmans *et al*., 2008). Hence, Barker *et al*. proposed the 'fetal origin of adult disease hypothesis', where embryos adapt to a deprived intrauterine environment by changing their epigenome, thereby affecting disease risks later in life (Barker and Osmond, 1986; MacLennan *et al*., 2004).

A further, truly transgenerational, epigenetic memory was described by Bygren and co-workers. While it has long been known that famine can have long-term adverse effects (de Rooij *et al*., 2010; Hayward *et al*., 2013), the use of demographical data from the remote northernmost part of Sweden, Norrbotten, where a bad harvest meant starvation in the 19th

century, showed that the opposite can also be true. They unveiled a significant connection between the availability of food during the prepubertal slow-growth period (5-12 years of age) and the life expectancy of the second generation offspring. Overall, boys enjoying a plentiful harvest had grandsons which lived on average 6 years shorter than the grandsons of starved boys (Bygren *et al*., 2001; Kaati *et al*., 2007; Bygren, 2013).

Transgenerational memory has been studied more intense in lower organisms. An impressive, however disputed, study in mice showed that a fearful memory can persist over at least two generations and that this memory is probably transmitted through alterations in DNA methylation (Dias and Ressler, 2014). Previous work by the lab of Anne Brunet has shown that a deficiency in H3K4me3 can be inherited from one generation to the next in *Caenorhabditis elegans*. This epigenetic memory can in fact induce longevity in up to three generations (Greer *et al*., 2010; Greer *et al*., 2011). Moreover, a recent study has shown that Polycomb repressive complex 2 (PRC2) is involved in the transmission of a H3K27 methylation-dependent cellular memory. Thus, H3K27 methylation patterns are sustained through several rounds of cell division, maintaining repressed chromatin patterns from mother to daughter cells. Interestingly, the H3K27me patterns introduced by PRC2 are even sustained during embryogenesis (Gaydos *et al*., 2014). In a yeast system, it has been demonstrated that drug-induced histone hyperacetylation and gene activation can be sustained over dozens of cell cycles (Ekwall *et al*., 1997). Lastly, a memory of environmental stimuli has also been observed in macrophages. Here, once stimulated cells show a faster and stronger response upon re-stimulation (Ostuni *et al*., 2013).

Overall, increasing evidence is emerging that changes in epigenetic landmarks can be inherited not only during mitosis but even on the level of whole organisms.

1.8 EPIGENETICS AND CANCER

Already 1983, a link between DNA methylation and cancer was described by Feinberg and Vogelstein. They showed that cancer genomes are generally hypomethylated compared to normal cells. This early loss of methylation during tumourigenesis can predispose cells to genomic instability inducing genetic mutations (Robertson, 2005).

However, CpG islands often become hypermethylated in cancers, which leads to transcriptional silencing. Thus, nearly all tumour types have been described to show hypermetylation in a range of tumour-suppressor genes (Esteller, 2007).

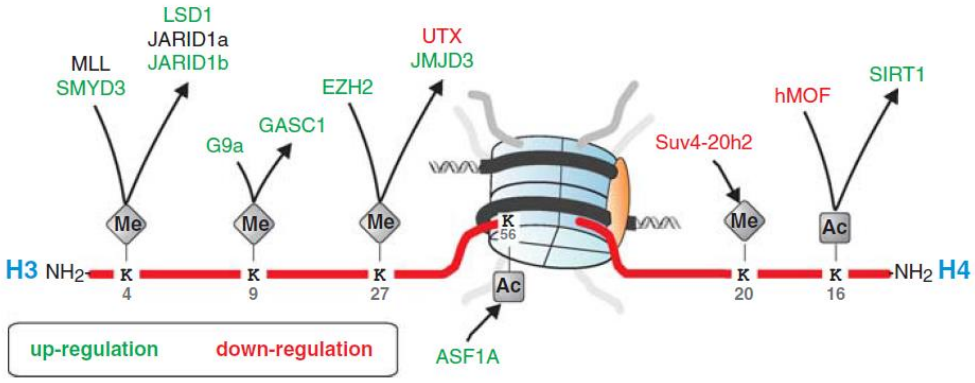

Figure 3. Histone 'writers' and 'erasers' altered in cancer cells. Green indicates an up-regulation in cancer cells compared to normal tissues and enzymes labeled in red were found to be down-regulated in cancer. Black indicates fusion proteins detected in cancer cells. Figure was adapted from Füllgrabe *et al.*, 2011.

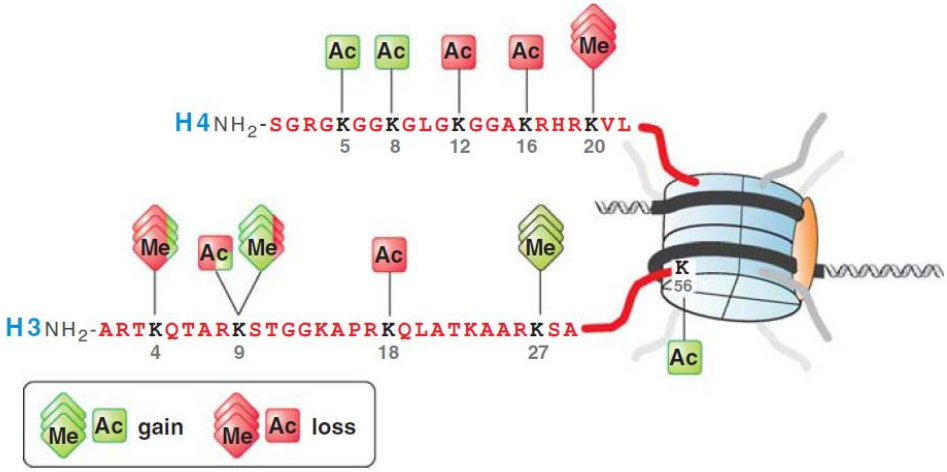

Figure 4. 'Histone onco-modifications'. Depiction of histone-modification patterns found in cancer cells. Green indicates up-regulation in cancer cells, while red indicates down-regulation. Histone modifications displayed in both colours indicate that depending on cancer type either up- or down-regulation has been described. Figure was adapted from Füllgrabe *et al.*, 2011.

Due to the correlation of specific histone modification with DNA methylation, it is not surprising that during the last years more and more histone modifications have been linked to an increasing number of cancer types (Ozdag *et al.*, 2006). However, until now only few of the more than 60 histone residues undergoing modifications have been described to be deregulated in cancer cells. Additionally, a range of histone modifying enzymes are known to be altered in cancer cells (reviewed in Sharma *et al.*, 2010 and Füllgrabe *et al.*, 2011). As specific histone modification patterns have a functional role in the life and death decision of cells, it is not surprising that these patterns influence tumour development and resistance against chemotherapy. The major histone modifications linked to cancer and the corresponding histone modifying enzymes known to be altered in a set of human cancers are displayed in **Figure 3 and 4**. Due to the importance of histone modification for cancer cells

and the impact that even the global alteration of a single histone modification can have on genome stability, we suggested the term ‘histone-onco modifications’ for the specific tumour-associated histone patterns (Füllgrabe *et al.*, 2011).

Several cancer-associated histone modifications are linked to chromatin aberrations facilitating cancer initiation and mutation (**Figure 5**) (Fuks, 2005). They are linked to aberrant gene expression (Turner, 2007; Jenuwein and Allis, 2001), genomic instability (Fournier *et al.*, 2002; Henckel *et al.*, 2009), DNA repair alterations (Li *et al.*, 2010b) or cell cycle checkpoint alterations (Liu *et al.*, 2010; Jorgensen *et al.*, 2007; Tardat *et al.*, 2007) (reviewed in Füllgrabe *et al.*, 2011).

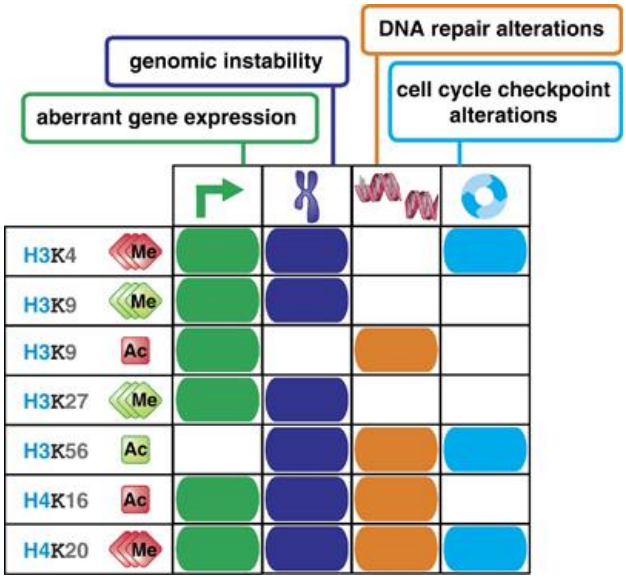

Figure 5. ‘Histone onco-modifications’ and their functional consequences. Alterations in several histone modification levels have been implied to have a role in cancer development. Figure was adapted from Füllgrabe *et al.*, 2011.

1.9 AUTOPHAGY

Macroautophagy, later referred to as autophagy, is a catabolic process which degrades the cytoplasmic contents of a cell. The word autophagy originates from the Greek terms ‘auto’ meaning self and ‘phagein’ meaning eating. Under baseline conditions autophagy serves as a quality control mechanism sustaining cellular homeostasis (Green *et al.*, 2011). Amongst other processes, autophagy has been shown to be involved in the degradation of intracellular bacteria and in the degradation of misfolded proteins (Klionsky, 2007). Autophagy-deficient mice die shortly after birth, probably due to a lack of nutrients. Autophagy is usually considered as a cell survival pathway, protecting cells from diverse stress inducers (Levine and Yuan, 2005). However, if autophagy is overstimulated it can also lead to cell death (Chen *et al.*, 2010; Levine and Yuan, 2005; Maiuri *et al.*, 2007; Platini *et al.*, 2010).

Autophagy is involved in numerous diseases, such as chronic inflammations, cancer, diabetes, cardiovascular disease, neurodegenerative diseases including Huntington, Alzheimer and Parkinson diseases. Autophagy also plays a role in physiological adaptation to

exercise, the development of the immune system and ageing (Klionsky, 2005; Mizushima *et al.*, 2008; Levine and Kroemer, 2008). Knowledge about the pathways involved in the autophagic life and death decision might help to improve autophagy-based clinical treatments.

1.10 THE AUTOPHAGY MACHINERY

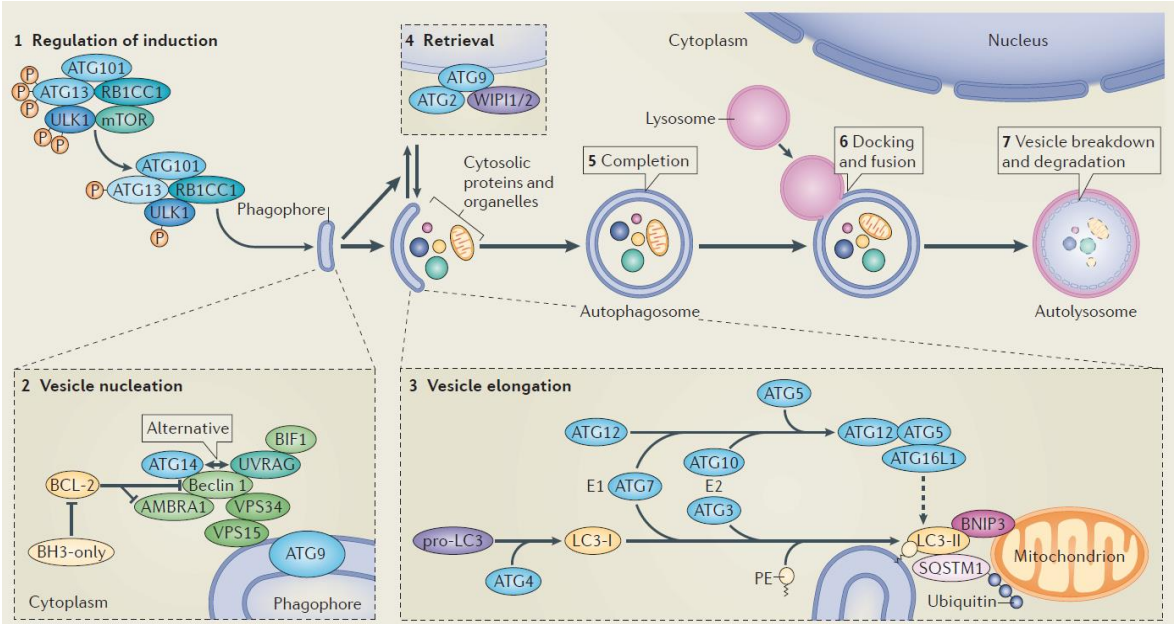

Figure 6. Depiction of the mammalian autophagy core machinery. Cytoplasmic events start with mTOR inhibition, leading to formation of autolysosomes. Previously, the nucleus was not considered to be a major autophagy regulator. Figure was adapted from Füllgrabe *et al.*, 2014b.

The basic autophagy machinery is displayed in **Figure 6**. Under basal conditions autophagy is repressed by the mTOR (mammalian target of rapamycin) kinase which phosphorylates and inactivates two autophagy-related proteins, unc-51-like kinase 1 (ULK1) and autophagy-related 13 (ATG13) (Dorsey *et al.*, 2009; He and Klionsky, 2009). Hence, most inducers of autophagy, like rapamycin, act as inhibitors of mTOR signalling. Upon activation of ULK1, vesicle nucleation is initiated, resulting in phagophore formation (Chang and Neufeld, 2010). Notably, the inhibitor of apoptosis BCL-2 (B-cell lymphoma 2) can also act as an inhibitor of autophagy by binding to beclin 1 and AMBRA1 (activating molecule in BECN1 regulated autophagy protein 1). Thus, the autophagic survival pathway and the apoptotic cell death pathway converge at the level of vesicle nucleation (He and Levine, 2010).

Vesicle elongation involves the action of two ubiquitin-like conjugation systems. In one of the pathways, phosphatidylethanolamine (PE) is conjugated to MAP1LC3 (LC3) by the protease ATG4, the E1-like enzyme ATG7 and the E2-like enzyme ATG3 (Satoo *et al.*, 2009; Sugawara *et al.*, 2005). This lipid conjugation allows LC3 to be attached to the

phagophore membrane (the lipidated form of LC3 is referred to as LC3-II). LC3 plays a role in phagophore extension and in recognizing molecules to be degraded by autophagy (Chen and Klionsky, 2011). Notably, the conversion of LC3 from processed LC3-I to the lipidated LC3-II is the main readout used in the investigation of autophagy (Klionsky *et al*., 2012).

In the second pathway, ATG12 becomes covalently bound to ATG5 by the action of the E1-like enzyme ATG7 and the E2-like enzyme ATG10. The complex of ATG5, ATG12 and ATG16-like1 (ATG16L1) could potentially act as an E3-like ligase on LC3 (Chen and Klionsky, 2011).

One of the interaction partners of LC3 is sequestosome 1 (SQSTM1, also called p62) which is a cargo receptor identifying ubiquitinated protein, organelles or even intracellular bacteria (Shvets *et al*., 2008). Once the autophagosome is formed, ATG proteins are released and recycled. The autophagosome then fuses with lysosomes creating the autolysosome. The acidic pH and the lysosomal enzymes in turn degrade the cargo of the autolysosome and its inner membrane (reviewed in Füllgrabe *et al*., 2014b).

1.11 AUTOPHAGY AND CANCER

The deregulation of autophagy is regarded as a hallmark of cancer cells (Hamai *et al*., 2014). Unlike apoptosis, where its inhibition is generally a survival advantage for cancer cells, the situation is complex in the case of autophagy (reviewed in Galluzzi *et al*., 2015). Initially, autophagy acts as a tumour suppressor preventing the accumulation of damaged proteins and organelles that have oncogenic potential (Liu and Ryan, 2012; Lorin *et al*., 2013). However, neoplastic cells often rely on autophagy for chemotherapy resistance and survival under conditions of nutrient limitation (Yang and Klionsky, 2010). Here, autophagy can promote tumour growth by protecting the cells from environmental or intracellular stimuli that would otherwise lead to cell death (Cai *et al*., 2014; Avivar-Valderas *et al*., 2013)

One example for deregulated autophagy in cancer cells is the monoallelic loss of beclin 1, which is often found in breast, prostate and ovarian cancer (Qu *et al*., 2003). Several cancer types show an increased basal level of autophagy for the maintenance of cellular energy production. However, their ability to further induce autophagy seems reduced at least in some cancer types (Yang *et al*., 2011a; Guo *et al*., 2011). Due to the common deregulation of autophagy in cancer cells and its role in chemotherapy resistance, autophagy inducers or inhibitors have a good potential to be applied for cancer treatment (Hamai *et al*., 2014; Yang and Klionsky, 2010).

1.12 THE RETURN OF THE NUCLEUS

Since the complete autophagy machinery is present in the cytoplasm (although many autophagy-related proteins like LC3 are also present in the nucleus), the nucleus was long

ignored in autophagy research (Huang *et al*., 2015). This focus on the cytoplasmic components of autophagy was encouraged by the work of Tasdemir *et al*., showing that even enucleated cells can form LC3-puncta (Tasdemir *et al*., 2008). While absolutely true, it made researchers think for a long time that the nucleus plays no regulatory role in autophagy. This was recently referred to as an 'accidental enucleation of autophagy' (Joseph, 2015). However, over the last years evidence accumulated that transcription factors, microRNAs (miRNAs) and histone modifications are all regulating autophagy on various levels (Füllgrabe *et al*., 2014b).

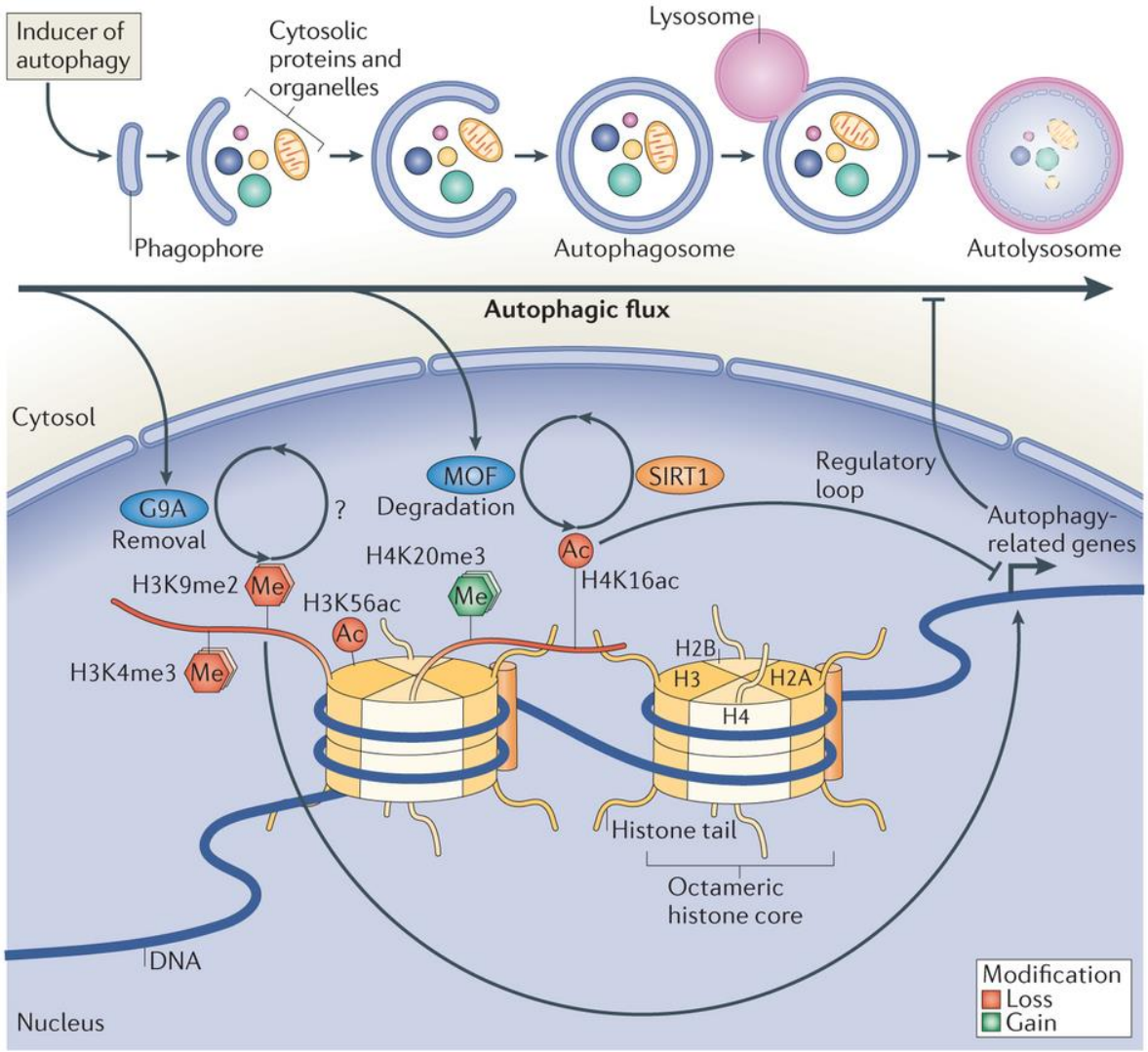

Figure 7. Histone modifications and the corresponding histone 'writers' and 'erasers' which have been linked to autophagy. Autophagy induction leads to the regulation of specific histone modifications which in turn can alter the expression of autophagy-related proteins involved in different phases of autophagy. The removal of G9a during autophagy leads to the up-regulation of LC3 and other autophagy-related genes while the loss of H4K16 acetylation late during autophagic flux acts as a negative regulatory feedback loop. Figure was adapted from Füllgrabe *et al*., 2014b.

More than 20 transcription factors have been linked to the control of autophagy-related genes (Pietrocola *et al*., 2013). One of the first impressive links between autophagy induction and transcriptional regulation was already shown in the year 2000 (Chan *et al*., 2001). Chan *et al*. showed an impressive up-regulation of ATG14 by Gln3p already 10 minutes after the induction of autophagy by rapamycin treatment. Since then, a growing number of transcription factors have been discovered to control autophagy-related genes and therefore to influence autophagic flux. These factors include β-catenin (Petherick *et al*., 2013), FOXO3 (Kenzelmann,-Broz *et al*., 2013), p53 (Zhou *et al*., 2012), SOX2 (Cho *et al*., 2013) and STAT1 (McCormick *et al*., 2012). The most prominent transcriptional regulator of autophagy is TFEB, even being referred to as the 'master regulator' of autophagy. Its translocation to the nucleus during autophagy up-regulates a whole set of autophagy-related genes involved in all stages of the process (Settembre *et al*., 2011). The counterpart of TFEB seems to be ZKSCAN3 which represses several autophagy-related genes under baseline conditions (Chauhan *et al*., 2013).

Most essential components of the autophagy-machinery have been described to be controlled by miRNAs (Frankel and Lund, 2012). Moreover, even the biogenesis of miRNA is regulated by autophagy. Autophagy promotes the degradation of inactive DICER1-AGO2 complexes which compete with correctly loaded complexes for additional factors (Gibbings *et al*., 2012; Kovaleva *et al*., 2012).

Additionally, several histone modifications have been linked to autophagy over the last years. At a local level the displacement of the H3K9 methyltransferase G9a/EHMT2 shortly after autophagy induction leads to the activation of autophagy-related genes, including MAP1LC3 (Artal-Martinez de Narvajas *et al.*, 2013). On a global level the autophagy inducer spermidine induces the loss of acetylation on histone H3 (Eisenberg *et al*., 2009). Moreover, an increase in histone H4 acetylation was described shortly after autophagy induction (Bowman *et al*., 2014). Hence, there appears to be a time- and location-dependent pattern of histone modifications, occurring after treatment with diverse autophagy inducers (Chen *et al*., 2012; Füllgrabe *et al*., 2014b)**. Figure 7** displays the specific histone modifications linked to autophagy and their suspected timing in the process. It is intriguing that the histone modifications that have already been linked to autophagy, usually seen as a survival pathway, are exactly opposing the apoptotic histone code (Füllgrabe *et al*., 2014a). Moreover, the histone modifications described to be altered during autophagy can also be changed during tumourigenesis (**Figure 8**). Overall, it appears that histone modifications participate in the cellular death or survival decision, presenting specific patterns depending on the cellular fate (Füllgrabe *et al*., 2011; Füllgrabe *et al*., 2014a).

Recently, the nuclear to cytosolic shuttling of essential autophagy-related proteins has turned out be an important regulator of autophagy. LC3 becomes deacetylated during starvation-induced autophagy by SIRT1, which results in its re-localization from the nucleus to the cytosol, where it participates in autophagosome formation (Huang *et al*., 2015). Since SIRT1

has been described to interact with a range of autophagy-related proteins, a similar mechanism can be imagined for several of them (Lee *et al*., 2008; Xie *et al*., 2015).

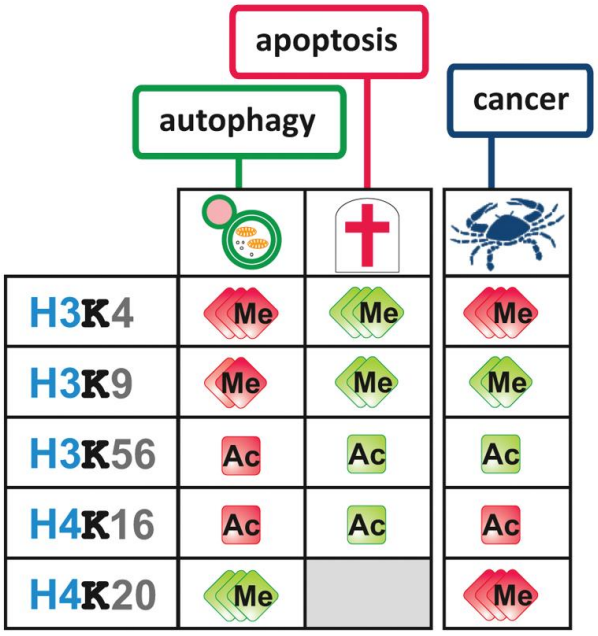

Figure 8. Comparison of the apoptotic and autophagic histone code. Histone modifications linked to autophagy show an inverse correlation to apoptotic histone modifications. Histone residues linked to autophagy have previously been described to be cancer-associated. Figure was adapted from Füllgrabe *et al*., 2014a.

In summary, autophagy regulation appears to be even more complicated than anticipated. Especially the timing of different transcription factors, histone modifications and shuttling of autophagy-related protein will be interesting to investigate in the future. Considering the recent discoveries it was speculated that autophagy is a tri-phasic process (Füllgrabe *et al*., 2014b). Initially autophagy proceeds using the proteins already present in the cytoplasm. However, transcription factors are quickly induced, leading to the transcriptional up-regulation of autophagy-related proteins needed to sustain autophagy. This involves a local loss of repressive histone marks like H3K9me2. However, to inhibit a potential cytotoxic level of autophagy, it is suppressed after prolonged activity by a global loss of active histone modifications, which shuts down autophagy-induced transcription. This inhibition of transcription could also be involved in energy preservation during times of nutrient starvation (Füllgrabe *et al*., 2014b).

1.13 CLINICAL APPLICATIONS FOR AUTOPHAGY REGULATORS

Interestingly, most anticancer agents currently in clinical use have an impact on autophagy, thus modulating autophagy seems to be a promising approach in cancer treatment (Kroemer *et al*., 2010). Hence, there have been several clinical trials exploring the possibilities of both, autophagy-inducing and autophagy-inhibiting drugs, for cancer treatment (Hamai *et al*., 2014). Especially the combination of the autophagy inhibitor hydroxychloroquine with

chemotherapy seems to be a promising lead in several tumour types (Yang *et al*., 2011b). Autophagy inducers like rapamycin and its analogues have shown success in renal cell and neuroendocrine carcinomas, and lymphoma (Meric-Bernstam and Gonzalez-Angulo, 2009). In this respect, it is also interesting that many chemotherapeutic drugs and also irradiation induce autophagy by themselves (Paglin *et al*., 2001; Li *et al*., 2010a; Amaravadi *et al*., 2007; Abedin *et al*., 2007).

Hitherto, only the cytoplasmic components of autophagy have been considered for the design of autophagy-based anti-cancer treatments. However, the newly discovered nuclear involvement in autophagy can open up new opportunities for treatment. Interestingly, combinations of rapamycin analogues and HDAC inhibitors, like suberoylanilide hydroxamic acid (SAHA), have already been employed for the treatment of solid tumours and lymphomas (ClinicalTrials.gov). The combination of autophagy inducers and modulators of the nuclear feedback loop could have the potential to widen the range of tumours responsive to treatment.

Notably, modulation of TFEB, the nuclear 'autophagy master regulator', has been successfully employed in rodent models of several diseases. Stimulation of autophagy by TFEB overexpression can reduce the accumulation of huntingtin or α-synuclein protein aggregates associated with Huntington and Parkinson diseases, respectively (Decressac *et al.*, 2013; Tsunemi *et al.*, 2012).

2 AIMS OF THE THESIS

The general aims of this thesis were to investigate a potential involvement of the nucleus during autophagy. Previously, no histone modifications or DNA methylations were known to be involved in autophagy. Moreover, I wanted to investigate the role of histone modifications in the cellular life and death decision. Hence, I am here trying to return the nucleus into the spotlight of autophagy research.

Specific aims of the four projects:

Paper I

To study the role of histone modifications, especially H4K16ac, during chemotherapy-induced cell death.

Paper II

To investigate a potential involvement of H4K16ac in the regulation of autophagic flux.

Paper III

To explore the potential of an additional histone modifying enzyme, KDM4A/Rph1 to be involved in autophagy.

Paper IV

To analyze how long an autophagy-induced epigenetic memory is maintained, and further if this memory alters the autophagic flux.

3 RESULTS

3.1 PAPER I

Opposing effects of hMOF and SIRT1 on H4K16 acetylation and the sensitivity to the topoisomerase II inhibitor etoposide

Various inhibitors of histone deacetylase activity, like SAHA, are in clinical use for cancer treatment (Bolden *et al*., 2006). These HDAC inhibitors have been described to reduce the resistance of cancer cells towards chemotherapy (Karagiannis and El-Osta, 2006; Hajji *et al*., 2008). We here investigated the common mechanism between two different HDAC inhibitors, valproic acid (VPA) and trichostatin A (TSA), in cancer chemotherapy sensitization.

In this study, we used various non-small cell lung carcinoma (NSCLC) cell lines known to be resistant to treatment with etoposide (VP16). Etoposide is an inhibitor of topoisomerase IIA, inducing the accumulation of DNA double strand breaks (Hajji *et al.*, 2008). However, in highly resistant NSCLC cell lines VP16 treatment alone only induces minimal DNA damage as evident by COMET assay and the DNA damage marker γ-H2AX. COMET assay measures the degree and type of DNA damage by the movement of free DNA in an agarose gel. Remarkably, both approaches show a massive increase in DNA damage when cancer cells were co-treated with VP16 and either VPA or TSA. This increase in DNA damage was accompanied by a significant increase in cell death.

Since both VPA and TSA are HDAC inhibitors, we wondered if there is a common set of histone residues which show increased acetylation levels after treatment. We found that amongst the histone marks tested especially histone H4 lysine 16 acetylation (H4K16ac) is increased when cancer cells are treated with either of these two HDAC inhibitors. H4K16ac is an interesting target as it was previously shown that H4K16 is deacetylated in various cancer types (Elsheikh *et al*., 2009; Fraga *et al*., 2005). Furthermore, there also exists a correlation between H4K16 deacetylation and tumour progression (Fraga *et al*., 2005). To investigate if acetylation of H4K16 is indeed part of the mechanism underlying chemotherapy sensitization, we altered the expression of the two enzymes regulating H4K16ac, hMOF and SIRT1. Notably, both hMOF and SIRT1 have also been found altered in cancer. While hMOF is reduced in some cancer types, SIRT1 is increased (Pfister *et al*., 2008; Chen *et al*., 2005; Hida *et al*., 2007; Huffman *et al*., 2007). As expected, we were able to sensitize NSCLC cells by overexpression of the H4K16 HAT hMOF or by specific chemical inhibition of the H4K16 HDAC SIRT1. Significantly, using small-interfering RNA (siRNA) targeting hMOF the VPA/TSA chemotherapy sensitization was inhibited while siRNA targeting SIRT1 mimicked VPA/TSA treatment.

In summary, we were able to show that H4K16ac is indeed one of the underlying mechanisms responsible for the VPA and TSA induced etoposide sensitization.

3.2 PAPER II

The histone H4 lysine 16 acetyltransferase hMOF regulates the outcome of autophagy

Previously, the nucleus was not considered to be a regulator of autophagy (Füllgrabe *et al*., 2014b; Joseph, 2015; Tasdemir *et al*., 2008). However, since a range of autophagy-related proteins have been described to be present in the nucleus we wanted to investigate a possible connection of histone modifications with the autophagic process. A likely candidate was H4K16ac as the H4K16 HDAC SIRT1 is known to play a role in starvation-induced autophagy (Lee *et al*., 2008). Furthermore, SIRT1 is known to directly interact and deacetylate several autophagy-related proteins (Lee *et al*., 2008; Morselli *et al*., 2010; Morselli *et al*., 2011).

When we investigated the level of H4K16ac after prolonged autophagy induction using rapamycin we discovered a massive down-regulation of H4K16ac on a global level. Remarkably, we observed a similar down-regulation in a panel of cell lines, ranging from human to yeast, using different autophagy-inducing drugs. This effect was significantly more pronounced in wild-type cells compared to autophagy-deficient cells showing that the H4K16 deacetylation is clearly linked to the autophagic process.

We looked into the underlying mechanism of H4K16 deacetylation and discovered that it was present in SIRT1 knockout cells to a similar degree as in wild-type cells. However, we found the H4K16 HAT hMOF to be down-regulated after prolonged treatment with autophagy inducers. We have previously seen that hMOF down-regulation is sufficient to reduce H4K16ac (Hajji *et al*., 2010). Notably, co-treatment with autophagy inhibitors like Bafilomycin A1 or chloroquine inhibited the autophagy-induced hMOF degradation.

Since H4K16 acetylation levels act as a transciptional regulator we further investigated the genomic locations of autophagy-induced H4K16 deacetylation. Using a genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) approach we identified H4K16ac peaks that were reduced after prolonged rapamycin treatment. To assess if these deacetylations have a functional output we ran another genome wide approach, global run-on sequencing (GRO-seq). GRO-seq detects genome-wide transcription occurring at a specific time point (Core *et al*., 2008). Comparing the ChIP-seq and GRO-seq results we found a significant overlap, especially in autophagy-related genes. Hence, the autophagy-induced H4K16 deacetylation seems to be at least partly responsible for the transcriptional down-regulation of autophagy-related genes.

To test the hypothesis that H4K16 deactylation is more than just correlating with autophagy, but has a functional implication, we inhibited H4K16 deacetylation using VPA treatment after autophagy induction. Notably, while VPA treatment did not induce autophagy itself, it increased autophagic flux, especially after rapamycin treatment. Thus, it appears that deacetylation plays a role in controlling autophagic flux after prolonged autophagy induction.

Since previous studies suggested that an overstimulation of autophagy can potentially lead to cell death we investigated cell death after inhibiting autophagy-induced H4K16 deacetylation (Chen *et al*., 2010; Levine and Yuan, 2005; Maiuri *et al*., 2007; Platini *et al*., 2010). None of the treatments we used to increase H4K16ac or induce autophagy resulted in cell death. However, induction of autophagy while inhibiting H4K16 deacetylation resulted in cell death in a range of cancer cell lines. This cell death was inhibited by various inhibitors of autophagy. Hence, a transcriptional inhibition by H4K16 deacetylation acts as a negative feedback loop during autophagy, preventing lethal levels of autophagy.

In summary, we discovered an autophagic feedback loop through H4K16 deacetylation resulting in reduced expression of autophagy-related genes. H4K16 deactylation only represents a first mark in a growing number of histone modifications discovered to be involved in autophagy regulation.

3.3 PAPER III

Rph1/KDM4 mediates nutrient-limitation signaling that leads to the transcriptional induction of autophagy

After discovering that H4K16ac is a regulator of autophagy we set out to find other nuclear regulators of autophagy. In a yeast system we screened over 150 mutants lacking either a transcription factor or a DNA-binding protein. This screen revealed Rph1 as a repressor of autophagy-related genes.

Rph1 is the only H3K36me3 demethylase in *Saccharomyces cerevisiae* (Tu *et al*., 2007). H3K36me3 is usually found in gene bodies and is associated with active transcription. When we investigated the expression levels of several autophagy-related genes involved in different steps of the autophagic pathway we found several of them up-regulated in the *rph1*Δ strains. Interestingly, in a mutant strain of the Rph1 paralogue Gis1, we also found an elevation of autophagy-related genes; however, to a lower extend than in the *rph1*Δ strain (Liang *et al*., 2013; Jang *et al*., 1999). The double mutation of both genes was even more efficient in the up-regulation of autophagy-related genes. We noted that this up-regulation was only present under baseline conditions but there was no difference in mRNA levels after autophagy induction in any of the mutant strains. Furthermore, a degradation of Rph1 seems to be naturally occurring during autophagy, leading to a release of autophagy-related genes from its inhibition. This down-regulation is dependent on a Rim15-dependent phosphorylation of Rph1.

As Atg7 mRNA was the most up-regulated mRNA discovered in the *rph1*Δ stain, we investigated the impact of different Atg7 expression levels on autophagy. We found that indeed, reduced levels of Atg7 alone are sufficient to inhibit autophagy under nitrogen starvation.

To further analyse the mechanism of autophagy regulation by Rph1, we overexpressed Rph1 in *Saccharomyces cerevisiae*. As expected, Rph1 overexpression inhibited the autophagy-induced up-regulation of autophagy-related genes, which in turn inhibited autophagy. We used two Rph1 mutants, one deficient for DNA binding activity and one deficient for histone demethylase activity. Surprisingly, and in contrast to our previous findings on H4K16ac, only its DNA binding, but not histone demethylase activity seem to be important for Rph1 to inhibit autophagy.

In summary, we found that Rph1 is required for the transcriptional inhibition of several autophagy-related genes under baseline condition. However, this function of Rph1 seems to be independent of its role as a H3K36 demethylase. Significantly, the role of Rph1 in autophagy regulation is conserved from yeast to mammals as we found that its homologue KDM4A acts similar in a human cancer cell line.

3.4 PAPER IV

The DNA methyltransferase DNMT3A contributes to autophagy long-term memory

Previous results from our lab and others have shown an involvement of a multitude of histone modifications and histone modifying enzymes in autophagy (Artal-Martinez de Narvajas *et al*., 2013; Füllgrabe *et al*., 2013). However, the true epigenetic nature of these autophagy-induced chromatin changes has never been investigated. According to the definition of epigenetic, the induced changes should be heritable (Berger *et al*., 2009). To investigate the possibility of an epigenetic memory of autophagy we treated cells for a short time with autophagy inducers after which we allowed them to recover for a prolonged period of time.

In order to induce autophagy, human cancer cells were either treated for four hours with rapamycin or Torin-1 or they were starved for amino-acids for four hours. We investigated changes in the cells two to four weeks after this initial autophagy induction. Most commonly autophagy is assessed by the accumulation of LC3-II by Western blot from (Klionsky *et al*., 2012). When we compared the protein level of LC3 between autophagy pre-treated and control cells we not only observed a reduction of LC3-II but also LC3-I in pre-treated cells. Therefore, we suspected that cells which have once undergone autophagy display a reduced ability to trigger autophagy again. To prove this hypothesis we transfected pre-treated and control cells with a GFP-RFP-LC3 plasmid and analysed the accumulation of LC3-puncta. While we saw no significant difference in autophagic flux, the Torin-1 pre-treated cells showed a clear reduction of LC3-puncta.

Furthermore, we continued to investigate which genes have been marked for an autophagic memory by the initial treatment. To this end we analysed the differential mRNA expression levels of 84 autophagy genes in parallel using the Qiagen RT Profiler. Overall, changes between autophagy-memory and control cells were minimal. Remarkably, amongst the 84 autophagy-related genes the only two genes found significantly down-regulated in pre-treated

cells were the two LC3 isoforms MAP1LC3A and MAP1LC3B. This correlates with the reduced level of LC3 found by Western blot and LC3-puncta formation.

To find an indication for the underlying epigenetic mechanism for the establishment of an autophagic long-term memory we performed GRO-seq. This allowed us to detect transcriptional changes shortly after autophagy induction. We found a subtle but consistent up-regulation of the *de novo* DNA methyl-transferase DNMT3A. An induction of stable DNA methylation on the MAP1LC3 gene could explain the persistent down-regulation of LC3 found in autophagy memory cells.

Therefore, we analysed genome-wide differences in DNA methylation levels between memory and control cells. As expected, we found an accumulation of DNA methylation patterns in autophagy-memory cells on the genes of MAP1LC3A and MAP1LC3B.

In summary, we have shown that four hours of autophagy stimulation are sufficient to induce an epigenetic memory of autophagy that can last at least one month in cell culture. The underlying mechanism involves a short up-regulation of DNMT3A. This autophagic memory results in an altered autophagic flux kinetic in the pre-treated cells when they are subjected to a second induction of autophagy. Our discoveries presented here might seem initially surprising, but there have already been several reports about a transgenerational epigenetic memory (Ekwall *et al*., 1997). In this respect, as several histone modifications have clearly been linked to autophagy, an epigenetic memory of autophagy should have been expected (Greer et *al.*, 2010; Gaydos *et al*., 2014).

4 GENERAL DISCUSSION

Ever since it was described that cells are able to induce autophagic LC3-puncta even in the absence of a nucleus, the nucleus was almost completely ignored in autophagy research (Tasdemir *et al*., 2008; Joseph, 2015). While an ever increasing number of transcription factors regulating autophagy-related genes were discovered over the past years, a true epigenetic regulation of autophagy was never speculated (Pietrocola *et al*., 2013).

Our findings, showing that H4K16 is deacetylated during prolonged treatments with autophagy inducers (**Paper II**), sparked new interest in the epigenetic regulation of autophagy with several papers following-up on autophagy regulatory histone modifications (Artal-Martinez de Narvajas *et al*., 2013; Huang *et al*., 2015; Füllgrabe *et al*., 2014b). Specifically the involvement of H4K16 is of interest as we have described previously that it is involved in cancer cell resistance to chemotherapy and apoptosis (**Paper I**). Notably, we have proven that the hMOF-dependent deacetylation of H4K16ac is more than a simple bystander of autophagy but an important regulatory inhibitor of a set of autophagy-related proteins (**Paper II**). Hence, this single histone modification is a regulator of a cell death (apoptosis) and a cell survival pathway (autophagy) rendering H4K16ac a key regulator of the cellular life and death decision.

Since chromatin modifications are often co-regulated and can have an impact on each other, we hypothesized that a wide range of epigenetic regulators are involved in autophagy. We have already seen that the H4K16ac associated H3K4me3 histone modification is globally down-regulated together with H4K16ac during autophagy (**Paper II**). Additionally, we have observed that the H3K36me3 demethylase KDM4A/Rph1 acts a repressor of autophagy under baseline conditions by limiting the expression of autophagy-related genes (**Paper III**). When autophagy is induced, KDM4A/Rph1 is degraded in a phosphorylation-dependent manner leading to increased expression of autophagy-related genes including ATG14 and ATG7. However, in the case of Rph1 its own histone demethylase activity does not seem to be required for the repression of autophagy-related genes. Possibly, it acts as a recruiter of other factors leading to specific gene silencing (**Paper III**).

We next investigated how long these autophagy-related chromatin changes are sustained, altering the cellular autophagic flux (**Paper IV**). Surprisingly, even a short autophagy induction leads to a prolonged alteration in baseline autophagy level. This ‘autophagic memory’ seems to be induced by a short induction of the *de novo* DNA methyltransferase DNMT3A leading to a stable increase in DNA methylation in autophagy-related genes. These long-lasting epigenetic changes seem to modify the cellular response towards a novel autophagy induction.

The stable down-regulation of autophagy-related genes seems to be reasonable from an evolutionary point of view. Cells that have experienced nutrient-limitation before would want to adapt their transcriptional program for a low-nutrient condition. Hence, these conditioned

cells might slow down autophagic flux to not induce a lethal level of autophagy. Moreover, these findings can be important for everyday cell culture experiments involving starvation of cells, for example during plasmid transfection. Thus, the transfection condition itself has the potential to alter transcription and cellular response for a long time (Man *et al*., 2010).

5 FUTURE PERSPECTIVE

The results by our lab and others have unveiled a network of chromatin modifications regulating autophagy induction and flux (reviewed in Füllgrabe *et al*., 2014b). However, given the young age of this field I expect H4K16ac, H3K4me3 and H3K9me2 to only represent the tip of the iceberg. In the near future, many more histone modifications will be linked to autophagy. Especially the local changes on chromatin landscape have not been investigated and will offer insight into the autophagic process.

Our results on DNA methylation also leave several questions to be answered. How is DNMT3A induced? Which factors are responsible for its specific targeting to autophagy-related genes? Moreover, giving the stable effects we are seeing it remains to be analysed if similar effects can be observed in whole organisms and how long an autophagic memory can be sustained *in vivo*.

To this end, results published by Bygren *et al.* are highly interesting. They showed that starvation can not only affect your own lifespan but also the lifespan of your children and grandchildren. They unveiled a significant connection between the availability of food during the prepubertal slow growth period (5-12 years of age) and the lifetime of the second generation offspring. The transgenerational memory on lifespan and disease risk produced by starvation indicates the induction of stable, heritable changes in the epigenome (Bygren *et al*., 2001). A direct link between environmental stimuli (hunger), regulation of histone acetylation and lifespan was established by Eisenberg *et al*. (Eisenberg *et al*., 2014). They have shown that depletion of the energy metabolite AcCoA, which acts as the acetyl-donor for histone acetylation, is sufficient to induce autophagy and prolong lifespan. Hence, it is intriguing to speculate that the 'autophagic memory' is at least partly underlying the transgenerational increase in lifespan observed after starvation. Remarkably, the two most efficient inducers of longevity in mice known up to this date, calorie restriction (Morselli *et al*., 2010 and reviewed in Chung *et al.*, 2013) and rapamycin are also the most classical inducers of autophagy (Harrison *et al.*, 2009). It was also shown that autophagy can delay age-associated phenotypes and age-associated disease (Rubinsztein *et al*., 2011; Gelino and Hansen, 2012). Interestingly, calorie restriction has already been shown to be able to increase promoter DNA methylation, inducing gene silencing (Chen *et al*., 2013). The direct link between DNA methylation and aging has been impressively described by Steve Horvath. He showed that the precise age of cells and individuals can be read by their DNA methylation patterns. Thus, cells display an 'epigenetic clock' highlighted by differential DNA methylation in 353 CpG sites where both, embryonic and induced pluripotent stem (iPS) cells mark age zero (Horvath, 2013). Given our results, one could speculate that DNA methylation induced by autophagy is one pathway how autophagy inducers can lead to longevity. Notably, the autophagy-associated histone modifications H4K16ac and H3K4me3 have also been linked to longevity (Dang *et al*., 2009; Greer *et al*., 2010). Hence, it would be interesting to investigate the exact relationship between autophagy inducers, chromatin modifications and lifespan in the future.

Furthermore, considering the role of autophagy in cancer and its link to cell death, the modulation of autophagy is a promising target for cancer therapy. Autophagy modulators, including rapamycin and chloroquine, are drugs already in clinical use with relatively mild side effects. The recently discovered epigenetic regulation of autophagy has the potential to lead to new drug combinations to be used against various cancer types.

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