Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XXVIII cycle - 2012–2015
Coordinator: Prof. Massimo Santoro

”HMGA1 knock-down induces autophagy through regulation of *ULK* gene expression in cancer cells”

Andrea Conte

University of Naples Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

Administrative Location

Dipartimento di Medicina Molecolare e Biotecnologie Mediche
Università degli Studi di Napoli Federico II

Partner Institutions

- Università degli Studi di Napoli "Federico II", Naples, Italy
- Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy
- Seconda Università di Napoli, Naples, Italy
- Università degli Studi di Napoli "Parthenope", Naples, Italy

Faculty

Francesco Beguinot
Roberto Bianco
Bernadette Biondi
Francesca Carlomagno
Maria Domenica Castellone
Gabriella Castoria
Angela Celetti
Annamaria Cirafici
Annamaria Colao
Gerolama Condorelli
Valentina De Falco
Vittorio De Franciscis
Sabino De Placido
Gabriella De Vita
Monica Fedele
Pietro Formisano
Alfredo Fusco
Fabrizio Gentile
Domenico Grieco
Michele Grieco
Maddalena Illario
Paolo Laccetti
Antonio Leonardi
Paolo Emidio Macchia
Rosa Marina Melillo
Claudia Miele
Nunzia Montuori
Roberto Pacelli
Giuseppe Palumbo
Giovanna Maria Pierantoni
Rosario Pivonello
Giuseppe Portella
Maria Fiammetta Romano
Giuliana Salvatore
Massimo Santoro
Donatella Tramontano
Giancarlo Troncone
Giancarlo Vecchio
Mario Vitale

“HMGA1 knock-down induces autophagy through regulation of *ULK* gene expression in cancer cells”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

1. Pierantoni GM*, **Conte A***, Rinaldo C, Tornincasa M, Gerlini R, Federico A, Valente D, Medico E, Fusco A. Deregulation of HMGA1 expression induces chromosome instability through regulation of spindle assembly checkpoint genes. Oncotarget. 2015 Jul 10;6(19):17342-53. (*Co-first authors)

2. **Conte A**, Pierantoni GM. Regulation of HIPK Proteins by MicroRNAs. Microrna. 2016;4(3):148-57

3. Anzilotti S, Tornincasa M, Gerlini R, **Conte A**, Brancaccio P Cuomo O, Bianco G, Fusco A, Annunziato L, Pignataro G, and Pierantoni GM. Genetic ablation of homeodomain interacting protein kinase 2 (HIPK2) selectively induces apoptosis of cerebellar Purkinje cells during adulthood and generates an ataxic-like phenotype. Cell Death Dis. 2015 Dec 3;6:e2004

4. **Conte A**, Procaccini C, Iannelli P, Kisslinger A, De Amicis F, Pierantoni GM, Mancini FP, Matarese G and Tramontano D. Effects of Resveratrol on p66Shc phosphorylation in cultured prostate cells. Transl Medi UniSa 2015, 13(8): 47-58

5. Pierantoni GM*, **Conte A***, Rinaldo C, Tornincasa M, Gerlini R, Valente D, Izzo A, Fusco A. Hmga1 null mouse embryonic fibroblasts display downregulation of spindle assembly checkpoint gene expression associated to nuclear and karyotypic abnormalities. Cell Cycle. 2016 Feb 18:0; 15(6):812-818 (*Co-first authors)

This dissertation is based upon the following paper:

Conte A, Bianco G, Paladino S, Gerlini R, Tornincasa M, Fasano D, Fusco A, Tramontano D, Pierantoni GM. The inhibition of High Mobility Group A1 protein expression induces autophagy. (Manuscript in preparation)

ABSTRACT

High Mobility Group A1 (HMGA1) is a chromatinic protein whose overexpression is a feature of malignant neoplasias. Many studies support its causal role in cell transformation and cancer progression. Indeed, HMGA1 is an architectural transcriptional factor that regulates, by binding DNA and interacting with various transcriptional regulators, the expression of several genes involved in critical biological processes, such as cell proliferation, apoptosis and migration. Autophagy is a self-degradative process that, providing energy sources, removing damaged organelles and misfolded or aggregated proteins, allows cell survival in stress conditions or, when iper-activated, leads to non-apoptotic programmed cell death. Autophagy is often deregulated in cancer cells in which plays an important and complex role, being mainly oncogenic during cancer initiation, and tumor-suppressive during cancer progression. Studying the effects of HMGA1 knock-down in skin cancer cells SCC-13, I have found that it increases autophagy, as assessed by both western blot and immunofluorescence analysis of several autophagic markers, such as pS6, LC3 and SQSTM1/p62. Interestingly, the ability of HMGA1 depletion to increase autophagy is not restricted to skin cancer cells, since similar results have been achieved also silencing HMGA1 expression in HeLa cells, and mouse embryonic fibroblasts null for *Hmga1* are more susceptible than the wild-type counterpart to undergo autophagy after starvation or treatment with rapamycin. Consistently, silencing of HMGA1 upregulates the two autophagy-initiating kinases Unc-51-like kinase 1 (ULK1) and Unc-51-like kinase 2 (ULK2), and functional experiments demonstrate that HMGA1 binds their promoter regions and negatively regulates their transcription. Accordingly, the block of ULK1 expression reduces the pro-autophagic effects induced by HMGA1 silencing indicating that they are, at least in part, mediated by ULK1. Taken together, these results clearly indicate that HMGA1 protects cancer cells from autophagy, thus suggesting, on one hand, a new mechanism through which HMGA1 can contribute to cancer progression and, on the other hand, a mechanism through which autophagy can be deregulated in cancer cells.

1.Introduction

1.1 HMGA proteins

The High Mobility Group (HMG) family is composed by heterogeneous chromatin non-histone proteins with the common feature of being smaller than 30 kDa (so they have an “high mobility” in electrophoresis gels) and rich in both acidic and basic amino acids. In mammals HMG proteins have been classified into three distinct sub-families: HMGB (formerly HMG-1/-2), HMGN (formerly HMG-14/-17) and HMGA (formerly HMG-I/-Y/-C). The characteristic functional motifs of HMGB, HMGN and HMGA sub-families are the “HMG-box”, the “nucleosomal binding domain” and the “AT-hook” respectively. HMGB and HMGN proteins are involved in development (Furusawa and Cherukuri 2010) and HMGB proteins play also an important role in inflammation and innate immune response (Yanai et al. 2009). HMGA proteins are involved in many biological process such as development, cell growth, differentiation, apoptosis and cancer. This sub-family is composed of four proteins: HMGA1a (Johnson et al. 1989), HMGA1b (Friedamnn et al. 1993), HMGA1c (Nagpal et al. 1999) and HMGA2 (Chau et al. 1995) (formerly HMGI, HMGY, HMG-I/R and HMGI-C respectively). They are encoded by two distinct genes: *HMGA1* and *HMGA2*. *HMGA1* encodes HMGA1a5, HMGA1b6 and HMGA1c through alternative splicing, whereas *HMGA2* encodes HMGA2. In addition, there are also numerous conserved pseudogenes in both mice and humans (De Martino et al. 2016).

The HMGA proteins are characterized by small size (10.6-12 kDa), solubility in dilute acids, high concentration of basic, acidic and proline amino acids, rapid mobility electrophoresis and by the ability to bind to the minor groove of short AT-rich DNA stretches (Lund et al. 1983). Spectroscopic techniques, such as circular dicroism and nuclear magnetic resonance spectroscopy, indicated that the HMGA proteins, as free molecules, had very little, if any, secondary structure. Nevertheless, when bound to other molecules, such DNA or protein substrates, the HMGA proteins assume

defined structural features (Reeves et al. 1987, Lehn et al. 1988). The HMGA proteins undergo a lot of different post-translational modifications (phosphorylation, acetylation, methylation and poly-ADP-ribosylation) which are dynamic and rapidly responding to both intra- and extracellular signaling events (Reeves and Beckerbauer 2001). These modifications also influence both the substrate-binding properties of the HMGA proteins and their biological activities. For example Cdc2 and Homeodomain-Interacting Protein Kinase 2 (HIPK2) phosphorylate HMGA1 resulting in a decrease in DNA binding (Zhang and Wang 2007).

The structure of each HMGA protein is characterized by three N-terminal AT-hook domains and a C-terminal domain involved in protein-protein interactions (see figure 1.1). The AT-hook domains display the consensus sequence of Pro-Arg-Gly-Arg-Pro flanked on either side by a number of positively charged lysine/arginine residues. The core of AT-hook motif is highly conserved in evolution and is also found in a large number of other, non-HMGA, proteins, especially transcription factors or components of chromatin remodeling complexes (Aravind and Landsman 1998). The HMGA proteins recognize DNA structure (rather than nucleotide sequences) like bent and supercoiled DNAs (Nissen and Reeves 1995, Bustin and Reeves 1996), synthetic fourway junctions (Hill et al. 1999), base-unpaired regions of AT-rich DNA (Liu et al. 1999) and restricted regions of DNA on the surface of nucleosomal core particles (Reeves and Nissen 1993). HMGA binding can induce structural changes in bound DNA substrates. Depending on the sequence, the organization, the topology or the length of the substrates, HMGA binding can bend, straighten, unwind and induce looping in linear DNA molecules (Esposito 2008). Exerting their role of architectural transcription factors, HMGA proteins also participate in protein-protein interactions and induce structural changes in protein substrates, many of which are gene regulatory factors.

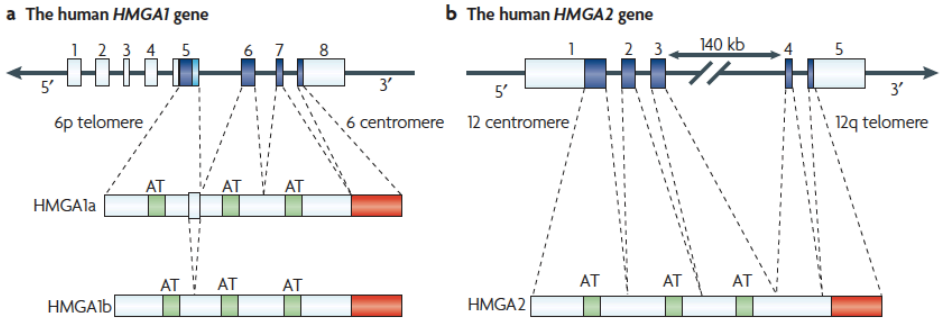

Figure 1.1 Schematic representations of *HMGA1* and *HMGA2* genes and HMGA1 and HMGA2 proteins. Each protein contains three basic domains, named AT hook (green box), with which they bind DNA, and an acidic carboxy-terminal region (red box) involved in protein-protein interactions (image from Fusco and Fedele 2007).

1.1.1 HMGA proteins regulate chromatin remodeling and gene expression

Chromatin structure plays a fundamental role in the regulation of gene expression in eukaryotic cells. Chromatin may exert a repressive effect on gene transcription, if either nucleosomes or other inhibitory chromatin proteins (as histone H1) are associated with critical regulatory regions of gene promoters or enhancers. Elaborate mechanisms, such as ATP-dependent chromatin remodeling machines, as well as precisely controlled biochemical modifications of histones and other regulatory protein complexes, have evolved to alter or modulate such repressive chromatin structures and allow active gene transcription (Fry and Peterson 2001).

HMGA proteins participate in gene expression regulation through different mechanisms. Even though they have not transcriptional activity *per se*, HMGA proteins act as architectural transcription factors, by interacting with the transcription machinery, altering chromatin structure and thereby regulating, negatively or positively, the transcriptional activity of several genes (Thanos and Maniatis 1992, Thanos and Maniatis 1993). In particular, HMGA proteins can modify DNA conformation and/or chromatin structure facilitating

the binding of several transcriptional factors. For example, HMGA proteins mediate the formation of an “enhanceosome”, a stereo-specific, multi-protein complex that includes HMGA proteins and other transcription factors making specific protein-DNA and protein-protein contacts in intricate but precise ways (Merika and Thanos 2001). The HMGA proteins can also influence gene transcription through direct protein-protein interactions with transcription factors by modifying their conformation and enhancing their binding affinity for DNA (see figure 1.2). When HMGA proteins act as negative regulators of gene expression, they often serve as inhibitors of enhanceosome formation, usually by sterically blocking the functional binding of other crucial transcription factors to their recognition sites in gene promoters (Esposito 2008).

There are reports of more than 60 different eukaryotic and viral genes whose transcriptional expression is regulated by HMGA proteins *in vivo* (Reeves and Beckerbauer 2001, Martinez Hoyos et al. 2004). The promoter regions of many of the regulated genes contain multiple stretches of AT-rich sequences that have been proposed to represent a gene-specific ”bar code” that is “read” by the AT-hooks during the process of transcriptional regulation.

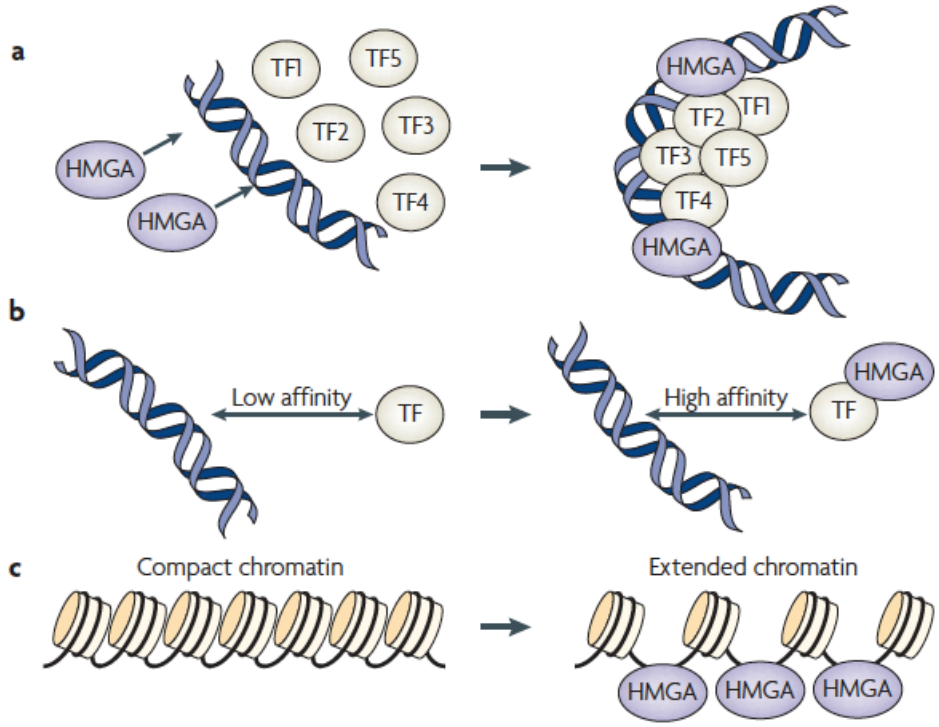

Figure 1.2 Mechanisms of action of HMGA proteins. The HMGA proteins participate in assembly or modulation of macromolecular complexes involved in the regulation of gene expression. In doing so, HMGA proteins directly bind the DNA, modifying its conformation and consequently facilitating the binding of a group of transcriptional factors (TF). HMGA proteins a) interact with both DNA and TFs to generate a multiprotein stereospecific complex bound to DNA; b) can influence gene transcription through direct protein-protein interactions with a TF by modifying its conformation and enhancing its DNA binding affinity or c) alter the chromatin structure (image from Fusco and Fedele 2007).

1.1.2 HMGA proteins are overexpressed in cancer cells

HMGA genes are physiologically expressed during embryogenesis at high levels in almost all tissues, whereas their expression is absent or low in adult healthy tissues. However the expression of both *HMGA* genes is high in malignant cells *in vitro* and *in vivo*, and it is clearly demonstrated that these genes play an important role in carcinogenesis (Fusco and Fedele 2007). The correlation between HMGA proteins and the neoplastic phenotype first emerged when two-dimensional electrophoresis found changes in nuclear proteins following the transformation of a rat thyroid cell line (FRTL5) by the Kirsten murine sarcoma virus (KiMSV) (Giancotti et al. 1985). The expression of HMGA1a, HMGA1b and HMGA2 was observed after cellular transformation. Subsequent studies validated the association of these proteins with high malignant phenotype and indicate that HMGA proteins have oncogenic activities, being causally involved in neoplastic transformation. When FRTL5 cells were transfected with an antisense construct against HMGA2 or against HMGA1 and then infected with KiMSV, they expressed significant levels of retroviral oncogene (v-ras-Ki) but did not grow in soft agar or form tumors in athymic mice. Other studies demonstrate that HMGA1a, HMGA1b or HMGA2 transforms rat fibroblast and human lymphoblastoid cells (Berlingieri et al. 1995, Berlingieri et al. 2002). Moreover it has been observed that transgenic mice over-expressing the wild type form of HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas (Fedele et al. 2005).

The induction of the *HMGA* genes in malignant transformation probably occurs through oncofetal transcriptional mechanisms which have not yet been well characterized. It is known that the elevated expression of HMGA1 in cancer cells requires a complex cooperation between SP1 family members and AP1 factors, induced by the activation of Ras GTPase signaling (Cleyen et al. 2007). Even though the molecular basis of *HMGA* genes upregulation are not fully understood so far, we know that they may change

among different tumour types, and may involve chromosomal rearrangements, micro-RNAs (miRNAs) and competitive endogenous-RNAs (ceRNAs) (De Martino et al. 2016).

Chromosomal rearrangements involving *HMGA1* or *HMGA2* genes have been found in a lot of different human benign tumor (expecially of mesenchymal origin). In particular lipomas, uterine leiomyomas, fibroadenomas of the breast, endometrial polyps, pleomorphic adenomas of the salivary glands and pituitary adenomas express a rearranged HMGA2 gene coding for a chimeric or a truncated protein that maintains its ability to bind DNA and interact with several proteins, but loses its carboxy-terminus, including the 3' untranslated region (3'UTR). Among the *HMGA2* fusion partners, tumor-suppressor genes have been described (*FHIT*, *RAD51L1* and *HEI10*). In all of these cases, the rearrangement of the partner gene results in a loss of function of the protein encoded by the tumor-suppressor gene itself, in addition to *HMGA2* overexpression (Fusco and Fedele 2007). The expression of a truncated or chimeric form of HMGA2 causes malignant transformation of NIH3T3, conversely wild-type HMGA2 did not transform cells. This difference is probably due to the absence of 3'UTR in truncated or chimeric forms of HMGA2. Without 3'UTR these forms can avoid the negative regulation of miRNAs which normally targets HMGA2 (let-7 and miRNA-98) (Lee et al. 2007, Hebert et al. 2007). In addition to HMGA2, rearrangements of HMGA1 gene have been frequently described in benign human tumors (including lipomas, uterine leyomiomas, pulmonary chondroid hamartomas, and endometrial polyps), but no intragenic rearrangements have been found (Tallini et al. 2000).

In malignant tumors HMGA proteins are expressed at high levels. In particular, HMGA 1 is expressed in all neoplastic tissues analyzed, including pancreas, thyroid, colon, breast, lung, ovary, uterine cervix and body, prostate and gastric carcinomas (Fusco and Fedele 2007). HMGA1 is expressed also in some form of leukaemia (see table 1.3). Importantly, overexpression of

HMGA1 correlated with malignancy and especially with metastatic ability, resistance to anti-cancer therapies and reduced survival. Indeed HMGA1 expression gradually increases from benign astrocytoma to the malignant glioblastoma. So HMGA1 expression might be a candidate biomarker for cancer diagnosis and prognosis (Pallante et al. 2015).

Recently, several studies reported a strong HMGA regulation by miRNAs in pituitary adenomas (mir-15, mir-16, miR-34b, mir-214, miR-326, miR-432, miR-548c- 3p, miR-570, miR-603 and mir-761), in thyroid carcinomas (let-7), and in breast cancer (mir-26a, miR- 33b). In addition, it is emerging that some HMGA-pseudogenes may "protect" HMGA transcripts from the inhibitory effects of these miRNAs by competing for the same miRNAs, acting as competitive ceRNAs (De Martino et al. 2016).

Table 1.3 Cancers associated with aberrant expression of HMGA proteins

Overexpression of full-length proteins

Lewis lung carcinoma (Giancotti *et al.* 1989)
Prostrate (Tamimi *et al.* 1993)
Thyroid neoplasias (Chiappetta *et al.* 1995)
Colorectal (Fedele et al.1996)
Squamous carcinoma of uterine cervix (Bandiera *et al.* 1998)
Non-small cell lung carcinoma (Rogalla *et al.* 1998)
Neuroblastomas (Giannini *et al.* 1999)
Burkitt's lymphoma (Wood *et al.* 2000)
Lipomas (Fedele *et al.* 2001)
Pancreatic duct cell carcinoma (Abe *et al.* 2002)
Breast (Baldassarre *et al.* 2003)
Lymphoblastic leukemia (Pierantoni *et al.* 2003)
Ovarian carcinoma (Masciullo *et al.* 2003)
Testicular germ cell tumours (Esposito *et al.* 2008)

Chromosomal translocations/AT hook rearrangements

Myeloid leukemias (Elton *et al.* 1986)
Thyroid neoplasias (Chiappetta *et al.* 1995)
Pulmonary chondroid hamartomas (Kazmierczak *et al.* 1996)
Uterine leiomyomas (Hennig *et al.* 1996)
Endometrial polyps (Hennig *et al.* 1996)
Breast hamartoma (Dal Cin *et al.* 1997)
Lipomas (Fedele *et al.* 2001)

Table 1.3 Cancers associated with aberrant expression of HMGA protein (table from Esposito 2008).

1.1.3 Oncogenic activity of HMGA1

Several mechanisms have been proposed to account for the transforming ability of HMGA proteins and most of them are based on the ability of these proteins to regulate the expression of genes that have a crucial role in the control of cell proliferation and invasion. For example, HMGA1 proteins increase the activity of several members of AP-1 transcription factors family (like JUNB and FRA1) which play an important role in the regulation of cell proliferation, tumorigenesis and metastasis (Angel et al. 1991). HMGA1 also enhance the transcriptional activity of NF-kB causing the expression of inflammatory proteins (iNOS, COX2, E-selectin, IL-2, IL-4 and GMCSF) and is demonstrated that inflammation represents a risk factor for most types of cancer (Mantovani et al. 1998). Moreover HMGA protein overexpression impairs DNA repair negatively modulating the expression of some genes involved in this process (for example *ERCC1*) and because they compete with p53 and human MutS homologue proteins for Holliday junction binding, exerting a negative influence on the DNA mismatch repair response (Subramanian et al. 2002). HMGA1 interacts with p53 and interferes with p53-mediated transcription of genes involved in regulation of apoptosis (*BAX*, *BCL2*) and cell cycle (*CDKN1A*) but promotes the transcription of the p53 inhibitor MDM2 (Esposito et al. 2010), reducing p53-dependent apoptosis. Moreover, the overexpression of HMGA1 promotes the reduction of Brn-3a binding to the *BCL2* promoter, thereby blocking the Brn-3a co-repressor function on BCL-2 expression following p53 activation (Esposito et al. 2010). HMGA1 alters the expression of genes involved in epithelial-mesenchymal transition (EMT) which is a common phenomenon in epithelial tumors (epithelial cells de-differentiate to a fibroblast-like state and regain the ability to invade, migrate and/or proliferate in an uncontrolled fashion) (Reeves et al. 2001).

1.1.4 HMGA1 and chromosome instability

Recently, we have identified a new mechanism by which HMGA1 exerts its oncogenic activity: the induction of chromosome instability (CIN) through the de-regulation of Spindle Assembly Checkpoint (SAC) genes (Pierantoni, Conte et al. 2015, Pierantoni, Conte et al 2016).

Genome instability, whose prominent form is represented by CIN, is a hallmark of cancer cells. CIN renders cancer cells prone to accumulate karyotypic alterations, such as gain or loss of whole chromosomes, translocation/deletion/duplication of chromosome segments or polyploidy. Alterations in chromosome number or aneuploidy can easily unbalance the equilibrium between oncogenes and tumour-suppressor genes, and are found in nearly all major human tumor types (Mertens et al. 1994). CIN is a feature often acquired by cancer cells during cancer progression, and is generally associated with the most aggressive forms of cancer. In cancer cells, one of the most frequent causes of CIN is the impairment of the SAC, the mechanism that prevents the anaphase onset until all chromosomes are attached with the proper amphitelic orientation to the mitotic spindle (Hartwell and Kastan 1994) (see figure 1.4). Interestingly, mutations of the genes coding for SAC proteins are quite rare in human cancers (reviewed by Rao et al. 2009), whereas their deregulation is more frequent, and it is widely demonstrated that both upregulation and downregulation of SAC genes can cause a checkpoint impairment leading to CIN. Indeed, downregulation of *Bub1* expression has been detected in a subset of acute myeloid leukemia (Lin et al. 2002), whereas upregulation of *Bub1* levels has been reported in lymphomas (Alizadeh et al. 2000), breast (van't Veer et al. 2002) and gastric cancers (Grabsch et al. 2003, Shigeishi et al. 2001). The molecular mechanisms of SAC genes regulation and de-regulation are almost still unknown.

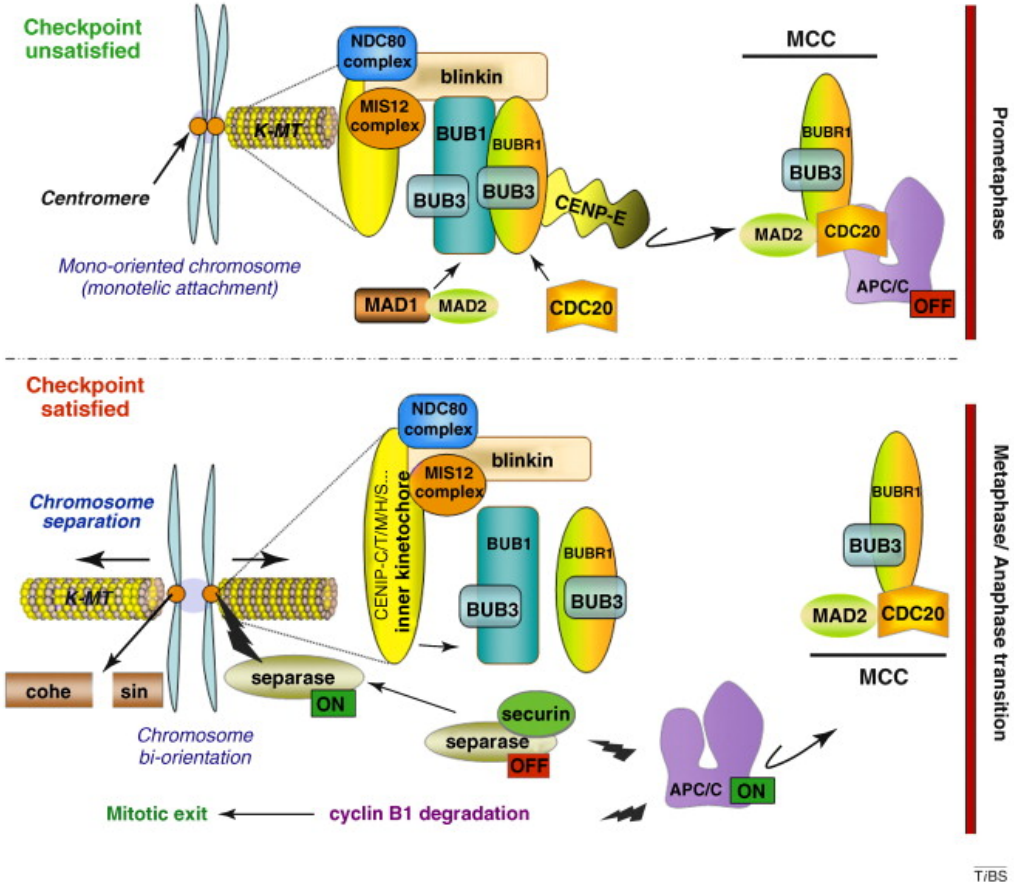

Figure 1.4 Schematic model of SAC. Cells with an unsatisfied checkpoint recruit BUB1, BUBR1, BUB3, MAD1, MAD2 and CDC20 to unattached kinetochores. An inactive open-MAD2 conformation is catalytically converted to a closed-MAD2 conformation that is able to bind to CDC20. The MAD2-CDC20 association prevents loss of cohesion of bi-oriented sister chromatids because it triggers the recruitment of BUBR1-BUB3 into an APC/C inhibitory complex. The function of SAC is linked to kinetochore-microtubule network through the physical interaction of BUB1 and BUBR1 with blinkin. In metaphase, when the checkpoint is satisfied (i.e. all chromosomes undergo bipolar attachment and are aligned at the center of the cell) it releases APC/C-CDC20 inhibition. Securin can be ubiquitylated by APC/C and degraded. This leads to the release and activation of separase that cleaves cohesin, the molecule that holds sister chromatids together at the centromere. The cleavage of cohesin at centromeres and chromosome arms is followed by chromosome separation and mitotic progression from metaphase to anaphase (image from Bolanos-Garcia and Blundell 2010).

We have demonstrated that HMGA1 increases the expression of *Bub1, Bub1b, Mad2l1* and *Mps1/Ttk* SAC genes at both mRNA and protein level, binding their promoter regions and increasing their transcriptional activity. We have found that HMGA1 knock-down compromises the mitotic checkpoint activity, and that HMGA1-depleted cells show a higher percentage of metaphases with unaligned chromosomes and reduced pro-metaphase time compared to control cells. The correlation between HMGA1 and SAC genes expression was confirmed in human tumor samples. In fact, human colon carcinomas show high SAC gene expression that correlates with HMGA1 levels, and this correlation further increases in liver metastasis of colon carcinomas (Pierantoni, Conte et al. 2015).

In mouse embryonic fibroblasts (MEFs), that physiologically express HMGA1, the genetic ablation of *HMGA1* gene causes down-regulation of SAC genes associated to features of CIN, such as nuclear abnormalities, bi-nucleation, micronuclei and karyotypic alterations (Pierantoni, Conte et al. 2016).

On the basis of these findings, HMGA1 emerges as an important factor for both physiological SAC genes regulation in embryonic development, and their pathological de-regulation in cancer cells.

1.2 Autophagy

The term ‘autophagy’ derives from the Greek, meaning ‘eating of self’, and was first coined over 40 years ago by Christian de Duve, who observed degradation of several cellular components within lysosomes of rat liver perfused with the pancreatic hormone glucagon (Deter et al. 1967). Nowadays, this term refers to a self-degradative process that provides energy sources in response to nutrient starvation, removes misfolded or aggregated proteins, cleare damaged organelles, and eliminates intracellular pathogens (Levine and Kroemer 2008). Autophagy plays a key role during embryonic development and tissue homeostasis, and it is generally considered a survival mechanism, although its deregulation may lead to non-apoptotic cell death. Moreover, autophagy has been linked to cellular senescence, antigen presentation, protection from genome instability and prevention of necrotic cell death, giving it a key role in the pathogenesis of several diseases, such as cancer, neurodegeneration, cardiomyopathy, diabetes, liver disease, autoimmune diseases and infections (Glick et al. 2010).

The main feature of autophagy is the proteolytic degradation of cytosolic components at the lysosome, and, basing on the mechanism by which the cargo reaches the lysosome, we distinguish three different types of autophagy: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. Macro-autophagy is the most common and best characterized form of autophagy, so that the term “autophagy” usually refers to macro-autophagy. During this process, cytoplasmic cargo is delivered to the lysosome through a double membrane-bound vesicle, the so-called “autophagosome”, that fuses with the lysosome to form an “autophagolysosome” or “autolysosome”; whereas, in micro-autophagy, cytosolic components are directly taken up by the lysosome itself through invagination of its membrane. Both macro-and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms (Glick et al. 2010). By contrast, chaperone-mediated autophagy (CMA) is a selective process in which target proteins are bound by

chaperones (such as Hsc-70) that mediate their translocation across the lysosomal membrane interacting with the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A) (Saftig et al. 2008). After lysosomal degradation, permeases and transporters export amino acids and other products of degradation back to the cytoplasm, where they can be re-used for building macromolecules and for metabolism (Mizushima 2007) (see figure 1.5). Thus, autophagy may be considered a cellular 'recycling factory' that represents an energy support through ATP generation and mediates damage control by removing non-functional proteins and organelles.

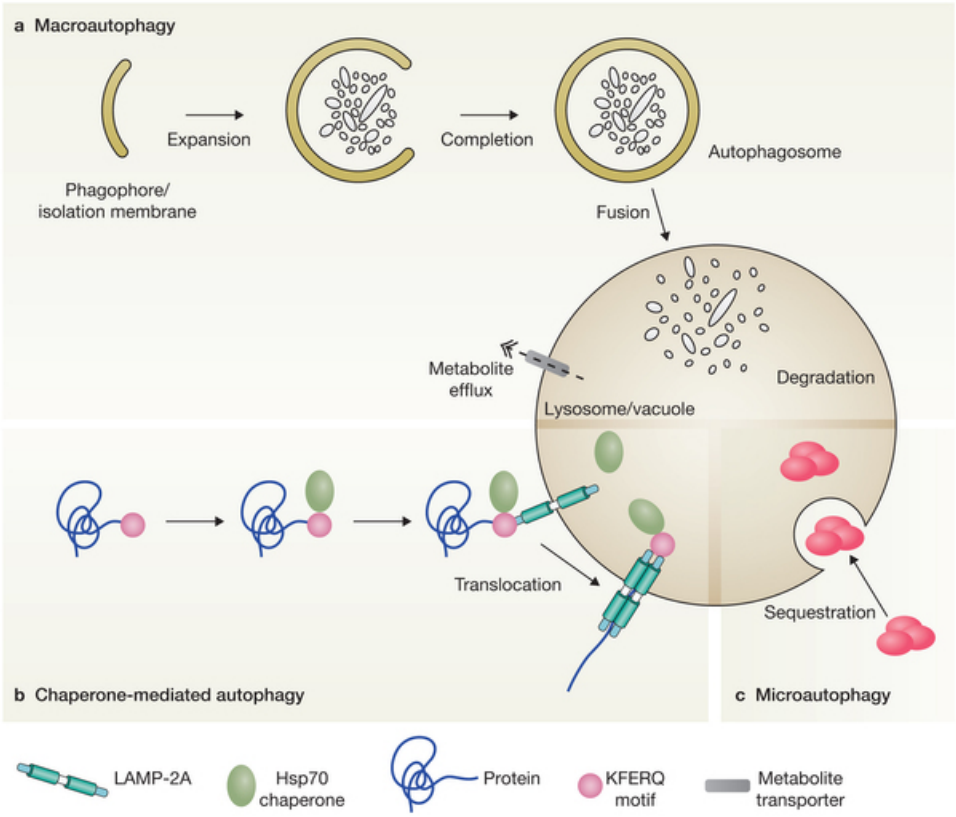

Figure 1.5 Schematic representation of macroautophagy (a), chaperone-mediated autophagy (b), and microautophagy (c) (image from Boya et al. 2013).

Genetic screenings in the budding yeast *Saccaromyces cerevisiae* have conducted to the identification of 32 different autophagy-related genes (*Atg*), coding for the components of the autophagic molecular machinery, that are highly conserved in plants, worms, flies and mammals, underlining the importance of autophagy during evolution (Nakatogawa et al. 2009). This machinery orchestrates at molecular level the different steps of autophagy. The first step of autophagy consists in the isolation of a membrane, the phagophore, deriving from lipid bilayer of the endoplasmic reticulum (ER) and/or of the trans-Golgi and endosomes, that expands to engulf intra-cellular cargo, such as protein aggregates, organelles and ribosomes (Hayashi-Nishino et al. 2009). The phagophore formation, regulated by the energy-sensing mTOR kinase, is not well characterized in mammalian cells, but we know that it involves the kinases ULK-1 and ULK-2 (two homologues of the yeast Atg1), the class III PI-3 kinase vesicular protein sorting 34 (Vps34), and its interactor Atg6/Beclin-1 (Kundu et al. 2008, Liang et al. 1999). In response to starvation signaling, Beclin-1 promotes the catalytic activity of Vps34, increasesing the levels of PI3P and promoting the formation of the phagofore (Backer 2008). The interaction between Vps34 and Beclin-1 is modulated by several other proteins to either promote autophagy, such as UVRAG, BIF-1, Atg14L and Ambra (Liang et al. 2006, Fimia et al. 2007), or to inhibit it, such as Rubicon and BCL-2 (Matsunaga et al. 2009, Pattingre et al. 2005).

The phagophore extention is mediated by the Atg5–Atg12–Atg16L complex, whose assembly requires the E1-ubiquitin activating enzyme Atg7 and the E2-like ubiquitin carrier Atg10. The Atg5–Atg12–Atg16L complex induces curvature into the growing phagophore through asymmetric recruitment of processed microtubule-associated protein light chain 3 (LC3), the mammalian homologue of Atg8 (Glick et al. 2010). In basal conditions, LC3 is a cytosolic protein that, upon induction of autophagy, undergoes a multi-step processing culminating in its recruitment at the autophagosome membrane. LC3 is first cleaved by Atg4, a cysteine protease, to generate LC3-

I, then activated in an ATP-dependent manner by Atg7, transferred to the E2-like carrier protein Atg3, and then conjugated to phosphatidylethanolamine (PE) to generate LC3-II, that is recruited to both the internal and external surfaces of the autophagosome. LC3-II is thought to be one of the molecular determinants of the selectivity of the autophagic process. In fact, it may act as a 'receptor' at the phagophore, promoting selective cargo uptake via interaction with 'adaptor' molecules that are bound to the targets (Kaminskyy and Zhivotovsky 2011). The most important adaptor protein, that interacts with LC3-II, is p62/SQSTM1, a multi-functional adaptor molecule that promotes turnover of poly-ubiquitinated protein aggregates, mediating their incorporation in autophagosomes. p62/SQSTM1 is mutated in the Paget's disease, in which abnormal turnover of bone results in bone deformation, arthritis and nerve injury (Ralston et al. 2008).

The fusion of the autophagosome with the lysosome is the final step of autophagy and it is still poor characterized. It has been described that this step involves the small G protein Rab7 (Jager et al. 2004), the Alzheimer's disease Presenilin protein (Eskelinen 2005) and the microtubules cytoskeleton (Webb et al. 2004) Moreover, there is evidence that autophagosomes can fuse with early and late endosomes, prior to fusion with the lysosome (Eskelinen 2005).

1.2.1 Signaling pathways that regulate autophagy

During nutrient deprivation, autophagosome formation is dramatically induced. In both yeast and mammalian cells, two well-characterized signaling cascades that sense nutrient status, activate cell division and growth, and negatively regulate autophagy are the target of rapamycin (TOR) and Ras-cAMP-PKA pathways (He and Klionsky 2009). TOR kinase is the major player in nutrient sensing and in regulating cell growth and autophagy; its activity is regulated by ATP levels, hypoxia, growth factor receptors and insulin signaling. In particular, in the presence of normal nutrients concentration, mammalian TOR (mTOR) is activated downstream of AKT

kinase, PI3-kinase and growth factor receptors, inhibiting autophagy and promoting cell growth, through induction of ribosomal protein expression and increased protein translation (see figure 1.6). The effect of mTOR on autophagy inhibition is mediated by its inhibitory effects on Atg1 kinase activity in yeast and *Drosophila*, whereas it is not yet clear how this is carried out in mammalian cells (Sabatini 2006). Signals that sense nutrient deprivation, including hypoxia, inhibit the activity of mTOR complex 1 (mTORC1), composed of mTOR, RAPTOR, DEPTOR, MLST8 and PRAS40, causing autophagy induction, and allowing the cells to adapt to environmental changes through reduced growth and increased catabolism. Negative regulators of mTOR are the TSC1 and TSC2 tumour suppressor proteins that inhibits Rheb, a small GTPase required for mTOR activity (Shaw 2009). TSC1 and TSC2 are activated by the adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), in response to low ATP concentration, or by the reduction of AKT activity, in response to decreased growth factor receptor signaling (Shaw 2009).

The Ras/cAMP-dependent protein kinase A (PKA) signaling pathway plays an important role in glucose sensing and down-regulates autophagy in parallel with the mTOR pathway acting on Atg1, which is a phosphorylation substrate of PKA (Budovskaya et al. 2005).

In addition to nutrient deprivation, other cellular stress factors, that are often correlated each other, such as ER stress, hypoxia, oxidative stress and pathogen infection, are able to activate specific signaling pathways that regulate autophagy. In particular, since hypoxia induces ER stress through the unfolded protein response, and reduced mitochondrial function, the induction of autophagy in this condition may allow the cell to eliminate portions of compacted ER, preventing wasteful ATP consumption, and to reduce mitochondrial mass, limiting production of reactive oxygen species (He and Klionsky 2009). Increased autophagy would also allow the cell to generate ATP from catabolism at a time when ATP production by oxidative

phosphorylation is limited. A key mediator of the hypoxia-induced autophagy activation is the hypoxia-inducible factor 1 (HIF-1) that regulates the expression of BNIP3 and BNIP3L, two autophagy regulators of the Bcl-2 superfamily (Zhang and Ney 2009).

Autophagy is known to induce cell cycle arrest and has been linked to proliferative senescence. This effect may be largely driven by nutrient deprivation-induced inhibition of mTOR activity and downstream effects on translation of key cell cycle genes, such as cyclin D1 (Liu et al. 2006), but it is not clear whether autophagy can induce cell cycle arrest also independently of mTOR signaling.

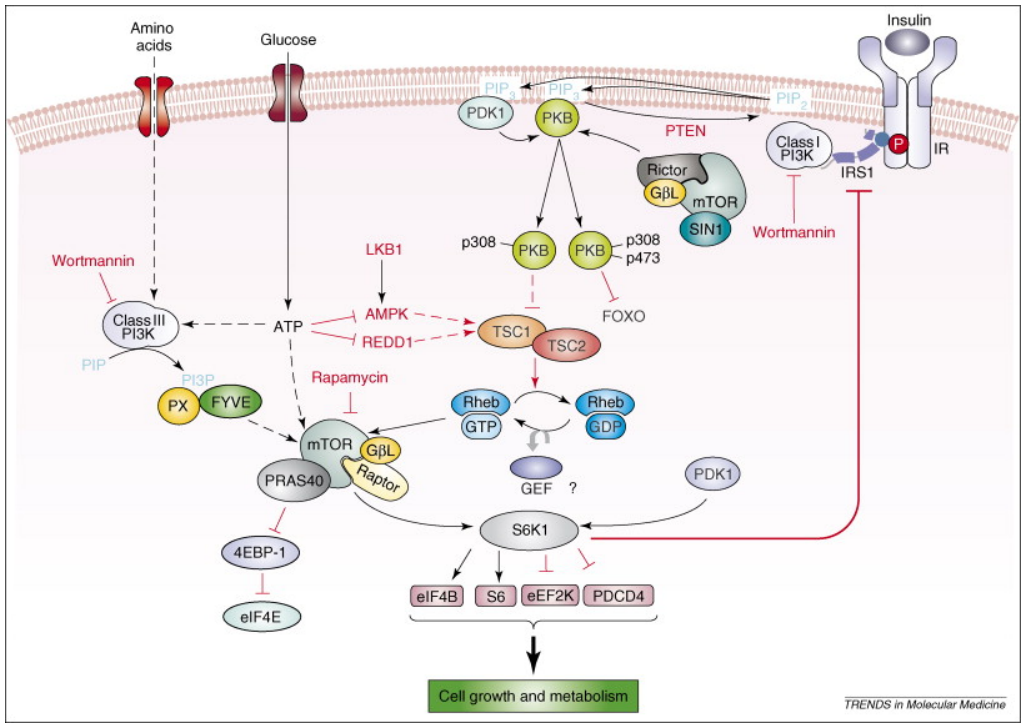

Figure 1.6 Schematic representation of mTOR pathway and its cross-talk with PI3K-AKT (PKB) and AMPK pathways (image from Dann et al. 2007).

1.2.2 Autophagy and cancer

De-regulation of the autophagic process is emerging to be an important etiopathological factor in several diseases, and modulation of autophagy may represent a promising therapeutic approach for neurodegenerative diseases and cancer. In the case of neurodegenerative diseases, defective autophagy seems to promote neurodegeneration. In fact, intracellular aggregate accumulation plays a particularly significant role in the aetiology of neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's diseases, in which aggregates respectively of Tau, α-synuclein and Huntingtin are dependent on autophagy for their clearance from neurons (Yue et al. 2009). By contrast, in the case of cancer, the role of autophagy is more complex. During neoplastic transformation and tumour progression, autophagy may represent both an oncogenic and a tumour-suppressive factor.

1.2.3 Tumor-suppressive functions of autophagy

The first connection between autophagy and cancer was due to the observation that the essential autophagy gene *ATG6*/*BECN1* seemed to be a tumour-suppressor. In fact, it is monoallelically lost in 40% to 75% of human prostate, breast, and ovarian cancers (Liang et al. 1999, Choi et al. 2013) and *Becn1* heterozygous mutant mice are prone to develop liver and lung tumors and lymphomas (Qu et al. 2003). Nowadays, the tumor suppressive functions of autophagy have been extensively investigated, and include: inhibition of necrosis and inflammation, contribution to tumor cell death, prevention of oxidative stress and genomic instability, and modulation of the anti-tumor immune response (Janji et al. 2013).

Since it is known that the inflammatory microenvironment plays a major role in tumor development, it is of note that activation of autophagy in tumor cells can inhibit the two forms of necrotic cell death necroptosis and poly-(ADP-ribose) polymerase (PARP)-mediated cell death that would, in turn, stimulate a robust inflammatory response (Shen and Codogno 2012).

Moreover, accumulation of p62/SQSTM1 in autophagy-deficient cells activates the pro-inflammatory transcription factor NF-κB and the stress-responsive transcription factor NRF2, thus favoring inflammation and tissue injury (Levine et al. 2011).

Consistently, autophagy-deficient tumors display an increased level of necrosis and inflammation. The anti-inflammatory effect of autophagy, together with its ability to negatively modulate epithelial-mesenchimal transition (EMT), plays also an anti-metastatic role (Lv et al. 2012).

Interestingly, autophagic cell death (or type II programmed cell death), induced by sustained autophagy, significantly contributes to tumour cell death, in particular when cancer cells have acquired resistance to apoptosis, and has been proposed as a possible tumor suppression mechanism (Kroemer and Levine 2008).

Moreover, autophagy helps normal cells to overcome several types of stress (e.g. metabolic, oncogenic) limiting their oncogenic potential. For example, autophagy can be activated in response to ER stress preventing the accumulation of ER chaperones, allows mitochondrial turnover avoiding accumulation of damaged mitochondria and reducing the risk of oxidative stress, and favors the degradation of damaged proteins involved in DNA replication, mitosis or repair preventing the accumulation of DNA damage and mutations. Autophagy is also able to mitigate the accumulation of genomic alteration by inducing mitotic senescence and limiting the proliferation of abnormal cells (Janji et al. 2013).

Finally, autophagy can favour the anti-tumor immune response, allowing the survival of immune cells in the hypoxic tumor microenvironment, and potentiating immune functions, such as antigen presentation and T-cell-mediated killing of tumor cells (Janji et al. 2013).

1.2.4 Oncogenic functions of autophagy

The ambivalence of autophagy in cancer is exemplified by the observation that liver-specific knock-out of essential autophagy genes *Atg5* or *Atg7* produces only benign liver tumors in mice, suggesting that autophagy may be important in liver to suppress tumor initiation, but also that autophagy may be required for progression from benign to malignant disease (Takamura et al. 2011).

In particular, autophagy may be oncogenic during the first steps of tumorigenesis, allowing cancer cells to survive under a variety of stresses and to cope the increased metabolic and biosynthetic demands imposed by deregulated proliferation. Before the beginning of neoangiogenesis, autophagy favors cancer cells survival in hypoxic and nutrient starvation conditions (White 2015).

1.2.5 *ULK* genes

The Unc-51-like kinase 1 (*ULK1*) and -2 (*ULK2*) are two mammalian homologs of the *S. cerevisiase Atg1* gene, whose name derives from the *C. elegans* hortologue *Unc-51*. These genes encode two high molecular weight Ser/Thr kinases, ULK1 and ULK2, crucial for autophagy initiation. In the absence of pro-autophagic stimuli, mTOR interacts with, phosphorylates, and inactivates ULK proteins. During starvation, or in the presence of other pro-autophagic stimuli, ULK1 and ULK2 are activated, undergo auto-phosphorylation and phosphorylate Atg13 and FIP200 (the homolog of yeast Atg17). ULKs, Atg13 and FIP200 form a molecular complex that localizes at the phagophore and is essential for autophagy initiation (Jung et al. 2009). The ULKs activity is regulated by a complex interplay between AMPK and mTORC1. In fact, AMPK directly phosphorylates both ULK1 and ULK2, positively regulating their kinase activity. mTORC1, in addition to inhibit ULKs activity, negatively regulates the interaction between AMPK and ULK1/2. Moreover, ULK1 and ULK2 are able to phosphorylate and negatively

regulate both their positive and negative regulators, AMPK and mTORC1 (Alers et al. 2012).

ULK1 and ULK2 are closely related, sharing 78% homology within their protein kinase domains, and seem to be functionally redundant. However, each ULK protein has a specific role in some particular cellular contexts. For example, ULK1, but not ULK2, is critical to induce the autophagic response of cerebellar granule neurons (CGN) to low potassium concentration, and has a cytoprotective function in neurons (Lee and Tournier 2011). Interestingly, ULK2 is essential for adipogenesis and for the regulation of adipocyte lipid metabolism, whereas ULK1 was dispensable for this processes (Ro et al. 2013).

Ulk1 knock-out mice display alterations in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation (Kundu et al. 2008), whereas *Ulk1* and *Ulk2* double knock-out induces severe defects of autophagy in the lung, causing pups death within 24 hours of birth for respiratory distress (Cheong et al. 2014).

As autophagy in general, *ULK* genes seem to have a variable role in cancer cells, that strongly depends on cellular context and stage of tumourigenesis. For instance, *ULK* genes are tumour-suppressive in glioma cells: in fact, *ULK2* epigenetic silencing is essential for astrocytes transformation, and both *ULK1* and *ULK2* are down-regulated in all grades of glioma (Shukla et al. 2014). On the other hand, hypoxia-induced ULK1 activation sustains the survival of MCF7 and A431 cancer cell lines (Pike et al. 2013).

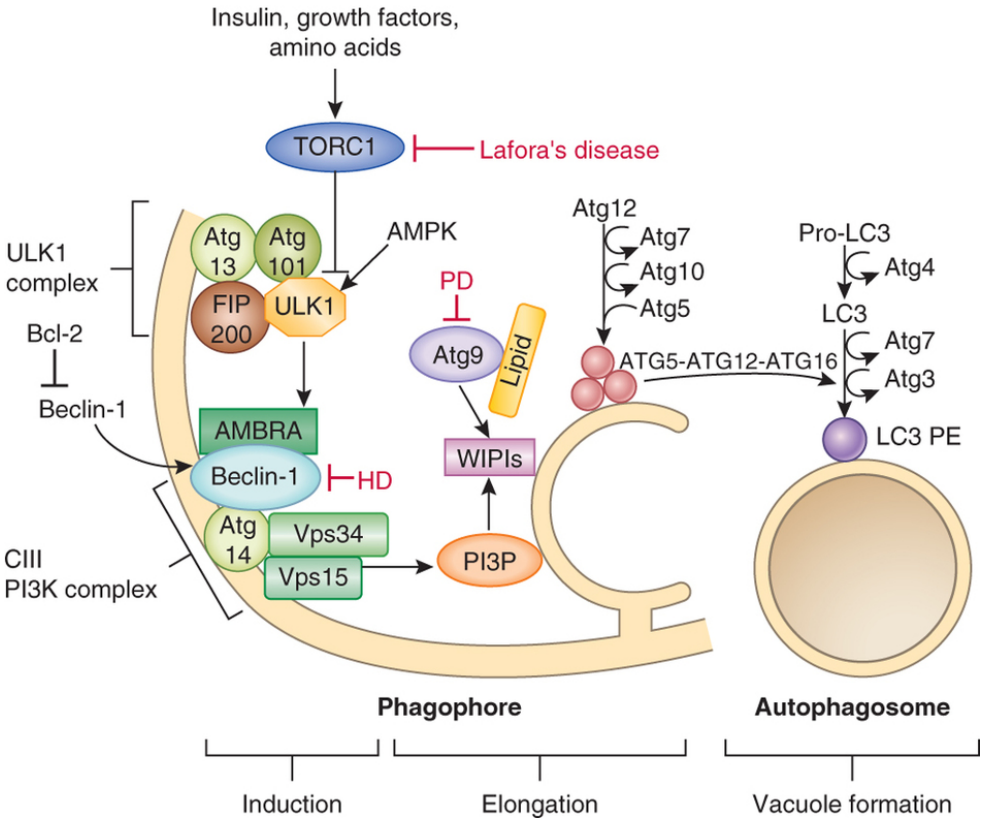

Figure 1.7 Schematic representation of autophagosome formation. Autophagosome formation can be initiated via mTOR inhibition or AMPK activation. This results in the activating phosphorylation of ULK1 and of its complex, composed of ULK1, ULK2, Atg13, FIP200 and Atg101. ULK1 also phosphorylates AMBRA, a component of the PI3K CIII complex I (Vps34, Vps15, Atg14, and beclin-1), enabling it to relocate from the cytoskeleton to the isolation membrane. Phosphatidylinositol 3-phosphate (PI3P), generated by Vps34 activity, specifically binds the PI3P effectors WD repeat domain phosphoinositide-interacting 1 (WIPI1) and WIPI2 and catalyzes the first of two types of ubiquitination-like reactions that regulate isolation membrane elongation. In this first reaction, Atg5 and Atg12 are conjugated to each other in the presence of Atg7 and Atg10. Attachment of the fully formed complex containing Atg5, Atg12 and Atg16L on the isolation membrane induces the second complex to covalently conjugate phosphatidylethanolamine to LC3, which facilitates closure of the isolation membrane (image from Nixon 2013).

2. Aims of the study

The above exposed considerations point out the importance of autophagy in cancer initiation and progression, but the mechanisms by which autophagy is deregulated in cancer cells are almost still unknown. Moreover, mutations in autophagy-related genes are rarely found in human cancers (White 2015), suggesting that they may not explain autophagy impairment in cancer cells. For this reason, investigation of transcriptional regulation and deregulation of autophagy-related genes appears a promising field of research to address this question. Considering that HMGA1 impacts on several biological processes by transcriptionally deregulating the expression of a lot of different target genes, and that overexpression of HMGA1 and impairment of autophagy are both common features of cancer cells, often acquired during cancer progression, we aimed to investigate whether there is a relationship between HMGA1 and autophagy in cancer cells. This hypothesis is supported by the evidence that HMGA1 has already been associated to some pathways (such as PI3K/AKT pathway) and biological processes (such as cellular proliferation, apoptosis, DNA repair and genomic instability) related to autophagy, and by some observations obtained studying the role of HMGA1 in skin cancer cells (described in the Results section). Therefore, we planned to evaluate the effects of HMGA1 knock-down on autophagy-regulating pathways and on autophagic flux in skin cancer cells SCC-13 and in other cell lines, and to identify new HMGA1-target genes that might account for the possible effect of HMGA1 on autophagy regulation which could represent a new mechanism by which HMGA1 exerts its oncogenic activity.

3. Materials and Methods

Cell cultures

HeLa cells were grown in RPMI supplemented with 10% FBS, L-glutamine, and antibiotics (Invitrogen, Carlsbad, CA). Human epidermal squamous carcinoma SCC-13 cell line was kindly provided by JG Rheinwald. Cells were cultured in keratinocyte serum-free medium (KSFM, Invitrogen) with 25 mg/mL bovine pituitary extract, penicillin, streptomycin, 0.2 ng/mL epidermal growth factor (EGF), and $CaCl_2$ to a final Ca2+ concentration of 0.4 mM. To maintain healthy confluent cultures, after cultures reached 40% confluence, they were refed daily with 1:1 medium (1:1 vol/vol Ca2+-free DMEM/KSFM, supplemented as above described). HEK293 cells were cultured in DMEM with 10% FBS, L-glutamine, and antibiotics (Invitrogen, Carlsbad, CA). MEFs were cultured in DMEM with 10% FBS, L-glutamine, and antibiotics (Invitrogen, Carlsbad, CA). MEFs have been isolated from 12.5 d.p.c. embryos. After head removing, embryos have been washed with PBS, incubated in trypsin 1% (Sigma) for 10 minutes at RT, pelletted and then resuspended in DMEM. MEFs have been genotyped for HMGA1 by PCR analysis with the following primers:

HMGA1-Fw 5'-AGAGACAAGAATGGGAGAGC-3'

HMGA1wt-Re 5'-TGTTACTAGGACCCTCATGG-3'

HMGA1KO-Re 5'-TAAAGCGACTGCTCCAGACT-3'

The wild-type allele is amplified using HMGA1-Fw + HMGA1wt-Re primers, whereas the knock-out allele is amplified using HMGA1-Fw + HMGA1KO-Re primers.

Transfections and plasmids

Cells were transfected with plasmids by Lipofectamine plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were transiently transfected with pcDNA3.1-*Hmga1b* (Pierantoni et al. 2003). Luciferase activity was analyzed by LightSwitch Luciferase Assay kit (Switch

Gear Genomics), according to manufacturer's instructions. ULK1-luc (cod. SKU:S707592) and ULK2-luc (cod. SKU:S709931) plasmids were from Switch Gear Genomics.

RNA interference, RNA extraction and quantitative Real-Time PCR (qRT-PCR)

RNA interference was obtained by specific siRNAs for HMGA1 [QiagenHs_HMGA1_5 (SI02662023) Sense strand and GGACAAGGCUAACAUCCCATT Antisense strand UGGGAUGUUAGCCUUGUCCAG] or ULK1 (LifeTechnologies, Cat. # AM51331) using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. As negative control, Qiagen AllStars control siRNA (SI03650318) was used. Total RNA was isolated using TRI-reagent solution (Sigma, St Louis, MO, USA) and treated with DNase (Invitrogen). Reverse transcription was performed according to standard procedures (Qiagen, Valencia, CA). qRT-PCR analysis was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) with following primer sequences:

human*HMGA1*-Fw 5'-CAACTCCAGGAAGGAAACCA-3'
human*HMGA1*-Re 5'-AGGACTCCTGCGAGATGC-3'
human*ULK1*-Fw 5'-CAGACAGCCTGATGTGCAGT-3'
human*ULK1*-Re 5'-CAGGGTGGGGATGGAGAT-3'
human*ULK2*-Fw 5'-TTTAAATACAGAACGACCAATGGA-3'
human*ULK2*-Re 5'-GGAGGTGCCAGAACACCA-3'
human*ACTB*-Fw 5'-CCAACCGCGAGAAGATGA-3'
human*ACTB*-Re 5'-CCAGAGGCGTACAGGGATAG-3'
mouse*Ulk1*-Fw 5'-GGATCCATGGTGTCACTGC-3'
mouse*Ulk1*-Re 5'-CAAGGGCAGCTGATTGTACC-3'
mouse*Ulk2*-Fw 5'-CACCATCTTGTCGCTTTGC-3'
mouse*Ulk2*-Re 5'-GGATAAGTTTTCTTCCTGAATATGCT-3'

mouse*Actb*-Fw 5'-CTAAGGCCAACCGTGAAAAG-3'
mouse*Actb*-Re 5'-ACCAGAGGCATACAGGGACA-3'

Growth curve and cell viability assay

SCC-13 cells were seeded in six-well plates, interfered and transfected as above described. Ctli and HMGA1i cells were counted after 48, 72 and 96 h. Cell viability of cells was quantified by MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega's CellTiter® 96 AQueous One Solution, Promega Fitchburg, WI, USA). Cells were seeded in 96-well plates at $5x10^3$ cells per well, then interfered and transfected as above described. After 72 h, absorbance was measured at 490 nm.

Western blotting and antibodies

Antibodies directed against HMGA1 proteins were already described (Pierantoni et al. 2003b). Commercial antibodies were: anti-pSer473-AKT (#9271), anti-AKT (#9272), anti-caspase 8 (1C-12), anti-caspase 7, anti-LC3A/B, anti-PARP (46D11) and anti-ULK1 (R600) antibodies from Cell Signaling Technology, MA, USA; anti-actin (I19), anti-p21(C-19), anti-phospho S6 ribosomal protein (Ser240/244), anti cyclin D1 A12 (sc8396) and anti cyclin E E20 (sc481) were from Santa Cruz Biotechnology, Santa Cruz; anti-p27 (610241) and anti-p62/SQSTM1 (610833) were from BD Biosciences (Franklin Lakes, NJ USA); anti-GAPDH was from ABM Materials (Richmond, BC, Canada). ECL System was purchased from Amersham Pharmacia (Buckinghamshire, UK).

Chromatin Immunoprecipitation (ChIP) Assay

After transfection, cells have been treated with formaldehyde 1%, washed and then lysed isolating the nuclei. Then the nuclei have been in turn lysed and chromatin has been sonicated. Then, samples have been processed

and immunoprecipitated using anti-HMGA1 antibody or normal rabbit IgG as negative control. For PCR analysis, 2µl out of 150µl immunoprecipitated DNA was used with primers described below. *GAPDH* promoter amplicon was used as negative control in all the experiments. Input DNA was used as positive control.

Primers used were:

ULK1-prom-Fw 5'-TGCCCTGTTCCATATTTTGC-3'

ULK1-prom-Re 5'-ACCCAAACCAACGACATAGC-3'

ULK2-prom-Fw 5'-AGCTGGGGATGGAGAGTACC-3'

ULK2-prom-Re 5'-AGAGACCGGAGCGGAAACT-3'

GAPDH-prom-Fw 5'-CCCAAAGTCCTCCTGTTTCA-3'

GAPDH-prom-Re 5'-GTCTTGAGGCCTGAGCTACG-3'

Fluorescence Microscopy

Lysotracker Probe **(**Molecular Probes**)** and monodansylcadaverin (MDC, 50 µM, Sigma) were used to label lysosomes and autophagosomes, respectively. Briefly, cells grown on coverslips were incubated with Lysotracker Probe for 1h at 37°C before fixation (4% PFA). Cells grown on bottom-glass dishes were incubated with MDC in PBS for 10 min at 37°C and imaged *in vivo* in PBS.

Cells stained with LC3 antibody (nanoTools) were fixed with methanol, quenched with 0.2% BSA/10% FBS in PBS for 30 min and permeabilized with 0.2% TX-100 for 7 min. Primary antibodies were detected with TRITC-conjugate secondary antibodies (Jackson ImmunoResearch Laboratories, Inc). Images were collected using a laser scanning microscope (LSM 510 META, Carl Zeiss Microimaging, Inc.) equipped with a Plan Apo 63x oil-immersion (NA 1.4) objective lens.

Quantification and morphometric analyses were carried out by using LSM 510 software. The mean fluorescence intensities in selected regions of interest of equal size were measured. For the quantification we acquired the

images, for each fluorophore, with the same setting (laser power, detector gain) as well as we kept the same threshold of fluorescence intensity in all experimental conditions (control and silenced cells). We evaluated the size of phagosome or lysosome compartments measuring the area occupied from each organelle marker that takes in account both the number and the dimension of these compartments.

Statistical analysis

Student's t-test was used to determine the significance for all the quantitative experiments. Error bars represent the standard deviation (SD) of the average.

4. Results

4.1 Knock-down of HMGA1 induces autophagy in skin cancer cells.

Skin cancer is the most frequently occurring among all types of human cancer, and cutaneous squamous cell carcinoma (CSCC), sometimes referred to as "epidermoid carcinoma" and "squamous-cell epithelioma", is the second most common of all the skin tumors, representing about 20% of non-melanoma skin cancers (NMSCs) (Uribe and Gonzalez 2011). Skin color, sunlight exposure and immunosuppression are risk factors for CSCC, with chronic sun exposure being the strongest environmental risk factor. CSCC is a serious health concern in Caucasian, displaying a mortality rate comparable to that of melanoma (Karia et al. 2012). In fact, similarly to melanoma, CSCC is more aggressive than other skin carcinomas, as 12% of cases metastasize and 1.5% of patients will succumb to this disease. The rising incidence and morbidity rates of CSCC have generated great research interest, in particular with regard to the progression and metastatization of this type of cancer (Sun et al. 2016). CSCC is a cancer of the squamous cells, the cells that compose all the different layers of the epidermis of the skin, with the exception of the basal layer. However, SCC can occur also in other squamous epithelia, such as those of lips, mouth, esophagus, urinary bladder, prostate, lung, vagina, and cervix. The SCCs of different body sites are highly heterogeneous, and can consistently differ in their symptoms, natural history, prognosis, and response to treatment.

The functional role and the oncogenic activity of HMGA1 has been extensively described in several types of cancer cells (Fusco and Fedele 2007), but it has not been investigated in skin cancer cells so far. To this aim, we focused our attention on CSCC, and selected as experimental model the human squamous carcinoma cell line SCC-13, established by Rheinwald and Beckett in 1981.

To evaluate the effects of the lack of HMGA1 in SCC-13 cells, we knocked-down its expression using an RNA-interference (RNAi) approach. In

particular, HMGA1 was silenced by transfecting short interfering RNAs (siRNAs) that target its mRNAs. As shown in figures 4.1A-B, a drastic reduction in HMGA1 expression was observed in the HMGA1-interfered cells (HMGA1i), at both mRNA and protein level. Then, we investigated whether HMGA1-interference affects cell proliferation, viability and apoptosis in SCC-13 cells, as described in other cell types. In particular, to compare the proliferation rate of control cells tranfected with a scrambled siRNA (Ctli), and HMGA1i cells, we performed a growth curve assay. As shown in figure 4.1C, a strong reduction in the number of HMGA1i with respect to Ctli cells was observed after 48, 72 and 96 hours post inhibition of HMGA1 expression. Interestingly, between 72 and 96 hours post-transfection, the number of Ctli cells continued to increase, whereas that of HMGA1i cells decreased, suggesting that HMGA1-knock down (KD) affects cell survival. The evidence that HMGA1i have a slower proliferation rate compared to Ctli cells is supported by the Western blot analysis of cell cycle modulators. We found that that HMGA1i cells express higher levels of the CDK inhibitors p27 and p21 and lower levels of cyclin D1 and cyclin E than control cells (figure 4.1D).

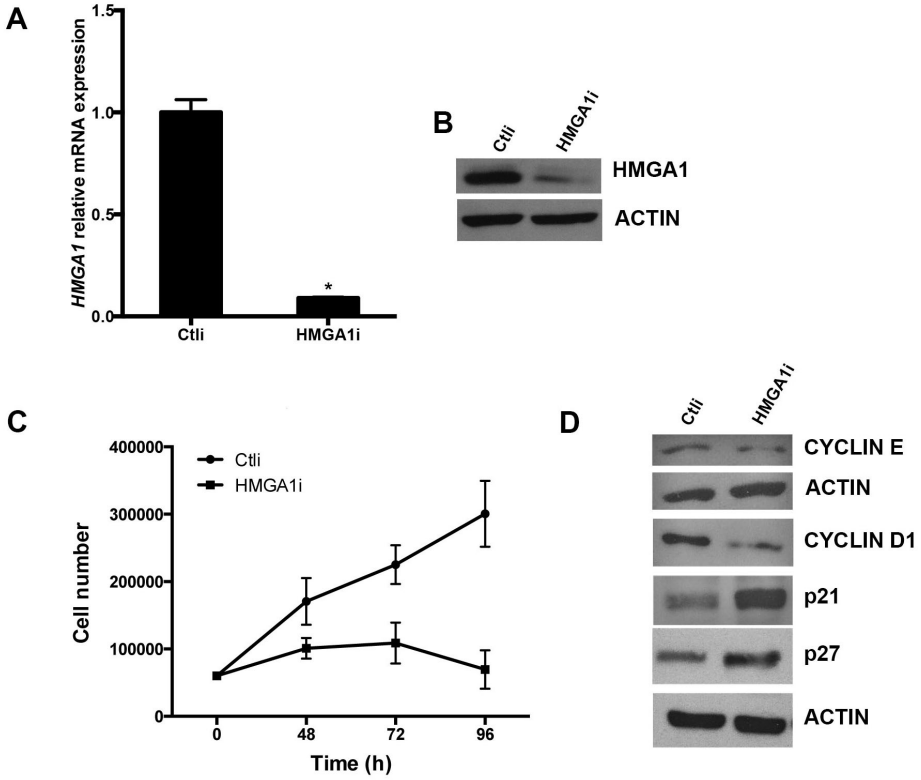

Figure 4.1 HMGA1 knock-down impairs proliferation of SCC-13 cell line. Control (Ctli) and HMGA1-interfered (HMGA1i) SCC-13 cells were tested for the expression of HMGA1 by qRT-PCR (A) and Western blotting (B). Actin was used as loading control. (C) Growth of HMGA1-interfered SCC-13 cells. Cells were plated as described in "Materials and Methods" and counted daily at 48, 72 and 96 h. (D) Proteins extracted from Ctli and HMGA1i cells were analyzed by Western blotting for Cyclin D1, Cyclin E, p21 and p27 protein levels. Actin was used as loading control.

Subsequently, viability of the HMGA1i cells has been evaluated performing a CellTiter assay, founding that it was significantly lower (about 40%) than that of control cells (figure 4.2A). To verify whether the reduced viability of HMGA1i is associated to type I programmed cell death (apoptosis), we analyzed PARP, caspase-7 and caspase-8 expression by Western blot. As shown in figure 4.2B, we observed neither a decrease in the full-length inactive forms of these apoptotic markers, nor an accumulation of the cleaved ones, deducting that HMGA1-KD is not sufficient to induce apoptosis in SCC-13 cells in the absence of pro-apoptotic stimuli.

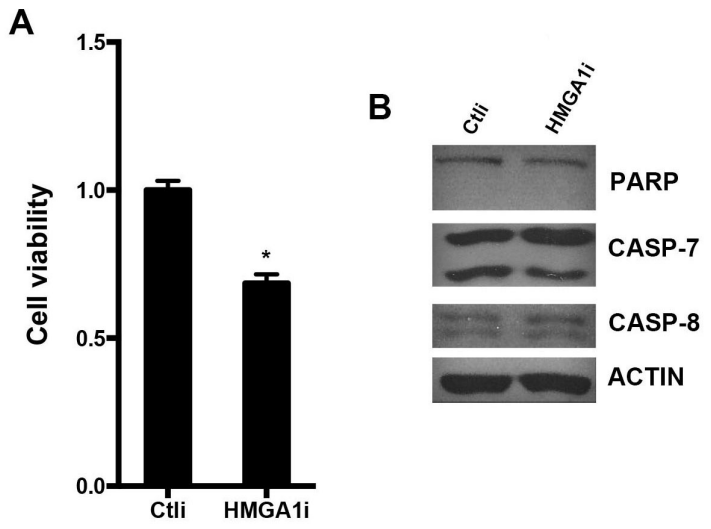

Figure 4.2 HMGA1 knock-down impairs viability of SCC-13 cell line without inducing apoptosis. (A) Cell viability of Ctli and HMGA1i cells was evaluated as described in "Materials and Methods" section. Error bars represent the mean ± S.D. of a representative experiment performed in triplicate. (B) The same extracts of (Fig. 4.1D) were tested by Western blotting with the indicated antibodies directed against proteins involved in the apoptotic process. Actin was used as loading control.

These data indicate that HMGA1 silencing impairs proliferation and viability of SCC-13 cells without inducing apoptosis, so we investigated whether these phenomena were associated to type II programmed cell death (autophagic cell death). A connection between HMGA1 and autophagy regulation has been suggested by the previous observations that HMGA1 overexpression is able to activate PI3-K/AKT cascade (Liau et al. 2007), a major signaling pathway regulating autophagy, and mammalian Target of Rapamycin Complex 1 (mTORC1) (Laplante and Sabatini 2012). Thus, we investigated whether HMGA1-KD is able to reduce the activation of AKT and of ribosomal protein S6, the final read-out of the mTOR pathway in SCC-13 cell line. Phosphorylation of AKT Ser473 and S6 Ser240/244 is reduced in HMGA1i with respect to Ctli cells, as shown in (figure 4.3A), suggesting that

the KD of HMGA1 expression may trigger traits of autophagy in skin cancer cells. Subsequently, to verify whether depletion of HMGA1 really affects the autophagic flux, we evaluated microtubule-associated protein light chain 3 (LC3) processing and degradation by Western blot analysis. The conversion-rate from LC3-I to LC3-II form is suggestive of autophagosomes assembly, since LC3-I>LC3-II conversion is due to the proteolysis and PE-conjugation of LC3-I that occurs during autophagosomes formation. On the other hand, since LC3-II itself is degraded inside autolysosomes during autophagy, LC3-II degradation rate indicates the intensity of the autophacic flux. For these reasons, when the autophagic flux is active, both LC3-I and LC3-II may decrease. Therefore, to better estimate the autophagic flux, it is appropriate to evaluate the LC3-II/LC3-I ratio in the presence and in the absence of lysosome inhibitors, which allow LC3-II to accumulate on the autophagosome membrane (Mizushima and Yoshimori 2007).

On these bases, to inhibit lysosomes, Ctli and HMGA1i cells were treated for 16 hours with NH_4Cl and leupeptin, at a concentration respectively of 20 mM and 100 μM, as previously described (Vitale et al. 2013). The ratio between LC3-II and LC3-I forms was increased in HMGA1i in comparison with the control cells in the presence of lysosomes inhibitors, whereas, in their absence, LC3-II was almost all degraded, and LC3-I was less abundant in HMGA1i than in Ctli cells.

The consistent degradation rate of LC3-II, observed in the absence of lysosomes inhibitors, indicates that both Ctli and HMGA1i SCC13 cells display high levels of autophagy. On the other hand, the increased LC3-II/LC3-I ratio in the presence of NH_4Cl and leupeptin, and the decreased levels of LC3-I in HMGA1i cells indicate that autophagosomes assembly and autophagolysosomes activity increase after HMGA1-KD (figure 4.3B). Consistently, the HMGA1-knockdown increased also the degradation of p62/SQSTM1, another protein that, as LC3, is recruited and degraded in the autophagolysosomes, representing an indicator of the autophagic flux (figure

4.3C). In fact, in the absence of lysosome inhibitors, p62/SQSTM1 levels were lower in HMGA1i than in Ctli cells, whereas the blockage of autophagolysosomes activity revealed a higher accumulation of this protein in the HMGA1-depleted cells.

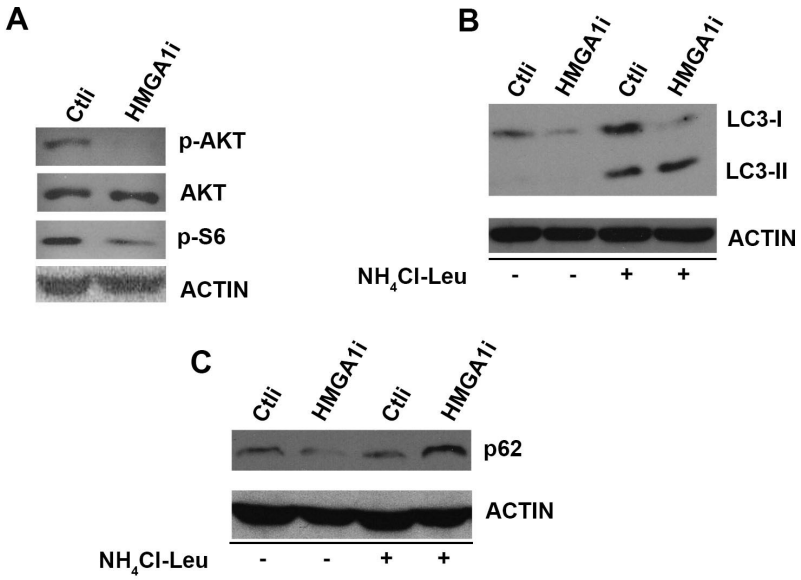

Figure 4.3 HMGA1 depletion induces autophagy. (A) Immunoblot detection of p-AKT (Ser473), total AKT and p-S6 expression levels in Ctli and HMGA1i cells. Actin was used as loading control. (B-C) Proteins extracted from Ctli and HMGA1i cells untreated or treated with 20 mM NH_4Cl and 100 μM leupeptin for 16 h were tested for LC3-I>II conversion levels (B) and for p62/SQSMT1 expression (C) by Western blotting. Actin was used as loading control.

The above described biochemical data indicate that HMGA1-KD induces down-regulation of AKT and mTOR pathways, associated to increased autophagic flux in SCC-13 cells. To confirm and support these data with another approach, we assessed the distribution of some markers of autophagy by confocal fluorescence microscopy. First of all, we evaluated the number and

size of autophagosomes, stained them *in vivo* using the fluorescent dye monodansylcadaverine (MDC). Autophagosomes of HMGA1i cells were more and surprisingly bigger than those of Ctli cells, to the point that the area occupied by MDC-positive structures were consistently higher in the HMGA1-depleted cells. Then, we evaluated the subcellular distribution of LC3 and p62/SQSTM1. The proportion of these proteins that has not been recruited to the autophagosomes gives a diffuse cytoplasmic staining, whereas the LC3- or p62/SQSTM1-positive dots (or *puncta*) represent autophagosomes or autophagolysosomes in which these proteins have been recruited. In agreement with Western blot analysis, both LC3 and p62/SQSTM1 were strongly recruited in autophagosome compartment in HMGA1i cells (figure 4.4A, middle panels), as demonstrated by the increase of LC3- and p62/SQSTM1 -positive *puncta*. Finally, we found that the depletion of HMGA1 caused also an increase in number and size of lysosomes, labelled with the lysostracker dye (figure 4.4A, upper panels). Interestingly, in HMGA1i cells there was a drastic expansion (3/4-fold increase) of the area occupied also by LC3 *puncta* and lysosomes (figure 4.4B).

Taken together, these data support the idea that the depletion of HMGA1 increases the activation of the autophagic pathway in SCC-13 cells.

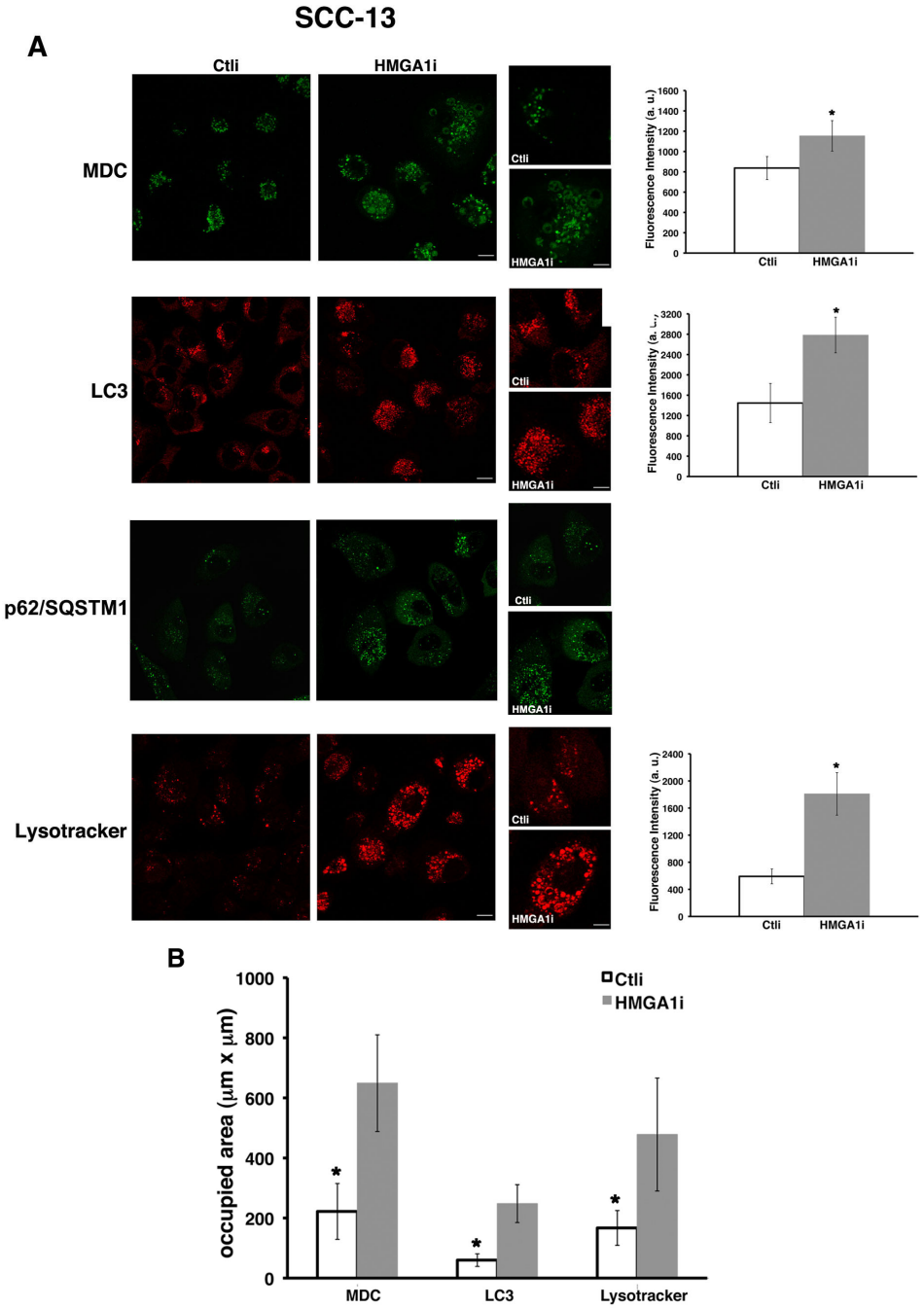

Figure 4.4 Evaluation of autophagic markers in HMGA1i-SCC-13 cells by immunofluorescence. (A) Ctli and HMGA1i cells were stained with monodansylcadaverin (upper panels, MDC, in green), LC3 specific antibody (middle panels, in red), p62/SQSTM1 specific antibody (middle panels, in green), or lysotracker (lower panels, in red) as described in "Materials and Methods". Serial confocal sections were collected. Bars, 11 µm. For each condition, image magnification was shown in the squares at right. Bars, 6 µm. Mean fluorescence intensity (arbitrary unit, a.u.) for each marker in Ctli and HMGA1i cells is shown. Experiments were performed at least two independent times (n ≥50 cells). Error bars, means ± SD; * $p < 0.0001$. (B) The area occupied by each organelle marker was measured in Ctli and HMGA1i cells. Experiments were performed at least two independent times (n ≥50 cells). Error bars, means ± SD; * $p < 0.0005$.

4.2 HMGA1 regulates autophagy not only in skin cancer cells.

Since HMGA1 is an architectural transcription factor that activates, recruits to or displaces from specific promoters other transcriptional regulators, its activity strongly depends on the set of proteins expressed by a particular cell type, thus its effects may vary on the basis of the cellular context. In order to verify whether the autophagy increase induced by the KD of HMGA1 expression is not restricted to skin cancer cells, we analyzed the effect of HMGA1 silencing on autophagic markers also in HeLa cervix cancer cells, a very common model of cancer cells in culture. As observed in SCC-13 cells, depletion of HMGA1 induces autophagy also in HeLa cells, as demonstrated by both Western blot analysis and confocal fluorescence microscopy experiments. In fact, phospho-S6 levels were lower in HMGA1-depleted cells with respect to the control cells (figure 4.5B), and LC3-I>II conversion levels and p62/SQSTM1 degradation were higher in proteins extracted from HMGA1i-HeLa cells untreated or treated with 20 mM NH_4Cl and 100 μM Leupeptin for 16 hours in comparison with the control transfected cells (figure 4.5C). Consistently, microscopy analysis of MDC, LC3, p62/SQSTM1 and lysotracker stainings confirmed these data (figure 4.6).

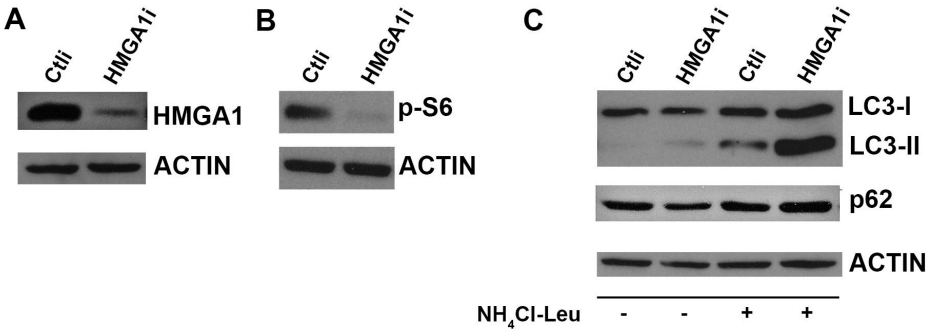

Figure 4.5 HMGA1 depletion induces autophagy also in HeLa cells. (B) Immunoblot detection of HMGA1 and (C) p-S6 expression levels in Ctli and HMGA1i cells. Actin was used as normalization. (D) Proteins extracted from Ctli and HMGA1i cells, untreated or treated with 20 mM NH_4Cl and 100 μM leupeptin for 16 h, were tested for LC3-I>II conversion and p62/SQSMT1 expression levels by Western blotting. Actin was used as loading control.

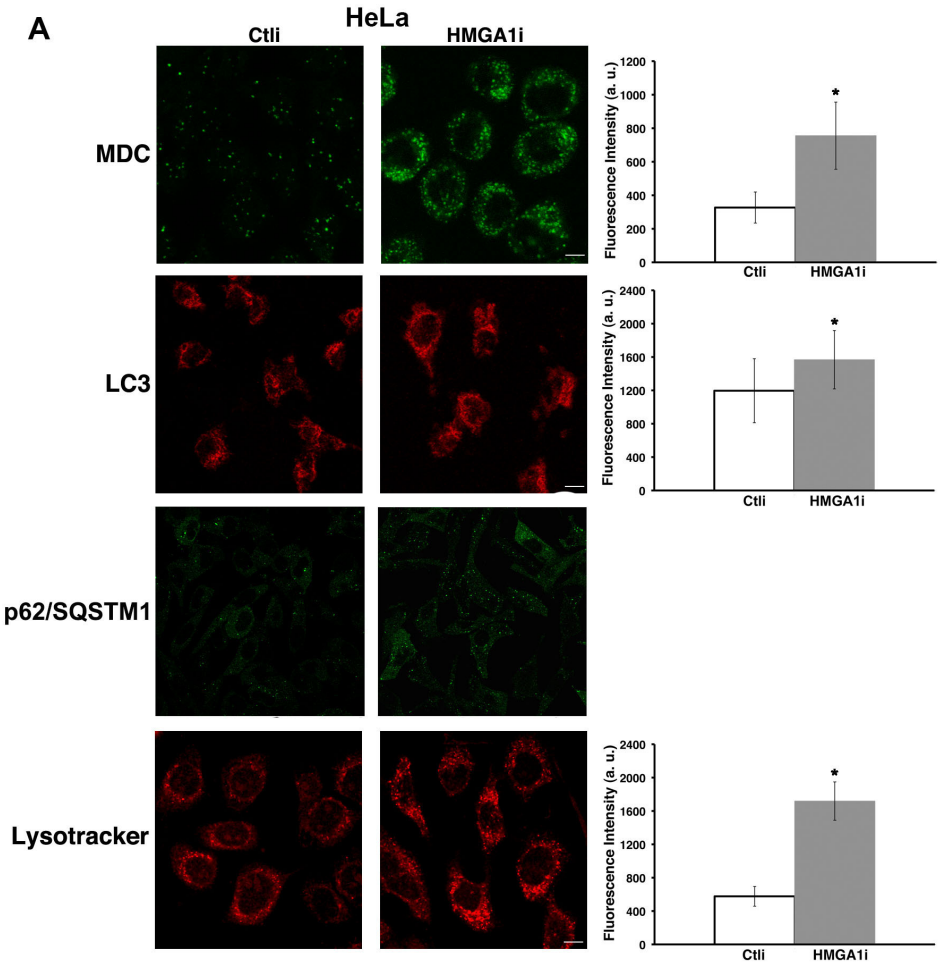

Figure 4.6 Evaluation of autophagic markers in HMGA1i-HeLa cells by immunofluorescence. Ctli and HMGA1i cells were stained with monodansylcadaverin (upper panels, MDC, in green), LC3 specific antibody (middle panels, in red), p62/SQSTM1 specific antibody (middle panels, in green), or lysotracker (lower panels, in red). Serial confocal sections were collected. Bars, 11 μm. Mean fluorescence intensity (arbitrary unit, a.u.) for each marker in Ctli and HMGA1i cells is shown. Experiments were performed two independent times ($n \geq 50$ cells). Error bars, means ± SD; * $p < e\text{-}7$ for MDC and lysotracker; $p < 0.01$ for LC3.

Moreover, we evaluated the autophagic flux also in wild-type (WT) MEFs, that physiologically express the HMGA1 proteins, and in their *Hmga1* null (KO) counterpart. Interestingly, confocal microscopy showed that KO MEFs did not display an appreciable enlargement of autophagosome-lysosome compartment with respect to their WT counterpart (figure 4.7A), but the treatment with several autophagy-inducers revealed that *Hmga1*$^{-/-}$ MEFs are more susceptible to autophagy in comparison with WT ones (figure 4.7B). In fact, after 6 hours of starvation in HBSS or after treatment with 1 μM of the mTOR-inhibitor rapamycin, LC3-I>II conversion was higher in KO MEFs with respect to WT.

These data clearly indicate that the involvement of HMGA1 proteins in the process of autophagy is not restricted to cancer cells, although it may depend on cellular context.

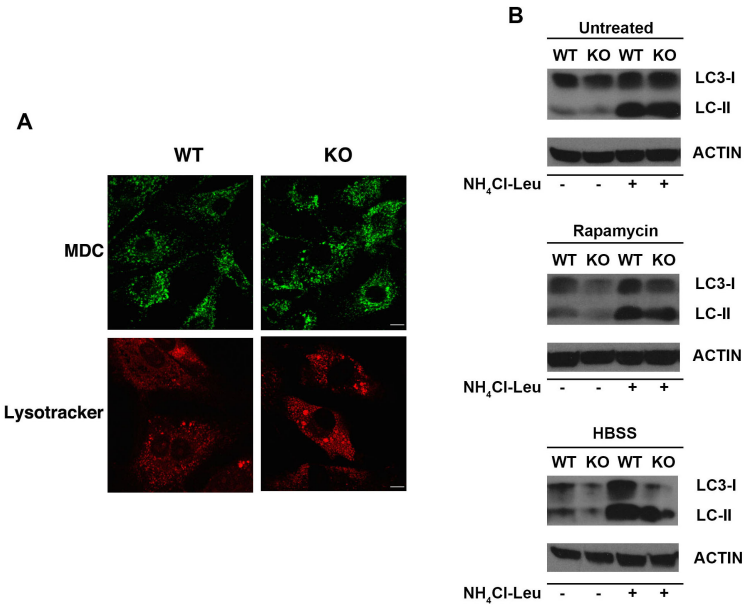

Figure 4.7 *Hmga1* genetic ablation induces autophagy in MEFs (A) *Hmga1* WT and KO MEFs were stained with monodansylcadaverin (upper panels, MDC, in green) or lysotracker (lower panels, in red). Bars, 11 μm. (B) Proteins extracted from WT and KO MEFs untreated or treated with HBSS (for 6 h) or rapamycin (for 16 H) in presence or absence of 20 mM NH4Cl and 100 μM leupeptin for 16 h were tested for LC3-I>II conversion levels by Western blotting. Actin was used as loading control.

4.3 HMGA1 negatively regulates the expression of *ULK1* and *ULK2*.

Even though HMGA1 has some "extra-nuclear" functions, such as regulation of mitochondrial function and mitochondrial DNA repair efficiency (Mao et al. 2009), the vast majority of its effects are due to the transcriptional regulation of specific target genes. To understand which are the HMGA1-regulated genes by which HMGA1 could regulate autophagy, we looked for autophagy-related genes in the data set of a microarray analysis that we had performed to compare the transcriptome of *Hmga1* KO and WT MEFs (unpublished data). Applying biostatistical analysis, we found several autophagy-related transcripts differentially expressed in KO compared to WT MEFs with a fold-change > 2 (data not shown). We focused our attention on the most upregulated gene in KO MEFs, that was Unc-51-like kinase 2 (*Ulk2*), whose gene product, the Ser/Thr kinase ULK2, is a recognized master regulator of autophagy, belonging to the autophagy-initiating complex (Mizushima 2010). Subsequently, the upregulation of *Ulk2* in *Hmga1* KO MEFs was confirmed by qRT-PCR, and we analyzed also the expression of its closely related paralogue *Ulk1*, founding that it is moderately overexpressed in KO vs WT MEFs (fold-change ≈1,9) (figure 4.8A).

To test whether HMGA1-KD induces *ULK* genes de-regulation also in human cancer cells, we analyzed by qRT-PCR the expression of *ULK1* and *ULK2* in HMGA1-depleted HeLa and SCC-13 cell lines. As shown in figure 4.8A, both *ULK1* and *ULK2* were upregulated following HMGA1 depletion. Interestingly, in both HeLa and SCC-13 cells, the fold change of *ULK1* (≈3-4) was higher than that of *ULK2* (≈2,5). In addition, we confirmed the upregulation of ULK1 also at protein level by Western blotting analysis (figure 4.8B).

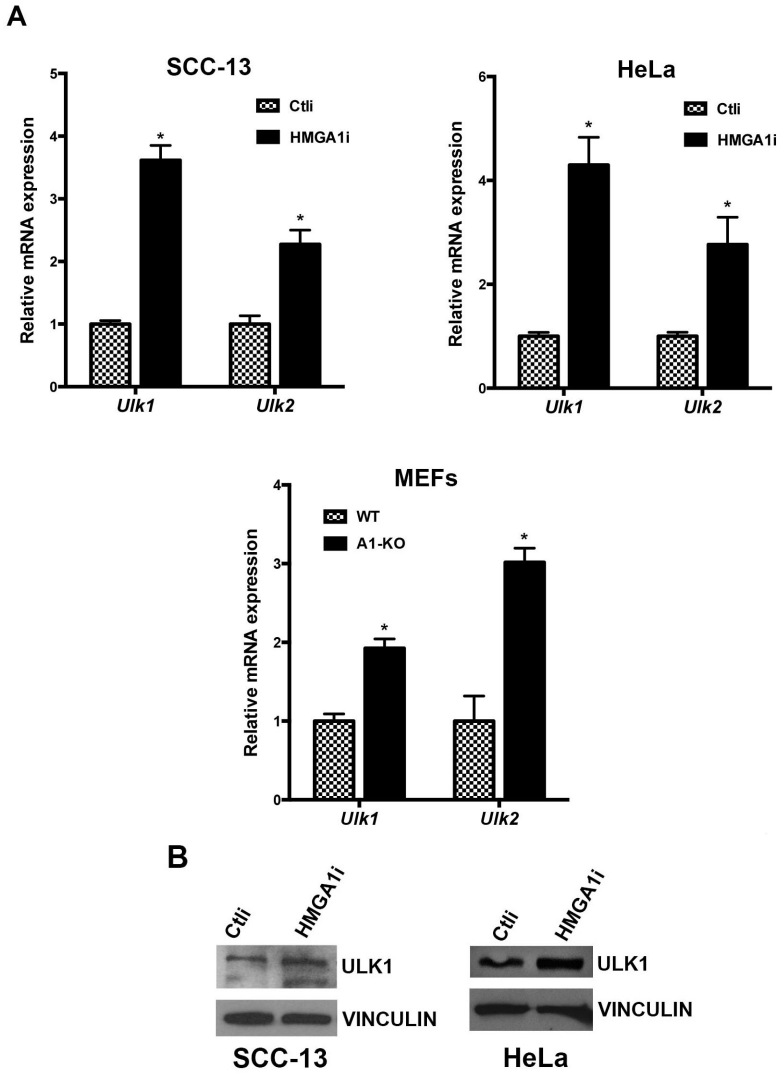

Figure 4.8 *Hmga1* modulates *ULK1* and *ULK2* mRNA expression levels in several cells. (A) RNA extracted from control (Ctli) or HMGA1-interfered (HMGA1i) SCC-13 or HeLa cells and from WT and KO MEFs, were analyzed by qRT-PCR for *ULK1* and *ULK2* expression. The actin expression level has been used for data normalization. Data are mean ± SD of a representative experiment performed in triplicate. (B) Proteins extracted from control and HMGA1i SCC-13 and HeLa cells were tested for ULK1 expression by western blotting experiment. Vinculin was used as loading control.

To verify whether *ULK1* and *ULK2* may be direct targets of HMGA1, we tested whether *ULK1* and *ULK2* promoter regions are bound by HMGA1. To this aim we identified putative AT-rich HMGA1 binding sites in the promoter regions of these genes, using bioinformatic methods (PROMO 3.0). Then we verify the binding of HMGA1 to these AT-rich regions of the *ULK* promoters by chromatin immunoprecipitation (ChIP) assays in HeLa cells. Thus, DNA-chromatin complexes were subjected to immunoprecipitation with anti-HMGA1 or aspecific rabbit polyclonal IgGs as negative control. The recovered DNA was subsequently analyzed by qRT-PCR, using primers spanning -878/-713 region of the *ULK1* promoter and +129/+352 region of the *ULK2* promoter. As shown in figure 4.9A, occupancy of *ULK1* and *ULK2* promoters by HMGA1 has been detected in the anti-HMGA1-precipitated chromatin from HeLa cells, whereas no amplification was observed in samples immunoprecipitated with rabbit IgGs. As negative control, the immunoprecipitated DNA has been amplified using primers for the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene promoter.

Finally, to evaluate the ability of HMGA1 to regulate the transcriptional activity of *ULK1* and *ULK2* promoters, we performed luciferase activity assays in HeLa cells. To this aim, Ctli and HMGA1i HeLa cells have been transfected with a reporter vector carrying the luciferase gene under the control of the *ULK1* or *ULK2* promoter. As shown in figure 4.9B, HMGA1 depletion increased the transcriptional activity of both *ULK1* and *ULK2* promoters. To further verify the ability of HMGA1 to regulate the activity of *Ulk1* promoter, we used HEK293 cells that represent a useful system to study the effects of both depletion and overexpression of HMGA1 protein. Accordingly, depletion of HMGA1 protein increased *ULK1* promoter activity also in these cells, whereas HMGA1 overexpression significantly reduced it (figure 4.9C). All these data strongly support a critical role of HMGA1 in the negative regulation of *ULK1* and *ULK2* gene expression exerted by binding *ULK1* and *ULK2* promoters and thereby decreasing their transcriptional activity.

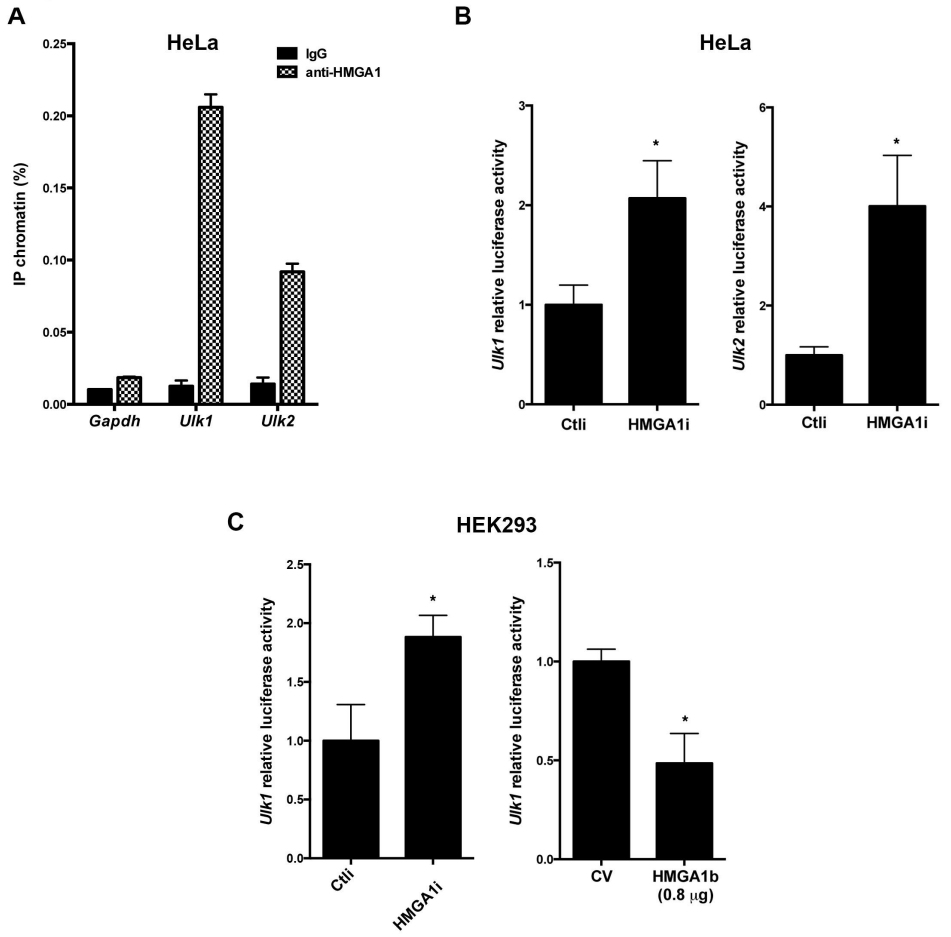

Figure 4.9 HMGA1 protein regulates *ULK1* and *ULK2* transcription. (A) ChIP was performed in HeLa cells. Soluble chromatin was immunoprecipitated with anti-HMGA1 antibodies. The DNAs were amplified by qPCR using primers covering specific regions of human *ULK1* and *ULK2* promoters (-878/-713 and +129/+352, respectively). IgG were used as negative control of immunoprecipitation. Amplification of the immunoprecipitated DNA using primers for the *GAPDH* gene promoter was used as control of specificity. Data are mean ± SD of a representative experiment performed in triplicate. (B) Analysis of *ULK1* and *ULK2* luciferase-reporter activity in Ctli and HMGA1i HeLa cells. All transfections were performed in triplicate. Data are mean ± SD of three independent experiments. The same analysis was performed for *ULK1* promoter activity in Ctli and HMGA1i HEK293 cells (C, left panel). Analysis of *ULK1* promoter activity was also evaluated in HEK293 cells transiently transfected with empty vector (CV) or 0.8 μg of pcDNA3.1-*Hmga1b* expression vector (C, right panel). All transfections were performed in triplicate. Data are mean ± SD of three independent experiments.

4.4 Knock-down of *ULK1* prevents autophagy induced by HMGA1-depletion.

The above described data indicate that HMGA1 is able to bind the promoters of *ULK* genes and to modulate their transcriptional activity. Since *ULK1* and *ULK2* are master regulators of autophagy, we hypothesized that the regulation of their expression may account for the induction of autophagy caused by HMGA1-KD. In particular, we focused on *ULK1*, which is the most up-regulated in response to HMGA1-depletion in both SCC-13 and HeLa cells.

To confirm that the effects of HMGA1 depletion on autophagy were mediated by *ULK1* upregulation, HeLa cells were interfered for both HMGA1 and ULK1 expression (figure 4.10A) using specific siRNAs. As shown in figure 4.10B, we confirmed the efficacy of HMGA1 and ULK1 silencing, and found that ULK1 silencing drastically reduced LC3I>II conversion induced by HMGA1 depletion. These data suggest that the autophagy induced by HMGA1-depletion is, at least in part, due to ULK1 upregulation.

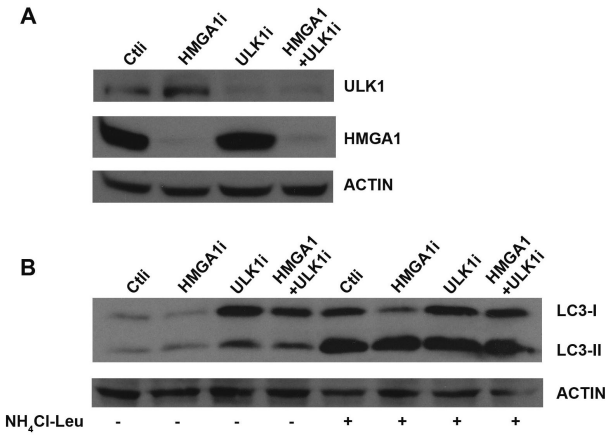

Figure 4.10 Effects of HMGA1 silencing in autophagy are mediated by Ulk1 depletion. (A) Proteins extracted from Ctli, HMGA1i, ULK1i and HMGA1i/ULK1i HeLa cells were tested for the expression of ULK1 and HMGA1 by Western blotting analysis. Actin was used as loading control. (B) The same samples untreated or treated with 20 mM NH_4Cl and 100 μM leupeptin for 16 h were tested for LC3-I>II conversion levels. Actin was used as loading control.

5. Discussion

Since HMGA proteins are overexpressed in several human malignancies, and their overexpression levels correlate with cancer progression, high aggressiveness, chemoresistance and poor prognosis (as reviewed in Fusco and Fedele 2007, Liau and Wang 2008), they represent promising prognostic markers and therapeutic targets for oncological patients. Plenty of studies have underlined the causal role of HMGA1 and HMGA2 in cancer initiation and progression, unrevealing several mechanisms by which HMGA proteins exert their oncogenic activity. In particular, it has been demonstrated that HMGA1 is able to: a) antagonize p53 function and inhibit p53-induced apoptosis (Frasca et al. 2006, Esposito et al. 2010); b) regulate cancer stem cell division (Puca et al. 2014); c) impair DNA repair machineries (Palmieri et al. 2011); c) transcriptionally regulate miRNAs and genes involved in the control of the cell cycle (Tessari et al. 2003, Mussnich et al. 2013) and induction of epithelial-mesenchymal transition (Reeves et al. 2001, Pegoraro et al. 2013); d) promote AP-1 activity (Vallone et al. 2007); e) induce chromosome instability (Pierantoni, Conte et al. 2015, Pierantoni, Conte et al. 2016). Nevertheless, as HMGA1 binds DNA throughout the genome, inducing massive chromatin remodeling and considerable changes in gene expression, it is general opinion that it can promote tumorigenesis also by other unidentified mechanisms.

Moving from the observation that HMGA1-knock-down (KD) decreases proliferation and survival, without inducing apoptosis, in human epidermal squamous cells carcinoma SCC-13 cell line, we demonstrate that HMGA1 plays an important role in the regulation of autophagy, a key process in cancer cells survival, thus suggesting a novel mechanism of HMGA1-mediated oncogenesis. Indeed, KD of HMGA1 increases autophagy in SCC-13 cells, as demonstrated by the appearance of the typical autophagic features. In fact, HMGA1-silenced cancer cells display both a reduced phosphorylation of AKT and of the final read-out of the mTORC1 pathway, the ribosomal S6

protein, and up-regulation of autophagic flux markers, such as LC3-I>LC3-II conversion-rate, p62/SQSTM1 degradation, and LC3 and p62/SQSTM1 redistribution from a diffuse pattern to cytoplasmic *puncta* representing autophagosomes or autophagolysosomes. Interestingly, the effect exerted by depletion of HMGA1 on autophagy is not restricted to SCC-13 cells. In fact, the induction of autophagy, assessed by both biochemical and morphological analysis, has been obtained also in HMGA1-KD cervix cancer HeLa cells. Morover, *Hmga1*-KO MEFs showed a higher susceptibility to autophagy in comparison to the WT counterpart, even though they do not display appreciable differences in the autophagosomal/lysosomal compartment under basal conditions. Considering that HMGA1 is an architectural transcription factor, whose activity strongly depends on its molecular partners and on cellular context, we cannot exclude that its effects on autophagy regulation could be different, or even absent, in other cell types. In particular, it would be of interest to investigate the relation between HMGA1 and autophagy also in non-transformed epidermal cells.

A microarray analysis, performed in *Hmga1* WT and KO MEFs, and confirmed by qRT-PCR, suggested that the expression of the two autophagy-initiating Ser/Thr kinases ULK1 and ULK2 is up-regulated as a consequence of *Hmga1* genetic ablation. On the basis of this evidence, to understand the mechanism underlying the increase in autophagy induced by HMGA1-silencing, we analyzed the expression of ULK1 and ULK2 in HMGA1i-SCC-13 and HMGA1i-HeLa cells. qRT-PCR analysis revealed that both *ULK1* and *ULK2* are upregulated with about 4- and 3-fold change, respectively, in HMGA1-interfered cells with respect to control cells. Then, ChIP experiments showed that HMGA1 protein is able to bind the promoter regions of these genes, and subsequently functional assays demonstrated that HMGA1 is also able to repress *UKL1* promoter activity. These data indicate that *ULK* genes are new direct targets of HMGA1 which is able to negatively modulate their expression. Finally, we were able to demonstrate that the block of ULK1

expression significantly reduces the autophagic effects induced by HMGA1 silencing in HeLa cells, indicating that HMGA1-driven autophagy regulation is, at least in part, due to *ULK1* transcriptional modulation.

It is worth of note that other studies suggested a possible functional relationship between HMGA1 and autophagy, and that this relation may be based also on other mechanisms, in addition to *ULK* genes regulation. Indeed, it has been reported that HMGA1 overexpression activates the phosphatidylinositol-4,5-bisphosphate-3 kinase (PI3K)/AKT signaling pathway, enhancing phosphorylation of AKT at serine 473 (Liau et al. 2007). In addition to AKT and PI3K, also BCL-2 and NF-kB are HMGA1-regulated oncogenic proteins that repress autophagy. In particular, BCL-2, upregulated in cancer cells by HMGA1 that antagonizes the p53-mediated transcriptional repression of the *BCL2* gene, inhibits autophagy and prevents autophagic cell death by sequestering the essential autophagy initiator Beclin 1 (Marquez and Xu 2012).

The NF-κB family of transcription factors plays a pivotal role in regulating inflammation and innate and adaptive immune responses. It is widely demonstrated that NF-κB promotes initiation and progression of certain cancers, because of its ability to upregulate genes involved in cell survival, invasion, angiogenesis, and metastasis. HMGA proteins interact with NF-κB and promote its activity, enhancing the binding to specific NF-κB target sequences (Trocoli and Djavaheri-Mergny 2011). NF-κB has a well characterized anti-apoptotic activity, and, recently, it is emerging a complex relationship between NF-κB and autophagy. In fact, on one hand, some NF-κB signaling components are degraded by autophagy or are regulated by autophagy-related proteins, and NF-κB protein is positively or negatively regulated by p62/SQSTM1, depending on environmental factors and cellular context. On the other hand, NF-κB itself regulates autophagy at several levels: NF-κB activates mTOR pathway and represses autophagy in Ewing's sarcoma,

breast, and leukemia cancer cell lines. Moreover, NF-κB directly regulates the transcription of some autophagy-related genes, such as *BECN1* coding for Beclin 1 (Trocoli and Djavaheri-Mergny 2011). On these bases, we can speculate that the physical and functional interaction between HMGA1 and NF-κB may be involved in the regulation of autophagy and autophagy-related genes.

More recently, it has been demonstrated that HMGA1 is able to positively regulate the transcription of the glucose transporter *SLC2A3/GLUT3* gene, thus increasing glucose uptake and ATP levels leading to AMPK inactivation with the consequent inhibition of autophagy (Ha et al. 2012).

Furthermore, a link with autophagy has been demonstrated also for HMGB1, a member of another HMG subfamily, that acts as both an architectural chromatin-binding factor and an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis. Extracellular HMGB1 is released from necrotic cells and secreted by immune cells, whereas HMGB1 nuclear sequestration is associated with apoptotic, but not necrotic, cell death. Recently, it has been demonstrated that cytosolic HMGB1 has pro-autophagic effects. In fact, cytosolic translocation of HMGB1, induced by stimuli that enhance ROS, induces autophagy because HMGB1 binds BECLIN1 in the cytoplasm, displacing it from BCL-2 and allowing the formation of the class III PI3K-BECLIN1 complex (Tang et al. 2010). It could be worth of note to investigate whether also the cytosolic proportion of HMGA1 is implied in autophagy regulation.

Interestingly, the role of extra-cellular HMGB1 has recently been studied in SCC-13 cells, founding that it enhances cell migration in a time- and dose-dependent manner, and activates the PI3K/AKT and mitogen-activated protein kinase (MAPK) signaling pathways. These data suggest the involvement of HMGB1 in the determination of the metastatic potential of CSCC cells (Sun et al. 2016).

In addition to the different possible mechanisms that link HMGA1 and autophagy, we have to discuss the complex role of autophagy in cancer, to hypothesize which can be the implications of HMGA1-mediated autophagy regulation in cancer cells. In fact, even though autophagy may promote the early phases of tumor growth, allowing survival of cancer cells under stress conditions, such as hypoxia, and providing new energy sources, it represents also a cell-autonomous mechanism of tumor suppression for its role in maintaining organelle homeostasis, reducing oxidative stress and inducing cell death (Matthew et al. 2007). In particular, stimulation of autophagy by exogenous and endogenous stress, including chemotherapy, radiotherapy and hypoxia, reduces cancer cell survival. However, autophagy impairment and resistance to autophagic cell death are features frequently acquired during cancer progression accounting, together with resistance to apoptosis, for enhanced cancer cell survival and chemoresistance (Sui et al. 2013). Therefore, we can speculate that HMGA1 overexpression may contribute to cancer progression by preventing cancer cells from dying for autophagy.

It is noteworthy that HMGA protein overexpression plays a critical role in inhibiting apoptosis mainly impairing p53-mediated regulation of apoptotic genes (Frasca et al. 2006). Intriguingly, since it has been reported that p53 positively regulates ULK1 expression (Gao et al. 2011), we can speculate that HMGA1 and p53 can regulate the expression of the same autophagy-related genes in an opposite way, as already described for apoptosis-related genes (Esposito et al. 2010). Moreover, resistance to autophagic cell death has also been correlated to other cellular processes in which HMGA1 proteins are involved, such as chromosomal instability and impairment of DNA repair (Palmieri et al. 2011, Matthew et al. 2007, Gao et al. 2011, Czarny et al. 2015). Therefore, we can envisage the possibility that HMGA1 would enhance the survival of cancer cell and, thereby, cancer progression by inhibiting, at the same time, autophagy and apoptosis. Moreover, it is worth of note to consider the possible interplay among HMGA1, autophagy and genomic instability in

cancer cells. In fact, on one hand, we have demonstrated that HMGA1 is able to impair DNA repair mechanisms and to induce chromosome instability, and, on the other hand, it is known that autophagy is able to counteract the accumulation of DNA damage and chromosomal abnormalities in cancer cells. In fact, autophagy-deficient cancer cells display both high levels of DNA damage, probably because of malfunctioning organelles, accumulation of toxic protein aggregates, oxidative stress, failure of energy homeostasis, and accumulation of aneuploidy. Despite the reduced cellular fitness caused by deficient autophagy, the superior adaptation due to increased mutation rate might be the key advantage that promotes tumorigenesis, because high mutational rate and genomic instability allow cancer cells to quickly adapt to environmental changes and to become resistant to chemo- and/or radiotherapy (Mathew et al. 2007). On the basis of these considerations, we can speculate that the inhibition of autophagy induced by HMGA1 is another mechanism, together with SAC and impairment, by which HMGA1 may contribute to genomic instability and, eventually, cancer progression.

Homeodomain-interacting protein kinase 2 (HIPK2) is a Ser/Thr kinase involved in several biological processes, such as cell proliferation, apoptosis and DNA damage response, that phosphorylates several transcription factors or co-regulators modulating their activity. HIPK2 binds and phosphorylate HMGA1 affecting its ability to bind DNA (Pierantoni et al. 2001, Zhang and Wang 2007). To evaluate the *in vivo* effects of the contemporary genetic ablation of both *Hmga1* and *Hipk2*, we have crossed *Hmga1* and *Hipk2* KO mice obtaining double KO mice (DKO). Interestingly, *Hmga1* and *Hipk2* DKO display an immature pulmonary phenotype, characterized by collapsed immature sac-like alveoli, which causes respiratory failure and perinatal mortality (Gerlini et al., unpublished data), strongly resembling the phenotype of *Ulk1* and *Ulk2* DKO that is due to defective autophagy (Cheong et al. 2013). These preliminary data suggest that HMGA1 and HIPK2 might cooperate in

the regulation of autophagy during lung development, but need to be confirmed by further studies.

6. Conclusions

In summary, the data reported here clearly evidence that depletion of HMGA1 increases autophagy by, at least in part, negatively regulating the expression of *ULK* genes, coding for master regulators of autophagy induction. These results would implicate that overexpression of HMGA1 may be a cause of autophagy impairment in cancer cells, and that autophagy inhibition may be a novel mechanism by which HMGA1 overexpression enhances survival of cancer cells and contributes to cancer progression, further supporting possible innovative antineoplastic therapies based on the inactivation of HMGA1 functions.

In order to rationalize the complexities of neoplastic diseases, Hanahan and Weimberg, in a very famous paper published in 2000 and updated in 2011, defined the so called "hallmarks of cancer": six biological capabilities acquired during the multistep development of human tumors that account for the "behaviour" of cancer cells (Hanahan and Weimberg 2000, Hanahan and Weimberg 2011). The hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism and evading immune destruction (Hanahan and Weimberg 2000, Hanahan and Weimberg 2011). Genomic instability is reputed the key determinant for the acquisition of hallmarks of cancer, and it is emerging that also autophagy impairment concurs to the establishment of some of these features. For these reasons, the findings that I have obtained during my PhD activity, demonstrating the involvement of HMGA1 both in the induction of genomic instability and in the regulation of the autophagic process, corroborate the idea that this protein has a central role in cancer initiation and progression and point out that HMGA1 may concur in the acquisition of almost all the hallmarks of cancer.

Acknowledgments

I would like to thank my tutor Dr. Giovanna Maria Pierantoni and Prof. Alfredo Fusco for giving me the possibility to do my research activity in their lab and for supporting me, our research partners Prof. Donatella Tramontano, Dr. Cinzia Rinaldo and Prof. Simona Paladino for improving our work with their expertise, Prof. Lienhard Schmitz and his group for hosting and supporting me in their lab in Germany, and my colleagues of the Fusco's lab for helping me and making more cheerful our days in the lab.

References

- Alers S, Löffler AS, Wesselborg S, Stork B.The incredible ULKs. Cell Commun Signal. 2012 Mar 13;10(1):7.
- Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000; 403:503–511.
- Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell proliferion and transformation. BiochimBiophysActa. 1991 Dec 10;1072(2-3):129-57.
- Aravind and Landsman. AT-hook motifs identified in a wide variety of DNA binding proteins. Nucleic Acids Res. 1998 Oct 1;26(19):4413-21.
- Backer JM. The reguation and function of Class III PI3Ks: novel roles for Vps34. Biochem J. 2008;410:1–17.
- Berlingieri MT, Manfioletti G, Santoro M, Bandiera A, Visconti R, Giancotti V, Fusco A. Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. Mol Cell Biol. 1995 Mar;15(3):1545-53.
- Berlingieri MT, Pierantoni GM, Giancotti V, Santoro M, Fusco A. Thyroid cell transformation requires the expression of the HMGA1 proteins. Oncogene. 2002 May 2;21(19):2971-80.
- Bolanos-Garcia VM, Blundell TL. Bub1 and BubR1: multifaceted kinases of the cell cycle, Trends Biochem Sci. 2011 Mar;36(3):141-50.
- Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. Nat Cell Biol. 2013 Jul;15(7):713-20.

- Budovskaya YV, Stephan JS, Deminoff SJ, Herman PK. An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. Proc Natl Acad Sci USA. 2005;102:13933–38.
- Bustin and Reeves. High mobility group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol. 1996;54:35-100.
- Chau KY, Patel UA, Lee KL, Lam HY, Crane-Robinson C. The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinc functional element. Nucleic Acids Res. 1995 Nov 11;23(21):4262-6.
- Cheong H, Wu J, Gonzales LK, Guttentag SH, Thompson CB, Lindsten T.Analysis of a lung defect in autophagy-deficient mouse strains. Autophagy. 2014 Jan;10(1):45-56.
- Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. N Engl J Med. 2013;368(7):651–662.
- Cleynen I, Huysmans C, Sasazuki T, Shirasawa S, Van de Ven W, Peeters K. Transcriptional control of the human high mobility group A1 gene: basic and oncogenic Ras-regulated expression. Cancer Res. 2007 May 15;67(10):4620-9.
- Czarny P, Pawlowska E, Bialkowska-Warzecha J, Kaarniranta K, Blasiak J. Autophagy in DNA damage response. Int J Mol Sci 2015; 16(2):2641-62.
- Dann SG, Selvaraj A, Thomas G.mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med. 2007 Jun;13(6):252-9.
- De Martino M, Forzati F, Arra C, Fusco A, Esposito F. HMGA1-pseudogenes and cancer. Oncotarget. 2016 Feb 16. doi: 10.18632/oncotarget.7427. [Epub ahead of print]

- Deter RL, De Duve C. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. J Cell Biol. 1967;33:437–449.
- Eskelinen EL. Maturation of autophagic vacuoles in mammaliam cells. Autophagy. 2005;1:1–10.
- Esposito F. High Mobility Group A1 (HMGA1) proteins regulate p53-mediated transcription of Bcl-2 gene. [Thesis] Naples, Italy: University of Naples Federico II; 2008, 103p. Available from: Department of Biology and cellular and molecular pathology.
- Esposito F, Tornincasa M, Chieffi P, De Martino I, Pierantoni GM, Fusco A. High-mobility group A1 proteins regulate p53-mediated transcription of Bcl-2 gene; Cancer Res. 2010 Jul 1;70(13):5379-88.
- Fedele M, Pentimalli F, Baldassarre G, Battista S, Klein-Szanto AJ, Kenyon L, Visone R, De Martino I, Ciarmiello A, Arra C, Viglietto G, Croce CM, Fusco A. Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. Oncogene. 2005 May 12;24(21):3427-35.
- Fimia GM, Stoykova A, Romagnoli AG, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, et al. Ambra1 regulates autophagy and development of the nervous system. Nature. 2007;447:1121–1125.
- Frasca F, Rustighi A, Malaguarnera R, Altamura S, Vigneri P, Del Sal G, Giancotti V, Pezzino V, Vigneri R, Manfioletti G. HMGA1 inhibits the function of p53 family members in thyroid cancer cells. Cancer Res 2006; 66(6):2980-9.
- Friedmann M, Holth LT, Zoghbi HY, Reeves R. Organization, inducible expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. Nucleic Acids Res. 1993 Sep 11;21(18):4259-67.

- Fry and Peterson. Chromatin remodeling enzymes: who's on first?. Curr Biol. 2001 Mar 6;11(5):R185-97.
- Furusawa T, Cherukuri S. Developmental function of HMGN proteins. BiochimBiophysActa, 2010 Jan-Feb;1799(1-2):69-73.
- Fusco and Fedele. Roles of HMGA proteins in cancer. Nat Rev Cancer. 2007 Dec;7(12):899-910.
- Gao W, Shen Z, Shang L, Wang X. Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death. Cell Death Differ 2011; 18(10):1598-607.
- Giancotti V, Berlingieri MT, DiFiore PP, Fusco A, Vecchio G, Crane-Robinson C. Changes in nuclear proteins following transformation of rat thyroid epithelial cells by a murine sarcoma retrovirus, Cancer Res. 1985 Dec;45(12 Pt 1):6051-7.
- Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010 May;221(1):3-12.
- Grabsch H, Takeno S, Parsons WJ, Pomjanski N, Boecking A, Gabbert HE, Mueller W. Overexpression of the mitotic checkpoint genes BUB1, BUBR1, and BUB3 in gastric cancer-association with tumour cell proliferation. J. Pathol. 2003; 200:16–22.
- Ha TK, Her NG, Lee MG, Ryu BK, Lee JH, Han J, Jeong SI, Kang MJ, Kim NH, Kim HJ, Chi SG. Caveolin-1 increases aerobic glycolysis in colorectal cancers by stimulating HMGA1-mediated GLUT3 transcription. Cancer Res 2012; 72(16):4097-109.
- Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar 4;144(5):646-74.
- Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000 Jan 7;100(1):57-70.
- Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994 Dec 16;266(5192):1821-8.

- Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol. 2009;11:1433–1437.
- He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67-93.
- Hebert C, Norris K, Scheper MA, Nikitakis N, Sauk JJ. High mobility group A2 is target for miRNA-98 in head and neck squamous cell carcinoma. Mol Cancer. 2007 Jan 14; 6:5
- Hill DA, Pedulla ML, Reeves R. Directional binding of HMG-I(Y) on fourway junctions DNA and molecular basis for competitive binding with HMG-I and histone H1. Nucleic Acids Res. 1999 May 15;27(10):2135-44.
- Jäger S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL. Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci. 2004;117:4837–4848.
- Janji B, Viry E, Baginska J, Van Moer K and Berchem G. Role of Autophagy in Cancer and Tumor Progression. In: Biochemistry, Genetics and Molecular Biology » "Autophagy - A Double-Edged Sword - Cell Survival or Death?", book edited by Yannick Bailly, ISBN 978-953-51-1062-0, Published: April 17, 2013
- Johnson KR, Lehn DA, Reeves R. Alternative processing of mRNAs encoding mammalian chromaosomal high mobility group proteins HMG-I and HMG-Y. Mol Cell Biol.1989 May;9(5):2114-23.
- Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009;20:1992–2003.
- Kaminskyy V, Zhivotovsky B.Proteases in autophagy. Biochim Biophys Acta. 2012 Jan;1824(1):44-50.

- Karia PS, Han J, Schmults CD. Cutaneous squamous cell carcinoma: Estimated incidence of disease, nodal metastasis and deaths from disease in the United States, 2012. J Am Acad Dermatol. 2013;68:957–966.
- Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol, 2008. 9(12): p. 1004-10.
- Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, Zhang J, et al. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood. 2008;112:1493–1502.
- Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell 2012; 149(2):274-93.
- Lee EJ, Tournier C.The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. Autophagy. 2011 Jul;7(7):689-95.
- Lee YS, Dutta A. A tumor suppressor miRNA let-7represses the HMGA2 oncogene. Genes Dev. 2007 May 1;21(9):1025-30.
- Lehn DA, Elton TS, Johnson KR, Reeves R. A conformational study of the sequence specific binding of HMGI(Y) with the bovine interleukine-2 cDNA. Biochem Int. 1988 May;16(5):963-71.
- Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell.2008;132:27–42.
- Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature, 2011. 469(7330): p. 323-35.
- Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol. 2006;8:688–699.
- Liang XH, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. 1999;402(6762):672–676.

- Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin1. Nature. 1999;402:672–676.
- Liau SS, Jazag A, Ito K, Whang EE. Overexpression of HMGA1 promotes anoikis resistance and constitutive AKT activation in pancreatic adenocarcinoma cells. Br J Cancer 2007; 96(6):993-1000.
- Liau SS, Wang E. HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma. Clin Cancer Res 2008; 14(5):1470-7.
- Lin SF, Lin PM, Yang MC, Liu TC, Chang JG, Sue YC, Chen TP. Expression of hBUB1 in acute myeloid leukemia. Leuk Lymphoma. 2002; 43:385–391.
- Liu L, Cash TP, Jones RG, Keith B, Thompson CB, Simon MC. Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol Cell. 2006;21:521–531.
- Liu WM, Guerra-Vladusic FK, Kurakata S, Lupu R, Kohwi-Shigematsu T. HMG-I(Y) recognizes base-unpairing regions of matrix attachment sequences and its increased expression is directly linked to metastatic breast cancer phenotype, Cancer Res. 1999 Nov 15;59(22):5695-703.
- Lund T, Holtlund J, Fredriksen M, Laland SG. On the presence of two new high mobility group-like proteins in HeLa S3 cells. FEBSLett. 1983 Feb 21;152(2):163-7.
- Lv Q, Wang W, Xue J, Hua F, Mu R, Lin H, Yan J, Lv X, Chen X, Hu ZW. DEDD interacts with PI3KC3 to activate autophagy and attenuate epithelial- mesenchymal transition in human breast cancer. Cancer Res, 2012. 72(13): p. 3238-50.
- Mantovani F, Covaceuszach S, Rustighi A, Sgarra R, Heath C, Goodwin GH, Manfioletti G. NF-kB transcriptional mediated activation

is enhanced by the architectural factor HMGAI-C, Nucleic Acids Res. 1998 Mar 15;26(6):1433-9.

- Mao L, Wertzler KJ, Maloney SC, Wang Z, Magnuson NS, Reeves R. HMGA1 levels influence mitochondrial function and mitochondrial DNA repair efficiency. Mol Cell Biol. 2009 Oct;29(20):5426-40.
- Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. Am J Cancer Res. 2012; 2(2): 214–221.
- Martinez Hoyos J, Fedele M, Battista S, Pentimalli F, Kruhoffer M, Arra C, Orntoft TF, Croce CM, Fusco A. Identification of the genes up- and down- regulated by the high mobility group A1 (HMGA1) proteins: tissue specificity of the HMGA1-dependent gene regulation, Cancer Res. 2004 Aug 15;64(16):5728-35.
- Matsunaga K, Saitoh T, Tabatra K, Omori H, Satoh T, Kurotori N, et al. Two Beclin1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol. 2009;11:385–396.
- Matthew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nature Rev Cancer 2007; 7(12):961–7.
- Merika and Thanos. Enhanceosomes. CurrOpin Genet Dev. 2001 Apr;11(2):205-8.
- Mertens F, Johansson B, Mitelman F. Isochromosomes in neoplasia. Genes Chromosomes Cancer. 1994; 10:221–230.
- Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007; 3(6):542-5.
- Mizushima N. Autophagy: process and function. Genes Dev. 2007;21:2861–2873.
- Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 2010; 22(2):132-9.

- Mussnich P, D'Angelo D, Leone V, Croce CM, Fusco A. The High Mobility Group A proteins contribute to thyroid cell transformation by regulating miR-603 and miR-10b expression. Mol Oncol 2013; 7(3):531-42.
- Nagpal S, Ghosn C, DiSepio D, Molina Y, Sutter M, Klein ES, Chandraratna RA. Retinoid-dependent recruitment of a histone H1 displacement activity by retinoic acid receptor. JBiol Chem. 1999 Aug 6;274(32):22563-8.
- Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol. 2009;10:458–467.
- Nissen and Reeves. Changes in superhelicity are introduced into closed circular DNA by binding of high mobility group protein I/Y. J Biol Chem. 1995 Mar 3;270(9):4355-60.
- Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013 Aug;19(8):983-97.
- Pallante P, Sepe R, Puca F, Fusco A. High mobility group a proteins as tumor markers. Front Med (Lausanne). 2015 Mar 25;2:15. doi:10.3389/fmed.2015.00015.
- Palmieri D, Valentino T, D'Angelo D, De Martino I, Postiglione I, Pacelli R, Croce CM, Fedele M, Fusco A. HMGA proteins promote ATM expression and enhance cancer cell resistance to genotoxic agents. Oncogene 2011; 30(27):3024-35.
- Pattingre S, Tassa A, Qu X, Garuti R, Linag XH, Mizushima N, et al. Bcl-2 anti-apoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122:927–939.
- Pegoraro S, Ros G, Piazza S, Sommaggio R, Ciani Y, Rosato A, Sgarra R, Del Sal G, Manfioletti G. HMGA1 promotes metastatic processes in basal-like breast cancer regulating EMT and stemness. Oncotarget 2013; 4(8):1293-308.

- Pierantoni GM, Agosti V, Fedele M, Bond H, Caliendo I, Chiappetta G, Lo Coco F, Pane F, Turco MC, Morrone G, Venuta S, Fusco A. High-mobility group A1 proteins are overexpressed in human leukaemias. Biochem J 2003; 372(Pt 1):145–50. (Referred to as "Pierantoni et al. 2003b")
- Pierantoni GM, Battista S, Pentimalli F, Fedele M, Visone R, Federico A, Santoro M, Viglietto G, Fusco A. A truncated HMGA1 gene induces proliferation of the 3T3-L1 pre-adipocytic cells: a model of human lipomas. Carcinogenesis 2003; 24(12):1861-9.
- Pierantoni GM, Conte A, Rinaldo C, Tornincasa M, Gerlini R, Federico A, Valente D, Medico E, Fusco A. Deregulation of HMGA1 expression induces chromosome instability through regulation of spindle assembly checkpoint genes. Oncotarget 2015; 6(19):17342-53.
- Pierantoni GM, Conte A, Rinaldo C, Tornincasa M, Gerlini R, Valente D, Izzo A, Fusco A. *Hmga1* null mouse embryonic fibroblasts display downregulation of spindle assembly checkpoint gene expression associated to nuclear and karyotypic abnormalities. Cell Cycle. 2016 Feb 18:0. [Epub ahead of print]
- Pierantoni GM, Fedele M, Pentimalli F, Benvenuto G, Pero R, Viglietto G, Santoro M, Chiariotti L, Fusco A. High mobility group I (Y) proteins bind HIPK2, a serine-threonine kinase protein which inhibits cell growth. Oncogene. 2001 Sep 27;20(43):6132-41.
- Pike LR, Singleton DC, Buffa F, Abramczyk O, Phadwal K, Li JL, Simon AK, Murray JT, Harris AL.Transcriptional up-regulation of ULK1 by ATF4 contributes to cancer cell survival. Biochem J. 2013 Jan 15;449(2):389-400.
- Puca F, Colamaio M, Federico A, Gemei M, Tosti N, Bastos AU, Del Vecchio L, Pece S, Battista S, Fusco A. HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels. Oncotarget. 2014; 5(10):3234-45.

- Qu X, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. 2003;112(12):1809–1820.
- Ralston SH. Pathogenesis of Paget's disease on bone. Bone. 2008;43:819–825.
- Rao CV, Yamada HY, Yao Y, Dai W. Enhanced genomic instabilities caused by deregulated microtubule dynamics and chromosome segregation: a perspective from genetic studies in mice. Carcinogenesis. 2009; 30:1469–1474.
- Reeves and Beckerbauer. HMGI/Y proteins: flexible regulators of transcription and chromatin structure. BiochimBiophysActa. 2001 May 28;1519(1-2):13-29.
- Reeves and Nissen. Interaction of high mobility group-I (Y) nonhistone proteins with nucleosome core particles. J Biol Chem. 1993 Oct 5;268(28):21137-46.
- Reeves R, Edberg DD, Li Y. Architectural trancription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol Cell Biol. 2001 Jan;21(2):575-94.
- Reeves R, Elton TS, Nissen MS, Lehn D, Johnson KR. Posttranscriptional gene regulation and specific binding of the nonhistone protein HMG-I by the 3' untranslated region of bovine interleukine 2 cDNA. ProcNatlAcadSci U S A. 1987 Sep;84(18):6531-5.
- Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res. 1981 May;41(5):1657-63.
- Ro SH, Jung CH, Hahn WS, Xu X, Kim YM, Yun YS, Park JM, Kim KH, Seo M, Ha TY, Arriaga EA, Bernlohr DA, Kim DH.Distinct functions of Ulk1 and Ulk2 in the regulation of lipid metabolism in adipocytes. Autophagy. 2013 Dec;9(12):2103-14.

- Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006;6:729–734.
- Saftig P, Beertsen W, Eskelinen EL. LAMP-2. Autophagy. 2008;4:510–512.
- Shaw RJ. LKB1 and AMP-activated kinase control of mTOR signalling and growth. Acta Physiol. 2009;196:65–80.
- Shen HM, Codogno P. Autophagy is a survival force via suppression of necrotic cell death. Exp Cell Res, 2012. 318(11): p. 1304-8.
- Shigeishi H, Oue N, Kuniyasu H, Wakikawa A, Yokozaki H, Ishikawa T, Yasui W. Expression of Bub1 gene correlates with tumor proliferating activity in human gastric carcinomas. Pathobiology. 2001; 69:24–29.
- Shukla S, Patric IR, Patil V, Shwetha SD, Hegde AS, Chandramouli BA, Arivazhagan A, Santosh V, Somasundaram K. Methylation silencing of ULK2, an autophagy gene, is essential for astrocyte transformation and tumor growth. J Biol Chem. 2014 Aug 8;289(32):22306-18.
- Subramanian D, Griffith JD. Interactions between p53, hMSH2-hMSH6 and HMGAI(Y) on Holliday junctions and bulged bases. Nucleic Acids Res. 2002 Jun 1;30(11):2427-34.
- Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q, Wang X, He C, Pan H. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013; 4:e838.
- Sun Y, Tu Y, He LI, Ji C, Cheng BO. High mobility group box 1 regulates tumor metastasis in cutaneous squamous cell carcinoma via the PI3K/AKT and MAPK signaling pathways. Oncol Lett. 2016 Jan;11(1):59-62.

- Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N. Autophagy-deficient mice develop multiple liver tumors. Genes Dev. 2011;25(8):795–800.
- Tallini G, Vanni R, Manfioletti G, Kazmierczak B, Faa G, Pauwels P, Bullerdiek J, Giancotti V, Van Den Berghe H, Dal Cin P. HMGI-C and HMGI(Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroidhamartomas, endometrial polyps and uterine leiomyomas and is compatible with rearrangment of the HMGI-C and HMGI(Y) genes. Lab Invest. 2000 Mar;80(3):359-69.
- Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, Hoppe G, Bianchi ME, Tracey KJ, Zeh HJ 3rd, Lotze MT. Endogenous HMGB1 regulates autophagy. J Cell Biol 2010; 190(5):881-92.
- Tessari MA, Gostissa M, Altamura S, Sgarra R, Rustighi A, Salvagno C, Caretti G, Imbriano C, Mantovani R, Del Sal G, Giancotti V, Manfioletti G. Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2. Mol Cell Biol 2003; 23(24):9104-16.
- Thanos and Maniatis. The high mobility group HMG I(Y) is an essential structural component of virus inducible enhancer complex. Cold Spring HarbSymp Quant Biol. 1993;58:73-81.
- Thanos and Maniatis. The high mobility group HMG I(Y) is required for NF-kB dependent virus induction of the human IFN-β gene. Cell. 1992 Nov27;71(5):777-89.
- Trocoli A, Djavaheri-Mergny M. The complex interplay between autophagy and NF-κB signaling pathways in cancer cells. Am J Cancer Res. 2011; 1(5): 629–649.
- Uribe P, Gonzalez S. Epidermal growth factor receptor (EGFR) and squamous cell carcinoma of the skin: Molecular bases for EGFR-targeted therapy. Pathol Res Pract. 2011;207:337–342.

- Vallone D, Battista S, Pierantoni GM, Fedele M, Casalino L, Santoro M, Viglietto G, Fusco A, Verde P. Neoplastic transformation of rat thyroid cells requires the junB and fra-1 gene induction which is dependent on the HMGI-C gene product. EMBO J 1997; 16(17):5310-21.
- van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002; 415:530–536.
- Vitale N, Kisslinger A, Paladino S, Procaccini C, Matarese G, Pierantoni GM, Mancini FP, Tramontano D. Resveratrol couples apoptosis with autophagy in UVB-irradiated HaCaT cells. PLoS One 2013; 8(11):e80728.
- Webb JL, Ravikumar B, Rubinsztein DC. Microtubule disruption inhibits autophagosome–lysosome fusion: implications for studying the roles of aggresomes in polyglutamine diseases. Int J Biochem Cell Biol. 2004;36:2541–2550.
- White E. The role for autophagy in cancer. J Clin Invest. 2015 Jan;125(1):42-6.
- Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, Nakasato M, Lu Y, Hangai S, Koshiba R, Savitsky D, Ronfani L, Akira S, Bianchi ME, Honda K, Tamura T, Kodama T, Taniguchi T. HMGB proteins function as universal sentinels for nucleic-acid mediated innate immune responses. Nature. 2009 Nov 5;462(7269):99-103.
- Yue Z, Friedman L, Komatsu M, Tanaka K. The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. Biochim Biophys Acta. 2009;1793:1496–1507.

- Zhang and Wang. Homeodomain-interacting protein kinase-2 (HIPK2) phosphorilates HMGAIa at Ser-35, Thr-22, and Thr-77 and modulates its DNA binding affinity. J Proteome Res. 2007 Dec;6(12):4711-9.
- Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy and mitophagy. Cell Death Diff. 2009;16:939–946.

Synopsis of publications

1. Pierantoni GM*, Conte A*, Rinaldo C, Tornincasa M, Gerlini R, Federico A, Valente D, Medico E, Fusco A. **Deregulation of HMGA1 expression induces chromosome instability through regulation of spindle assembly checkpoint genes.** Oncotarget. 2015 Jul 10;6(19):17342-53. (*Co-first authors)

The mitotic spindle assembly checkpoint (SAC) is an essential control system of the cell cycle that contributes to mantain the genomic stability of eukaryotic cells. SAC genes expression is often deregulated in cancer cells, leading to checkpoint impairment and chromosome instability. The mechanisms responsible for the transcriptional regulation and deregulation of these genes are still largely unknown. Herein we identify the nonhistone architectural nuclear proteins High Mobility Group A1 (HMGA1), whose overexpression is a feature of several human malignancies and has a key role in cancer progression, as transcriptional regulators of SAC genes expression. In particular, we show that HMGA1 proteins are able to increase the expression of the SAC genes Ttk, Mad2l1, Bub1 and Bub1b, binding to their promoter regions. Consistently, HMGA1-depletion induces SAC genes downregulation associated to several mitotic defects. In particular, we observed a high number of unaligned chromosomes in metaphase, a reduction of prometaphase time, a delay of anaphase, a higher cytokinesis time and a higher percentage of cytokinesis failure by using live-cell microscopy. Finally, a significant direct correlation between HMGA1 and SAC genes expression was detected in human colon carcinomas indicating a novel mechanism by which HMGA1 contributes to cancer progression.

2. Conte A, Pierantoni GM. **Regulation of HIPK Proteins by MicroRNAs.** Microrna. 2016;4(3):148-57

The homeodomain-interacting protein kinase (HIPK) family consists of four evolutionarily conserved and highly related nuclear serine/threonine kinases of recent discovery. They interact with homeobox proteins and other transcription factors, as well as transcriptional coactivators or corepressors depending on the cellular context. HIPK proteins are sensors for various extracellular stimuli, which control key cellular functions such as signal transduction to downstream effectors that regulate apoptosis, embryonic development, DNA-damage response, and cellular proliferation. Thus, HIPKs are involved in proliferative diseases such as cancer and fibrosis. mRNA levels and protein stability tightly regulate expression levels of HIPKs. Here, we review recent works investigating the regulation of HIPKs expression by microRNAs (miRNAs) that are involved in the control of cell proliferation, sensitivity to chemotherapeutic drugs, epithelial-mesenchymal transition, and glucose-stimulated insulin secretion. It appears that HIPK family members, and their related miRNAs, may be considered as novel therapeutic targets for treating cancer, renal fibrosis and type 2 diabetes.

3. Anzilotti S, Tornincasa M, Gerlini R, Conte A, Brancaccio P Cuomo O, Bianco G, Fusco A, Annunziato L, Pignataro G, and Pierantoni GM. **Genetic ablation of homeodomain interacting protein kinase 2 (HIPK2) selectively induces apoptosis of cerebellar Purkinje cells during adulthood and generates an ataxic-like phenotype.** Cell Death Dis. 2015 Dec 3;6:e2004

Homeodomain-interacting protein kinase 2 (HIPK2) is a multitalented coregulator of an increasing number of transcription factors and cofactors involved in cell death and proliferation in several organs and systems. As *Hipk2*(-/-) mice show behavioral abnormalities consistent with cerebellar dysfunction, we investigated whether *Hipk2* is involved in these neurological symptoms. To this aim, we characterized the postnatal developmental expression profile of *Hipk2* in the brain cortex, hippocampus, striatum, and cerebellum of mice by real-time PCR, western blot analysis, and immunohistochemistry. Notably, we found that whereas in the brain cortex, hippocampus, and striatum, HIPK2 expression progressively decreased with age, that is, from postnatal day 1 to adulthood, it increased in the cerebellum. Interestingly, mice lacking *Hipk2* displayed atrophic lobules and a visibly smaller cerebellum than did wild-type mice. More important, the cerebellum of *Hipk2* (-/-) mice showed a strong reduction in cerebellar Purkinje neurons during adulthood. Such reduction is due to the activation of an apoptotic process associated with a compromised proteasomal function followed by an unpredicted accumulation of ubiquitinated proteins. In particular, Purkinje cell dysfunction was characterized by a strong accumulation of ubiquitinated β-catenin. Moreover, our behavioral tests showed that *Hipk2* (-/-) mice displayed muscle and balance impairment, indicative of *Hipk2* involvement in cerebellar function. Taken together, these results indicate that *Hipk2* exerts a relevant role in the survival of cerebellar Purkinje cells and that *Hipk2* genetic ablation generates cerebellar dysfunction compatible with an ataxic-like phenotype.

4. Conte A, Procaccini C, Iannelli P, Kisslinger A, De Amicis F, Pierantoni GM, Mancini FP, Matarese G and Tramontano D. **Effects of Resveratrol on p66Shc phosphorylation in cultured prostate cells.** Transl Medi UniSa 2015, 13(8): 47-58

There is increasing evidence that diet plays a crucial role in age-related diseases and cancer. Oxidative stress is a conceivable link between diet and diseases, thus food antioxidants, counteracting the damage caused by oxidation, are potential tools for fight age-related diseases and cancer. Resveratrol (RSV), a polyphenolic antioxidant from grapes, has gained enormous attention particularly because of its ability to induce growth arrest and apoptosis in cancer cells, and it has been proposed as both chemo-preventive and therapeutic agent for cancer and other diseases. Even though the effects of RSV have been studied in prostate cancer cells and animal models, little is known about its effects on normal cells and tissues. To address this issue, we have investigated the effects of RSV on EPN cells, a human non-transformed prostate cell line, focusing on the relationship between RSV and p66Shc, a redox enzyme whose activities strikingly intersect those of RSV. p66Shc activity is regulated by phosphorylation of serine 36 (Ser36) and has been related to mitochondrial oxidative stress, apoptosis induction, regulation of cell proliferation and migration. Here we show that RSV inhibits adhesion, proliferation and migration of EPN cells, and that these effects are associated to induction of dose- and time-dependent p66Shc-Ser36 phosphorylation and ERK1/2 de-phosphorylation. Moreover, we found that RSV is able to activate also p52Shc, another member of the Shc protein family. These data show that RSV affects non-transformed prostate epithelial cells and suggest that Shc proteins may be key contributors of RSV effects on prostate cells.

5. Pierantoni GM*, Conte A*, Rinaldo C, Tornincasa M, Gerlini R, Valente D, Izzo A, Fusco A. ***Hmga1*** **null mouse embryonic fibroblasts display downregulation of spindle assembly checkpoint gene expression associated to nuclear and karyotypic abnormalities.** Cell Cycle. 2016 Feb 18:0; 15(6):812-818 (*Co-first authors)

The High Mobility Group A1 proteins (HMGA1) are nonhistone chromatinic proteins with a critical role in development and cancer. We have recently reported that HMGA1 proteins are able to increase the expression of spindle assembly checkpoint (SAC) genes, thus impairing SAC function and causing chromosomal instability in cancer cells. Moreover, we found a significant correlation between HMGA1 and SAC genes expression in human colon carcinomas. Here, we report that mouse embryonic fibroblasts null for the *Hmga1* gene show downregulation of Bub1, Bub1b, Mad2l1 and Ttk SAC genes, and present several features of chromosomal instability, such as nuclear abnormalities, binucleation, micronuclei and karyotypic alterations. Interestingky, also MEFs carrying only one impaired *Hmga1* allele present karyotypic alterations. These results indicate that HMGA1 proteins regulate SAC genes expression and, thereby, genomic stability also in embryonic cells.