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"Role of the Oncogene KDM2B in the Epigenetic Regulation of the Cytoskeleton"

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MSc Report

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ABSTRACT

Cancer can evolve from a combination of epigenetic and genetic abnormalities resulting in dysregulated gene expression and function. In cancer, many physiological cellular activities are altered; epithelial cells lose their polarity and cell-cell adhesion, and gain migratory and invasive properties, in a process that involves a dramatic reorganization of the cytoskeleton, accompanied by the formation of membrane protrusions required for invasive growth. Many molecules have been shown to be implicated in cancer. Several lines of evidence suggest that alterations in chromatin regulators have been linked to deregulated cytoskeletal functions and cell-cell adhesion, but the mechanisms behind these processes are now beginning to emerge. Focusing on the role of KDM2B and based on our hypothesis that in cancer, epigenetic alterations frequently result in the deregulation of the mechanisms maintaining normal cytoskeleton organization and cell-cell adhesion function, we will try to understand how chromatin regulation and epigenetic mechanisms influence the onset and progression of cancer.

In DU145 and HCT116 cells expressing either an shKDM2B or overexpressing KDM2B, we found that the expression levels of several proteins involved in the cytoskeleton and in cell-cell adhesion are altered. Especially, the expression levels of EZH2 upon depletion of KDM2B were downregulated and the expression levels of the cell adhesion protein E-cadherin upregulated. Also, we saw a significant change on the expression levels of the RhoGTPases family proteins, RHOA, RHOB, RHOC, RAC1 and CDC42. Especially, in both cell lines, the expression levels of RhoA, RhoB, RhoC and Cdc42 were downregulated upon knockdown of KDM2B, whereas Rac1 was upregulated. The altered expression levels of several proteins of the cytoskeleton and of adhesion junction upon overexpression or knockdown of KDM2B lead us to propose a model for increased migration rate for the cells that overexpress KDM2B. This was confirmed in a wound-healing assay and in a proliferation assay. Based on our results, we can conclude that KDM2B may directly regulate major signaling molecules controlling cellular physiology and may promote somehow motility and invasion.

By elucidating the underlying mechanism of how histone demethylase KDM2B interconnect with cytoskeleton organization and cell to cell adhesion, it will be established a basic framework of how epigenetic regulation controls the structure and function of

the cytoskeleton and adhesion junction and how deregulated chromatin regulators can impact basic aspects of cellular physiology. This research is highly innovative, as it constitutes the first focused effort towards elucidation of the cross-regulation between chromatin and the cytoskeleton. Using the mechanism of cytoskeleton regulation by KDM2B as an example, this project addresses fundamental questions and creates significant added value by developing important tools and expertise in this area. Future studies on the cancer epigenome and its regulation will redefine our understanding of the cancer cell and may reveal new targets for therapeutic intervention.

Chapter 1
INTRODUCTION

Chromatin is the macromolecular complex of DNA and histone proteins, which provides the scaffold for the packaging of our entire genome. The basic functional unit of chromatin is the nucleosome. It contains 147 base pairs of DNA, which is wrapped around a histone octamer, with two each of histones H2A, H2B, H3, and H4 *(Dawson & Kouzarides, 2012)*. All of its components are subject to covalent modification, which fundamentally alters the organization and function of these basic tenants of chromatin *(Nomenclature & Enzymes, 2007)*. These modifications are functionally linked and crossregulated, and their combination form a code for the interpretation of the genetic information. Some of these modifications are transferred from mother to daughter cells or even from one generation of a multicellular organism to the next. These heritable alterations in chromatin that are not accompanied by changes in the DNA sequence are termed epigenetic, and are necessary for the temporal and spatial interpretation of the genome. Alterations in chromatin structure impact fundamental cellular processes and play a central role in human disease, and, in particular, cancer.

Cancer can evolve from a combination of epigenetic and genetic abnormalities resulting in dysregulated gene expression and function *(Jones & Baylin, 2007)*. In cancer, many physiological cellular activities are altered, resulting in uncontrolled cell proliferation, unrestricted survival, and pronounced defects in cellular morphogenesis. In the metastatic process, which is a multi-step process, epithelial cells lose their polarity and cell-cell adhesion, and gain migratory and invasive properties, in a process termed epithelial-mesenchymal transition (EMT), which involves a dramatic reorganization of the cytoskeleton accompanied by the formation of membrane protrusions required for invasive growth. Many molecules have been shown to be implicated in cancer. Several lines of evidence suggest that alterations in chromatin regulators have now been linked to deregulated cytoskeletal functions and cell-cell adhesion, but the mechanisms behind these processes are now beginning to emerge. Several lines of evidence suggest that the cytoskeleton is regulated by epigenetic mechanisms. For example, daughter 3T3 fibroblast cells have similar actin-filament stress-fiber organization and motile behavior *(Albrecht-Buehler, 1977a; Albrecht-Buehler, 1977b)*, while cells that are genetically identical to wild-type cells pass on

alterations in the orientation of their cilia for hundreds of generations *(Beisson and Sonneborn, 1965; Sonneborn, 1964)*.

Actin cytoskeleton reorganization and signaling play an essential role in promoting cell movement and establishing intracellular adhesion, and are thus implicated in malignant cell growth and invasion *(Winder, 2005)*. The regulation of actin assembly and disassembly is under the control of complex signaling systems in which key actin regulators become deregulated during cancer progression *(Papakonstanti & Stournaras, 2008)*. The members of the Rho GTPase family are mainly responsible for integrating and transmitting signals for actin remodeling *(Hall, 2012; Kardassis et al., 2009)*. Rho GTPases work as sensitive molecular switches existing either in an inactive, GDP-bound form or an active GTP-bound form. Their activation is tightly controlled by three groups of regulatory proteins, guanine nucleotide exchange factors (GEF), GTPase-activating proteins (GAP), and guanine nucleotide dissociation inhibitors (GDI). GEF are responsible for the activation of Rho GTPases *(Hall, 2012; Papadimitriou et al., 2011)* by promoting the exchange of Rho-bound GDP by GTP. This is counteracted by GAP, which raises the intrinsic GTPase activity of Rho GTPases and the hydrolysis of bound GTP to GDP. Finally, GDI bind inactive Rho-GDP and prevent the interaction with RhoGEFs and thus its activation. The best-studied members of the Rho GTPase family are RhoA, Rac1 and Cdc42. In a simplified view, RhoA induces actin stress fiber formation and regulates cytoskeletal changes affecting cell-cell or cell-matrix adhesion, Rac1 is involved in lamellipodia and membrane ruffle formation, and Cdc42 is involved in filopodia formation *(Bishop and Hall, 2000)*. Downstream effectors of Rho include the serine/threonine kinase ROCK which is mainly involved in the formation of stress fibers and focal adhesions *(Papadopoulou et al., 2008)*. ROCK phosphorylates downstream myosin light chain (MLC) leading to actin–myosin contractility *(Konstantinidis et al., 2011)*. Another member of the RhoGTPase family, RhoB, acts as a tumor suppressor and is generally downregulated in cancers. RhoB is involved in the stability of integrin-mediated adhesions and integrin activity *(Ridley, 2013)*. Many studies until now suggest that RhoA, RhoB and RhoC act as tumor suppressors and are generally downregulated in cancers. In addition, depleted cells migrate faster *(Chen et al., 2000; Jiang et al., 2004 Liu et al., 2001 Croft & Olson, 2011; Prendergast, 2001)*.
Polarization of epithelial cells results from cadherin-induced

cytoskeletal organization utilizing the small GTPases Rho, Rac and Cdc42 *(Miyoshi and Takai, 2008)*. During progression to invasive carcinomas, epithelial cells undergo morphogenetic changes *(Yilmaz and Christofori, 2009)*, which are often associated with loss of junction components (such as E-cadherin).

The Trans-membrane glycoprotein E-Cadherin is one of the most important molecules that mediate intercellular adhesion in the epithelium *(Van Roy & Berx, 2008)*. Is localized on the surfaces of epithelial cells in regions of cell-cell contact known as adherens junctions *(Gumbiner, 1996)*. Besides its role in normal cells, this highly conserved gene can play a major role in malignant cell transformation, and especially in tumor development and progression. The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion. Most tumors have abnormal cellular architecture, and loss of tissue integrity can lead to local invasion. Thus, loss of function of E-cadherin tumor suppressor protein correlates with increased invasiveness and metastasis of tumors, resulting in it being referred to as the "suppressor of invasion" gene *(Vleminckx, 1991)*.
Whereas the extracellular domain of E-cadherin is responsible for the formation of this cell-cell binding, the adhesive function of the molecule is dependent on its interaction with the actin cytoskeleton through intracellular linker molecules, alpha- beta- and gamma-catenins *(Pecina Slaus, 2003)*.

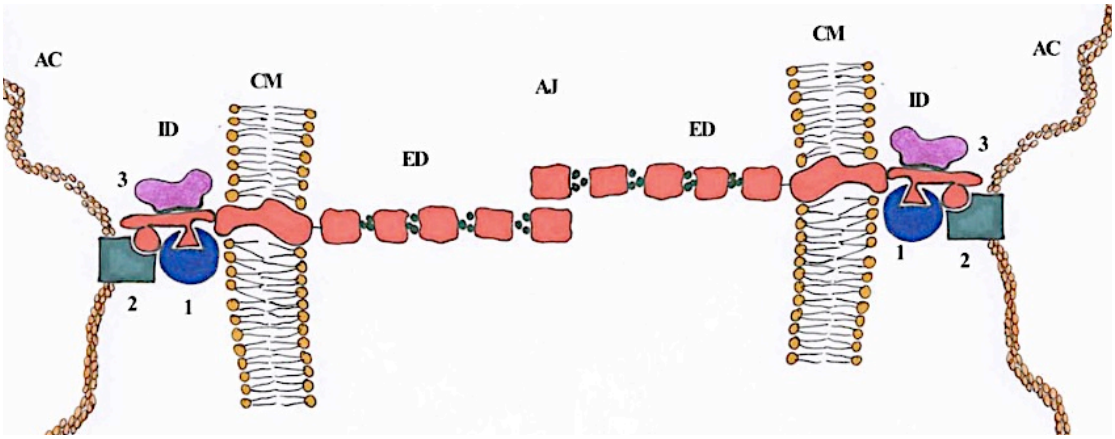


Figure 1: Schematic illustration of E-cadherin in adherens junction. E-cadherin homodimer on the cytoplasmic membranes of adjacent cells is shown. The juxtamembrane region with the interacting molecules is also shown. CM – cytoplasmic membrane; AJ – adherens junction; ED – extracellular domain; ID – intracellular domain; AC – actin cytoskeleton; 1-beta-catenin; 2-alpha-catenin; 3-p120 (Nives Pećina-Šlaus, 2003).

Tumor invasion is characterized by the disruption of cell-to-cell adhesion. Lots of evidence suggests that loss of E-cadherin expression is associated with the acquisition of invasiveness that contributes to metastasis *(Theveneau E. and Mayor R., 2012, Pecina Slaus, 2003, Chen et al., 1997)*. Therefore, E-cadherin is an important tumor suppressor gene.

KDM2B/NDY1

(i) Molecular Characterization of KBM2B/NDY1

The KDM2B, also known as NDY1, FBXL10 or JHDM1B, was first being isolated in a genome-wide screen for novel oncogenes, as a target of provirus integration in MoMuLV-induced rat T cell lymphomas *(Pfau et al., 2008)*.

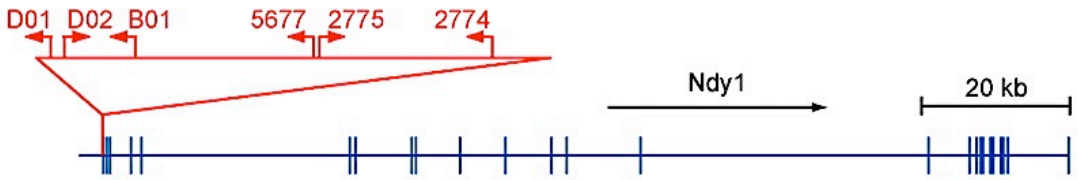


Figure 2: Provirus insertion activates the *Kdm2b1* gene. Sites and orientation of provirus integration at the 5' end of the *Kdm2b* gene. The numbers above the arrows showing the sites of provirus integration identify the tumors in which the integrations were detected *(Pfau et al., 2008)*.

It belongs to the family of KDM2 histone demethylases, which has two members, NDY1/KDM2B and NDY2/KDM2A. KDM2B is a lysine-specific demethylase protein that targets primarily histone H3K36me2 and H3K36me1. It also has a weak demethylase activity against histone H3K4me3 *(Tzatsos, Pfau, Kampranis, & Tsichlis, 2009)*, marks that lead to transcriptional repression.
Both members of this family are nuclear proteins that contain an N–terminal JmjC domain, a CXXC zinc–finger domain, a PHD zinc–finger, an F–box, and a leucine–rich repeat (LRR). In addition, they both contain a region rich in proline residues (PRR), which is located N–terminally to the F–box *(Figure 3)*. The CXXC motif binds unmethylated CpG DNA and contributes to the association of these proteins with their target genes *(Koyama–Nasu et al., 2007; Yamagishi et al., 2008)*. The PHD domain is a protein–protein interaction domain. By analogy with the PHD domains of other histone demethylases, it may mediate the binding of NDY1 and NDY2 to methylated histone tails *(Iwase et al., 2007; Shi et al., 2006; Taverna et al., 2007; Vermeulen et*

al., 2007; Wysocka et al., 2006b), or to other regulators of chromatin structure, such as histone deacetylases *(Barrett et al., 2007)*. The F-box domain identifies NDY1 and NDY2 as components of ubiquitin ligase complexes. The F-box is frequently coupled with C-terminal LRRs, and both domains function coordinately in the assembly of the ubiquitination machinery and the recognition of the target protein *(Kipreos and Pagano, 2000)*.

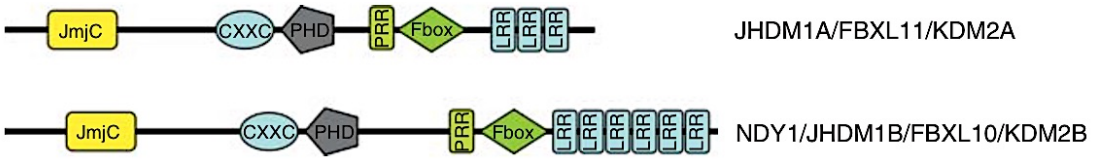


Figure 3: Domain structure of the JHDM1/KDM2 family *(Kampranis & Tsichlis, 2009)*.

Differential transcriptional initiation and alternative splicing give rise to several NDY1 isoforms. One of these isoforms, termed the short isoform *(Pfau et al., 2008)*, is particularly interesting because it is relatively abundant and lacks the JmjC-demethylase domain. The relative roles of these isoforms in cell biology and animal physiology remain to be determined.

The overexpression and the knockdown of NDY1 have diametrically opposite phenotypes in different cell types. Thus, in normal cells, such as mouse embryonic fibroblasts (MEFs), NDY1 promotes cell proliferation and functions as a physiological inhibitor of senescence *(Pfau et al., 2008)*. In HeLa cells, on the other hand, overexpression of NDY1 inhibits, and knockdown promotes, cellular proliferation *(Frescas et al., 2007; Koyama-Nasu et al., 2007)*. Interestingly, some of the cell proliferation inhibitory effects of NDY1 appear to be JmjC-domain independent *(Koyama-Nasu et al., 2007)*. Differences in phenotype correlate with differences in the subcellular distribution of the protein in the two cell types. In both types of cells the protein exhibits primarily a nuclear distribution. However, in MEFs it is excluded from the nucleoli *(Pfau et al., 2008)*, whereas in HeLa cells it is primarily nucleolar *(Frescas, Guardavaccaro, Bassermann, Koyama-Nasu, & Pagano, 2007)*. The subcellular distribution of NDY1 may explain the phenotypic differences of its overexpression in different cell types. In addition, it suggests that the protein may normally shuttle between the nucleoplasm and the nucleoli and that in some tumor cells the protein may be sequestered in the nucleolus because of defects in nucleolar retention or transport.

(ii) Catalytic mechanism and Substrate Specificity of JmjC-Domain-Containing Histone Demethylase KDM2B

JmjC–domain histone demethylase KDM2B catalyze histone lysine demethylation through an oxidative reaction that requires Fe(II) and α–ketoglutarate as cofactors. The catalytic reaction begins with the coordination of molecular oxygen (O_2) by Fe(II) and the conversion of α–ketoglutarate to succinate and CO_2 with the concomitant hydroxylation of the methyl group of the peptide substrate. The resulting carbinolamine is unstable and degrades spontaneously to unmethylated peptide and formaldehyde *(Figure 4) (Ozer and Bruick, 2007; Shi and Whetstine, 2007).*

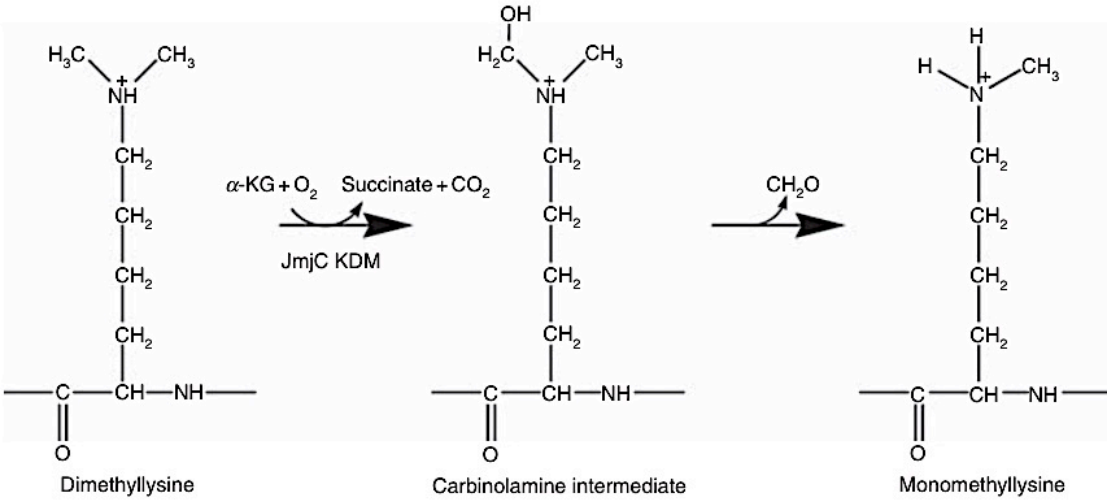


Figure 4: The reaction mechanism of the JmjC–domain–containing family of histone demethylases. The catalytic mechanism of JmjC–domain demethylases involves molecular oxygen (O_2) in the conversion of α–ketoglutarate to succinate and CO_2 with the concomitant hydroxylation of the methyl group of the peptide substrate. The resulting carbinolamine is unstable and breaks down to the unmethylated peptide with the release of formaldehyde (Ozer and Bruick, 2007; Shi and Whetstine, 2007).

(iii) KDM2B-Mediated Immortalization; Inhibition of Senescence

In normal cells, such as MEFs, KDM2B promotes cell proliferation and functions as a physiological inhibitor of senescence. Expression of single domain mutants of NDY1 in MEFs revealed that the immortalization phenotype depends on the JmjC domain and the CxxC-ZF motif, which mediates its genome-wide binding to unmethylated CpG islands, but is independent of the PHD domain, the F–box, or the C–terminal LRR domain *(Pfau et al.,*

2008). KDM2B is downregulated during senescence in mouse embryonic fibroblasts, while knockdown of endogenous KDM2B promotes senescence *(Tzatsos et al., 2009)*. Ectopic expression of KDM2B enables MEFs to undergo immortalization and bypass replicative senescence, while when KDM2B is expressed together with the oncogene Ras, it also inhibits oncogene-induced senescence *(Pfau et al., 2008; Tzatsos et al., 2009)*.
Cellular senescence is due to progressive telomere shortening, to the activation of the Ink4a–Arf–Ink4b locus, and to the activation of the DNA damage response *(Figure 5)*.

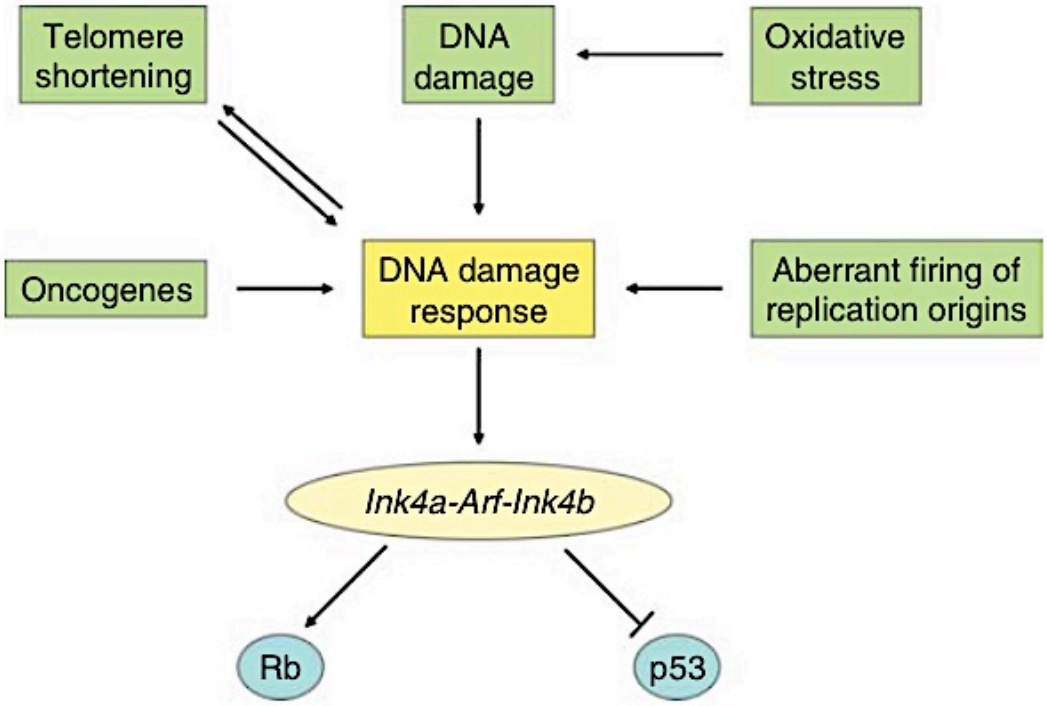


Figure 5: Different mechanisms promote cellular senescence through the Ink4a–Arf–Ink4b locus (Kampranis and Tsichlis, 2009).

KDM2B bypasses replicative and oncogene-induced senescence by co-operating with PRC complexes in the repression of the *Ink4a-Arf-Ink4b* locus. This locus encodes tree critical cell cycle cyclin-dependent kinase inhibitors, p16, p15 and p19, which in turn regulate the Rb pathway preventing G1/S phase transition in cells, and the p53 pathway respectively, that results to growth arrest or apoptosis. Both pathways result to senescence.
KDM2B function in this pathway downstream of the DNA damage response ((Tzatsos et al., 2009) and binds directly to this tumor suppressor locus, inhibiting its activation. This inhibition results in repression of the encoding by the locus proteins and to an upregulation of Rb phosphorylation, which results to

immortalization of the cells. KDM2B manage to repress this locus by an elaborate mechanism *(Figure 6)* in which EZH2 has also a role. EZH2 is associated with Polycomb Repressor Complex 2 and increased levels of it have been observed in solid tumors. It catalyzes the tri- and di-methylation of H3K27. These marks are specifically recognized by Polycomb Repressive Complex 1, in which KDM2B is a member.

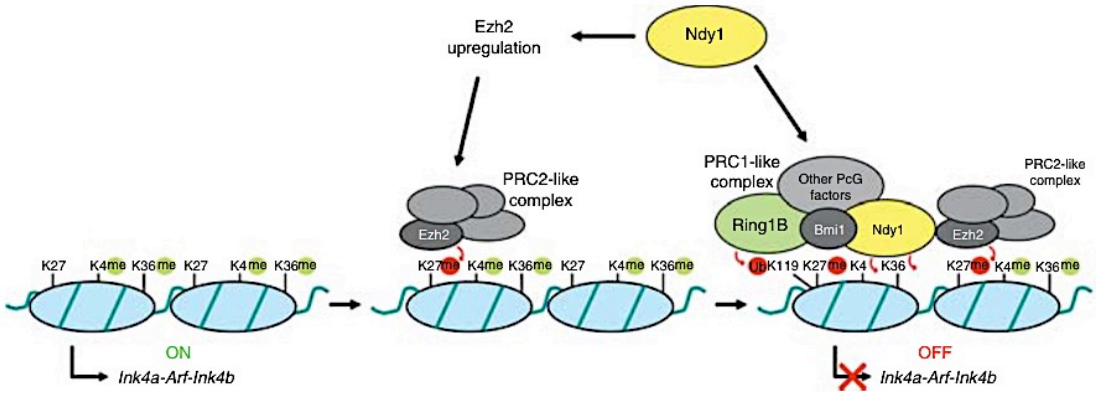


Figure 6: Schematic representation of the coordinated action of NDY1/KDM2B that leads to the repression of the Ink4a–Arf–Ink4b locus (Kampranis and Tsichlis, 2009).

On the one hand, KDM2B expression counteracts the senescence-associated downregulation of EZH2, resulting in elevated global H3K27me3 levels. Increased trimethylation of H3K27 is also observed specifically in the *Ink4a-Arf-Ink4b* locus and results in the recruitment of PRC1 to the locus. In addition, KDM2B is specifically recruited to the *Ink4a-Arf-Ink4b* locus via a PRC1-type complex and it removes local H3K36me2 and H3K4me3 marks. The F-box domain of KDM2B also participates in the ubiquitination of H2A by PRC1-associated Ring proteins *(Farcas et al., 2012; Wu et al., 2013)*. These histone modifications when combined, interfere with the binding of RNApolymerase II and contribute to the silencing of the locus (Figure 7) *(Tzatsos et al., 2009)*.

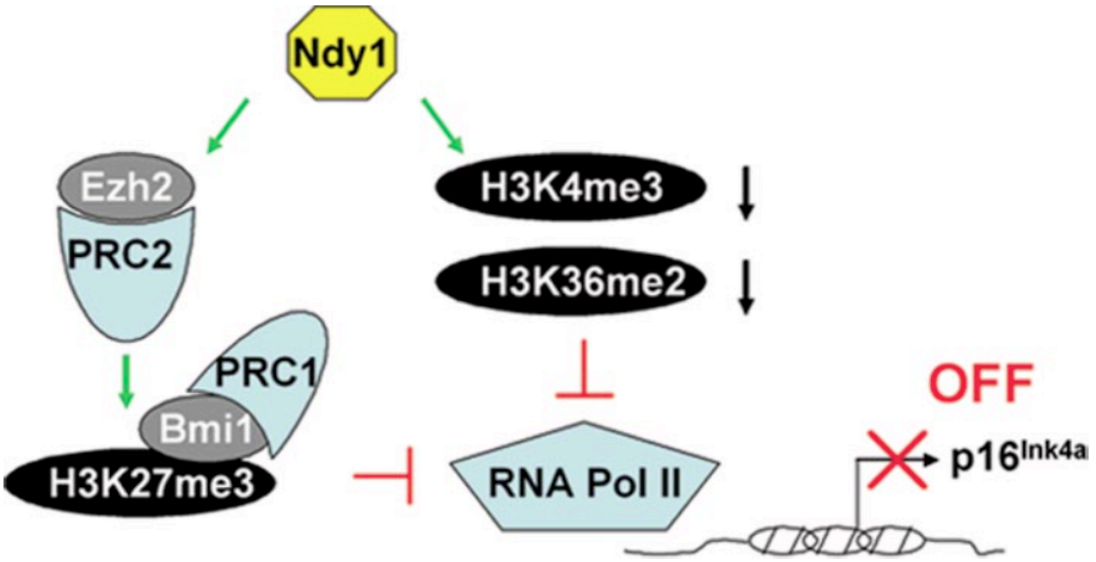


Figure 7: The histone demethylase KDM2B represses the Ink4a/Arf locus. KDM2B represses the Ink4a/Arf locus by 2 distinct mechanisms: it up-regulates EZH2 and promotes histone H3K27 trimethylation and Bmi1 binding within the Ink4a/Arf locus, and it binds to and demethylates H3K36me2 and H3K4me3 within the Ink4a/Arf locus. These histone modifications, when combined, interfere with the binding of RNA Pol II and contribute to the silencing of the Ink4a/Arf locus (Tzatsos et al., 2009).

Repression of the *Ink4a-Arf-Ink4b* by KDM2B is required for initiation and maintenance of acute myeloid leukemia *(He et al., 2011)*. Forced expression of KDM2B maintains self-renewing hematopoietic stem cells *(Konuma et al., 2011)*, and is sufficient to transform hematopoietic progenitors, while, depletion of KDM2B in hematopoietic progenitors significantly impairs Hoxa9/Meis1-induced leukemic transformation. In leukemic stem cells, knockdown of KDM2B impairs their self-renewing capability in vitro and in vivo *(He et al., 2011)*. KDM2b maintains murine embryonic stem cell status by recruiting PRC1 to CpG islands of developmental genes *(He et al., 2013; Wu et al., 2013)*. Kdm2b is highly expressed in mESCs and regulated by the pluripotent factors Oct4 and Sox2 directly. Depletion of Kdm2b in mESCs causes de-repression of lineage-specific genes and induces early differentiation *(He et al., 2013)*. As we said before, the function of KDM2B depends on its CxxC-ZF domain, which mediates its genome-wide binding to CpG islands *(Farcas et al., 2012; He et al., 2013; Wu et al., 2013)*. KDM2B interacts with Ring1B and Nspc1, forming a noncanonical PRC1 that is required for H2AK119ub1 in mouse ESCs. ESCs lacking KDM2B cannot differentiate properly *(Wu et al., 2013)*. Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) resets the epigenome to an embryonic-like state. KDM2B accelerates cell

cycle progression and suppresses cell senescence during vitamin C-induced reprogramming by repressing the *Ink4a-Arf-Ink4b* locus *(Wang et al., 2011)*. KDM2B also has the capacity to promote iPSC generation in a vitamin C-independent manner, by co-operating with reprogramming factors Oct4, Sox2, and Klf4. This capacity depends on its demethylase and DNA-binding activities, but is largely independent of its role in antagonizing senescence. KDM2B functions at the beginning of the reprogramming process and enhances activation of early responsive genes in reprogramming *(Liang et al., 2012)*.

As we said before, many molecules have been shown to implicate in cancer. Several lines of evidence suggest that alterations in chromatin regulators have now been linked to deregulated cytoskeletal functions and cell-cell adhesion *(Asensio-Juan et al., 2012; Kottakis et al., 2011; Su et al., 2005)*, but the detailed mechanisms behind these processes are now beginning to emerge.
The long-term goal of this project is to understand how chromatin regulation and epigenetic mechanisms influence the onset and progression of cancer. For this we will focus on the epigenetic regulation of one of the main cellular machineries involved in the metastatic process, the cytoskeleton. Our central hypothesis is that, in cancer, epigenetic alterations frequently result in the deregulation of the mechanisms maintaining normal cytoskeleton organization and cell-cell adhesion function, which in turn promotes motility and invasiveness. To address this hypothesis, it is important to elucidate the chromatin factors that are involved in the regulation of the cytoskeleton, to decipher the epigenetic mechanisms by which these critical cellular factors regulate the expression of cytoskeleton genes, and to establish how these mechanisms contribute to tumor survival, invasiveness and migration. By addressing these specific questions we will establish a basic framework of how epigenetic regulation controls the structure and function of the cytoskeleton and how deregulated action of chromatin regulators can impact basic aspects of cellular physiology through the cytoskeleton. In the long term, this research will enable us to determine the contribution of specific epigenetic mechanisms to cancer and may provide novel targets for intervention.
Focusing on the role of KDM2B and based on our hypothesis that in cancer, epigenetic alterations frequently result in the deregulation of the mechanisms maintaining normal cytoskeleton organization and cell-cell adhesion function, we will try to

understand how chromatin regulation and epigenetic mechanisms influence the onset and progression of cancer.

Chapter 2
MATERIALS AND METHODS

2.1 Cell Culture

Human DU145 and HCT116 cells will be cultured in RPMI, containing 10% FBS and 1%penicillin/streptomycin.

2.2 Lentiviral and Retroviral packaging and transduction

The cells will be transduced with retroviruses and lentiviruses, in order to either overexpress or to silence genes of interest and evaluate their impact.

Virus Production:

-Day 1: Plate HEK 293T cells in 100mm dishes

-Day 2: Transfection

The total volume of complexes (DNA + transfection reagent) is 600ul

For fugene use 20ul/transfection

DNA volume +20ul Fugene + OptiMEM = 600ul (for example 25 ul DNA + 20ul fugene + 555ul optiMEM)

1. For retro virus: 6ug construct + 2ug amphopac (amphopac: 0.5ug/ul)
   For lenti virus: 3ug construct + 1ug pVSV-G + 2ug + pΔ8.1 (pVSV-G: 0.8ug/ul, pΔ8.1: 0.4ug/ul)
   In an eppendorf combine the appropriate plasmids and bring volume of DNA to the minimum possiible with TE or optiMEM (for example 25ul)
2. In an eppendorf add the optiMEM (for example 555ul) and then 20ul fugene (be carefull not to touch the tube sides). Incubate at RT for 5min
3. Add to the fugene mixture the DNA, incubate at RT for 15min
4. Change the medium to DMEM + 10% FBS –Pen/Strep (8ml/dish).
5. Add complexes to the cells dropwise and incubate O/N

-Day3: Change to complete medium (DMEM + 10% FBS + 1% Pen/Strep), 10ml/dish

-Day4: Collect 24hrs virus

Carefully collect the culture supernatant and replace with fresh complete medium. Centrifuge at 2500rpm, 4˚C, 10min (optional). Filter supernatant through 0.45um filter. Aliquot (approx. 3ml/tube) in 15ml tubes and store at -80 ˚C

-Day 5: Collect 48hrs virus

Same procedure as for 24hrs

For overexpression of KDM2B: pBABEpuro-KDM2B and control empty vector (pBABEpuro) RETRO VIRUS

For depletion of KDM2B: pLKO.1-KDM2B-sh and control pLKO.1-scrambled-sh LENTI VIRUS

2.3 Protein extraction

DU145s and HCT116s cells will be plated in RPMI containing 10% FBS and they will be grown to confluency. Confluent cultures will be washed three times to remove floating cells with PBS 1%. Following washing, the cells will be scratched and collected after adding 700μl of the lysis buffer solution. Lysis buffer solution contains protease inhibitors 25x, NaF 1M, β-glycerophosphate 1M, Na3NO4 1M in Tris-HCl 50mM pH7.5, NaCl 150mm Ph7.5, triton X-100 1%, SDS 0,2% and Deoxycholate 0,1%.

2.4 Protein extraction by sonication

The cells were disrupted by sonication with a microprobe for minimal sonication time, using short bursts to prevent heating of the sample. The sonication was repeated 3 times, for 30 seconds each and 10% pulse. It is important to make sure that the cell suspension is kept chilled packed with crushed ice during sonication. The samples were then stored at -80°C.

2.5 Determination of protein concentration with Lowry Assay

Protein quantification is often necessary prior to handle protein samples for isolation and characterization. It is a required step before submitting protein samples for chromatographic, electrophoretic and immunochemical separation or analyses.

Lowry assay is a popular, simple, rapid, inexpensive, sensitive and reproducible assay for the quantification of proteins. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01–1.0 mg/mL and is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction).

In a 1,5ml eppendorff tube, 195μl of water were mixed with 5μl of the protein solution and 100μl reagent A and 800μl reagent B of the kit. After incubation for 15mins in 25°C, the mixture was analyzed by spectrophotometry at OD_{750} nm using lysis buffer mixed with Lowry solution as a blank. Known BSA concentrations were used to generate a linear curve in order to calculate the unknown concentrations of our samples.

2.6 RNA extraction

Solutions: TRIZOL Reagent, 75% Ethanol, Chloroform, Isopropanol (2-propanol), RNase free water.

DU145s and HCT116s cells were plated in RPMI containing 10% FBS and were grown to confluency. Confluent cultures were washed three times to remove floating cells with PBS 1%. Following washing was added Trizol Reagent in the plates and incubated at 15-30°C for 5 minutes. The cells were scratched with a cell scraper and collected in

eppendorffs. Once homogenized, samples were centrifuged at 11,000rpm for 5 minutes at 4°C to remove cell debris. The supernatant was transferred to new tube. After that, was added 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. The samples were vortexed vigorously for 15 seconds and were incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 11,000rpm for 5 minutes at 4°C and the upper aqueous phase was transferred carefully without disturbing the interphase into fresh tube. In the supernatant was added 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization and the samples were incubated at room temperature for 10 minutes. After centrifuged at max speed for 5 minutes at 4°C and the removal of the supernatant completely, the RNA pellet was washed with 75% ethanol at 7,000rpm for 5 minutes at 4°C. The above washing procedure was repeated twice and all leftover ethanol was after removed. The RNA was dissolved in RNase free water.

2.7 Western Blot

In a Western blot, proteins that are separated on polyacrylamide gels on the basis of size are transferred to a membrane and then specifically detected in the immunoassay step using specific antibodies to the protein. The technique involves first the transfer of the proteins from the gel onto a membrane made of nitrocellulose or PVDF, then the blocking, where the transfer membranes are blocked with a concentrated protein solution (e.g. non-fat milk powder) to prevent further non-specific binding of proteins. The blocking step is followed by the incubation of the membrane in a diluted antibody solution, the washing of the membrane, the incubation in a diluted conjugated probe antibody or other detecting reagent, further washing, and finally the detection.

Solutions:

100% Methanol

Running Buffer 10x: in 1L Water was added 30,3 gr Tris, 10gr SDS and 144,2 gr Glycine. The pH was adjusted to 8,3.

Transfer Buffer: 70% Water, 10% Running buffer 10x and 20% Methanol 100%.

Blocking solution: 5% powder milk in 1X TBS-T.

Primary antibody stock: the primary antibodies were prepared in 1X TBS-T solution containing 5% BSA and 0,02% NaN_3. The antibody was then stored at 4°C. The antibody was prepared at 1:100 or 1:1000 dilution.

Secondary antibody stock: secondary antibodies were prepared in 1X TBS-T solution containing 5% milk. HRP-anti mouse and anti-rabbit antibody was prepared at 1:10000 dilution.

Western blotting involves transferring of electrophoretically separated components from a gel to a solid support, such as a nitrocellulose or PVDF. Western blotting is extremely useful for the identification and

quantitation of specific proteins in a complex mixture of proteins. A 1-5 ng of an average sized protein can be detected by western blotting.
The gel cassette was opened and lifted gently and the gel was cut, stacking gel was thrown away, resolving gel was immersed in transfer buffer. A piece of the nitrocellulose or the PVDF membrane was cut to the dimension of the gel. One corner of the membrane was notched for later correspondence with the corner of the gel. The PVDF membrane was then activated by wetting in 100% methanol for 5 minutes. The 3 MM paper, the PVDF membrane and the gel were assembled in the cassette in the following order starting from the black part: foam pad, paper, gel, membrane, paper, foam pad. Care should be taken to exclude bubbles between gel and nitrocellulose, and between nitrocellulose and paper. The cassette was closed and placed in the tank blotting apparatus so that the side of the cassette holder with the gel was facing the cathode. Transfer buffer was then added to the blotting apparatus until the cassette was totally covered. The tank was then connected to the power output and the system was turned on for 1 hour at 400 mA. The tank was soaked in ice to prevent overheating of the buffer during the transfer process. After removing the foam pad and filter papers, the membrane was blocked in 5% milk to allow the saturation of all non-specific protein-binding sites on the blots, for 1 hour and then washed three times for 5 minutes each in 1X TBS-T. The membrane was then transferred to a plastic bag containing the primary antibody, and it was incubated slowly shaking at over-night at 4^{o}C. The membrane was washed 3 times for 5 minutes each in 1X TBS-T. The membrane was transferred to a new plastic bag, and the HRP second antibody was added and incubated with the membrane for 2 hours at room temperature. The membrane was washed again 3 times for 5 minutes each in 1X TBS-T. The membrane was incubated with 1 ml of each detection buffer (BioRAD Laboratories) for 5 minutes, away from light. The bands were developed and were visible. The size of protein of interest was identified by comparison with the molecular marker.

2.8 Real-time RT-PCR

Total cell RNA will be isolated using Trizol. cDNA will be synthesized from 1.0 μg of total RNA, using oligo-dT priming and an appropriate reverse transcription kit. Specific primers will be used to amplify the genes of interest so their expression levels will be evaluated. Real-time PCR will be performed in triplicate using the Universal SYBR Green PCR master mix kit and a—7500 Real-Time System (Applied Biosystems). mRNA levels will be normalized to *ACTIN* which will be used as an internal control.

2.9 Actin cytoskeleton analysis by Immunofluorescence

For direct fluorescence microscopic analysis of the actin cytoskeleton, cell monolayers under different conditions will be serum starved for 24 hours. Staining will be performed using Rhodamine-phalloidin at 1:100

dilution. All secondary antibodies will be used in a 1:100 dilution. Slides will be mounted using the ProLong Gold Antifade Reagent. Photomicrographs will be obtained with a LEICA microscope and will be photographed with a LEICA DC 300F digital camera, using the Zeiss Plan-neofluar 40×/0.75 objective lens. The images will be analyzed with the instrument's software.

2.10 Nondirectional migration – Wound healing assay

DU145s will be plated in RPMI containing 10% FBS and they will be grown to confluency. Confluent cultures will be serum-starved for 24 hours, and serum-starved, cofluent cell monolayers will be wounded with a plastic pipette tip and they will be washed three times to remove floating cells with PBS 1%. Following washing, the cells will be cultured in RPMI without FBS. The wounded area will be photographed 6, 12, 24 and 30 hours later.

Chapter 3
RESULTS

Based on our hypothesis and on preliminary data, indicating that deregulation of the histone demethylase KDM2B results in alterations of several proteins involved in cytoskeleton organization (*Kottakis et al., 2011)*, we decided to investigate further the underlying mechanism.
We have worked with two cell lines; human prostate cancer DU145 cells and human colon cancer HCT116 cells *(Figure 1)*.

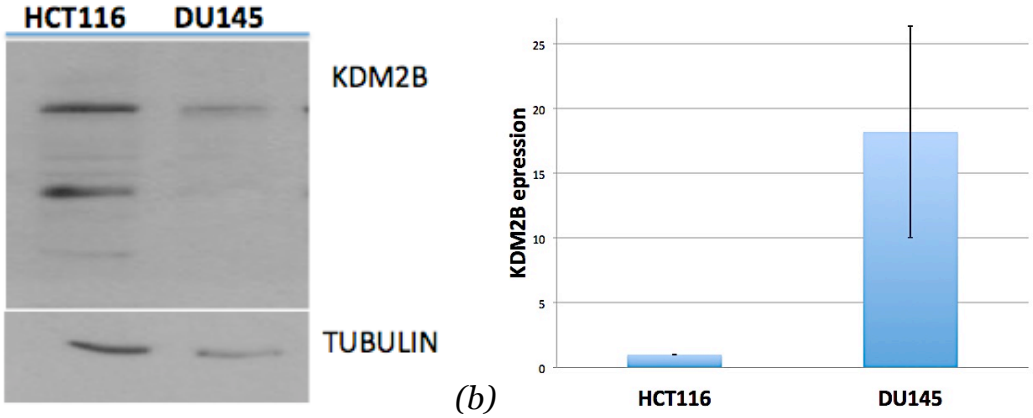
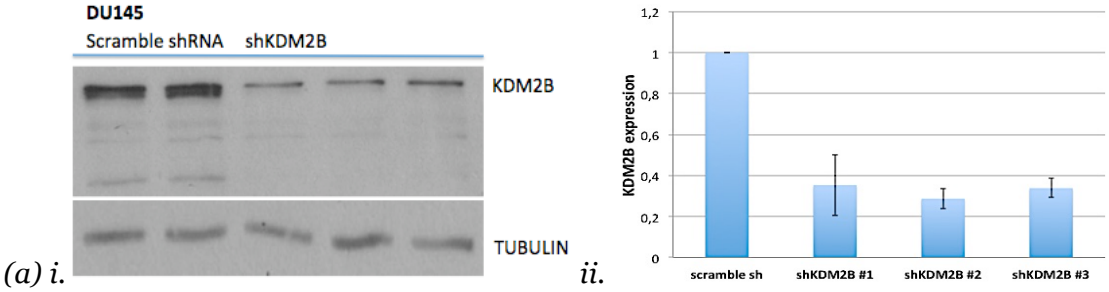


Figure 1: Basal expression levels of KDM2B in DU145 and HCT116 cell. (a) Western Blot of KDM2B in DU145 and HCT116 cells. (b) mRNA expression levels of KDM2B in DU145 and HCT116 cells.

Starting with knockdown and overexpression of KDM2B in DU145 and HCT116 cells *(Figure 2),* we examined a number of proteins, which their expression levels may possible be altered upon different expression levels of KDM2B. The knockdown and overexpression of KDM2B in both cell lines were confirmed with qRT-PCR, also *(Figure 2)*.



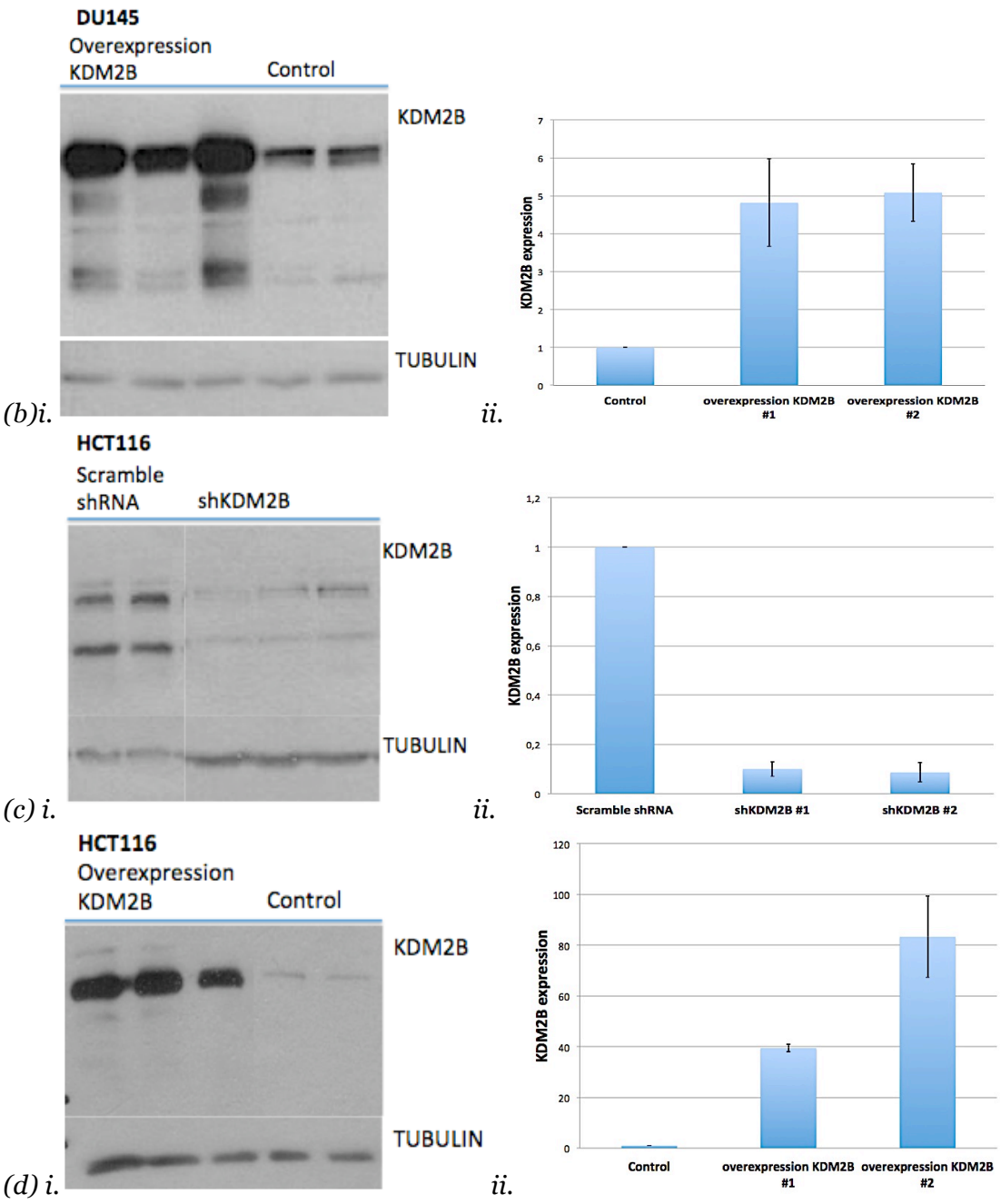


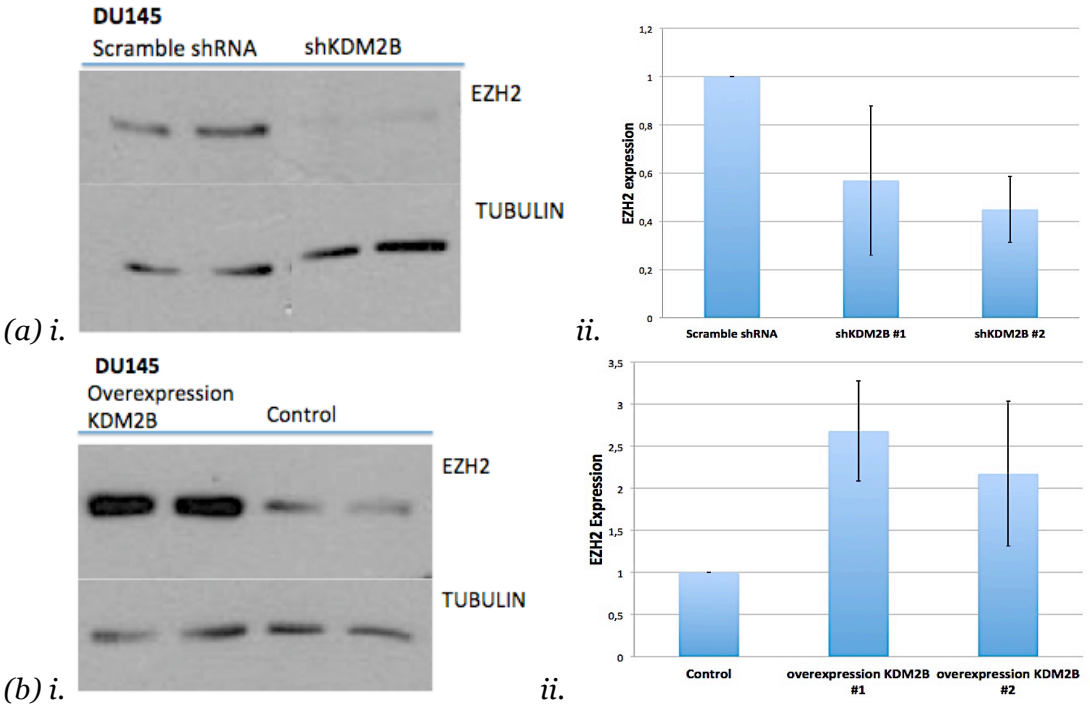
Figure 2: Western blot of KDM2B expression and results from RT-PCR showing mRNA expression of KDM2B in DU145 and HCT116 cells.
(a) i. Expression levels of KDM2B in shKDM2B and scramble shRNA DU145 cells. ii. mRNA expression levels of KDM2B in shKDM2B and scramble shRNA in DU145 cells.
(b) i. Expression levels of KDM2B in overexpression of KDM2B and control DU145 cells. ii. mRNA expression levels of KDM2B in overexpression of KDM2B and control DU145 cells.
(c) i. Expression levels of KDM2B in shKDM2B and scramble shRNA HCT116 cells. ii. mRNA expression levels of KDM2B in shKDM2B and scramble shRNA in HCT116 cells.

(d) i. Expression levels of KDM2B in overexpression of KDM2B and control HCT116 cells. ii. mRNA expression levels of KDM2B in overexpression of KDM2B and control HCT116 cells.

The levels of KDM2B in the shKDM2B DU145 cells were decreased almost 60%, whereas in the overexpression of KDM2B samples, the expression levels of KDM2B were upregulated 3 to 5 times. In HCT116 cells, the levels of KDM2B in the shKDM2B samples were decreased almost 90% and in the overexpression of KDM2B samples, the expression levels of KDM2B were increased 40 to 80 times.

<u>KDM2B alters EZH2 and E-CADHERIN expression</u>

EZH2 is a transcriptional repressor that has a crucial function in maintaining the delicate homeostatic balance between gene expression and repression, the disruption of which may lead to oncogenesis. As we expected, the expression levels of Ezh2 were changed in the same manner as those of KDM2B, in both cell lines. Upon depletion of KDM2B the levels of Ezh2 were decreased and upon overexpression the levels of Ezh2 were upregulated *(Figure 3)*. The same results were confirmed with qRT-PCR, also.



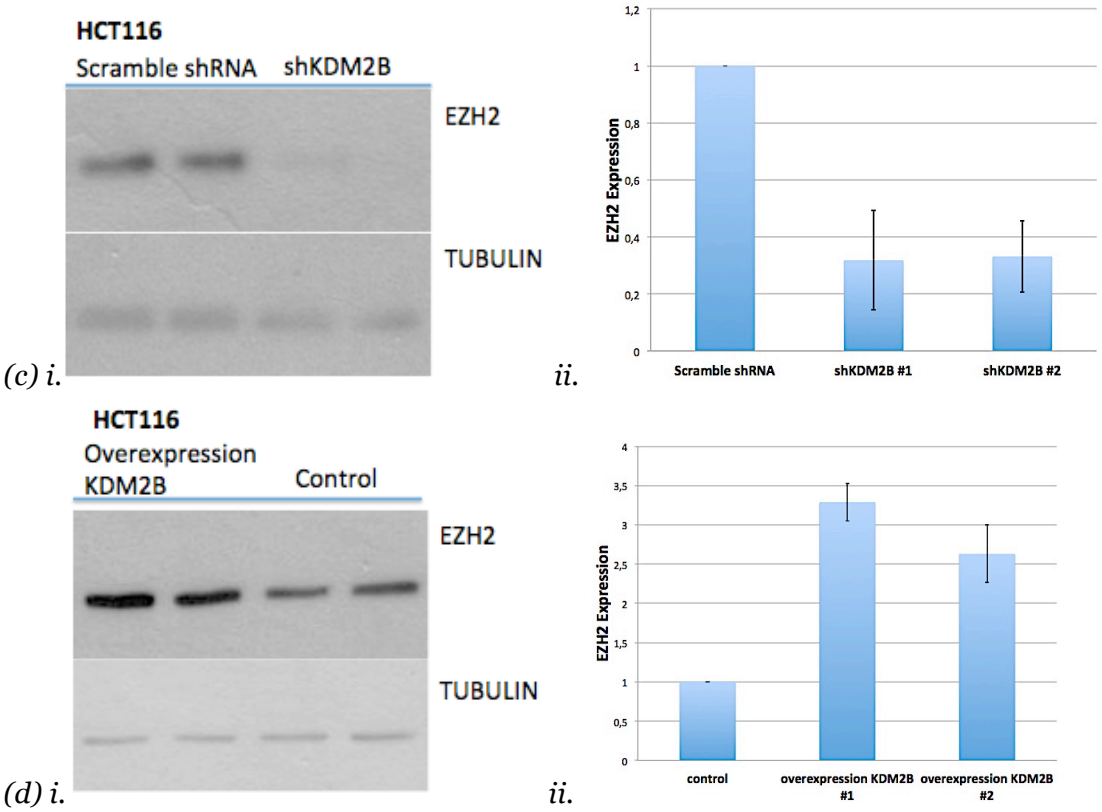
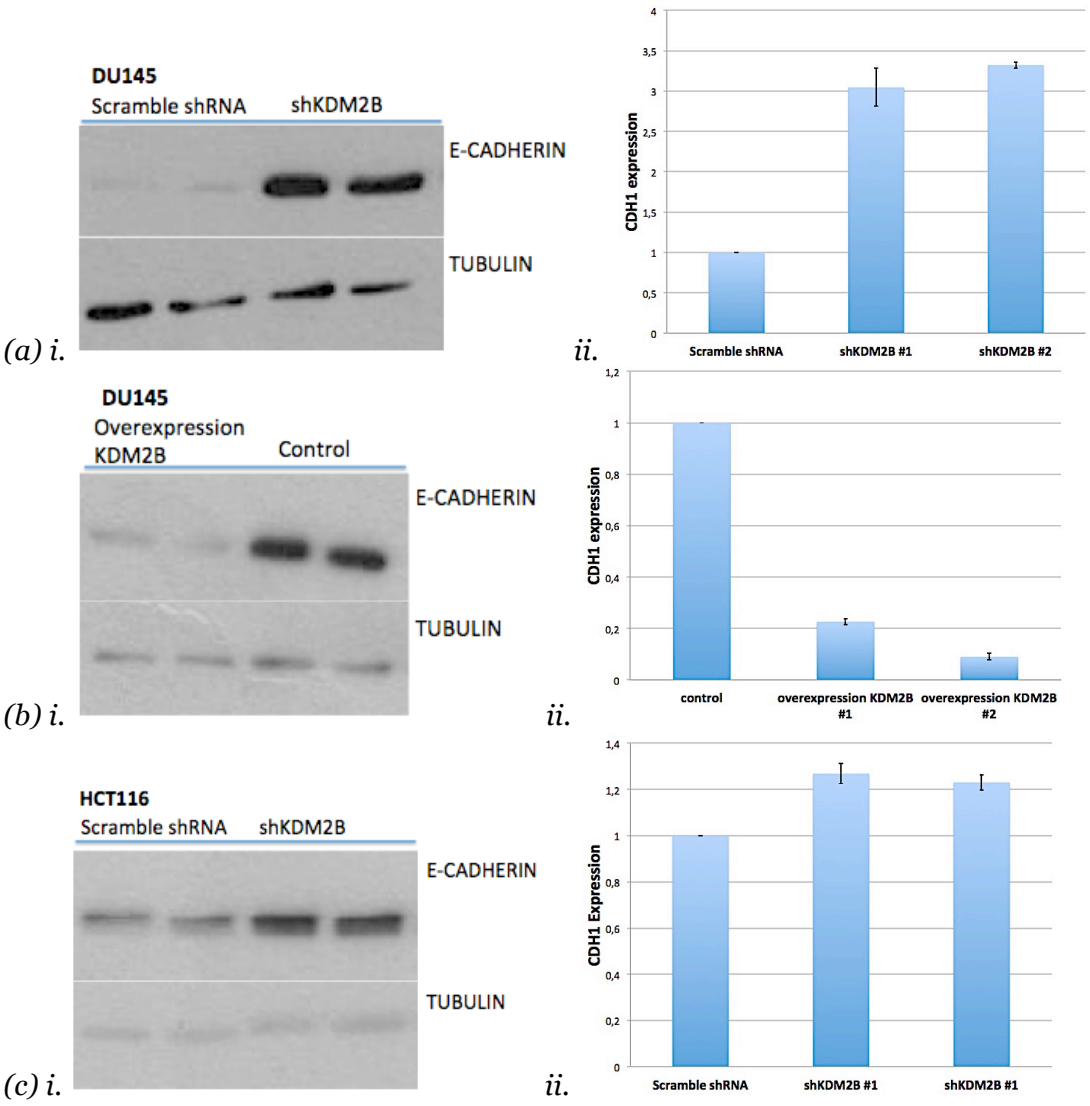
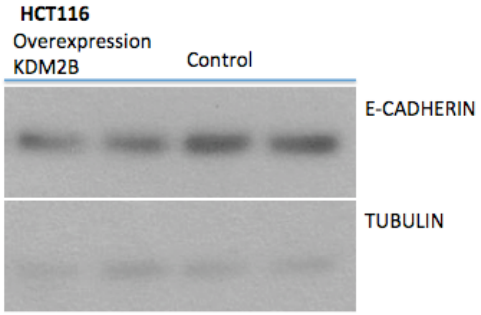


Figure 3: Western blot of EZH2 expression and results from RT-PCR showing mRNA expression of EZH2 in DU145 and HCT116 cells.
(a) i. Expression levels of EZH2 in shKDM2B and scramble shRNA DU145 cells. ii. mRNA expression levels of EZH2 in shKDM2B and scramble shRNA in DU145 cells.
(b) i. Expression levels of EZH2 in overexpression of KDM2B and control DU145 cells. ii. mRNA expression levels of EZH2 in overexpression of KDM2B and control DU145 cells.
(c) i. Expression levels of EZH2 in shKDM2B and scramble shRNA HCT116 cells. ii. mRNA expression levels of EZH2 in shKDM2B and scramble shRNA in HCT116 cells.
(d) i. Expression levels of EZH2 in overexpression of KDM2B and control HCT116 cells. ii. mRNA expression levels of EZH2 in overexpression of KDM2B and control HCT116 cells.

Ezh2 expression increases with tumor progression and alteration in its expression levels changes the invasive phenotype of the cells. In addition, knockdown Ezh2 cells have less invasive properties *(Cao et al., 2008)*. Also, pre-clinical studies showed that Ezh2 is able to silence several anti-metastatic genes (e.g., E-cadherin), thereby favoring cell invasion and anchorage-independent growth *(Crea et al., 2012)*. Tumor invasion is characterized by the disruption of cell-to-cell adhesion. One of the most important molecules that mediate intercellular adhesion in the epithelium is the trans-membrane glycoprotein E-Cadherin. Lots of evidence

suggests that loss of E-cadherin expression is associated with the acquisition of invasiveness that contributes to metastasis.
So, based on this, we examined the protein levels of E-cadherin in both cell lines. As expected, the levels of the cell adhesion protein E-cadherin were increased upon KDM2B knockdown and decreased in the overexpression samples, in both cell lines *(Figure 4)*. The different expression levels of E- cadherin upon knockdown and overexpression of KDM2B were confirmed in both cell lines with qRT-PCR, also.



(d) i. 

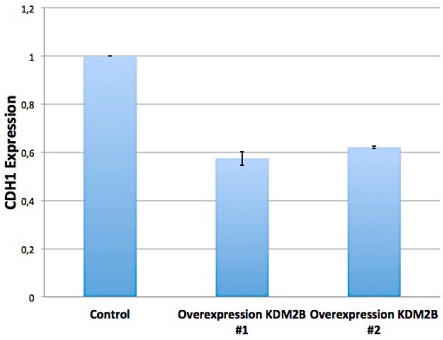
ii. 

Figure 4: Western blot of E-CADHERIN expression and results from RT-PCR showing mRNA expression of E-CADHERIN in DU145 and HCT116 cells.
(a) i. Expression levels of E-CADHERIN in shKDM2B and scramble shRNA DU145 cells. ii. mRNA expression levels of E-CADHERIN in shKDM2B and scramble shRNA in DU145 cells.
(b) i. Expression levels of E-CADHERIN in overexpression of KDM2B and control DU145 cells. ii. mRNA expression levels of E-CADHERIN in overexpression of KDM2B and control DU145 cells.
(c) i. Expression levels of E-CADHERIN in shKDM2B and scramble shRNA HCT116 cells. ii. mRNA expression levels of E-CADHERIN in shKDM2B and scramble shRNA in HCT116 cells.
(d) i. Expression levels of E-CADHERIN in overexpression of KDM2B and control HCT116 cells. ii. mRNA expression levels of E-CADHERIN in overexpression of KDM2B and control HCT116 cells.

After seeing this significant upregulation of E-cadherin upon KDM2B knockdown, we wanted to see if this change is obvious in immunofluorescence. We fixed DU145 cells expressing either shKDM2B or scramble shRNA with Meth or PFA/triton and stained for E-cadherin and nuclei. The increased levels of E-cadherin upon KDM2B knockdown, compared to the control samples, were obvious *(Figure 5)*.

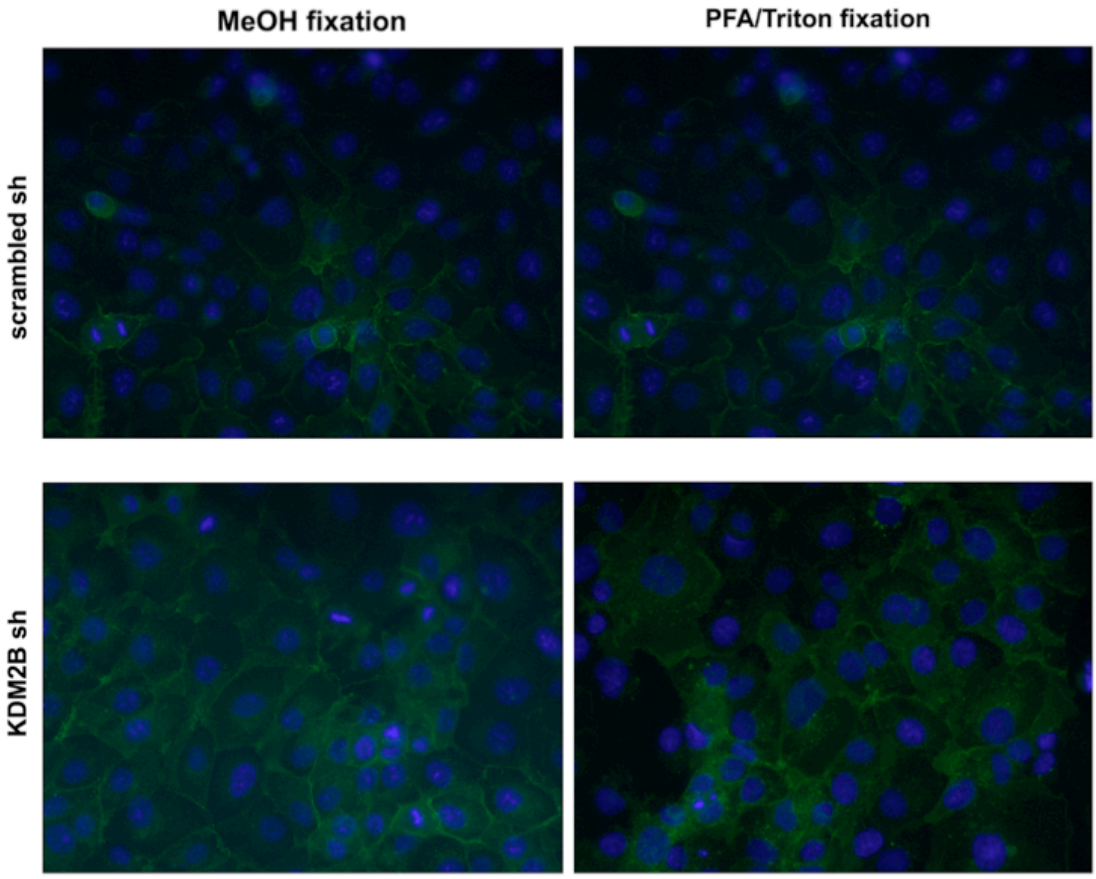


Figure 5: Immunofluorescence of E-CADHERIN in DU145. DU145 cells expressing either shKDM2B or scramble shRNA were fixed with Meth or PFA/triton and stained for e-cadherin (green) and nuclei (blue).

HCT116 overexpressing KDM2B cells were also fixed with PFA/triton and stained for E-cadherin and nuclei. Now, the lower levels of E-cadherin were clearly in the overexpressing of KDM2B cells, compared to the control *(Figure 6)*.

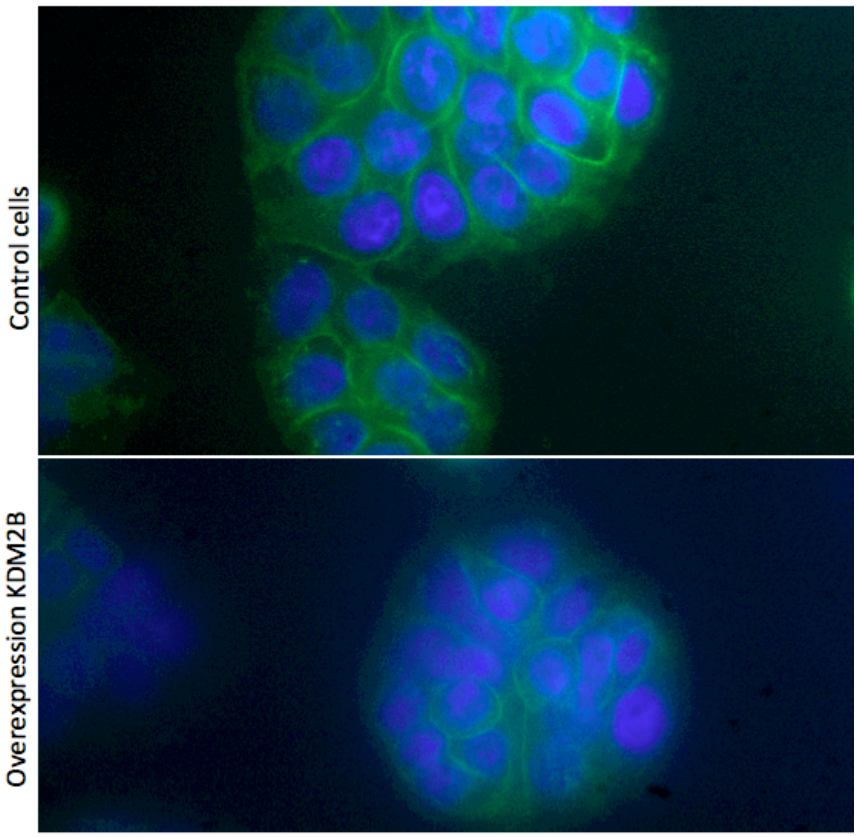


Figure 6: Immunofluorescence of E-CADHERIN in overexpressing KDM2B HCT116 cells. HCT116 cells were fixed with PFA/triton and stained for e-cadherin (green) and nuclei (blue).

KDM2B alters the expression levels of RhoGTPases

The adhesive function of E-cadherin is dependent on its interaction with the actin cytoskeleton through intracellular linker molecules. In this view, we started to check the expression levels of proteins of the cytoskeleton, such as PAXILLIN, VINCOULIN, FAK, ACTIN, but we did not see any differences in the protein levels between the control and the shKDM2B or overexpressing of KDM2B cells, in neither of the two cell lines *(Figure 7)*.

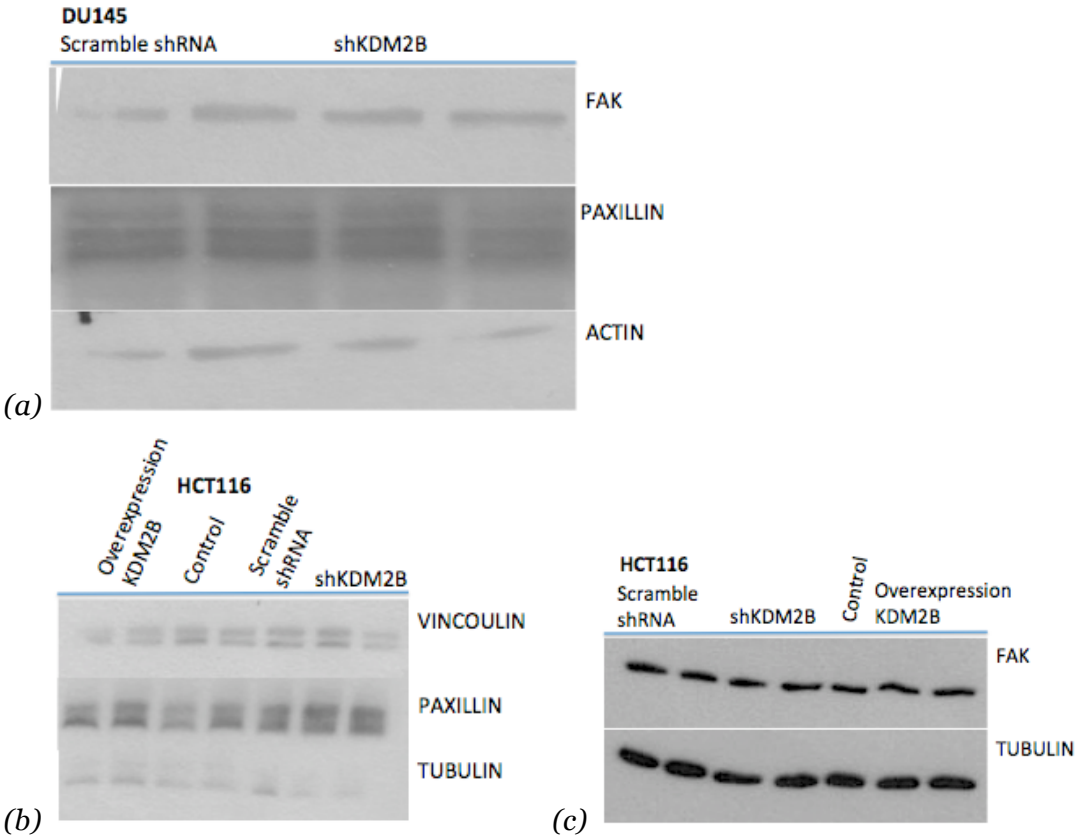
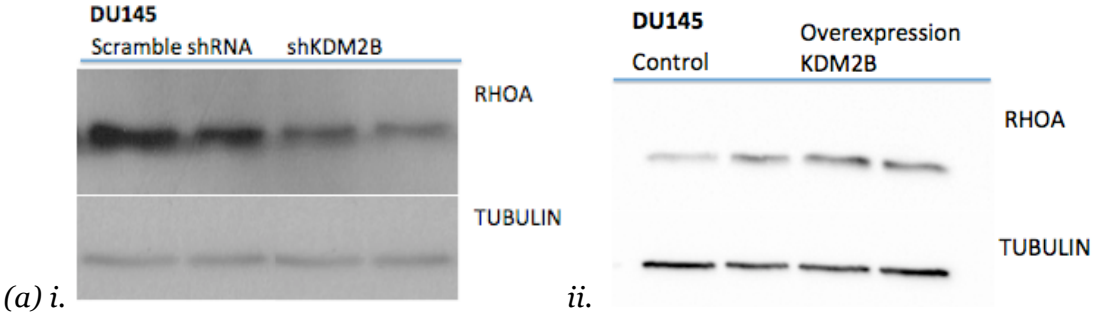


Figure 7: Western blot of cytoskeleton proteins expression. (a) Expression levels of FAK and PAXILLIL in shKDM2B and scramble shRNA DU145 cells. (b) Expression levels of VINCOULIN and PAXILLIN in overexpression of KDM2B, control, shKDM2B and scramble shRNA HCT116 cells. (c) Expression levels of FAK in shKDM2B, scramble shRNA, overexpression of KDM2B and control HCT116 cells.

However, despite the unchanged expression levels of the above cytoskeleton proteins, we saw a significant change on the expression levels of the RhoGTPases family proteins, RHOA, RHOB, RHOC, RAC1 and CDC42. Especially, in both cell lines, the expression levels of RhoA, RhoB, RhoC and Cdc42 were downregulated upon knockdown of KDM2B, whereas Rac1 was upregulated *(Figure 8 and 9)*.



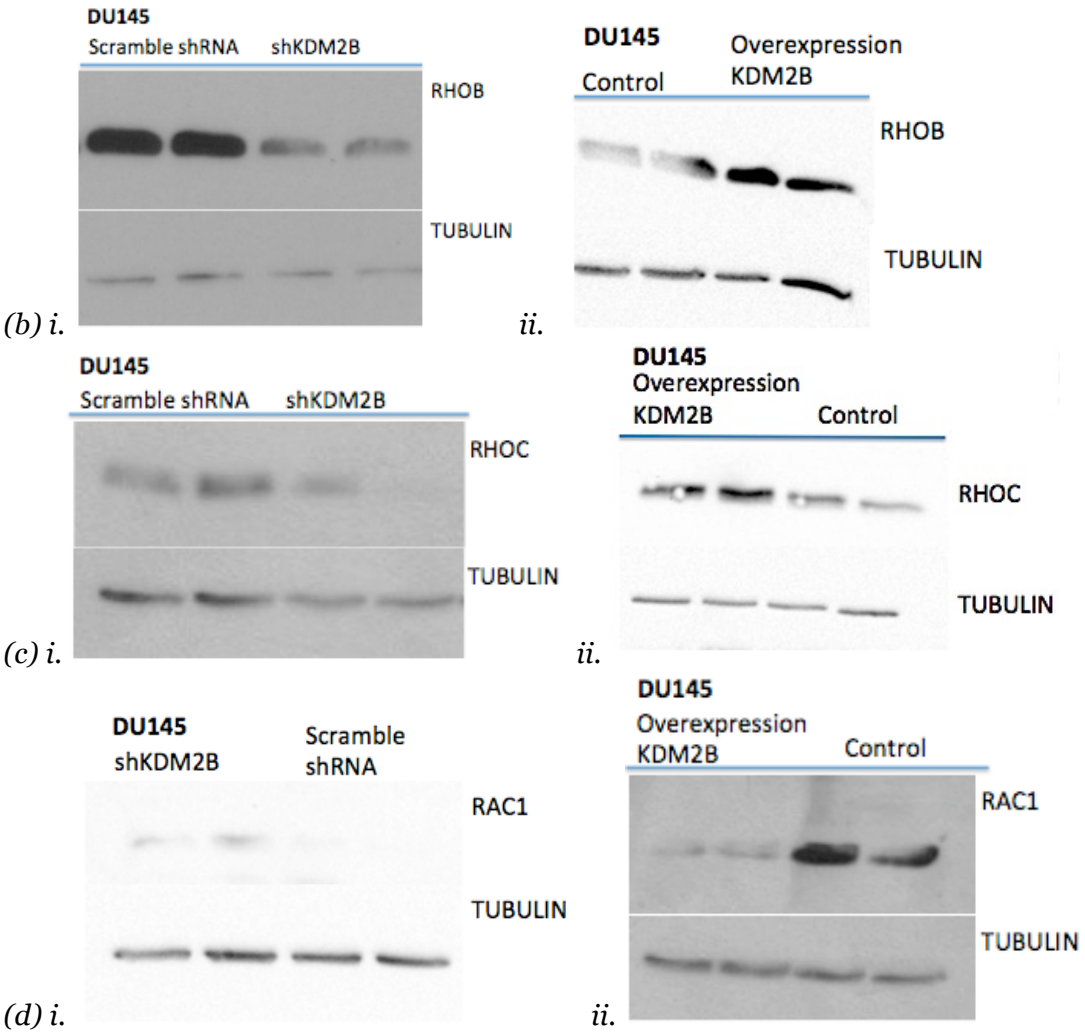


Figure 8: Western blot of RhoGTPases family proteins expression in DU145 cells.
(a) i. Expression levels of RHOA in shKDM2B and scramble shRNA DU145 cells. ii. Expression levels of RHOA in overexpression of KDM2B and control DU145 cells.
(b) i. Expression levels of RHOB in shKDM2B and scramble shRNA DU145 cells. ii. Expression levels of RHOB in overexpression of KDM2B and control DU145 cells.
(c) i. Expression levels of RHOC in shKDM2B and scramble shRNA DU145 cells. ii. Expression levels of RHOC in overexpression of KDM2B and control DU145 cells.
(d) i. Expression levels of RAC1 in shKDM2B and scramble shRNA DU145 cells. ii. Expression levels of RAC1 in overexpression of KDM2B and control DU145 cells.

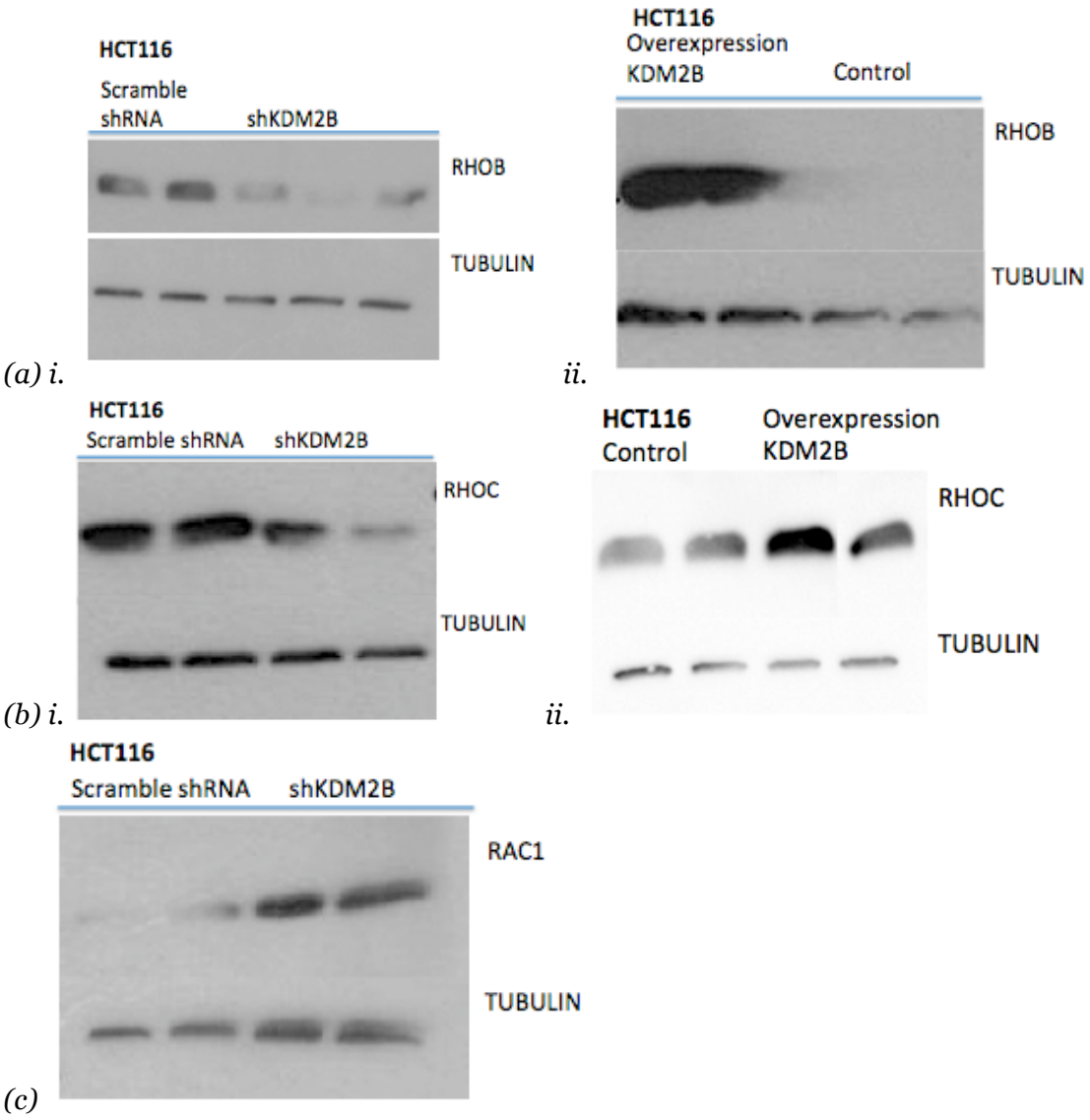


Figure 9: Western blot of RhoGTPases family proteins expression in HCT116 cells.
(a) i. Expression levels of RHOB in shKDM2B and scramble shRNA HCT116 cells. ii. Expression levels of RHOB in overexpression of KDM2B and control HCT116 cells.
(b) i. Expression levels of RHOC in shKDM2B and scramble shRNA HCT116 cells. ii. Expression levels of RHOC in overexpression of KDM2B and control HCT116 cells.
(c) Expression levels of RAC1 in shKDM2B and scramble shRNA HCT116 cells.

It is known that activation of CDC42 induce actin-rich surface protrusions, called filopodia and Rho proteins are involved in stress fibers formation, actomyosin assembly and adhesion. After fixing overexpressing KDM2B DU145 cells with PFA/triton and stained for actin and nuclei *(Figure 10, 11)*, we observed that the

overexpressing KDM2B cells have more and bigger filopodia and more stress fibers compared to the control ones.

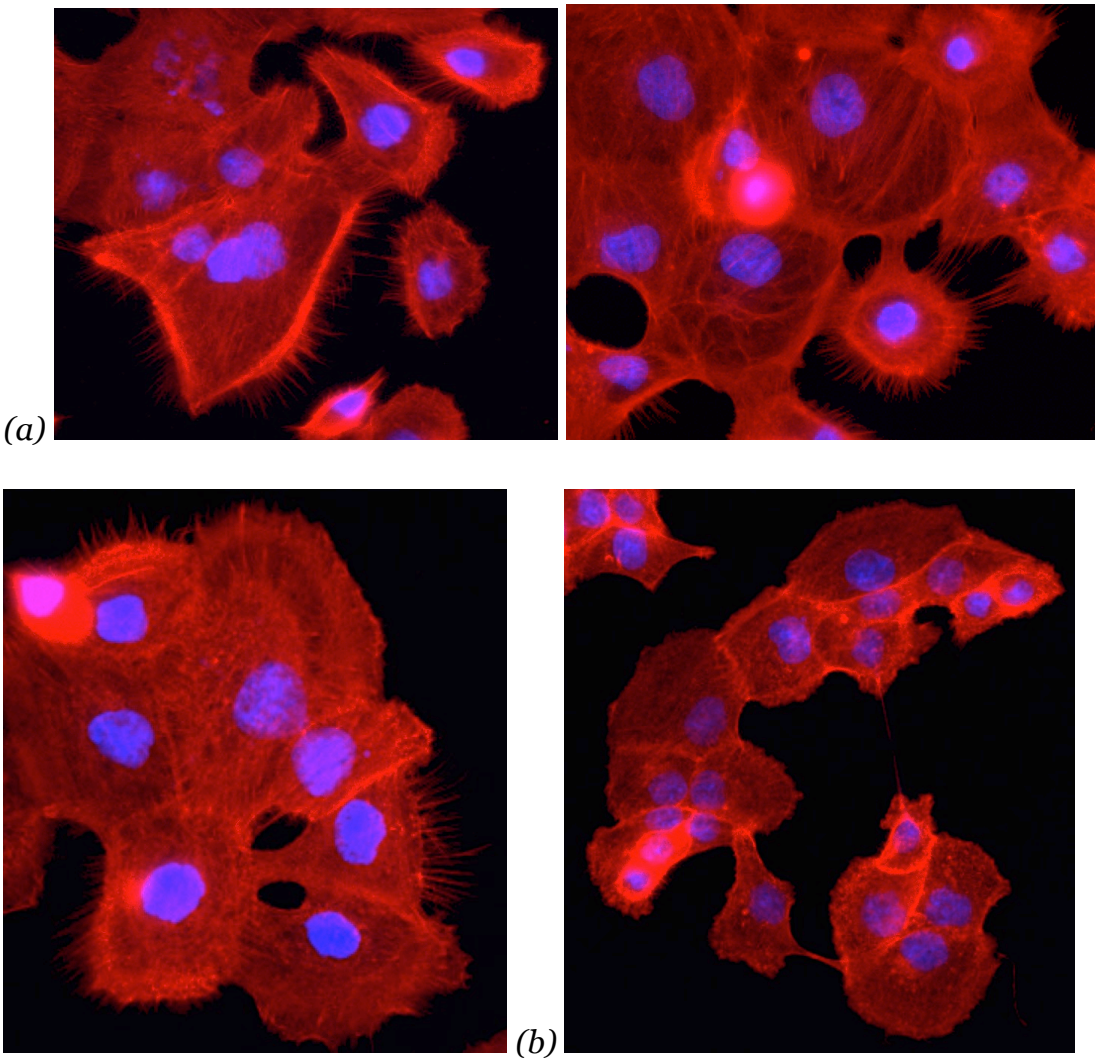


Figure 10: Immunofluorescence of actin in DU145. (a) Overexpressing KDM2B and (b) control DU145 cells were fixed with PFA/triton and stained for actin (red) and nuclei (blue).

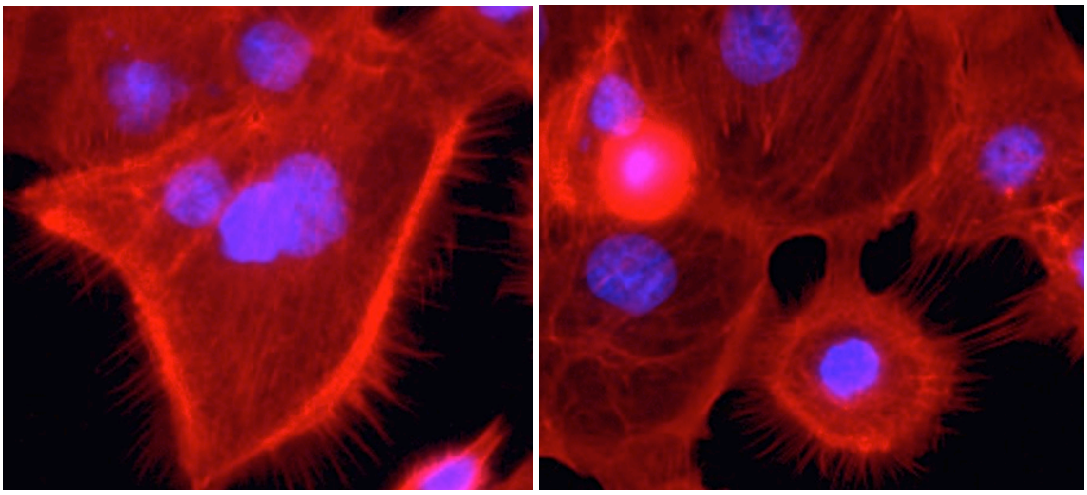
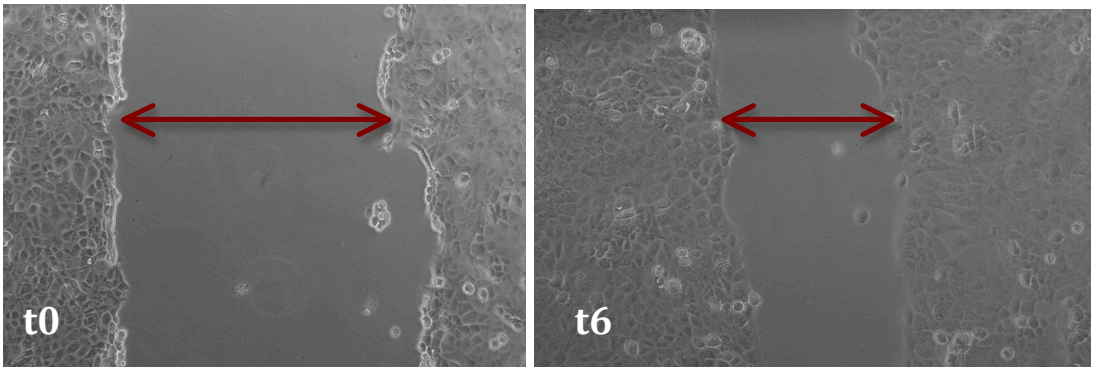


Figure 11: Immunofluorescence of actin in DU145 in a more detailed view. Overexpressing KDM2B cells were fixed with PFA/triton and stained for actin (red) and nuclei (blue).

So, until now, we can conclude that KDM2B knockdown resulted in the downregulation of EZH2, RAC1 and CDC42 and in the upregulation of the cell-cell adhesion protein E-cadherin and the cytoskeleton regulators RHOA, RHOB, RHOC. These results agree with already published data and fit with a model, which proposes less migration for shKDM2B cells. Also, it can be suggested that KDM2B may directly regulate major signaling molecules controlling cellular physiology.

In order to confirm the different migration rate upon knockdown and overexpression of KDM2B, we perform a wound-healing assay. This method mimics cell migration during wound healing in vivo and allows studying cell migration and cell-cell interactions *(Figure 12)*. As a result, we found that overexpression of KDM2B leads to an increased migration rate.



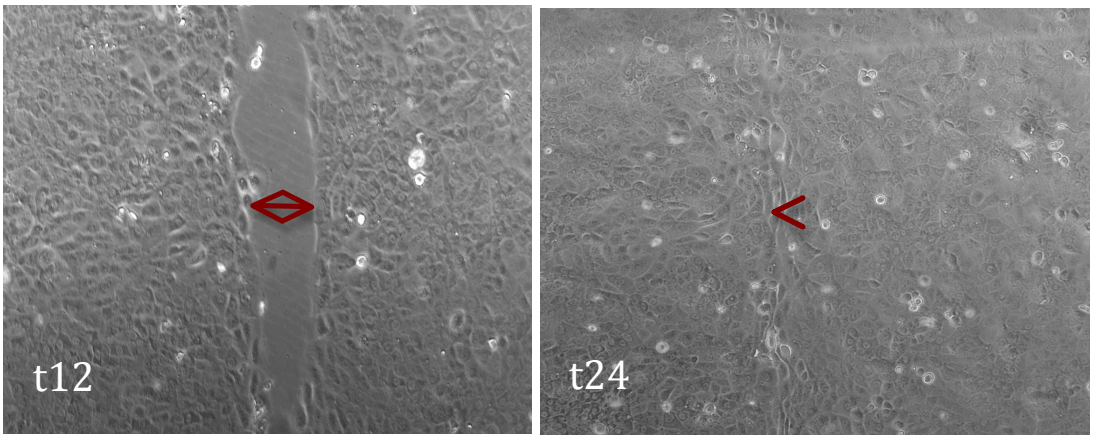


Figure 12: Wound Healing Assay. Pictures were taken at the beginning (t0), after 6 (t6), 12 (t12) and 24 (t24) hours after the creation of the wound, in DU145 overexpression of KDM2B monolayer.

Between the scramble shRNA and the shKDM2B DU145 cells, the cells that migrate faster are the scramble shRNA cells. We can see from the diagram that the wound in these cells is totally closed after 30 hours, whereas for the knockdown cells, in the same time the closure is approximately 70% *(Figure 13)*.

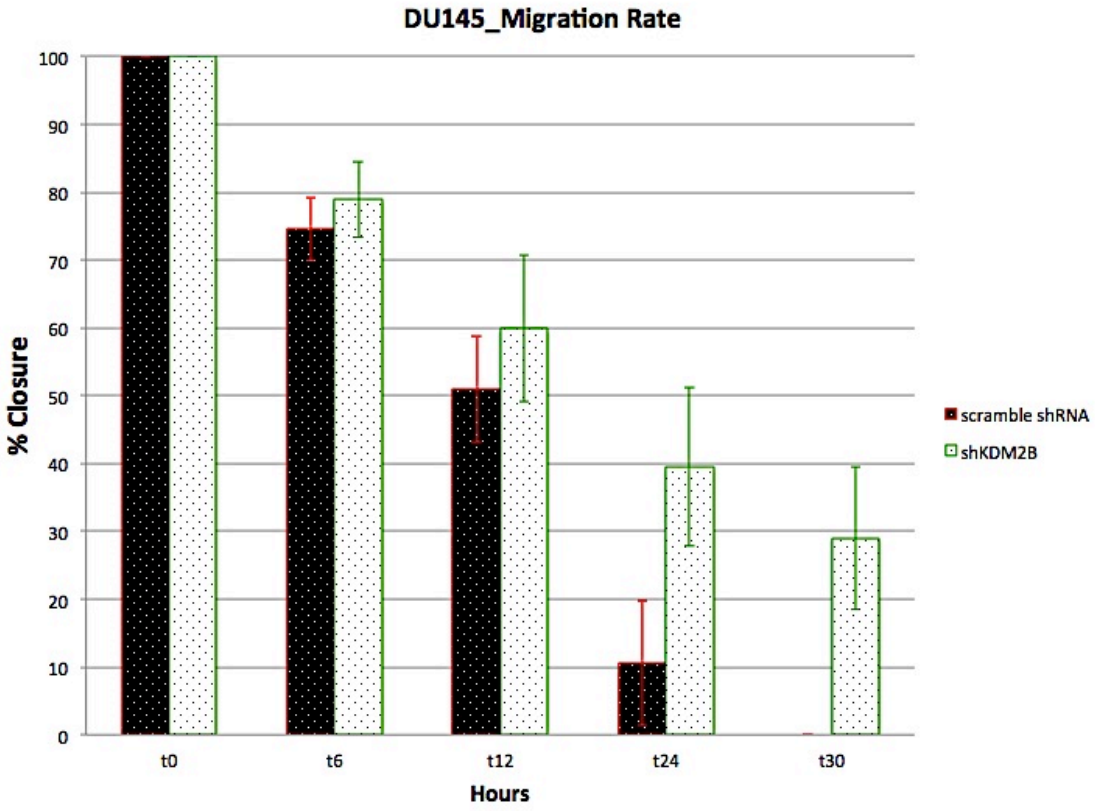


Figure 13: Graph showing the % closure of the wound in shKDM2B and scramble shRNA DU145 cells.

Between the overexpressing of KDM2B cells and the control cells, the cells that migrate faster are the overexpressing KDM2B. After 24 hours the wound in the overexpressing KDM2B wells is totally closed *(Figure 14)*.

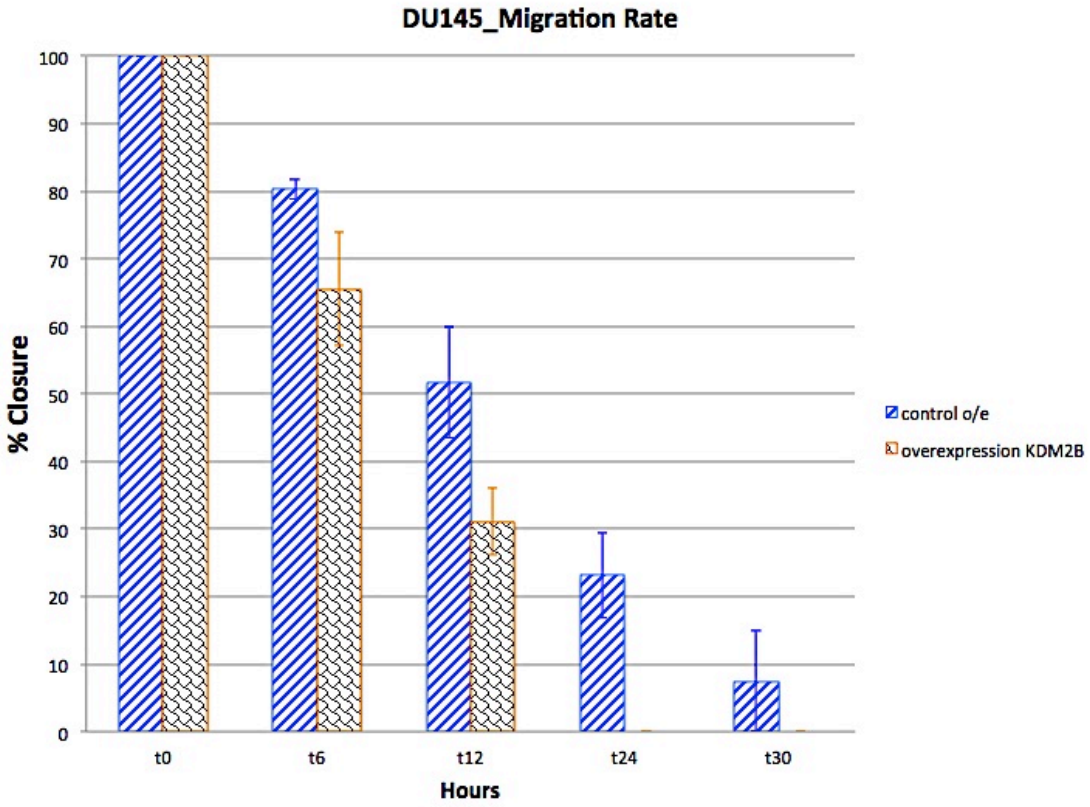


Figure 14: Graph showing the % closure of the wound in overexpressing KDM2B and control DU145 cells.

To confirm that the closure of the wound was due to the migration of the cells and not due to their proliferation, we performed a proliferation assay with the same cells *(Figure 15 and 16)*. The cells that proliferate faster were the shKDM2B cells and the proliferation rate of the overexpressing KDM2B cells was significant slow.

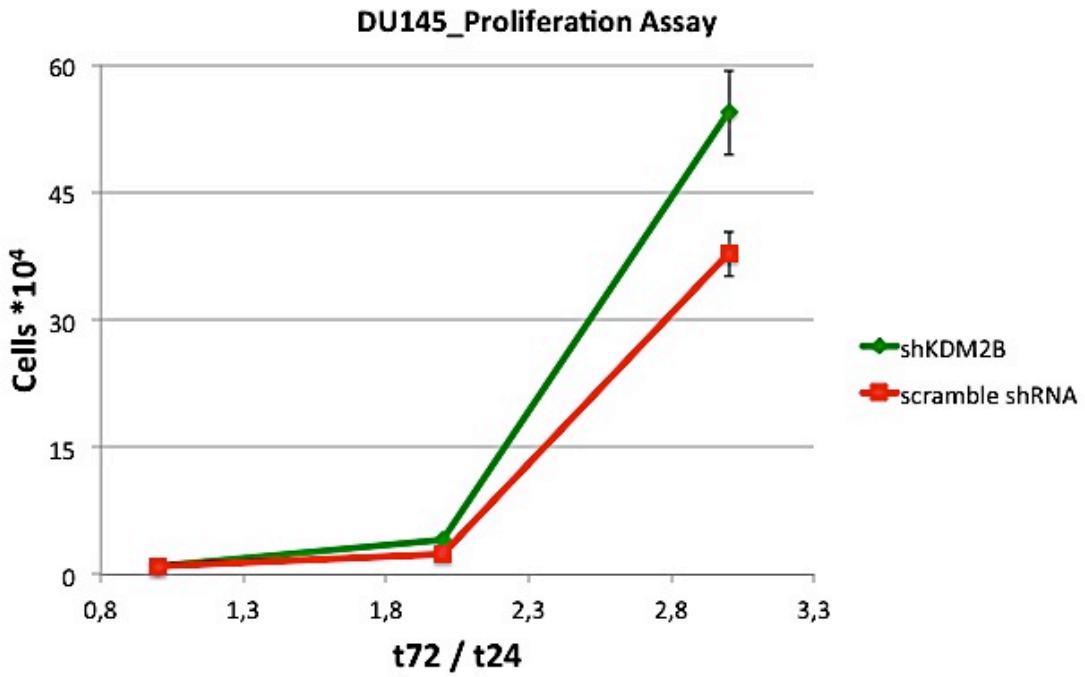


Figure 15: Graph showing the proliferation rate of shKDM2B and scramble shRNA DU145 cells.

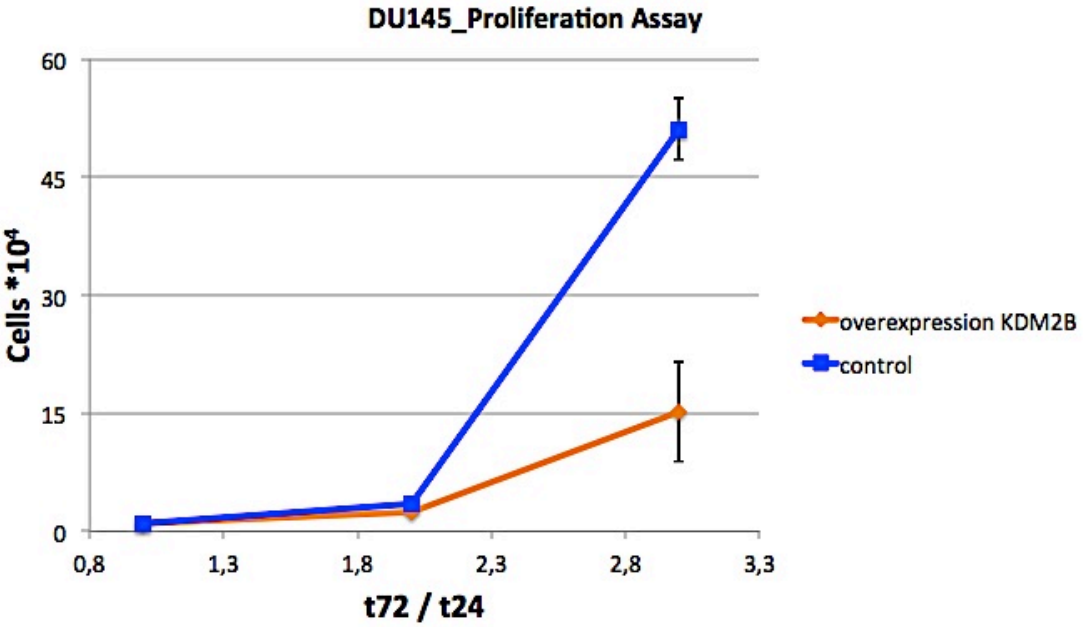


Figure 16: Graph showing the proliferation rate of overexpressing KDM2B and control DU145 cells.

These findings suggest that the different expression of KDM2B in DU145 cells may promote motility and invasion and specifically, shKDM2B cells fit with less migration and invasion. Also, our findings suggest that KDM2B may directly regulate major signaling molecules controlling cytoskeleton and cell-cell adhesion proteins.

The next step of this project will focused on to elucidate the underlying mechanism of how these key regulators of cancer cell migration, invasion and endothelial interaction interconnect. From the bibliography and from our results until now, an hypothetical diagram of how these molecules interconnect is represented in *figure 17* and *figure 18*.

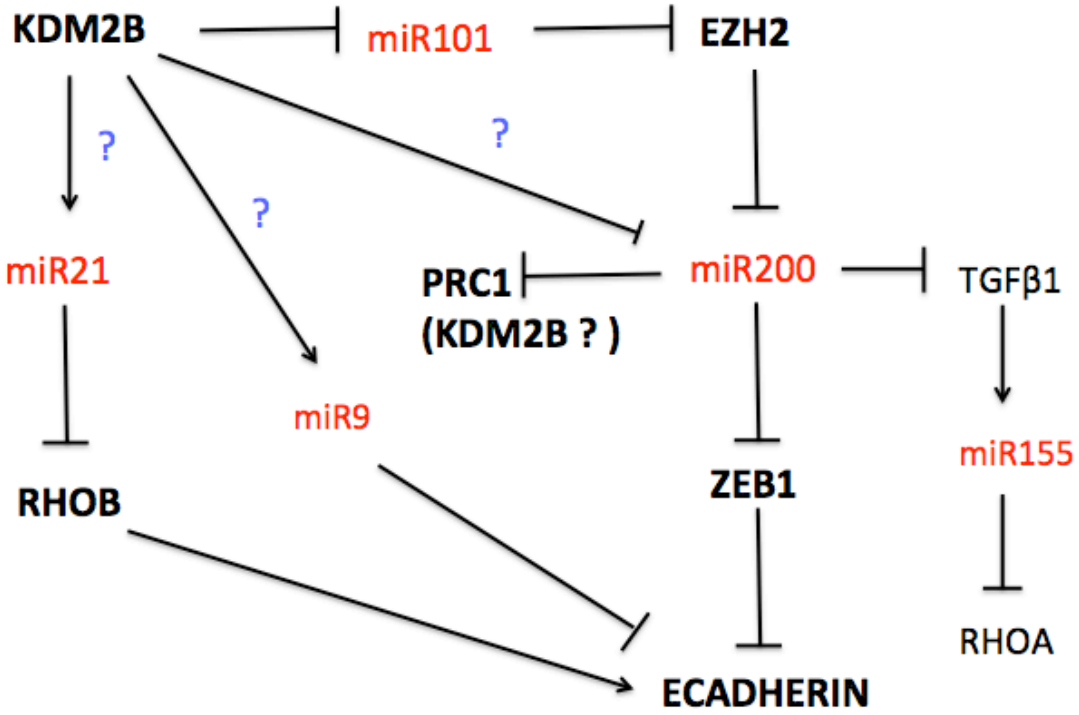


Figure 17: Hypothetical diagram of the underlying mechanism.

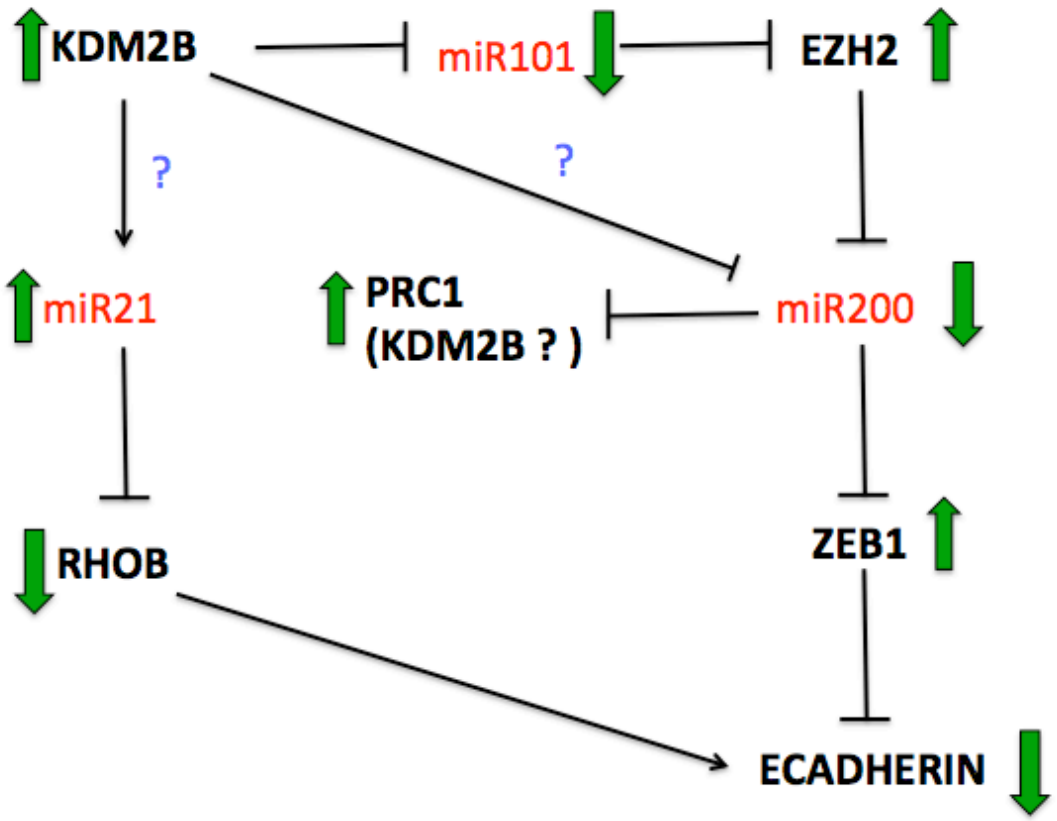


Figure 18: Hypothetical diagram of how the molecules interconnect in a cancer cell that overexpressed KDM2B.

By elucidate the underlying mechanism we will establish a basic framework of how epigenetic regulation controls the structure and function of the cytoskeleton and adhesion junction and how deregulated chromatin regulators can impact basis aspects of cellular physiology.

Chapter 4
DISCUSSION

Cancer is a genetic disease. Emerging evidence, however, is challenging this concept by showing that non-genetic heritable changes affecting chromatin structure and gene expression may play an equally important role in the biology of the cancer cell. Based on this evidence, tumor development and evolution depends on the combination of genetic and epigenetic changes *(Kampranis C. S. and Tsichlis N. P., 2009)*. The two of course are not independent. Thus, epigenetic silencing of genes involved in the response to DNA damage may give rise to genetic instability and the accumulation of genetic changes in cancer cells. Genetic changes affecting genes involved in epigenetic regulation, on the other hand, may alter the epigenome of the cancer cell. The central role of epigenetic abnormalities in tumor evolution has prompted efforts for the development of therapeutic approaches that aim to reverse epigenetic changes. The cytosine analogs 5–azacytosine (azacytidine) and 2–deoxy–5–azacytidine (decitabine) are currently the most advanced drugs for epigenetic cancer therapies. These DNA methyltransferase inhibitors have shown remarkable efficiency in the de-repression of epigenetically silenced tumor suppressor genes *(Constantinides et al., 1977; Santi et al., 1983)*. Following the success of DNA methyltransferase inhibitors, the antitumor potential of another class of proteins involved in epigenetic regulation, that of histone demethylases. With inhibitors for other main components of the epigenetic machinery already showing clinical promise, histone demethylases are emerging as new possible drug targets. Several lines of evidence suggest that histone demethylases may indeed be good candidates for the development of epigenetic cancer therapies. For example, PLU–1/KDM5B is implicated in breast cancer and its depletion from MCF–7 breast cancer cells resulted in growth inhibition and promoted the derepression of several genes *(Yamane et al., 2007)*. Also, the knockdown of NDY1 and the ablation of exogenous NDY1 in MEFs, downregulated the expression of EZH2, induced the expression of $p16^{INK4A}$ and promoted senescence *(Pfau et al., 2008; Tsukada et al., 2006; Tzatsos et al., 2009)*. Histone demethylase inhibitors, either alone or in combination with histone deacetylase or DNA methyltransferase inhibitors, may prove to be effective epigenetic therapeutic agents.

In our project, focusing on the role of KDM2B and based on our hypothesis that in cancer, epigenetic alterations frequently result

in the deregulation of the mechanisms maintaining normal cytoskeleton organization and cell-cell adhesion function, we tried to understand how chromatin regulation and epigenetic mechanisms influence the onset and progression of cancer. The underlying mechanism of how key regulators of cancer cell migration, invasion and endothelium interaction interconnect downstream of the histone demethylase KDM2B, will open a new window of research in this field.

In DU145 and HCT116 cells expressing either an shKDM2B or overexpressing KDM2B *(Figure 2)*, we found that the expression levels of several proteins involved in the cytoskeleton and in cell-cell adhesion are altered. Especially, the expression levels of Ezh2 upon depletion of KDM2B were downregulated and upon overexpression of KDM2B upregulated *(Figure 3)*. Ezh2 expression increases with tumor progression and alteration in its expression levels changes the invasive phenotype of the cells. In addition, knockdown Ezh2 cells have less invasive properties *(Cao et al., 2008)* and high Ezh2 expression predicts poorer prognosis and higher metastatic risk in several cancer types *(Crea et al., 2012)*. KDM2B is proposed to be an oncogene *(Pfau et al., 2008)* and ectopic expression of it enables MEFs to undergo immortalization and bypass replicative senescence *(Pfau et al., 2008; Tzatsos et al., 2009)*. So, as we expected, taken together these data (overexpressing KDM2B leads to upregulation of Ezh2 and knockdown of KDM2B leads to downregulation of Ezh2), they indicate that Ezh2 expression and activity increase in parallel with the expression of KDM2B and together with human tumor progression, predict treatment resistance and poor prognosis. Ezh2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which methylates histone H3 lysine 27, thereby silencing several tumor-suppressor genes and anti-metastatic genes (e.g., E-cadherin) *(Cao et al., 2008)*.

In the last years the already intricate network of gene expression and epigenetic regulation has been further complicated by the discovery of the crucial role of microRNAs *(Iorio, Piovan, & Croce, 2010)*. MicroRNAs are able to control gene expression at a post-transcriptional level, by pairing with specific partially complementary 3'-UTR regulatory elements on mRNAs, thus representing a new important class of regulatory molecules. MicroRNAs play pivotal roles in numerous biological processes, and their deregulation is a common feature of human cancer *(Malumbres et al., 2013, Melo and Esteller, 2011)*.

Almost every gene is regulated by a microRNA. For example, the

microRNAs miR-31, miR-21 and miR-138 have been shown to suppress RhoA, RhoB and RhoC mRNAs, respectively. Also, the microRNAs miR-151 and miR-155 result in increased basal activation of RhoA, Rac1 and Cdc42 *(Iorio M. V. et al., 2005, Meng F. et al., 2007, Asangani I.A. et al., 2008)*. Last but not least, the microRNA miR-101 has been shown to regulate E-cadherin levels, through Ezh2, a member of Polycomb Repress Complex which expression is regulated by KDM2B *(Aamer M. Qazi et al., 2012)*.
MicroRNA expression can be affected by the same mechanisms modulating protein-coding genes (PCGs), including epigenetic regulation. Not only miRNAs are modulated by epigenetic regulation, but also several miRNAs themselves can directly target and regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism *(Iorio, Piovan, & Croce, 2010)*. Thus, miRNAs and epigenetic pathways appear to form a complex regulatory circuit that modulates the expression of an increasing number of genes in the genome. The disruption of this circuit contributes to various diseases including cancer *(Iorio, Piovan, & Croce, 2010)*.
Molecular mechanisms of Ezh2-dependent metastatization have been extensively investigated, and a comprehensive picture of the process is proposed. First, Ezh2 acts together with histone deacetylases (HDACs) to repress CDH1 expression, a critical step for EMT (epithelial-to-mesenchymal transition) *(Tong et al., 2012)*. Moreover, Ezh2 is at the crossroad of a complex microRNA network. Some anti-metastatic microRNAs, like miR-101 and let7, are frequently silenced in metastatic tumors *(Lovat, Valeri, & Croce, 2011)*. Several studies showed that miR-101, let 7, and miR-26a inhibit Ezh2 expression in cancer cells *(Kong et al., 2012; Zhang et al., 2011)* and that this action is essential for its tumor-suppressing activity *(Varambally et al., 2009; Wang, Ruan, He, Ma, & Jiang, 2010)*. In turn, EZH2 silences some anti-metastatic microRNAs (including miR-101, let-7c, miR-200b, miR-139-5p, and miR-125b) *(Au et al., 2012; Cao et al., 2011)* which in turn targets tumor-suppressor genes. One of these genes is E-cadherin. Reduced expression of E-cadherin is regarded as one of the main molecular events involved in dysfunction of the cell-cell adhesion system, triggering cancer invasion and metastasis *(Nives Pećina-Šlaus, 2003)*. As we expected, when we checked the expression levels of the cell adhesion protein E-cadherin there were increased upon KDM2B knockdown and decreased in the overexpression samples, in both cell lines *(Figure 4)*. The increased and decreased levels of E-cadherin upon KDM2B knockdown in DU145 and

overexpressing of KDM2B HCT116 cells, respectively, were obvious in immunofluorescence, also *(Figure 5 and 6)*.
The next step was to check the expression levels of proteins of the cytoskeleton due to the fact that the function of E-cadherin is dependent on its interaction with the actin cytoskeleton and because cytoskeleton organization changes in cancer. We saw a significant change on the expression levels of the RhoGTPases family proteins, RHOA, RHOB, RHOC, RAC1 and CDC42. Especially, in both cell lines, the expression levels of RhoA, RhoB, RhoC and Cdc42 were downregulated upon knockdown of KDM2B, whereas Rac1 was upregulated *(Figure 8 and 9)*.
The Rho family of GTPases regulates a wide variety of cellular processes, including apoptosis, cell cycle progression and migration via changes in the actin cytoskeleton. Given that Rho GTPases regulate such a wide range of cellular processes, it is not surprising that Rho protein activity is tightly regulated. However, Rho GTPase signaling may become de-regulated in cancer *(Croft & Olson, 2011)*. Rho GTPases are well known for their roles in cell migration, through effects on actin and microtubule dynamics, myosin activity, and cell-ECM and cell–cell adhesions *(Parsons et al., 2010; Ridley, 2011)*. Especially, RhoA is upregulated in a variety of human tumour types and stimulates cell cycle progression and cytokinesis, as well as regulating cell migration *(Karlsson et al., 2009; Vega & Ridley, 2008)*. RhoA depletion induces the elongated phenotype with narrow lamellipodia. RhoB is postulated to be a tumour suppressor because its expression is decreased in a number of tumour cell types *(Huang & Prendergast, 2006)*. RhoB expression promotes apoptosis in epithelial cancer cells and fibroblasts *(Croft & Olson, 2011; Prendergast, 2001)* and contributes to directional cell migration. It normally regulates cancer cell migration by increasing the stability of integrin-mediated adhesions and integrin activity, thereby promoting lamellipodial protrusion and migratory polarity. RhoB depletion inhibits cell spreading and stable lamellipodium extension, and promotes migration *(Vega, Colomba, Reymond, Thomas, & Ridley, 2012)*. Also, RhoB has been implicated in the recycling and delivery of receptors and signaling proteins to the plasma membrane via its action on endosomal trafficking *(Qualmann & Mellor, 2003)*. Like RhoA, RhoC expression is upregulated at the mRNA and protein levels in a number of human malignancies RhoC has been shown to promote invasion in many cancer cell types *in vitro*, including hematoma, prostate and breast cancer cells *(Fingleton, 2007)*. Its expression correlates positively

with metastasis in a number of cancer types, including melanoma *(Karlsson et al., 2009)* and several studies have shown that RhoC is important for metastasis *in vivo*. RhoA and RhoC, like other GTPase family members such as Rac1, and Cdc42, promote oncogenesis, invasion, and metastasis *(Khosravi-Far et al., 1995; Westwick et al., 1997; Pruitt and Der 2001; Ridley, 2004)*. RhoA, RhoB, and RhoC can all induce stress fibers when overexpressed. RhoA, RhoB and RhoC play different roles in cancer and are differentially regulated, although so far no studies have directly compared their functions in a single cancer cell type. A simplified model is that Rac proteins are involved in lamellipodia formation: Cdc42 induces actin polymerization to generate filopodia often seen at the front of migrating cells *(Nobes and Hall, 1995)* and Rho in stress fibers formation, actomyosin assembly and adhesion. Rho activity induces high levels of stress fibers and substrate adhesions preventing migration *(Raftopoulou & Hall, 2004)*. The formation of stress fibers and numerous filopodia was obvious in our results, also, in agreement with the bibliography. As we were expected, the overexpressing KDM2B cells have more and bigger filopodia and more stress fibers compared to the control ones *(Figure 10, 11)*.

Based on our results, we can conclude that KDM2B may directly regulate major signaling molecules controlling cellular physiology. Also, the altered expression levels of several proteins of the cytoskeleton and of adhesion junction upon overexpression or knockdown of KDM2B leads to propose a model for increased migration rate for the cells that overexpress KDM2B. This was confirmed in a wound-healing assay; in which there was obvious that the cells that migrate faster are the overexpressing KDM2B cells whereas the knockdown KDM2B cells were moving significant slow *(Figure 13 and 14)*. The closure of the wound was indeed due to the migration rate of the cells and not due to their proliferation, based on our results from the proliferation assay *(Figure 15 and 16)*. These findings suggest that the different expression of KDM2B in DU145 and in HCT116 cells may promote somehow motility and invasion.

Summarizing, the goal of the project is to identify genes that are involved in cytoskeleton function and are regulated by KDM2B and to elucidate the mechanism of transcriptional regulation of these genes. KDM2B can regulate the expression of genes either directly by binding at their loci or indirectly by regulating the expression of regulatory proteins. The direct binding of KDM2B on their loci can be confirmed by ChIP. The very next step is to perform ChIP assay for KDM2B and E-cadherin and KDM2B and RhoB. For the non

direct binding of KDM2B, bioinformatics analysis will identify common regulators for sets of genes and after that we can examine the direct binding of KDM2B at the regulators' loci. The direct involvement of each specific regulator can be confirmed by knocking down or overexpressing the regulator under conditions of KDM2B knockdown or overexpression and determining the mRNA levels of the cytoskeleton regulating genes. Also, we can check the expression levels of specific miRNAs, like miR101, miR21, miR200, and see if there are any significant changes upon knockdown or overexpression of KDM2B. So, by answering these questions we will have a framework of how epigenetic regulation controls the structure and function of cytoskeleton.

By elucidating the underlying mechanism of how histone demethylase KDM2B interconnect with cytoskeleton organization and cell to cell adhesion junction, it will be established a basic framework of how epigenetic regulation controls the structure and function of the cytoskeleton and adhesion junction and how deregulated chromatin regulators can impact basic aspects of cellular physiology. Due to the fact that the field of histone demethylases is now beginning to emerge, we can understand that our project is innovative, as it constitutes the first focused effort towards elucidation of the cross-regulation between chromatin and the cytoskeleton.

This research will enable us to determine the contribution of specific epigenetic mechanisms to cancer and may provide novel targets for intervention. Also, epigenetics is a new chapter in genome biology that we are just starting to understand its in vivo relevance but there is no doubt that its potential in therapeutics opens up new opportunities in the future of cancer therapy. So, modulation of epigenetic mechanisms is a promising novel strategy in cancer therapy. Also, a possible use of microRNAs to either directly regulate gene expression, modulating for instance the levels of oncogenes and oncosuppressor genes, or to control the epigenetic machinery targeting the effector enzymes and thus affecting the expression of a wide range of modulated molecules, is certainly a fascinating and promising perspective. It is anticipated that additional study of the relationship between epigenetic regulation and miRNAs will lead to the discovery of new biomarkers as well as therapeutic targets. It is clear that epigenetic mechanisms are interdependent and integrated into the regulatory machinery of the cell. Epigenetic regulators can be modified by the complex signaling pathways that become deregulated in the cancer cell. Future studies on the cancer epigenome and its regulation will

redefine our understanding of the cancer cell and may reveal new targets for therapeutic intervention.

APPENDIX

Primers

KDM2B

F 5' TCT ACG AGA TCG AGG ACA GGA 3'
R 5' ACC AGC ACA TCT CAT AGT AGA AGG 3'

E-CADHERIN

F 5' TAC AAT GCC GCC ATC GCT TA 3'
R 5' CGT AGG GAA ACT CTC TCG CT 3'

EZH2

F 5' ACG GGG ATA GAG AAT GTG GGT TTA 3'
R 5' AGG TGG GGC GGC TTT CTT TAT CAT C 3'

GAPDH

F 5' GGA GTC AAC GGA TTT GGT CGT A 3'
R 5' GGC AAC AAT ATC CAC TTT ACC AGA GT 3'

Primary antibodies

RHOA: Sc-418
RHOB: Sc-180
RHOC: Cell Signaling Technologies, D40E4
RAC1: Sc-95
CDC42: Sc-8401
FAK: Sc-557
KDM2B: Millipore, 09-864
E-CADHERIN: BD Biosciences, 610181
EZH2: Cell Signaling Technologies, D2G9
PAXILLIN: BD Biosciences, P13520
VINCOULIN: Cheminon, MAB1674
RHODAMINE-PHALLOIDIN: Invitrogen, R415

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