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Tristetraprolin restoration in colorectal cancer cells
inhibits epithelial-to-mesenchymal transition through
the suppression of multiple specific targets

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Abstract

Tristetraprolin (ZFP36) is a RNA binding protein which recognizes AU-rich sequences (ARE) in the 3' untranslated region (3'UTR) of specific transcripts and thereby inhibits their expression.

ZFP36 and the other members of its family exert a fundamental role in limiting the expression of pro-inflammatory and pro-proliferative genes. Impairment of their function can indeed lead to neoplastic transformation as observed in a variety of cancerous malignancies, including colorectal cancer (CRC). Experimental evidence show a progressive down-regulation of ZFP36 (in vivo) starting from the early stages of CRC development, together with concomitant up-regulation of some of its target genes. Nevertheless, its role in the development of cancer hasn't been fully elucidated. Our aim is to identify new targets of ZFP36 and to further characterize its biological role in colon physiology and colorectal carcinogenesis, with a particular focus on the more advanced stages of CRC progression.

We selected three putative ZFP36 target genes having an overt role in colon tumorigenesis: MACC1, ZEB1 and SOX9. These genes display metastasis-inducing activities and a positive correlation with epithelial-to-mesenchymal transition (EMT). In order to validate these genes as new ZFP36 targets, we performed ZFP36 over-expression in CRC cell lines which resulted in a significant decrease of their mRNA and protein levels. These data were confirmed through luciferase reporter assays where the presence of the 3'UTRs of these genes strongly decreased luciferase activity after ZFP36 over-expression. At the light of this observation we decided to assess the expression of ZFP36 in various CRC-related samples uncovering that Zfp36 expression is downregulated in metastatic samples if compared to carcinoma samples.

Subsequently we analyzed the effects of ZFP36 restoration on EMT-related processes. Consistently with the role of its novel target genes, we observed that ZFP36 overexpression upregulates the epithelial markers E-Cadherin and ZO-1(Zona Occludens 1) and determines in parallel a reduction of the mesenchymal markers Vimentin and N-cadherin. Moreover, we observed that ZFP36-overexpressing cells show a higher impedance of the cellular monolayer consistent with a more epithelial phenotype. These findings were confirmed by immuno-fluorescence technique showing

how E-cadherin is not only expressed at higher levels, but it also accumulated in the cellular junctions compared to controls.

Successively, we asked whether this modification of the cellular phenotype could affect cellular behavior. Therefore, we analyzed the migratory ability of CRC cells but, surprisingly, we could not detect any significant difference between control and ZFP36 overexpressing cells in both traditional and impedance-based scratch assays. Since another feature of EMT is the loss of intercellular contacts and the ability to grow in the absence of substratum, we decided to assess the anchorage-independent growth through soft-agar assays. These experiments revealed that ZFP36 overexpression inhibits the colony-forming abilities of cells cultured in semi-solid medium resulting in a reduction in number and size of colonies. Moreover, we observed by specific assays that ZFP36 overexpressing cells have a higher susceptibility to anoikis compared to the control populations.

Eventually, we examined the relation between ZFP36 and the Wnt pathway. Interestingly, we found that pharmacological silencing of TCF7L2, a transcriptional effector of β -catenin, increased the expression of ZFP36 in a time dependent-manner and, conversely, the treatment with a Wnt3a-conditioned medium, lowers its expression both at protein and mRNA levels.

Overall, our data indicate that the down-regulation of ZFP36 observed in colorectal cancer alters the expression of oncogenic target genes involved in EMT, thereby weighing upon the pathological phenotype of cells. Moreover, we observed that ZFP36 downregulation might be related to the constitutive activation of Wnt pathway which is a milestone of colon cancer.

Introduction

Regulation of messenger RNA turnover

Regulation of gene expression is a fundamental process and is therefore regulated at multiple levels. In particular, post-transcriptional regulation plays an indispensable role in the control of gene expression, complementing regulation of transcription and influencing the expression of a broad variety of genes, especially the stress-regulated genes (1). The stages of posttranscriptional regulation are multiple and include splicing, maturation, transport, editing, localization and storage, turnover, and translation (2). The control of mRNA stability and translation are not only well characterized but also very rapid and effective processes of the adaptive response. This level of regulation is particularly important to ensure the production of proteins in the appropriate time, amount, and localization, and all of these aspects may have profound effects on protein activity.

At the light of these observations it is not surprising that dysregulation of mRNA stability and turnover underlies a number of diseases, directly contributing to the overexpression of many genes encoding growth factors, inflammatory cytokines, and proto-oncogenes (3, 4).

This kind of regulation requires two main elements: the first are the trans-acting factors, represented mainly by noncoding RNA (i.e. micro(mi)RNAs] or RNA-binding proteins (RBPs), which associate to RNA and regulating its stability or availability. The other elements involved are the cis-factors, represented by the sequences of the RNA which are recognized and bound by the trans-acting factors.(2)

RBPs usually shuttle between cellular compartments, upon various stimuli, and may undergo post-translational modifications (phosphorylation and methylation) to ensure their proper subcellular localizations and functions (5).

ARE sequences

Among the best studied cis-elements regulating post-transcriptional control are the adenosine and uridine (AU)-rich elements (ARE), located in the 3' untranslated region (3'-UTR) of many unstable

mRNAs. The relevance of these conserved sequences is apparent in their frequency, currently estimated at approximately 8% of the human transcripts (3, 6).

Usually, the presences of ARE sequences target the mRNAs for rapid decay but it could also allow the stability and translation of mRNAs to be regulated in response to extracellular cues. In fact these sequences can recruit both stabilizing and destabilizing RBP, and the final effect on mRNA stability depends on the presence and the interaction of these protein (7).

The ARE sequences are composed by pentamers of the sequence AUUUA, which is often found tandemly arranged, or by the nonamer UUAUUUAUU, which is a pentamer flanked by two uridylates. AREs are generally considered instability elements, since they typically interact with RBPs that subsequently recruit mRNA degradation machinery in a process defined as ARE mediated decay (AMD). (8, 9)

Molecular studies reveal that the first step of AMD in eukariotes is the removal of poly-Adenine tail. This process is performed by at least three different protein complexes comprising deadenylating enzymes that are: the Ccr4-Not complex, the Pan2/3 complex and the poly (A)-specific ribonuclease (PARN). Once the poly-A tail is removed a complex composed by exonucleases and helicases, called exosome, is allowed to degrade the mRNA in the 3'–5' direction (10). Another possibility is the removal of the 7 methyl guanosine (7mG) cap at the 5' end by decapping enzymes, allowing the exoribonuclease Xrn1 to degrade the transcripts in the 5'-3' orientation.(11)

ARE-binding proteins : ZFP36

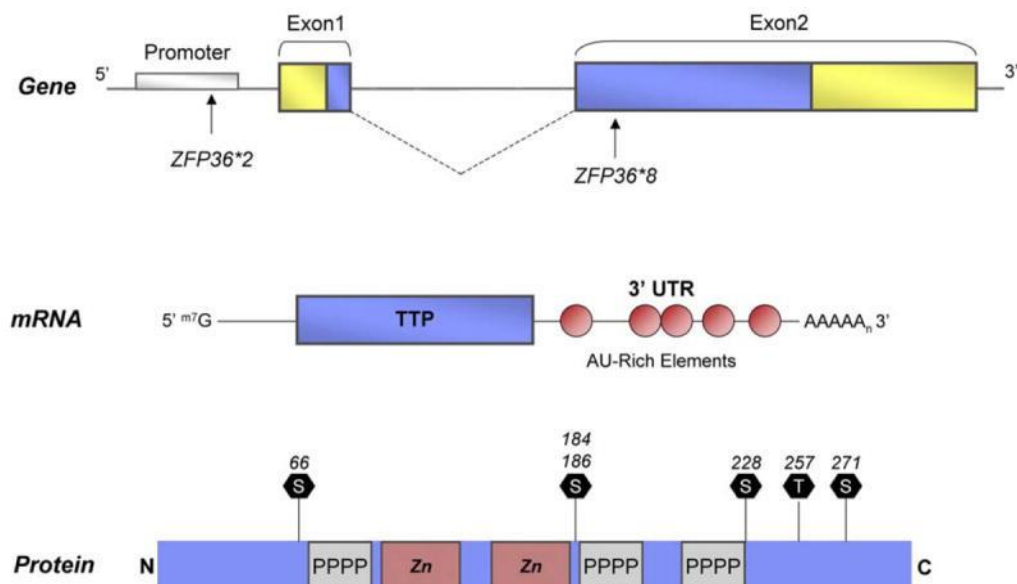
The regulation function performed by AREs sequences firstly requires the interaction with specific RBPs that recognize them.

Among all the RBP there is a small family of zinc finger RNA-binding proteins which consists of three members: Tristetraprolin, encoded by TIS11/ZFP36, BRF-1 encoded by TIS11b/ZFP36L1 and BRF-2 by TIS11d/ZFP36L2. The main member of this family is Tristetraprolin, also known as Zfp36 or TTP, which was originally identified and cloned in 1990 from mouse fibroblasts stimulated with insulin or serum and its mRNA showed the expression pattern of immediate-early response genes (12,

13). For reason of brevity, in this thesis Tristetraprolin will be called Zfp36, even if in some figures belonging to other works it may be called TTP. The other two members of this family were later identified thanks to the high percentage of amino acid identity >70 % and to the similar trend in response to growth factors. All the members of this family directly bind ARE sequences promoting degradation of the target transcript (14, 15), even if with different timing.

Zfp36 mRNA and protein structure:

ZFP36 gene is located on chromosome 19q13.1, and composed by just two exons and one intron and encodes for a 1745 bp long mRNA. It contains AREs in its own 3' UTR which can be recognized by Zfp36 protein generating a negative feedback loop and allowing for Zfp36 expression to rapidly return to low levels after an initial induction (13). The resulting protein has a molecular weight of 36 kDa and own some structural features found exclusively in the members of this family. (12, 16). These proteins are characterized by three repeats of four prolins, which give rise to the more commonly used name of tristetraprolin, and by two tandem CX8CX5CX3H zinc finger domains needed for the



specific recognition of mRNA sequences (13, 17). In quiescent cells, ZFP36 is weakly expressed and predominantly nuclear while, under mitogenic stimulation, its expression is quickly induced and the

Figure 1. Schematic representation of Zfp36 gene, mRNA and protein. Human ZFP36 gene consist of two exons and one intron with yellow portion of exon representing the non-coding region while the purple one representing the coding-region. Arrows represent two catalogued SNPs (single nucleotide polymorphism). Zfp36 mRNA (here called TTP) contains ARE motifs (red dots) in 3'UTR. Protein structure displays unique features namely three tetra-proline (PPPP) repeats, two CCCH-type zinc finger domains and several serine/threonine phosphorylation sites. Adapted from: *"The role of tristetraprolin in cancer and inflammation."*. Sanduja S. et al. 2012.

protein rapidly shuttles from the nucleus into the cytoplasm.

ZFP36 protein is subject of multiple phosphorylations, which cause an increase of the molecular weight and a consequent shift in its electrophoretic mobility(18). Site-directed mutagenesis, together with mass spectrometric experiments, identified the major sites of phosphorylation and they include S60, S66, S184, S186, S228, T257, and T271.

Various protein kinases, such as p38 MAPK, p38 MAPK-activated protein kinase 2 (MK2), and ERK2, have been reported that can directly phosphorylate Zfp36 in vitro and/or in vivo, and is a putative target of PKA, glycogen synthase kinase 3 (GSK3 β), and ERK1 based on their phosphorylation motifs (19, 20).

This mitogen-stimulated phosphorylation affects ZFP36 localization, inducing its rapid shuttling to the cytoplasm, but, up to date, the phosphorylatable residue involved in this phenomenon has not been identified yet.

The role of p38 MAPK/MK2-mediated Zfp36 phosphorylation at serine residues S52 and S178 in mouse Zfp36 (S60 and S186 in human Zfp36 (21, 22)) has also been extensively analyzed in different publications. These works reveal how this phosphorylation enhances the interaction between ZFP36 and 14-3-3 adapter proteins, increases its stability and induces its cytoplasmic localization (23), suggesting an higher activity of ZFP36. Interestingly, different studies reveal that phosphorylated ZFP36 shows a reduced RNA-degrading activity if compared to the un-phosphorylated protein, and that his phenomenon is due to a decreased recruitment of CAF1 deadenylase complex and not to a lower binding affinity. Un-phosphorylatable ZFP36 mutants show an enhanced down-regulation of ZFP36 target genes, which produces a minor inflammatory response in transgenic mice expressing these proteins. At the light of these observations it can be reasoned that, after a mitogenic stimulus characteristic of the initial phase of an inflammatory response, Zfp36 expression is induced and it accumulates in an inactive form into the cytoplasm. As the stimulus decreases, phosphorylation-dependent inhibition of Zfp36 ceases resulting into a targeted decay of ARE containing mRNAs. Collectively these data suggest how phosphorylation on these two Serine exerts a negative regulation of ZFP36 activity, but little is known about the other phosphorylatable residues.(24)

Zfp36 binding to ARE sequences

The first description of the ZFP36 binding site was provided by Carballo and colleagues, who discovered how Zfp36 could directly bind to the ARE sequences found in the 3'UTR of TNF- α mRNA and target it for degradation (10). Precisely, further works revealed that Zfp36 preferentially binds the nonameric sequence UUAUUUAUU, a destabilizing motif found in many ARE-containing mRNAs.

The RNA-binding specificity of ZFP36 resides in the central tandem zinc finger domain (TZF), which interacts with ARE sequences mostly arranged in the form of pentamer AUUUA or the nonamer UUAUUUAUU. A single mutation of any of the eight cysteines or histidines in the CX8CX5CX3H TZF domain completely abrogates RNA binding (25).

Also the ZFP36 target sequence seems to be very conserved. In fact, modifications in the number of U residues between the two A result in a remarkable increasing of Kd (26). The other Zfp36 family members, TISS11b/ZFP36-L1 AND TIS11d/ZFP36-L2 recognize the same optimal RNA sequences and are able to promote mRNA decay in a similar manner(25).

The interaction between ZFP36 and RNA displays two important characteristics. Firstly, in order to allow the binding, the RNA must totally devoid of secondary structures, raising the possibility that these structures might indeed interfere with this association. Moreover, the RNA strand occupies a single side of the TZF domain structure, leaving open the possibility that other proteins or small molecules could interact with other faces of the TZF domain and perhaps affect RNA binding affinity (26).

Zfp36-mediated mRNA decay

The main mechanism underlying ARE-mRNA decay resides in the capacity of ZFP36 to promote mRNA deadenylation. This activity requires the presence of the two zinc-finger domains through which ZFP36 specifically binds to RNA .

As previously anticipated, mammalian mRNA decay is executed from three major deadenylation complexes: the Caf1 complex, the poly A-specific ribonuclease (PARN) complex, and the Pan2/Pan3 complex (10, 27).

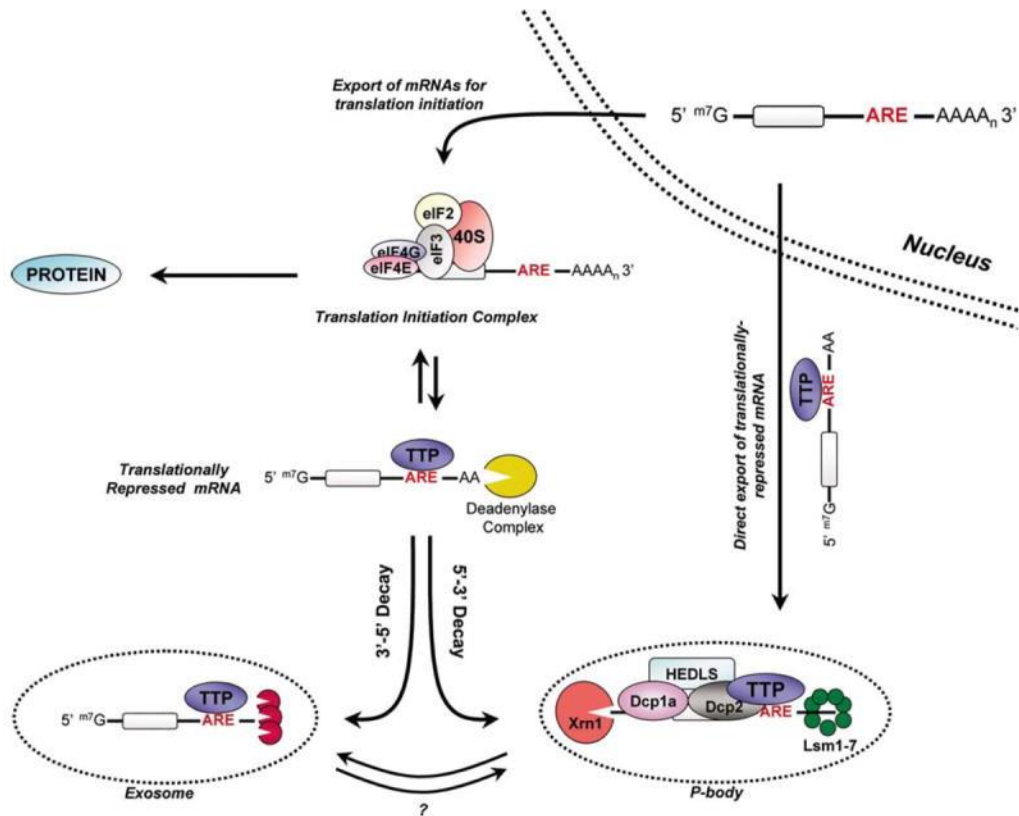


Figure 2: Zfp36-mediated mRNA decay. Processed mRNA transcripts are exported from the nucleus into the cytoplasm wherein the translation initiation complex is assembled. Cytoplasmic Zfp36 (TTP) associates with these transcripts via ARE binding and further recruits the deadenylase complex. Following deadenylation, the mRNA transcript can undergo 5'-3' decay in P-bodies, where the transcript is decapped and the mRNA is stored or decayed by the exonuclease Xrn1. Alternatively, Zfp36 can facilitate 3'-5' exosome-mediated decay of target mRNAs. Under stress conditions, Zfp36 can be recruited to stress granules and may facilitate delivery of translationally-repressed ARE-containing mRNAs from stress granules to P-bodies for degradation. Adapted from: "The role of tristetraprolin in cancer and inflammation.". Sanduja S. et al. 2012.

The Caf1 deadenylase complex has been implicated in ARE-mediated decay and co-immunoprecipitation experiments identified a direct association between ZFP36 and Caf1 (14, 28). In vitro studies have shown that Zfp36 can promote PARN-dependent deadenylation of ARE-containing mRNAs, however Zfp36 mediated stimulation of PARN activity was independent of direct physical association, indicating a possible indirect interaction (14).

In eukaryotes, deadenylated mRNAs may be degraded through 5'-3' or 3'-5' decay pathways (Figure 2) (1, 29). The mechanisms regulating the choice between the two pathway is still unclear, but the current consensus is that the 5'-3' decay pathway predominates in ARE-dependent mRNA decay (30, 31).

The 3'-5' decay is mediated by the multi-protein complex exosome (1) and initial studies indicated that ARE-mRNAs are degraded primarily in the 3'-5' direction by this complex. ZFP36 plays a key

role in this mechanism through its capacity to interact with the exosome and to recruit it to the AU-rich mRNAs (32).

Up to date, increasing body of evidence highlights the importance of the 5'-3' pathway in AMD. The first step of this process is the removal of 7-methyl guanosine cap by the decapping complex Dcp1/Dcp2, generating an unstable RNA susceptible to degradation by the 5'-3' exonuclease Xrn1 (33, 34) (Figure 2). The components of the 5'-3' pathway co-localize at small cytoplasmic structures defined as processing (P)-bodies (PBs), which contain factors involved in translational silencing, and miRNA- and siRNA mediated mRNA silencing (35, 36). ZFP36 is able to associate to P-body members thus promoting AMD localized at P-bodies (37, 38). Interestingly, different publications demonstrate how ZFP36 can also localize to discrete sub-cellular structures called stress granules (SGs). These structures assemble after cellular stress, and contain a subset of translationally arrested RNAs, stalled translational machinery and other ARE-binding proteins such as TIA-1 and TIAR (39, 40). This capacity of ZFP36 to interact with components of P-bodies and stress granules suggests a potential mechanism by which it could mediate dynamic shuttling of mRNAs between these two structures, for example after cellular stress, establishing it as a versatile link in the mRNA turnover (41).

Zfp36 regulatory pathways

Expression of ZFP36 can be stimulated by a broad variety of stimuli. It was firstly described as a TPA (12-O-tetradecanoylphorbol-13-acetate) inducible sequence in murine fibroblasts (42, 43). Further works demonstrated how ZFP36 mRNA display the same expression pattern of immediate-early response genes, showing low levels of Zfp36 under quiescent growth and a rapid transient induction after stimulation with different substances like insulin, serum or growth factors (12, 13, 43).

This characteristic expression pattern is largely due to a modulation in the transcriptional regulation of this gene. In fact Lai et al demonstrated how a conserved region in ZFP36 promoter is essential for the serum-inducibility of ZFP36 and how it contains multiple binding sites for several transcription factors like SP1, EGR-1, and AP2 (12, 44). Additional regulatory elements were identified in the intron of

murine Zfp36 which cooperate with 5' regions to promote serum-stimulation of ZFP36 expression(44).

Subsequently a increasing body of evidence report that various growth-inhibitory cytokines as well as anti-inflammatory drugs and natural products such as glucocorticoids, green tea, and cinnamon can enhance ZFP36 transcription, suggesting that the anti-inflammatory activity of these factors is in part mediated by ZFP36. For example transforming growth factor-beta (TGF- β) was demonstrated to induce ZFP36 expression, probably through the trans-activation of human ZFP36 promoter by Smad3/4 transcription factors (45-48).

Zfp36 biological activity: role in cancer and inflammation

The first description of a ZFP36 specific knockout mice provided a great amount of information about the biological role of ZFP36. Following deletion of Zfp36 gene, mice appear normal at birth, but before 8 weeks of life they start to develop an autoimmune inflammatory syndrome characterized by a broad plethora of symptoms, such as cachexia, conjunctivitis, alopecia, severe arthritis and dermatitis. The authors also demonstrated how this syndrome was due to excess of circulation TNF- α , a potent pro-inflammatory cytokine, since anti-TNF antibodies totally prevented the ZFP36 deficiency syndrome (49). Interestingly, this phenotype require the complete loss of Zfp36 since heterozygous mice do not show sign of disease.

These mice also displayed hematopoietic abnormalities such as myeloid hyperplasia and a consequent enlargement of spleen and lymph nodes, and thymic atrophy. These observations provided the first evidence about the tremendous importance of this protein in inflammatory response, and also highlight the pleiotropic activity of Zfp36.

After this pioneer study, the list of Zfp36 target genes is massively increased together with our knowledge about its role in different biological contexts. Among the more studied ZFP36 target genes are: GM-CSF, VEGF, COX2, HIF1-alpha and various cytokines (50, 51).

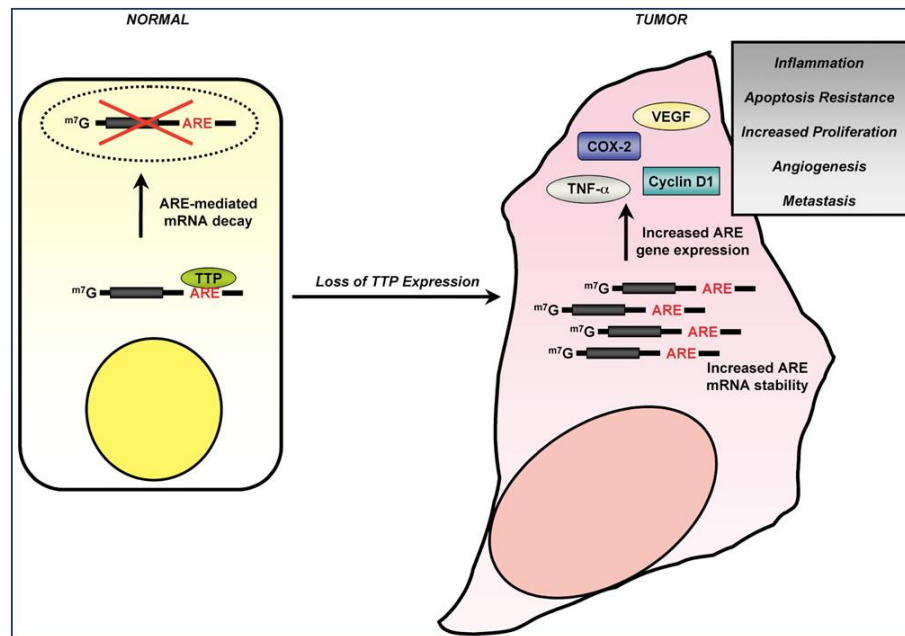


Figure 3: Loss of Zfp36 (TTP) allows the upregulation of multiple oncogenes and facilitates tumor progression.
Adapted from "The roles of ZFP36 and BRF proteins in regulated mRNA decay." Sanduja S. 2011

These genes, as well as a great part of the potential ZFP36 target genes, encode for proteins having a positive role in inflammation and-or cancer progression. Consistent with this, multiple lines of evidence indicated that loss of ZFP36 expression is a common feature of a broad range of human malignancies, and that its restoration induces a variety of anti-oncogenic effects, which depends among the analyzed cell type, implicating ZFP36 as a potential tumor suppressor. The anti-oncogenic effects of ZFP36 restoration have been demonstrated in a variety of cancers, like gliomas, breast cancer, liver and colon cancer.

In particular Young and colleagues showed that loss of ZFP36 expression occurs at early stages of colorectal tumorigenesis, and is concomitant with elevated COX-2 expression (52). Other studies have identified tumor-suppressive effects of ZFP36 in colon cancer cells through its ability to target VEGF and inhibit tumour vascularisation (53).

Oncogenic mechanisms that suppress Zfp36

Several publications have shown that ZFP36 expression is suppressed in many cancers, and that there is frequently an inverse correlation between ZFP36 levels and tumor aggressiveness. This is consistent with the observation that ZFP36 inhibits the expression of multiple oncogenes, but the mechanisms responsible for ZFP36 down-regulation in cancer is still poorly understood(50).

One of the few examples consists in an epigenetic mechanism described in hepatocellular carcinoma. Authors demonstrated how, in this kind of tumor, a CpG site in TGF- β 1 response element of the ZFP36 gene promoter is frequently methylated and how this methylation is sufficient to repress ZFP36 expression in a hepatocellular cancer cell line (54).

A second possible mechanism involves the miR-29 which is reported to directly inhibits ZFP36 expression. This molecule in fact is overexpressed in metastatic breast cancer, and is sufficient to induce invasiveness in murine mammary cancer cell (55).

Finally Zfp36 activity can be down-regulated through hyper-phosphorylation by the p38 MAP kinase pathways, as previously described (56). Since the MAP Kinase pathway usually is highly activated in a variety of cancers, this mechanism could affect Zfp36 activity without necessarily decreasing its expression.

Colorectal cancer:

Colorectal cancer (CRC) is the third most common human tumor, and is one of the leading cause of cancer-related deaths worldwide (57). Although recent advantages in the detection and in the therapy of this neoplasm have dramatically increased the survival of CRC patients, it still remains often a fatal disease (58). According to NIH, survival after 5 years is largely affected by the stage at the diagnosis, ranging from 90% for the localized tumors to 13% for the metastatic tumors. The risk factors for CRC development are multiple and include mainly intrinsic parameters such as age and genetic predisposition together with environmental factors such as diet, alcohol, smoke, obesity and diabetes (59). Approximately 5% of the cases are due to genetic mutations, while the remaining 95% are sporadic, with 20% of the cases with family history for CRC development (60) .

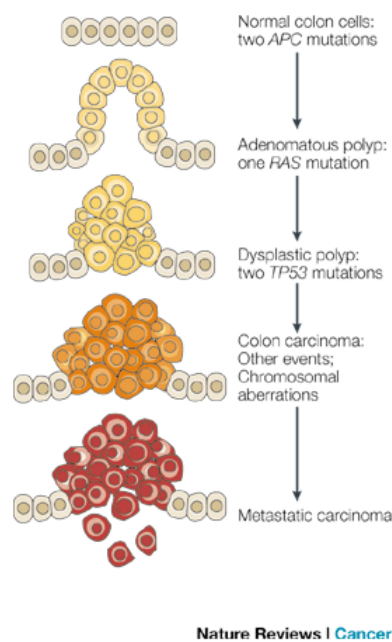


Figure 4: Schematic representation of Vogelstein model. Following this model each step of CRC development is caused by a specific genetic mutation. Adapted from: “Two genetic hits (more or less) to cancer”. Knudson A, 2001

CRCs evolve through a stepwise accumulation of genetic and epigenetic alterations, resulting in the transformation of normal mucosa into invasive cancer. Due to these characteristic, development of this disease is really slow and may continue for 10-15 years. The adenomatous polyp is the precursor lesion to cancer but only a small fraction of the lesions progress to cancer and patients affected by syndromes (i.e. familial adenomatous polyposis) that facilitate adenomas development have a dramatically increased risk to develop CRC.

CRC is characterized by the accumulation of genetic mutations and epigenetic alterations which follow the multiple stages pattern theorized by Fearon and Vogelstein (61). According to this model, such alterations are directly responsible for a specific event within the sequence that leads to CRC development. They may contribute to the “initiation” of neoplastic transformation or in the “progression” towards more malignant stages of the disease, as summarized in Figure.

The genetic alterations responsible for this disease can be divided in different pathways, characterized by different mechanisms leading to genetic instability, clinical manifestations, and pathological behavior.

Most CRC follows the chromosomal instability (CIN) pathway, characterized by an abnormal loss of heterozygosis and chromosomal abnormalities , which accounts for the majority of CRCs (65-70%)(62, 63).

CIN is characterized by gain or loss of whole chromosomes or chromosomal regions resulting in the loss or gain of function of genes involved in process of CRC carcinogenesis. It is often caused by defects in chromosome segregation with subsequent aneuploidy, telomere dysfunction, impairment of the DNA damage response mechanisms. Higher levels of CIN are constantly associated with poorer disease free survival (DFS)(64)

The second pathway is responsible for approximately 15% of CRC and is due to the impairment of the DNA Mismatch Repair (MMR) system and the resulting microsatellite instability (MSI).

Recently other systems and pathways have been reported to actively contribute to the carcinogenic process such as abnormal DNA methylation, inflammation and, microRNA (miRNA).

CIN pathway

APC mutations. CIN pathway is characterized by allelic losses on chromosome 5q (APC), 17p (p53), and 18q (SMADs). Inactivating mutations in the adenomatous polyposis coli (APC) tumor suppressor gene, are considered the initial events of CRC development in both sporadic CIN and in Familial Adenomatous Polyposis (FAP) Syndrome. This syndrome arise from a germline mutation of APC gene in the 80% of the cases and is characterized by the development of hundreds to thousands polyps in the large intestine during early adult life (65, 66).

APC is an important component of Wnt pathway and its inactivation stimulate the shuttling of β -catenin from the cytoplasm to the nucleus where it drives the transcription of multiple genes implicated in tumor growth and invasion. Extensive description of this signalling cascade is reported later.

K-ras mutations. The early mutations of APC are followed by multiple events that promote new mutations and facilitate the tumor progression. The adenoma-carcinoma sequence is determined by activating mutations in the K-ras gene, a proto-oncogene that encodes for a small GTPase involved in the transduction of extracellular signals (i.e. mitogen-activated protein kinase (MAPKs) (67). These alterations confers resistance to apoptosis and a selective advantage which depends of the codon affected by the mutations (68).

p53 mutations. P53 is the most commonly mutated tumor suppressor gene in various kinds of cancers and its loss of function is generally found in the later stages of colorectal tumorigenesis. Mutations in this gene is considered one of the key steps in colorectal carcinogenesis and stimulates high proliferative activity through the loss of cell cycle control and apoptosis (69, 70).

Microsatellite instability pathway

The MSI pathway accounts for approximately the 15% of sporadic CRCs and for >95% of Hereditary Non Polyposis Colorectal Cancer(HNPCC) syndrome. MSI is caused by the inactivation of the DNA Mismatch Repair (MMR) system which causes a 100-fold increase in the mutation rate in colorectal cells (71). In human, the MMR system is composed of multiple proteins including the human MutS homologue (MSH) 2, and human MutL homologue (MLH) 1 (23, 72). The HNPCC syndrome is determined by germline mutations in one of the MMR components and is characterized by development of CRC, as well as other malignant tumors, in early adult life (73).

Interestingly, MSI-based CRCs exhibits peculiar clinical features: more often located in the proximal colon, with a poorly differentiated and mucinous histotype, often presents abnormal lymphocytic infiltrations. They usually display a better prognosis and survival in comparison of patients affected by CIN-based CRCs, but they are resistant to 5-fluorouracyl chemotherapy (74, 75).

The genomic instability and high mutation rate finally result in the inactivation of target genes such as BRAF, TGF- β Receptor II and SMAD4 (76-78). These oncogenes belongs from the the TGF- β /SMAD pathway which is composed by TGF- β receptor type I and II (TGF β RI and TGF β RII) and SMAD proteins. When the pathway is activated a complex composed by R-SMAD (regulatory-SMAD) and Co-SMAD (common partner) translocates into the nucleus and induces the expression of Cdk inhibitors leading to growth arrest.

Finally, the analysis of the colon cancer epigenome revealed that virtually all CRCs have aberrantly methylation pattern and that the average CRC methylome has hundreds to thousands of abnormally methylated genes. It revealed the existence of a unique molecular subgroup of CRCs **called CpG island methylator phenotype (CIMP)**, which has a distinct epigenome with a high frequency of methylated genes (79, 80). In addition to DNA hypermethylation of promoter regions, epigenetic regulation of this kind of CRC also includes post-translational histone modifications, such as histone acetylation and methylation that also play critical roles in regulation of expression of oncogenes and tumor suppressor genes.

Canonical Wnt/ β -catenin signaling pathway:

Wnt/ β -catenin is an ancient and evolutionary conserved pathway controlling myriad biological phenomena throughout development and adult life of all animals with a prominent role in embryogenesis, cell differentiation, and stem cell maintenance self-renewal (81). As previously anticipated, the main effector of Wnt pathway is a co-transcription factor named β -catenin. β -catenin subcellular localization and activity are finely regulated by a group of proteins called destruction complex and composed by tumor suppressor adenomatous polyposis coli (APC) and Axins along with the Ser/Thr kinases casein kinase 1 α (CK1 α) and glycogen synthase kinase 3 β (GSK3 β). APC and Axin act as scaffolding proteins on which CK1 α and GSK3 β phosphorylate β -catenin leading its degradation through poly-ubiquitination and proteasome complex recruitment (82). Indeed β -catenin is not detectable in the cytoplasm or nucleus of unstimulated cells and is accumulated in the adherens junctions, in association with E-cadherin, α -catenin, and actin filaments of the cytoskeleton (83).

In physiological conditions, the pathway is activated from the binding of Wnt proteins to the extracellular domain of its specific receptor called Frizzled (Fzd). Upon binding of Wnt to Fzd, the co-receptor Low density Lipoprotein 5/6 (LRP5/6) interacts with Fzd and protein dishevelled (DVL). DVL promote the phosphorylation of a LRP intracellular domain conserved motif leading to the recruitment of APC and Axin to the plasma membrane along with CK1 α and GSK3 β . This mechanism brings to a decrease of GSK3 β -dependent phosphorylation of β -catenin and then to its transient stabilization and accumulation of into the cytoplasm. Cytoplasmic accumulation of β -catenin facilitates its translocation into the nucleus where, by displacing the co-repressor Groucho, enhances the expression of specific genes through the interaction with the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins (84, 85). In the adult intestine, TCF7L2 (also known as TCF4) is the main member involved in mediating β -catenin signalling (86). Once it is bound to DNA β -catenin/TCFs complex trans-activates the expression of a myriad of genes that are critical for cellular proliferation (c-Myc, cyclinD1), or for tumor invasiveness (MMPs, Twist, CD44) or stemness (Nanog, Oct4) (87). Although β -catenin target genes varies in function of the biological context, the Axin2

gene is generally regarded as a global transcriptional target and therefore a general indicator of Wnt pathway activity (88).

When the activating stimulus ceases, the transcription complex is disassembled and nuclear APC exports β -catenin to the cytoplasm where it is degraded (89).

Considering the importance of Wnt pathway in regulation of stem cell renewal and proliferation, is reasonable to find that Wnt pathway mutations are frequently involved in cancer formation and progression, especially in those tissues where Wnt controls self-renewal and tissue repair.

Indeed, aberrant activation of β -catenin/TCF signaling has been involved in a number of tumors, most notably colorectal carcinomas (CRCs) where 80% of sporadic CRCs and the vast majority of cases of familial (FAP). In addition, oncogenic β -catenin mutations occurs in 10-15% of the remaining cases (90). This indicates that activating mutations in one of the members of b-catenin pathway are found in more than 90% of CRCs.

Finally, it has been recently observed that the increase of β -catenin and its stable interaction with TCF/LEF lead to a mechanism well known as “Epithelial-Mesenchymal Transition” or EMT, an important process in tumorigenic cells for acquisition of invasiveness.

Epithelial-to-Mesenchymal transition

Epithelia are defined as a single cell layer or multilayer tissues lining the lumens of different organs. Epithelial cells adhere with each other through intercellular bridges, display an apical–basal polarity, and are located on a basement membrane that helps to maintain their physiology. These tissues exert multiple functions ranging from the protection from external factors to the control of permeability.

The Epithelial-Mesenchymal transition is a process defined as the trans-differentiation of epithelial cells in mesenchymal-like cells. To achieve this result, cells need to undergo a drastic change in gene expression profile in order to lose epithelial structure and acquire mesenchymal properties such as migratory and invasive ability (91). This phenomenon is involved in different physiological situations. During embryonic development it coordinates many different steps, from gastrulation to development of highly organized organs such as heart. Moreover EMT also occurs as a physiological response to injury, in fact keratinocytes at the border of the wound acquire a mesenchymal-like intermediate phenotype which allows them to move collectively without losing cellular contacts rather than migrating as individual cells (92).

EMT not only occurs during development or during physiological wound healing, but is also an important process in cancer progression and other degenerative pathologies such as organ fibrosis. At the molecular and cellular level, pathological and physiological EMT are very close and are regulated by the same signalling pathways.

In particular, EMT plays a fundamental role in development and progression of solid tumors. Cancer cells undergoing EMT acquire the capacity to escape immune-surveillance, to resist apoptosis and chemotherapeutic drugs and to disseminate throughout the body. EMT activation provides cancer cells with migratory and invasive properties and also promotes tumor progression gradually transforming a weakly-invasive tumor cell into a metastatic cell (93). Data from human solid cancers, xenograft animal models, and cell culture demonstrate that the EMT phenotype leads to poor outcome and enhances cell migration and invasiveness of cancer cells (94). Both *in vitro* and *in vivo* studies demonstrated how the progression from a primary-tumor cell to a metastatic one frequently involves EMT process and the changes towards a migratory mesenchymal-like phenotype, a phenomenon particularly evident at the invasive front of primary tumors (95). Another interesting feature of EMT is

the ability to generate tumor stromal cells like cancer-associated fibroblasts (CAFs), which significantly enhance tumor formation (96).

To be truthful, despite a huge amount of literature regarding the positive role of EMT in metastatization, in the scientific community the effective importance of EMT in the process on *in vivo* metastatization, is still fiercely debated (97).

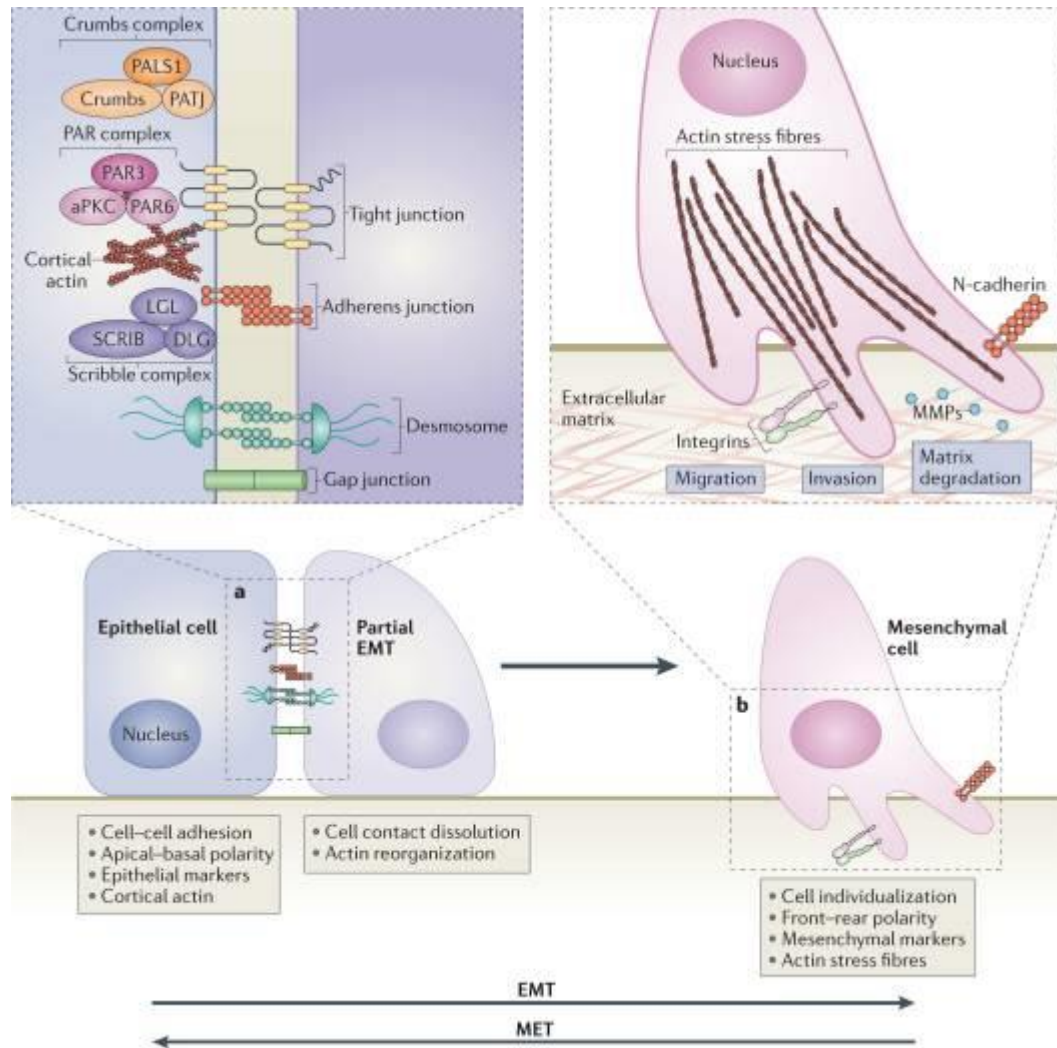


Figure 5: Schematic representation of Cellular events during EMT. First steps are disassembly of epithelial cell-cell contacts (tight junctions, adherens junctions, desmosomes and gap junctions) and the loss of cell polarity. Next, the epithelial actin architecture reorganizes, and cells acquire motility and invasive capacities. Adapted from: *Molecular mechanism of Epithelial-to-Mesenchymal transition*. Lamouille S. 2014

In all tissue contexts, key events in EMT are the dissolution of the epithelial cell–cell junctions; loss of apical–basal polarity and acquisition of a front–rear polarity; reorganization of the cytoskeletal architecture; downregulation of an epithelial gene expression signature and activation of genes that help to define the mesenchymal phenotype; increased cell protrusions and motility. In metastasis-related EMT cells also acquire further properties such as the ability to degrade extracellular matrix (ECM) proteins, resistance to senescence, apoptosis. The reduced sensibility to apoptosis is a key event since allows tumour cells to survive in absence of adhesion to basal membrane, a process defined as anoikis, and to become resistant to chemotherapeutic agents used in cancer therapy (98)

Deconstructing cell junctions and polarity

Epithelial tissues have a typical cobblestone structure which is maintained by strong inter-cellular contacts. These structures can be divided in adherens junctions, sub-apical tight junctions (TJ), desmosomes, and scattered gap junctions at lateral surfaces.

As previously anticipated EMT process requires the dissolution of intercellular contacts. In the early steps of EMT, adherens junctions are partially degraded and the junction proteins are relocalized and/or degraded. For example E-cadherin is cleaved at the plasma membrane and subsequently degraded, while the tight junction protein ZO-1 diffuses into the cytoplasm (94, 99). As EMT progresses, the expression of these proteins is transcriptionally repressed, which stabilizes the loss of epithelial junctions (100).

Instructed through contacts with the basement membrane, epithelial cells display apical–basal polarity, which is maintained by polarity complexes that are physically and functionally integrated with the cell junction architecture. In mammalian epithelium there are mainly two “apical-complex” which are associated with tight junctions and define the apical compartment: partitioning-defective (PAR) complexes and Crumbs complexes, while Scribble complexes define the basolateral compartment. The dissolution of tight junctions causes a mislocalization of the apical complexes and the consequent loss of apical–basal polarity . As EMT progress, the expression of proteins composing the polarity complexes is repressed, resulting in a further impairment of the polarized phenotype (101).

Cytoskeletal changes and motility

Together with the dissolution of intercellular connections, cells undergoing EMT also revolutionize their shape, losing the poliedric structure in favor of a less regular appearance, and rearrange their cytoskeleton in order to facilitate cell motility and dynamic cell elongation. (91, 94) **(FIG. 1)**

Cells develop some new actin-rich protusions called lamellipodia and filopodia which dynamically adhere to the substrate, like ECM, and act as the motor of cell migration. These structures display also the expression of multiple receptors which allows them to acquire information from the extracellular environment. Invadopodia are a different kind of extracellular protrusion also able to degrade the matrix and thereby to facilitate cellular invasion (102, 103). These dynamic changes are largely mediated by RHO protein, and RHO-associated kinase (ROCK) which promote actin polymerization and actomyosin contraction.

At the light of these observations, It is easy to understand how the acquisition of cellular motility also requires the loss of apical–basal polarity and in favor of a front– rear polarity.

The conversion to front-rear polarity involves the interplay between RHO GTPases and the of apical-basal polarity complex (104, 105). To achieve directional polarity, the PAR and Scribble complexes together with PATJ of the Crumbs complex re-localize to the leading edge of the cell, where RAC1 and CDC42 activate actin polymerization and membrane protrusion formation. By contrast, RHOA localizes at the rear of the cell and promotes the disassembly of adhesion complexes and cell retraction, in a mechanism highly dependent on PI3K (105).

The reorganization of the cytoskeletal architecture and polarity complexes, which result in cell shape changes, cell elongation, membrane protrusions and front–rear polarity are essential in EMT and enable directional migration.

It is important to note that before these cancer cells are able to metastasize, they must first overcome anoikis, a form of programmed cell death initiated when anchorage-dependent cells (integrins) detach from the surrounding ECM (106-108). Under normal conditions, when integrins on the epithelial cell surface come in contact with the ECM, FAK is activated by phosphorylation, which in turn triggers a phosphorylation cascade ending with the activation of Akt, thus promoting cell survival. If the integrin lose contact with the ECM, the cell survival signals cease, leaving pro-apoptotic proteins such as Bad uninhibited and able to initiate cell death(109). Cancer cells can overcome anoikis in a variety of ways

that are often related to EMT. For example, a loss of E-cadherin expression and an increase in N-cadherin expression is correlated with anoikis resistance and increased invasiveness. It has also been shown that dysregulation of growth factor receptors can lead to anoikis resistance (109).

Overview of changes in gene expression

To support all the morphological and phenotypical changes associated to EMT, epithelial cells need to modulate their gene expression profile. Even if this phenomenon is tissue and time dependent, the common steps seem to be the down-regulation of epithelial proteins found in cellular junctions, such as desmosome TJ and adherens junctions, together with an increase in the adhesion to mesenchymal cells and an alteration in the interaction with the ECM.

One of the hallmark of EMT is the destabilization of adherens junction achieved by the inhibition of E-cadherin expression, through proteolytic cleavage before and transcriptional silencing after (99). Desmosome and TJ also are impaired through the down-regulation of claudins, occludins, desmoplakin and plakophilin.

The silencing of these proteins prevents the *de novo* formation of epithelial intercellular junctions and results in a impairment of the epithelial barrier function (110).

The repression of epithelial genes is accompanied by the increase in the expression of proteins which promote mesenchymal adhesion. Typically, the downregulation of E-cadherin is balanced by the increase in the expression of neural cadherin (N-cadherin), the main mesenchymal adhesion molecule. This phenomenon is defined as “cadherin switch” and is necessary in order to allow the cells to lose their binding with epithelial cells and acquire an affinity for mesenchymal cells (94, 111).

The newborn homotypic N-cadherin interaction is weaker than homotypic E-cadherin binding and allows cell migration and invasion. EMT also induces the expression of neural cell adhesion molecule (NCAM), which cooperates with N-cadherin to modulate the activity of receptor tyrosine kinases (RTKs), such as platelet-derived growth factor (PDGF) and fibroblast growth factor receptors (FGFRs), thus enhancing the assembly of focal adhesions, invasion and migration .

EMT also requires a rearrangement of the cytoskeleton. The pattern of intermediate filament expression undergoes to a drastic change in composition with the silencing of cytokeratin and the activation of vimentin expression. Keratins, expressed exclusively by epithelial tissues, are able to carry E-cadherin to the plasma membrane in contrast to mesenchymal vimentin filaments. On the other hand vimentin allows cell motility, possibly through the interaction with motor proteins (94).

Moreover, in order to allow directional movement, cells inhibit the expression of the apical complex proteins such as Crumbs3 and PATJ, and of baso-lateral Scribble complex, such as LGL2.

Another important aspect of cellular homeostasis which is affected by EMT process is the interaction between cells and ECM. Integrins are transmembrane molecules which interact with extracellular proteins and consequently transactivate a intracellular cascade. When epithelial cells move from their original site, they lose contact with basement membrane and need to communicate with a different ECM. Consequently, cells down-regulate some epithelial integrins, but enhance the expression of others. For instances, the epithelial $\alpha 6 \beta 4$ integrin, which interacts with the basal membrane, is repressed through epigenetical silencing of the gene coding for $\beta 4$. By contrast, increased $\alpha 5 \beta 1$ integrin expression during EMT allows cellular adhesion to fibronectin promoting cell migration into the connective tissue (112).

In order to migrate toward a different site, epithelial cells also acquire the ability to degrade ECM. To achieve this they activate the expression of some proteases, such as the matrix metalloproteinases MMP2 and MMP9 thus enabling degradation of ECM proteins. As a “side affect”, MMPs also perform the cleavage of some important epithelial proteins such as E-cadherin, thus contributing to the loss of adherens junctions.

Transcription factors driving EMT

The Modifications in gene expression occurring during EMT are orchestrated by few transcription factors: including TWIST, SNAIL and zinc-finger E-box-binding (ZEBs) (110). The induction of these master regulators occurs early during EMT, both in physiological and pathological contexts, and their role depends on the cell type and tissue involved. Their activity results in the concomitant

repression of epithelial genes and the activation of mesenchymal ones, and often the same transcription factors direct both effects.

The binding of these transcription factors to chromatin results in epigenetic regulation of gene expression through post-translational modifications of histone proteins. In particular it results in the methylation and acetylation at histone H3 Lys 4 (H3K4), H3K9 and H3K27 and the creation of repressive or activating epigenetic marks in regulatory regions, like gene promoters. The presence of both activating and repressive marks may occur in the same promoter region and creates a poised state which maintains transcriptional repression, but also enables a timely activation of the gene in presence of the right stimulus. This phenomenon may contribute to the reversible nature of EMT and to the concomitant repressive and activating activity of some EMT master regulators (113).

SNAIL family

SNAIL1 (also known as SNAIL) and SNAIL2 (SLUG) are highly involved in EMT and activate the EMT program during development, fibrosis and cancer.

They repress transcription through the binding of their carboxy-terminal Zinc-finger domains to E-box sequences found in promoter of multiple epithelial genes. In particular SNAIL recruits the Polycomb repressive complex 2, which contains chromatin modifying enzymes such as methyltransferases, enhancer of zeste homologue 2 (EZH2) histone deacetylases 1, 2 and/ or 3, and the Lys-specific demethylase 1 (LSD1) (94, 114). The expression and activity of SNAILs are enhanced by multiple pathway such as TGF β , β -catenin, Notch pathway, RTKs(110). For example WNT and PI3K–AKT pathways inhibit SNAIL1 phosphorylation through GSK3 β while Notch and nuclear factor- κ B (NF- κ B) disrupt the GSK3 β –SNAIL1 interactions, increasing SNAIL1 stability. On the other hand tumor suppressor p53 causes SNAIL2 degradation through its interaction with the ubiquitin ligase mouse double minute 2 (MDM2), resulting in inhibition of invasiveness.

bHLH transcription factors.

bHLH transcription factors function as master regulators of lineage specification and cellular differentiation in different contexts. Among these, TWIST1 and TWIST2 and inhibitor of differentiation (ID) proteins have fundamental roles in EMT process (110, 115). TWIST1 represses

E-cadherin and induces N-cadherin independently of SNAIL and probably through the association with other factors. From a molecular point of view, TWIST recruits the methyltransferase SET8 which causes H4K20 monomethylation, a histone mark associated with repression at E-cadherin promoters and with activation at N-cadherin (115).

As well as Snail, TWISTs expression is activated by multiple signalling pathways during development and tumorigenesis. For example the transcription factor hypoxia-inducible factor 1 α (HIF1 α) increases TWIST expression under hypoxic conditions, thus promoting tumor cell migration from the necrotic and hypoxic core of primary tumors. The stability of TWIST1 is regulated by phosphorylation; MAPKs phosphorylate TWIST1 at Ser68, preventing it from ubiquitin-mediated degradation and increasing its protein levels.

ZEB transcription factors. ZEBs transcription factors binds to regulatory gene sequences at E-boxes and are considered one of the EMT master regulators. Extensive discussion about their role in EMT and cancer is reported in the following chapters of this thesis.

Novel transcription factors regulating EMT. In addition to the previously described master regulators, other transcription factors were shown to induce or regulate EMT. Many of these are forkhead box (FOX) transcription factors, which are characterized by a DNA-binding forkhead domain, while others belong to the GATA family, and promote EMT by regulating the expression of genes coding for protein of cell junctions or polarity complexes. Moreover inducers of EMT are SRY box (SOX) transcription factors that associate with SNAIL in driving EMT and cell invasion (116).

The role of transcription factors in EMT induction is indeed prominent, but also changes in the post-transcriptional regulation of mRNAs are acquiring increasing importance in this process(117), the adhesion protein cluster of differentiation 44 (CD44) and FGFR2 . This is due to the down-regulation of the epithelial splicing regulatory protein 1 (ESRP1) and ESRP2, two RNA binding proteins that control the splicing of many mRNAs .

The role of miRNAs in EMT is also attracting increasing attention, since they can regulate EMT at different levels. Some of these control the expression of EMT master transcription factors, as miR-29b

and miR-30a which repress SNAIL1 expression or members of the miR-200 and miR-205 family which inhibit ZEB1 and ZEB2 expression. In many cases, the transcription factors involved in this kind of regulation, directly target miRNAs expression resulting in a double negative feedback loop (118, 119).

Moreover miRNAs can also directly inhibit the expression of genes encoding for adhesion junction and polarity complex proteins such as E-cadherin and N-cadherin. Finally several microRNAs control the expression of RHOA, affecting actin organization and tight junction stability. For example miR-155, which is induced from TGF β , targets the RHOA transcript, resulting in the dissolution of tight junctions(119).

EMT-inducing pathways

One of the most potent EMT-inducing stimulus is TGF β , which orchestrate EMT by acting through SMAD-mediated and non-SMAD signalling. EMT process is regulated at the transcriptional, post-transcriptional, translational and post-translational levels and TGF β signals can virtually affect all of these steps. This cytokine acts through a tetrameric receptor and activates intracellular SMAD proteins. After SMADs oligomerization the trimeric SMAD complex translocates into the nucleus and cooperates with transcription regulators thereby activating or inhibiting the expression of multiple target genes. TGF β –SMAD pathway activates the expression of EMT master regulators, and additionally SMADs cooperate with these transcription factors to increase their transcriptional activities. TGF β –SMADs can also induce EMT through the regulation of several miRNAs or the regulation of splicing. TGF β can also regulate EMT through non-SMAD signalling pathways, for example by activating PI3K–AKT signalling, or by regulating RHO-GTPases and, consequently, affecting cytoskeletal changes previously described. Consistently this pathway is involved in multiple EMT-related process such as carcinoma metastatization, organs fibrosis and development (120).

Tyrosine kinase receptors family is a broad group of transmembrane proteins which usually mediates a proliferative and anti-apoptotic intracellular signal. Ligand binding induces the autophosphorylation of these receptors and the consequent activation of multiple intracellular pathways such as PI3K–AKT,

MAPK and SRC signalling. Growth factors are potent inducers of EMT and the role of many of these cytokines (such as EGF,FGF,IGF, VEGF and PDGF) in this process has been extensively demonstrated in multiple contexts(121, 122).

EMT process is induced also by different signalling pathways, including Wnt/ β -catenin pathway. Multiple members of this signaling cascade can regulate EMT at different levels. For example, GSK3 β phosphorylation promotes SNAIL cytoplasmic export and its proteasomal degradation [4]. Therefore the inhibitory effects of Wnt pathway on this kinase, globally results in a stabilization of SNAIL protein. Furthermore it is demonstrated how β -catenin directly or indirectly enhances the transcription of Twist1, Snail1 and ZEB1 expression. (110, 123)[45,46]. Finally the transcriptional effectors of TGF β 3 and Wnt pathway (i.e. SMADS and TCFs) may associate and cooperate to promote EMT related genes.

Putative Zfp36 targets involved in EMT process

The scientific work developed in this thesis was designed in order to analyze the role of ZFP36 in EMT process. During this study we identified three novel putative ZFP36 target genes, which displayed EMT-inducing capabilities in the context of colon cancer. In order to allow the reader to perform a critical analysis of the work we provide a brief summary of the state-of-art about these factors.

ZEB1:

Zinc-fingers are among the most common DNA binding domains found in eukaryotic transcription factors and ZEB family belongs to this group. It includes two members ,ZEB1 and ZEB2 encoded by ZFHX1A and ZFHX1B genes respectively, both characterized by the presence of two zinc-finger clusters. The N-terminal cluster (NZF) contains four Zn-fingers (three CCHH fingers and one CCHC finger)while the C terminal one (CZF), contains three CCHH zinc fingers. Between these two domains resides a central homeodomain mainly involved in protein-protein interactions(124).

DNA-binding sequence of ZEBs proteins is composed by bipartite E-boxes, a common motif also recognized by other proteins such as basic helix-loop-helix (bHLH) factors. Usually these sequences

are represented by the consensus palindromic sequence CANNTG and, precisely, ZEBs binding site is composed by the CACCT and CACCTG sequences variously arranged in space(125, 126).

After the binding to gene promoters ZEB1 exerts its inhibitory effects through the recruitment of the co-repressor C-terminal-binding protein (CtBP1/2), histone deacetylase (HDAC1)((127), and through the NC2 (DR1/DRAP1) negative cofactor of TATA-binding protein (128). Even if it is mainly considered a transcriptional repressor, recently multiple publications highlights its role as a transcriptional activator. This switch is due to the association with co-activators like PCAF (CBP/p300-associated factor) or p300(129) or different transcription factors (such as YAP or TCF7L2) (130, 131). The ability to associate to other transcription factors highlights the importance of this protein in the crosstalk and the coordination of different intracellular pathways.

The effects of ZEB are prominent in many patho-physiological contexts such as in cartilage, bone and muscle formation and hematopoietic development and, importantly, it has been identified as one of the more potent EMT activators together with Snail and Twist proteins (132, 133).

ZEB1 is associated with aggressive behaviour, metastasis, treatment resistance and poor prognosis in different tumour types, including colorectal, pancreatic and lung cancer (134-137). In colorectal cancer ZEB1 is a negative prognostic factor, correlates with liver metastasis, and it is found to be highly expressed at the invasive front of the tumor (138, 139).

These effects on tumor transformation are mediated by the repression of multiple epithelial genes, such as E-cadherin and proteins of cell polarity complex, which are considered founding events in EMT process and are responsible for the development of aggressive cellular phenotype (124, 138, 140). Thanks to its dual role in transcriptional regulation, ZEBs are also able to induce some genes involved in mesenchymal phenotype as N-cadherin, Vimentin and MMPs (134, 141).

ZEB 1 expression and activity is stimulated by multiple oncogenic pathways like. Wnt/ β -catenin signalling pathway which directly enhances its transcription(133). Also TGF- β , Hippo and miRNA 200 family can induce its expression and-or activity (141-143).

SOX9

SOX9 (Sex-determining Region Y (SRY) box 9) is a transcription factor characterized by a high mobility group(HMG) box domain responsible for DNA binding specificity. It shares a high sequence similarity with SRY, and during development cooperation of these two proteins is fundamental for normal testis differentiation. This protein is also important in chondrogenic differentiation, as it is found to be expressed from chondrogenic progenitors but must be silenced in order to complete ossification. Germline mutations in this gene cause a syndrome called campomelic dysplasia, which affects all cartilage-derived structures and is characterized by skeletal malformations and is often associated with sex reversal and problems in reproductive system (144, 145). Multiple scientific evidences demonstrate the involvement of this protein in development of others important tissues including pancreas, heart valves, liver, brain ad derivatives of neuronal crests (146, 147).

In particular, SOX9 plays a significant role in neural crest development of vertebrates (148-150). Cells from neural fold need to acquire migratory ability in order to give rise to the neural crest (151). SOX9, in cooperation with Snail, is fundamental in the induction of this particular type of EMT in a mechanism dependent on Wnt pathway signaling (152).

Recently the involvement of this protein in cancer progression has also been proposed. In fact it is found highly up-regulated in many premalignant lesions and in different tumors (153, 154). In breast cancer SOX9 overexpression, in association with Slug, induces the de-differentiation of epithelial cells in tumor-initiating motile cells, promotes the tumorigenic and metastatic abilities of breast cancer cells, and is associated with poor patient survival (155, 156).

It also plays a critical role in lineage commitment of intestinal epithelium and also during the process of carcinogenesis. In this context SOX9 in fact exhibits multiple oncogenic properties such as stimulation of proliferation, inhibition of senescence, and cooperation with other oncogenes in neoplastic transformation. Moreover downregulation of this protein leads to decreased invasive and metastatic abilities in colon cancer cells (154, 155). Finally its expression is upregulated in cancerous lesions and in CRC patients, where higher SOX9 levels have been associated with poor prognosis

(157). A relationship between SOX9 and Wnt pathway has been reported, but it still slightly controversial. In fact β -catenin activation through mutations in APC gene results in a induction of, while SOX9 overexpression results in β -catenin activation, suggesting the existence of a positive feedback loop (158-160).

MACC1

Metastasis-associated in colon cancer-1 or MACC1 has been recently identified as a highly upregulated gene in primary and metastatic carcinomas described for the first time in 2009 by Stein and colleagues (161). In these years evidences about its role in solid tumours are impressively increasing and it has become clear how its expression is a negative prognostic factor in a broad range of cancers (for reviews see (162)).

MACC1 is located on human chromosome 7 (7p21.1). Chromosomal aneuploidy is a characteristic feature of carcinomas and chromosome 7, and in particular 7p21, is one of the most frequently gained chromosomal regions in intestinal cancers (163, 164). This suggests that chromosomal gain of function could be one of the mechanisms underlying its over-expression in CRC. From a structural point of view it contains multiple domains for putative protein interactions, such as a Clatrin box, a ZU5 domain and a SH3 domain and a SH3 binding domain , which are indispensable for the transcriptional activity and consequently for the biological effects of this protein (161, 165). Surprisingly the C-terminus also contains two Death Domains, usually involved in apoptosis, but their functions has not been fully elucidated. The DNA binding domain of this protein has not been described yet.

This transcription factor directly activates the expression of the HGF receptor protein MET. This pathway trans-activates intracellular signals via the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-Akt pathways and causes increased migration, invasion and in general induces tumorigenesis and metastasis in multiple solid tumors (166, 167). Besides MET, MACC1 has been shown to transactivates the expression of Spondin 2, the pluripotency markers Oct-4

and (168, 169) and MMP2 and MMP9 (170). A positive link between MACC1 and β -catenin pathway has also been reported (171, 172).

Concordantly with these observations its abnormal expression induces tumorigenesis and metastatization through a vast plethora of oncogenic properties such as increased cellular proliferation, invasion, migration, resistance to apoptosis and to chemotherapeutic agents (i.e. cisplatin) in a variety of solid cancers. (173-178)

Interestingly different experimental evidences highlight the importance of post-transcriptional regulation of this genes. Its expression in fact is down-regulated from different micro-RNAs such as MiR-338-3p (179), miR-200a (180) and miRNA-143 (181).

Moreover a couple of works report the presence of high levels of MACC1 transcripts in plasma of CRC and lung cancer patients, and demonstrated that the diagnostic capability of circulating MACC1 transcripts was higher than that of carcinoembryonic antigen CAE cytokeratins (182, 183). Moreover author demonstrated that high MACC1 expression is associated with poor survival and disease-free survival. Altogether, these data indicates that MACC1 is a promising candidate gene that can be used to predict the risk of metastasis and guide further diagnostic and therapeutic decisions (165).

Aim of the thesis:

As discussed in the Introduction, Zfp36 is a RNA binding protein involved in many biological processes. This protein inhibits the expression of multiple transcripts through the binding to specific sequences found in the 3'UTR of multiple transcripts. This activity enables Zfp36 to downregulate a plethora of oncogenes, and it is therefore not surprising that Zfp36 expression appears to be lost in a variety of tumors.

Previous reports show that the expression of Zfp36 gradually decreases during adenoma-carcinoma transition in CRC development, resulting in the upregulation of multiple target genes, such as VEGF and COX-2. Despite of that, little is known about the role of this protein in the last and more important step of CRC development, which is metastatization. Therefore, the aim of this project is to verify if Zfp36 inactivation is indeed relevant for this process. To pursue this I decided to study different points.

Firstly, since Zfp36 tumor suppressor activities are linked to its ability to inhibit the expression of specific transcripts, I decided to verify if among of its negatively-regulated target genes some could have a role in metastasis development. During this research I identified and validated three novel Zfp36 target genes, MACC1, SOX9 and ZEB1, which are well known to have metastatic-inducing activities and to be inducers of epithelial-to-mesenchymal transition (EMT).

Then, I verified whether ZFP36 expression is decreased during all the steps of CRC progression including metastatization. For this purpose I took advantage of a database containing gene expression profiles belonging to normal mucosa, carcinomas and metastatic samples. Moreover I assessed Zfp36 mRNA and protein levels in two cell lines derived from primary tumors and the matched metastatic lesions. In both cases Zfp36 expression is found to be downregulated in metastatic samples.

Third, I decided to analyze the effects that ZFP36 restoration could induce on the phenotype and behavior of CRC cell lines. In particular I decided to focus my work on the analysis of EMT, which is the process underlying metastatization. Consistently with the previous data, I found that ZFP36

restoration inhibits EMT process and induces a more epithelial-like phenotype and a higher susceptibility to anoikis.

In the last part of this work, I decided to study the mechanism underlying ZFP36 downregulation in CRC. For this purpose I focused on β -catenin pathway which is constitutively activated in this kind of tumors. Modulations in the activity of this pathway, achieved by pharmacological inhibitors or natural agonists, actually affected Zfp36 expression uncovering the possibility that β -catenin activity negatively regulates the expression of this protein.

Materials and Methods

Cell culture and treatment

SW480 and SW620 cell lines were kindly gifted by Prof Alberto Bardelli, (University of Turin). HCT116 and HEK293T cells were purchased from ATCC. All cell lines were grown in DMEM supplemented with 10% FBS (Sigma), 2 mM L-glutamine and penicillin/streptomycin (100 µg/ ml) (Euroclone) at 37°C under a 5% CO₂ atmosphere.

FH535 (Sigma) was dissolved in DMSO and diluted in culture medium at a concentration of 20 µM. Cyclohexymide (Sigma) was dissolved in DMSO and diluted in culture medium at concentration of 25 µM. In both cases, control cells received the same amount of DMSO.

Plasmids

Zfp36 coding sequences was previously cloned into pCDNA3.1 vector and pRRL lentiviral vector(184). The ARE-containing regions of the 3'UTRs of MACC1, SOX9 and ZEB1 were amplified by RT-PCR on total RNA extracted from HCT116 cells using specific primers:(**MACC1 DP:** AGTTCCTCAGGCTTTTGTCT with **MACC1 RP:** AGAACTTCACTCTCACTAAACC ; **SOX9 DP:** CCTGTGCCTCTCAGAACACC with **SOX9 RP:** GCTGGGAGGGAAACAAGTGA; **ZEB1 DP:** TGGCTCATAACTGTTTCCAAATGT with **ZEB1 RP:** AAGGCAATAGAAAAAGAAGGCATAA). The amplified fragments were then inserted into the pCR2.1-TOPO T/A cloning vector and fully sequenced. These fragments were then excised with KpnI and XhoI and cloned into pGL3-Promoter Vector (Promega) that had previously been modified in order to transfer the multiple cloning region downstream the luciferase reporter gene.

Human WNT3a coding sequence was amplified by RT-PCR on total RNA extracted from HCT116 cells using specific primers (**DP:** CGATGGCCCCACTCGGATAC and **RP:** GCGGCCGGTGCCTACT). The amplified fragment were then inserted into the pCR2.1-TOPO T/A

cloning vector and fully sequenced. Wnt3a coding sequences was then excised with XhoI and KpnI and cloned into pCDNA3.1 vector digested with the same enzymes.

CRC samples database

We considered the expression profiles of ZFP36, MACC1, SOX9 and ZEB1 genes in CRC patients, using a dataset available at the GEO database (GSE35834). This dataset reports expression estimations, obtained with GeneChip Human Exon 1.0 ST (Affymetrix) platform, of 22,517 genes in 80 samples, including 23 normal colon mucosa (Normal), 30 primary colorectal tumors (CRC) and 27 liver metastases (Mts) obtained from 46 patients diagnosed with colorectal carcinoma at TNM stage IV, who underwent surgery at the University of Padova (Surgery Unit, Department of Surgery, Oncology and Gastroenterology) in the period 1994-2008. See Pizzini et al. 2013 (184) for additional details on patients characteristics as sex, age, and tumor site). Differential expression analysis was performed using limma package, available from BioConductor for the R statistics environment (BioConductor, www.bioconductor.org), setting p-value<0.05 and using Benjamini Hochberg (BH) method to adjust p-value.

Western blot analysis

Total protein extracts were obtained resuspending and vortexing cell pellets in small volumes of RIPA buffer containing 50 mM TRIS-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM sodium deoxycholate, 1 mM sodium orthovanadate and with added 1 mM EDTA and Complete Protease Inhibitor Cocktail (Roche Applied Science) and quantified. Equal amounts of proteins were loaded onto a SDS-Polyacrilamide gels and transferred to a nitrocellulose sheet. The membranes were then blocked with 5% nonfat milk in TBST 0,1% and immunoblotted overnight at 4°C with different primary antibodies listed below together with their working dilution:

N-cadherin (910920 BD Transduction Laboratories); Vimentin (Dako MO725); E-Cadherin (610181 BD); ZO-1 (Zymed 61-7300); MACC1 (Bioss bs-4293R); SOX9 (Bioss bs-4177R); Zeb1 (Sigma HPA027524); Zfp36 (S. Cruz Biotechnology sc-14030)

Blots were then incubated either with anti-Rabbit or anti-Mouse (Santa Cruz Biotechnology, sc-2054 and sc-2005 respectively) IgG-HRP antibodies and detected using BM Chemiluminescence Blotting Substrate (Roche Applied Science).

RNA extraction and qRT-PCR

Total RNA was extracted with PureLink RNA mini Kit (Invitrogen), quantified with Nanodrop 2000 and retro-transcribed with High Capacity cDNA KIT. The obtained cDNA was qPCR quantified using TaqMan® Gene Expression Master Mix (Applied Biosystems) with specific primer pairs using the ABI PRISM 7900 HT Detection System (Applied Biosystems). TaqMan Gene Expression assays for GAPDH, Zfp36, TCF7L2 and Axin2 transcripts were purchased from Invitrogen.

Anchorage-independent growth assays

Anchorage-independent growth assays were performed in triplicate in 35mm well plates.

72hours after infection, cells were trypsinized in order to achieve a single-cell suspension and diluted at the same concentrations. Equal amounts of cells were then resuspended in complete DMEM supplemented with 0.3% of Bacto Agar (Sigma) and seeded on the top of a thick layer of DMEM supplemented with 0.6% Bacto Agar. For SW620 and HCT116 were seeded 5000 cells and 10000 for SW480. After 2-3 weeks colonies were quantified by taking photographs of 10 random fields. For crystal violet staining dishes were fixed with methanol, stained for 3 hours with 0.01% crystal violet in PBS 40% methanol and destained with PBS overnight.

Lentivirus production and infection

Lentiviral particles production was carried out as previously described (185). Briefly pRRL vectors were co-transfected with packaging plasmids in HEK293T and the resulting supernatant was concentrated through ultracentrifugation at 19000 rpm for 2.5 hours. Target cell lines was infected at MOI 3 overnight with Polybrene. Unless otherwise stated, infected cells were subject to different

experimental procedures after 72 hours from infection and with a maximum of one week from infection.

Anoikis assay

Infected cells were seeded in a 24 wells plate at a density of 200000 cells/well in 0,5 ml of DMEM supplemented with 2 U/ml of Dispase (BD Biosciences) which prevents cell adhesion. Subsequently cells were grown for 72hours and cellular viability was assessed every day through Trypan blue exclusion assay. Number of cells expansion were normalized on the number of cells seeded at 0 hours.

Wound healing (scratch) assay.

Infected cells were grown in 12 wells plates until complete confluence and then scratched with a p200 pipette tips. Photographs were taken at 0 hours and every day, until gap closure.

Luciferase assay

For luciferase assays, HEK293T cells were plated at a density of 50,000 cells/well the day before transfection in 24 wells plates. In a typical assay, each well received 200 ng of pGL3-based reporter constructs (described in Plasmids chapter), 200 ng of CMV- b-galactosidase plasmid (Clontech Laboratories) and 10 ng of pcDNA3.1 o pCDNA3.1-Zfp36. Transfections were carried out with Lipfectamine 2000 (Invitrogen). After 48 hours, cells were harvested and cell lysates were assayed for luciferase and b-galactosidase activity. Luciferase values was normalized on protein concentration and b-galactosidase activity. Each transfection was done in duplicate, and the plotted results represent the average of four different experiments, error bars represent SEM (*p < 0.05; **p < 0.01).

Immunofluorescence

Briefly, infected cells were grown for 48-72h on uncoated coverslips then fixed with 4% paraformaldehyde for 10 min and incubated with blocking solution containing 0.1% Saponin(Sigma) and 2% BSA(Sigma) in PBS for 30 min. Primary antibody was diluted 1:50 in blocking solution and incubated overnight in humified chamber at 4°C. Subsequently slides were incubated with fluorescently labeled secondary antibody Alexa Fluor (Invitrogen) and nuclei were counterstained by DAPI (Sigma-Aldrich). Finally slides were analyzed using a Carl Zeiss Axioskop 40 fluorescent microscope.

Wnt3a conditioned medium production

pCDNA-WNT3a vector (described in section Plasmids) was linearized with SspI and transfected into HCT116 cells. In order to obtain stably transfected cell lines HCT116 was selected with 0,9ug/ml G418. After 2 weeks of selection, cells were splitted at 1:8 ratio in absence of G418 and supernatant was collected twice, after 72h and after further 48h. The same procedure was performed with pCDNA3.1 empty vector. The so obtained medias were filtered 0,45 um and freezed at -80°C.

Epithelial barrier functions experiments

The assessment epithelial barrier functions was performed with Electric Cell-substrate Impedance Sensing (ECIS) Z θ instrument (Applied Biophysics). Briefly this instrument applies a small alternating current and records the impedance, and thus the resistance, of the cellular layer.

In this case, infected cells were seeded at 10⁵cells/cm² onto uncoated gold microelectrodes in ECIS cultureware (8W10E+) and impedance was recorded immediately after seeding and for at least 48 hours. The results display the resistance recoded at 4000 Hz and normalized on the values recorded at 0 hours and represents the mean of three independent experiments +/- SEM.

Transwell assays

Infected cells were serum starved for 6 hours and then resuspended in DMEM 0,5% FBS, 0,1% BSA. 60000 SW480 were then seeded in the upper chamber of 8 μ m-pored transwell inserts (Corning code)

and DMEM 10% FBS was added in the lower chamber in order to provide a chemoattractant stimulus. After 16 hours migrated cells were removed with a cotton swab and the inserts were fixed with cold methanol for 5 minutes and stained with DAPI for 10 minutes. Permeable membranes were then excised with a blade and mounted on a glass slide with mounting medium. Quantification of migrated cells were performed by counting the migrated nuclei on 10 random fields and normalized over pRRL values. Results are the mean of two independent experiments $\pm$ SEM.

Impedance based scratch assays

Infected cells were seeded on ECIS cultureware (8W1E array) and allowed to reach confluence. Then a wound in the cellular monolayer was generated in by applying a 3-mA, voltage pulse for 20 s, and recovery of the layer was monitored by measuring Resistance at 4000 Hz

Results

Analysis of Zfp36 expression in CRC progression.

The expression of Zfp36 in normal colonic mucosa and CRC samples has been previously analyzed in a couple of works, as reported in the Introduction. These publications show that the expression of this protein is strongly downregulated in adenomas and adenocarcinomas, consistently with its anti-oncogenic effects. However, up to date, we could not find any report about Zfp36 expression in the final and more relevant step of CRC development which is metastatization. In order to obtain data about this topic we took advantage of a database containing gene expression profiles of normal mucosa, primary carcinomas and liver metastasis. Specifically it contains the profile of 80 bioptic samples including 23 normal colon mucosa (Normal), 30 primary colon carcinoma (CRC) and 27 liver metastases (Mts).

The results depicted in Figure 1 show that the mRNA levels of Zfp36 are significantly lower in the primary tumor if compared to the normal counterpart and that there is a further decrease in the metastatic samples. In particular the median value is 8.7 in normal mucosa and decrease to 8.3 in carcinomas and to 8.2 in liver metastasis. The results of the differential expression tests (data not shown) demonstrate that Zfp36 results significantly differentially expressed in primary tumor and metastatic samples, if compared to normal mucosa. Conversely, the comparison between metastasis and normal tissue does not result significant. The relatively small size of this database could be the reason of this poor statistical significance but, in our opinion the decreasing trend in Zfp36 expression during CRC progression is still evident.

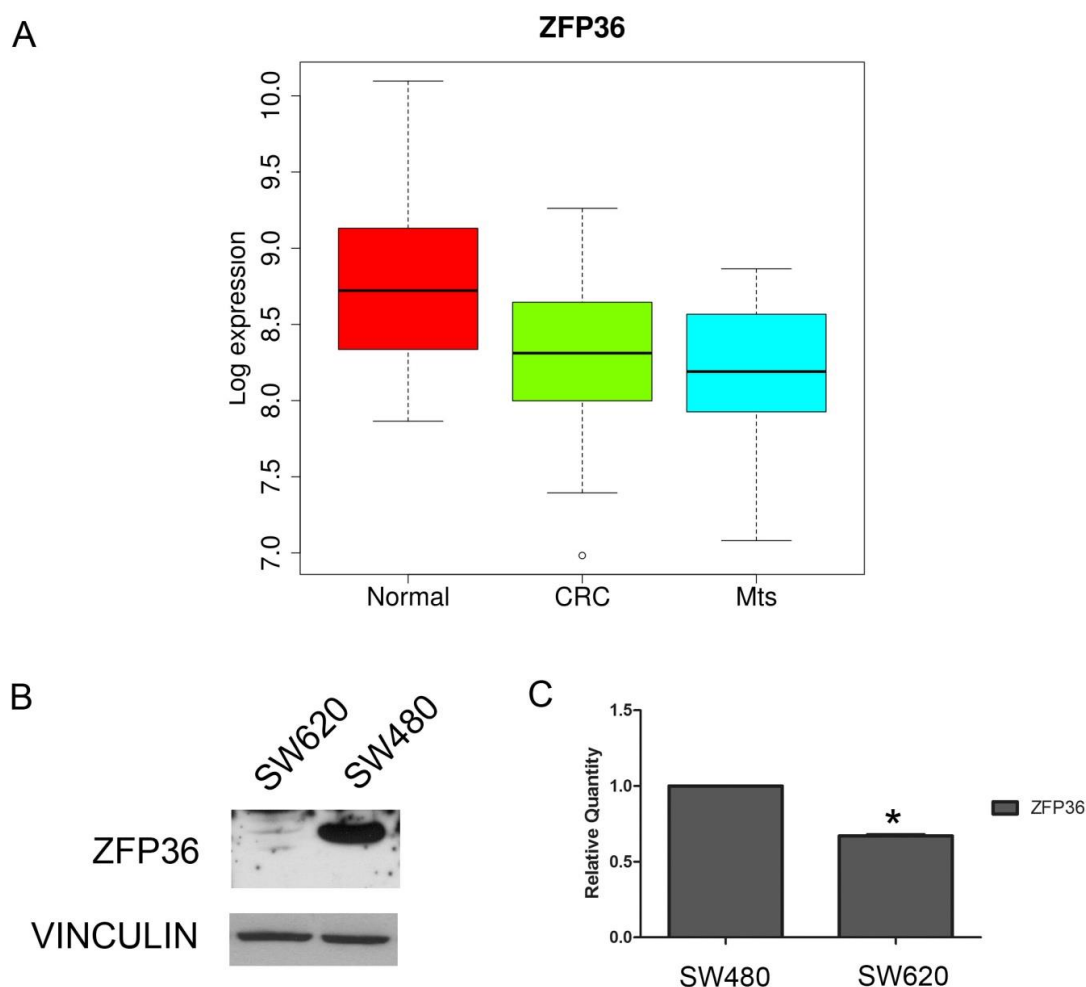


Figure 6 Analysis of Zfp36 expression in CRC-related samples.

Panel A. Zfp36 mRNA levels evaluated in a GEO database containing gene expression profiles derived by 23 normal colon mucosa (Normal), 30 primary colon carcinoma (CRC) and 27 liver metastases (Mts) samples, expressed as median Log2 expression.

Panel B. Zfp36 protein expression in CRC cell lines derived from primary tumor (SW480) and matched metastatic lesion (SW620). Equal amounts of total protein extracts were loaded on a SDS PAGE, transferred to a nitrocellulose sheet and blotted with antibody directed against Zfp36. Actin was used as loading control.

Panel C. Zfp36 mRNA levels detected in SW480 and SW620 cell lines through qRT-PCR. Results are represented as means of two experiments (+/-SEM) and GAPDH was used as calibrator. * $p < 0.05$

MACC1, SOX9 and ZEB1 are directly and post-transcriptionally downregulated by Zfp36.

The hypothesis that Zfp36 could have a role in EMT was firstly generated from the observation that at least three transcription factors involved in this process carry ARE sequences in their 3'UTR and are

therefore putative Zfp36 targets, as shown in panel B. We analyzed the expression of these genes in the same database of gene expression profile previously described. Consistently with their functions, MACC1 and SOX9 mRNA levels increase during CRC progression with a median log2 expression value rising from 6.8 to 8.7 and from 8.0 to 8.8 in Normal and CRC respectively. Surprisingly ZEB1 expression shows an opposite behavior and appears be downregulated in tumor versus normal mucosa (median log2 expression value from 7.6 of normal to 6.9 of tumor and 6.6 of metastasis). This result is controversial since ZEB1 is widely recognized as an Epithelial to Mesenchymal Transition (EMT) master gene and huge amount of literature reports its up-regulation in cancer progression.

To validate these genes as Zfp36 targets we firstly performed over-expression experiments in two CRC cell lines: HCT116 and SW480. As shown in panels C and D of Figure 2, Zfp36 over-expression results in a strong decrease in mRNA and protein levels in both cell lines. To further strengthen this hypothesis we performed a luciferase reporter assay using a pGL3-based construct modified in order to generate a luciferase (Luc) mRNA carrying the 3' UTRs of MACC1, ZEB1 and SOX9 after the Luc coding sequence. The results reported in panel D show that the luciferase activity of each of these constructs is significantly lower in presence of Zfp36 overexpression, indicating that the presence of the analyzed 3'UTRs is sufficient to decrease Luc protein levels. These data indicate that Zfp36 is capable to directly bind and degrade the mRNAs of MACC1, ZEB1 and SOX9 by either decreasing mRNA stability or by inhibiting translation.

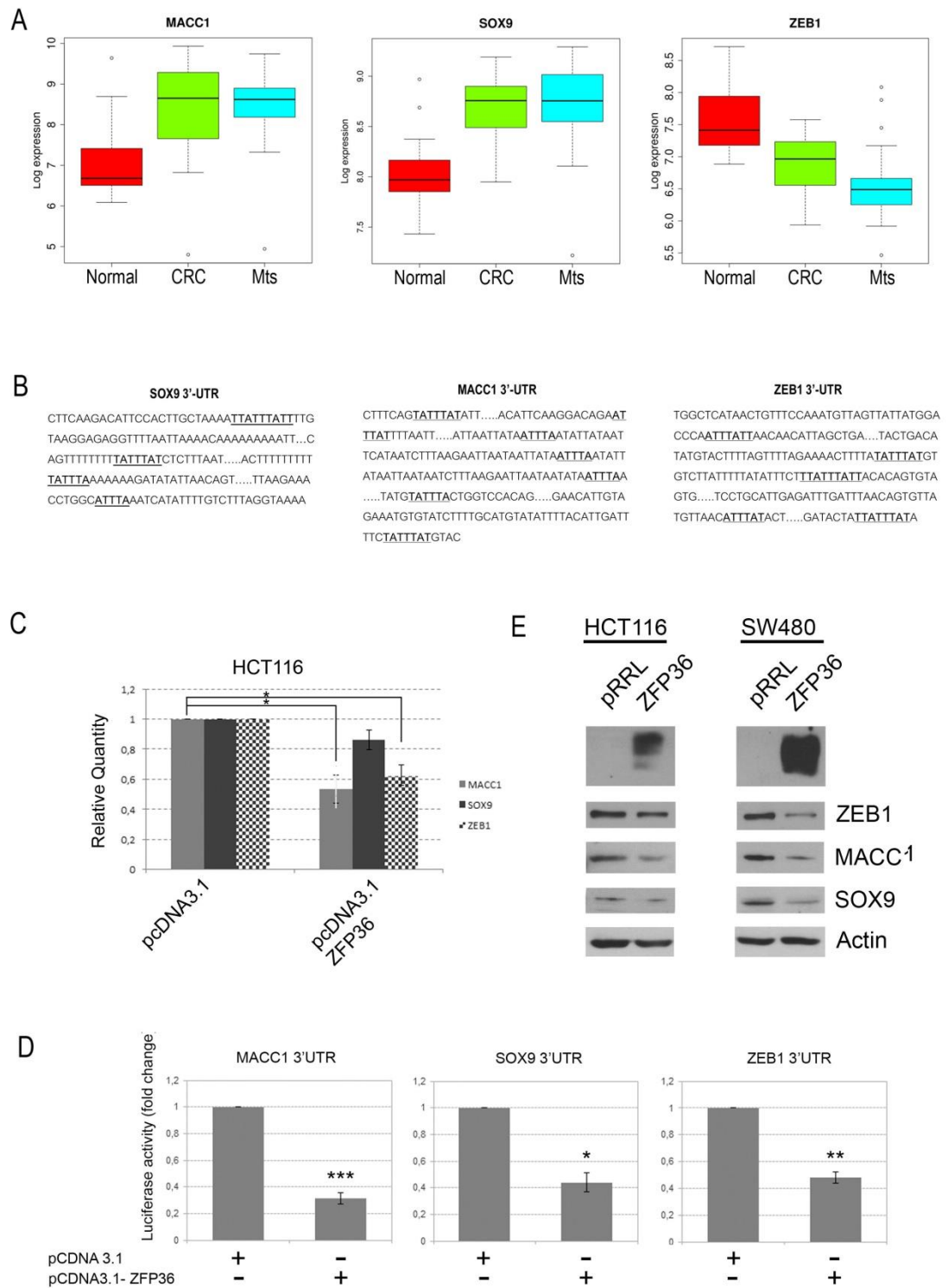


Figure 7: Validation of three novel Zfp36 target genes involved in EMT.

Panel A shows the median Log2 expression values of MACC1, SOX9 and ZEB1 evaluated in normal colon mucosa (Normal), primary carcinoma (CRC) and liver metastases (Mts) samples.

Panel B. represents the 3'UTRs sequences of MACC1, SOX9 and ZEB1. A-U rich sequences (ARE) are highlighted in bold.

Panel C. HCT116 cells were transfected with empty vector (pCDNA3.1) or ZFP36 overexpressing vector (pCDNA3.1-ZFP36). After 48 hours RNA was extracted and MACC1, SOX9, ZEB1 mRNA levels were assessed through qRT-PCR analysis. Results are represented as means of three experiments (+/-SEM) and GAPDH was used as calibrator.

Panel E. HCT116 and SW480 were infected with empty vector (pRRL) or ZFP36 overexpressing vector (ZFP36) and corresponding total protein lysates were analysed through Western blotting techniques with antibodies against MACC1, SOX9, ZEB1 and ZFP36. Actin was used as loading control.

Panel D. A fragment of the 3'UTRs of MACC1, SOX9 and ZEB1 was cloned in a pGL3 vector, downstream of the Luciferase gene. These constructs were co-transfected with a β -gal reporter construct and with empty vector (pCDNA3.1) or ZFP36 overexpressing vector (pCDNA3.1-ZFP36) in HEK293T cells. After 48 hours cells were harvested, luciferase activity was measured and normalized over β -gal signals. Results are represented as means of three independent experiments $\pm$ SEM. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

Zfp36 restoration in CRC cell lines inhibits EMT and induces a shift towards a more epithelial phenotype.

At the light of these observations we decided to verify if Zfp36 restoration could have some effects on EMT-related processes. For this purpose we overexpressed this protein in SW480 and HCT116 through lentiviral transduction. Western blot analysis reported in figure 2 show the protein levels of epithelial markers (E-cadherin and ZO-1) and mesenchymal markers (N-cadherin and vimentin) in cells infected with empty vector (pRRL) or vector carrying Zfp36 coding sequence (ZFP36).

In presence of Zfp36 overexpression, SW480 display an augment of E-cadherin and ZO-1 together with a concomitant decrease in N-cadherin and vimentin levels. Importantly, the most prominent effect appears to be the increase of E-cadherin protein. On the other hand, HCT116 cell line shows similar results on the cadherins and ZO-1 expression, together with a strong decrease of vimentin.

In normal epithelium, E-cadherin exerts its functions as a component of the adherens junctions, and is thereby located on plasma membrane. Thus we decided to gain more information about its sub-cellular localization by analyzing its protein levels through immunofluorescence experiments. Results obtained are depicted in panel C. In agreement with the previous observation E-cadherin protein levels appear to be higher in Zfp36 overexpressing cells, if compared to control cells. Moreover, it is also apparent that Zfp36 induces a noticeable accumulation of this protein in inter-cellular contacts, suggesting an increase in the functions and strength of epithelial junctions. Again, effects on E-cadherin levels were more prominent in SW480 rather than HCT116 cells.

To test whether the activity of intercellular junctions was increased by Zfp36, we performed impedance-based experiments aimed to measure the barrier functions of the cellular monolayer. Thus, we recorded the resistance (4000 Hz) opposed from the cellular monolayer to an small electric field, which is the better condition to assess the strength of intercellular epithelial junctions, in this kind of experiments. In this setting, in fact, the electric current preferentially flows in the spaces between the cells, which are usually occupied by intercellular junctions in the case of an epithelial monolayer. Therefore, the stronger are the cellular contacts, the higher is the resistance opposed to the flow.

The results of these experiments, resembled in panels C and D, reveal how Zfp36-overexpressing cells exhibit an augmented resistance of the cellular monolayer in comparison to control cells. In Panel C, changes in normalized resistance are reported in function of time, where 0h correspond to the moment of the seeding. In panel D, the same parameter is recorded for 5 hours after the establishment of complete cellular confluence, and then averaged and expressed as a fold changes. Precisely, cellular resistance significantly increase of the 40% in SW480 and of 20% in HCT116 cells

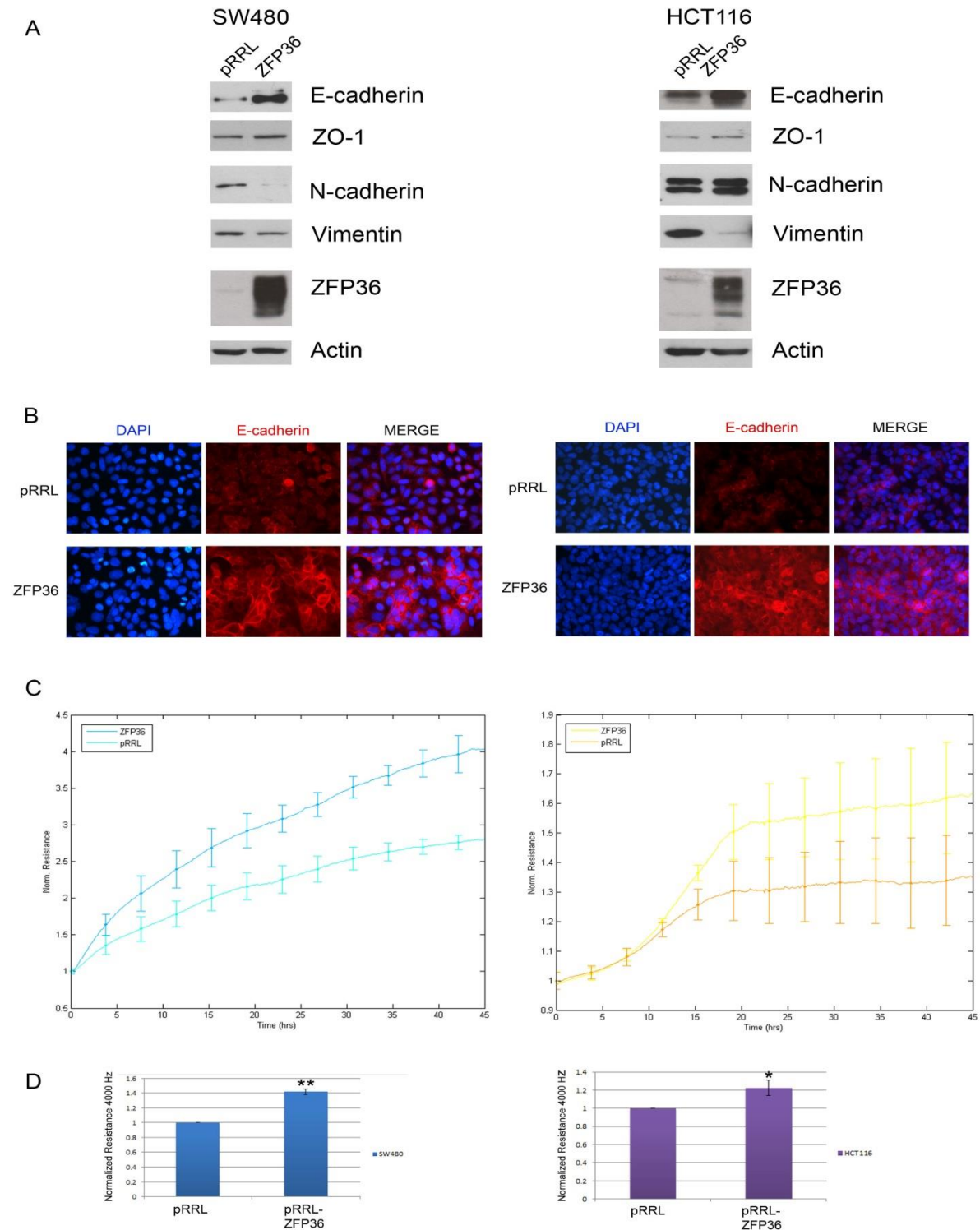


Figure 8: Zfp36 overexpression induces a shift towards epithelial phenotype.

Panel A. HCT116 and SW480 were infected with empty vector (pRRL) or Zfp36 overexpressing vector (ZFP36) and corresponding total protein lysate were analysed through Western blot with antibodies against E-cadherin, ZO-1, N-cadherin, Vimentin and ZFP36. Actin was used as a loading control.

Panel B. HCT116 and SW480 infected with pRRL and ZFP36 vectors were seeded on glass dishes and subsequently subjected to immunofluorescence analysis with antibody against E-cadherin (red), to assess its sub-cellular localization. Nuclei were counterstained with DAPI (blue).

Panel C. HCT116 and SW480 infected with pRRL and ZFP36 vectors were seeded on uncoated gold microelectrodes and subjected to a small alternating current. Immediately after seeding, resistance at 4000Hz was recorded every 5 minutes for at least 45 hours. Graphs show the mean of the normalized resistance of three different experiments plotted in function of time (+/-SEM).

Panel D. Histograms summarizing the results described in panel C. Approximately 48 hours after seeding, resistance at 4000 Hz became stable and was recorded for 5 more hours. Values obtained during the last 5 hours were averaged and normalized on pRRL values. * $p < 0.05$, ** $p < 0.001$,

Zfp36 restoration in CRC cell lines impairs anchorage independent growth and increases susceptibility to anoikis.

Having described the phenotypic changes induced from Zfp36 ectopic overexpression, we decided to examine the effects on cellular behavior. As previously described, resistance to anoikis together with the ability to grow in absence of a substrate, are two key features which epithelial cells need to acquire in order to give rise to metastasis. For this reason we analyzed these cellular abilities through different experiments.

Results of the soft agar assays, reported in panel A,B,C show that Zfp36 ectopic induction strongly diminishes the ability of the cells to form colonies inside a semi solid medium, thus in absence of anchorage. This phenomenon was more prominent in SW480 cell lines where, after ectopic induction of Zfp36, we observed a decrease of almost 70% in colony formation efficiency, while HCT116 displayed a decrease of 40%. These results were confirmed also in SW620 cell line, which is the metastatic counterpart of SW480. Although the decrease observed in this case is minor (33%), this last result is particularly relevant, since this cell line is considered highly malignant and strongly resistant to anoikis.

Subsequently we assessed the effects of Zfp36 overexpression on sensitivity to anoikis. For this purpose we cultured cells in presence of Dispase, thereby preventing cell adhesion. The analysis of cellular viability, reported in panel D, displays that Zfp36 overexpressing cells survive with minor efficiency in these conditions, indicating that presence of Zfp36 sensitizes cells to anoikis. For

HCT116 cell line, pRRL cells survive almost two times better than Zfp36 overexpressing cells both at 24 and 48 hours. SW480 cells display a significant difference just at 24 hours.

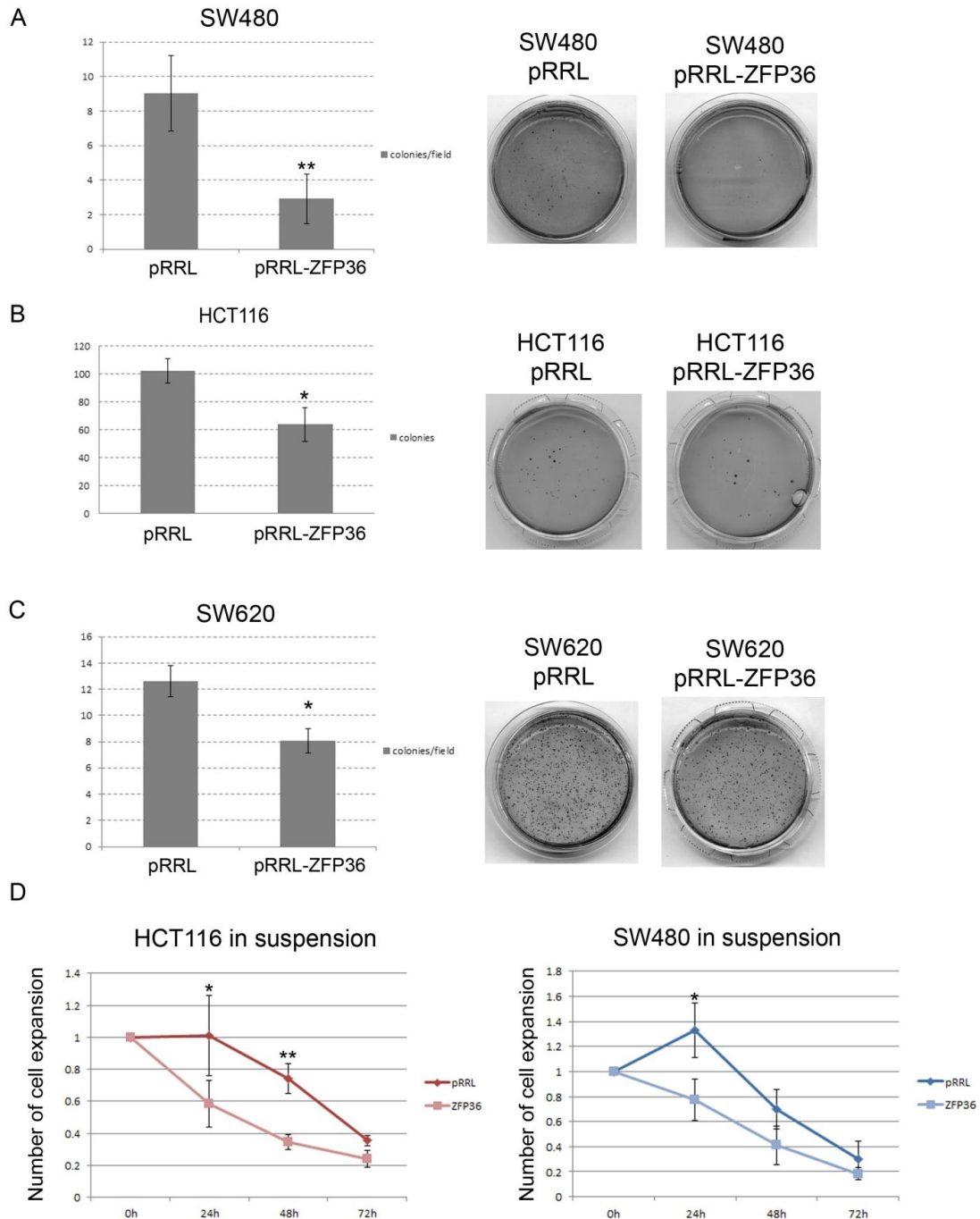


Figure 9: Zfp36 overexpression impairs anchorage-independent-growth and sensitize to anoikis.

Panel A,B,C. HCT116, SW480 and SW620 infected with empty vector (pRRL) or Zfp36 overexpressing vector (ZFP36) were seeded in soft-agar medium (DMEM + 0,3% Agar) and allowed to form colonies. Histograms report the number of colonies in one optical field and represent the mean of three independent experiments +/- SEM. For HCT116 the total number of colonies is reported Photographs show the crystal-violet staining of one representative experiment. Panel D. Equal amounts of HCT116 and SW480 infected with pRRL or ZFP36 vectors were seeded in presence of Dispase to prevent cell adhesion (in suspension). Vital cells were then counted through Trypan blue exclusion assay. Results were normalized on the number of cells seeded at 0 hours and reported as the mean of three independent experiments (+/- SEM). * $p < 0.05$, ** $p < 0.001$

Zfp36 restoration in CRC cell lines exerts minor effects on cellular migration

Another important feature of cells undergoing EMT is the acquisition of migratory abilities. For this reason we speculated that Zfp36 overexpression could decrease cellular motility. Surprisingly, the results of traditional scratch assays shown in Panel B demonstrate that, comparing control cells and Zfp36 overexpressing cells, there is no significant difference in the time required for the gap closure. In an attempt to further verify this result we performed a similar assay with the ECIS machinery. In this case the wound in cellular monolayer was achieved by an high voltage pulse, and the efficiency of gap closure was assessed by measuring the cellular impedance. This assay, although it was repeated just one time, confirmed the previous result (Panel A).

Finally we performed a further kind of test, the transwell assay. This kind of experiment assesses the ability of the cells to migrate towards a chemoattractant stimulus, in contrast to scratch assays which measure the random migration of cells. Our data in this case are quite preliminary, but they show a difference in the migration abilities between control cells and Zfp36 cells. In fact, the count of migrated nuclei shown in panel C, reveals that SW480-Zfp36 migrated with half the efficiency of control cells. Although these data require further investigations, in our opinion they indicate that Zfp36 restoration exerts a weak effect on cellular migration and has major effects on anoikis sensitivity and anchorage independent growth.

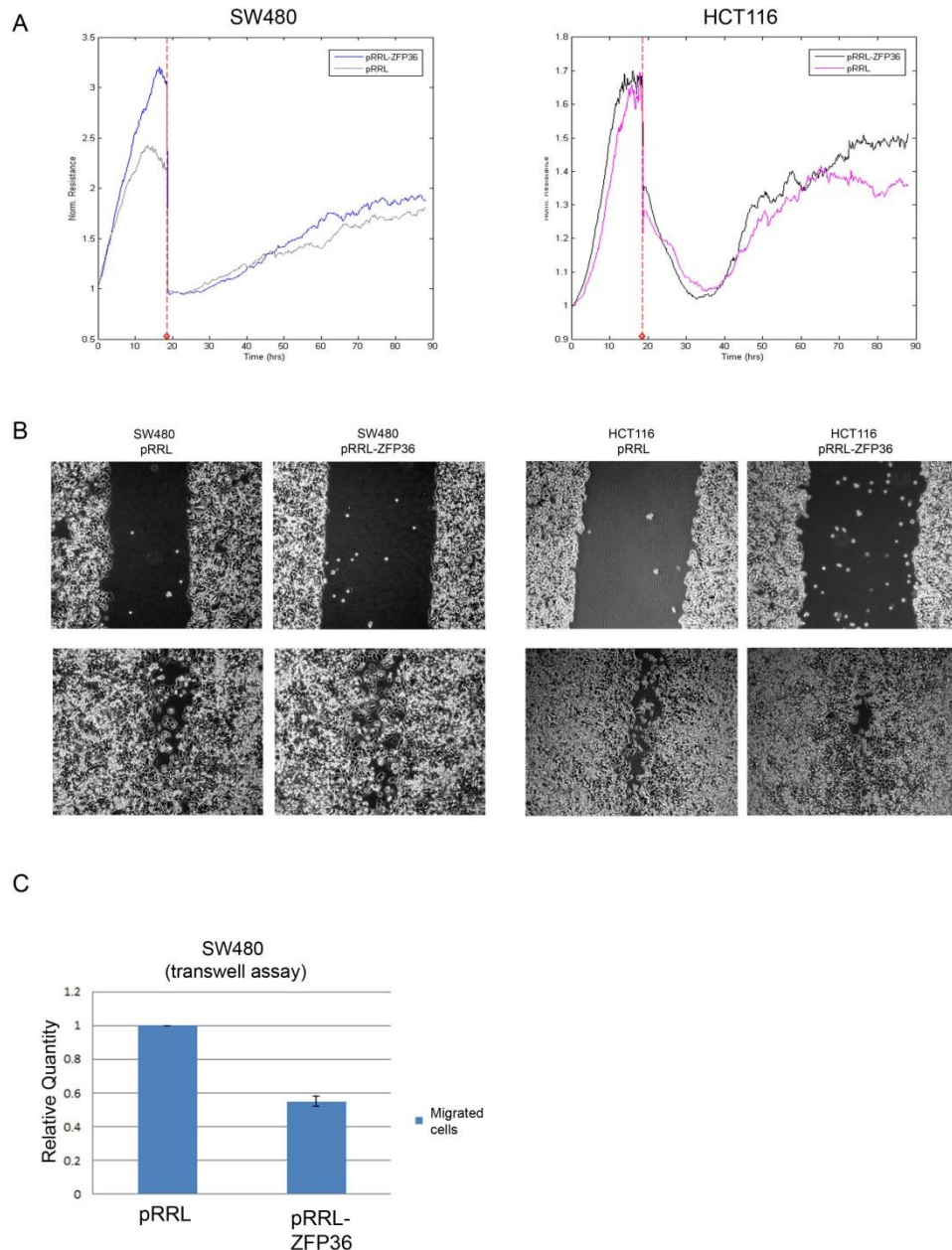


Figure 10 Zfp36 restoration in CRC cell lines exerts minor effects on cellular migration:

Panel A. SW480 and HCT116 infected with empty vector (pRRL) or ZFP36 expressing vector (pRRL-ZFP36) were seeded on uncoated gold microelectrodes and subjected to a small alternating current. After reaching complete confluence, a wound in the cellular layer was produced by applying a high voltage pulse and the recovery of the cellular monolayer was monitored by measuring Resistance at 4000 kHz. The graphs shown represent a single experiment.

Panel B. SW480 and HCT116 infected with empty vector (pRRL) or ZFP36 overexpressing vector (pRRL-ZFP36) were grown until complete confluence, and a scratch on cellular monolayer was performed with a pipette tip. The results of one representative experiment are shown in the photographs.

Panel C. SW480 and HCT116 infected with empty vector (pRRL) or Zfp36 overexpressing vector (pRRL-ZFP36) were serum starved and seeded inside the upper chamber of 8 μ m-pore transwell insert. Cells were then allowed to migrate towards a chemo-attractant stimulus (FBS) for 16 hours. Migrated nuclei were stained with DAPI and quantified by taking random pictures. Results obtained were normalized on the pRRL values and represented as the mean of three independent experiments

Zfp36 expression in CRC cell lines is inversely correlated to Wnt/ β -Catenin pathway activity

Today, multiple scientific reports demonstrate the silencing of Zfp36 during CRC progression(186, 187). Despite of that, evidences about the mechanistical explanation of this phenomenon are still lacking. Thus, we hypothesized that this phenomenon could be a downstream effect of the abnormal activation of a molecular pathway involved in CRC. In particular, the funding importance of Wnt/ β -Catenin pathway activation in CRC development led us to analyze the relationship between the state of activity of this signaling cascade and Zfp36 expression. In order to address this point we designed different experiments aimed to inhibit or stimulate the activity of β -catenin pathway.

Firstly we decided to repress this pathway through the use of a small molecule (FH535) which inhibits β -catenin mediated transcription through silencing of TCF7L2 (188). Results of these experiments, reported in Figure 6 panel A, show that treatment of HCT116 with FH535 20 μ M resulted in a time-dependent induction of Zfp36 mRNA if compared to cells treated with DMSO alone, as assessed

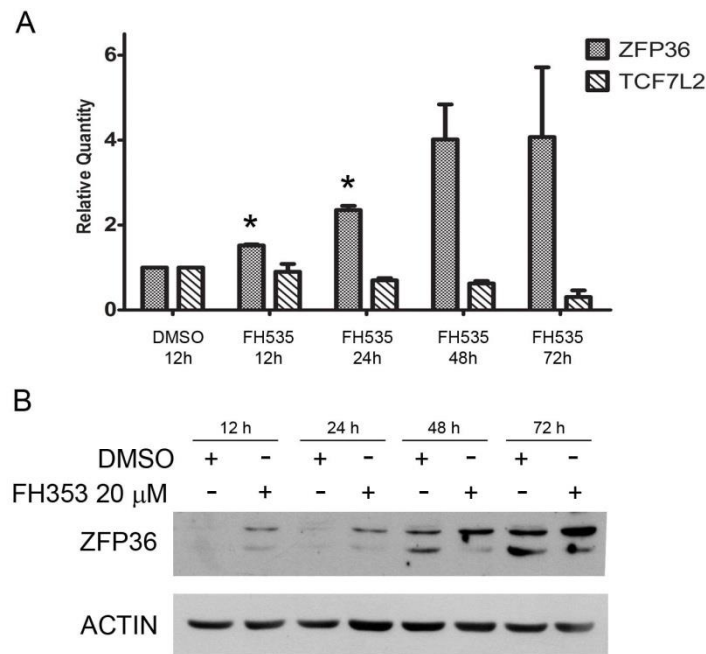


Figure 11: Pharmacological inhibition of β -catenin pathway results in an increase of ZFP36 expression.

Panel A. HCT116 were treated with FH535 20 μ M or vehicle (DMSO) for the indicated time. The RNA was extracted, retro-transcribed and used for real-time qRT-PCR analysis of ZFP36 and TCF7L2 transcripts. Results were normalized on DMSO values at each time point. Histograms represent the mean of two independent experiments ($\pm$ SEM), GAPDH was used as calibrator. *p < 0.05

Panel B. HCT116 treated as in Panel A were lysed and equal amounts of total protein extracts were loaded on a SDS-PAGE and subsequently analysed through Western blot techniques with an antibody against Zfp36. Actin was used as loading control

through real-time PCR. This increase ranged between 1.8 and 4 fold changes induction, after 12h and 72 hours respectively, and is statistically significant at 12 and 24 hours. We also assessed the expression of TCF7L2 which, as expected, progressively decreases during time. The time-dependent induction of Zfp36 mRNAs resulted in a concomitant increase of its protein levels, as displayed in Figure 6, panel C.

The second part of these experiments, designed to achieve the stimulation of β -catenin pathway activity, was performed through the use of Wnt3a conditioned medium, described in Materials and Methods. The use of Wnt3a cytokine was intended to activate the pathway in a physiological and specific manner. Western blot analysis displayed Panel A (Figure 12) demonstrates that protein levels of Zfp36 are strongly decreased in Wnt3a-treated cells, if compared control cells, both in HCT116 and SW480.

This observation generated two main hypotheses: the first is that Zfp36 transcription could be downregulated from a repressor induced by Wnt3a treatment, while the second one is that β -catenin could directly repress Zfp36. A third hypothesis, which requires further investigations, consists in the possibility that Wnt3a treatment could affect the stability of Zfp36 mRNA. In the attempt to clarify this issue we took advantage of Cycloheximide (CHX), which inhibits protein synthesis. Evaluation of expression levels of Axin2, a well-known β -catenin target gene, was used as positive control of the effectiveness of Wnt3a usage. Treatment with Wnt3a conditioned medium alone for 3, 6 and 9 hours resulted in a time dependent increase of Axin2 transcripts, and to a slight decrease in Zfp36 mRNA. The addition of CHX in culture medium surprisingly resulted in a strong augment of Zfp36 mRNA (up to 10 folds), a phenomenon previously described in a couple of papers and not linked to an increase in protein levels (189, 190). Interestingly, also in presence of CHX, Wnt3a treated cells displayed reduced levels of Zfp36 transcript, compared to cells receiving E.V. medium, with a decrease of 30% after 6 hours of treatment. Curiously, also Axin2 induction was enhanced by CHX treatment. These results were confirmed also through Western Blot analysis, displayed in panel B. These data demonstrate that inhibition of protein synthesis does not affect Wnt3a-mediated repression of Zfp36, thus suggesting that this could be a direct effect of β -catenin/TCF complex. Another clue in favor of this hypothesis is represented by the presence of some TCF7L2 putative binding sites in the promoter

of Zfp36, and by the proof of the effective binding of TCF7L2 to some of these sequences, provided by Encode project data (data not shown) .

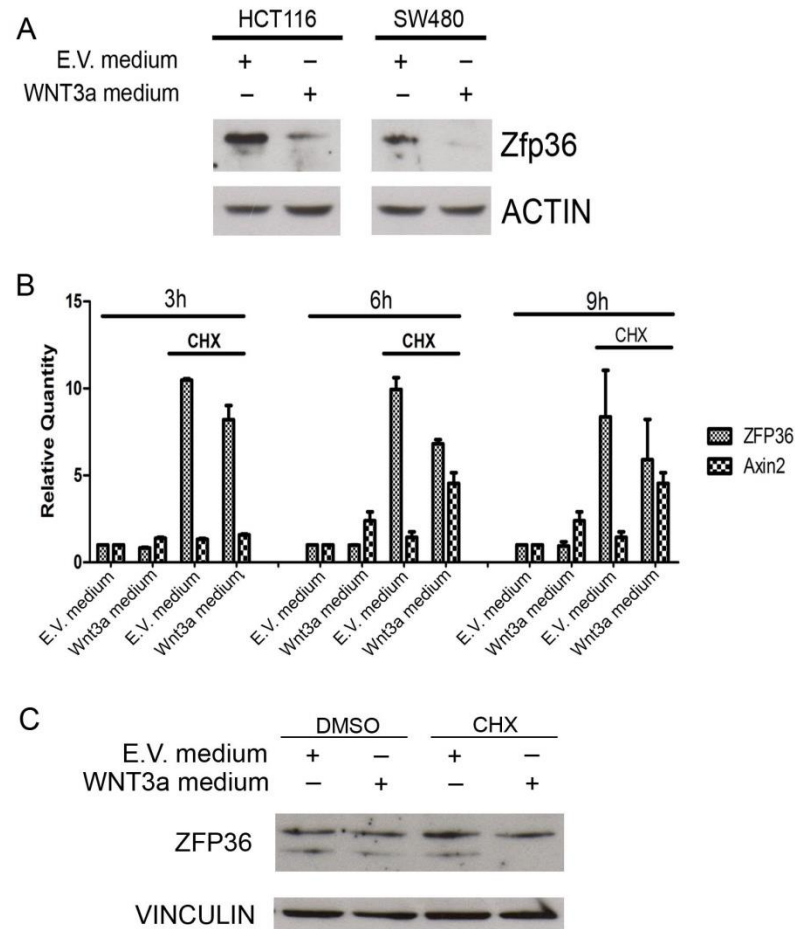


Figure 12: Stimulation of β -catenin pathway down-regulates Zfp36 independently of protein synthesis.

Panel A: HCT116 and SW480 were treated with conditioned medium obtained from empty vector cells (E.V medium) or Wnt3a overexpressing cells (Wnt3a medium). After 24 hours cells were collected, lysed and equal amounts of protein were loaded on SDS-PAGE and subsequently analysed through Western blot technique with an antibody against Zfp36. Actin was used as loading control. Panel B.

Panel B: HCT116 was treated with E.V. medium and Wnt3a medium supplemented with Cyclohexymide (CHX) or DMSO (not indicated). After the indicated amount of time, RNA was extracted, retro-transcribed and used for real-time PCR analysis of Zfp36 and Axin2 transcripts. Results were normalized on E.V medium values at each time point. Histograms represent the mean of two independent experiments ($\pm$ SEM), GAPDH was used as calibrator.

Panel C: HCT116 were treated with E.V. medium and Wnt3a medium supplemented with (CHX) or DMSO for 6 hours. Cells were then collected, lysed and equal amounts of protein were loaded on SDS-PAGE and subsequently analysed through Western blot techniques with antibody against Zfp36. Vinculin was used as loading control.

Discussion

Colorectal cancer is one of the most common malignancies in developed countries and accounts globally for the 12% of the cancer-related deaths in Europe. The survival after 5-years dramatically drops from 90% of localized tumors to 10% of the metastatic tumors, implying that development of metastasis is fatal in almost the totality of the cases (79). Assumed this, it is easy to understand why a great attention is currently pointed into understanding the basis of this process and into the development of novel therapies aimed to treat this last step of tumorigenesis. Trans-differentiation of epithelial cells into mesenchymal-like cells, the Epithelial-to-Mesenchymal (EMT) process, is considered necessary for metastasis development and for the acquisition of pharmacological resistance to chemotherapy, which is one of the biggest issues in metastasis treatment (94).

Zfp36 is a RNA binding protein whose expression appears to be downregulated in different tumors. This protein directly interferes with the expression of a broad range of oncogenes and its role as a tumor suppressor has been reported in multiple publications. Interestingly, we observed that three genes highly involved in metastasis development contain some Zfp36 binding sequences in their 3'UTR, namely MACC1 SOX9 and ZEB1. In particular ZEB1 is considered one of the EMT master regulators, together with Snail and Twist (110). MACC1 is of more recent discovery but its association with metastasis is higher than that of other well-known proteins (such as CAEs) (182, 183). Finally, SOX9 enhances metastasis formation in a broad range of tumors and regulates EMT in physiological situations such early embryogenesis (154). Through overexpression experiments and luciferase assays, we demonstrated that Zfp36 directly mediates the decay of these transcription factors and the consequent diminishing of the corresponding proteins in CRC cell lines.

The identification of these novel Zfp36 target genes led us to hypothesize that Zfp36 inactivation could facilitate metastasis development in CRC. On this ground we analyzed Zfp36 expression in a database of CRC related samples. This analysis shows that Zfp36 is downregulated in CRC carcinoma

samples in comparison to normal tissue, confirming previous reports. In metastatic samples Zfp36 mRNA levels appear to be further diminished, even if this observation seems to be not significant. Moreover the analysis of Zfp36 protein levels in two CRC cell lines derived from a primary tumor and from the corresponding metastatic lesion enforced this result and revealed a strong silencing of Zfp36 in metastatic cells. Since Zfp36 expression is subject to extensive post-transcriptional regulation, the analysis of its protein levels should be considered more relevant rather than the analysis of its mRNA levels. Assumed that Zfp36 inhibits the expression of at least three transcription factors involved in EMT regulation, we speculated that Zfp36 ectopic expression could affect phenotype and behavior of CRC cell lines, inducing a reversion of the EMT process. These cells, in fact, are no longer uniquely epithelial and have gained some features distinctive of the mesenchymal phenotype, such as the expression of mesenchymal markers and motility. As expected, Zfp36 overexpression resulted in the induction of epithelial markers and in the repression of mesenchymal markers, collectively indicating the acquisition of a more epithelial-like phenotype. This finding was also confirmed through functional assays aimed to measure the barrier functions of cellular layer. Surprisingly, in our experimental context, we could not detect a strong effect on cellular migration assessed through scratch assays. Conversely, we could observe major effects on different EMT-related process. In fact, ectopic induction of Zfp36 resulted in an impairment of anchorage-independent growth of the cells, together with an increased sensitivity to anoikis, which are two key features necessary for the spreading of metastatic cells into the organism.

Nowadays it is quite recognized that Zfp36 is downregulated in CRC development, but the mechanisms underlying this event are still undisclosed. Scientific evidences about specific mutations or genetic loss of Zfp36 locus in CRC are lacking. Since the silencing of Zfp36 expression is a common event observed in a broad range of tumors and being few the common aspects between these malignancies, we speculated that Zfp36 could be specifically repressed by different oncogenic stimuli in different cellular contexts. The most relevant oncogenic pathway in CRC pathogenesis is surely Wnt/ β -catenin pathway, therefore we decided to verify if the activity of this signaling cascade could affect Zfp36 expression. Interestingly, experiments performed both with pharmacological inhibitors

and with an endogenous agonist revealed that Zfp36 expression is inversely correlated to Wnt/ β -catenin pathway activity in CRC cell lines. In an attempt to further elucidate this point, we performed experiments with Cycloheximide which revealed that this effect on Zfp36 mRNA is independent of protein synthesis. These results allowed to further hypothesize on this mechanism. The first possibility is that β -catenin might directly regulates the expression of Zfp36 mRNA. This hypothesis is strengthened by some data from the Encode project which demonstrate the direct binding of TCF7L2 to multiple sites on Zfp36 promoter region. In this case it would be one of the few described cases in which β -catenin would act as a transcriptional repressor (191, 192). The other hypothesis consist in the possibilities that β -catenin could act through the upregulation of a non-coding RNA (i.e. a miRNA) and, in general, through the modulation of Zfp36 mRNA stability.

Collectively, these data indicate that Zfp36 negatively regulates EMT process by inducing a more epithelial phenotype together with an increased sensitivity to anoikis. Similar observations were made by a very recently published work demonstrating that also Snail and Twist are Zfp36 target genes (193). On this ground we can affirm that Zfp36 is a potent “epithelial factor” which controls the expression of multiple EMT master regulators thereby affecting the expression of a plethora of target genes .

Finally this work provides, for the first time, evidences of a relationship between β -catenin pathway and Zfp36 expression and, more importantly, a possible explanation underlying the loss of Zfp36 in colorectal cancers.

The last observation regard the possible clinical relevance of this work. In fact, although it is a merely speculative remark, the use of chemical and natural compounds capable of inducing the expression of Zfp36 could be useful in the different aspects of metastasis treatment. Firstly, the Zfp36-inducing activities of these compounds could inhibit metastasis formation by inducing anoikis or inhibiting cellular spreading and could therefore find a practical application as preventive measures in patients with increased risk for CRC development. Moreover, we speculate that the use of these compounds could be useful in order to sensitize cells to chemotherapy. In fact, metastatic cells often acquire a

pharmacological resistance, and the synergy between these molecules and chemotherapeutic drugs could be useful in overcoming this issue resulting in a definitive improvement in CRC therapy (194).

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