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Identification of potential oncogenes as novel therapeutic targets by RNAi screening

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ABSTRACT

Colorectal cancer (CRC) represents one of the leading causes of cancer worldwide, with a prevalence of more than a million cases every year. Despite the decreasing incidence in the last few years, as a result of the implementation of early diagnosis screening programmes, the mortality rate is still about 50%. Consequently, new treatments of CRC patients are demanded, especially in advanced stages. Nowadays the most common therapeutic options are cytotoxic drugs, such as 5-fluorouracil and irinotecan, associated to biologics, such as cetuximab, according to the severity of the neoplasia. However, metastatic CRC do not respond very well to the available therapies, therefore better therapies should be taken into account.

In this context, we have conducted an RNAi screening in order to identify new oncogenes that could be used as therapeutic target of new drugs. Starting from a list of amplified genes retrieved from a publicly available database, provided by The Cancer Genome Atlas, we analysed which gene could negatively influence cell viability in different CRC cell lines, which would indicate its possible involvement in CRC. After a careful evaluation of the silencing specificity, we functionally analysed the amplified genes and find that one gene called STARD3, which is frequently co-amplified with HER2, is of particular interest. Given the phenotype resulting from its inhibition, the finding was confirmed by a cell viability time course and further supported by cell cycle analysis, which demonstrated an significant increase in subG1 populations. In order to identity the nature of the processes involved in the cell death increment, we analysed different apoptotic markers, which revealed a significant increase of apoptosis upon STARD3 knockdown. In addition, we detected reduction of migration rate and the reduction of anchorage-independent growth after STARD3 downregulation as well, which both corroborated with the hypothesis that STARD3 is an oncogene in colorectal cancer. Finally, despite the mechanism by which STARD3 exerts its function is still not clear, we showed that its ablation may result in suppression of p53 oncogenic activity, which also reinforce a possible relationship between STARD3 and cell cycle regulation. Besides the variability amongst the cell lines, which may be imputable to their different genetic background, STARD3 seems to possess a different mechanism of action in CRC in comparison to the proposed mechanism in breast cancer. Thereby, we can conclude that our approach toward the identification of oncogenes has been successful and amongst the amplified genes, STARD3 has an oncogenic potential and this is supported by the phenotypes of its

silencing. Furthermore, this result shed light on the role of STARD3 in colorectal cancer, opening a possibility to use it not only as cancer biomarker, but also as a therapeutic target, which would provides a better treatment for colorectal cancer.

1 - INTRODUCTION

1.1 Colorectal Cancer

Colorectal cancer (CRC) is a global burden with an incidence of 1.5 million diagnoses every year. It is the fifth most common cancer worldwide, and almost half of the patients die from the disease (Ferlay et al., 2015) (Figure 1).

Tumours of colon and rectum are very variable, and hyperplastic mass that protrude from the mucosa are called polyps (Bosman et al., 2010). Even if most of the polyps are hyperplastic and have a dimension of <5mm, few of them progress in carcinoma (Zlobec et al., 2012). Instead, the precancerous lesion develops from adenoma, which arises from glandular epithelium and presents dysplastic morphology and altered cellular differentiation (Zlobec et al., 2012). Adenomas are detected from 20 to 40% in people of >50 years of age and increase with old age, nevertheless only a small amount of adenomas develop in CRC. Surgical resection of the polyps reduces significantly the risk (Liljegren et al., 2003).

Even if early stages of invasive cancer are still curable with surgery and chemotherapy, nonetheless if untreated they metastasize to other organs, a stage where CRC is usually incurable. Novel therapeutic options have improved the survival at the early stages, however later stages are still less responsive to the therapies (Markowitz and Bertagnolli, 2009).

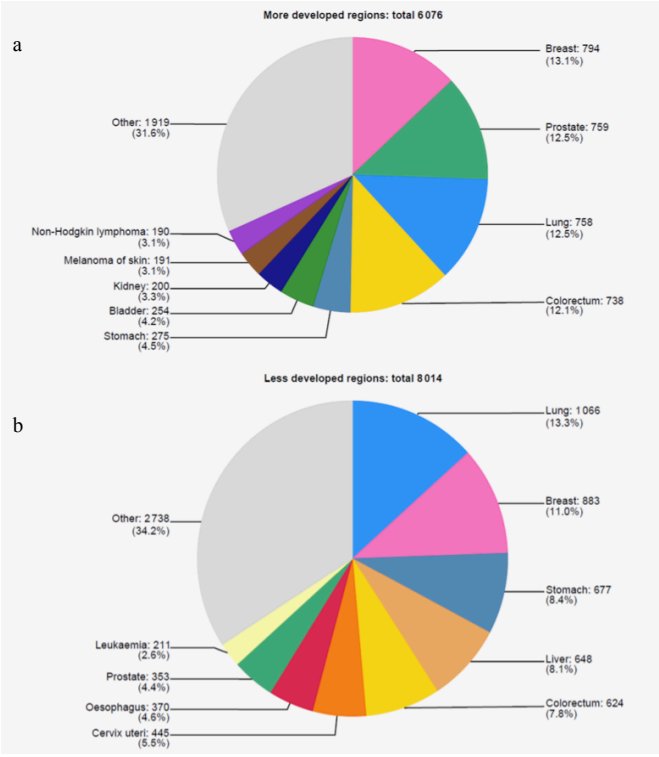

Figure 1 Estimated global numbers of new cases (thousands) with proportions for (a) more developed and (b) less developed regions, both sexes combined, 2012. The area of the pie is proportional to the number of new cases. Adapted from Feraly et al., 2015

In CRC, different and complex factors play a fundamental role in its onset and progression. On one hand, the environmental factors have a significant role on CRC. Particular dietary regiment and lifestyle, such as a diet rich in unsaturated fats and red meat, total energy intake, excessive alcohol consumption, and reduced physical activity appear to be important risk factors for the disease (Huxley et al., 2009). Conversely, nonsteroidal anti-inflammatory drugs, estrogens, calcium, and perhaps some statins protect against CRC (Thun et al., 2012). These and other factors, for example gut microbiota and inflammation, have been suggested to be potential external risk factors. These could indeed help reduce CRC incidence, but neither dietary nor other environmental specific risk factors have been well defined, yet (Chan and Giovannucci, 2010).

On the other hand, the study of the genetics and the molecular basis have identified several pivotal genes in CRC oncogenesis, such as p53, APC and KRAS (Rodrigues et al., 1990; Fodde et al., 2001; Lievre et al., 2006). Somatic and inherited alterations of these genes can be regarded as risk factors, since they increase predisposition to CRC and are currently used as biomarkers, for instance, to predict progression or resistance to specific therapies (Prenen et al., 2013). The genetic defects have different nature, they could be point mutations, chromosome rearrangements, deletions or amplifications, and can be divided in two different types of alteration: oncogenic activation or tumour suppressor inactivation (Cancer Genome Atlas Network, 2012). This plethora of different defects is then shaped by clonal selection, which results in different subsets of colorectal cancer with very different and distinctive, often overlapping, features (Siravegna et al., 2015; Greaves and Maley, 2012).

Finally, another key aspect of CRCs is its familiarity. Eighty-five percent of colorectal cancer cases are sporadic without family history or genetic predisposition, whilst in approximately 15% of cases a causative inherited genetic event has been identified, which highlight the importance of preventive genetic screening (Taylor et al., 2010).

1.1.1 Classification

In the recent years, with advancements in personalised medicine, colorectal cancer patient management has associated to traditional classification, based on morphology and histopathology, also immunohistochemistry and genetics.

Colorectal cancer is primarily classified by using TNM staging system, recommended by the WHO. It takes into account tumour site and invasiveness, status of lymph nodes, and location and number of metastases (Hamilton et al., 2000). Besides TNM staging, from histopathological point of view, can be classified in adenocarcinomas, neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas. Among all CRCs, more than 90% are adenocarcinomas originating from epithelial cells of the colorectal mucosa, generally with a moderate differentiation. (Bosman et al., 2010). The precise classification is a fundamental aspect of clinical management of colorectal cancers, since based on it the clinicians decide the more suitable therapy (Fleming et al., 2012)

1.1.2 Molecular Classification of Colorectal Cancer

The CRC multistep transformation from adenoma to carcinoma, as presented in the early ’90, is a model of colorectal cancerogenesis in which selective mutations are consequentially acquired (PDQ Cancer Genetics Editorial Board, 2002; Fearon and Vogelstein, 1990), and this concept is still valid today for most of CRCs. Whereas once few mutations were believed to be sufficient to progress to carcinoma, colorectal cancer genome screenings have revealed that a wide array of different mutations can instead occur to the tumours, but only a small subset (<15) are likely to be indeed driver mutations (Wood et al., 2007; Cancer Genome Atlas Network, 2012) (Figure 2)

One of the main characteristics of all colorectal cancers is the acquisition of a certain/specific level of genomic instability at some point during the onset and progression of the disease. Notwithstanding, the precise molecular origin, both familial and sporadic CRCs share most of the pathological mechanisms.

The recent advancements in the genomics of CRC discovered previously unknown genetic and molecular features, allowing not only a better classification of CRCs but also an improved prognosis and treatment. These data suggest that CRCs are not a single disease, but a series of different tumours with different and frequently overlapping features (Bogaert and Prenen, 2014) .

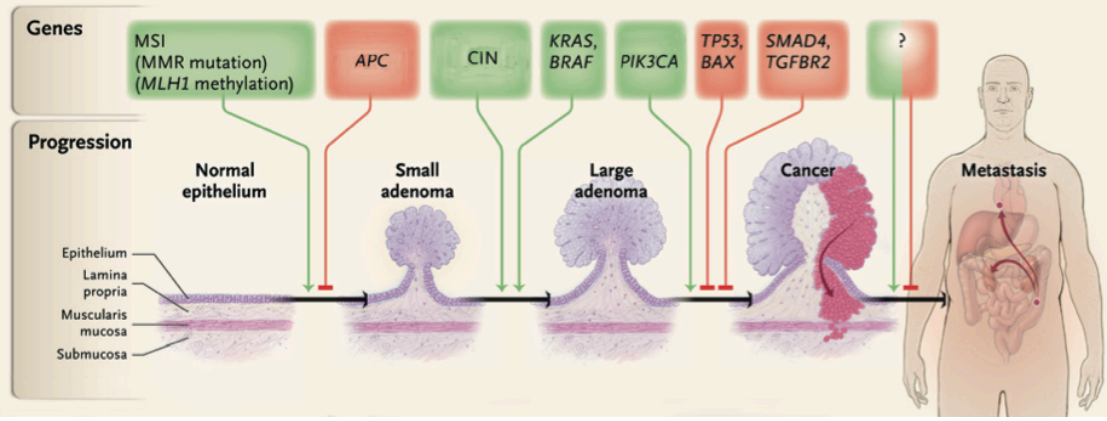

Figure 2 Genes and pathways involved in the multistep carcinogenesis of colorectal cancer. This model represents some of the genes involved in the step-wise progression of CRC. The aberrant activity of these genes activation of oncogenes, such as MMR, or inactivation of tumour suppressors, such as TP53, lead to the formation of a tumour from the normal epithelium, which eventually evolve in metastasis. Oncogenic alterations are depicted in green, oncosuppressive in red. Adapted from Markowitz and Bertagnolli, 2009.

1.1.2.1 Molecular subtypes

CRCs are mainly molecularly classified as microsatellite instable (MSI), caused by defective function of DNA mismatch repair system (MMR), microsatellite stable (MSS), which is mostly associated with chromosomal instability (CIN), and hypermethylated, also known as CIMP (CpG island methylation phenotype). MSI phenotype is characterised by a clear molecular origin and specific clinical-pathological features. CIN tumours are characterised by chromosomal instability, resulting in aneuploidy, with both chromosomal gains and losses, and by a highly heterogeneity from clinical-pathological and prognostic traits (Bogaert and Prenen, 2014). Another layer of complexity of the classification is added by the methylation status. The analysis of different methylation marker on CpG islands, identified a different phenotype, which is CpG island methylation phenotype (CIMP). This phenotype is characterised by the methylation of TSG promoters and, according to the specific extension and location of the methylation on the genome, the activity of different oncosuppressive pathways could be hampered, which could lead in turn to the onset of very diverse tumours from both biological and clinical perspective (Issa, 2004).

1.1.2.1.1 Chromosomal Instability - CIN

CIN represents the most frequent and most heterogeneous phenotype detected in CRC, accounting for about 85% of the total CRCs (van Geel et al., 2015). Whereas this subtype has well defined genetic alterations, it is unclear whether the genomic

alterations are the cause of cancer initiation and progression or the result of it; moreover, its molecular basis seems to much more complex and heterogeneous than MSI (Pino and Chung, 2010).

The pathogenesis of this type of CRC is described by the model of multisteps carcinogenesis. At the beginning of CRC tumourigenesis, aberrant lesion of the intestinal crypt is formed, that is generally due to APC inactivation. The progression to adenoma and early carcinoma needs instead first KRAS activating mutation, then TP53 mutation and chromosome 18q loss of heterozygosity (LOH). Other mutations, such as PI3KCA activation, can occur at the later stages in a small subset of CRCs. During the progression of tumourigenesis, chromosomal instability increases (Takayama et al., 1998).

Furthermore, CIN cancers are also characterised by other genomic alterations: chromosomal imbalances in terms of number and size, frequent LOH, copy number amplification and altered chromosomal segregation (Bogaert and Prenen, 2014). Genotyping studies have also found that CIN cancers, besides presenting large genomic alteration or LOH, are associated with low or no microsatellite instability, also referred as microsatellite stable (MSS) (Vilar and Tabernero, 2013).

The disruption of genomic stability can have different consequences on other pathways, such as a defective mitotic spindle assembly, after mutation of BUB1 for example, an abnormal telomerase activity, caused by either TERC overexpression or telomere shortening, or LOH, regarding mainly APC and TP53 (Pino and Chung, 2010).

1.1.2.1.2 MicroSatellite Instability – MSI

Microsatellite instability in colorectal cancer is defined by the dysfunction of MMR pathway, which leads to genetic hypermutation. The phenotype is characterized by a poorly differentiated histotype, a usually clear molecular origin, enriched with BRAF mutations, and a favourable prognosis in early-stage of the disease. Besides being one of the main features in hereditary non-polyposis colorectal cancers (HNPCC), which I am going to describe later, MSI is detected mainly in sporadic CRC (Merok et al., 2013; Timmermann et al., 2010).

Microsatellites are short nucleotide repeat of 1-6 bp, which, in MSI tumours, tend to accumulate mutations, insertion and/or deletions because of the improper activity of DNA polymerase. MMR defective genes give rise to genetic abnormalities that are

hugely amplified, in fact it has been identified a difference of 100-fold in MSI cancers compared to non cancerous tissues (Thomas et al., 1996). The first gene to be identified to play a role in MSI cancers, in MSH2, a fundamental component of the MMR machinery, that is located in the short arm of chromosome 2. Other important genes found in MSI CRCs are MLH1, PMS1, PMS2, GTBP and MSH6 (Rustgi, 2007). Considering the defective MMR pathway, an MSI phenotype accumulates a high number of mutations, which, if occur in a TSG, such the case of APC (Giannakis et al., 2014), drive rapidly the progression from benign tumour to cancer (Vilar and Tabernero, 2013). MSI cancers can be also divided in 2 sub-phenotypes: high frequency MSI (MSI-H) and low frequency MSI (MSI-L).

MSI-H cancers, in which more than 40% microsatellite loci are mutated, represent the 15% of all MSI cancers and have generally better prognosis and more precise alterations, whereas MSI-L, in which the mutation are accumulated in less than 40%, are the bulk of MSI cancers, and not only have an unclear association with MMR pathway, but also a worse prognosis (Wright et al., 2005; Vilar and Tabernero, 2013).

However, sporadic MSI phenotype, specifically MSI-H, can also be explained by the epigenetic silencing of MMR genes promoters (Shen et al., 2007). The consequences of the methylation lead to a MSI phenotype where the microsatellite of genes like MLH1 are not truly mutated, but their function is defective as well (Weisenberger et al., 2006).

1.1.2.1.3 CpG Island Methylation Pathway – CIMP

Epigenetic changes on genes, specifically the methylation of their promoters, represent a third phenotype identified across CRCs (Fearon, 2011).

In the normal genome, methylation occurs at the promoter in regions called CpG islands. Although during cancerogenesis the total methylation levels decrease, there is a local hypermethylation of promoters that, in turn, can lead to a downregulation of expression of tumour suppressor genes, such as APC, p16INK4alpha and MSH1 (Ng and Yu, 2015; Serra et al., 2014).

CIMP cancers are often localised in the proximal site of the colon and can be identified in 15% of all CRCs. They present a particular adenoma morphology, called sessile serrated adenoma (SSA)(Kim et al., 2011). Moreover, CIMP phenotype is detected in nearly all CRCs that bear MSH1 aberrant methylation, which constitute a major part of sporadic MSI (Shen et al., 2007). CIMP cancers can be further divided

in two categories, depending on the level of methylation, that are CIMP high, which are mainly related to BRAF activating mutations and MLH1 methylation, and CIMP low, related to KRAS mutations instead. As observed, in CIMP cancers KRAS and BRAF mutations are mutually exclusive (Corso et al., 2013). CIMP-low-KRAS cancers are also usually associated with MGMT and PIK3CA mutations, which causes them to acquire a peculiar hybrid phenotype where adenomatous and serrated polyps are present (Zlobec et al., 2012).

In the last few years, also microRNAs (miRNA) have been found to be implicated in CRC. miRNA, that are short non-coding RNA, are able to transiently repress gene expression, therefore acting as either oncogenes or tumour suppressors. Even though their role in cancerogenesis is not completely defined, some miRNAs were found to be involved in CRC. For example, miR-143, which binds KRAS, and miR-145, which is a negative regulator of WNT pathway, were both downregulated in adenomatous polyps at precancerous stages, compared to normal tissue, suggesting that these miRNAs may indeed play a role in CRC early development (Luo et al., 2011).

Thence, considering the three major CRC phenotypes, the non-entirely mutual exclusivity caused by CRC heterogeneity and the different overlap of the aforementioned phenotypes, according to (Fleming et al., 2012) which took into account also pathological and clinical classification, CRCs can be classified as in Figure 3:

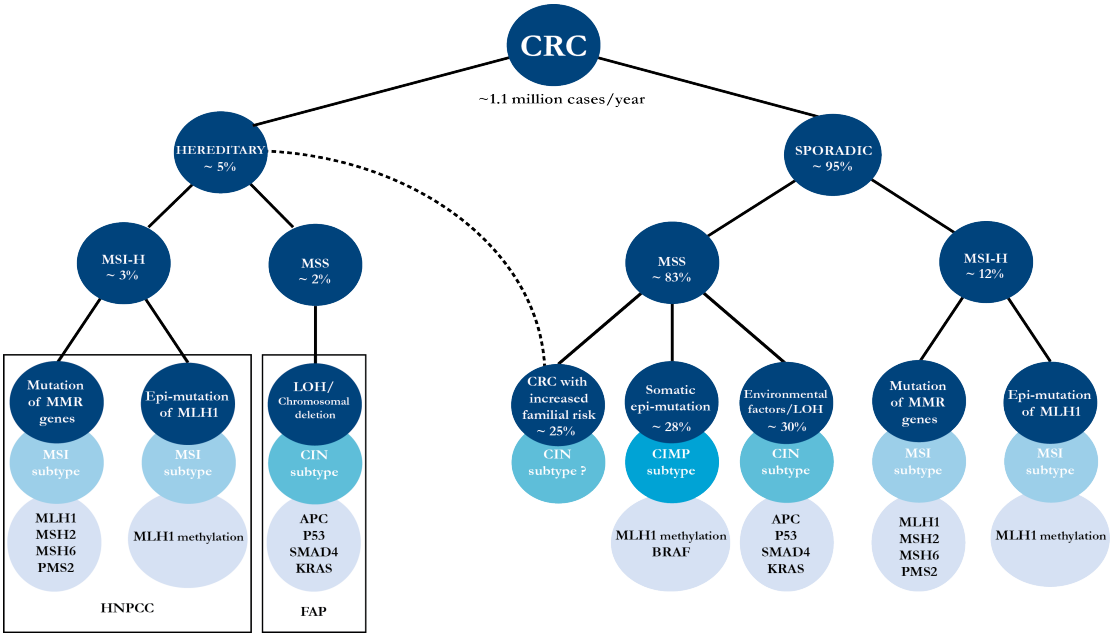

Figure 3 Molecular classification of colorectal cancer. Colorectal cancer could be primarily divided in inherited and in sporadic. Each branch is then further divided in tumours with microsatellite instability (MSI-H) and with chromosomal instability (MSS or CIN). According to the particular combination of genetic alterations three major subtypes could arise: CIN, MSI and CIMP. Specifically, an hereditary CRC with CIN subtype lead to familial adenomatous polyposis (FAP), whilst MSI-H subtypes usually lead to hereditary non-polyposis colorectal cancer syndrome (HNPCC). The genes mainly involved in each one of the subtypes are within the light blue circles. Adapted from Fleming et al., 2012.

1.1.2.1.4 Consensus on Colorectal Cancer subtype classification

Thanks to the genomics revolution, cancer sequencing project have started to shed more light on the biological complexity of CRC. Analysing chromosomal and gene alterations, gene expression and pathway activation of different cohorts of CRCs, several research groups proposed their own profiling of CRCs ; Marisa et al., 2013; Roepman et al., 2014; De Sousa E Melo et al., 2013; Sadanandam et al., 2013; Schlicker et al., 2012). Whereas the thorough examination of various classification proposal could not address all the discrepancies amongst the tumoural subtypes, lately Guinney and colleagues suggested an integration and improvement of the previous subtyping projects, in particular an integration of data processing and of algorithms used to analyse CRCs features, proposing another, more comprehensive CRC classification. The newly redacted classification has been divided in 4 consensus molecular subtypes (CMSs) (Figure 4):

CMSs not only could specifically classify 87% of the analysed samples (4,151), but as well identified more precisely the role and the clinical impact of the molecular pathways involved in CRC. Even though this study could not link a specific set of

mutations to a unique CMS, thus not allowing a clinical stratification for CRC, it identified that CMS1 cancers had worse survival after relapse and CMS4 with tumours had increased risk of metastasis and worse overall survival, therefore it could represent a starting point for the translation of integrative analyses into clinical practice, improving both stratification and subtype-based targeted interventions, in similar fashion to what has been done in breast cancer (Sotiriou and Pusztai, 2009).

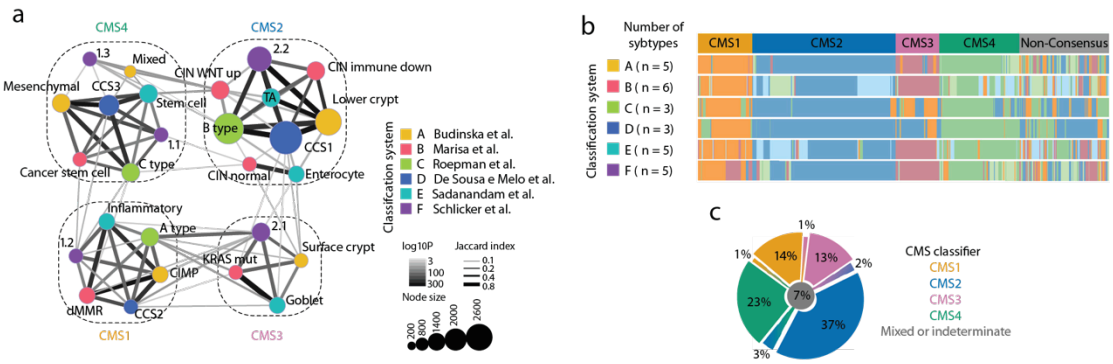

Figure 4 Identification of the consensus subtypes of colorectal cancer and application of classification framework in non-consensus samples. (a) Network of CRC subtypes across six classification systems: each node corresponds to a single subtype (colored according to group) and edge width corresponds to the Jaccard similarity coefficient. The four primary clusters, identified from the Markov cluster algorithm, are highlighted and correspond to the four CMS groups. (b) Per sample distribution of each of the six CRC subtyping systems (A–F), grouped by the four consensus subtyping clusters, and the unlabeled non-consensus set of samples. Colours within each row represent a different subtype. The n values shown in b correspond to the number of subtypes in the original independent classification published by each group. (c) Final distribution of the CMS1–4 groups (solid colours), 'mixed' samples (gradient colours) and indeterminate samples (gray colour) resulting from the classification framework. Adapted from Guiney et al., 2015.

1.1.3 Hereditary Colorectal Cancer

As said, CRC occurrence could be inherited or sporadic. Although a roughly 20% of CRCs have a hereditably component, the study of familial cancer syndrome improved the understanding of the molecular basis and the mechanisms that contribute to CRC development (Taylor et al., 2010).

The main familial colorectal cancer syndromes are hereditary nonpolyposis colorectal cancer (HNPCC) syndromes and familial adenomatous polyposis (FAP).

1.1.3.1 Familial adenomatous polyposis - FAP

Familial adenomatous polyposis is an autosomal dominant syndrome that represents the 0.5% of all CRCs. The disorder presents a high number of adenomas (>10^5) at the age of 35, but only few of them progress to cancer, with the occurrence of nearly

100%. The only preventive option is surgical removal of the adenomas (Galiatsatos and Foulkes, 2006). Even though in 25% of FAP cases de-novo mutations occur, that is counterintuitive for this type of disorder, the gene mostly identified as mutated in FAP is APC (Adenomatous Polyposis Coli), a known tumour suppressor gene. APC is affected by different disruptive events, 95% of which are frameshift mutations that result in a truncated protein. Most of the times truncated APC behaves as a dominant negative, resulting in a haploinsufficiency (Venesio et al., 2003), but depending on the site of truncation the number of polyps can differ.

1.1.3.2 Hereditary non-polyposis colorectal cancer syndrome - HNPCC

Hereditary non-polyposis colorectal cancer syndrome is the first colorectal cancer syndrome historically described and represents the 2-5% of all CRCs (Barrow et al., 2008). It is an autosomal dominant disease, localised mainly in proximal colon, and presents an increased lymphocyte invasion, mucinous differentiation and low occurrence of polyposis (Bosman et al., 2010). Similarly to other CRCs, HNPCC shows genomic instability, specifically microsatellite instability. In fact, more than 90% of HNPCCs present MSI (Timmermann et al., 2010), and most of these cancers have inherited a mutated allele of the gene encoding MSH2. The inherited mutation predispose to early onset of CRCs, since "another hit" in MSH2 generally, but not exclusively, occur in MSI cancer phenotypes (Nagasaka et al., 2010). Nevertheless, just 40% of HNPCCs present a clear mutational profile, which in turn lead to a higher risk of CRC compared to unclear profiles.

1.1.4 Key genes and pathways in CRC

As stated above, several pathways play a role in CRC development, simultaneously, most of the times. Although new molecular and sequence-based investigations have elucidated the critical role of different genes and pathways in the various CRC subtypes, there are long known pivotal gene alterations, such as APC and KRAS, which not only have elucidated fundamental aspects of the diseases, but also improved the clinical management of cancer patients.

1.1.4.1 APC – Wnt/ß-catenin pathway

As mentioned earlier, APC mutations are found in 70-80% of all CRCs, both familial and sporadic. APC inactivation, often biallelic, represents one of the early steps of

colorectal carcinogenesis and it is probably an event that limits the amount of transformation in small adenomas (Wood et al., 2007; Cancer Genome Atlas Network, 2012).

APC is a 300KDa protein which regulates multiple cellular functions, such as cell adhesion, chromosomal segregation and apoptosis in normal intestinal crypts (Aoki and Taketo, 2007). There are 4 major sites of mutation on APC gene, localised mainly between codon 1309 and 1450, which result in protein truncation, therefore inactivation. Moreover, given the size, the protein contains different motifs, thus acting as hub protein, of which other proteins can interact, such as AXIN2 and GSK3ß. But amongst the binding partners, ß-catenin constitutes the most known and important of them, and its interaction with APC represent a fundamental node of Wnt classic pathway. In physiological conditions, when Wnt signalling is not active, APC induces a phosphorylation of ß-catenin, which leads to its ubiquitination and consequent degradation. Conversely, once Wnt signalling is activated, Wnt interaction with its coreceptors, Frizzled and LRP5/6, blocks the activity of APC, thereby allowing ß-catenin to translocate into the nucleus and to act as a transcription factor,

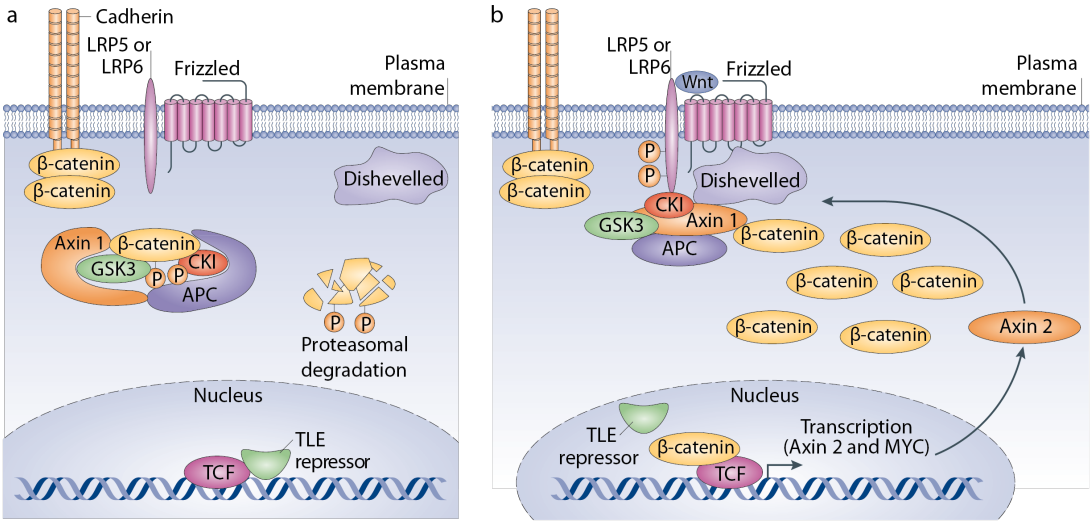

Figure 5 The canonical Wnt signalling pathway. (a) In the absence of a signal, APC promote ubiquitination of β-catenin, which is then degradated by the proteasome. In the nucleus, and transducin-like enhancer (TLE) repress the transcription mediated by T cell factor (TCF). (b) The binding of a Wnt ligand to its Frizzled receptor and lipoprotein receptor-related proteins (LRPs) induces a change in conformation that results in the disruption of the destruction complex. β-catenin can then accumulate and associate with the TCF, hence promoting transcriptional activation of genes, including *MYC* and axin 2. Axin 2 feeds back to inhibit the pathway by promoting the assembly of more destruction complexes (McNeill and Woodgett, 2010).

inducing the expression of genes like MYC and cyclin D1 (Tetsu and McCormick, 1999; Zhang et al., 2012; White et al., 2012). The consequences of transcriptional

activity of ß-catenin are different, for instance, it could influence self renewal and regulate migration of intestinal crypt cells and activating stemness transcriptional programme (Armaghany et al., 2012)(Figure 5).

Since its role of WNT pathway effector, ß-catenin has been found to be mutated, although in a small subset of tumours. This somatic mutation does not allow its phosphorylation, which leads, in turn, to its constitutive transcriptional activity (Segditsas and Tomlinson, 2006). The fundamental role of WNT signalling in CRCs is finally proved by the notion that this pathway, and its components, were identified altered someway in more than 90% of the tumours (Cancer Genome Atlas Network, 2012)

1.1.4.2 TP53

Another important regulator of CRC development is p53. This gene represents one of principal tumour suppressor genes and is implicated in nearly all types of cancers. P53 is a transcription factor that is involved in several processes concerning the physiological proliferation of cells, such as the control of checkpoints G1/S and G2/M (Levine and Oren, 2009) (Figure 6). LOH is commonly linked to TSG inactivation process and loss of 17p chromosome is found in 70% of CRCs. Since p53 is localised in chromosome 17p, this genetic loss is thought to target it specifically (Fearon, 2011). LOH of p53 is often associated, but not exclusively, with somatic mutations of p53, which are mainly missense. The alteration of p53 is a fundamental step in the classic model on colorectal cancer development (Fearon and Vogelstein, 1990), and whereas it is mainly associated to a CIN phenotype, p53 can also be found mutated in MSI tumours (Bogaert and Prenen, 2014). Early adenomas usually have a normal status of p53, whereas in carcinoma at least p53 mutations can be identified. This suggest that p53 could play decisive role in adenoma transformation, especially in the later steps of the carcinogenesis (Baker et al., 1990). Mutations, along with LOH of p53, result in deregulation of genomic integrity, cell cycle, angiogenesis and apoptosis, which lead to aberrant growth and invasiveness. This is mainly due to p53 loss of function as transcription factor, that in turn reduce the levels of p21, BAX and BUBR1, and AMPK (Ogino et al., 2009; Baba et al., 2010; Sturm et al., 1999). Nonetheless, mutations could induce also a gain of function of p53. In particular, mutant p53 can act promoting chronic inflammation through NFκB signalling, which can cooperate to

CRC development (Cooks et al., 2013). Mutations of p53 are mainly detected in tumours located in the distal regions of the intestine (Russo et al., 2005). These aberrations are linked to a poorer survival rate, but, even though it is not clear yet whether mutations could improve drug sensitivity or not, p53 mutations have been shown to improve the clinical outcome of cetuximab-treated metastatic CRC (Naccarati et al., 2012).

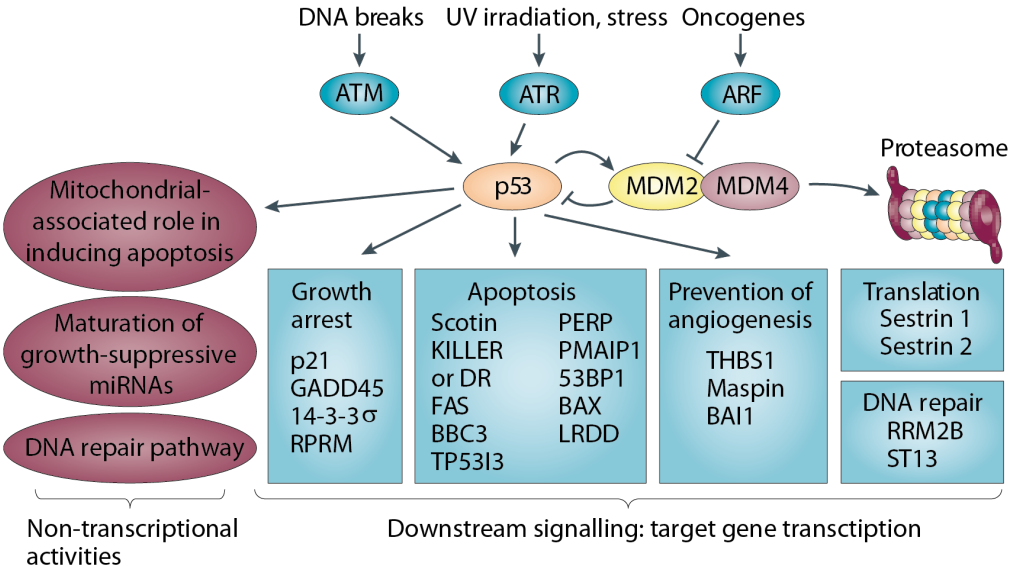

Figure 6 The p53 pathway. The schematic representation of p53 activity show it at the centre of a complex web of biological interactions that relays stress signals into cell cycle arrest or apoptosis. Upstream signalling to p53, such as Ataxia Telangectasia Mutated (ATM), increases its level and activates its function as a transcription factor in response to a wide variety of stresses, whereas downstream components execute the appropriate cellular response, such as p21. The principal regulator of p53 levels is MDM2. In non-stressed conditions it targets p53 for degradation by the proteasome. In stressed conditions MDM2 action is inhibited by its phosphorylation. Due to its importance the identification of a functional p53 signalling is particularly relevant from a therapeutic perspective (Brown et al., 2009).

1.1.4.3 MMR pathway

DNA can be subjected to spontaneous and environmental mutations**.** Another source of errors is the DNA replication itself, despite being a highly precise process. Due to the potential dangerous consequences, during evolution cells have developed a system that repairs these errors: mismatch repair pathway. MMR pathway is mainly composed by MSH1, MLH1, MSH2, MSH6 and PMS2. The protein complexes formed by the combination of the various pathway components, such as hMutL, a dimer made of MLH1 and PMS2, by recognition, excision and restoration the original sequence, resolve the genetic mutations (Hewish et al., 2010)(Figure 7).

Disruption of MMR is the main feature of the HNPCC tumours, and, one of the principal aspects of sporadic MSI CRCs, although representing only the 15-20% of all CRCs (Geiersbach and Samowitz, 2011). As mentioned above, almost all MSI cancers are caused by inefficacy of MMR activity. Whereas deficient MMR results in a hypermutator phenotype regardless of CRC oncogenesis, its components could be subjected to different mutations or epigenetic silencing accordingly to the different

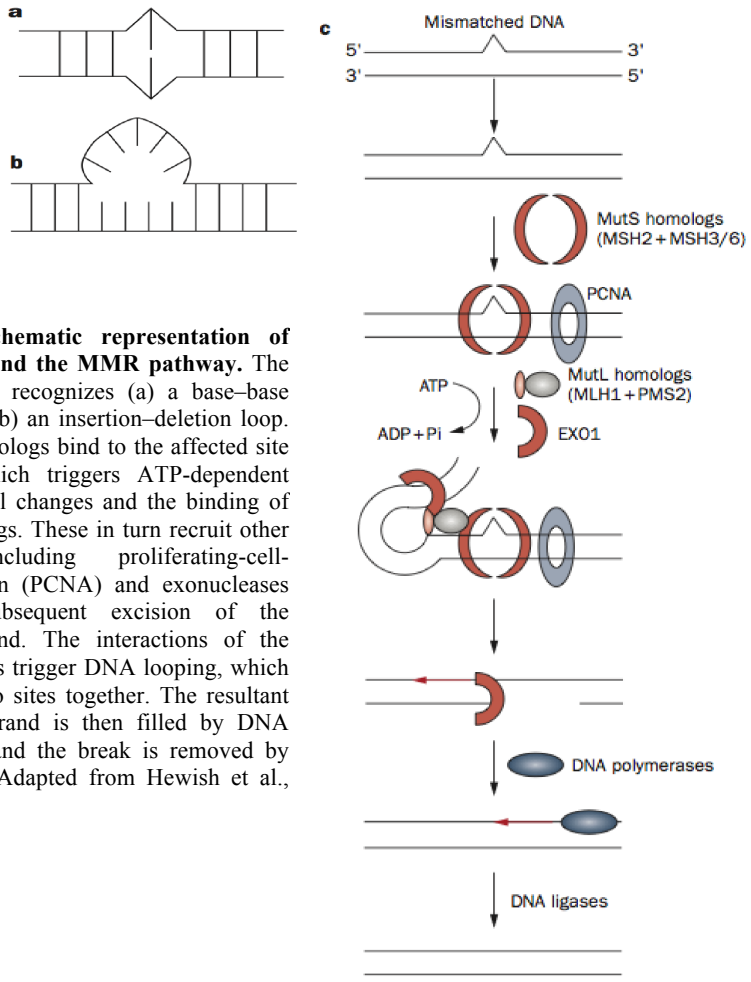

Figure 7 Schematic representation of mismatches and the MMR pathway. The MMR system recognizes (a) a base–base mismatch or (b) an insertion–deletion loop. (c) MutS homologs bind to the affected site of DNA, which triggers ATP-dependent conformational changes and the binding of MutL homologs. These in turn recruit other proteins, including proliferating-cell-nuclear-antigen (PCNA) and exonucleases with the subsequent excision of the damaged strand. The interactions of the bound proteins trigger DNA looping, which brings the two sites together. The resultant gap in the strand is then filled by DNA polymerases and the break is removed by DNA ligase. Adapted from Hewish et al., 2010

CRC subtypes. In fact, methylation of MLH1 promoter and MSH3 mutations are commonly found in sporadic MSI/CIMP subtypes, conversely in HNPCC, MLH1 is mutated along with MSH2 (Vilar and Tabernero, 2013). The effect of MMR pathway alterations, thus MSI CRCs, are not very clear in terms of both prognostic values and response to therapy. MSI indeed displays a lower rate of recurrences in CRC early stages, but the poor prognosis in later stages is controversial, since it is still debated whether the associated BRAF mutations could instead be accounted for the worse

outcome (Vilar and Tabernero, 2013). Moreover, defective MMR cancers have shown an unclear and unpredictable response to DNA damaging agents, the elective therapy for CRCs, such as 5-fluorouracil. Despite the lack of consensus, for example, National Comprehensive Cancer Network recommends not to use 5-fluorouracil in MSI/MSI-H tumours, since several observations have shown no benefit from this treatment (Hewish et al., 2010 ; Cancer, 2016).

1.1.4.4 Epidermal Growth Factor Receptor - EGFR pathway

Epidermal Growth Factor Receptor is a transmembrane receptor with tyrosin-kinase activity and member of a superfamily of the receptor tyrosine-kinase (RTK). EGFR is the first component of its pathway, which play a fundamental role in cellular/tissutal homeostasis and, when altered, in the biology of different kinds of cancers, among which there is also CRC (Akbani et al., 2014) .

The importance of this pathway is highlighted not only by the identification of numerous alterations of its components, such as RAS, PI3K and BRAF, but also by the efficacy of the anti-EGFR therapies, in particular with cetuximab and panitumumab (Finnberg et al., 2015) (Figure 8).

1.1.4.4.1 RAS

RAS, a small family of Rho-GTPase, are pro-oncogenes and act as a molecular switches in cells. They are activated mainly by the members of the EGFR family, transducing the signals of cell survival to the nucleus through different pathways, such as MAPK pathway. The 3 members of the family, KRAS, NRAS and HRAS, are found to be frequently mutated in CRC. In particular, KRAS is mutated in nearly 40% of CRCs, with the majority of these mutations affecting codon 12, while those at codon 13 and 61 are more rare and less oncogenic. The effect of the mutations turn KRAS into a defective switch, which permanently activates EGFR pathway (Tan and Du, 2012) Even though present in a substantial part of CRCs, KRAS is not needed for cancer onset, but rather for the subsequent development. Actually, KRAS mutations are often localised in flat colonic epithelial lesions and in hyperplastic polyps as well. Even more, the mutations are correlated to the size of the adenoma, since mutations are found in ~10% of smaller adenomas, whereas in 50% of larger adenomas. The relevance of KRAS in CRC is further supported by the connection with WNT

pathway, by which MAPK signalling can be activated, and p53 pathway (Armaghany et al., 2012)

..

KRAS mutations represent also an important biomarker for anti-EGFR therapy response. Owing to KRAS position in the EGFR signalling pathway, its mutations prevent any therapeutic effect of cetuximab, a monoclonal antibody against EGFR. Even so, KRAS wt protein does not mean a clinical response in 100% of CRCs, suggesting that KRAS or other components of the pathway may be involved in the resistance to anti-EGFR therapies (Misale et al., 2015).

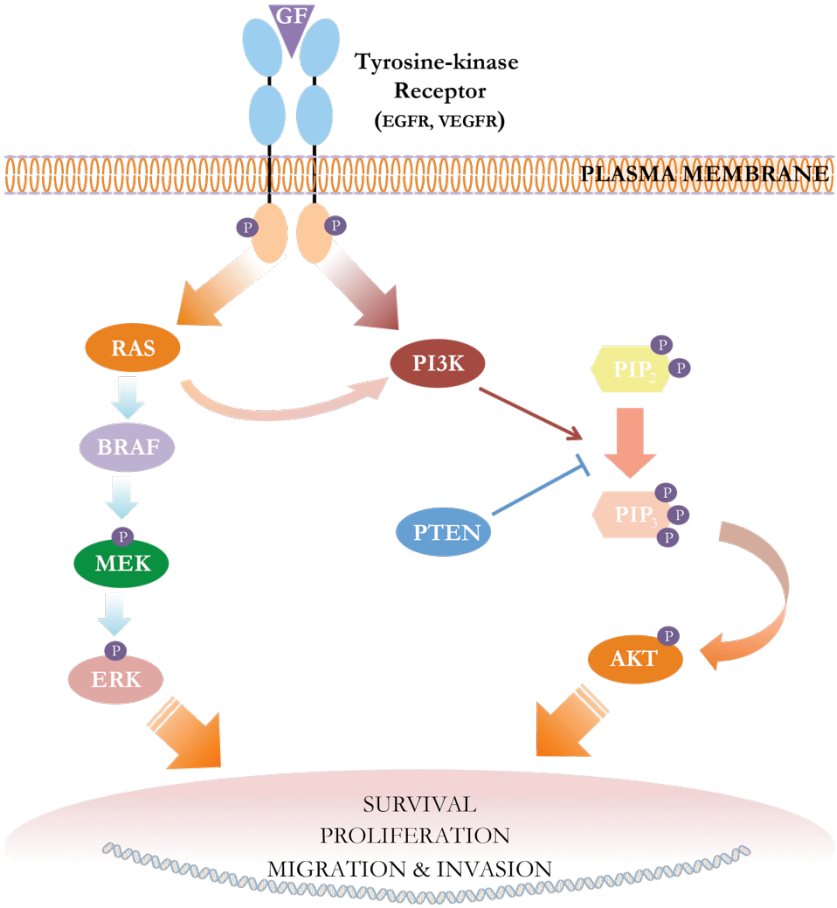

Figure 8 Overview of Tyrosine-Kinase Receptor pathways. The transmembrane receptors, such as EGFR, is usually activated by autophosphorylation upon interaction with its ligand (EGF, VEGF etc). The activated recteptor transduces the external stimuli through a signalling cascade to the nucleus via different downstream effectors. Two of the most studied transductors are RAS and PI3K, since their overactivation is frequently identified in many types of cancer, such as CRC. Once the signal reaches the nucleus, genes involved in survival, proliferation and invasion are consequently transcribed. Adapted from Siena et al., 2009 and Walther et al., 2009.

1.1.4.4.2 BRAF

Another important component of EGFR pathway is BRAF, a small kinase similar to Ras proteins. BRAF is activated by KRAS and, in turn, activate MEK1 and 2, the main downstream effectors of MAPK pathway. The main mutation affecting BRAF is the V600E substitution and it can be found in approximately 10% of all CRC. BRAF mutation is commonly detected in CIMP-MSI-H sporadic cancers that originate from serrated lesions of the epithelium, and it is very rarely co-mutated with KRAS, in an almost total mutual exclusive fashion. Since BRAF role in EGFR signalling pathway, its mutation could lead to resistance of anti-EGFR drugs, probably shunting the signalling to another oncogenic pathway (such as Wnt), therefore reducing the therapeutic option and predisposing to a worse prognosis (Corso et al., 2013; Vaughn et al., 2011).

1.1.4.4.3 PI3K/AKT/PTEN

EGFR activation can be transduced by different routes, amongst which PI3K/AKT pathway can be found. The importance of this pathway is testified by alterations of its components found in nearly 50% of all CRCs (Cancer Genome Atlas Network, 2012). Phosphoinositide 3-kinases (PI3Ks) are a class of protein complexes involved into the signal transduction of RTK pathway. Upon activation of RTK or RAS, class I PI3Ks can phosphorylate PIP_2 to PIP_3 and the resulting higher levels of PIP_3 lead to the activation of downstream effectors, such as AKT and MAPKs, which eventually promote protein and DNA synthesis and cell growth, proliferation and survival (Danielsen et al., 2015).

Several studies have pointed out that different cancers bear mutations in class I PI3Ks, of which the most common are those affecting the p110α catalytic subunit, encoded by PIK3CA gene, or other regulatory subunits (Danielsen et al., 2015). These mutations result in a constitutive activation of the kinase activity and are present in 15-35% of non-hypermutator and hypermutator CRCs respectively (Cancer Genome Atlas Network, 2012). Whereas PI3K works downstream to KRAS, it is not infrequent found them co-mutated, probably because KRAS is a mild activator of PI3K/AKT pathway. Overall, the alterations of PI3K/AKT pathway are found in nearly 50% of all CRCs, in both hypermutator and non hypermutator phenotypes. PI3K activity is physiologically negatively regulated by PTEN (Phosphatase and

tensin homologue), a well-known tumours suppressor gene. Its mutation or LOH are found in CRC, with a particular enrichment in MSI-H phenotypes (20%), suggesting a particular relationship of PI3K/AKT pathway with this phenotype. Despite the importance of this pathway, its impact on prognosis is still uncertain, given the frequent co-presence of BRAF and KRAS mutations (Danielsen et al., 2015). AKT, another component of this pathway, is affected by mutations in 1% of the CRCs. Its alteration lead to protein overexpression and constitutive activation, which may promote cancer invasiveness and metastasis, with the cooperation of PTEN inactivation (Suman et al., 2014).

1.1.5 Therapy

In the past few decades, accordingly to the International Agency for Research on Cancer, the incidence of CRC world-wide has not increased and concurrently the mortality decreased. This improvement is due to new and better therapeutic regiments, preventive screening and general awareness about the risk factors, which were able to reduce the death of the this disease (especially the early stages)(Howlander et al., 2014). Nevertheless, CRC diagnosed at later stages and metastatic tumours have still therapeutic options with limited efficacy and a poorer prognosis, compared to early diagnosed cancers, then representing one of the main caveats to be overcome in the future (Howlander et al., 2014).

1.1.5.1 Current therapeutic management of colorectal cancer

Regardless of the stage, the main therapy for colorectal cancer is surgery. Generally speaking, CRC at early stages and with low risk (i.e. no genetic predisposition to CRC) benefit more from local resection and sometimes there is no need to proceed with other therapies, especially the MSI-H subset (Ribic et al., 2003; André et al., 2009). Lately, diverse evidences showed that chemotherapy administered after the surgical resection, also called adjuvant therapy, significantly improved the survival of the patients with the disease at medium or late stages. In particular, fluorouracil-based therapies (5-fluorouracil/leucovorin or capecitabine) have shown survival benefits to medium stage CRCs with moderate risk, and even more to later stages of the disease when associated to oxaliplatin (André et al., 2009; Haller et al., 2011). Colorectal cancers display metastases in about 50% of the cases, most of which localise to the liver. Liver metastases are often unresectable, therefore deadly, however up to 30% of

the patients present a resectable liver tumour, whose removal could improve significantly the short- and long-term survival (Van Cutsem et al., 2006; Muratore et al., 2007; Taylor et al., 2012). In several clinical trials, pre-operative chemotherapy, also called neoadjuvant therapy, has been reported to downstage cancer, allowing its surgical resection. In fact, a study from Pozzo et al. reported that a combination of 5-fluorouracil/leucovorin and irinotecan allowed about 30% of the patients to have a resectable tumour, which increased the survival of 19 months. Moreover, the association of classic cytotoxic drug to biological agents, such as cetuximab, provided evidences of increased resectability, whereas little improvement of survival was observed, taking into account also the hepatotoxicity of the therapeutic regiment (Petrelli et al., 2012). Nevertheless, therapy for metastatic colorectal cancer (mCRC) encompasses combination of fluoropyrimidines, irinotecan, oxaliplatin and bevacizumab, an anti-VEGF monoclonal antibody, cetuximab or panitumumab, anti-EGFR monoclonal antibodies, sometime associated with regorafenib or aflibercept (VEGFR inhibitors). The therapeutic regiment is chosen according to the characteristic of the disease, such as MSI-H subtype and prior therapies (Cancer, 2016). Association of target therapeutics to 5-fluorouracil/leucovorin and irinotecan have indeed displayed clear benefits in patients with unresectable mCRC compared to 5-fluorouracil/leucovorin monotherapy, improving their overall survival (Hurwitz et al., 2004; Van Cutsem et al., 2009; Van Cutsem et al., 2007). Promising results have been reported by new chemotherapeutic regiments and novel targeting molecules, mostly in the past few years, but 5-year survival of patients with advanced or metastatic disease, that account for 19% of all CRC at the time of diagnosis, is only the 11% (Siegel et al., 2012; Howlander et al., 2014)(Figure 9). At these stages, patients cannot be cured and can be treated only to prolong survival. Novel and innovative target therapy could improve the outcome of CRC, especially in later stages, similarly to the impact that targeting agents has had on breast cancer (Swain et al., 2015). In truth, current available therapies have already shown clear efficacy, even if limited, although new therapeutic advancement should be carried out, rationalising the application of these treatments, and, furthermore, understanding how to target the specific molecular defects onto which CRC cells rely, with innovative drugs and delivery systems (Finnberg et al., 2015; Posocco et al., 2015).

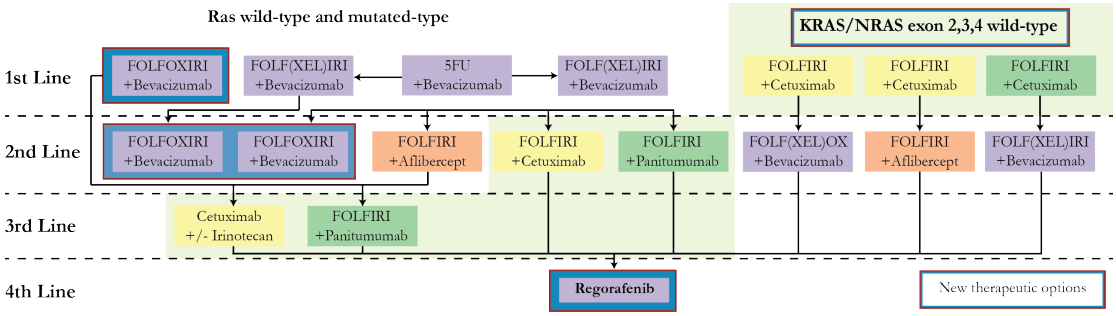

Figure 9 New proposed approach to therapeutic management of metastatic colorectal cancer. Proposal for a new algorithm for CRC treatment selection based on new clinical and molecular data according to Schmoll and Stein

1.1.5.2 Targeting oncogenic signalling pathways

Altered oncogenic pathways drive cancer development and metastatic processes, hence they are being exploited in anticancer therapy by using molecules able to block their signalling. For this purpose, in the previous decades, several targeted drugs, have been developed allowing the inhibition of specific pathways. The blockade of their signalling can not only have an anticancer effect *per se,* but also be implemented synergistically with other drugs whose efficacy would be hampered by the activation of pathways involved in drug resistence, when administered alone.

1.1.5.2.1 EGFR

EGFR is a transmembrane tyrosine kinase receptor (RTK), whose activation, by auto-phosphorylation, is triggered mainly by EGF, and in turn can activate both RAS and PI3K downstream pathways (Merla and Goel, 2012). This receptor is overexpressed in 65-70% of all CRCs and its status is associated with tumour's advanced stages, although only a subpopulation of patient benefit from the blockade of the receptor, manifesting how the expression cannot be used as a therapeutic response prediction biomarker to anti-EGFR drugs (Yarom and Jonker, 2011). CRCs with wild-type EGFR pathway's components benefit the most from anti-EGFR therapeutics, in fact any mutation affecting the signalling decreases the efficacy of its inhibition (Sood et al., 2012),

Currently, EGFR signalling can be blocked by preventing its activation from external stimuli or by inhibiting its kinase activity. Cetuximab, a IgG1 recombinant chimeric monoclonal antibody, and panitumumab, a IgG2 recombinant human monoclonal antibody, are the two FDA-approved antagonist antibodies targeting EGFR used in

the CRC clinical practice (Cancer, 2016). They act by binding the extracellular part of the receptor, inducing its internalisation and degradation, thus inhibiting its activation and downstream signalling. Both cetuximab and panitumumab demonstrated a significant but limited effect survival of metastatic CRC. In fact on one hand, cetuximab association with irinotecan and fluoropyrimidine shown a significant increase in mCRC survival (Van Cutsem et al., 2009), but on the other hand, the association with oxaliplatin have not replicated the same effect, despite a slight increase of survival (Bokemeyer et al., 2009). Similarly, panitumumab displayed efficacy only in association with irinotecan and 5-fluorouracil (Peeters et al., 2014). The benefit of these therapeutic regiments was nullified when administered to patients with KRAS mutations, with sometime a detrimental effect on survival (Bokemeyer et al., 2009; Cutsem et al., 2010). Along with KRAS mutation assessment, also NRAS, BRAF and PI3K mutations should be evaluated. Since their role in the EFGR pathway, these components could all participate to cetuximab or panitumumab resistance (Misale et al., 2015). Bertotti et al. reported that in a preclinical settings, the efficacy of anti-EGFR therapies could be improved with a better stratification of patients, based on the expression of other upstream regulator of EGFR pathway, such as HER2. They demonstrate that the association of pertuzumab, an anti-HER2 monoclonal antibody used in breast cancer treatment, and lapatinib, an EGFR/HER2 inhibitor, can not only overcome resistance, but also decrease tumour growth in xenograft models of mice with cetuximab-resistant CRCs. This suggests that a better stratification and the implementation of gene expression analysis in CRC clinical management could be beneficial for patient survival.

Novel antibodies and EGFR kinase inhibitors have been proposed and are a promising class of drugs in CRC therapy. Despite none has been yet approved, several clinical trials suggested that their application in therapy could provide clinical benefit to patients. GA201, a humanised monoclonal antibody against EGFR, have demonstrated to trigger an antibody-dependent cellular toxicity (ADCC) in several cancers, including mCRC, representing an improvement of cetuximab activity (Oppenheim et al., 2014). Also necitumumab, another anti-EGFR antibody, demonstrated encouraging results in clinical trials with patients with advanced colorectal cancer when associated to chemotherapy (Elez et al., 2016). Lastly, afatinib is a selective inhibitor of EGFR and HER2 kinase activity, which has already displayed promising results in breast and pancreatic cancer patients. Despite its

promising activity in association with conventional chemotherapy, phase II clinical trial shows a limited clinical efficacy when administered in combination to BIBF, an angio-kinase inhibitor (Bouche et al., 2011). A clinical trial using an association of cetuximab and afatinib is still ongoing (UNICANCER, 2013)

1.1.5.2.2 RAS/RAF/MEK

RAS proteins are the main downstream transducers of EGFR pathway signalling. They belong to a family of GTPases, critical in CRC development. Mutations affecting normal RAS activity occur in 40% of CRCs, which cause its constitutive activation. Mutations predict lack of responsiveness to EGFR signalling (Armaghany et al., 2012). RAS proteins are required to be bound to the plasma-membrane by a lipidic anchor in order to become active GTPases. This post-translational modification is carried out by farnesyl transferase (FTase) and geranylgeranyl-transferase (GGTase) in tandem. Attempts to block RAS activity by inhibiting the activity of these two enzymes have been successfully accomplished, but with very limited therapeutic effects on CRC (Shimoyama, 2011; Armaghany et al., 2012). Since the precursor of FTase and GGTase substrate are produced by HMG-CoA reductase, statins have been considered as possible options as RAS inhibitors. Preclinical data indicate that statins can indeed block cell proliferation and induce cell death, and synergise with 5-FU activity (Lochhead and Chan, 2013).

As mentioned above, RAF proteins are the effectors downstream to RAS in the RTK pathways. RAF mutations, 10% of which are V600E, have been reported to be poor prognostic markers of response to cetuximab and irinotecan combination (Bokemeyer et al., 2012). Considering that RAF mutations are usually mutually exclusive with KRAS mutations, RAF could be a suitable target for pharmacological inhibition. Clinical trials performed on metastatic melanoma demonstrated encouraging results, but response was hampered by acquired resistance to the inhibition, which reactivated MAPK activity, for example by novel mutation of MEK or NRAS (Flaherty et al., 2012). Combination of MEK and BRAF inhibitors has indeed displayed promising results in metastatic melanoma clinical trials. Moreover, targeting of EGFR and BRAF simultaneously have identified sustained suppression of MAPK pathway and enhanced anti-cancer effect in xenograft models of mutant BRAF CRCs (Corcoran et al., 2012).

Finally, given the difficulty to inhibit RAS or RAF signalling, MEK inhibitors have been taken into consideration. MEKs are serine/threonine kinase activated by a different set of external stimuli, relayed by RAS and RAF. Their action is to control the phosphorylation of several transcription factors, which are involved in cell proliferation, for instance. As a consequence, an aberrant MEK activity leads to cancer progression and drug resistance, although in CRC they are not frequently affected my mutations, probably because of aberrant upstream signalling (Luca et al., 2012). MEK inhibitors have been developed, but some of them failed to demonstrate a significant clinical activity (Rinehart et al., 2004), however selumetinib administration had the same effect of capecitabine toward the maintenance of progression free survival (Bennouna et al., 2011). As mentioned above, association of MEK inhibitors and BRAF inhibitors may improve patient survival as suggested by the results of their application in BRAF-mutated metastatic melanoma (Flaherty et al., 2012).

1.1.5.2.3 PI3K/AKT

PI3K is ones of the downstream effectors of the EGFR pathway, as aforementioned. Fifteen percent of all CRC present a mutations that affects the behaviour of PI3K, which usually result in its overactivation, therefore rendering its signalling independent from upstream stimuli (i.e. cetuximab-resistant)(Jonker et al., 2007). The application of PI3K inhibitors in CRC should synergise with EGFR blockade, thus hampering tumour growth and improving patient clinical benefits. Several inhibitors are available, but none of them has been approved. Pan-PI3K inhibitors, which block both wt and mutated form of the protein, have exhibited induction of apoptosis in colon cancer cell line models, with a preferential activity towards mutated cells. Notwithstanding the limitation of PI3K inhibition in presence of AKT mutations, many PI3K inhibitors are subjected to clinical trial, such as PKI-587 (Tabernero et al., 2015). Since 10% of PI3K mutated CRCs present also RAS/RAF mutations, the dual inhibition could have the potentiality of blocking the transduction of external signals and prevent circumvention of one or the other transduction pathway. Data from a phase I study suggests that it may be a viable option, given the positive response of the combined administration of PI3K/AKT and RAS/RAF/MEK pathway inhibitors compared to monotherapy (Shimizu et al., 2012).

1.1.5.2.4 VEGF and VEGFR

Aberrant angiogenetic signalling is one of the hallmarks of cancer (Hanahan and Weinberg, 2011) This process is regulated by several extracellular growth factors, amongst which there is Vascular and Endothelial Grow Factor (VEGF). VEGF exerts a pro-angiogenetic function upon binding to VEGF receptor family, which belong to the tyrosine kinase receptors family. The interaction induces its activation, which in turn induces endothelial proliferation and blood vessels to grow, via PI3K and RAS signalling. Clinical studies have suggested a role for angiogenic pathways in the growth and lethal potential of colorectal cancer. Current therapeutic options are the inhibition of the pathway signalling by administration of monoclonal antibodies against VEGF, decoy receptors or inhibitors of kinase activity of VEGFR (Troiani et al., 2012). Bevacizumab is a recombinant humanised monoclonal antibody against VEGF, approved from FDA for CRC therapy. These targeted agents have shown to confer clinical benefit of mCRC patients when associated to 5-fluorouracil and leucovorin (5-FU/LV), in short-term survival. In addition, association of oxaliplatin and irinotecan to 5-FU/LV and bevacizumab improved the outcome, increasing both short- and long-term survival rate (Loupakis et al., 2014). Regorafenib is a novel oral multikinase inhibitor that blocks the activity of several protein kinases, including kinases involved in the regulation of tumour angiogenesis such as VEGFR, and the tumour microenvironment. In pre-clinical studies, regorafenib has shown antitumour activity, including in colorectal cancer models (Wilhelm et al., 2011) . Understanding the clear mechanism by which patients respond positively to VEGF inhibition remains a challenge.

1.1.5.2.5 Reactivation of TSG

Inactivation of tumour suppressor genes is one of the main features displayed by cancer cells (Hanahan and Weinberg, 2011). Despite the potential impact of restoring the TSGs capability on anti-neoplastic therapy, TSG reactivation has been reported to be very difficult to achieve. Among the several proposed drugs, p53 “reactivators” have been reported to be successful, but in a limited number of studies. Preclinical studies has observed that PRIMA1, a p53 re-activator, and its analogues could reduce cancer growth and improve the efficacy of standard chemotherapy in preclinical models (Roh et al., 2011; Li et al., 2015).

Although direct TSG reactivation was not very successful, anti-cancer therapy may take advantage of TSG inactivation. Since MMR pathway is frequently inactivated in CRCs (10%), synthetic lethality approaches have been pursued. MSI-H CRCs have no benefit from 5FU/LV chemotherapy, probably because 5-FU metabolites are excised more efficiently than in MSS cells (Zhang et al., 2011). The association of irinotecan, which is able to induce double strand breaks, to 5-FU/LV has conversely shown an improvement of the survival of some late-stage CRC patient, compared to 5-FU/LV alone (Bertagnolli et al., 2009). In this context, preclinical data suggested that PARP inhibitors could potentiate the activity of chemotherapy. The inhibition of PARP, an enzyme family which repair single strand breaks (Murai et al., 2012), associated to irinotecan have demonstrated a synergistic effect on *in vitro* and *in vivo* models (Donawho et al., 2007; Davidson et al., 2013).

Current CRC therapeutic management has demonstrated to clearly ameliorate the patient's outcome, in term of both progression-free survival and overall survival (Haller et al., 2011). Moreover, the association of targeted therapy to conventional chemotherapy have also exhibited its efficacy, but with milder results than expected (Dutta and Maity, 2007). Despite the extensive knowledge about colorectal cancer biology, so far it is not clear how to efficiently target the specific defects of tumoural cells. Anti-EGFR therapies are an example: although overexpressed, the blockade of EGFR activity has small, but substantial effect on cancer growth, hence survival (Jonker et al., 2007). Given the importance of the pathway in CRC, since the multiple mutations identified in its various components, a multi-inhibition approach may be a way to improve targeted therapy efficacy, as seen in preclinical studies (Bertotti et al., 2011). The approach could be guided by genomic datasets and genomic makeup of the patients. Furthermore, genomic datasets can not only improve our understanding of the processes of cancer cells, but also provide new therapeutic targets, on which new drugs could be design.

1.2 RNAi screening

Specific gene silencing through RNA interference (RNAi) has allowed genome-wide functional screenings both in cells and in organisms. The information derived from the screening led to the identification of new cellular pathways and potential drug targets (Perwitasari et al., 2013).

RNAi is a physiological pathway that acts reducing, but not eliminating, mRNA target expression by double strand RNA (dsRNA), which is delivered into the cells, in a sequence-specific manner (Fire et al., 1998). The discovery and the application of this technology have led to impressive new research in several fields. In fact, RNAi has been harnessed as a molecular tool for precise downregulation of mRNA levels, which have fundamentally contributed to large-scale studies of functional genomics in a wide variety of cells and organisms, including mammalian cells, fruit fly and mouse (Martin and Caplen, 2007; Armknecht et al., 2005).

1.2.1 Applications

Coupling RNAi technologies with genomic sequence databases, genome-scale libraries of RNAi reagents have been created by which high-throughput screenings (HTS) have been carried out multiple type of cells (Echeverri and Perrimon, 2006). This powerful tool has allowed systematic functional analyses in several cells or model organisms, which, because of their technical limitations, were previously carried out only in few sets of genetically modifiable biological systems. Amongst the wide range of application, RNAi has been proven particularly useful in performing genome-scale cell-based RNAi screening in mammalian cells (Gondi and Rao, 2009). Actually, several biomedical discoveries have been made possible by the use of RNAi, including the identification of novel oncogenes and potential targets on which design new therapeutics (Kim and Hahn, 2007; Wolters and MacKeigan, 2008).

1.2.2 Methodology

The outcome of the application of RNAi could be compared to a loss-of-function genetic screening. Indeed, RNAi, by knocking down a gene, can analyse the importance of the given gene on a specific function. If the function was affected by the absence of the gene, directly or indirectly, a phenotype may arise, that could be detected by a conventional test for the specific function (Root et al., 2006). Since its unique mechanism of action, the implementation of this tool has improved the analysis of both small-scale studies and HTSs. For instance, HTSs made possible the simultaneous analysis of a wide set of gene functions, in a way that it is possible to identify various members of functional pathways or involve new genes in a known function or process (Lord et al., 2009). These types of RNAi screenings are usually subdivided in two formats: the pool format, where the silencing occurs in the cells

randomly, and the arrayed format, where instead each gene is targeted by the silencing molecule in a multi-well plate. On one hand, in the pool screen the library of

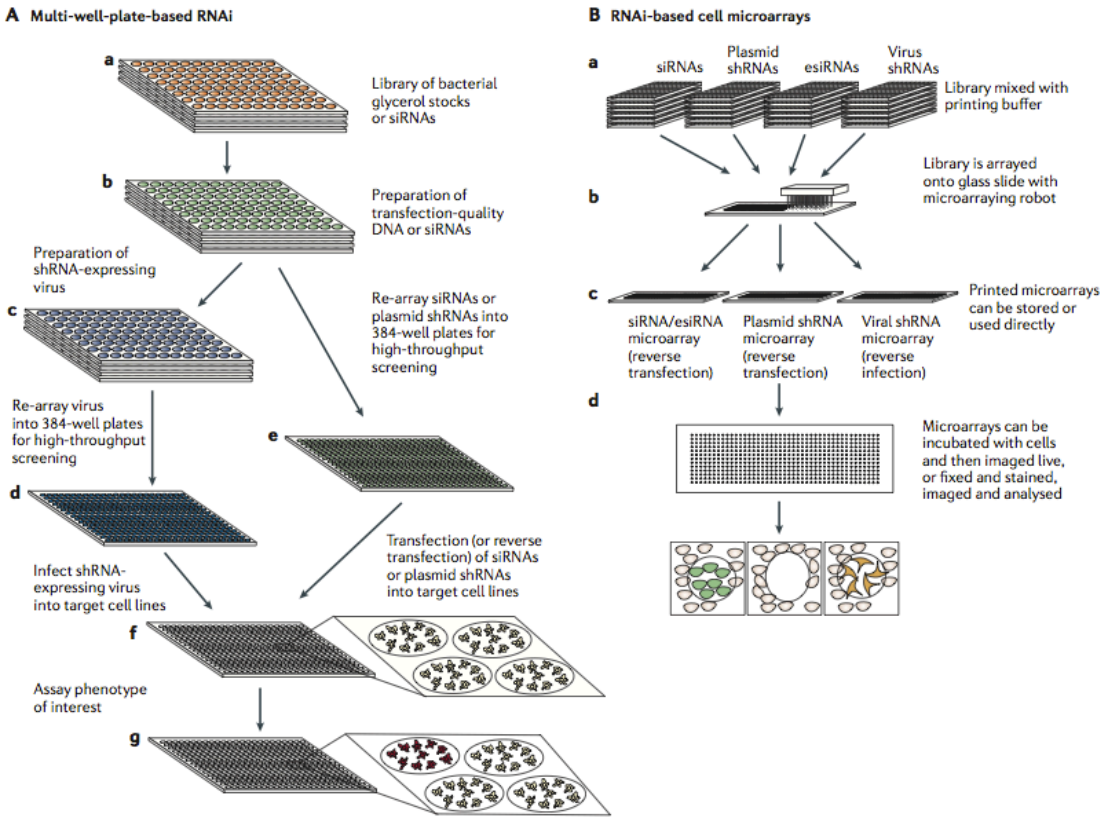

Figure 10 Formats for high-throughput mammalian RNAi screens: some examples. (A) Well-based RNA interference (RNAi) screening. (Aa) Libraries of gene-targeting reagents (bacterial glycerol stocks siRNAs are kept in multi-well plates. (Ab) The libraries of gene-targeting reagents are converted into transfection-quality DNA (plasmid-based shRNAs) or siRNAs. A strategy that is commonly used is to pool multiple siRNAs that target the same gene and array these gene-specific pools into multi-well plates. (Ac) Transfection-quality DNA from viral plasmid-based libraries can be used to make viruses in multi-well-plate format that, in turn, can be used for infection-based screening. Viruses (Ad) or nucleic acids (Ae) are then re-arrayed into 384-well plates for high-throughput screening. (Af) Transduction, transfection or reverse transfection of the appropriate gene-targeting reagents into target cells results in gene-specific knockdown. (Ag) Phenotypic plate-based assays can be performed, and wells where the target cells show a dramatic response to the perturbation can be identified simply by their plate position (see red cells). (B) Various mammalian RNAi approaches, which are compatible with cell microarrays. (Ba) Libraries can be of several formats including siRNAs, plasmid-based shRNAs, enzymatically derived siRNAs (esiRNAs) or virus-based shRNAs. (Bb) Library constituents can be printed onto glass microscope slides at high densities. (Bc) RNAi microarrays can be stored for long periods of time or cells can be cultured on top of these arrays and then processed in an image-based assay. (Bd) The cells on top of 'spots' that represent specific gene knockdowns are examined automatically by analysis software (Moffat and Sabadini, 2006).

RNAi reagents, usually against the whole genome, is introduced randomly, in such a way that statistically only one gene is targeted in one cell. The readout of these screens is based on the selection of a phenotype displayed by the cells, for instance the survival or the expression of a particular protein, or by the comparison. Consequently, the identification of the genes responsible for the phenotype, also called deconvolution, could be carried out in different ways, such as next generation sequencing (Luo et al., 2009). Due to the complexity of the application of this format,

in particular the deconvolution process, the whole-genome library was divided in smaller pools. On the other hand, in arrayed formats the number of genes to analyse is more adaptable, ranging from few hundreds to the entire genome. The RNAi reagents are delivered each one in one well of the multiwell plate, which facilitates both the manipulation and the readouts. The detection of the phenotype is usually done by assessing cell viability, the response to a drug or the localisation of a protein, by luminescence or fluorescence readouts (Echeverri and Perrimon, 2006; Conrad and Gerlich, 2010). In addition, the advantage of arrayed formats is the ability to assay multiple phenotypes in each gene or well during a single screen, in order to improve the specificity of the system (Figure 10).

1.2.3 The role of bioinformatics

Disregarding the type, the meaningful information retrieved from these screenings could be integrated with bioinformatic approaches. Bioinformatics analyses carried out on the entire genome can identify a subset of candidate genes, which can be subsequently tested with the experimental setup of RNAi reagents. As such, a subset of genes sharing a biochemical function (i.e. kinase) have been analysed, showing that the activity of JNK, a transcription factor, could be regulated by different, previously unrelated, kinases (Bakal et al., 2008). Bioinfomatics can be also used indirectly, retrieving the information about a specific subset of genes from the data available from literature. These literature-based analyses are then followed by the screen of the retrieved gene subset. An example of this is the identification new regulation mechanisms of haematopoietic stem cell activity. RNAi screening evaluated a small subset of genes involved in stem cell homeostasis, identifying Msi2, a RNA binding protein, as positive regulator of stem cell self-renewal, speculating on its implication in leukaemic cells (Hope et al., 2010). Hence, the further integration of RNAi screenings with bioinformatic platforms and data derived from protein-protein interaction or gene expression studies can shed more light on the complexity of gene networks (Mohr and Perrimon, 2012).

1.2.4 Specificity

Given the high throughput of the RNAi screenings, an important issue that has not been fully addressed yet is the false discovery (FD). During the initial years of RNAi screens, several inconsistencies amongst primary screenings have been detected,

which were mainly attributed to false positive "hits". The cause of these false discoveries was the off-target effect (OTE) (Birmingham et al., 2006). The improvement of the technologies and the understanding of the mechanism of OTE, such as implementation of gene annotation, improved reagent design coupled with statistical analysis, have demonstrated to effectively minimise FD (Birmingham et al., 2009). Although it is not clear if OTE can be ruled out, there are different methods to limit FD. Perform the screening using a different readout, which reduces the intrinsic error of a specific assay, such as proceeding with fluorescence-based assay when previously a luminescence-base was used, as well as choose the right cut-off according to the purpose of the screening, and, lastly, using a multiple RNAi reagents or cell lines are different ways proven useful to minimise the OTE (Mohr and Perrimon, 2012).

1.2.5 Discoveries

The efficacy of systematic interrogation of gene function that RNAi application has provided is presented by a plethora of studies and it led to a wider comprehension of the function of genes, and their role, in pathways and networks of different cells and organisms (Bakal et al., 2008; Luo et al., 2009; Toyoshima et al., 2012; Rudalska et al., 2014). RNAi screens have played a significant role in elucidating cancer biology and the mechanisms of anticancer drug resistance. The study of Luo et al. (Luo et al., 2009) demonstrated the sensibility of colorectal cancer cells to alteration of mitosis comparing KRAS wt and mutated cells, in particular the susceptibility of KRAS mutated cell to proteasomal degradation of Polo-like kinase 1 (PLK1). This synthetic lethal approach could lead to the application of PLK1 inhibitors in CRCs with mutated KRAS. A similar recent approach pointed out instead, how pharmacologically circumvent the oncogenetic activity of MYC. By the screening of thousand of targetable genes, the group identified hundreds genes required for MYC-driven oncogenesis, such as casein kinase 1 epsilon. This particular kinase was then inhibited by a small molecule, which resulted in reduced cell viability of both MYC-overexpressing cell models and *in vivo* models, providing another possible tool to anticancer therapy. Finally, a new research has identified, by a pooled RNAi screen, that among the gene amplified in hepatocellular carcinoma (HCC) one, Mapk14, was associated to poor cancer survival and resistance to sorafenib treatment. In this experimental setup, inhibition of Mapk14 sensitised both *in vitro* and *in vivo* models

to the action of sorafenib by blocking gene's downstream signalling. This discovery not only provided a new therapeutic target but also a new approach to therapy of drug-resistant HCC (Rudalska et al., 2014). Hitherto, literature presented the power of RNAi to elucidate various aspects of biology of the cells and how this tool could be useful to understand cancer biology and identify new therapies.

1.3 Steroidogenic Acute Regulatory Protein–Related Lipid Transfer D3 - STARD3

STARD3 is a cholesterol-binding protein belonging to the START (steroidogenic acute regulatory protein–related lipid transfer) protein family and it is involved in the shuttling of cholesterol across the different membranes of the cellular compartments. Whereas its precise mechanism of action is still elusive, STARD3 has been recognised to play a role in endosomal dynamics and in the last years several studies have shown also its involvement in cancer, specifically HER2-overexpressing breast cancer, as an oncogenic partner of HER2-driven carcinogenesis.

1.3.1 START Superfamily

Lipids consist in a wide a diverse array of molecules and their activity is crucial in maintaining different aspects of cellular homeostasis, such as membrane integrity, energy balance and signal transduction (Santos and Schulze, 2012). As a result of their physico-chemical features, transportation of lipids in aqueous environment is needed for their action and it is achieved by both vesicular and non-vesicular mechanisms. Vesicular transportation takes place by formation, shipping and fusion of vesicles between cellular organelles and plasma membrane, whilst non-vesicular transport consist of simple transfer of lipid molecules from a membrane to another, through the cytoplasm (Lev, 2010). The second process is carried out by a series of amphipathic proteins of which the START domain proteins represents a one of the major families (D'Angelo et al., 2008).

The START domain protein family (STARD) is composed by 15 different members, each one sharing one START domain. This domain, which is constituted of about 210 aminoacids, contains the lipid-binding capability, due its hydrophobicity and the special arrangement of ß-sheets and α-helixes. The resulting pocket binds to the lipid,

which varies accordingly to the specific arrangement of the STARD protein (Clark, 2012). These proteins can be classified in six subgroups according to their sequence homology: organelle-bound cholesterol carriers, START-only cholesterol carriers, phospholipid/ceramide carriers, RhoGAP-START group, thioesterase group and STARD9 (Figure 11) Organelle-bound cholesterol subgroup is composed by STARD1 and STARD3, the first STARD proteins to be identified. These proteins present a similar biochemical functions, such as binding to cholesterol, but differ in the structure, subcellular localisation and tissue expression. STARD1 is basically the C-term START domain with a N-term mitochondrial localisation signal (Tsujishita and Hurley, 2000) and it is mainly found expresses in gonads and adrenal cortex. Its expression is regulated by a cAMP-dependent mechanism in response to trophic hormones, which underline the importance played in cellular steroidogenic regulatory pathways (Lin et al., 1995). Indeed, the activity of STARD1 is pivotal in transporting cholesterol to mitochondria, where it is metabolised to the hormone precursor pregnenolone (Clark, 2012). This has been further demonstrated by the STARD1 knock-out mice. The animals died few days after birth because of the absence of hormonal production, showing an accumulation of cytoplasmic lipid deposit in adrenal and gonad steroidogenic cells (Caron et al., 1997). Moreover, despite its internalisation in the mitochondria is not necessary for its activity, the absence of targeting signal showed an slightly aberrant STARD1 function, resulting in modest lipid accumulation in both adrenal and gonads, which is probably due to the regulatory property of mitochondrial localisation motif (Arakane et al., 1998; Bose et al., 2002). STARD3 will be discussed in detail later in the paragraph.

Another group of sterol-binding STARD proteins are START-only sterol subgroup, which in composed by STARD4, D5 and D6. The peculiarity of these proteins is that their structure is made of only a START domain, which has a similar specificity of STARD1 and D3. STARD4 is the main component of the subfamily and seems to play a role in delivering sterols to endoplasmic reticulum (ER) (Alpy and Tomasetto, 2005; Rodriguez-Agudo et al., 2011), a central event in cellular cholesterol-sensing. In fact, the cellular cholesterol levels are strictly linked to ER cholesterol and an increase induces esterification of cholesterol, whereas a decrease lead to the activation of sterol regulatory element-binding protein-2 (SREBP2) (Goldstein et al., 2006; Chang et al., 2009). In high-cholesterol conditions, SREBP2, a transcription factor precursor, is inactive and sequestered in ER, but when sterol levels are too low it

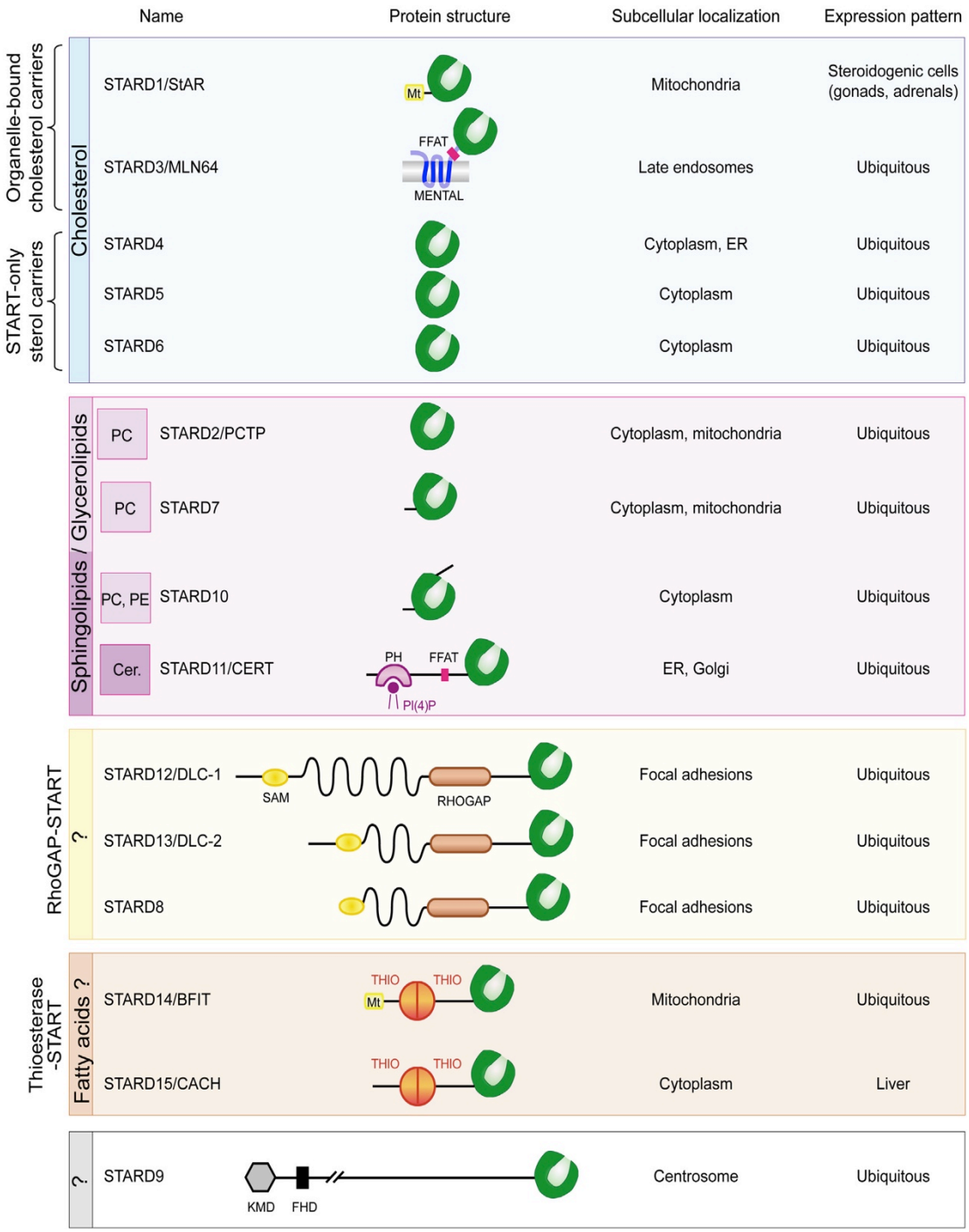

Figure 111 START protein family structure. The START protein family is divided into sub-families based on sequence homology within the START domain. Mt: mitochondria targeting signal; MENTAL: MLN64 N-terminal domain; PH: pleckstrin homology; FFAT: two phenylalanines in an acidic tract; THIO: hotdog-fold acyl-CoA thioesterase domain; SAM, sterile alpha motif; RHOGAP: GTPase activating protein; KMD: kinesin motor domain, FHD: FHA phosphoprotein binding domain (Alpy and Tomasetto, 2014).

induces the transcription of genes involved in cholesterol uptake, synthesis, and, by a mechanism of negative feedback, STARD4 itself. This is further demonstrated by STARD4 overexpression, which results in SREBP2 retention in ER (Goldstein et al., 2006; Rodriguez-Agudo et al., 2011). Moreover, STARD4 can also transport other sterol derivatives to the ER, since its function can be substituted by methyl-ß-

cyclodextrin injection when STARD4 is silenced, demonstrating its non-selective transporter activity in equilibrating cholesterol levels between the membranes (Mesmin et al., 2011). Lastly, STARD4-deficient mice have shown limited alteration of lipid metabolism, suggesting probable redundancies in STARD4 action (Mesmin et al., 2013). Conversely, STARD5 binds with greater affinity to cholic and chenodeoxycholic acid, components of bile acids, than cholesterol and hydroxycholesterol (Létourneau et al., 2012; Mesmin et al., 2013). Nevertheless, evidences suggest that its role may be transporting cholesterol to mitochondria and ER, increasing free cholesterol levels and inducing the transcription of SREBP2 (Borthwick et al., 2010; Rodriguez-Agudo et al., 2008). Despite a still controversial role, STARD5 may transport cholesterol to the ER, increasing its stress, or act as a cholesterol buffer to reduce free-cholesterol induced lipotoxicity (Clark, 2012). The last component is STARD6. Little is know about this transporter, which it is expressed almost exclusively in male gonads and can transport cholesterol to the mitochondria more efficiently than STARD1 (Bose et al., 2008)

The phospholipid/ceramide subgroup is characterized by a higher level of heterogeneity than the other. This is due to the structural and ligand-affinity differences of the components. Whereas STARD2/PCTP, STARD7 and STARD10, STARD-only proteins, bind to phosphatidylcholine (PC), STARD11/CERT, which present two membrane-interacting domains, binds to ceramides. All these proteins are expressed ubiquitously (Hanada et al., 2003; Horibata and Sugimoto, 2010; Kanno et al., 2007; Olayioye et al., 2005). STARD2, or PC-TP, is a protein that binds and rapidly transport PC to the plasma membrane and the mitochondria (Kang et al., 2010). It is usually localised in cytoplasm, but it can be also detected in mitochondria. In this regard, observations showed that its localisation can be regulated by the phosphorylation of serine 110 (de Brouwer et al., 2002). Even though its affinity for PC, STARD2 interacts also with fatty acids, such as palmitoyl acid. Given its broad specificity, the protein can have a differential preference of interaction with a different membranes (Kang et al., 2010). The second component of the subgroup is STARD7. This protein is present in two isoforms, a longer one which is usually localise to mitochondria and shorter one, derivative from the previous, which instead is cytoplasmic (Horibata and Sugimoto, 2010). Due to its structure, it is involved in PC shuttling to the mitochondria (Flores-Martin et al., 2013). STARD10 has affinity not only for PC but also for phosphatidylethanolamine (PE). Its function as lipid

transporter is regulated by the phosphorylation, by casein kinase II, of the serine 284. In particular, phosphorylation of S284 or/and removal of few aminoacids at the C-term prevents both the interaction with the membrane, therefore the delivery of the lipids (Olayioye et al., 2007). The last component of the group is STARD11, also known as CERT. Current data propose that this protein acts as a ceramide carrier from ER to Golgi, where in turn ceramide is converted into sphingomyelin or ceramide derivatives (Hanada et al., 2003). The peculiarity of STARD11 compared to the other member of the subgroup is the multi-domain structure. Actually, it is composed by a plecktrin homology domain (PH), a middle region of two phenylalanines in an acidic tract (FFAT), and a C-term where START domain is found (Holthuis and Menon, 2014). This particular arrangement of domains has been demonstrated, *in vivo*, to be fundamental for its localisation, between ER and Golgi, and function, that is to transport ceramide from ER, where ceramide is synthesised, to Golgi, where it is converted in sphingomyelin, despite *in vitro* START domain alone was sufficient to carry out its activity (Hanada et al., 2003; Hanada et al., 2009). Thereby, STARD11 is bound to Golgi through PH domain, specifically to the phosphatidylinositol 4-phosphate (PI4P) in the Golgi membrane, and to ER through FFAT, leaving START domain free to swap lipids from one membrane to the other. The activity of STARD11 is regulated by phosphorylation of serine-repeat motif: the hyperphosphorylation induces a globular folding, resulting in an inactive form of STARD11, whilst the hypo-phosphorylation leads to the active open conformation (Hanada et al., 2009). Finally, the importance of STARD11 on ceramide transportation is also supported by the phenotype of knock-out animals. The lack of STARD11 suppresses the delivery of ceramide in Golgi, thus decreasing the amount of sphingomyelin and induces mitochondrial alteration, which lead to the death of the animals during embryogenesis (Wang et al., 2009).

The subfamily of RhoGAP-START, referred also as deleted in liver cancer (DLC), is composed by STARD12/DLC-1, STAD13/DLC-2, and STARD8/DLC-3. These proteins act as tumour suppressors in different types of cancer and share a unique multidomain structure, which consists of an N-terminal sterile alpha motif (SAM), a RhoGAP domain and a C- terminal START domain (Lukasik et al., 2011). SAM domain activity is not entirely clear, having been only reported to play a role within STARD12 in regulation of cytoskeletal network and migration (Zhong et al., 2009). Conversely, RhoGAP domain activity has been well studied. Since its GAP function,

this domain plays an important role in the regulation of the RhoGTPases, inducing a change in the state of activation. In particular, STARD12 regulates Rho A, and C, whilst STARD8 and 13 only Rho A and Cdc42 (Ching et al., 2003; Healy et al., 2008; Kawai et al., 2007). Given the Rho proteins action in cytoskeletal actin, this subfamily is likely to play a role in the rearrangement of the cytoskeleton and a modification of the cellular structure, a feature further supported by the disruption of actin stress fibers after the overexpression of both STARD8, STARD12, and STARD13 (El-Sitt and El-Sibai, 2013). However, different observations have shown that also an unstructured region between START and RhoGAP influences the cellular morphology. This region promotes the interaction of STARD8/D12/13 with focal adhesion, specifically with resident protein CTEN, tensin 1 and 2, all actively involved in the regulation of cytoskeletal structure (Kawai et al., 2007, 2009; Liao et al., 2007). Taken together, all these observations show the high level of similarity of these 3 proteins, suggesting a redundant function, especially because mice KO for STARD13 have a normal development (Yau et al., 2009). However, diverse reports have pointed out the non complete overlap of function of these proteins, for example STARD12 silencing induces an early stop in embryonic development in mouse (Durkin et al., 2005). Lastly, considering the activity toward RhoGTPases, which regulate actions such as adhesion, polarity, and cell division, and given their localisation near focal adhesions, it has been speculated that STARD8, 12 and 13 could play a role in regulating these processes in a lipid-dependant fashion. This seems to be supported by the fact that STARD13 mediates ceramide signalling, probably inducing PGP synthase in mitochondria, a role previously considered of Rho A (Hatch et al., 2008). The exact role of START domain in these proteins is still unknown.

The last group is represented by thioesterase subfamily, which is composed by two proteins: STARD14 and 15. They belong to the acyl-CoA thioesterase (ACOT) from which they differ only for the presence of the START domain, and possess two hotdog-fold/thioesterase domains (THIO)(Kirkby et al., 2010). STARD14 acts as a dimer, favouring the binding to long-chain fatty acyl-CoAs. The enzymatic activity is carried out by the THIO domains, but the START domain promote the hydrolisation. Although the presence of a mitochondrial targeting signal, its localisation has been identified mainly in cytosol and microsomes (Han and Cohen, 2012). On the other

hand, STARD15, despite the similarities with STARD14, is specific toward acetyl-CoA, probably shuttling it to other ACOT proteins (Suematsu and Isohashi, 2006).
Finally, the last member of the START-domain containing protein is STARD9, the most diverse of the STARDs. Strikingly, it outweighs all the other proteins, weighting 500 kDa, and presents a N-terminal kinesine domain along with an FHA phosphoprotein binding domain, and a C-terminal START domain. Its role is not fully understood, but it localises to the centrosomes and seems to be important for mitosis, since STARD9 knock-down induces aberrant mitosis and, in turn, apoptosis. Moreover, its silencing promoted apoptosis after taxol treatment, therefore suggesting a possible target of therapeutic intervention (Torres et al., 2011).

Despite some of the START proteins are not well examined, several proofs of their function in cancer have been found. As such, STARD1 was referred as gene possibly involved in HCC. In particular, elevated mitochondrial cholesterol levels have been linked to decreased intrinsic apoptosis activation (Henry-Mowatt et al., 2004). Since in HCC cells STARD1 has been shown to be highly expressed and correlates with cholesterols levels, it could play a role in the oncogenesis of liver cancer (Montero et al., 2008). Moreover, STARD10 has been detected as overexpressed in breast cancer and more frequently, but not exclusively, associated to EGFR and HER2 overexpression, by whom it can promote malignant transformation (Olayioye et al., 2004). Interestingly, STARD10 may possess also some oncosuppressor property, since not only its lost correlated with a poorer breast cancer prognosis, but also it is downregulated during epithelial-to-mesenchymal transition (Murphy et al., 2010; Vetter et al., 2010). In line with this, the RhoGAP-START proteins have been shown to be selectively deleted in liver cancer, hence their name (DLC). Specifically, STARD12 is down regulated in several cancer cell lines besides breast and liver cancer (Plaumann et al., 2003; Yuan et al., 2003), whereas its overexpression slow cellular growth (Goodison et al., 2005). Along with STARD12/DLC1, STARD8 and 13 have potential oncosuppressor effects. These proteins are frequently lost in HCC as well. However, since their function as regulator of RhoGTPases, the tumour suppressor activity may occur by RhoGAP domain. At the end, also STARD11/CERT has been demonstrated to be involved in cancer, precisely in the resistance to taxol agents. This activity seems to be caused by the alteration of ceramide levels of the membranes, which could prevent chemotherapy-induced apoptosis (Lee et al., 2012).

As such, STARD11 could represent a novel biomarker for drug resistance in cancer and a therapeutic target as well (Hullin-Matsuda et al., 2012).

1.3.2 STARD3

STARD3 has been discovered during a screening of lymph nodes derived from metastatic breast cancer, hence the name MLN64. In these samples, the protein was identified to be overexpressed as the result of the amplification of the region q11-q21 of chromosome 17, specifically co-overexpressed with HER2 (Tomasetto et al., 1995). The sequencing of the gene revealed that MLN64 had a high homology with STARD1, which suggested a possible involvement in cholesterol transportation as well as STARD1. Despite its similarity with STARD1 and its cholesterol-binding property, STARD3 role as cholesterol carrier is not yet fully understood.

1.3.2.1 Structure and molecular biology

STARD3 is formed by two major domains, MENTAL, which has a FFAT motif, and START (Figure 122).

On the C-terminal side, it is localised the START domain, highly similar to STARD1, by which it exerts steroidogenic activity, as aforementioned. For this activity, START domain has been proven fundamental, since its removal blocked steroidogenic activity, diversely MENTAL removal has not resulted in the same block, showing instead an increase of steroidogenesis (Watari et al., 1997). This steroidogenetic event seems effectively to take place in the mitochondria. It has been reported that mitochondrial proteases could process STARD3 producing a 28 KDa fragment corresponding to the START domain, which stimulates the synthesis of steroids, such as progesterone (Esparza-Perusquía et al., 2015). The precise conformation of the aminoacids in this domain creates hydrophobic lipid-binding pocket, by which STARD3 binds cholesterol in an equimolar ratio (1:1) and carry out its activity as specific cholesterol transporter (Reitz et al., 2008; Tsujishita and Hurley, 2000). Several roles of START have already been discussed extensively in the previous section.

MENTAL domain is located at the N-terminal of the protein and it is present also only in STARD3 N-teminal like (STRAD3NL), with whom it shares more than 80% of homology. The MENTAL domain is highly conserved in all the animal kingdom and present four transmembrane helices linked by three inverted loops, which are arranged in a unique fashion (Alpy et al., 2003, 2005). It prompts the localisation of

STARD3, by the interaction with 14-3-3, in late endosome (LE), with a particular accumulation in the limiting membrane (Liapis et al., 2012). Whereas no proper signal for this localisation has been identified, a mutated version of MENTAL showed the inability of STARD3 to bind to the LE. Moreover, by fluorescence microscopy, the MENTAL conformation was shown to be fundamental for the orientation toward the cytoplasmic side of LE membrane (Alpy et al., 2001, 2002).

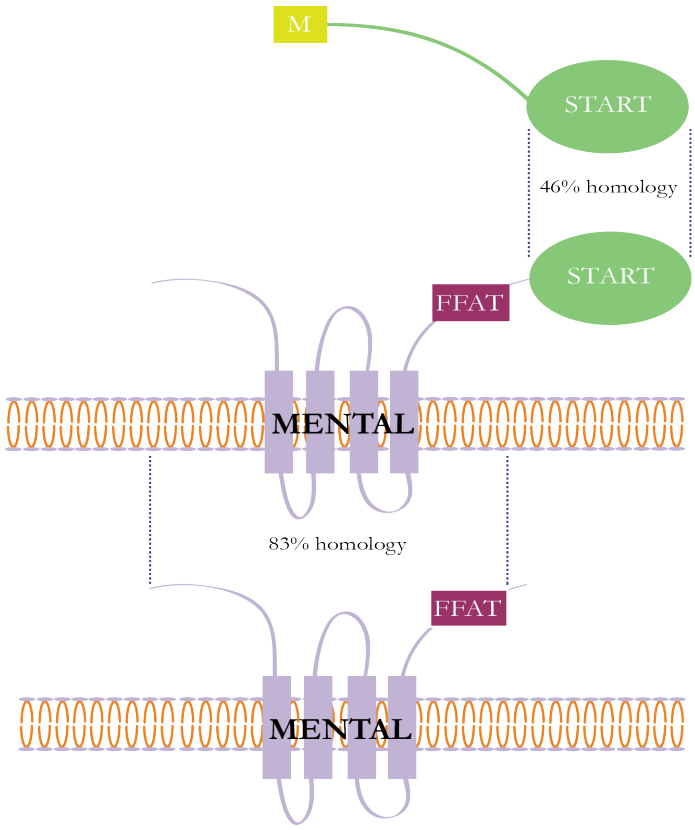

Figure 122 STARD3 contain two domains and shares some sequence homology. A schematic representation of STARD1, STARD3 and STARD3NL shows the high grade of homology between these proteins. Adapted from Alpy and Tomasetto 2014.

The particular disposition of the transmembrane helices confers to MENTAL domain dimerisation properties, that could be either homo- or etero-dimerisation between STARD3 and STARD3NL on LE membrane, in a similar way of tetraspandin oligomerisation (Alpy et al., 2005). In addition, MENTAL seems to bear cholesterol-binding activity as well as START domain, promoting the formation of cholesterol microdomain between the helices (Alpy et al., 2005). The consequences of this particular characteristic are still not clear. Finally, MENTAL domain has been also

demonstrated to be involved in the association between LE and ER. Precisely, by fluorescence and electron microscopy, it has been identified that the interaction is elicited by the FFAT motif that, acting as a molecular tether, binds to VAP1, a protein found in ER membrane, bringing about the LE and ER (Figure 133). The interaction between FFAT and VAP1 leads to the formation of special site of connection, also called membrane contact sites (MCS), resulting in the surrounding of endosome by ER. A prolonged interaction of STARD3 and VAP1, thus LE and ER, leads to the alteration of endosomal compartment dynamics (Alpy et al., 2013). In these settings, STARD3 is believed to transferring cholesterol from LE to ER across the MCS, as a consequence of swinging of cholesterol from a membrane to the other, similarly to STARD11. Given the huge similarity, the process can be carried out by STARD3NL, suggesting a possible role executed in tandem. Although the role in different processes, STARD3-deficient mouse did not displayed any serious phenotype, but a slight sterol metabolic alteration (Kishida et al., 2004).

As previously encountered, START proteins can have some redundant function. of the many examples is STARD3. Since STARD1 is not ubiquitously expressed as STARD3, the last has been suggested to compensate for STARD1 absence in steroidogenic organs, such as placenta and brain (King et al., 2006; Moog-Lutz et al., 1997; Watari et al., 1997). Whereas the role in placenta has been referenced byseveral studies (Esparza-Perusquía et al., 2015; Olvera-Sanchez et al., 2011), STARD3 role may be compensated by alternative steroidogenic pathways in KO mouse models (Kishida et al., 2004; Miller, 2013). Conversely, in brain STARD3 is unlikely to be involved in synthesis of neurosteroids (King et al., 2006). Low density lipoproteins (LDLs) possess high concentration of cholesterol and are internalised in the cell by a clathrin-mediated endocytic mechanism, which directs the lipid component first to the LEs and/or Lysosomes (LE/Ly), then to other regions (Goldstein and Brown, 2009; Midzak and Papadopoulos, 2014). In cholesterol redistribution are involved two proteins, Neimann-Pick C1 and C2 (NPC1, NPC2), whose deficiency causes a neurodegenerative disorder (Neimann-Pick type C disease) characterised by lipid storage disruption. The two proteins act together in promoting the efflux of cholesterol from LE/Ly: NPC2, localised in the LE, binds and transfers luminal cholesterol to NPC1, which carries it out of the endosome (Midzak and Papadopoulos, 2014). To test whether there are connection between STRAD3, NPCs and cholesterol, STARD3 was overexpressed. Its overexpression showed that STARD3 may promote

transportation of cholesterol from LE to plasma membrane along with ABCA3, alternatively to mitochondria when coupled with NPC2 (Charman et al., 2010; van der Kant et al., 2013). However, no alteration was reported in NPC-deficient cells (Alpy et al., 2001; Hölttä-Vuori et al., 2005), whereas lipid accumulation, apoptosis and liver damage have been reported in *in vivo* models (Tichauer et al., 2007).

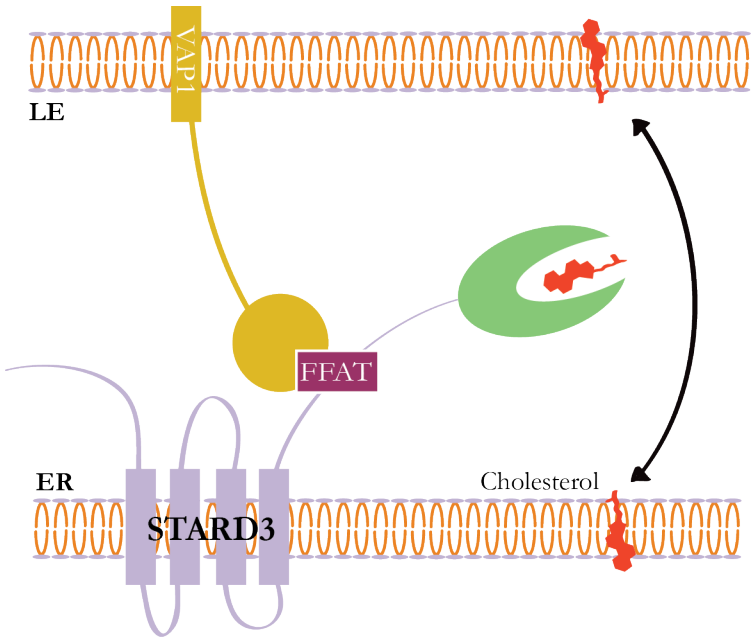

Figure 133 STARD3 mechanism of action. Accordingly to Alpy and Tomasetto 2014, STARD3 could act tethering ER to LE via the interaction of VAP1, a LE-resident protein, with the FFAT motif presents in STARD3. This closure between the two compartments could allow an exchange of cholesterol between the membrane mediated by STARD3.

Although the role of STARD3 in cholesterol transfer seems to be paradoxical, its role in LE-ER transportation appears to be clearer and consistent (Alpy et al., 2013). The redundancies resulting from the similarities between START domain proteins coupled with different expression levels in different cell types might account for the difficulty in elucidating the role of STARD3. Whereas lacking a proper signal of LE localisation, it interacts with endosome-lysosomes, its activity slow the maturation of LE down into lysosome.

1.3.2.2 STARD3 implication in cancer

STARD3 was first identified in the minimal HER2 amplification region in samples of breast cancer (Tomasetto et al., 1995). Whereas many other genes were co-amplified, only few were also co-overexpressed with HER2, such as growth-factor-bound-7 (GRB7) and STARD3 itself (Staaf et al., 2010; Vincent-Salomon et al., 2008). STARD3 lies about 30Kb far from Erbb2 gene, centromerically, and its co-overexpression with HER2 could be the consequence of Sp1 transcription factor, whose binding sites were found in both their promoters (Alpy et al., 2003). Although the co-overexpression was observed more frequently in breast cancer, new evidences suggested a possible HER2-STARD3 overexpression also in colorectal and gastric cancers (Cancer Genome Atlas Network, 2012; Qiu et al., 2014).

The HER2-positive breast cancer are about the 20% of all breast cancers and the application of targeted therapy with trastuzumab have demonstrated to improve clinical outcome (Higgins and Baselga, 2011). However, 50% of the cases do not respond or develop resistance to the treatment (Sahlberg et al., 2013), and STARD3, due to the relationship with HER2, has been proposed as a possible cause of these responses (Dave et al., 2011). Thus far is not clear how STARD3 and HER2 are connected, nevertheless the association between these genes is strong, even considering the reduced survival resulting from their overexpression (Cai et al., 2010; Lamy et al., 2011; Vassilev et al., 2015). This has been supported by the effect of STARD3 on cancer cell models. In fact, silencing of STARD3 in HER2-positive breast cancer cell lines resulted in reduced cell proliferation and increased cell death, conversely no effect was detected in cell who did not overexpress HER2 (Kao and Pollack, 2006). Strikingly, overexpression of STARD3 in cell with normal HER2 levels produced an similar effect, inducing cell death and toxicity (Tichauer et al., 2007). In addition, Sahlberg et al., through a loss-of-function study in HER2-positive breast cancer cell line, identified several genes in amplicon of HER2, which reduced cell viability, amongst which were found STARD3 and GRB7. Moreover, it was observed a synergic effect of silencing of HER2 and STARD3 on cell viability, which induced apoptosis, suggesting that the HER2-positive tumours may depend on genes present in HER2 amplicon.

How STARD3 relates to HER2 is still controversial, nonetheless different studies addressed its mechanism of action. Cai et al. showed, by RNAi approach, that in breast cancer cell lines, STARD3 promoted proliferation and adhesion, proposing

STARD3 a regulator of focal adhesion kinase (FAK), main regulator of adhesion processes. Interestingly, this evidence has been further supported by a recent study in breast cancer cell lines (Vassilev et al., 2015) where STARD3 have been shown to regulate Scr and FAK phosphorylation in response to serum and insulin levels, that in turn led to higher adherence and plasma membrane cholesterol. Although these models used breast cancer cell lines in which HER2 is not overexpressed, therefore the relationship between HER2 and STARD3 is yet to be explained. Considering the multistage nature of cancer development and the heterogeneity of cancer cells, the unclear, sometime paradoxical, activity of STARD3 should not surprise. Further investigations should be carried out to elucidate the mechanism by which it exerts its functions

2 - RESULTS AND DISCUSSION

2.1 Rationale

Colorectal cancer is one of the leading causes of cancer-related mortality world wide and, although the current treatments have been able to reduce mortality, we still need better therapies to improve patient survival, especially of patients with metastatic tumours (Ferlay et al., 2015; Gallagher and Kemeny, 2010)

Keeping in mind the power of RNAi screening to interrogate the function of a large gene sets (Luo et al., 2009) and to find new pharmacological targets (Cole et al., 2011; Rudalska et al., 2014), we thought to identify new important regulators of CRC development by the aid of screening with therapeutic potential. To increase the speed and the efficiency of this process, we selected a subset of genes found to be amplified in colorectal cancer in a study carried out by The Cancer Genome Atlas (TCGA, 2012). In this study, TCGA analysed 257 cancer genomes, of which 97 were analysed in further detail for somatic copy number variation (SCNV). Amongst the significant SCNV identified, we have selected those with amplified genes, which more probably contain genes with oncogenic features (Pierotti et al., 2003). The total amount of amplified gene retrieved was 212.

2.2 Screen of specificity and activity of shRNA library.

To interrogate the gene set, in order to identify potential oncogenes, we have chosen to proceed by carrying out a RNAi screening in an arrayed format, since it has already been proven a useful tool to identify genes involved in oncogenesis. The RNAi reagents chosen were short hairpin RNA (shRNA), by which a more stable and reproducible gene silencing could be obtained (Mohr and Perrimon, 2012). RNAi has an intrinsic issue with specificity, also known as off-target effect (OTE), which can be successfully reduced when RNAi is integrated with gene expression data (Booker et al., 2011). Based on this, we analysed the expression of the gene set by qPCR in parallel with specificity of the silencing. By the evaluation of mRNA levels (Figure 14), we identified that in HCT116 cell line about 70% of the genes were indeed expressed and specifically silenced by the action on shRNA compared to the control (cell transduced with pLKO empty vector), considering an arbitrary cut-off of 50%

silencing. The remaining about 30% was either not amplified or not enough silenced. As a consequence, other shRNAs and primers have been ordered.

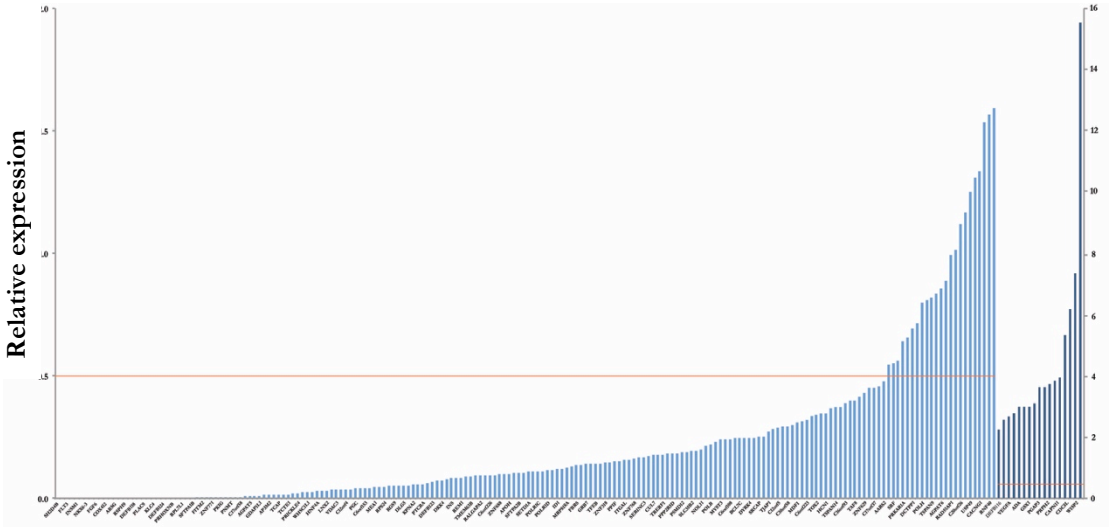

Figure 14. Validation of gene list specific silencing. Relative expression of amplified genes in HCT116. RNA was analysed by qPCR. The orange line correnspponds to the arbitrary cut-off of 50% of silecing. Genes whose expression was less than 50% of control (1.0, pLKO) were considered silenced, otherwise not silenced. A subset of genes was not expressed (left side of the chart).

Taking into account that the major part of the genes was expressed in our cell line model, we proceeded to test the phenotypical effects of the shRNA library, in particular the effect on cell viability, one of most conspicuous characteristic of cancer cells (Hanahan and Weinberg, 2011). To minimise the influences derived from a specific cell line, we used 4 different colorectal cancer cell lines, HCT116, HT29, DLD1 and CACO2, each one corresponding to a different molecular subtype (Guinney et al., 2015). These cell lines were seeded and after 96 hours, cell viability was assessed by luminescence assay. The results (Figure 15) displayed great variation of cell viability amongst and within the cell lines, resulting from shRNA activity. In particular, whereas in DLD1 and CACO2 the shRNA library slightly affected cell viability, in HCT116 and HT29, the genes whose silencing reduces cell viability of an arbitrary cut-off of 50% compared to the control, were 53 and 14 respectively. Considering the levels of reduction of cell viability, the druggability of the genes (i.e. the presence of enzymatic active sites) and the relative involvement in cancer (i.e. number of publications retrieved from pubmed.org at the query: "gene's identifier" AND cancer), we identified a set of 12 genes, which, after further validation (data not

shown) were downsized to 1, given its strong and more reproducible phenotype: STARD3.

Our approach showed that coupling shRNA specificity validation and loss-of-function assays could be effectively used to investigate the function of subsets of cancer-related genes derived from literature, in line with previous findings, substantiating the feasibility of these type of small/medium-scale RNAi screening. Despite not yet complete, thus far the screen has allowed us to identify a candidate oncogene. In fact, STARD3 has been shown to be involved in HER2-positive breast cancer as a gene that synergycally support HER2 activity (Sahlberg et al., 2013). Moreover, given its cholesterol-binding pocket on the START domain, STARD3 represents an ideal protein on which an inhibitor could be design to block its action.

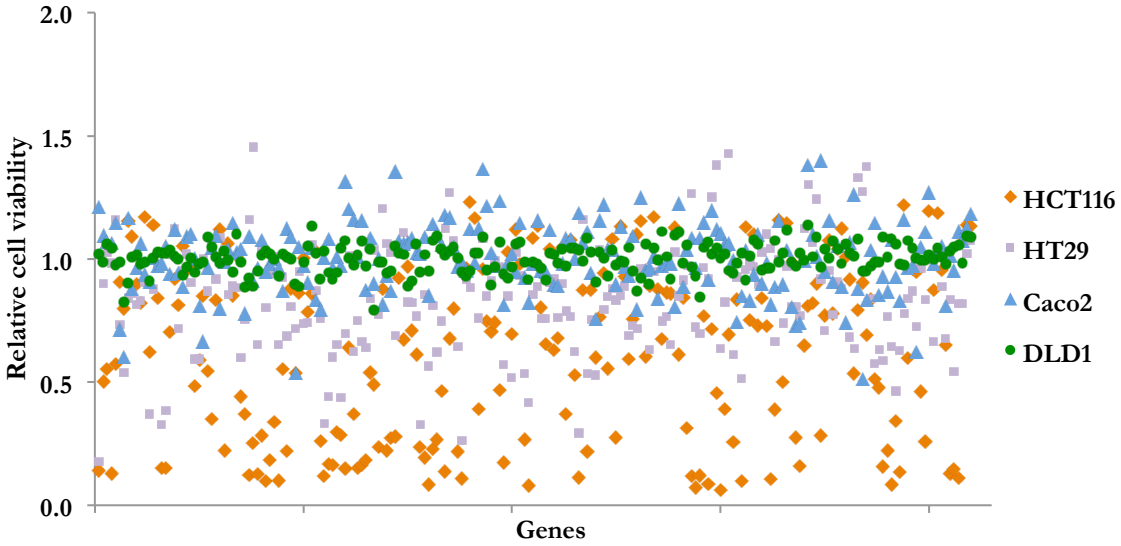

Figure 15. Functional screening of candidated genes following RNAi. Relative viability of HCT116, HT29, Caco2 and DLD1. The cells were seeded at 500 cell/well (HCT116) and 1000 cells/well (HT29, Caco2, DLD1) and the vitality was measured after 96-hours. The realtive cell viability was normalised on the values of cells transduced with empty vector (pLKO).

2.3 STARD3 affects cell viability of CRC cell lines.

To evaluate the extent of the effect of STARD3 silencing and to rule out possible cell-dependent effect, we tested the effect of STARD3 knockdown evaluating the variation in terms of cell viability not only in HCT116 and HT29, but also in COLO201 and COLO205, two cell lines that correspond to different molecular subtypes. In these experimental settings, to reduce the probability of OTE we used a second shRNA (shSTARD3_2) in addition to the previous, which paired to a different sequence on STARD3 messenger RNA. The effect of knockdown on the viability was monitored

every 24 hours, for 96 hours and from the analysis we observed a substantial and robust reduction of vitality in all the cells knockdown for STARD3, (Figure 16 a, b, cd). Moreover, to confirm that STARD3 was effectively and specifically reduced, we analysed its protein levels by western blot, detecting a considerable decrease of the protein, indicating the strength and the specificity of shRNA activity (Figure 16 e). By the analysis, it was evident that STARD3 downregulation had a fundamental effect on cell proliferation. In fact, its ablation caused a decrease of >50% of vitality in all the different cell lines, although the variation of growth rate and timing amongst them, which could have been imputable to the diverse genetic and mutational background of the cell lines (i.e. HCT116 shows a microsatellite instability and HT29 chromosomal instability instead) and experimental variability

The activity and the specificity of STARD3 silencing was effectively validated and the results showed a remarkable effect on cell viability, even if the differences between the cell lines are considered. Although previously observed in breast cancer, we identified the effect of STARD3 on viability of colorectal cancer cells, and given its profound consequences when silenced, we speculated on its possible involvement in carcinogenesis as an oncogene

2.4 STARD3 silencing alters cell cycle distribution and induces an increase of sub-G1 population.

Cell proliferation is highly dependent on cell cycle activity, and is tightly regulated in normal cells, whereas in many cancers it is frequently deregulated resulting in uncontrolled cell division, which sustains cancer progression. For these reasons, cell proliferation is considered an hallmark of cancer (Hanahan and Weinberg, 2011). Given the intimate connection between cell growth and cell cycle and the drastic effect of STARD3 silencing on cell viability, in order to look deeper into the mechanisms on which STARD3 may impinge, we analysed the distribution of cell cycle phases. Implementing the previously used experimental settings, STARD3-knockdown cells and the relative control cells were harvested along with the culture medium, and stained with propidium iodide, to evaluate DNA content by flow cytometry. In all the cellular models (Figure), we saw that STARD3 ablation caused a significantly different distribution of cell cycle phases. In particular, after STARD3

silencing, cell populations in G1 and S phases have a mild often discordant variation amongst the cell lines, however that was not the case of the population in

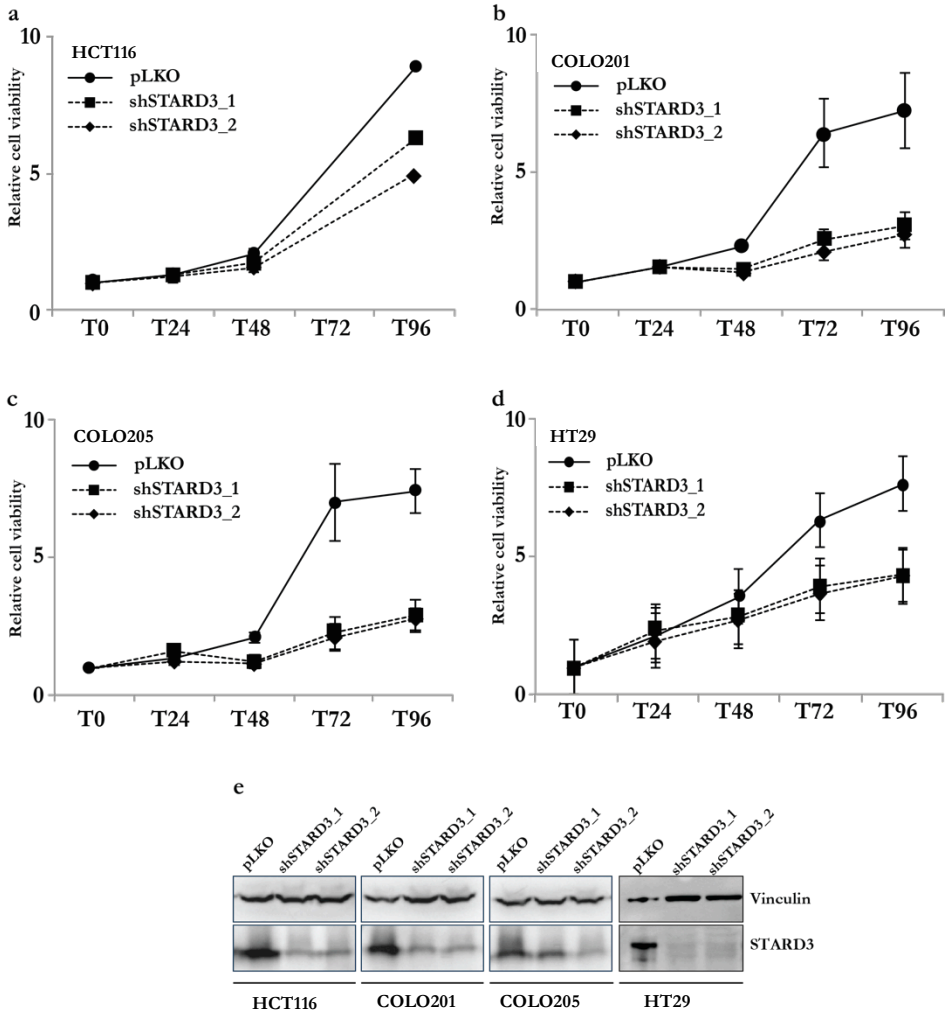

Figure 16 STARD3 silencing hampers cell viability. Relative viability of HCT116 (a), COLO201 (b), COLO205 (c) and HT29 (d) transduced with two different shRNAs compared to the control. The cells were seeded at 500 cells/well (HCT116) and 1000 cells/well (COLO201, COLO205, HT29) and the vitality was measured by luminescence over a 96-hours period, every 24 hours (except HCT116 at 72 hours), using a microplate reader. The realtive cell viability was normalised on the values of cells transduced with empty vector (pLKO). (e) Western blot to evaluate STARD3 expression in HCT116, COLO201, COLO205 and HT29 after transduction with two different shRNA and the relative control

G2/M phase. In fact, the phase saw a significative increment, though variable, of more than 50% compared to the control levels. Interestingly, despite not ubiquitously, the number of cells with a DNA content below 1*n*, hence in subG1 "phase", was greatly affected by the silencing of STARD3 as well, which increased the amount up to 24 folds.

These observations of STARD3 effect on cell cycle phases, suggest that it may not influence the cells in phase G1 and S, but instead play a role in the activation in G2/M checkpoint, since the increases amount of cell detected in phase G2/M. This thoroughly tuned checkpoint prevents cells with DNA aberrations to be enter mitosis, whilst its deregulation, usually detected in different types of cancers, allow the cells to maintain the mutations which render the cells genomicallyunstable (Talos and Moll, 2010). As a consequence of prolonged activation, thus a non-resolution of the DNA damage, the checkpoint leads to permanent cell cycle arrest and in turn cell death (Löbrich and Jeggo, 2007). We speculated that these could have been the conditions, as we have seen a significant increase amongst the cell lines of subG1 population, a marker of cell death, as well (Löbrich and Jeggo, 2007; Lukas et al., 2004). With these results. we showed that STARD3 may generally act by promoting a favourable resolution of the G2/M checkpoint, allowing the cells to progress toward mitosis, regardless of the G1 and S phases, which substantiate its oncogenic behaviour in colorectal cancer.

2.5 STARD3 silencing induces apoptosis.

G2/M checkpoint is a crucial step in cell cycle and its activation trigger the cell cycle arrest and cell death. Since the effect of STARD3 silencing led to a substantial increase of the subG1 population of cells, we investigated whether this process could result in apoptotic activation. Using HCT116, COLO201, COLO205 and HT29 transduced with shRNA against STARD3, and the relative control (empty vector), we harvested the cells, including the culture medium, and evaluate the expression of the apoptotic marker Annexin V, by flow cytometry. Interestingly, the analysis revealed that after STARD3 knockdown, the cells started to express more apoptotic markers compared to the control cells and, despite differences among the cell lines, both single positive (Annexin V alone) and double positive (Annexin V and 7AAD) populations were from at least twice more concentrated upon STARD3 silencing than the control (Figure 188). The increase in the apoptotic population was detected in all the cell lines analysed, however only in HCT116, COLO 201 and COLO205 the difference between the silenced and control cells was significant. In HT29, a trend toward an apoptotic increase was seen, despite the wide variation of the standard deviation.

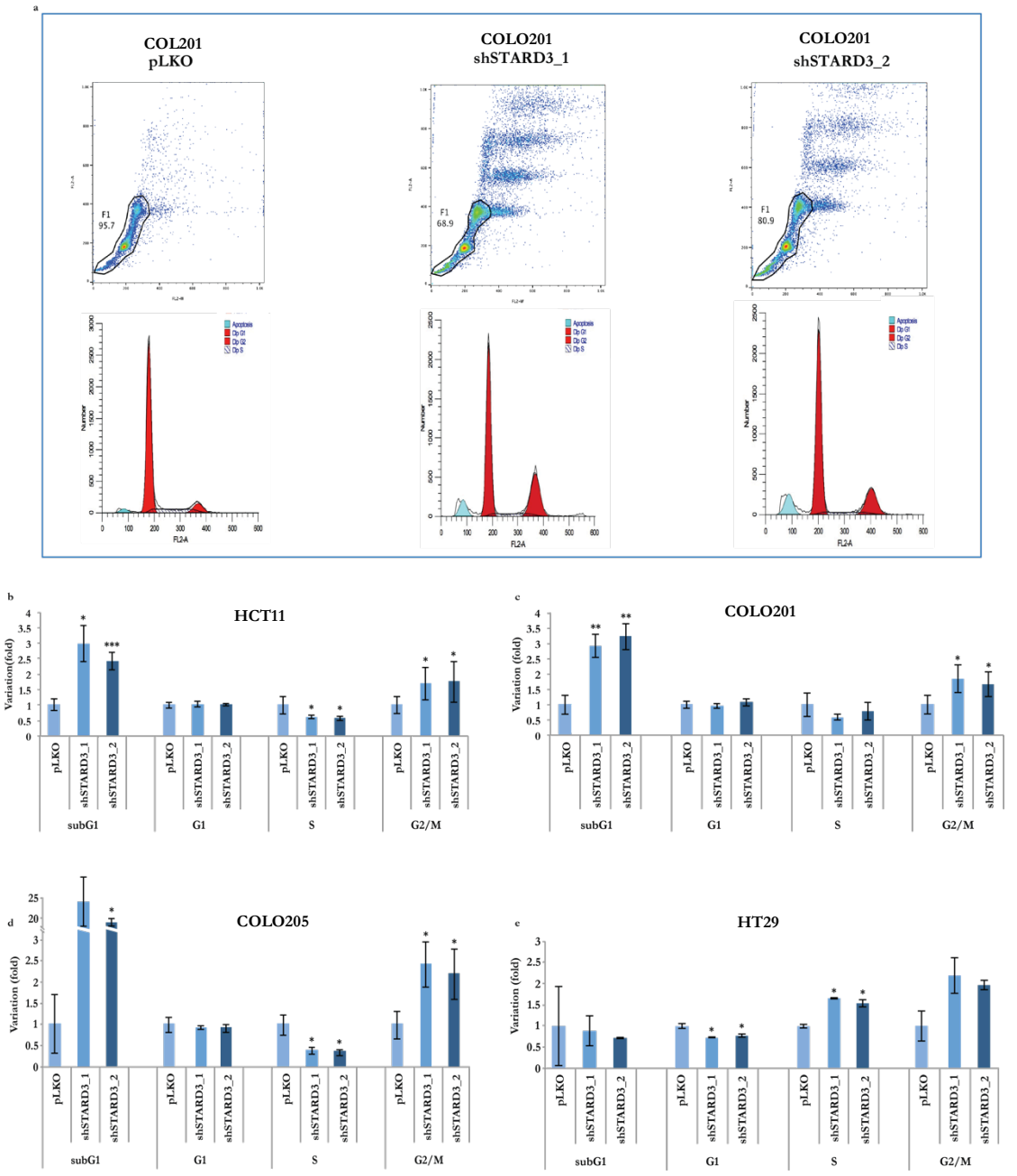

Figure 17 STARD3 effect on cell cycle prevents cell death. (a) Upper panel – Example of PI-positive COLO201 control and silenced cells plotted on an area vs. height chart, to distinguish single cell from aggregates. Lower panel – Example of PI-positive cells plotted on an area vs. events chart, to identify cells in G1 phase (at about 200 realtive intensity units (RIU) on the FL2-A axis), G2/M phase (at about 400 RIU on the FL2-A axis), S phase (between G1 and G2/M phases) and subG1 "phase" (labeled as "Apoptosis" in light blue). Fold variation of cell cycle phases of HCT116 (b), COLO201 (c), COLO205 (d), HT29 (e) control and silenced cells. HCT116 and COLO201, COLO205 and HT29 were collected 3 days and 5 days after transduction, respectively, stained with PI, then analysed by flow cytometery. The fold variation was normalised on the values of the contols. p values were calculated with two tailed t-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

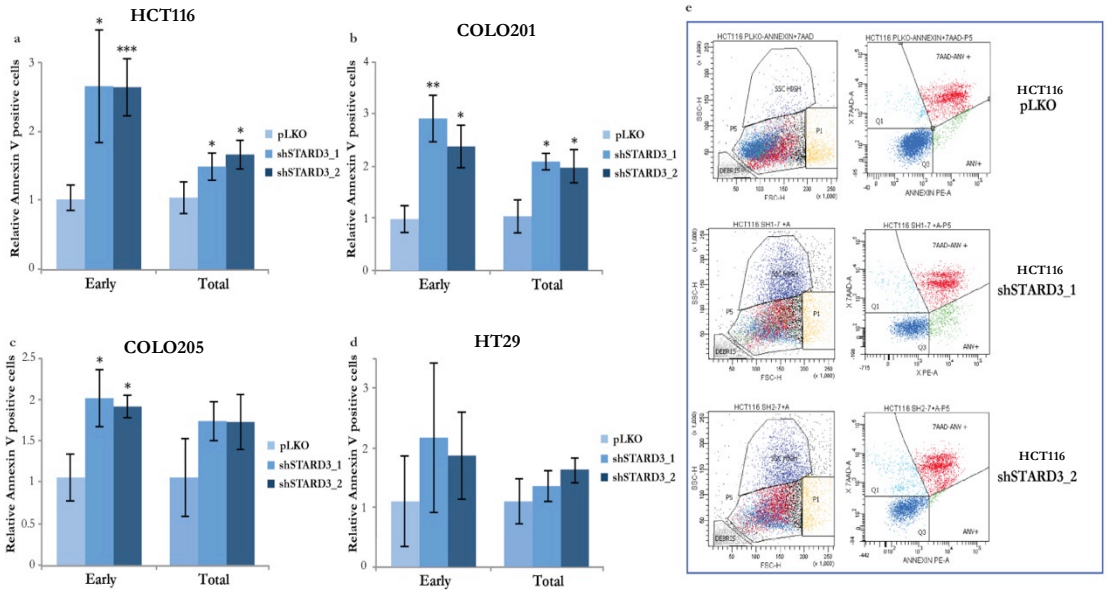

Figure 18 STARD3 prevents cell death inhibiting apoptosis. Relative amount of Annexin-V positive HCT116 (a), COLO201 (b), COLO205 (c), HT29 (d) silenced and control cells. HCT116 and COLO201, COLO205 and HT29 were collected 3 days and 5 days after transduction, respectively, stained with 7AAD and Annexin-V, then analysed by flow cytometery. (e) Left panel - Example of HCT116 plotted on an intensity vs. complexity chart. Right panel – Example of HCT116 plotted on a 7AAD vs. Annexin-V chart. The amount of Annexin-V positive cells was normalised on the values of the contols. p values were calculated with two tailed t-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

The pro-apoptotic phenotype resulting from the ablation of STARD3 was further supported by the detection of the activation of caspase-3 and -7, the main and final effectors of apoptotic pathway (Elmore, 2007). Using a luminescence assay that detected caspase activity, apoptosis levels were analysed in HCT116 (Figure 19a), COLO201 (Figure 19b) and COLO205 (Figure 19c) upon STARD3 silencing, showing a significant increase of caspase activity compared to the control cells and, in line with these observations, a narrow variation after its overexpression. Doxorubicin was used as positive control (Figure 19a). A slight perturbation of caspase activity was detected in HT29 though, which indicated however a trend toward an increased caspase activity, suggesting that STARD3 absence could be compensated by other proteins regarding apoptosis inhibition, and this could be dependent on the particular gene expression levels and genetic background (Figure 19d).

Despite the variability amongst different cell lines, we showed a clear involvement of STARD3 in the apoptotic process, which suggest that STARD3 may play a role as anti-apoptotic protein, another hallmark of cancer, thus supporting the oncogenic potential of this gene. The phenotype resulting from the inhibition of its activity could

be the consequence of the circumvention of G2/M checkpoint activation, therefore allowing the cells to proliferate without control. Whereas from this process, cancer cells could acquire new oncogenic features, possibly evolving in more aggressive cells, thus more aggressive tumour (Greaves and Maley, 2012), we may also argue, considering the results of STARD3 overexpression, that the accumulation of a DNA aberration derived from a improper resolution of G2/M checkpoint may lead to unsustainable genomic instability, which led to cell death, especially if we also consider a non-perfectly responsive G1/S checkpoint (Yasutis and Kozminski, 2013)

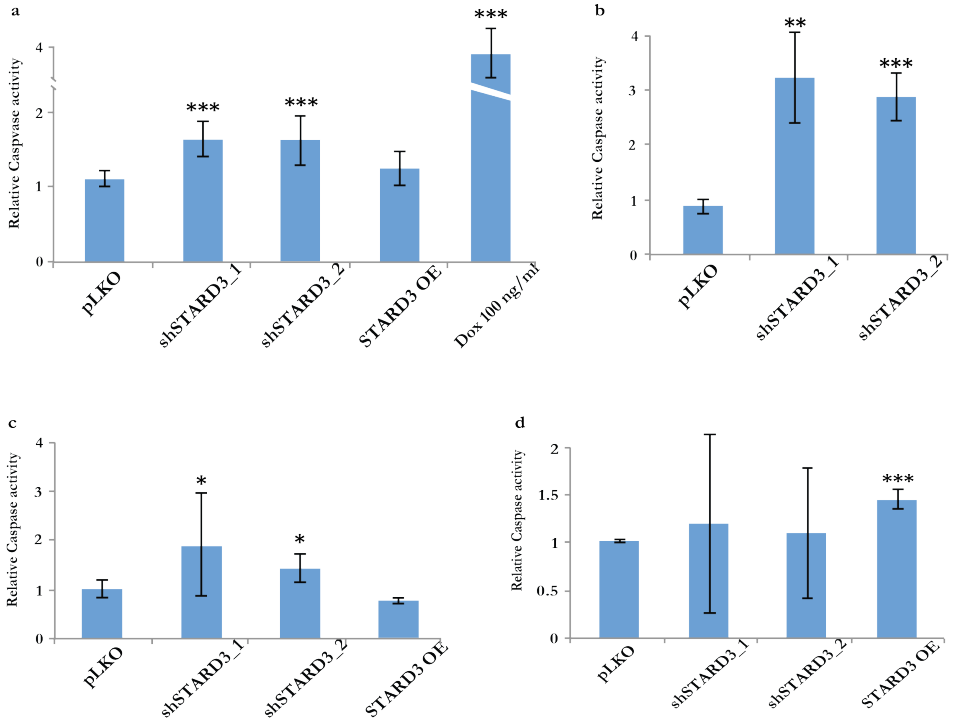

Figure 19. STARD3 prevents apoptosis by inhibiting Caspase-3 and -7 activity. Relative caspase activity of HCT116 (a), COLO201 (b), COLO205 (c), HT29 (d), silenced and control cells, analysed by luminescence assay. HCT116 and COLO201, COLO205 and HT29 were collected 3 days and 5 days after transduction, respectively. The proteic lysates were incubated with Caspase-Glo 3/7® kit reagents and luminescence measured by microplate reader. HCT116 were treated with doxorubicin for 5 hours. p values were calculated with two tailed t-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

2.6 STARD3 induces anchorage-independent growth

A hallmark of oncogenic behaviour is the ability of sustaining cell growth independently from external mechanical stimuli, such as interaction with a solid substrate, which otherwise would induce cell death, also called anoikis. In particular, anchorage-independent growth is a key feature of oncogenic transformation, and can be measured by soft agar assay (Guadamillas et al., 2011). For this purpose, shSTARD3-transduced HCT116 cell line, and relative control, were seeded into a soft

agar matrix and cultured for 20 days. Pictures were taken during the culture and afterwards cells were stained with crystal violet and counted in term of size of the cell clusters (Figure 20a). The analyses shown that STARD3 played a pivotal role in cell cluster growth when not anchored to a firm substrate, since where it is silenced, colonies were more rare. Furthermore, the assay was carried out on NIH3T3 cell line, immortalised but not transformed murine fibroblasts. Using the same experimental setup, we overexpressed STARD3 in the cells, using cells transduced with an empty vector as reference. We observed that the overexpression led to the formation of colonies (Figure 20b), conversely none were identified in control cells, that is in line with literature (Guadamillas et al., 2011).

The experiments demonstrated that STARD3 played a fundamental role in supporting cancer progression and in the acquisition of oncogenic features of non-cancer cells, hence we may infer that STARD3 has a more general effect toward carcinogenesis, promoting not only the deregulation of G2/M checkpoint, but also sustaining anchorage-independent growth of cells of the normal colonic mucosa.

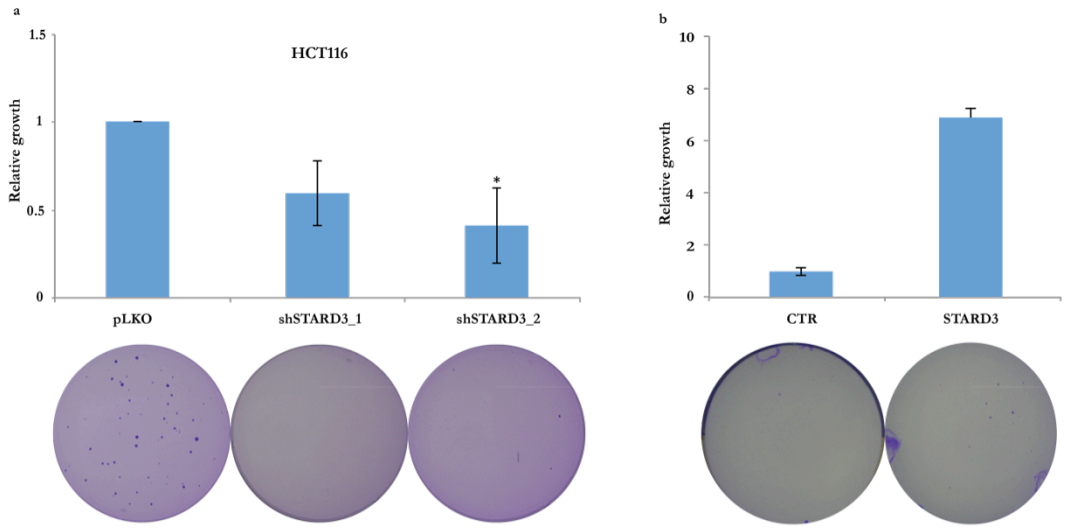

Figure 20. STARD3 expression prevents anoikis. (a) Upper Panel - Relative growth of HCT116 silenced and control cells. Lower panel – Colonies of HCT116 silenced and control cell on soft agar matrix stained with crystal violet (b) Upper Panel - Relative growth of NIH3T3 silenced and control cells. Lower panel – Colonies of NIH3T3 overexpressed and control cell on soft agar matrix stained with crystal violet. The cells were embedded in soft-agar matrix, seeded in a multiwell plate, and cultured adding the proper medium to prevent the soft agar to dry. After 20 days cells were washed, fixed with PFA, and stained. After destaining, the images were acquired and analysed with ImageJ. The number of colonies was normalised on the values of the controls. p values were calculated with two tailed t-test, *, $p < 0.05$.

2.7 STARD3 is involved in cell migration.

As previously stated, overcoming anoikis is an hallmark of cancer and this ability could lead to cell migration and invasion, that is in turn associated with metastasis formation (Guadamillas et al., 2011). Following the anchorage-independent assay, we investigated whether STARD3 could be involved in these cellular processes. To test that, STARD3 was knockdown in HCT116, COLO201 and DLD1 cell line and their migration potential was assessed by evaluating the cell capability to migrate through the pores of a semi-permeable membrane, a widely used migration assay. Consistently with the earlier results, the cells knockdown for STARD3 showed significantly less migration capability compared with the control cells (Figure 21a, b, c), although with noticeable variability amongst the cell line migration kinetic. Subsequently, we also tested STARD3 effect on cell invasion, by analysing the capability of the cells to migrate across the semi permeable membrane and a layer of Matrigel®, which mimics extracellular matrix. Despite preliminary results, cells with impaired STARD3 showed a trend toward fewer invasions although not significant (Figure 21d, e, f).

The data showed that STARD3 is involved in the regulation of cell migration, since the effect of the silencing on the migratory capability. The ability to promote migration, coupled with the results of anchorage-independent assays, reinforce the idea of STARD3 behaves as an oncogene, therefore suggesting its possible involvement in the regulation of the metastasis process. However, the mild effect on invasiveness detected upon STARD3 knockdown may mean that STARD3 requires additional supporting partners to pass through the extracellular matrix

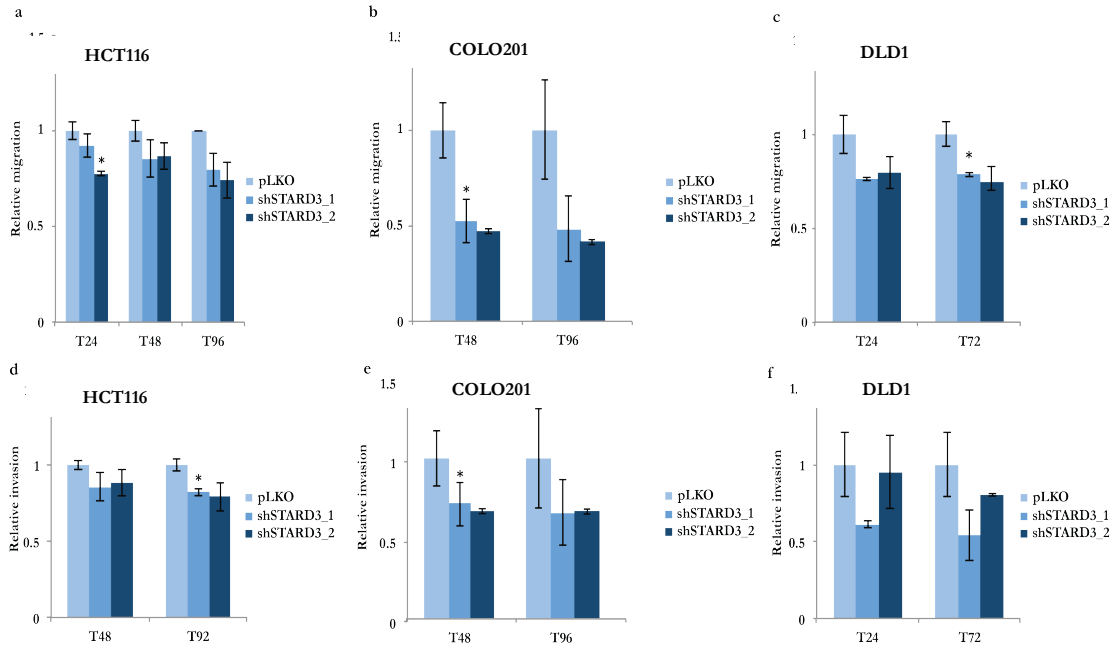

Figure 21. STARD3 promote migration, but not invasion. Relative migration of HCT116 (a), COLO201 (b), DLD1 (c) silenced and control cell lines, analysed by fluorescence assay. Relative migration of HCT116 (d), COLO201 (e), DLD1 (f) silenced and control cell lines, analysed by fluorescence assay. Cells stained with DiI were seeded in the transwell basket and placed in contact with complete medium in a multiwell plate. After 24, 48 and 96 hours, for HCT116 and COLO201, after 24 and 72 hours, for DLD1, the fluorescence was measured by microplate reader. The fluorescence intensity was normalised on the values of control cells. p values were calculated with two tailed t-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

2.8 STARD3 influences p53 expression

Thus far, although the mechanism by which STARD3 exerts its function has been partially elucidated (Alpy et al., 2013; van der Kant et al., 2013), it is not fully understood. Considering the involvement of the tumour suppressor gene *TP53* in many types of cancer, especially in colorectal, and the particular connection with cell cycle and cell death, we decided to analyse its expression. By western blot, we were able to identify an upregulation of p53 in STARD3-silenced HCT116 when compared with control cells (Figure 22a, b, left panels). This indicated that p53 may be involved in the promotion of G2/M checkpoint-derived apoptosis. Curiously, in COLO201 and COLO205 p53 was conversely downregulated following STARD3 silencing (Figure 22a, b, central and right panels). Although the apparently contradicting observations, the results may be explained by p53 status in these cells. In fact, in both COLO201 and COLO205 p53 is homozygously mutated in the DNA-binding domain (DBD), which usually results in the production of an oncogenic version of p53, whereas in HCT116 p53 gene is wild type (Mouradov et al., 2014). The mutations affecting the

DBD of p53 are usually pro-oncogenic (Brosh and Rotter, 2009), therefore from this perspective, the downregulation of p53 in COLO201 and COLO205 could be in line with HCT116 upregulation toward an antitumoural phenotype triggered upon STARD3 silencing.

Overall, the results suggested that STARD3 influence oncogenic p53 activity, whatever its mutational status, which might not only support its oncogenic feature, but also might explain the G2/M checkpoint arrest and consequent induction of apoptosis after STARD3 silencing, given the involvement of p53 in the regulation of this checkpoint (Stark and Taylor, 2006). In order to confirm the influence of STARD3 on p53 activity, the results should be further supported by the evidence of activation of p53 downstream effectors, such as p21, the decreasing levels and activity of cyclin B and cdc2, two proteins that control and promote G2/M transitions, and the status of other regulators of G2/M checkpoint, such as Chk1 and 2, to have a more comprehensive picture of the effect of STARD3 on this biological network.

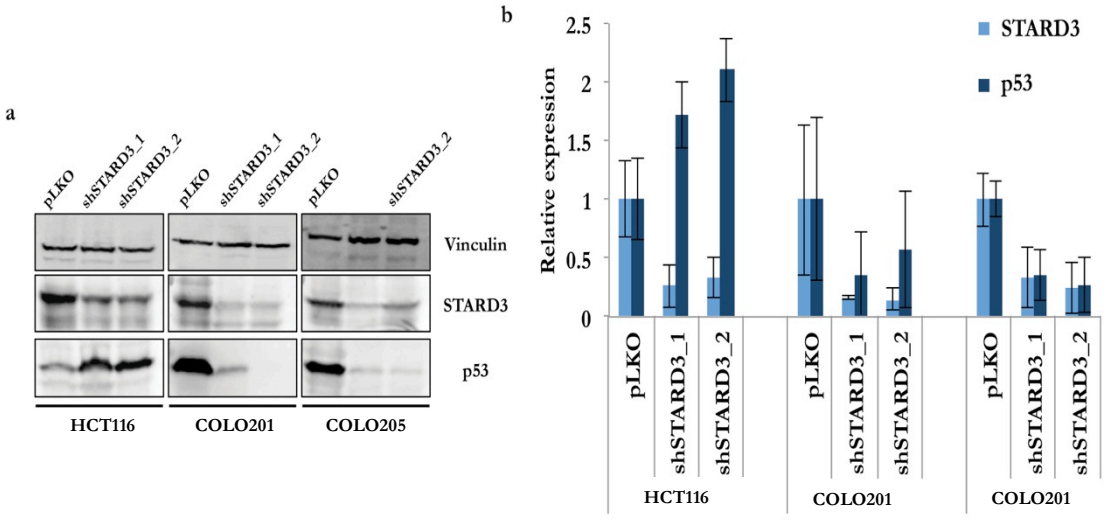

Figure 22. p53 oncogenic activity follows STARD3 expression. (a) Expression of p53 and STARD3 in HCT116, COLO201 and COLO206. Cells were transduced with STARD3 shRNAs or pLKO and the expression was detected by Western blot (b) Relative expression and quantification of p53 and STARD3 normalised over vinculin expression. The expression values of the cells were normalised on the control cells.

3 - CONCLUSIONS AND FUTURE PERSPECTIVES

The current therapeutic options regarding the treatment of colorectal cancer have demonstrated a certain grade of efficacy, however the benefit in terms of survival are still limited (Cancer, 2016). The necessity of better therapeutics has been addressed by multiple studies but remains one of the major challenges to deal with for the improvement of CRC therapy (Finnberg et al., 2015). On these bases, we wanted to investigate whether new therapeutic targets could be retrieved by carrying out a systematic RNAi screening. Starting from a set of amplified genes in CRC derived from literature, by loss-of-function assay we successfully identified a new potential oncogene: STARD3. This gene encodes a cholesterol binding protein, which thought to regulate cholesterol shuttling between various subcellular compartments. STARD3 has been frequently detected co-amplified with HER2 in HER2-positive breast cancer (Sahlberg et al., 2013; Tomasetto et al., 1995; Vassilev et al., 2015) thus its involvement in cancer is not new, however, to the best of our knowledge, we are the first to report its oncogenic potential in colorectal cancer. Indeed, in *in vitro* models, STARD3 was shown to possess the typical features of an oncogene (Hanahan and Weinberg, 2011), such as ability to migrate, to promote proliferation, to suppress pro-apoptotic signals and to evade cell cycle arrest. Interestingly, these cancer-promoting capabilities were detected in almost all the models used, even if some has been derived from CIN/MSS (HT29) and some from MSI (HCT116, COLO201, COLO205, DLD1 and Caco2) colorectal cancers. Since in MSI cancers both deletions and amplifications are extremely rare (Mouradov et al., 2014) , we can speculate that STARD3 may act regardless from its amplification and from the amplification of HER2 amplification as well, therefore having an effect much wider that previously thought. Perhaps, upstream factors could differently upregulate its expression or its activity, which could also explain the difference of the results between the cell lines and the apparent discrepancies between our data and literature. Nevertheless, both the relationship with HER2 and the putative alternative mechanism by which STARD3 exerts its function are yet to be found, and their fundamental analysis would be a major issue to be addressed in the future.

Whereas so far the mechanism of STARD3 is still elusive, our data suggest that it might promote the “loosening” of G2/M checkpoint. A defective checkpoint results in the progression of the cell into mitosis phase disregarding the resolution of any DNA

damages, which is generally associated to the promotion of the accumulation of random mutations, that eventually lead cancer cells to acquire a more aggressive phenotypes (Löbrich and Jeggo, 2007). The evidence that in our experimental setup the silencing of STARD3 led to an increase of cells arrested in phase G2/M represents an opportunity to exploit this apparent advantage of cancer cells in which STARD3 is overactive. In fact, on one hand we showed that the inhibition of STARD3 led to a persistence of G2/M arrest, thus increased rate of apoptotic cell death. On the other hand, we could infer that this could synergise with the administration of DNA damaging agents toward cell death. Several evidences have shown in fact that a massive accumulation of DNA damages could lead to a permanent arrest of the cell cycle by the activation of G2/M checkpoint or activation of mitotic catastrophe, therefore promoting cell death (Surova and Zhivotovsky, 2013). On these bases, the inhibition of STARD3 could represent another interesting aspect to be further elucidated, since it not only could increase cell death by itself, but also synergise with currently implemented CRC therapies. Moreover, the presence of a cholesterol-binding pocket, considering also the availability of the crystallographic structure (Tsujishita and Hurley, 2000), makes STARD3 an ideal targetable candidate for pharmacological inhibition on which design a drug, whereas the silencing mediated by antisense oligonucleotides could be an intriguing option to be explored as well.

This type of approach to identify previously unknown gene participating in carcinogenesis has been proven successfully useful, since the identification of STARD3. This gene is an example of a new promising oncogenic target whose inhibition may provide benefits to colorectal cancer patients. Despite promising, its role is far to be well understood, therefore further investigations need to be carried out to better comprehend how and where to apply these body of knowledge in colorectal cancer therapy.

4 - MATERIALS AND METHODS

4.1 Genes and shRNAs selection

Genes were selected amongst the significant ($p<0.05$) focal amplification identified by (Cancer Genome Atlas Network, 2012), in supplementary table 4. The miRNAs were excluded from the selection.

We interrogated the Mission® library of shRNA provided by Sigma-Aldrich, which use the lentiviral-based vector pLKO.1-puro. For each gene, up to 12 shRNA variants were available, but just 1 was selected at the beginning. The selection procedure preferentially included the shRNA with the following characteristics:

- sequence complementarity to all splice variants of a mRNA
- validated shRNA
- sequence complementarity at the 3' UTR region.

Consequently to the validation processes, we selected another shRNA against STARD3 with a different target sequence.

4.2 shRNA plasmid production

The shRNA clones were purchased in bacterial glycerol stock format. Each bacterial clone, correspondent to one shRNA, was streaked on LB agar plate, additioned with ampicillin (100 μg/ml, Sigma), and left to grow overnight at 37° C. The resulting colonies were picked, inoculated in LB medium (ThermoScientific), and cultured overnight at 37° C in an orbital incubator. The bacterial culture was centrifuged, in order to recover the pellet, at 4500 rpm for 10 min, then the supernatant was discarded. The plasmid DNA was purified from the bacterial pellet by Plasmid Midi Kit (Qiagen) following manufacturer's recommendation, and the quantity was assessed by nanodrop (Thermo Fisher Scientific) and quality with both nanodrop and on 1% agarose gel. The plasmids were stored at -20°.

4.3 Cell culture

HCT116, HT29, Colo201 and Colo205 cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC), DLD1, Lovo, Caco2, SW48, HEK293T from American Type Culture Collection (ATCC) and NIH3T3 were kindly donated by Gustavo Baldassarre. Unless otherwise stated, cells were maintained in

their appropriate growth medium and plastic support at 37°C and 5% CO2. NIH3T3 colon cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with 10% foetal bovine serum (FBS, Euroclone), Non Essential AminoAcids (Lonza) and antibiotics (penicillin and streptomycin, Lonza). HCT116 colon cancer cells were grown in McCoy's 5A medium supplemented with 10% FBS and antibiotics. HT29 colon cancer cells was grown in MEM medium supplemented with 10% FBS, Non Essential AminoAcids and antibiotics. Colo201 and Colo205 colon cancer cells were grown in RPMI medium supplemented with 10% FBS and antibiotics. Lovo colon cancer cells were grown in RPMI medium supplemented with 10% FBS and antibiotics. Upon reached the confluence of 85-90%, the cells were detached by trypsin (Lonza). After trypsin inactivation, cells were resuspended in the appropriate medium and centrifuged 5 minutes at 1000 rpm. The resulting cellular pellet was resuspended using fresh medium and the cells counted by Bürker haemocytometer. The cells were seeded accordingly.

4.4 Virus production, transduction and stable cell line production

To produce lentivirus, 7×10^5 HEK293T packaging cells per pool were seeded in 2 wells of 6 multiwell plate 1 day before transfection. For each well, we diluted 2 μg of Sigma-Aldrich custom library plasmid (i.e. shSTARD3), 0.5 μg of pMD2G, 1 μg of psPAX2 in 100 μl of plain DMEM incubated 20 min at RT, plus 16 μl of transfection agent (FuGENE® HD, Promega). The cells were incubated for 24 h at 37 °C, after which the medium was refreshed. Lentivirus-containing supernatants were collected at 48 and 72 hours post-transfection with 20% of FBS. The two collections of lentiviral particles were pooled, filtered through a 0.45 μm membrane (Sartorius Stedim/PVDF) and stored at −80 °C. Cell lines were transduced with lentivirus supernatants supplemented with 8 μg/ml hexabromide (Sigma). At 24 h post-infection, medium was replaced and cells were selected with 2 μg/ml of puromycin or 5 ug/ml blasticidin (Gibco). Antibiotic selection was stopped as soon as no surviving cells remained in the no-transduction control plate.

4.5 Primer Selection

Primers were selected using IDT technologies "Designer Tool" software. The selected primers were designed to anneal in exons separated by an intron. Primers were resuspended at 100μM in TE buffer 1X (10 mM Tris-HCl pH8 and 1mM EDTA in

ddH_2O), diluted at the working concentration of 10µM, and used at the final concentration of 0.5µM.

4.6 RNA isolation and real-time PCR (qPCR)

Cells were collected, washed with PBS and stored at -80°. Then, cellular pellet was thaw and total RNA was extracted using Quick-RNA miniprep (Zymo Research) and following the manufacturer's protocol. RNA quantity was assessed by nanodrop (Thermo Fisher Scientific) and quality with both nanodrop and on 2% agarose gel. cDNA was produced with GoScript™ Reverse Transcription (Promega) using 400ng of total RNA per reaction. qPCR reaction was performed with GoTaq® qPCR Mater Mix (Promega) in 7500 Real-Time PCR system (Applied Biosystem). Data analysis was performed by comparative ΔΔCt methodology, using ß-actin as normaliser. Each gene was analysed in duplicate as well as the normaliser, along with one negative control. The amplification products were subsequently analysed by agarose gel electrophoresis.

4.7 Western blot

At the right confluence, the cells were collected and washed in PBS, to remove the left traces of medium. Cells were then lysed in RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories), diluted 1:5 in H_2O, on which 2 µl of sample was diluted and placed in a plastic cuvette. The protein concentration was assessed by plotting the result of absorbance of the solution, read at 595 nm, on a titration curve made using BSA. Equal amounts of protein (20-30µg) were separated by 8, 10 or 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE, Euroclone) and transferred to nitrocellulose membrane (GE Healthcare) by miniprotean (Bio-Rad Laboratories) and detected with either LiteABlot ECL (Euroclone) or Amersham ECL by Chemidoc (Bio-Rad Laboratories), then the results analysed by ImageLab (Bio-Rad Laboratories) and ImageJ (v2.0.0, NIH).

The primary antibodies used were: STARD3 (1:500, SantaCruz), vinculin (1:1000, Sigma), Vinculin (1:1000, Santa Cruz), p53 (1:500, Santa Cruz). The secondary

antibodies used were: anti-mouse (1:5000, Thermo Fisher Scientific), anti-rabbit (1:5000, Thermo Fisher Scientific) and anti-goat (1:5000, Thermo Fisher Scientific).

4.8 Cell viability

Cells were seeded in 96 multiwell transparent microplate (Falcon) and, after experimental timing, cell viability was measured using the CellTiter-Glo® assay system (Promega) according to the manufacturer's instructions. Luminescence was assessed with microplate reader (Infinite F200 Pro, Tecan). The resulting values were normalised on the control, that is cells transduced with pLKO empty vector, in the experiments at 96h end point, whilst in time course experiments, each cell line was normalised on its T0.

4.9 Cell cycle analysis and propidium iodide

Adherent cells were detached by trypsin and collected along with floating cells and debries at the proper timing. 10^6 cells were counted, and fixed in ice-cold 70% ethanol for 2 hours at 4° degrees, followed by 1 hour of Rnase A (Roche) treatment and PI staining (BD). Cells were acquired by flow cytometer FACScan (BD). The recorded events were 20000 per sample, gated using Forward Scatter Channel (FSC) and Side Scatter Channel (SSC) to select cells with the most homogeneous features, and were analysed only the PI-positive single cells identified by plotting the events on a Area/Height chart (FL2-A/FL2-H) chart. These events were subsequentially plotted on a chart with FL2-A/events on which cell cycle and cell death were analysed by ModFit (Verity Software House).

4.10 Cell death analysis and Annexin V

Adherent cells were detached by trypsin and collected along with floating cells and derbies at the proper timing. 10^6 cells were counted, then incubated with 5µl of Annexin V-PE and 10µl of 7AAD reagents for 30 minutes at RT (PE Annexin V Apoptosis Detection Kit I - BD). Cells were acquired by flow cytometer FACS Canto (BD). The recorded events were 10000 per sample, gated using Forward Scatter Channel (FSC) and Side Scatter Channel (SSC) to select cells with the most homogeneous features, and were analysed in term of Annexin-V and 7AAD fluorescence intensity detected by flow cytometer using FACS Diva software (BD).

4.11 Caspase-3, -7 activity assay

Cells were collected, lysed in NP-40 buffer (Igepal 0.5%, Hepes pH7 50 mM, NaCl 250 mM, EDTA 5 mM) then the protein content was quantified by Bradford assay (reported above). Equal amount of protein (20µg) were added to a 96 multiwell transparent microplate. Each sample was incubated with 20 µl of Caspase-Glo 3/7® kit reagents (Promega) and after 30 minutes the luminescence was monitored by microplate reader. A higher level of luminescence was indicative of higher caspase activity.

4.12 Soft Agar

Cells were plated in 6 multiwell plate, in duplicate, at the concentration of 20000 and 50000 per well, in 1.5 ml of Soft Agar matrix, composed by DMEM 2X (Millipore), TBP buffer (Sigma), Noble Agar (BD) and FBS (Euroclone), 0.35% final, over a bottom layer of Soft Agar matrix, 0.75% final. Cells were kept in culture and medium was added weekly. Picture of the cells were taken weekly. After the proper time, cells were fixed with 4% PFA (Sigma) and stained with Crystal Violet 0.05% (Sigma). The images were acquired after extensive destaining with ddH_2O and analysed by ImageJ, using the "Analyse particle" tool.

4.13 Migration and invasion assay

For migration assay, 10^6 cells were collected and resuspended in 100 µl of PBS. The cells were incubated with 1 µl of Fast DiI oil (ThermoFisher) for 1 hour at 37° C, washed with PBS and resuspended in (150µl) DMEM without phenol red and FBS (Sigma) moved in a Fluoro Block transwell basket (Falcon). The basket was consequently inserted in a well of a 24 multiwell plate in which 700 µl of complete DMEM without phenol red were added. Invasion assay was carried out with the same setup, with the only addition of 50 µl of Matrigel® Growth Factor reduced (BD) coating of transwell basket. The flourescence of the cells was measured from the top and the bottom of the plate after cell seeding, by microplate reader, using an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

4.14 Statistical Analysis

All the results were the average of at least 3 experiments. The p-value was calculated using two-tailed Student's T-test. The software adopted for the statistical analysis was Microsoft Excel.

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