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TITOLO TESI DI DOTTORATO DI RICERCA

NF-Y AND LAMIN A IN CELL PROLIFERATION AND CANCER

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ABSTRACT

Nuclear transcription factor (NF-Y) is a sequence-specific transcription factor that binds DNA on a sequence spanning the CCAAT a common element present on several gene promoters. It has long been considered an activator of genes involved in growth promotion including cell cycle regulatory genes. NF-Y is composed of three subunits (-YA –YB –YC) all required for the DNA binding and transactivation activity. NF-YA is the regulatory subunit of the NF-Y protein. Numerous findings highlight that NF-Y is involved in cancer.

In manuscript I, to get clues on NF-Y function(s) in cancer cells, we performed a mass spectrometry screening of a pool of proteins that co-precipitate with the NF-YA subunit. By this screening we identified lamin A as a novel putative NF-Y interactor. This result was validated by co-IP experiments using different cell lines. Confocal analysis confirmed this interaction in different phases of mitosis. In order to investigate the possible involvement of lamin A/NF-Y complex in gene regulation, we generated the chromatin fractions. Co-IP experiments confirmed the occurrence of lamin A /NF-Y complex in the chromatin. We further isolated euchromatin from proliferating cells in order to evaluate the possible involvement of lamin A in actively transcribed genes involved in cell cycle progression where NF-Y plays a key role as transcription factor. Our data demonstrated that NF-Y and lamin A co-localize in transcriptionally active region. We performed ChIP experiments to explore the possible recruitment of lamin A on promoters of NF-Y target genes demonstrating that lamin A physically interacts with several promoter regions carrying CCAAT-boxes, such as *CCNB2*, *DHFR*, *CCNA2*, *CDK1*, *CCNB1*, *CDC25C*, *TOPO2a* and *PCNA*. Gain and loss of function experiments revealed that lamin A counteracts NF-Y transcriptional activity impacting on cell cycle progression. We performed luciferase assays using the *CCNB2* promoter driven luciferase reporter construct as a sensor of NF-Y activity in cellular and mouse models. These experiments revealed that lamin A counteracts NF-Y activity on *CCNB2* promoter. Moreover, data obtained from experiments performed under serum deprivation or oxidative stress conditions emphasized the importance of lamin A activity in cell proliferation in the tumour microenviroment characterized by low nutrient supply and excessive reactive oxygen species (ROS) production.

In manuscript II, we analysed the possible involvement of NF-Y and lamin A in stratifying endometrial cancer (EC). EC is a major cause of mortality for patients worldwide. EC is classified as type I or type II based on histologic properties. Type I, also called the endometrioid type (EEC) because of its histologic similarity to the endometrium, accounts approximately 70–80% of sporadic

EC. Most type I tumours occur in the setting of unopposed estrogen stimulation, leading to endometrial hyperplasia. According to FIGO definition, type I ECs include lower grade EECs (grade 1 and 2 EEC). Unlike type I tumors, type II lesions are not related to estrogen exposure or endometrial hyperplasia and include high risk malignancies, as poorly differentiated high-grade EEC (G3), and non endometrioid endometrial carcinomas (NEM) such as serous papillary and clear cell carcinomas. In general, patients with EC have a good prognosis since early diagnosis is frequent and the disease has usually not spread beyond the uterus. However, the prognosis for recurrent or metastatic EC remains poor. Although most cases of low grade ECs do not behave aggressively, in rare instances, even low-grade, well-differentiated ECs can progress in a highly aggressive manner. In this study, we analysed several EC tissues to find novel clinical and biological features to help the diagnosis and consequently the treatment of early EEC. A retrospective cohort of several formalin-fixed, paraffin-embedded (FFPE) specimens from patients with EC were analysed. Total RNA and proteins were extracted and analyzed, respectively, by quantitative PCR and western blotting. Our correlation studies identify NF-YAs, a splicing isoform of NF-YA, , and lamin A as two novel potential biomarkers in ECs. It has been recently demonstrated that NF-YAs belongs to the embryonic stem cell transcription factor circuitry. Lamin A has been shown to be involved in cancer development and tumor aggressiveness. We observed that NF-YAs is exclusively expressed in EC tissues, while lamin A is strongly down-modulated in EC compared with benign tissues and its loss of expression correlates with higher histologic grade and aggressiveness. Results obtained in low grade EEC (grade 1) tissues demonstrated that NF-YAs expression is heterogeneous, with 55% of samples expressing the short isoform compared to 100% in G2 and G3 EEC and NEM. Interestingly, the presence of NF-YAs was related with lower lamin A protein and mRNA levels. It is worth to note that the presence on NF-YAs and loss of lamin A expression was consistently associated with lower estrogen receptor (ERs) expression and related with miR-200 family upregulation and ZEBs decreased expression, indicators of EC aggressiveness, thus supporting the potential role on NF-YAs and lamin A as novel prognostic correlation biomarkers with a potential for a more systematic integration in clinical practice for individualized therapy in EC, in particular in low grade malignancy.

Our studies help to promote our understanding of the mechanisms of NF-Y activity providing a molecular evidence for the direct transcriptional modulation of cell cycle related genes by lamin A/NF-Y nuclear protein complex. Moreover, they open up a possibility to use lamin A and NF-YAs expression, in combination with ERs status, in the diagnosis and treatment of early EC .

CONTENTS

1 INTRODUCTION

1.1 NF-Y

NF-Y (also called CBF, a-CP1 and CP1) is a ubiquitous protein, composed of 3 subunits, NF-YA, -YB, and -YC, whose genes are highly conserved from yeast to mammals. All 3 subunits are required for NF-Y binding to the consensus sequence, the CCAAT-box. The CCAAT box is one of the most common cis-acting elements found in the promoter and enhancer regions of a large number of genes in eukaryote. The CCAAT box location within promoters is fixed at −60/−100 nucleotides from the transcriptional start site (TSS) and, whenever tested, has been shown to be crucial for promoter activity. The structure and the DNA-binding mode of the NF-YB/NF-YC dimer are highly reminiscent of that of other histone-fold domains (HFDs), and an activation domain is present in the NF-YA subunit (1). NF-YA is the regulatory subunit of the trimer. The crystal structure of NF-Y bound to a 25 bp CCAAT oligonucleotide shows that the HFD dimer binds to the DNA sugar-phosphate backbone, mimicking the nucleosome H2A/H2B-DNA assembly. First, NF-YB and NF-YC interact to form heterodimers through their HFDs. The NF-YB/NF-YC heterodimer then interacts with NF-YA to form the heterotrimeric NF-Y transcription factor. The absence of any of the NF-Y subunits results in loss of binding of the NF-Y complex to DNA and NF-Y-directed transcription (Figure 1).

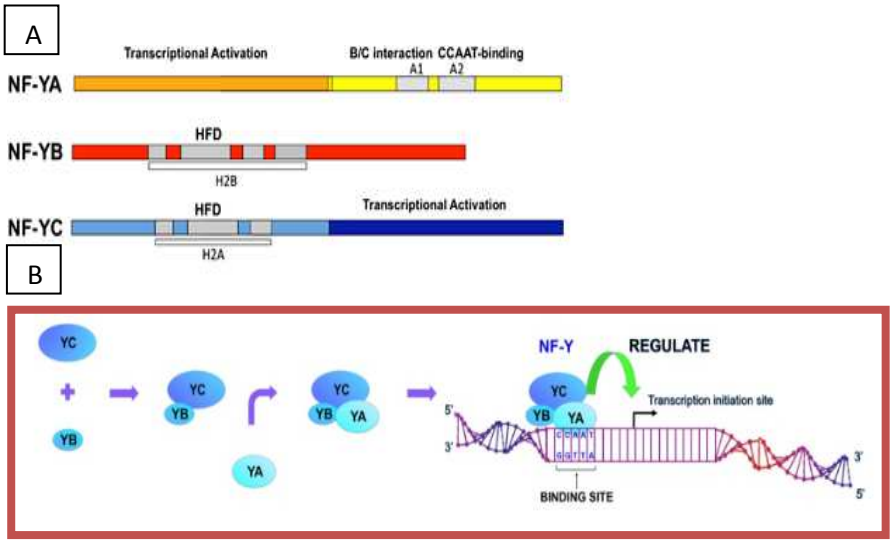

Figure 1: **(A)** Scheme of NF-Y subunits. **(B)** NF-Y complex formation. NF-Y consists of three different subunits, NF-YA, NF-YB and NF-YC, which are all necessary for formation of NF-Y complexes and binding to CCAAT boxes to activate transcription. The arrow with bar indicates transcription initiation site

A bioinformatic analysis of promoters of cell-cycle regulatory genes shows an abundance of CCAAT boxes in promoters regulated during the G2/M transition progression (Figure 2), among which are mitotic cyclin complexes (3-10). Taken together, these studies demonstrate that the binding of NF-Y to cellular promoters is essential for cell proliferation.

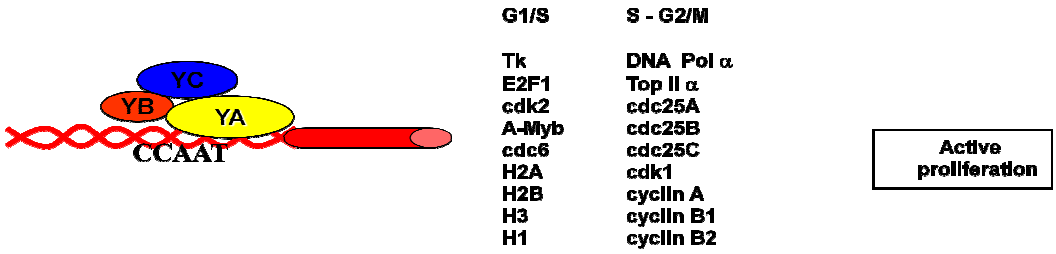

Figure 2: The NF-Y complex support basal transcription of a class of regulatory genes responsible for cell cycle progression, among which are mitotic cyclin complexes**.**

It has been reported the presence of two major NF-YA isoforms, "long" (347 aa) and "short" (318 aa), the short isoform lacking a 28-amino acid within the NF-YA amino-terminal domain (11), that are present at different levels in various cellular context (Figure 3). NFYA-long (NF-YAl) contains an exon encoding the majority of a glutamine-rich transactivation domain, whereas NFYA-short (NF-YAs) lacks this. NFYAl and NFYAs show distinct expression patterns; the former is preferentially expressed in epithelial cells and the latter in lymphoid cells (12,13).

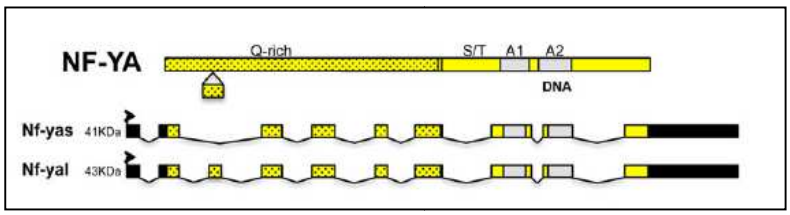

Figure 3: Description of the two isofroms of NF-YA originating from alternative splicing of exon 3.

The level of NF-YAl increases through mouse and human embryonic stem (ES) cell differentiation, whereas NF-YAs is significantly downregulated (14). Functional significance of the two NF-YA isoforms remains to be elucidated, but recent data indicate that NF-YAs promotes self-renewal of hematopoietic stem cells (15).

Although mutations in NF-Y subunits have never been specifically identified in tumours, systematic examination of expression profiles indicates that NF-Y targets are upregulated in different types of cancer. Expression of NF-YA in normal cells is modulated during the cell cycle (4) and its

abrogation plays an important role in downregulating several cell-cycle control genes in differentiated cells (5,7,9,10). Previous studies aimed at understanding the biological role of NF-Y took advantage of a loss of function approach, such as expression of dominant-negative NF-YA mutants and conditional deletion of the mouse NF-YA gene. When a dominant-negative NF-YA mutant that interacts with -YB/YC but does not bind DNA is expressed in mouse fibroblasts, retardation of cell growth is observed (16). The knock out of the NF-YA subunit in mice leads to embryo lethality; moreover, inactivation of the NF-YA gene in mouse embryonic fibroblasts results in inhibition of cell proliferation and growth arrest at various phases of the cell cycle (17-18). It has been demonstrated that NF-Y modulates the promoter activity of several genes in response to DNA-damaging agents (19,20), and NF-Y overexpression increased the proliferation rate of cancer cells harbouring endogenous mutant p53 (Figure 4).

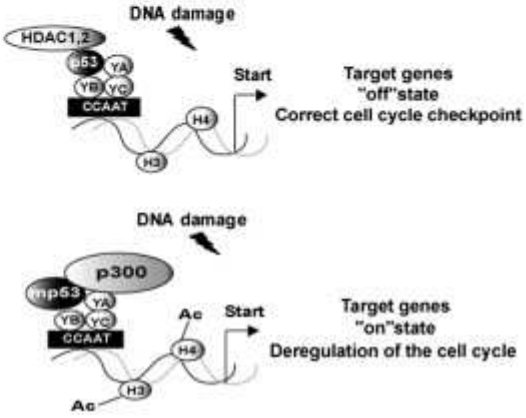

Figure 4: Model proposing the molecular mechanism underlying the transcriptional control of cell cycle-related genes by mut-p53/NF-Y or wtp53/NF-Y protein complexes.

Next, it has been shown that NF-Y interacts in vivo with mutant p53 and increases DNA synthesis, which is impaired upon abrogation of NF-YA expression (8,21). Clinical studies have indicated that patients with upregulated expression of NF-Y target genes have poor prognosis in multiple cancers (22). Using global gene expression profiles, the involvement of NF-Y in cancer-associated pathways has been recently reported across human cancers (23). In agreement with its wide involvement on human cancers, previous studies described that NF-Y interacts with different partners. Indeed, in normal cells NF-YA binds to deacetylase enzymes (HDACs) while in transformed cells the acetylase p300 is preferentially recruited (8,9). Although some NF-Y interactors are already known, several partners through which NF-Y exerts its role still need to be characterized.

1.2 Lamin A

Lamins are type V intermediate filament (IF) proteins and the major components of the nuclear lamina that scaffold adjacent to the inner nuclear membrane (Figure 5).

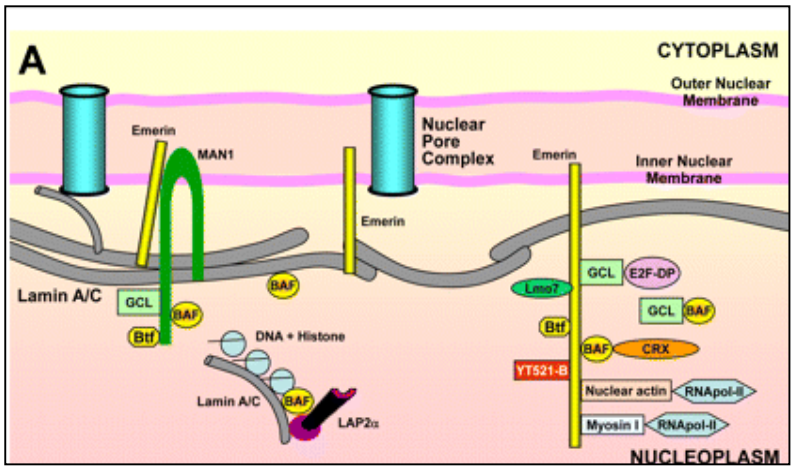

Figure 5: Scheme of interactions of A-type lamins and NE-associated proteins with DNA, chromatin complexes, and related transcription factors.

As IF proteins, lamins exhibit a typical tripartite structure consisting of an α-helical rod domain flanked by globular N-terminal head and C-terminal tail domains. The C terminus contains a nuclear localization signal (NLS) and a structural motif similar to a type s immunoglobulin fold (Ig-fold), likely involved in protein–protein interactions (Figure 6).

A-type lamins, whose most represented isoforms are lamin A and C, are alternatively spliced products of the same gene, LMNA, and are found in roughly similar amounts in most tissues. The lamin A gene is 57.6 kb long and consists of 12 exons, encoding two globular domains and a central-helical coiled-coil rod domain. Lamin C is encoded by exons 1 to 9 and a portion of exon 10. Lamin A results from alternative splicing, which adds exons 11 and 12 and removes the lamin-C-specific portion of exon 10. Diseases caused by mutations in genes encoding nuclear lamins are generally termed laminopathies (24).

Lamins play important roles in nuclear architecture, mechanosignaling (25) and chromatin dynamics (26) and impact on stem cell proliferation and differentiation (27,28). Disruption of one or more of these functions due to lamin mutations cause a group of inherited diseases affecting various tissues and organs or causing accelerated ageing (26).

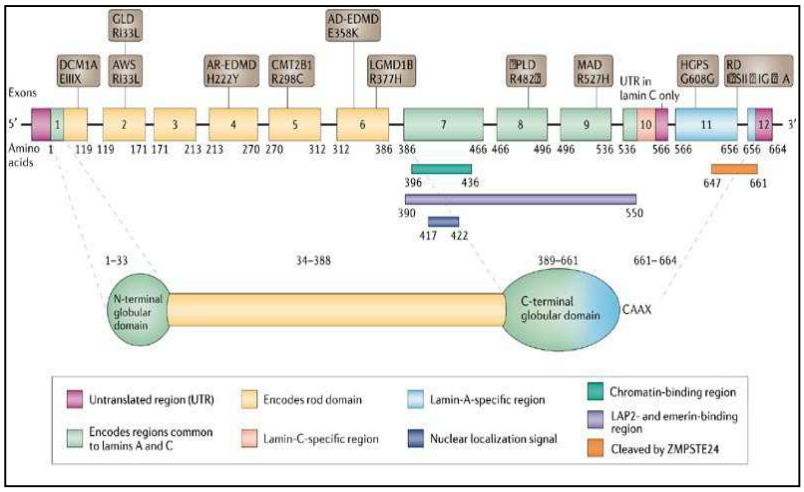

Figure 5: Scheme of the LMNA gene. Examples of the main mutations that cause laminopathies are shown above the gene; not all mutations are listed. In the case of HGPS, MAD, FPLD, AR-EDMD, RD and CMT2B1, the most common causative LMNA mutation (or only mutation) is shown. In the cases of AD-EDMD, AWS, LGMD1B, GLD and DCM1A, a representative mutation among multiple causative mutations is included. AD-EDMD, autosomal dominant Emery–Dreifuss muscular dystrophy; AR-EDMD, autosomal recessive Emery–Dreifuss muscular dystrophy; AWS, atypical Werner syndrome; CMT2B1, Charcot–Marie–Tooth disorder, type 2B1; DCM1A, dilated cardiomyopathy, type 1A; FPLD, Dunnigan familial partial lipodystrophy; GLD, generalized lipodystrophy; HGPS, Hutchinson–Gilford progeria syndrome; LGMD1B, limb girdle muscular dystrophy, type 1B; MAD, mandibuloacral dysplasia; RD, restrictive dermopathy.

Unlike lamin C, lamin A is translated as prelamin A and undergoes posttranslational processing steps at the C-terminal CaaX motif (Figure 7). Farnesylation of prelamin A occurs at a key aminoacid, cysteine 661, within the C-terminal CaaX box. Cysteine 661 is farnesylated by the dimeric protein farnesyl transferase (29). The modification is necessary for further processing of the lamin A precursor, consisting of methylation of the same residue by the enzyme Icmt, and double cleavage leading to production of mature lamin A. Mature lamin A and lamin C are solubilized in mitosis and can also localize throughout the nucleoplasm in interphase cells.

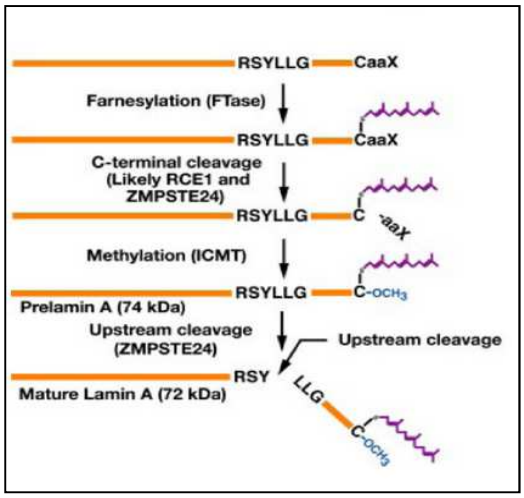

Figure 7: Normal Prelamin A processing.

It has been shown that lamin A stabilizes the nuclear lamina and chromatin, with implications for epigenetic stabilization and limiting of DNA breaks. Interactions of lamins with chromatin occur through domains termed lamina-associated domains (LADs) with the implication that lamins associate with chromatin at the nuclear lamina at the nuclear periphery. LADs are often located in repressive chromatin structures with an enrichment of this compartment at the nuclear periphery (24 30). Although most lamins are found near the nuclear membranes, nucleoplasmic populations also exist, which may have distinct roles (31-34). Lamins are often aberrantly expressed or localized in tumours. With respect to its multiple functions, it is convincible to presume that change of lamin A protein levels may contribute to tumourigenesis and progression.

The expression of the A-type lamins is often reduced or absent in cells that are highly proliferative, including various human malignancies such as colon cancer, cervical cancer, lung cancer, prostate cancer, gastric cancer, ovarian cancer and leukemia and lymphoma (35-39). Several studies reported that miR-9 is able to target and reduce lamin A expression (40,41). miR-9 has been identified as both an oncogene and a tumor suppressor depending on different cancer types. In gastric cancer (42), endometrial cancer (43), brain cancer (44) and leukemia (45) miR-9 is observed upregulated and oncogenic, whereas in cervical cancer (46.), colorectal cancer (47) and ovarian cancer (48) it is observed downregulated and anti-tumorigenic. miR-9 overexpression was also correlated with cancer progression, metastasis and poor prognosis (43). Moreover, cells lacking lamin A proliferate faster and display inefficient cell cycle arrest upon contact inhibition (48). Recent data highlight the specific functions of a small pool of lamina-independent A-type lamins, located throughout the nucleoplasm, in the regulation of early tissue progenitor cell proliferation and commitment (31,50,51).

1.3 Endometrial cancer

Endometrial cancer (EC) is the most common genital tract malignancy and occurs in reproductive and postmenopausal women. EC develops in the inner lining of the uterus, also called the endometrium (Figure 8). The human endometrium is stratified into two functional layers: the transient superficial stratum functionalis and the permanent deeper stratum basalis adjacent to the myometrium. The superficial stratum functionalis is lined by luminal epithelium, contains superficial glandular epithelium and stroma and is completely shed and regenerated during the monthly menstrual cycle and after childbirth.

The development of EC is most prevalent in postmenopausal women. For populations within this category, it is highly recommended to have a pelvic exam every year and to report any vaginal bleeding as soon as possible to prevent the cancer metastasis.

The growing obesity epidemic in recent decades had a major impact on EC incidence in most developed countries. In 2013, 49.560 new cases of endometrial cancer were diagnosed with a 3% death rate in the USA (52). In EC, myometrial invasion is considered one of the most important prognostic factors. Traditionally, the main treatment of EC is surgery where it includes abdominal total hysterectomy, salpingo-oophorectomy and eventually pelvic and/or paraortic lymphadenectomy.

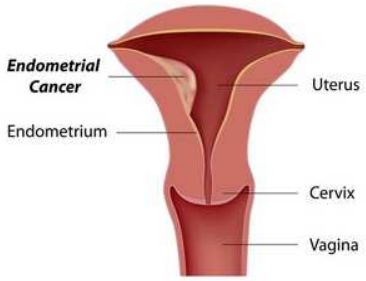

Figure 8: Endometrial cancer.

TYPE I TUMORS
80% of cases
Estrogen-dependent
Endometrioid, well-differentiated morphology
Usually good prognosis
Estrogen receptor positive, diploid, microsatellite unstable
Include KRAS and/or PTEN mutations
TYPE II TUMORS
10% of cases
Less differentiated phenotype
Often serous papillary histology
Worse prognosis
Often aneuploid with alterations in CDK2A, p53, and ERBB2

Table I: Classification of endometrial cancers.

Most EC cases are sporadic, with only 10% considered familiar (53,54). In general, patients with EC have a good prognosis since early diagnosis is frequent and the disease has usually not spread beyond the uterus. However, women with recurrent and/or metastatic EC of either type have a poor prognosis, with a median survival of 7–12 months (55). These patients require more effective systemic therapy than is presently available. Currently, adjuvant and systemic treatment of recurrent and metastatic EC are based on conventional chemotherapy and anti-hormonal treatment. In order to improve therapy it is important to understand the processes which inhibit and stimulate cancer

progression. EC is classified as type I or type II based on histologic parameters, clinical behavior and epidemiology (Table I). Type I, also called the endometrioid type (EEC) because of its histologic similarity to the endometrium, accounts approximately 70–80% of sporadic EC. Most type I tumors occur in the setting of unopposed estrogen stimulation, leading to endometrial hyperplasia. The cellular action of estrogens is mediated trough the estrogen receptors (ERs) that belong to the nuclear steroid receptor superfamily. Two distinct ERs, defined as ER-α and ER-β, have been identified. In the human uterus, ER-α is the predominant subtype. Unlike type I tumors, type II lesions are not related to estrogen exposure or endometrial hyperplasia and include high risk malignancies, as high grade EECs, serous papillary and clear cell carcinoma. Some biological molecules have been identified as prognostic markers in EC, such as *KRAS, PTEN, EGFR, FGFR, P53, HER2,* and *ER*s. (56). Expression of ERs has been correlated with stage, histologic grade and survival. Loss of ERs has been significantly associated with aggressive phenotype and poor survival in EC patients (57). In particular, early stage, well differentiated ECs usually retain ERs expression, whereas advanced stage, poorly differentiated tumours often lack one or both receptors. Recently, it has also been observed an association between lack of ER-α and epithelial-mesenchymal transition (EMT) (58,59).

1.4 Epithelial-mesenchymal transition (EMT) in EC.

EMT enables epithelial cells to acquire a like mesenchymal potential with increase motility and ability to extravasate and circulate. A classification into three types of EMT has been proposed (60)(Figure 9).

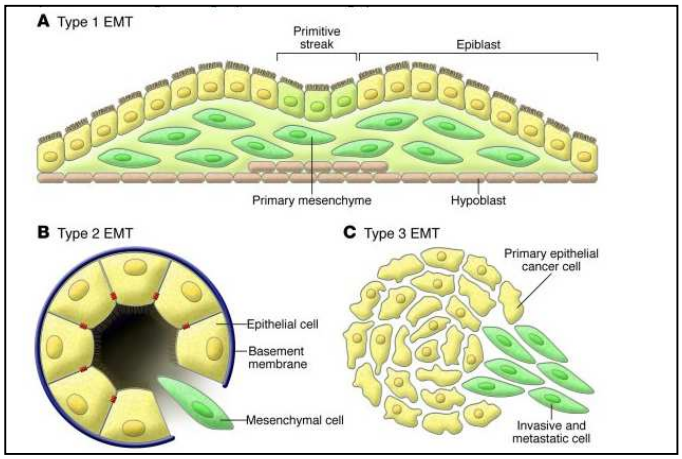

Figure 9: Classification of EMT.

Type 1 EMT is used during development to generate cells with mesenchymal features out of epithelial cells. The primitive epithelium, specifically the epiblast, gives rise to primary mesenchyme via an EMT. This primary mesenchyme can be re-induced to form secondary epithelia by a MET. It is speculated that such secondary epithelia may further differentiate to form other types of epithelial tissues and undergo subsequent EMT to generate the cells of connective tissue, including astrocytes, adipocytes, chondrocytes, osteoblasts, and muscle cells. It is a "clean" and entirely physiological process and not associated with inflammation, fibrosis, or an invasive phenotype. Type 2 EMT, in contrast, occurs during tissue repair in response to traumatic or inflammatory injury. Under normal circumstances, type 2 EMT is limited to an acute repair process (e.g., wound healing) and can be beneficial, as it provides tissue replacement. Unlike the type 1 EMT, the type 2 EMT is expressed over extended periods of time and can eventually destroy an affected organ if the primary inflammatory insult is not removed or attenuated. Finally, type 3 EMT is associated with migratory and invasive features of tumor cells. A characteristic of type 3 EMT is that it originates from cells that have already undergone malignant transformation. Thus, the genetic and epigenetic changes typical for cancer cells, such as the activation of oncogenes and the inactivation of tumor suppressors, can act in concert with the EMT program. The composition of the basement membrane also changes, altering cell-ECM interactions and signaling networks. The next step involves EMT and an angiogenic switch, facilitating the malignant phase of tumor growth. Progression from this stage to metastatic cancer also involves EMTs, enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastases, which may involve METs and thus a reversion to an epithelial phenotype. After the transition to a mesenchymal state, cells can also change back to an epithelial state in a process known as mesenchymal-epithelial transition (MET) (Figure 10).

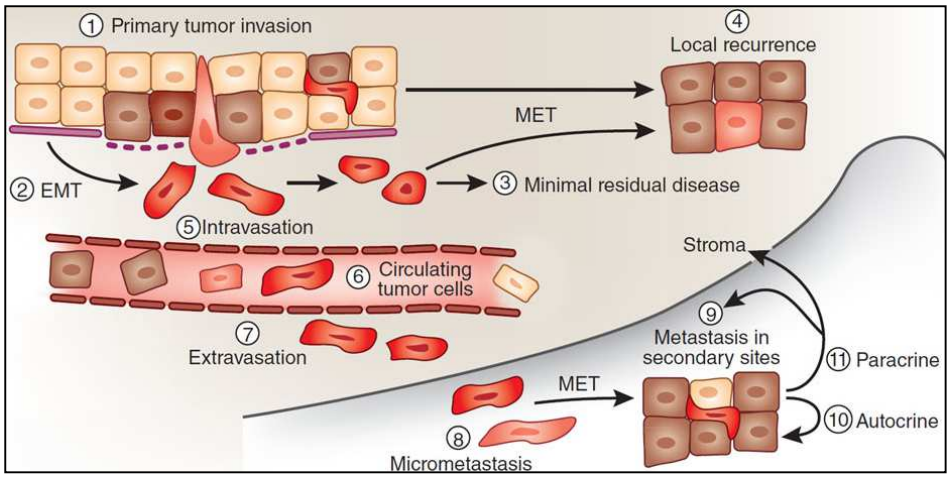

Figure 10: Contribution of EMT to cancer progression.

In EC, alteration of EMT markers have been identified in metastatic disease and associated with reduced survival (Table II). Importantly, loss of epithelial markers such as E-cadherin is associated with adverse prognosis in both EEC and NEM tumor types (61-63). Increased expression of the E-cadherin transcription repressors Twist, Snail, and Slug has been demonstrated in EC cell lines and in tumor samples, and down-regulation of E-cadherin immunoreactivity has been described in both EEC and nonendometrioid malignancies (64). It has been shown that E-cadherin repressors Slug, ZEB1, and HMGA2 were expressed preferentially at the myoinvasive front of EEC and that EMT-like changes could be induced in vitro through extracellular signal-regulated kinases ERK) 1/2 phosphorylation (65).

Table II: Factors involved in EMT in endometrial cancer	
Transcriptional regulators	Snail/Slug Zeb1 Twist1/Twist2 KLF17 ETV5 HMGA2
Growth factors	TGF-β EGF VEGF IGF1
Oncogenes	BMI-1
Tumor suppressor	P53
miRNAs	miR-200 family miR-155 miR-130
Other molecular factors	ERα TrkB PR

Table II: EMT markers in EC

miRNAs are small non-coding RNA elements that control cellular function by modulating the stability and translation of multiple target mRNAs at the post-transcriptional level (Figure 11). They play important roles in development, cellular differentiation, proliferation, cell-cycle control, and cell death.

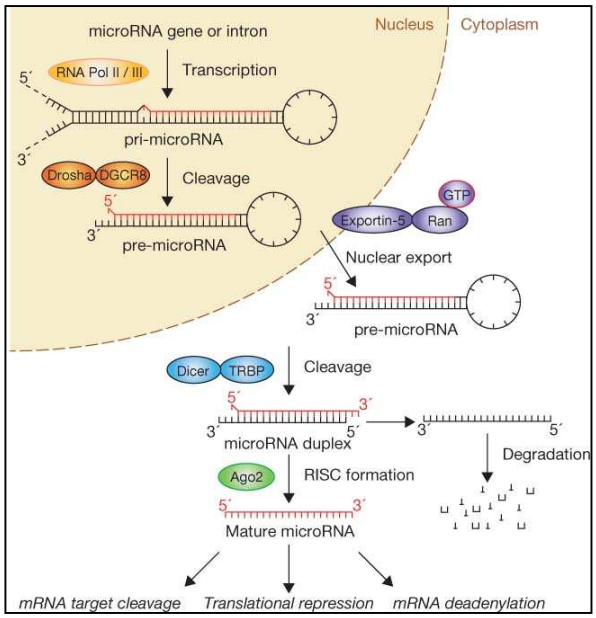

Figure 11: The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. In this review we discuss the many branches, crossroads and detours in miRNA processing that lead to the conclusion that many different ways exist to generate a mature miRNA.

It is recognized that, in order for tumors to develop, cells must acquire cellular characteristics that are very different from those of healthy cells. Since individual miRNAs have potentially hundreds of target genes, miRNA dysregulation will have a profound effect on the regulation of the cellular machinery and contribute to enabling cancer hallmarks. Several studies have identified miRNAs, which are differentially expressed in EC compared to healthy endometrial tissues (66). For example, miR-205 (67-70), and miR-96 cluster (hsa-miR-96, hsa-miR-182 and hsa-miR-183) (70,71) were found upregulated in EC compared to benign tissues. Other studies identified three miRNAs (miR-499, miR-135b and miR-205) as upregulated and five (miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21) as downregulated (72). In particular, the miR-200 family members have been extensively studied with respect their role in EMT in various tissues, where they target the expression of many genes, such as the transcription factors ZEBs (73,74). It has been already shown that elevated levels of all miR-200 family, in all stages of EC, inversely correlates with the expression of ZEBs. miR-200s upregulation has been demonstrated in type I EEC compared to normal endometrial tissues (75,76) in keeping with observations in other tumours, such

as melanoma (77,78), ovarian cancer (79), and colorectal carcinoma (80). Recently, it has also been suggested that miR-200 family, under influence of estrogen, maintains an epithelial phenotype in lower grade EEC (81). However, based on hormone status, miR 200a upregulation has been linked with outcome of EC patients. A recent study correlated miR-200a with prolonged survival in ERs positive subgroup, whereas an inverse trend was observed in the ERs negative soubgroup (82).

2 THESIS AIMS AND RESULTS

2.1 MANUSCRIPT I

The lamin A/NF-Y protein complex reveals an unknown transcriptional mechanism of cell cycle regulation.

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The analysis of global regulatory perturbations across human cancers pointed at NF-Y as one of the transcription factors responsible for oncogenic transcriptional changes. Although mutations in NF-Y subunits have never been specifically identified in tumours, systematic examination of protein expression profiles indicates that NF-YA transcriptional activity is upregulated in different types of cancer. Thus, identification of NF-Y protein partners can help to the characterization of mechanisms associated with its tumorigenic potential. In the present study, starting with a mass-spectrometry screening, we identified a novel nuclear protein complex formed by lamin A and NF-Y involved in chromatin binding and cell proliferation. Using a combination of biochemical, cell biology and molecular imaging techniques, we demonstrated that NF-Y, physically interacting with lamin A, strongly impacts on cancer cell proliferation. Changes in lamin A expression have been reported in a variety of cancers, correlating with tumorigenic potential and more aggressive phenotype. In our study, ChIP experiments demonstrate that lamin A physically interacts with several promoter regions of cell cycle genes in a NF-Y dependent manner. In particular, we detected lamin A binding to promoter regions encompassing CCAAT boxes of actively transcribed NF-Y target genes, such as *CCNB2, DHFR, CCNA2, CDK1, CCNB1, CDC25C, TOPO2a* and *PCNA*, as demonstrated by histone methylation marks and pol II recruitment. Moreover, we showed that lamin A has a role in transcriptional regulation of several NF-Y target genes impairing its transcriptional activity. Numerous studies showed that lamin A can modulate cell signaling through several mechanisms, for example, by sequestering transcription factors in inactive complexes, modulating post-translational modifications and degradation, and regulating transcriptional complexes. We hypothesizes that lamin A hinders the targeting of NF-Y to its consensus sites and highlight a dose-dependent effect of lamin A binding. In fact, we observed an increased NF-Y transcriptional activity in LMNA silenced cells and a basal *CCNB2* promoter activity inversely correlated to lamin A expression. Moreover, gain and loss of function experiments revealed that LMNA counteracts NF-Y transcriptional activity impacting on cell cycle progression. It has already been observed that lamin A interactions often appear to be confined to promoter subregions rather than to entire promoter regions. Our data support a view of lamin A as modulator of NF-Y transcriptional activity

by its interaction with NF-YA, and are consistent with a locus-specific regulation of lamin A interactions with promoters important for cell cycle regulation and tumor progression. To demonstrate *in vivo* the impact of LMNA on NF-Y transcriptional activity we took advantage of MITO-Luc mouse model, that we recently developed, harbours a strictly NF-Y dependent promoter in front of a luciferase reporter allowing us to monitor the NF-Y activity in a spatiotemporal manner within the entire living organism. Data obtained strongly support the physiological impact of lamin A expression in cell proliferation. We suggest that changes in lamin A expression could modulate NF-Y activity and, consequently, its oncogenic transcriptional potential. To investigate the role of lamin A in cell cycle progression we compared the ability of SW-480 and SW-480 LMNA-KD cells to grow under low nutrient or oxidative stress conditions Our data indicate that the lamin A/NF-Y complex strongly impacts on cancer cell proliferation under cellular stress conditions, Further exploration to uncover the molecular mechanism(s) by which lamin A/NF-Y complex acts as crucial regulator in diverse cellular processes and, in particular, in cancer could be important to improve and potentially provide new clues into new therapeutic approaches for cancer treatment.

2.2 MANUSCRIPT II

Prognostic role of NF-YA splicing isoforms and lamin A status in low grade endometrial cancer.

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Endometrial cancer (EC) is a major cause of mortality for patients worldwide. Although most cases of low grade ECs do not behave aggressively, in rare instances, even low-grade, well-differentiated ECs can progress in a highly aggressive manner. Current clinical approaches in the treatment of EC mainly relies on surgical FIGO classification, histologic subtype, and histologic grade. Identification of novel molecular markers may be helpful to avoid risk of over-and under treatment of EC patients and to overcome recurrence. In this study we analyzed several EC tissues to find novel clinical and biological features to help the diagnosis and treatment of early ECs.

In this study, a retrospective cohort of FFPE specimens from patients with EC and benign (NE) specimens from patients who underwent a hysterectomy to treat other benign disease (n=13) were collected. According with the histologic grade, we analysed 29 low grade (G1), 49 high grade endometrioid (G2-G3) and 10 non endometrioid EC tissues (NEM). Biopsies were sampled for primary tumors in hysterectomy specimens.

A recent study based on both informatics analysis and microarray expression profile of the motifs of known transcription regulators and experimental evidence from ENCODE, identified NF-Y as one of the key components the key transcription factors involved in gynecological cancers. NF-Y is composed of three different subunits: YA, YB and YC. The association between NF-YB and NF-YC provides a docking site for NF-YA, and NF-YA is the regulatory subunit of the complex responsible for sequence-specific DNA binding. Subunit NF-YA has two different isoforms, NF-YAl (long) and NF-YAs (short), resulting from alternative splicing. It has recently demonstrated that NF-YAs belongs to the embryonic stem cell transcription factor circuitry. We analysed the expression of NF-Y in several FFPE specimens by comparing the protein expression level of two subunits of NF-Y complex, NF-YA and NF-YB, in EC and benign endometrial tissues. We found that the exclusive expression of NF-YAl characterizes benign endometrial tissues, whereas the appearance of the NF-YAs is specifically associated with a tumour phenotype. In fact, the short form was detectable only in EC tissues. Very interestingly, NF-YAs was expressed in all high grade EEC and NEM tissues, whereas it was detected only in 55% of our low grade G1 EEC samples. This result suggests that NF-YAs could represent a diagnostic marker in early EC. To explore the potential role of NF-YAs in EC aggressiveness, we stratified G1 EEC tissues in two subgroups: one expressing only NF-YAl (NF-YAs-) and another expressing both isoforms (NF-YAs+).

Concomitantly, we analysed the ERs status, whose loss of expression has been well documented in advantage stages and poorly differentiated tumours, in all our cohort of FFPE EC samples. A massive down-modulation of ESR1 mRNA in 61,2% high grade EC and in NEM tissues was observed, in agreement with literature data indicating a strong correlation between the downmodulation of this gene with advanced stage of EC. Focusing on our G1 subgroups (NF-YAs- and NF-YAs+), very interestingly we observed a correlation with the loss of ESR1 and the presence of NF-YAs. In fact, expression of ESR1 was lower in NF-YAs+ compared with NF-YAs- tissues. These evidences indicate a possible involvement of NF-YAs expression in EC aggressiveness.

Lack of ESR1 has been recently associated with epithelial to mesenchymal transition (EMT). We analysed mRNA levels of several EMT markers, such as E-Cadherin, N-Cadherin, miR-200 family and its direct targets, ZEB1 and ZEB2. Analysis of the qRT-PCR data showed that an augmented percentage of EC tissues exhibits a low E-cadherin/N-cadherin (E/N) ratio, an index of differentiated phenotype, together with an increase of E-Cadherin mRNA (CDH2) expression compared with benign tissues, and this modulation correlated with a more aggressive clinophatologic phenotype. The same analysis, performed in our G1 subgroups, revealed that both E/N ratio and CDH2 mRNA levels are not related with NF-YAs expression, since both groups showed the same expression pattern. miR-200s has already been demonstrated to be differentially expressed in EC compared to healthy endometrial tissues and associated with EMT in EC. Our analysis confirmed that all members of the family (miR-200a, miR- 200b, miR-200c, and miR-141) were up-regulated in all stages of EC compared to benign tissues and their expression inversely correlates with ZEB1 and ZEB2 mRNA expression. In our G1 subsgroups, we observed a consistent increase of miR-200 family expression inversely related to ZEB1 mRNA levels in G1 EEC NF-YAs+ compared with NF-YAs-, thus indicating a possible involvement of NF-YAs in miR-200 family regulation. Several studies identified A-type lamins as an indicator of differentiated tumour cells and demonstrated to represent a potential biomarker for various types of cancer. We observed a significant correlation of loss of lamin A expression with stage and histologic grade in EC. Interestingly, clustering of NF-YA isoforms in G1 EEC indicated that NF-YAs+ samples consistently exhibited lower lamin A expression compared with NF-YA-. Our findings indicate NF-YAs and lamin A as molecules with a potential for a more systematic stratification of low grade EC malignancy.

3 CONCLUDING REMARKS

One of the issues of our laboratory is to address the role of NF-Y in cancer. NF-Y is one of the transcription factors responsible for aberrant oncogenic transcription occurring in several cancers.

We firstly focused our study on the identification of novel NF-Y protein partners in order to better characterize the mechanism associated with its tumorigenic potential. Starting with a mass-spectrometry screening, we identified a nuclear protein complex formed by lamin A and NF-Y involved in chromatin binding, cell proliferation and cancer progression. Besides its localization to the nucler lamina, we observed that a small fraction of lamin A is also present in the nucleoplasm. We focused our study on the occurrence of lamin A/NF-Y association in the nucleoplasm compartment and, in particular, on chromatin where NF-Y exerts its role as transcription factor.

We demonstrated that lamin A binds to promoter regions encompassing CCAAT boxes of NF-Y target genes, such as *CCNB2, DHFR, CCNA2, CDK1, CCNB1, CDC25C, TOPO2a* and *PCNA*, and this binding is mediated by NF-Y. We clearly demonstrate that lamin A physically interacts with NF-Y target genes actively transcribed, as demonstrated by histone methylation marks and pol II recruitment, and that lamin A inhibits NF-Y transcriptional activity modulating transcription in a manner dependent on local chromatin marks. Our results are consistent with previous evidences demonstrating that down-regulation of lamin A/C leads to dissociation of lamin A/C from promoters by enhancing transcriptional permissiveness (83). It has been observed that lamin A interactions often appear to be confined to promoter subregions rather than to entire promoter regions. Our data support a view in which lamin A, through its ability to bind NF-Y, exerts a locus-specific interaction with promoters important for cell cycle regulation and tumor progression.

In our study, an inverse correlation between lamin A and several NF-Y target genes expression level was observed, thus supporting the role of lamin A as regulator of NF-Y transcriptional function. We validated these evidences by in vivo imaging involving the use of a genetically engineered mouse model called MITO-Luc (for mitosis-luciferase), in which an NF-Y–dependent promoter controls luciferase expression. Data obtained strongly support the physiological impact of lamin A expression in cell proliferation. Interestingly, our data obtained treating cancer cells under low nutrient and oxidative stress conditions (Figure 10) indicate that loss of lamin A in cancer cells may confer the ability to grow under low nutrient supply and oxidative stress and to adjust to a changed environment in vivo by inducing gene expression so that the tumor continues to grow.

Figure 10

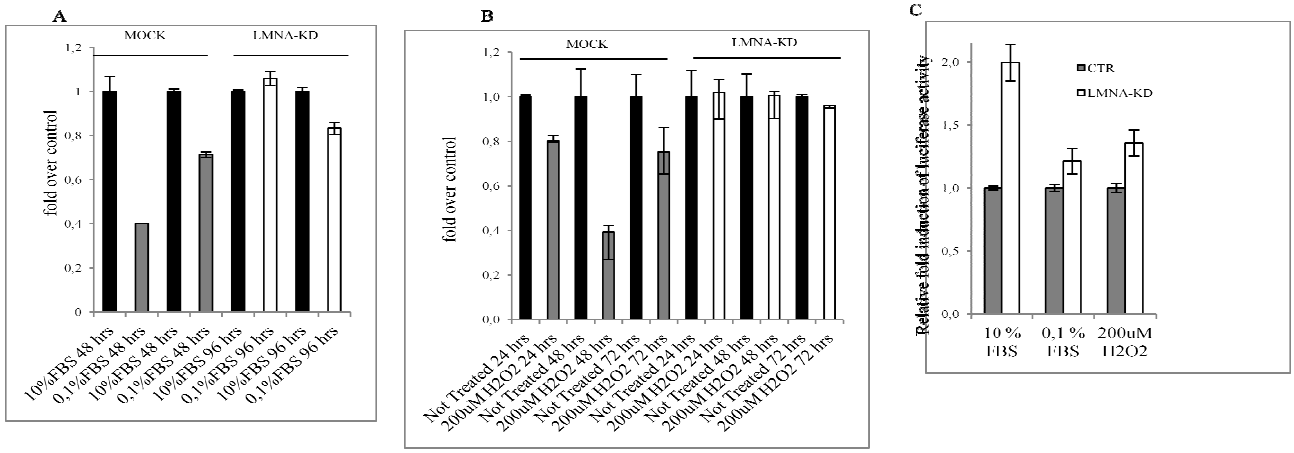

Figure 10: To investigate the role of LMNA in cell cycle progression, we performed time course experiments and compared the ability of SW-480 and SW-480 LMNA-KD cells to grow in 0,1% serum or after hydrogen peroxide treatment (200µM H_2O_2). As shown in this figure, the growth of SW-480 cells was partially reduced upon growth factor deprivation (A) or upon oxidative stress conditions (B) compared with control cells, whereas the growth of SW-480 LMNA-KD cells was always not impaired. This results, together with the higher basal *CCNB2* promoter luciferase activity observed both under serum deprivation and oxidative stress conditions in LMNA-KD cells compared with control cells, even if at low extent (C), suggest a rate proliferation gain of function related to the increase NF-Y translational acitvity for the cells with reduced lamin A levels.

Cancer cells from solid tumors become metabolically stressed, when nutrients are insufficient within poorly vascularized regions. Metabolic stress results from severe deprivation of oxygen, glutamine and glucose, partly through excessive reactive oxygen species (ROS) production. Oxygen radicals may augment tumor invasion and metastasis by increasing the rates of cell migration. During transformation into invasive carcinoma, epithelial cells undergo profound alterations in morphology and adhesive mode, resulting in a loss of normal epithelial polarization and differentiation, and a switch to a more motile, invasive phenotype. A better understanding of these adaptations might reveal cancer cell liabilities that can be exploited for therapeutic benefit. Further exploration to uncover the molecular mechanism(s) by which NF-Y/ lamin A complex acts as crucial regulator in diverse cellular processes and, in particular, in cancer could be important to improve and potentially provide new clues into new therapeutic approaches for cancer treatment.

Loss of lamin A expression have been reported in a variety of cancers, correlating with tumorigenic potential and more aggressive phenotype (35-39). Our data suggest that changes in lamin A expression could modulate NF-Y activity and, in particular, lamin A downmodulation may increase its oncogenic transcriptional potential.

To understand the clinical impact of lamin A expression in cancer and its possible correlation with NF-Y expression, we translated our findings in a clinical study focused on the identification of

novel molecular biomarkers in EC. Current clinical approaches in the treatment of EC mainly relies on surgical FIGO classification, histologic subtype, and histologic grade. Identification of novel molecular markers may be helpful to avoid risk of over-and under treatment of EC patients and to overcome recurrence. A recent study based on both informatics analysis of the motifs of known transcription regulators and experimental evidence from ENCODE, identified NF-Y as one of the key components of the transcription regulation factories of gynecological cancer (84). We analyzed NF-Y expression levels in a cohort of formalin-fixed, paraffin-embedded (FFPE) EC tissues. In our study we identified a specific splicing isoform of the regulatory subunit of NF-Y, NF-YAs, as a new potential indicator of aggressiveness in G1 endometrial endometrioid adenocarcinoma (EEC). We observed that NF-YAs protein was undetectable in benign tissues, whereas it was consistently expressed in high grade EEC and in NEM subtypes. Interestingly, only in G1 EEC a heterogeneous expression of NF-YA isoforms was observed with some samples expressing exclusively the long form (NF-YAl) and others samples expressing both isoforms. This results prompted us to stratify G1 EEC in two subgroups: one expressing only NF-YAl (NF-YAs-), and another, including 40 % of G1 EEC tissues analysed, expressing both isoforms (NF-YAs+). It is worth to note that patients with G1 tumors involving only endometrium and no evidence of intraperitoneal disease have a low risk (<5%) of nodal involvement. Although most cases of G1 EEC do not behave aggressively, in rare instances, even low-grade, well-differentiated endometrial adenocarcinomas can progress in a highly aggressive manner. We hypothesize that the molecular feature related to NF-YA isoforms expression could be a relevant biomarker to predict the outcome of these cancers. The exclusive presence of NF-YAl form in benign tissues suggests that it may represent a marker of differentiation and that the presence of NF-YAs may be linked with an increase of a pool of poorly differentiated cells in tumors tissues. Lamin A has been demonstrated to play a key role in sensing tissue elasticity in differentiation and the reduction in its expression frequently correlates with cancer subtypes and cancer aggressiveness, proliferative capacity and differentiation state (85). To evaluate the possible involvement of lamin A also in EC, we analyzed its protein and mRNA expression levels. Interestingly, lamin A was consistently down-modulated in EC both at mRNA and protein level. Lamin A loss was further increased in high grade EEC and non-endometrioid endometrial adenocarcinoma (NEM), thus indicating lamin A as a novel potential marker of EC aggressiveness. It is worthwhile to note that decreased lamin A levels were observed in our subgroup of NF-YAs expressing G1 EECs, thus further supporting the hypothesis of a possible involvement of NF-YAs in tumor differentiation and aggressiveness. We confirmed this correlation by evaluating several indicators of EC aggressiveness, such as estrogen receptor status, miR-200 family expression and EMT markers, miR-200 family members and ZEBs.

Our studies presented in this thesis indicate NF-Y/lamin A complex as an important regulator of cancer cells proliferation. In particular, we identified NF-YAs, a specific isoform of NF-YA, and lamin A as two novel potential targets and predictive markers for new therapeutic approaches in EC, in particular in low grade EEC, which may contribute in determining a patient's prognosis and in tailoring adjuvant therapies.

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6 MANUSCRPIT I

The laminA/NF-Y protein complex reveals an unknown transcriptional mechanism of cell cycle regulation.

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ABSTRACT

Lamin A is a component of the nuclear matrix that also control proliferation by largely unknown mechanisms. NF-Y is a ubiquitous protein involved in cell proliferation composed of three subunits (-YA -YB -YC) all required for the DNA binding and transactivation activity. To get clues on new NF-Y partner(s) we performed a mass spectrometry screening of proteins that co-precipitate with the regulatory subunit of the complex, NF-YA. By this screening we identified lamin A as a novel putative NF-Y interactor. Co-immunoprecipitation experiments and confocal analysis confirmed the interaction between the two endogenous proteins. Interestingly, this association occurs on transcriptionally active chromatin regions, too. ChIP experiments demonstrate that lamin A physically interacts with several promoter regions of cell cycle genes in a NF-Y dependent manner. Gain and loss of function experiments reveal that lamin A counteracts NF-Y transcriptional activity impacting on cell cycle progression. Taking advantage of a recently generated transgenic reporter mouse, called MITO-Luc, in which an NF-Y–dependent promoter controls luciferase expression,

we demonstrate that lamin A counteracts NF-Y transcriptional activity not only in culture cells but also in living animals. Altogether, our data demonstrate lamin A as a novel repressor of NF-Y activity and indicate its role as a suppressor of cell proliferation.

INTRODUCTION

NF-Y is a ubiquitous protein, composed of 3 subunits, NF-YA, -YB, and -YC, whose genes are highly conserved from yeast to mammals. All 3 subunits are required for NF-Y binding to the consensus sequence, the CCAAT-box. The NF-YB and -YC subunits contain histone-like domains, and an activation domain is present in the NF-YA subunit (1). A bioinformatic analysis of promoters of cell-cycle regulatory genes shows an abundance of CCAAT boxes in promoters regulated during the G2/M transition (2). Consistent with this, the NF-Y complex supports basal transcription of a class of regulatory genes responsible for cell-cycle progression, among which are mitotic cyclin complexes (3-10). Taken together, these studies demonstrate that the binding of NF-Y to cellular promoters is essential for cell proliferation. NF-YA is the regulatory subunit of the trimer. It has been reported the presence of two major NF-YA isoforms, “long” and “short”, the short isoform lacking a 28-amino acid within the NF-YA amino-terminal domain (11). Expression of NF-YA in normal cells is modulated during the cell cycle (4) and its abrogation plays an important role in downregulating several cell-cycle control genes in differentiated cells (5,7,9,10). Previous studies aimed at understanding the biological role of NF-Y took advantage of a loss of function approach, such as expression of dominant-negative NF-YA mutants and conditional deletion of the mouse NF-YA gene. When a dominant-negative NF-YA mutant that interacts with -YB/YC but does not bind DNA is expressed in mouse fibroblasts, retardation of cell growth is observed (12). The knock out of the NF-YA subunit in mice leads to embryo lethality; moreover, inactivation of the NF-YA gene in mouse embryonic fibroblasts results in inhibition of cell proliferation and growth arrest at various phases of the cell cycle (13-14). Abundant evidence indicates that NF-Y is involved in cancer. We have demonstrated that NF-Y modulates the promoter activity of several genes in response to DNA-damaging agents (15, 16), and NF-Y overexpression increased the proliferation rate of cancer cells harbouring endogenous mutant p53. Next, we have shown that NF-Y interacts in vivo with mutant p53 and increases DNA synthesis, which is impaired upon abrogation of NF-YA expression (8, 17). Clinical studies have indicated that patients with upregulated expression of NF-Y target genes have poor prognosis in multiple cancers (8, 18). Using global gene expression profiles, the involvement of NF-Y in cancer-associated pathways has been recently reported across human cancers (19). In agreement with its wide involvement on human cancers, we have described that NF-Y interacts with different partners.

Indeed, we have shown that in normal cells NF-YA binds to deacetylase enzymes (HDACs) while in transformed cells the acetylase p300 is preferentially recruited (8-9). Although some NF-Y interactors are already known, several partners through which NF-Y exerts its role still need to be characterized.

Lamins are components of the nuclear lamina that play important roles in nuclear architecture, mechanosignaling (20) and chromatin dynamics (21) and impact on stem cell proliferation and differentiation (22, 23). Disruption of one or more of these functions due to lamin mutations cause a group of inherited diseases affecting various tissues and organs or causing accelerated ageing (21). Interactions of lamins with chromatin occur through domains termed lamina-associated domains (LADs) with the implication that lamins associate with chromatin at the nuclear lamina at the nuclear periphery. LADs are often located in repressive chromatin structures with an enrichment of this compartment at the nuclear periphery (24). Although most lamins are found near the nuclear membranes, nucleoplasmic populations also exist, which may have distinct roles (25-28). A-type lamins, whose most represented isoforms are lamin A and C, are alternatively spliced products of the same gene, LMNA, and are found in roughly similar amounts in most tissues. The expression of the A-type lamins is often reduced or absent in cells that are highly proliferative, including various human malignancies (29-33). Moreover, cells lacking A-type lamins proliferate faster and display inefficient cell cycle arrest upon contact inhibition (34). Recent data highlight the specific functions of a small pool of lamina-independent A-type lamins, located throughout the nucleoplasm, in the regulation of early tissue progenitor cell proliferation and commitment (25, 35, 36).

Using a combination of biochemical, cell biology and molecular imaging techniques, we demonstrate here that NF-Y, a master regulator of cell proliferation, physically interacts with a component of the nuclear lamina, lamin A and this interaction strongly impacts on cancer cell proliferation.

MATERIALS & METHODS

Cell lines:

Human breast cancer cell lines MCF-7 and SKB-R3, human colorectal carcinoma cell lines HCT-116 and SW-480, human osteosarcoma with osteoblastic properties cells Saos-2, human primary fibroblasts (HF), and human cervical adenocarcinoma HeLa cells were all cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum and antibiotics in a humidified 5% CO_2 atmosphere.

Total lysates extraction:

Cells, harvested in cold phosphate-buffered saline, were extracted for 30 min at 4˚C in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40) containing 1 mM DTT, protease inhibitor cocktail (Roche) and phosphatase inhibitors (50 mM NaF, 0.2 mM Na3VO4). After centrifugation, supernatants were collected as the total protein extract and stored at -80˚C. Protein concentrations were measured using the Bradford-type protein assay (Bio-Rad).

Lamins solubilization:

Cells were resuspended in hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 % NP-40, 0.5 mM DTT, and protease inhibitors) and nuclei separated by centrifugation. Nuclei were then washed in 1 KCk and then incubated 30 min in moderate-salt buffer buffer (300 mM KCl, 2% Triton X-100, 10% sucrose, 20 mM MES-KOH pH 6.0, 2 mM EDTA, 1 mM DTT) and centrifuged (3,300g, 15 min, 4°C) to obtain a lamin-enriched pellet. This pellet was incubated on ice 30 min in high pH/high detergent buffer (300 mM KCl, 2% Triton X-100, 20 mM Tris-HCl pH 9.0, 2 mM EDTA, 1 mM DTT) followed by centrifugation (6,000g, 20 min) to yield a supernatant of solubilized lamins (soluble lamins) and an insoluble pellet.

Chromatin extraction:

Cells were lysed by mechanical homogenization in isotonic- sucrose based buffer (15 mM Tris-HCl pH 7.5, 15 mM NaCl, 60 mM KCl,5 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM Sucrose, 0.3 % NP-40, and protease inhibitors) followed by centrifugation (5 min, 1,300 g, 4°C). The pellet, containing the nuclei, was resuspended and lysed in lysis buffer (20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 150 mM KOAc, 3 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40 and protease inhibitors) and centrifuged (5 min, 1,700 g, 4°C). The supernatant corresponds to the nucleoplasmic fraction and the pellet to the chromatin fraction.

Euchromatin isolation:

Chromatin fraction, obtained as described above, was digested im MNase buffer (20 mM Tris pH 7.5, 15 mM NaCl, 1 mM CaCl2) with Micrococcal nuclease to a final concentration of 1.2 units/mL for 5 minutes, and the reaction was quenched by 1 mM EGTA on ice for 10 minutes. The sample was centrifuged at 1,000× g for 5 minutes at 4°C to generate the supernatant, corresponding to the euchromatin fraction. The insoluble chromatin pellet was resuspended in 15 mM Tris, pH = 7.5, 0.5% SDS. Extraction of DNA

DNA was isolated from chromatin samples by extraction with an equal volume of phenol/chloroform (1:1), extraction with an equal volume of chloroform, ethanol-precipitated and then resuspended in water and loaded onto a 2% agarose gel.

Immunoprecipitation and immunoblotting:

For immunoprecipitation experiments, lysates were clarified and immunoprecipitated at 4˚C overnight in lysis buffer by adding protein G-agarose after 2 h of incubation with 2 μg of antibody. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Each membrane was blocked with 5% non-fat dry milk in Tris buffered saline-Tween-20 (TBST) for 1 h at room temperature and subsequently incubated with primary antibody for 16 h at 4˚C. The following antibodies were used: anti-NF-YA polyclonal (Santa Cruz), anti NF-YA monoclonal (Santa Cruz) and anti-lamin A (Santa Cruz) and anti-lamin A/C (Santa Cruz), anti-β actin (sigma-aldrich). Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary antibody.

Plasmids and transfections

Plasmids used in transfections were as follows: NF-YA and empty vector (Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene 1999; 239: 15–27), B2-Luci and mutant Y1,2m-luci constructs (Bolognese F.et al (1999) Oncogene 18:1845–1853), pMXIH-V5 and PMXIH-lamin A res (Nitta RT, Smith CL, Kennedy BK. PLoS One. 2007 Sep 26;2(9):e963), pcDNA6.2-GW/EmGFP-miR-LMNA (Invitrogen)

Cells were transfected with Lipofectamine LTX and Plus reagent (Invitrogen) following the manufacturer's instructions. For reporter assay luciferase activity was measured using the dual luciferase assay system (Promega) according to the instructions of the manufacturers. All transfections were done as cotransfections with a CMV-driven plasmid expressing Renilla luciferase as internal control to standardize transfection efficiencies.

ChIP assay

1% formaldehyde was added directly to the cells and incubated at 22 °C for 10 min. The reaction was stopped adding 0.125 m glycine. Then, the cells were rinsed with cold 1× PBS, incubated with 0.2× trypsin-EDTA in 1× PBS, and scraped. cells were centrifuged, washed in cold 1× PBS plus 0.5 mm PMSF and resuspended in lysis buffer (5 mm piperazine N,N bis zethone sulfonic acid (pH 8.85) mm KCl, 0.5% Nonidet P-40). Next, nuclei were solicited in the sonication buffer (0.1% SDS, 10 mm EDTA, 50 mm Tris-HCl (pH 8), 0.5% deoxycholic acid) for 10 min by using a microultrasonic cell disruptor. The chromatin was sheared to an average size of 500 base pairs, and immunoprecipitation was performed with protein G-agarose (KPL). The chromatin solution was precleared by adding protein G for 1 h at 4 °C and incubated at 4 °C overnight with 4 μg of antibody or non-specific immunoglobulins (IgGs, Santa Cruz Biotechnology) as negative control. Input was collected from a control sample supernatant (not immunoprecipitated antibody). Immunoprecipitates were recovered by incubation for 2 h at 4 °C with protein G-agarose precleared previously in immunoprecipitation buffer (1 μg/μl bovine serum albumin, 1 μg/μl salmon testis

DNA, protease inhibitors, and PMSF). Reversal of formaldehyde cross-linking, RNase A, and proteinase K treatments were performed. DNA was phenol-extracted, ethanol-precipitated, and analyzed by PCR. DNA representing 0.005–0.01% of the total chromatin sample (input) or 1–10% of the immunoprecipitates was amplified using specific primers indicated below. The following antibodies were used: anti-Pol II phospho ser 3 (Upstate), anti Pol II phospho ser 5 (Upstate), anti-H3K14ac (Abcam), anti-H4K20me3 (Abcam), anti-NF-YA polyclonal (Santa Cruz), anti NF-YA monoclonal (Santa Cruz) and anti-lamin A (Santa Cruz) and anti-lamin A/C (Santa Cruz). PCR analysis was performed with HOT-MASTER Taq (Eppendorf). Quantitative PCR (qPCR) was performed using SYBR Green (Applied Biosystems) on an ABI Prism 7500 apparatus (Applied Biosystems). Primers used are the following:

PCR primers for ChIP assay		Amplicon length (bp)
CCNB2 FWD	ACCGGCTGT TGTGACAATCA	76 bp
CCNB2 REV	GGCCAACACAAGATGCACTCT	
DHFR FWD	CTGGAGACCTAAGGGCAGCTT	81 bp
DHFR REV	TTGGTGGTCGAAGAGTTTTACTGA	
CCNA2 FWD	GCCCCAGCCAGTTTGTTTC	71 bp
CCNA2 REV	GGCGAGTGAAGGGTAAACCA	
CDK1 FWD	CGTAGCTGGGCTCTGATTGG	93 bp
CDK1 REV	CAAACTCACCGCGCTAAAGG	
CCNB1 FWD	GCCCTGGAAACGCATTCT C	78 bp
CCNB1 REV	CCTCCTTATTGGCCTGTTCGT	
CDC25C FWD	GCTGGTGGGCCAAACACT A	72 bp
CDC25C REV	TGTGCTTGCTCTGGAAATGG	
TOPO 2A FWD	TGGCCAGATTCCCTGTCAAT	81 bp
TOPO2A REV	AGGTTAGGGAGGCGGGACTA	
PCNA FWD	CACATATGCCCGGACTTGTTC	94 bp
PCNA REV	CAGGTCTCCCCGCCTCTT	
CXCR4 FWD	AGTGGTTTGACCTCCCCTTT	150 bp
CXCR4 REV	ACTTGCACCTGCCAGTCTTC	

RNA extraction and RT-PCR

Total RNA was extracted using the Trizol reagent (Gibco BRL) following the manufacturer's instructions. The first-strand cDNA was synthesized according to the instructions for the M-MLV

RT kit (Invitrogen). Quantitative PCR (qPCR) was performed using SYBR Green (Applied Biosystems) on an ABI Prism 7500 apparatus (Applied Biosystems). mRNA expression was normalized for β-actin levels. Primers used are listed below. Relative mRNA expression was calculated using the comparative Ct method ($2^{--\Delta\Delta Ct}$).

PCR primers for mRNA quantification		Amplicon length (bp)
CDK1 FWD	GCGGAATAATAAGCCGGGATC	104 bp
CDK1 REV	CCCTTATACACAACTCCATAGGT	
CDC25C FWD	TCCTGGAGAGAGACACTTCC	118 bp
CDC25C REV	CAACGTTTTGGGGTTCCTCC	
CCNB1 FWD	TGCAGAAGATGGAGCTGATC	123 bp
CCNB1 REV	GTGACTTCCCGACCCAGTAG	
CCNB2 FWD	GCACATGGCCAAGAATGTGGTG	149 bp
CCNB2 REV	TCAGTGGGGAGGCAAGGTCTT	
DHFR FWD	AAACTGCATCGTCGCTGTGTC	148 bp
DHFR REV	ACCCATAATCACCAGATTCTGT	
LMNA FWD	GGACAATCTGGTCACCCGC	96 bp
LMNA REV	TGGCAGGTCCCAGATTACATG	
ACTIN FWD	GGACTTCGAGCAAGAGATGG	134 bp
ACTIN REV	AGCACTGTGTTGGCGTACAG	

FACS cell-cycle analysis

Cells were harvested, washed in PBS, and fixed in MetOH:acetic acid solution (4:1) for 60 minutes at +4°C. Cells were then incubated in 500 μL of staining solution (50 μg/mL of propidium iodide, 50 μg/mL of RNAase, 0.1% Triton X-100 in PBS 1×) for 1 hour at 4°C and analyzed by flow cytometry.

Retroviral infection.

Phoenix-ampho cells (American Type Culture Collection) were transfected with pMXIH-V5 or pMXIH-V5 Lamin A. The supernatant medium containing the emerging retrovirus was collected 48 and 72 h after transfection. The supernatants were pooled and the retroviral particles were concentrated by ultracentrifugation at 22.000 rpm for 2 h, resuspended in cold PBS and stored at -80°C. The viral titer was determined infecting NIH3T3 murine fibroblasts at different serial

dilutions. The retroviral vectors were injected into the tail vein in adult mice with 5.25x10^2/mouse.

***In vivo* BLI and experimental animals.** Light emission was detected using the IVIS Lumina II CCD camera system and analyzed with the Living Image 2.20 software package (Caliper Life Sciences). Mice were anesthetized and 150 mg/kg or 75 mg/kg of D-luciferin were injected IP. Ten minutes later, quantification of light emission was performed in photons/second and visualized in a pseudo-color scaling. Time exposure ranged from 1 to 5 minutes depending on light intensity.

Animal experiments performed in this study were conducted according to the "Giudelines for Care and Use of Experimental Animals" and the Italian low DL 116/92.

RESULTS

NF-Y interacts *in vivo* with lamin A

To get clues on NF-Y function(s) in cancer cells, we performed a mass spectrometry screening of a pool of proteins that co-precipitate with NF-YA subunit overexpressed in human breast cancer, SKBR3 cells. By this screening we identified lamin A as a novel putative NF-YA interactor (Supplementary Figure S1).

This result was validated by coimmunoprecipitation experiments between endogenous proteins. As shown in figure 1A, both lamin A and NF-YA are expressed in all tested cell lines. Lysates from these growing cells were immunoprecipitated with anti-NF-YA antibody and subjected to western blot analysis. Endogenous Lamin A coimmunoprecipitated with both differentially spliced forms of endogenous NF-YA protein in all cell culture lines (Figure 1B).

Reciprocal immunoprecipitation experiments performed with anti-laminA/C antibody further validated the occurrence of endogenous lamin A/NF-YA in all tested cell lines (Figure 1C). As expected NF-YB subunit is present in the complex, too. As shown in supplementary figure S2A, an antibody against NF-YB protein immunoprecipitated lamin A, further confirming the involvement of this subunit in the complex and thus indicating that lamin A associates with the NF-Y complex and not with the NF-YA subunit alone. Transfection with plasmid encoding the long form of NF-YA in SW-480 cells confirmed this result (Supplementary Figure S2B). We also overexpressed the dominant negative mutant of NF-YA subunit (dnNF-YA) that carrying a triple aminoacid substitution in the DNA binding domain does not bind DNA and impairs NF-YA transcriptional activity (37). Results demonstrate that dnNF-YA mutant form is still able to efficiently bind lamin A thus suggesting that the DNA binding domain of NF-YA is not necessary for its interaction with lamin A.

Confocal analysis performed on several proliferating cells confirmed lamin A/NF-YA interaction both in interphase (Figure1C and Supplementary Figure 2C) and in different mitosis phases (Figure 1D). Interestingly, we observed a colocalization of lamin A and NF-YA both in the nucleoplasm and in the nuclear lamina.

Taken together, our data reveals the existence of a lamin A/NF-Y nuclear complex on several human cell lines.

NF-YA localizes in lamin enriched nuclear fractions. Accurate identification of the cellular compartment(s) where an interaction takes place is often critical to understand the function of that interaction. To get clues on lamin A and NF-YA physical interaction, we isolated different lamin enriched nuclear fractions (Supplementary Figure S3A). We isolated nuclei in hypotonic buffer. Firstly, we verified the presence of lamin A by western blot on resuspended nuclei (Supplementary Figure S3B). We treated isolated nuclei with moderate-salt buffer to solubilize most nuclear proteins, and then pelleting to obtain a lamin-enriched pellet. The lamin-enriched pellet was solubilized by high pH and high detergent and, upon centrifugation, we separated soluble lamins from insoluble pellet (38). The two fractions were studied for the presence of lamin A and NF-YA. As expected, both lamin A and C were present on soluble lamin-enriched fractions and they are still present, on insoluble pellet (Figure 2A). Interestingly, NF-YA was found both in the lamin-enriched fractions and on insoluble pellet thus suggesting NF-YA as a nucleoskeletal protein (Figure 2A). Taken together, these results strongly support the scenario that lamin A and NF-YA together localize in specific nuclear compartments.

Recent data support a role for lamin A in gene regulation through its interaction with chromatin (39). Thus, we asked whether the interaction of lamin A and NF-YA occurs on chromatin. To this purpose, we generated chromatin and nucleoplasm fractions from different cell lines. We isolated nuclei in isotonic-sucrose based buffer and, upon resuspension in potassium acetate, we separated nucleoplasm and chromatin (Supplementary Figure S4A). As shown in figure 2B, both NF-YA and lamin A were found in the chromatin fraction. As expected, NF-YA was present in nucleoplasm deprived of chromatin while lamin A was almost undetectable in this fraction. Next, we performed reciprocal co-immunoprecipitation experiments using chromatin fractions from several cell lines. The antibody against NF-YA immunoprecipitates lamin A and viceversa in all tested cell lines (Figure 2C). These results confirmed that lamin A/NF-YA association occurs on chromatin, too.

Lamin A binds *in vivo* NF-Y target genes in promoter regions encompassing CCAAT boxes in a NF-Y-dependent manner.

To start to investigate whether lamin A localizes on euchromatin, where NF-Y plays a key role as transcription factor, we first isolated euchromatin fraction from several cell lines (Supplementary

Figure S4A). To this purpose, we subjected the above isolated chromatin to mild MNase digestion to separate euchromatin, more accessible to MNase digestion. A time course for MNase digestion up to 30 minutes was performed on chromatin from SW-480 and MCF-7 cells in order to determine the minimal incubation time necessary for NF-YA extraction in the euchromatin fraction. Our data showed that after 5 minutes NF-YA was already detected in the supernatant. Of note, also lamin A, was detected in this fraction, although the majority of this protein was still associated with undigested chromatin after 30 minutes of MNase treatment thus indicating that the majority of lamin A is heterochromatin associated (Supplementary Figure S4B).

Based on this result, we digested for 5 minutes chromatin from several cell lines and analyzed it by agarose gel (Supplementary Figure S4C). In all employed cell lines we observed an enrichment of mono-, di- and tri-nucleosomes thus indicating an enrichment of transcriptionally active chromatin in these fractions. Interestingly, by western blot we demonstrated that NF-YA and LMNA are present in this fraction in all tested cell lines (Figure 2D and Supplementary Figure S4D). Taken together, the results obtained so far demonstrate the presence of a lamin A/NF-Y interaction on chromatin and the two proteins localize on the same open chromatin fraction thus suggesting the possibility that they cooperate on gene regulation.

Based on the observation that lamin A interacts with NF-Y on chromatin and they reside also on euchromatin regions, we asked whether lamin A binds NF-Y target promoters. To answer this question we performed ChIP experiments followed by quantitative real time pcr (ChIP-qPCR). Specific primers were used to amplify DNA regions encompassing NF-Y consensus sites on several NF-Y target promoters. Our experiments performed with two different antibodies against lamin A, demonstrate that it physically interacts with several promoter regions carrying the CCAAT-boxes, such as *CCNB2*, *DHFR*, *CCNA2*, *CDK1*, *CCNB1*, *CDC25C*, *TOPO2a* and *PCNA* promoters (Figure 3A). In contrast, when using primers corresponding to sequence of an unrelated promoter that do not contain CCAAT boxes, we did not find any specific lamin A in vivo recruitment (Supplementary Figure S4E). As expected (9), NF-Y binds all these promoter regions (Figure 3B). To directly assess the role of NF-Y in the recruitment of lamin A to the NF-Y promoter regions carrying CCAAT boxes, we performed ChIP experiments upon overexpression of dnNF-YA mutant protein in SW-480 cells. ChIP-qPCR analysis shows that the overexpression of dnNF-YA leads to a decrease in the recruitment of NF-Y (Figure 3D) and this correlates with a reduction of lamin A binding (Figure 3C), although at different extent depending on the analyzed promoter. These data, demonstrate that the binding of lamin A to several promoter regions carrying CCAAT-boxes is largely dependent on the binding of NF-Y complex, and indicate that lamin A binds these promoter regions through its ability to bind NF-Y.

Lamin A impacts on NF-Y transcriptional activity.

To characterize the functional role of the lamin A binding to NF-Y target promoters, we investigated the chromatin structure of the promoter regions bound by lamin A and NF-Y. To this purpose, we performed ChIP-qPCR experiments using antibodies against H4K20me3 and H3K14ac, hallmarks of closed and open chromatin structure, respectively. We observed an enrichment of H3K14ac on these regions thus demonstrating their open chromatin configuration and transcription-permissive state (Figure 4A). Next, to investigate the transcriptional activity of these promoter regions we performed ChIP-qPCR experiments using antibodies against RNA polymerase II active isoforms. Of note, the transcriptionally active phosphorylated forms of RNA Polymerase II (Ser-2 and Ser-5) are recruited to all the analyzed regions, thus further indicating gene activation (Figure 4B). The same results were obtained by ChIP followed by semiquantitavie PCR, amplifying cyclin B2 promoter as prototype of NF-Y target gene (Supplementary Figure S5A,B). Altogether, the results shown until now clearly demonstrate that lamin A physically interacts with NF-YA on chromatin of NF-Y target genes in an open conformation status and actively transcribed.

We confirmed the involvement of lamin A on NF-Y target gene transcription using *CCNB2* promoter driven luciferase reporter construct as a sensor of NF-Y activity. We overexpressed this construct in SW-480 and SAOS-2 cells together with a vector expressing NF-YA and/or a vector expressing an artificial miRNA targeting the lamin A mRNA (34). Our data suggest that down-modulation of lamin A, although not complete (Supplementary Figure S6A), led to a significant increase of *CCNB2* promoter activity upon NF-YA overexpression (Figure 4C) thus supporting the hypothesis that lamin A may interfere with NF-Y activity. As shown in supplementary figure 6B, NF-Y localization on euchromatin was not affected by lamin A silencing. This result suggests that, very likely, the enhanced NF-Y transcriptional activity observed by lamin A silencing depends on a decreased number of lamin A molecules able to associate with NF-Y and inhibits its activity

To validate these data, we produced stable siLMNA SW-480 and SAOS-2 cells (LMNA-KD cells) showing a reduction in the lamin A protein of approximately 60% and 70% , respectively, compared to control cells transfected with the same vector carrying a non-targeting artificial EmGFP-miRNA (Supplementary Figure S6C). We observed an increase of cyclin B2 promoter activity in LMNA-KD cells (Figure 4D). We also employed a *CCNB2* promoter construct carrying Y1,2 mutated CCAAT boxes (mut cyclin B2 promoter). As already shown (4), the basal CCAAT-less promoter activity of the Y1,2 mutant was significantly reduced compared to the wt construct but lamin A modulation did not affect significantly the residual activity (Figure 4D). In agreement

with the above results we observed in LMNA-KD cells an increase of the amount of several NF-Y target mRNAs such as *CCNB2*, *DHFR*, *CCNB1*, *CDC25C* and *CDK1*(Figure 4E).

Next, we stably transduced in SW-480 cells a human lamin A (lamin A-res) expressing vector resistant to the endogenous siRNAs (40) (Supplementary Figure S6D). Consistent with our previous results, introduction of lamin A-res led to a reduction of the basal *CCNB2* promoter activity of approximately 30% (Figure 4F) and to a decrease of *CCNB2*, *DHFR*, *CCNB1*, *CDC25C* and *CDK1* mRNA levels (Figure 4G). Also in these cells, *CCNB2* promoter construct carrying two mutated CCAAT boxes (mut *CCNB2* promoter) was not affected by lamin A modulation (Figure 4F).

To demonstrate *in vivo* the impact of lamin AA on NF-Y transcriptional activity we took advantage of the MITO-Luc mouse model, that we recently developed, harbours a strictly NF-Y dependent promoter in front of a luciferase reporter allowing us to monitor the NF-Y activity in a spatiotemporal manner within the entire living organism (41). Thus, we injected MITO-Luc mice intravenous with a retrovirus expressing lamin A-res (40), and we followed luciferase activity in the entire animals for several days. Of note, lamin A-res injection results in a inhibition of total body luciferase activity emitted by mice at any tested time (Figure 5A). Representative images of a control and injected mouse are shown in figure 5B.

All together these experiments demonstrate that lamin A impacts on NF-Y transcriptional activity in cell cultures and living animals. Interestingly, in MITO-Luc mice bioluminescence imaging of NF-Y activity visualizes areas of physiological cell proliferation (41-44). Thus, our findings strongly support a role for lamin A as a suppressor of cell proliferation.

To investigate the role of lamin A in cell cycle progression we compared the ability of SW-480 and SW-480 *LMNA*-KD cells to grow in 0,1% serum. As shown in figure 5C, the growth of SW-480 cells, as expected, is partially reduced upon growth factor deprivation compared with those of cells cultured in presence of growth factors. Of note, the growth of SW-480 *LMNA*-KD cells was not impaired at all by growth factor deprivation, indicating a rate proliferation gain of function for the cells with reduced lamin A (Figure 5C). Cell cycle of either the exponentially proliferating cells or the starved-cells was analyzed by flow cytometry at the indicated times. This analysis revealed that the fraction of cells in S-phase in SW-480 cells was decreased by 50% after 48 and 72 hours serum deprivation compared to control, whereas in *LMNA*-KD cells the percentage of cell number in S-phase were comparable under 10% and 0,1% FBS (Figure 5D).

Altogether, our data demonstrate lamin A as a novel repressor of NF-Y activity and indicate its role as a suppressor of cell proliferation. Moreover, we hypothesize a role of lamin A as a sensor of cellular stress, such as serum deprivation.

DISCUSSION

In the present study, starting with a mass-spectrometry screening, we identified a novel nuclear protein complex formed by lamin A and NF-Y involved in chromatin binding and cell proliferation.

NF-Y is a sequence-specific transcription factor that binds the common CCAAT element and has long been considered a modulator of genes involved in growth promotion including cell cycle regulatory genes (7,9). Numerous findings highlight that NF-Y is involved in cancer. Although mutations in NF-Y subunits have never been specifically identified in tumours, analysis of global regulatory perturbations across human cancers pointed at NF-Y as one of the transcription factors responsible for oncogenic transcriptional changes (19). Thus, identification of NF-Y protein partners can help to characterize the mechanism associated with its tumorigenic potential. Here, we provide evidences that lamin A associates with NF-Y on promoter regions encompassing CCAAT boxes of NF-Y target genes modulating its transcriptional activity.

Besides its localization to the nucler lamina, we observed that a small fraction of lamin A is also present in the nucleoplasm. Although there are some evidences that lamin A binds DNA, directly or through the histone proteins, the role of nucleoplasmic lamin A is not completely understood, so far (29,45). Thus, we focused our study on the occurrence of lamin A/NF-Y association in the nucleoplasm compartment and, in particular, on chromatin where NF-Y exerts its role as transcription factor.

In our study, we detected lamin A binding to promoter regions encompassing CCAAT boxes of NF-Y target genes, such as ccnb2, cdk1, cdc25c and dhfr and this binding is mediated by NF-Y. These genes are actively transcribed as demonstrated by histone methylation marks and pol II recruitment. Lamin A has been described to have a negative role on transcription. Although, we clearly demonstrate that lamin A physically interacts with NF-Y target genes actively transcribed, we observed that lamin A inhibits NF-Y transcriptional activity. These results suggest, therefore, that lamin-promoter interactions, per se, do not have a causative role on gene repression but may be able to modulate transcription in a manner dependent on local chromatin marks.

Numerous studies showed that lamin A can modulate gene expression through several mechanisms, for example, by sequestering transcription factors in inactive complexes, modulating their post-translational modifications and degradation, and regulating transcriptional complexes (46-51). Interestingly, we observed that the NF-Y target cyclin B2 promoter activity inversely correlated to lamin A expression and NF-Y transcriptional activity increases in lamin A silenced cells. Of note NF-Y localization on euchromatin was not affected by lamin A silencing (supplementary figure 7b) thus excluding the hypothesis that lamin A modulates NF-Y target

promoters by sequestering NF-Y in inactive complexes. This result suggests that, very likely, the enhanced NF-Y transcriptional activity observed in lamin A silenced cells depends on a decreased number of lamin A molecules able to associate with NF-Y and inhibit its activity. These results are consistent with previous evidences demonstrating that down-regulation of lamin A/C leads to dissociation of lamin A/C from promoters by enhancing transcriptional permissiveness (49). It has been observed that lamin A interactions often appear to be confined to promoter subregions rather than to entire promoter regions. Our data support a view in which lamin A, through its ability to bind NF-Y, exerts a locus-specific interaction with promoters important for cell cycle regulation and tumor progression.

In our study, an inverse correlation between lamin A and several NF-Y target genes expression level was observed, thus supporting the role of lamin A as regulator of NF-Y transcriptional function. We validated these evidences by in vivo imaging involving the use of a genetically engineered mouse model called MITO-Luc (for mitosis-luciferase), in which an NF-Y–dependent promoter controls luciferase expression. Data obtained strongly support the physiological impact of lamin A expression in cell proliferation.

NF-Y is one of the transcription factors responsible for aberrant oncogenic transcription occurring in several cancers (19). Since loss of lamin A expression have been reported in a variety of cancers, correlating with tumorigenic potential and more aggressive phenotype (29-31,33,52-54), our data suggest that changes in lamin A expression could modulate NF-Y activity and, consequently, its oncogenic transcriptional potential. Further exploration to uncover the molecular mechanism(s) by which NF-Y/ lamin A complex acts as crucial regulator in diverse cellular processes and, in particular, in cancer could be important to improve and potentially provide new clues into new therapeutic approaches for cancer treatment.

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FIGURES

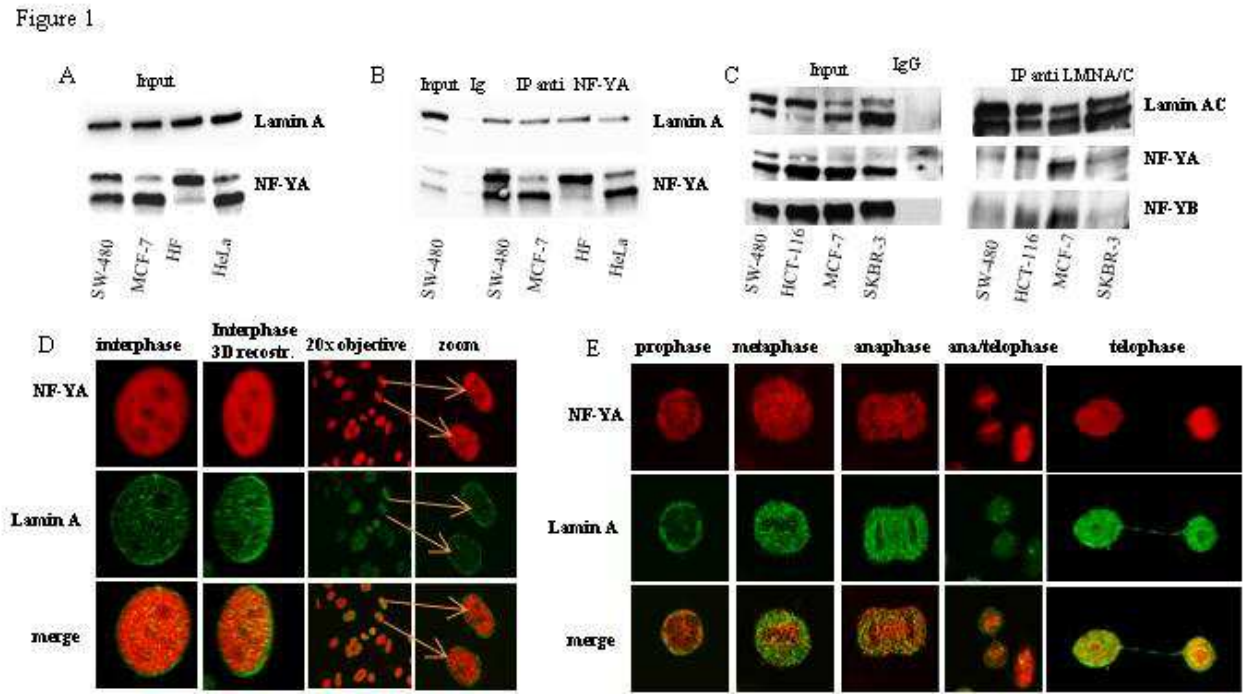


Figure 1: Analysis of the occurrence of lamin A/NF-Y complex in several cell lines. (A) Whole cell lysates from several cell lines were analyzed by western blotting with anti-NF-YA and lamin A antibody. (B) Whole cell lysates were immunoprecipitated with an antibody against NF-YA, and western blotting was performed with an antibody to lamin A and NF-YA. (C) Whole cell lysates from several cell lines were immunoprecipitated with an antibody against lamin A/C, and western blotting was performed with an antibody to lamin A/C, NF-YA and NF-YB. As negative control, in (B) and (C) were used SW480 cell lysates immunoprecipitated with an anti-IgG antibody. As a reference, in (B) and (C) 1/20 of whole cell extract used in the immunoprecipitations was loaded (input). (D) Confocal analysis performed on proliferating SW480 cells using antibodies against NF-YA (tritc) and lamin A (fitc). Different optical fields are shown. Colocalization (yellow) of endogenous NF-YA (red) with lamin A (green) was analyzed by indirect immunofluorescence combined with Confocal Scanning Laser Microscopy. Confocal analysis of single optical section is shown. The images have been collected with a 60x oil objective. Different optical fields are shown. (E) Confocal analysis of mitotic phases associated localization of NF-YA and lamin A in SW-480 cells. Colocalization (yellow) of endogenous NF-YA (red) with lamin A (green) was analyzed by indirect immunofluorescence combined with Confocal Scanning Laser Microscopy.

Figure 2

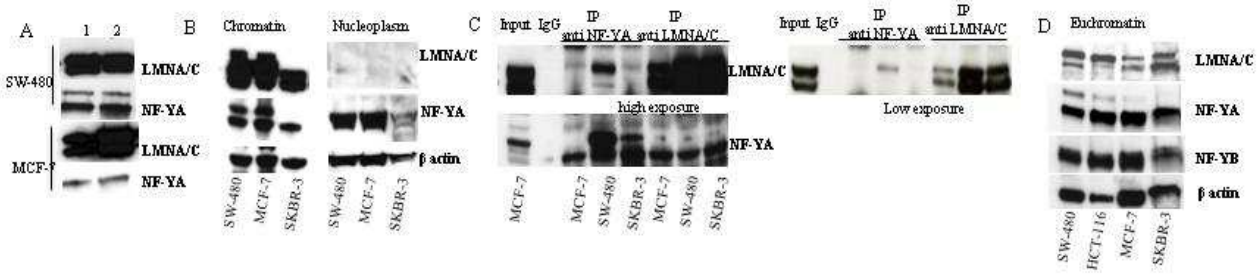


Figure 2: NF-Y localizes in lamin-enriched nuclear fraction and associates with lamin A in the chromatin fraction. (A) Western blotting of soluble (1) and insoluble (2) lamin enriched fractions obtained following the procedure described in figure S3 from SW480 and MCF7 cell lines using anti-NF-YA or -LMNA antibodies. (B) Chromatin and nucleoplasm fractions obtained following the procedure described in figure S4A from SW480 and MCF7 cell lines were subjected to western blotting analysis using anti-NF-YA or -LMNA antibodies. β actin was used as loading control. (C) Immunoprecipitation experiments using chromatin fraction isolated from SW480, MCF7 and SKBR3 cell lines obtained following the procedure described in figure S4A using anti-NF-YA and –lamin A antibody followed by western blotting analysis using antibodies against the indicated proteins. (D) Euchromatin fractions were produced by digestion of chromatin with Micrococcal nuclease as described in figure S4. These fractions were loaded onto a SDS polyacrylamide gel and analyzed by western blotting using the antibodies against the indicated proteins.

Figure 3

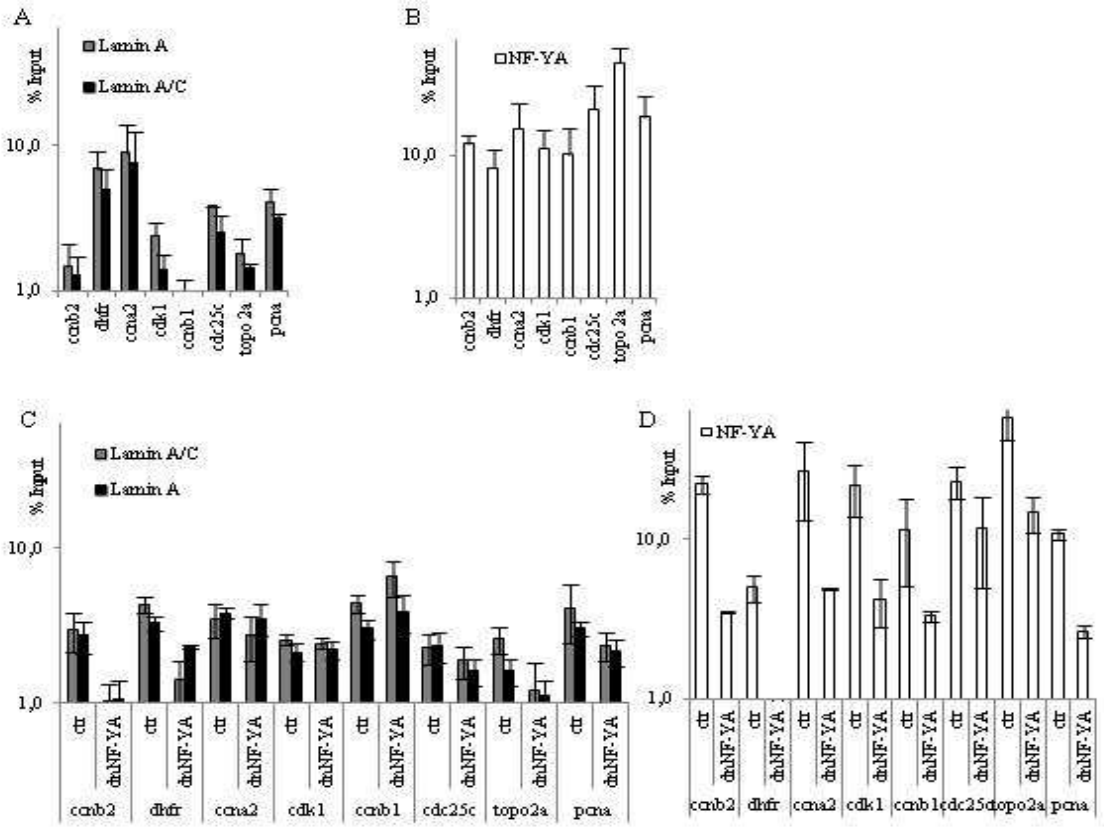


Figure 3: lamin A physically binds to promoter regions encompassing NF-Y consensus sites on several NF-Y targets. ChIP experiments using anti lamin A/C, and lamin A (A), and anti-NF-YA (B) antibodies followed by quantitative real time pcr (ChIP-qPCR) using specific primers to amplify DNA regions encompassing NF-Y consensus sites on ccnb2, dhfr, ccna2, cdk1, cdc25c, topo2a, and pcna promoters. ChIP-qPCR analysis on *CCNB2, DHFR, CCNA2, CDK1, CDC25C, TOPO2A, AND PCNA* promoters with anti-lamin A/C and lamin A (C) and anti-NF-YA (D) antibodies using mock transfected (CTR) and dnNF-A (dnNF-YA) transfected SW-480 cells. The ChIP results obtained by 3 independent replicate experiments are represented as percentage of input (% Input) on a logaritmic scale, the error bars indicate the standard error. No antibody values were subtracted. The error bars indicate the standard error.

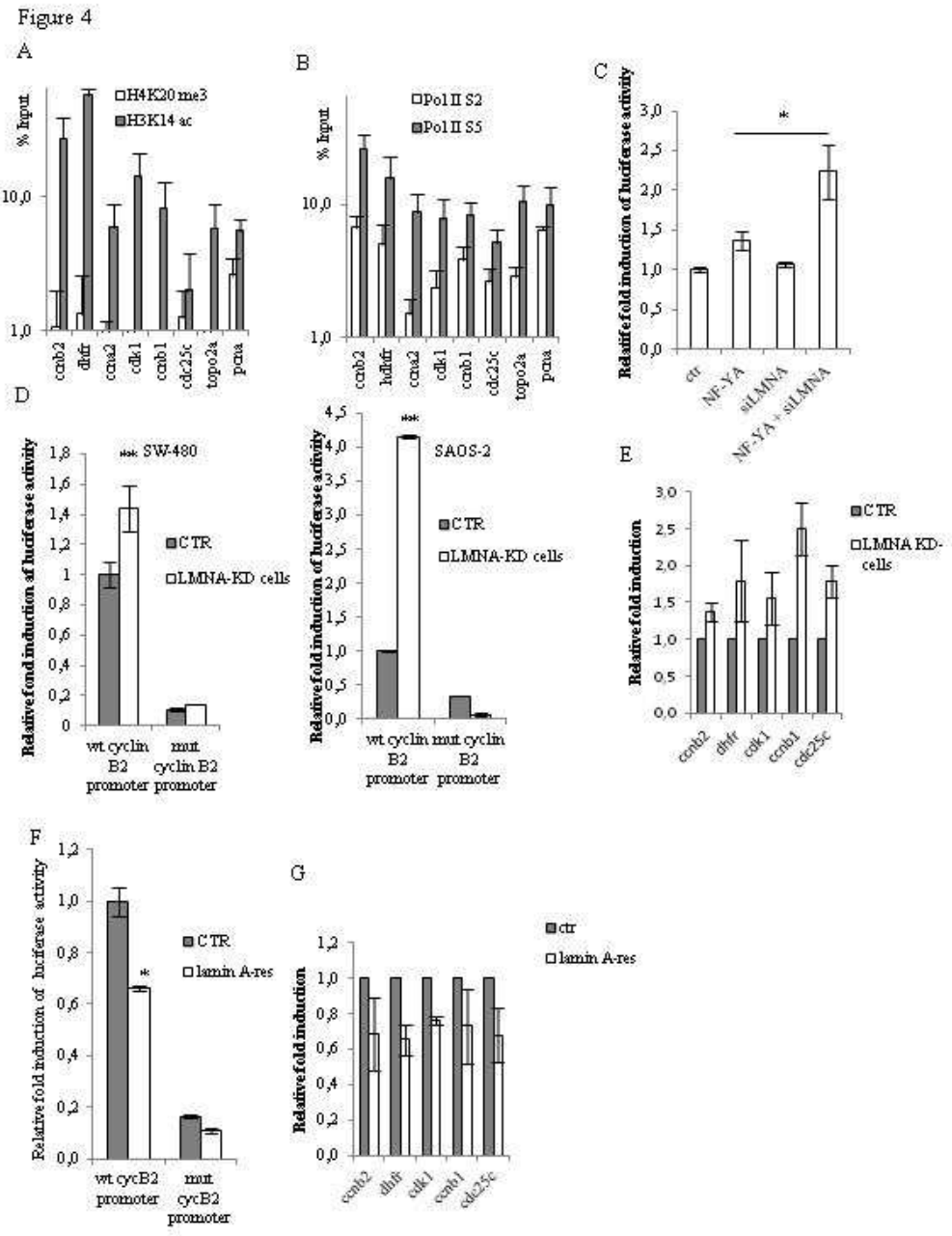


Figure 4: lamin A is involved in NF-Y transcriptional activity. ChIP-qPCR analysis using anti-H3K4me3 and H3K14ac (A) and anti-pol II phospho-ser 3 (Pol IIS2) and pol II phosphor-ser 5 (Pol IIS5) (B) antibodies using specific primers to amplify DNA regions encompassing NF-Y consensus sites on *CCNB2, DHFR, CCNA2, CDK1, CDC25C, TOPO2A, AND PCNA* promoters. The ChIP results obtained by 3 independent replicate experiments are represented as percentage of input (% Input) on a logaritmic scale, the error bars indicate the standard error. No antibody values were subtracted. (C) Luciferase assays performed in SW480 cells transfected with cyclin B2 promoter construct driven luciferase gene. Cells were transiently co-transfected with the indicated vectors and luciferase assays were performed after 48 hrs. Promoter activity is expressed as fold change of firefly/Renilla luciferase ratio. Results were obtained by eight independent replicate

experiments. (D) Luciferase assays performed with *LMNA* knock-down in SW-480 cells (*LMNA*-KD), transfected with the cyclin B2 luciferase construct or *CCNB2* promoter carrying mutated CCAAT boxes (mut *CCNB2* promoter). Promoter activity is expressed as fold change of firefly/Renilla luciferase ratio. Results were obtained by 10 independent replicate experiments. The error bars indicate the standard error. (E) qPCR analysis of expression levels of the indicated mRNA in *LMNA*-KD versus Mock cells (n=3). (F) Luciferase assays performed in lamin A-res SW480 cells (LMNA-res) versus mock cells (CTR) transfected with the *CCNB2* promoter luciferase construct (wt *CCNB2* promoter) or cyclin B2 promoter carrying mutated CCAAT boxes (mut *CCNB2* promoter) (n=3). (G) Expression levels by qPCR of the indicated mRNA in lamin A-res SW480 cells (lamin A-res) versus mock cells (CTR) (n=3). In all experiments, the error bars indicate the standard error. Statistical significance: *p<0.05, **p<0.01.

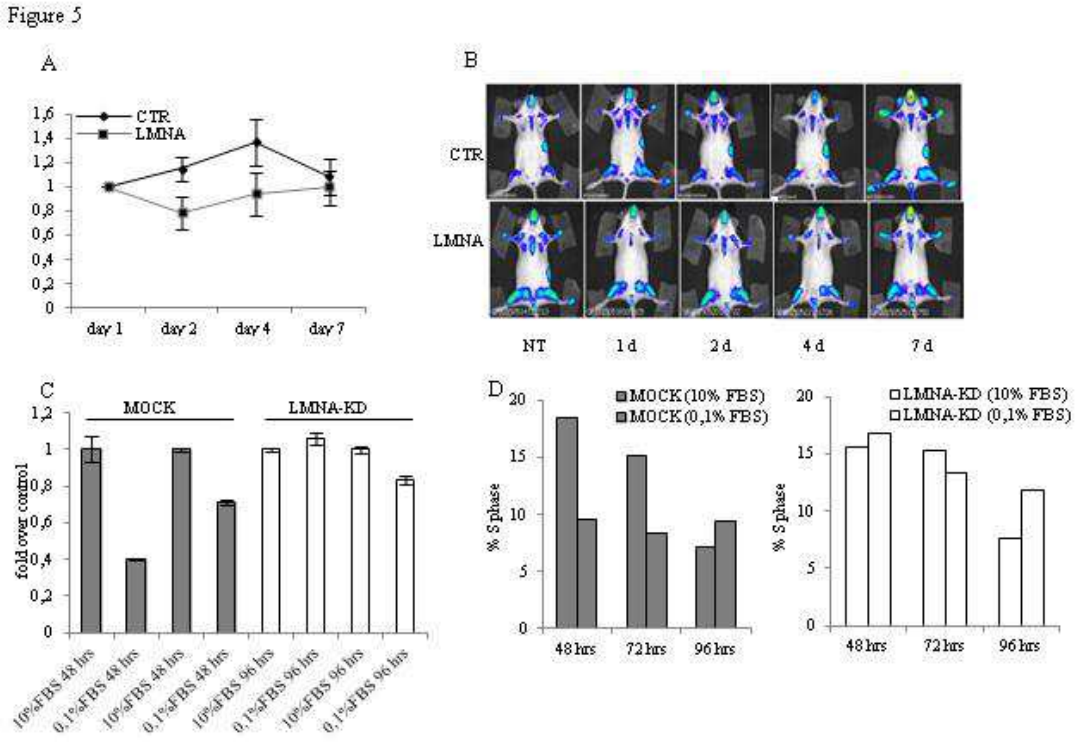
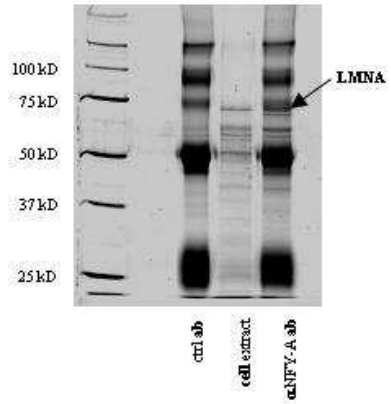


Figure 5: lamin A impact on NF-Y activity *in vivo.* (A) Mean in vivo of emitted light from MITO-Luc mouse. The retroviruses pMXIH-V5(CRT), and pMXIH-human lamin A-res (lamin A) were used for infections. (B) Quantification of emitted light from MITO-Luc mice were determined at 1, 2, 4, and 7 days after injection by whole-body imaging. Bioluminescence was expressed as p/s/cm2/sr. (B) Bioluminescence imaging of a representative MITO-Luc mouse before (NT) and after (1,2,4, and 7 days) after infection. The experiments were conducted in five animals. (C) Histogram shows number of cells grown in 0,1% FBS normalized over number of cells grown in 10% FBS (fold over control), as mean S.E. from three independent experiments at 48 and 96 hrs. (D) Cell cycle of either the normally proliferating cells or the starved-cells was analyzed by flow

cytometry at the indicated times. Histograms show the percentage of cells seeded in 0,1% or 10% FBS with S phase DNA content determined by flow cytometry after 48,72, and 96 hrs.

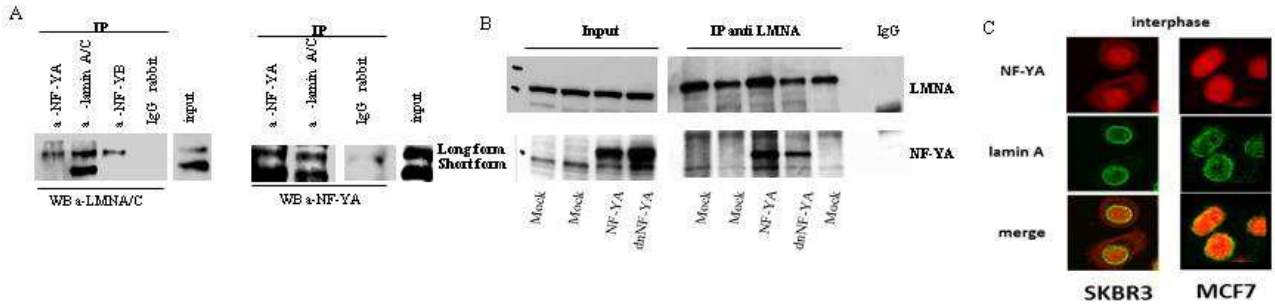
SUPPLEMENTARY FIGURES

Suppl. Figure 1



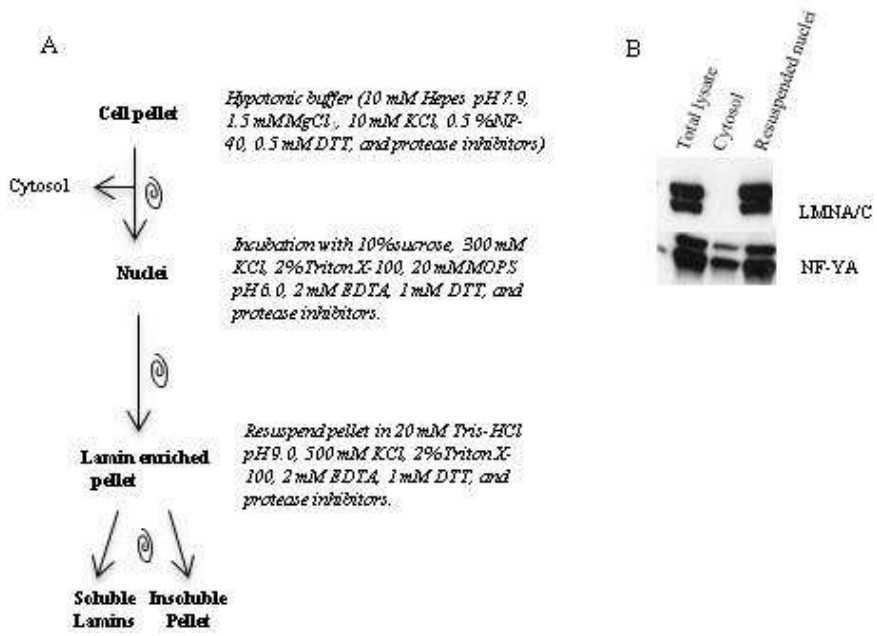
Suppl. Figure 1: LMNA interacts with NF-YA. Silver-stained SDS-PAGE gel of the immunoprecipitates with the indicated antibodies from SKBR3 cells. Arrow indicates the presence of LMNA. IgG immunoprecipitate was used as control (ctr ab).

Suppl. Figure 2



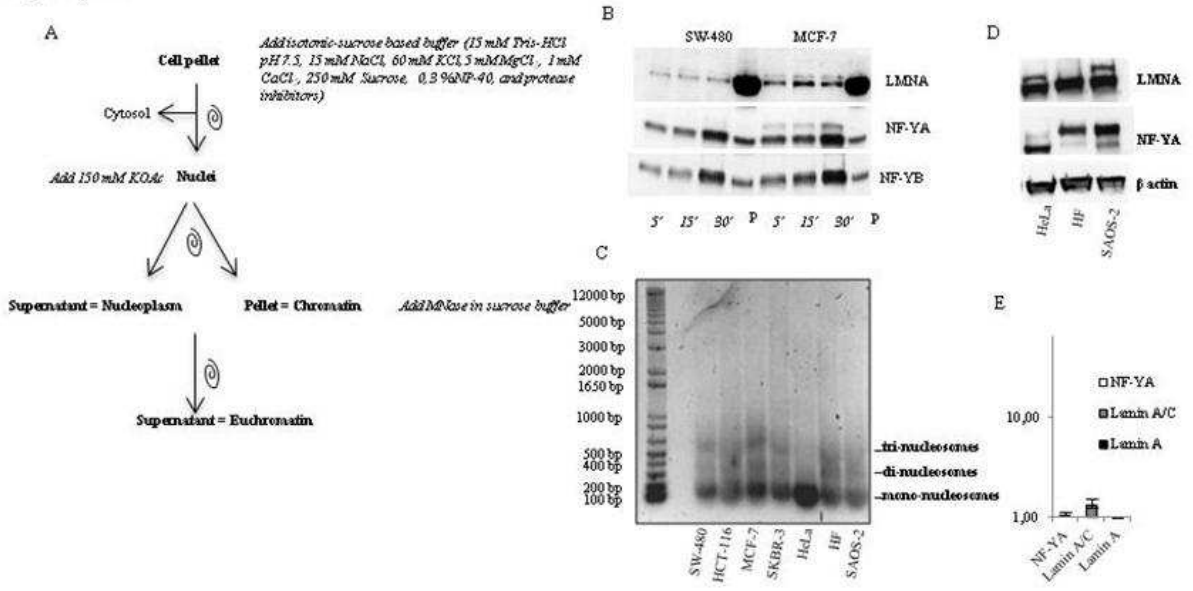
Suppl. Figure 2: NF-Y/LMNA colocalization. (A) Immunoprecipitation experiments using whole cell lystes produced from SKBR-3 cells with antibodies specific to the indicated proteins followed by western blotting with anti-LMNA/C or NF-YA antibody. IgG immunoprecipitate was used as control (IgG). **(B)** Confocal analysis of interphase associated localization of NF-YA and LMNA in SK-BR3 and MCF-7 cells. Colocalization (yellow) of endogenous NF-YA (red) with LMNA (green) was analyzed by indirect immunofluorescence combined with Confocal Scanning Laser Microscopy.

Suppl. Figure 3



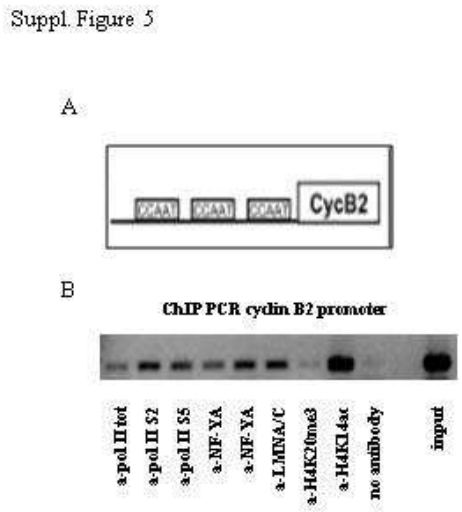
Suppl. Figure 3: NF-Y localization in lamin enriched nuclear fraction. (A) Diagrammatic representation of a procedure for lamin solubilization by high-salt and high-detergent buffer. **(B)** Western blotting with anti-LMNA and anti-NF-YA antibody of whole cell lysates (total lysate), cytosolic and nuclear (resuspended nuclei) fractions produced as described above obtained from SW-480 cells.

Suppl. Figure 4

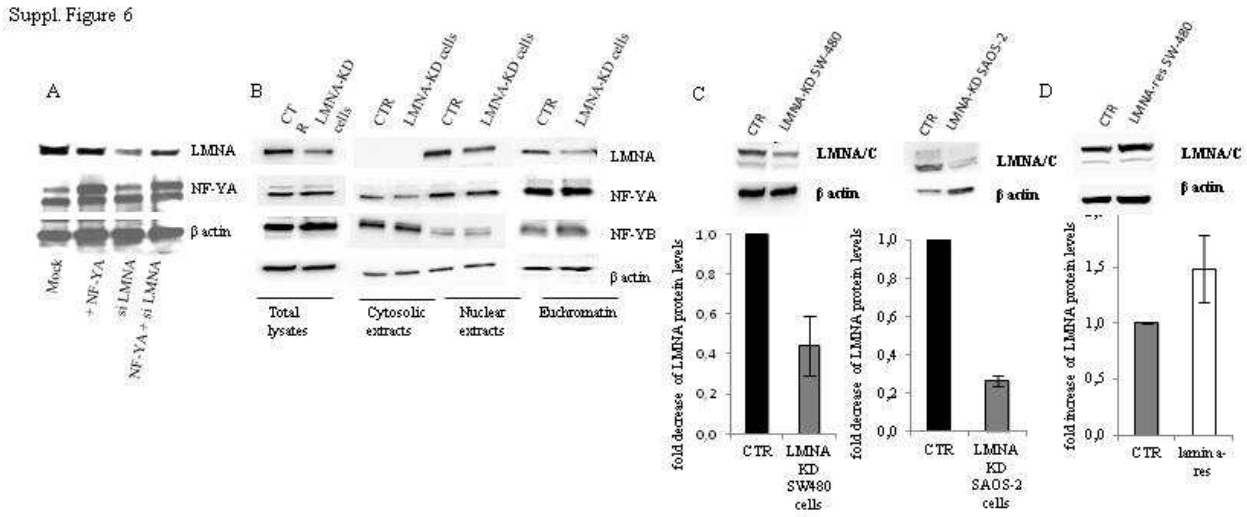


Suppl. Figure 4: LMNA localizes in euchromatin fraction (A) Chromatin and euchromatin purification diagram. **(B)** Western blotting analysis with the indicated antibodies of euchromatin

and heterochromatin (P) fractions obtained from a time course of MNase digestion on chromatin from SW480 and MCF7 cells produced as described above. The insoluble chromatin pellet (P) was isolated after 30 minutes by centrifugation (1,700× g for 5 minutes at 4°C) and then resuspended in 15 mM Tris, pH = 7.5, 0.5% SDS. Euchromatin fractions were collected after 5, 15 and 30 minutes of MNase digestion. **(C)** Euchromatin fractions obtained from the indicated cell lines run on 2% gel agarose collected after 5 minutes of Mnase digestion. Euchromatin fraction is composed mostly of mononucleosomes running at approximately 150 bp. **(D**) Western blotting analysis with anti-LMNA/C and NF-YA antibodies of euchromatin fractions produced as described above obtained from human fibroblasts (HF), HeLa and SAOS-2 cells after 5 minutes of digestion.



Suppl. Figure 5: LMNA binding activity on cyclin B2 promoter. (A) Schematic representation of the CCAAT boxes on cyclin B2 promoter amplified in ChIP analysis. **(B)** Representative agarose gel of ChIP-PCR experiment performed using SW480 cells with the indicated antibodies. Sample without antibody (no antibody) was used as control.



Suppl. Figure 6: Analysis of LMNA and NF-YA expression. (A) Western blotting showing the expression levels of LMNA and NF-YA proteins in SW480 transiently transfected cells used for luciferase assays. β actin was used as loading control. **(B)** Western blotting analysis with the indicated antibodies of whole cell lysate (total lysates), cytosolic and nuclear extracts and euchromatin fraction with the indicated antibodies produced from mock transfected (CTR) or LMNA-KD stable transfected SW-480 cells. **(C)** Western blotting of total cell lysates from SW-480 and SAOS-2 mock transfected (CTR) or LMNA-KD stable transfected cells (LMNA-KD) with LMNA/C antibodies. Bands were quantified by densitometry using UVI-1D quantification module. Histogram shows the quantitative densitometry of LMNA protein (fold over control) normalized over β actin expression, as mean S.E. from three independent experiments. **(D)** Western blot of mock or stably transduced human LMNA (LMNA-res) SW-480 cells with LMNA/C antibody. β actin was used as control. Bands were quantified by densitometry using UVI-1D quantification module. Histogram shows the quantitative densitometry of LMNA protein (fold over control) normalized over β actin expression, as mean S.E. from three independent experiments.

7 MANUSCRPIT II

Prognostic role of NF-YA splicing isoforms and lamin A status in low grade endometrial cancer.

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Running title: NF-YAs and lamin A as novel molecular prognostic biomarkers for EC.

ABSTRACT

Background: Endometrial cancer (EC) is a major cause of mortality for patients worldwide. Although most cases of low grade ECs do not behave aggressively, in rare instances, even low-grade, well-differentiated ECs can progress in a highly aggressive manner. In this study we analyzed several formalin-fixed, paraffin-embedded (FFPE) EC tissues to find novel clinical and biological features to help the diagnosis and treatment of early EC in order to better stratify patient risk of recurrence.

Methods: A retrospective cohort of FFPE specimens from patients with EC (n=87) and benign tissue specimens (NE) from patients who underwent a hysterectomy to treat other benign disease (n=13) were collected. Biopsies were sampled for primary tumors in hysterectomy specimens. Total RNA and proteins were extracted and analyzed, respectively, by quantitative PCR and western blotting.

Results: We identified two novel potential EC biomarkers, NF-YAs and lamin A. We observed that NF-YAs is exclusively expressed in EC tissues, while lamin A is strongly down-modulated in EC compared with benign tissues and its loss of expression correlates with tumor aggressiveness, as indicated by comparative analysis with estrogen receptor (ER) status and epithelial-mesenchymal transition (EMT) markers. In grade 1 EC, NF-YAs expression is heterogeneous and related with

lower lamin A levels, thus suggesting its potential role as biomarker of tumor aggressiveness in low grade EC.

Conclusions: Loss of lamin A correlates with higher histologic grade of EC. In grade 1, NF-YAs expression is heterogeneous and related with lower lamin A levels, thus suggesting its potential role as biomarker of tumor aggressiveness in low grade EC. Our findings indicate NF-YAs and lamin A as novel biomarkers with a potential for a more systematic integration in clinical practise for individualised therapy in EC, in particular in low grade malignancy.

Keywords: Endometrial cancer, lamin A, NF-Y, Estrogen receptor, EMT, miR-200 family.

BACKGROUND

Endometrial cancer (EC) is the most common genital tract malignancy and occurs in reproductive and postmenopausal women. EC is a type of uterine cancer that involves the lining of the uterus (the endometrium). Treatment for EC usually includes surgical removal of the uterus, cervix, ovaries, and fallopian tubes; it may also involve sampling or removal of the surrounding lymph nodes. Most EC cases are sporadic, with only 10% considered familiar (1). In general, patients with EC have a good prognosis since early diagnosis is frequent and the disease has usually not spread beyond the uterus. However, the prognosis for recurrent or metastatic EC remains poor and in order to improve therapy it is important to understand the processes which inhibit and stimulate cancer progression. Meanwhile, several prognostic factors such as histological type, histological grade, surgical stage, pelvic lymph node involvement and myometrial invasion have been established (1-5). EC is classified as type I or type II based on histologic properties. Type I, also called the endometrioid type (EEC) because of its histologic similarity to the endometrium, accounts approximately 70–80% of sporadic EC. Most type I tumors occur in the setting of unopposed estrogen stimulation, leading to endometrial hyperplasia. According to FIGO definition, type I ECs include lower grade EECs. Unlike type I tumors, type II lesions are not related to estrogen exposure or endometrial hyperplasia and include high risk malignancies, as serous papillary and clear cell carcinoma, generally. Some biological molecules have been identified as prognostic markers in EC, such as KRAS, PTEN, EGFR, FGFR, P53, HER2, and estrogen receptors (ERs) (6).

Nuclear transcription factor (NF-Y) is a sequence-specific transcription factor that binds the common CCAAT element and has long been considered an activator of genes involved in growth promotion including cell cycle regulatory genes. Numerous findings highlight that NF-Y is involved in cancer. A bioinformatic analysis of promoters of cell-cycle regulatory genes shows an abundance of CCAAT boxes in promoters regulated during the G2/M transition progression, among

which are mitotic cyclin complexes (7-14). NF-Y is composed of three different subunits: YA, YB and YC. The association between NF-YB and NF-YC provides a docking site for NF-YA, and NF-YA is the regulatory subunit of the complex responsible for sequence-specific DNA binding. Subunit NF-YA has two different isoforms, NF-YAl (long) and NF-YAs (short), resulting from alternative splicing. Previous studies demonstrated that NF-YAl is down-regulated, whereas NF-YAs is up-regulated during differentiation of hESCs, mouse ES cells, and hematopoietic stem cells (15-17). Although mutations in NF-Y subunits have never been specifically identified in tumours, systematic examination of protein expression profiles indicates that NF-YA targets are upregulated in different types of cancer. Indeed, there is evidence from previous studies that the levels of NF-Y vary in different cell types and under different growth conditions. Recently, informatics analysis and microarray expression profile studies conducted in various gynecological cancers, revealed that NF-Y is one of the key transcription factors involved in endometrial, cervical and vulvar cancer development (18).

Expression of ERs has been correlated with EC stage, histologic grade and survival (19). Loss of ERs has been significantly associated with aggressive phenotype and poor survival in EC patients (20). In particular, early stage, well differentiated ECs usually retain ERs expression, whereas advanced stage, poorly differentiated tumours often lack one or both receptors. In the human uterus, ER-α is the predominant subtype (21), and ER-β is supposed to play an important role by modulating ER-α function (22,23). Recently, it has also been observed an association between lack of ER-α and epithelial-mesenchymal transition (EMT) (24).

EMT enables epithelial cells to acquire a like mesenchymal potential with increase motility and ability to extravasate and circulate (25). In EC, alteration of EMT markers, including several miRNAs, have been identified in metastatic disease and associated with reduced survival (26-32).

miRNAs are small non-coding RNA elements that control cellular function by modulating the stability and translation of multiple target mRNAs at the post-transcriptional level. They play important roles in development, cellular differentiation, proliferation, cell-cycle control, and cell death. In particular, the miR-200 family members have been extensively studied with respect their role in EMT in various tissues, where they target the expression of many genes, such as the transcription factors ZEBs (33-36). A recent report has already shown that elevated levels of all miR-200 family, in all stages of EC, inversely correlates with the expression of ZEBs (37). miR-200s upregulation has been demonstrated in type I EEC compared to normal endometrial tissues in keeping with observations in other tumours, such as ovarian cancer (38), melanoma (39,40) and colorectal carcinoma (41). Recently, it has also been suggested that miR-200 family, under influence of estrogen, maintains an epithelial phenotype in lower grade EEC (42). However, based

on hormone status, miR 200a upregulation has been linked with outcome of EC patients. A recent study correlated miR-200a with prolonged survival in ERs positive subgroup, whereas an inverse trend was observed in the ERs negative soubgroup (43).

Lamins are type V intermediate filament proteins that are often aberrantly expressed or localized in tumours. A-type lamins, whose most represented isoforms are lamin A and C, are alternatively spliced products of the same gene. Several studies identified A-type lamins as an indicator of differentiated tumour cells and demonstrated to represent a potential biomarker for various types of cancer (44). Loss of lamin A expression has been reported for colon cancer, cervical cancer, lung cancer, prostate cancer, gastric cancer, ovarian cancer and leukemia and lymphoma (45-49). It has been shown that lamin A stabilizes the nuclear lamina and chromatin, with implications for epigenetic stabilization and limiting of DNA breaks. With respect to its multiple functions, it is convincible to presume that change of lamin A protein levels may contribute to tumourigenesis and progression (50-54). Expression of miR-9 has been reported to reduce lamin A expression (55,56). Moreover, miR-9 overexpression has been observed in several cancers, including EC (57) and this overexpression was correlated with cancer progression, metastasis and poor prognosis (58-63).

In the present study, we characterised NF-YA isoforms and lamin A expression in several EC samples and identified them as novel potential prognostic EC biomarkers. We report for the first time that a specific NF-YA splicing isoform, NF-YAs, is associated with EC development. In our cohort of grade 1 EECs, NF-YAs was expressed in about 55% of samples, whereas it was detectable in all higher grade and non endometrioid (NEM) ECs. Also, our data indicate that an association between NF-YAs isoform and lower ER-α mRNA (ESR1) expression occurs, suggesting its involvement in estrogen response. Moreover, we observed a significant correlation of lamin A expression with stage and histologic grade. Interestingly, clustering of NF-YA isoforms in grade 1 EEC indicated an inverse association between NF-YAs and lamin A expression and a direct correlation with an increase of miR-200 levels inversely related to ZEBs expression, well known markers of aggressiveness in EC (37,42,43).

Our findings suggest NF-YAs and lamin A as novel biomarkers with a potential for a more systematic integration in clinical practise for individualised therapy in EC, in particular in low grade malignancy.

METHODS

Patients

A retrospective cohort of formalin-fixed, paraffin-embedded (FFPE) specimens from patients with EC (n=87) and NE specimens from patients who underwent a hysterectomy to treat other benign disease (n=13) were collected. According with the histologic grade, we analysed 29 low grade (G1), 49 high grade endometrioid (high grade EEC) and 9 non endometrioid EC tissues (NEM). Biopsies were sampled for primary tumors in hysterectomy specimens.

RNA extraction and RT-PCR

Total RNA derived from FFPE tissues was extracted using the PureLink™ FFPE Total RNA Isolation Kit (Invitrogen) following the manufacturer's instructions and reverse-transcribed using PrimeScript RT reagent kit (Takara). The quality of the total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington DE, USA). Quantitative PCR (qPCR) was performed using SYBR Select (Applied Biosystems) on an ABI Prism 7500 apparatus (Applied Biosystems). mRNA expression was normalized for 18S rRNA levels. Relative mRNA expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

PRIMERS

NF-YAs fw	ACAGATTCAGCAGCAGGTCC
NF-YAs rv	ATGGGTTGGCCAGTTGATGT
NF-YAl fw	CAGGGTGGTGTCACTGCTG
NF-YAl rv	TACCTGGAGGGTCTGGACTT
LMNA fw	GGACAATCTGGTCACCCGC
LMNA rv	TGGCAGGTCCCAGATTACATG
ESR1 fw	TACTGACCAACCTGGCAGACAG
ESR1 rv	TGGACCTGATCATGGAGGGT
ESR2 fw	AGTTGGCCGACAAGGAGTTG
ESR2 rv	CGCACTTGGTCGAACAGG
ZEB1 fw	AACCACCCTTGAAAGTGATCCA
ZEB1 rv	CTTGTCTTTCATCCTGATTTCCATT
ZEB2 fw	CAAAGGAGAAAGTACCAGCGGA
ZEB2 rv	CATCAAGCAATTCTCCTGAAATCC
CDH1 fw	CCCACCACGTACAAGGGTC
CDH1 rv	ATGCCATCGTTGTTCACTGGA
CDH2 fw	AGAAGAAGACCAGGACTATGACTTGAG
CDH2 rv	ACAGTGTCAGGCTGCTGCAG
18S rRNA fw	CCTGGATACCGCAGCTAGGA
18S rRNA rv	GCGGCGCAATACGAATGCCCC

MicroRNA analysis

Reverse transcription and qRT-PCR amplification were performed in two steps. In the first reverse transcription step, 10 ng of RNA was used in reactions with specific stem-loop RT primer for miR-200a, miR-200b, miR-200c, miR-141, and miR-9 and endogenous control primer for small nuclear

RNA U6. Reaction was performed with TaqMan MicroRNA Reverse Transcription Kit, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). In the second step, cDNA samples were amplified in Real Time PCR instrument 7500 (Applied Biosystems) with the specific TaqMan miR-200a, miR-141, and miR-205 assay and small nuclear RNA U6 as endogenous control. The relative quantity (RQ) of each miRNA was calculated by the comparative CT (2-ΔΔCT) method, in which ΔΔCT was calculated as follows: ΔΔCT = (CTmiR-of-interest - CTU6)cancer - (CTmiR-of-interest - CTU6)benign.

Immunoblotting

The paraffin from thin sections of FFPE specimens was melted at 72°C for 20 minutes using heat in the presence of a specially designed Melting Buffer contained in the PureLink™ FFPE Isolation Kit used for RNA extraction (Invitrogen). Tissues were then separated from the melted paraffin by centrifugation. Proteins were extracted in a high pH lysis buffer (20 mM Tris HCl pH 9.0, 0.2 M Glycine, 2% (w/v) SDS) as described by Guo et al (Proteome Science 2012, 10:56). The samples were first incubated on ice for 5 min, and mixed by vortexing, then boiled at 100°C for 20 min followed by an l hour incubation at 80° C for 2 hours. After extraction, any remaining unsolubilized material was pelleted at 14000 × g for 20 minutes, and protein concentration of total protein extracted was determined by the BCA Protein Assay (Pierce Chemicals Co., Rockford, IL, USA). The Pierce BCA Protein Assay is a detergent compatible formulation and the protein standards were prepared using the same lysis buffer as the samples. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Each membrane was blocked with 5% non-fat dry milk in Tris buffered saline-Tween-20 (TBST) for 1 hour at room temperature and subsequently incubated with primary antibody for 16 hours at 4˚C. The following antibodies were used: anti-NF-YB monoclonal (Santa Cruz), anti-NF-YA monoclonal (Santa Cruz), anti-Lamin A (Santa Cruz), and anti-β actin (sigma-aldrich). Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary antibody.

Statistical analysis

Data were reported as mean and standard deviation. Differences were considered statistically significant when $P \leq 0.05$. Student T test was performed for the comparison of results from qRT-PCR (*P<0.05, **P<0.01, ***P<0.001).

RESULTS

NF-YAl and NF-YAs splicing isoforms are differentially expressed in EC tissues.

The analysis of global regulatory perturbations across human cancers pointed the NF-Y matrix is enriched in promoters of genes over-expressed in cancer cells. We analysed the expression of NF-

Y in several EC FFPE specimens, whose clinicopathological features are described in Table 1, by comparing the protein expression level of two subunit of NF-Y complex, NF-YA and NF-YB, in EC and benign endometrial tissues (Figure 1A). We did not detect any significant modulations of NF-YA and NF-YB protein levels in ECs compared to benign tissues but, very interestingly, we observed a different and specific electrophoretic profile of NF-YA splicing isoforms. We found that the long isoform of NF-YA (NF-YAl) is the main form expressed in benign tissues, whereas the short isoform (NF-YAs) is almost absent in this samples being only barely detectable in few benign endometrial tissues. Interestingly, we observed that NF-YAs is specifically associated with a tumour phenotype being clearly detectable in EC tissues (Figure 1A). Very interestingly, NF-YAs was expressed in all higher grades (G2-G3) EEC and NEM tissues, whereas it was detected only in 55% of our grade 1 samples (G1). This result suggests that NF-YAs could represent a marker of EC and, also, an indicator of tumour aggressiveness. We hypothesises that NF-YAl form may represent a marker of differentiation and that the short isoform may be related to an increase of poorly differentiated cells in tumours tissues. NFYAs and NF-YAl mRNA status in normal and EC tissues was also examined by qRT-PCR using primes designed to specifically amplify only NF-YAl or NF-YAs mRNA. Results showed that the expression level of the two splicing isoforms was not correlated with protein expression. In fact, the ratio of mRNA expression of NF-YAs and NF-YAl (NF-YAs/NF-YAl) was very similar in benign and low grade EC tissues (Figure 1B), thus indicating that differences observed of NF-YA protein profile between benign and EC FFPE specimens are very likely due to the occurrence of different post transcriptional and/or stabilization mechanisms.

NF-YAs detection in low grade EC tissues correlates with decreased ERs mRNA expression

ER status has been shown to represents a relevant prognostic marker in EC since loss of ERs has been consistently associated with aggressive clinicopathologic phenotypes and poor survival in EC patients (20). We firstly analysed ERs status to evaluate its prognostic values in our patient cohort (Table 3). In grade 1 EEC, 27,5% and 31% of tissues were found to express low ER-α (ESR1) and ER-β (ESR2) mRNA levels, respectively. A massive down-modulation of ESR1 and ESR2 mRNA, in 61,2% and 77,5% of high grade EC and 77,7% and 88,8% NEM samples respectively, was observed (Table 3), thus confirming that reduction of ERs level is related with advanced stage of EC.

A significant correlation between ER-α protein and ESR-1 mRNA expression has been previously estimated by microarray and qPCR analysis (64). Based on this evidence in the following experiments we focus our attention on ESR-1 mRNA expression. To explore if ESR1 status was correlated with the expression of NF-YA isoforms, we focused our analysis on grade 1 EECs where

a heterogeneous NF-YA expression of the two isoforms was observed. Firstly, analysis of ESR1 mRNA in our cohort of specimensconfirmed the correlation of loss of ESR1 expression with an aggressive clinopathologic phenotype but, more interestingly, demonstrated an association between the presence of NF-YAs and lower ESR1 mRNA levels (Figure 2 and Table 3). These evidences indicate a possible involvement of NF-YAs in EC aggressiveness. It is worth to note that ESR1 mRNA levels in NF-YAs positive tissues were very similar to that of higher grades (G2 and G3), whereas in NF-YAs negative (NF-YAs-) specimens were comparable to those of benign tissues (Figure 2A and Table 3). These data strongly support a correlation between ESR1 and NF-YAs expression.

Upregulation of miR-200 family inversely correlates with ZEBs expression in ECs an related to NF-YAs expression in grade 1.

Lack of ER-α has been recently associated with epithelial to mesenchymal transition (EMT) (64). The miR-200 family has been extensively studied with respect its role in regulating genes of EMT. Analysis of the qRT-PCR data showed that all members of the miR-200 family analysed (miR-200a, miR- 200b, miR-200c, and miR-141) are up-regulated in all stages of EC compared to benign tissues (Figure 3A), which confirms results reported in previous studies (37,42,43). In order to validate the activity of miR-200s in EC, we also analysed the expression level of ZEB1 and ZEB2 (Figure 3B), well established as direct targets of miR-200 family. In EC, levels of ZEB1 and ZEB2 were lower and further decreased in NEM, thus supporting the hypothesis of a augmented translational activity of miR-200 family members in more aggressive phenotype. Then, we focused our analysis in our subset of G1 samples. Interestingly, we observed a consistent increase of miR-200 family expression (Figure 3C) inversely related to ZEBs mRNA levels in NF-YAs positive compared with negative NF-YAs grade 1 EEC samples. (Figure 3D). It is worth noting that upregulation of miR-200a and ERs loss have been associated with poor prognosis (43). Our data indicated that in grade 1 EEC specimens higher levels of miR-200s are related with NF-YAs expression and, also, with lower ESR1 expression, thus supporting the hypothesis of a correlation between NF-YAs expression and tumour aggressiveness. Finally, tobetter characterize EMT involvement in EC development and aggressiveness, we analyzed N-cadherin mRNA expression (*CDH2*), a mesenchymal marker, by qRT-PCR. Levels of *CDH2* were augmented in all EC tissues and a further significant increase was observed in more aggressive phenotypes (Figure 3E). We also evaluated level of the ratio of E-cadherin to N-cadherin (E/N), an index of differentiated phenotype, in our samples. An augmented percentage of EC tissue exhibits a low E/N ratio compared with benign tissues and this increase correlated with a more aggressive clinophatologic phenotype (Table

3), whereas we did not detect any modulation of *CDH2* mRNA levels and E/N ratio in NF-YAs positive compared with NF-YAs negative grade 1 EEC tissues (Figure 2B and Table 3).

Decreased lamin A levels are associated with EC progression and aggressiveness and NF-YAs expression in grade 1 EEC.

A protein recently identified as an indicator of differentiation and involved in cancer progression is lamin A. In fact, lamin A is absent in embryonic stem cells and some adult stem cell types but is expressed in the majority of cell types in adults. Loss of lamin A expression has been reported for colon cancer, cervical cancer, lung cancer, prostate cancer, gastric cancer, ovarian cancer and leukemia and lymphoma (44-49). It is worth noting that lamin A has been reported to be a direct target of miR-9 and that upregulation of miR-9 expression occurs in EC (57). In order to evaluate lamin A involvement in EC, we analysed lamin A protein and mRNA expression and miR-9 levels in our cohort of EC tissues. Lamin A protein expression was consistently lower in all EC than that in benign endometrial samples (Table 2 and Fig.1A).Results of qPCR revealed that lamin A down-modulation occurs at transcriptional level since a significant decrease ($P \leq .001$) of *LMNA* mRNA expression was observed in all EC subtypes analysed (fig. 4A). Our data clearly show that reduced expression of lamin A is associated with tumour development and correlates with higher histologic grading of EC (Table 2 and 3). Interestingly, data obtained by comparing *LMNA* mRNA levels in the two subgroups of grade 1 EEC tissues indicated that *LMNA* reduction was significantly higher ($P \leq .01$) in NF-YAs positive with respect to NF-YAs negative samples (Fig.4A), comparable to that of high grade EC. This modulation in miR-9 expression was consistently higher in all EC than that in benign endometrial samples (Fig. 4B) while the two grade 1 subgroups were not associated with miR-9 expression

Our evidences further support the potential role of NF-YAs and lamin A as molecular indicator of tumour aggressiveness in early stage EC.

DISCUSSION

Current clinical approaches in the treatment of EC mainly relies on surgical FIGO classification, histologic subtype, and histologic grade. Identification of novel molecular markers may be helpful to avoid risk of over-and under treatment of EC patients and to overcome recurrence. A recent study based on both informatics analysis of the motifs of known transcription regulators and experimental evidence from ENCODE, identified NF-Y as one of the key components of the transcription regulation factories of gynecological cancer (12). In our study we identified a specific splicing isoform of the regulatory subunit of NF-Y, NF-YAs, as a new potential indicator of aggressiveness in grade 1 EEC. We observed that NF-YAs was mostly undetectable in benign

tissues, whereas it was consistently expressed in high grade EEC and in NEM subtypes. Interestingly, only in grade 1 EEC a heterogeneous expression of NF-YA isoforms was observed with some samples expressing exclusively the long form (NF-YAl) and others samples expressing both isoforms. This results prompted us to stratify grade 1 EEC in two subgroups: one expressing only NF-YAl, NF-YA negative (NF-YAs-), and another expressing both isoforms, NF-YA positive (NF-YAs+). Patients with grade 1 tumors involving only endometrium and no evidence of intraperitoneal disease have a low risk (<5%) of nodal involvement. Although most cases of grade 1 EEC do not behave aggressively, in rare instances, even low-grade, well-differentiated endometrial adenocarcinomas can progress in a highly aggressive manner. We hypothesize that the molecular feature related to NF-YA isoforms expression could be a relevant biomarker to predict the outcome of these cancers. To this aim, to better characterize the significance of the heterogeneous expression of NF-YA isoforms in grade 1 EEC, we analyzed the ER status. It has been well documented that higher level of ERs are significantly associated with good prognosis (65) and that early stage-well differentiated EC usually retain their expression, whereas advantage stages and poorly differentiated tumors often lack one or both of these receptors (18, 64, 66).

In the current study, we confirmed this correlation. Moreover, clustering of the samples expressing and not expressing NF-YAs in grade 1 EEC allowed as to identify a strong correlation with the specific loss of ERs and the presence of NF-YAs. Interestingly, a recent study reported that lack of ER-α is linked with EMT in EC (64). It has been recently demonstrated that miR-200 family, under influence of estrogen, maintains an epithelial phenotype in lower grade EEC, and that the miR-200 family is regulated by estrogens (35,67). miR-200 upregulation has been demonstrated in type I EEC compared to normal endometrial tissues in keeping with observations in other tumours, such as ovarian cancer, melanoma and colorectal carcinoma (38-41). The function of miR-200 family have been well documented in various tissues, where they target the expression of many genes, such as the transcription factors ZEBs. In turn, ZEB1 and ZEB2 bind to a conserved pair of ZEB-type E-box elements located at the promoter region of the miR-200s, both in the pri-miR-200a/b/429 and pri-miR-200c/141 loci. Thus, a double-negative feedback loop exists between ZEB1/ZEB2 and miR-200s in EMT regulation (33-36). A recent report has already shown that elevated levels of all miR-200 family, in all stages of EC, inversely correlates with the expression of ZEBs (36). It has also recently observed that in ER positive EC miR-200a upregulation was correlated with a prolonged patient survival, whereas an inverse trend existed in ER negative group (24). Very interestingly, in grade 1 EC we observed an association between NF-YAs expression, loss of ERs and upregulation of miR-200 family members, thus suggesting a possible involvement of this isoform in tumour aggressiveness.

The exclusive presence of NF-YAl form in benign tissues suggests that it may represent a marker of differentiation and that the presence of NF-YAs may be linked with an increase of a pool of poorly differentiated cells in tumors tissues. The degree of tumor differentiation has an important impact on the natural history of this disease and on treatment selection. It has been shown a strong correlation between a more aggressive phenotype and poor outcome in EC expressing and alteration of EMT-related markers, such as an over-modulation of N-cadherin expression and a decrease of the ratio of E-cadherin to N-cadherin (E/N), an index of the epithelial phenotype (25). In the present report, a significant increase of N-cadherin mRNA expression and a lower E/N index was observed in more aggressive EC tissues, thus confirming the association of high EMT signature with tumor aggressiveness and poor prognosis. However, this analysis revealed that levels of N-Cadherin are not correlated with NF-YAs status in grade 1 EEC.

We, also, investigated the expression of *LMNA* (68,69). and its regulator, miR-9, both involved in EC development (57) and its target gene.Numerous studies showed that lamin A is expressed at very low levels early in embryonic development but is expressed at high levels in most differentiated cells and indicated that reduced or absent lamin A expression is a common feature of a variety of different cancers (44-49). Lamin A has been demonstrated to play a key role in sensing tissue elasticity in differentiation and the reduction in its expression frequently correlates with cancer subtypes and cancer aggressiveness, proliferative capacity and differentiation state (70). To evaluate the possible involvement of lamin A also in EC, we analyzed its protein and mRNA expression levels in our cohort of EC FFPE tissues. Interestingly, lamin A was consistently down-modulated in EC both at mRNA and protein level, and this decrease was inversely associated with miR-9 expression levels. Interestingly, lamin A loss was further increased in high grade EEC and NEM, thus indicating lamin A as a novel potential marker of EC aggressiveness. It is worthwhile to note that an increased loss of lamin A expression levels was observed in our subset of NF-YAs positive grade 1 EEC tissues compared with NF-YAs negative, thus further supporting the hypothesis of a possible involvement of NF-YAs in tumor differentiation and aggressiveness.

CONCLUSIONS

All together, these evidences indicate NF-YAs and lamin A as two novel potential targets and predictive markers for new stratification approaches in EC. Further investigations are needed to evaluate and better characterize the role of NF-YAs in low grade EC and its association with lamin A, miR-200s expression, and ERs status, in particular focusing in recurrent low grade EECs to improve identification and stratification of patients at risk of recurrence. A retrospective analysis in our cohort of grade 1 EEC showed that two patients developed recurrence in the liver and in the

lung and, interestingly, both belonged to the NF- YAs positive subgroup. Considering the relative rarity of distant metastasis in grade 1 EEC (< 5%), and our low number of cases, further studies are needed to evaluate the possible correlation between NF-YAs expression and risk of recurrence.
Also, our study suggests that a better evaluation of lamin A expression and ERs status in patients that experience recurrence according to site of recurrence, prior radiation therapy, and initial disease characteristics may help focus treatment guidelines for these patients.

DECLARATIONS

List of abbreviations

Endometrial cancer = EC
Endometrioid endometrial adenocarcinoma = EEC
Non endometrioid endometrial adenocacinome = NEM
Benign tissue= NE
Formalin-fixed, paraffin-embedded = FFPE
NF-YA short form = NF-YAs
NF-YA long form=NF-YAl
Estrogen receptor = ER
Epithelial-mesenchymal transition = EMT

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TABLES

Table 1 ***Clinicopathological features***	**Total EECs**	**G1 EECs**	**G2-G3 EECs**	**NEM**
No of cases	78	29	49	9
Age	Median 63 years, range 42-88	Median 58 years, range 42-76	Median 66 years, range 43-88	Median 65 years, range 50-74
BMI > 30	14 (17,9%)	8 (27,5%)	6 (12,2%)	7 (77,7%)
MI > 50%	33 (42,3%)	5 (17,2%)	28 (57,1%)	7 (77,7%)
Lymph node metastases	4 (5,1%)	0 (0,0%)	4 (8,1%)	2 (22,2%)
Stage				
I and II	40 (51,2%)	29 (100%)	21 (42,8%)	6 (66,6%)
III and IV	28 (35,9%)	0 (0,0%)	28 (57,1%)	3 (33,3%)
RT	29 (37,1%)	3 (10,3%)	26 (53,0%)	4 (44,4%)
CHT	15 (19,2%)	0 (0,0%)	15 (30,6%)	9 (100%)

Table 1: Clinicopathological features of 87 ECs. RT= adjuvant radioteraphy; CHT=adjuvant chemoteraphy. BMI= body mass index; MI= myometrial infiltration.

Table 2: ***LMNA protein expression in EC***	**Low LMNA n (%)**
NE	0 (0%)
G1 EEC	21(72,4%)
G1 EEC NF-YA- (n=13)	9(69,2%)
G1 EEC NF-YA+ (n=16)	12 (75,0%)
G2-G3 EEC	49 (100%)
NEM	9 (100%)

Table 2: Lamin A protein levels. Proteins were extracted from FFPE specimen tissues and subjected to western blot analysis.
Cut off= lamin A expression in EC over benign samples (NE) $\leq 0,5$.

Table 3: ***ERs, LMNA and E/N mRNA expression in EC***	**Low ESR1 n (%)**	**Low ESR2 n (%)**	**Low LMNA n (%)**	**Low E/N n (%)**
NE (n=13)	0 (0%)	6(46,1%)	0 (0%)	2 (15,3%)
G1 EEC (n=29)	8 (27,5%)	9 (31,0%)	20(68,9%)	9(31,0%)
G1 EEC NF-YA- (n=13)	3(23,0%)	3 (23%)	5 (38,5%)	4 (30,7%)
G1 EEC NF-YA+ (n=16)	5 (31,2%)	6 (37,5%)	15 (93,7%)	5 (31,2%)
G2-G3 EEC (n=49)	30 (61,2%)	38 (77,5%)	43 (87,7%)	25(52,0%)
NEM (n=9)	7 (77,7 %)	9 (88,8%)	7 (77,7%)	4 (44,4%)

Table 3: EC histologic grade in relation to low levels of LMNA, ESR1, ESR2 expression, and E/N index. Lamin A, ESR1, ESR2, CDH1 and CDH2 mRNA was examined by qRT-PCR.
Cut off=EC over benign samples $\leq 0,5$.

FIGURES

Fig. 1

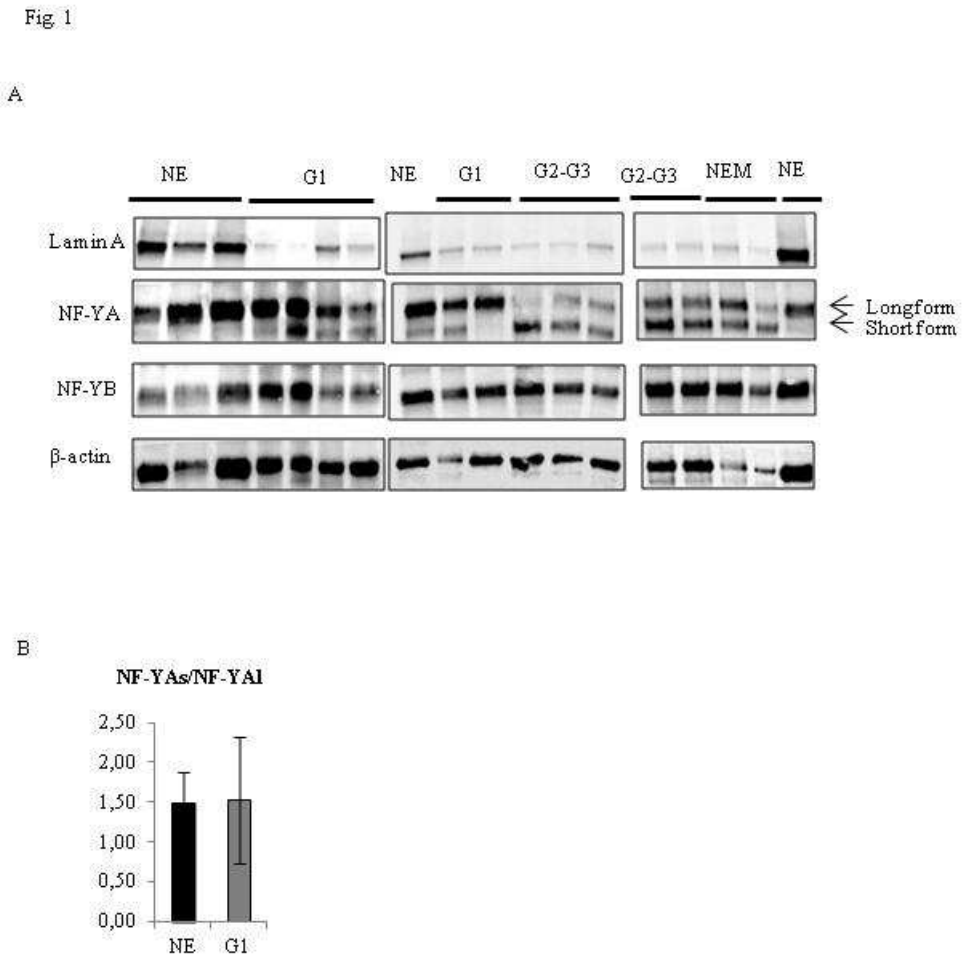

Figure 1: **Analysis of NF-YA isoforms and lamin A protein expression in benign and EC tissues.** A: Representative immunoblottings of proteins extracted from benign (NE), low grade G1 (G1) and high grade endomentrial endometrioid cancer (G2-G3) and non endometrioid (NEM) EC FFPE tissues with anti NF-YA, anti-NF-YB, anti-Lamin A antibodies. Anti-β actin was used as loading control. B: Average expression of the ratio NF-YAs to NF-YAl mRNA expression examined by qRT-PCR±SD in G1 EEC tissues. mRNA expression was normalized for 18S rRNA levels. The error bars indicate the standard error.

Fig. 2

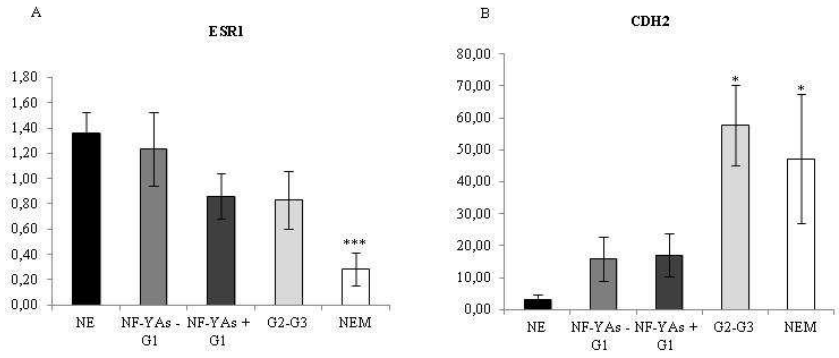

Figure 2: ESR1 decreased mRNA levels are associated with increase CDH2 expression. Average of expression of the ESR-1 mRNA (A), and of CDH2 (B) mRNA in EC tissues examined by qRT-PCR±SD. mRNA expression was normalized for 18S rRNA levels as endogenous control. Statistical significance: *P<0.05, **P<0.01, ***P<0.001. The error bars indicate the standard error.

Fig. 3

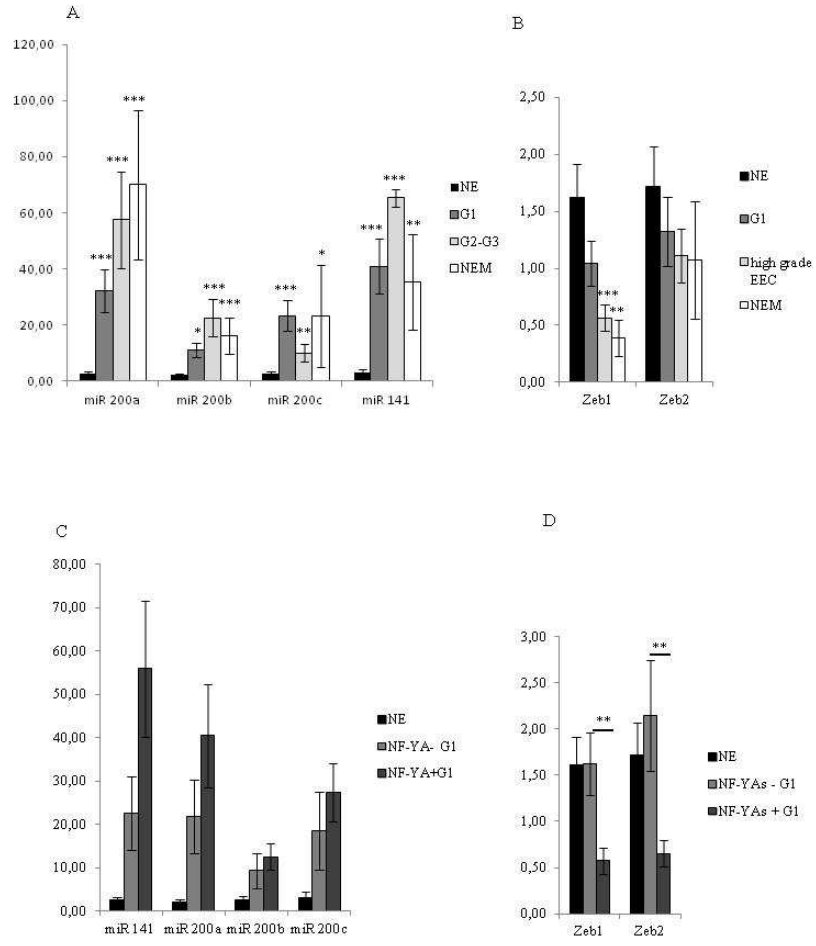

Figure 3: miR-200s and ZEBs expression in EC FFPE tissues. Average of miR-200 family members (miR-200a, miR-200b, miR-200c, and miR-141) expression (A), of ZEB1 and ZEB2 (B), mRNA in benign (NE), G1 EEC, G2-G3 EEC, and NEM FFPE tissues. Average of miR-200 family members (miR-200a, miR-200b, miR-200c, and miR-141) expression (C), and of ZEB1 and ZEB2 (D), mRNA in benign (NE), NF-YAs positive (NF-YA+) and negative (NF-YAs-) G1 subsets of EEC FFPE tissues. Analysis was performed by qRT-PCR±SD. miRNA expression was normalized using small nuclear RNA U6 as endogenous control. mRNA expression was normalized for 18S rRNA levels as endogenous control. Statistical significance: *P<0.05, **P<0.01, ***P<0.001. The error bars indicate the standard error.

Fig 4

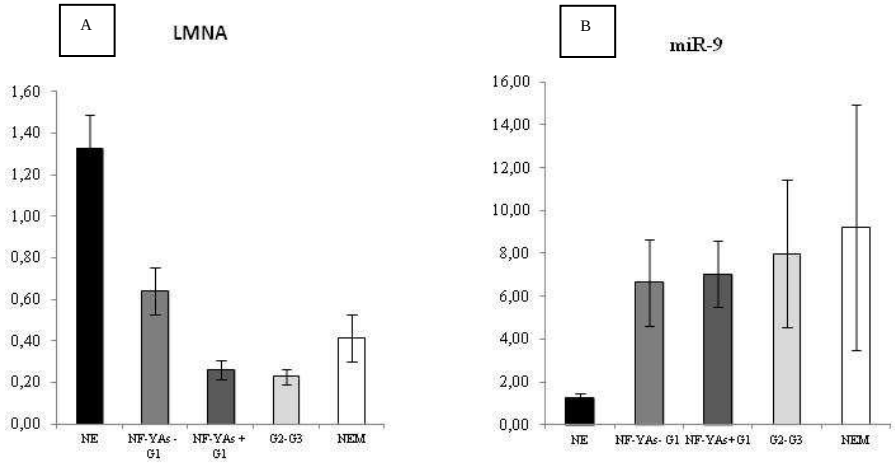

Figure 4: Evaluation of lamin A and mir-9 expression in ECs. Average of expression of LMNA mRNA (A) and miR-9 (B) in benign (NE), in the two subsets of FFPE G1 EEC tissues (NF-YAs- and NF-YAs+) G1 EEC, G2-G3 EEC, and NEM FFPE tissues examined by qRT-PCR±SD. mRNA expression was normalized for 18S rRNA levels. miRNA expression was normalized using small nuclear RNA U6 as endogenous control. mRNA expression was normalized for 18S rRNA levels as endogenous control. Statistical significance: *P<0.05, **P<0.01, ***P<0.001. The error bars indicate the standard error.