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

XXVI cycle - 2010–2013

Coordinator: Prof. Massimo Santoro

"*HMGA1 expression controls the symmetric/asymmetric division ratio of colon cancer stem cells by regulating p53 and NUMB.*"

Francesca Puca

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

Italian 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
Università degli Studi del Sannio, Benevento, Italy
Università degli Studi di Genova, Genova, Italy
Università degli Studi di Padova, Padova, Italy
Università degli Studi "Magna Graecia", Catanzaro, Italy
Università degli Studi di Udine, Udine, Italy

Foreign Institutions
Université Libre de Bruxelles, Bruxelles, Belgium
Universidade Federal de Sao Paulo, Brazil
University of Turku, Turku, Finland
Université Paris Sud XI, Paris, France
University of Madras, Chennai, India
University Pavol Jozef Šafàrik, Kosice, Slovakia
Universidad Autonoma de Madrid, Centro de Investigaciones Oncologicas (CNIO), Spain
Johns Hopkins School of Medicine, Baltimore, MD, USA
Johns Hopkins Krieger School of Arts and Sciences, Baltimore, MD, USA
National Institutes of Health, Bethesda, MD, USA
Ohio State University, Columbus, OH, USA
Albert Einstein College of Medicine of Yeshiwa University, N.Y., USA

Supporting Institutions
Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Naples, Italy
Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy
Istituto Superiore di Oncologia, Italy

Italian Faculty

Salvatore Maria Aloj
Vittorio Enrico Avvedimento
Francesco Beguinot
Maria Teresa Berlingieri
Roberto Bianco
Bernadette Biondi
Francesca Carlomagno
Maria Domenica Castellone
Gabriella Castoria
Angela Celetti
Annamaria Cirafici
Annamaria Colao
Gerolama Condorelli
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
Maria Giovanna Pierantoni
Rosario Pivonello
Giuseppe Portella
Maria Fiammetta Romano
Giuliana Salvatore
Massimo Santoro
Donatella Tramontano
Giancarlo Troncone
Giancarlo Vecchio
Giuseppe Viglietto
Mario Vitale

“*HMGA1 expression controls the symmetric/asymmetric division ratio of colon cancer stem cells by regulating p53 and NUMB*”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the submitted publication:

Puca F, Colamaio M, Federico A, Gemei M, Tosti N, Uchimura Bastos A, Del Vecchio L, Pece S, Battista S and Fusco A. ***HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels.***

LIST OF ABBREVIATIONS

CSCs = Cancer stem cells
ESCs = Embryonic Stem cells
CTSCs = Colon Tumor Stem Cells
MSCs = Mesenchymal Stem Cells
HMGA = High Mobility Group A
SFE = Sphere forming efficiency
EMT = Epithelial-Mesenchymal Transition
TICs = Tumor Initiating Cells
EBs = Embryoid bodies
ACD = Asymmetric cell division
TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling
ChIP = Chromatin Immunoprecipitation
qRT-PCR = quantitative RT-PCR

ABSTRACT

High-mobility group A1 (HMGA1) proteins are architectural chromatinic proteins that are abundantly expressed during embryogenesis and in most cancer tissues, but are expressed at low levels or are absent in normal adult tissues. Several studies have demonstrated that HMGA1 proteins play a causal role in neoplastic cell transformation. HMGA1 has been shown to induce stem cell-like properties in colon cancer cells, suggesting that HMGA1 may be a key regulator in the maintenance of a stem cell-like state. The aim of this study was to investigate the role of these proteins in the control of cancer stem cells (CSCs), which have emerged as a preferred target in cancer therapy, because of their role in tumor recurrence. First, we observed that HMGA1 is overexpressed in colon tumor stem cell (CTSC) lines with respect to normal and colon cancer tissues. We demonstrated that the inhibition of HMGA1 expression in CTSCs increases the percentage of PKH26-positive cells, indicating increased stem cell quiescence. Moreover, HMGA1 silencing induces a drastic reduction in their self-renewal and sphere-forming efficiency (SFE). This effect, together with the finding of an asymmetric NUMB distribution in interfered cells, is indicative of the recovery of an asymmetric division pattern, characteristic of normal stem cells. Indeed, we have been able to demonstrate that HMGA1 negatively regulates p53 and NUMB expression at transcriptional level, thereby accounting for their increased expression at protein level in CTSC-HMGA1-silenced cells. Indeed, p53 has been recently found to regulate the balance between symmetric and asymmetric division; on the other hand, NUMB is known for its role as cell fate determinant in stem cells and effector in the stabilization of p53. The HMGA1 transcriptional regulation of NUMB and p53 likely accounts for the reduced SFE of silenced CTSCs. Preliminary data suggest that targeting HMGA1 makes resistant CTSCs more sensitive to chemotherapeutic agents. Therefore, our data indicate a critical role for HMGA1 in regulating both self-renewal and the balance of symmetric/asymmetric division in colon CSCs and suggest that blocking HMGA1 function may be an effective anti-cancer therapy.

1. BACKGROUND

1.1. Tumors and Stemness

Cancer arises from a small set of stem cells, or tumor-initiating cells, that differ from normal stem cells in their deregulated self-renewal and differentiation programs.
Although researchers had long hypothesized that cancers may arise from stem or stem-like cells, it was in 1994 that a stem cell-like population was isolated from a human cancer. John Dick and colleagues showed that human acute myeloid leukemia (AML) contains a small percentage of cells enriched on the basis of cell surface marker expression ($CD34^+/CD38^-$) that were capable of transferring human AML into immunodeficient hosts. The resulting leukemia recapitulates the morphologic and immunophenotypic heterogeneity of the original disease, and engrafted blasts were able to reproduce the tumor in secondary recipients (Lapidot et al. 1994).

1.1.1. The CSCs hypotesis

First studies of AML stem cells laid the foundation for the CSC hypotesis which holds that, like normal tissue, cancers are originated by a population of stem-like cells that exibit the ability to self-renew as well as differentiate into downstrem, non self-renewing progenitors and mature cells. Further these interesting findings arose great interest through the discovery of cells with stem cell properties in other tumors. So, human CSCs were identified also in solid tumors, including breast (Al-Hajj et al. 2003), brain (Singh et al. 2003), colon (O'Brien et al. 2007), pancreas (Li et al. 2007), lung, prostate cancer (Collins et al. 2005) and melanoma.
Expression of cell surface markers such as CD44, CD24, CD29, CD90, CD133, epithelial-specific antigen (ESA), and aldehyde dehydrogenase1 (ALDH1) have been used to isolate and enrich CSCs from different tumors (Al-Hajj, Wicha et al. 2003), (Singh et al. 2003), (Ginestier et al. 2007). Notably, the expression of CSC surface markers is tissue type-specific and tumor subtype-specific. For example, $CD44^+CD24^-$/low lineage and $ALDH^+$ are characteristic of breast CSCs; $CD133^+$ of colon, brain and lung; $CD34^+CD8^-$ of leukemia; $CD44^+$ of head and neck; $CD90^+$ of liver; $CD44^+/CD24^+/ESA^+$ of pancreas CSCs (Ginestier et al. 2007).
There are several different theories regarding the origin of CSCs (Fig. 1.1).
One theory holds that CSCs arise from normal stem/progenitor cells which gain the ability to generate tumors when encountering a special genetic mutation or environmental alteration. Some CSCs exhibit similarities to normal stem/progenitor cells in cellular property, phenotype, function, and even cell

surface markers (Li et al. 2007), (Kucia and Ratajczak 2006). Another theory suggests that they arise from normal somatic cells which acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. For example, cancer cells gain stem-like characteristics through epithelial-mesenchymal transition (EMT). The induction of EMT in immortalized human mammary epithelial cells results in the acquisition of mesenchymal traits and expression of stem-cell markers (Yu et al. 2007) (Takahashi et al. 2007). Given the different hypotesis about the origin of CSCs, they are often defined preferentially as Tumor Initiating Cells (TICs) to avoid confusion.

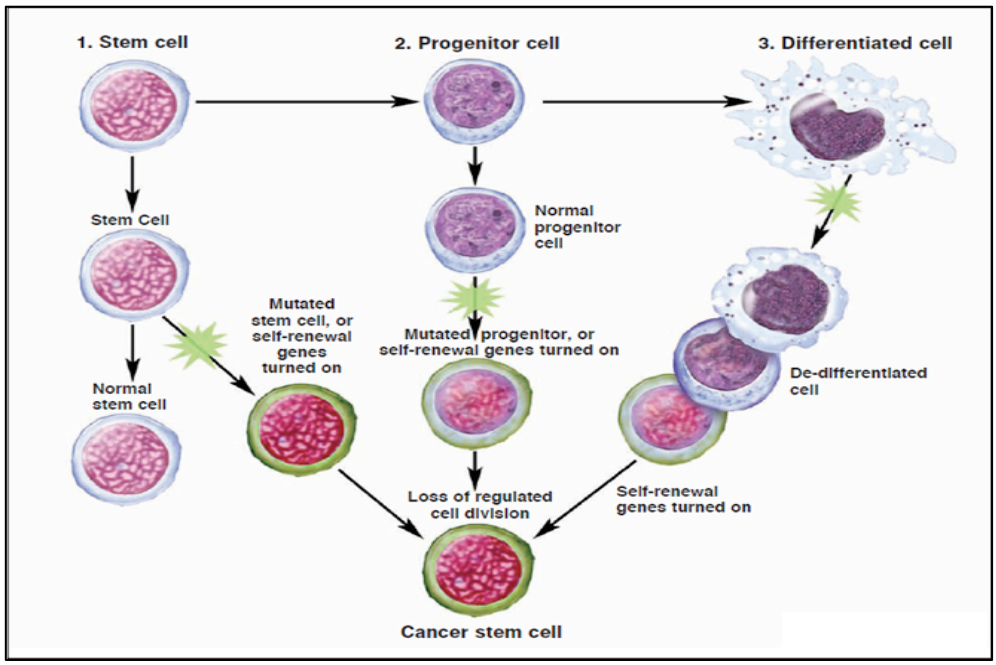


Figure 1.1. Summary scheme of the hypotesis on the CSCs origin. A CSC could take origin from a normal stem cell which, after mutations in genes involved in the regulation of fundamental cell function such as proliferation, differentiation and self renewal acquire malignant properties. Also CSCs could derive from a differentiated cell which gain proliferation potential and turns on self-renewal genes resulting in a stem-like cell (from *Are Stem Cells Involved in Cancer? Stem Cell Information 2011)*.

1.1.2. CSCs feautures

CSCs are defined by the American Association of Cancer Research as “the cells within a tumor that possesse the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor". Indeed, CSCs

are characterized by some particular features. They are able to generate more SCs (self-renewal) and to produce cells that differentiate. Asymmetric cell division achieves both tasks, as one progeny retains SC identity and the other undergoes rounds of cell division and subsequent post-mitotic differentiation. Also, CSCs are responsible of the initiation and the maintainance of tumor growth; indeed, in serial transplantation in mice they are able to reproduce the tumor with the same molecular and phenotypic characteristics of the tumor from which they derived. Further, CSCs are defined by their unlimited proliferation potential. Still debated are the criteria for classifying CSCs and, therefore, it has not been possible to definitively define the proportion of CSCs subpopulation in a given tumor, the relevance of CSCs to clinical outcome, and the origin of CSCs (Yu, Vodyanik et al. 2007) (Hope et al. 2004).

1.1.3. CSCs and therapies

Despite decades of research and many novel therapeutic approaches, cancer remains one of the leading causes of mortality in humans at all ages. New drugs are available, but few of these provide a durable response, and even fewer can readily eradicate tumors in a small number of malignancies (Sakariassen et al. 2007). Surrogate end point parameters such as 'progression-free survival', 'disease-free survival', or 'recurrence-free survival' reflect the temporary pause in the progression of the disease, seldom lasting more than a few months. Subsequently, the cancer typically relapses with even more aggressive characteristics due to the presence of a bulk of CSCs which, because of their intrinsic chemoresistance, are spared and “naturally selected” by the routinely used anti-cancer drugs. CSCs resistance to conventional therapies is generally attributed to specific features such as high levels of molecules involved in the DNA damage repair, high expression of drug transporters and resistance to apoptosis. After the isolation of CSCs from human acute myeloid leukemia (AML), the greatest clinical implication evolving around the CSC hypothesis is that one may need to develop targeted treatment regimes against the unique CSC population for the cure of cancer (Tang et al. 2007). The identification of biomarkers that distinguish CSCs from their normal counterparts and a better understanding of the biological processes operating predominantly in the CSC subpopulation would be good challenges in the development of specific therapies to eradicate cancer.

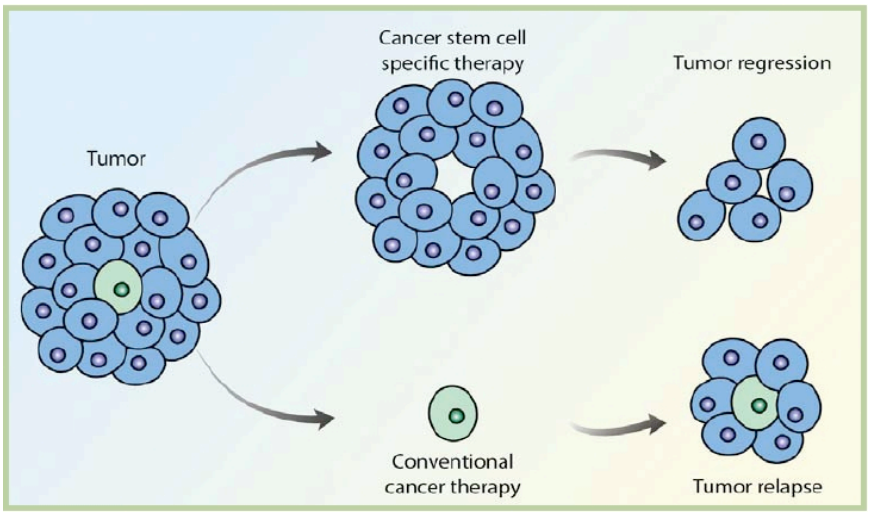


Figure 1.2. CSCs drug resistance. CSCs are resistant to conventional therapies, thereby leading to tumor relapse. Specific strategies directed against these few cells could kill specifically the repopulating nucleus of the tumor and eradicate the malignancy. (from *Post Chemotherapy Stem Cells Treatment For Cancer, March 2013.)*

1.1.4. Asymmetric cell divisions and cancer

Asymmetric divisions are a key mechanism to ensure tissue homeostasis. In normal stem cells they are controlled by intrinsic and extrinsic mechanisms. Intrinsic mechanisms involve partitioning of cell components that determine cell fate, such as cell polarity factors or cell fate determinants. A classic example comes from the *C. elegans* zygote asymmetric localization of the PAR-3, PAR-6 and atypical protein kinase C (PAR–aPKC) complex at the cortex. The asymmetrically localized PAR proteins in turn govern both mitotic spindle orientation and asymmetric segregation of cytoplasmic cell fate determinants. In *Drosophila* neuroblasts a related mechanism involves the evolutionarily conserved cell fate determinant, NUMB, which is asymmetrically localized to daughter cells that are committed to differentiate by antagonizing the Notch signaling (Bultje et al. 2009). Extrinsic mechanisms involve the asymmetric placement of daughter cells relative to external stimuli. In this mechanism of regulation has great importance the stem cell niche, defined as a 'microenvironment' that promotes stem-cell maintenance. In *Drosophila*, for example, germline stem cell divides with a reproducible orientation to generate one daughter that remains in the stem-cell niche and retains stem-cell identity, and one daughter that is placed away from the niche and begins to differentiate (Doe and Bowerman 2001). Symmetric stem-cell divisions have been observed during the development of both invertebrates and vertebrates but it has been found still present in the adult *C. elegans* germ line. Also in mammals, some adult stem cells seem to divide asymmetrically under

steady-state conditions, but they retain the ability to divide symmetrically to restore stem-cell pools depleted by injury or disease. In normal stem and progenitor cells, asymmetric divisions balance proliferation and self-renewal with cell-cycle exit and differentiation (Morrison and Kimble 2006). An interesting model of how adult stem cells can divide both symmetrically and asymmetrically comes from epithelia (Fig. 1.3). The capacity for symmetric stem-cell self-renewal may confer developmental plasticity and enhanced regenerative capacity, but has also implications with cancer (Fig. 1.3F). The first suggestion that the loss of asymmetric divisions might be involved in tumorigenesis came from *Drosophila* neuroblasts. Studies of loss-of-function mutations in key regulators of asymmetric divisions revealed hyperproliferative phenotypes in situ. In these mutants, presumably due to defective asymmetric divisions, cells divide more symmetrically and generate progeny that fails to exit the cell cycle and differentiate, but rather proliferates continuously. Molecular mechanisms underline defects in polarity and asymmetric divisions in human cancer initiation and progression remain poorly understood, but it is now clear that the asymmetric division acts as tumor suppressor mechanism by impairing aberrant self-renewal, invasion and matastasis, promoting differentiation and regulating cell polarity (Morrison and Kimble 2006).

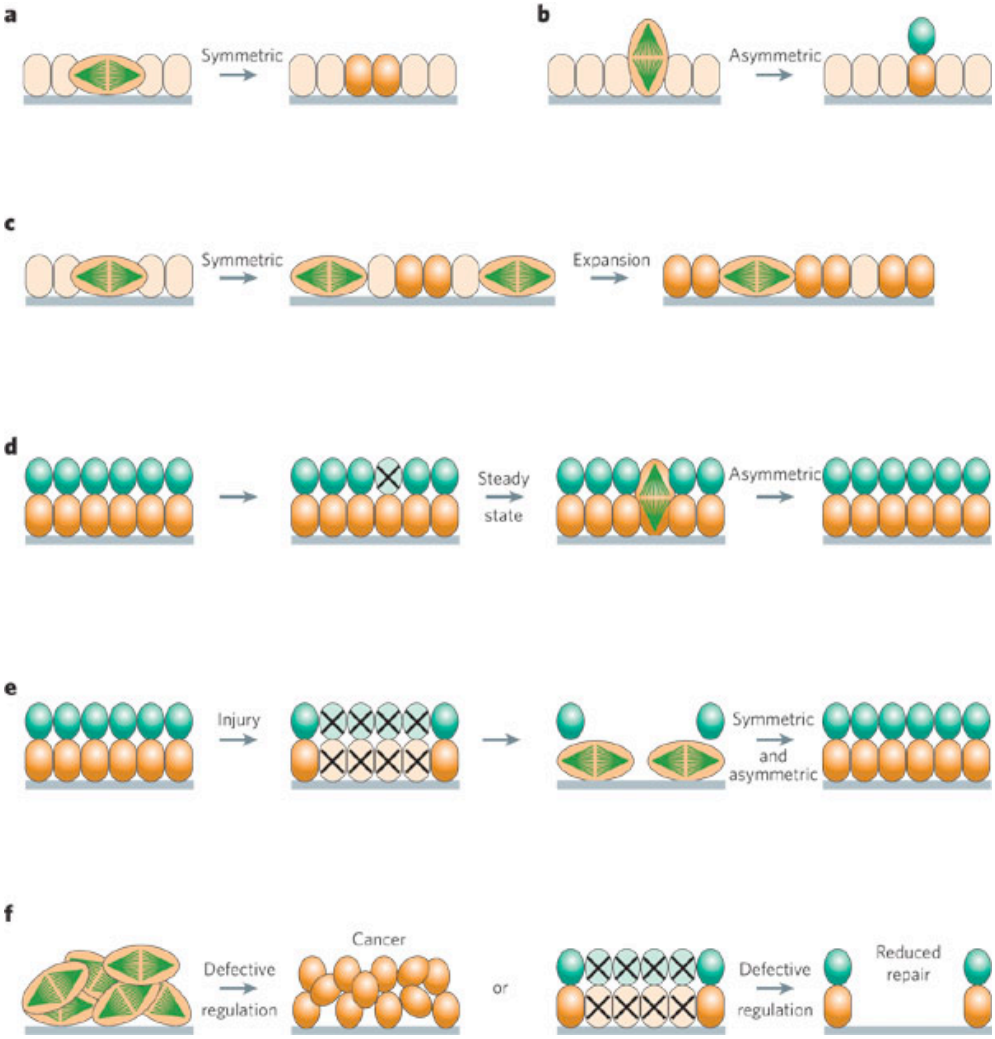


Figure 1.3. Schematic overview of symmetric/asymmetric divisions in epithelia. A. Division in the plane of the epithelium generates two morphologically similar daughter cells that are both likely to be stem cells (orange). Grey line, basement membrane. **B.** Division perpendicular to the plane of the epithelium generates one stem cell and one differentiated daughter (green). **C.** During development, symmetric divisions expand the stem-cell pool. **D.** In healthy adults, divisions perpendicular to the epithelial plane typically maintain normal numbers of stem cells and differentiated cells in the basal layer of epithelia. **E.** In healthy adults, cells can be lost to injury (X). Symmetric divisions are proposed to regenerate additional stem cells, and asymmetric divisions to regenerate differentiated daughters. **F.** Defects in the balance between symmetric and asymmetric divisions can lead to tumorigenesis (left). A defect favouring asymmetric divisions results in decreased capacity for tissue repair (right). (*From Morrison and Kimble, Nature reviews, 2006.*)

1.1.5. SCs regulatory networks

Polarity is a fundamental property of eukaryotic cells and cell-fate determination is necessary in a range of contexts. It is often specified by asymmetric cell division, in which molecular pathways exert an important regulatory function and fate determinants become differentially distributed between daughter cells. SC self-renewal and differentiation is tightly controlled by multiple regulatory networks, including cytokines from the cancer cell microenvironment, also refferred as stem cell niche. Many signaling pathways including Wnt/β-catenin, Notch, Hedgehog and microRNAs, have a close relationship with the cell polarity and the regulation of the symmetric/asymmetric division ratio.

1.1.5.1. Wnt/β-catenin

The canonical Wnt cascade has emerged as a critical regulator of stem cells. In many tissues, activation of Wnt signalling has also been associated with cancer and later found tightly associated with self-renewal in stem and progenitor cells and subverted in cancer cells to allow malignant proliferation (Blank et al. 2008).

Wnt signaling is initiated when a ligand binds to the Frizzled and lipoprotein receptor-related protein (LRP) receptors at the cell surface. In the absence of Wnt ligands, the downstream signal transducer β-catenin is trapped by adenomatous polyposis coli (APC) and Axin in a destruction complex, where it is phosphorylated by casein-kinase 1α (CK1α) and glycogen synthase kinase (GSK3β). Phosphorylation ultimately leads to ubiquitination and degradation of β-catenin. On ligand binding, Frizzled forms a complex with Disheveled (Dsh), whereas LRP is phosphorylated, resulting in Axin relocation to the cell membrane. Subsequently, the destruction complex is dispersed and β-catenin accumulates and translocates to the nucleus where it interacts with the T-cell factor/lymphoid enhancer factor (TCF) transcription factors to regulate gene expression (Blank et al. 2008).

Increased Wnt signaling gives rise to pituitary tumors in mice (Gaston-Massuet et al. 2011) and Wnt drives symmetrical cell divisions in stem cells (Le Grand et al. 2009).

1.1.5.2. Notch

Notch receptors are single pass transmembrane proteins. The receptor is processed in the ER and Golgi resulting in cleavage, producing a glycosylated Ca^{2+} stabilized heterodimer. The processed receptor is translocated to the membrane where it binds ligands, members to the Delta-like and Jagged family, located in the signal-sending cells. When ligands of the Delta (Delta1-3) or Jagged (Jagged 1-2) families bind to the Notch receptor, proteolytic events involving γ-secretase lead to release and translocation of the intracellular domain of the receptor (NICD) to the nucleus. Subsequently,

NICD will form a complex with the transcription factor CSL and cofactors of the Mastermind-like (MAML) family to activate transcription of target genes (Chiba 2006).
The role of Notch as cell fate determinant has been extensively studied in particular in neural development. Indeed, Notch signaling can promote glial determination, in addition to inhibiting the determination of neurons. Its activity often specifies the most undifferentiated state during cell fate decisions and is thought to help in maintaining the multipotent character of stem cells as they divide. Notch activation contributes to the expansion of a variety of stem and early progenitor cells (Chiba 2006). The activation of the Notch signaling pathway have been demonstrated to increase the self-renewal capacity of long-term *in vivo* repopulating HSCs (Stier et al. 2002; Kunisato et al. 2003) or even to immortalize primitive hematopoietic progenitor cells (Varnum-Finney et al. 2000). Given its role in the regulation of stem cells expansion by counteracting the asymmetric cell division, the de-regulation of Notch activation is tightly correlated with cancer development (Moeini et al. 2012).

1.1.5.3. Hedgehog

Hedgehog (Hh) is involved in a signaling cascade that regulates development and expansion of tissue progenitor or stem cells. The Hh ligand is translated as a precursor, which undergoes autocatalytic processing to form an N-terminal fragment. Secretion and paracrine signaling requires participation of the Dispatched proteins. The family comprises Indian hedgehog, desert hedgehog and Sonic hedgehog. These proteins bind the patched receptor (PTCH1), depressing its constitutive repression of Smoothened (Smo), leading to activation of the Gli transcription factors (Yu et al. 2012). Hh signaling is crucial for self-renewal of neural SCs, and its deregulation is often associated with the tumorigenic progression of neural SCs. Not surprisingly, the subversion of the Hh pathway has been implicated in a variety of developmental abnormalities, and in different types of cancers (Teglund and Toftgard 2010).

1.1.5.4. p53

The tumor suppressor protein p53 functions mainly as a transcription factor, inducing the expression of genes involved in cell-cycle arrest, senescence and apoptosis in response to cellular stress. In unstressed cells, therefore, p53 is kept inactive, mainly through proteasomal degradation induced by the E3 ubiquitin ligase MDM2 polyubiquitination. Loss of MDM2 binding or inhibition of its E3 ligase activity allows p53 to be rapidly stabilized and activated in response to a variety of cellular stresses that are associated with tumor development and progression, including DNA damage, oncogene activation, hypoxia and metabolic stress. The tumor suppressor activity of p53 is affected in most, if not all, cancers. This is frequently achieved by mutations in p53 (Hainaut and Hollstein 2000) or, less commonly, by amplification of the

gene for MDM2 (Momand et al. 1998). In some tumors other machanisms can act to prevent the activation of the p53 pathway.
Recently, interesting findings highlighted an important role of p53 in regulating the symmetric/asymmetric division ratio in CSCs. Indeed, using the ErbB2 transgenic model of breast cancer, first, it was found that CSCs are characterized by more frequent self-renewing divisions compared to their normal counterparts, thus contributing to increasing numbers of SCs in tumoral tissues. Further, primary SCs with mutation in p53 revealed the same self-renewal properties of CSCs, and their number increases progressively in the p53 null premalignant mammary gland. After pharmacological reactivation by using Nutlin-3, which acts by displacing p53 from its E3-ligase mdm2, the asymmetric divisions in CSCs was restored and tumor growth reduced (Cicalese et al. 2009).
In mouse embryonic stem cells (ESCs) p53 is highly abundant, it was found to be inactive and localized mainly in the cytoplasm (Han et al. 2008). Several studies have highlighted the importance of p53 in regulating suppression of self-renewal and induction of differentiation after DNA damage. Indeed, master transcription factor Nanog and the pluripotency factor Oct4 are known to drive self-renewal and the maintenance of an undifferentiated state in mouse ESCs (Mitsui et al. 2003). In DNA damaged ESCs mouse, p53 binds and suppress their promoters, thus forcing differentiation into cell types that can be subjected to classical p53 processes such as cell-cycle arrest or apoptosis. Moreover, p53 was found to activate the expression of miR-34a and miR-145, which in turn repress stem cell factors Oct4, KLF4, LIN28A and Sox2 and prevent backsliding to pluripotency (Jain et al. 2012). It was also found that the compromization of p53 enhance the iPSC generation. Furthermore, it was suggested that p53 may induce cell cycle arrest and apoptosis and thus function as a barrier to select exclusively perfect reprogrammed SCs. In mesenchymal stem cells (MSCs) mutations in oncogenes or tumor suppressors may function as tumor initiating cells (TICs) leading to tumor formation. Transformation of MSCs seems to be highly dependent on alterations in the p21/p53 pathway, mainly by the abolishment of WT p53, but not on the retinoblastoma. Heterozygous p53 SCs are at the junction between normal SCs and CSCs, which concomitantly express a functional WT p53 and a mutant p53. The tumor transformation follows the loss of the WT p53 generally due to loss of heterozygosity (LOH), but in the 40% it is due to diverse mechanisms such as promoter hypermethylation, increased activity of Mdm2, the E3 ligase responsible for p53 ubiquitination and so on (Aloni-Grinstein et al. 2014). All these findings support the idea that p53 has an important role as barrier to the cancer stem cells formation.

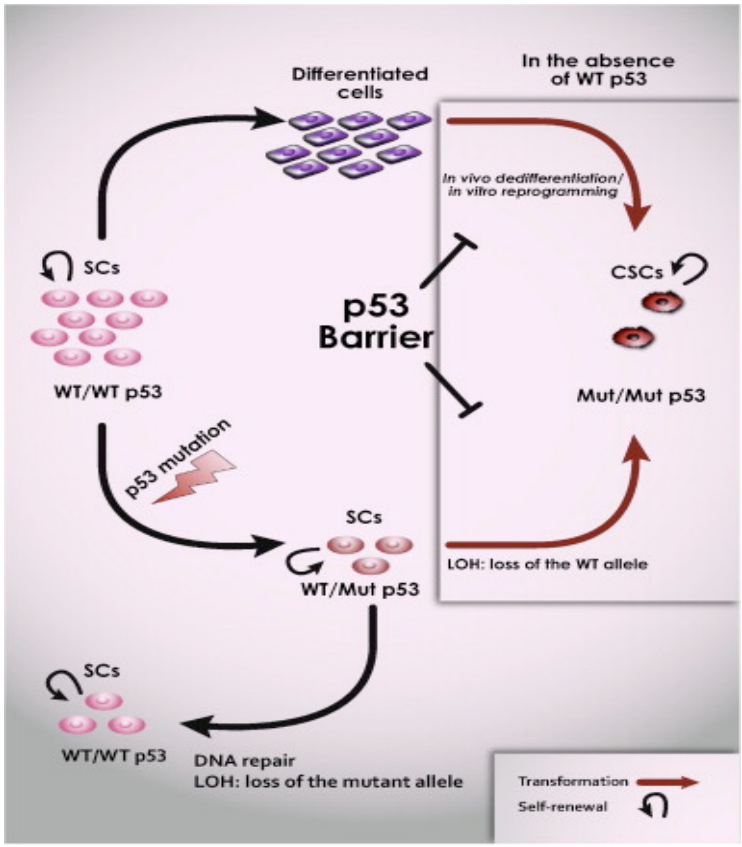


Figure 1.4. p53 as barrier to CSCs formation. p53 restricts processes of *in vivo* dedifferentiation and *in vitro* reprogramming, preventing the transformation and dedifferentiation of differentiated cells into CSCs. SCs have the potential to undergo mutation in p53. In heterozygous p53 SCs, LOH can occur as a DNA repair process, leading to the loss of the mutant allele and ensuring the quality of the SCs. In the case where the WT allele is lost, CSCs will be formed. *(from Aloni-Grinstein et al., FEBS letters, 2014)*

1.1.5.5. Numb

Numb is an important cell fate determinant which acts at different levels in the development and in the stem cell division. Mice lacking Numb exhibit profound defects in angiogenic remodelling and neural-tube closure, and lack several neuronal cell lineages (Zhong et al. 2000) (Zilian et al. 2001). An asymmetric distribution of Numb has been reported in mitotic neural progenitors in the mouse forebrain (Zhong et al. 1996). In mammalian epithelial cells, endogenous Numb is localized primarily to the basolateral cortex (Dho et al. 2006), indicating that Numb also maintains a polarized distribution in non-mitotic cells. During mitosis, the cell-fate is modulated by the Baz/Par6/aPKC complex that directs the basal distribution of the cell-fate determinant NUMB; after aPKC phosphorylation, Numb is released from the

apical cortex and localizes to the basal side of the cell. (Gomez-Lopez et al. 2014)

Numb regulates endocytosis by participating to cargo internalization and recycling. Further, by interacting with PAR complex it has an important role in determining cell polarity, since the asymmetrical localization of PAR proteins governs both mitotic spindle orientation and asymmetric segregation of cytoplasmic cell fate determinants. Numb is also connected with signaling molecules, by regulating Notch and Hedgehog activated pathways (Pece et al. 2011). Particular importance it has been attributed to its role in antagonizing Notch. Numb's interaction with endocytic proteins has led to the hypothesis that it may regulate Notch by promoting removal of the Notch receptor from the plasma membrane into endosomes. Indeed, the Notch receptor trafficking through the endocytic pathway is important for Notch signaling regulation(Fortini and Bilder 2009). This hypotesis has been replaced with another more complex mechanism by which Numb regulates Notch through Sanpodo, which is expressed only in asymmetrically dividing cells by associating with Numb and Notch, thus regulating Notch signaling only during asymmetric divisions (Hutterer and Knoblich 2005). Notch and Sanpodo act together in opposition to Numb in cell fate determination (Skeath and Doe 1998), Numb inhibits the membrane localization of Sanpodo and toghether with α-adaptin controls the endocytosis of Sanpodo. Besides this mechanisms, in mammalian cells, Numb is able to bind the E3 ubiquitin-ligase, Itch, and to cause the ubiquitination of Notch (Qiu et al. 2000).

In stem cells, Numb activity is regulated by asymmetric partitioning at mitosis, leading to unequal distribution of Numb in daughter cells that are then destined for different fates. In particular the daughter cell which inherits Numb will be committed to differentiate. This particular feature together with its role in regulating cell fate, self-renewal and differentiation, leads to correlate Numb with tumor development. Also, it has been found that the loss of NUMB occurs frequently in breast tumors, leading to activation of oncogenic Notch signaling (Pece et al. 2004) and to the inactivation of the p53 tumor suppressor pathway, as more recently demonstrated (Colaluca et al. 2008). Primary tumor cells with low or normal levels of NUMB, revealed that loss of NUMB correlates with reduced levels of p53 and also with resistance to chemotherapeutic agents, more aggressive neoplastic disease and poor prognosis. Toghether with these findings Colaluca et al. showed that NUMB is able to interact *in vivo* with endogenous MDM2 and p53, resulting in a trimeric complex between the three proteins. This interaction appears to regulate the stability of p53, and consequently the levels of the protein (Colaluca et al. 2008). Since, as described above, also p53 has a fundamental role in regulating asymmetric cell division, the tight connection between p53 and NUMB confirm their importance in the prevention of CSCs formation.

1.1.5.6. microRNAs in CSCs

In recent years it has emerged that miRNAs are implicated in the regulation of a broad range of biological processes including embryonic development, cell cycle, cell proliferation, tumor initiation and progression, cancer metastasis, self-renewal, and differentiation of stem cells (Yu et al. 2007). The first two miRNAs found in the stem cells regulation were *lin-4* and *let-7*, involved in regulating the timing of larval to adult cell fates in *C. elegans* (Lee et al. 1993). miRNA play important functional roles in the establishment and maintenance of a core network of transcription factors and RNA binding proteins. Transcriptional factors important for the ES identity such as OCT4, Sox2, Nanog, Klf4, c-Myc, Tcf3, Lin28 are regulated by the miR-290, 302, 371; further they also regulates Lin28 and c-Myc. Anti-stemness miRNAs includes miR-134, miR296, miR-200c, miR-203, miR-183 and let-7 miRNAs. let-7 over-expression inhibits mammosphere formation, and metastasis of breast CSC (Yu et al. 2007), whereas reduced let-7 expression maintains the undifferentiated state of breast CSCs. let-7 may also regulate self-renewal by targeting *H-RAS* and the differentiation via *HMGA2* in breast CSC. Recently it has been found able to regulate self-renewal in colon CSCs. miR-34a loss of function and gain of function alter the balance between self-renewal versus differentiation both *in vitro* and *in vivo*. Indeed it is able to regulate Notch by sequestering Notch mRNA. This activity, togheter with the modulation of multiple targets besides Notch determines cell-fate asymmetry in a robust and precise way during CSCs division (Bu et al. 2013).

Further, there is an important interplay between miRNAs and the stem cell niche, since they can regulate immune cells, cancer associated fibroblasts, cytokines and chemokine in the tumor microenvironment. For instance, miR-17/20 inhibits IL-8 secretion to block tumor stem cell migration and metastasis (Yu et al. 2012).

1.2. High Mobility Group A Proteins

Life depends on the ability of a biological system to store, retrieve, process and translate the genetic instructions required to make and maintain a living organism. The packaging of all the information into chromosomes is achieved thanks to a series of molecules which both contribute in maintaining this ordered structure and regulate the accessibility to regulatory factors. Many studies have focused on histone proteins, but it is now clear that besides histones there is a second class of proteins, which are either part of, or associated with, the chromatin fiber. These proteins are referred to as non-histone chromosomal proteins.
The high mobility group (HMG) proteins are the largest and best characterized group of non-histone chromosomal proteins. They are defined as nuclear proteins with a high electrophoretic mobility in poly-acrylamide gels and typically, they have a high content of charged amino acids and a molecular mass <30 kDa (Cleynen and Van de Ven 2008).
In 1983, two new high-mobility group-like proteins were found in HeLa cells and named Y and I. Goodwin and co-workers (Goodwin et al. 1973)identified two proteins in the nuclei of rat thymus and of fibroblasts transformed with avian sarcoma virus. These proteins were named Y and I′ because of their resemblance to the two proteins previously found by Lund et al. In the same period, A. Varshavsky's group studied the binding to the DNA of a mammalian protein called α subsequently found to be an HMG protein. Later, these small DNA-binding proteins were categorized and named HMGA proteins (Lund et al. 1983).

1.2.1. HMGA genes and protein structure

HMGA family comprises three members named HMGA1a, HMGA1b, and HMGA2.

HMGA1 gene is located on human chromosome 6 (6p21) and on mouse chromosome 17. The human *HMGA1* gene is constituted of eight exons, distributed over a region of about 10 kb whereas the mouse *hmga1* orthologue contains six exons spanning about 7 kb (Pedulla et al. 2001) (Johnson et al. 1993). The human *HMGA2* gene is located at chromosomal band 12q14-15 and contains five exons dispersed over a genomic region of ≥160 kb. The mouse *hmga2* gene locates on chromosome 10 and contains five exons spanning more than 110 kb. Both human and mouse *HMGA2/hmga2* genes are much larger than their *HMGA1/hmga1* counterparts, mainly because of longer 5' and 3' untranslated regions and because of the extremely long third intron of *HMGA2/hmga2* (Zhou et al. 1995).

The *HMGA1* gene undergoes alternative splicing, resulting in the generation of

three different mRNAs encoding HMGA1a (107 amino acids, 11.7 kDa), HMGA1b (96 amino acids, 10.6 kDa) and the more recently identified HMGA1c (179 amino acids, 19.7 kDa). The HMGA1a and HMGA1b isoforms differ by only 11 internal amino acids present in HMGA1a but not in HMGA1b and are encoded by the most abundant splice variants of the *HMGA1* gene. HMGA1c is produced from the *HMGA1* gene by alternative splicing using non-canonical splice donor and acceptor sites. This alternative splicing results in a frameshift so that the two proteins are identical in their first 65 amino acids but differ thereafter.

HMGA1a, HMGA1b and HMGA2 are polypeptides of about one hundred amino acid residues characterized by a modular sequence organization. These proteins presents highly conserved regions, rich in positively charged regions, called AT-hooks, since they bind A-T rich regions in the minor groove of B-form DNA; their feature is to recognize structure, rather than a particular nucleotide sequence. The AT-hook motif is a positively charged stretch of 9 amino acids containing the invariant repeat Arg-Gly-Arg-Pro (R-G-R-P), flanked by other positively charged residues, usually Arg (R) and Lys (K). Free in solution, A T -hooks possess little, if any secondary structure. Upon binding to DNA, the AT-hook undergoes a disordered-to-ordered conformational change. Depending on the number and spacing of AT-rich binding sites in DNA, HMGA proteins can influence the conformation of bound DNA substrates in different ways.

The C-terminus domain has completely different features since it contains a high percentage of negatively charged acidic residues. Among different HMGA proteins, the three AT-hooks are differently spaced along the protein molecules, resulting in an interactive modular system constituted by a set of three proteins able to establish interactions with differently spaced AT-rich DNA regions. The HMGA proteins show an unusual capability to bind other nuclear proteins; indeed, they are able to bind to a great number of partners, by which they are involved in regulating and in performing a myriad of functions (Sgarra et al. 2004) (Cleynen and Van de Ven 2008).

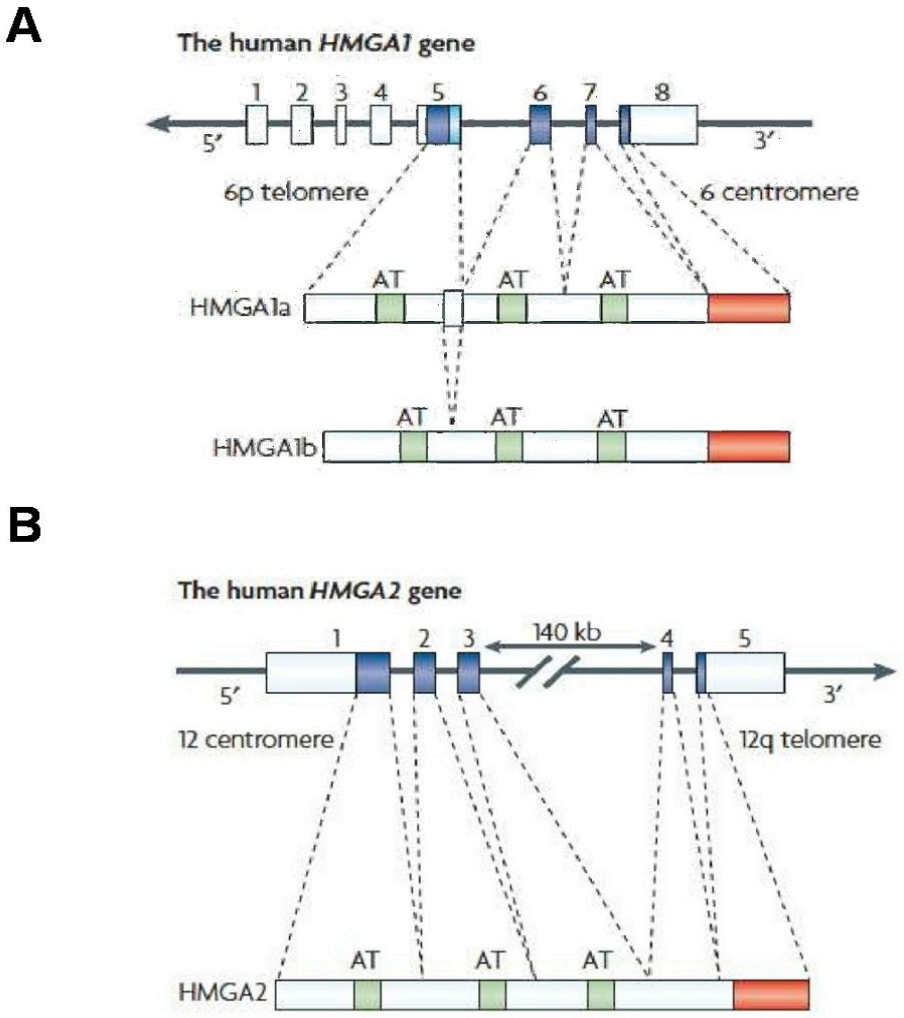


Figure 1.5. Structure of human HMGA1/HMGA2 genes and proteins. HMGA1(**A**) and HMGA2 (**B**) proteins have a similar structure and are well conserved during evolution. In this picture AT-hooks are represented in green, in red the C-terminus domains of the proteins. (*from Fusco and Fedele, Nature Reviews, 2007*)

1.2.2. HMGA mechanisms of action

As mentioned above, HMGA proteins do not exhibit transcriptional activity *per se*, but they regulate the activity of several genes by interacting with the transcription machinery and altering the chromatin structure (Reeves and Beckerbauer 2001). The levels of HMGA proteins are low or absent in normal cells and adult tissues but are elevated in many tumors, neoplastically transformed cells, and embryonic cells (Fedele and Fusco 2010). HMGA

proteins participate in a wide variety of nuclear and cytoplasmatic processes, ranging from chromosome and chromatin dynamics, such as chromosome condensation, to architecture-mediated transcriptional regulation.

1.2.2.1. Transcriptional regulation

HMGA proteins seem to function as an anti-repressor of transcription since they are able to induce the remodeling of the inhibitory chromatin complexes. Thus they control the global structure of large loops or domains of chromatin. In one important machanism, HMGA proteins bind to the SARs at the base of repressed chromatin loops and concomitant displacement/exclusion of histone H1 from these sequences leads to a local opening of chromatin and initiation of the gene activation process. Further, HMGA proteins are proposed to be involved in the initial remodelling of the inhibitory nucleosomes and the subsequent formation of an enhanceosome (Cleynen and Van de Ven 2008).

1.2.2.2. Long-range chromatin interactions

The HMGA proteins have been proposed to be involved in long-range enhancer-promoter interactions. For example, inducible expression of the chicken *ß-globin* gene requires a functional interaction between a specialized TATA-box motif located at -30 bp in the gene 5' promoter and a distal 3' enhancer element located ±1.9 kb downstream of the start of the *ß-globin* coding region (Fong and Emerson 1992). Bagga *et al* demonstrated that HMGA1 proteins mediate chromatin looping and long-range interactions of these widely spread regulatory regions to regulate the chicken *ß-globin* gene (Bagga et al. 2000)

1.2.2.3. Protein-protein interactions

HMGA proteins can also influence gene transcription, without first binding to DNA, via direct protein-protein interactions with transcriptional factors. HMGA proteins, indeed, are able to bind to many transcriptional factors. After the binding, HMGA proteins induce conformational changes in the substrate. These interaction sites are distributed along their entire length. Generally the site of interaction includes part, or the whole, of one or more AT -hook motifs, plus flanking regions. The region between the second and third AT-hook of the HMGA1 protein, for example, has the most of identified interacting partners (Reeves and Beckerbauer 2001).

1.2.2.4. Viral integration

It has been demonstrated an involvement of HMGA proteins during infectious processes; they act, for example, as co-factors during the infection of HIV-1. It has been proposed a mechanism by which HMGA proteins both bind multiple AT-rich sites on retroviral cDNAs in pre-integration complexes, favouring the viral integration, and act on AT -rich regions of viral promoter/enhancer regions, cooperating with both viral copies and host proteins in the control of viral gene transcription (Li et al. 2000).

1.2.3. Physiological functions of HMGA proteins

1.2.3.1. Growth and differentiation

In normal conditions the expression of HMGA proteins is restricted to embryogenesis, until 8.5 dpc, it decreases with organogenesis and in normal adult cells is very low or almost absent. HMGA1 expression is confined to specific organs of ectodermal, mesodermal and endodermal origin, while HMGA2 expression is restricted to mesenchimal tissues. A role for both factors in development has been demonstrated. Indeed, genetic studies in mice have isolated four viable, spontaneous mutants showing dwarfism (Zhou, Benson et al. 1995). Three of the four phenotypes could be explained by aberrations in the growth hormone insulin-like growth factor endocrine pathway. One of the phenotypes, called 'pygmy'results from the disruption of the *hmga2* gene.

Further, it has been found that HMGA2 is correlated to adipogenesys, since the deficiency of the Hmga2 gene in mice results in resistance to obesity induced by diet (Anand and Chada 2000) .The phenotype of HMGA1 knockout mice has not been reported possibly because the more general expression of this factor could severely impair development. Indeed, suppression of HMGA1 expression impairs differentiation of pre-adipocytic cells, lymphohematopoietic differentiation and normal sperm development (Sgarra et al. 2004). To better clarify the role of *hmga1* proteins in embryonic development, Battista *et al* used ES cells carrying disruption of one or both alleles of the *hmga1* gene. It appeared that hmga1 proteins are involved in different hematopoietic lineage commitment checkpoints. In *hmga1*$^{-/-}$ embryoid bodies (EBs), myeloid differentiation was shown to be impaired, while megakaryocytic and erythroid differentiation was shown to be increased (Battista et al. 2003). An important role of hmga1 in mice developmemente was highlighted also by Fedele *et al.* Heterozygous and homozygous mice for the *hmga1*$^{-/-}$ allele showed cardiac hypertrophy due to the direct role of HMGA1 on cardiomyocytic cell growth regulation. These mice also developed hematologic malignancies, including B cell lymphoma and myeloid granuloerythroblastic leukemia (Fedele et al. 2006).

1.2.3.2. Senescence

Recently it has been proposed a new role for HMGA proteins in promoting senescence. Cellular senescence is a stable state of proliferative arrest that provides a barrier to malignant transformation and contributes to the antitumor activity of certain chemotherapies. Senescent cells can accumulate senescence-associated heterochromatic foci (SAHFs), which may provide a chromatin buffer that prevents activation of proliferation-associated genes by mitogenic transcription factors. It was found that HMGA proteins cooperate with the $p16^{INK4a}$ tumor suppressor to promote SAHF formation and proliferative arrest and stabilize senescence by contributing to the repression of proliferation-associated genes. This function can be accomplished only in the absence of two factors, HDM2 and CDK4, which are generally amplifyed during tumorigenesis (Narita et al. 2006).

1.2.3.3. DNA repair

HMGA proteins are involved in the regulation of the DNA repair mechanisms in at least two ways. First, it has been demonstrated that HMGA1 is involved in the nucleotide excision repair. HMGA1 can hamper nucleotide excision repair both by physically inhibiting access of DNA repair-associated proteins to cyclobutane pyrimidine dimer (CPD) lesions, by directly binding to the UV-induced lesions (Adair et al. 2005), (Maloney et al. 2007) and by physical interacting with repair factors, thereby inhibiting their function.

Second, HMGA1 and HMGA2, interact and are phosphorylated by ATM in response to DNA damage. Interestingly, RT-PCR and Western Blot analysis on Mouse Emrbyonic Fibroblasts (MEFs) lacking HMGA1, HMGA2 or both genes, revealed that, in the absence of HMGA proteins, mRNA and protein levels of ATM are strongly reduced. Moreover, p53 phosphorylation and the consequent activation of p21 transcription, induced by ATM kinase activity, were significantly affected by the absence of HMGA proteins (Pentimalli et al. 2008).

1.2.3.4. Cell cycle regulation

Many studies have highlighted a role for HMGA proteins in regulating cell cycle. As previously described, they are susceptible to phosphorylation mediated by important kinases involved in the control of cell cycle check points, such as Cdc2 kinase, thus modulating the affinity of HMGA proteins and enhancing their activity (Reeves and Nissen 1995). In 2001 it has been found that the overexpression of HMGA1b protein in rat thyroid cells is able to deregulate their cell cycle; indeed cells enter S-phase earlier and the G2-M transition is delayed (Fedele et al. 2001). Further, a role for HMGA proteins has been proposed in the alteration of the cell cycle as a critical event in

pituitary adenomas. *HMGA2* overexpression is responsible of the upregulation of cyclin B2 gene transcription, leading to overexpression of cyclin B2 in pituitary adenomas developed by mice carrying *hmga* transgenes. Since cyclin B2, complexed with CDK1, plays a critical role in regulating the G_2/M phase transition of the cell cycle, it is reasonable to hypothesize that cyclin B2 induction by HMGA proteins may contribute to increase cell proliferation of the pituitary cells (Fedele and Fusco 2010). The role of HMGA proteins in neoplastic transformation will be further discussed in the following sections.

1.2.3.5. Apoptosis

The role of HMGA proteins in the regulation of apoptosis is controversial. It seems that in wild type, not transformed cellular systems, HMGA proteins function promoting the programmed cell death upon mitogenic stimuli. Indeed, it was shown that normal rat thyroid PC Cl 3 cells overexpressing HMGA1, which pushes cells through the S-phase, undergo apoptosis through a pathway involving caspase-3 activation, probably consequent to the conflict between mitogenic pressure and the inability to proceed through the cell cycle. It was also found that the third AT-hook domain and the acetylation site K60 are the protein regions required for induction of apoptosis in PC Cl 3 cells (Fedele et al. 2001).

Further, our group have highlighted a role of HMGA proteins as anti-apoptotic factors in transformed systems. They act mainly on the p53 apoptotic pathway. In response to DNA damage, p53 induces either cell-cycle arrest or apoptosis. HMGA proteins act by inhibiting this pathway both by regulating at the transcriptional level the E3 ligase specific of mdm2/HDM2, thus promoting the p53 proteasome degradation and by direct interaction with the p53 protein. Also, recent studies have shown that in pancreatic carcinoma cell lines HMGA1 overexpression inhibits the phosphorylation of Akt, thus avoiding the activation of caspases 3, 8 and 9 upon exposure to chemotherapeutic agents These mechanisms by which HMGA proteins negatively regulate the apoptotic pathway in cancer cells will be further discussed below.

1.2.4. Regulation of HMGA proteins

HMGA genes expression can be regulated in response to a large array of cellular stimuli. Many growth factors are able to induce HMGA gene expression. Moreover, HMGA expression can be repressed by activation of a differentiation program. For example, in neuroblastoma and in embryonic carcinoma cells, HMGA1/2 expression can be repressed by retinoic acid. As mentioned above, the deregulation of the HMGA proteins expression is frequently associated to neoplastic transformation.

1.2.4.1. Transcriptional regulation of the HMGA1 gene

Many studies focused on the role of HMGA1 in the regulation of cellular functions, but little is known about transcriptional regulation of HMGA1 gene. This is due to the extremely complex structure and transcriptional regulation of both the mouse and human HMGA1 gene. It comprises four different transcription start sites, of which the first two are considered to be the major ones (Ogram and Reeves 1995). A few cis-elements were carachterized on the HMGA1 promoter. Among these, a multiple *cis*-acting elements located near the first three transcription start sites seems to be important for the HMGA1 trans-activation performed by MYCN in neuroblastoma. Also studies in Burkitt's lymphoma reported that the oncogene c-myc is able to activate the HMGA1 gene expression. Further, it has been shown that the HMGA1 promoter is strongly inducible by the oncogenic Ras, via a distal regulatory region (Cleynen and Van de Ven 2008). More recently it has been demonstrated that HMGA1 is a downstream target of the Wnt/β-catenin/TCF-4 signalling pathway. Indeed, the deregulation of this pathway in colon carcinoma is associated with high levels of the HMGA1 expression, whereas the restoration of the Wnt pathway resulted in HMGA1 down-regulation. In the same study were highlighted two regions in the 5'-flanking sequence of HMGA1 that specifically bind the β-catenin/TCF-4 complex *in vitro* and *in vivo* (Bush et al. 2013).

1.2.4.2. Transcriptional and post-transcriptional regulation of the HMGA2 gene

HMGA2 promoter also contains multiple transcription initiation sites, which are differentially used. In addition, they show that *HMGA2* 5'-flanking sequences had constitutive promoter activity in cell lines which express high levels of HMGA2, and in cell lines which do not express HMGA2. Thus probably, HMGA2 gene undergoes a negative regulation by cis-elements and/or a post-transcriptional regulation. In particular, the post-transcriptional regulation seems to be fundamental, since some rearrangements involving the 3' UTR but leaving the ORF intact, lead to an HMGA2 overexpression (Schoenmakers et al. 1995) (Geurts et al. 1997), probably due to the loss of repressive elements in its 3' UTR. Within the last few years, miRNAs have emerged as important regulators of both transcriptional and post-transcriptional gene silencing. HMGA2 overexpression has been found correlated with the down-regulation of miR-15 and miR-16 (Palmieri et al. 2012). Recently, it was found that the HMGA2 transcript can be negatively regulated by let-7 miRNA (Fig. 1.6). As previously discussed, through the regulation of HMGA2, let-7 is able to modulate the self-renewal and asymmetric cell divisions in stem cells. The let-7 inhibition enhances cell proliferation in H1299 (Lee and Dutta 2007) cells and causes transformation in NIH-3T3 cells (Mayr et al. 2007; Cleynen and Van de Ven 2008).

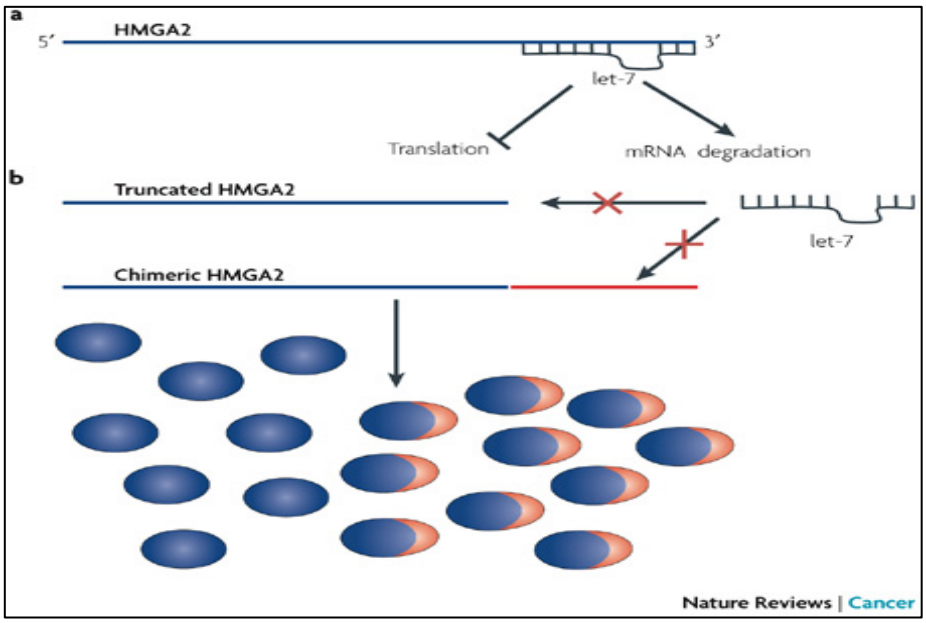


Fig 1.6. The let-7 mechanism. A. The let-7 microRNA binds to the 3' region of the *HMGA2* gene and thus downregulates HMGA2 expression by inhibiting translation and/or causing the degradation of the mRNA. **B.** Following truncation or fusion with ectopic sequences, *HMGA2* loses the sites previously recognized by let-7, resulting in increased HMGA2 mRNA levels. Here, HMGA2 is in blue and its fusion partner is in red *(Fusco, Fedele, Nature reviews, 2007).*

1.2.4.3. Post translational modifications

All the mechanisms of action proposed above are influenced by post translational modifications. Indeed, HMGA proteins are among the most highly adducted proteins in the nucleus, exhibiting complex patterns of phosphorylations, acetylations, methylations and less frequently also sumoylation and ribosylation (Fig. 1.6). It is clear that after modifications HMGA proteins bind with different affinity both DNA and proteins, thus influencing their biological activities. Among the post-translational modifications, the more frequent is the phosphorylation. Many effectors such as protein kinase C, cdc2 kinase and Casein Kinase 2 are able to phosphorilate HMGA proteins in different sites of their structure and in specific phases of the cell cycle. Further It has recently been demonstrated that HMGA1 proteins also undergo methylation. Examples include Arg^{24} within the first AT-hook of HMGA1a, which has been found methylated in tumor cell lines. This kind of methylation is involved in the apoptosis process. Recently HMGA1a has been identified as a target of the protein arginine methyltransferase PRMT6, which specifically methylates HMGA1a on Arg^{56} and Arg^{58} within the second A T - hook domain (Sgarra et al. 2006).

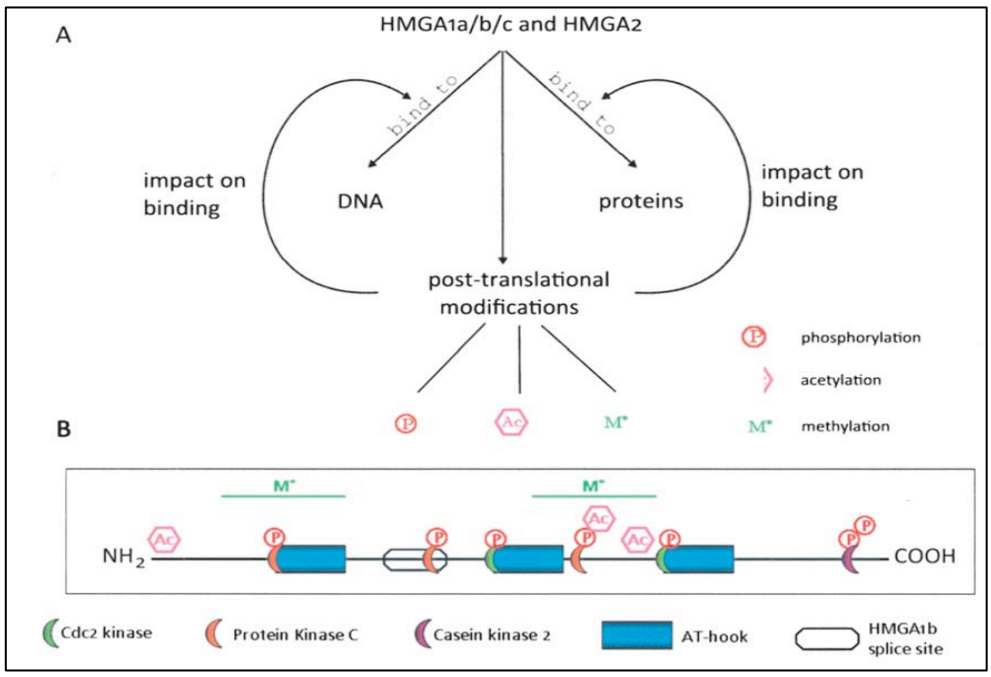


Figure 1.7. HMGA modifications. A. Schematic overview of the effect of post-translational modifications on HMGA binding properties. **B.** Simplified diagram of some of the post-translational modifications of the HMGA1 protein. (*from Cleynen and Van De Ven, review 2007*)

1.2.5. HMGA Proteins And Cancer

HMGA1 and *HMGA2* are hardly detectable in adult human tissues, as well as *hmga1* and *hmga2* in mouse tissues. HMGA expression is very high during embryogenesis, whereas it is undetectable or very low in differentiated adult tissues (Zhou et al. 1995; Chiappetta et al. 1996), being confined, at least for HMGA2, to the staminal compartment (Rommel et al. 1997) (Anand and Chada 2000) (Nishino et al. 2008). Studies of mice models have revealed a crucial role of HMGA proteins in adipogenesis, somatic growth (Battista et al. 1999), cardiac cell growth control (Fedele et al. 2006), and glucose homeostasis (Foti et al. 2005) (Chiefari et al. 2009). However, there are some exceptions to the general repression of these genes in adult tissues. For example, there is a burst of synthesis of the HMGA1a protein during the immune response (Shannon et al. 1998). Beside these, their overexpression represents a constant feature of human malignancies, and correlates with a poor prognosis.

1.2.5.1. HMGA proteins in benign tumors

HMGA dysregulation, as a result of specific chromosomal rearrangements, occurs in a broad variety of common benign mesenchymal tumors, making HMGA genes among the most commonly rearranged genes in human neoplasms. They include lipomas (Tallini et al. 1997), uterine leiomyomas (Medeiros et al. 2007), pulmonary chondroid hamartomas (Kazmierczak et al. 1996), fibroadenomas of the breast (Staats et al. 1996), endometrial polyps (Dal Cin et al. 1998), pleomorphic adenomas of the salivary glands (Persson et al. 2009) and vulvar aggressive angiomyxoma (Nucci et al. 2001). As mentioned above, many rearrangements involving the HMGA2 gene lead to the gene overexpression, truncation or, more frequently, generation of fusion genes encoding chimeric transcripts. As a consequence, the rearranged HMGA2 gene codes for a chimeric or a truncated HMGA2 protein that maintains its capacity to bind DNA and interact with several proteins, but loses its C-terminal tail, including the 3′ UTR. Concerning HMGA1, chromosomal breakpoints of rearrangements are located either upstream or downstream of the gene sequence, but no intragenic rearrangements have been found. As mentioned above the involvement of HMGA gene mutations in mesenchymal tumors seems to be correlated to deletions of its 3′ UTR. The consequent HMGA overexpression is due to the loss of the negative regulation exerted by miRNAs, such as let-7, miRNA-98 and miR-16 (Mayr et al. 2007) (Hebert et al. 2007) (Kaddar et al. 2009), on the 3′ UTR sequences of the HMGA genes. Many studies focused on the role of HMGA proteins in pituitary adenomas. Indeed mice expressing an Hmga2 transgene either in its wild-type or truncated form, developed pituitary adenomas that secrete prolactin and growth hormone by the age of 6 months (Fedele et al. 2002). The same phenotype was also shown by mice over- expressing Hmga1 (Fedele et al. 2005). Studies in nine human prolactinomas revealed an increased dosage of the HMGA2 locus and its overexpression in most of them, whereas no HMGA2 expression was detected in normal pituitary cells (Finelli et al. 2002). Interesting and recent findings showed that a possible mechanism by which HMGA2 is overexpressed, involves the down-regulation of miR-15 and miR-16 that occurrs in human prolattinomas; HMGA proteins were demonstrated to be targets of these two miRNAs (Palmieri et al. 2012).

Studies conducted on HMGA2-trangenic mice allowed to clarify a critical role of E2F1 activation in pituitary adenoma. In fact, mice obtained by crossing Hmga2-transgenic with E2F1 knockout mice showed a milder pituitary phenotype compared to the Hmga2 transgenic mice in a wild-type E2f1 genetic background (Fedele et al. 2006). It was also demonstrated that the E2F1 activation is dependent on overexpression of HMGA2 that causes a displacement of HDAC proteins from the RB/E2F complex and, consequently leads to the acetylation and stabilization of the E2F-free factor (Fedele et al. 2006). This model provides a very nice example of the mechanism of action of

HMGA proteins to activate transcription and of the effect of post-translational modifications on their function.

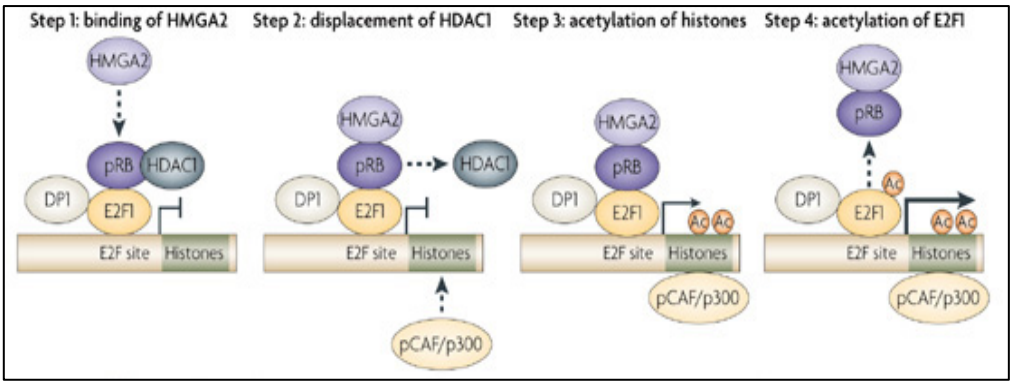


Figure 1.8. E2F transcriptional regulation. The transcriptional activity of the E2F family of transcription factors is crucial for the expression of several genes required to enter the S-phase of the cell cycle. Hmga2 acts by displacing HDAC1 from E2F1 target promoters, hereby recruiting histone acetylases which first acetylate the histones - relieving transcriptional repression - and then also acetylate E2F1 causing its stabilization. (*from Fusco, Fedele. Nature reviews, 2007*).

1.2.5.2. HMGA proteins in malignancies.

The most studied and remarkable observation about HMGA proteins is that a generalized overexpression of HMGA1 and often HMGA2 characterizes neoplastic cells. Overexpression of members of the HMGA family, in particular of HMGA1, was first observed in HeLa cells (Lund et al. 1983) and in cultured rat thyroid transformed by the Kirsten murine sarcoma virus. The latter revealed the specific presence in KiMSV transformed cells of HMGA1a, HMGA1b and HMGA2 proteins (Giancotti et al. 1985). Further, it was found that rat thyroid PC Cl 3 transformed by the v-ras oncogene, which does not confer to them the tumorigenic phenotype, did not express HMGA proteins, revealing that the presence of HMGA proteins is associated with an aggressive phenotype. The confirmation of the presence of HMGA proteins in thyroid carcinomas and metastases but not in normal thyroid tissue (Chiappetta et al. 1995) prompted further studies to investigate of HMGA proteins expression in human thyroid neoplasms. It was found that HMGA1 protein was detectable in 121 of 126 thyroid carcinomas analyzed, in about 20% of benign adenomas, and essentially in no hyperplastic thyroid tissues. Moreover, a correlation between HMGA1 expression levels and aggressiveness of the tumors was observed, since the highest HMGA1 expression was detected in the anaplastic histotype, that represents one of the most aggressive and invariably lethal

cancers in the humans (Chiappetta et al. 1998).
Overexpression of HMGA proteins has been described in different types of cancer. These include prostate (Tamimi et al. 1996) (Tamimi et al. 1993) colorectum (Kim et al. 1999) (Fedele et al. 1996) (Chiappetta et al. 2001), lung (Sarhadi et al. 2006), breast (Flohr et al. 2003) (Chiappetta et al. 2004), pancreas (Abe et al. 2000) (Abe et al. 2002). sarcomas (Berner et al. 1997), oral squamous cell carcinomas (Miyazawa et al. 2004), and non-small cell lung cancer (Meyer et al. 2007). In most of them, increasing levels of HMGA1 proteins are correlated with increasing degrees of malignancy or metastatic potential. For example, in breast carcinoma it was found a correlation between HMGA1 and c-erbB-2, whose expression represents an indicator of a poor prognosis (Chiappetta et al. 2004).
Many *in vitro* and *in vivo* studies over years supported the role of HMGA1 and HMGA2 overexpression in tumor development, also providing evidence of the causal involvement of HMGA proteins in cancer. Indeed, it has been found that the block of HMGA1 protein synthesis by an antisense methodology prevents rat thyroid malignant cell transformation induced by myeloproliferative sarcoma virus or Kirsten murine sarcoma virus, carrying oncogenes v-mos and v-ras-Ki, respectively, providing evidence that HMGA1 is required for thyroid cell tranformation (Berlingieri et al. 1995) (Berlingieri et al. 2002). Further, the HMGA1 silencing induces cell death in two human thyroid anaplastic carcinoma cell lines, but not in normal thyroid cells (Scala et al. 2000). It was also demonstrated that increased expression of both HMGA1 and HMGA2 proteins leads to neoplastic transformation of Rat1a fibroblasts with anchorage-independent cell growth (Wood et al. 2000), whereas in lung and pancreatic carcinoma cell lines HMGA1 silencing reduced the anchorage-independent proliferation in soft agar and increased susceptibility to anoikis (Liau et al. 2007). Moreover, in human pancreatic cancer cells the knockdown of HMGA2 inhibited cell proliferation, leading to an epithelial-state transition that restores cell-cell contact due to E-cadherin up-regulation (Watanabe et al. 2009).
Hmga1a, hmga1b and *hmga2* transgenic mice were developed. Both *hmga1b* and *hmga2* transgenic mice develop mixed growth hormone/ prolactin cell pituitary adenomas, abdominal/pelvic lipomatosis and/or an abnormally high incidence of lipomas and NK-T/NK cell lymphomas. A crucial step in the onset of pituitary adenomas in *hmga2* transgenic mice, and probably also in humans, is E2F1 activation, as described above (Fedele et al. 2006).

Further, transgenic mice that misexpressed full-length or truncated human HMGA2 transcript under the control of the promoter of the adipocyte P2 (Fabp4) gene, specific of mesenchymal differentiated cells, produced neoplastic phenotype, including fibroadenomas of the breast and salivary gland adenomas (Zaidi et al. 2006). *Hmga1a* transgenic mice develop aggressive, highly penetrant lymphoid malignancy and *HMGA1a* is also overexpressed in human lymphoid leukemia (Pierantoni et al. 2003; Xu et al. 2004). In addition to lymphoid malignancy, *Hmga1a* transgenic mice develop uterine tumors,

which resemble human uterine adeno-sarcomas (Tesfaye et al. 2007). Further, *hmga1* null mice are less susceptible to chemically induced skin carcinogenesis (Baldassarre et al. 2001).

1.2.5.3. Mechanisms of HMGA proteins oncogenicity

The role of HMGA proteins in cell transformation is essentially based on its ability to modulate the expression of genes critical for the tumorigenic process, involved in fundamental cellular functions, such as cell proliferation and invasion.

1.2.5.3.1. Activation of the AP1 complex

After discovering the importance of HMGA1 for the establishment of the neoplastic phenotype, our group also showed its ability to control the AP-1 complex in retrovirally transformed thyroid cell lines. This complex is constituted of three Jun proteins (JUN, JUNB and JUND) and four Fos proteins (FOS, FOSB, FRA1 (also known as FOSL1) and FRA2 (also known as FOSL2) and it is responsible for the activation of various target genes involved in the control of cell proliferation, tumorigenesis and metastasis(Angel and Karin 1991; Karin et al. 1997) It was found that HMGA1 exerts a positive regulation activity on this complex, in particular on JUNB and FRA-1, which are strongly up-regulated in the neoplastic transformation induced by the HMGA1 overexpression. Conversely, the HMGA1 antisense is able to prevent JUNB and FRA-1 overexpression. These results indicated that HMGA1 is essential for the JUNB and FRA-1 transcriptional induction associated with neoplastic transformation. (Vallone et al. 1997).

1.2.5.3.2. Cell cycle regulation

HMGA proteins exert their control on the cell cycle both directly and indirectly. Indeed, as previously discussed, HMGA2 is able to activate E2F1 by displacing histone deacetylase 1 from the pRB–E2F1 complex; it leads to an enhanced acetylation of both E2F1 and DNA-associated histones. Further, HMGA2 regulates the expression of several cyclins through E2F1, including cyclin A and other proteins required for the cell-cycle progression, both at the S-phase entry and at the G2/M transition (Pagano et al. 1992). Moreover, HMGA2 directly induces the cyclin A gene *CCNA2* as well as its association with the transcriptional repressor $p120^{E4F}$ and through the activation of the AP1 transcriptional complex (Tessari et al. 2003).

1.2.5.3.3. Modulation of the apoptosis

HMGA proteins are able to inhibit the apoptosis by regulating the p53 activity at a post-transcriptional level. This effect is achieved throught different mechanisms. First, HMGA1 regulates the transcription of many p53 effectors, such as BCL2-associated X protein and cyclin-dependent kinase inhibitor 1; further, it cooperates with p53 to activate the transcription of the p53 inhibitor MDM2. It has also found in thyroid cancer cells that HMGA1 associates *in vivo* with p53 family members, and that this interaction results in the inhibition of their tumor suppressor activity. (Frasca et al. 2006) Moreover, HMGA1 is able to relocalize the proapoptotic p53 activator homeodomain-interacting protein kinase 2 (HIPK2) from the nucleus to the cytoplasm, thus preventing its interaction with p53 (Pierantoni et al. 2007).

1.2.5.3.4. HMGA proteins impair DNA repair

As previously described HMGA proteins have been demonstrated to preferentially bind to, and inhibit the nucleotide excision repair, UV-induced CPDs in stretches of AT-rich DNA both *in vitro* and *in vivo*. Further, it has been found that HMGA2 binds the promoter of the nucleotide excision-repair gene *ERCC1* and negatively modulates its activity (Borrmann et al. 2003). Reeves *et al.* using transcriptional microarrays described a number of genes involved in DNA repair that were negatively regulated in MCF7 cells by HMGA1 overexpression, suggesting that HMGA proteins can influence DNA repair by negatively regulating the transcriptional activity of genes involved in various aspects of DNA-damage recognition and removal (Reeves and Adair 2005). A role for HMGA1 has been reported also in the double-strand break (DSB) repair. Indeed, in 2003 our group reported that HMGA1b protein binds to and inhibits the activity of both human and mouse BRCA1 promoters both *in vitro* and *in vivo*, and that there is an inverse correlation between HMGA1 and BRCA1 expression in human breast carcinomas. Interestingly, murine ES cells with the hmga1 gene deleted showed higher Brca1 mRNA and protein levels than do wild-type ES cells and stable transfection of MCF-7 cells with the HMGA1b cDNA results in a decrease of BRCA1 gene expression (Baldassarre et al. 2003). Consistent with these data, it has been shown that HMGA proteins potentiate genotoxic stress induced by different DNA-damaging agents causing DSBs, such as cisplatin, bleomycin, doxorubicin and X-ray irradiation (Baldassarre et al. 2005; Boo et al. 2005). Moreover, the cyclin A, indirectly regulated by HMGA1, complexed with the cyclin dependent kinase 2, as well in the regulation of DSB repair (Muller-Tidow et al. 2004).

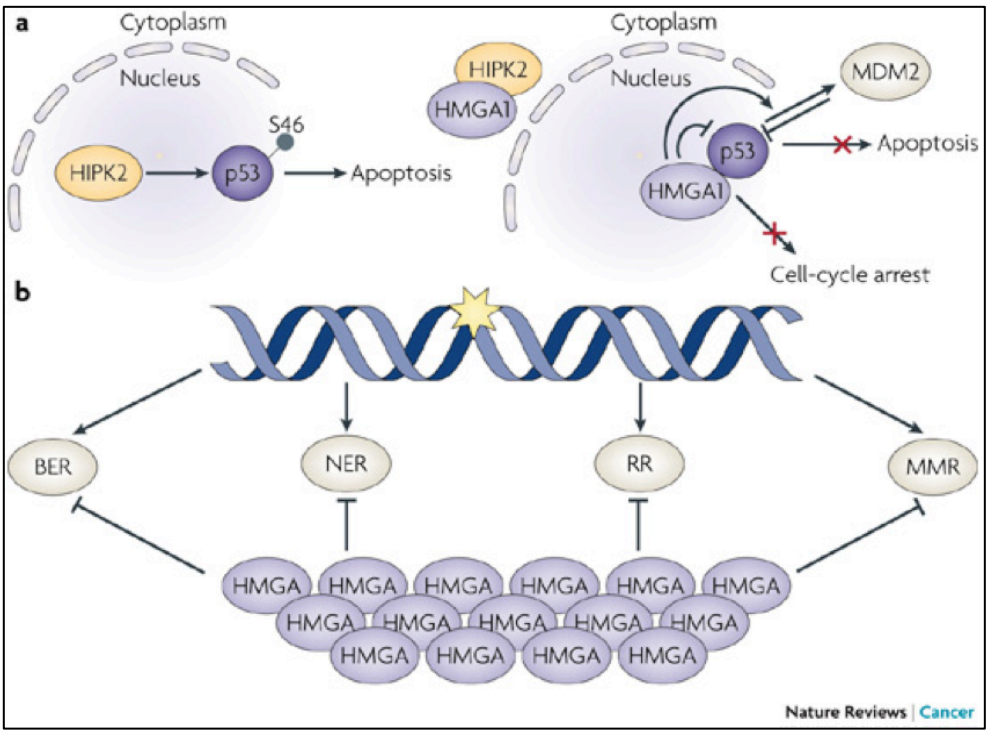


Figure 1.9. HMGA1 in cancer. A. Schematic representation of p53 inhibition by HMGA1. **B.** Schematic illustration of HMGA proteins in DNA repair. (*From Fusco, Fedele. Nature reviews, 2007*)

1.2.5.3.5. Regulation of genes involved in inflammation

The inflammatory process is known to sustain the tumor growth and it is at the basis of the cancer progression. HMGA proteins are able to stimulate inflammatory pathways, which in turn can promote cancer progression by suppressing apoptosis and inducing proliferation, angiogenesis, invasion and metastasis (Mantovani 2005). Indeed HMGA1 regulates the transcription of a number of genes such as inducible nitric oxide (NO) synthase (iNOS, also known as NOS2A) (Perrella et al. 1999), cyclooxygenase 2 (COX2) (Ji et al. 1998), (Whitley et al. 1994) , E-selectin (Kim et al. 1995), immunoglobulin E (Chuvpilo et al. 1993), IL4 (Himes et al. 1996), IL2 (Kim et al. 2001), (Mantovani et al. 1998) and granulocyte-macrophage colony-stimulating factor. The induction of these genes by HMGA1 likely occurs through its ability to enhance the transcriptional activity of nuclear factor κB (NF-κB) (Zhang and Verdine 1999) (Bogdan 2001).

1.2.5.3.6. Induction of STAT3

In 2008, it was highlighted a mechanism by which HMGA proteins have a

causal role in hematopoietic malignancies through the regulation of STAT3 (Hillion et al. 2008). Indeed, the STAT3 gene is a critical downstream target of HMGA1a and STAT3 mRNA and protein are up-regulated in fibroblasts overexpressing HMGA1a and activated STAT3 recapitulates the transforming activity of HMGA1a in fibroblasts. Further, HMGA1a transgenic mice developed aggressive lymphoid malignancy and an overexpression of STAT3 was found in the leukemia cells but not in control cells. Blocking STAT3 function induced apoptosis in the transgenic leukemia cells but not in controls and led to decreased cellular motility and foci formation in human leukemia or lymphoma cells. Consistent whith these data, it was found a positive correlation between HMGA1a and STAT3 mRNA in primary human leukemia samples (Hillion et al. 2008).

1.2.5.3.7. Regulation of the epithelial–mesenchymal transition (EMT)

The phenomenon of EMT is a common feature of both embryonic development and advanced epithelial tumors, where epithelial cells de-differentiate to a more fibroblast-like state and regain the ability to invade, migrate and/or proliferate in an uncontrolled fashion (Huber et al. 2005). Several studies revealed that in MCF7 cells overexpressing HMGA1, many EMT markers were up or down-regulated in function of the HMGA1 overexpression. Further, transcriptomic analysis showed that the HMGA2 gene is induced by the Smad pathway during EMT and endogenous HMGA2 mediates EMT through the TGF-beta pathway, whereas ectopic HMGA2 causes irreversible EMT, characterized by severe E-cadherin suppression. Moreover, specific knockdown of HMGA2 inhibited cell proliferation, leading to an epithelial-state transition that restores cell-cell contact due to up-regulation of E-cadherin (Thuault et al. 2006), (Thuault et al. 2008). Consistently, an inverse correlation between HMGA2- and E-cadherin- positive cells was found in cancer tissues (Donato et al. 2004).

1.2.5.3.8. Modulation of miRNA expression

One mechanism recently highlighted concerns the ability of HMGA1 to regulate the expression of miR-181b, which is overexpressed in several malignant neoplasias. A direct correlation between HMGA1 and miR-181b expression was found in human breast carcinomas. Further, it was shown that miR-181b modulates the CBX7 expression, which is generally down-regulated in cancer. In particular, it was demonstrated that HMGA proteins are involved in the down-regulation of CBX7, which in turn negatively regulates miR-181b expression. All toghether these data highlight the existance of a pathway involving HMGA1, miR-181b and CBX7, which is at the basis of the cancer progression (Mansueto et al. 2010).

1.2.5.3.9. Down-regulation of tumor suppressor genes

Recently, our group has described a role of HMGA1 in the transcriptional regulation of HAND1 gene, which codes for a transcription factor crucial for differentiation of trophoblast giant cells and heart development. Hand1 was found upregulated in hmga1 minus embryonic stem cells and HMGA1 was demonstrated to bind directly to its promoter, thus regulating its activity. Further, the analysis of human thyroid carcinoma cell lines and tissues overexpressing HMGA1, revealed an inverse correlation between HMGA1 and HAND1 and it was consistent with the repression of HAND1 and its promoter hypermethylation in anaplastic carcinomas. These studies highlighted a role of HMGA1 in the silencing of a tumor suppressor gene in later stages of thyroid tumor progression, and that it is achieved through the HAND1 promoter hypermethylation (Martinez Hoyos et al. 2009).

1.3. HMGA PROTEINS AND STEMNESS

The apparent parallels between tumor cells and normal stem cells have generated great interest in the possible links between these two classes of cells. Feautures of stem cells, such as self-renewal and differentiation capacity can find their counterpart in the high proliferative capacity and phenotypic plasticity of tumor cells. Moreover, tumor cells often lack the terminal differentiation ability that characterizes normal cells. Many regulatory networks controlling the function of stem cells may also be active in certain tumors. These networks have been the focus of much recent interest, and particular attention has been paid to the study of ES cells.

1.3.1. HMGA proteins in stemness networks

Current evidence indicates that some of the key regulators of stem cells identity in ES cells, for example Oct4, Sox2 and Nanog, Stat3 and Lin28, are expressed in several human cancer types. These studies have revealed the existence of a so called ES-like signature in which HMGA proteins appear as master regulator of up and down-regulated genes; the presence of these signature directly correlates with a poor prognosis in many human cancers. (Ben-Porath et al. 2008), (Shah et al. 2012).

Pegoraro et al showed that in breast cancer cells, HMGA1 activates stemness and key migration-associated genes which are linked to the Wnt/beta-catenin, Notch and Pin1/mutant p53 signalling pathways. They studied an HMGA1 signature in which they found different genes known to be involved in processes related to the EMT and the formation of stem cells, including CD24, FAM83H, IL1R1, SERPINE1, CALD1, TUBB, LIFR, LEF1 and SET8. Interestingly, LEF1 and SET8 are regulatory elements of the Wnt/beta-catenin pathway that cooperate in a complex with beta-catenin and function as coactivators to sustain the EMT and stem properties (Pegoraro et al. 2013). An important role of HMGA proteins in ES cells was highlighted in 2003. Indeed, Battista et al found that HMGA1 double knock-out ES cells have reduced self-renewal ability, demonstrating for the first time the importance of HMGA1 in stemness. They reported that Hmga1 null ES cells generate fewer T-cell precursors than their wild-type counterpart and the differentiation is preferentially into B cells lineage, probably as a consequence of decreased interleukin 2 expression and increased interleukin 6 expression. Moreover, a lack of HMGA1 expression induces changes in hemopoietic differentiation, i.e., a reduced monocyte/macrophage population and an increase in megakaryocyte precursor numbers, erythropoiesis, and globin gene expression. More recently it has been reported that HMGA1 promotes the cellular reprogramming of adult somatic cells to undifferentiated, fully pluripotent stem

cells (iPSCs). Since it is known that HMGA1 directly activates specific genes involved in tumor growth and progression, including proliferation, migration, invasion, angiogenesis, resistance to cell death, immune evasion, and an epithelial-mesenchymal transition in cancer cells, it has been explored its role also in human embryonic stem cells (hESCs). First, it was found that during differentiation, the expression levels of HMGA1 are closely parallel those of the embryonic stem cell and pluripotency factors such as NANOG, SOX2 and OCT4. Then bone-marrow derived, commercial, adult mesenchymal stem cells (MSCs) were transduced by using the standard retroviral reprogramming technology with the four Yamanaka factors (OCT4, SOX2, KLF4, and cMYC or OSKM) plus HMGA1 or control. The addition of HMGA1 to OSKM resulted in an enhancement of the reprogramming rate, stem cell survival, proliferation, or a combination of these factors during iPSC generation (Shah, Kerr et al. 2012). According to these findings, HMGA1 has been demonstrated able to bind the promoter of pluripotency genes *in vivo*.
HMGA1 also drives proliferative changes and polyp formation in the intestines of transgenic mice and induces stem-like properties in colon cancer cells (Belton et al. 2012). The observation of HMGA1 upregulation in colon cancer dates back to 1996, when Fedele et al detected the HMGA1 protein in human colorectal cancer cell lines and tissues but not in normal intestinal mucosa (Fedele et al. 1996). Subsequently, it was found that HMGA1 protein expression was associated with the early stages of the neoplastic transformation of colon cells but only rarely with colon cell hyperproliferation (Chiappetta et al. 2001). HMGA1 also correlates with the degree of cellular atypia in adenomas in transgenic mice and colon cancer cells. Recently, a causative role of HMGA1 in proliferative changes, aberrant crypt formation, and intestinal polyposis it has been showed in transgenic mice. Further, in colon cancer cell lines from poorly differentiated, metastatic tumors, knock-down of HMGA1 blocks anchorage-independent cell growth, migration, invasion and xenograft tumorigenesis while the presence of HMGA1 is responsible of the metastatic progression and stem cell-like properties in metastatic colon cancer cells (Belton et al. 2012). Interesting findings on the role of HMGA1 in regulating stemness came also from the hmga1 null mice phenotype. Indeed our group demontrated that the hmga1 double knock-out generates pigmy mice. It was compared the tumor susceptibility of mice wild-type or Hmga1-null mice by using a two-stage chemical skin carcinogenesis protocol. Transgenic mice exhibited a decreased number and a delayed onset of skin papillomas in comparison with wild-type mice (Visone et al. 2008). A possible explanation for all these findings is that the hmga1 knock-out leads to the depletion of the stem cell number in transgenic mice, suggesting a role for HMGA1 in regulating the stem cell pool during development and growth.

An important role in the maintainance of the stem-like state has been reported also for HMGA2. Indeed, by comparing breast tumor-initiating cells (BT-IC) and non-BT-IC from 1 degrees breast cancers, Yu et al found that let-7

miRNAs were markedly reduced in BT-IC and increased with differentiation. The restoration of let-7 by lentivirus infection reduced proliferation, mammosphere formation, the proportion of undifferentiated cells *in vitro* and tumor formation and metastasis in NOD/SCID mice, while antagonizing let-7 by antisense oligonucleotides enhanced *in vitro* self renewal of non-T-IC. It is known, as previously described that HMGA2 is one of the main target of let-7 miRNA and the specific silencing of HMGA2 induced differentiation in BT-IC, suggesting its role in the stemness maintainance (Yu et al. 2007).

2. AIM OF THE STUDY

HMGA1 is a chromatin-binding protein highly expressed in several malignant tumors. It is expressed during embryogenesis, whereas it is absent or at very low levels in adult tissues. Several studies have shown a correlation between the over-expression of HMGA1 and a highly malignant phenotype (Chiappetta et al. 2004). Moreover, it has been exstensively demonstrated that HMGA1 have a causal role in cell transformation, since blocking HMGA expression hampers transformation (Berlingieri et al. 2002). In recent years HMGA proteins have emerged as stemness factors; indeed, HMGA1 double knock-out ES cells show reduced self-renewal ability (Battista et al. 2003) and HMGA1 transgenic mice develop polyps and expansion in the stem cell compartment (Belton et al. 2012). Further, HMGA1 is a key regulator both in metastatic progression and in the maintenance of a stem-like state.
Given its role in cancer and its involvement in stemness, the aim of this study is to clarify whether and how HMGA1 regulate the biology of CSCs. CSCs are the real tumor initiating cells and are emerging as election targets for cancer eradication therapies. The main purpose of my study has been to characterize the molecular machanisms underlying HMGA1 function in CSCs. In particular, in this study I focused on the role of HMGA1 in colon cancer stem cells.
This project, which has been developed during my PhD program, represents the main body of my thesis and the subject of the following publication:

Puca F, Colamaio M, Federico A, Gemei M, Tosti N, Uchimura Bastos A, Del Vecchio L, Pece S, Battista S and Fusco A. ***HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels.***

3. MATERIALS AND METHODS

3.1. Cell cultures and culture conditions

Colon tumor stem cell lines were kindly donated by Prof. Ruggero De Maria (Istituto Superiore di Sanità, Rome, Italy) and have been described elsewhere, together with their culturing conditions (Ricci-Vitiani et al. 2007). Cytokines added to the medium includedrecombinant human FGF-basic and EGF (Peprotech, Rocky Hill, NJ). HEK293 cells and their culture conditions have been described elsewhere.

3.2. Colon samples

Normal intestinal mucosa was kindly provided by Dott. Marina De Rosa (Facoltà di Medicina e Chirurgia, University "Federico II", Naples, Italy). Colon cancer samples have been described elsewhere (Fedele, Bandiera et al. 1996).

3.3. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was reverse transcribed using the QuantiTectâ Reverse Transcription Kit (Qiagen), and qRT-PCR was performed by using Power SYBRâ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. *G6PD* was used to normalise RNA levels. The primers used were as follows:

hsaHMGA1 FW: CAACTCCAGGAAGGAAACCA,

Hsa HMGA1 hsa Rev: AGGACTCCTGCGAGATGC;

Hsa_-G6PD_for: 5'-ACAGAGTGAGCCCTTCTTCAA, and

Hsa_G6PD_rev: 5'-ATAGGAGTTGCGGGCAAAG.

The 2^{-}DDCt formula was used to calculate the differential gene expression.

3.4. Immunostaining and cell sorting

For cell sorting, the cells were trypsinised, washed twice with PBS, and incubated with anti-human CD133 (CD133/2 (293C3)-PE, Miltenyi Biotech) for 20 minutes at 4°C. After washing twice with PBS, the cells were FACS sorted with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett-Packard computer (Palo Alto, CA).

3.5. Western blots and antibodies

Total protein extraction, western blotting, and anti-HMGA1 antibodies have been described elsewhere (Melillo et al. 2001).

Differential nuclear and cytoplasmic cell lysates were obtained as reported previously (Schreiber et al. 1989).

The following other antibodies were used: anti-GAPDH (Santa Cruz Biotechnology, CA, USA) and anti-p21 (Cell Signaling Technology, Inc., Danvers, MA). Anti-NUMB antibodies were obtained from Abcam (Cambridge, UK) and were used at 1:5000. Blots were visualised using western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

3.6. Plasmids

The hairpin RNA interference plasmid for human HMGA1 (pLKO.1-HMGA1, TRCN0000018949) and the scramble control pLKO.1-Puro plasmid (SHC002) were obtained from Sigma-Aldrich. The sequence of the short hairpin RNA targeting the human HMGA1 gene was:

5′-
CCGGCAACTCCAGGAAGGAAACCAACTCGAGTTGGTTTCCTTCCTG
GAGTTGTTTTT–3′, (shHMGA1 targets coding region positions 446-466 of HMGA1 mRNA transcript variant 2).

The HMGA1 antisense consists of the expression vector pRc/CMV (Invitrogen) coding sequence of the HMGA1 gene in the antisense orientation as described elsewhere (Melillo et al. 2001).

The pGL3-luci vector containing the p53 promoter was kindly provided by Prof. David Reisman (Center for Colon Cancer Research Tissue Repository, University of South Carolina, Columbia) (Durland-Busbice and Reisman, 2002).

The pCDNA3.1-HMGA1 expression vector has been described previously (Battista et al. 2003).

3.7. Transfections

CTSCs were electroporated using the Neon® Transfection System (Invitrogen). Cells were trypsinised with TrypLE™ Express (GIBCO) and counted; 1 x 10^6 cells were subjected to the electric field (1400 V, 20 msec; 1 pulse).

After 48 h, CTSCs transfected with the short hairpin-expressing constructs were selected with puromycin (2 μg/μl).

3.8. Growth curves and TUNEL assay

Approximately 5 x 10^3 stably transfected cells were plated in 96-well plates. Cells were counted in triplicate at daily intervals with a Burker hemocytometer chamber. A TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche) according to manufacturer's instructions.

3.9. Flow cytometry

After trypsinisation, the cells were washed in PBS and fixed in 70% ethanol. Staining for DNA content was performed with 0.1% NP-40, 50 μg/ml propidium iodide, and 25 μg/ml ribonuclease A for 20 min. For each measurement, 10,000 events were analysed. We used a FACScanto II flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle data were analysed with the ModFit LT 2.0 software (Verity Software) in a semiautomatic analysis procedure. Briefly, we manually selected the cell population in an FSC versus SSC dot plot and discarded debris, and then we gated single cells in a PI-height versus PI-area dot plot, excluding all doublets. The MODFIT algorithm was used to analyse our files, calculating the percentages of cells in each cell cycle phase. Statistical analyses to evaluate the significance of the variation in the G1

and S phases were performed using the Mann-Whitney U-test.

3.10. Sphere formation assays

Sphere-forming assays in methylcellulose-based medium were performed as previously described (Liu et al. 2011) with some modifications. Briefly, medium containing 0.8% methylcellulose was used instead of liquid medium, and other conditions were the same as in liquid medium. Three percent methylcellulose was purchased from R&D Systems (Minneapolis, USA), and a stock solution was made of 2% methylcellulose in DMEM/F12. A final concentration of 0.8% methylcellulose in DMEM/F12 was used for cell culture. Approximately 2 x 10^4 cells from disaggregated CTSC spheres were resuspended in a semisolid medium and plated in 6-well plates. After 7 days, the spheres were microscopically visualised, and the diameters were measured.

Serial passage experiments were conducted as described previously (Cicalese, Bonizzi et al. 2009) with some modifications. Briefly, 5,000 cells from disaggregated CTSC spheres were plated on 150-mm poly-HEMA-treated cell culture plates. After 10 days, the spheres were disaggregated and re-plated at the same density. The sphere-forming efficiency (SFE) at each passage was obtained by calculating the percentage of the number of spheres divided by the number of cells plated.

3.11. PKH staining and flow cytometric analysis

PKH staining was performed as previously described (S. Pece et al., Cell 2010).

CTSCs were trypsinised, filtered through a 40-μm cell strainer, resuspended in PBS (approximately 500,000 cells/ml), labelled with PKH26 (Sigma, 10−7 M, 5 min), washed twice, and plated.

For the flow cytometric analysis of PKH26-stained cells, CTSCs were trypsinised, filtered through a 40-μm cell strainer, and resuspended in PBS at a concentration of 1x106/ml. The cells were subdivided into 5-ml polystyrene tubes (Falcon, Becton Dickinson). The BD FACSAria cytometer, equipped with four excitation laser lines (633 nm, 488 nm, 405 nm, and 375 nm) (Becton Dickinson) was used for FACS analysis, and the BD FacsDIVA software was used for data analysis.

PKH26 staining was evaluated by selecting the appropriate cell population according to the following gating strategy: cells were first gated on physical parameters (forward scatter [FSC] and side scatter [SSC]) to exclude most of the debris and dead cells; doublets and aggregates were eliminated using the FSC-area vs. FSC-height pattern. We gated 10-15% of the brightest PKH26+ cells in a PKH26 versus empty channel dot plot.

3.12. Immunofluorescence

Whole spheres were centrifuged and fixed with 4% paraformaldehyde, permeabilised, with 0.1% Triton X-100 and 3% BSA, and stained with anti-NUMB (kindly donated by Prof. Salvatore Pece and described in (Cicalese et al. 2009), followed by anti-mouse Alexa 488 (Jackson Laboratories) antibodies. Confocal analysis was performed with a Leica TCS SP2 AOBS microscope.

3.13. Luciferase assays

Transfections for luciferase assays were performed in HEK293 cells using the Lipofectamine 2000 method (Invitrogen). For p53 luciferase assays, approximately 2 × 105 cells were transiently transfected with 200 ng of pGL3-luci vector containing the p53 promoter (kindly provided by Professor David Reisman, Center for Colon Cancer Research Tissue Repository, University of South Carolina, Columbia) and with the indicated amounts of the pCDNA3.1-HMGA1 expression vector or the corresponding empty vector together with 0.5 μg of Renilla. Various amounts of the pCDNA3.1 plasmid were co-transfected to keep the total DNA concentration constant. Transfection efficiencies were normalised using Renilla luciferase expression assayed with the dual luciferase system (Promega). All transfection experiments were performed in duplicate. For NUMB luciferase assays, 2 × 105 cells were transiently transfected with 200 ng of GoClone vector containing the NUMB promoter (SwitchGear Genomics) or the corresponding empty vector. For overexpression was used pCDNA3.1-HMGA1 expression vector. The silencing was achieved by using the expression vector pRc/CMV (Invitrogen) coding sequence of the HMGA1 gene in the antisense orientation as previously described(Melillo, Pierantoni et al. 2001). Transfection efficiencies were normalised using the Cypridina TK control construct (pTK-Cluc) expression assayed with the BioLux® Cypridina Luciferase Starter Kit according to manufacturer instructions.

3.14. Chromatin immunoprecipitation

ChiP was performed as described previously(Federico et al. 2009). As a negative control, ChIP experiments were performed with isotype-matched preimmune IgG. The promoter occupancy was calculated with respect to the input as the percentage of anti-A1-immunoprecipitated DNA subtracted from the IgG-immunoprecipitated DNA. The P53 promoter regions assayed for HMGA1 binding refer to the nucleotide sequence published in Durland-Busbice and Reisman, 2002: Region I: nt 401-557; region II: nt 538-650; region III: nt 631-751; region IV: nt 767-890; and region V: nt 989-1090.

The primers for each region were as follows:

Prom_hQ_tp53_1_Fw CAGGCTTCAGACCTGTCTCC

Prom_hQ_tp53_1_Rev GCTTTCAGTACATGGAAACGTAA

Prom_hQ_tp53_2_Fw CGTTTCCATGTACTGAAAGCAA

Prom_hQ_tp53_2_Rev CCCTAACGTTTTCTCCCAGA

Prom_hQ_tp53_3_Fw TCTGGGAGAAAACGTTAGGG

Prom_hQ_tp53_3_Rev AAGGGTGGAAGGAAGAAAGC

Prom_hQ_tp53_4_Fw GCAGGATTCCTCCAAAATGA

Prom_hQ_tp53_4_Rev GAGGGTGCAGAGTCAGGATT

Prom_hQ_tp53_5_Fw GTTGATGGGATTGGGGTTTT

Prom_hQ_tp53_5_Rev AGCTACCTGCTCCCTGGAC

To study the NUMB promoter occupancy of HMGA1 six different regions

were selected for AT richeness after in silico analysis. Region I: nt 401-557; region II: nt 538-650; region III: nt 631-751; region IV: nt 767-890; and region V: nt 989-1090.

The primers for each region were as follows:

Prom_hQ_NUMB1_F	GAGTGGATCTGGAAGCTGGA
Prom_hQ_NUMB1_R	CCTCAATGCTAAAGGGCAAA
Prom_hQ_NUMB2_F	TTTGCCCTTTAGCATTGAGG
Prom_hQ_NUMB2_R	AGCCCTGGGGAGAAAGGTAT
Prom_hQ_NUMB3_F	CAGGGCTTGGAAACACTTCT
Prom_hQ_NUMB3_R	TCCACTTCTCCAGCTCACTTC
Prom_hQ_NUMB4_F	GCTTCTCCTTGCAGGAAGTG
Prom_hQ_NUMB4_R	CAGTTTGGTTGCGCAGTAGA
Prom_hQ_NUMB5_F	TCTACTGCGCAACCAAACTG
Prom_hQ_NUMB5_R	TGGGCAATTCGAAGTTATGA
Prom_hQ_NUMB6_F	TAACTTCGAATTGCCCAACC
Prom_hQ_NUMB6_R	TAGTATACCCGCCCGTCATC

3.15. Chemoresistance to Gefitinib

The effect of Gefitinib on cell survival was assessed by plating single cells in 96-well plates at a density of 3 x 10^3 cells/well. CTSCs were treated with different concentrations of Gefitinib (5, 10 and 15 μM); DMSO was used as control. After 24, 48, 72 and 96 hours the cell viability was assessed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer instructions. After incubating for 3 hours at 37°C, the absorbance was read at a wavelenght of 490 nm by using a Microplate Reader 480 (Biorad). Results are expressed as absorbance at 490 nm.

3.16. Statistical analysis

Statistical analyses were performed using the Kruskal-Wallis test or the Mann-Whitney *U*-test. If the Kruskal-Wallis test was positive ($P<0.05$), a pairwise comparison of subgroups was performed using Dunn's or Conover post-hoc test.

4. RESULTS

4.1. HMGA1 is overexpressed in CTSCs and in the CD133$^+$ sub-population

We first analysed HMGA1 expression by Western Blot in normal colonic mucosa (NM), colon cancer samples, colon cancer cell lines, and CTSC lines. As shown in Fig. 4.1, HMGA1 is expressed at higher levels in CTSCs compared with NM, colon cancer tissues (Tumor #3), and colon cancer cell lines (GEO, SW480, SW48, CACO2). At the protein level, HMGA1 was undetectable in NM (Fig. 4.1.A), whereas it was expressed in colon cancer samples (Tumor #3), in 3 colon cancer cell lines (SW48, SW480 and CACO2), and CTSCs (CTSC#18 and CTSC#1.1), which exhibited the highest HMGA1 expression. Interestingly, when CTSCs were stained for the cancer stem cell marker CD133 and then sorted, HMGA1 expression was enriched in CD133$^+$ cells (Fig. 4.1.B). These data indicate that HMGA1 is overexpressed in CTSCs and is more abundant in stem cells than in precursors.

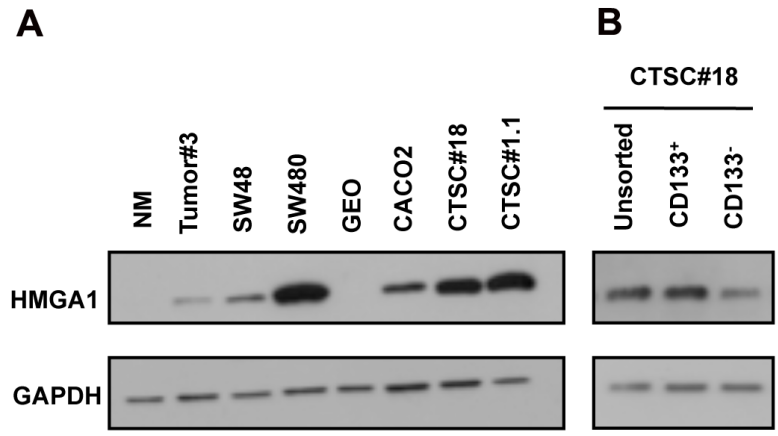


Figure 4.1. HMGA1 expression in CTSCs
A) Western blot for HMGA1 in normal colonic mucosa (NM), colon cancer sample (T#3), colon tumor-derived cell lines, SW48, SW480, GEO, and CACO2, and colon tumor stem cells (CTSC#18 and CTSC#1.1). **B**) Western blot for HMGA1 in unsorted CTSC#18 and sorted CD133$^+$ and CD133$^-$ cells. GAPDH was used as a loading control.

4.2. HMGA1 knockdown impairs CTSCs growth and induces apoptosis

To understand the role of HMGA1 in CTSC, we silenced HMGA1 expression in the CTCS#18 cell line, using a short hairpin interfering construct (see the Materials and Methods section), leading to an HMGA1 knockdown efficiency of approximately 50%-80% in stable transfectants (Fig. 4.2.A). Then, we characterized interfered CTSCs at different levels. Growth curves performed on single-cell suspensions demonstrated that the knockdown of HMGA1 significantly reduced CTSC proliferation. (Fig. 4.2.B)

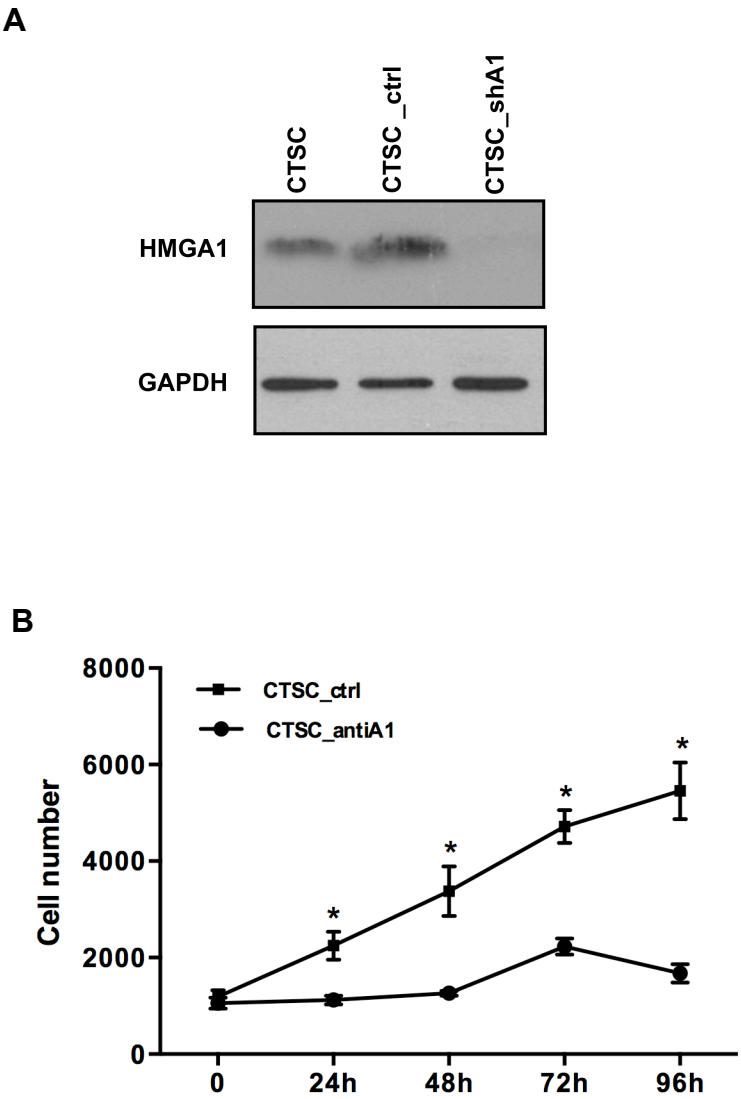


Figure 4.2. HMGA1 interference in CTSCs. A) Western blots for HMGA1 in untransfected, HMGA1-knockdown (CTSC_shA1), and scramble-transfected (CTSC_ctrl) cells. GAPDH is used as a loading control.
B) Growth curve of CTSCs stably transfected with HMGA1 antisense (CTSC_antiA1) and empty vector-RcCMV (CTSC_ctrl). Data are the mean

value ± SD of one representative experiment, performed in quadruplicate (*, $p < 0.05$, Mann-Whitney U-test).

The analysis of cell cycle progression, performed by flow cytometric analysis, demonstrated that HMGA1 knockdown reproducibly altered cell cycle progression, inducing a mean increase of 5% ($P < 0.05$) in the G1 phase population and a concomitant mean reduction of 4% ($P < 0.05$) in the S phase (Fig. 4.3).
As previously reported (Shah, Kerr et al. 2012), HMGA1 knockdown reduced the expression of stem cell/pluripotency genes, such as SOX2 and NANOG (Fig. 4.4).

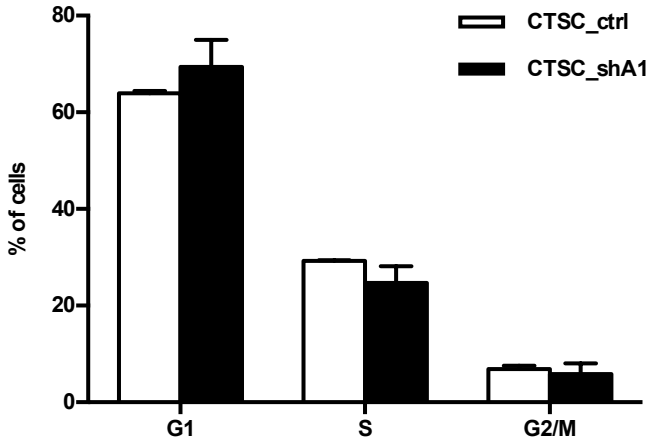


Figure 4.3. Cell cycle analysis. A) Histogram of the FACS analysis in CTSC_ctrl and CTSC_shA1 cells. Data are the mean value ± SD of 3 independent experiments.

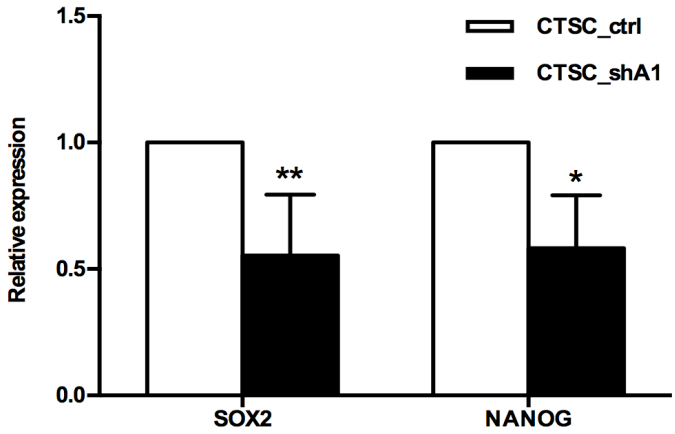


Figure 4.4. Differentiation markers analysis. qRT-PCR for SOX2 and NANOG gene expression in CTSC_ctrl and CTCS_shA1 cells. The expression level of each gene was normalized to the G6PD gene expression (*, $p < 0.05$, Mann-Whitney U-test).

Because the alteration of the cell cycle only partially accounts for the reduction in CTSC proliferation induced by HMGA1 knockdown, we investigated apoptotic cell death. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays performed on stable transfectants exhibited a 7-fold increase in apoptotic cell numbers in CTSC_shA1 with respect to control cells (Fig. 4.5.A, B). These data suggest that, as in other cell systems (Fedele and Fusco 2010), HMGA1 plays a key role in CTSC proliferation by affecting cell cycle progression and apoptosis.

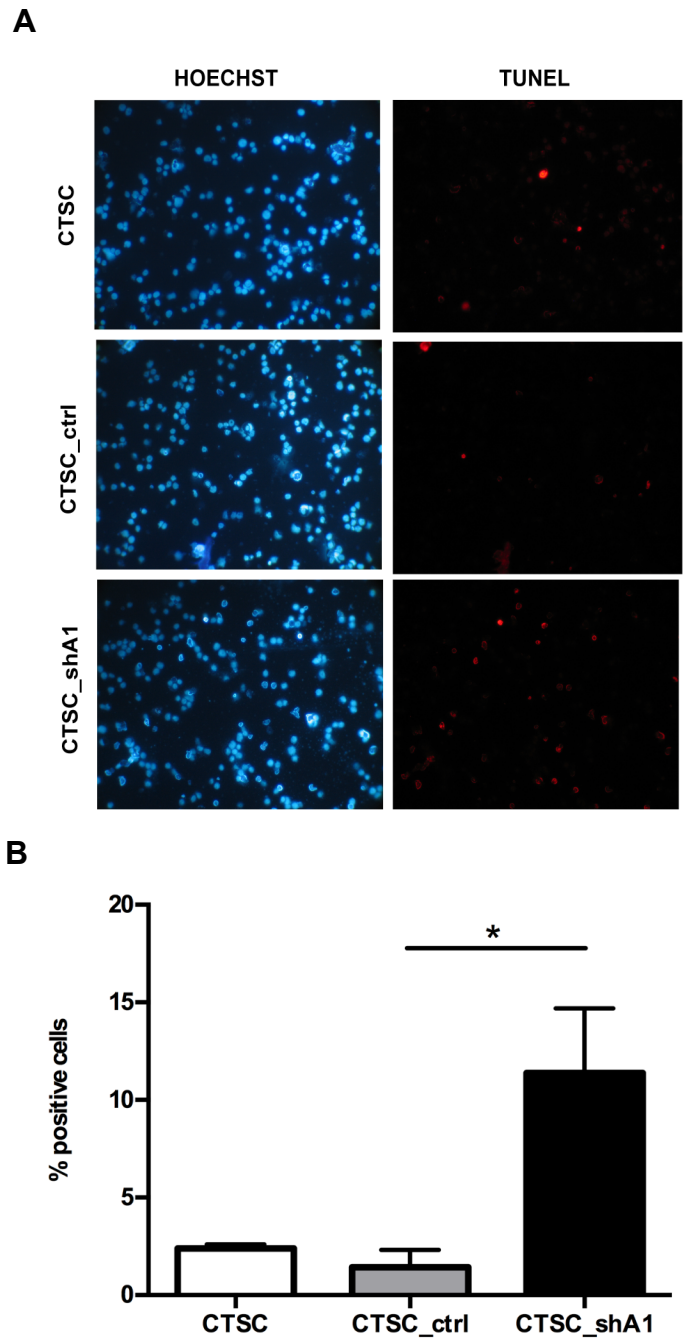


Figure 4.5. HMGA1 knockdown effects on apoptosis in CTSCs.
A) Fluorescence micrographs of TUNEL assays performed on non-transfected CTSCs, CTSC_ctrl and CTSC_shA1 cells, double-stained with Hoechst dye (left) to identify total nuclei and with TMR red UTP (right) to identify apoptotic, TUNEL-positive cells. B) Bar chart representation of the number of TUNEL-positive cells per 100 Hoechst-positive nuclei in the samples shown in A. Each bar represents the mean ± SD of 10 arbitrary fields. An asterisk indicates the significance of the difference between

CTSC_shA1 and CTSC_ctrl (*, p = 0.0014; Kruskal-Wallis test followed by Dunn's post-hoc test).

4.3. HMGA1 silencing impairs CTSC self-renewal and sphere-forming efficiency in serial passages

CTSCs, as other types of cancer stem cells, are characterised by their ability to form spheres in suspension cultures (Ricci-Vitiani et al. 2007) or in a semisolid medium. The number of spheres reflects the quantity of cells capable of *in vitro* self-renewal, whereas the number of cells/sphere measures the self-renewal capacity of each sphere-generating cell (Dontu et al. 2004). Therefore, we assayed the ability of cells with reduced HMGA expression to form spheres in methylcellulose-based medium. A dramatic reduction in the number of spheres (Fig. 4.6.A,B) and in their diameter (Fig. 4.6.C) was observed in CTSC_shA1 cells compared with control CTSC_ctrl, thus indicating that HMGA1-interference affects CTSC self-renewal.

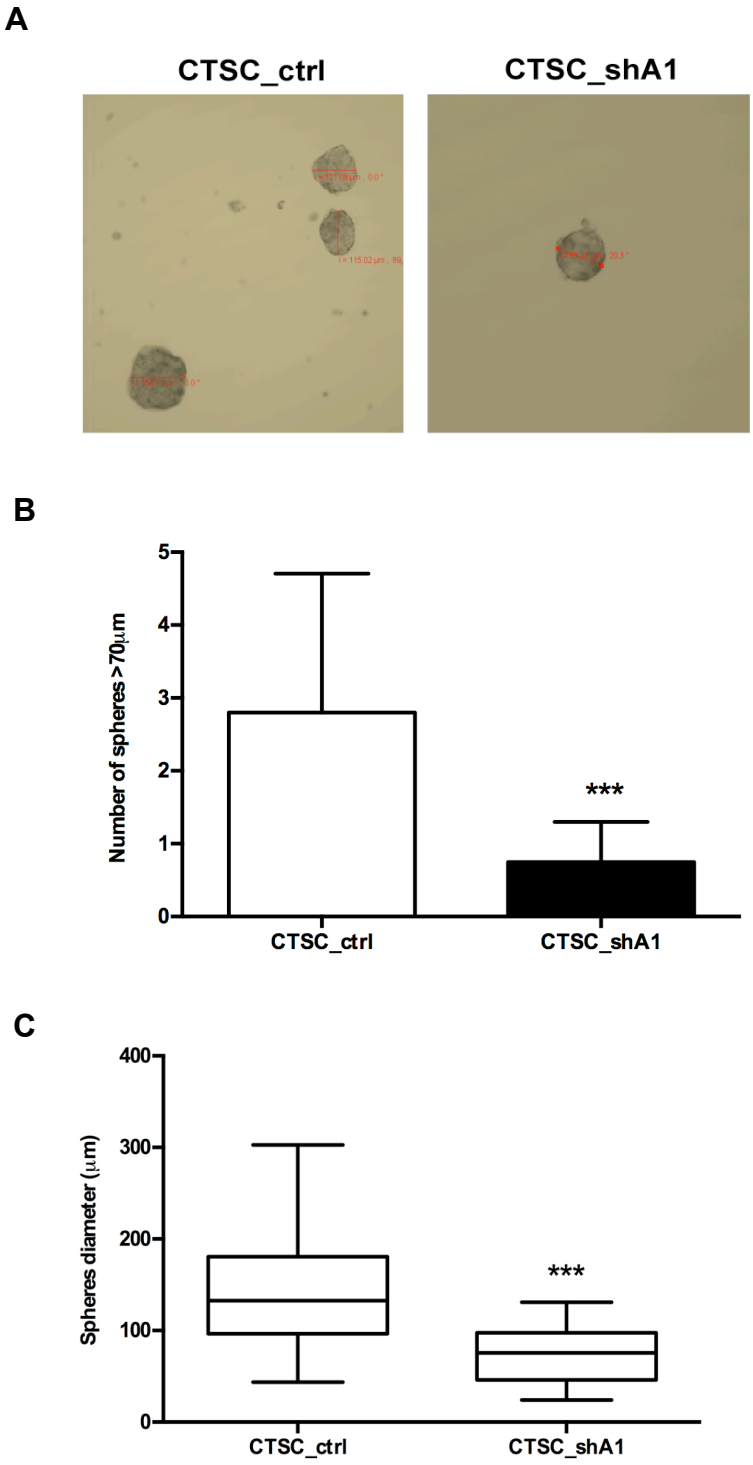


Figure 4.6. Effects of HMGA1 knockdown on the sphere-formation ability of CTSCs. A) Sphere formation was determined by plating single cells in methylcellulose-based medium. We characterized spheres formed from CTSCs silenced for HMGA1 compared to control. **B**) The diagram

represents the average number of spheres 7 days after plating. Spheres with diameters > 70 μm were counted in each of 10 representative fields. Triple asterisks indicate the significance of the difference in the number of spheres formed by CSC_ctrl and CTSC_shA1 cells (***, $p < 0.001$; Mann-Whitney U-test).
C) Diagram showing the sphere diameter distribution in CTSC_ctrl and CTSC_shA1 cells. Each bar represents the mean ± SD diameter found in 10 representative fields. Mann-Whitney U-test.

CSCs are known to differ from normal stem cells both in their deregulated self-renewal and cell division pattern, and an evaluation of their sphere-formation efficiency (SFE) in serial passages allows the assessment of the rate at which CSCs divide symmetrically (Dontu, El-Ashry et al. 2004) (Deleyrolle et al. 2011). As shown in Fig. 4.7., the SFE of CTSC_shA1 cells decreased at every passage, whereas the number of spheres derived from parental (not shown) or CTSC_ctrl cells increased progressively.

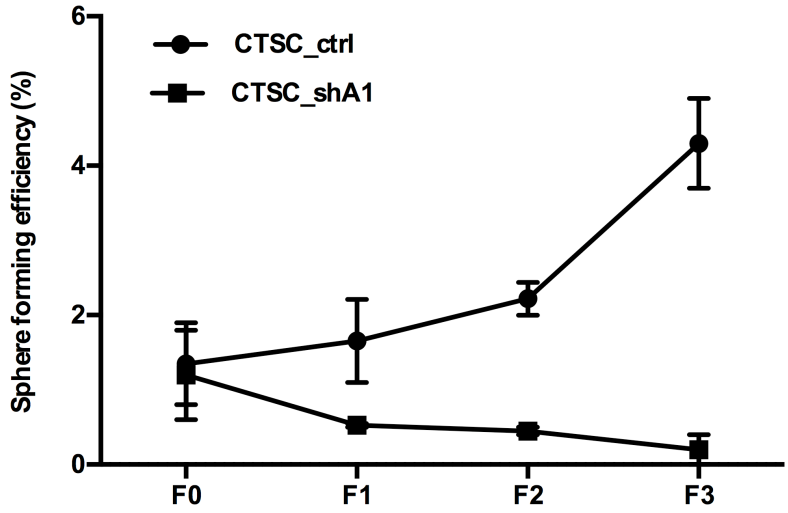


Figure 4.7. Sphere forming efficiency in CTSCs silenced for HMGA1. Diagram showing the sphere-formation efficiency (SFE) ± SD in scramble- and HMGA1-knockdown CTSCs in serial passages (from F0 to F3). The spheres were disaggregated every 10 days. SFE is measured as the percentage of the number of spheres per plated cell at every passage. The data represent the results of two independent experiments.

Interestingly, previous studies have demonstrated that the knock-out of p53 leads to increases in SFE and the symmetric division of mammary stem cells in serial passages, whereas the p53 stabiliser Nutlin-3 is able to reduce the SFE of

ErbB2 mammary stem cells in serial passages (Cicalese et al. 2009). Consistent with these studies, we observed that the treatment of parental CTSC#18 with 5 μM Nutlin-3 was able to maintain the SFE over subsequent passages, similar to what was observed for CTSC_shA1 cells. Conversely, SFE increased in DMSO-treated cells. Therefore, these results indicate that HMGA1 silencing not only restores the ability to divide asymmetrically but also exhibits a more dramatic effect than p53 stabilisation alone.

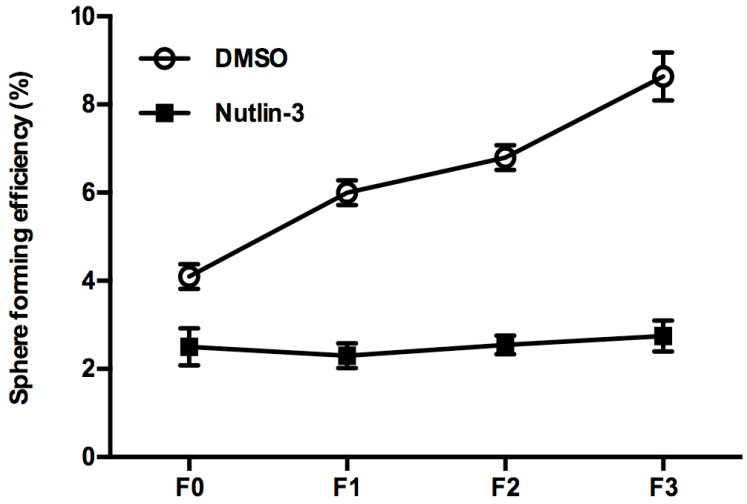


Figure 4.8. Sphere forming efficiency in CTSCs after p53 stabilization with Nutlin-3. Sphere-formation efficiency (SFE) in parental CTSC#18 cells treated with DMSO or Nutlin-3 (5 μM) in serial passages (from F0 to F3). Spheres were disaggregated every 7 days. The data represent the mean value ± SD of two independent experiments.

4.4. HMGA1 silencing induces quiescence in CSCs

Long-term label retention of PKH26 dye is frequently used as an indicator of normal stem cell quiescence (Pece et al. 2010). Indeed, rapidly and symmetrically dividing CSCs tend to quickly lose PKH26 (Cicalese, Bonizzi et al. 2009), which irreversibly binds to the lipid bilayers on cell membranes and is equally distributed among daughter cells during each cell division. Conversely, normal quiescent stem cells divide asymmetrically in one proliferating progenitor and one self-renewing PKH26-retaining quiescent stem cell. Therefore, we stained CTSC_shA1 and control CTSC_ctrl cells with PKH26 and performed a FACS analysis after 10 days. As shown in Fig. 4.9.A, knockdown of HMGA1 expression led to a drastic increase in $PKH26^{bright}$ cells (1.5 % in CTSC_ctrl cells versus 4.8 % in CTSC_shA1 cells), suggesting that

the reduction in HMGA1 expression confers properties of quiescence to the stem cell compartment. Interestingly, very similar results were obtained when HMGA1-knockdown brain tumor stem cells (BTSCs) were stained with PKH26 (Colamaio et al., manuscript in preparation).

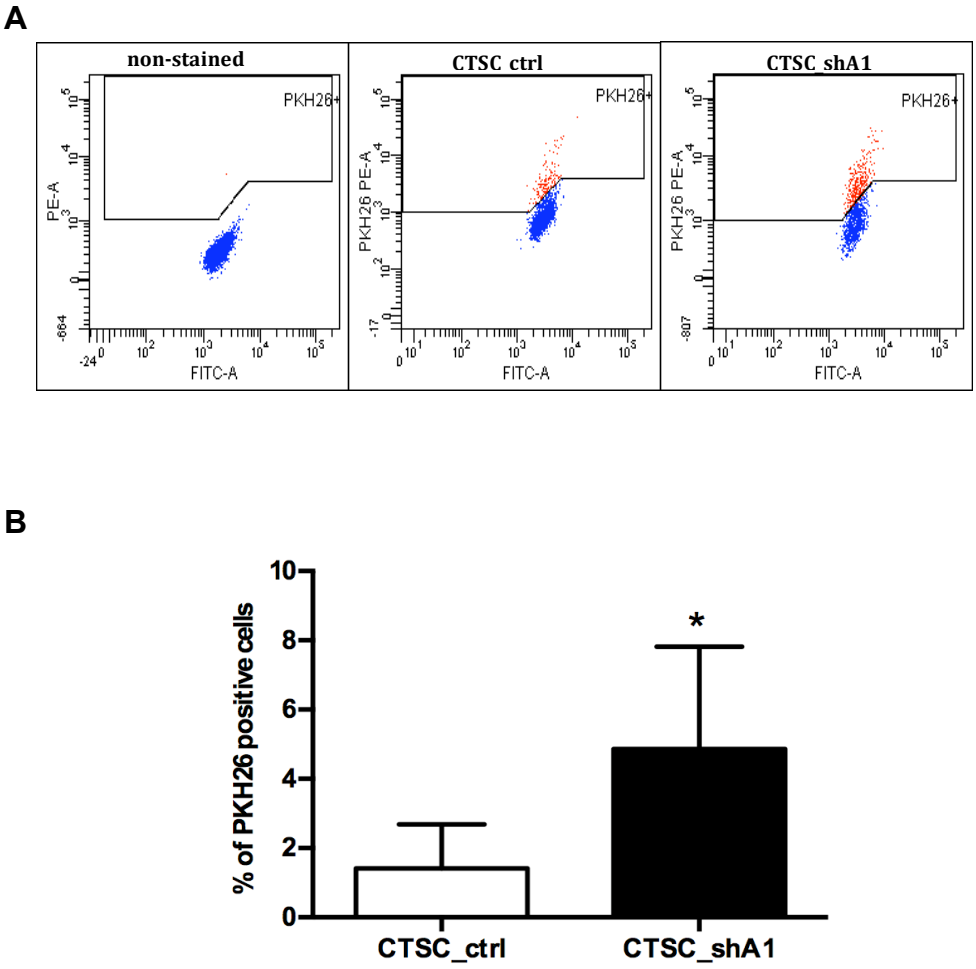


Figure 4.9. HMGA1 knockdown induces stem cell quiescence.
A) FACS plots of double-coloured (PKH26-phycoerythrin [PE] and fluorescein isothiocyanate [FITC-A]) CTSC_ctrl and CTSC_shA1 cells (one representative experiment). The left-most panel shows cells non-stained with PKH26, gated on physical parameters (forward scatter [FSC] and side scatter [SSC]) to exclude most of the debris and dead cells.
B) Mean percentage of PKH26^{bright} cells in CTSC_ctrl and CTSC_shA1 populations, after 10 days from staining. Each bar represents the mean ± SD of 5 independent experiments (*, $p < 0,05$; Mann-Whitney U-test).

4.5. HMGA1 regulates p53 expression at the transcriptional level

Because it has been previously shown that an imbalance between asymmetric and symmetric division can be determined by loss of the p53 tumor suppressor (Cicalese, Bonizzi et al. 2009), we analysed p53 expression in CTSC_shA1 cells. Western blots performed on untransfected, scramble-transfected, and HMGA1–knockdown CTSCs demonstrated that the downregulation of HMGA1 increased p53 protein expression and was associated with an increase in the p53-regulated p21 protein (Fig. 4.10), indicating the presence of a functional p53.

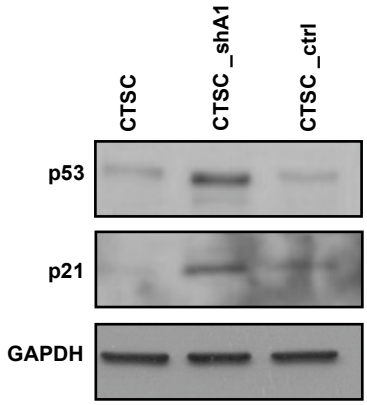


Figure 4.10. p53 expression in CTSCs interfered for HMGA1. Western blot analysis for p53 and p21 expression in non-transfected, HMGA1-knockdown, and scramble-transfected cells. GAPDH was used as a loading control.

Then, to verify whether the HMGA1 knock down affects the p53 expression also at the transcriptional level, we analyzed the p53 mRNA level by qRT-PCR in CTSCs_shA1 compared to CTSCs_ctrl. We found that the silencing of HMGA1 induces an increased expression of p53 also at the mRNA level.

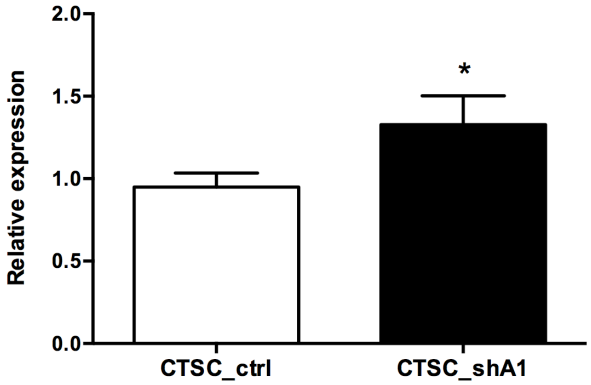


Figure 4.11. p53 mRNA level. qRT-PCR for p53 gene expression in CTSC_ctrl and CTCS_shA1 cells. The expression level of each gene was normalized to the G6PD gene expression (* $p < 0.05$, Wilcoxon signed-rank test).

Subsequently, to assess the direct action of HMGA1 on p53 transcription, we evaluated HMGA1 protein binding to the p53 promoter *in vivo* by performing ChIP assays. Therefore, CTSC_ctrl and CTSC_shA1 cells were cross-linked and immunoprecipitated with anti-HMGA1 or anti-IgG antibodies. Immunoprecipitation of chromatin was then analysed by quantitative PCR, using primers spanning 5 different regions of the p53 promoter (see the Materials and Methods section). Occupancy of the p53 promoter regions II and III by HMGA1 was clearly detectable, whereas no amplification was observed in regions I, IV, and V, indicating the specificity of the binding to regions II and III. Conversely, CTSC_shA1 cells exhibited a negligible enrichment for regions II and III and no enrichment at all for the remaining regions (Fig. 4.12). These results indicate that HMGA1 protein binds the p53 promoter region *in vivo*.

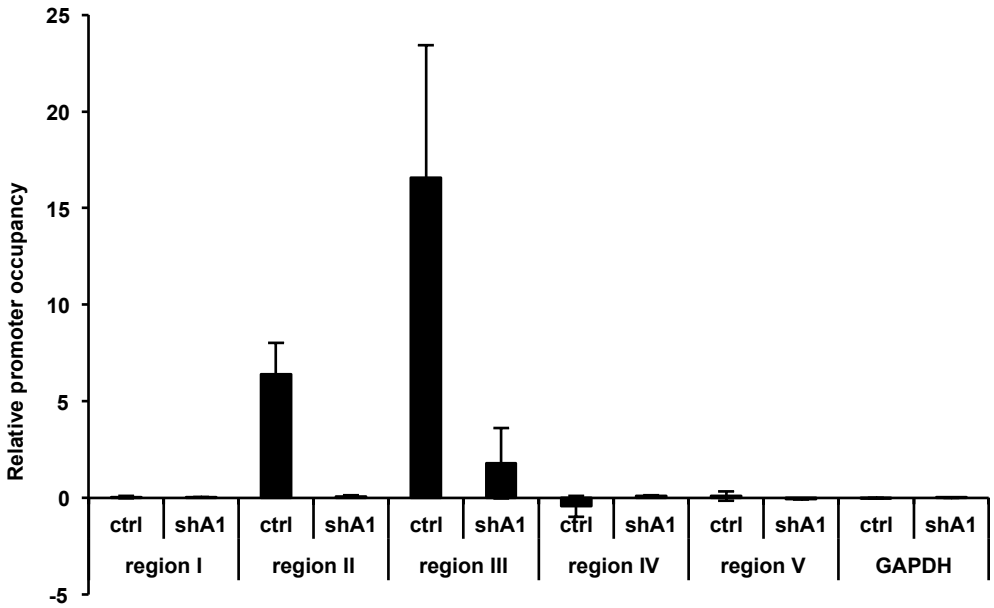


Figure 4.12. HMGA1 direct binding of the p53 promoter. ChIP assay, revealed by qPCR, detecting the *in vivo* binding of HMGA1 to the 5 sub-regions in the p53 promoter in CTSC_ctrl and CTSC_shA1 extracts. The relative occupancy of the p53 promoter regions by HMGA1 is indicated as vertical bars. GAPDH promoter amplicon was used as a negative control.

To define the functional importance of HMGA1 binding to the p53 promoter, we evaluated the activity of the p53 promoter in the presence or absence of HMGA1 by performing luciferase assays. Therefore, HEK293 cells were transfected with a reporter gene construct carrying the human p53 promoter (Durland-Busbice and Reisman 2002) or a control backbone vector (pGL3-basic) in the presence of an HMGA1-expressing construct. As shown in Fig. 4.13, HMGA1 repressed the p53 promoter, and this effect was dose-dependent. Consistently, this repressive effect was abolished by the pharmacological blocking of the HMGA1-DNA interaction with distamycin (not shown), a drug known to displace HMGA1 from its AT-rich DNA regions (Smith and Buchmueller 2011). Therefore, these results clearly demonstrate that HMGA1 transcriptionally regulates p53 expression.

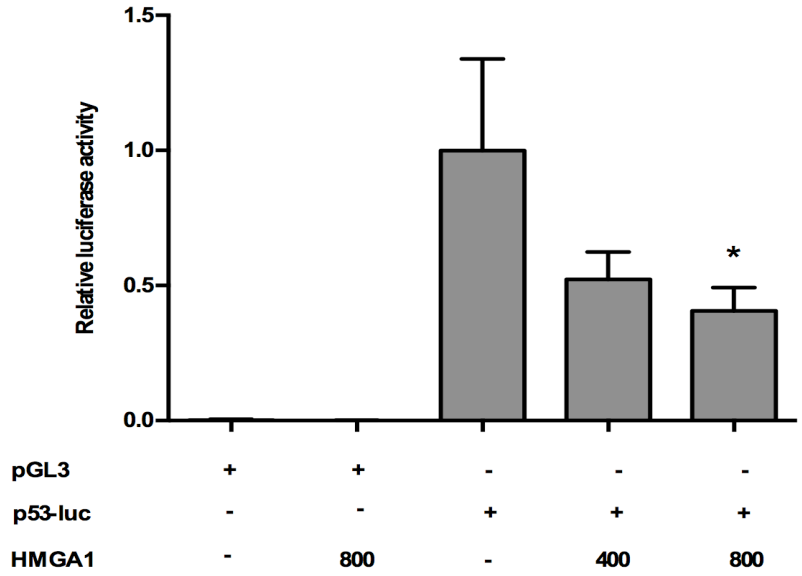


Figure 4.13. HMGA1 directly regulates the p53 promoter. Luciferase activity of the p53 promoter in HEK 293 cells in the presence or absence of an HMGA1-expressing vector. The amounts of the HMGA1-expressing vector are indicated. The data are the results of three independent experiments performed in duplicate. The relative luciferase activity was normalised with Renilla luciferase and was expressed as the fold induction over the activity of the p53 promoter (*, $p < 0.05$). pGL3-basic activity in the presence or absence of the HMGA1-expressing construct was used as a negative control.

4.6. HMGA1 silencing induces asymmetric distribution of NUMB in CSCs

Asymmetric cell division can be demonstrated by the asymmetric partitioning of the cell fate determinant NUMB (Pece, Tosoni et al. 2010). During asymmetric cell division, the part of the cell that inherits high levels of NUMB will undergo differentiation, whereas the part that inherits low levels will produce a stem cell; conversely, in symmetrically dividing CSCs, NUMB is expressed at lower levels and is uniformly distributed in such a way that both daughter cells will be equal in terms of NUMB expression and function (Morrison and Kimble 2006).
Therefore, we analysed NUMB expression/distribution in HMGA1-knockdown and control cells. Western blot analysis for NUMB revealed higher levels of NUMB in CTSC_shA1 cells with respect to untransfected and CTSC_ctrl cells (Fig. 4.14).

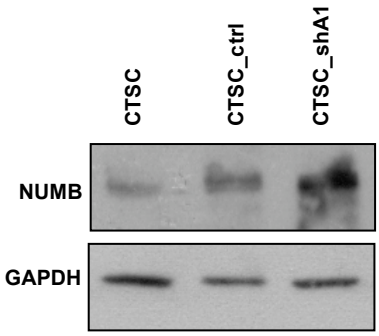


Figure 4.14. NUMB expression in CTSCs interfered for HMGA1. Western blot for NUMB in non-transfected, CTSC_shA1, and CTSC_ctrl cells. GAPDH was used as a loading control.

Moreover, immunofluorescence of the spheres (Fig. 4.15.A) demonstrated that whereas NUMB was distributed almost uniformly in the cell cytoplasm of CTSC_ctrl cells, it formed an asymmetric punctuate crescent close to the cell membrane in CTSC_shA1 cells, where it also undergoes partial nuclear localisation (asterisks). The frequency of NUMB crescents increased from approximately 6% in control cells, to approximately 32% in CTSC_shA1 cells ($p = 0{,}0048$) (Fig. 4.15.B). Together, these data demonstrate that HMGA1-silencing restores normal stem cell properties in CSCs, such as quiescence and asymmetric division.

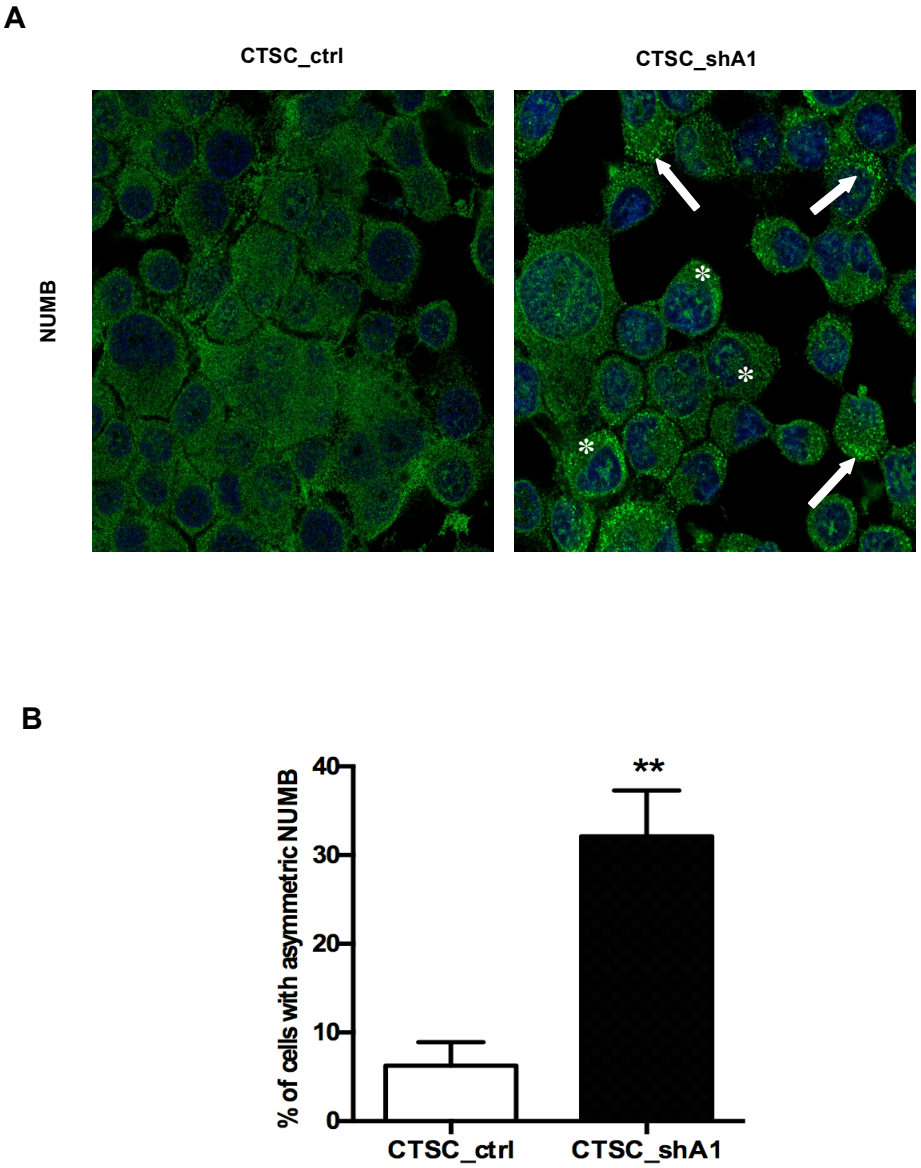


Figure 4.15. NUMB detection by immunofluorescence. A. Immunofluorescence for NUMB in CTSC_ctrl (left) and CTSC_shA1 (right) CTSCs. The nuclei are stained in blue with DAPI. Arrows denote the crescent-shaped NUMB distribution whereas asterisks indicate nuclear-localised NUMB. **B.** The histogram shows the percentage of cells with crescent-shaped, asymmetric NUMB distribution, obtained in immunofluorescence analyses. Data are the mean value ± SD of 7 arbitrary fields for each sample. (** , p = 0.0048; Mann-Whitney U-test).

4.7. HMGA1 regulates NUMB expression at the transcriptional level

Since HMGA1 knock-down induces an increased expression of NUMB in CTSCs, we asked whether HMGA1 could have a role in the transcriptional regulation of NUMB as well.
First, we analyzed NUMB expression at mRNA level. qRT-PCR analyses demonstrated an increased expression of NUMB in CTSCs_shA1 compared to CTSCs_ctrl (Fig. 4.16).

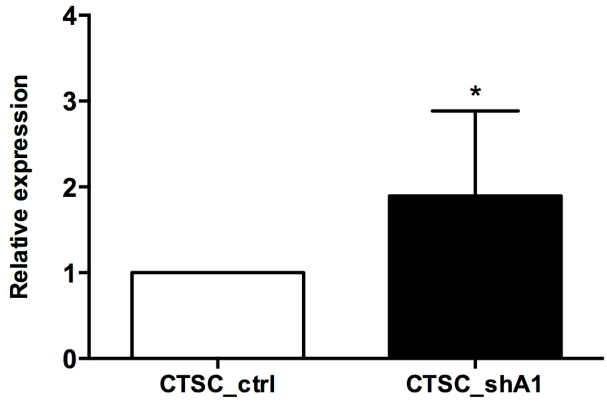


Figure 4.16. NUMB mRNA level. qRT-PCR for NUMB gene expression in CTSC_ctrl and CTCS_shA1 cells. The expression level of each gene was normalized to the G6PD gene expression (* $p < 0.05$, Wilcoxon signed-rank test).

Then, to assess whether HMGA1 directly binds the NUMB promoter, we performed ChIP assays. CTSC_ctrl and CTSC_shA1 cells were cross-linked and immunoprecipitated with anti-HMGA1 or anti-IgG antibodies. In this case, immunoprecipitation of chromatin was analysed by quantitative PCR, using primers spanning 6 different regions of the NUMB promoter (see Materials and Methods).
We detected occupancy of the NUMB promoter regions I and II by HMGA1 (Fig. 4.17), whereas no amplification was observed in the remaining regions, indicating the specificity of the binding. Conversely, CTSC_shA1 cells exhibited a negligible enrichment for regions I and II and no enrichment at all for the remaining regions. These results indicate that HMGA1 protein binds also the NUMB promoter *in vivo*.

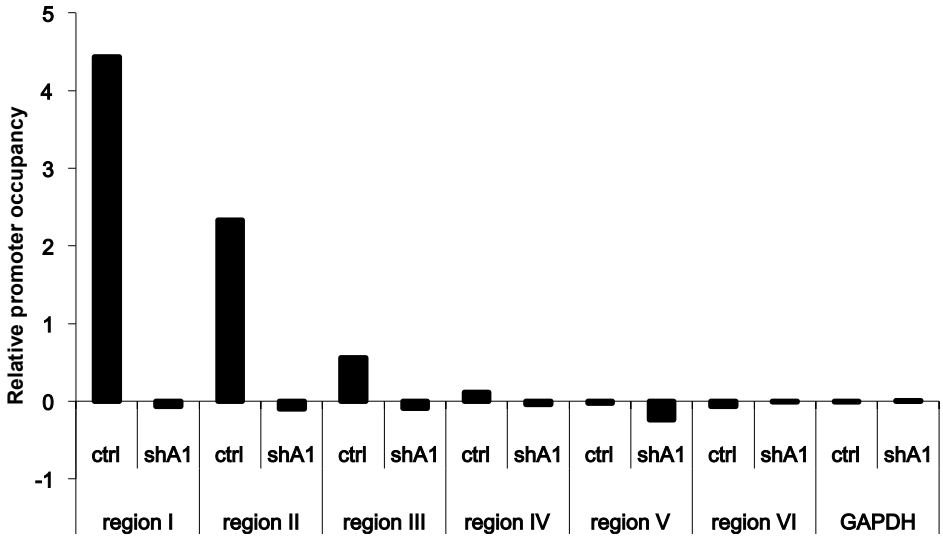


Figure 4.17. HMGA1 direct binding of the NUMB promoter. ChIP assay, revealed by qPCR, detecting the *in vivo* binding of HMGA1 to the 6 sub-regions in the NUMB promoter in CTSC_ctrl and CTSC_shA1 extracts. The relative occupancy of the NUMB promoter regions by HMGA1 is indicated as vertical bars. GAPDH promoter amplicon was used as a negative control.

The functional significance of the binding of HMGA1 to the NUMB promoter was examined by luciferase assays, performed in HEK293 cells transfected with a NUMB promoter-reporter construct, in presence of a vector expressing the HMGA1 cDNA in sense or antisense orientation (see Materials and Methods). Strikingly, we found that the silencing of HMGA1 induced an increase of the luciferase activity, whereas its overexpression reduced the reporter activity (Fig. 4.18). These data clearly indicate that HMGA1 is able to negatively control NUMB expression at transcriptional level.

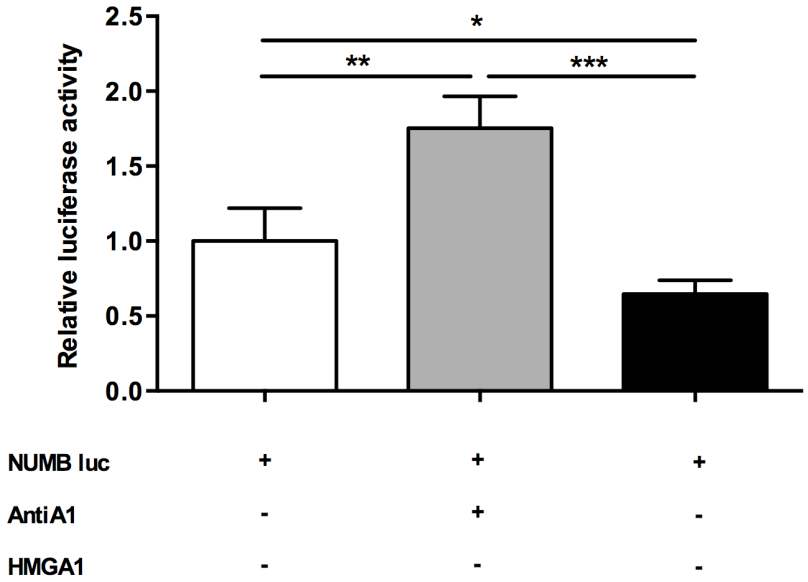


Figure. 4.18. Regulation of NUMB promoter by HMGA1. Luciferase activity of the NUMB promoter (NUMB luc) in HEK 293 cells in presence of the HMGA1-antisense (AntiA1), the HMGA1-expressing (HMGA1) vector or the corresponding empty vectors. NUMB-specific effects are shown as fold induction respect the NUMB promoter activity. Values are average of four independent experiments and error bars denote standard deviation (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Kruskal-Wallis test, Conover post-hoc test).

4.8. HMGA1 modulates NUMB localization in CTSCs

Since, as shown above, immunofluorescence suggested that HMGA1 knock-down induced NUMB nuclear delocalization, we better investigated the different distribution of NUMB in CTSCs silenced for HMGA1 by Western blot analysis of nuclear/cytoplasmic extracts.
We found that there is no variation of NUMB expression levels in the cytoplasmic extracts in CTSC_shA1 compared to CTSC_ctrl; conversely NUMB was found increased in the nuclei of HMGA1 knock-down cells (Fig. 4.19).
These preliminary data suggest that, besides its effect on the transcriptional regulation, HMGA1 could be implicated also in the regulation of NUMB localization. Further experiments are required to confirm these data and to better characterize the mechanism by which HMGA1 could achieve this function.

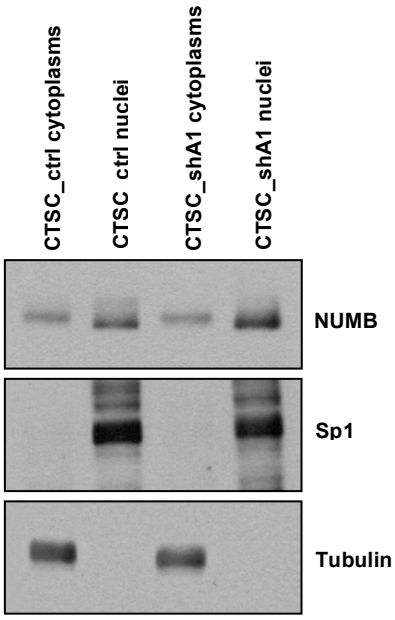


Figure 4.19. NUMB relocalization in CTSCs after HMGA1 silencing. Western blot analysis for NUMB expression in fractionated protein extract from CTSCs_ctrl and CTSC_shA1. 8 μg of cytoplasmic and 20 μg of nuclear extracts were loaded. Sp1 was used as a loading control for nuclear protein extracts. Tubulin was used as a loading control for cytoplasmic extracts.

4.9. HMGA1 knock-down enhances chemosensitivity to Gefitinib in CTSCs

Several studies have found a correlation between HMGA1 and chemoresistence. This is due, at least in part, to its involvement in anti-apoptotic pathways. Since CSCs, because of their resistance to conventional therapies, are identified as responsible of tumor relapse after chemoterapy, we decided to investigate whether HMGA1 could be related to this CSCs feature. To this aim, we treated CTSCs with Gefitinib, used in colon cancer treatment. We evaluated the cell proliferation after treatment with Gefitinib concentrations ranging from 5 to 15 μM. Interestingly, we found that CTSCs_ctrl showed resistance to 5 μM and 10 μM and sensitivity to drug starting from 15 μM; conversely, in CTSCs_shA1 we observed a cytotoxic effect starting from 10 μM and increasing at 15 μM (Fig. 4.20). These data suggest that HMGA1 contributes to determine the resistance of CTSCs to a drug conventionally used in clinical practice.

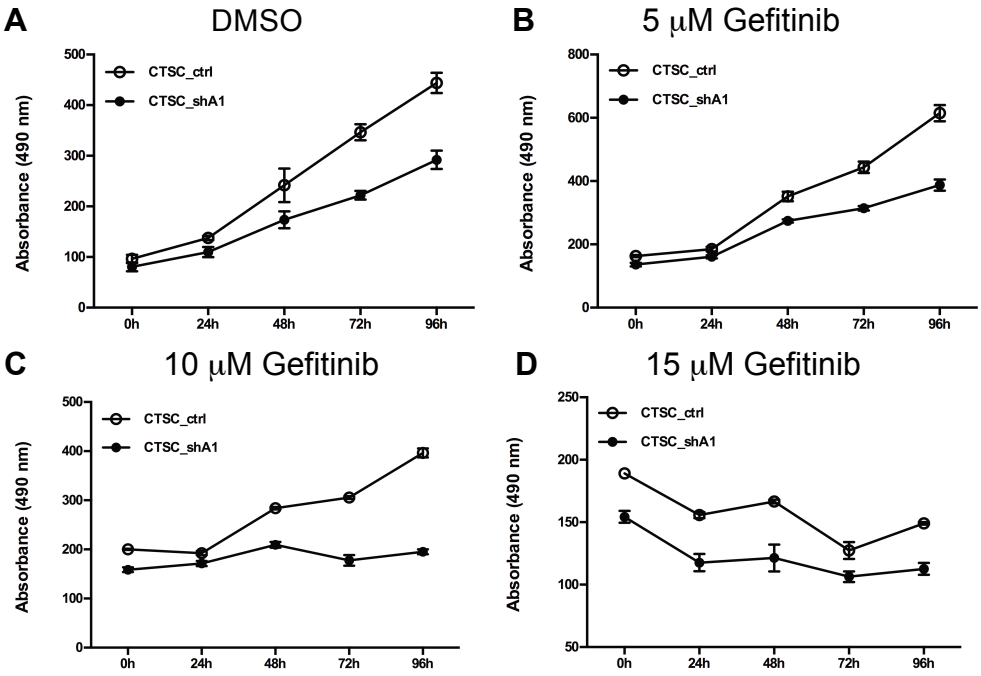


Figure 4.20. HMGA1 interference on chemoresistance.
Growth curve of scramble (CTSC_ctrl) and HMGA1-knockdown (CTSC_shA1) CTSCs treated with Gefitinib at different concentrations. **A.** CTSCs treated with DMSO, as control. **B.** Treatment with 5 μM **C.** 10 μM **D.** 15 μM. Data are the mean value ± SD of one representative experiment, performed in quadruplicate.

5. DISCUSSION

CSCs are a distinct subset of cancer cells with the ability to self-renew through symmetric division and to generate a repertoire of various cell types, thus initiating and perpetuating tumor growth. CSCs are often endowed with distinctive surface markers, such as CD133, which, in colon cancer and in gliomas and other cancers, decorates tumor-initiating cells versus cells that are unable to initiate tumors in transplantation experiments (O'Brien et al., 2007). In human cancers, CSCs have been demonstrated to be a major cause of cancer treatment resistance, invasion, metastasis, and relapse. Thus, eliminating cancer cells with stem cell properties is of prime importance for the successful treatment of cancer, regardless of the tissue of origin (O'Brien et al. 2007).
Recent studies have suggested a unique role for the HMGA1 protein as a master regulator in both cancer stem cells and normal embryonic stem cells. Indeed, it has been recently shown that HMGA1 reprograms somatic cells into pluripotent stem cells, by inducing stem cell transcriptional networks (Shah et al. 2012). Moreover, it has been directly or indirectly demonstrated that HMGA1 proteins are tightly associated with stemness (Belton et al. 2012), and have a critical role in EMT transition (Pegoraro et al. 2013).
Here, we present a new work unveiling a central role for HMGA1 in CSCs self-renewal.
First of all, we demonstrate that HMGA1 is enriched in colon CSCs compared to tumors and cancer cell lines; moreover, in CSCs, it is more abundantly expressed in $CD133^+$ cells than in $CD133^-$ cells, strengthening its association with tumor initiating cells.
We show that the knockdown of the HMGA1 protein in colon CSCs (CTSCs) induces a drastic reduction in proliferation, a slight increase in G1 phase and a more evident increase in apoptosis, consistently with our previous findings in different cell systems (Baldassarre et al. 2001). Moreover, CTSC_shA1 regain two properties typical of normal adult stem cells: reduced SFE, suggestive of a reduction in symmetric self-renewing divisions (Deleyrolle et al. 2011), and increased percentage (and brightness) of PKH26 positive cells, which is suggestive of increased quiescence. Quiescence is one of the most important characteristics distinguishing normal SCs from CSCs.
Normal stem cells are characterized by a balance between symmetric and asymmetric divisions. In a typical outcome of an asymmetric division, the stem cell generates a copy of itself, which retains self-renewing ability and differentiation potential, and one daughter cell that enters the path of differentiation. Thus, by balancing self-renewal with differentiation, asymmetric division maintains the stem and progenitor cell pool while allowing the generation of diverse functional cells (Gomez-Lopez et al. 2014), so that the number of stem cells in the stem cell niche do not change overtime; in *in vitro* cultures this property results in a progressive decrease of their efficiency to form spheres, in serial passages. Conversely, in cancer stem cells the balance between symmetric and asymmetric divisions is completely

compromised. CSCs mainly divide symmetrically and, due to their proliferation potential, the number of stem cells increases over generations. Genetic cancerogenic mutations affect molecular pathways that are important regulators of asymmetric cell division. A genome-wide screen for micro-deletions across a range of primary and cultured human tumor cells identified the deletion of polarity regulators in numerous human epithelial tumor cells. Such deletions include PARD6 (PAR6) in lung cancer, PARD3 (PAR3) in lung, head and neck, esophagus, prostate and bladder cancers, and DLG2 in lung and cervical cancers (Kunnev et al. 2009). Disruption of asymmetric cell divisions leads to aberrant self-renewal and impairs differentiation, and could therefore constitute an early step in the neoplastic transformation of stem and progenitor cells.

Our data, and data from other laboratories, suggest a pivotal role of HMGA1 in regulating self renewal and the symmetric/asymmetric division ratio in CTSCs. Indeed, CTSCs, isolated from patients and stabilized in suspension cultures, behave exactly as expected for cancer stem cells, since their efficiency in forming spheres in culture increases over generations. The reduction in the SFE and proliferation found in HMGA1 knock-down cells makes CTSCs more similar to normal stem cells and demonstrate the importance of detecting HMGA1 in both diagnosis and choice of the therapeutic strategy, since higher levels of HMGA1 in tumors could imply the presence of more CSCs, possibly endowed with higher proliferation ability and aggressiveness. Notably, we have achieved comparable results, in terms of SFE reduction and PKH26 increase, in HMGA1-interfered BTSCs, suggesting a critical role of the HMGA1 protein in the regulation of CSCs of different tissue origin.

From our data, it can be postulated that HMGA1 overexpression levels in normal stem cells might induce symmetric division and open the way to the transformation process. As a corollary, one would speculate that the lack of HMGA1 would protect from cancer initiation, due to the reduction in the cell population responsible of tumor initiation. Actually, even if a reduction in the stem cell compartment has not been thoroughly investigated, it has previously shown in our lab that Hmga1 null mice are less susceptible to chemically induced skin carcinogenesis (Visone et al. 2008), possibly due to a lower number of stem cells/potentially tumor initiating cells.
Recently, it has been demonstrated that ErbB2 transgenic mice harbor CSCs with increased frequency of self-renewing divisions, higher replicative potential and preponderance of symmetric versus asymmetric division, highlighted by the symmetric distribution of the cell fate determinant NUMB, compared to stem cells from wild-type mice (Cicalese et al. 2009). In these cells, Nutlin 3-mediated stabilization of p53 was able to restore asymmetric division, also evidenced by the recovering of the asymmetric distribution of NUMB (Cicalese et al. 2009). The similarity of the CTSC_shA1 phenotype with those obtained by treating CSCs with Nutlin-3 and the tight correlation

between HMGA1 and ErbB2 expression (Chiappetta et al. 2004), prompted us to investigate the effect of HMGA1 interference on the HMGA1-p53 axis. Strikingly, we found that CTSC_shA1 cells have increased p53 (and p21cip) expression levels, compared to control cells. Further, we found that the HMGA1 knock-down induces also an increase of the p53 mRNA level. Luciferase assays and chromatin immunoprecipitation analysis revealed that HMGA1 exerts its regulation on p53 expression at transcriptional level, by binding to its promoter region. Therefore, the restoration of p53 functions induced by HMGA1 knockdown likely accounts for the effects of the HMGA1 silencing on CSC cells. Accordingly, in p53-null BTSCs, HMGA1 interference does not affect sphere formation ability, even though it has dramatic consequences on CSCs migration and differentiation (Colamaio et al., manuscript in preparation).
On the other hand, the stabilization of p53 in CTSCs by treating cells with Nutlin-3 also affected their self-renewal ability, even though with lower efficacy than HMGA1 knock-down. Indeed, the SFE of Nutlin-treated CTSCs remains constant overtime, whereas it decreases in HMGA1 knock-down CTSCs, probably due to the additional effects on apoptosis (Cicalese et al. 2009).
Even though we demonstrate, for the first time, a direct role of HMGA1 on p53 activity in CSCs, the inhibitory activity of HMGA1 on p53 at different levels and in different cell systems, is not new: HMGA1 has been shown to interfere with p53-mediated transcription of p53 effectors Bax and p21(waf1) and to transcriptionally activate the p53 inhibitor mdm2; at a second level of regulation, HMGA1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2. Therefore, the data presented here, add a new level of regulation on the already described HMGA1-dependent p53 control. rev in (Fedele and Fusco 2010).
All these findings concerning the regulation of the asymmetric cell division exerted by HMGA1 and the tight correlation with p53 prompted us to further investigate the importance of the HMGA1 knock-down on other molecular regulators of polarity and cell fate.

Mounting evidence points to a role for the cell-fate determinant NUMB as a tumor suppressor in distinct types of human cancers and recently this role has been correlated with its function in the regulation of the asymmetric cell division. In breast cancers, NUMB protein levels are frequently reduced or lost and inversely correlate with tumor grade and patient prognosis. In primary breast tumor cell cultures, those with low or no NUMB expression, referred to as class 1, not only display increased NOTCH activity but also a reduction of p53 protein levels. Forced-expression of NUMB in class 1 cells significantly reduces NOTCH activity and cell proliferation and restores normal p53 levels (Gomez-Lopez et al. 2014).

Cicalese et al. demonstrated that in primary ErbB2-driven mouse mammary carcinoma cells, p53 is unstable, and attenuated p53 levels switch the mode of

cell division from asymmetric to symmetric, leading to geometric cell expansion (Cicalese et al. 2009). Although it is not clear whether in this model the levels of NUMB protein are also affected, these observations suggest that downregulation of NUMB-p53 in human breast cancers may cause a bias towards symmetric divisions.

Interestingly, it has been shown an interaction *in vivo* between overexpressed NUMB and HDM2; the latter, as already mentioned, is transcriptionally regulated by HMGA1 (Fedele and Fusco 2010). Colaluca *et al*. demonstrated that NUMB physically interacts with and stabilizes p53, by preventing its ubiquitination and degradation induced by the E3 ubiquitin ligase HDM2. Indeed, NUMB co-immunoprecipitates with p53 and these data led to postulate the existence *in vivo* of a tricomplex NUMB–HDM2–p53. *In vitro* binding assays confirmed the direct interaction between the three proteins. The presence of Nutlin-3, which inhibits the HDM2–p53 interaction, prevented the formation of the HDM2–p53 complex, but not of the HDM2–NUMB or p53–NUMB complexes. Thus, the NUMB–p53 and NUMB–HDM2 surfaces of interaction are distinct, at least in part, from that of the HDM2–p53 interaction, confirming the formation of the tricomplex *in vivo*. (Colaluca et al. 2008)

Reduced NUMB levels are also observed during the progression of human chronic myeloid leukemia (CML) to blast crisis and this is associated with increased NOTCH signaling and reduced p53 activity (Ito et al. 2010).
Consistently with all these findings, here we report an increased expression of NUMB in the CTSC after the HMGA1 knock-down. We confirmed a role for HMGA1 in regulating NUMB at transcriptional level by revealing both a physical interaction with the NUMB promoter by ChIP assay, and a functional regulation by luciferase assays. Moreover, in CTSC_shA1, we observed a different distribution of NUMB. Immunofluorescence of the spheres demonstrated that whereas NUMB was distributed almost uniformly in the cell cytoplasm of CTSC_ctrl cells, it formed an asymmetric punctuate crescent close to the cell membrane in CTSC_shA1 cells. The frequency of NUMB crescents increased from approximately 6% in control cells, to approximately 32% in CTSC_shA1 cells, indicating an unexpected effect of HMGA1 knock-down on NUMB localization. Further, we observed that NUMB undergoes partial nuclear localization in presence of HMGA1 knock-down. This was confirmed by Western blot analysis of nuclear/cytoplasmic extracts.
Therefore, altogether, these data suggest the existence of an HMGA1-NUMB-p53 axis. Given, as previously discussed, the significant role of NUMB and p53 in the regulation of the asymmetric cell division and in the protection from CSCs formation, one could hypotesize that HMGA1 participates in these processes by increasing the CSCs self-renewal, either by directly acting on p53 and NUMB or by enhancing their interaction in the nucleus through NUMB relocalization.
The interaction between p53 and NUMB has been found implicated in chemoresistance. Indeed, NUMB overexpression increases p53 stability and

activity, and in MCF10A it has been found to correlate with an approximately three-fold higher level of activated caspase-3 in response to cisplatin-induced DNA damage (Colaluca et al. 2008). Deficient p53 activity is associated with resistance to the cytotoxic effects of chemotherapy. Thus, also NUMB showed an inverse correlation with chemoresistance in breast tumors. Further, re-expression of NUMB in class 1 cells restored responsiveness to cisplatin to levels comparable to those of control class 3 cells. Accordingly, NUMB-silencing in class 3 cells increased resistance to the drug to levels comparable to those of control class 1 cells (Colaluca et al. 2008). HMGA1 has been demonstrated to enhance chemoresistance in tumors by promoting ATM expression, or by regulating the Akt phosphorylation, leading to an increased resistance to apoptosis (Liau and Whang 2008; Palmieri et al. 2011). In agreement with these findings, we evaluated the cell proliferation after treatment with Gefitinib, a selective inhibitor of the tyrosine kinase receptor of epidermal growth factor (EGFR). We observed that HMGA1 knock-down sensitizes CTSCs to the drug; indeed, we achieved a cytotoxic effect at lower doses in CTSC_shA1 compared to CTSCs_ctrl. In addition, our preliminary data showed that CTSC_ctrl cells decreased for few days in their number, after treatment with cytotoxic doses of Gefitinib, but they regained normal proliferation after one week from the treatment. Conversely, in CTSC_shA1 we observed a long-lasting cytotoxic effect. A possible explanation for this phenomenon is that, thanks to their self-renewal ability, resistant CSCs rapidly replace those that were destroyed by Gefitinib; this behaviour is at the basis af tumor recurrence. HMGA1 knock-down, instead, sensitizes CSCs to Gefitinib and restrains the CSCs self-renewal capacity, making the treatment more effective. These findings are of great importance, since targeting HMGA1 could be a potent strategy in clinical practice to achieve cancer eradication at lower drug doses.

Many questions await answers. It remains to be clarified how HMGA1 modulates the asymmetric relocalization of NUMB. During mitosis, the Baz/Par6/aPKC complex directs the distribution of the cell-fate determinant and the aPKC phosphorylation is a foundamental step to release NUMB from the apical cortex and to relocalize it (Gomez-Lopez et al. 2014). One can postulate an involvement of HMGA1 in the regulation of the Baz/Par6/aPKC complex; for instance, it might be interesting to investigate whether HMGA1 overexpression can inhibit aPKC phosphorylation, blocking the NUMB relocalization. Another open question to be solved is whether HMGA1 is able to directly interact with NUMB, and to enter in the three-complex NUMB-HDM2-p53. HMGA1, HDM2 and p53 are nuclear proteins and it has been demonstrated the direct interaction with p53 and its regulatory activity on HDM2. NUMB is in the cytoplasm, mostly associated to biomembranes. Since the presence of HMGA1 regulates the NUMB relocalization in the nucleus, one could hypotesize an indirect effect of HMGA1 on its localization by regulating other effectors that promote the NUMB relocalization. For instance, it has been found that mdm2, which is regulated by HMGA1, can associate

with NUMB promoting its relocalization in the nucleus (Juven-Gershon et al. 1998). Further, it is known that post-translational modifications have great importance for NUMB activity. Recently, the lysine methyltransferase Set8 has been found to inhibit the NUMB-p53 interaction and NUMB-mediated apoptosis by methylating NUMB. Doxorubicin promotes apoptosis and reduces the mRNA and protein levels of Set8. Thus, targeting Set8 or a NUMB demethylase may prove an effective strategy for cancer treatment (Dhami et al. 2013). Our preliminary data showed that in BTSCs the HMGA1 silencing causes decreased mRNA levels of Set8, so we can postulate another molecular mechanism by which HMGA1 could regulate NUMB activity and localization by modulating its post-translational modifications.

In conclusion, on the basis of the results shown here, indicating the cancer specific expression of HMGA1 and its peculiar role in CSCs, HMGA1 targeting may represent a promising CSC-specific anti-cancer therapy.

6. CONCLUSIONS

Several studies provide evidence that HMGA1 has an important role in the neoplastic transformation and it is causally involved in carcinogenesis. In recent years HMGA1 has also emerged as stemness factor, since it is able to regulate the self-renewal of ESCs; further its expression in adult tissues contributes to the maintainance of a "stem-like" state.
The aim of our study has been to clarify the importance of HMGA1 in CSCs, which are the real tumor initiating cells, emerging as election targets for cancer eradication. We focused on colon tumor stem cells (CTSC) as a model for our study and we demonstrated that:

- HMGA1 expression was higher in CTSCs compared to cancer cell lines and samples of tumoral and normal mucosa; the correlation with stemness is confirmed by the finding that, in $CD133^+$ CTSCs, HMGA1 expression was higher than in $CD133^-$ CTSCs.

- HMGA1 has a pivotal role in CTSCs self-renewal and its silencing increases stem cell quiescence.

- The silencing of HMGA1 decreases the sphere forming efficiency (SFE) and leads to an asymmetric distribution of NUMB, indicating that HMGA1 is able to influence the choice between symmetric and asymmetric divisions.

- HMGA1 is able to transcriptionally repress NUMB and p53, which are known to regulate the asymmetric cell division.

- All together, these data suggest that HMGA1 regulates the division pattern of CSCs by modulating the expression and/or the localization of polarity and cell fate determinants.

7. ACKNOWLEDGMENTS

At the end of my PhD years, I would like to thank all the people that accompanied me, someone professionally and someothers becoming part of my life.

I thank prof. Alfredo Fusco for giving me the possibility to join his lab at the beginning of these three years and to professionally grow up.

I thank Prof. Massimo Santoro, coordinator of the Molecular Oncology and Endocrinology Doctorate program, for the opportunity to work at the Department of Molecular Medicine and Medical Biotechnology at the University of Naples "Federico II".

I warmly thank Dr. Sabrina Battista for her guidance and her advices as my supervisor during all my PhD.

I would like to thank all my labmates, colleagues and friends.
Thanks to Nadia for technical support and special thanks to Andrè for his statistical contribution and friendship.

Thanks to my family and to all friends, the old ones and the ones, who I met during these three years: everyone has taught me something.

A very special thanks to Giuseppe, for always believing in me.

Finally, I would like to thank all those who, even in small things, gave me the strenght to go on during this journey.

BIBLIOGRAFY

Abe, N., T. Watanabe, et al. (2002). "Diagnostic significance of high mobility group I(Y) protein expression in intraductal papillary mucinous tumors of the pancreas." Pancreas **25**(2): 198-204.

Abe, N., T. Watanabe, et al. (2000). "Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins." Cancer Res **60**(12): 3117-3122.

Adair, J. E., Y. Kwon, et al. (2005). "Inhibition of nucleotide excision repair by high mobility group protein HMGA1." J Biol Chem **280**(37): 32184-32192.

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-3988.

Aloni-Grinstein, R., Y. Shetzer, et al. (2014). "p53: The barrier to cancer stem cell formation." FEBS Lett.

Anand, A. and K. Chada (2000). "In vivo modulation of Hmgic reduces obesity." Nat Genet **24**(4): 377-380.

Angel, P. and M. Karin (1991). "The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation." Biochim Biophys Acta **1072**(2-3): 129-157.

Bagga, R., S. Michalowski, et al. (2000). "HMG I/Y regulates long-range enhancer-dependent transcription on DNA and chromatin by changes in DNA topology." Nucleic Acids Res **28**(13): 2541-2550.

Baldassarre, G., S. Battista, et al. (2003). "Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma." Mol Cell Biol **23**(7): 2225-2238.

Baldassarre, G., B. Belletti, et al. (2005). "HMGA1 protein expression sensitizes cells to cisplatin-induced cell death." Oncogene **24**(45): 6809-6819.

Baldassarre, G., M. Fedele, et al. (2001). "Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway." Proc Natl Acad Sci U S A **98**(14): 7970-7975.

Battista, S., V. Fidanza, et al. (1999). "The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis." Cancer Res **59**(19): 4793-4797.

Battista, S., F. Pentimalli, et al. (2003). "Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation." FASEB J **17**(11): 1496-1498.

Belton, A., A. Gabrovsky, et al. (2012). "HMGA1 induces intestinal polyposis in transgenic mice and drives tumor progression and stem cell properties in colon cancer cells." PLoS One **7**(1): e30034.
Ben-Porath, I., M. W. Thomson, et al. (2008). "An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors." Nat Genet **40**(5): 499-507.
Berlingieri, M. T., G. Manfioletti, et al. (1995). "Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells." Mol Cell Biol **15**(3): 1545-1553.
Berlingieri, M. T., G. M. Pierantoni, et al. (2002). "Thyroid cell transformation requires the expression of the HMGA1 proteins." Oncogene **21**(19): 2971-2980.
Berner, J. M., L. A. Meza-Zepeda, et al. (1997). "HMGIC, the gene for an architectural transcription factor, is amplified and rearranged in a subset of human sarcomas." Oncogene **14**(24): 2935-2941.
Blank, U., G. Karlsson, et al. (2008). "Signaling pathways governing stem-cell fate." Blood **111**(2): 492-503.
Bogdan, C. (2001). "Nitric oxide and the immune response." Nat Immunol **2**(10): 907-916.
Boo, L. M., H. H. Lin, et al. (2005). "High mobility group A2 potentiates genotoxic stress in part through the modulation of basal and DNA damage-dependent phosphatidylinositol 3-kinase-related protein kinase activation." Cancer Res **65**(15): 6622-6630.
Borrmann, L., R. Schwanbeck, et al. (2003). "High mobility group A2 protein and its derivatives bind a specific region of the promoter of DNA repair gene ERCC1 and modulate its activity." Nucleic Acids Res **31**(23): 6841-6851.
Bu, P., K. Y. Chen, et al. (2013). "A microRNA miR-34a-regulated bimodal switch targets Notch in colon cancer stem cells." Cell Stem Cell **12**(5): 602-615.
Bultje, R. S., D. R. Castaneda-Castellanos, et al. (2009). "Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex." Neuron **63**(2): 189-202.
Bush, B. M., A. T. Brock, et al. (2013). "The Wnt/beta-catenin/T-cell factor 4 pathway up-regulates high-mobility group A1 expression in colon cancer." Cell Biochem Funct **31**(3): 228-236.
Chiappetta, G., V. Avantaggiato, et al. (1996). "High level expression of the HMGI (Y) gene during embryonic development." Oncogene **13**(11): 2439-2446.
Chiappetta, G., A. Bandiera, et al. (1995). "The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias." Oncogene **10**(7): 1307-1314.

Chiappetta, G., G. Botti, et al. (2004). "HMGA1 protein overexpression in human breast carcinomas: correlation with ErbB2 expression." Clin Cancer Res **10**(22): 7637-7644.
Chiappetta, G., G. Manfioletti, et al. (2001). "High mobility group HMGI(Y) protein expression in human colorectal hyperplastic and neoplastic diseases." Int J Cancer **91**(2): 147-151.
Chiappetta, G., G. Tallini, et al. (1998). "Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma." Cancer Res **58**(18): 4193-4198.
Chiba, S. (2006). "[Normal and deregulated notch signaling in regulation of hematopoietic stem cells and development of leukemia]." Rinsho Ketsueki **47**(5): 371-378.
Chiba, S. (2006). "Notch signaling in stem cell systems." Stem Cells **24**(11): 2437-2447.
Chiefari, E., F. Paonessa, et al. (2009). "The cAMP-HMGA1-RBP4 system: a novel biochemical pathway for modulating glucose homeostasis." BMC Biol **7**: 24.
Chuvpilo, S., C. Schomberg, et al. (1993). "Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter." Nucleic Acids Res **21**(24): 5694-5704.
Cicalese, A., G. Bonizzi, et al. (2009). "The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells." Cell **138**(6): 1083-1095.
Cleynen, I. and W. J. Van de Ven (2008). "The HMGA proteins: a myriad of functions (Review)." Int J Oncol **32**(2): 289-305.
Colaluca, I. N., D. Tosoni, et al. (2008). "NUMB controls p53 tumor suppressor activity." Nature **451**(7174): 76-80.
Collins, A. T., P. A. Berry, et al. (2005). "Prospective identification of tumorigenic prostate cancer stem cells." Cancer Res **65**(23): 10946-10951.
Dal Cin, P., S. Wanschura, et al. (1998). "Amplification and expression of the HMGIC gene in a benign endometrial polyp." Genes Chromosomes Cancer **22**(2): 95-99.
Deleyrolle, L. P., G. Ericksson, et al. (2011). "Determination of somatic and cancer stem cell self-renewing symmetric division rate using sphere assays." PLoS One **6**(1): e15844.
Dhami, G. K., H. Liu, et al. (2013). "Dynamic methylation of Numb by Set8 regulates its binding to p53 and apoptosis." Mol Cell **50**(4): 565-576.
Dho, S. E., J. Trejo, et al. (2006). "Dynamic regulation of mammalian numb by G protein-coupled receptors and protein kinase C activation: Structural determinants of numb association with the cortical membrane." Mol Biol Cell **17**(9): 4142-4155.

Doe, C. Q. and B. Bowerman (2001). "Asymmetric cell division: fly neuroblast meets worm zygote." Curr Opin Cell Biol **13**(1): 68-75.
Donato, G., J. Martinez Hoyos, et al. (2004). "High mobility group A1 expression correlates with the histological grade of human glial tumors." Oncol Rep **11**(6): 1209-1213.
Dontu, G., D. El-Ashry, et al. (2004). "Breast cancer, stem/progenitor cells and the estrogen receptor." Trends Endocrinol Metab **15**(5): 193-197.
Durland-Busbice, S. and D. Reisman (2002). "Lack of p53 expression in human myeloid leukemias is not due to mutations in transcriptional regulatory regions of the gene." Leukemia **16**(10): 2165-2167.
Fedele, M., A. Bandiera, et al. (1996). "Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins." Cancer Res **56**(8): 1896-1901.
Fedele, M., S. Battista, et al. (2002). "Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas." Oncogene **21**(20): 3190-3198.
Fedele, M., V. Fidanza, et al. (2006). "Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice." Cancer Res **66**(5): 2536-2543.
Fedele, M. and A. Fusco (2010). "HMGA and cancer." Biochim Biophys Acta **1799**(1-2): 48-54.
Fedele, M. and A. Fusco (2010). "Role of the high mobility group A proteins in the regulation of pituitary cell cycle." J Mol Endocrinol **44**(6): 309-318.
Fedele, M. and A. Fusco (2010). "Role of the high mobility group A proteins in the regulation of pituitary cell cycle." J Mol Endocrinol **44**(6): 309-318.
Fedele, M., F. Pentimalli, et al. (2005). "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 **24**(21): 3427-3435.
Fedele, M., G. M. Pierantoni, et al. (2001). "Overexpression of proteins HMGA1 induces cell cycle deregulation and apoptosis in normal rat thyroid cells." Cancer Res **61**(11): 4583-4590.
Fedele, M., G. M. Pierantoni, et al. (2006). "E2F1 activation is responsible for pituitary adenomas induced by HMGA2 gene overexpression." Cell Div **1**: 17.
Fedele, M., R. Visone, et al. (2006). "HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity." Cancer Cell **9**(6): 459-471.
Federico, A., P. Pallante, et al. (2009). "Chromobox protein homologue 7 protein, with decreased expression in human carcinomas,

positively regulates E-cadherin expression by interacting with the histone deacetylase 2 protein." Cancer Res **69**(17): 7079-7087.
Finelli, P., G. M. Pierantoni, et al. (2002). "The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas." Cancer Res **62**(8): 2398-2405.
Flohr, A. M., P. Rogalla, et al. (2003). "High mobility group protein HMGA1 expression in breast cancer reveals a positive correlation with tumor grade." Histol Histopathol **18**(4): 999-1004.
Fong, T. C. and B. M. Emerson (1992). "The erythroid-specific protein cGATA-1 mediates distal enhancer activity through a specialized beta-globin TATA box." Genes Dev **6**(4): 521-532.
Fortini, M. E. and D. Bilder (2009). "Endocytic regulation of Notch signaling." Curr Opin Genet Dev **19**(4): 323-328.
Foti, D., E. Chiefari, et al. (2005). "Lack of the architectural factor HMGA1 causes insulin resistance and diabetes in humans and mice." Nat Med **11**(7): 765-773.
Frasca, F., A. Rustighi, et al. (2006). "HMGA1 inhibits the function of p53 family members in thyroid cancer cells." Cancer Res **66**(6): 2980-2989.
Gaston-Massuet, C., C. L. Andoniadou, et al. (2011). "Increased Wingless (Wnt) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans." Proc Natl Acad Sci U S A **108**(28): 11482-11487.
Geurts, J. M., E. F. Schoenmakers, et al. (1997). "Molecular characterization of a complex chromosomal rearrangement in a pleomorphic salivary gland adenoma involving the 3'-UTR of HMGIC." Cancer Genet Cytogenet **95**(2): 198-205.
Giancotti, V., M. T. Berlingieri, et al. (1985). "Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sarcoma retrovirus." Cancer Res **45**(12 Pt 1): 6051-6057.
Ginestier, C., M. H. Hur, et al. (2007). "ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome." Cell Stem Cell **1**(5): 555-567.
Gomez-Lopez, S., R. G. Lerner, et al. (2014). "Asymmetric cell division of stem and progenitor cells during homeostasis and cancer." Cell Mol Life Sci **71**(4): 575-597.
Goodwin, G. H., C. Sanders, et al. (1973). "A new group of chromatin-associated proteins with a high content of acidic and basic amino acids." Eur J Biochem **38**(1): 14-19.
Hainaut, P. and M. Hollstein (2000). "p53 and human cancer: the first ten thousand mutations." Adv Cancer Res **77**: 81-137.
Han, M. K., E. K. Song, et al. (2008). "SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization." Cell Stem Cell **2**(3): 241-251.

Hebert, C., K. Norris, et al. (2007). "High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma." Mol Cancer **6**: 5.

Hillion, J., S. Dhara, et al. (2008). "The high-mobility group A1a/signal transducer and activator of transcription-3 axis: an achilles heel for hematopoietic malignancies?" Cancer Res **68**(24): 10121-10127.

Himes, S. R., L. S. Coles, et al. (1996). "High mobility group protein I(Y) is required for function and for c-Rel binding to CD28 response elements within the GM-CSF and IL-2 promoters." Immunity **5**(5): 479-489.

Hope, K. J., L. Jin, et al. (2004). "Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity." Nat Immunol **5**(7): 738-743.

Huber, M. A., N. Kraut, et al. (2005). "Molecular requirements for epithelial-mesenchymal transition during tumor progression." Curr Opin Cell Biol **17**(5): 548-558.

Hutterer, A. and J. A. Knoblich (2005). "Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs." EMBO Rep **6**(9): 836-842.

Ito, T., H. Y. Kwon, et al. (2010). "Regulation of myeloid leukaemia by the cell-fate determinant Musashi." Nature **466**(7307): 765-768.

Jain, A. K., K. Allton, et al. (2012). "p53 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells." PLoS Biol **10**(2): e1001268.

Ji, Y. S., Q. Xu, et al. (1998). "Hypoxia induces high-mobility-group protein I(Y) and transcription of the cyclooxygenase-2 gene in human vascular endothelium." Circ Res **83**(3): 295-304.

Johnson, K. R., S. A. Cook, et al. (1993). "Identification and genetic mapping of the murine gene and 20 related sequences encoding chromosomal protein HMG-17." Mamm Genome **4**(2): 83-89.

Juven-Gershon, T., O. Shifman, et al. (1998). "The Mdm2 oncoprotein interacts with the cell fate regulator Numb." Mol Cell Biol **18**(7): 3974-3982.

Kaddar, T., J. P. Rouault, et al. (2009). "Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation." Biol Cell **101**(9): 511-524.

Karin, M., Z. Liu, et al. (1997). "AP-1 function and regulation." Curr Opin Cell Biol **9**(2): 240-246.

Kazmierczak, B., S. Wanschura, et al. (1996). "Ten pulmonary chondroid hamartomas with chromosome 6p21 breakpoints within the HMG-I(Y) gene or its immediate surroundings." J Natl Cancer Inst **88**(17): 1234-1236.

Kim, D. H., Y. S. Park, et al. (1999). "Expression of the HMGI(Y) gene in human colorectal cancer." Int J Cancer **84**(4): 376-380.

Kim, H. P., J. Kelly, et al. (2001). "The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements." Immunity **15**(1): 159-172.

Kim, J., R. Reeves, et al. (1995). "The non-histone chromosomal protein HMG-I(Y) contributes to repression of the immunoglobulin heavy chain germ-line epsilon RNA promoter." Eur J Immunol **25**(3): 798-808.

Kucia, M. and M. Z. Ratajczak (2006). "Stem cells as a two edged sword--from regeneration to tumor formation." J Physiol Pharmacol **57 Suppl 7**: 5-16.

Kunisato, A., S. Chiba, et al. (2003). "HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo." Blood **101**(5): 1777-1783.

Kunnev, D., I. Ivanov, et al. (2009). "Par-3 partitioning defective 3 homolog (C. elegans) and androgen-induced prostate proliferative shutoff associated protein genes are mutationally inactivated in prostate cancer cells." BMC Cancer **9**: 318.

Lapidot, T., C. Sirard, et al. (1994). "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice." Nature **367**(6464): 645-648.

Le Grand, F., A. E. Jones, et al. (2009). "Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells." Cell Stem Cell **4**(6): 535-547.

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

Lee, Y. S. and A. Dutta (2007). "The tumor suppressor microRNA let-7 represses the HMGA2 oncogene." Genes Dev **21**(9): 1025-1030.

Li, C., D. G. Heidt, et al. (2007). "Identification of pancreatic cancer stem cells." Cancer Res **67**(3): 1030-1037.

Li, F., B. Tiede, et al. (2007). "Beyond tumorigenesis: cancer stem cells in metastasis." Cell Res **17**(1): 3-14.

Li, L., K. Yoder, et al. (2000). "Retroviral cDNA integration: stimulation by HMG I family proteins." J Virol **74**(23): 10965-10974.

Liau, S. S., A. Jazag, et al. (2007). "Overexpression of HMGA1 promotes anoikis resistance and constitutive Akt activation in pancreatic adenocarcinoma cells." Br J Cancer **96**(6): 993-1000.

Liau, S. S. and E. Whang (2008). "HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma." Clin Cancer Res **14**(5): 1470-1477.

Liu, S., S. Liu, et al. (2011). "The PI3K-Akt pathway inhibits senescence and promotes self-renewal of human skin-derived precursors in vitro." Aging Cell **10**(4): 661-674.

Lund, T., J. Holtlund, et al. (1983). "On the presence of two new high mobility group-like proteins in HeLa S3 cells." FEBS Lett **152**(2): 163-167.

Maloney, S. C., J. E. Adair, et al. (2007). "Gene-specific nucleotide excision repair is impaired in human cells expressing elevated levels of high mobility group A1 nonhistone proteins." DNA Repair (Amst) **6**(9): 1371-1379.

Mansueto, G., F. Forzati, et al. (2010). "Identification of a New Pathway for Tumor Progression: MicroRNA-181b Up-Regulation and CBX7 Down-Regulation by HMGA1 Protein." Genes Cancer **1**(3): 210-224.

Mantovani, A. (2005). "Cancer: inflammation by remote control." Nature **435**(7043): 752-753.

Mantovani, F., S. Covaceuszach, et al. (1998). "NF-kappaB mediated transcriptional activation is enhanced by the architectural factor HMGI-C." Nucleic Acids Res **26**(6): 1433-1439.

Martinez Hoyos, J., A. Ferraro, et al. (2009). "HAND1 gene expression is negatively regulated by the High Mobility Group A1 proteins and is drastically reduced in human thyroid carcinomas." Oncogene **28**(6): 876-885.

Mayr, C., M. T. Hemann, et al. (2007). "Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation." Science **315**(5818): 1576-1579.

Medeiros, F., M. R. Erickson-Johnson, et al. (2007). "Frequency and characterization of HMGA2 and HMGA1 rearrangements in mesenchymal tumors of the lower genital tract." Genes Chromosomes Cancer **46**(11): 981-990.

Melillo, R. M., G. M. Pierantoni, et al. (2001). "Critical role of the HMGI(Y) proteins in adipocytic cell growth and differentiation." Mol Cell Biol **21**(7): 2485-2495.

Meyer, B., S. Loeschke, et al. (2007). "HMGA2 overexpression in non-small cell lung cancer." Mol Carcinog **46**(7): 503-511.

Mitsui, K., Y. Tokuzawa, et al. (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." Cell **113**(5): 631-642.

Miyazawa, J., A. Mitoro, et al. (2004). "Expression of mesenchyme-specific gene HMGA2 in squamous cell carcinomas of the oral cavity." Cancer Res **64**(6): 2024-2029.

Moeini, A., H. Cornella, et al. (2012). "Emerging Signaling Pathways in Hepatocellular Carcinoma." Liver Cancer **1**(2): 83-93.

Momand, J., D. Jung, et al. (1998). "The MDM2 gene amplification database." Nucleic Acids Res **26**(15): 3453-3459.

Morrison, S. J. and J. Kimble (2006). "Asymmetric and symmetric stem-cell divisions in development and cancer." Nature **441**(7097): 1068-1074.

Muller-Tidow, C., P. Ji, et al. (2004). "The cyclin A1-CDK2 complex regulates DNA double-strand break repair." Mol Cell Biol **24**(20): 8917-8928.

Narita, M., M. Narita, et al. (2006). "A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation." Cell **126**(3): 503-514.

Nishino, J., I. Kim, et al. (2008). "Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression." Cell **135**(2): 227-239.

Nucci, M. R., S. Weremowicz, et al. (2001). "Chromosomal translocation t(8;12) induces aberrant HMGIC expression in aggressive angiomyxoma of the vulva." Genes Chromosomes Cancer **32**(2): 172-176.

O'Brien, C. A., A. Pollett, et al. (2007). "A human colon cancer cell capable of initiating tumor growth in immunodeficient mice." Nature **445**(7123): 106-110.

Ogram, S. A. and R. Reeves (1995). "Differential regulation of a multipromoter gene. Selective 12-O-tetradecanoylphorbol-13-acetate induction of a single transcription start site in the HMG-I/Y gene." J Biol Chem **270**(23): 14235-14242.

Pagano, M., R. Pepperkok, et al. (1992). "Cyclin A is required at two points in the human cell cycle." EMBO J **11**(3): 961-971.

Palmieri, D., D. D'Angelo, et al. (2012). "Downregulation of HMGA-targeting microRNAs has a critical role in human pituitary tumorigenesis." Oncogene **31**(34): 3857-3865.

Palmieri, D., T. Valentino, et al. (2011). "HMGA proteins promote ATM expression and enhance cancer cell resistance to genotoxic agents." Oncogene **30**(27): 3024-3035.

Pece, S., S. Confalonieri, et al. (2011). "NUMB-ing down cancer by more than just a NOTCH." Biochim Biophys Acta **1815**(1): 26-43.

Pece, S., M. Serresi, et al. (2004). "Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis." J Cell Biol **167**(2): 215-221.

Pece, S., D. Tosoni, et al. (2010). "Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content." Cell **140**(1): 62-73.

Pedulla, M. L., N. R. Treff, et al. (2001). "Sequence and analysis of the murine Hmgiy (Hmga1) gene locus." Gene **271**(1): 51-58.

Pegoraro, S., G. Ros, et al. (2013). "HMGA1 promotes metastatic processes in basal-like breast cancer regulating EMT and stemness." Oncotarget **4**(8): 1293-1308.

Pentimalli, F., D. Palmieri, et al. (2008). "HMGA1 protein is a novel target of the ATM kinase." Eur J Cancer **44**(17): 2668-2679.

Perrella, M. A., A. Pellacani, et al. (1999). "High mobility group-I(Y) protein facilitates nuclear factor-kappaB binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer." J Biol Chem **274**(13): 9045-9052.

Persson, F., Y. Andren, et al. (2009). "High-resolution genomic profiling of adenomas and carcinomas of the salivary glands reveals amplification, rearrangement, and fusion of HMGA2." Genes Chromosomes Cancer **48**(1): 69-82.

Pierantoni, G. M., V. Agosti, et al. (2003). "High-mobility group A1 proteins are overexpressed in human leukaemias." Biochem J **372**(Pt 1): 145-150.

Pierantoni, G. M., C. Rinaldo, et al. (2007). "High-mobility group A1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2." J Clin Invest **117**(3): 693-702.

Qiu, L., C. Joazeiro, et al. (2000). "Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase." J Biol Chem **275**(46): 35734-35737.

Reeves, R. and J. E. Adair (2005). "Role of high mobility group (HMG) chromatin proteins in DNA repair." DNA Repair (Amst) **4**(8): 926-938.

Reeves, R. and L. Beckerbauer (2001). "HMGI/Y proteins: flexible regulators of transcription and chromatin structure." Biochim Biophys Acta **1519**(1-2): 13-29.

Reeves, R. and M. S. Nissen (1995). "Cell cycle regulation and functions of HMG-I(Y)." Prog Cell Cycle Res **1**: 339-349.

Ricci-Vitiani, L., D. G. Lombardi, et al. (2007). "Identification and expansion of human colon-cancer-initiating cells." Nature **445**(7123): 111-115.

Rommel, B., P. Rogalla, et al. (1997). "HMGI-C, a member of the high mobility group family of proteins, is expressed in hematopoietic stem cells and in leukemic cells." Leuk Lymphoma **26**(5-6): 603-607.

Sakariassen, P. O., H. Immervoll, et al. (2007). "Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies." Neoplasia **9**(11): 882-892.

Sarhadi, V. K., H. Wikman, et al. (2006). "Increased expression of high mobility group A proteins in lung cancer." J Pathol **209**(2): 206-212.

Scala, S., G. Portella, et al. (2000). "Adenovirus-mediated suppression of HMGI(Y) protein synthesis as potential therapy of human malignant neoplasias." Proc Natl Acad Sci U S A **97**(8): 4256-4261.

Schoenmakers, E. F., S. Wanschura, et al. (1995). "Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumors." Nat Genet **10**(4): 436-444.

Schreiber, E., P. Matthias, et al. (1989). "Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells." Nucleic Acids Res **17**(15): 6419.

Sgarra, R., J. Lee, et al. (2006). "The AT-hook of the chromatin architectural transcription factor high mobility group A1a is arginine-methylated by protein arginine methyltransferase 6." J Biol Chem **281**(7): 3764-3772.

Sgarra, R., A. Rustighi, et al. (2004). "Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer." FEBS Lett **574**(1-3): 1-8.

Shah, S. N., C. Kerr, et al. (2012). "HMGA1 reprograms somatic cells into pluripotent stem cells by inducing stem cell transcriptional networks." PLoS One **7**(11): e48533.

Shannon, M. F., S. R. Himes, et al. (1998). "A role for the architectural transcription factors HMGI(Y) in cytokine gene transcription in T cells." Immunol Cell Biol **76**(5): 461-466.

Singh, S. K., I. D. Clarke, et al. (2003). "Identification of a cancer stem cell in human brain tumors." Cancer Res **63**(18): 5821-5828.

Skeath, J. B. and C. Q. Doe (1998). "Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the Drosophila CNS." Development **125**(10): 1857-1865.

Smith, A. E. and K. L. Buchmueller (2011). "Molecular basis for the inhibition of HMGA1 proteins by distamycin A." Biochemistry **50**(38): 8107-8116.

Staats, B., U. Bonk, et al. (1996). "A fibroadenoma with a t(4;12) (q27;q15) affecting the HMGI-C gene, a member of the high mobility group protein gene family." Breast Cancer Res Treat **38**(3): 299-303.

Stier, S., T. Cheng, et al. (2002). "Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome." Blood **99**(7): 2369-2378.

Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell **131**(5): 861-872.

Tallini, G., P. Dal Cin, et al. (1997). "Expression of HMGI-C and HMGI(Y) in ordinary lipoma and atypical lipomatous tumors: immunohistochemical reactivity correlates with karyotypic alterations." Am J Pathol **151**(1): 37-43.

Tamimi, Y., H. G. van der Poel, et al. (1993). "Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization." Cancer Res **53**(22): 5512-5516.

Tamimi, Y., H. G. van der Poel, et al. (1996). "A retrospective study of high mobility group protein I(Y) as progression marker for prostate

cancer determined by in situ hybridization." Br J Cancer **74**(4): 573-578.
Tang, C., B. T. Ang, et al. (2007). "Cancer stem cell: target for anti-cancer therapy." FASEB J **21**(14): 3777-3785.
Teglund, S. and R. Toftgard (2010). "Hedgehog beyond medulloblastoma and basal cell carcinoma." Biochim Biophys Acta **1805**(2): 181-208.
Tesfaye, A., F. Di Cello, et al. (2007). "The high-mobility group A1 gene up-regulates cyclooxygenase 2 expression in uterine tumorigenesis." Cancer Res **67**(9): 3998-4004.
Tessari, M. A., M. Gostissa,

. (2003). "Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2." Mol Cell Biol **23**(24): 9104-9116.
Thuault, S., E. J. Tan, et al. (2008). "HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition." J Biol Chem **283**(48): 33437-33446.
Thuault, S., U. Valcourt, et al. (2006). "Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition." J Cell Biol **174**(2): 175-183.
Vallone, D., S. Battista, et al. (1997). "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 **16**(17): 5310-5321.
Varnum-Finney, B., L. Xu, et al. (2000). "Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling." Nat Med **6**(11): 1278-1281.
Visone, R., R. Iuliano, et al. (2008). "Hmga1 null mice are less susceptible to chemically induced skin carcinogenesis." Eur J Cancer **44**(2): 318-325.
Watanabe, S., Y. Ueda, et al. (2009). "HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells." Am J Pathol **174**(3): 854-868.
Whitley, M. Z., D. Thanos, et al. (1994). "A striking similarity in the organization of the E-selectin and beta interferon gene promoters." Mol Cell Biol **14**(10): 6464-6475.
Wood, L. J., J. F. Maher, et al. (2000). "The oncogenic properties of the HMG-I gene family." Cancer Res **60**(15): 4256-4261.
Xu, Y., T. F. Sumter, et al. (2004). "The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia." Cancer Res **64**(10): 3371-3375.
Yu, F., H. Yao, et al. (2007). "let-7 regulates self renewal and tumorigenicity of breast cancer cells." Cell **131**(6): 1109-1123.

Yu, J., M. A. Vodyanik, et al. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science **318**(5858): 1917-1920.

Yu, Z., T. G. Pestell, et al. (2012). "Cancer stem cells." Int J Biochem Cell Biol **44**(12): 2144-2151.

Zaidi, M. R., Y. Okada, et al. (2006). "Misexpression of full-length HMGA2 induces benign mesenchymal tumors in mice." Cancer Res **66**(15): 7453-7459.

Zhang, X. M. and G. L. Verdine (1999). "A small region in HMG I(Y) is critical for cooperation with NF-kappaB on DNA." J Biol Chem **274**(29): 20235-20243.

Zhong, W., J. N. Feder, et al. (1996). "Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis." Neuron **17**(1): 43-53.

Zhong, W., M. M. Jiang, et al. (2000). "Mouse numb is an essential gene involved in cortical neurogenesis." Proc Natl Acad Sci U S A **97**(12): 6844-6849.

Zhou, X., K. F. Benson, et al. (1995). "Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C." Nature **376**(6543): 771-774.

Zilian, O., C. Saner, et al. (2001). "Multiple roles of mouse Numb in tuning developmental cell fates." Curr Biol **11**(7): 494-501.

HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels

Francesca Puca[a], Marianna Colamaio[a], Antonella Federico[a], Marica Gemei[b], Nadia Tosti[a], André Uchimura Bastos[a], Luigi Del Vecchio[b], Salvatore Pece[c], Sabrina Battista[a] and Alfredo Fusco[a]

[a]Istituto di Endocrinologia ed Oncologia Sperimentale - CNR e/o Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Naples, Italy
[b]CEINGE, Biotecnologie Avanzate - Napoli, & SEMM - European School of Molecular Medicine - Naples Site, Naples, Italy
[c]Istituto Europeo di Oncologia, Milan, Italy

Running title: HMGA1 silencing in colon tumour stem cells

Key words: HMGA1, cancer stem cells, p53, colon carcinoma, NUMB

Corresponding authors:

Alfredo Fusco
Istituto di Endocrinologia ed Oncologia Sperimentale del CNR,
via Pansini 5, 80131 Naples, Italy
Tel/Fax: 39-081-7463749
E-mail: alfusco@unina.it

Sabrina Battista
Istituto di Endocrinologia ed Oncologia Sperimentale del CNR,
via Pansini 5, 80131 Naples, Italy
Tel: 39-081-7464550
Fax: 39-081-2296674
E-mail: sabattis@unina.it

Abstract

High-mobility group A1 (HMGA1) proteins are architectural chromatinic proteins, abundantly expressed during embryogenesis and in most cancer tissues, but expressed at low levels or absent in normal adult tissues. Several studies have demonstrated that HMGA1 proteins play a causal role in neoplastic cell transformation. The aim of this study was to investigate the role of these proteins in the control of cancer stem cells (CSCs), which have emerged as a preferred target in cancer therapy, because of their role in cancer recurrence. We observed that HMGA1 is overexpressed in colon tumour stem cell (CTSC) lines compared to normal and colon cancer tissues. We demonstrated that HMGA1 silencing in CTSCs increases stem cell quiescence and reduces self-renewal and sphere-forming efficiency (SFE). The latter, together with the upregulation and asymmetric distribution of NUMB, is indicative of the recovery of an asymmetric division pattern, typical of normal stem cells. We further found that HMGA1 transcriptionally regulates p53, which is known to control the balance between symmetric and asymmetric divisions in CSCs. Therefore, our data indicate a critical role for HMGA1 in regulating both self-renewal and the symmetric/asymmetric division ratio in CSCs, suggesting that blocking HMGA1 function may be an effective anti-cancer therapy.

Introduction

Cancer arises from a small set of stem cells, or tumour-initiating cells, that differ from normal stem cells in their deregulated self-renewal and differentiation programs (1). Chemotherapy improves the 5-year survival of adult cancer patients by only 2.3% in Australia and 2.1% in the USA (2). Surrogate end point parameters such as 'progression-free survival,' 'disease-free survival,' or 'recurrence-free survival' reflect the temporary pause in the progression of the disease, seldom lasting more than a few months. Subsequently, the cancer typically returns with even more aggressive characteristics due to a few tumour-founding cells (the cancer stem cells or CSCs), which, because of their intrinsic chemoresistance, are spared and "naturally selected" by the routinely used anti-cancer drugs. This common trend makes the identification of CSC-specific targets and tightly related CSC-specific drugs necessary for the development of new effective anti-cancer therapies.

The High-Mobility Group A (HMGA) family includes three proteins: HMGA1a, HMGA1b, and HMGA2. These proteins are encoded by two distinct genes; the HMGA1a and HMGA1b proteins are products of the same gene through alternative splicing (3). The HMGA proteins bind the minor groove of AT-rich DNA sequences through their DNA binding domains, the so-called "AT-hooks." HMGA proteins do not exhibit transcriptional activity *per se*, but they regulate the activity of several genes by interacting with the transcription machinery and altering the chromatin structure (4). The levels of HMGA proteins are low or absent in normal cells and adult tissues but are elevated in many tumours, neoplastically transformed cells, and embryonic cells (4). Their overexpression is largely associated with a highly malignant phenotype and also represents a poor prognostic marker, as HMGA overexpression often correlates with the presence of metastasis and reduced survival (5). Moreover, several studies indicate a causal role for HMGA gene expression in the process of carcinogenesis. Indeed, it has been reported that the blockage of their expression prevents thyroid cell transformation and promotes the death of malignant cells (6-7). Transgenic mice overexpressing either HMGA1 or HMGA2 develop uterine tumours, haematopoietic tumours, and pituitary adenomas (8-11).

The observation of HMGA1 upregulation in colon cancer dates back to 1996, when our group detected the HMGA1 proteins, previously called HMGI(Y), in human colorectal cancer cell lines and tissues but not in normal intestinal mucosa (12). Subsequently, we reported that HMGA1 protein expression was associated with the early stages of the neoplastic transformation of colon cells but only rarely with colon cell hyperproliferation (13), closely correlating with the degree of cellular atypia in adenomas. Very recently, Belton and colleagues (14) reported that HMGA1 overexpression induces cell proliferation and polyp formation in the intestines of HMGA1 transgenic mice and leads to metastatic progression and stem cell-like properties in colon cancer cells (14), suggesting that HMGA1 is a key regulator both in metastatic progression and in the maintenance of a stem cell-like state (14). Therefore, the aim of our studies was to investigate the role of the HMGA proteins in colon cancer stem cells by silencing their expression.

Here, we report that HMGA1 silencing dramatically affects the survival of colon tumour stem cells and shifts stem cell division to an asymmetric pattern**.** The ability of HMGA1 to negatively regulate p53 promoter activity at the transcriptional level at least partially accounts for the effects induced by its inhibition on CTSCs.

Results

HMGA1 is overexpressed in CTSCs and in the CD133^{+} sub-population

We first analysed HMGA1 expression by Western Blot in normal colonic mucosa (NM), colon cancer, colon cancer cell lines, and CTSC lines. As shown in Figure 1A, HMGA1 was undetectable in NM, whereas it was expressed in colon cancer (Tumour #3), in 3 colon cancer cell lines (SW48,

SW480 and CACO2), and CTSCs (CTSC#18 and CTSC#1.1), which exhibited the highest HMGA1 expression (Figure 1B). Interestingly, when CTSCs were stained for the cancer stem cell marker CD133 and then sorted, HMGA1 expression was enriched in $CD133^+$ cells (Figure 1B). These data indicate that HMGA1 is overexpressed in CTSCs and is more abundant in stem cells than in precursors.

HMGA1 knockdown impairs CTSC growth and induces apoptosis

To understand the role of HMGA1 in CTSC, we silenced HMGA1 expression in the CTCS#18 cell line, using a short hairpin interfering construct (see the Materials and Methods section), leading to an HMGA1 knockdown efficiency of approximately 50%-80% in stable transfectants (Figure 2A). Growth curves performed on single-cell suspensions demonstrated that the knockdown of HMGA1 significantly reduced CTSC proliferation ($p < 0.05$) (Figure 2B). The analysis of cell cycle progression, performed by flow cytometric analysis, demonstrated that HMGA1 knockdown reproducibly altered cell cycle progression, inducing a mean increase of 5% in the G1 phase population and a concomitant mean reduction of 4% in the S phase (Figure 2C). As previously reported (Shah et al., 2012), HMGA1 knockdown reduced the expression of stem cell/pluripotency genes, such as SOX2 and NANOG (Figure 2D).

Because the alteration of the cell cycle only partially accounts for the reduction in CTSC proliferation induced by HMGA1 knockdown, we investigated apoptotic cell death. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays performed on stable transfectants exhibited a 7-fold increase in apoptotic cell numbers in CTSC_shA1 with respect to control cells (Figures 3A and B). These data suggest that, as in other cell systems (4), HMGA1 plays a key role in CTSC proliferation by affecting cell cycle progression and apoptosis.

HMGA1 silencing impairs CTSC self-renewal and sphere-forming efficiency in serial passages

CTSCs, as other types of cancer stem cells, are characterised by their ability to form spheres in suspension cultures (15) or in a semisolid medium. The number of spheres reflects the quantity of cells capable of *in vitro* self-renewal, whereas the number of cells/sphere measures the self-renewal capacity of each sphere-generating cell (16). Therefore, we assayed the ability of cells with reduced HMGA expression to form spheres in methylcellulose-based medium. A dramatic reduction in the number of spheres (Figure 4A) and in their diameter (Figure 4B) was observed in CTSC_shA1 cells compared with control CTSC_ctrl, thus indicating that HMGA1-interference affects CTSC self-renewal.

CSCs are known to differ from normal stem cells both in their deregulated self-renewal and cell division pattern, and an evaluation of their sphere-formation efficiency (SFE) in serial passages allows the assessment of the rate at which CSCs divide symmetrically (16-17). As shown in Figure 4C, the SFE of CTSC_shA1 cells decreased at every passage, whereas the number of spheres derived from parental (not shown) or CTSC_ctrl cells increased progressively. Interestingly, previous studies have demonstrated that the knock-out of p53 leads to increases in SFE and the symmetric division of mammary stem cells in serial passages, whereas the p53 stabiliser Nutlin-3 is able to reduce the SFE of ErbB2 mammary stem cells in serial passages (18). Consistent with these studies, we observed that the treatment of parental CTSC#18 with 5 μM Nutlin-3 was able to maintain the SFE over subsequent passages (Figure 4C, lower panel), similar to what was observed for CTSC_shA1 cells. Conversely, SFE increased in DMSO-treated cells (Figure 4C, lower panel). Therefore, these results indicate that HMGA1 silencing not only restores the ability to divide asymmetrically but also exhibits a more dramatic effect than p53 stabilisation alone.

HMGA1 silencing induces quiescence and the asymmetric distribution of Numb in CSCs

Long-term label retention of PKH26 dye is frequently used as an indicator of normal stem cell quiescence (19). Indeed, rapidly and symmetrically dividing CSCs tend to quickly lose PKH26

(18), which irreversibly binds to the lipid bilayers on cell membranes and is equally distributed among daughter cells during each cell division. Conversely, normal quiescent stem cells divide asymmetrically in one proliferating progenitor and one self-renewing PKH26-retaining quiescent stem cell. Therefore, we stained CTSC_shA1 and control CTSC_ctrl cells with PKH26 and performed a FACS analysis after 10 days. As shown in Figure 5A, knockdown of HMGA1 expression led to a drastic increase in $PKH26^{bright}$ cells (1.5 % in CTSC_ctrl cells versus 4.8 % in CTSC_shA1 cells) (Figure 5A), suggesting that the reduction in HMGA1 expression confers properties of quiescence to the stem cell compartment. Interestingly, very similar results were obtained when HMGA1-knockdown brain tumour stem cells (BTSCs) were stained with PKH26 (Colamaio et al., manuscript in preparation).

Asymmetric cell division can be demonstrated by the asymmetric partitioning of the cell fate determinant Numb (19). During asymmetric cell division, the part of the cell that inherits high levels of Numb will undergo differentiation, whereas the part that inherits low levels will produce a stem cell (20); conversely, in symmetrically dividing CSCs, Numb is expressed at lower levels and is uniformly distributed in such a way that both daughter cells will be equal in terms of Numb expression and function (20).

Therefore, we analysed Numb expression/distribution in HMGA1-knockdown and control cells. Western blot analysis for Numb revealed higher levels of Numb in CTSC_shA1 cells with respect to untransfected and CTSC_ctrl cells (Figure 5B). Moreover, immunofluorescence of the spheres (Figure 5C) demonstrated that whereas Numb was distributed almost uniformly in the cell cytoplasm of CTSC_ctrl cells, it formed an asymmetric punctuate crescent close to the cell membrane (Figure 5C, right panels, arrows) in CTSC_shA1 cells, where it also undergoes partial nuclear localisation (asterisks). The frequency of Numb crescents increased from approximately 6% in control cells, to approximately 32% in CTSC_shA1 cells ($p = 0,0048$) (Figure 5C, right panel). Together, these data demonstrate that HMGA1-silencing restores normal stem cell properties in CSCs, such as quiescence and asymmetric division.

HMGA1 regulates p53 expression at the transcriptional level

Because it has been previously shown that an imbalance between asymmetric and symmetric division can be determined by loss of the p53 tumour suppressor (18), we analysed p53 expression in CTSC_shA1 cells. Western blots performed on untransfected, scramble-transfected, and HMGA1–knockdown CTSCs demonstrated that the downregulation of HMGA1 increased p53 protein expression (Figure 6A, upper panel) and was associated with an increase in the p53-regulated p21 protein (Figure 6A, lower panel), indicating the presence of a functional p53.

Subsequently, to assess the direct action of HMGA1 on p53 transcription, we evaluated HMGA1 protein binding to the *p53* promoter *in vivo* by performing ChIP assays. Therefore, CTSC_ctrl and CTSC_shA1 cells were cross-linked and immunoprecipitated with anti-HMGA1 or anti-IgG antibodies. Immunoprecipitation of chromatin was then analysed by quantitative PCR, using primers spanning 5 different regions of the p53 promoter (see the Materials and Methods section). Occupancy of the p53 promoter regions II and III by HMGA1 was clearly detectable (Figure 6B), whereas no amplification was observed in regions I, IV, and V, indicating the specificity of the binding to regions II and III (Figure 6B). Conversely, CTSC_shA1 cells exhibited a negligible enrichment for regions II and III (Figure 6B) and no enrichment at all for the remaining regions. These results indicate that HMGA1 protein binds the p53 promoter region *in vivo*.

Finally, to define the functional importance of HMGA1 binding to the p53 promoter, we evaluated the activity of the p53 promoter in the presence or absence of HMGA1 by performing luciferase assays. Therefore, HEK293 cells were transfected with a reporter gene construct carrying the human p53 promoter (21) or a control backbone vector (pGL3-basic) in the presence of an HMGA1-expressing construct. As shown in Figure 6C, HMGA1 repressed the p53 promoter, and this effect was dose-dependent. Consistently, this repressive effect was abolished by the pharmacological blocking of the HMGA1-DNA interaction with distamycin (not shown), a drug

known to displace HMGA1 from its AT-rich DNA regions (22). Therefore, these results clearly demonstrate that HMGA1 transcriptionally regulates p53 expression.

Discussion

CSCs are a distinct subset of cancer cells with the ability to self-renew through symmetric division and to generate a repertoire of various cell types, thus initiating and perpetuating tumour growth. CSCs are often endowed with distinctive surface markers, such as CD133, which, in colon cancer and in gliomas and other cancers, decorates tumour-initiating cells versus cells that are unable to initiate tumours in transplantation experiments (23). In human cancers, CSCs have been demonstrated to be a major cause of cancer treatment resistance, invasion, metastasis, and relapse. Thus, eliminating cancer cells with stem cell properties is of prime importance for the successful treatment of cancer, regardless of the tissue of origin.

Recent studies have suggested the critical role of the HMGA1 protein as a master regulator in both cancer stem cells (14; 24) and normal embryonic stem cells (25; 24). Indeed, HMGA1 has recently been shown to reprogram somatic cells into pluripotent stem cells by inducing stem cell transcriptional networks (26). Moreover, several studies have directly or indirectly demonstrated that HMGA proteins are tightly associated with stemness and play a critical role in the epithelial-mesenchymal transition (EMT) (27).

Here, we present data unveiling a central role for HMGA1 in CSC self-renewal. First, we demonstrated that HMGA1 is enriched in CTSCs compared with colon tumours and cancer cell lines and is more abundantly expressed in $CD133^+$ cells than in $CD133^-$ cells, strengthening its association with tumour-initiating cells.

Next, we demonstrated that the knockdown of HMGA1 by antisense methodology in colon CSCs induces a drastic reduction in cell proliferation due to a slight increase in the G1 phase population and a more evident increase in apoptosis, consistent with our previous findings (7). Moreover, CTSC_shA1 cells regain two properties typical of normal adult stem cells: an increased percentage (and brightness) of PKH26-positive cells, which indicates increased quiescence, and reduced SFE, suggestive of the recovery in asymmetric division at the expense of symmetric self-renewing division (17). This shift in the division modality, induced by the depletion of HMGA1 in CSC cells might be of practical clinical relevance because asymmetric division plays a tumour-suppressive role (20). Notably, we have achieved comparable results, in terms of SFE reduction and PKH26 increase, in HMGA1-knockdown BTSCs (Colamaio et al., manuscript in preparation), suggesting the critical role of the HMGA1 protein in the regulation of CSCs of different tissue origin.

Recently, it has been demonstrated that ErbB2 transgenic mice harbour CSCs with an increased frequency of self-renewing divisions, higher replicative potential, and a preponderance of symmetric versus asymmetric division, highlighted by the symmetric distribution of the cell fate determinant Numb, compared with stem cells from wild-type mice (18). In these cells, Nutlin 3-mediated stabilisation of p53 was able to restore asymmetric division (as evidenced by the recovery of the asymmetric distribution of Numb) (18). The striking similarity of the phenotype obtained in CTSC_shA1 with Nutlin-3 treated CSCs and the tight association of HMGA1 and ErbB2 expression (28) prompted us to investigate the effect of HMGA1 interference on the p53-Numb axis. Indeed, we demonstrated that in CTSC_shA1 cells, the expression levels of both p53 and its downstream target p21cip are increased with respect to control cells. Moreover, we demonstrate that HMGA1 directly binds to a discrete region in the p53 promoter, and this binding has functional consequences, leading to p53 transcriptional downregulation.

It is worth noting that HMGA1 overexpression may lead to p53 inactivation by other mechanisms, such as through interfering with p53-mediated transcription of the p53 effectors Bax and p21 (29), transcriptionally activating the p53 inhibitor mdm2, and cytoplasmically relocalising its proapoptotic activator HIPK2 (30; 4). Therefore, the restoration of p53 functions exerted by HMGA1 knockdown likely accounts for the effects of HMGA1 silencing on CSC cells.

However, we cannot exclude the possibility that in addition to a p53-mediated mechanism, HMGA1 silencing might affect CSCs through other mechanisms. In fact, we have demonstrated that whereas the SFE of Nutlin-3-treated CTSCs remains constant overtime, the SFE of HMGA1-knockdown cells progressively decreases in serial passages.

Consistent with the data of Cicalese, the increased expression of NUMB was also observed in CTSC_shA1 concomitant with the nuclear relocalisation of the protein (F. Puca, unpublished preliminary results). The increased expression of NUMB would also enhance the stabilisation of p53 (31). Therefore, taken together, these data suggest the existence of an HMGA1-Numb-p53 axis in which HMGA1 could play a significant role in regulating self-renewal either by directly acting on p53 and NUMB or by enhancing their interaction in the nucleus. In conclusion, the results presented herein indicate that HMGA1 has a specific role in CSC self-renewal, and thus, targeting HMGA1 may represent a promising CSC-specific anti-cancer therapy strategy.

Materials and methods

Cell cultures and culture conditions
Colon tumour stem cell lines were kindly donated by Prof. Ruggero De Maria (Istituto Superiore di Sanità, Rome, Italy) and have been described elsewhere, together with their culturing conditions (15). Cytokines added to the medium included recombinant human FGF-basic and EGF (Peprotech, Rocky Hill, NJ). HEK293 cells were maintained in DMEM medium containing 10% fetal calf serum (Invitrogen).

Colon samples
Normal and cancerous intestinal mucosa samples were kindly provided by Dott. Marina De Rosa (Facoltà di Medicina e Chirurgia, University "Federico II", Naples, Italy) and have been described elsewhere (32).

Quantitative reverse transcription PCR (qRT-PCR)
Total RNA was reverse transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen), and qRT-PCR was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. *G6PD* was used to normalise RNA levels. The primers used were as follows: Hsa_HMGA1_Fw: 5'-CAACTCCAGGAAGGAAACCA, Hsa_HMGA1_Rev: 5'-AGGACTCCTGCGAGATGC; Hsa_G6PD_Fw: 5'-ACAGAGTGAGCCCTTCTTCAA, and Hsa_G6PD_Rev: 5'-ATAGGAGTTGCGGGCAAAG; SOX2_Fw: 5'- GCACATGAACGGCTGGAGCAACG; SOX2_Rev: 5'-TGCTGCGAGTAGGACATGCTGTAGG; NANOG_Fw: 5'- CAAAGGCAAACAACCCACTT; NANOG_Rev: 5'- TCTGGAACCAGGTCTTCACC. The $2^{-\Delta\Delta Ct}$ formula was used to calculate the differential gene expression.

Immunostaining and cell sorting
For cell sorting, the cells were trypsinised, washed twice with PBS, and incubated with anti-human CD133 (CD133/2 (293C3)-PE, Miltenyi Biotech) for 20 minutes at 4°C. After washing twice with PBS, the cells were FACS sorted with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett-Packard computer (Palo Alto, CA).

Western blots and antibodies
Total protein extraction, western blotting, and anti-HMGA1 antibodies have been described elsewhere (33).
The following other antibodies were used: anti-GAPDH (Santa Cruz Biotechnology, CA, USA) and anti-p21 (Cell Signaling Technology, Inc., Danvers, MA). Anti-Numb antibodies were obtained from Abcam (Cambridge, UK) and were used at 1:5000. Blots were visualised using western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

Plasmids
The hairpin RNA interference plasmid for human HMGA1 (pLKO.1-HMGA1, TRCN0000018949) and the scramble control pLKO.1-Puro plasmid (SHC002) were obtained from Sigma-Aldrich. The sequence of the short hairpin RNA targeting the human HMGA1 gene was 5′–CCGGCAACTCCAGGAAGGAAACCAACTCGAGTTGGTTTCCTTCCTGGAGTTGTTTTT–3′, (shHMGA1 targets coding region positions 446-466 of HMGA1 mRNA transcript variant 2).
The pGL3-luci vector containing the p53 promoter was kindly provided by Prof. David Reisman (Center for Colon Cancer Research Tissue Repository, University of South Carolina, Columbia) (21).

The pCDNA3.1-HMGA1 expression vector has been described previously (25).

Transfections
CTSCs were electroporated using the Neon® Transfection System (Invitrogen). Cells were trypsinised with TrypLE™ Express (GIBCO) and counted; 1 x 10^6 cells were subjected to the electric field (1400 V, 20 msec; 1 pulse). After 48 h, CTSCs transfected with the short hairpin-expressing constructs were selected with puromycin (2 µg/µl).

Growth curves and TUNEL assay
Approximately 5 x 10^3 stably transfected cells were plated in 96-well plates. Cells were counted in triplicate at daily intervals with a Burker hemocytometer chamber.
A TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche) according to manufacturer's instructions.

Flow cytometry
After trypsinisation, the cells were washed in PBS and fixed in 70% ethanol. Staining for DNA content was performed with 0.1% NP-40, 50 µg/ml propidium iodide, and 25 µg/ml ribonuclease A for 20 min. For each measurement, 10,000 events were analysed. We used a FACScanto II flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle data were analysed with the ModFit LT 2.0 software (Verity Software) in a semiautomatic analysis procedure. Briefly, we manually selected the cell population in an FSC versus SSC dot plot and discarded debris, and then we gated single cells in a PI-height versus PI-area dot plot, excluding all doublets. The MODFIT algorithm was used to analyse our files, calculating the percentages of cells in each cell cycle phase.

Sphere formation assays
Sphere-forming assays in methylcellulose-based medium were performed as previously described (34) with some modifications. Briefly, medium containing 0.8% methylcellulose was used instead of liquid medium, and other conditions were the same as in liquid medium. Three percent methylcellulose was purchased from R&D Systems (Minneapolis, USA), and a stock solution was made of 2% methylcellulose in DMEM/F12. A final concentration of 0.8% methylcellulose in DMEM/F12 was used for cell culture. Approximately 2 x 10^4 cells from disaggregated CTSC spheres were resuspended in a semisolid medium and plated in 6-well plates. After 7 days, the spheres were microscopically visualised, and the diameters were measured.
Serial passage experiments were conducted as described previously (18) with some modifications. Briefly, 5,000 cells from disaggregated CTSC spheres were plated on 150-mm poly-HEMA-treated cell culture plates. After 10 days, the spheres were disaggregated and re-plated at the same density. The sphere-forming efficiency (SFE) at each passage was obtained by calculating the percentage of the number of spheres divided by the number of cells plated.

PKH staining and flow cytometric analysis
PKH staining was performed as previously described (19).
CTSCs were trypsinised, filtered through a 40-µm cell strainer, resuspended in PBS (approximately 500,000 cells/ml), labelled with PKH26 (Sigma, 10^{-7} M, 5 min), washed twice, and plated.
For the flow cytometric analysis of PKH26-stained cells, CTSCs were trypsinised, filtered through a 40-µm cell strainer, and resuspended in PBS at a concentration of 1x10^6/ml. The cells were subdivided into 5-ml polystyrene tubes (Falcon, Becton Dickinson). The BD FACSAria cytometer, equipped with four excitation laser lines (633 nm, 488 nm, 405 nm, and 375 nm) (Becton Dickinson) was used for FACS analysis, and the BD FacsDIVA software was used for data analysis.
PKH26 staining was evaluated by selecting the appropriate cell population according to the following gating strategy: (i) cells were first gated on physical parameters (forward scatter [FSC]

and side scatter [SSC]) to exclude most of the debris and dead cells; (ii) doublets and aggregates were eliminated using the FSC-area vs. FSC-height pattern. We gated 10-15% of the brightest PKH26+ cells in a PKH26 versus empty channel dot plot.

Immunofluorescence
Whole spheres were centrifuged and fixed with 4% paraformaldehyde, permeabilised, with 0.1% Triton X-100 and 3% BSA, and stained with anti-Numb (kindly donated by Prof. Salvatore Pece and previously described (18), followed by anti-mouse Alexa 488 (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) antibodies. Confocal analysis was performed with a Leica TCS SP2 AOBS microscope.

Luciferase assay
Transfections for luciferase assays were performed in HEK293 cells using the Lipofectamine 2000 method (Invitrogen S.r.l., S. Giuliano Milanese, MI, Italy). Approximately 2×10^5 cells were transiently transfected with 200 ng of pGL3-luci vector containing the p53 promoter (kindly provided by Professor David Reisman, Center for Colon Cancer Research Tissue Repository, University of South Carolina, Columbia) and with the indicated amounts of the pCDNA3.1-HMGA1 expression vector or the corresponding empty vector together with 0.5 μg of *Renilla*. Various amounts of the pCDNA3.1 plasmid were co-transfected to keep the total DNA concentration constant. Transfection efficiencies were normalised using *Renilla* luciferase expression assayed with the dual luciferase system (Promega Italia, Milan, Italy). All transfection experiments were performed in duplicate.

Chromatin immunoprecipitation
ChiP was performed as described previously (35). As a negative control, ChIP experiments were performed with isotype-matched preimmune IgG. The promoter occupancy was calculated with respect to the input as the percentage of anti-A1-immunoprecipitated DNA subtracted from the IgG-immunoprecipitated DNA. The P53 promoter regions assayed for HMGA1 binding refer to the nucleotide sequence previously published (21) and are indicated as follows: Region I: nt 401-557; region II: nt 538-650; region III: nt 631-751; region IV: nt 767-890; and region V: nt 989-1090.
The primers for each region were as follows:
Prom_hQ_tp53_1_F: 5'-CAGGCTTCAGACCTGTCTCC
Prom_hQ_tp53_1_R: 5'-GCTTTCAGTACATGGAAACGTAA
Prom_hQ_tp53_2_F: 5'-CGTTTCCATGTACTGAAAGCAA
Prom_hQ_tp53_2_R: 5'-CCCTAACGTTTTCTCCCAGA
Prom_hQ_tp53_3_F: 5'-TCTGGGAGAAAACGTTAGGG
Prom_hQ_tp53_3_R: 5'-AAGGGTGGAAGGAAGAAAGC
Prom_hQ_tp53_4_F: 5'-GCAGGATTCCTCCAAAATGA
Prom_hQ_tp53_4_R: 5'-GAGGGTGCAGAGTCAGGATT
Prom_hQ_tp53_5_F: 5'-GTTGATGGGATTGGGGTTTT
Prom_hQ_tp53_5_R: 5'-AGCTACCTGCTCCCTGGAC
Prom_GAPDH_1F: 5'-CCCAAAGTCCTCCTGTTTCA
Prom_GAPDH_R: 5'-GTCTTGAGGCCTGAGTACG

Statistical analysis
Statistical analyses were performed using the Kruskal-Wallis test or the Mann-Whitney *U*-test. If the Kruskal-Wallis test was positive ($p < 0.05$), a pairwise comparison of subgroups was performed using Dunn's post-hoc test.

Figure 1

HMGA1 expression in CTSCs

A) Western blot for HMGA1 in normal colonic mucosa (NM), colon cancer sample (T#3), colon tumour-derived cell lines, SW48, SW480, GEO, and CACO3, and colon tumour stem cells (CTSC#18 and CTSC#1.1). **B)** Western blot for HMGA1 in unsorted CTSC#18 and sorted $CD133^{+}$ and $CD133^{-}$ cells. GAPDH was used as a loading control.

Figure 2

HMGA1 knockdown affects the proliferation and cell cycle of CTSCs. A) Western blots for HMGA1 in untransfected, HMGA1-knockdown (CTSC_shA1), and scramble-transfected (CTSC_ctrl) cells. GAPDH is used as a loading control. **B)** Growth curve of stable scramble (CTSC_ctrl) and HMGA1-knockdown (CTSC_shA1) CTSCs. Data are the mean value ± SD of one representative experiment, performed in quadruplicate (*, $p < 0.05$, Mann-Whitney *U*-test). **C)** Histogram of the FACS analysis in CTSC_ctrl and CTSC_shA1 cells. Data are the mean value ± SD of 3 independent experiments. **D)** qRT-PCR for SOX2 and NANOG gene expression in CTSC_ctrl and CTCS_shA1 cells. The expression level of each gene was normalized to the G6PD gene expression (*, $p < 0.05$, Mann-Whitney *U*-test).

Figure 3

HMGA1 knockdown effects on apoptosis in CTSCs.

A) Fluorescence micrographs of TUNEL assays performed on non-transfected CTSCs (CTSC_NT), CTSC_ctrl and CTSC_shA1 cells, double-stained with Hoechst dye (left) to identify total nuclei and with TMR red UTP (right) to identify apoptotic, TUNEL-positive cells. **B)** Bar chart representation of the number of TUNEL-positive cells per 100 Hoechst-positive nuclei in the samples shown in A. Each bar represents the mean ± SD of 10 arbitrary fields. An asterisk indicates the significance of the difference between CTSC_shA1 and CTSC_ctrl (*, $p = 0.0014$; Kruskal-Wallis test followed by Dunn's post-hoc test).

Figure 4

Effects of HMGA1 knockdown on the sphere-formation ability of CTSCs.

A) The diagram represents the average number of spheres in the methylcellulose-based medium after 7 days. Spheres with diameters > 70 μm were counted in each of 10 representative fields. Triple asterisks indicate the significance of the difference in the number of spheres formed by CSC_ctrl and CTSC_shA1 cells (***, $p < 0.0001$; Mann-Whitney *U*-test).

B) Diagram showing the sphere diameter distribution in CTSC_ctrl and CTSC_shA1 cells. Each bar represents the mean ± SD diameter found in 10 representative fields. Mann-Whitney *U*-test. **C)** Diagram showing the sphere-formation efficiency (SFE) ± SD in scramble- and HMGA1-knockdown CTSCs in serial passages (from F0 to F3). The spheres were disaggregated every 10 days. SFE is measured as the percentage of the number of spheres per plated cell at every passage. The data represent the results of two independent experiments.

D) Sphere-formation efficiency (SFE) in parental CTSC#18 cells treated with DMSO or Nutlin-3 (5 μM) in serial passages (from F0 to F3). Spheres were disaggregated every 7 days. The data represent the mean value ± SD of two independent experiments.

Figure 5

HMGA1 knockdown induces stem cell quiescence and asymmetric Numb distribution.

A) FACS plots of double-coloured (PKH26-phycoerythrin [PE] and fluorescein isothiocyanate [FITC-A]) CTSC_ctrl and CTSC_shA1 cells (one representative experiment). The left-most panel shows cells non-stained with PKH26, gated on physical parameters (forward scatter [FSC] and side scatter [SSC]) to exclude most of the debris and dead cells.

B) Mean percentage of $PKH26^{bright}$ cells in CTSC_ctrl and CTSC_shA1 populations, after 10 days from staining. Each bar represents the mean ± SD of 5 independent experiments (*, $p < 0,05$; Mann-Whitney *U*-test).

C) Western blot for Numb in non-transfected, CTSC_shA1, and CTSC_ctrl cells. GAPDH was used as a loading control.

D) Immunofluorescence for Numb in CTSC_ctrl (left) and CTSC_shA1 (right) CTSCs. The nuclei are stained in blue with DAPI. Arrows denote the crescent-shaped Numb distribution whereas asterisks indicate nuclear-localised Numb. The histogram shows the percentage of cells with crescent-shaped, asymmetric Numb distribution, obtained in immunofluorescence analyses, as in C). Data are the mean value ± SD of 7 arbitrary fields for each sample. (** , $p = 0.0048$; Mann-Whitney U-test).

Figure 6

HMGA1 negatively regulates p53 expression at the transcriptional level.

A) Western blot analysis for p53 (upper panel) and p21 (lower panel) expression in non-transfected, HMGA1-knockdown, and scramble-transfected cells. GAPDH was used as a loading control.

B) ChIP assay, revealed by qPCR, detecting the *in vivo* binding of HMGA1 to the 5 sub-regions in the p53 promoter in CTSC_ctrl and CTSC_shA1 extracts. The relative occupancy of the p53 promoter regions by HMGA1 is indicated as vertical bars. GAPDH promoter amplicon was used as a negative control.

C) Luciferase activity of the p53 promoter in HEK 293 cells in the presence or absence of an HMGA1-expressing vector. The amounts of the HMGA1-expressing vector are indicated. The data are the results of three independent experiments performed in duplicate. The relative luciferase activity was normalised with *Renilla* luciferase and was expressed as the fold induction over the activity of the p53 promoter (*, $p < 0.05$). pGL3-basic activity in the presence or absence of the HMGA1-expressing construct was used as a negative control.

Acknowledgments
This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC) (IG 5346), the Project "Medicina Personalizzata" of the National Research Council (CNR) and from the Italian Ministry of Economy and Finance to the CNR for the Project FaReBio di Qualità. We are grateful to Prof. David Reisman for the p53 promoter reporter construct. We are grateful to Prof. Pece staff for the kind technical assistance.
The authors declare no conflict of interest.

References

1. Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annu Rev Cell Dev Biol. 2007; 23: 675-699. Review.

2. Morgan G, Ward R, Barton M. The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies. Clin Oncol (R Coll Radiol). 2004; 16: 549-560.

3. 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; 21: 4259-4267.

4. Fedele M, Fusco A. HMGA and cancer. Biochim Biophys Acta. 2010; 1799: 48-54. Review.

5. Liau SS, Whang E. HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma. Clin Cancer Res. 2008; 14: 1470-1477.

6. 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; 15: 1545-1553.

7. Scala S, Portella G, Fedele M, Chiappetta G, Fusco A. Adenovirus-mediated suppression of HMGI(Y) protein synthesis as potential therapy of human malignant neoplasias. Proc Natl Acad Sci U S A. 2000; 97: 4256-4261.

8. Baldassarre G, Fedele M, Battista S, Vecchione A, Klein-Szanto AJ, Santoro M, Waldmann TA, Azimi N, Croce CM, Fusco A. Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. Proc Natl Acad Sci U S A. 2001; 98: 7970-7975.

9. Fedele M, Battista S, Kenyon L, Baldassarre G, Fidanza V, Klein-Szanto AJ, Parlow AF, Visone R, Pierantoni GM, Outwater E, Santoro M, Croce CM, Fusco A. Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. Oncogene. 2002; 21: 3190-3198.

10. Tesfaye A, Di Cello F, Hillion J, Ronnett BM, Elbahloul O, Ashfaq R, Dhara S, Prochownik E, Tworkoski K, Reeves R, Roden R, Ellenson LH, Huso DL, Resar LM. The high-mobility group A1 gene up-regulates cyclooxygenase 2 expression in uterine tumorigenesis. Cancer Res. 2007; 67: 3998-4004.

11. Ikeda K, Mason PJ, Bessler M. 3'UTR-truncated Hmga2 cDNA causes MPN-like hematopoiesis by conferring a clonal growth advantage at the level of HSC in mice. Blood. 2011; 117: 5860-5869.

12. Fedele M, Bandiera A, Chiappetta G, Battista S, Viglietto G, Manfioletti G, Casamassimi A, Santoro M, Giancotti V, Fusco A. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. Cancer Res. 1996; 56: 1896-1901.

13. Chiappetta G, Manfioletti G, Pentimalli F, Abe N, Di Bonito M, Vento MT, Giuliano A, Fedele M, Viglietto G, Santoro M, Watanabe T, Giancotti V, Fusco A. High mobility group HMGI(Y) protein expression in human colorectal hyperplastic and neoplastic diseases. Int J

Cancer. 2001; 91: 1447-1451.

14. Belton A, Gabrovsky A, Bae YK, Reeves R, Iacobuzio-Donahue C, Huso DL, Resar LM. HMGA1 induces intestinal polyposis in transgenic mice and drives tumor progression and stem cell properties in colon cancer cells. PLoS One. 2012; 7: e30034.

15. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007; 445: 111-115.
16. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res. 2004; 6: R605-615.

17. Deleyrolle LP, Ericksson G, Morrison BJ, Lopez JA, Burrage K, Burrage P, Vescovi A, Rietze RL, Reynolds BA. Determination of somatic and cancer stem cell self-renewing symmetric division rate using sphere assays. PLoS One. 2011; 6: e15844.

18. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, Brisken C, Minucci S, Di Fiore PP, Pelicci PG. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell. 2009; 138: 1083-1095.

19. Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, Bernard L, Viale G, Pelicci PG, Di Fiore PP. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell 2010; 140: 62-73.

20. Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature. 2006; 441: 1068-1074.

21. Durland-Busbice S, Reisman D. Lack of p53 expression in human myeloid leukemias is not due to mutations in transcriptional regulatory regions of the gene. Leukemia. 2002; 16: 2165-2167.

22. Smith AE, Buchmueller KL. Molecular basis for the inhibition of HMGA1 proteins by distamycin A. Biochemistry. 2011; 50: 8107-8116.

23. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007; 445: 106-110.

24. Shah SN, Cope L, Poh W, Belton A, Roy S, Talbot CC. HMGA1: a master regulator of tumor progression in triple-negative breast cancer cells. PLoS One. 2013; 8: e63419.

25. Battista S, Pentimalli F, Baldassarre G, Fedele M, Fidanza V, Croce CM, Fusco A. Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation. FASEB J. 2003; 17: 1496-1498.

26. Shah SN, Kerr C, Cope L, Zambidis E, Liu C, Hillion J, Belton A, Huso DL, Resar LM. HMGA1 reprograms somatic cells into pluripotent stem cells by inducing stem cell transcriptional networks. PLoS One. 2012; 7: e48533.

27. 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: 1293-308.

28. Chiappetta G, Botti G, Monaco M, Pasquinelli R, Pentimalli F, Di Bonito, Vento MT, Giuliano A, Fedele M, Viglietto G, Santoro M, Watanabe T, Giancotti V, Fusco A. HMGA1 protein overexpression in human breast carcinomas: correlation with ErbB2 expression. Clin Cancer Res. 2004; 10: 7637-7644.

29. 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: 2980-2989.

30. Reeves, R. Nuclear functions of the HMG proteins. Biochim Biophys Acta. 2010; 1799: 3-14. Review.

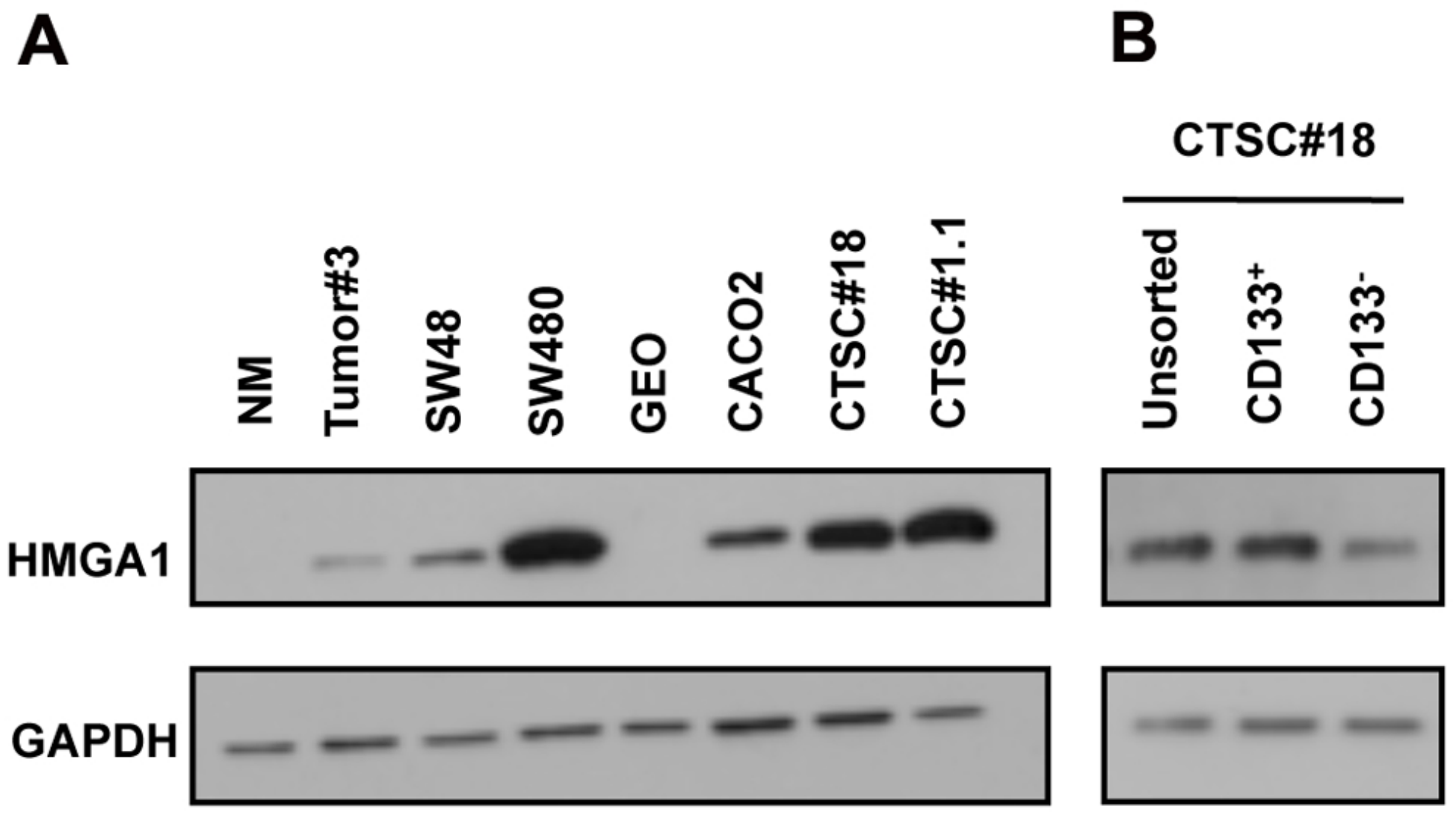
31. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP. NUMB controls p53 tumour suppressor activity. Nature. 2008; 451: 76-80.

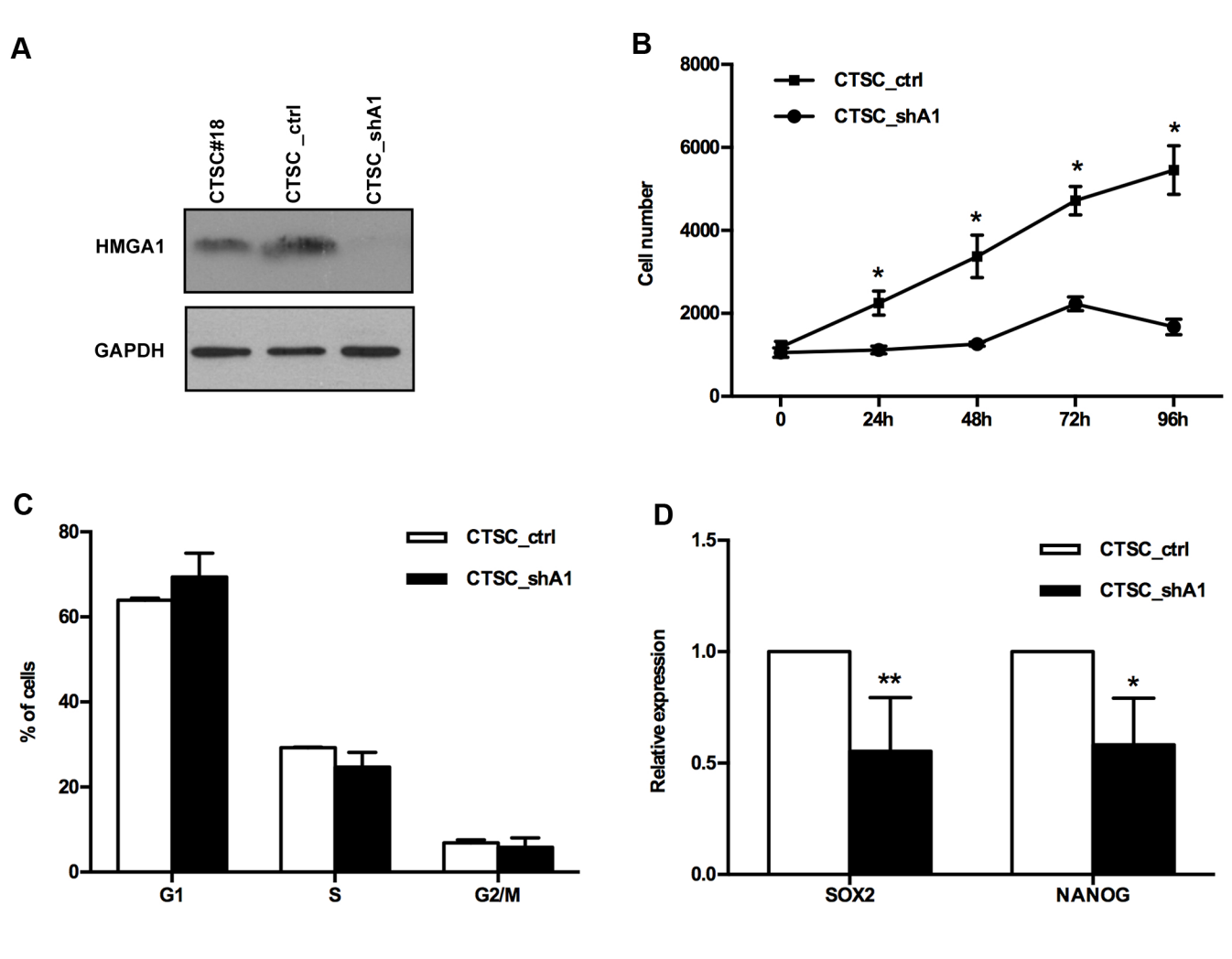
32. Galatola M, Miele E, Strisciuglio C, Paparo L, Rega D, Delrio P, Duraturo F, Martinelli M, Rossi GB, Staiano A, Izzo P, De Rosa M. Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis. World J Gastroenterol. 2013; 19: 8659-8670.

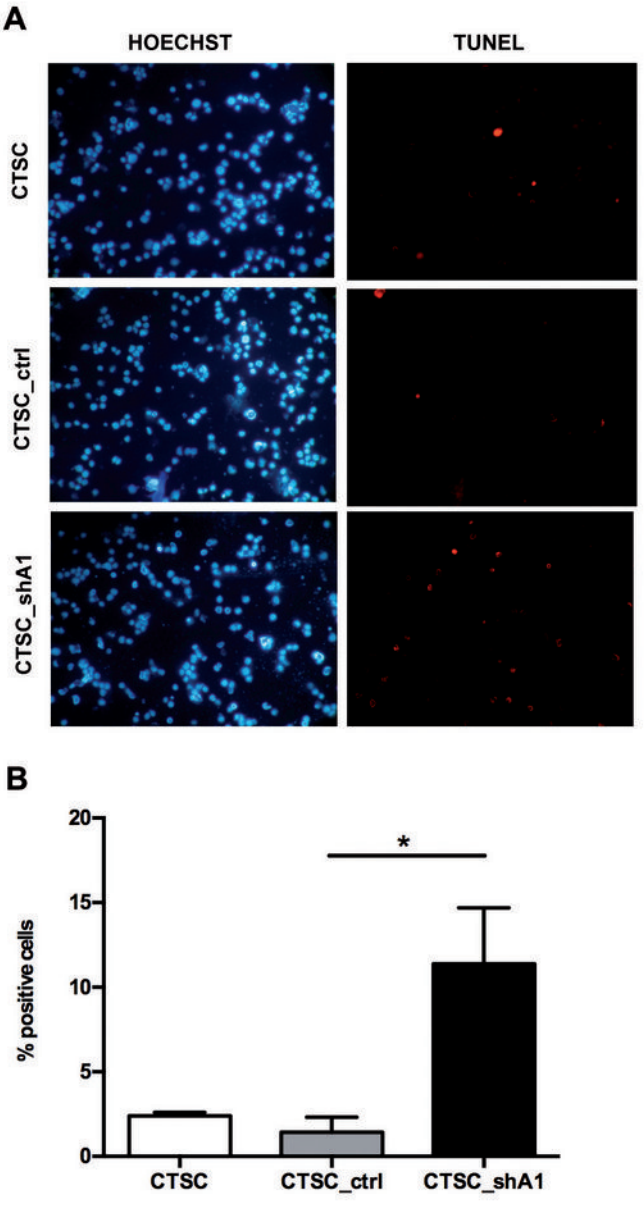
33. Melillo RM, Pierantoni GM, Scala S, Battista S, Fedele M, Stella A, De Biasio MC, Chiappetta G, Fidanza V, Condorelli G, Santoro M, Croce CM, Viglietto G, Fusco A. Critical role of the HMGI(Y) proteins in adipocytic cell growth and differentiation. Mol Cell Biol. 2001; 21: 2485-2495.

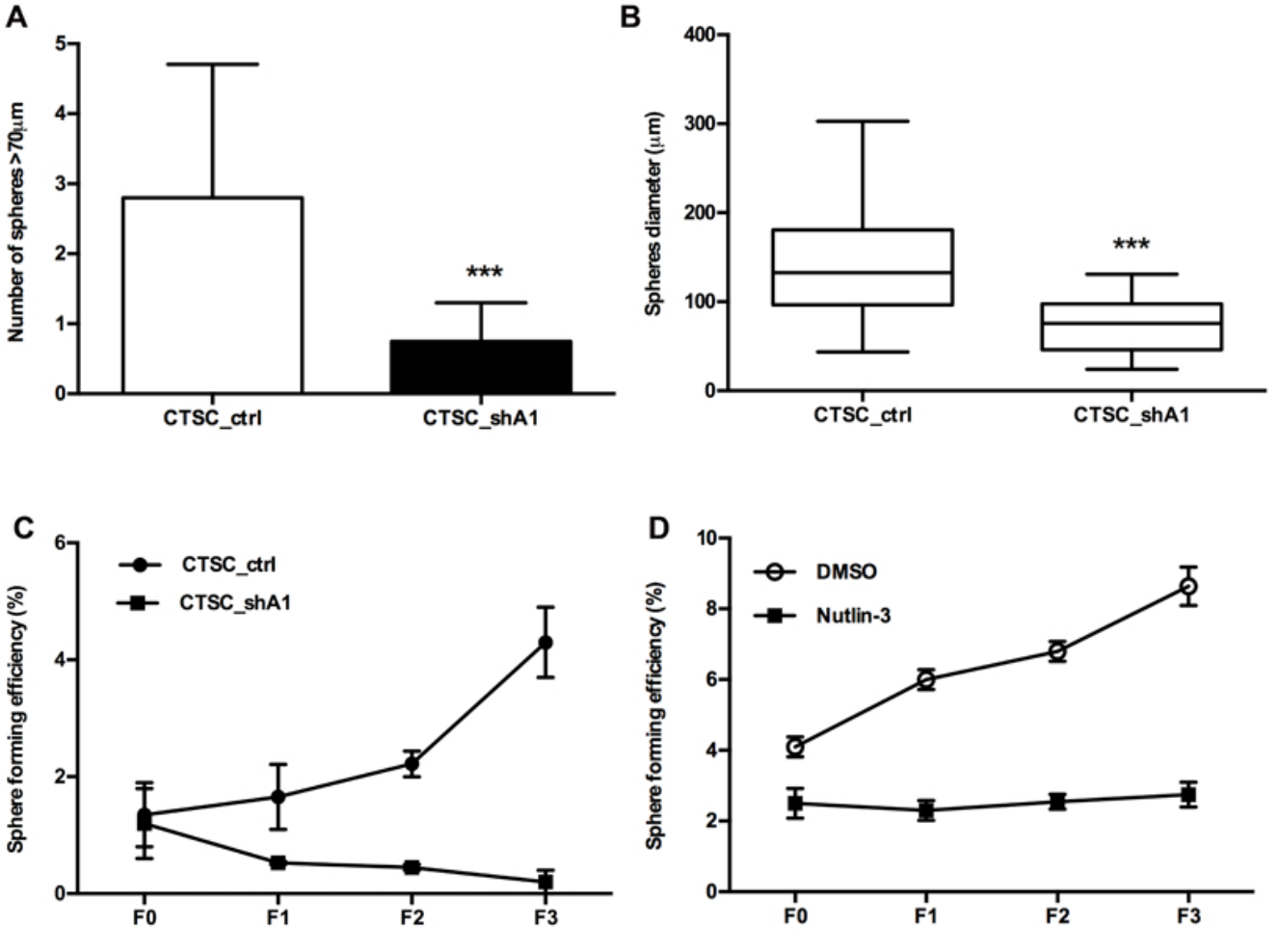
34. Liu S, Liu S, Wang X, Zhou J, Cao Y, Wang F, Duan E. The PI3K-Akt pathway inhibits senescence and promotes self-renewal of human skin-derived precursors in vitro. Aging Cell. 2011; 10: 661-674.

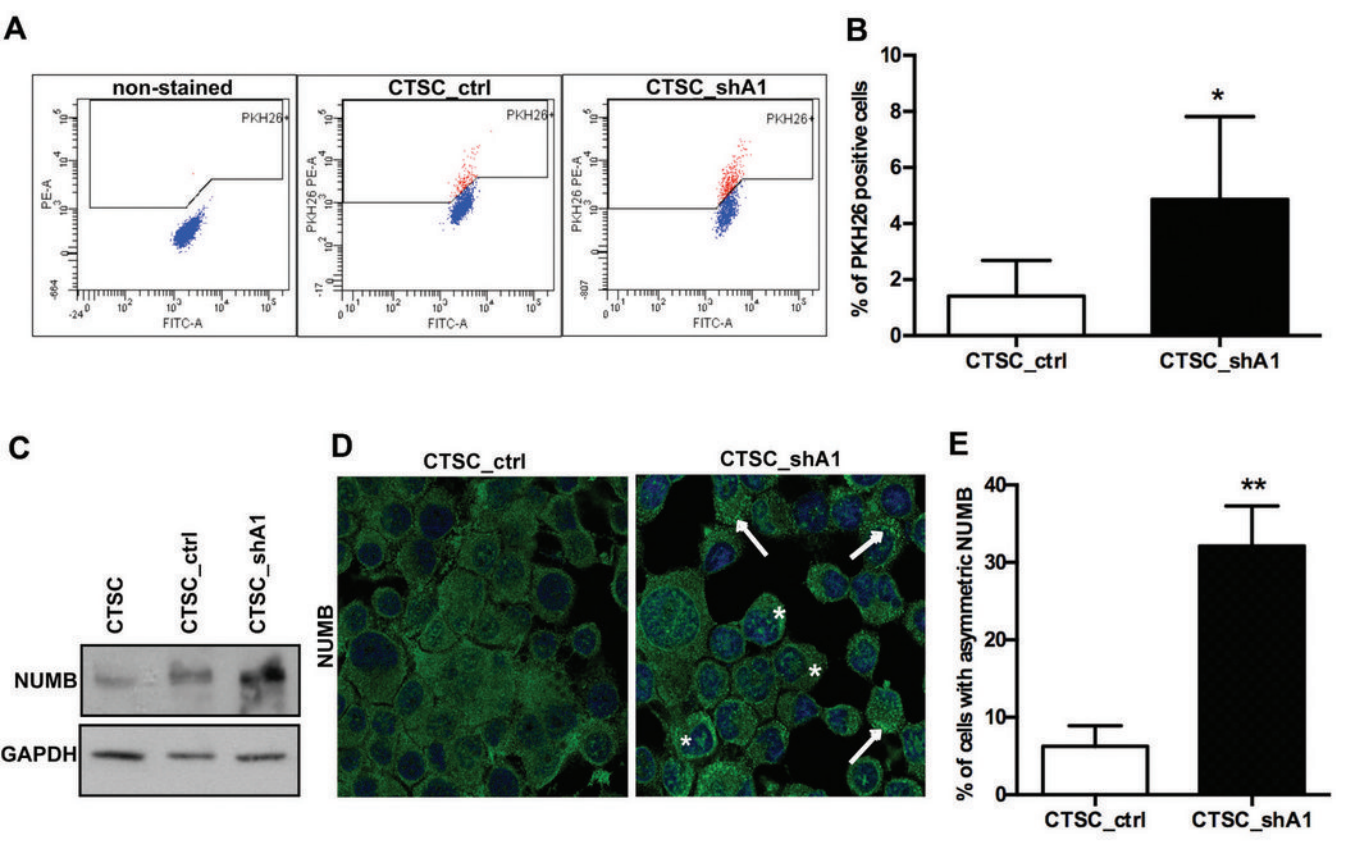
35. Federico A, Pallante P, Bianco M, Ferraro A, Esposito F, Monti M, Cozzolino M, Keller S, Fedele M, Leone V, Troncone G, Chiariotti L, Pucci P, Fusco A. Chromobox protein homologue 7 protein, with decreased expression in human carcinomas, positively regulates E-cadherin expression by interacting with the histone deacetylase 2 protein. Cancer Res. 2009; 69: 7079-7087.

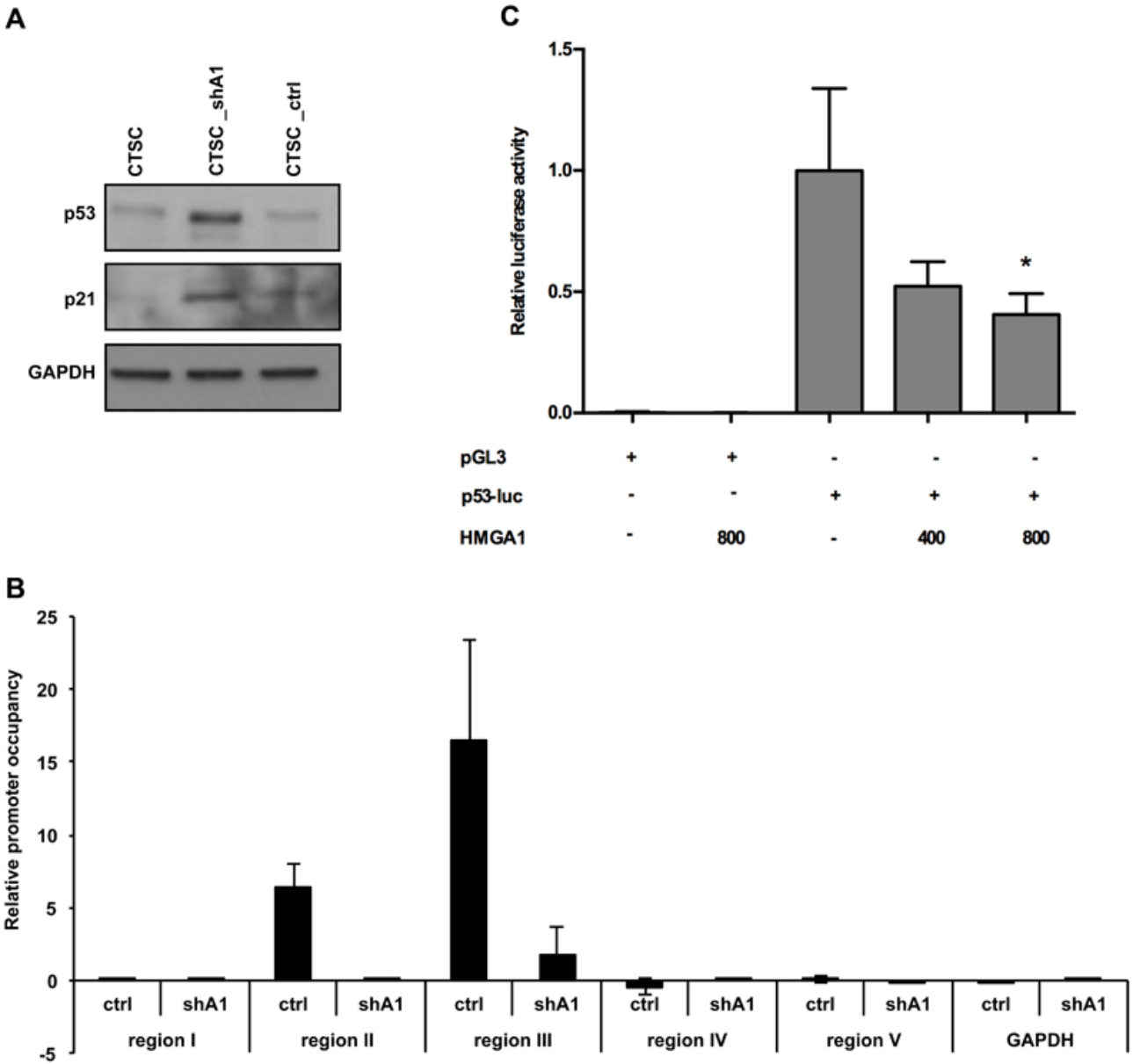

A
B
CTSC#18
NM
Tumor#3
SW48
SW480
GEO
CACO2
CTSC#18
CTSC#1.1
Unsorted
CD133+
CD133-
HMGA1
GAPDH


A
CTSC#18
CTSC_ctrl
CTSC_shA1
HMGA1
GAPDH
B
CTSC_ctrl
CTSC_shA1
Cell number
8000
6000
4000
2000
0
0
24h
48h
72h
96h
*
C
CTSC_ctrl
CTSC_shA1
% of cells
80
60
40
20
0
G1
S
G2/M
D
CTSC_ctrl
CTSC_shA1
Relative expression
1.5
1.0
0.5
0.0
**
*
SOX2
NANOG


A
HOECHST
TUNEL
CTSC
CTSC_ctrl
CTSC_shA1
B
% positive cells
20
15
10
5
0
*
CTSC
CTSC_ctrl
CTSC_shA1


A
Number of spheres >70µm
5
4
3
2
1
0
***
CTSC_ctrl
CTSC_shA1
B
Spheres diameter (µm)
400
300
200
100
0
***
CTSC_ctrl
CTSC_shA1
C
Sphere forming efficiency (%)
6
4
2
0
CTSC_ctrl
CTSC_shA1
F0
F1
F2
F3
D
Sphere forming efficiency (%)
10
8
6
4
2
0
DMSO
Nutlin-3
F0
F1
F2
F3


A
non-stained
CTSC_ctrl
CTSC_shA1
PKH26+
PE-A
PKH26 PE-A
FITC-A
B
% of PKH26 positive cells
CTSC_ctrl
CTSC_shA1
*
C
CTSC
CTSC_ctrl
CTSC_shA1
NUMB
GAPDH
D
CTSC_ctrl
CTSC_shA1
NUMB
E
% of cells with asymmetric NUMB
**


A
CTSC
CTSC_shA1
CTSC_ctrl
p53
p21
GAPDH
C
Relative luciferase activity
1.5
1.0
0.5
0.0
*
pGL3 + + - - -
p53-luc - - + + +
HMGA1 - 800 - 400 800
B
Relative promoter occupancy
25
20
15
10
5
0
-5
ctrl
shA1
region I
region II
region III
region IV
region V
GAPDH