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A novel oncogenic axis involving the ETS factor ESE3/EHF, miR-424, COP1 and STAT3 drives prostate tumor progression

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**A novel oncogenic axis involving the ETS factor  
ESE3/EHF, miR-424, COP1 and STAT3 drives prostate  
tumor progression**

**THÈSE**

*présentée à la Faculté des sciences de l'Université de Genève  
pour obtenir le grade de Docteur ès sciences, sciences pharmaceutiques*

par

**Cecilia Dallavalle**

de

Valmorea (Italie)

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intitulée :

**"A Novel Oncogenic Axis Involving the ETS Factor ESE3/EHF,  
miR-424, COP1 and STAT3 Drives Prostate Tumor Progression"**

La Faculté des sciences, sur le préavis de Monsieur L. SCAPOZZA, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), Madame G. CARBONE, docteure et codirectrice de thèse (Institute of Oncology Research, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland), Monsieur Y. KALIA, professeur associé (Section des sciences pharmaceutiques), Monsieur Z. CULIG, professeur (Department of Urology, University of Innsbruck, Austria) et Monsieur P. WILD, professeur (Institute of Surgical Pathology, University of Innsbruck, Austria), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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*List of abbreviations*

aa	amino acid
Ab	Antibody
AR	Androgen Receptor
bp	base pair
CSC	Cancer Stem-like Cells
CRPC	Castration-Resistant Prostate Cancer
ECM	Extra-Cellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ESE	Epithelium Specific ETS
ERG	ETS Related Gene
ETS	E26 Transformation Specific
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HGF	Hepatocyte Growth Factor
Kb	Kilobases
KDa	KiloDalton
hTERT	human Telomerase Reverse Transcriptase
LNA	Locked Nucleic Acid
nt	nucleotide
PBS	Phosphate-Buffered Saline
PCa	Prostate Cancer
PDGF	Platelet-Derived Growth Factor
PS	Prostato-sphere
RNA	RiboNucleic Acid
RNase	Ribonuclease
shRNA	short hairpin RNA
SV40	Simian Virus 40
STAT3	Signal Transducer and Activation of Transcription 3
TF	Transcription Factor
TGF- $\beta$	Transforming Growth Factor $\beta$
TSS	Transcription Start Site
UTR	UnTranslated Region

## ***Summary***

**Background.** Dissecting the diverse pathways that promote and sustain tumorigenesis of different PCa subtypes is an important task to identify new molecular markers and guide the development of novel therapeutic approaches for this disease. Altered expression of ETS factors has emerged as a key event during PCa progression. For instance, we previously reported that a subgroup of prostate tumors characterized by loss of the ETS factor ESE3/EHF (ESE3<sub>low</sub>) is associated with aggressive clinical features and enrichment of epithelial-mesenchymal transition (EMT) and cancer stem-like cell (CSC) features. MicroRNAs play important roles in cell proliferation, differentiation and self-renewal regulating gene expression at a post-transcriptional level. In addition, aberrant miRNA expression has been correlated with tumor development, cancer progression and the acquisition of an EMT phenotype in tumor cells. The identification of key miRNAs involved in the acquisition of EMT and CSC phenotypes in aggressive subtypes of prostate cancer could provide new therapeutic targets as well as diagnostic and prognostic markers for the management of this disease.

**Principal findings.** To understand the mechanisms controlling prostate epithelial cell differentiation and transformation, we profiled microRNA (miRNAs) expression in tissue samples of human primary prostate tumors and normal prostate. miRNAs profiling showed that miR-424 was overexpressed in prostate tumors compared to normal samples and specifically upregulated in ESE3<sub>low</sub> tumours subgroup. Notably, prostate tumours with high miR-424 expression were enriched of EMT and CSC transcriptional features. In normal prostate epithelial cells miR-424 was directly repressed by ESE3/EHF by binding to an ETS binding site in the pri-miRNA promoter. Consistent with an oncogenic role of miR-424, overexpression of the miRNA promoted malignant transformation and CSCs properties in prostate epithelial cells. Mechanistically, we found that miR-424 targets the E3 ubiquitin ligase COP1 repressing its expression. Silencing of COP1 by miR-424 resulted in reduced turnover and increased level of several oncogenic transcription factors, including known COP1 substrates like c-Jun and ETV1. Moreover, we identified STAT3 as a novel substrate of COP1 promoting tumor-initiating and metastatic properties. COP1 induced STAT3 ubiquitilation and degradation by the ubiquitin-proteasome system (UPS). Therefore, COP1 silencing in prostate cancer cells resulted in increased STAT3 stabilization and enhanced STAT3 signaling. COP1 knockdown phenocopied the effects of miR-424 deregulation on oncogenic phenotypes and STAT3 signaling. On the other hand, STAT3 silencing prevented the transforming effects of miR-424 *in vitro*. Inhibition of miR-424 in metastatic prostate cancer cells prevented tumor initiation and metastatic spread *in vivo*, suggesting a possible therapeutic strategy based on miRNA antagonists for prostate cancer. In addition, these findings have prognostic



implications, since low COP1 and high STAT3 protein level were significantly associated and predictive of biochemical relapse in primary prostate tumors.

**Conclusions.** In this study we defined a novel oncogenic pathway of broad biological and clinical significance involving miRNA deregulation and altered protein ubiquitination. Collectively, our results establish miR-424 as a key oncogenic effector in prostate cancer and uncover a novel mechanism controlling STAT3 protein level and activity. This oncogenic pathway is preferentially activated in a subgroup of prostate tumors characterized by loss of the ETS factor ESE3/EHF and is associated with disease progression and poor clinical outcome in prostate cancers. Furthermore, monitoring the state of miR-424/COP1/STAT3 this oncogenic axis could provide new insights for the development of specific therapeutic strategies and the identification of patients more likely to have aggressive tumor and disease recurrence.

## ***Résumé***

**Introduction.** Étudier les diverses voies qui favorisent et soutiennent la tumorigenèse de différents sous-types de tumeur de la prostate est une tâche importante afin d'identifier de nouveaux marqueurs moléculaires et d'orienter l'élaboration de nouvelles approches thérapeutiques de cette maladie. Il apparaît qu'une expression altérée des facteurs de transcription ETS est un événement clé dans la progression du cancer de la prostate. Dans des études précédentes, nous avons indiqué qu'un sous-groupe de tumeurs de la prostate, caractérisé par la perte du facteur ETS ESE3/EHF (ESE3<sub>low</sub>), est associée à des caractéristiques spécifiques aux formes cliniques agressives et à l'enrichissement des propriétés typiques de la transition épithélio-mésenchymateuse (EMT) ainsi qu'à celles des cellules souches cancéreuses (CSC). Les microRNA (miRNA) jouent un rôle important dans la prolifération, la différenciation et l'auto-renouvellement cellulaire en régulant l'expression des gènes au niveau post-transcriptionnel. En outre, l'expression aberrante des miRNA a été corrélée avec le développement des tumeurs, la progression du cancer et l'acquisition d'un phénotype EMT dans les cellules tumorales. L'identification des principaux miRNA impliqués dans l'acquisition d'un phénotype typique de l'EMT et des CSC dans des sous-types agressifs de cancer de la prostate pourrait fournir de nouvelles cibles thérapeutiques ainsi que des marqueurs diagnostiques et pronostiques pour la gestion de cette maladie.

**Principaux résultats.** Pour mieux comprendre les mécanismes contrôlant la différenciation et la transformation des cellules épithéliales de la prostate, nous avons évalué le profil d'expression de microRNA (miRNA) dans des échantillons de tissu prostatique tumoral et normal. Le profilage des miRNA a montré que le miR-424 était surexprimé dans les tumeurs de la prostate comparativement aux échantillons normaux et spécifiquement surexprimé dans le sous-groupe des tumeurs ESE3<sub>low</sub>. Notamment, les tumeurs de la prostate avec haute expression du miR-424 ont été enrichies des propriétés transcriptionnelles typiques aux CSC et à l'EMT. Dans les cellules épithéliales de la prostate, miR-424 est directement réprimé par ESE3/EHF par l'interaction avec un site de liaison des facteurs ETS sur le promoteur du pri-miRNA. Compatible avec le rôle oncogénique du miR-424, la surexpression du miRNA promeut la transformation maligne et l'acquisition de propriétés des CSC dans les cellules épithéliales de la prostate. Nous avons constaté que miR-424 agit sur l'ubiquitine ligase COP1 et réprime son expression. Le silençage de COP1 par miR-424 est responsable de la réduction de la dégradation de plusieurs facteurs de transcription oncogéniques, y compris certains substrats de COP1 déjà connus, comme c-Jun et ETV1. En outre, nous avons identifié STAT3 comme un nouveau substrat de COP1, capable de

promouvoir les propriétés tumorigènes et métastatiques. COP1 induit l'ubiquitination du facteur transcriptionnel STAT3 et sa dégradation par l'intermédiaire du système ubiquitine-protéasome (UPS). En conséquence, le silençage de COP1 dans les cellules du cancer de la prostate a conduit à une augmentation de la stabilisation de STAT3 et de son activité transcriptionnelle. Le silençage de COP1 simule les effets de miR-424 sur les phénotypes oncogènes aussi. D'autre part, le silençage du STAT3 a empêché la transformation maligne liée à l'expression de miR-424 *in vitro*. L'inhibition de miR-424 dans les cellules métastatiques du cancer de la prostate permet de bloquer l'initiation des tumeurs et la propagation métastatique *in vivo*, suggérant une stratégie thérapeutique éventuelle fondée sur les antagonistes des miRNA pour le cancer de la prostate. En outre, ces résultats ont des implications pronostiques, car un faible niveau de COP1 et un niveau élevé de STAT3 ont été associés de manière significative et prédictive à la rechute biochimiques des tumeurs primaires de la prostate.

**Conclusions.** Dans cette étude, nous avons défini une nouvelle voie oncogénique d'importance biologique et clinique impliquant la déréglementation de miRNA et l'altération de l'ubiquitination des protéines. Collectivement, nos résultats établissent miR-424 comme un effecteur oncogénique impliqué dans le cancer de la prostate et révèlent un nouveau mécanisme de contrôle de l'expression et de l'activité de la protéine STAT3. Cette voie oncogénique est activée préférentiellement dans un sous-groupe de tumeurs de la prostate caractérisé par la perte du facteur ETS, ESE3/EHF, et associée à la progression de la maladie ainsi qu'à un pronostic défavorable pour le cancer de la prostate. En outre, la surveillance de l'état de l'axe oncogénique miR-424/COP1/STAT3 pourrait fournir de nouvelles perspectives pour le développement de stratégies thérapeutiques spécifiques et l'identification des patients plus susceptibles d'avoir des tumeurs agressives et la réapparition de la maladie.

## ***Introduction***

## **1. Prostate Cancer**

Prostate cancer (PCa) is the fourth most common tumor type worldwide and the second most common cancer in men (Ferlay et al., 2013). Despite recent progress, PCa continues to represent a major cause of cancer-related mortality, especially in elderly men. More than half of all men diagnosed with cancer are over 70 years, with PCa constituting about 50% of cancers in this age group (Detchokul and Frauman, 2011; Ferlay et al., 2013). In 2015, 220,800 of new prostate cancer cases and 27,540 related deaths were estimated in the United States alone (Siegel et al., 2015). Multiple genetic and demographic factors, including age, family history, genetic susceptibility and race, contribute to the incidence of this disease (Al Olama et al., 2014).

The availability of highly accessible blood test for prostate-specific antigen (PSA) has revolutionized the diagnosis of prostate cancer over the past three decades, opening the possibility to detect the disease in its early stages. PSA is a serine proteases produced in normal prostate secretions, but released into the blood as a consequence of disruption of normal prostate architecture (Lilja et al., 2008). Men showing elevated PSA levels typically undergo biopsy to assess the potential presence of PCa. Following biopsy, histopathological grading of prostate tissue is performed by Gleason scoring that classifies tumors from 1 to 5 based on their architecture. Once prostate cancer is diagnosed, conventional treatment regimens include surgical excision of the prostate (radical prostatectomy), or irradiation. In the case of advanced cancer, these regimens are usually followed or substituted with androgen deprivation therapy, which initially reduces tumor burden and circulating PSA. However, the disease recurs in most of the cases. This recurrent disease is termed “castration resistant” and it is essentially untreatable (Shen and Abate-Shen, 2010). Therefore, a major clinical challenge is the elucidation of pathways of castration resistance, which could lead to the identification of new therapeutic approaches.

Furthermore, a second major clinical challenge is the inability to distinguish indolent from aggressive tumors, in order to avoid the overdiagnosis and overtreatment of PCa. Indeed, due to PSA screening, nearly 90% of prostate cancers are clinically localized at the time of their diagnosis (Penney et al., 2013). However, the clinical behavior of localized prostate cancer is highly variable: while some men develop aggressive cancer that leads to metastasis and death for the disease, many others show indolent cancers that can be cured with initial therapy or may be safely observed (Nelson et al., 2003) . Therefore, the widespread use of PSA testing has led to a vast increase in the diagnosis of patients with clinically localized low Gleason grade carcinomas that may not require treatment, since their tumor are indolent. On the other hand, for sensitivity limitations, it has been found that 15% of men with PSA considered in “normal” range, have PCa in their biopsy.

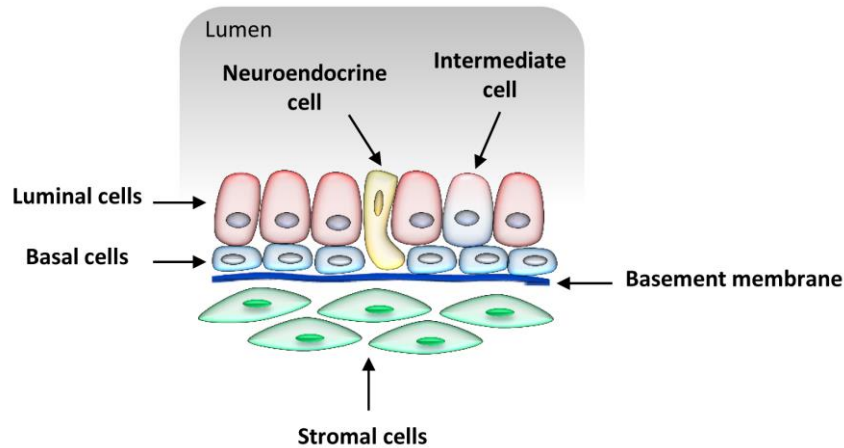
To solve these issues, multiple risk stratification systems have been developed, combining the best currently available clinical and pathological parameters (such as Gleason score, PSA levels, and clinical and pathological staging). However, these tools still do not adequately predict patients outcome and new biomarkers for a PCa diagnosis and prognosis are urgently needed. In this scenario, a better understanding of the molecular basis of prostate cancer initiation and progression could significantly improve PCa diagnosis and treatment.

### ***Development of the prostate gland: Anatomy and Histology***

The normal human prostate is a tubular-alveolar gland composed of a well-developed stromal compartment containing nerves, fibroblasts, infiltrating lymphocytes and macrophages, capillaries with endothelial and smooth muscle cells surrounding glandular acini. The glandular epithelium is pseudostratified and characterized by three differentiated layers: luminal, basal and neuroendocrine (Figure 1). The luminal layer is composed of polarized columnar cells that are responsible for the production and secretion of PSA, Prostatic Acid Phosphatase (PAP) and human kallikrein-2, which are secreted as part of the seminal fluid. The luminal cells are well differentiated non-proliferative cells expressing characteristic markers, such as cytokeratin 8 and 18, as well as high levels of androgen receptor (AR) and p27 (Barbieri et al., 2012). The basal layer is located beneath the luminal epithelium and represents the proliferative compartment of the prostatic tissue. Basal cells are un-differentiated cells expressing p63 and high-molecular-weight cytokeratin 5 and 14, but expressing low or undetectable level of AR.

Between these two layers is present the population of so called intermediate cells that partially retain the phenotype of basal progenitors, but also show some features of the differentiated luminal cells (Shen and Abate-Shen, 2010).

An additional cell type is represented by neuroendocrine cells, which are rare cells of unknown function that express endocrine markers such as chromogranin A and synaptophysin, but are AR-negative. It is thought that they play a role in growth and differentiation of the developing prostate. These cells do not properly generate a layer because they are spread within the prostate epithelium and are terminally differentiated cells (Shen and Abate-Shen, 2010).



**Figure 1. Schematic representation of the cellular architecture of the human prostate epithelium.**

### ***Molecular Mechanisms of Prostate Cancer initiation and progression***

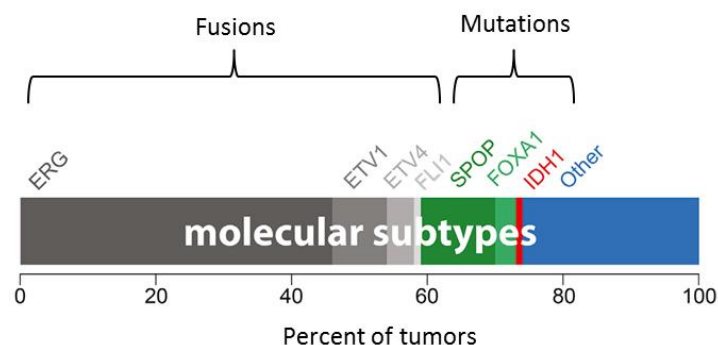
Like most other epithelial cancers, the first stage of prostate cancer progression is usually the Prostatic Intraepithelial Neoplasia (PIN). PIN is generally characterized by the appearance of luminal epithelial hyperplasia, reduction in the number of basal cells, and nuclear atypia. Generally, high-grade PIN lesions also display marked elevation of cellular proliferation markers (Shappell et al., 2004). Recent evidence has suggested that the majority of the alterations involved in the progression to prostate cancer occur during transition from benign epithelium to PIN rather than from PIN to prostate cancer (Zynger and Yang, 2009). Starting from PIN precursor lesions, PCa progresses to locally invasive disease and ultimately metastasis.

The majority of prostate cancers is morphologically classified as adenocarcinoma that express AR and are characterized by a luminal phenotype, although human PCa displays significant phenotypic heterogeneity (Shen and Abate-Shen, 2010). Indeed, a notable difference between prostate cancer and other epithelial tumors is the lack of clearly distinguishable subtypes that differ in their prognosis and treatment response. The most significant histological variant is neuroendocrine prostate cancer (NEPC) that is characterized by the presence of small, round, blue neuroendocrine cells, which do not express AR or secrete PSA but usually express neuroendocrine markers (Beltran et al., 2011). Focal regions of neuroendocrine differentiation are commonly observed in prostate adenocarcinoma following recurrence after prostatectomy and androgen deprivation therapy (Komiya et al., 2009). Accordingly, expression of neuroendocrine markers is also associated with the development of castration-resistant tumors. The poor molecular characterization of NEPC in the past accounted in part for the lack of disease-specific therapeutics. Recent evidence demonstrated that NEPC is an aggressive subtype of prostate



cancer that clonally arises from prostate adenocarcinoma after hormonal therapy and provided new insight into molecular alterations that can distinguish castration-resistant tumors with neuroendocrine features from adenocarcinomas (Beltran et al., 2016; Beltran et al., 2011).

The histological and clinical heterogeneity of PCa is reflected also at a molecular level. Thus, the identification of key molecular alterations responsible for prostate carcinogenesis and PCa susceptibility genes has always represented a major issue. However, during the last years the emergence of Next Generation Sequencing provided novel insights into cancer genomes and several recent studies that explored the molecular basis of primary prostate cancer have identified multiple recurrent genomic alterations (Baca et al., 2013; Barbieri et al., 2012; Berger et al., 2011; Pflueger et al., 2011; Taylor et al., 2010; Tomlins et al., 2007; Wang et al., 2011). Thanks to these new discoveries, PCa is transitioning from a poorly understood, clinically heterogeneous disease to a collection of different subtypes of prostate tumors identifiable by molecular criteria (Tomlins et al., 2008; Barbieri et al., 2012). The first molecular classification of PCa identified four major classes characterized by ETS rearrangements, SPINK1 overexpression, SPOP mutations and PTEN deletion (Barbieri et al., 2012). Very recently a more comprehensive molecular taxonomy of primary prostate cancers has been proposed by The Cancer Genome Atlas (TCGA) Research Network integrating the results from somatic mutations, gene fusions, somatic copy-number alterations, gene expression, and DNA methylation (TCGA, 2015). This analyses uncovered that the majority of tumors are assignable to one of seven molecular classes based on distinct oncogenic drivers (Figure 2): fusions involving ERG (46%), ETV1 (8%), ETV4 (4%) or FLI1 (1%); mutations in SPOP (11%), FOXA1 (3%) or IDH1 (1%). Nevertheless, due to the heterogeneous natural history of the disease, a significant diversity in DNA copy-number alterations, gene expression and DNA methylation was observed within the groups.



**Figure 2. Molecular Taxonomy of PCa based on oncogenic drivers.** The seven molecular subtypes identified in the analysis of primary prostate cancer samples by the TCGA Network are shown: tumors can be classified based on ETS fusions or mutations in SPOP, FOXA1 or IDH1. (Modified from TCGA, 2015).

Collectively, the recent studies show that prostate cancer genomes display low mutation rates compared with other cancers. On the other hand, PCa is characterized by a large numbers of chromosomal rearrangements and extensive copy number alterations.

The most recurrent non-synonymous mutations involve the tumor suppressor gene SPOP, which encodes for the substrate-recognition component of a Cullin3-based E3-ubiquitin ligase. These mutations inactivate SPOP function by disrupting the interaction with substrate proteins and thus resulting in increased invasion in prostate cell lines (Barbieri et al., 2012; Theurillat et al., 2014; Geng et al., 2013). In addition to SPOP, other frequently mutated genes in primary prostate cancers are TP53, FOXA1 and PTEN (Barbieri et al., 2012; TCGA, 2015). The study performed by TCGA Network identified new clinically relevant genes with lower mutation frequencies, including genes involved in kinase signaling pathways (BRAF, HRAS, AKT1), beta-catenin pathway (CTNNB1), and the DNA repair pathway (ATM). NKX3.1, a key regulator of prostate epithelial differentiation and stem cell function, was also somatically mutated in this cohort. Besides point mutations, other molecular mechanisms, such as genomic deletion and epigenetic alterations, frequently trigger down-regulation of NKX3.1 promoting prostate cancer initiation (Asatiani et al., 2005; Shen and Abate, 2010).

Alterations involving AR gene are also relevant events in prostate carcinogenesis, particularly in the progression towards more aggressive diseases. The TCGA analysis showed that AR activity in primary prostate cancer varied widely and in a subtype-specific manner, with SPOP and FOXA1 mutant tumors having the highest levels of AR-induced transcripts (TCGA 2015). AR is a nuclear hormone receptor that is critical for the regulation of prostate development, as well as cell growth and survival programs in prostate carcinoma. When prostate cancer become castration-resistant, AR activation can remain sustained despite androgen ablation through a variety of molecular mechanisms. Although recent data show AR amplification in about 40% and point mutations in an additional 10% of treated metastatic tumors, these lesions are usually absent in clinically localized prostate cancers (Barbieri and Tomlins, 2014). However, several other mechanisms apart from genomic aberrations can affect AR expression or activity in PCa, such as the expression of alternative splice isoforms encoding constitutively active AR variants (Dehm et al., 2008; Watson et al., 2010) or the endogenous expression of androgen synthetic enzymes by tumor tissue (Montgomery et al., 2008). Nevertheless, metastatic castration-resistant PCa (CRPC) is usually constituted by a mixture of cells that display a wide range of AR expression levels (Crnalic et al., 2010) or can also acquire a complete independence from AR signaling. For instance, CRPC with neuroendocrine features is completely AR-independent and displays marked epigenetic differences from castration-resistant adenocarcinomas, despite the substantial

genomic similarity. Thus, epigenetic alterations, such as DNA-methylation, may play a role in the induction of androgen-independent state (Beltran et al., 2016).

Frequent copy-number alterations in PCa include genomic gains of chromosome 7 and 8q and heterozygous losses of 8p, 13q, 16q and 18 (TCGA, 2015). In addition, recurrent focal amplifications involve relevant oncogenes such as CCND1, MYC and FGFR1. Homozygous deletion spanning the PTEN locus occurred at one of the highest rates of any tumor type studied so far (15%). Several studies support the conclusion that PTEN undergoes copy number loss as an early event in prostate carcinogenesis, and is correlated with progression to aggressive, castration-resistant disease (Shen and Abate 2010). Overall, indolent and low-Gleason tumors have few copy-number alterations, whereas more aggressive primary and metastatic tumors have extensive genome-wide alterations (Taylor et al., 2010; Lalonde et al., 2014).

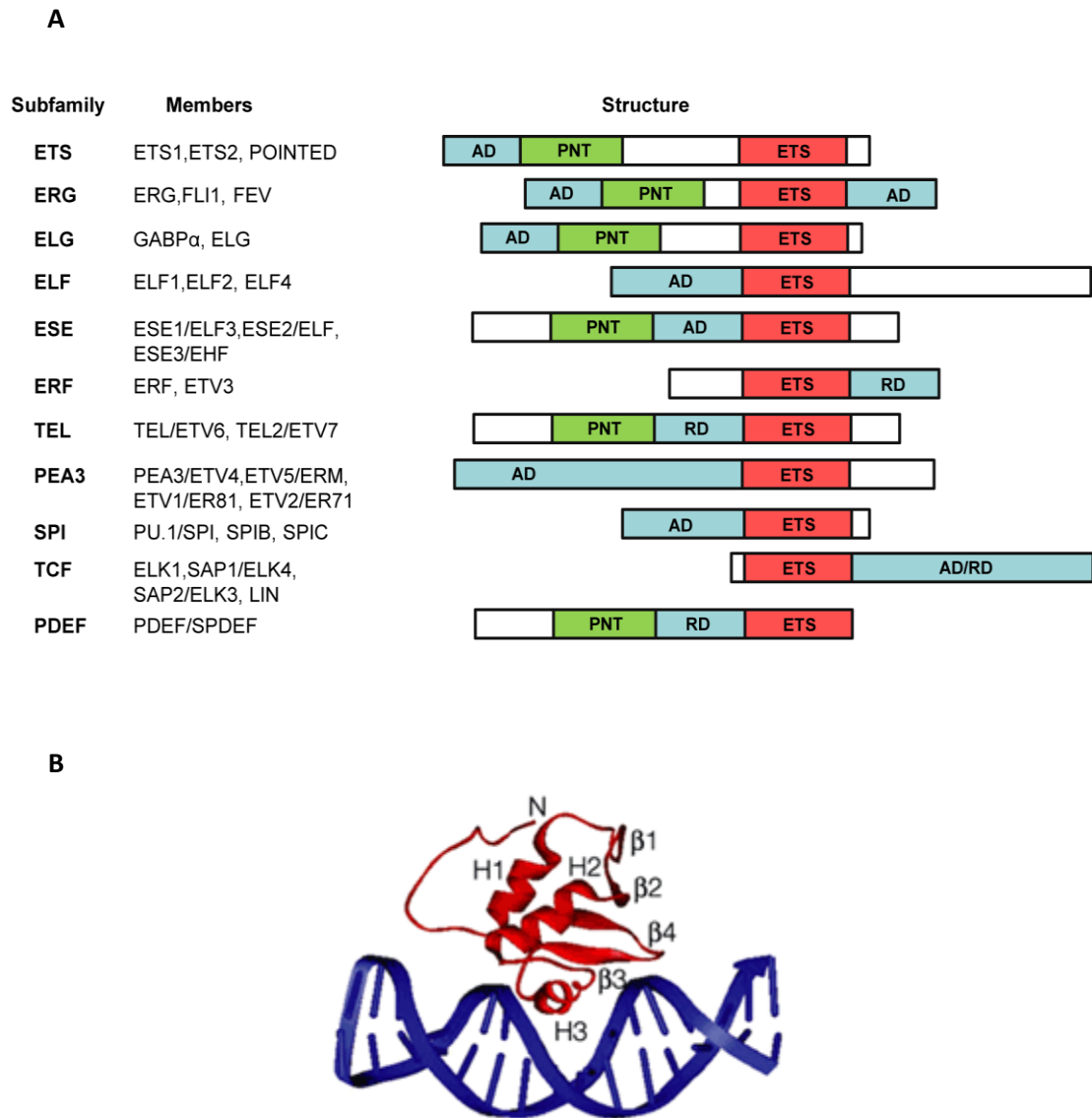
As already mentioned, a large number of rearrangements is usually identified in all prostate cancer genomes. Indeed, the most common alterations in PCa are fusions of androgen-regulated promoters with members of the ETS family. Different studies have identified chromosomal rearrangements that activates members of this family of transcription factors, such as ERG, ETV1, ETV4, in the majority of prostate carcinomas (Tomlins et al., 2007; Mosquera et al., 2008; Saramaki et al., 2008; TCGA, 2015). TMPRSS2 gene, encoding for an androgen-dependent serine-protease, is the most frequent fusion partner of ETS proteins. In particular, the TMPRSS2-ERG fusion, resulting in expression of ERG protein under the control of the androgen-responsive promoter, is the most common of these rearrangements as present in 40-50% of prostate cancers (Tomlins et al., 2005; Clark et al., 2007). Starting from this evidence, alterations involving ETS genes emerged to be relevant in prostate carcinogenesis. A number of studies suggested that activation of oncogenic ETS promotes tumor-initiating and tumor-invasive properties (Tomlins et al., 2007, 2008; Kunderfranco et al., 2010; Longoni et al., 2013b). Additional reports uncovered the altered expression of ETS factors acting as tumor-suppressor in prostate cancer (Gu et al., 2007; Rostad et al., 2007; Cangemi et al., 2008; Albino et al., 2012). Considering these findings, ETS proteins represent potential targets for the development of new cancer therapy and the identification of aberration involving ETS genes could provide new insights on the molecular mechanisms driving prostate tumorigenesis.

## **2. ETS transcription factors**

ETS (E26 Transformation Specific) transcription factors represent one of the major families of transcriptional regulators, including 28 members that share a highly conserved DNA-binding domain. The first component of this family was *v-ets* or *ETS-1*, originally identified as fusion oncogene *gag-myb-ets* in the avian transforming retrovirus E26 (E Twenty-Six), which induces both erythroblastic and myeloblastic leukemias in chickens (Sharrocks, 2001). The identification of ETS-1 as an oncogenic fusion protein in the E26 virus was the first link between ETS proteins and cancer. Based on the sequence homology with the DNA-binding domain of ETS-1, many other ETS domain proteins from various organisms have been identified (Sharrocks, 2001).

ETS proteins are subdivided in 11 subfamilies (Figure 3A) on the basis of sequence similarities within the binding domain and the presence of additional conserved domains that might be reflected in similar molecular and biological functions. One of such region is the Pointed (PNT) domain, which is the second evolutionary-conserved domain and has been found in a subset of ETS proteins consisting of 11 out of 28 human members. This 65–85 amino acids domain belongs to the sterile alpha motif (SAM) family and shows multiple functions: homo-oligomerization, hetero-dimerization and docking site for signaling pathways, such as the MAPK cascade (Foulds et al., 2004).

The ETS domain is composed of about 85 amino acids and mediates the binding to purine-rich DNA sequences (ETS binding sites), characterized by a GGAA/T core with an additional flanking nucleotides important to discriminate the binding of different ETS (Sharrocks, 2001). This winged helix-turn-helix DNA binding domain is conserved among all the family members and it is composed of three alpha helices and a four-stranded antiparallel beta sheet (Figure 3B). The two invariant amino acid residues in helix 3, residues in the turn between helices H2 and H3, as well as those in the wing between beta strands 3 and 4, provide key ETS protein interactions with the major groove of DNA (Oikawa and Yamada 2003).



**Figure 3. Structure of ETS proteins (A) and 3D structure of ETS domain (B).** (A) ETS domain (ETS); transactivation domain (TAD); inhibitory domain (ID); A/T hook domain; and repressor domain (RD). (B) The location of helices (H) and  $\beta$ -strands ( $\beta$ ) within the structures of the ETS domain is shown. Red, ETS domain; Blue, DNA major groove. (Modified from Sharrocks, 2001).

Genome wide occupancy studies revealed that a single ETS protein can bind to hundreds of ETS binding sites (EBS) in a particular cell type. Moreover, these studies also show that multiple ETS factors can occupy the same ETS binding site, indicating redundant binding (Kar and Gutierrez Hartman, 2013; Kunderfranco et al., 2010). Importantly, many of these redundant binding regions are characterized by the consensus ETS sequence ACCGGAAGT and are mostly found on the proximal promoters of housekeeping genes (Barski and Zhao, 2009; Farnham, 2009; Hollenhorst et al., 2007). By contrast, non-redundant ETS sites are more often atypical and low affinity EBS, flanked by recognition sequence for other transcription factors, such as AP-1 or PAX5, that provide a stabilizing binding surface (Wheat et al., 1999; Hollenhorst et al., 2011).

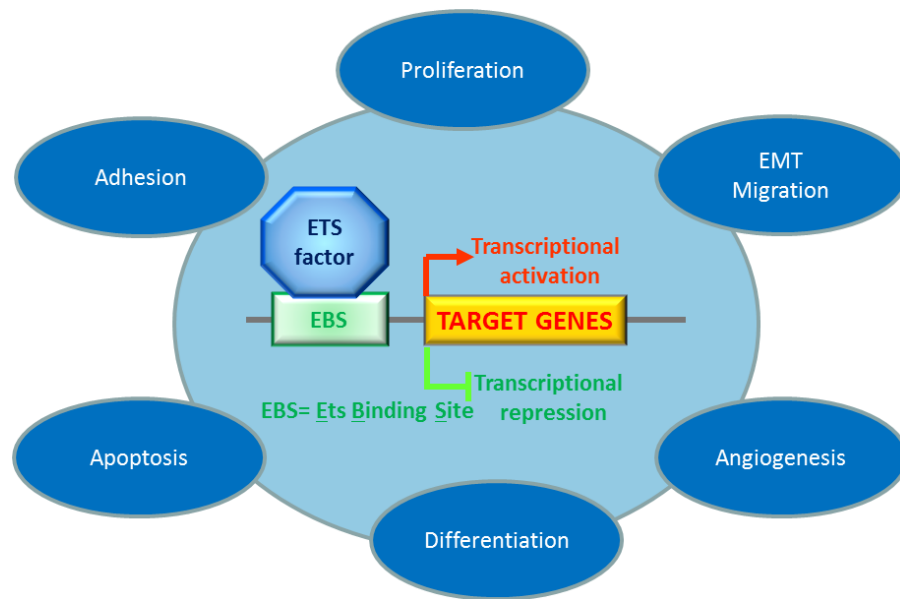
Although there are several similarities between ETS proteins and minimal differences in their consensus DNA binding sequences, each transcription factor show a distinct mechanism of action. Indeed, ETS target specificity and functional activity can be modulated at multiple levels. *In vitro* binding assays show that the specificity is often determined by the amino acids surrounding the ETS domain of the protein and by DNA sequences flanking the central GGAA/T core in the genome (Wei et al., 2010). The interaction with other transcription factors and cofactors also affects target gene specificity and additionally enhances ETS DNA-binding domain activity (Garvie et al., 2001; Goetz et al., 2000; Kar and Gutierrez Hartman, 2013). Thus, maximal transcriptional activation of target genes often depends on the simultaneous expression of ETS proteins and their partner transcription factors. Furthermore, specific intracellular signaling pathways and post-translational modifications, such as phosphorylation, glycosylation and acetylation, directly affect the activity of several ETS proteins by regulating subcellular localization, DNA-binding, transactivation potential and stability (Findlay et al., 2013). For example, inhibition of TCF (Ternary Complex Factor) subfamily members ELK-1, ELK-3 and ELK-4 can be reversed by phosphorylation of the transactivation domain mediated through MAPK (Sharrocks, 2002; Stinson et al., 2003). Phosphorylation of ESE1 increases its protein stability, thus enhancing the transforming potential (Manavathi et al., 2007). Acetylation of multiple relevant sites in the C-terminus of the fusion protein EWS-FLI1 increases its binding ability, protein stability and transcriptional potency (Schlottmann et al., 2012).

These structural and functional differences, together with different spatial and temporal expression patterns, allow each ETS factor to regulate a distinct set of target genes and thereby control distinct biological functions. Therefore, ETS proteins constitute a complex network of transcriptional regulators with specific biological responses depending on the balance between factors with similar or opposite roles (Seth and Watson, 2005; Kunderfranco et al., 2010). Overall, the ETS family of transcription factors positively and/or negatively regulates the expression of genes involved in key cellular processes (Figure 4), such as development, proliferation, differentiation, migration, apoptosis, angiogenesis, invasion and metastasis (Oikawa et al., 2003; Seth and Watson, 2005; Turner et al., 2007). Based on this notion, it is not surprising that alterations involving ETS genes can have a huge impact on cellular homeostasis and contribute to tumor initiation and progression.

Indeed, deregulated expression of ETS genes has been documented in several human malignancies. The transforming potential of different ETS factors has been particularly investigated in several experimental systems. The strongest evidence for oncogenic activation mediated by amplification or overexpression comes from breast, prostate and hematologic tumors (Kalyuga et al., 2012; Seth and Watson, 2005; Kar and Gutierrez-Hartmann, 2013). For

example, multiple ETS factors, including ESE1, PEA3, ETS1, ETS2, ERM and ETV1, are up-regulated in breast cancer and are associated with poor prognosis and metastasis (Seth and Watson, 2005). Increased expression of ETS1 gene is correlated with enhanced cancer metastasis and invasion in several others solid tumors (Nakayama et al., 2001; Dittmer, 2003). A number of studies show that ESE1 overexpression is sufficient to drive cell transformation and tumorigenesis in different tissues (Prescott et al., 2004; Wang et al., 2014). Furthermore, overexpression of ERG (Tomlins 2005), ETV1 (Shin et al., 2013) and ELF1 (Shaikhibrahim et al., 2011) frequently occurs in prostate cancer due to different molecular mechanisms. Several ETS genes are often rearranged to produce chimeric oncoproteins both in solid and hematologic malignancies (Riggi and Stamenkovic, 2007). A well-known example is the fusion protein EWS-FLI1 derived from the translocation t(11;22)(q24,q12) that is found in up to the 95% of human Ewing's sarcomas. As a consequence, the N-terminal region of EWS, an RNA binding protein, and C-terminal portion of Fli-1 are rearranged. The derived chimeric protein possesses increased transactivation potential, compared to the wild type Fli-1, suggesting its contribution in developing the malignant phenotype of the cells (Davis et al., 2006).

While in the past it has been presumed that the primary function of ETS factors in tumorigenesis is to promote the malignant phenotype, it is now clear that in certain tissues ETS proteins serve to suppress tumorigenesis (Jedlicka et al., 2009; Albino et al., 2012). For example, PU.1 is involved in myeloid and lymphoid differentiation and has been recently reported to act as a potent tumor suppressor in Hodgkin lymphoma (Yuki et al., 2013). Other ETS including PDEF, ESE2 and ESE3 were found to be downregulated during breast cancer progression. Functional studies showed the impact of altered expression patterns of these ETS family members on the regulation of genes associated with proliferation, transformation, migration, invasion, apoptosis and angiogenesis (Turner et al., 2007).



**Figure 4. Biological functions controlled by ETS factors.** Proteins belonging to the ETS family can act as transcriptional activator or repressor regulating the expression of several target genes involved in oncogenic pathways.

#### ***Role of ETS factors in prostate cancer progression***

Several genetic, epigenetic and chromosomal alterations involving ETS transcription factors have been identified in PCa and have a crucial role in the pathogenesis of this disease (Table 1).

The discovery of ETS genes chromosomal rearrangements in prostate cancer, has given new insight to the understanding of prostate tumorigenesis. Indeed, the translocations leading to fusions of the 5'-untranslated region (UTR) of the androgen regulated gene TMPRSS2 (21q22.3) with ETS genes, like ERG (21q22.2), ETV1 (7p21.2), ETV5 (3q28) or ETV4 (17q21), are the most recurrent and provide a mechanism for androgen-mediated over-expression of these transcription factors in prostate cancer (Kumara-Sinha et al., 2008; Tomlins et al., 2008). The gene fusion TMPRSS2-ERG, which occurs in approximately 50% of cases (Kumara-Sinha et al., 2008) is the best described. By now, seventeen different types of TMPRSS2-ERG fusion transcripts involving various regions of the two genes have been identified. Eight of these transcripts are unlikely to encode functional ERG proteins owing to the introduction of premature stop codons. The fusion between exon 1 of TMPRSS2 and exon 4 or 5 of ERG gene are the most frequently expressed (Tomlins et al., 2005). Although TMPRSS2-ERG rearrangement has been found in high-grade prostatic intraepithelial neoplasia (HGPIN), indicating that it is an early event in prostate tumorigenesis (Perner et al., 2007), its fusion product appears to be unable to transform benign prostatic epithelial cell lines or induce the development of an adenocarcinoma in mouse prostate. However, recent studies have demonstrated that overexpression of ERG fusion proteins facilitates prostate cancer progression by promoting cell migration and invasion (Carver et al., 2009; Tomlins



et al., 2008). Other studies involving mice model of ERG have reported that ERG needs to cooperate with other oncogenic events such as PTEN loss to induce transformation (Carver et al., 2009; Chen et al., 2013; Sreenath et al., 2011).

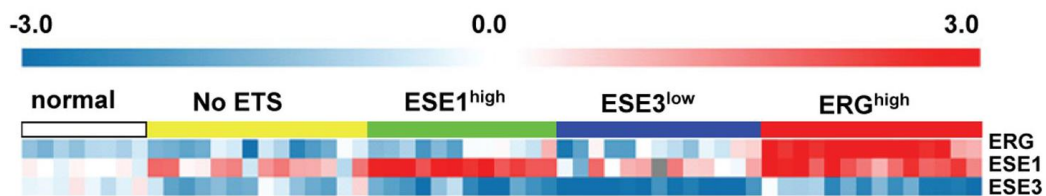
Other ETS factors have been found overexpressed in prostate cancer independently from the presence of translocation. For example, ETS1 protein expression is significantly increased in clinical and latent PCa relative to benign prostatic hyperplasia and normal prostate (Turner et al., 2007). ETS1 overexpression has been associated with poor tumor differentiation and with the progression of the disease (Shaikhibrahim and Wernert, 2012). ETS2 is overexpressed in high Gleason score primary and metastatic PCa. Interfering with ETS2 expression or activity has a negative impact on cell proliferation and survival (Sementchenko et al., 1998; Carbone et al., 2004).

Few studies reported on ETS factors having tumor suppressor role in prostate cancer. PDEF (Prostate-Derived ETS Factor) was reported to act as negative regulator of carcinogenesis promoting migration and invasiveness of PCa cells. Indeed, inhibition of PDEF expression triggers a transcriptional program of genes involved in migration, invasion, adhesion, and epithelial dedifferentiation (Gu et al., 2007). Our group has reported a tumor suppressor role of the ETS factor ESE3/EHF. This gene controls prostate epithelial cell differentiation and its loss induces transforming phenotypes (see specific section for details).

ETS Factor	Molecular alterations involved in PCa
ERG	Overexpression of TMPRSS2-ERG fusion protein and androgen dependent activation of ERG target genes; relevant role in invasion and cancer progression (Tomlins et al. 2005; Kunderfranco et al. 2010).
ESE1	Overexpression in both primary and metastatic cancers; Role in transformation and progression. Associated with inflammatory pathways (Longoni et al. 2013).
ETS1	Overexpression and association with poor tumor differentiation (Alipov et al. 2005); Role in transformation and cancer progression (Shaikhibrahim and Wernert 2012).
ETS2	Overexpression in metastatic prostate cancers; involved in cell proliferation and survival (Sementchenko et al. 1998; Carbone et al. 2004).
ESE3	Downregulation in primary prostate cancer; important role in epithelial cell differentiation; its loss induces dedifferentiation, epithelial-mesenchymal transition, cancer stem-like properties and tumorigenesis (Cangemi et al. 2008; Longoni et al. 2013b, Albino et al. 2012).
PDEF	Downregulation in prostate cancer cells; involved in the regulation of epithelial markers; Its loss induces migratory capability and invasiveness (Gu et al. 2007).
ETV1	Overexpressed following translocation; promote aggressive phenotypes in prostate cancer (Mesquita et al., 2015; Tomlins et al. 2005)
ETV4	Overexpressed following translocation; promote aggressive phenotypes in prostate cancer (Mesquita et al., 2015; Tomlins et al. 2005)

**Table 1. Summary of most relevant molecular alterations involving ETS factors in prostate cancer.**

Recent data from our group provided new information on the deregulated expression of multiple ETS genes in primary prostate tumors compared to normal prostate (Kunderfranco et al., 2010). About 75-80% of tumors evaluated had at least one aberrantly expressed ETS gene, often with multiple ETS simultaneously affected. In addition to the translocated ETS genes (ERG, ETV1 and ETV4), other ETS were frequently deregulated. In particular, ERG, ESE1 and ESE3 were the most significantly affected genes (Kunderfranco et al., 2010). Based on these data, the tumors were classified in three different subgroups with predominant deregulation: tumors with high ERG expression (ERG<sub>high</sub>), tumors with high ESE1 expression (ESE1<sub>high</sub>) and tumors with low ESE3 expression (ESE3<sub>low</sub>). A fourth group included tumors with normal-like expression of ETS genes (NoETS) (Figure 5).



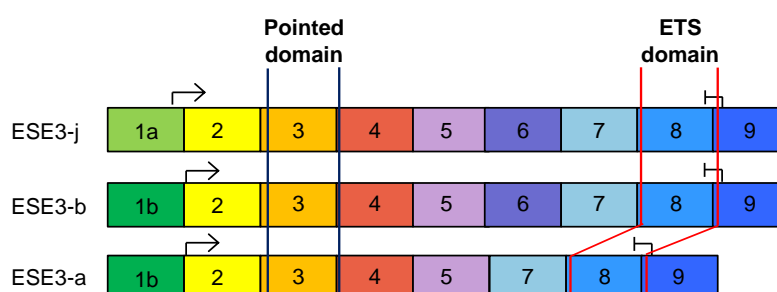
**Figure 5. Analysis of ETS factors expression in prostate samples.** ERG, ESE1/ELF3 and ESE3/EHF expression levels were evaluated in normal prostate and prostate tumors. Columns represent individual patients and rows report the level of the indicated ETS genes. Samples were classified in four groups based on the specific ETS factor aberrantly expressed compared to normal samples (Kunderfranco et al., 2010).

The properties of the ETS-based subgroups were further investigated by our laboratory attempting to link their biological and molecular characteristics to specific clinical features. These additional studies revealed a pivotal role of specific ETS factors in prostate carcinogenesis and identified genes and pathways affected by the aberrant activity of ERG, ESE1 and ESE3 proteins. Collectively, it was reported that these ETS factors directly control the transcription of multiple genes involved in cell differentiation, proliferation, migration, neoplastic transformation and tumor progression and, thus, their abnormal expression has relevant consequences on the phenotype of normal and prostate cancer cells (Albino et al., 2012; Kunderfranco et al., 2010; Longoni et al., 2013b).

In particular, tumors characterized by loss of ESE3 showed distinctive biological features, including increased expression of mesenchymal and cancer stem cell-related genes (Albino et al., 2012). These results were consistent with previous findings by our group that showed for the first time reduced expression of ESE3 in clinical samples of prostate cancer and suggested a tumor suppressor role of this transcription factor in prostate tumorigenesis (Cangemi et al., 2008).

### ***The transcription factor ESE3***

ESE3 (also known as EHF, ETS Homologous Factor) belongs to the epithelial specific subset (ESE) of ETS transcription factors, including also ESE1 (or ELF3) and ESE2 (ELF5). ESE transcription factors play a critical function in the differentiation and proliferation of epithelial cells and share a high homology in their protein sequences (Seth and Watson, 2005). Three ESE3 transcript variants encoding for different isoforms have been identified: ESE3-a, ESE3-b and ESE3-j (Figure 6). ESE3-a is the shortest transcript lacking exon 6 and encoding for a 32 kDa protein, ESE3-b has a molecular weight of 35 kDa, while ESE3-j is the longest one encoding a 37 kDa protein (Kas et al., 2000). ESE3-b is considered the most abundant in different cellular contexts (Sprater et al., 2012). Exons 2 and 3 encode the Pointed domain responsible for protein-protein interaction, while exons 8 and 9 encode the DNA-binding ETS domain.



**Figure 6. Exon composition of ESE3 transcript variants.** Exons are marked with numbers 1-9. The relevant domains and translational start (→) and stop (⊥) are marked. An alternative exon (1a) distinguishes ESE-j from ESE3-a and ESE3-b.

Various studies showed that ESE3 is widely expressed in human tissues with high epithelial content, in particular ESE3 expression is high in salivary gland, prostate and trachea, and low in colon, mammary gland, pancreas, lung and stomach (Tugores et al., 2001; Kas et al., 2000; Kleinbaum et al., 1999). Immunohistochemical analysis revealed that this ETS member is a nuclear protein expressed exclusively in differentiated epithelial cells and absent in most of the epithelial carcinomas examined (Tugores et al., 2001). Concerning the activity of ESE3, this transcription factor has been reported to act as an activator or a repressor depending on the target genes and the interaction with other transcriptional partner. For instance, Kas et al. showed that ESE3 induces the expression of genes involved in cell differentiation (Kas et al., 2000). On the other hand, Tugores et al. found that ESE3 acts as a transcriptional repressor of Ras-responsive genes involved in migration and cell interaction (Tugores et al., 2001). Based on these studies, ESE3 was defined an epithelial-specific factor, relevant for the maintenance of epithelial cell differentiation (Kas et al., 2000; Tugores et al., 2001).

Studies performed by our group revealed that ESE3 was highly expressed in normal prostate epithelial cells (LHS and RWPE1) and in cancer cells with low tumorigenic potential (LNCaP). By

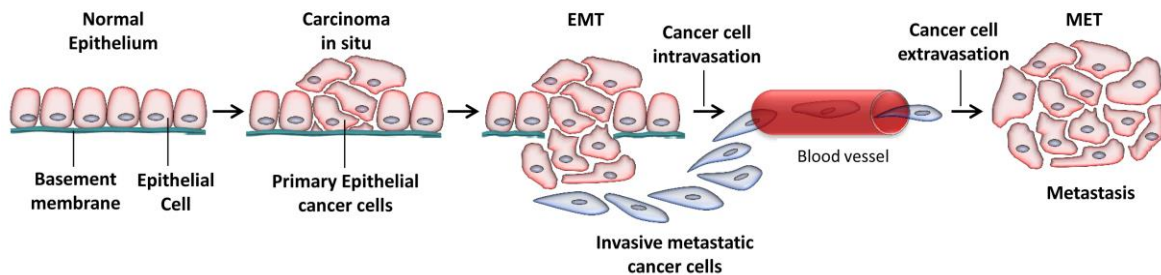
contrast, in metastatic prostate cancer cell lines (DU145 and PC3) ESE3 is epigenetically silenced (Cangemi et al., 2008). Thus, ESE3 silencing was inversely associated with the acquisition of a malignant transformed phenotype and with metastatic potential (Cangemi et al., 2008). Accordingly, it was shown that ESE3 acts as a tumor suppressor by playing an important role in the regulation of apoptosis (Cangemi et al., 2008). Consistent with a tumor suppressor function, ESE3 acts in opposite direction to the oncogene ERG in the regulation of EZH2 and NKX3.1, two key genes in prostate cell differentiation and tumorigenesis (Kunderfranco et al., 2010).

More recent findings demonstrate that ESE3 is a key factor determining the fate of prostate epithelial cells and that its loss determines epithelial-mesenchymal transition (EMT) and expansion of the cancer stem-like cell (CSC) compartment (Albino et al., 2012). Furthermore, gene expression profile studies in ESE3<sub>low</sub> tumors and ESE3kd cell lines showed that loss of ESE3 determines a broad reprogramming of the cell transcriptome. ESE3 controls the transcription of several genes associated with EMT, such as TWIST1, ZEB2, Vimentin and E-cadherin, and (CSC) markers, such as NANOG, POU5F1 and BMI1 (Albino et al., 2012). Consistently, ESE3kd cell lines show typical features of EMT, including migratory capability and elongated shape, as well as metastatic properties *in vivo*. In addition, loss of ESE3 confers sphere-forming potential to prostate epithelial cells (Albino et al., 2012). The capability to generate prostatespheres *in vitro* starting from single cells in anchorage-independent conditions has been widely used to enrich for stem-like cells in heterogeneous cell populations and assess their self-renewal potential (Dontu et al., 2003; Gupta et al., 2009). Indeed, prostatospheres-forming cells derived from ESE3kd cells generated tumors once injected subcutaneously in mice (Albino et al., 2012). On the other hand, re-expression of ESE3 in DU145 prostate cancer cells reduced the sphere-forming capability and *in vivo* tumor-growth (Albino et al., 2012). Moreover, our group found that ESE3 loss is associated with increased biochemical recurrence and reduced overall survival after prostatectomy (Kunderfranco et al., 2010; Albino et al., 2012). Collectively, these data point to a key role of ESE3 in the pathogenesis of prostate tumors by inducing mesenchymal, stem-like and tumor-initiating properties to prostate cells.

### **3. Epithelial-Mesenchymal Transition and cancer progression**

Cancer associated deaths are generally attributed to the spread of cancer cells to secondary organs by a process known as metastasis. Among other factors, the metastatic dissemination of cancer cells is attributed to the reactivation of an evolutionary conserved developmental program known as Epithelial-Mesenchymal Transition. EMT is an essential process that allows a fully differentiated and polarized epithelial cell, which normally interacts with basement membrane, to undergo multiple biochemical and morphological changes that enable it to assume a less

differentiated mesenchymal phenotype. EMT normally occurs as a physiological process during embryogenesis and organ development (Type 1 EMT) (Nakaya and Sheng , 2013). However, this program can also be activated during tissue regeneration and organ fibrosis associated with inflammatory processes (Type 2 EMT) or during cancer progression and metastasis (Type 3 EMT). Indeed, a growing body of evidence establishes the role of EMT in promoting cell migration, invasion and metastasis in multiple cancer types, including prostate cancer (Nauseef and Henry, 2011; Thiery et al., 2009). The expression of EMT-related molecules was found to be correlated with high grade tumors especially those with poor prognosis (Tsai and Yang, 2013). Moreover, various mouse studies and cell culture experiments have demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers (Ye and Weinberg, 2015). These cells are typically observed at the invasive front of primary tumors and are considered the cells that eventually enter into subsequent steps of the invasion-metastasis process, including intravasation, transport through the circulation, extravasation and ultimately metastatic colonization. The last step of metastasis formation probably requires a process of reversion to an epithelial phenotype called Mesenchymal to Epithelial Transition (MET). This plasticity allow the EMT-derived migratory cancer cells to establish secondary colonies at distant sites that resemble, at the histopathological level, the primary tumor from which they arose (Kalluri and Weinberg, 2009) (Figure 7).



**Figure 7. The role of EMT in cancer progression.** Progression from normal epithelium to invasive and metastatic carcinoma goes through several stages. In the first stage epithelial cells lose their polarity and detach from the basement membrane. In the next step cancer cells undergoing EMT acquire the ability to invade through the basal membrane, intravasate and extravasate at a remote site. The last step of metastasis formation requires a MET process and thus a reversion to an epithelial phenotype.

### ***EMT and acquisition of stem-like properties***

It has recently been proposed that cells undergoing EMT also display cancer stem-like cells (CSCs) features. CSCs constitute a distinct subset of tumor cells characterized by stem-like properties, unlimited self-renewal, high metastatic potential and resistance to therapy (Rybak et al., 2015; Sharpe et al., 2013; Singh and Settleman et al., 2010). In addition, CSCs are defined by

their ability to initiate tumors upon implanting in immunocompromised mice (Reya et al., 2001; Visvader and Lindeman, 2008) and thereby they are also termed tumor-initiating cells (TIC). This distinct population of cells was initially identified in leukemias (Bonnet and Dick, 1997) and more recently in many solid cancers, including PCa (Wang and Shen, 2011). Multiple markers for the identification and characterization of CSCs have been proposed, including cell surface proteins and markers of self-renewal and pluripotency (Sharpe et al., 2013). Regarding PCa, putative CSCs were isolated from different cancer cell lines, such as PC3, DU145 and LNCaP, based on the expression of the surface marker CD44 (Rybak et al., 2015). Indeed, several reports suggest that CD44<sup>high</sup> prostate cells display increased tumorigenicity *in vivo* (Rybak et al., 2015). Moreover, Hurt et al. proposed that CD44<sup>high</sup>/CD24<sup>low</sup> PCa cells are stem-like cells responsible for tumor initiation (Hurt et al., 2008). Embryonic stem cell genes, such as Nanog, Oct4, Sox2, represent others typical CSCs markers, consistently with the finding that these genes are overexpressed in poorly differentiated tumors (Ben-Porath et al., 2008; Kong et al., 2010). However, the mechanisms underlying the origin of CSCs within a tumor are still matter of debate. Different scenarios are likely to exist depending on the context (Singh and Settleman, 2010): while in some tumors CSCs can arise from existing stem progenitor cells following a malignant transformation, in others they may originate from terminally differentiated cells that acquire oncogenic alterations resulting in dedifferentiation to a primitive stem-like state. The compelling evidence relating EMT to the emergence of a cancer-stem like phenotype indicates a possible mechanism of EMT-induced formation of CSCs. Indeed, Mani et al. showed for the first time that immortalized human mammary epithelial cells undergoing EMT are also CSCs, as characterized by CD44<sup>high</sup>/CD24<sup>low</sup> phenotype, self-renewal and mammospheres-forming capacity *in vitro* and tumor-initiating ability *in vivo* (Mani et al., 2008). Consistently, the sphere-forming capability of breast CSCs is abrogated after EMT shut-down (Shimono et al., 2009). Moreover, signaling pathways that regulate EMT, such as TGF- $\beta$  and Notch1 pathways, also drive CSCs self-renewal and maintenance (Singh and Settleman, 2010). Therefore, the induction of an EMT program can result in cell dedifferentiation to a mesenchymal state and concomitantly in the expression of stem-cell markers (Mani et al., 2008). Consistently with this notion, emerging evidences suggest that the EMT program serve as a driver of drug resistance and disease recurrence in patients (Ye and Weinberg, 2015; Oliveras-Ferraros et al., 2012). For instance, the EMT process was shown to confer drug resistance against conventional chemotherapy, such as docetaxel and cisplatin (Puhr et al., 2012; Haslehurst et al., 2012), and the resistance of EMT tumor cells was related to CSCs features (Puhr et al., 2012). In addition, recent studies support a strong connection between EMT induction and enhanced tumorigenicity (Ye and Weinberg, 2015; Chaffer et al., 2013), indicating a more causal role of EMT in the development of tumor malignancy.

***Molecular mechanisms mediating EMT in PCa***

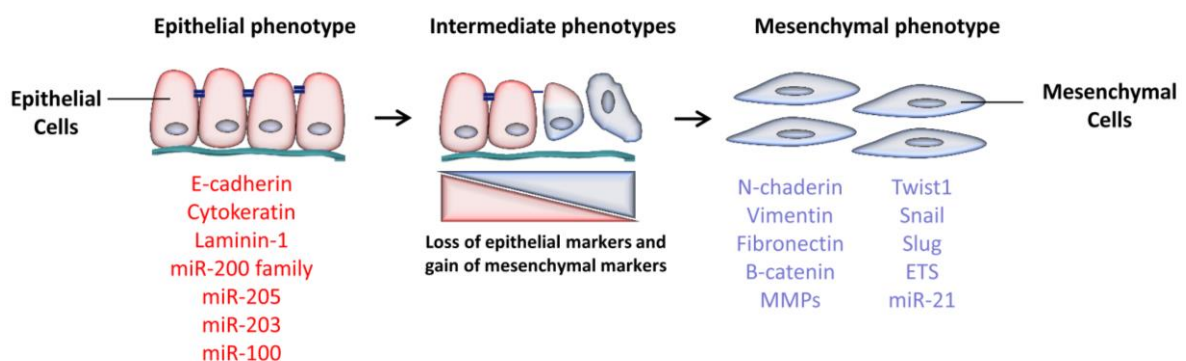
A number of distinct molecular events are engaged in order to initiate and complete the EMT process. These include activation of transcription factors, expression of specific cell-surface proteins, reorganization of cytoskeletal proteins, production of extracellular matrix (ECM)-degrading enzymes. All the components of the EMT molecular machinery are generally classified into three categories: *inducers*, *regulators* and *effectors*. Some of these molecules have also been used as markers of the EMT process in several tumor types, including PCa (Tsai and Yang, 2013; Banyard and Bielenberg, 2015; Sethi et al., 2010) (Figure 8).

*The EMT inducers* are usually stimuli provided by the tumor microenvironment, such as growth factors and cytokines. The most relevant growth factors known to contribute in EMT in prostate cancer are transforming growth factor beta (TGF- $\beta$ ), endothelial growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). TGF- $\beta$  is considered the most potent inducer of EMT and high levels of TGF- $\beta$  have been linked to invasiveness and metastasis in prostate cancer (Papageorgis, 2015). For instance, TGF- $\beta$  was shown to promote the expression of Slug, a well-known transcription factor involved in EMT in benign prostatic hyperplasia (Slabakova et al., 2011). Moreover, TGF- $\beta$  treatment can induce STAT3 activation and HIF1 $\alpha$  stabilization that in turn promote TWIST1 expression leading to morphological changes towards a mesenchymal phenotype and prostate cancer invasion (Cho et al., 2013; Cho et al., 2014). Other *EMT inducers* known to promote EMT in PCa are cytokines secreted in the cancer-associated stroma following inflammatory signals, like TNF- $\alpha$ , INF- $\gamma$ , IL6 and IL1 $\beta$  (López-Novoa and Nieto, 2009). In addition, other stimuli coming from the tumor microenvironment, such as hypoxia, can promote this process by inducing HIF1 $\alpha$  and VEGF (Philip et al., 2013; Nauseef and Henry, 2011). Recent reports also established that AR-related signaling act as a potent modulator of EMT in PCa by regulating ZEB2 expression (Jacob et al., 2014). However, the interplay between AR signaling and expression of EMT related transcription factors needs to be further explored.

*The EMT regulators* are mainly transcription factors that convert all these environmental stimuli into intracellular signals driving the changes associated with EMT. The main function of these EMT related transcription factors is to repress the expression of epithelial associated genes and induce mesenchymal genes (Puisieux et al., 2014). Indeed, most of these proteins, including Snail 1, Slug, Zeb 1/2 and Twist1, directly or indirectly repress the transcription of E-cadherin, a key gene involved in cell migration and invasion. Increased expression of Twist1 is particularly relevant in PCa, because it is indicative of high-grade tumors, malignant disease progression and resistance to anti-cancer therapies (Yuen et al., 2007). Smad proteins are also key signal transducers in TGF- $\beta$ -driven EMT. Activated Smad translocate into the nucleus and interact with

various transcription factors/coactivators to regulate the transcription of target genes (Papageorgis, 2015).

The combined actions of inducers and regulators lead to changes in expression or activity of a variety of genes, named *EMT effectors*, that finally carry out the phenotypical modifications typically linked to EMT, such as alterations in cell adhesion and morphology, acquisition of migratory and invasive capability, as well as increased cell survival. For instance, loss of E-cadherin and up-regulation of N-cadherin, typical markers of the EMT process, are associated with increased ability to migrate and invade in prostate cells. Importantly E-cadherin loss correlates with prostate tumor progression and Gleason grade, establishing this EMT-related protein as a prognostic factor for disease progression (Gravdal et al., 2007). Furthermore, the alterations in cytoskeletal intermediate filament proteins, such as vimentin and cytokeratins, act to shift the shape and behavior from stable interconnected cells to separate, spindle-shaped motile mesenchymal cells (Banyard and Bielenberg, 2015). The upregulation of many enzymes involved in the degradation of ECM, including collagenases MMP2 and MMP9, allow transformed cells to invade through the ECM and basement membrane and thus enter the blood vessels (Nistico et al., 2012).



**Figure 8. Epithelial and mesenchymal-related molecules commonly evaluated as markers of the EMT process in prostate cancer.** Red, epithelial markers. Blue, mesenchymal markers.

#### 4. microRNAs

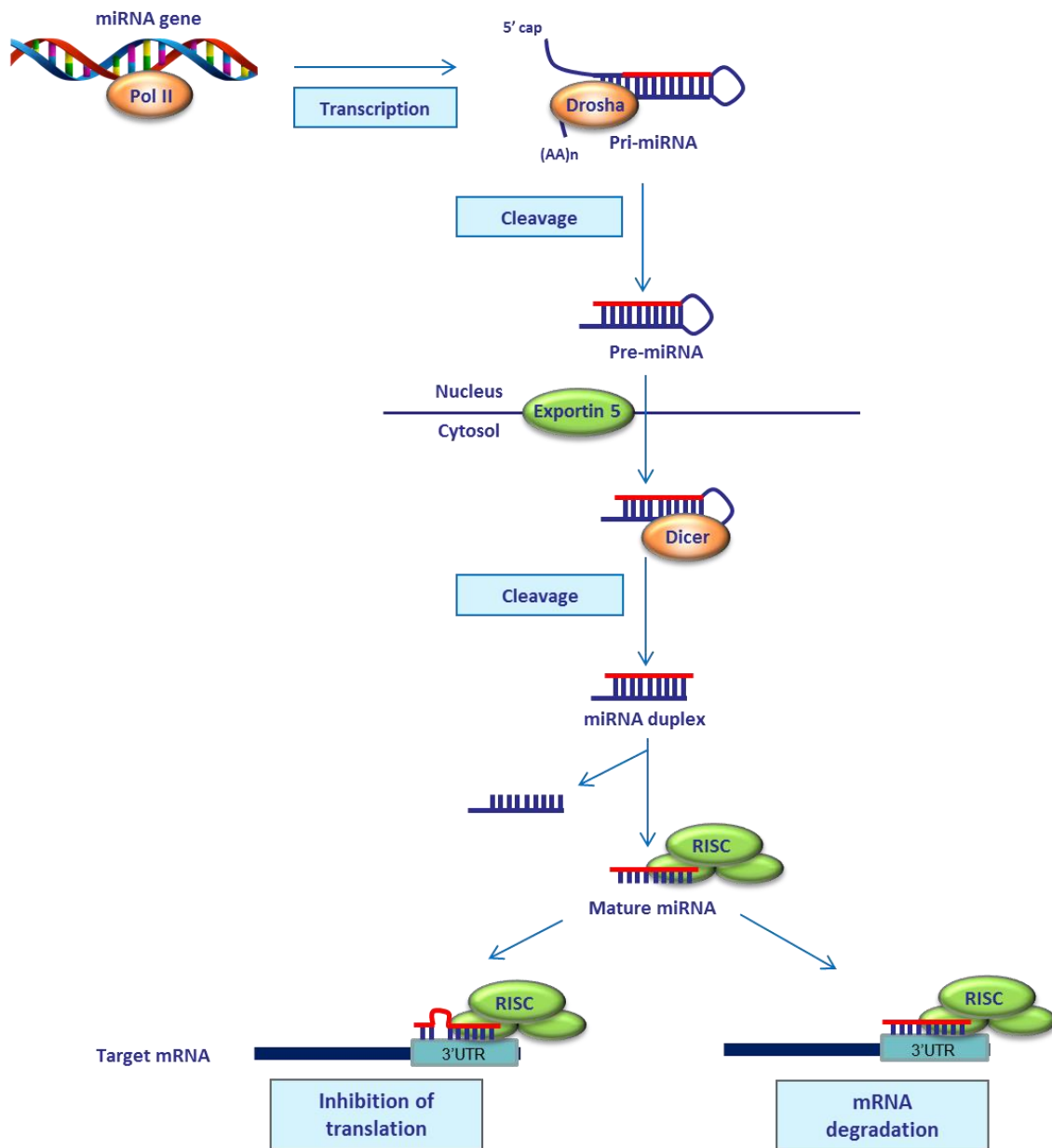
miRNAs are single-stranded non-coding RNAs, 19-25 nucleotides long, that regulate gene expression at the post-transcriptional level (Esquela-Kerscher and Slack, 2006; Filipowicz et al., 2008). miRNA genes are phylogenetically conserved and usually have multiple isoforms, which were probably generated by duplication events (Di Leva et al., 2014). Most of miRNA genes are located in intergenic regions, distant from other annotated genes, and thus represent single transcriptional units (Di Leva et al., 2014, Rodriguez et al., 2004). A minority of miRNAs is



considered intragenic, because they are located inside another gene and have the same transcriptional orientation. The host gene can be coding or non-coding, and the miRNA gene can be found both in intronic and exonic regions. More than 50% of miRNA genes are clustered together (e.g the miR-17/92 cluster) and normally transcribed as multicistronic RNA transcripts that enable their coordinate expression (Di Leva et al., 2014).

The biogenesis of mature miRNAs follows a multistep process that starts in the nucleus and ends in the cytoplasm (Figure 9). Most of miRNA genes are transcribed in the nucleus by RNA Polymerase II and retain mRNA features such as the 5'-cap structure and 3'-poly(A)-tail (Lee et al., 2004). Only a minor group of miRNAs associated with Alu repeats is transcribed by RNA Polymerase III (Di Leva et al., 2014). These first transcripts (pri-miRNAs) are several kilobases long and are characterized by a stem-loop structure that harbors the mature miRNA in the 5' or 3' half of the stem. Still in the nucleus, Drosha/DGCR8 heterodimer cleaves the pri-miRNA at the stem and releases a hairpin-structured precursor (pre-miRNA) of 60-100 nucleotides. The 3'-end of the pre-miRNA hairpin is recognized by Exportin-5 and its partner Ran-GTP, which enables its nuclear export. The pre-miRNA is then cleaved in the cytoplasm near the terminal loop by the RNase III enzyme Dicer, releasing the 19-25 nt miRNA duplex. Once produced, the miRNA duplex is bound by the RNA-Induced Silencing Complex (RISC), composed of the Transactivation-responsive RNA-Binding Protein (TRBP) and Argonaute2 (Ago2). While the active mature strand, named guide strand (or miRNA), is retained by the RISC complex, the other strand, named passenger strand (or miRNA\*), is removed and degraded (Thimmaiah et al., 2005; Diederichs and Haber, 2007). Subsequently, the mature miRNA guides the RISC complex to the targets mRNAs. miRNAs mainly bind to the 3'-untranslated region (UTR) of their target mRNAs. The specificity of the binding mainly depends on the complementarity between the so called "seed region" (2-8 nucleotides of 5'-end) of the miRNA and the target mRNA. Depending on the homology to the 3'-UTR target sequence, miRNAs can induce translational repression or degradation of mRNAs: a perfect complementarity leads to the degradation of target mRNAs, while an imperfect matching inhibits its translation into protein (Bartel, 2009; Djuranovic et al., 2011). Regardless of which of these two events occur, the result is usually a decrease in the amount of protein encoded by the target mRNA. However, recent studies have shown other "non-canonical" mechanisms of miRNA-mediated regulation of gene expression. Some miRNAs can bind to the open reading frame of the target mRNAs (Qin et al., 2010) or to the 5'-UTR region and in a few cases they have been shown to enhance gene expression (Orom et al., 2008). A completely different mechanism of action was also recently reported for some miRNAs that can bind to ribonucleoproteins interfering with their RNA binding functions (Eiring et al., 2010; Beitzinger et al., 2010).

Through these mechanisms each miRNA can usually regulate hundreds of target mRNAs (Bartel, 2004). Bioinformatic analysis for miRNA targets prediction shows that 60% of human messenger RNAs harbor miRNAs binding sites (Friedman et al., 2009). Moreover, multiple reports demonstrate that the 3'-UTR of a single gene is frequently bound by several miRNAs, suggesting that different miRNAs can cooperate in the regulation of gene expression (Ivanoska and Cleary, 2008). Therefore, these small non-coding RNAs are considered key regulators of gene expression. Nevertheless, the identified and validated miRNA targets represent only a small fraction of the predicted mRNAs. Thus, the identification of new targets is needed to improve our understanding of the mechanisms of miRNA-mediated gene regulation and the specific function of these non-coding RNAs. In addition, miRNAs can have distinct targets in different cell types and their biological effects likely depend on the sets of genes that are affected in the specific cell context (Bartel, 2009). Overall, it is now clear that miRNA-mediated modulation of gene expression represent a complex mechanism of regulation impacting several cellular processes, including apoptosis, proliferation, cell cycle, differentiation, stem cell maintenance and metabolism (Xu et al., 2004; Karp et al., 2005; Cheng et al., 2009; Garzon et al., 2010). Indeed, it is not surprising that in the last decade the role of aberrant miRNAs expression in the development of several human diseases has been thoroughly investigated and great progress has been particularly made in the identification of miRNAs involved in cancer onset and progression.



**Figure 9. Schematic view of miRNA biogenesis and target repression modalities.** miRNA genes are transcribed by RNA polymerase II (Pol II) into long primary miRNA transcripts (pri-miRNA) that are recognized and cleaved in the nucleus by the RNase III enzyme Drosha, resulting in a hairpin precursor called pre-miRNA. Pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 and is further processed by another RNase enzyme called Dicer that produces a transient 19–25 nucleotide duplex. Only one strand of the miRNA duplex, the mature miRNA, is incorporated into a large protein complex called RNA-induced silencing complex (RISC). Subsequently, the mature miRNA leads RISC to cleave target mRNAs or to inhibit their translation, depending on the degree of complementarity between miRNA and target.

***microRNAs in cancer***

Precise control of miRNA level is essential for maintaining normal cellular homeostasis (Di Leva et al., 2014). The initial indication that miRNAs play a role in cancer came from the observation that miR-15a/miR-16-1 cluster is deleted or downregulated in the majority of B-cell patients with chronic lymphocytic leukemia (CLL) (Calin et al., 2002). This miRNA cluster is located in the non-coding gene DLEU2 (deleted in lymphocytic leukemia 2). The chromosome region 13q14 that contains this gene was found to be heterozygously deleted in 65% of CLL, but also in other hematologic and solid tumors (Calin et al., 2002), suggesting a tumor-suppressive role for these miRNAs. Later on, it has been reported that more than half of miRNA genes is localized within minimal LOH or amplification regions or fragile sites (Calin et al., 2004). Starting from this discovery, several studies identified significant alterations of miRNA levels in tumor tissues compared with their normal counterparts and defined a role for miRNAs in the pathogenesis of cancer. The majority of tumors analyzed exhibited a specific miRNA signature that characterizes the malignant state and defines some of their clinic-pathological features (Calin et al., 2006). Indeed, recent studies showed that miRNA profile may discriminate between different tumors subgroups and predict outcome or response to therapy (Carmuta et al., 2010; Giovannetti et al., 2010; Acunzo et al., 2015)

Extensive analyses have highlighted the causal role of many miRNAs in cancer development and progression by using either human cancer cells or genetically engineered animal models. For example, transgenic expression of miR-155 (Costinean et al., 2006) or miR-21 (Medina et al., 2010) and deletion of miR-15/16 are sufficient to initiate tumorigenesis in mice (Klein et al., 2010; Di Leva et al., 2014). The identification of overexpression or downregulation of a specific miRNA in several tumor types further confirmed the relevance of these non-coding RNAs in the pathogenesis of the disease. One major example is miR-21, which is found to be overexpressed and plays an oncogenic role in colon, lung, breast, stomach, pancreas and prostate cancer (Garzon et al., 2009b). On the contrary, miR-29 displays a tumor suppressor role and is lost in both hematologic malignancies (e.g. CLL and AML) and solid tumors (e.g lung, breast and liver cancer) (Garzon et al., 2009a). Overall, aberrant expression of miRNAs is now considered a common feature of almost all human cancers. Deregulated miRNAs can be up- or down-regulated in malignant tissues compared to normal and can be considered oncogenes or tumor suppressors depending from their target genes.

Nevertheless, some miRNAs may have also different functions and thus act as either oncogenes or tumor suppressors depending on the cell type in which they are expressed. For example, miR-221 and miR-222 target the oncogene *KIT* and inhibit the growth of erythroblastic leukemia (Felli et al., 2005), thereby functioning as tumor suppressors in erythroblastic cells.

However, they also repress at least four important tumor suppressors, PTEN, p27, p57, and TIMP3, thereby functioning as oncogenic miRNAs in various human solid tumors (Garofalo et al., 2012). For these reasons, understanding the specific miRNA functions in different tissues and the mechanisms causing their aberrant expression is a relevant aspect for the development of new therapeutic strategies.

### ***microRNAs involved in EMT***

microRNAs (miRNAs) represent well-established component of the cellular signaling circuitry that regulates the EMT program. Indeed, miRNAs can control each of the molecules and pathways mediating EMT (Zaravinos, 2015). Some of the most studied miRNAs in this context include miRNA-200 family and miR-205, that inhibit the repressors of E-cadherin expression, ZEB1 and ZEB2, thereby helping in the maintenance of an epithelial phenotype (Korpala et al., 2008). miRNA-200 family is significantly down-regulated also during PCa progression (Watahiki, 2011; Fang, 2014) and act as tumor suppressor in prostate cancer cells (Kong et al., 2009). miRNA-203 and 205 were also found to prevent EMT in PCa (Khan et al., 2015). Recently, Gandellini et al demonstrated that miRNA-205 is down-regulated in PCa cells upon cancer-associated fibroblasts (CAF) stimulation, and forced expression of miR-205 reversed CAF-induced EMT (Gandellini et al., 2014). In the opposite direction acts miR-21, that is overexpressed in multiple tumors and promotes EMT and CSCs phenotypes by targeting several key genes, including PTEN (Zavadil et al., 2007; Han et al., 2012). In addition, miR-143/145 cluster is down-regulated in metastatic PCa and the expression of both miRNAs is known to reverse EMT in PC3 cells by repressing fibronectin and enhancing E-cadherin expression (Peng et al., 2011). Furthermore, several miRNAs can control the same EMT regulator, as it occur for the transcription factors Snail1 that was reported to be repressed by miR-30a (Kumarswamy et al., 2012), miR-34a (Siemens et al., 2011) and miR-203 (Moes et al., 2012) in different cancers. These findings highlight a pivotal role of miRNAs in the regulation of EMT. Based on this evidences, miRNAs are now considered important prognostic markers and potential therapeutic targets in EMT-associated PCa progression (Khan et al., 2015).

### ***Causes of miRNAs dysregulation in cancer***

In the past years several mechanisms responsible for dysregulation of miRNAs in cancer have been identified, including both genetic and epigenetic abnormalities (Figure 10).

Chromosomal rearrangements, genomic amplifications, deletions or mutations can alter miRNA genes, as much as they affect protein-coding genes. Deletion of miR-15a/16-1 cluster (Calin et al., 2002) and amplification of miR-17/92 cluster in lymphomas (Ota et al., 2004) represent the major examples of genetic alterations involving relevant miRNAs in hematologic

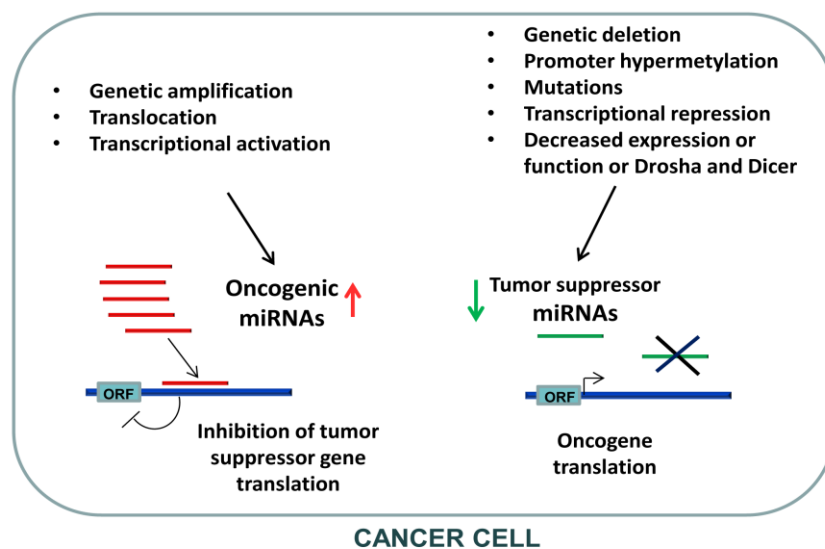
malignancies. Regarding solid tumors, miRNAs belonging to the let-7 family are located at genomic fragile sites and thus lost in a number of different tumors (Calin et al., 2004). Interestingly, they act as tumor suppressor miRNAs inhibiting the expression of relevant oncogenes, such as Ras, c-Myc (Chang et al., 2007) and Lin28 (Hagan et al., 2009). On the other hand, *miR-21*, the most commonly overexpressed miRNA, has been located in a genomic region frequently amplified in different types of tumor (Calin and Croce, 2007).

Epigenetic modifications represent another common mechanism associated with the alteration of miRNA expression in cancer (Lopez-Serra and Esteller, 2012; Fabbri et al., 2013). Indeed, several reports have shown that in cancer cells tumor suppressor miRNAs are frequently silenced by increased methylation of their promoter region, thereby allowing overexpression of the oncogenic targets. This is the case for miR-127, which is epigenetically repressed in 75% of primary prostate and bladder tumors due to DNA hyper-methylation of the promoter and histone acetylation. Re-activation of miR-127, following treatment with inhibitors of DNA-methylation and histone-acetylation, represses the expression of the proto-oncogene BCL6 (Saito et al., 2006).

Aberrant expression of miRNAs can also be caused by alterations in their transcriptional control. Indeed, dysregulation of the transcription factors regulating miRNAs expression during cancer progression will lead to aberrant activation or repression of specific miRNAs (Garzon et al., 2010; Di Leva et al., 2014). One of the first examples of cancer-associated transcriptional regulation of miRNA genes was the transcriptional induction of the miR-17/92 cluster mediated by the oncogene Myc (O'Donnell et al., 2005). Importantly, Myc was also involved in the regulation of let-7 and miR-29 families (Chang et al., 2007). Another important transcriptional program deregulated in cancer involves miRNAs controlled by the tumor-suppressor p53, which is frequently lost in tumor tissue. Transcriptionally induced by p53, miR-34 recapitulates the biological effects of p53 activation, including apoptosis and senescence, and its expression is decreased following loss of this tumor suppressor in ovarian tumors (Corney et al., 2007).

Moreover, many components of the miRNA biogenesis machinery could be either aberrantly expressed or mutated in tumors, contributing to dysregulation of miRNA expression or function (Di Leva et al., 2014). As described above, several enzymes and regulatory proteins are involved in the correct maturation of the primary miRNA precursor, therefore controlling miRNA expression at a post-transcriptional level. For instance, a number of evidence uncovered the altered expression or function of Drosha and Dicer, the major enzymes responsible for miRNA maturation, in human cancer. A decrease in Drosha and Dicer mRNA level has been identified in patients with ovarian cancer (Meritt et al., 2008). In 2009 Suzuki and colleagues demonstrated that p53 associates with Drosha, promoting Drosha-mediated processing of certain tumor-suppressor miRNA in response to DNA damage. Thus, loss of p53 in cancer reduces the levels of

miRNAs regulated by p53-induced Drosha processing (Suzuki et al., 2009). An interesting mechanism of deregulation in Dicer function that involves inactivating mutations of TRBP gene was identified in tumors with microsatellite instability. Loss of TRBP destabilizes Dicer leading to an impairment of miRNA processing and the enhancement of cellular transformation (Melo et al., 2009). The control of let-7 family members maturation by the RNA-binding protein LIN28 is another important post-transcriptional mechanism of miRNA regulation frequently altered in cancer (Wang et al., 2015). Several studies have determined that LIN28 protein can interact in the cytoplasm with pre-let-7 inhibiting its processing by Dicer and promoting its decay (Hagan et al., 2009). Indeed, overexpression of the oncogenic protein LIN28 in tumor cells causes down-regulation of let-7 contributing to malignant transformation and cancer progression (Liu et al., 2013).



**Figure 10. miRNA dysregulation in cancer.** The figure describes the main mechanisms causing increased expression of oncogenic miRNAs or reduced expression of tumor suppressor miRNAs in cancer cells.

### ***Roles of miRNAs during prostatic tumorigenesis and progression***

The miRNA biology of prostate cancer has been significantly understudied in the past years compared with other primary malignancies, despite evidence suggesting that dysregulation of miRNA expression is involved in both prostate cancer pathogenesis and treatment resistance (O’Kelly et al., 2012; Fang and Gao, 2014). Recently, the urgent need to find new diagnostic and prognostic biomarkers and to develop more powerful therapeutic strategies for prostate cancer management, led to a deeper investigation of miRNA aberrations involved in prostatic tumorigenesis. In 2005, Volinia et al. used for the first time a microarray platform to define the microRNA signature of six human solid tumors, including PCa (Volinia et al., 2006). In 2007 Porkka

et al. specifically carried out a miRNA expression profile on PCa samples leading to the identification of different miRNAs both up- or down-regulated in prostate cancer compared to normal tissue (Porkka et al., 2007). At present, several studies have been reported evaluating altered expression of miRNAs in PCa in order to identify tumor-specific miRNA signatures (Ambros et al., 2008; Lu et al., 2005; Ozen et al., 2008; Porkka et al., 2007; Szczyrba et al., 2010; Volinia et al., 2006). Despite the variability among the different results, some common signatures have been reported. Collectively, these studies confirmed deregulated expression of miRNAs in prostate cancers and suggest the potential of miRNA signatures to discriminate tumor from normal tissue.

However, still limited information is available on the differential expression of miRNAs within distinct prostate tumor subsets or at different stages of the disease progression (Fang and Gao, 2014). Some miRNAs correlated with biochemical recurrence within two years of surgery were identified by Tong et al. performing a paired analysis of 40 prostatectomy specimens (Tong et al., 2009). Spahn et al. assessed the global expression of miRNAs in benign prostatic hyperplasia and metastatic prostate cancer showing that miR-221 was down-regulated in tumor samples and that its level is a significant predictor of clinical recurrence (Spahn et al., 2010). Schaefer et al. evaluated the prognostic significance of miRNAs in radical prostatectomy specimens and found an association of high miR-96 expression with reduced recurrence-free survival (Schaefer et al., 2010). Low expression of miR-143 and miR-145 was related with development, metastatic progression and also Gleason score in PCa (Zaman et al., 2010). Starting from these observations, active research is ongoing with the attempt to identify tumor subtype-specific miRNAs and miRNAs profile that could be useful as markers for diagnostic and prognostic purposes in PCa management. The recent discovery of detectable and quantifiable amounts of miRNAs in numerous body fluids, such as plasma, serum and urine, has opened up new possibilities in this field. The presence of miRNAs in plasma and serum has been especially evaluated in the last years, providing evidence for its correlation with hematologic and solid tumors, including prostate cancer (Brase et al., 2010; Wittmann and Jack, 2010). As an example, serum levels of miR-141 could distinguish patients with prostate cancer from healthy subjects (Mitchell et al., 2008). In 2011, Yaman Agaoglu et al. tested the possible diagnostic utility of three specific PCa-associated miRNAs (Yaman Agaoglu et al., 2011). A combination of serum PSA and plasma expression levels of a miRNA signature including let-7c, miR-30, miR-141 and miR-375 has been proposed as potential better diagnostic marker for PCa (Kachakova et al., 2015). Indeed, circulating miRNAs could be used as early detection biomarkers of the disease in alternative to invasive tissue sampling (Acunzo et al., 2015; Fabris et al., 2016).

Despite the high number of miRNAs found to be aberrantly expressed in prostate tumors, the assessment of their specific role in prostate tumorigenesis is still incomplete. Some miRNAs that



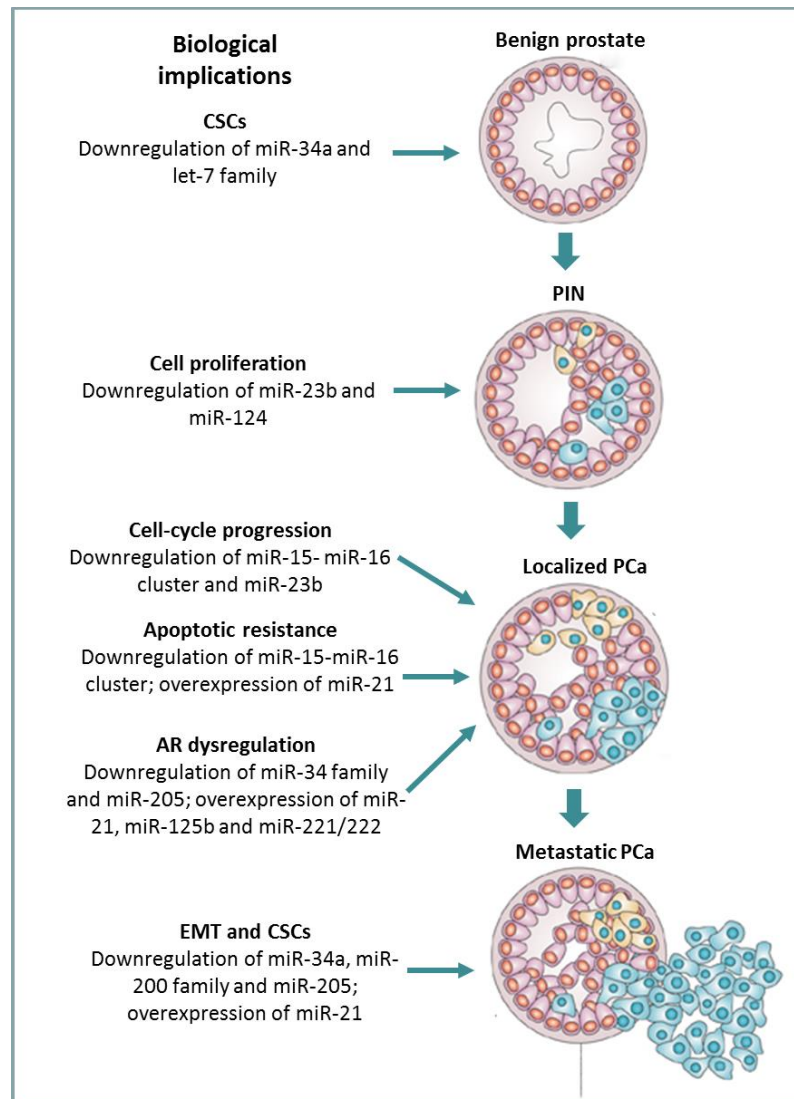
act as suppressors of PCa growth and metastasis (Liu et al., 2013; Nadiminty et al., 2012; Ozen et al., 2008) or on the contrary promote PCa tumorigenesis (Coppola et al., 2013; Hudson et al., 2013; Shi et al., 2011) have been identified (Table 2).

miRNA	Role in PCa	Relevant Targets	Altered function
Let-7 family	TS	RAS, LIN28, EZH2,	Apoptosis, proliferation
miR-125b	OG	BAK1, PUMA	Apoptosis, AR signaling
miR-143/145	TS	MYC KRAS, CD44, OCT4	Metastasis, apoptosis, CSC properties
miR-205	TS	E2F1, ZEB2 MAPK	Cell proliferation, EMT
miR-221/222	TS	p27Kip1	Cell cycle
miR-15/16	TS	BCL2, CCND1, CDK1/2, FGFR1	Cell cycle, apoptosis
miR-21	OG	PTEN, PDCD4, LATS2	Apoptosis, metastasis
miR-101	TS	EZH2	Metastasis
miR-203	TS	ZEB2, BMI1	EMT
miR-200 family	TS	ZEB1/2, Snail, Slug	EMT
miR-124	TS	AR	Proliferation
miR-34	TS	CD44, MYC, NOTCH1, AR	Proliferation, CSC properties

**Table 2. Summary of the most relevant miRNAs deregulated in PCa.** The identified targets and the related functions involved in prostate tumorigenesis are also shown. TS, tumor suppressor; OG, oncogenic

The role of these miRNAs has mainly been associated to resistance to apoptosis, cell proliferation, cell cycle progression and deregulation of AR pathway (Figure 11). Moreover, recent studies support the notion that specific miRNAs are involved in the acquisition of EMT and CSC features also during prostate tumor progression, suggesting a relevant impact of these small non-coding RNAs in metastatic spread, disease recurrence and treatment failure (Fang and Gao, 2014). The most relevant tumor suppressor and oncogenic miRNAs involved in PCa are better described in the paragraphs below. Overall, understanding the consequences of miRNAs dysregulation in

prostate cancer development and progression and investigating the ways to restore their normal expression or function could contribute to the development of novel therapeutic strategies.



**Figure 11. Biological functions affected by miRNAs dysregulated during prostate cancer progression.**

### ***miRNAs with tumor suppressor role in PCa***

The miR-15a and miR-16-1 are relevant examples of tumor suppressor miRNAs in many tumors, including PCa. Allelic loss of the miR-15a/miR-16-1 cluster on chromosome 13 is correlated with progression of prostate cancer from early stage to metastatic disease (Bonci et al., 2008). Antagomirs designed to specifically sequester and inhibit miR-15a and miR-16 activity resulted in increased proliferation, migration, and survival in non-tumorigenic prostate cells *in vitro* and *in vivo*. On the contrary, restoration of miR-15a and miR-16 expression resulted in growth arrest, apoptosis and regression of prostate tumor xenografts (Bonci et al., 2008). CCND1,

WNT3a, BCL2 (Bonci et al., 2008) and CDK1/2 (Takeshita et al., 2010) are validated targets of miR-15a and miR-16, indicating that this miRNA cluster contributes to prostate carcinogenesis by targeting multiple oncogenic pathways. Other miRNAs were shown to act as tumor suppressor in PCa due to their capability to regulate EMT and CSCs related genes. miR-205 is one the best-characterized tumor suppressor miRNA in this context. DNA hypermethylation of miR-205 leads to its silencing in prostate cancer cell lines and tissue (Hulf et al., 2013). As described above, miR-205 plays an important role in reducing cell migration and invasion by inactivating the EMT regulators ZEB1 and ZEB2 (Gregory et al., 2008; Gandellini et al., 2009). Both miR-205 and miR-200 family were reported to be down-regulated and to play a tumor suppressor role in the metastatic prostate cancer cell line PC3 (Tucci et al., 2012; Gandellini et al., 2012; Kong et al., 2009). In addition, reduced expression of miR-205 and miR-200c was associated to resistance to chemotherapy in prostate cancer (Puhr et al., 2012). In particular Puhr et al. showed that the development of docetaxel resistance mediated by the down-regulation of these miRNAs is characterized by the appearance of EMT and the induction of stem cell-like properties in resistant cells (Puhr et al., 2012). Another example in this context is miR-34a that inhibits CSCs and metastasis by directly repressing CD44 marker in different solid tumors, including prostate cancer (Liu et al., 2011). Down-regulation of the let-7 family is also involved in the acquisition of CSC features. In PCa cells, loss of let-7 is inversely correlated with expression of EZH2 and forced expression of let-7 results in reduced clonogenic capability and sphere formation (Kong et al., 2012).

### ***miRNAs with oncogenic role in PCa***

miR-21 is the best characterized oncogenic miRNA in several human cancers, including PCa. Different results demonstrate the function of miR-21 in the regulation of anti-apoptotic genes, such as PDCD4, in prostate cells (Programmed Cell Death 4) (Li T et al., 2009; Shi et al., 2010). In addition, miR-21 represses PTEN inducing HIF1 $\alpha$ , VEGF and AKT, which synergistically leads to tumor angiogenesis and progression (Liu et al., 2011).

Several oncogenic miRNAs have also been implicated in the development and progression of CRPC. Many studies have investigated the interaction between AR signaling and miRNAs demonstrating differences in miRNA expression between androgen-sensitive and castration-resistant tissues. miR-125b was found to be overexpressed in prostate cancer cell lines and to promote the growth of androgen-independent cells by suppressing BAK1 expression (Shi et al., 2007). Indeed, overexpression of miR-125b confers resistance to androgen withdrawal to prostate cancer cells and conversely inhibition of miR-125b sensitized cancer cells to different therapeutic intervention (Shi et al., 2007). Ribas et al. found that AR binds to the promoter of miR-21 and

inhibition of miR-21 can repress androgen-induced prostate cancer growth (Ribas et al., 2009). In addition, overexpression of miR-21 in mice leads to the evasion of castration-mediated growth arrest. (Yang et al., 2010). miR-221/miR-222 represent another example of miRNA frequently overexpressed in PCa. The aberrant elevation of miR-221/miR-222 is highly correlated with metastatic CRPC phenotypes. Several studies demonstrated that miR-221/miR-222 target the tumor suppressor p27(Kip1), leading to cell cycle progression from G1 to S phase, increased clonogenicity *in vitro* and enhanced tumorigenicity *in vivo* (Mercatelli N. et al., 2008; Galardi et al., 2007).

### ***The therapeutic potential of miRNAs***

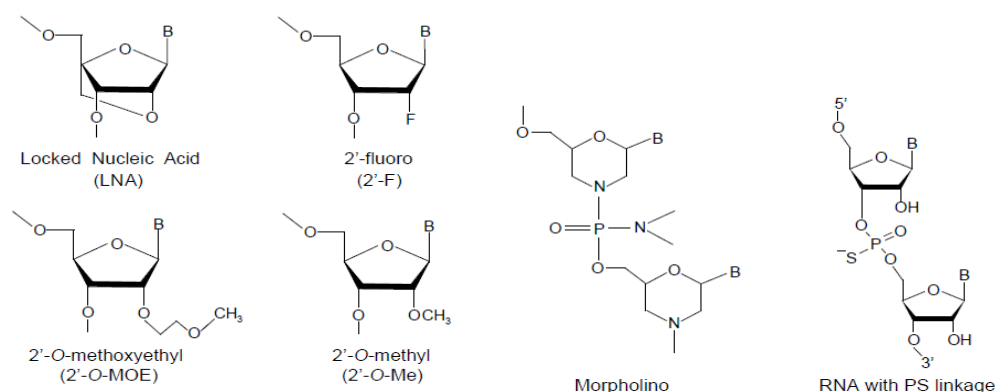
The increasing evidence that associate aberrant expression of miRNAs with the development of various types of cancers renders these molecules new potential therapeutic targets for the treatment of this disease. One of the most appealing properties of miRNAs as therapeutic agents is their ability to target multiple genes, frequently in the context of the same molecular pathway (Garzon et al., 2010). miRNAs can function as master coordinators of gene expression, efficiently regulating multiple cellular processes (Di Leva et al., 2014). Therefore, restoring cell homeostasis by modifying miRNA expression may be easier and more powerful than targeting individual genes or proteins.

There are two main strategies to target miRNA expression in cancer. Direct strategies involve the use of oligonucleotides to either substitute for the loss of tumor suppressor miRNAs or block the expression of oncogenic miRNAs. The indirect strategy involves the use of drugs to modulate miRNA expression by targeting their transcription and their processing.

An effective solution to restore the normal function or expression of a tumor suppressor miRNA is the introduction of synthetic molecules identical to the selected miRNA, known as miRNA mimics. miRNA mimics are small, chemically modified RNA duplexes that are processed into single-strand forms inside the cells and loaded into RISC to achieve the downstream inhibition of the target mRNAs (Shah and Calin, 2014). Several studies show that transfection of miRNA mimics with tumor suppressor function in cancer cells effectively induces cell death and blocks proliferation (Xiong et al., 2010; Bonci et al. 2008; Garzon et al., 2009a). However, the improvement of *in vivo* delivery system is needed to ensure the stability and uptake of miRNA mimics. Indeed, few reports of effective *in vivo* delivery of miRNA mimics are present so far. For example, intra-tumoral injections of miR-29 mimics can effectively decrease tumorigenicity in different murine models (Xiong et al., 2010; Garzon et al., 2009a). Systemic delivery of synthetic miR-16 was shown to inhibit the growth of metastatic prostate tumors (Takeshita et al., 2010).

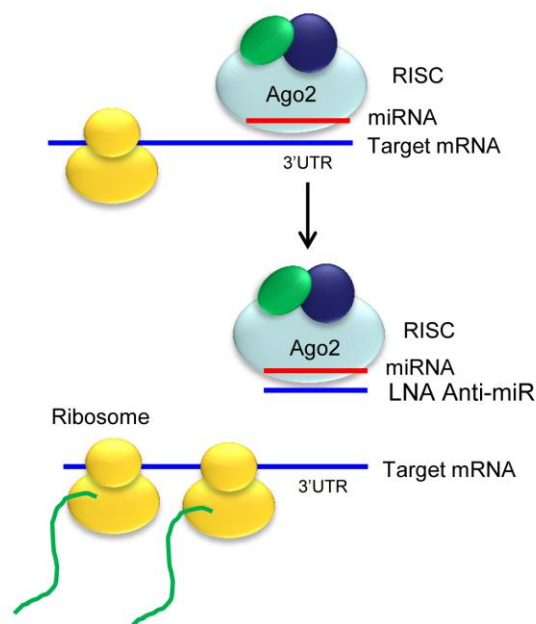
On the other hand, oncogenic microRNAs could be therapeutically targeted by repression of

their expression or inhibition of their function. The most used approach to inhibit miRNAs is the use of antisense oligonucleotides complementary to the mature miRNA. These molecules function as competitive inhibitors by annealing to the mature miRNA and preventing its interaction with target mRNAs. Subsequently, increased expression of the target can be achieved. The introduction of modifications to the chemical structure of these oligonucleotides was applied to increase stability, nuclease resistance, binding affinity and specificity. These chemically modified oligonucleotides targeting miRNAs are called anti-miRs. Anti-miRs are used to study loss of function effects of specific miRNAs *in vitro* and *in vivo* and have been developed as therapeutics during the last years. A variety of chemical modifications have been applied so far (Figure 11). First generation anti-miRs were complementary to full-length miRNA and included 2'-O-methyl-modified bases (Hutvagner et al., 2004; Krutzfeldt et al., 2005). The introduction of 2'-O-methyl groups contributes to nuclease resistance and improved binding affinities to RNA (Hutvagner et al., 2004). In 2005, Krutzfeldt et al. showed that these oligonucleotides can be conjugated to a cholesterol moiety in order to facilitate the tissue uptake and improve their pharmacokinetic (Krutzfeldt et al., 2005). Anti-miRs containing cholesterol, conjugated via a 2'-O-methyl (2'-OMe) linkage, are known as antagomiRs. For instance, following injection of antagomiRs complementary to miR-122 into the tail vein of mice, a specific targeting of miR-122 was observed in the liver after 24 hours and the silencing achieved was efficient and long lasting, since the effects were still observed 23 days after injection (Krutzfeldt et al., 2005). In addition, nuclease resistance of anti-miRs was improved by backbone modification with phosphorothioate (PS) linkages, or by using morpholino oligomers (Figure 12). Phosphorothioate linkages also promote plasma protein binding, thereby reducing the clearance of antimiRs by glomerular filtration and urinary excretion, which facilitates tissue delivery of antimiRs *in vivo* (Krutzfeldt et al., 2005). Furthermore, the miRNA antagonist chemistries currently in development use unconjugated phosphorothioate antisense molecules with various additional 2'-sugar modifications, such as 2'-O-methoxyethyl (2-MOE), or Locked-nucleic acid (LNA) nucleosides (Figure 12).



**Figure 12. Structures of the most commonly used chemical modifications in anti-miR oligonucleotides.** Locked nucleic acid (LNA) is a bicyclic RNA analogue in which the ribose is locked in a C3'-end conformation by introduction of a 2'-O,4'-C methylene bridge. The 2'-fluoro (2'-F), 2'-O-methoxyethyl (2'-MOE) and 2'-O-methyl (2'-O-Me) nucleotides are modified at the 2' position of the ribose moiety, whereas a six-membered morpholine ring replaces the sugar moiety in morpholino oligomers. In the phosphorothioate (PS) linkage, sulfur replaces one of the non-bridging oxygen atoms in the phosphate group. Modified from Stenvang et al., 2012.

LNAs anti-miR are a class of nucleic acid analogues in which the ribose ring is “locked” by a methylene bridge connecting the 2'-O atom and the 4'-C atom. These oligonucleotides are particularly interesting since they are characterized by unprecedented hybridization affinity toward complementary single-stranded RNA and high stability. In addition, they display excellent mismatch discrimination and high aqueous solubility (Garzon et al., 2010). LNA anti-miRs have been used successfully in several *in vitro* and *in vivo* studies to block specific miRNAs and are the compounds that have already reached the clinic. In 2008 Elmén and colleagues showed that the simple systemic delivery of an unconjugated LNA anti-miR (SPC3649 or Miravirsén) effectively antagonizes the liver-expressed miR-122 in non-human primates (Elmén et al., 2008a). The same group investigated the potential of miR-122 antagonism by SPC3649 as a new anti-HCV therapy in a chimpanzee model of chronic infection without any evidence of LNA-associated toxicities or histopathological changes (Elmén et al., 2008b). Very recently, a phase 2 clinical study demonstrated the efficacy and safety of Miravirsén in treating this viral disease following five weekly subcutaneous injections in patients with chronic HCV. This compound sequesters miR-122 in a highly stable heteroduplex preventing its interaction with HCV RNA and the replication of the virus (Janssen et al., 2013). Therefore, Miravirsén could be the first miRNA-based therapeutic brought to the market. In 2014 Hogan et al. clarified the mechanism of action of LNA anti-miRs suggesting that they specifically associate with target miRNAs in Argonaute complex via the “seed region” (Figure 13). Binding of anti-miR to the complementary miRNA prevents the interaction with target mRNAs, leading to subsequent stabilization and thus increased expression of the targeted mRNAs (Hogan et al., 2014).



**Figure 13. Model of LNA anti-miR mechanism of action.** miRNAs loaded onto Argonaute (Ago2), guide the RISC complex to target mRNA transcripts. The anti-miR specifically associates with Argonaute-bound target miRNA. As a consequence the miRNA-Argonaute complex can no longer bind and regulate target mRNAs. The mRNA targets are stabilized and can now be translated.

Although chemical modifications have improved multiple aspects of these molecules, several challenges remain for microRNA-based therapies, including issues of delivery, potential off-target effects and safety (Rotschild, 2014). The major obstacles for the use of miRNA therapeutics *in vivo* relates to tissue-specific delivery and cellular up-take of sufficient amounts of synthetic oligonucleotides to achieve sustained target inhibition (Garzon et al., 2010). The recent clinical progress achieved with therapeutic small interfering RNAs (siRNAs) using lipid-, liposomal- and polymer-based delivery technologies (Aagaard and Rossi, 2007) could be applied with success also for clinical development of miRNA therapeutics. Indeed, new delivery methods for miRNA mimics have been recently developed using these technologies. For instance, systemic delivery of neutral lipid-based vehicle for miR-34a and let-7 mimics reduced tumor burden in KRAS-activated non-small cell lung cancer mouse model (Trang et al., 2011). In addition, the first miRNA replacement therapy, performed using an intravenously injected liposome-based miR-34 mimic (MRX34), is currently in Phase I clinical trials for patients with advanced hepatocellular carcinoma (Bader, 2012).

Given that miRNAs concomitantly regulate many genes, the potential off-target effects of miRNA therapeutics represent another major concern. Therefore, there is the need to find effective systems that ensure the delivery of miRNA therapeutics specifically to the diseased tissue of interest and only to cancer cells. This problem could be solved by incorporating miRNA

mimics or anti-miRs into liposome-nanoparticles binding tumor-specific ligands, which can be directed to tumor cells via active targeting (Zhao et al., 2009). For example, nanocarriers based on hyaluronic acid could be used taking advantage of the binding between hyaluronic and the cancer stem cell marker CD44, which is overexpressed in various tumor cells (Orian-Rousseau et al., 2010). The development of miR-15a/miR-16 oligonucleotide nanoparticles coated with CD20-specific antibodies was also proposed as a potential strategy to target lymphocytic leukemia cells and overcome off-targets effect in hematologic malignancies (Garzon et al., 2010).

Overall, the application of miRNA-based therapeutics to reprogram miRNAs networks in cancer constitutes a reasonable and evidence-based strategy with a strong potential for success (Garzon et al., 2010). Not surprisingly, miRNA-targeting therapies are an area of increasing interest for pharmaceutical companies and many compounds are now in preclinical or clinical development (Table 3).

MicroRNA	Oligonucleotide format	Indications	Companies	Developmental stage
miR-122	LNA-modified antisense inhibitor	HCV infection	Santaris Pharma	Phase II
miR-122	GalNAc-conjugated antisense inhibitor	HCV infection	Regulus Therapeutics	Phase I
miR-34	miRNA mimic replacement	Liver cancer or metastasized cancer involving liver	miRNA Therapeutics	Phase I
Let-7	miRNA mimic replacement	Cancer (details undisclosed)	miRNA Therapeutics	Preclinical
miR-21	2'-F and 2'-MOE bicyclic sugar modified antisense inhibitor	Cancer, fibrosis	Regulus Therapeutics	Preclinical
miR-208	Antisense inhibitor	Heart failure, cardiometabolic disease	miRagen/Servier	Preclinical
miR-195 (miR-15 family)	Antisense inhibitor	Post-myocardial infarction remodelling	miRagen/Servier	Preclinical
miR-221	Antisense inhibitor	Hepatocellular carcinoma	Regulus Therapeutics	Preclinical
miR-103/105	Antisense inhibitor	Insulin resistance	Regulus Therapeutics	Preclinical
miR-10b	Antisense inhibitor	Glioblastoma	Regulus Therapeutics	Preclinical

**Table 3. Selected miRNA-based therapeutics currently in development.** 2'-F, 2'-fluoro; 2'-MOE, 2'-O-methoxyethyl; GalNAc, N-acetylgalactosamine. Modified from Li and Rana 2014.



## ***Aim of the work***

Cancer of the prostate is a leading cause of cancer death in developed countries. Clinical behavior of prostate cancer ranges from indolent disease to very aggressive tumors that rapidly progress to castration-resistance. However, the molecular mechanisms contributing to this heterogeneity are still poorly understood. Epithelial-mesenchymal transition (EMT) and acquisition of cancer stem-like cells (CSCs) properties within the primary tumors are important processes that contribute to metastasis, treatment failure and aggressive clinical behavior. Deregulation of endogenous ETS transcription factors, like ESE3/EHF, has been shown to play a relevant role in prostate tumorigenesis. Indeed, our group previously reported that in ETS translocation negative primary prostate tumors reduced expression of ESE3/EHF was associated with increased expression of mesenchymal and stem cell-related genes as well as clinical features of aggressive disease (Albino et al., 2012). Defining the mechanisms leading to dedifferentiation and acquisition of stem-like properties in this context could provide relevant insights for the management of prostate cancer. A critical role of microRNAs (miRNAs) in the regulation of EMT and stemness has been established in the last years (Wang et al., 2010). miRNAs are naturally occurring small non-coding RNAs that regulate gene expression at a post-transcriptional level. Deregulated expression of miRNAs is very frequent in human cancers, including prostate cancer. Indeed, aberrant miRNA expression has been correlated with tumor development and cancer progression (Bonci et al., 2008; Klein et al., 2010; Di Leva et al., 2014). However, little is known about prostate cancer progression specific miRNAs and their association with tumor aggressiveness. Moreover, the development of miRNA-based therapeutics has emerged as a potential strategy for cancer treatment (Soifer et al., 2007; Garzon et al., 2010; Takeshita et al., 2010; Cheng et al., 2014).

The overall goal of the studies described in this thesis work was to understand mechanisms leading to dedifferentiation and CSCs properties in prostate tumors and the role of microRNAs in these processes. In particular our final aim was to identify miRNAs relevant to prostate cancer progression and associated with aggressive tumor subgroups. Dissecting the role of microRNAs in prostate tumorigenesis, using both *in vitro* and *in vivo* approaches, could provide the rational for the development of novel therapeutic strategies and in addition may have diagnostic and prognostic applications.

## ***Materials and Methods***

***In vitro studies******Cell cultures***

LNCaP and DU145 were obtained from ATCC and maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum. Immortalized prostate epithelial cells LHS were maintained in PrEC growth medium (PrEGM; Cambrex, Lonza Group Ltd.), and RWPE1 in Keratinocyte serum-free growth medium (KSF; Gibco) with specific supplements (Albino et al., 2012) (Table 4). Cell lines with stable ESE3 knockdown (ESE3kd) were established and maintained in presence of 400 µg/ml G418 as previously described (Albino et al., 2012). LNCaP stably expressing miR-424 (LNCaP-424) and empty vector (EV) as control (LNCaP-EV) were established by infection with the pCDH lentiviral vector containing both GFP and a puromycin-resistant gene (vector kindly provided by Dr. Soleimani, Tehran, Iran) (Rahimian et al., 2011). HEK293T cell line was used for lentiviral amplification and viruses were harvested at 48 and 72 h after transfection, filtered and used for infecting cells in presence of 5 µg/ml polybrene (Sigma). After drug selection with puromycin (0.5µg/ml), resistant LNCaP-424 and control cells were subsequently infected with the pMMP-LucNeo retrovirus and selected with 2 mg/ml of G418 (Gibco). Monoclonal cell lines (LNCaP-424mono) were obtained by serial dilution in a 96-well plate. Clones were selected based on highest GFP expression measured by FACS and miR-424 expression levels by qRT-PCR. For *in vivo* experiments DU145-Luc were established by infecting cells with a constitutively expressing luciferase vector (pMMP-LucNeo retrovirus). LucNeo-expressing cells were selected with 2 mg/ml G418 (Gibco) as described above.

Name	Origin	Tumorigenic	Comments
RWPE1	Human prostate normal epithelial cells	No	AR positive Androgen sensitive
LHS	Human prostate normal epithelial cells	No	AR positive Androgen sensitive
LNCaP	Human prostate carcinoma derived from left supraclavicular lymph node metastasis	Yes	AR positive Androgen sensitive ETV1 translocation positive
DU145	Human prostate carcinoma derived from brain metastasis	Yes	Androgen independent ESE3 silenced by promoter methylation

**Table 4. Summary of the main characteristics of the cell lines used in the study.**

***miRNA precursor, antagomiR and siRNAs***

For miR-424 overexpression, RWPE1 and LNCaP cells were transiently transfected with 30 nM of miR-424 precursor (miR-424; AM17100-PM10306, Ambion) or negative control #1 (Ctr; AM17110, Ambion). For miRNA inhibition, DU145 and ESE3kd cells were transiently transfected with 40 nM of a specific LNA anti-miR (Anti-424; Mercury LNA Power Inhibitor, Exiqon) or a Scrambled control (Scr; Negative Control A, Exiqon). For RFWD2/COP1 knockdown cells were transfected with 50 nM of two distinct COP1-directed siRNAs (siCOP1#1 and siCOP1#2, Ambion) and siRNA directed to luciferase gene (siGL3, Ambion) or Negative Control#1 siRNA (n.4611, Ambion) as controls. For STAT3 knockdown, cells were transfected with 50 nM of STAT3 directed siRNA (siSTAT3, Ambion). All transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. *In vitro* cellular assays were performed 24 h following the indicated transfection. Unless otherwise specified, cell lysates were prepared 72 h after miRNA precursor or anti-miR transfection and 48 h after siRNAs transfection.

***Expression vectors, reporter constructs and luciferase assays***

FLAG-tagged COP1 wild-type (pCOP1) and RING (pRING) overexpressing plasmid (kindly provided by Christoph Marine)(Migliorini et al., 2011) were transfected using jetPRIME® (Polyplus) according to manufacturer's protocol. Overexpression of ESE3/EHF in DU145 cells was performed as previously described (Albino et al., 2012). Control cells were transfected with pcDNA3.1 empty vector (EV). Full length wild type EGFP-STAT3 was provided by Nadya I. Tarasova (Georgetown University, Washington, DC, USA). The EGFP-STAT3 Y705F mutant construct was previously generated by site-directed mutagenesis using GENEART® Site-Directed mutagenesis (Invitrogen). The COP1 3'UTR reporter construct was generated by cloning the 3'UTR region of the gene downstream to the luciferase reporter gene in the pMIR-REPORT vector (Life Technologies). The specific region was amplified by PCR from human genomic DNA extracted from LNCaP (primers: COP1-SacI FW: 5'-GAGCTCAGTCAAATTGTAAGTCTGCTG-3', COP-HindIII REV: 5'-AAGCTTCAAGCTC AACTGTGGCTCAA-3'), cloned in pGEM-T Easy vector and then subcloned in pMIR-REPORT into the SacI and HindIII sites. Point mutations were introduced in the putative binding site of COP1 reporter using GENEART Site Directed Mutagenesis System (Invitrogen) to generate COP1-MUT using the following primers: COP1 FWD 5'-TGTAAGTCCATGGA CATTAAATCTTGGTGGTGTATC-3', COP1 REV 5'-GATAACACCAAGATTATTAATGT CCATGGAGTTACA-3'. For STAT3 activity we used the STAT3 responsive luciferase reporter (pGL4.32/luc2P/STAT3-RE/Hygro from Promega, ID CS154201). Luciferase reporter assays were performed using Dual-Glo Luciferase kit (Promega) as previously described (Albino et al., 2012).

Renilla pRLSV40 (Promega) was used as control to monitor transfection efficiency. Results were expressed as Relative Luciferase Activity (RLA) normalized for Renilla luciferase activity and represented as percentage to the indicated control. Luciferase assays were performed in triplicate and repeated in three independent experiments.

### **Cellular Assays**

All the *in vitro* cellular assays were performed on cells transiently or stably transfected with the indicated molecule (miR-424, Anti-424, siCOP1 or pCOP1) compared to cells transfected with an irrelevant molecule used as control (Ctr, Scr, EV, siGL3 or NC).

#### **Colony formation in soft agar (or Soft agar assay)**

Growth in anchorage-independent condition was assessed by colony formation in soft agar as previously described (Albino et al., 2012). Cells were plated ( $2 \times 10^4$  cells per dish) in low-grade agar (1,8%) then incubated at 37°C. After 14-20 days, colonies were fixed and stained with 0,01% crystal violet in 20% ethanol and counted (larger than 100  $\mu$ m in size). Each experiment was carried out in triplicate and repeated three times. One representative experiment is shown.

#### **Cell migration and invasion assays**

Cell migration was assessed using scratch/wound-healing assay as previously described (Albino et al., 2012). Cells were grown to confluence in a 12-well plate and serum-starved overnight before introducing a scratch in the cell monolayer. Following the scratch pictures of cell migration were taken at different time points (0-24-48-72-96 h) until the closure of the wound using a Zeiss microscope with a Canon EOS 450D camera (100X magnification). Differences in wound width are shown as percentage relative to time 0 (0 h).

Cell motility and invasion were further evaluated in Boyden Chamber assays using 6.5 mm transwell chambers with 8.0  $\mu$ m pore polycarbonate membrane (Corning Incorporated, n.3422) coated with rat tail Collagen Type I Solution (60  $\mu$ g/mL, Sigma). Briefly, following transfection with the miR-424 precursor or miR-424 inhibitor and relative controls, cells were incubated for 24 h in RPMI without serum (DU145) or in KSF without supplements (RWPE1). Then cells were plated ( $5 \times 10^5$  cells/well in 24 well plates) on the top chamber of transwells in the medium described above with the addition of 0.1% BSA. RPMI with 10% FBS or KSF with supplements was added to the lower chamber and cells were incubated at 37°C. After 20 h (DU145) or 24 h (RWPE1) of incubation, non-migrated cells were carefully removed from the top of each insert with a cotton swab. Migrated cells were fixed in 4% formaldehyde at room temperature for 15 min and stained with 0.1% crystal violet. The number of cells in five random fields (200x magnification) was counted for each chamber. The stained cells were dissolved in 10% acetic acid and absorbance (proportional to the number of cells) was measured at 590 nm. The migrated cells were

represented as percentage of absorbance value relative to the control (Scr or Ctr). Representative images of invading cells stained with crystal violet were taken. The assay was done in triplicate and repeated in two independent experiments.

#### **Cell proliferation assay**

For cell proliferation assay, cells were seeded in 12-well plates, collected and counted at the indicated time points using an automated cell counter (Beckman Coulter Counter). Each experiment was carried out in triplicate and repeated twice.

#### **In vitro and ex-vivo Sphere formation assays**

For sphere-forming assay (SFA) single cell suspensions were plated in poly-HEMA treated dishes in serum-free Mammary Epithelial Basal Medium (MEBM, Cambrex) supplemented with specific supplements (B27, FGF, EGF, Insulin) for the selection of CSCs as previously described (Albino et al., 2012). Prostate-spheres  $\geq 100 \mu\text{m}$  in diameter were counted after 7 days and the sphere-forming efficiency (SFE) was evaluated as previously described (Albino et al., 2012). Representative pictures were taken using Zeiss Microscope with Canon EOS 450D. Each experiment was carried out in triplicate. Representative results are shown. For ex vivo SFA, xenografts tissues were chopped and incubated with Collagenase-Dispase mix solution (1:2) in Hanks Buffer for 3 h at 37°C, passed through a 40  $\mu\text{m}$  filter and single cells were counted by hemocytometer. Single cell suspensions were used to perform ex vivo SFA ( $8 \times 10^3$  cells/mL).

#### **Flow cytometry**

Cell cycle analysis was performed by staining with 7-AAD (25 mg/ml, Sigma-Aldrich). Samples were analyzed with a FACS Fortessa (BD Biosciences) and Flowjo software as previously described (Civenni et al., 2013).

#### **RNA extraction, quantitative real-time PCR and miRNAs expression analysis**

Total RNA extraction was performed using Trizol® (Invitrogen) and subsequently by Direct-zol RNA Mini-prep kit (Zymo Research) that allows the recovery of small RNAs. Quantitative real-time RT-PCR (qRT-PCR) was carried out using 20ng of RNA as template for SYBR Green Fast One Step kit (Quiagen). Primers sets used for qRT-PCR (designed using primer3, <http://frodo.wi.mit.edu/primer3/>) are reported in Table 5. The expression of each gene was normalized to  $\beta$ -actin. For miRNA expression analysis, 400ng of purified RNA were retro-transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem) with specific primers (TaqMan® MicroRNA Assays ID:4427975-000604, Applied Biosystem) and the cDNA was subjected to TaqMan Probe-based Real Time PCR (TaqMan® Universal PCR Master Mix, Applied

Biosystem). Each samples was analyzed in triplicate. The expression was normalized to RNU6 (Control miRNA assay, ID: 4427975-001093, Applied Biosystem). Relative expression was quantified using 2- $\Delta$ Ct method. Fold change compared to the indicated control (Ctr, Scr, EV) was measured using 2- $\Delta\Delta$ Ct method.

GENE	SEQUENCE
<i>COP1 FWD</i>	5'-ACGACCTTTAGCCACATTGT-3'
<i>COP1 REV</i>	5'- TAACTCCAGCAATCGCAAAA-3'
<i>ACTIN FWD</i>	5'-ATTGGCAATGAGCGGTTC-3'
<i>ACTIN REV</i>	5'-GGATGCCACAGGACTCCAT-3'
<i>MYC-C FWD</i>	5'-GGTGCTCCATGAGGAGACA-3'
<i>MYC-C REV</i>	5'-CCTGCCTCTTTTCCACAGAA-3'
<i>STAT3 FWD</i>	5'-GGAGGAGTTGCAGCAAAAAG-3'
<i>STAT3 REV</i>	5'-GATTCTCTCCTCCAGCATCG-3'
<i>IL6 FWD</i>	5'-CCACACAGACAGCCACTCAC-3'
<i>IL6 REV</i>	5'-TTTCAGCCATCTTTGGAAGG-3'
<i>DDIT4 FWD</i>	5'-GGTTCGCACACCCATTCAAG-3'
<i>DDIT4 REV</i>	5'-TAGGCATGGTGAGGACAGAC-3'
<i>NANOG FWD</i>	5'-CAGTCTGGACACTGGCTGAA-3'
<i>NANOG REV</i>	5'-CTCGCTGATTAGGCTCCAAC-3'
<i>POU5F1 FWD</i>	5'-AGCGATCAAGCAGCGACTAT-3'
<i>POU5F1 REV</i>	5'-TAGCCTGGGGTACCAAAATG-3'
<i>SOX2 FWD</i>	5'-AACCCCAAGATGCACAACCTC-3'
<i>SOX2 REV</i>	5'-GCTTAGCCTCGTCGATGAAC-3'

**Table 5. Primer sets used for qRT-PCR.**

### ***miRNA target prediction***

miR-424 targets prediction was performed using *miRWalk* (<http://mirwalk.uni-hd.de/>) with a comparative analysis of different prediction programs. We selected targets predicted by at least five of the eight prediction tools included in *miRWalk*. miR-424 binding site on COP1 3'UTR was identified by six out of the eight programs (miRWalk, miRanda, Targetscan, Diana, Pictar4, PITA) with a 8-bp perfect match between the “seed region” and the mRNA target sequence. The miR-424/COP1 binding site was further confirmed by *RNAhybrid* tool, which takes in to account the minimum free energy of hybridization between the two RNA molecules (Kruger and Rehmsmeier, 2006).



**Chromatin immunoprecipitation (ChIP)**

Computational search for ETS binding sites on miR-424 promoter was performed using *MotifViz* (biowulf.bu.edu/MotifViz). For ChIP experiments cells were exposed to formaldehyde to cross-link protein-DNA complexes and processed as previously described (Albino et al., 2012; Kunderfranco et al., 2010). Chromatin was immunoprecipitated with antibodies anti-ESE3 (Clone 5A5.5, Lab Vision, Fremont, CA USA), anti-H3K27me3 (Millipore), anti-H3Ac (Millipore), anti-H3K9me2 (Millipore) and IgG antibody (Millipore) as control. Samples were analyzed by qPCR performed with SYBR® Green PCR Master Mix (Applied Biosystems) using primers for the miR-424 promoter: FWD 5'-GGACGAAGGCATATGCAAAA-3', REV 5'-TCTGAACTGCAATGTGTTTGG-3'. The amount of immunoprecipitated DNA was calculated in reference to a standard curve and normalized to input DNA (Cangemi et al., 2008). In addition to the selected region of miR-424 promoter, the analysis of TWIST1 promoter including an ETS binding site (FWD 5'-GAATTTCTGCCGCATTCTCT-3'; REV 5'-GCCGCTGCCTTACTGTAAAA-3') and of GAPDH promoter region (FWD 5'-TCCTCTGTTTCATCCAAGC-3'; REV 5'-TAGTAGCCGGGCCCTACTTT-3') were performed as positive and negative control respectively by end-point PCR performed as previously described (Cangemi et al., 2008) using Taq Gold (Roche). PCR products were analyzed by gel electrophoresis.

**Immunoblotting and immunoprecipitation**

Cell lysates were prepared using RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 0.25% Na-deoxycholate, 1mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF, 0.1% SDS) with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (PhosStop, Roche) as previously described (Albino et al., 2012). Total cell extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (PROTRAN). The following antibodies were used for immunoblot analysis: anti-COP1 (ab56400, Abcam), anti-STAT3 (124H6 cat.9139, Cell Signaling), anti-pSTAT3 Tyr705 (D3A7 cat.9145, Cell Signaling), anti-ETV1 (ab81086, Abcam), anti-c-Jun (Calbiochem), anti-glyceraldehyde-3-phosphatedehydrogenase (GAPDH, Millipore), anti-Ubiquitin (ENZO), anti-FLAG (F3165, Sigma), anti-CUL2 (C-4, sc-166506, Santa Cruz). Immunoprecipitation (IP) was performed as previously described (Longoni et al., 2013a). Cell lysates were incubated with antibody against STAT3 (sc-482, Santa Cruz) or FLAG tag (F3165, Sigma) and immunoblotting was performed using antibody against COP1 and STAT3. To detect ubiquitylated STAT3, cells were lysate in denaturing buffer (1% SDS, 20 nM HEPES pH 7.2, 2 mM EGTA, 20 nM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>) supplemented with 20 mM NEM (N-Ethylmaleimide, Sigma), protease inhibitor and phosphatase inhibitor cocktails. After incubation for 10 minutes at 65°C, lysates were sonicated and diluted 10 times in 1% Tryton buffer (1% Tryton, 20 nM HEPES pH 7.2, 2 mM EGTA, 20 nM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>) and subjected to

immunoprecipitation with anti-STAT3 antibody (Santa Cruz). Where indicated, PS-341 treatment was done for 5 h at 10  $\mu$ M concentration.

### **In vivo studies**

#### ***Animals and tumor xenografts***

Athymic nude mice (Balb/c nu/nu, 4–6 weeks old, Harlan Laboratories) were used for *in vivo* experiments.  $2 \times 10^6$  RWPE1 cells were subcutaneously injected (n=4/group) with Matrigel (1:1) and UGSM2 ( $2 \times 10^6$ ) cells 24h after *in vitro* transfection of 30 nM PremiR-424 or Negative control. Tumor growth was monitored every 2 days with a caliper and final tumor weight was measured.  $1 \times 10^6$  DU145-Luc cells (n=6/group) were subcutaneously injected after *in vitro* transfection of 40 nM LNA Anti-424 or Scrambled control. Tumor growth was monitored every 2 days with a caliper and IVIS Spectrum (LifeSciences). Results were analyzed using the Living Image software 4.2 (LifeSciences). To evaluate the lung metastatic capacity, *in vitro* transfected DU145-Luc cells ( $1.5 \times 10^6$ ), were injected into tail vein of nude mice (n=7/group). Animals were sacrificed after 5 weeks. Formation of lung metastasis was monitored by *in vivo* and post-necropsy bioluminescence imaging with the IVIS Spectrum.

#### ***Immunohistochemistry and immunocytochemistry***

Immunohistochemistry (IHC) was performed as previously described (Longoni et al., 2013b) using anti-STAT3 (124H6, Cell Signaling, cat. 9139); anti-pSTAT3 Tyr705 (D3A7, Cell Signaling, cat. 9145); anti-COP1 (B-12, Santa Cruz, cat. sc-166799); anti-ki67 (Lab Vision Corporation, ready-to-use #RT-9106-R7); anti-Cleaved Caspase 3 (Cell Signaling, #9664); anti-CD31(PECAM-1, Cell Signaling, #3528). Cell nuclei were counterstained with haematoxylin solution. Slides were evaluated by at least two investigators in a blinded manner. Immunocytochemistry (ICC) was performed on cells ( $4\text{--}5 \times 10^6$  cells/mL in PBS) fixed in Acetone:Methanol (1:1) using anti-STAT3 antibody. Cell nuclei were counterstained by haematoxylin solution.

#### ***Tissue samples for Immunohistochemistry***

Single tissue sections from primary prostate tumors (n=15), were obtained from the Cancer Genomics laboratory (Biella, Italy). Tissue microarrays (TMA) were constructed from formalin fixed paraffin-embedded tissue specimens as previously described (Prtilo et al., 2005). Tissue samples were collected with the approval of the Institutional Ethics Committees at Insespital (Bern, Switzerland) and patient written-informed consent. Histopathological and clinical data relative to the TMA specimens were collected and continuously updated (Insespital, Bern).

### ***Bioinformatic Analysis***

#### ***Patient samples and data sets***

The in-house patient cohort (*Biella* cohort) was used for miRNA expression profiling and included snap-frozen tissue samples collected from normal prostate biopsies (n=21) and primary prostate tumors (n=48). Gene expression profile data from these patients were previously described (Kunderfranco et al., 2010). The protocol for tissue collection was approved by the Ethics Committee of Regione Piemonte (Italy). The *TCGA* (The Cancer Genome Atlas) data set was downloaded from <http://gdac.broadinstitute.org/>, and included 497 primary prostate tumors. Data for other tumors were downloaded from datasets publicly available on the GEO database (GSE60371).

#### ***miRNA expression profiling***

miRNA expression profiling was performed by the Cancer Genomics laboratory (Biella, Italy). The analysis was carried out in normal (n=21) and primary prostate tumors (n=48) and in ESE3kd and control cells using the one-color labelling method and following the manufacturer's protocols (Agilent Technologies). Briefly, 100 ng of total RNA were dephosphorylated and denatured; then a ligation and labeling step with Cy3 was performed. Samples were hybridized to oligonucleotide glass arrays (Agilent Human miRNA Microarray 8x15K,V3) with sequences representing probes for 866 human and 89 human viral miRNAs from the Sanger database v12.0 (Human miRNA Microarray V3, Agilent Technologies). Slides were washed and scanned using an Agilent B dual-laser microarray scanner. Images obtained were analyzed using Feature Extraction software. Raw data were processed using R; briefly, background subtraction was not applied and log<sub>2</sub> intensities were normalized using the "invariants" methodology (Pradervand et al., 2009). Differentially expressed miRNAs were obtained by selecting probes with absolute log<sub>2</sub> fold change >0.37 and adjusted p-value <0.01. Data are MIAME-compliant and have been deposited in GEO (GSE60371).

#### ***Hierarchical clustering***

Hierarchical clustering with Pearson correlation as similarity metric and "complete" as linkage method was applied to the log intensity matrix of microRNAs x samples, with selection of miRs based only on their coefficient of variation along samples (> 0.016, i.e., greater than the median of all CVs). This selection yielded to 1367 probes (corresponding to 698 miRNAs), including those for hsa-miR-424-5p.

***Gene set enrichment analysis (GSEA)***

Gene set enrichment analysis (GSEA) was conducted as previously described (Albino et al., 2012; Longoni et al., 2013b). To identify gene signatures associated with increased miR-424 expression, we used the gene set enrichment analysis (GSEA) algorithm and the Molecular Signatures Database (MSigDB) C2 collection (version 4) of chemical and genetic perturbations (n=3402 gene sets). The metric used for gene ranking was the correlation of the expression values of a gene with the miR-424 expression values across the human prostate samples. The metric was calculated for all genes within the microarray, after collapsing the probes to the one having maximum absolute value. GSEA results are shown using the Enrichment Score (ES). Then, Normalized Enrichment Score (NES), that accounts for differences in gene set size and correlations between gene sets in different datasets, and False Discovery Rate (FDR) were calculated.

***Gene expression profiling***

RNA was collected from RWPE1 cells 48 h following transfection with 30 nM of pre-miR-424 or Negative control #1. We performed 8x60k Sure Print G3 Human GE Arrays. RNA was amplified, labeled, and hybridized according to the two-color microarray-based gene expression analysis protocol (Agilent Technologies). Slides were scanned with the dual-laser scanner Agilent G2505B and analyzed as described (Albino et al., 2012). Differentially expressed genes were obtained by selecting probes with absolute log2 fold change >0.37 and adjusted P value <0.05. Data are MIAME-compliant and have been deposited in GEO (GSE60371).

***Comparative transcriptomic analysis of miR-424***

For comparative transcriptomic analysis of miR-424-5p in several datasets (lung, gastric, breast and prostate tumors versus normal matched controls), gene expression datasets publicly available on the GEO database were examined. Moderated t-test p values were obtained using LIMMA (linear models for microarray analysis). In case of multiple probes for miR-424-5p, the one with the lowest p-value was used. In addition we retrieved RNA-Seq data from The Cancer Genome Atlas project (TCGA). Level of miR-424-5p were expressed as RPM=Reads per million.

***Extraction of miR-424 signatures in primary prostate tumors***

RNA-Seq data were retrieved from TCGA as described above. Samples were sub-divided according to the level of miR-424-5p. Two class comparison was performed on PCa with high (n=50) versus low (n=50) miR-424-5p levels, using the Limma package available within Bioconductor. For the

*Biella* dataset two class comparison was performed on PCa with high (n=17) versus low (n=17) miR-424-5p level and two class comparison was performed as described above.

### ***Functional annotation and transcription factor enrichment analysis***

For Functional annotation we used ENCODE project database to search for the most enriched KEGG pathways in selected gene lists. In addition we used Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/summary.jsp>) to search for the most enriched biological processes of the Gene Ontology (GoBP) terms. Enrichment scores were used to indicate the enrichment level of genes participating to the indicated GoBP in each subgroup. To select for putative transcription factors (TFs) involved in the regulation of selected gene lists (e.g., genes induced by miRNA-424) we used *Enrichr*. *Enrichr* is a database within the ENCODE project including a collection of genes whose promoters are bound by specific TFs as determined by ChIP-seq. In addition, we used ChIP Enrichment Analysis (ChEA) software, which search for TF enrichment in the ChIP-X database containing a collection of ChIP-chip, ChIP-seq, ChIP-PET and DamID experiments.

### ***Patient Survival analysis***

Differential survival between patient subgroups was plotted and calculated using Kaplan-Meier (KM) curves. P-values were calculated using log-rank test. For TMA staining, patient samples were stratified based on percentage of positive cells in primary PCA. The "high STAT3" group is defined as samples being within the percentiles 75 to 100 of % positive cells. The "low COP1" group is defined as samples being within the percentiles 0-25 of % positive cells. Patients included in the KM analysis had been treated with radical prostatectomy, had complete clinical record and follow-up data and evaluable IHC staining. Biochemical recurrence was defined as a 0.2 ng/ml increase in PSA with a second confirmatory PSA measurement > 0.2 ng/ml or recurrence of disease after prostatectomy, such as development of metastatic cancer, if biochemical recurrence information was not available. For biochemical recurrence-free survival we included all the deaths occurred within 10 years after radical retropubic prostatectomy.

### ***Statistical analysis***

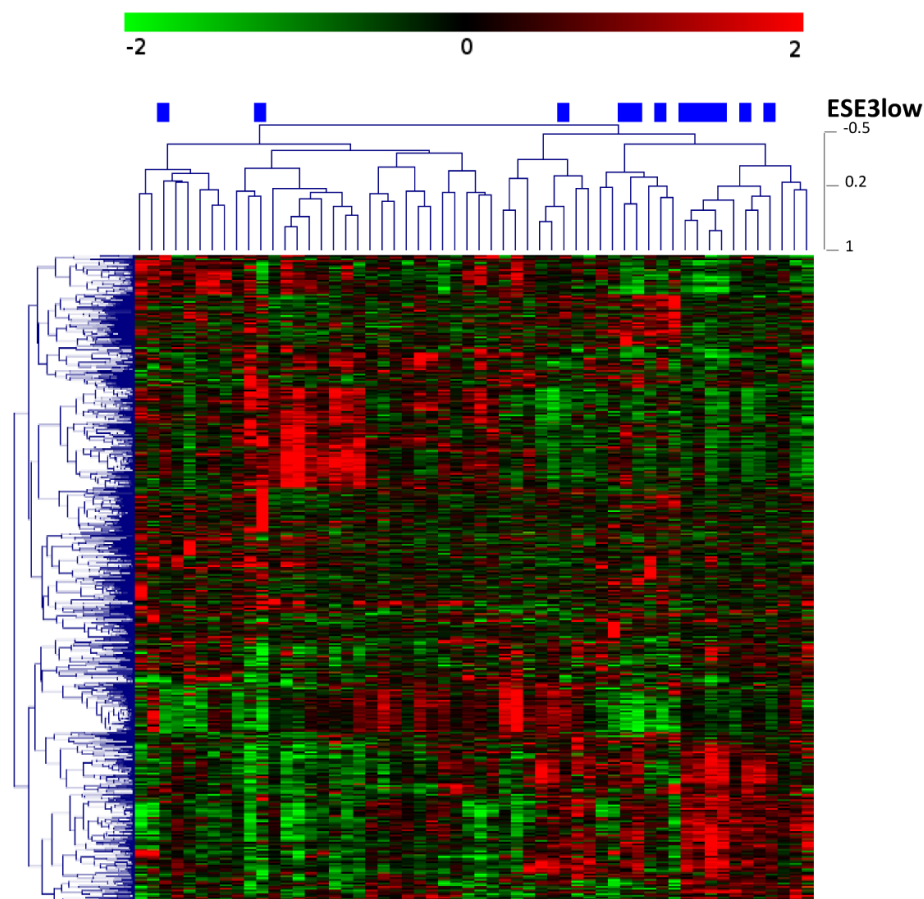
The significance of differences between groups was measured with two-tailed Student's t-test and p-values of less than 0.05 were considered statistically significant. Significance in overlapping gene lists was calculated using Fisher's exact test and the statistical analysis was done using Stata 12.1® and SPSS software. For correlation analysis the Pearson correlation was calculated in Stata

13 software. The t-test is used to establish if the correlation coefficient is significantly different from zero, and, hence that there is evidence of an association between the two variables.

## ***Results***

### Identification of miRNAs controlled by ESE3

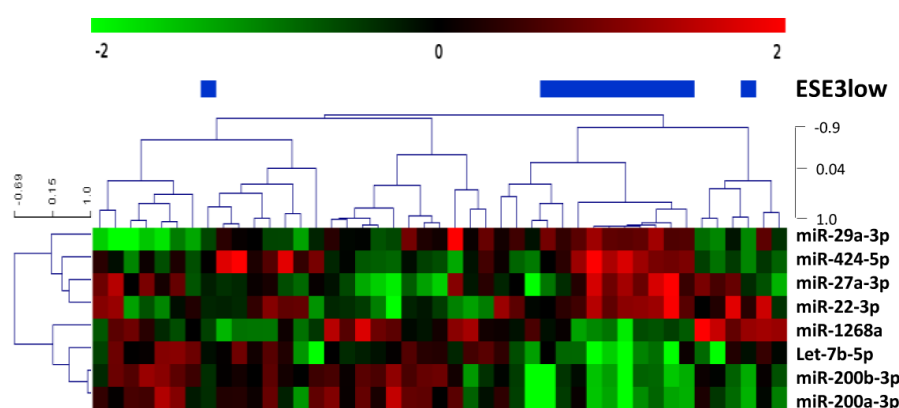
To understand the mechanisms leading to dedifferentiation and stemness in prostate tumors and the role of ETS factors in this context, we profiled miRNA expression in human normal prostate ( $n=21$ ) and prostate tumor ( $n=48$ ) tissue samples that we previously analyzed for the global gene expression and ETS gene status (Albino et al., 2012; Kunderfranco et al., 2010). In addition to miRNAs differentially expressed between normal and tumor samples, we observed a substantial heterogeneity of the miRNA profiles across tumor samples in unsupervised hierarchical clustering (UHC) analysis, in line with tumor subtype-specific miRNA deregulation. Interestingly, most of the ESE3<sub>low</sub> tumors clustered together displaying a similar miRNA profile (Figure 14). We reported previously that these tumors represent a distinct subgroup characterized by low ESE3/EHF expression and the absence of ETS gene fusion. Furthermore, ESE3<sub>low</sub> tumors show distinctive biological features, including increased expression of EMT-related and stem-like genes (Albino et al., 2012). Low ESE3/EHF expression was also associated with more aggressive clinical features and poor prognosis (Albino et al., 2012).



**Figure 14. ESE3<sub>low</sub> tumors have a unique miRNA signature.** Unsupervised Hierarchical Clustering (UHC) of primary prostate tumors from the *Biella* dataset according to their microRNA profile. Columns represent tumor samples and rows report microRNAs expression level compared to normal samples. Blue squares, ESE3<sub>low</sub> tumors. Red color, upregulated miRNAs; Green color, downregulated miRNAs.



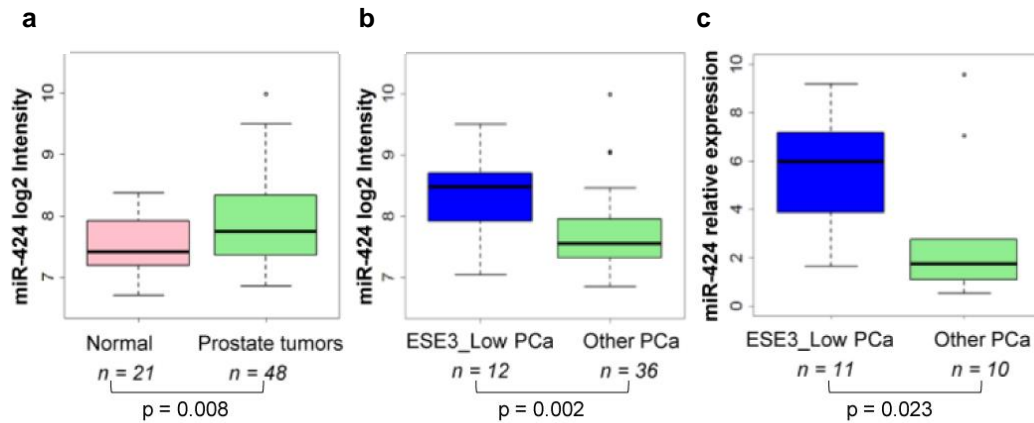
In order to identify miRNAs whose altered expression was specifically linked to ESE3/EHF status we profiled miRNAs in normal prostate epithelial cells with stable knockdown of ESE3/EHF (ESE3kd cells). Stable ESE3kd cells recapitulated the EMT and stem-like features observed in ESE3<sub>low</sub> tumors and thus provided an experimental model to study the transition from normal to transformed and dedifferentiated prostate epithelial cells (Albino et al., 2012). Notably, the miRNA profile of ESE3kd cells resembled that of ESE3<sub>low</sub> tumors and identified those tumors as a distinct cluster in UHC analysis (Figure 15). This indicated that ESE3/EHF could have a key role in driving the specific miRNA signature associated with this tumor subgroup.



**Figure 15. miRNA signature of ESE3kd prostate cells discriminates ESE3<sub>low</sub> tumors.** Unsupervised Hierarchical Clustering (UHC) of primary prostate tumors from the *Biella* dataset using the list of most significantly deregulated miRNAs in ESE3 knockdown prostate epithelial cells (LHS ESE3kd) compared to control cells. Columns represent tumor samples and rows report microRNAs expression level. Blue squares, ESE3<sub>low</sub> tumors. Red color, upregulated miRNAs; Green color, downregulated miRNAs.

### ***miR-424 is up-regulated in prostate tumors and prevalently in ESE3<sub>low</sub> tumor subgroup***

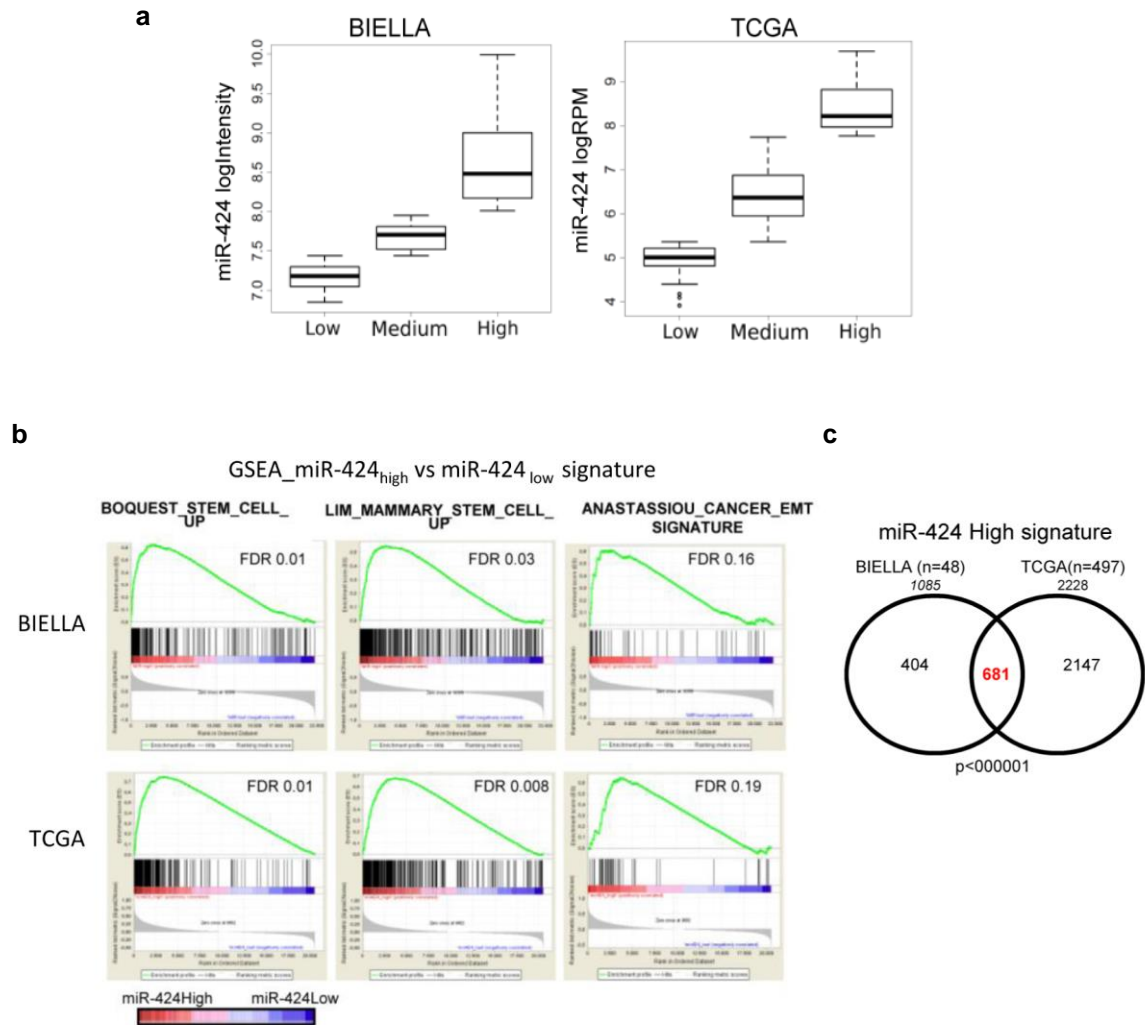
Among the top miRNAs up-regulated both in ESE3kd cells and ESE3<sub>low</sub> tumors, we focused on miR-424. In fact, this miRNA was found over-expressed in primary tumors compared to normal prostate in the microarray analysis (Figure 16a). Moreover, miR-424 was preferentially upregulated in the ESE3<sub>low</sub> tumor subgroup as indicated by both microarray (Figure 16b) and qRT-PCR analysis (Figure 16c).



**Figure 16. miR-424 is specifically overexpressed in ESE3low prostate tumors.** (a and b) miR-424 log2 intensity levels evaluated by microarray in normal and prostate tumor samples from the *Biella* dataset (a) and in ESE3 low prostate tumors (ESE3\_low PCa) compared to the other prostate cancer samples (Other PCa)(b). (c) Relative expression levels evaluated by qRT-PCR (c) in indicated samples. Dots represent outliers.

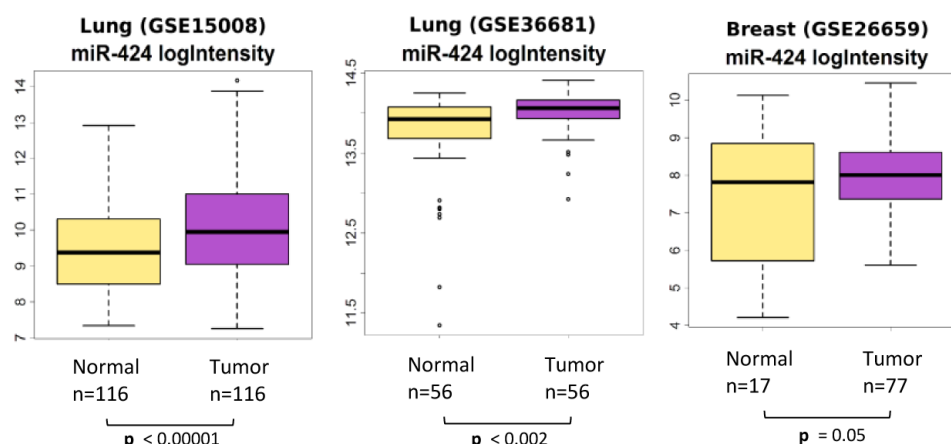
### ***miR-424 elevation is associated with EMT and stem cell-like signatures in prostate tumors***

To understand the clinical relevance of miR-424 elevation in prostate tumors, we also evaluated miR-424 level in a large set of primary prostate cancer publicly available, The Cancer Genome Atlas project (TCGA, n=497). Both in our patient cohort (*Biella*, n=48) and in TCGA dataset expression of miR-424 was highly heterogeneous with tumors exhibiting low (TCGA, n=50; *Biella*, n=17), intermediate (TCGA, n=397; *Biella* n=14) or high (TCGA, n=50; *Biella*, n=17) level of the miRNA (Figure 17a). To understand the potential impact of miR-424 upregulation on the tumor phenotype, we applied differential gene expression analysis and compared tumors with high and low miR-424 level to identify molecular and biological differences between the two subgroups. Gene Set Enrichment Analysis (GSEA) of the extracted miR-424 signatures revealed that miR-424 high tumors were significantly enriched of gene sets associated with mesenchymal transition (Anastassiou\_cancer mesenchymal transition) and stem-like features (Boquest\_stem cell up and Lim\_mammary stem cell\_up) (Figure 17b). Strikingly, the gene signatures of miR-424 high tumors were significantly ( $p < 0.001$ ) convergent in these two datasets, supporting miR-424 as a key driver of these features (Figure 17c).



**Figure 17. miR-424 is associated with EMT and stem cell-like signatures** (a) Prostate samples divided according to miR-424 expression in two independent primary prostate tumor datasets, The Cancer Genome Atlas project (TCGA) and the in house *Biella* dataset (see Methods for details). (b) Gene set enrichment analysis (GSEA) comparing miR-424<sub>high</sub> to miR-424<sub>low</sub> prostate tumors in the two microarray datasets (total n=545), using the indicated stem-like and EMT-related gene signatures. False Discovery Rate (FDR) is shown. (c) Venn diagram crossing genes upregulated in miR-424<sub>high</sub> signatures extracted from *Biella* and TCGA datasets and showing a significantly high convergence (p<0.001).

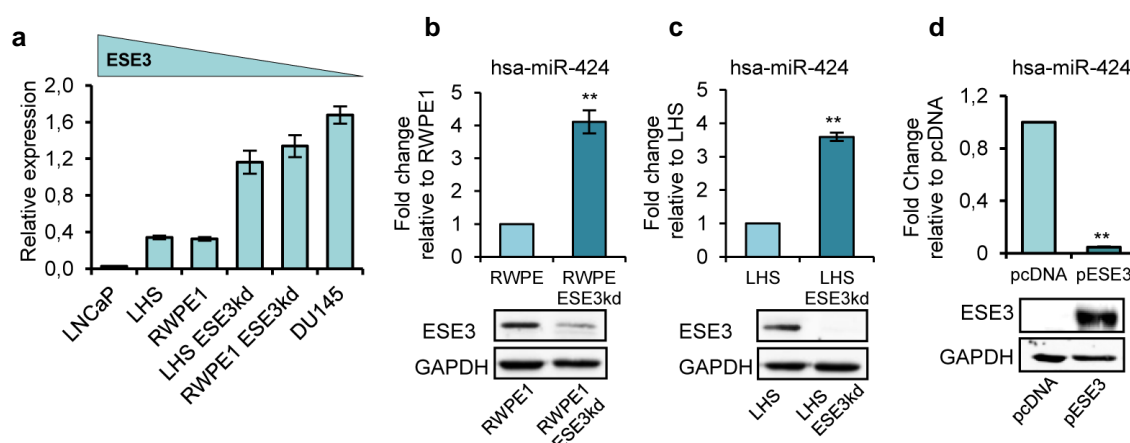
Notably, analysis of miRNA expression datasets from additional tumor types revealed that miR-424 was significantly up-regulated also in other epithelial tumors, including lung and breast cancer (Figure 18). Collectively, these findings indicated that miR-424 could act as an oncogenic miRNA deregulated in prostate tumors and potentially in multiple other epithelial cancers.



**Figure 18. miR-424 is overexpressed in other epithelial tumor types.** Comparative transcriptomic analysis of miR-424 level evaluated by microarray in indicated tumor datasets publicly available on the GEO database versus normal matched controls. p values of modified t-test are indicated. Dots represent outliers.

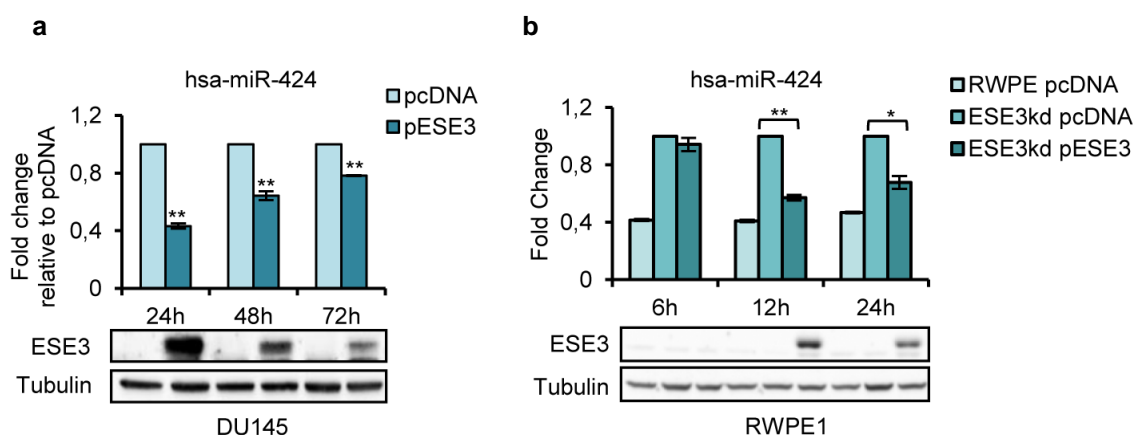
### *miR-424 is repressed by ESE3/EHF in prostate epithelial cells*

The expression of miR-424 was inversely correlated with the level of ESE3/EHF not only in the prostate tumors analyzed (Figure 16), but also in cell lines (Figure 19a). In particular, a very high level of miR-424 was observed in the metastatic prostate cancer cell line DU145 (Figure 19a), in which ESE3/EHF expression is silenced due to methylation of the promoter region (Cangemi et al., 2008). In addition, miR-424 was up-regulated in the immortalized normal prostate epithelial cell lines LHS and RWPE1 after stable ESE3/EHF knockdown (Figure 19b and c). Conversely, stable expression of ESE3/EHF reduced miR-424 level in the metastatic DU145 cell line (Figure 19d).



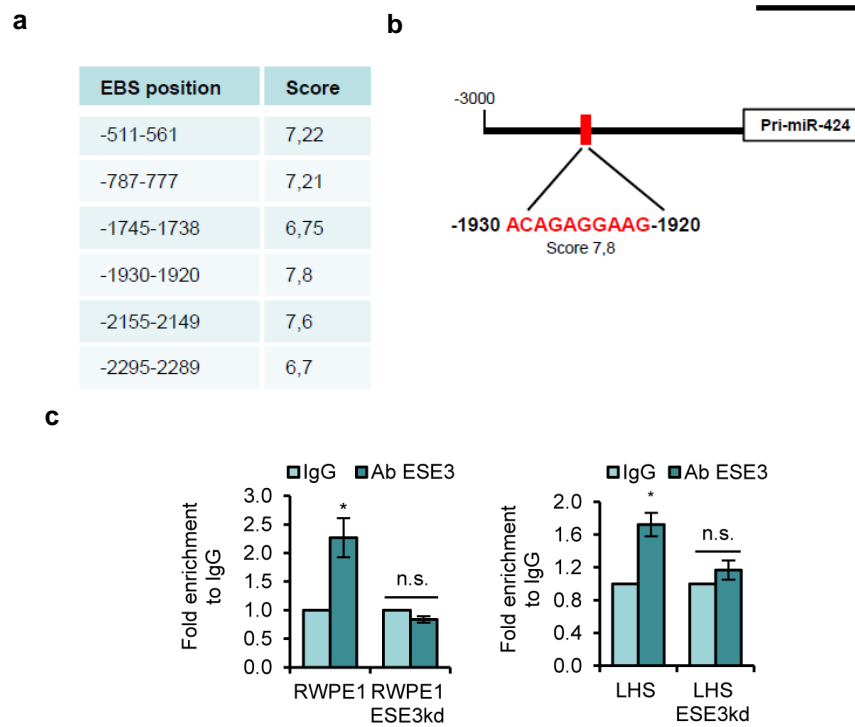
**Figure 19. miR-424 level is inversely correlated ESE3/EHF in prostate cells.** (a) miR-424 level evaluated by qRT-PCR in indicated prostate cell lines. The results were normalized to RNU6 internal control and represented as miR-424 relative expression. (b-d) miR-424 level in RWPE1 (b) and LHS with stable ESE3kd (c) and in DU145 with stable ESE3/EHF (pESE3) expression (d) compared to control cells. The results were normalized to RNU6 and represented as fold change relative to indicated control cells (RWPE1, LHS, pcDNA) set to 1. Lower panels, immunoblot of ESE3/EHF. \*p ≤ 0.05; \*\*p ≤ 0.01 by two-tailed Student's t-test.

Furthermore, transient transfection of ESE3/EHF reduced miR-424 expression in a time dependent manner both in DU145 and RWPE1 ESE3kd cells (Figure 20a and b). These data suggested that ESE3/EHF directly controlled the transcription of miR-424.

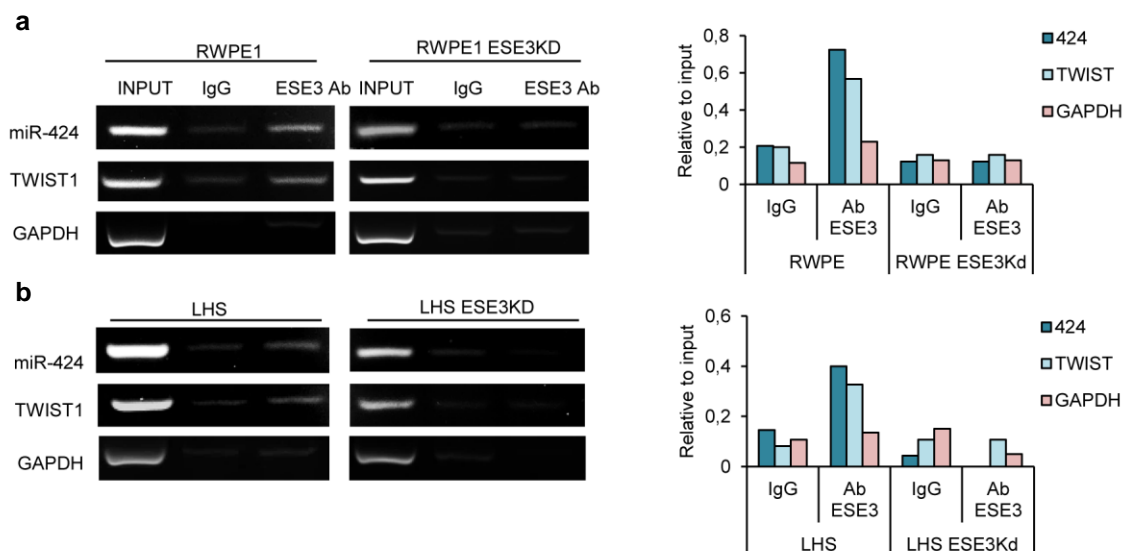


**Figure 20. ESE3/EHF overexpression represses miR-424 transcription in DU145 and ESE3kd cancer cells in a time-dependent manner.** (a) miR-424 level evaluated by qRT-PCR in DU145 following transfection with pESE3 or control vector (pcDNA) at the indicated time points (*upper*) and immunoblot of ESE3/EHF (*lower*). The values are normalized to RNU6 and represented as fold change relative to pcDNA transfected cells at each time point. (b) miR-424 level evaluated by qRT-PCR in RWPE1 control and ESE3Kd cells following ectopic expression of ESE3 (pESE3) or control vector (pcDNA) at the indicated time points. *Lower panels*, Immunoblot of ESE3/EHF at indicated time points following overexpression of pESE3 (*left*) and in RWPE1 control and ESE3kd cells (*right*). The values are normalized to RNU6 and represented as miR-424 expression relative to pcDNA transfected RWPE1 ESE3kd cells at each time point. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

Consistently, ETS binding site (EBS) with high score were predicted in the miR-424 promoter by using *MotifViz* tool (Figure 21a). In order to evaluate ESE3/EHF occupancy, we analyzed the promoter region encompassing the EBS predicted with the highest score (Figure 21b). ChIP-qPCR showed binding of ESE3/EHF to miR-424 promoter region in RWPE1 and LHS prostate epithelial cells and not in the derivative ESE3kd cell lines used as negative control (Figure 21c). To confirm the specificity of the binding in the same experiment we used as positive control the TWIST1 promoter region, previously shown to be regulated by ESE3/EHF (Albino et al., 2012), and the GAPDH promoter region as negative control (Figure 22a and b). The binding for TWIST1 promoter was observed in RWPE1 and LHS cells and not in the derivative ESE3kd cells, similarly to what observed for miR-424 promoter.

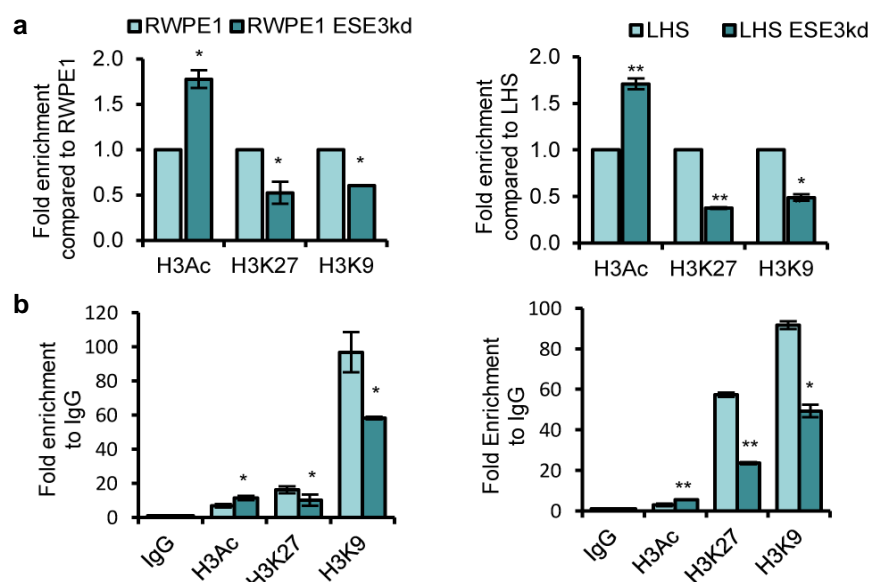


**Figure 21. ESE3/EHF occupies miR-424 promoter region.** (a) Table showing position and scores of ETS binding sites on miR-424 promoter identified using *MotifViz*. (b) Schematic diagram of miR-424 promoter and position of the evaluated ETS binding site relative to the 5' end of pri-miR-424 (RefSeq NR\_029946). (c) ESE3/EHF occupancy on miR-424 promoter evaluated by ChIP and qPCR in indicated cell lines. The results are represented relative to IgG that was used as negative control. \* $p \leq 0.05$  by two-tailed Student's t-test. n.s. not significant.



**Figure 22. Positive and negative control for ESE3/EHF occupancy on miR-424 promoter region.** ESE3/EHF occupancy on TWIST1 promoter and GAPDH promoter were evaluated by end point PCR as positive and negative control respectively in RWPE1 (a) and LHS (b) cells compared to ESE3kd cells (left). Densitometric analysis (relative to input) of the single experiment is also shown (a and b right).

To further confirm the consequences of ESE3/EHF occupancy, we evaluated activating and repressive chromatin marks on the same promoter region. Consistent with a repressive role of ESE3/EHF, the miR-424 promoter was enriched of active chromatin marks and depleted of repressive marks in ESE3kd cells compared to parental ESE3/EHF expressing RWPE1 and LHS cells (Figure 23a and b). Thus, ESE3/EHF prevented expression of miR-424 in normal prostate epithelial cells and loss of ESE3/EHF triggered miR-424 upregulation.

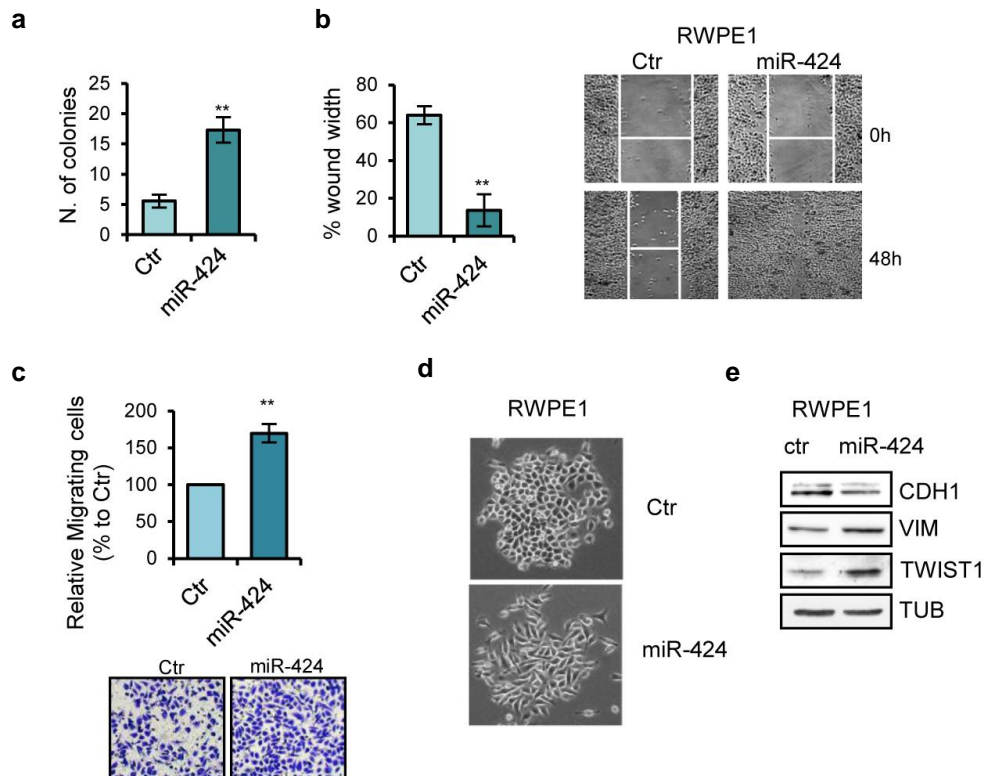


**Figure 23. ESE3/EHF induces repressive histone marks on the miR-424 promoter region containing the EBS.** (a, Left and right) Histone 3 acetylation (H3Ac), lysine 27 and lysine 9 methylation (H3K27 and H3K9) occupancy on miR-424 promoter evaluated by ChIP in indicated cell lines. The results were normalized on IgG and represented as fold enrichment relative to the parental cell line. (b, Left and right) ChIP data presented as fold enrichment relative to IgG. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

### ***miR-424 promotes malignant phenotypes and tumor-initiating properties in prostate epithelial cells***

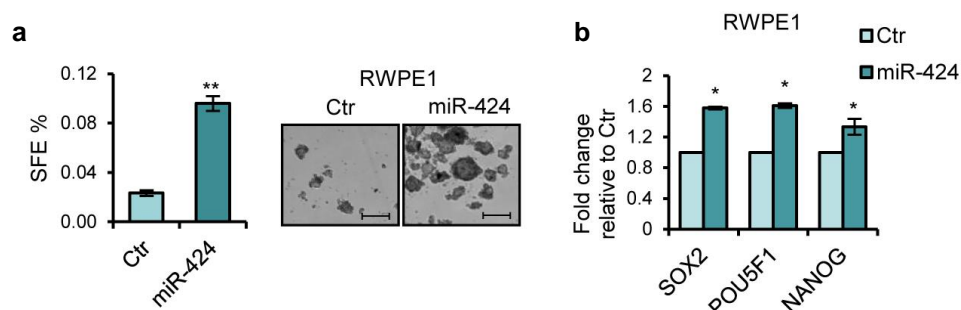
Our data identified miR-424 as a potential oncogenic miRNA in prostate tumors. Consistently, we found that transient over-expression of miR-424 in normal RWPE1 cells promoted anchorage-independent growth as assessed by colony formation in soft-agar (Figure 24a) and also cell migration and invasion as shown by wound healing and Boyden chamber assays (Figure 24b and c). miR-424 transfected RWPE1 cells exhibited also a dramatic change in morphology and altered expression of E-cadherin, vimentin and TWIST1, supporting a reprogramming toward the EMT phenotype (Figure 24d and e).





**Figure 24. miR-424 promotes transforming phenotypes in normal prostate epithelial cells.** (a-c) Colony formation in soft agar (a), cell migration by wound healing (WH) (b) and Boyden chamber assay (c) following transient transfection of miR-424 precursor (miR-424) or negative control (Ctrl) in RWPE1 cells. Representative images of wound width at 0 and 48 h after the scratch in WH assay (b right) and pictures of migrating cells stained with crystal violet from Boyden chamber assay (c lower) are shown. (d) Phase-contrast pictures showing cell morphology following miR-424 overexpression. (e) Immunoblot for indicated EMT markers evaluated 72 h following miR-424 overexpression. CDH1, E-cadherin. VIM, Vimentin. TUB, Tubulin. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  by two-tailed Student's t-test.

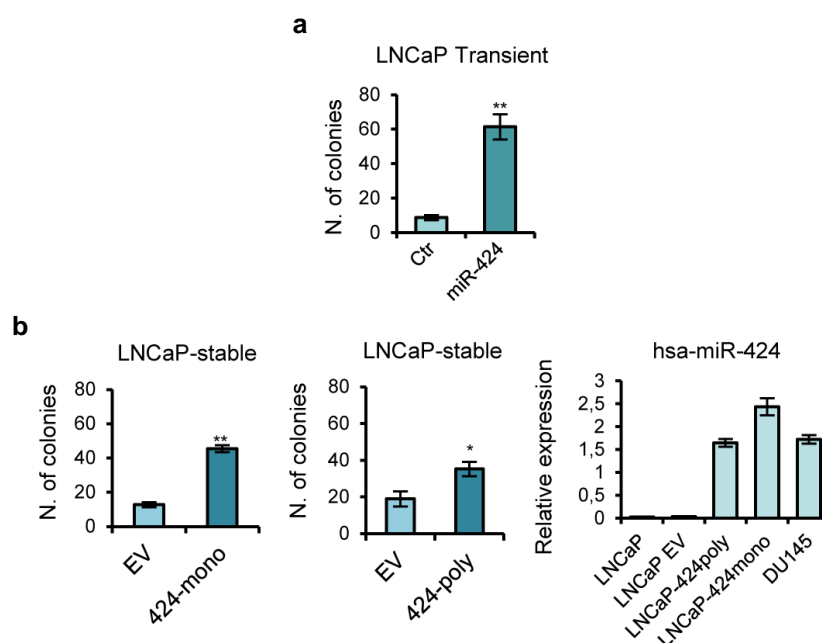
Furthermore, formation of prostato-spheres (Figure 25a), a feature associated with the stem-like phenotype (Albino et al., 2012; Civenni et al., 2013), and the level of stem cell markers (Figure 25b) were significantly increased by miR-424 in RWPE1 cells.



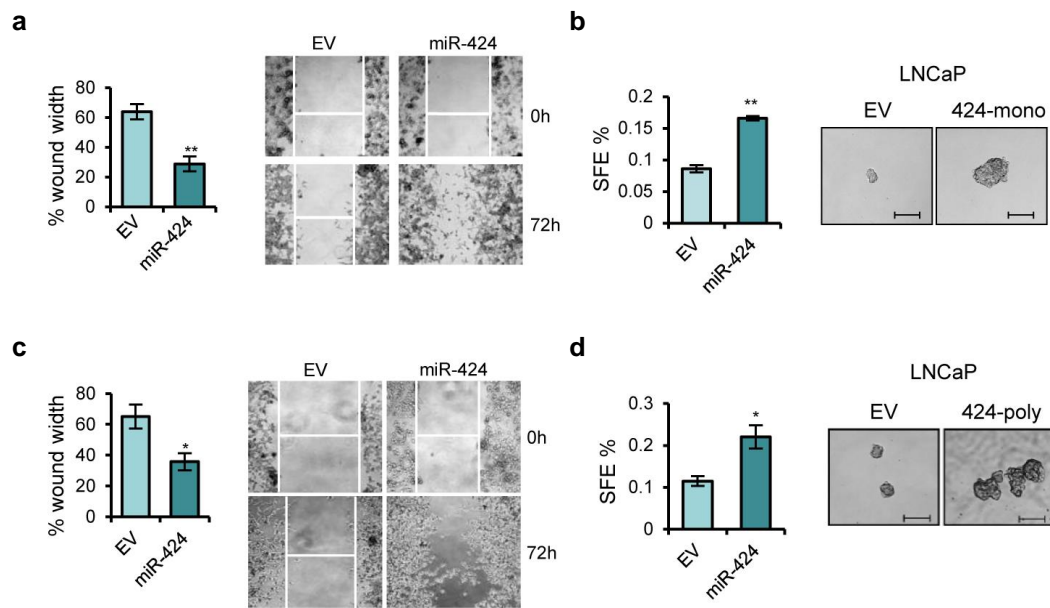
**Figure 25. miR-424 promotes cancer stem cell-like features in normal prostate epithelial cells.** (a) Sphere forming efficiency (SFE, left) with representative images of spheroids (right) following transient transfection of miR-424 precursor (miR-424) or negative control (Ctrl) in RWPE1 cells. Scale bars: 200 $\mu$ m. (b) Expression of cancer stem cell-like markers evaluated by qRT-PCR in RWPE1 72h following miR-424 overexpression. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.



Moreover, both transient and stable transfection of miR-424 in tumorigenic LNCaP cells significantly increased anchorage-independent growth in soft agar (Figure 26a and b). Stable expression of miR-424 also induced migration and prostato-sphere formation in LNCaP cells (Figure 27a-d).

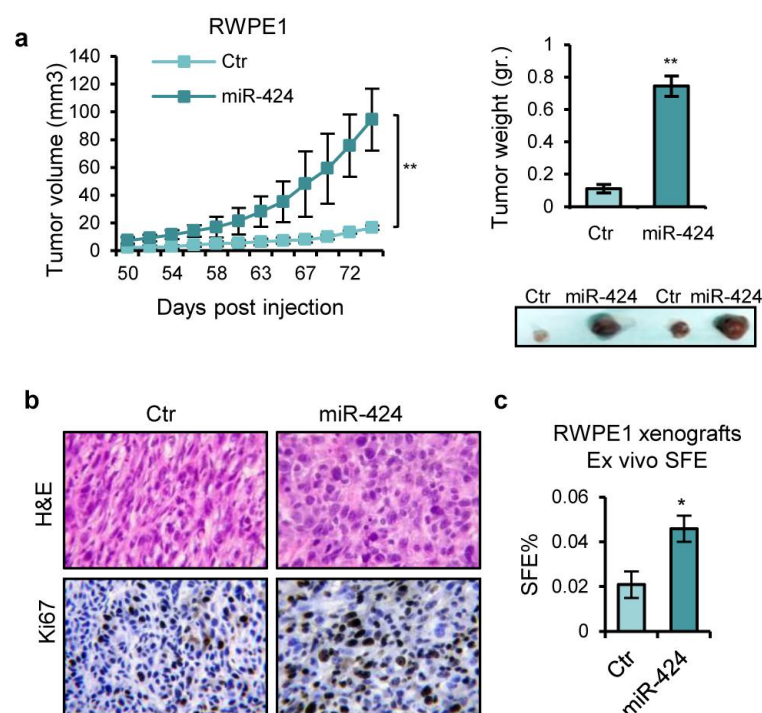


**Figure 26. Transient and stable expression of miR-424 induces anchorage-independent growth in LNCaP cells.** (a) Colony formation in LNCaP cells following transient transfection of miR-424 precursor (miR-424) or negative control (Ctr). (b) LNCaP-424 monoclonal (LNCaP-424mono) and polyclonal (LNCaP-424poly) cell lines compared to control cells expressing empty vector (LNCaP EV). (b) Overexpression of miR-424 in LNCaP stable cell lines evaluated by qRT-PCR. Data show mean  $\pm$  s.d. (n=3). \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.



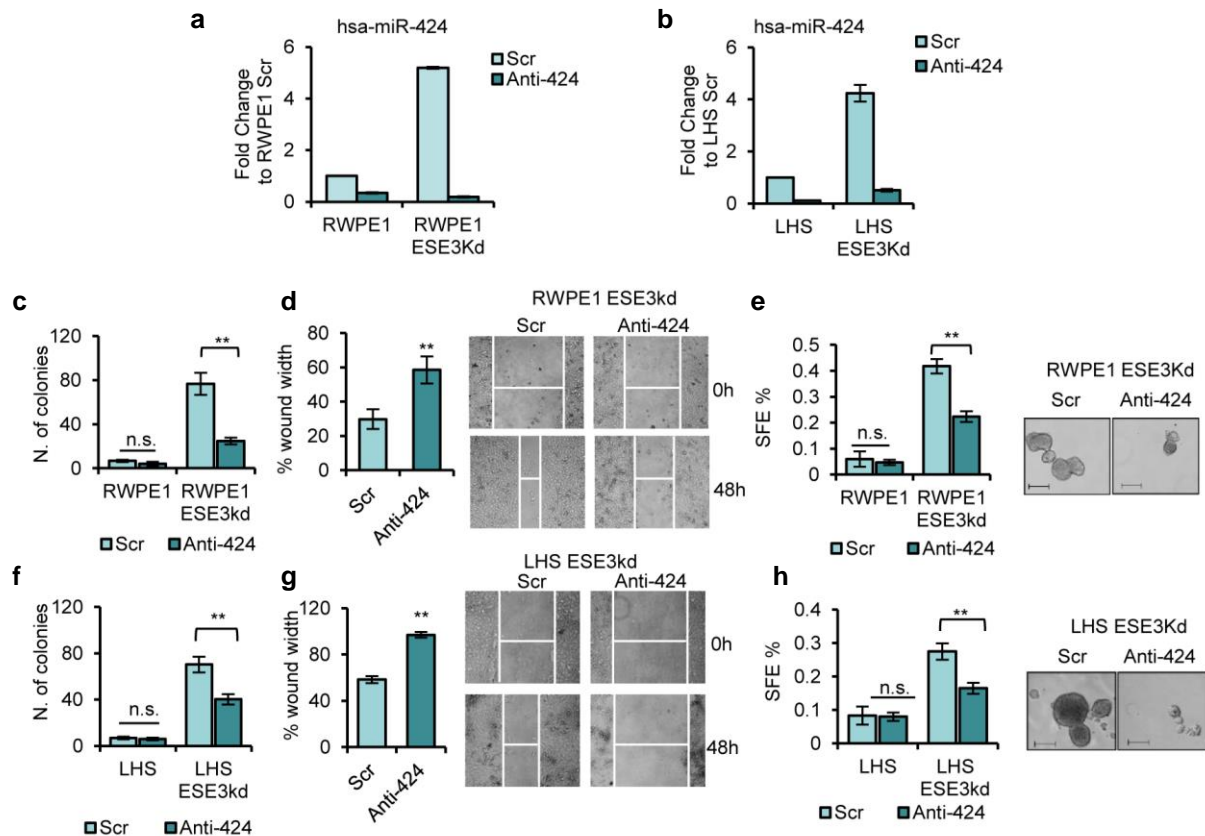
**Figure 27. Stable expression of miR-424 sustains malignant phenotypes in LNCaP cells.** (a and b) Cell migration evaluated by WH (a) and SFE (b) in LNCaP-424 monoclonal cells compared to control. EV, Empty-vector. (c and d) Cell migration by WH (c) and SFE (d) evaluated in LNCaP-424 polyclonal cells. Representative images of wound closure (a and c right) and spheroids (b and d right) are shown. Data show mean  $\pm$  s.d. (n=3) of one representative experiment. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test. Scale bars: 200 $\mu$ m

Based on the *in vitro* data showing that elevation of miR-424 even in a transient condition induced a persistent malignant phenotype, we assessed whether an acute increase of miR-424 level could be sufficient to induce tumor-initiating capability in normal prostate epithelial cells *in vivo*. Consistent with the effects observed *in vitro*, RWPE1 cells transfected with miR-424, unlike control transfected cells, acquired the ability to form tumors when engrafted subcutaneously in nude mice, indicating that miR-424 was an inducer of tumor-initiating properties in prostate epithelial cells (Figure 28a and b). Moreover, we observed increased ex vivo prostato-sphere formation from cells harvested from xenografts formed by miR-424 transfected RWPE1 cells, suggesting acquisition of stem-like and self-renewal properties (Figure 28c). These results suggest that an acute transient expression of miR-424 is sufficient to induce a stable malignant transformation of prostate epithelial cells.



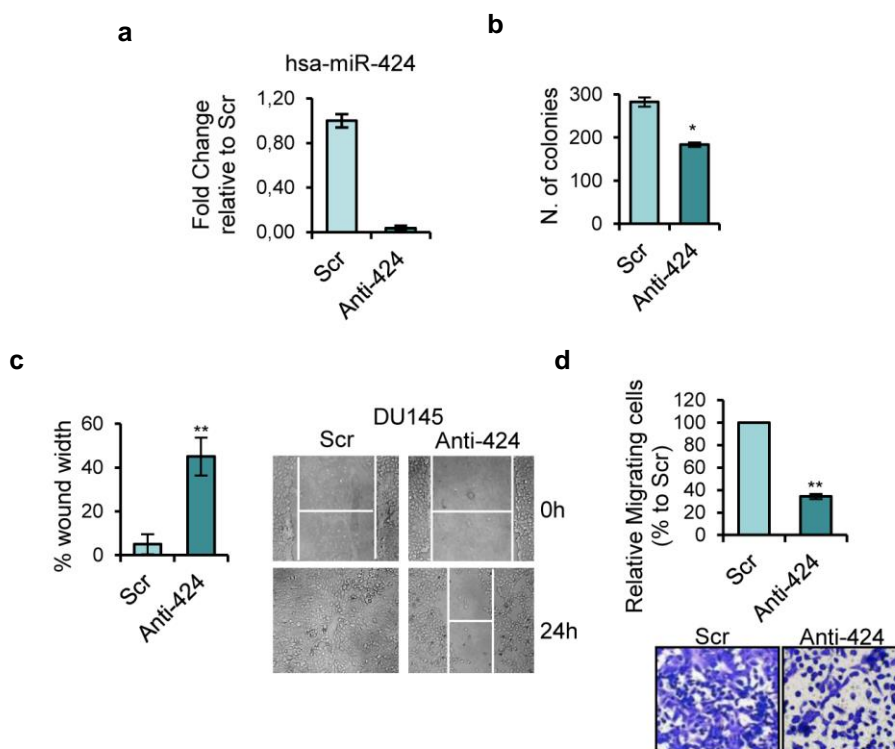
**Figure 28. miR-424 induces tumor-initiation and tumor-growth *in vivo*.** (a) Tumor growth (*left*), tumor weight (*right*) and representative images of tumors (*lower panel*) of subcutaneous xenografts in nude mice from RWPE1 cells transfected with miR-424 (n=4) or Ctr (n=4). Data show mean  $\pm$  s.d. (b) Images of Hematoxylin and Eosin (H&E) stain and immunohistochemical stain for Ki67 proliferation marker of control and miR-424 ensued xenografts described above. (c) Ex vivo SFE from RWPE1 xenografts ensued from control and miR-424 as described above. Xenografts were dissociated and sphere-forming assay was carried out as described in Methods. \* $p \leq 0.05$  \*\* $p \leq 0.01$  by two-tailed Student's t-test.

Conversely, a miR-424 antagonist (anti-miR-424) decreased selectively anchorage-independent growth, migration and prostate-sphere forming ability of ESE3kd-RWPE1 (Figure 29a and c-e) and ESE3kd-LHS cells (Figure 29b and f-h), sustaining the role of miR-424 as a key oncogenic driver in the context of ESE3<sub>low</sub> tumors.

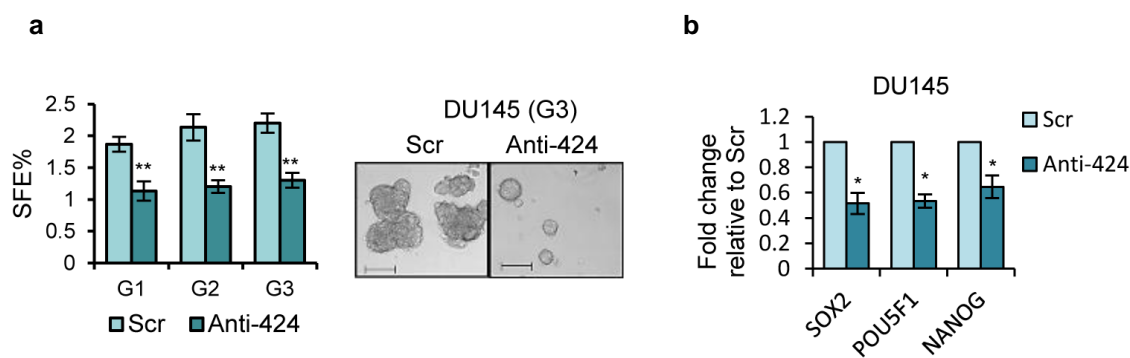


**Figure 29. miR-424 inhibition reduces malignant phenotypes in ESE3kd cells.** (a and b) miR-424 level evaluated by qRT-PCR 24 h following transfection of anti-miR-424 in the indicated cell lines. The miRNA level was normalized to RNU6 and represented as fold change relative to the indicated scrambled control (Scr). (c-h) Colony formation in soft agar (c and f), WH (d and g) and SFE (e and h) in ESE3kd cell line models following inhibition of miR-424 by anti-miR-424 (Anti-424) or scrambled control (Scr). Representative images of wound closure (d and g right) and spheroids (e and h right) are also shown. Scale bars: 200  $\mu$ m. Data show mean  $\pm$  s.d. (n=3) of one representative experiment. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

Furthermore, treatment with anti-miR-424 decreased significantly anchorage-independent growth, migration and prostato-sphere formation in metastatic DU145 cells, expressing high level of miR-424 (Figure 30a-d and Figure 31a). In order to evaluate the self-renewal capability, primary prostato-sphere forming cells were further propagated for different generations. Interestingly, the effect on prostate-sphere formation persisted after the first generation, indicating stable impairment of self-renewal of the stem-like subpopulation after miR-424 inhibition (Figure 31a). Consistently, the expression of cancer stem cell-like markers (Figure 31b) was significantly reduced by anti-miR-424.

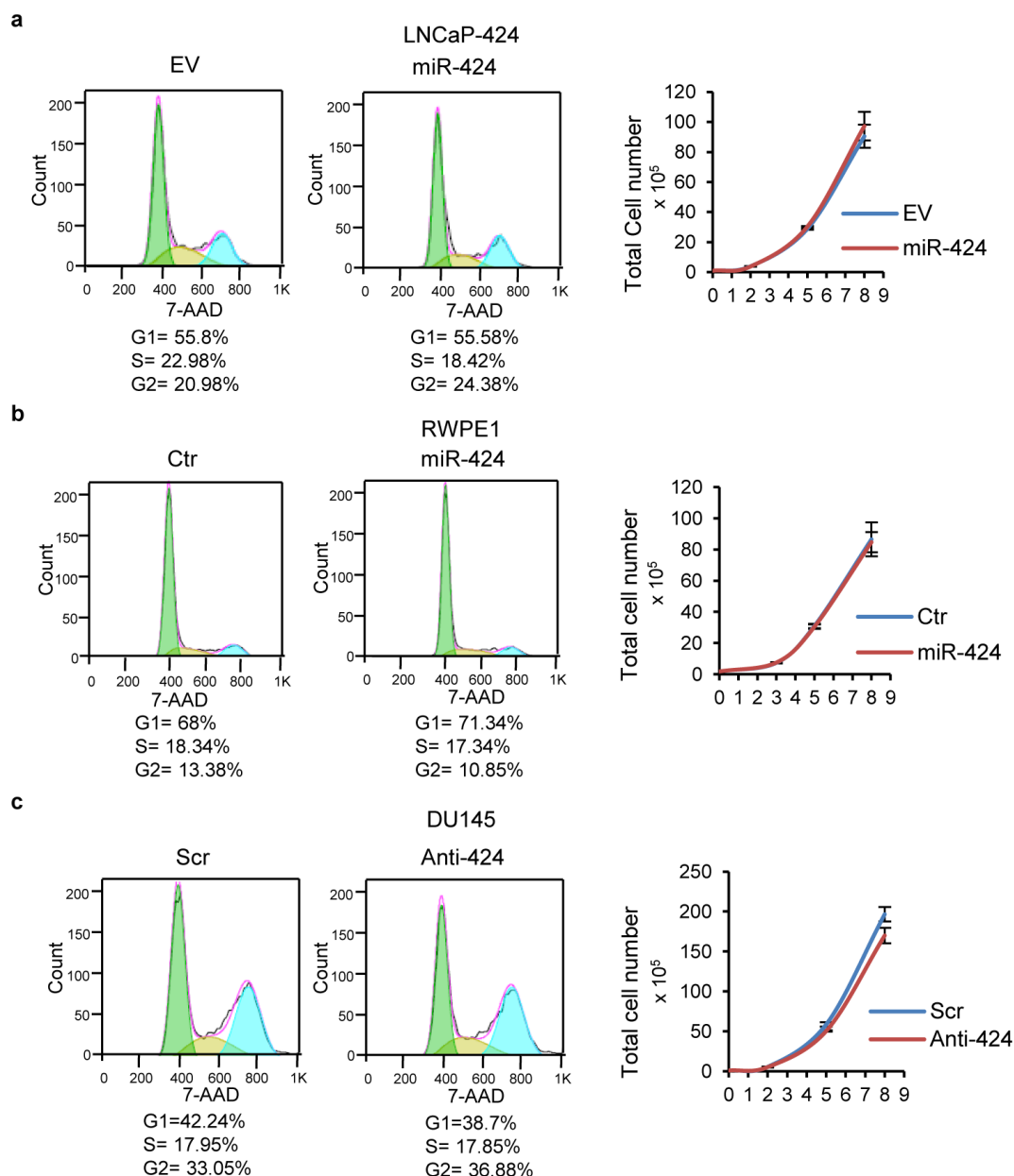


**Figure 30. miR-424 inhibition reduces malignant phenotypes in metastatic prostate cancer cells.** (a) miR-424 level evaluated by qRT-PCR 24h following transfection of anti-miR-424 (Anti-424) in DU145 cells. The miRNA level was normalized to RNU6 and represented as fold change relative to the Scrambled control (Scr). (b-d) Colony formation in soft agar (b), cell migration and invasion by WH (c) and Boyden chamber (d) assay in DU145 cells transfected with Anti-424 compared to control cells (Scr). Representative images of wound width taken at 0 and 24 h after the scratch in WH assay (c right) and pictures of migrating cells stained with crystal violet from Boyden chamber assay (d lower) are shown. Data show mean  $\pm$  s.d. (n=3) of one representative experiment. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.



**Figure 31. miR-424 inhibition reduces cancer stem cell-like properties in metastatic prostate cancer cells.** (a) SFE through different generations (G) with representative images of spheroids in DU145 cells following transfection with anti-miR-424 (Anti-424) or scrambled control (Scr). Spheroids from G1 were dissociated and replated in sphere-forming conditions up to G3. Data show mean  $\pm$  s.d. (n=3) of one representative experiment. Scale bars: 200 $\mu$ m. (b) Expression of cancer stem cell-like markers evaluated by qRT-PCR in RWPE1 72h following miR-424 overexpression. Data show mean  $\pm$  s.e.m. of three independent experiments. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

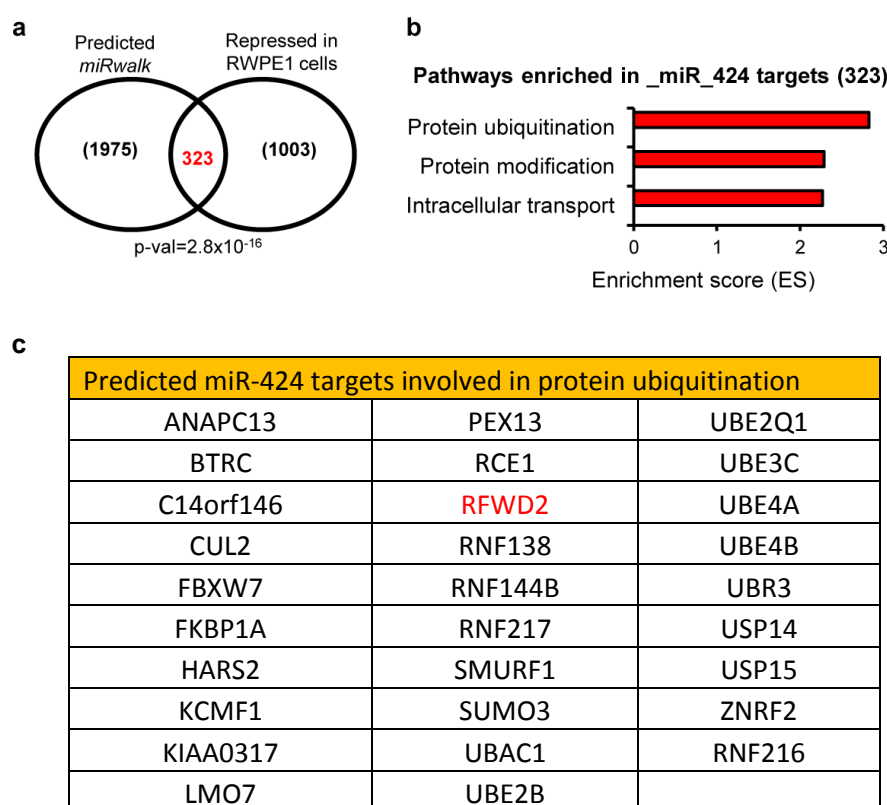
Notably, overexpression of miR-424 level in RWPE1 and LNCaP cells or inhibition of miRNA function in DU145 cells did not affect overall cell proliferation and cell cycle (Figure 32a-c), indicating that miR-424 impacted prominently on specific properties of malignant cells by inducing EMT and stem-like phenotypes.



**Figure 32. miR-424 does not affect cell cycle and cell proliferation *in vitro*.** Cell cycle analysis by 7AAD staining (*left panels*) and cell proliferation assay (*right panels*) were performed in stable LNCaP-424 monoclonal cell line (**a**) and in RWPE1 cells following miR-424 overexpression (**b**) and DU145 cells following miR-424 inhibition by anti-miR-424 (**c**). Cell cycle analysis was performed 72h after cell seeding or transfection and percentage of cells in G1, S and G2 are indicated. Cell counts for cell proliferation assay were done at the indicated time points up to 8 days following cell seeding. Data show mean  $\pm$  s.d of triplicate.

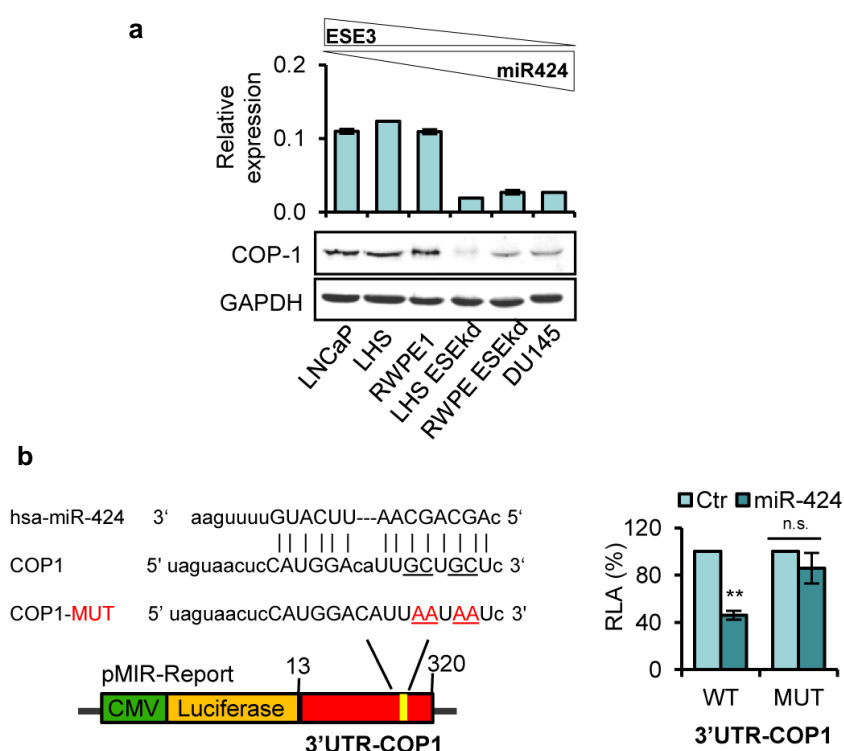
### miR-424 targets the E3 ubiquitin ligase COP1

To identify direct miR-424 targets relevant in prostate epithelial cells, we performed gene expression analysis in RWPE1 cells transfected with or without miR-424. The list of miR-424 repressed genes was then merged with predicted miR-424 targets nominated using multiple prediction algorithms to enrich for putative direct targets. Predicted targets converged significantly with miR-424 repressed genes ( $p\text{-value} = 2.8 \times 10^{-16}$ ) yielding a total of 323 putative targets (Figure 33a). Intriguingly, functional annotation analysis revealed among the 323 putative targets a prevalence of genes encoding factors involved in protein ubiquitination and particularly components of the E3 ubiquitin ligase complexes (Figure 33b and c). E3 ubiquitin ligase complex components are emerging as important tumor suppressor or oncogenic factors and are deregulated as consequence of genetic events in various cancers, including prostate cancer (Migliorini et al., 2011; Theurillat et al., 2014; Vitari et al., 2011).



**Figure 33. Analysis of miR-424 putative targets.** (a) Venn diagram of targets predicted using *miRWalk* database and genes repressed by miR-424 in RWPE1 cells. (b) Functional annotation analysis by DAVID of the putative miR-424 target genes. (c) miR-424 putative target genes encoding for protein involved in ubiquitination processes as obtained by DAVID analysis. The predicted target evaluated in this study (RFWD2/COP1) is highlighted in red.

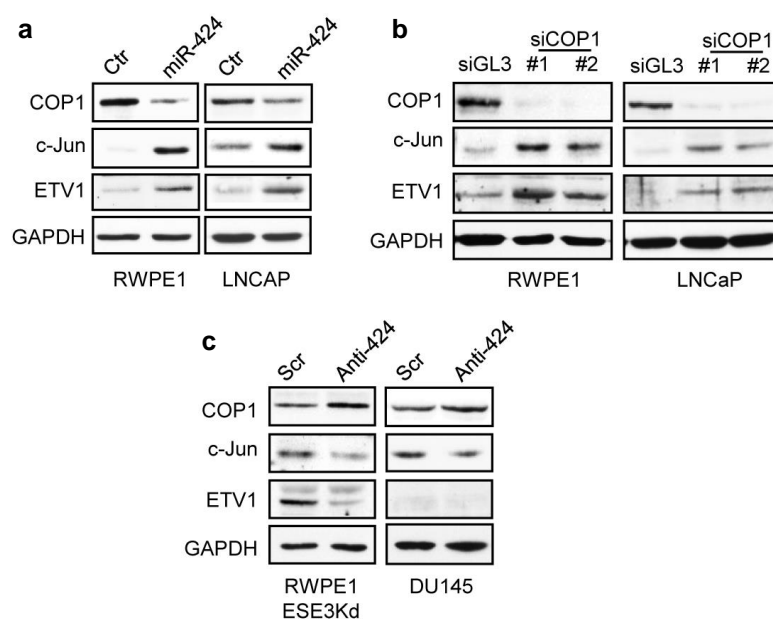
Among the top miR-424 putative targets we focused our attention on the E3 ubiquitin ligase COP1 (also called RFWD2, Ring Finger And WD Repeat Domain 2) (Figure 33c) that was already reported to act as tumor suppressor in prostate cancer negatively regulating the stability of oncogenic proteins, such as c-Jun and ETV1 (Migliorini et al., 2011; Vitari et al., 2011). Furthermore, COP1 expression was directly correlated with ESE3/EHF and inversely correlated with miR-424 levels in our panel of prostate cell lines (Figure 34a) suggesting that ESE3/EHF and miR-424 deregulation resulted in down-regulation of COP1. In support of this hypothesis, we identified a highly conserved miR-424 target sequence in the 3'UTR of COP1 mRNA using *miRWalk* browser (Figure 34b *left*). In order to validate COP1 as a target of the miRNA, we generated a reporter construct containing COP1 3'UTR fused to the luciferase gene. miR-424 overexpression inhibited the activity of COP1 3'UTR reporter containing the wild type sequence, while the mutation of the target sequence prevented this effect, indicating that miR-424 acted directly on the 3'UTR to block COP1 expression (Figure 34b *right*).



**Figure 34. miR-424 targets the E3 ubiquitin ligase COP1.** (a) mRNA level of COP1 evaluated by qRT-PCR (*upper*) and immunoblotting (*lower*) in prostate cell lines. (b) Diagram of 3'UTR COP1 reporter construct with wild type (WT) and mutated (MUT) sequence of the miR-424 binding site (*left*) and Relative Luciferase Activity (RLA) following transfection of miR-424 or control negative (Ctrl) in LNCaP cells (*right*). \*\*p ≤0.01; n.s. not significant.

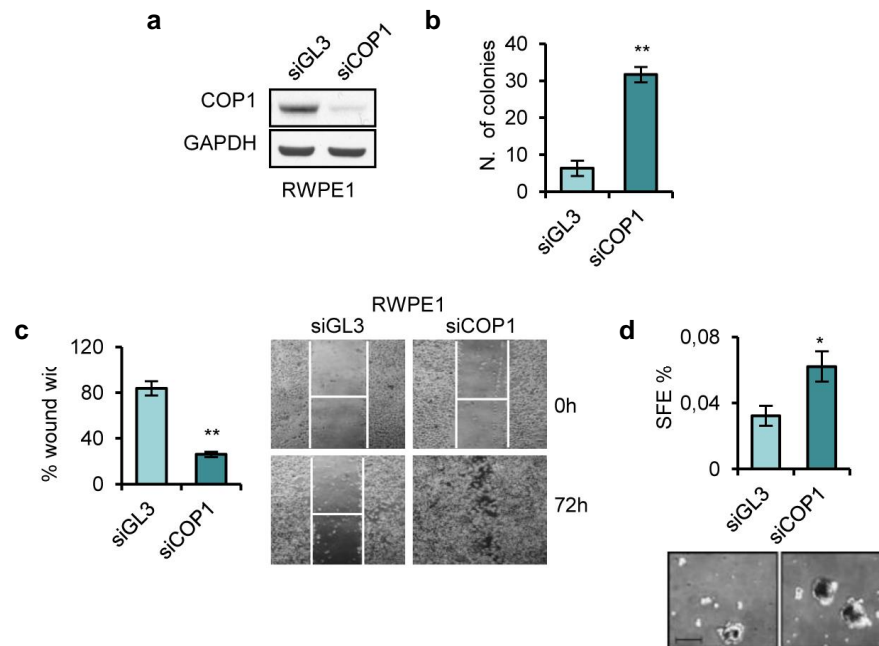


Consistently, transfection of miR-424 decreased COP1 protein level and increased concomitantly the known COP1 targets c-Jun and ETV1 (Migliorini et al., 2011; Vitari et al., 2011) in RWPE1 and LNCaP cells (Figure 35a). Knockdown of COP1 had the same effect on c-Jun and ETV1 expression (Figure 35b). On the other hand, anti-miR-424 increased COP1 and decreased ETV1 or c-Jun in the miR-424 expressing cells RWPE1-ESE3kd and DU145 cells (Figure 35c).

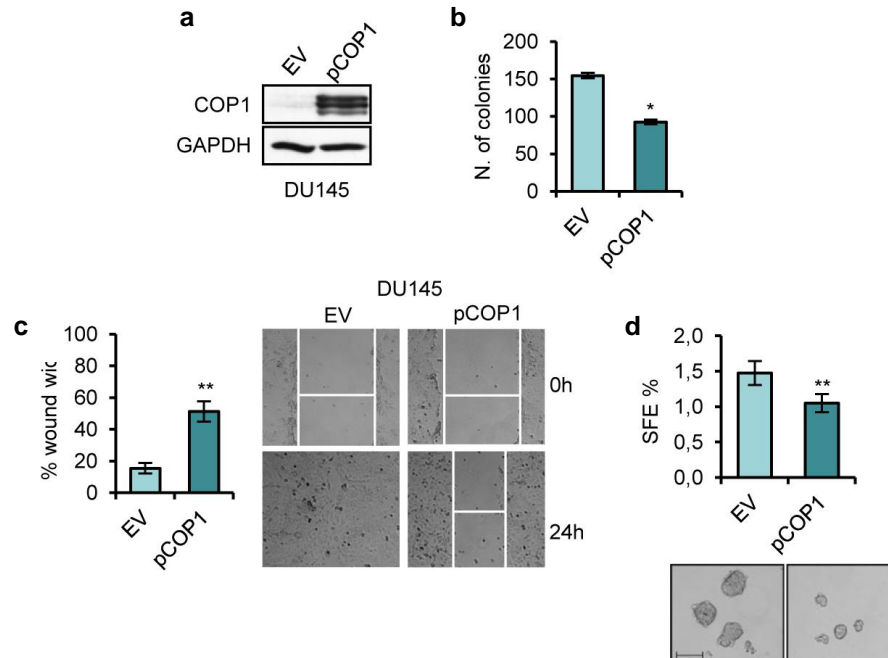


**Figure 35. miR-424 regulates COP1 expression in prostate cells.** (a) COP1, c-Jun and ETV1 expression in RWPE1 cells following transient transfection of miR-424 (*left*) and in LNCaP cells stably overexpressing miR-424 (*right*). (b) COP1, c-Jun and ETV1 expression following transfection of siRNAs targeting COP1 (siCOP1 #1 and #2) or control siRNA (siGL3) in RWPE1 (*left*) and LNCaP cells (*right*). (c) COP1, c-Jun and ETV1 expression following transfection of Anti-424 or Scr in RWPE-ESE3kd (*left*) and DU145 cells (*right*).

Notably, COP1 down-regulation was important for the phenotypic effects of miR-424. Indeed, knockdown of COP1 by siRNA reproduced the effect of miR-424 overexpression on anchorage-independent growth and migration and affected prostate-sphere formation in RWPE1 cells (Figure 36a-d). Conversely, expression of COP1 in DU145 cells reduced anchorage-independent growth, migration and prostate-sphere formation (Figure 37a-d). Thus, modulation of COP1 level affected the major phenotypes associated with deregulation of miR-424, indicating that COP1 was a key target of this oncogenic axis.



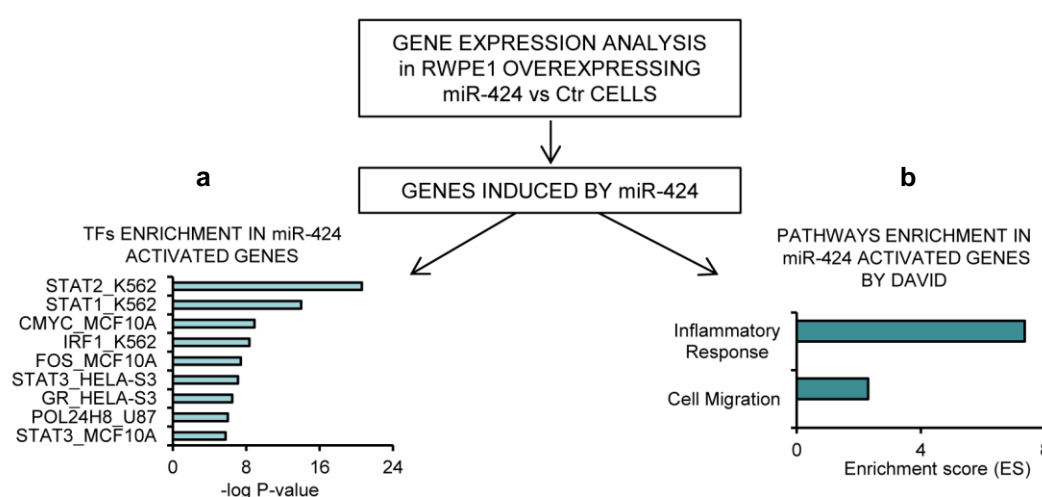
**Figure 36. Knockdown of COP1 mimics the effects of miR-424 overexpression.** (a) Immunoblot of COP1 48h following transfection of siRNA against COP1 (siCOP1#1) and control (siGL3) in RWPE1 cells. (b-d) Colony formation in soft agar (b), cell migration by WH (c), SFE (d) in RWPE1 cells following knockdown of COP1 by siCOP1 compared to control cells (siGL3). Representative pictures of wound width (c right) and images of spheroids are shown (d lower). Data show mean  $\pm$  s.d. (n=3) of one representative experiment. \*p $\leq$ 0.05; \*\*p $\leq$ 0.01 by two-tailed Student's t-test.



**Figure 37. COP1 overexpression reverts malignant phenotypes in DU145 prostate cancer cells.** (a) Immunoblot of COP1 48h following transfection of FLAG-COP1 (pCOP1) or empty-vector (EV) in DU145 cells. (b-d) Colony formation in soft agar (b), cell migration by WH (c) and SFE (d) in DU145 cells following overexpression of pCOP1 compared to EV. Representative pictures of wound width (c right) and images of spheroids are shown (d lower). Data show mean  $\pm$  s.d. (n=3) of one representative experiment. \*p $\leq$ 0.05; \*\* p  $\leq$ 0.01 by two-tailed Student's t-test.

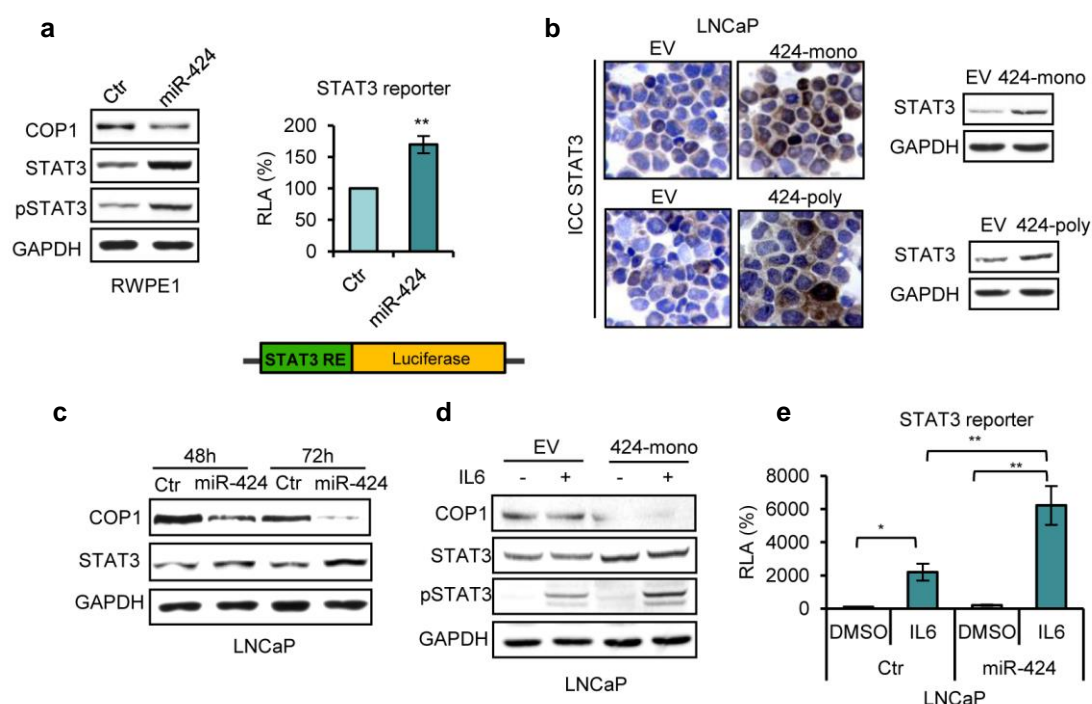
**miR-424 increases STAT3 protein level**

Our results show that down-regulation of the E3 ubiquitin ligase COP1 by miR-424 resulted in increased levels of c-Jun and ETV1 and had major phenotypic effects in prostate epithelial cells. Both c-Jun and ETV1 are transcription factors (TFs) with known oncogenic functions in human cancers, including prostate cancer (Chi et al., 2010; Migliorini et al., 2011; Tomlins et al., 2007; Vitari et al., 2011). Interestingly, our gene expression analysis revealed that miR-424 up-regulation is associated with a broad transcriptional reprogramming both in prostate epithelial cells and tumors. Therefore, we hypothesized that miR-424 overexpression could lead to accumulation and increased activity of other oncogenic TFs through a similar mechanism. To further investigate this aspect, we applied the Encode TF-Chip tool to identify the transcription factors (TFs) preferentially associated with the genes significantly up-regulated following miR-424 overexpression (miR-424 activated genes) in RWPE1 cells (Figure 36a). Interestingly, the top enriched TFs included most of the members of the Signal Transducer and Activator of Transcription (STAT) family of TFs, such as STAT3 (Figure 38a). Abnormal activation of STAT3 signaling is implicated in the progression of many types of cancer, including PCa (Kroon et al., 2013; Mora et al., 2002; Schroeder et al., 2014; Yu et al., 2014a). In addition, functional annotation analysis of miR-424 activated genes in RWPE1 cells showed significant enrichment of inflammatory and migration-related pathways (Figure 38b), features known to be associated with STAT3 activation (Yu et al., 2009). Based on these findings we decided to assess whether STAT3 could be involved in the oncogenic activity of miR-424 in prostate tumors.



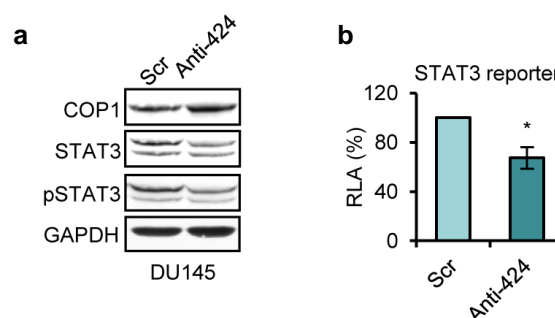
**Figure 38. miR-424 activated genes are associated with TFs belonging to the STAT family and with inflammatory pathways. (a)** TF analysis performed by Encode-ChIP tool and **(b)** most enriched biological processes of the Gene Ontology (GoBP) terms obtained using DAVID tool on genes induced by miR-424 in RWPE1 cells. See Method for details.

In support of this hypothesis, transfection of miR-424 in RWPE1 cells increased STAT3 and pSTAT3 (Tyr705-phosphorylated STAT3) protein level and concomitantly enhanced STAT3 transcriptional activity evaluated by a reporter construct (Figure 39a). A significant increase of total STAT3 protein was observed also in LNCaP cells stably expressing miR-424 (Figure 39b) and following miR-424 transient transfection (Figure 39c). In this cell line pSTAT3 expression is not detectable in basal condition, but only following cytokine stimulation. Thus, in order to observe the effect of miR-424 on STAT3 phosphorylation in this context, we stimulate LNCaP cells with the cytokine interleukin 6 (IL6). Consistently, a stronger induction of pSTAT3 protein level by IL6 was observed in miR-424 expressing LNCaP cells compared to controls (Figure 39d), resulting in hyper-induction of STAT3 reporter activity (Figure 39e). These results suggest that the presence of a higher level of STAT3 protein enhances STAT3 activity in response to IL6 stimulation.



**Figure 39. miR-424 increases STAT3 level and enhances basal and cytokine-induced STAT3 activation.** (a) Immunoblot (IB) of COP1, STAT3, and pSTAT3 (left) and Relative Luciferase Activity (RLA) of STAT3 reporter (right) in RWPE1 cells following transfection of miR-424 or control (Ctr). A schematic diagram of the reporter construct is also shown. RE, Responsive element. (b) STAT3 level in LNCaP-424 monoclonal and polyclonal stable cell lines by immunocytochemistry (ICC, left) and IB (right). (c) IB of COP1 and STAT3 in LNCaP cells after transient transfection of miR-424 or control (Ctr) at the indicated time point. (d) IB of COP1, STAT3 and pSTAT3 in control (EV) and LNCaP monoclonal cells with or without IL6 (10 ng/mL) stimulation. (e) RLA of STAT3 reporter in cells transfected with miR-424 or negative control (Ctr) with or without IL-6 stimulation (10 ng/mL). Values are show relative to the control cells (Ctr) treated with DMSO. \*p<0.05; \*\*p ≤0.01 by two-tailed Student's t-test.

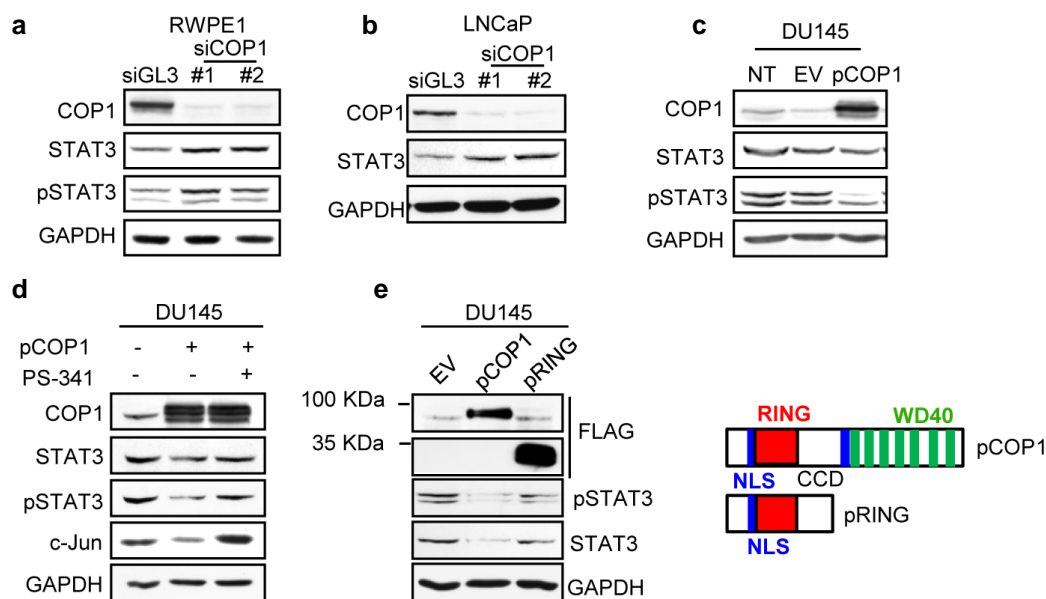
Conversely, anti-miR-424 slightly reduced total STAT3 and pSTAT3 along with STAT3 reporter activity in DU145 cells (Figure 40a and b). Overall, these results confirmed that miR-424 overexpression leads to increased STAT3 expression and activity.



**Figure 40. miR-424 inhibition reduces STAT3 expression and activity in DU145 prostate cancer cells.** Immunoblot of COP1, STAT3 and pSTAT3 (a) and RLA of STAT3 reporter (b) in DU145 cells following transfection with Anti-424 or Scr control. \*p ≤ 0.05 by two-tailed Student's t-test.

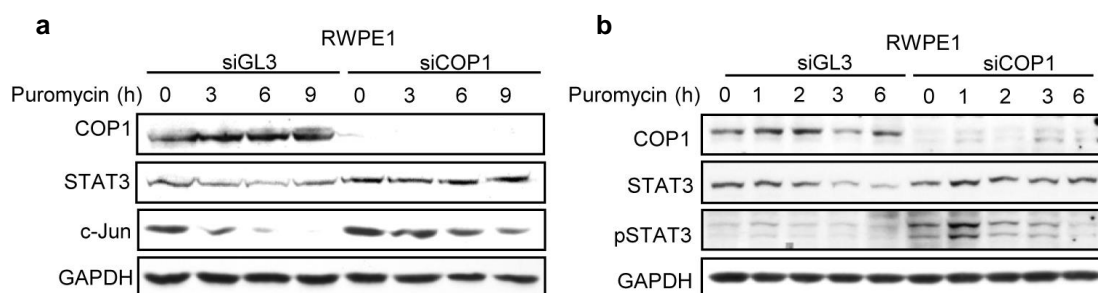
### ***The E3 ubiquitin ligase COP1 controls STAT3 protein level and turnover***

The effects observed on STAT3 were concomitant with the reduction of COP1 mediated by miR-424 (Figure 39a and c, 40a). Thus, we hypothesized that these effects could depend on reduced COP1 ubiquitin ligase activity. In support of a direct link between COP1 and STAT3, knockdown of COP1 by siRNAs increased STAT3 level in RWPE1 (Figure 41a) and LNCaP cells (Figure 41b). Conversely, ectopic expression of COP1 reduced STAT3 in DU145 cells similarly to anti-miR-424 treatment (Figure 41c). This suggested that STAT3 could be a substrate of the E3 ligase activity of COP1 leading to its ubiquitination and degradation by the ubiquitin-proteasome system (UPS). Consistently, proteasome inhibition by PS-341 prevented STAT3 down-regulation induced by COP1 in DU145 cells (Figure 41d). Furthermore, expression of a deletion mutant of COP1 (pRING) unable to bind and ubiquitinate substrate proteins (Migliorini et al., 2011) did not affect STAT3 level (Figure 41e).



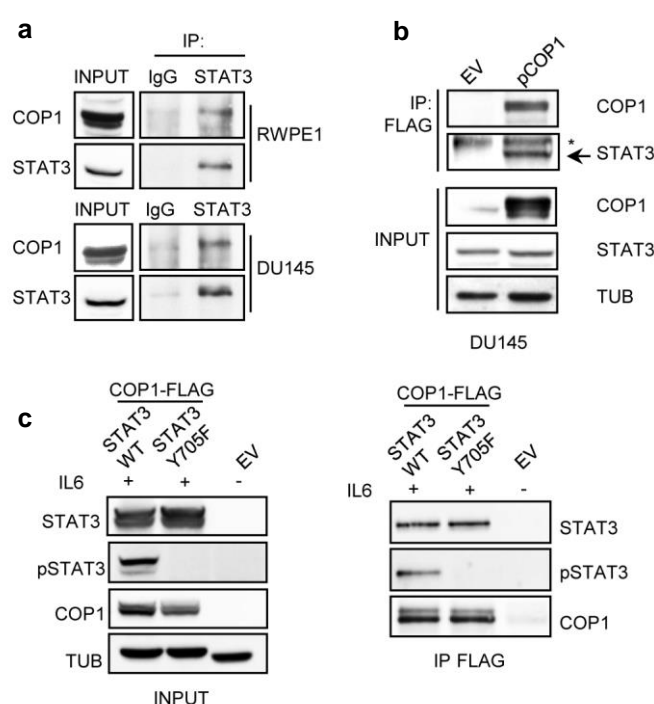
**Figure 41. The E3 ubiquitin ligase COP1 regulates STAT3 protein level.** (a and b) IB of COP1, STAT3 and pSTAT3 in RWPE1 (a) and LNCaP (b) cells after COP1 silencing. (c) IB of COP1, STAT3 and pSTAT3 in DU145 cells after transfection with full length FLAG-COP1 (pCOP1) or empty vector (EV). (d) IB of DU145 cells transfected with pCOP1 with or without treatment with proteasome inhibitor PS-341 (10 $\mu$ M) for 5h. c-Jun was used as control. (e) IB of FLAG, STAT3 and pSTAT3 in DU145 transfected with EV, pCOP1 or a deletion mutant (pRING). A schematic diagram of the constructs used is also shown; NLS, nuclear localization sequence; CCD, coiled coil domain; RING, RING-finger domain; WD40, WD40 repeat-containing domain.

To further assess the role of COP1 in the regulation of STAT3 degradation, we evaluated the effects of COP1 silencing on STAT3 protein stability. COP1 knockdown delayed significantly the turnover of STAT3 in RWPE1 cells treated with an inhibitor of protein synthesis (puromycin), indicating that COP1 is an important regulator of STAT3 degradation in prostate epithelial cells (Figure 42a and b).



**Figure 42. COP1 silencing delays STAT3 turnover.** (a and b) Immunoblot of COP1 and STAT3 in RWPE1 cells transfected with siCOP1#1 or siGL3 and treated with puromycin (50 $\mu$ M) for the indicated time points. In (a) c-Jun was evaluated as control for the effectiveness of the treatment. In (b) the experiment was done at different time points in order to observe also the effect on the pSTAT3, which has a shorter half-life.

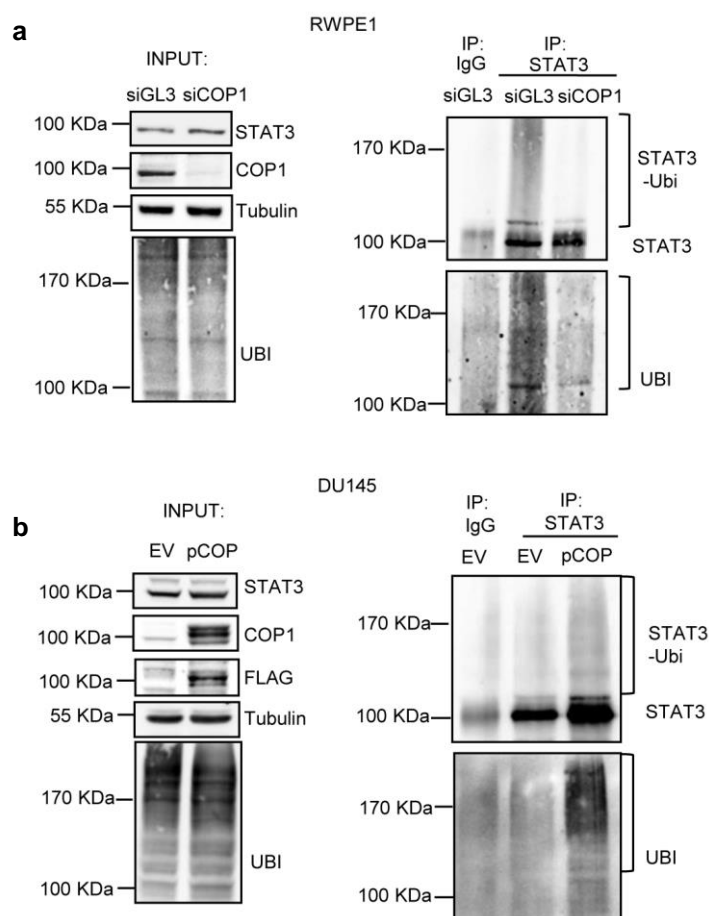
Consistently with a direct effect on STAT3, COP1 and STAT3 interacted in RWPE1 and DU145 cells in co-immunoprecipitation experiments (Figure 43a and b). Indeed, immunoprecipitation with anti-STAT3 antibody led to co-precipitation of endogenous COP1 (Figure 43a). Similarly, immunoprecipitation of exogenous COP1 led to co-precipitation of STAT3 (Figure 43b). Interestingly, co-immunoprecipitation experiments performed with wild type STAT3 and a phosphorylation defective STAT3 mutant (STAT3 Y705F), in which the tyrosine residue essential for STAT3 activation was mutated, showed that COP1 binds to both phosphorylated and unphosphorylated protein. Therefore, the interaction with COP1 did not depend on the STAT3 phosphorylation status (Figure 43c).



**Figure 43. COP1 physically interacts with STAT3.** (a) Immunoprecipitation (IP) of endogenous STAT3 followed by immunoblot (IB) of COP1 and STAT3 in RWPE1 and DU145 cells. IgG antibody was used as negative control. (b) IP of exogenous COP1 (pCOP1) with an anti-FLAG antibody followed by IB with anti-STAT3 antibody in DU145 cells transfected with the indicated plasmids and treated with PS-341 (10 $\mu$ M for 6h). Asterisk indicates non-specific bands. (c) IB of STAT3 and COP1 in HEK293T cells transfected with FLAG-COP1 and EGFP-STAT3 wild type (WT) or the mutant construct lacking phosphorylation on Y705 (Y705F). COP1 was immunoprecipitated with anti-FLAG antibody and IB was performed using indicated antibodies. Cells were stimulated with 10 ng/mL IL6 for 45 minutes and treated with PS-341 10 $\mu$ M for 3h. TUB, tubulin. INPUT, total cell lysate.

To confirm that STAT3 was a direct target of COP1-mediated ubiquitination we performed immunoprecipitation of STAT3 in denaturing conditions following COP1 modulation. Ubiquitinated STAT3 was detected in RWPE1 cells and its level was reduced after COP1 knockdown (Figure 44a). Conversely, ubiquitinated STAT3 increased in DU145 cells following expression of COP1 (Figure

44b). Collectively, these data provided evidence of COP1 dependent ubiquitination and proteasome-mediated degradation of STAT3 in prostate epithelial cells.



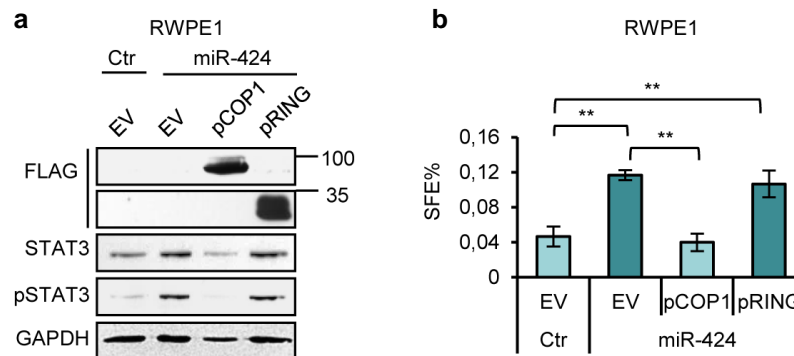
**Figure 44. COP1 E3 ubiquitin-ligase mediates STAT3 ubiquitination in prostate cells.** (a) STAT3 ubiquitination analyzed in RWPE1 cells transfected with siCOP1#1 or siGL3 for 48h. Denatured lysates were subjected to IP with anti-STAT3 antibody or IgG followed by IB with the indicated antibodies. UBI, Ubiquitin. (b) STAT3 ubiquitination analyzed in DU145 cells transfected with control vector (EV) or COP1 (pCOP1) for 48h and treated with PS-341 (10 $\mu$ M for 3h). Denatured lysates were subjected to IP with anti-STAT3 antibody or IgG followed by IB with the indicated antibodies. UBI, Ubiquitin. STAT3-Ubi, ubiquitinated STAT3. INPUT, total cell lysate.

### ***miR-424 induces STAT3 expression and promote malignant transformation by COP1 repression***

Based on these evidences, we hypothesize that the increase in STAT3 expression following miR-424 overexpression could be mediated by the repression COP1 E3 ubiquitin ligase. In line with our hypothesis, transfection of COP1 in RWPE1 cells prevented the induction of STAT3 and pSTAT3 resulting from miR-424 transient expression (Figure 45a) and concomitantly blocked the prostatosphere-forming capability promoted by the miRNA (Figure 45b). On the other hand, the COP1 deletion mutant construct was ineffective underlying that COP1 interaction to the target and its ubiquitin ligase activity are relevant in mediating these effects (Figure 45a and b). These results

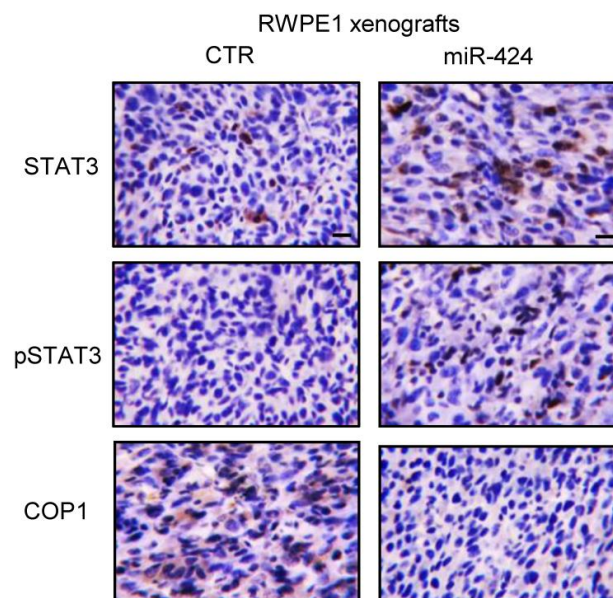


additionally indicated that increased STAT3 expression due to COP1 repression is a critical event in the induction of malignant phenotypes by miR-424.



**Figure 45. miR-424 induces STAT3 and promotes malignant phenotypes by COP1 repression.** (a) IB of STAT3 and pSTAT3 in RWPE1 cells following sequential transfection of miR-424 or control and pCOP1, the mutant construct (pRING) or empty vector (EV). (b) SFE of RWPE1 cells transfected as described in a. \*\*p ≤ 0.01 by two-tailed Student's t-test.

Consistently, we confirmed by immunohistochemistry that tumor xenografts generated by RWPE1 cells transfected with miR-424 exhibited increased levels of STAT3 and pSTAT3 and reduced level of COP1 (Figure 46) recapitulating *in vivo* the relationship between miR-424, COP1 and STAT3 observed *in vitro*. Collectively, these results demonstrate that repression of the E3 ubiquitin ligase COP1 represents an important mechanism in miR-424 induced oncogenesis.

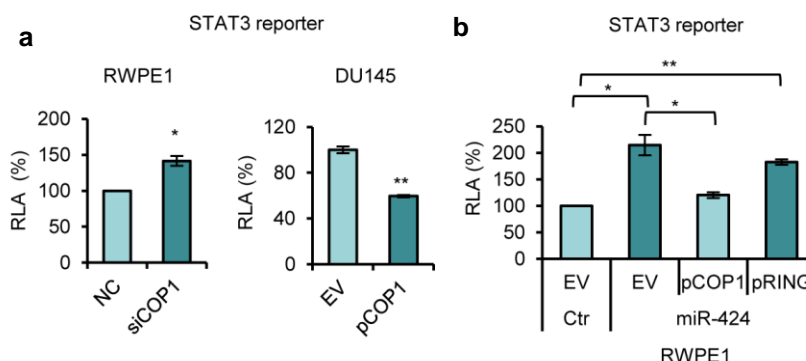


**Figure 46. Xenografts derived from miR-424 overexpressing cells show increased level of STAT3 and pSTAT3 and reduced level of COP1.** Immunohistochemical staining of STAT3, pSTAT3 and COP1 (brown) in xenografts derived from RWPE1 cells transiently transfected with miR-424 or control (Ctr). Cell nuclei were counterstained with haematoxylin (blue). Scale bars: 10 μm.

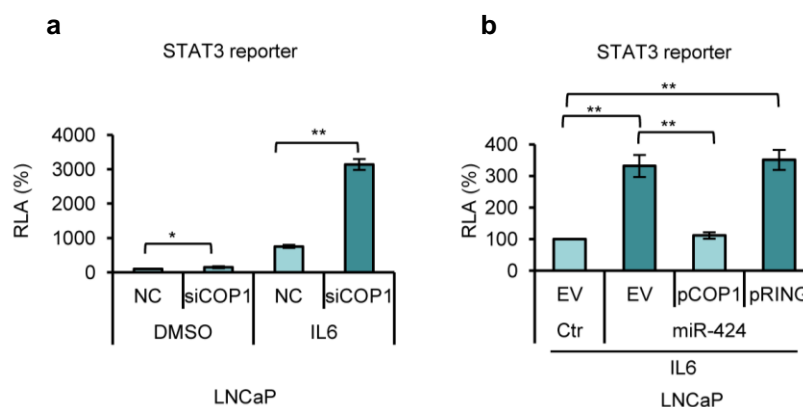
### ***COP1-mediated alteration of STAT3 turnover affects basal and cytokine-induced STAT3 activity***

Next, we assessed whether the increase in protein level induced by loss of COP1 resulted also in increased STAT3 activity, as observed following miR-424 overexpression. Knockdown of COP1 by siRNA increased the reporter activity in RWPE1 cells (Figure 47a *left*), while COP1 expression in DU145 cells decreased it (Figure 47a *right*). Moreover, in RWPE1 cells miR-424-induced STAT3 activity was blocked by the concomitant expression of wild type COP1 but not by the RING deletion mutant (Figure 47b). These results confirmed that the increase in STAT3 activity induced by miR-424 overexpression was a direct consequence of COP1 repression and impaired STAT3 degradation.

We similarly assessed the role of COP1 on cytokine-induced STAT3 activity in LNCaP cells. Knockdown of COP1 slightly increased STAT3 activity in unstimulated LNCaP cells (Figure 48a). However, COP1 silencing followed by treatment with IL6 resulted in hyper-induction of the reporter activity, mimicking the effect of miR-424 on STAT3 described above. Furthermore, expression of wild type COP1, but not the RING mutant, prevented STAT3 activation by IL6 in LNCaP cells transfected with miR-424 (Figure 48b). Thus, controlling STAT3 protein turnover, COP1 affected the level of both basal and cytokine-induced STAT3 activity.



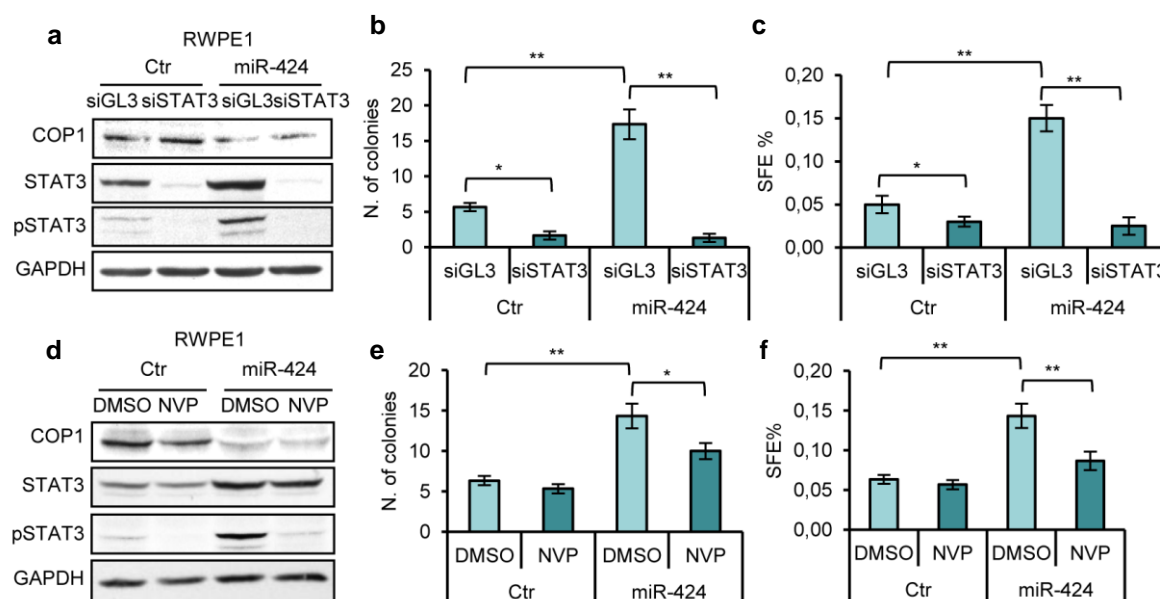
**Figure 47. miR-424 induces STAT3 activity by targeting COP1.** (a) RLA of STAT3 reporter in RWPE1 cells following transfection of siCOP#1 or negative control (NC) (*left*) and in DU145 cells after transfection with pCOP1 or empty vector (EV) (*right*). (b) RLA of STAT3 reporter following sequential transfection of miR-424 and pCOP1, the mutant construct (pRING) or EV. The values are normalized to the control cells (Ctr) transfected with EV. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.



**Figure 48. miR-424 increases cytokine-induced STAT3 activity by targeting COP1.** (a) RLA of STAT3 reporter following transfection with siCOP1#1 or negative control (NC) with or without IL6 stimulation (10 ng/mL). The values are normalized to the control cells (NC) treated with DMSO. (b) RLA of STAT3 reporter following sequential transfection of miR-424 and pCOP1, pRING or EV in presence of IL6 stimulation (10 ng/mL). The values are normalized to the control cells (Ctr) transfected with EV. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

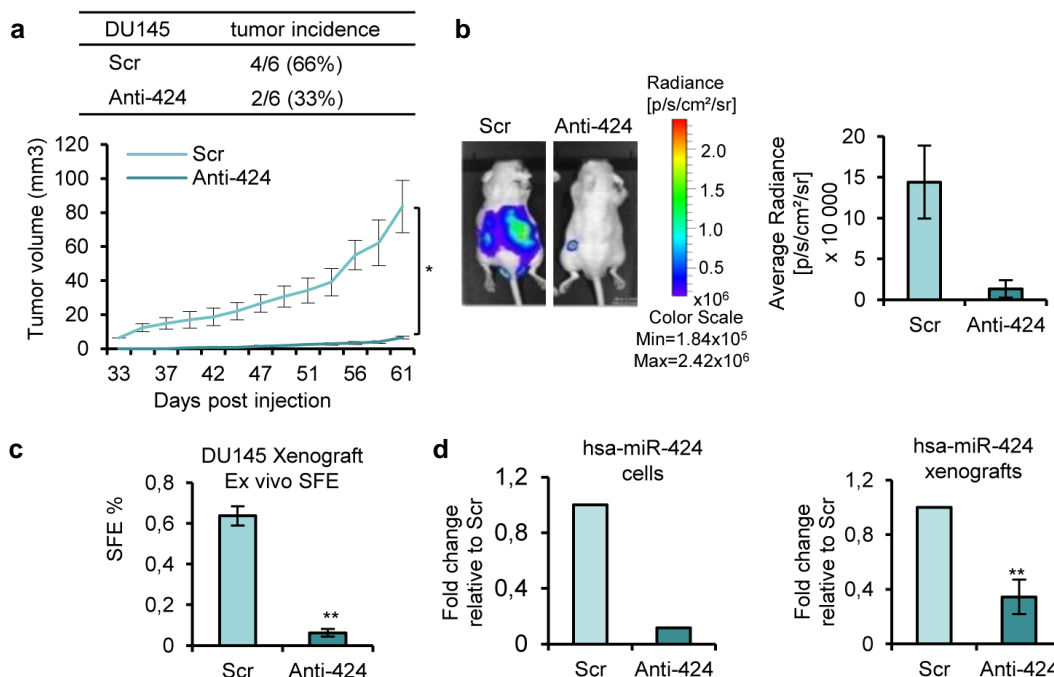
### ***Therapeutic strategies for miR-424 high prostate cancers***

Our data indicated that decreased STAT3 protein degradation by COP1 led to STAT3 activation and enhanced responsiveness to cytokine stimulation in miR-424 expressing cells. STAT3 is known to confer tumorigenic and cancer stem cell-like properties in various tumor types, including prostate cancer (Kroon et al., 2013; Schroeder et al., 2014; Yu et al., 2014a). Consistent with a key role of STAT3 in mediating the effects of miR-424, knockdown of STAT3 by siRNA blocked the induction of anchorage-independent growth and prostate-sphere formation resulting from miR-424 overexpression in RWPE1 cells (Figure 49a-c). Interestingly, the Janus kinase (Jak) inhibitor NVP-BSK805, which prevented selectively STAT3 phosphorylation (Baffert et al., 2010), also partially decreased the effects of miR-424 on growth in soft agar and prostato-sphere formation (Figure 49d-f). Thus, depletion or inhibition of STAT3 could be an effective therapeutic strategy in tumors with deregulated miR-424/COP1/STAT3 axis.



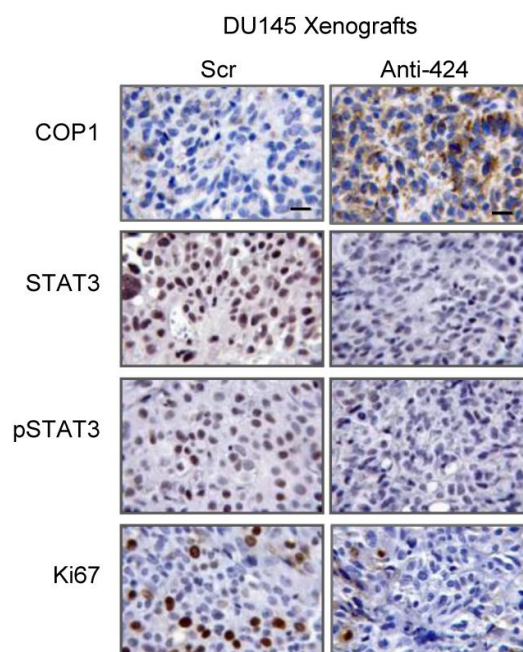
**Figure 49. Genetic and pharmacologic inhibition of STAT3 revert miR-424 induced transformation.** (a-c) Immunoblot (IB) of COP1, STAT3 and pSTAT3 (a), colony formation in soft agar (b) and SFE (c) in RWPE1 cells following co-transfection with miR-424 or Ctr and siRNA targeting STAT3 (siSTAT3) or control siRNA (siGL3). (d-f) IB of COP1, STAT3 and pSTAT3 (d), colony formation in soft agar (e) and SFE (f) in RWPE1 cells 48 h following transfection with miR-424 or Ctr and treatment with NVP 5  $\mu$ M or DMSO for 16h. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  by two-tailed Student's t-test. The experiments were performed in triplicate and data are shown as mean  $\pm$  s.d of one representative experiment.

In line with this notion, we investigated whether targeting miR-424 and reactivating COP1-mediated degradation of STAT3 using a miRNA antagonist could be a powerful therapeutic strategy in this context. To test this hypothesis, we assessed the tumor initiating capability of DU145 prostate cancer cells transfected with anti-miR-424 or a scrambled control and subcutaneously injected in immunodeficient mice. Anti-miR-424 transfected cells showed a reduced tumor incidence compared to control cells (Figure 50a). Moreover, *in vivo* growth of anti-miR-424 transfected cells was considerably impaired (Figure 50a and b). Furthermore, tumor cells harvested from anti-miR-424 treated tumors, exhibited *ex vivo* considerably reduced prostato-sphere forming ability, indicating a persistent loss of cancer stem cell-like and self-renewal properties (Figure 50c). Notably, miR-424 level was persistently low in anti-miR-424 treated xenografts compared to control xenografts (Figure 50d).



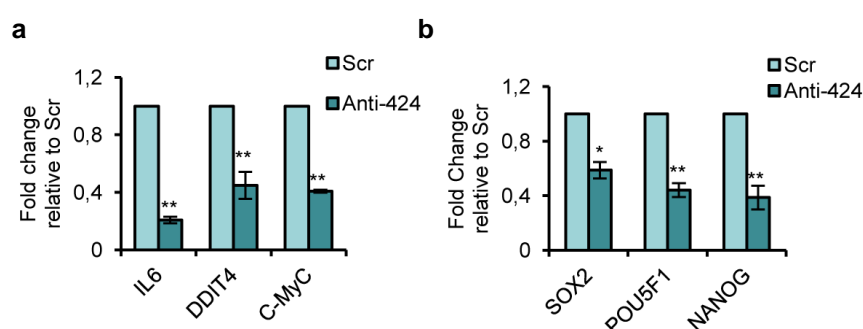
**Figure 50. miR-424 inhibition reduces tumor initiation and tumor growth in vivo.** (a and b). Tumor incidence (a, upper) of subcutaneous xenografts of DU145-Luc cells pretreated *in vitro* with anti-miR-424 (n=6) or Scr control (n=6) and tumor growth monitored by tumor size (mean  $\pm$  s.e.m) (a, lower) and *in vivo* bioluminescence (b). (c) *Ex vivo* SFE from Scr or Anti-424 dissociated tumor xenografts. (d) miR-424 level was evaluated by qRT-PCR in DU145 cells transfected with anti-miR-424 or Scr control before the injection in nude mice (left) and in tumor cells harvested from the xenografts obtained at the end of the experiment (right). The results were normalized to RNU6 level and represented as fold change compared to control (Scr) treated cells. \*p  $\leq$  0.05; \*\*p  $\leq$  0.01 by two-tailed Student's t-test.

Immunohistochemistry confirmed the reactivation of COP1 expression and reduced levels of both STAT3 and pSTAT3 in tumor xenografts of anti-miR-424 treated cells (Figure 51), further demonstrating *in vivo* the relationship between miR-424, COP1 and STAT3 and the effects of miR-424 inhibition shown *in vitro*. Furthermore, staining for the proliferation marker Ki67 was reduced in tumor xenografts generated by anti-miR-424 treated cells.



**Figure 51. Tumor xenografts derived from Anti-424 treated cancer cells show increased COP1 expression and reduced level of both STAT3 and pSTAT3.** Immunohistochemical staining for STAT3, pSTAT3, COP1 and Ki67 (brown) in tumor xenografts of DU145 cells treated with Anti-424 or Scr. Cell nuclei were counterstained with haematoxylin (blue). Scale bars: 10  $\mu$ m.

Consistently with these findings, expression of STAT3 target genes and cancer stem cell-like markers was significantly reduced in DU145 tumor xenografts from anti-424 treated cells (Figure 52 a and b).

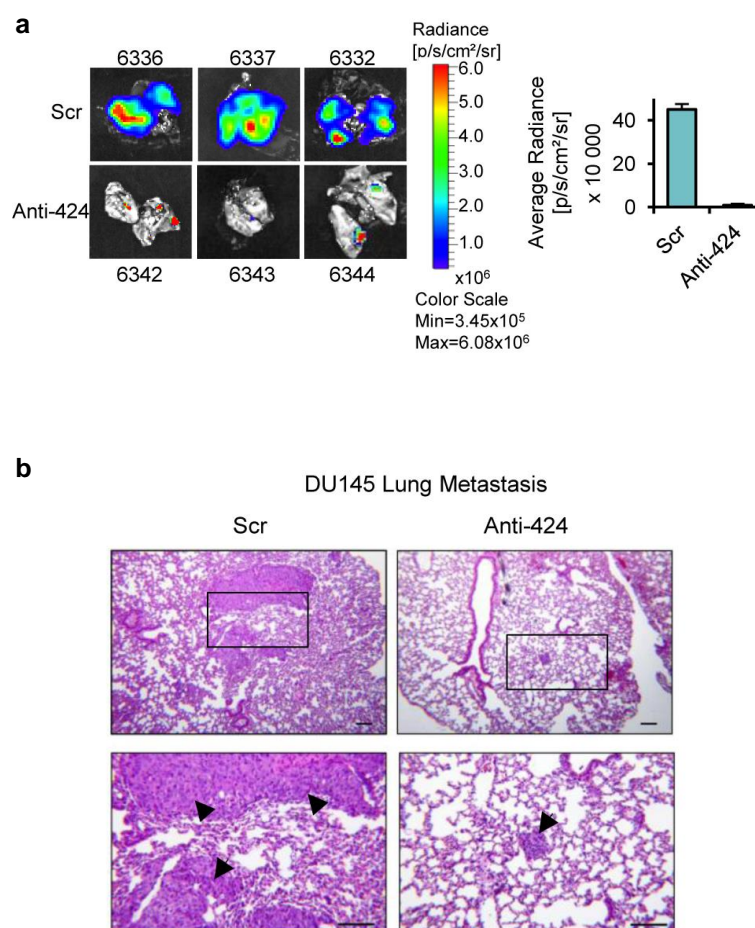


**Figure 52. Anti-424 treatment of prostate cancer cells reduces the expression of STAT3 target genes and cancer stem cell-like markers *in vivo*.** qRT-PCR evaluation of selected STAT3 targets (a) and selected cancer stem cell markers (b) in xenografts derived from control (Scr) and miR-424 ablated (Anti-424) cells. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  by two-tailed Student's t-test.

Importantly, inhibition of miR-424 by anti-miR compromised also the ability of DU145 cells to metastasize to the lung following injection into the tail vein of mice. Formation of lung metastasis by anti-miR-424 treated cells was almost completely prevented as shown by bioluminescence imaging and histopathology evaluation (Figure 53 a and b). These results indicated that miR-424



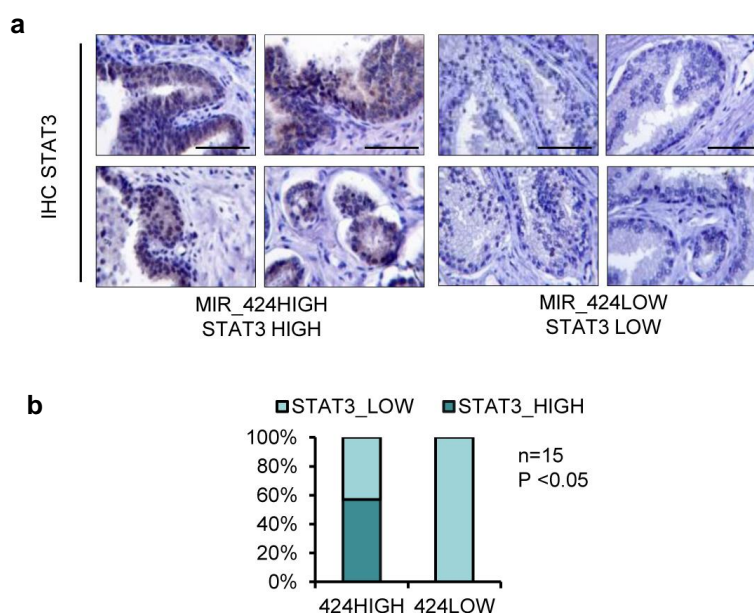
had a relevant impact on *in vivo* tumor growth, tumor-initiating and metastatic properties of prostate cancer cells. Collectively, these findings strongly support miR-424 as a potential therapeutic target in prostate cancer and suggest also the development of novel miRNA-based therapeutic strategies specific for prostate tumors characterized by elevation of miR-424 expression.



**Figure 53. miR-424 inhibition reduces metastatic capability of prostate cancer cells in vivo.** (a) Representative bioluminescent images (*left*) and relative quantification post-necropsy (*right*) of lung metastasis from DU145-Luc cells pretreated *in vitro* with Anti-424 or Scr and injected into the tail vein of nude mice. (b) Representative images of H&E staining of lung metastasis generated by DU145. Areas within the boxes in upper panels are magnified in the lower images. Arrowheads indicate metastatic lesions. Scale bars: 100 μm.

### ***COP1/STAT3 deregulation is associated with adverse prognosis in prostate cancer***

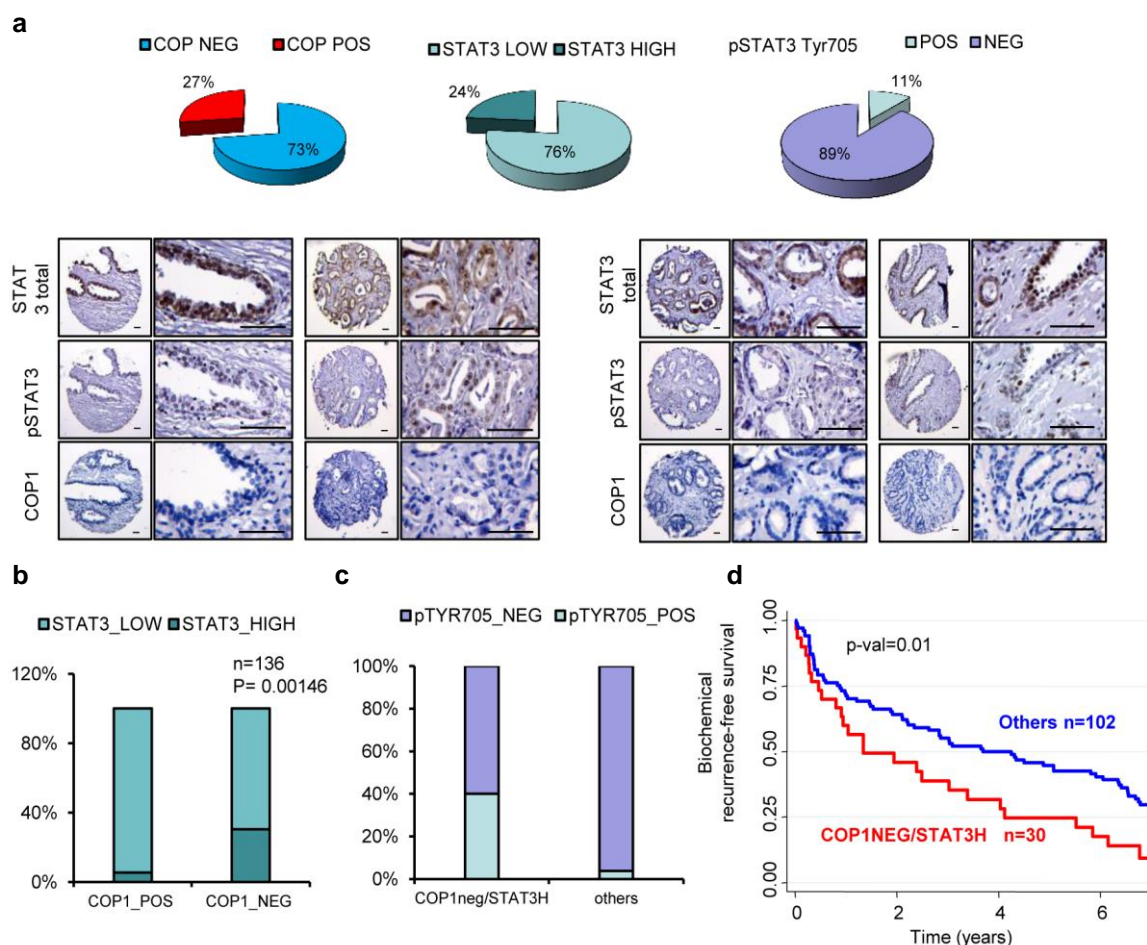
In support of the link between miR-424 and STAT3 observed in experimental models, we found a positive correlation (Fisher test  $p=0.025$ ) between high miR-424 expression determined by RT-qPCR and STAT3 level assessed by immunohistochemistry in a set of prostate tumors ( $n=15$ ) (Figure 54 a and b).



**Figure 54. miR-424<sub>high</sub> prostate tumors are associated with high STAT3 level.** (a) Representative IHC images of total STAT3 (brown staining) in a set of prostate tumors samples of the *Biella* dataset ( $n=15$ ), in which miR-424 level was previously evaluated by qRT-PCR. Cell nuclei were counterstained with haematoxylin (blue). (b) STAT3/miR-424 high/low association in the set prostate tumor samples analyzed. The t-test p-value is shown. Scale bars: 100 $\mu$ m.

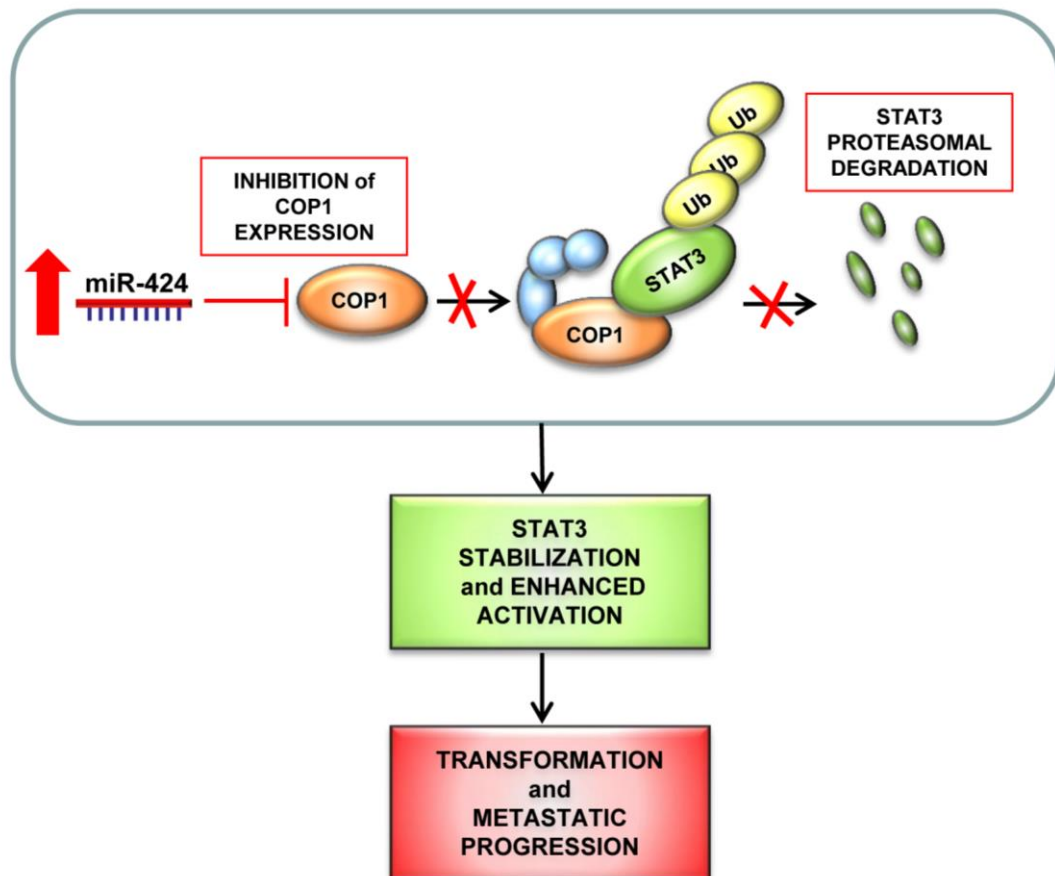
To further assess the clinical relevance of our findings, we examined the level of COP1, STAT3 and pSTAT3 by immunohistochemistry in primary prostate tumors ( $n=136$ ) from patients with long-term clinical follow up (Figure 55a). Low COP1 expression was significantly associated with high STAT3 level (Fisher test  $p<0.001$ ) (Figure 55b). In line with our *in vitro* data, pSTAT3 was prevalently observed in the group of COP1 negative/STAT3 high tumors indicative of STAT3 activation (Figure 55c). Furthermore, the combination of low COP1 and high STAT3 expression identified patients with a significantly higher risk of biochemical relapse after prostatectomy ( $p\leq 0.01$ ) (Figure 55d). Thus, loss of COP1 was associated with increased STAT3 level and their association had prognostic relevance.





**Figure 55. Prognostic impact of COP1 loss and STAT3 activation in prostate tumors.** (a) Immunohistochemistry (IHC) score of COP1 and STAT3 and pSTAT3 Tyr705 in Tissue Microarray (TMA) of primary prostate tumors ( $n=136$ ) (upper) and representative IHC images (lower). Scale bars: 100  $\mu\text{m}$ . (b) STAT3/COP1 and COP1negative/STAT3/pSTAT3 association analysis in the TMA. The t-test p-value is shown. (c) Association between pSTAT3 Tyr705 negative and positive tumors with COP1 and STAT3 staining (t test  $p<0.00001$ ). (d) Survival plots showing significant increase in biochemical recurrence in COP1 negative/STAT3 high tumors compared to the other tumors in the TMA cohort analyzed above. Log-rank test p-value is shown.

Collectively, these data support the notion that miR-424 has key oncogenic functions in prostate tumors largely related to the silencing of COP1 and activation of STAT3 and that miR-424/COP1/STAT3 oncogenic axis promote clinically aggressive prostate tumors (Figure 56).



**Figure 56. Proposed model for miR-424/COP1/STAT3 oncogenic axis.** Overexpression of miR-424 in prostate cells represses the expression of COP1 E3 ubiquitin ligase. Consequently, COP1-mediated ubiquitination and proteasomal degradation of STAT3 are reduced, leading to STAT3 protein stabilization. The increased STAT3 expression allows a stronger transcriptional activity on its target genes, thereby promoting cell transformation and metastatic progression.

## ***Discussion***

Clinical behavior of prostate cancer (PCa) ranges from indolent disease to very aggressive tumors that rapidly progress to castration-resistance (Nelson et al., 2003). However, the molecular mechanisms contributing to progression and clinical heterogeneity of PCa are still poorly understood (Shen and Abate-Shen, 2010). Thus, dissecting the diverse pathways that promote and sustain tumorigenesis in different tumor subtypes is an important task to identify new molecular markers and guide the development of novel therapeutic approaches for this disease (Beltran and Rubin, 2013). It is more and more accepted that induction of an Epithelial-Mesenchymal Transition (EMT) program and the acquisition of cancer stem-like properties play an important role in tumor progression and represent critical processes underlying metastatic spread, disease recurrence and treatment failure (Frank et al., 2011; Mani et al., 2008; Thiery et al., 2009). These features are also associated with aggressive, clinically resistant and metastatic subtype of prostate cancers (Kong et al., 2010; Sethi et al., 2010; Wang and Shen, 2011). A growing body of evidence establishes microRNAs as relevant components of the cellular signaling circuitry that regulates the EMT and cancer stem-like properties (Wang et al., 2010). In addition, aberrant miRNA expression has been correlated with tumor development, cancer progression and the acquisition of an EMT phenotype in tumor cells (Bonci et al., 2015; Wang et al., 2010). Importantly, miRNA expression patterns can distinguish different tumor subtypes and distinct stages of the disease providing clinically relevant diagnostic and prognostic information (Bonci et al., 2015; Iorio and Croce, 2012; Li et al., 2015).

Based on this evidence, in this study we particularly investigated miRNA-mediated mechanisms leading to dedifferentiation and stemness in prostate tumors. We previously reported that a subgroup of prostate tumors characterized by loss of the ETS factor ESE3/EHF (ESE3<sub>low</sub>) is associated with aggressive clinical features and enrichment of EMT and cancer stemness-related genes (Albino et al., 2012; Kunderfranco et al., 2010). In the present analysis, we evaluated the signature of miRNAs aberrantly expressed in ESE3<sub>low</sub> tumors and we identified miR-424 as a miRNA specifically overexpressed in this subgroup of prostate cancers and in cell lines with silencing of ESE3/EHF. Consistently, Gene Set Enrichment Analysis (GSEA) indicated that miR-424 was associated with mesenchymal and stem cell-like phenotypes in PCa samples evaluated. According to this findings, miR-424 over-expression in prostate cells induced EMT and tumorigenic properties, while inhibition of miR-424 reversed the transformed phenotype. Importantly, *in vitro* and *in vivo* experiments showed that transient expression of the miRNA is sufficient to induce a persistent malignant transformation in normal prostate epithelial cells. On the contrary, miR-424 inhibition impaired tumor-initiating and metastatic capability of prostate cancer cells, highlighting a strong oncogenic role of miR-424 in this context.

Furthermore, our results suggest that the ETS factor ESE3/EHF binds to the promoter region of miR-424 and represses its transcription in prostate epithelial cells. Therefore, loss of ESE3/EHF results in increased expression of the miRNA with the consequent acquisition of tumorigenic and stem-like properties. Interestingly, we found that miR-424 was also up-regulated in other epithelial cancers, such as breast and lung cancer, suggesting that it could exert similar functions in these tissues. ESE3/EHF is expressed in many normal epithelia and it is frequently down-regulated in carcinomas (Tugores et al., 2001), thus it is likely that the link between ESE3/EHF and miR-424 might exist in other epithelial normal and cancer tissues. However, the correlation of miR-424 and ESE3/EHF expression in other tumor types would need to be further examined.

It is relevant to notice that this is the first study identifying miRNAs regulated by the transcription factor ESE3/EHF. At present, only a handful of reports on miRNAs modulated by ETS factors have been published. As an example, ETV4 was shown to repress miR-125a and miRNA-200b, two negative regulators of EMT, in ovarian (Cowden Dahl et al., 2009; Nicholson et al., 2001) and breast cancer (Zhang et al., 2013), respectively. miR-126 presents multiple ETS factor binding sites (EBS) in its promoter region and was reported to be controlled by ETS1 and ETS2 (Harris et al., 2010), suggesting that the same miRNA can be regulated by different ETS factors. Similarly, another ETS transcription factor, PU.1, was previously reported to regulate miR-424 expression in monocytes and endothelial cells (Ghosh et al., 2010; Rosa et al., 2007) by binding to the same miR-424 promoter region described in our study. These findings are consistent with the notion that different ETS factors can control the transcription of the same gene, even sharing the binding region, and suggest that the transcription of miR-424 can be modulated depending on the network of ETS proteins expressed in a specific cell context.

miR-424 belongs to the miR-15/107 group (Finnerty et al., 2010). These miRNAs share a similar “seed region” in their 5'-UTR, which may result in some overlapping targets. Members of this family, such as miR-15 and miR-16, have been shown to act as tumor suppressor miRNAs in different tumor types and also in prostate cancer (Bonci et al., 2015; Fang and Gao, 2014). However, we found that miR-15, miR-16 and other members of the miR-15/107 family were not modulated in ESE3<sub>low</sub> tumors and cell lines, suggesting a different biological function and emphasizing the role of miR-424 in this specific context. Accordingly with our results, other recent studies showed that miR-424 is overexpressed in cancer patients and exert oncogenic functions. For instance, Zhang et al. demonstrated that miR-424 level is increased in breast cancer cells under hypoxic conditions and enhances tumor cell resistance to chemotherapy by targeting the anti-apoptotic gene PCDC4 (Zhang et al., 2014). An involvement of miR-424 in chemoresistance was also reported in ovarian cancer by Park and colleagues that found this miRNA overexpressed in a subpopulation of cancer stem cells (Park et al., 2013). A body of

evidence particularly supports the involvement of miR-424 in inducing cancer-related EMT and promoting metastasis. Li et al. showed that miR-424 expression is induced by TGF- $\beta$  in metastatic breast tumors and in turn enhances TGF- $\beta$  signaling suppressing two inhibitors of this pathway, Smad7 and Smurf2 (Li et al., 2014). Recently, Drasin et al. showed that this miRNA can be induced by the EMT regulator TWIST1 and its up-regulation is associated with the expression of EMT and cancer stem cell-like genes and increased cellular motility in breast cancer (Drasin et al., 2015). However, in this study miR-424 level was higher in primary tumor compared to normal samples, but lower in metastatic tissue. Thereby, they hypothesized that miR-424 plays distinct roles in tumor progression by regulating an EMT-MET axis and suggested the need for a biphasic expression of the miRNA: at first, increased expression of miR-424 in the primary tumor could allow cancer cells to undergo epithelial-mesenchymal transition and acquire the capability to disseminate, while later its down-regulation is needed for the metastatic colonization to occur. On the other hand, a tumor suppressor role was suggested for miR-424 in colon cancer (Oneyama et al., 2013) and in hepatocellular carcinoma (Yang et al., 2015; Yu et al., 2014b). In these types of tumor downregulation of miR-424 was reported to promote growth and invasion by upregulating different targets, such as c-Myb (Yu et al., 2014b), Akt3/E2F (Yang et al., 2015) and Rictor (Oneyama et al., 2013). These apparently conflicting observations suggest distinct functions of miR-424 in different tissues and cell types. Indeed, miRNAs can have multiple targets and their biological effects likely depend on the sets of genes that are affected in a specific cell context (Bartel, 2009). For example, no detectable alteration in Rictor expression was found in breast cancer cells following miR-424 overexpression, in contrast to what observed in colon cancer cells (Li et al., 2014). Therefore, the same miRNA may have oncogenic or tumor suppressor function depending on the cell type or tissue considered (Garzon et al., 2009b). Regarding prostate cancer, very few reports on miR-424 expression in this tumor type are present so far. In line with our data, miR-424 was recently reported to be expressed in metastatic DU145 cells promoting dramatic changes in morphology and in the expression of EMT-related markers, such as E-cadherin and Vimentin (Banyard et al., 2013). The authors suggested a pro-metastatic role of miR-424, however the possible targets mediating this effect were not elucidated.

In this scenario, our aim was to identify miRNA targets that could be specifically relevant in prostate epithelial cells. Given that the biological functions of miRNAs are mainly mediated by their ability to attenuate the expression of target mRNAs (Bartel, 2009), we integrated computational target predictions with gene expression analysis to retrieve mRNAs directly repressed by miR-424 in prostate cells. Interestingly, among the putative targets that showed reduction in mRNA levels following miR-424 overexpression, we identified several genes encoding proteins involved in ubiquitination processes, especially E3 ubiquitin ligase components. The

ubiquitin-proteasome system (UPS) regulates the ubiquitination, and thus the degradation and turnover of many important cellular proteins. In particular the role of E3 ubiquitin ligases in the modulation of cancer-related processes has emerged during the last decade (Micel et al., 2013) and some ubiquitin ligases with tumor suppressor function have been identified (Satija et al., 2013; Theurillat et al., 2014; Wang et al., 2012). Among the identified genes, we focused on the E3 ubiquitin ligase COP1 (or RFWD2) because its involvement in prostate cancer progression was recently hypothesized (Migliorini et al., 2011; Vitari et al., 2011). Through molecular and functional studies, we demonstrated that COP1 is a new biologically relevant target of miR-424 in prostate epithelial cells. Indeed, COP1 silencing mimicked the effects of miR-424 overexpression on anchorage independent growth, cell migration and prostato-sphere formation, while COP1 ectopic expression reverted these malignant phenotypes. COP1 was previously reported to act as a tumor suppressor by promoting the destruction of oncogenic proteins (Migliorini et al., 2011; Vitari et al., 2011). Genetic studies have shown that COP1 deficiency leads to spontaneous tumor formation in mice and have identified mutations in COP1 and its substrates in various human cancers (Migliorini et al., 2011; Vitari et al., 2011). More recently, COP1 tumor suppressor activity was also confirmed in breast cancer by *in vitro* and *in vivo* studies (Shao et al., 2013). Accordingly with our results, COP1 overexpression blocked *in vitro* cell migration and *in vivo* metastasis of invasive breast cancer cells. In addition, higher COP1 expression correlated with better recurrence-free survival in breast cancer patients (Shao et al., 2013). Although a significant decrease in COP1 expression was previously observed in several tumor types, including prostate carcinoma (Migliorini et al., 2011), the mechanism of COP1 down-regulation was in many cases unknown (Marine, 2012). Rare focal COP1 deletions (less than 8% in all cancer types analyzed) have been found in several types of cancer by Migliorini et al. (Migliorini et al., 2011). Additional analysis established the loss of one COP1 allele in these tumors and provided evidence for transcriptional silencing of the second allele by mechanisms that remained to be elucidated (Migliorini et al., 2011; Vitari et al., 2011). Our study further supports a tumor suppressor role of COP1 in prostate cancer progression and identifies miR-424 induced post-transcriptional silencing as a novel mechanism leading to inactivation of COP1.

From a mechanistic point of view, previous researches showed that COP1 exerts its tumor suppressor function by negatively regulating the stability of oncogenic transcription factors, the most important being c-Jun (Migliorini et al., 2011; Shao et al., 2013) and ETV1 (Vitari et al., 2011). C-Jun is frequently overexpressed in advanced human prostate cancers and induces tumorigenic capability in prostate cancer cells (Chen et al., 2006; Ouyang et al., 2008). Moreover, c-Jun expression promotes cell invasion and stem cell expansion in breast cancer cells (Jiao et al., 2010). Similarly, ETV1 overexpression induces cell migration and invasion and it is associated with

aggressive prostate cancer (Baena et al., 2013). In line with these notions, we found that miR-424 up-regulation increased c-Jun and ETV1 protein level by suppressing COP1 expression. In addition, the induction of migration, EMT and cancer stem-like phenotypes by miR-424 in prostate cells is consistent with the oncogenic properties of these transcription factors. Given that ETV1 translocation occurs in 2-6 % of prostate cancer (Tomlins et al., 2007), it is interesting to notice that we observed an increase in ETV1 level also in translocation negative RWPE1 cells. Thus, our data suggest an alternative mechanism leading to ETV1 up-regulation.

More importantly, in this study we identified STAT3 as a new substrate of COP1 E3 ubiquitin ligase. We provide several lines of evidence demonstrating that COP1 is responsible for STAT3 ubiquitination and proteasome-mediated degradation. Indeed, COP1 depletion by siRNAs enhanced STAT3 protein stability and diminished its ubiquitination in normal epithelial cells. On the contrary, COP1 ectopic expression increased STAT3 ubiquitination in metastatic cancer cells. In addition, we show that increased STAT3 protein level and altered turnover following miR-424 over-expression and silencing of COP1 increases STAT3 basal activity and enhances responsiveness to cytokine stimulation. Aberrant activation of STAT3 signaling is frequent in human tumors, including prostate cancer, and is generally associated with metastatic progression and poor clinical outcome (Mora et al., 2002; Yu et al., 2014a; Yu et al., 2009). Indeed, STAT3 is known to modulate the transcription of a variety of genes involved in the regulation of critical functions, including cell proliferation and differentiation, apoptosis, angiogenesis and inflammation (Johnston and Grandis, 2011; Yu et al., 2009). Moreover, STAT3 contributes to the expansion and maintenance of stem-like cancer cells and progression to castration-resistant prostate tumors (Kroon et al., 2013; Mora et al., 2002; Schroeder et al., 2014). The oncogenic activation of this transcription factor is generally ascribed to the induction of JAK tyrosine kinases that phosphorylate STAT3 at Tyr705 (Yu et al., 2014a; Yu et al., 2009). However, alternative pathways of activation that are independent or cooperate with this canonical pathway are emerging (Qin et al., 2008; Sellier et al., 2013; Yu et al., 2014a). In this scenario, our study highlights a new possible mechanism leading to aberrant induction of STAT3 in prostate cancer.

Accordingly, our results suggest that increased STAT3 expression can mediate the transformation induced by miR-424 in prostate cells, as genetic or pharmacologic inhibition of STAT3 significantly affected miRNA-related malignant phenotypes. Furthermore, the induction of cancer stem cell-like properties and inflammatory pathways mediated by miR-424 is consistent with the effects of aberrant STAT3 activity and compatible with aggressive features in human prostate tumors. Consistently, we observed high STAT3 level in tumor samples with high miR-424 expression. Moreover, the association of COP1 loss and high STAT3 level with increased risk of biochemical relapse in prostate cancer patients gives clinical and prognostic relevance to our



findings. Based on this evidence, tumors with high miR-424 expression might be more prone to aberrant activation of STAT3 in response to inflammatory stimuli, like cytokines, and thereby have an increased risk to progress and metastasize.

Importantly, the abnormal activation of STAT3 signaling could explain our observation that miR-424 transient expression is sufficient to induce a persistent malignant transformation of prostate epithelial cells. Consistently, increased total STAT3 and Tyr705 pSTAT3 and reduced COP1 levels were observed in tumor xenografts generated by RWPE1 following miR-424 overexpression. The induction of persistent phenotypic changes upon transient modulation of tumorigenic signaling has been described by others investigators. In particular, Iliopoulos et al. reported that an “epigenetic switch” from non-transformed to cancer cells can be mediated by a feedback loop involving NF- $\kappa$ B, Lin28, let-7 and IL6 (Iliopoulos et al., 2009). Moreover, the same authors demonstrated that STAT3 takes part in this self-sustaining process that links inflammation to cancer by directly activating the transcription of two miRNAs, miR-21 and miR-181-b (Iliopoulos et al., 2009; Iliopoulos et al., 2010). Similarly to our observation, transient expression of miR-21 and miR-181-b was sufficient to induce a stable transformed state (Iliopoulos et al., 2010). Additional studies suggest that STAT3 activation can result in a stable genetic reprogramming by inducing the expression of several cytokines associated with cancer-promoting inflammation. Indeed, many of these cytokines, including IL6, further activate STAT3 forming a feedforward loop (Ogura et al., 2008; Samavati et al., 2009; Yu et al., 2009). Considering this evidence together with the alteration in IL6 levels that occurs following miR-424 modulation, we hypothesize that miR-424 transient over-expression can lead to the establishment of a stable reprogramming mediated by aberrant activation of STAT3 and the consequent induction of IL6 expression. Further experiments should be performed in order to confirm such a mechanism.

To our knowledge, few reports regarding STAT3 ubiquitination and proteasome-dependent degradation are present so far (Tanaka et al., 2011; Ulane et al., 2003). The E3 ubiquitin ligase PDLIM is the only one previously demonstrated to promote STAT3 proteasomal degradation with consequent inhibition of its activity (Tanaka et al., 2011). Similarly to what we observed, PDLIM silencing resulted in increased STAT3 level and enhanced STAT3-mediated gene activation, due to nuclear accumulation of STAT3 following cytokine-stimulation. Interestingly, Liu et al. recently showed that miR-221 and miR-222 reduced ubiquitination and degradation of RelA and STAT3 proteins by targeting PDLIM and concomitantly affected the activity of these transcription factors in colorectal cancer cells (Liu et al., 2014). However, the molecular mechanism accounting for the effects on STAT3 activity was not clearly described. Therefore, our study unveils a novel miRNA-mediated pathway leading to STAT3 activation and gives relevant mechanistic details on the regulation of STAT3 activity by proteasome-dependent degradation.

In agreement with the causal role of COP1 and STAT3 in miR-424 mediated oncogenesis and more broadly in prostate cancer, we showed that a miR-424 antagonist restored COP1 expression and reduced STAT3 protein level and activity both *in vitro* and in tumor xenografts. Decreased expression of STAT3 target genes was observed in these tumors further confirming the impairment of STAT3 activity *in vivo*. In addition, anti-miR-424 treatment was highly effective in reversing the malignant phenotype of miR-424 expressing cancer cells, with a prominent and persistent effect on the stem-like cancer cell subpopulation both *in vitro* and *in vivo*.

These findings also have important therapeutic implications. Indeed, our results suggest that compounds inactivating or depleting STAT3 could represent an effective strategy in prostate tumors exhibiting miR-424/COP1/STAT3 deregulation. Accordingly, interfering with JAK/STAT3 signaling has been proposed as treatment option for prostate cancer (Hedvat et al., 2009; Kroon et al., 2013; Schroeder et al., 2014). However, the identification and development of novel drugs that can target effectively deregulated STAT3 activation remains an important scientific and clinical challenge (Siveen et al., 2014). The ability of miR-424 antagonist to promote STAT3 degradation and to reduce its protein level may represent a new approach to effectively block STAT3 signaling in miR-424 expressing prostate tumors. In addition, given that miR-424 up-regulation can result in the induction of multiple oncogenic transcription factors by suppressing the E3 ubiquitin ligase COP1, it is proper to think that the inhibition of miR-424 function could represent a more powerful approach than simply blocking STAT3. This aspect is further relevant because one of the major properties of miRNAs is their ability to target multiple genes, frequently in the same network (Garzon et al., 2010). In line with this notion, although our data suggest a prominent role of COP1 in mediating the effects of miR-424 up-regulation, it is likely that additional targets might contribute in the induction of the oncogenic phenotype observed. Indeed, other E3 ubiquitin ligases having tumor suppressor activity were identified from our analysis of miRNA putative targets. For instance, the scaffolding protein CUL2 that represents a core component of the E3 ubiquitin ligase complex regulating HIF-1 $\alpha$  degradation was previously reported as miR-424 target in endothelial cells. In this study, the authors showed that miR-424 up-regulation promotes angiogenesis by suppressing the expression of CUL2, thereby stabilizing HIF-1 $\alpha$  isoforms (Ghosh et al., 2010). This observation supports the possibility that multiple genes encoding for E3 ubiquitin ligases could be targeted by miR-424 contributing to its oncogenic role in prostate cells. Deciphering the network of genes regulated by the miRNA represent an important issue that should be further explored in future studies in order to fully understand the role of miR-424 during prostate cancer development and progression. Moreover, considering the potential therapeutic application of miR-424 inhibition, the identification of the most relevant targets will be necessary to avoid negative side effects or treatment failure.

Anti-miRNA strategies are an area of active investigation and innovative therapeutic opportunities (Cheng et al., 2014; Soifer et al., 2007). In the present study we used an anti-miR oligonucleotide based on LNA technology to block miR-424 function in prostate cells. LNA anti-miRs have been used successfully in several *in vitro* and *in vivo* studies and represent the miRNA-inhibitors most powerful and least toxic (Stenvang et al., 2012). The efficacy and safety recently demonstrated for anti-miR-122 (also called Miravirsen) in a phase 2 clinical study strongly support the feasibility and high efficacy of this approach (Janssen et al., 2013). Therefore, *in vivo* experiments using systemic delivery of anti-miR-424 in mice could be envisioned in order to evaluate the therapeutic potential of this compound for prostate cancer treatment.

In conclusion, our study uncovered a novel oncogenic pathway of broad biological and clinical significance involving deregulation of miR-424 and leading to altered stability of STAT3 and other key oncogenic transcription factors. Induction of this oncogenic axis in prostate cells promotes tumor-initiating and metastatic properties, thereby sustaining cancer progression. Moreover, our results strongly support the relevance of miR-424 targeting in the context of prostate cancers characterized by loss of ESE3/EHF and deregulation of the miR-424/COP1/STAT3 axis. Monitoring the state of this oncogenic axis could provide new insights for the development of specific therapeutic strategies and identify patients more likely to have aggressive tumor and higher risk of disease recurrence.

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