UNIVERSITY OF TURIN

Phd School in life and Health Sciences
Molecular Medicine

PDK1 targeting in mutated KRAS metastatic colorectal cancer

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XXIX Cycle
Academic Years: 2014-2017

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ABSTRACT

Colorectal cancer (CRC) is one of the most frequent neoplastic disease diagnosed in the world. The progressive accumulation of genetic and epigenetic alterations drives the transformation and progression of normal colon epithelial cells into aberrantly growing cells. RAS/MAPK and PI3K/AKT pathways are frequently altered in colorectal cancer and such alterations constitute one of the leading causes of tumor progression toward an aggressive and metastatic phenotype. Despite several efforts, no definitive therapies targeting these pathways exist to treat such type of cancer. Since developing drugs against KRAS is a daunting task, one promising strategy is represented by targeting its downstream effectors. Among those, 3-phosphoinositide-dependent kinase 1 (PDK1) is a serine/threonine kinase, belonging to the AGC kinase family and able to bind PIP3 produced by PI3K. Upon binding to PIP3, PDK1 phosphorylates and activates Akt. Furthermore, PDK1 phosphorylates and activates RSK, which is a RAS/MAPK downstream effector. For these reasons, PDK1 is a particularly interesting target as it is a common effector of RAS/MAPK and PI3K/AKT pathway. Our work is aimed at understanding whether PDK1 targeting could be suited to treat colorectal cancer.

As a first experimental model, we used established cell lines of colorectal cancer (CRC) harboring different genetic lesions, including mutations in RAS/MAPK and PI3K/Akt pathways. In these cell lines we inhibited PDK1 kinase activity and we tested cell survival both in adhesion and in anchorage-independent conditions. PDK1 inhibition poorly affected cell survival in adhesion while efficiently abrogated anchorage-independent growth, with different sensitivity among the cell lines. Then, we evaluated the effects of PDK1 inhibition on two of its downstream effectors. In particular, we focused on Akt and RSK, both belonging to the AGC kinase family, and we tested the effect of their inhibition on the anchorage-independent growth ability of CRC cell lines. We observed that only RSK inhibition by BI-DI1870, but not AKT inhibition with MK2206, correlates with PDK1 inhibition.

In order to demonstrate that the role of PDK1 in oncogenic *KRAS*-driven tumors is mediated by RSK, we cloned a RSK phosphomimetic mutant, designed to be constitutively active, independently of PDK1. Unexpectedly, instead of an active kinase we obtained an inactive mutant, which was not able to phosphorylate its substrate, YB1. Other similar cases have been indeed reported in the literature, where phosphomimetic mutants of some other proteins belonging to the AGC kinase family turned out to be inactive. However, this phenomenon has never been extensively investigated. We have therefore conducted a biochemical and structural analysis to elucidate the reasons of the impossibility of phosphomimetic substitution in the activation loop in AGC kinases. We demonstrated by means of molecular dynamics simulations that the phosphorylated serine in the wild type protein is in a fixed and stable conformation, while the aspartate in the phosphomimetic mutant destabilize the structure of the kinase domain making it completely inactive. It is therefore not possible to decouple RSK activation from phosphorylation mediated by PDK1.

To get a more realistic model of metastatic colorectal cancer (mCRC) we exploited the model of patient-derived organoids (PDOs). PDOs were cultured *in vitro* embedded in a

3D matrix, which mimics the *in vivo* microenvironment, and grow recreating similar features of the *in vivo* tumor. We verified PDK1 role in a collection of mutated KRAS mCRC PDOs obtained from mCRC patient-derived xenografts (PDXs). We found that PDK1 inhibition with both BX795 and BX912 profoundly alters the viability and 3D morphology of mutated KRAS mCRC PDOs.

In summary, PDK1 is able to impair anchorage-independent growth of colorectal cancer cell lines and to alter viability of mCRC patient-derived organoids in the presence of mutations in RAS/MAPK pathway and, in some cases, also in PI3K/AKT pathway. Thus, our data suggest that PDK1 could be a possible therapeutic target in colorectal cancer. Indeed, by impairing both RAS/MAPK and PI3K/AKT pathways, the inhibition of PDK1 could potentially be a more effective solution compared to therapies singularly targeting one of these two pathways.

Colorectal Cancer (CRC)

Colorectal cancer (CRC) is a common health problem, representing the third most commonly diagnosed cancer worldwide and causing a significant burden in terms of morbidity and mortality, with annual deaths estimated at 700,000 (Hadjipetrou et al., 2017). CRC global incidence and mortality rates appear to be substantially higher for males than for females, with 21 new cases and 10.5 deaths for 100,000 males and 17.6 new cases and 9.2 deaths for 100,000 females. In males, CRC ranks third in incidence, following lung and prostate cancers, and in females it ranks second, following breast cancer (Torre et al., 2015). CRC is a multifactorial disease (Tiwari et al., 2016) as both genetic and environmental factors play an important part in the aetiology (Kuipers et al., 2015). It is estimated that about 70–80% cases of CRC are sporadic whereas the remaining 20–30% of affected individuals carry inherited mutations (DA Silva et al., 2016; Hahn et al., 2016; Tiwari et al., 2016). The 'rise' of CRC in developed countries can be attributed to the increasing ageing population, the increasing worldwide adoption of the Western diet, accompanied to rise in obesity rates, and an increase in risk factors, such as smoking and low physical exercise. However, a specific subgroup of the patient population is formed by those affected by a hereditary colorectal cancer syndrome (Kuipers et al., 2015). Inherited forms of CRC are usually subdivided into nonpolyposis syndromes characterized by the development of few or no polyps and polyposis syndromes characterized by the development of multiple (usually tens, hundreds or more) colorectal polyp. Identification of the genetic defects underlying most hereditary CRC syndromes has been a major step forward in cancer research and has had very important consequences for clinical practice (Vasen et al., 2015). The most common syndrome in this category is Lynch syndrome (a hereditary non-polyposis type of CRC). This syndrome is caused by a mutation in one of the DNA mismatch-repair (MMR) genes: *MLH1*, *MSH2*, *MSH6*, *PMS2* or *EPCAM*. Impaired mismatch repair during replication gives rise to the accumulation of DNA mutations, which occur, in particular, in microsatellite DNA fragments with repetitive nucleotide sequences. The second most common hereditary colorectal cancer syndrome is familial adenomatous polyposis (FAP). This syndrome is caused by mutations in the adenomatous polyposis coli (*APC*) gene, which controls the activity of the Wnt signalling pathway. Most patients with familial adenomatous polyposis develop very large numbers

of colorectal adenomas and subsequent colorectal cancer at a young age (Kuipers et al., 2015; Vasen et al., 2015) (Fig. 1).

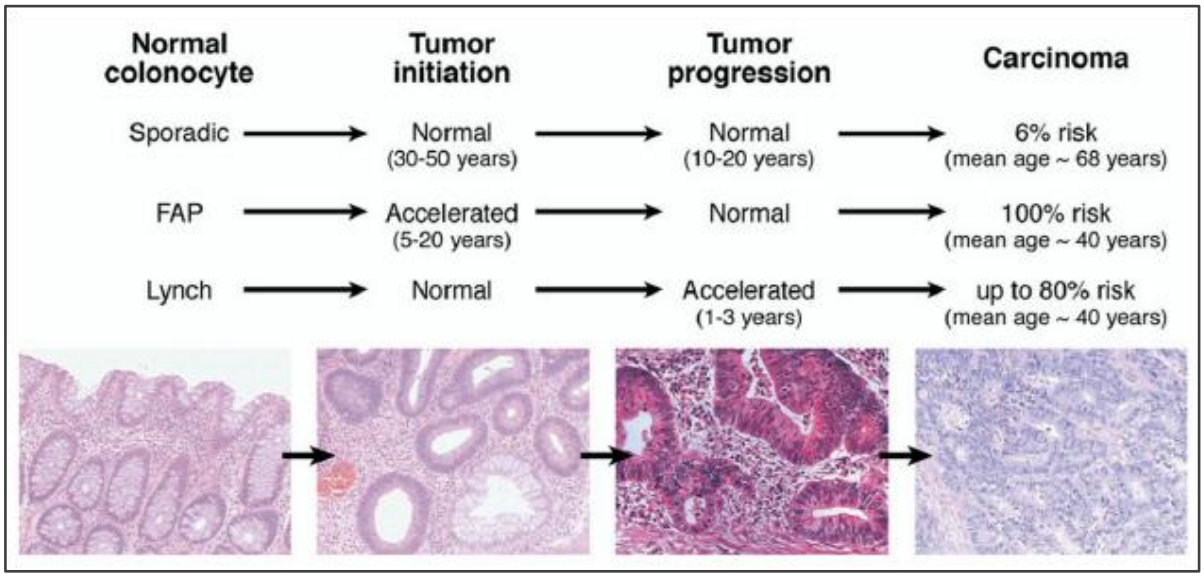

Fig. 1 | Colorectal tumor progression in sporadic and hereditary syndromes. The general paradigm is that a tumor is initiated from a normal colonocyte stem cell that has sustained genetic damage over time due to the local environment and any germline genetic mutation that has been inherited. The damaged DNA provides a growth advantage that drives tumor progression as successive clonal outgrowths are generated, ultimately forming carcinoma. In FAP, tumor initiation is accelerated with the inheritance of a germline *APC* mutation; in Lynch syndrome, tumor initiation might be normal to slightly accelerated, but tumor progression is greatly accelerated due to the hypermutable phenotype that occurs with loss of DNA MMR. Photomicrographs depict, in order, normal colon, tubular adenoma, high grade dysplasia, and cancer (Grady and Carethers, 2008).

- *Intestinal Morphology*

The cecum, colon and rectum constitute the large intestine. They are responsible for digestion and the uptake of food (Vries et al., 2010). The colon is a muscular tube about 1.5 meters long, which is anatomically subdivided into 4 sections: ascending colon, transverse colon, descending colon, and sigmoid colon. The final sections of the large intestine are the rectum and the anal canal, where the waste matter, called feces or stool, is stored until it passes out of the body[1].The wall of the colon is organized into four histologically distinct layers: *serosa*, *muscularis mucosae* (MM), *submucosal* and *mucosa* (Ricci-Vitiani et al., 2009). The inner luminal lining consists of a single layer of columnar epithelial cells, which form finger-like invaginations into the underlying connective tissue of the lamina propria (LP) to form the basic functional unit of the intestine (Humphries and Wright, 2008), the crypt of Lieberkűhn (Fig. 2). Active proliferation takes place in these crypts because of the presence of multipotent stem cells (SCs) at crypt bottoms. The

remainder of the crypts is made up of transit-amplifying (TA) cells, which rapidly divide 4 to 5 times before differentiation (Schuijers and Clevers, 2012) (Fig. 3A).

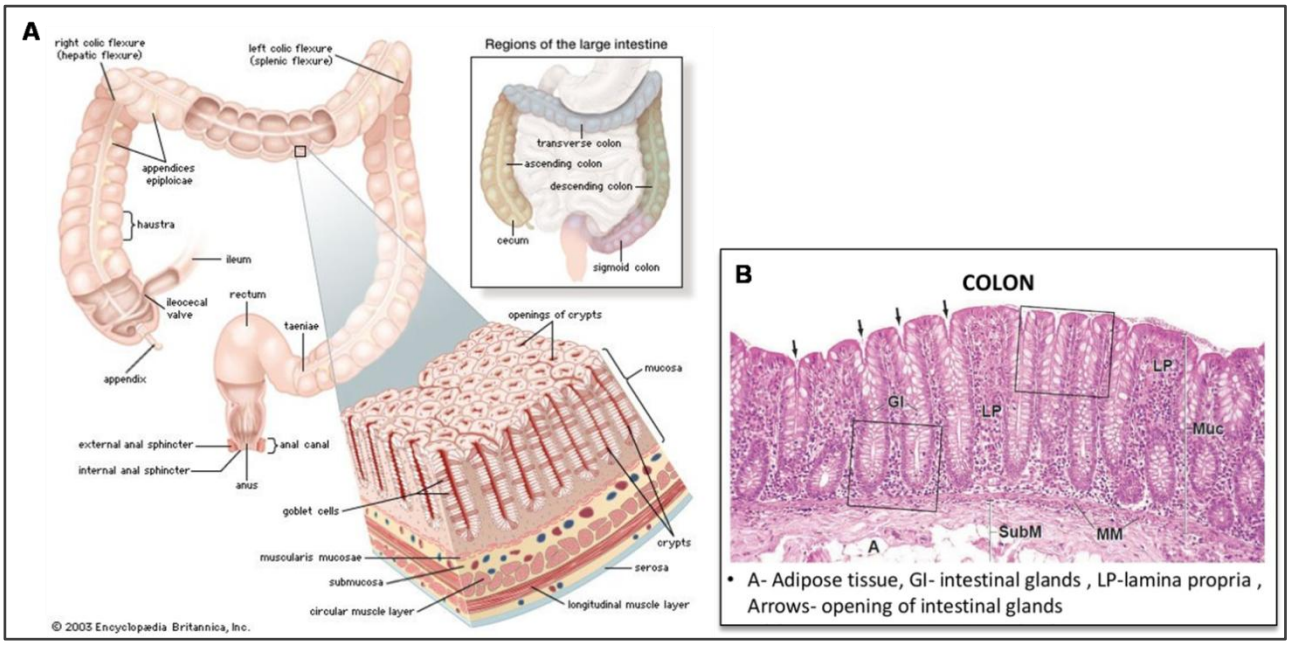

Fig. 2 | Structures of the human large intestine, rectum, and anus. A. The mucosa of the large intestine (or colon) is punctuated with numerous crypts, named crypts of Lieberkűhn, that absorb water and are lined with mucus-secreting goblet cells. At the lower end of the rectum, the circular and longitudinal muscle layers terminate in the internal and external anal sphincters (*Encyclopædia Britannica, Inc.*[2]*);* **B.** Simple columnar epithelium intestinal glands (GI) are simple tubular structures (crypts of Lieberkűhn), which extend from the muscularis mucosae (MM) through the thickness of the lamina propria (LP) and open onto the luminal surface of the intestine (arrows). The mucosal epithelium of the large intestine, or colon, contains columnar absorptive cells, that predominate (4:1) over goblet cells (DR.DEEPAK N.KHEDEKAR/LTMMC/2016 [3]).

There are three major terminally differentiated epithelial lineages in the colon: the colonocytes, also termed absorptive enterocytes (Ricci-Vitiani et al., 2009), which express a layer of microvilli with their apical membranes (Papailiou et al., 2011; Vries et al., 2010); the mucus-secreting goblet cells; and the less abundant enteroendocrine cells, also called peptide hormone-secreting endocrine cells (Humphries and Wright, 2008). The terminally differentiated cells are literally pushed up along the crypt axis from proliferating stem cells. When they reach the tip of the villus they undergo apoptosis and are extruded into the lumen (Ricci-Vitiani et al., 2009; Schuijers and Clevers, 2012). Therefore, tissue homeostasis and the replacement rate of intestinal epithelium (5 days) is regulated by SCs and is under microenvironmental influence (Medema and Vermeulen, 2011; Ricci-Vitiani et al., 2009). Adult stem cells use three different types of cell division: 1) self-renewing symmetric division, generating two daughter stem cells to expand the stem cell population; 2) asymmetric divisions, generating one stem cell and one progenitor cell (Vries et al., 2010), called transit-amplifying cell, that, upon migration upward the crypt, proliferate and

differentiate into one of the epithelial cell types of the intestinal wall (Ricci-Vitiani et al., 2009); or 3) non self-renewing symmetric division, resulting in the generation of two progenitors (Vries et al., 2010) (Fig. 3B). Stem cells have the ability to switch between these options in response to their microenvironment (Papailiou et al., 2011), which consists of cellular and extracellular components that ensure the optimal conditions for stem cells maintenance through the secretion of various cytokines, growth factors, and direct interactions (Potten et al., 2009; Todaro et al., 2010).

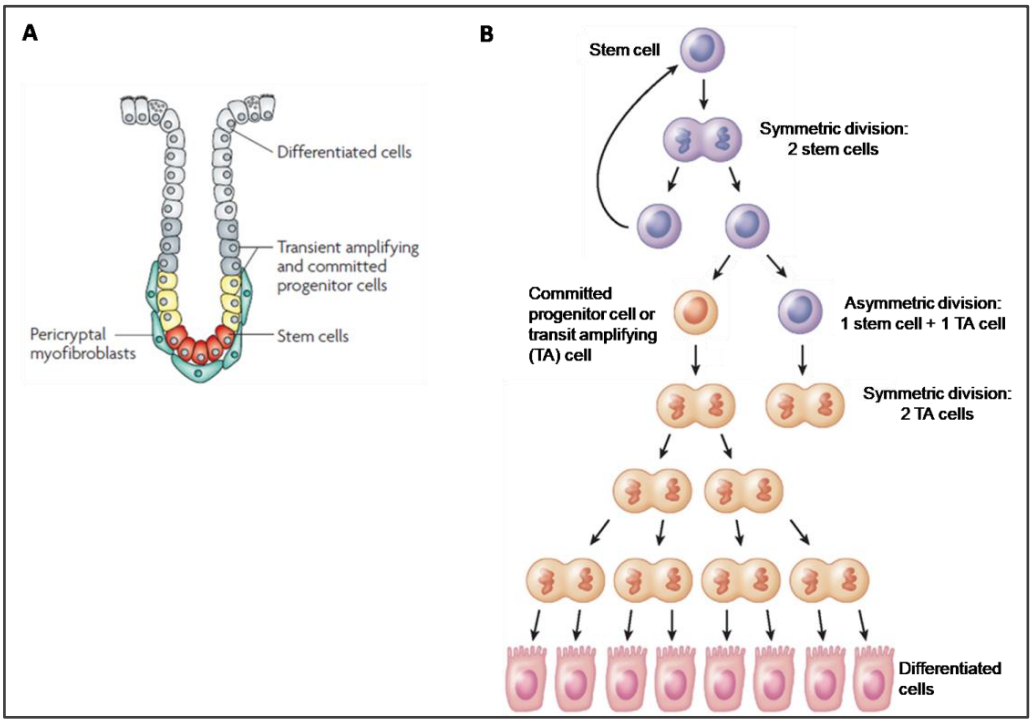

Fig. 3 | Colonic crypt organization and patterns of stem cell divisions. A. A diagrammatic representation of the colonic crypt of Lieberkűhn. Stem cells are housed in the base of the crypts where they communicate with the niche cells — the pericryptal myofibroblasts, which are outside the crypt but communicate by cell signaling. Stem cells feed the transit amplifying compartment, where most cell production occurs. This portion of the crypt is thought to also house the committed progenitor cells: cells committed to one or more cell lineages (Humphries and Wright, 2008); **B.** In rapidly growing adult tissues and perhaps in other tissues there are slowly dividing populations of stem cells. Stem cells could divide symmetrically, producing two stem cells, or asymmetrically, producing one cell that remains as a stem cell and another that becomes committed to a differentiative pathway but divides a few more times at a more rapid rate. Such cells have been termed progenitor cells or "transit amplifying cells", each of which eventually stops dividing and becomes fully differentiated. Stem cells could also divide symmetrically, giving rise to two transit amplifying cells (adapted from Mesher AL: Junqueira's Basic Histology, 13th Edition [4]).

This hierarchical organization of adult intestinal tissue is suggested to help the organism recover from injury and to slow aging, and to protect the cells from accumulating damage that would ultimately lead to cancer (Vries et al., 2010). The maintenance of the stem cell compartment and the transition from proliferation to differentiation are finely coordinated by the Wnt signaling (Fevr et al., 2007) in conjunction with other pathway such as Notch and

BMP (Vries et al., 2010). The Wnt pathway direct a specific set of genes that strictly control temporal and spatial regulation of cell growth, movement and cell survival. Signaling proteins of the Wnt family, that are secreted by mesenchymal cells, bind to specific Frizzled (Fzd) receptor complexes on the surface of target cells, activating distinct intracellular pathways. These pathway are classified in canonical or non-canonical Wnt signaling pathway, depending on the composition of the Wnt/Fzd complex formed (Barker and Clevers, 2006; Logan and Nusse, 2004). The canonical pathway is the better characterized and the more relevant for cancer development. In the absence of a Wnt signal, a protein complex comprising Axin, Adenomatous Polyposis Coli (APC), casein-kinase 1α (CK1α) and Glycogen Synthase Kinase 3B (GSK3B) is activated and form a scaffold that facilitates β-catenin phosphorylation at several N-terminal serine/threonine residues. Once phosphorylated, β-catenin is recognized and ubiquitinylated, resulting in its proteasomal degradation. Subsequently, because of the low levels of β-catenin, the DNA-binding T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors bound to target gene promoters and/or enhancers in the nucleus form a transcriptional repressor complex with Groucho proteins and silence the Wnt target genes (Fig. 4A). In the presence of the Wnt signal, the β-catenin destruction complex is deactivated. Consequently, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it forms an active transcription complex with the TCF/LEF transcription factor family (Barker and Clevers, 2006; Vries et al., 2010) (Fig. 4B).

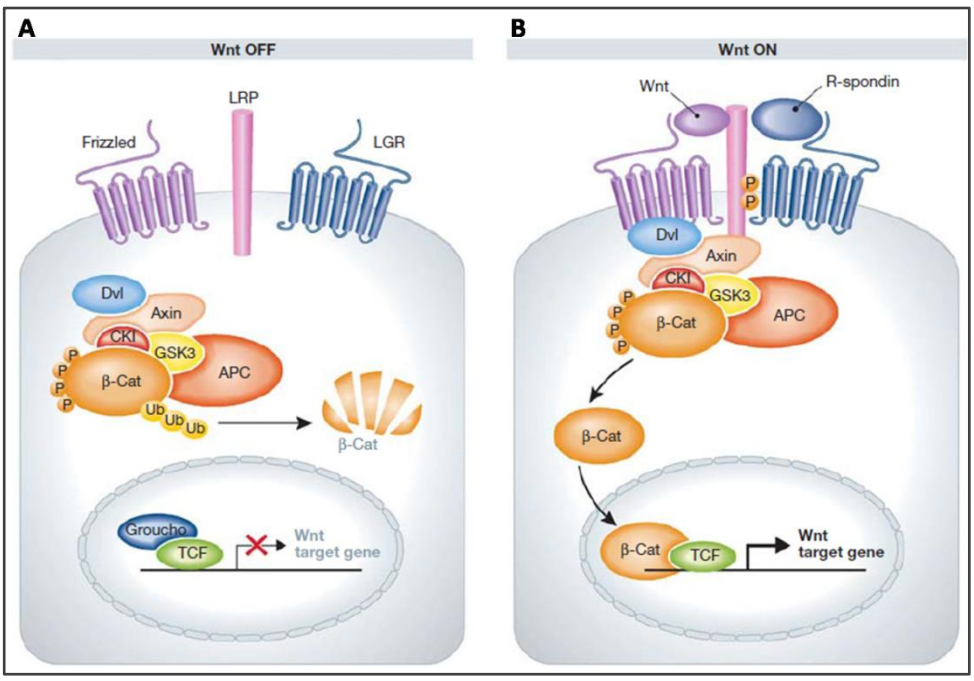

Fig. 4 | An overview of the Wnt signalling pathway. A. In the absence of a Wnt signal, β-catenin is captured by APC and axin within the destruction complex, facilitating its phosphorylation by the kinases

CK1α and GSK3β. CK1α and GSK3β then sequentially phosphorylate a conserved set of serine and threonine residues at the amino terminus of β-catenin. This subsequently mediates the ubiquitinylation and efficient proteasomal degradation of β-catenin. The resulting β-catenin 'drought' ensures that nuclear DNA-binding proteins of the TCF/LEF transcription factor family (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoters and/or enhancers. **B.** Interaction of a Wnt ligand with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dvl–Fzd complexes and the phosphorylation of LRP, facilitating relocation of axin to the membrane and inactivation of the destruction box. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family. In the nucleus, β-catenin converts the TCF proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of coactivator proteins. This ensures efficient activation of TCF target genes such as c-MYC, which instruct the cell to actively proliferate and remain in an undifferentiated state. Following dissipation of the Wnt signal, β-catenin is evicted from the nucleus by the APC protein and TCF proteins revert to actively repressing the target gene program. APC, adenomatous polyposis coli; CK1α, casein kinase 1α; Fzd, Frizzled; LEF, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related protein; TCF, T-Cell Factor (Barker and Clevers, 2006; Schuijers and Clevers, 2012).

This canonical pathway is permanently activated in both inherited FAP and spontaneous forms of colon cancer (Korinek et al., 1997). FAP is a syndrome characterized by one defective APC allele and the spontaneous inactivation of the second APC allele at low frequency in the intestinal epithelial cells (Kinzler et al., 1991; Nishisho et al., 1991). The resulting chronic activation of the Wnt pathway in these cells drives their expansion into benign adenomas (also termed polyps), which frequently progress to invasive colon carcinoma following additional genetic mutations later in life (Barker and Clevers, 2006). Indeed, loss of APC in a crypt cell induces maintenance of EphB expression in the resulting transformed clone (Batlle et al., 2002; Cortina et al., 2007). As a consequence, the clone fails to migrate out of its crypt and eventually invades the subepithelium (Barker et al., 2009; Cortina et al., 2007). Less frequently, sporadic colon cancers show aberrant Wnt signaling activity because of mutations in β-catenin or Axin2, which compromise their function within the β-catenin destruction complex (Barker and Clevers, 2006).

In the later years, a new theory about colon cancer was developed: the "cancer stem cell theory" (Papailiou et al., 2011). This theory states that tumor growth is fueled by small numbers of tumor stem cells hidden in cancers. It explains clinical observations, such as the almost inevitable recurrence of tumors after initially successful chemotherapy and/or radiation therapy, the phenomenon of tumor dormancy, and metastasis (Batlle and Clevers, 2017) (Fig. 5).

Up to now, identification, isolation and characterization of stem cells of the colonic crypt remain elusive due to the fact that cancer stem cells (CSCs) do not exhibit any morphological criteria for identification (Papailiou et al., 2011).

In the first decade of this century, CSCs were identified in many common cancer types, including leukemia (Bonnet and Dick, 1997; Lapidot et al., 1994; Uckun et al., 1995),

breast cancer (Al-Hajj et al., 2003), colorectal cancer (Dalerba et al., 2007b; O'Brien et al., 2007; Ricci-Vitiani et al., 2007), and brain cancer (Singh et al., 2004), inspiring innovative treatment strategies for these cancers with the aim to exterminate CSCs, the cell population that sustains long-term growth (Batlle and Clevers, 2017).

Several studies have attempted to identify intestinal SCs within colonic crypts by using indirect techniques based on biological features restricted to the stem cell compartment. Long-term retention of label DNA has been exploited as surrogate marker of stemness based on the observation that SCs in adult tissues usually divide at a slow rate when compared to the progenitor population (Cotsarelis et al., 1990; Ricci-Vitiani et al., 2009). However, it should be noted that terminally differentiating cells will also retain DNA labels (Barker et al., 2007). Furthermore, over the past few years, it has gradually become clear that CSCs (similarly to normal stem cells) can be abundant and can proliferate vigorously (Batlle and Clevers, 2017).

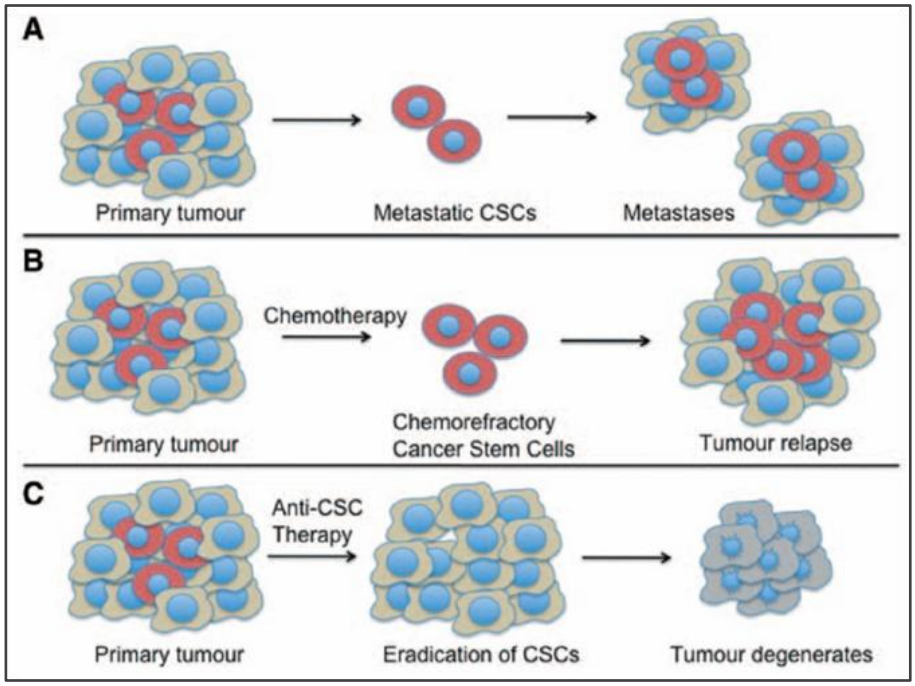

Fig. 5 | CSC hypothesis—potential implications. A. CSCs are thought to be the subset of cancer cells that are capable of forming new metastases and are capable of forming the full range of differentiated cells that comprise the tumor; **B.** CSCs are often resistant to standard chemotherapy and radiotherapy. Treatments that fail to eradicate the CSC subpopulation are likely to lead to relapse of disease; **C.** Successful CSC-directed therapies may improve clinical outcomes by reducing the portion of tumor cells most likely to persist through standard therapies and most likely to cause relapse or metastasis. CSC, cancer stem cell (O'Flaherty et al., 2012).

The recognition of specific cell surface markers has allowed the identification of SCs in many tissues including the hematopoietic system (Kiel et al., 2005) and mammary gland (Ricci-Vitiani et al., 2009; Shackleton et al., 2006). The choice of testable markers for

CSCs in solid cancers is often based on differential expression between different tumor-cell subpopulations and/or on knowledge of stem cell–specific expression of the marker in an unrelated tissue (Batlle and Clevers, 2017). Several molecules have been proposed as markers of SCs in the intestine including CD133, CD44, and the RNA-binding protein musashi-1 (Msi-1) (Nakamura et al., 1994). Other putative biomarkers have been evaluated to distinguish the SC population within the colon, such as members of the integrin superfamily of transmembrane glycoproteins including α2 and β1 subunits and aldehyde dehydrogenase-1 (ALDH-1) (Fujimoto et al., 2002; Ricci-Vitiani et al., 2009).

The initial approach to study the capacity of a given cell to reconstitute its specific lineage, so to evaluate its stem cell potential, was based on tumor-cell transplantation assay, which consisted in the inoculation of an isolated cell populations into immune-deficient mice (Doulatov et al., 2012). However, such xenotransplantation approach used to investigate the properties of CSCs carries inherent technical and conceptual limitations (Batlle and Clevers, 2017; Clevers, 2011; Meacham and Morrison, 2013), especially regarding epithelial cancers, such as colorectal cancers (CRCs) (Nguyen et al., 2012; Plaks et al., 2015; Zeuner et al., 2014), which show poor engraftment. Sorted epithelial cells undergo anoikis during detachment from the tissues and their reallocation into unrelated recipient environments could perturb the original stem cell hierarchy (Plaks et al., 2015; Sato et al., 2011b).

As mentioned above, Wnt signals constitute the principal driving force behind the biology of the crypt (Korinek et al., 1998). Therefore, some Wnt target genes may be specifically expressed in the stem cells. Of approximately 80 selected intestinal Wnt target genes (Van der Flier et al., 2007), it was demonstrated that Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as *Gpr49*) showed exclusive expression in cycling columnar (CBC) cells at the very base of the crypt (Barker et al., 2007). Lgr5 is a seven-transmembrane receptor protein which serves as a co-receptor for canonical Wnt/β-catenin signaling, potentiating it (Barker et al., 2007; Morgan et al., 2017). The $Lgr5^+$ CBC cells represent the genuine stem cells of the small intestine, and also $Lgr5^+$ colon cells fulfilled the stem-cell requirements in being pluripotent and capable of maintaining epithelial self-renewal over long periods of time.

The small intestinal $Lgr5^+$ cells are generally not quiescent, but are rapidly cycling, and seem to divide more actively than their colonic counterparts, probably reflecting differences in the rate of epithelial turnover between the two organs. Lgr5 expression is also highly restricted in a variety of other tissues. Thus, it represents a more general

marker of adult stem cells (Barker et al., 2007). Moreover, Lgr5 was identified as a gene expressed in colon cancer cells (van de Wetering et al., 2002) and in other cancers (McClanahan et al., 2006; Yamamoto et al., 2003) and it is also expressed in scattered cells in pre-malignant mouse adenomas. These $Lgr5^+$ malignant cells may represent cancer stem cells (Barker et al., 2007). These cancer stem cells may be refractory to current therapies and may also be the cells that most likely metastasize (Barker et al., 2009). Selective ablation of $Lgr5^+$ CSCs, obtained with a CRISPR-Cas9 approach used to insert an inducible version of the suicide-gene caspase 9 (iCasp9) into the *Lgr5* locus in human CRC organoids (Batlle and Clevers, 2017), leads to tumour regression. This regression is followed by tumour regrowth driven by re-emerging $Lgr5^+$ CSCs (Shimokawa et al., 2017). Lineage-tracing experiments from differentiated tumor cells that express Keratin 20 ($KRT20^+$), demonstrated that these cells regained proliferative potential (Batlle and Clevers, 2017), constantly diminishing in tumor tissues, while reverting to $Lgr5^+$ CSCs and thus contributing to tumor regrowth after $Lgr5^+$ CSC ablation. Therefore, despite several potential CSC-targeted agents for CRC (Shimokawa et al., 2017; Todaro et al., 2008; Zeuner et al., 2014) a single CSC-targeting therapy could not be sufficient to eradicate cancers. Lgr5 targeting is effective temporarily, since it is probably overcome by robust plasticity of non-targeted cancer cells (Shimokawa et al., 2017).

- Histopathogenesis and neoplastic conditions

CRC survival is highly dependent on the stage of disease at diagnosis (Haggar and Boushey, 2009). The pathogenesis of CRC varies according to genetic or epigenetic changes, which contribute to the initiation of neoplastic transformation of healthy epithelium and/or determine the development and the progression towards more malignant stages of the illness (Colussi et al., 2013). CRC is generally classified into four stages, I to IV, characterized by submucosal invasion (stage I), penetration of the outer colonic wall (II), lymph node invasion (III) and metastasis (IV) (Strubberg and Madison, 2017). The tumors arising in the epithelial tissue, called carcinoma, constitute about 80% of life-threatening cancer (Valastyan and Weinberg, 2011), and 30% of patients with CRC present with a metastatic disease (Martini et al., 2017; Torre et al., 2015).

CRC usually begin as benign polyps that grow from the mucosa. Most polyps remain benign and are often termed hyperplastic polyps. The likelihood that hyperplastic polyps will become cancer is very low. Other benign polyps have a chance of becoming cancerous if not removed. Examples include adenomatous and hamartomatous polyps,

which are usually associated with hereditary CRC syndromes. Adenomas that are malignant are called adenocarcinomas[1]. The metastases spawned by carcinomas are formed following a complex succession of cell-biological events, termed the invasion-metastasis cascade (Valastyan and Weinberg, 2011). Epithelial cells in primary tumors grow and divide within the polyp. Then, they can eventually invade nearby colon tissue, through surrounding extracellular matrix (ECM) and stromal cell layers, and grow into and beyond the wall of the colon or rectum. If the cancer becomes advanced, the tumors will grow though all of the tissue layers of the colon rectum, and may metastasize, shedding cells into the lumina of blood vessels and spreading the cancer to other organs such as the liver and lungs[1]. Therefore, cancer cells are able to extravasate into the parenchyma of distant tissues, survive in these foreign microenvironments and reinitiate their proliferative programs at metastatic sites, thereby generating macroscopic, clinically detectable neoplastic growths (Friedl and Wolf, 2003) (Fig. 6).

Histologically, hyperplastic polyps contain an increased number of glandular cells with decreased cytoplasmic mucus, but lack nuclear hyperchromatism, stratification, or atypia (Tsai and Lu, 1995). On the contrary, adenomatous nuclei are usually hyperchromatic, enlarge, cigar-shaped, and crowed together in a palisade pattern. Adenomas are classified as tubular or villous. Histologically, tubular adenomas are composed of branched tubules, whereas villous adenomas contain digitiform villi arranged in a frond (Cappell, 2005).

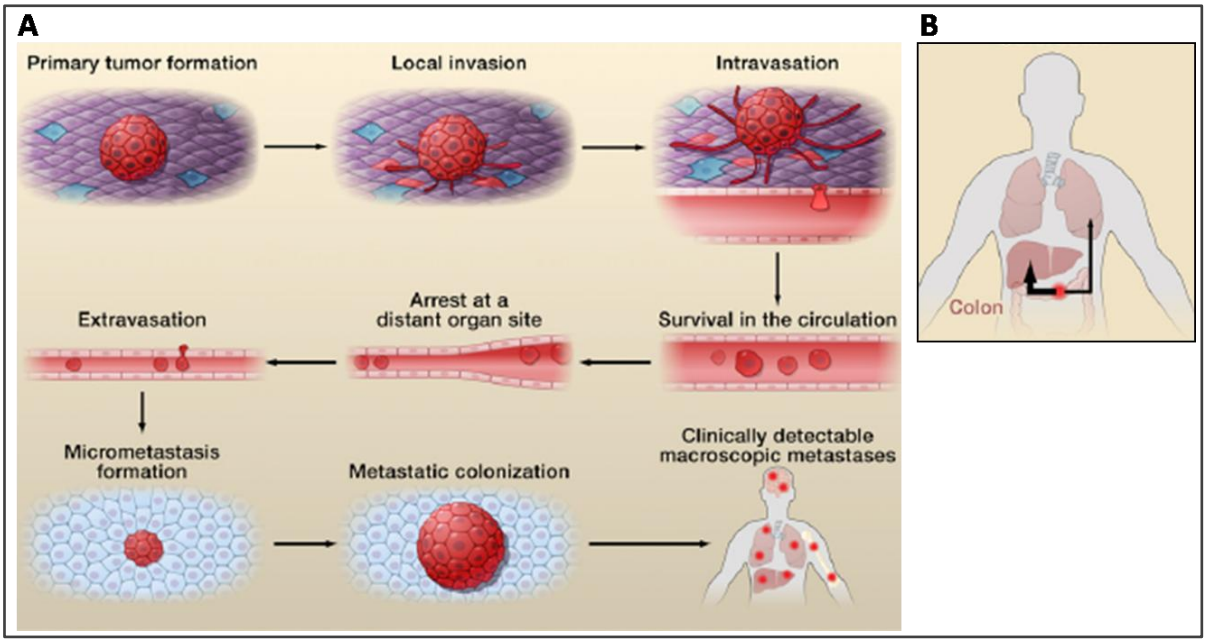

Fig. 6 | The Invasion-Metastasis Cascade. A. Clinically detectable metastases represent the end products of a complex series of cell-biological events, which are collectively termed the invasionmetastasis cascade. During metastatic progression, tumor cells exit their primary sites of growth (local invasion, intravasation), translocate systemically (survival in the circulation, arrest at a distant organ site, extravasation), and adapt to survive and thrive in the foreign microenvironments of distant tissues (micrometastasis formation, metastatic

colonization). Carcinoma cells are depicted in red; **B.** Carcinomas originating from a particular epithelial tissue form detectable metastases in only a limited subset of theoretically possible distant organ sites. Primary tumor in the colon is depicted in red. Thickness of black lines reflects the relative frequencies with which this primary tumor type metastasizes to the indicated distant organ site (lung and liver) (Valastyan and Weinberg, 2011).

Colonoscopy is considered the main method for detecting and removing precursor lesions, through screening and surveillance for CRC (Nishihara et al., 2013). Surveillance colonoscopy is often recommended according to the characteristics of polyps, mainly the size and number, determined at a baseline colonoscopy (Bond, 2003). Although the number of strategies for screening could increase, due to emerging technologies in molecular marker applications (Carethers, 2015; Imperiale and Ransohoff, 2010), to date, no molecular information has been useful in predicting whether new lesions will be detected at follow-up (Juárez et al., 2017).

➢ Molecular pathogenesis and genetic alterations involved in CRC

Cancer is fundamentally a genetic disease caused by mutational alterations (gene mutations, gene transformation, and so on) or epigenetic alterations (aberrant DNA methylation, chromatin modifications, and so on) in DNA. The loss of genomic stability and resulting gene alterations are key molecular pathogenic steps that occur early in tumorigenesis; they permit the acquisition of a sufficient number of alterations in tumor suppressor genes and oncogenes that transform cells and promote tumor progression (Grady and Carethers, 2008). From a molecular point of view, colorectal carcinoma is one of the best-characterized cancers (Ricci-Vitiani et al., 2009). It is presented as a multistep genetic disorder characterized by specific mutations in signal transduction pathways (Markowitz and Bertagnolli, 2009). There has been a remarkable expansion of the molecular understanding of colonic carcinogenesis in the last 30 years and this understanding is changing many aspects of CRC care (Obuch and Ahnen, 2016). The regulation of colonic epithelial growth is mainly mediated by the epidermal growth factor receptor (EGFR) and by the activation of several pathways downstream of EGFR, including the RAS/MAPK (mitogen-activated protein kinases) and PI3K/Akt pathways, and also the PLC, STAT and SRC/FAK pathways (Saif and Chu, 2010). All these pathways are a key event in tumor proliferation, angiogenesis and cell survival, and they are frequently altered in CRC. Indeed, they form an interconnected network, which involves phosphorylation of proteins that active transcription factors triggering carcinogenesis through deregulation of protein synthesis, cell-cycle progression, apoptosis, angiogenesis and altered metabolism (Shaw and Cantley, 2006).

The earliest identifiable lesion in colon cancer formation is the aberrant crypt focus (ACF). Some of the lesions harbor mutations in *KRAS* or *APC.* In particular, dysplastic ACF frequently carry inactivating mutations in *APC* and appear to have the highest potential for progressing to colon cancer. Mutated APC leads to the overactivation of the Wingless/Wnt signaling pathway (Grady and Carethers, 2008). Then, according to the "adenoma–carcinoma model", which was proposed by Vogelstein *et al.* in the 1990s, the neoplastic process progresses through sequential activating mutations in the EGF receptor (EGFR) pathway, inactivating mutations in the tumor suppressor gene *TP53*, and mutations in the transforming growth factor (TGF)-β pathways (Fig. 7), in the context of a growing chromosomal instability (Drost et al., 2015; Fearon, 2011; Fearon and Vogelstein, 1990; Ricci-Vitiani et al., 2009; Villalba et al., 2017; Walther et al., 2009).

A recent study has shown that deregulation (by retroviral expression of short hairpin RNAs [shRNAs] or cDNA) of *APC*, *P53*, *KRAS* and *SMAD4* is sufficient for transformation of cultured mouse colon into tumours with adenocarcinoma-like histology (Drost et al., 2015; Li et al., 2014).

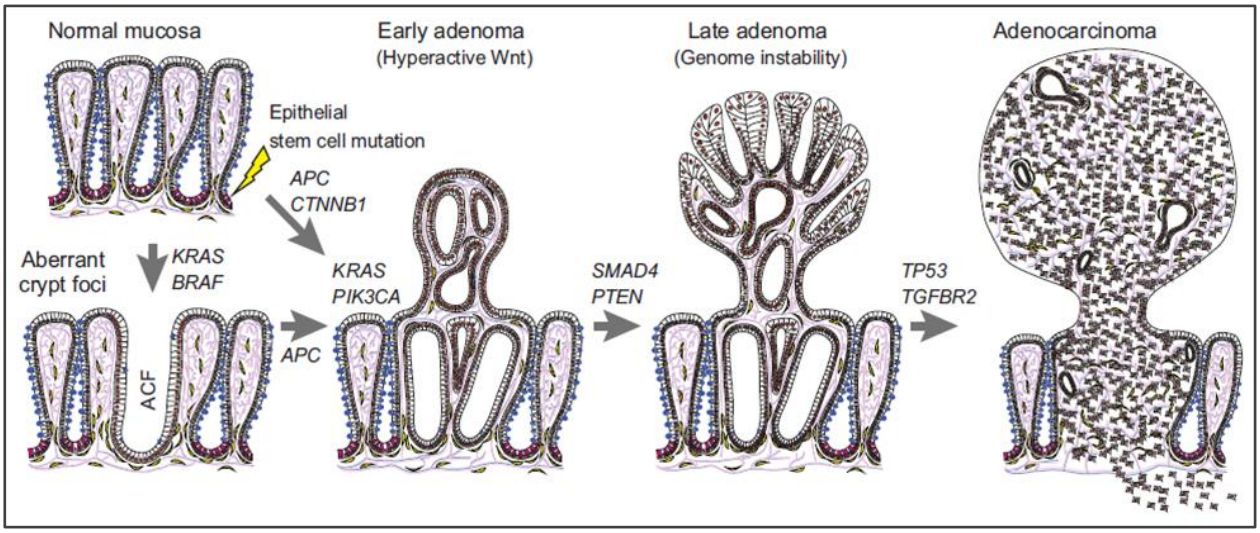

Fig. 7 | Step-wise tumorigenesis in colorectal cancer. Cartoon of the large intestine showing the structure of the normal colonic mucosa, with many mucous-secreting goblet cells (blue) at top left. Tumorigenesis begins with the mutation of intestinal epithelial stem cells (shown in magenta) in the colon or rectal mucosa, with mutations often occurring first in the *APC*, *KRAS* or *BRAF* genes. Mutations in *BRAF* or *KRAS* (in the absence of Wnt pathway mutations) are often associated with the formation of aberrant crypt foci (ACF). Most adenomas are associated with mutations in Wnt pathway components, such as *APC* or *CTNNB1*, which result in hyperactivation of Wnt signaling in early adenomas. Deregulation of Wnt signaling often co-occurs with mutations in *KRAS*, *PIK3CA*, or other mutations, leading to activation of the PI3K-Akt signaling cascade. Adenomas then progress with additional mutations (e.g. *SMAD4*) and frequently acquire genomic instability. Lastly, mutations in *TP53* and *TGFBR2* are associated with later stages of cellular transformation and with invasive characteristics of adenocarcinomas. Official human gene symbols and full names: *AKT*, AKT serine/threonine kinase 1; *APC*, adenomatous polyposis coli or Wnt signaling pathway regulator; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *KRAS*, Kirsten rat sarcoma viral oncogene homolog or proto-oncogene and GTPase; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit

alpha; *SMAD4*, Mothers against decapentaplegic homolog family member 4; *TGFBR2*, transforming growth factor beta receptor 2; *TP53*, tumor protein p53 (Strubberg and Madison, 2017).

Therefore, the genes involved in colorectal carcinoma are well known and the most important are the following:

* *EGFR*

The epidermal growth factor receptor (EGFR) is a member of the HER family (Bos, 1989). The natural ligands for EGFR are growth factors, which include EGF and transforming growth factor-α (TGF-α). On ligand binding, the EGFR can either undergo receptor dimerization by binding to a second EGFR molecule or preferentially heterodimerize with others members of HER family with the greatest affinity to ErbB-2. Then, this event mediates the activation of a complex signaling network (Lièvre et al., 2008). Two pivotal intracellular pathways activated by EGFR are the Ras/Raf/MEK/ERK and the PIK3CA/PTEN/Akt signaling pathways (Neumann et al., 2013) that regulate cell growth, proliferation, differentiation, survival, and even angiogenesis (Lièvre et al., 2008) (Fig. 8). EGFR is widely expressed in the gastrointestinal tract and stimulates proliferation of a range of cell types, including epithelial cells (Dignass and Sturm, 2001).

Mutations of the EGFR and its downstream signaling pathways are common events in the development and progression (in particular invasion and migration) of human tumors, including CRC (Fearon, 2011) (Fig. 8). The classic mutations of the EGFR kinase domain result in ligand-independent activation of the pathway. Tyrosine kinase inhibitors, such as erlotinib and gefitinib, interfere with the kinase activity of the gene and prevent downstream signaling (Gazdar, 2009).

EGFR is differentially expressed in normal, premalignant, and malignant tissue and its overexpression has been documented in 80% of cases of CRC (Arteaga, 2001; Bos, 1989; Milano et al., 2008). This is associated with increased metastatic potential and poor prognosis (Nicholson et al., 2001; O'dwyer and Benson, 2002; Radinsky et al., 1995).

High-affinity EGFR sites represent the biological target of anti-EGFR therapies (Defize et al., 1989). EGFR expression and gene modifications have been examined in CRC as possible predictive markers of treatment outcome under anti-EGFR therapies (Milano et al., 2008). Clinical studies have reported that a large number of *EGFR* gene copies may be predictive of clinical responsiveness to EGFR target therapies (Moroni et al., 2005; Sartore-Bianchi et al., 2007). However, *EGFR* gene copy number in colorectal tumors varies up to 4.7-fold between patients (from 0.44 to 2.06 units) (Milano et al., 2008) and only a small fraction of

EGFR-positive tumors are associated with gene amplification (Shia et al., 2005). Therefore, it is no longer clinically relevant (Rankin et al., 2016; Ross, 2011).

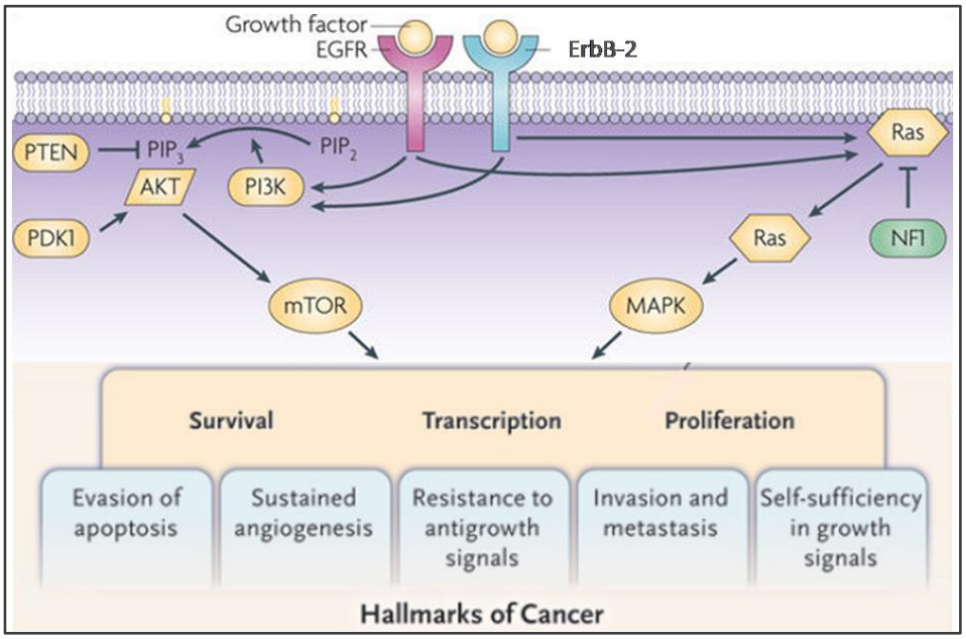

Fig. 8 | Schematic Epidermal Growth Factor Receptor (*EGFR*) Signaling Pathway. The family of surface-receptor tyrosine kinases encoded by *EGFR* consists of four members that form homodimers or heterodimers after ligand binding. Dimerization results in the activation of tyrosine kinases, which is followed by stimulation of signaling pathways, such as the PI3K–Akt–mTOR and Ras–MAPK networks. These signaling pathways eventually lead to the activation of five of the six hallmarks of cancer (independence of growth signals, insensitivity to growth inhibitory signals, resistance to programmed cell death, angiogenesis, and metastasis, with the exception of limitless replication). EGFR, epidermal growth factor receptor; ErbB-2, Erb-B2 Receptor Tyrosine Kinase 2, also called HER2; NF1, neurofibromin 1; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP2 , phosphatidylinositol-4,5-bisphosphate; PIP3 , phosphatidylinositol-3,4,5-trisphosphate; Akt denotes protein kinase B, PI3K phosphatidylinositol 3-kinase, PTEN phosphatase and tensin homologue, mTOR mammalian target of rapamycin, MAPK mitogen activated protein kinase (adapted from (Gazdar, 2009; Huse and Holland, 2010).

* *APC*

Adenomatous polyposis coli (APC) regulates a number of cellular functions, including mitosis, migration, and the maintenance of genome stability (Nelson and Näthke, 2013). APC, together with Axin1, GSK3β and CKI (Logan and Nusse, 2004), is part of a multi-protein complex which controls the Wnt signalling pathway. It promotes the degradation of β-catenin and, as a consequence, it limits the transcription of Wnt target genes involved in regulating the cell cycle. More than 90% of CRC harbor alterations that affect it (Thorstensen et al., 2005; Walther et al., 2009). Loss of function of the tumor suppressors *APC* leads to accumulation of nuclear β-catenin, which then induces the transcriptional activation of a variety of genes and oncogenes, including *c-Myc* (a transcription factor that activates several genes controlling cell growth and division) (Nieuwenhuis et al., 2009).

Individuals with *APC* polyposis (also known as familial adenomatous polyposis or FAP), an inherited autosomal dominant syndrome, develop hundreds of colorectal adenomas before the age of 35 (Dow et al., 2015; Nieuwenhuis et al., 2009). The identification of *APC* loss-of-function mutations in early, pre-malignant disease indicates that it is the initiating and driving event in tumor progression (Dow et al., 2015).

The severity of polyposis depends on the mutation site in the *APC* gene. If patients are not treated in a timely manner, almost all will develop CRC (Vasen et al., 2015). APC mutant CRC accounts for more than 600,000 deaths annually worldwide.

In a recent work, it has been shown that re-establishing normal control of APC-β-catenin pathway through endogenous APC restoration is sufficient to induce rapid and sustained tumor regression without deleterious effect on normal crypt function (Dow et al., 2015). Moreover, disruption of the MYC oncogene, a key factor supporting APC-driven intestinal disease (Sansom et al., 2007), can induce tumor regression via multiple mechanisms, such as apoptosis, senescence and/or terminal differentiation, depending on cellular context (Felsher, 2008).

However, because of the large number of APC mutations already described and the frequency of genetic changes in the β-catenin degradation pathway, neither APC nor β-catenin is a useful prognostic marker able to differentiate between patients (Walther et al., 2009).

* *RAS*

RAS proteins are small GTPases of 21-kDa, which belong to the monomeric small G protein family (Saif and Chu, 2010) and consist of different members such as KRAS, NRAS, and HRAS (Wilson and Tolias, 2016). They possess GDP/GTP-binding and intrinsic GTPase activities (Konstantinopoulos et al., 2007) that cycle between "on" and "off" conformations, which are conferred by the binding of GTP and GDP, respectively. Under physiological conditions, the transition between these two states is regulated by guanine nucleotide exchange factors (GEFs), which promote the activation of RAS proteins by stimulating GDP for GTP exchange, and by GTPase-activating proteins (GAPs), which accelerate RAS-mediated GTP hydrolysis (Pylayeva-Gupta et al., 2011) (Fig. 9A.). RAS signaling pathway mediates cellular responses to external growth signals (Juárez et al., 2017). Indeed, its activation occurs after binding of a ligand to a receptor tyrosine kinase (RTK), such as the EGFR (Wilson and Tolias, 2016). The extracellular signal is transmitted through the transmembrane domain resulting in EGFR dimerization

and activation. Activated EGFR recruits the son of sevenless (SOS), a GEF, to its phosphorylated C-terminal tail via the adaptor proteins, SH2- adaptor protein (SHC) and growth factor receptor-bound protein 2 (Grb2). GEF exchanges GDP by GTP, activating Ras. Active, GTP-loaded Ras dimerizes and binds Raf, thereby promoting Raf dimerization and activation. Active Raf dimer phosphorylates and activates mitogen-activated protein kinase kinase 1 and 2 (MEK1/2), which induces ERK1/2 activation (Nussinov et al., 2014). Then, ERK1/2 can phosphorylate both cytosolic and nuclear substrates, which include transcription factors like Jun and Elk-1 (Yordy and Muise-Helmericks, 2000), to regulate various cellular responses including proliferation, survival and differentiation (Wilson and Tolias, 2016). Elk-1 binds to its cofactor, a dimer of serum response factor (SRF), leading to transcription activation and cell proliferation (Nussinov et al., 2014). Ras–GTP also binds the catalytic subunit of type I PI3Ks (Pacold et al., 2000; Rodriguez-Viciana et al., 1994). This binding results in the translocation of PI3K to the plasma membrane and subsequent activation (Bader et al., 2005) (Fig. 9B). Furthermore, KRAS modulates other downstream effectors, that include RaIGDS/p38MAPK and Rac/Rho (Bahrami et al., 2017).

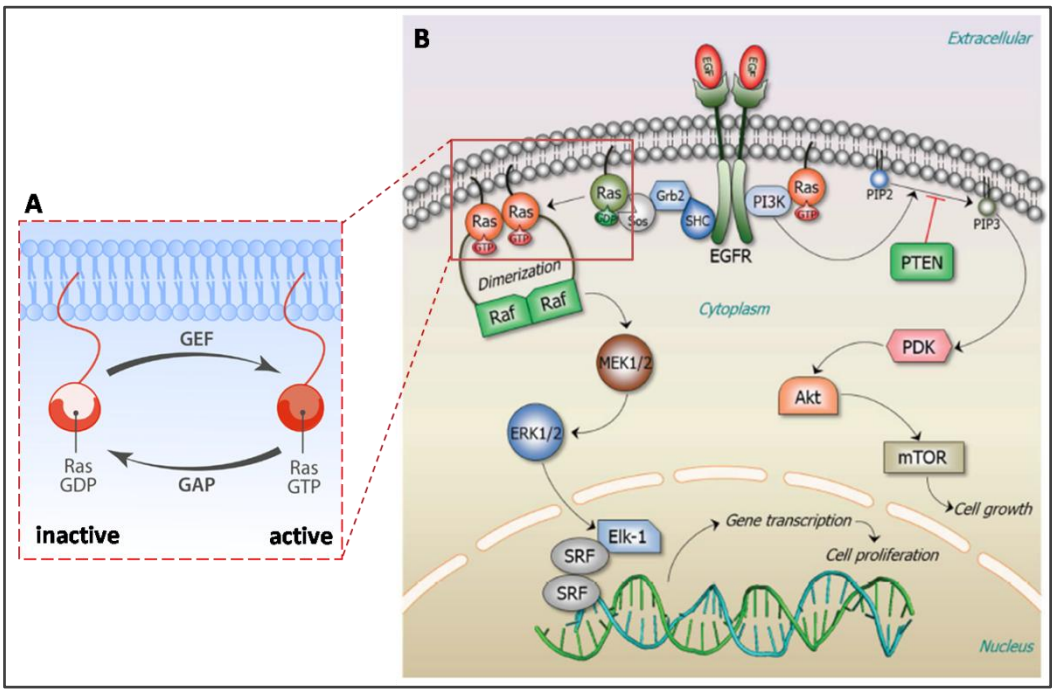

Fig. 9 | Regulation of Ras family proteins and Ras signaling pathway. A. The Ras GTPases cycle between GDP-bound inactive and GTP-bound active forms. Activation of Ras is regulated by the balance of opposing actions of two classes of Ras regulatory enzymes. Guanine nucleotide exchange factors (GEFs) promote GTP-bound Ras state by enhancing exchange of GDP with GTP. GTPase activating proteins (GAPs) enhance slow rate of intrinsic Ras GTPase activity, promoting the inactive GDP-bound state of Ras (Jun et al., 2013); **B.** Ras is normally activated in response to the binding of extracellular ligands to various receptors. Among these is epidermal growth factor (EGF) binding to its cognate receptor EGFR. Upon EGF binding to the extracellular domain of EGFR, the intracellular domain of EGFR forms homo- or hetero-dimers

with another ERBB receptor family member. Downstream signaling proceeds through Ras in the Raf-MEK-ERK and/or PI3K-Akt-mTOR pathways, which regulate various cellular responses including proliferation and survival. SOS, son of sevenless; Grb2, growth factor receptor-bound protein 2; SHC, SH2- adaptor protein; SRF, serum response factor (Nussinov et al., 2014).

RAS signaling pathways are activated during tumorigenesis by a number of different mechanisms, including mutations in RAS, loss of GAP proteins, overexpression of RTKs (EGFR, ErbB-2), and also mutations or amplifications in any of their effector molecules (Hennessy et al., 2005; Jia et al., 2009; Shaw and Cantley, 2006; Vivanco and Sawyers, 2002). Particularly, RAS gene have long been known as proto-oncogenes mutated in various type (30%) of human cancer (Bos, 1989), including 90% of pancreatic, 45% of colon and 35% of lung cancers (Kwak et al., 2017; Wilson and Tolias, 2016). These human tumors have activating point mutations in *RAS* (Downward, 2003). *KRAS* is the most frequently mutated isoform, present in more than 20% of all human cancer, followed by *NRAS* (about 8%) and *HRAS* (3.3%) (Baines et al., 2011). These point mutations of the RAS gene are mainly localized in codons 12 and 13 (Juárez et al., 2017), and rarely, in codons 61. These mutations lock RAS proteins into a constitutively activated state in which they signal to downstream effectors even in the absence of extracellular stimuli (Castellano and Downward, 2011). In particular, oncogenic mutations of Q61 (e.g. Q61K) impair the GTP hydrolysis reaction by interfering with the coordination of a water molecule that is required for the nucleophilic attack on the γ-phosphate (Buhrman et al., 2010; Scheidig et al., 1999). Similarly, oncogenic substitutions in residues G12 and G13 (e.g. G12D, G12V, G12C and G13D) (Wilson and Tolias, 2016) prevent the formation of van der Waals bonds between RAS and the GTPase-activating proteins (GAP) through steric hindrance and so perturb the proper orientation of the catalytic glutamine (Q61) in RAS, which results in the pronounced attenuation of GTP hydrolysis (Scheffzek et al., 1997). The residue Q61 is mutated within HRAS with a frequency of 36%, and within KRAS and NRAS with a frequency of 1% and 62%, respectively; while G12 is mutated with frequencies of 55%, 85% and 25%; and G13 9%, 14% and 13% [5].

Mutations are also observed in the *BRAF* gene encoding the BRAF protein located downstream of KRAS (Davies et al., 2011). BRAF is frequently activated by mutation in human tumors, such as melanomas (~70%) and colon carcinoma (~15%). In particular, mutated BRAF is found in 7-10% of patients with metastatic CRC (Roth et al., 2010; Samowitz et al., 2005; Strickler et al., 2017; Tie et al., 2011; Tol et al., 2009). Mutations in *BRAF* occur in a very limited number of residues in its kinase domain (Downward, 2003), associated with elevated kinase activity (Davies et al., 2011). The most common BRAF

mutation, occurring in about 90% of cases, is the single substitution missense mutation V600E (Bamford et al., 2004; Network, 2012; Strickler et al., 2017).

BRAF and KRAS mutations are mutually exclusive (Rajagopalan et al., 2002). Because of BRAF mutations potently stimulate MAPK signaling, co-mutations in the MAPK signaling cascade offer no selective advantage for developing tumors (Tol et al., 2009); therefore, BRAF mutations rarely occur concomitantly with KRAS or NRAS mutations (Tie et al., 2011).

Activating KRAS mutations occur during the early to advanced stages of the polyp-to-carcinoma sequence (Davies et al., 2002). Patients with at least one polyp that harbours a KRAS mutation are at higher risk of developing advanced polyps, specifically, advanced adenomas, compared to patients with polyps that harbour BRAF mutations or no mutation. Therefore, the KRAS mutation is an independent predictor of the development of advanced polyps and advanced adenomas, stronger than other characteristics, like the size or number of lesions at baseline. These considerations establish the potential utility of molecular markers for stratifying risk among patients with colonic polyps (Juárez et al., 2017).

The high occurrence of the mutations in RAS in different tumors, make it one of the most important drug targets in oncology. Unfortunately, for the past few decades, efforts to target RAS have been unsuccessful in generating drug therapies, suggesting that it might represent an ‘undruggable’ target. Targeting RAS directly through small molecule binding presents challenges as well because some small molecules are too small to fully block the surface area involved in protein–protein binding. Therefore, researchers shifted their focus to new targets (Wilson and Tolias, 2016).

* *PIK3CA*

PIK3CA encodes the p110α catalytic subunit of the class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks are heterodimers composed of a p110 catalytic and a p85 regulatory subunits and can be activated by recruitment to the cell surface by growth factor receptor tyrosine kinases (RTKs), such as EGFR and the insulin receptor (Cantley, 2002; Vanhaesebroeck and Waterfield, 1999) and by G protein-coupled receptors (GPCRs). Upon binding of the ligand (likes EGF) to the receptor (Castellano and Downward, 2011) the regulatory subunit of PI3K, p85, directly binds to phosphotyrosine residues and other adaptors found on the RTKs, triggering activation of the p110 catalytic subunit of PI3K (Courtney et al., 2010). Active PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate

(PIP_2) at the 3-position of the inositol ring, generating the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Samuels et al., 2005; Vanhaesebroeck and Alessi, 2000) and drives the various downstream pathways that regulate a number of cellular functions, like evasion of apoptosis (Courtney et al., 2010), cell growth, cell cycle entry, cell survival, cytoskeleton reorganization, metabolism (Castellano and Downward, 2011) proliferation, glucose homeostasis and vesicle trafficking (Engelman et al., 2006). Once PIP_3 is formed, it acts as a docking site for pleckstrin homology (PH)-containing proteins, such as Akt (also called PKB, protein kinase B) and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Vanhaesebroeck and Alessi, 2000). Therefore, PIP3 recruits the cytosolic proteins, PDK1 and Akt, to the plasma membrane in close proximity to each other (Lien et al., 2017; Zenonos and Kyprianou, 2013). Then, PDK1 activates Akt by phosphorylation at residue threonine 308 (T308) (Alessi et al., 1997; Currie et al., 1999; Majumder and Sellers, 2005). Akt becomes fully activated after the additional phosphorylation of serine 473 (S473) by mTORC2 (Sarbassov et al., 2005) and, in turn, it phosphorylates numerous (>200) substrates, including mTOR, 4E-BP1, p70-S6K, Tuberin and GSK3β (Ebner et al., 2017; Hers et al., 2011; Manning and Cantley, 2007; Samuels et al., 2005). PI3K is negatively regulated at the level of PIP_3 by the tumor suppressor phosphatase and tensing homolog (PTEN), which dephosphorylates PIP_3 into its inactive PIP_2 form, thereby terminating PI3K-dependent signaling (Carracedo and Pandolfi, 2008) (Fig. 10).

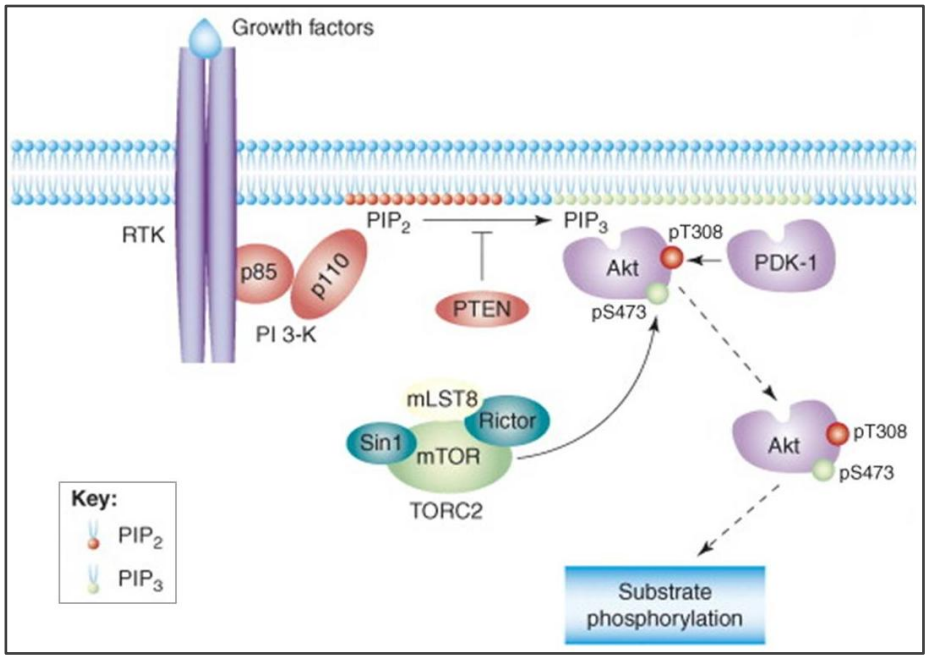

Fig. 10 | The PTEN/PI3K/Akt signaling pathway. In the classical growth-factor-stimulated pathway, PI3K activation is stimulated by binding of the p85 subunit to activated receptor tyrosine kinases (RTKs), bringing

the catalytic p110 subunit in proximity to the PtdIns(4,5)P_2 (PIP_2) substrate. Interconversion into PtdIns(3,4,5)P_3 (PIP_3) can be reversed by the tumor suppressor PTEN. PtdIns(3,4,5)P_3 then recruits inactive Akt to the membrane where Thr308 phosphorylation is catalyzed by PDK1, whereas Ser473 phosphorylation is mediated by the TORC2 complex comprising the protein kinase mTOR and the adapter proteins rictor, stress-activated protein kinase interacting protein 1 (Sin1), and mLST8 (mammalian lethal with sec-13). Fully active Akt leaves the membrane to catalyze the phosphorylation of several predominantly cytoplasmic substrates (adapted from (Toker, 2008).

Several studies have established that the PI3K signaling pathway is constitutively active in several cancer types. It has a central role in numerous processes critical for cancer development and progression, including metabolism, growth, survival, and motility (Castellano and Downward, 2011; Saif and Chu, 2010). Gene amplifications, deletions, and somatic missense mutation in the *PIK3CA* gene have been reported in many human cancer types, including colon, breast, brain, liver, stomach, prostate and lung cancer (Castellano and Downward, 2011; Samuels et al., 2004). The genetically deregulation of PI3K pathway is also due to the deletion or mutation of PTEN (Carracedo and Pandolfi, 2008; Samuels et al., 2005). Breast, endometrial, bladder, and colorectal carcinomas have been found to have a high rate of *PIK3CA* gene mutations, with frequencies of 26%, 22%, 21%, and 12%, respectively[5]. *PIK3CA* mutations generally occur during the adenoma to carcinoma transition (Samuels et al., 2004). The hotspots mutated within *PIK3CA* are three: two in exon 9, codons 542 and 545 (E542K, E545K), and one in exon 20, codon 1047 (H1047R), which correspond to the helical and kinase domains of p110α subunit of PI3K, respectively (Castellano and Downward, 2011; Marone et al., 2008). Mutation at any one of these sites results in a gain of enzymatic function and promotes oncogenic transformation *in vitro* and *in vivo* (Ikenoue et al., 2005). Indeed, it has been shown that *PIK3CA* mutations conferred resistance to apoptosis and a dramatic ability to migrate and invade *in vitro* as well *in vivo* (Samuels et al., 2005). The coexistence of mutations in both exons 9 and 20 of the same p110α molecule leads to a synergistic gain of function, with a potent transforming capacity *in vitro* (Zhao and Vogt, 2008) and results in more aggressive tumor behavior compared with cancers with wild-type *PIK3CA*, or with a single mutation in either exon 9 or exon 20 (Liao et al., 2012).

PI3K pathway could also be activated via RAS. Some RTKs, such as members of the EGF or FGF receptor families lack the consensus p85-binding motifs necessary for direct PI3K interaction. The discovery of the direct interaction between RAS and PI3K provides a mechanism with which to bridge this gap in the RTK signaling pathway. Oncogenic RAS is thought to exert its tumorigenic activities through multiple downstream effector pathways.

Indeed, one of the RAS-driven tumorigenic mechanisms is mediated by this interaction (Bardelli and Siena, 2010).

Although the vast majority of studies have focused on the protein kinase Akt as the dominant effector of PI3K signaling, other PIP_3-dependent signaling proteins critically contribute to cancer progression. Indeed, it has recently been shown the importance of Akt-independent signaling branches downstream of PI3K, which have roles in promoting phenotypes associated with malignancy. They include the PDK1-mTORC2-SGK3 axis, mTORC2/NF-κB pathway, mTORC2/c-Myc pathway, Rac signaling, and the TEC family kinases (TEC, BMX, BTK, ITK, and TXK) (Faes and Dormond, 2015; Lien et al., 2017) (Fig. 11).

Given the high frequency of PI3K mutations, but also in many of its downstream effectors, in human cancers, PI3K signaling has clearly emerged as a highly attractive drug target (Lien et al., 2017). Therefore, significant efforts are being made to generate inhibitors for this pathway to treat human cancers. Small molecule inhibitors of PI3K have entered into clinical trials in the past few years (Castellano and Downward, 2011; Marone et al., 2008).

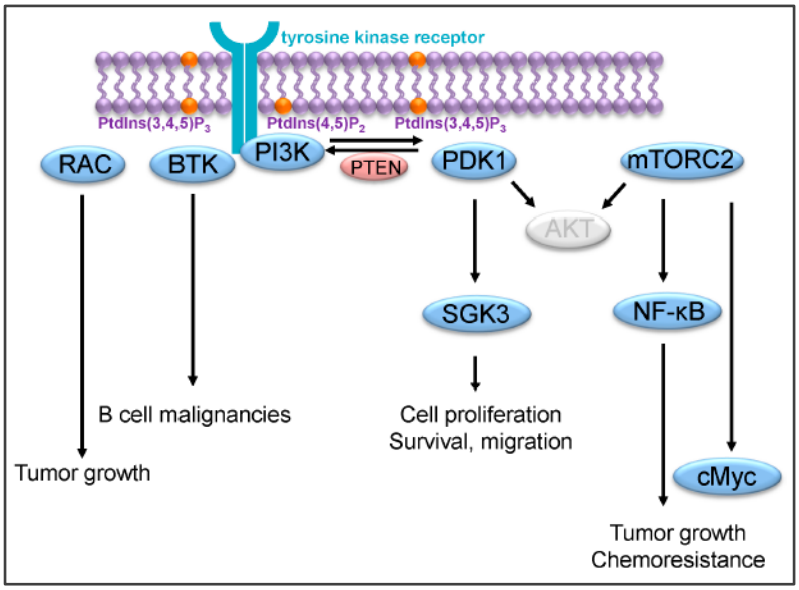

Fig. 11 | AKT-independent PI3K signaling in cancer. Several AKT-independent mechanisms used by PI3K to promote cancer growth have been described. They include activation of RAC, BTK in B cell malignancies, PDK1/SGK3 and mTORC2/NF-κB; mTORC2/c-Myc. Light gray ovals imply components of the signaling pathway that are not activated or bypassed (Faes and Dormond, 2015).

* *TGF-β/SMAD*

The transforming growth factor-beta (TGF-β) signaling pathway is involved in several key biologic process, including cell proliferation, differentiation, immune regulation, migration, and apoptosis (Massagué, 2012; Massagué et al., 2000; Xu and Pasche, 2007). Three

mammalian isoforms of TGF-β are known, namely TGF-β1, TGF-β2 and TGF-β3 (Wakefield and Hill, 2013). TGF-β1 is expressed in most tissues, TGF-β2 is highly expressed in heart and eye and TGF-β3 is expressed in the developing lung. Their functional role is similar, suggesting functional redundancy (Hinck and O'Connor-McCourt, 2011). TGF-β secreted from monocytes, macrophages, lymphocytes and fibroblasts is sequestered in the extracellular matrix in a biologically inactive, latent form. Latent TGF-β activation is catalyzed by αvβ6 integrin on epithelial cell membranes (Annes et al., 2003).

TGF-β signaling is initiated by binding of released TGF-β factor to type II TGF-β receptor (TGF-βRII), which is a Ser/Thr protein kinase. Then, TGF-βRII recruits and phosphorylates TGF-βRI, which subsequently phosphorylates two downstream transcription factors, SMAD2 and SMAD3. This phosphorylation gives rise to a hetero-oligomeric complex with SMAD4, that undergoes nuclear shuttling (Saif and Chu, 2010; Villalba et al., 2017) and interacts with a number of key transcription factors. Several downstream targets of TGF-β signaling pathway include cell cycle checkpoint genes and their activation leads to growth arrest. Therefore, TGF-β seems to function as a tumor suppressor. However, TGF-β signaling can also directly stimulate the production of several mitogenic growth factors, such as TGF-α, FGF, and EGF. As a consequence, RAS/Raf/MEK/ERK and PI3K/Akt pathways can be activated. Finally, TGF-β has been shown to promote angiogenesis as well as regulate cell adhesion, motility, and the extracellular matrix (Saif and Chu, 2010).

SMAD phosphorylation and signal relay is negatively regulated by the binding of inhibitory SMAD6/7, which can bind to both TGF-β and BMP receptors and can trigger their degradation by E3 ubiquitin ligases upon internalization through caveolin-mediated vesicles (Massagué, 2012; Villalba et al., 2017; Wakefield and Hill, 2013) (Fig. 12).

TGF-β participates in a large variety of processes including cell proliferation, differentiation, immune regulation, apoptosis and the maintenance of homeostasis under normal conditions. Moreover, it is involved in the development of CRC and its metastatic process. The role of TGF-β1 in cancer has been investigated and this isoform is considered the most relevant member in physiological and malignant conditions. On the contrary, TGF-β2 and TGF-β3 have been much less studied (Villalba et al., 2017). Levels of TGF-β are increased in advanced CRC patients (Picon et al., 1998). Indeed, high circulating levels of TGF-β are predictors of liver metastasis after CRC resection (Tsushima et al., 2001). Several studies, carried out on metastatic models of CRC and other cancer types, highlights that metastasis are reduced after the targeting of TGF-β

signaling (Gonzalez-Zubeldia et al., 2015; Sheen et al., 2013; Villalba et al., 2017; Wakefield and Hill, 2013).

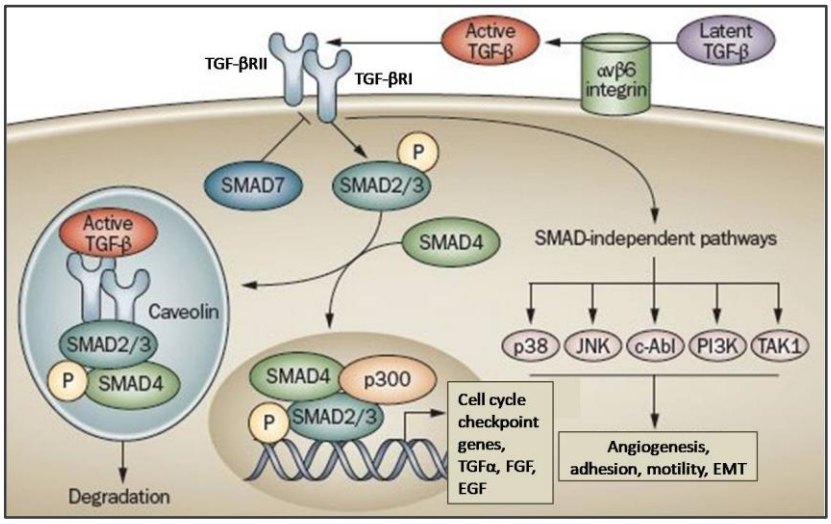

Fig. 12 | TGF-β signaling pathway. TGF-β secreted from monocytes, macrophages, lymphocytes and fibroblasts is sequestered in the extracellular matrix in a biologically inactive, latent form. Latent TGF-β activation is catalyzed by αvβ6 integrin on epithelial cell membranes. Active TGF-β binds to serine/threonine kinase cell surface receptors that phosphorylate downstream SMAD2/3. Phosphorylated SMAD2/3 complexes with SMAD4 and accumulates within the nucleus, where it collaborates with other transcription factors, and recruits cofactors, such as p300, to target genes, resulting in the transcription of many genes among which those involved in the cell cycle checkpoint or those codifying growth factors. SMAD7 is a TGF-β-inducible endogenous SMAD inhibitor that negatively regulates TGF-β signaling. TGF-β can also induce cellular responses, such as angiogenesis, cell adhesion, motility and EMT, via SMAD-independent pathways involving the kinases JNK, p38, PI3K, c-Abl and TAK1. The duration of the TGF-β signal is regulated by the uptake of the TGF-β receptor–ligand complex into caveolin-lined endosomes that promote degradation. Abbreviations: c-Abl, c-Abelson; EMT, epithelial–mesenchymal transition; JNK, Jun N-terminal kinase; PI3K, phosphoinositide 3 kinase; TAK1, TGF-β-activated kinase 1; TGF-β, transforming growth factor β (adapted from (Varga and Pasche, 2009).

Mutations in TGF-βRII are one of the most common alterations in TGF-β signaling in CRC cells with microsatellite instability (MSI). The overall incidence of TGF-βRII mutations is 25–30% in this disease (de Caestecker et al., 2000), although inactivating mutations in this receptor have also been reported in CRCs that do not present MSI (Bellam and Pasche, 2010; Villalba et al., 2017). Furthermore, alterations in the downstream effectors of the TGF-β signaling pathway are also known to be involved in tumor progression. Loss of SMADs expression, either through deletion or through mutation, is present in 10% of CRC (Bellam and Pasche, 2010; Saif and Chu, 2010).

Clinical trials targeting TGF-β in various tumor types by using a variety of strategies are currently evaluating the therapeutic response and toxicity in patients (Villalba et al., 2017).

* *TP53*

The human *TP53* gene codes a nuclear protein that induces cell cycle arrest, apoptosis, or cellular senescence in response to endogenous and exogenous stress signals (Riley et al., 2008). Indeed, the p53 protein product is up-regulated after cell stress from radiation exposure, DNA injury, or other noxious events to prevent new DNA synthesis and halt cell division. Thus, it permits either DNA repair if the damage is correctable, or apoptosis if the damage is too severe (Baker et al., 1989; Robbins and Itzkowitz, 2002). Normal cells express low levels of p53 (Feng et al., 2008) and its stabilization, with the subsequent accumulation enable a checkpoint response to resolve the initiating damage or induce apoptosis to protect cells from becoming tumorigenic (Oren and Rotter, 2010). However, this gene is the target of mutation in over 50% of human cancers, including CRC (Feng et al., 2008). The stabilization of mutant p53 enables it to accumulate to high levels but to the opposite effect to that shown in normal cells, promoting growth, invasive/metastatic properties, and genetic instability (Oren and Rotter, 2010). It has been shown that although mutant p53 is constitutively stable in cancer cells (Midgley and Lane, 1997; Terzian et al., 2008), it does not possess inherent stability in normal cells (Terzian et al., 2008), suggesting that cancer cells provide an environment that converts mutant p53 into a more stable form (Frum and Grossman, 2014).

In CRC cells, p53 signaling is frequently dysregulated (Terranova-Barberio et al., 2017). The elimination of p53's tumor suppression ability, usually due to a missense point mutation of one allele and deletion of the other wild type allele (Baker et al., 1989; Robbins and Itzkowitz, 2002), seems to be a late but necessary occurrence for the transition of transformed colorectal epithelial cells into a malignant tumor (Feng et al., 2008; Prives, 1998; Riley et al., 2008). The loss of a complete functional p53 is often associated with resistance to current therapies and poor prognosis (Terranova-Barberio et al., 2017). In turn, the presence of increased nuclear p53 seems to be related to nodal affectation, distant metastasis and a poor prognosis (Soussi, 2007).

* *Myc*

The *MYC*-family of cellular proto-oncogenes encodes three highly related nuclear phosphoproteins, namely c-Myc, N-Myc, and L-Myc (Mukherjee et al., 1992). c-Myc is a basic-helix-loop-helix leucine zipper (Sipos et al., 2016) transcription factor, which coordinates the expression of about 15% of genes (Casey et al., 2017; Knoepfler, 2007)

involved in cell cycle, protein synthesis, metabolism, cell adhesion, cytoskeleton, survival and microRNA expression (Dang et al., 2006). Myc expression is tightly controlled by post transcriptional mechanisms in response to growth factor-dependent signals, nutrient supply and metabolic stress in normal cells (Dejure and Eilers, 2017; Gagliardi et al., 2017). Myc protein levels are frequently increased in human cancers through different mechanisms such as chromosomal amplifications and translocations, augmented c-Myc mRNA transcription, increased mRNA and protein stability (Gagliardi et al., 2017). c-Myc overexpression in cancer causes increased proliferation rates, metabolic transformation, metastatic capacity and altered cell fate decisions (Dang, 2012). High Myc protein levels are not only able to drive tumor initiation and progression, but also essential for tumor maintenance (Koh et al., 2016). Notably, effects on cellular metabolism depend both on oncogene activity and on the tissue of tumor origin and on interaction with microenvironment components (Davidson et al., 2016; Mayers and Vander Heiden, 2017; Yuneva et al., 2012). Metabolic changes induced by deregulated Myc can therefore vary among different tumor types (Dejure and Eilers, 2017).

c-Myc is phosphorylated on threonine 58 and serine 62. Phosphorylation on serine 62, is mediated by ERK (Sears et al., 2000), Polo-like kinase 1 (PLK1) (Tan et al., 2013), cyclin E/cyclin-dependent kinase 2 (CDK2) (Hydbring et al., 2010) and c-Jun N-terminal kinase (JNK) (Noguchi et al., 1999), and stabilizes the protein. On the contrary, phosphorylation on threonine 58 is mediated by GSK-3β and promotes c-Myc degradation (Gregory et al., 2003) (Fig. 13). The opposite roles of threonine 58 and serine 62 phosphorylation are in agreement with their opposite roles in tumors: serine 62 phosphorylation is pro-tumorigenic, while threonine 58 phosphorylation is anti-tumorigenic (Wang et al., 2011).

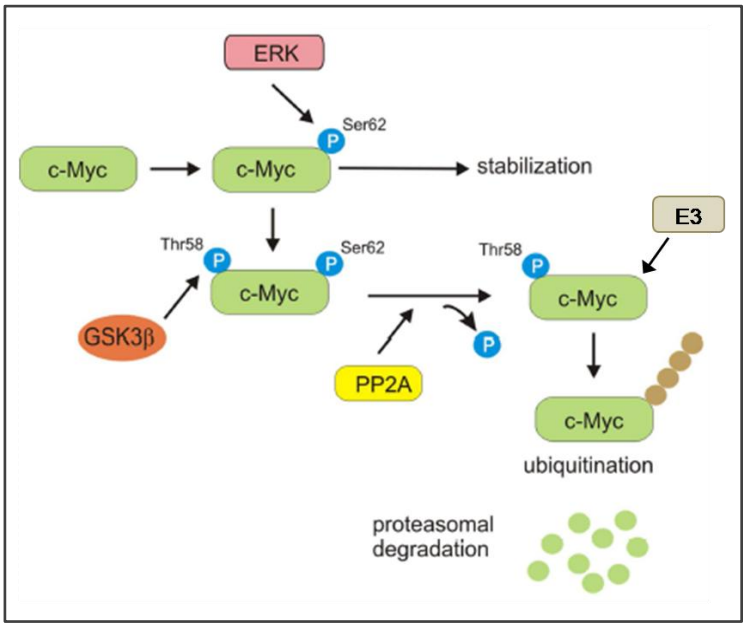

Fig. 13 | c-Myc stability regulation. Stability of c-Myc is controlled by phosphorylation of specific sites. Activated extracellular receptor kinase (ERK) stabilizes c-Myc by phosphorylation at Ser62. Once c-Myc phosphorylated at Ser62, it is recognized by GSK3β, which phosphorylates it at Thr58. At that time, dephosphorylation of Ser62 is mediated by protein phosphatase 2A (PP2A). c-Myc phosphorylated at Thr58, but not at Ser62 is recognized by the E3 ligase, which ubiquitinates c-Myc at the N-terminus and targets it for proteasome-dependent degradation (adapted from (Jóźwiak et al., 2014).

Many pieces of evidence demonstrate that enhanced Myc expression is a major driving force of tumorigenesis and that both Myc-driven tumors and tumors driven by other oncogenes, for example, by mutant RAS, continuously depend on elevated Myc levels for growth (Annibali et al., 2014; Felsher and Bishop, 1999; Shachaf et al., 2004; Soucek et al., 2008).

Given the critical role of Myc overexpression in promoting a multitude of human cancers (Cunningham and Ruggero, 2013) it turns out to be an ideal drug target in oncology (Koh et al., 2016). Multiple approaches are currently being used to target both the *Myc* oncogene directly, as well as specific cellular/molecular targets upstream or downstream of Myc that contribute to tumor formation upon Myc hyperactivation (Cunningham and Ruggero, 2013).

➢ Therapeutic approaches

CRC is one of the most frequent tumor types in Western countries. Approximately 20% of patients show metastatic disease at the time of diagnosis, with the liver being one of the most affected organs (Villalba et al., 2017), and 25-30% of patients present with stage I/II disease will have a recurrence within 5 years of a curative intent surgery (Loree and Kopetz, 2017; Shah et al., 2016).

Previous studies have suggested that primary tumor location (PTL) may affect treatment outcomes (Lee et al., 2015; Yahagi et al., 2016). CRC can be divided into distinct disease entities according to the PTL. Tumors of the right side of the colon, including the cecum, the ascending colon, and the transverse colon, tend to more frequently exhibit a poorly differentiated histology, BRAF mutation, a hypermethylated phenotype, and MSI, while c-Myc expression occurs more commonly in tumors of left colon and rectum (Kim et al., 2017; Lee et al., 2015; Rothberg et al., 1985; Shen et al., 2015). However, it is not clear the role of PTL in treatment decisions. Recently, it has been reported that right colon primary might be associated with a poor survival outcome in patient with wild type KRAS metastatic CRC treated with a specific drug targeting EGFR receptor as salvage treatment (Kim et al., 2017).

At present, anticancer therapies for CRC include surgery, radiation, chemotherapy, and anti-VEGF (vascular endothelial growth factor) or anti-EGFR monoclonal antibodies. Whereas surgical resection and adjuvant therapy can cure well-confined primary tumors, metastatic disease is largely incurable, due to its systemic nature (Valastyan and Weinberg, 2011). Carcinoma patients frequently harbor significant numbers of

disseminated tumor cells in their blood or distant organ sites (Nagrath et al., 2007; Pantel et al., 2008). In addition, these disseminated tumor cells show resistance to existing therapeutic agents (Valastyan and Weinberg, 2011). This explains why > 90% of mortality from cancer is attributable to metastases and not to the primary tumors from which these malignant lesions arise (Gupta and Massagué, 2006; Steeg, 2006). Consequently, truly effective anti-metastatic therapeutics must be capable of impairing the proliferation and survival of already disseminated carcinoma cells, rather than trying to block the escape of these cells from primary tumors (Valastyan and Weinberg, 2011). Moreover, although anti-tumor strategies eliminate the vast majority of cancer cells, leading to a reduction in tumor size and disease remission, they fail to prevent relapse by leaving intact the cancer stem cells (CSCs) reservoir. On the contrary, although a CSC-targeted therapy does not cause tumor-size shrinkage in short term, it could prevent future regrowth and metastasis (Dalerba et al., 2007a; Massard et al., 2006). Identification of CSC in colorectal cancer with reliable markers may aid the development of stem cell gene therapy, by the administration of antagonistic growth factors or by immunotherapy directed towards those unique overexpressed cellular markers. Therefore, CSC research provides the foundation for therapeutic advancement in CRC treatment (Papailiou et al., 2011).

The survival of metastatic CRC has gradually been improved with advancements in medical therapy, which include not only the development of new drugs but also the discovery of predictive biomarkers (Kim et al., 2017). A wide range of solid tumors over-expresses EGFR. As previously reported, this receptor is involved in their growth and proliferation through various mechanisms. Therefore, it has an important role in the development and progression of cancers. Based on this consideration, EGFR signaling pathway represents a rational target for drug development (Lee and Chu, 2007; Vokes and Chu, 2006). Cetuximab and panitumumab are anti-EGFR monoclonal antibodies presently approved by the United States FDA for the treatment of metastatic CRC (mCRC). These antibodies bind with higher affinity to the EGFR than its natural ligands, and competitively inhibit binding of the natural ligands to EGFR, thereby blocking receptor phosphorylation and downstream growth signaling, inducing receptor internalization, and reducing the level of EGFR expression on the cell surface (Kim et al., 2001). They exert their antitumor effects through inhibition of cell proliferation by inducing cell cycle arrest and apoptosis, and inhibiting tumor angiogenesis (Saif and Chu, 2010). However, mutation of downstream effectors of EGFR was established as a mechanism of drug resistance (Zhang et al., 2017). Indeed, recent studies indicated that primary and acquired drug resistance induced

by aberrant mutations in oncogenes or tumor suppressor genes could reduce cetuximab efficacy in some patients. In particular, all of the clinical studies conducted to date have shown that anti-EGFR antibody therapies are essentially inactive in patients with metastatic CRC whose tumors express mutant KRAS (Bertotti et al., 2015; De Roock et al., 2011; De Roock et al., 2010; Konstantinopoulos et al., 2007; Schirripa et al., 2015). However, also 40% of patients with wild type KRAS do not respond. In these patients mutant BRAF, which is present in 5–10% of the tumors, may affect response outcome (Arvelo et al., 2015; Loree and Kopetz, 2017; Morkel et al., 2015). Moreover, about 40% of malignant tumors carry known activating PI3K/Akt alterations (Danielsen et al., 2015). PI3K activation has been identified in CRC as a potential mechanism of resistance to chemotherapy, hormonal therapy, radiation therapy, and to various therapies targeting certain signaling pathways, such as trastuzumab and lapatinib. Therefore, various individual components of this pathway are attractive therapeutic targets for drug development (LoPiccolo et al., 2007). All these considerations emphasize the importance to understand the cross-talks between KRAS/MAPK and PI3K/Akt pathways. Information about the tumor genotype and, in particular, about mutations in these molecules are currently used to predict the success of systemic chemotherapy of different types of tumors, including CRC, and are used to stratify patients for treatment options (Fritsche-Guenther et al., 2016; Yokota et al., 2011).

Mutations in the TGF-β and TP53 genes are also responsible for clonal expansion of the colorectal carcinoma, as well cellular potential for invasiveness and metastasis, and therefore they are implicated in the therapeutic resistance (Hodgkinson et al., 2017).

Furthermore, other possible causes of cetuximab resistance have been proposed, such as tumor cell heterogeneity, tumor microenvironment and cellular interactions (Bardelli and Siena, 2010; Hartmann et al., 2016; Schmitz and Machiels, 2016) .

In addition, according to the recent data obtained by means of mCRC patient derived xenografts (PDXs), even in the responder cases, cetuximab therapy fails to eradicate all the cells (Bertotti et al., 2015). In these cases, some of the cells become drug tolerant and form the so-called minimal residual disease (MRD), playing potentially a role in disease relapse and formation of metastases (Meads et al., 2009).

Several therapeutic options are currently under clinical investigation (Villalba et al., 2017). However, there is an urgent need to identify new therapeutic strategies for targeting mutated KRAS metastatic colorectal cancer (mCRC).

3-phosphoinositide-dependent protein kinase-1 (PDK1)

3-phosphoinositide-dependent protein kinase-1 (PDPK1 or PDK1) is a key element of signaling transduction activated by extracellular ligands, such as growth factors (Gagliardi et al., 2015). PDK1, and the majority of its substrates, belong to the AGC family of kinases (related to c**A**MP-dependent protein kinase 1, cyclic **G**uanosine monophosphate-dependent protein kinase and protein kinase **C**) (Bayascas, 2010), and control a plethora of cellular processes, downstream either to PI3K or to other pathways, such as RAS/MAPK. Interestingly, PDK1 has been demonstrated to be crucial for the regulation of each step of cell migration, by activating several proteins such as protein kinase B/Akt (PKB/Akt), myotonic dystrophy-related CDC42-binding kinases alpha (MRCKα), PAK1, Rho associated coiled-coil containing protein kinase 1 (ROCK1), phospholipase C gamma 1 (PLCγ1) and integrin β3 (Di Blasio et al., 2017; Gagliardi et al., 2015).

The family of the conserved AGC kinase superfamily includes 63 serine and threonine kinases with a common phylogenetic origin, such as PDK1, p70 ribosomal protein S6 kinase (p70S6K) (Pullen et al., 1998), serum/glucocorticoid regulated kinase (SGK) (Kobayashi and Cohen, 1999), p90 ribosomal protein S6 kinase (p90RSK) (Jensen et al., 1999) and the members of protein kinase C (PKC) family (Dutil et al., 1998; Gagliardi et al., 2017; Le Good et al., 1998; Leroux et al., 2017). Many of these kinases carry two phosphorylation sites that regulate their activation: one in the kinase domain, named activation loop; and the other in the hydrophobic motif. Importantly, phosphorylation of the activation loop of all these kinases is mainly mediated by PDK1. Phosphorylation of both regulatory sites leads to enzymatic full activation.

PDK1 is a soluble and globular medium-size protein of 556 amino acids constituted by an N-terminal catalytic domain and a C-terminal pleckstrin homology (PH) domain (Alessi et al., 1997; Di Blasio et al., 2017; Gagliardi et al., 2017). The N-terminal catalytic domain includes two important regulatory sites: the PDK1 interacting fragment (PIF) pocket, which is a hydrophobic pocket essential for PDK1 interaction with phosphorylated hydrophobic motif of some of its targeted protein (Biondi et al., 2000; Biondi et al., 2001; Collins et al., 2003); and the activation loop, containing the serine 241 (Fig. 14A, 14B and 14C). The phosphorylation of this serine is catalyzed by an auto-trans-phosphorylation reaction (Di Blasio et al., 2017; Gagliardi et al., 2015; Wick et al., 2003). Following the biochemical interaction between PDK1 and its substrates, PDK1 phosphorylates the activation loop on these substrates, consequently leading to their activation (Biondi et al., 2001; Gagliardi et al., 2017).

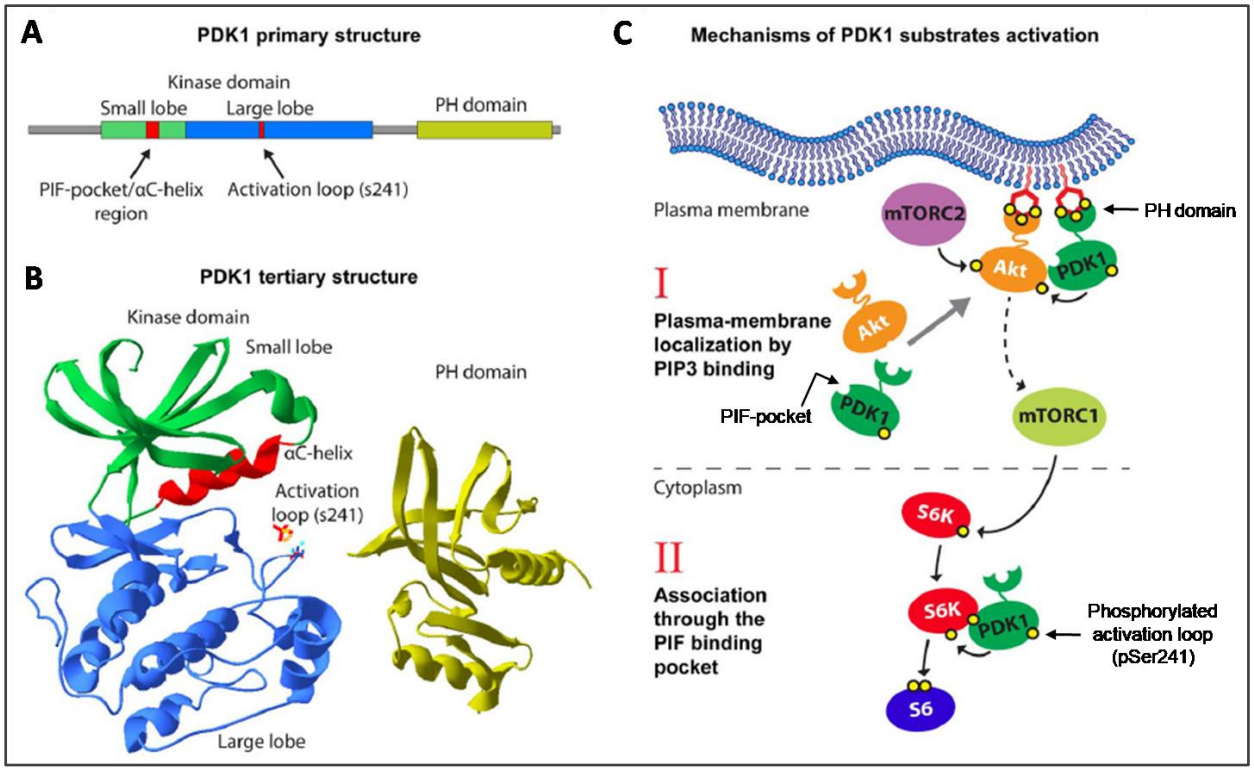

Fig. 14 | PDK1 structure and regulation. A. The primary structure of PDK1 protein (556 aminoacids long); **B.** 3D structures of the kinase domain (Protein Data Bank ID 4RRV) and the PH domain (Protein Data Bank ID 1W1G); **C.** PDK1 substrates phosphorylation is regulated by two different mechanisms. The first one (I) is responsible for the phosphorylation of Akt and depends on membrane localization. The second mechanism (II) is based on the binding of PDK1 PIF-binding pocket to the phosphorylated hydrophobic motif on its substrates (adapted from (Gagliardi et al., 2017).

Since phosphorylation on the serine 241 in the PDK1 activation loop is catalyzed by PDK1 itself, it is poorly accessible to phosphatases, making de-phosphorylation of this site very inefficient. For these reasons PDK1 has been considered constitutively active (Casamayor et al., 1999). The physiological role of PDK1 has been extensively investigated *in vivo* in murine models (Di Blasio et al., 2017). Knockout of PDK1 is lethal, indicating its requirement for normal embryo development (Lawlor et al., 2002). PDK1 knockout mice die at the E9.5 embryonic stage, showing lack of branchial arches, defects in neural crest-derived tissues and forebrain development, as well as defective assembly of a functional vascular system. To understand the role of PDK1 during development, hypomorphic mice for PDK1 have been generated, in which the expression of PDK1 is reduced by 80%–90% in all tissues. These mice are viable and show a decreased body size, but no significant differences in the activation of Akt, p70S6K, and p90RSK (Di Blasio et al., 2017). Notably, some of the defects found during development of knockout embryos might be due to deficient migration.

By studying these animal models, a number of roles of PDK1 were discovered, including the management of gastric acid secretion levels (McManus et al., 2004) and the

stimulation of the Na+/H+ exchanger via the serum- and glucocorticoid-inducible kinase 1 (SGK1) and the consequent regulation of the transport of electrolytes in the intestine (Mora et al., 2003). Decreased PDK1 activity leads to the distortion of amino acid transport in the jejunum and increased amino acid excretion in the urine, implying defective renal reabsorption and decreased transport of amino acids in the kidneys and the intestine of PDK1 hypomorphic mice (Emmanouilidi and Falasca, 2017).

➢ PDK1 role in cancer

It is well established that many tumors arise by the dysregulation of key signaling pathways. Due to its property to be the master regulator of AGC kinases, PDK1 is exactly situated in between the most commonly altered pathways in cancer (Gagliardi et al., 2015). The aberrant activation of PDK1 and many of its downstream effectors (Akt, p90RSK, p70S6K, PKC and SGK) has been reported to be involved in pathological phenotypes such as uncontrolled cell replication, apoptosis escape, metabolic reprogramming and abnormal angiogenesis (Arencibia et al., 2013; Gagliardi et al., 2017). The constitutively active state of PDK1 suggests that it cannot be activated further by genetic mutations and indeed, despite massive sequencing efforts of tumor genomes, no PDK1-activating mutations have been identified so far. However, many human tumors show high protein expression levels of PDK1, as consequence of gene amplification (Gagliardi et al., 2017). Indeed, PDK1 was found overexpressed both at protein and mRNA levels in tumors of different histological origins (Fig. 15) including breast (Maurer et al., 2009), prostate (Choucair et al., 2012), esophageal squamous cell carcinoma (Yang et al., 2014), primary and metastatic melanoma (Scortegagna et al., 2014) and acute myeloid leukemia (Zabkiewicz et al., 2014). Notably, PDK1 amplification or overexpression correlates with a more aggressive phenotype and worse prognosis. Evidence of the contribution of PDK1 to tumorigenesis comes from the experimental increase of PDK1 in cellular models. The ability to survive in the absence of adhesion is usually precluded for normal epithelial and also for several tumor cells (Gagliardi et al., 2015). However, in breast cancer cells it was observed that overexpression of PDK1 strongly increased adhesion-independent growth ability (Gagliardi et al., 2012; Xie et al., 2006; Zeng et al., 2002). Therefore, high PDK1 expression may contribute to the ability of cancer cell to survive in suspension in the blood stream and to grow in matrices different to the original basal membrane (Di Blasio et al., 2017).

Furthermore, PDK1 overexpression in cells causes an increased tumor growth *in vivo*, such as in human breast cancer cells xenografted in immunocompromised mice (Gagliardi et al., 2012). Likewise both motility and invasive ability of tumor cells are sustained by artificially increased expression levels of PDK1 (Gagliardi et al., 2014; Maurer et al., 2009; Xie et al., 2006).

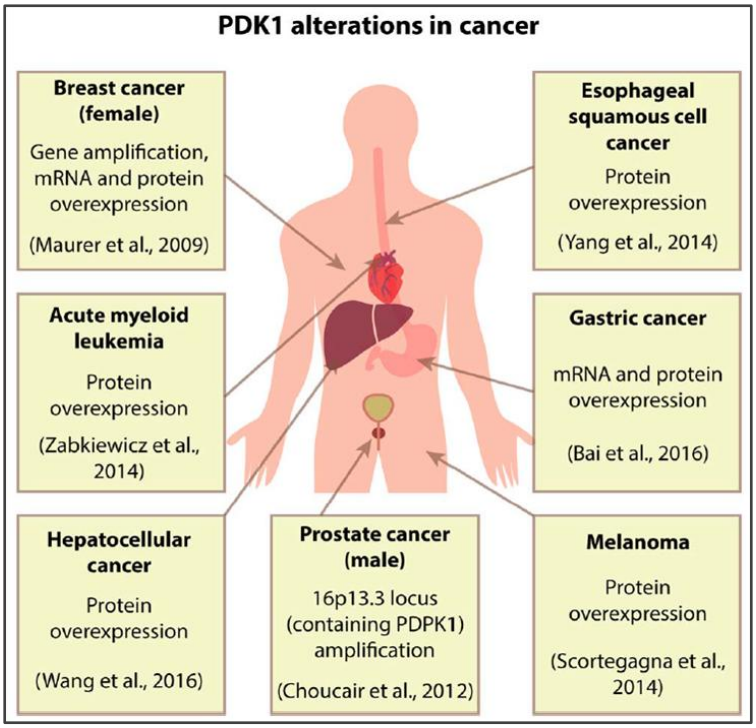

Fig. 15 | PDK1 alterations in tumors. The sketch shows PDK1 tumors alterations in each tissue of origin, including gene amplification, increased mRNA and protein expression. Data have been gathered by published PDK1 targeted studies indicated in the figure (Gagliardi et al., 2017).

PDK1 is a common effector of two of the pathways mainly involved in the development and progression of cancers: PI3K/Akt pathway and RAS/MAPK pathway (Bayascas, 2010). In fact, beside the canonical role of PDK1 in PI3K/Akt pathway, an increasing number of observations point at an involvement in RAS/MAPK pathway in cancer (Gagliardi et al., 2017). The most compelling evidence for the existence of a PDK1 role downstream of RAS comes from experiments in murine KRAS-driven pancreatic cancer. It has been shown that expression of $KRAS^{G12D}$ or $KRAS^{G12V}$ in the murine pancreas gives rise to lesions called pancreatic intraepithelial neoplasia (PanIN), which progress to metastatic pancreatic ductal adenocarcinoma (PDAC). In this murine model of pancreatic cancer, PDK1 has been found to play an important role in both pancreatic cancer initiation and progression. Indeed, PDK1 knockout in epithelial compartment of the pancreas completely blocks PanIN and PDAC formation. In contrast, deletion of PDK1 in a $KRAS^{G12D}$-driven non-small-cell lung carcinoma (NSCLC) model has no effect on lung

tumor formation (Eser et al., 2013). PI3K/Akt and RAS/MAPK pathways are therefore critical targets for therapy in different types of tumors. To this aim, a plethora of inhibitors has been developed to target their components. However, not less relevant, but much less explored, is the possibility to exploit PDK1 targeting to block the aberrant activation of these pathways (Gagliardi et al., 2017).

Moreover, PDK1 was identified as an upstream regulator of c-Myc in cancer. The activation of PDK1–PLK1–Myc signaling promotes cell growth, cell survival and cancer stem cell self-renewal in several cancer cell lines (Gagliardi et al., 2017; Tan et al., 2013).

Despite its importance in all these altered signal transduction pathways, PDK1 still remains an underestimated therapeutic target in cancer (Gagliardi et al., 2017). Indeed, accumulating evidence demonstrates that PDK1 is a valid therapeutic target and suggests that PDK1 inhibitors may be useful to prevent cancer progression and abnormal tissue dissemination (Raimondi and Falasca, 2011). Its targeting by newly developed small molecule inhibitors would provide a valid alternative for rational target therapies. However further studies are required to further increase the knowledge of the mutational context that would benefit from a PDK1 inhibition (Gagliardi et al., 2017).

Recently, it has been shown that PDK1 is directly or indirectly implicated in chemoresistance, with its inhibition resulting in re-sensitization of cancer cells to chemotherapeutic agents. It was observed in multiple types of cancer, such as ovarian cancer, breast cancer, and acute myeloid leukemia (Emmanouilidi and Falasca, 2017).

➢ PDK1 downstream effectors

As previously mentioned, PDK1 is a master kinase that phosphorylates the activation loop site and is required for the activity of at least 23 other AGC kinases, including Akt, SGK, S6K, some PKC isoforms and RSK (Leroux et al., 2017; Pearce et al., 2010; Raimondi and Falasca, 2011). All these proteins are activated downstream of a wide range of extracellular stimuli by distinct mechanisms (Pearce et al., 2010). The mechanism by which PDK1 activates some of these substrates is controlled by the binding specificity of the PH domain to the PIP_3 (Dieterle et al., 2014; Leroux et al., 2017). Instead, the activation of other AGC kinases (up to 20) by PDK1 requires the docking interaction of their hydrophobic motif with the PIF-pocket of PDK1 (Leroux et al., 2017). Moreover, PDK1 is able to transduce signals through kinase independent mechanisms, such as by activating ROCK1 (Pinner and Sahai, 2008) or MRCK (Gagliardi et al., 2014) or through Myc (Cunningham and Ruggero, 2013) and YAP (Fan et al., 2013).

In some cancer cases, PDK1 exerts its oncogenic properties through all these downstream effectors (Emmanouilidi and Falasca, 2017).

* PDK1 - Akt

Akt is a 57 kDa Ser/Thr kinase (Staal, 1987), also called PKB (Protein kinase B). To date, three human isoforms of Akt, coded by three different genes, have been identified, Akt-1, -2, and -3 (Coffer et al., 1998). They contain a PH domain in the N-terminus, a central kinase domain, and a C-terminal regulatory domain (Osaki et al., 2004). The N-terminal PH domain interacts with high affinity with PIP_3. This interaction leads to its recruitment to the plasma membrane and induces a major conformational change, which enables thephosphorylation of Thr308 in the activation segment of Akt by membrane-localized PDK1 (Pearce et al., 2010). Full Akt activation is achieved by phosphorylation of Ser473 within the hydrophobic motif via the mammalian target of rapamycin complex 2 (mTORC2) (Dieterle et al., 2014) (Fig. 16). Akt is also constitutively phosphorylated at its turn motif (Thr450 in AKT1), which stabilizes it and protects the hydrophobic motif from dephosphorylation (Hauge et al., 2007). Activated Akt is implicated in mediating a variety of biological responses including cell growth, proliferation, survival and protein synthesis, and it has also a key role in controlling metabolism (Cicenas, 2008; Pearce et al., 2010).
Several studies have found Akt2 to be amplified or overexpressed at them RNA level in various tumor cell lines and in a number of human malignancies such as colon, pancreatic and breast cancers. Nevertheless, activation of Akt isoforms by phosphorylation appears to be more clinically significant than Akt2 amplification or overexpression (Cicenas, 2008).

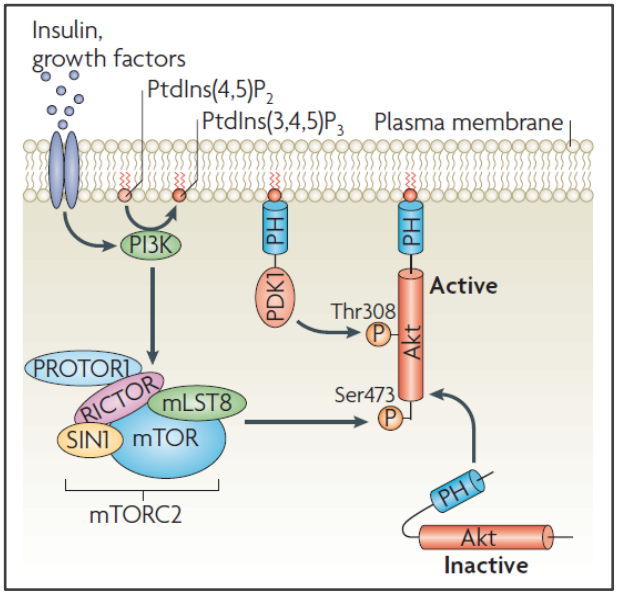

Fig. 16 | Mechanism of activation of Akt. Phosphoinositide 3-kinase (PI3K) is activated downstream of receptor tyrosine kinases, growth factor receptors and the insulin receptor. The generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) by PI3K recruits Akt to the membrane. Here, it is phosphorylated by 3-phosphoinositide dependent kinase (PDK1), and mammalian target of rapamycin complex 2 (mTORC2)-mediated phosphorylation is required for full Akt activation. PH, pleckstrin homology; PROTOR1, protein observed with RICTOR 1; RICTOR; rapamycin-insensitive companion of TOR; SIN1, SAPK-interacting protein kinase 1 (Pearce et al., 2010).

∗ PDK1 - S6K and PDK1 - SGK

Similarly to Akt, the kinases p70S6K and serum- and glucocorticoid-induced protein kinase (SGK) are activated by phosphorylation of their activation segment and hydrophobic motif in response to agonists such as insulin and growth factors. S6K activity, unlike SGK or Akt isoforms, is stimulated by nutrients such as amino acids, and is suppressed by low cellular energy levels (Pearce et al., 2010). However, they lack of a PH domain. S6K isoforms are phosphorylated at their hydrophobic motif (on Thr389) by mTORC1 (Hara et al., 2002; Kim et al., 2002) following PI3K activation, whereas SGK1 is phosphorylated by mTORC2 (on Ser422) (García-Martínez and Alessi, 2008; Pearce et al., 2010). The hydrophobic motif phosphorylation alone is not sufficient for activate p70S6K and SGK, but regulates their interaction with PDK1 (Pearce et al., 2010). PDK1 binds to the phosphorylated hydrophobic motif via its PIF-pocket and consequently phosphorylates their activation segment and thereby activates these kinases (Dieterle et al., 2014; Pearce et al., 2010).
S6K has a vital role in controlling protein synthesis. Although the roles of SGK are not well defined, SGK can stimulate sodium transport into epithelial cells by enhancing the stability and expression of the epithelial sodium channel (Loffing et al., 2006; Pearce et al., 2010).

∗ PDK1 - PKC

There are 12 isoforms of PKC termed conventional (PKCα, PKCβ and PKCγ), novel (PKCδ, PKCε, PKCη and PKCθ), atypical (PKCζ and PKCλ) and PKN and PKC-related (PKN1, PKN2 and PKN3) forms. PKC isoforms are also activated following the phosphorylation of the hydrophobic motif and activation segment by mTORC2 and PDK1, respectively. Phosphorylated PKC is maintained in an inactive state (House and Kemp, 1987) and its full activation occurs through the binding to the second messengers diacylglycerol and Ca^{2+}. PKC isoforms are differentially sensitive to these second messengers. They have a wide range of functions and mediate the effects of growth factors and hormones (Pearce et al., 2010).
During melanoma initiation, PDK1 is the intermediate for the PKC regulation by the TIMP metallopeptidase inhibitor 1 (Timp1), and in later stages of progression and metastasis, it promotes resistance to *anoikis* (Toricelli et al., 2017).

* PDK1 - RSK

The best characterized RAS effector pathway is the RAF-MEK-ERK pathway (MAPK cascade) (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). p90 ribosomal protein S6 kinase (RSK) is one of the downstream effectors of these pathway. RSK controls cell growth, cell proliferation, cell motility, protein synthesis and survival (Carriere et al., 2008). RSK family of protein is a group of highly conserved Ser/Thr kinases and includes 4 isoforms, coded by 4 different genes (Anjum and Blenis, 2008). The most striking feature of RSK is the presence of two functional and non-identical phosphotransferase domains within the same polypeptide (Fisher and Blenis, 1996; Jones et al., 1988). The kinase domains are connected by a linker region containing essential regulatory sites, including hydrophobic motif. After the activation of MAPK cascade, ERK1/2 binds RSK on a specific consensus sequence (called KIM, Kinase interaction motif) at the C-terminal tail (Gavin and Nebreda, 1999; Smith et al., 1999) (Fig. 17A) and phosphorylates two residues in the hydrophobic motif and one in the C-terminal kinase domain (CTKD). The phosphorylation on CTKD causes a conformational rearrangement in the active form, which phosphorylates a serine (Ser380 in human RSK1 or Ser386 in human RSK2) in the hydrophobic motif, the docking site for PDK1. As a consequence, PDK1 binds the phosphorylated hydrophobic motif through its PIF pocket and then phosphorylates a serine (Ser221 in RSK1 or Ser227 in RSK2) in the activation loop of N-terminal kinase domain (NTKD) (Jensen et al., 1999). RSK is now fully active and it phosphorylates its own substrates. NTKD was also described to phosphorylate a serine in the C-terminal tail of RSK causing RSK inactivation (Anjum and Blenis, 2008) (Fig. 17B). Activation of RSK occurs in the absence of the PH domain of PDK1, indicating that RSK activation is not restricted to PIP3-containing membrane domains (Jensen et al., 1999; McManus et al., 2004). Activated RSK phosphorylates several transcription factors, such as NF-*κ*B (nuclear factor *κ*B), regulating growth-related transcription initiation. RSK controls cell proliferation through the regulation of mediators of the cell cycle. RSK2 was shown to promote cell cycle progression by phosphorylating c-Fos, a transcription factor that positively regulates expression of cyclin D1 during G1/S transition (David et al., 2005). Moreover, RSK was shown to phosphorylate Raptor, an important mTORC1 (mTORcomplex 1) scaffolding protein, providing a link between the Ras/MAPK and mTORsignalling pathways (Carriere et al., 2008). RSK may also regulate mRNA translation through the phosphorylation of GSK3*β* (Angenstein et al., 1998; Sutherland et

al., 1993). Phosphorylation by RSK on Ser9 inhibits GSK3*β*kinase activity and thereby releases inhibition of the translation-initiation factor eIF2B (Wang et al., 2002).

RSK1 and RSK2 recently emerged as essential regulators of tumorigenesis. Overexpression of RSK2 was demonstrated to increase proliferation as well as anchorage-independent transformation (Cho et al., 2007). Targeting RSK in cancer may be avaluable and more specific alternative to the inhibition of upstream components of theRas/MAPK pathway, such as MEK1/2 (Romeo and Roux, 2011).

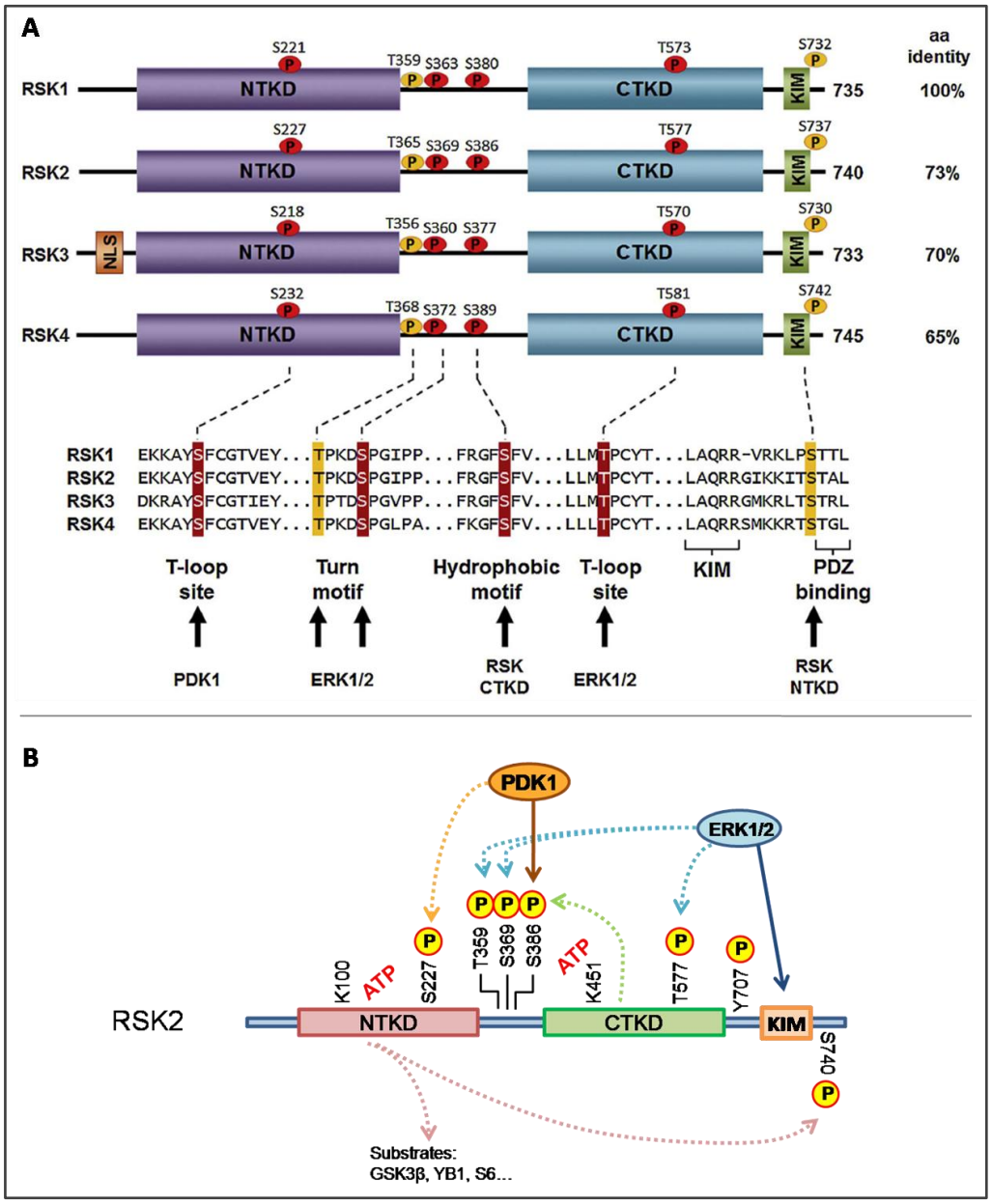

Fig. 17 | Structural features, phosphorylation sites and mechanism of activation of RSKs. A. Top, schematic representation of RSK. RSK1-4 are characterized by the presence of two functional domains, the N-terminal kinase domain (NTKD) and the C-terminal kinase domain (CTKD), which are connected by a linker region. The C-terminal end contain an extracellular signal-regulated kinase 1/2 (ERK1/2)-docking domain resembling a KIM motif. In addition, RSK3 contains a nuclear localization signal (NLS) in its N-terminal region. Activation of RSK is associated with increased phosphorylation at six sites (each shown as colored circles). Middle, these phosphorylation sites are highly conserved in RSK1–4 and were shown to be

either essential (red) or accessory (orange) for RSK activation. Bottom, conserved phosphorylation sites required for RSK activation and their phosphorylating kinases. The extreme C-terminus comprises a PDZ-binding motif. Right, percentage of amino-acid identity refers to human RSK1; **B.** The RSK protein kinases are activated after direct phosphorylation mediated by ERK1/2 and PDK1 following activation of the Ras/MAPK pathway. This leads to phosphorylation of functionally diverse RSK substrates in the cytosol as well as in the nucleus following its translocation (Houles and Roux, 2017).

* PDK1 - Myc

Myc is a well-studied oncogene and its respective protein is involved in the ability of the cancer cells, as well of the stem cells, to self-renew (Wong et al., 2008). PDK1 directly phosphorylates the threonine 210 of Polo-like kinase 1 (PLK1) activation loop, which is also upregulated in many cancers. Activated PLK1 phosphorylates c-Myc on serine 62 promoting its stability and accumulation in cancer cells (Gagliardi et al., 2017; Tan et al., 2013) (Fig. 18). PDK1–PLK1–Myc signaling axis was also shown to drive resistance to target therapy. Indeed, PDK1-mediated stabilization of c-Myc is responsible for the resistance to the mTOR inhibitor Rapamycin in CRC, when PI3K/Akt/mTOR pathway is inhibited by a drug (Tan et al., 2010). As a consequence, combinatorial treatment with mTOR and PLK1 inhibitors shows a synergistic effect on CRC cells, preventing compensatory c-Myc induction (Tan et al., 2013). Moreover, Myc-driven breast cancer is shown to be more responsive to PDK1/PLK1 inhibitors than is Myc-independent one, and taking into consideration that a Myc inhibitor is not currently available in the clinic, targeting the PDK1-PLK1-Myc axis reveals a new potential therapeutic approach against Myc-induced cancers (Emmanouilidi and Falasca, 2017).

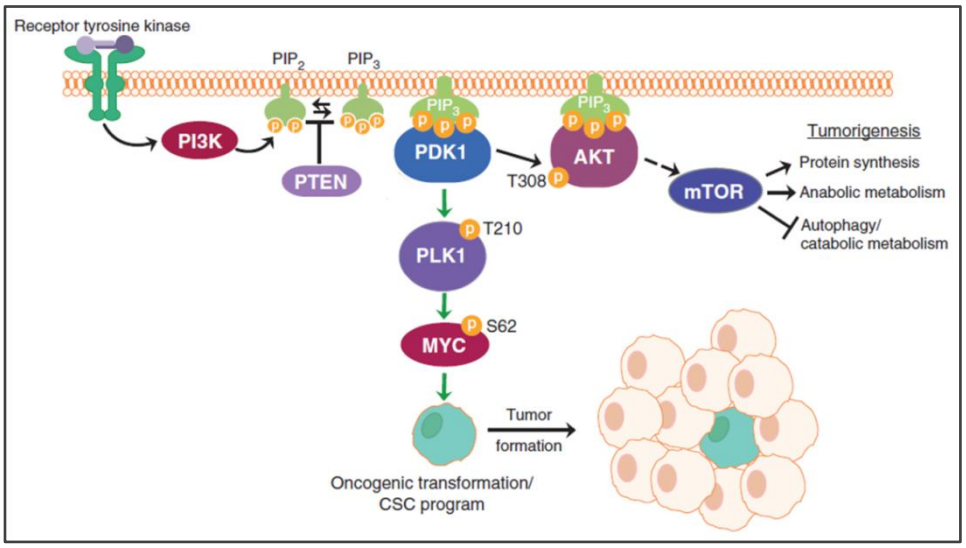

Fig. 18 | Classical and novel PI3K signaling pathway. Tumors with hyperactive PDK1 signaling, in addition to signaling through the classical downstream effector AKT, induce a novel signaling pathway through phosphorylation of PLK1. Phosphorylation and activation of PLK1 results in phosphorylation of serine 62 of MYC, leading to stabilization of MYC protein, which promotes cellular transformation, in part

through transcriptional upregulation of a cancer stem cell (CSC)-like gene signature (adapted from (Cunningham and Ruggero, 2013).

Target Therapy and Precision Medicine

Genetic and molecular profiling of tumor specimens have revealed potential targets for personalized anticancer therapy and seen a shift toward an emerging molecular taxonomy of cancer (Biankin and Hudson, 2011; Moris and Pawlik, 2017).

Like many cancers, colorectal liver metastasis (CRLM) is a heterogeneous malignant disease probably due to variabilities in genomic profile, molecular and signal transduction network, and microenvironment discrepancies (Kawamata et al., 2015).

Multidisciplinary management of patients with CRLM represents the best strategy to increase their survival. Future advances for these patients will depend on a better understanding of genomics and molecular biology to facilitate characterization of a specific tumor “identity”. Hence, the individualized treatment for each CRLM patient will be possible (Moris and Pawlik, 2017).

The development of novel and effective therapeutic strategies require the use of CRC models that recapitulate key features of human disease. Classic methods of modeling CRC such as human cell lines and xenograft mice, despite being useful for many applications, carry significant limitations. Recently developed *in vitro* models, such as three-dimensional "organoid" culture systems, and *in vivo* models overcome some of these deficiencies (Golovko et al., 2015).

➢ Cell lines

Cell lines are useful tools in the study of cell biology and in the development and testing of new therapeutic modalities (Park et al., 2004). They have played a significant role in elucidating signaling pathways in cancer since the derivation of the HeLa cervical cancer line in 1951 (Golovko et al., 2015). A large bank of well-characterized cell lines should reflect the diversity of tumor phenotypes and provide adequate models for tumor heterogeneity. CRC cell lines can be developed from ascitic effusions, metastatic tissues (regional lymph nodes and distant metastatic sites, such as liver), and primary tumors (Park et al., 2004). Human cell lines are commercially available, inexpensive to use, and provide rapid experimental results. For all these reasons, they have been extensively used in drug discovery (Shoemaker, 2006). Most of the widely used cell lines have been genomically characterized and represent the genetic landscape of human CRC. Thus, a

panel of lines with activating KRAS mutations can be easily compared to a panel lines with wild-type KRAS. Alternatively, cell lines can be genetically manipulated through homologous recombination, short hairpin RNA (shRNA) gene knockdown, or CRISPR-Cas9 gene editing (Golovko et al., 2015). However, this model presents several important limitations. Cell lines represent a clonal population of tumor cells which are selected to grow in culture plates and media. Therefore, they likely differ from the original tumor and do not recapitulate the functional and genetic heterogeneity of human cancers, which is a significant factor in resistance to targeted therapies (Gerlinger et al., 2012). Furthermore, cell lines are difficult to create from individual patient tumors and cannot be derived from matching normal tissue. Thus, traditional cancer cell lines are not well suited for personalized clinical application (Golovko et al., 2015).

- Mouse models

The most common *in vivo* model is the patient-derived xenograft (PDXs) (Golovko et al., 2015). In the PDX model, portions of patient tumor tissue are obtained during surgery and implanted into an immunodeficient mouse (P0). Once the tumor is grown, it is surgically removed and implanted into other mice (P1). This is repeated until enough animals are obtained for the experiment (i.e., P2, P3, etc.). Notable, in PDXs tumor stroma will grow with the tumor cells, thus allowing for tumor-stroma cross-talk (Rosfjord et al., 2014). Therefore, these i*n vivo* systems are essential to assess the role of the tumor microenvironment, host immune system, and angiogenesis in tumor response to therapy (Golovko et al., 2015).

Mouse models of CRC were first used in 1928 and have played an important role in understanding CRC biology, providing insights into pathogenesis mechanisms (Karim and Huso, 2013). Commonly used mouse strains include classically nude (athymic) and severe combined immunodeficient (SCID) mice, which are devoid of T lymphocytes (Croy et al., 2001) or both B and T lymphocytes, respectively (Blunt et al., 1996). NOD/SCID mice, unlike SCID mice, also have deficient NK cells. Injection of human CRC cells subcutaneously into an immunodeficient mouse typically results in growth of tumor at the injection site (Golovko et al., 2015). This is currently the best available model to study prevention strategies targeting early events in CRC development. Many models have been developed to monitor the invasiveness and metastasis of the implanted or injected tumors (Nanda et al., 2006), but one disadvantage is that progression to malignant cancer and metastases occur late in the course of disease, so it is infrequently observed (Halberg et

al., 2000; Moser et al., 1990). Moreover, the use of immunodeficient animals eliminates the important role of the host immune system in tumorigenesis. Given the difference in species, interactions between cancer cells and stromal cells cannot be studied (Golovko et al., 2015).

Cell line xenografts consist instead in the injection of a large and homogenous cell population in order to form tumor. Therefore, this model does not reflect the cellular heterogeneity seen in most human CRCs (Golovko et al., 2015), and the tumor development is not exactly the same as human CRC development due to species differences (Nanda et al., 2006). In addition, cells in xenografts are typically injected into the subcutaneous space, a microenvironment that is very different than the tumor original site (Golovko et al., 2015). Therefore, orthotopic implantation has been used to produce a model more similar to human cancers than subcutaneous xenografts. In this model, in the case of CRC, the implant (colon cancer cell lines) is directly placed into serosal surface of the cecum (Céspedes et al., 2007; Nanda et al., 2006). The advantage is that the metastatic site (local lymphatics, lung, and liver) can be monitored by imaging. The disadvantage is that the orthotopic implantation is a challenging procedure and can be associated with inflammation of the implanted site if stringent surgical technique is not followed (Karim and Huso, 2013). A colonoscopy system has therefore been developed for implanting human colorectal cancer cells into the mouse colonic submucosa. This model is non-invasive, fast, and is not associated with significant inflammation (Zigmond et al., 2011). Finally, tumors derived from transplanted CRC cell lines do not recapitulate the histological features of human cancer. Perhaps due to all these factors, drug response in xenograft models correlate poorly with drug response in clinical trials (Voskoglou-Nomikos et al., 2003).

On the contrary, PDXs preserve important aspects of tumor histology, vascularity and architecture of primary CRCs (Golovko et al., 2015). In our Institute, samples from hepatic metastatic colorectal cancer (mCRC) were xenografted in NOD-SCID mice in order to build a platform of PDXs to test their different sensitivity to target therapies. PDXs were validated as faithful representatives of the original tumors, able to retain their genetic and phenotypic features across multiple serial passages (Bertotti et al., 2011). Indeed, important driver mutations appear to be consistent along passages of PDXs, such as *KRAS* and *PIK3CA* (Tignanelli et al., 2014), and these mouse models are therefore an essential tool used to unveil novel CRC driver genes with therapeutic potential (Clark and Starr, 2016). Furthermore, PDX models predict clinical response to therapy (Hidalgo et al.,

2011; Siolas and Hannon, 2013; Voskoglou-Nomikos et al., 2003), and an important application of PDXs is the personalized cancer treatment. Following surgery, an individual patient's cancer might be implanted into a mouse, passaged, and studied with various chemotherapy agents to determine a clinical approach to treat that patient (Hidalgo et al., 2011).

➢ Patient-derived organoids

Monolayer cell cultures were established more than 40 years ago. Nevertheless, a key limitation of traditional monolayer cell culture is that normal tissue cannot be efficiently cultured normally. The Clevers lab recently described a technology consisting of an *in vitro* three-dimensional (3D) culture model in which murine intestinal crypts (characterized by self-renewing stem cells) are cultured in three-dimensional matrix (mimicking the *in vivo* environment) and specific media containing Wnt3, the Wnt activator R-spondin1, the BMP inhibitor Noggin, epidermal growth factor (EGF) and other growth factors. The crypts grow into "mini-intestines" or organoid structures that contain stem cells and the differentiated cell types of the intestine (Golovko et al., 2015; Sato et al., 2011a) (Fig. 19). This technology therefore allows the establishment of long-term stem cell-based organotypic cultures in the absence of feeder cells by supplementing the culture medium with well-defined stem cell niche factors. A variety of different organoids culture methods have been described over the past years (Kretzschmar and Clevers, 2016). Single CRC cells can also be immobilized in Matrigel and their clonal CRC organoids can be tracked on a real time basis (Fig. 20), which may enable visualization of self-renewal of CSCs in a dish. Their clonal expansion capacity could be applied to various biomedical analyses including deep sequencing. Combined with integrated molecular information, establishing "living biobanks" would be a useful resource for both basic research and clinical applications (Ohta and Sato, 2014).

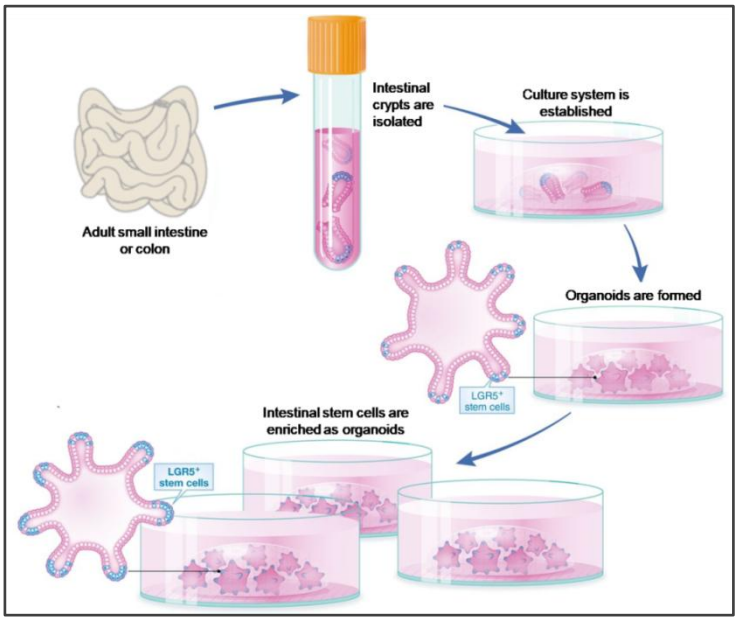

Fig. 19 | A schematic of the process from harvesting of the intestinal crypt to organoid formation. Intestinal stem cells (ISCs) can be isolated from intestinal epithelium and enriched *in vitro* as organoids. They are embedded into a 3D

matrix (like Matrigel) and cultured in the presence of niche factors, for example, EGF, Noggin, and R-spondin-1. Organoids generated by this approach most closely resemble the adult organ of origin containing differentiated cell types (adapted from (Holmberg et al., 2017).

The identification of leucine-rich repeat containing G-protein-coupled receptor 5 (Lgr5) as intestinal stem cell-specific marker gene allowed the characterization and the purification of the stem cells and, subsequently, the understanding that these adult intestinal stem cells can be both proliferative and long lived *in vitro* (Barker et al., 2007; Kretzschmar and Clevers, 2016). FACS (fluorescence activated cell sorting)-sorted stem cells from *Lgr5-EGFP-creERT2* mice (in which Lgr5+ stem cells are labeled with GFP) could be cultured and led to the derivation of epithelial organoid cultures (Golovko et al., 2015; Sato et al., 2009). Stem cell proliferation first creates cystic spheroids, which then forms crypt-like structures that within 2 weeks further develops into ‘‘mini-guts’’ with distinct crypt-villus compartmentalization as seen *in vivo* (Kretzschmar and Clevers, 2016).

Primary intestinal organoids could be maintained in culture for greater than 1.5 years (Sato et al., 2011a). The Clevers lab also reported cultured of murine colon organoids (Sato and Clevers, 2013). Intestinal organoids have been extensively used as *in vitro* study models of normal intestinal functions (Golovko et al., 2015).

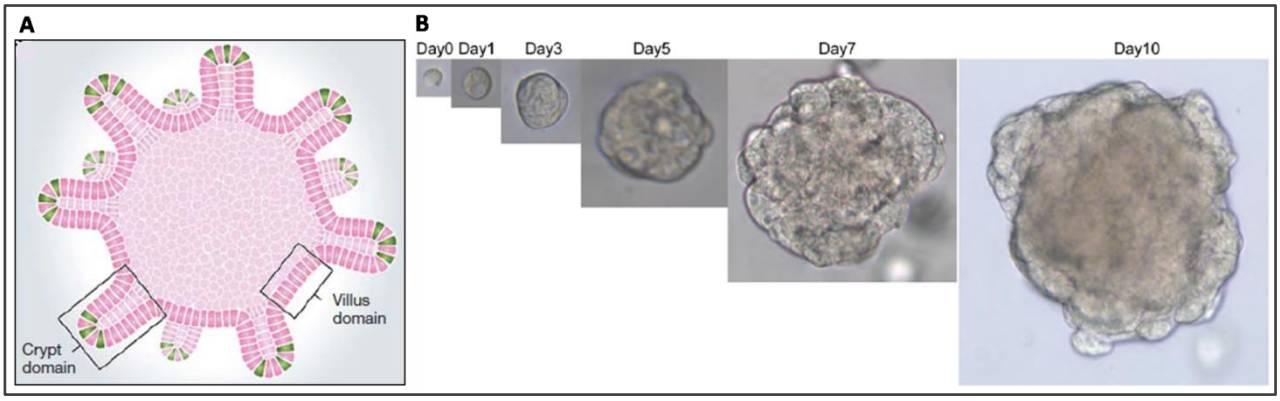

Fig. 20 | Three-dimensional *in vitro* cultures of intestinal organoids A. Schematic representation of an intestinal organoid with the lumen corresponding to the intestinal lumen and crypts (Schuijers and Clevers, 2012); **B.** Time course culture of human colon adenocarcinoma cells (Sato et al., 2011a).

Currently, organoids are also being established from a variety of tumors with colorectal cancer leading the way. They can be directly derived from patient tumor samples or from samples propagated and amplified in mice as patient-derived xenografts (PDXs) (Sato et al., 2011a) (Fig. 21). In CRC organoid culture condition, the success rate of establishing culture is superior to that of previously reported culture systems (Ohta and Sato, 2014). This innovative culture approach of tumor cells is aimed to preserve *in vitro* the tumor features present *in vivo*, allowing to perform more rapid and variegated studies, including mid-throughput screenings and multiple drug combinations, and thus more translational

studies (Sato et al., 2011a). Organoids could be cultured from different sections of tumors to model tumor heterogeneity, or from primary and metastatic sites to identify mechanisms of cancer metastasis (Golovko et al., 2015).

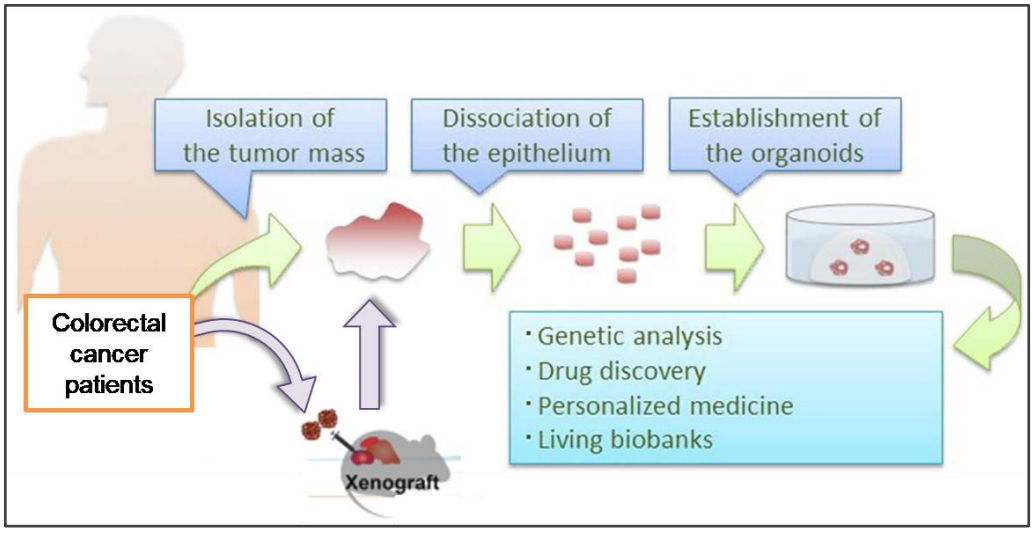

Fig. 21 | Development and application of CRC organoid technology. Patient-derived CRC organoids are applied to basic and clinical research: deep sequencing of pure epithelial cancer cells, drug development, prediction of clinical responses inpatients, and establishment of living biobanks (adapted from (Ohta and Sato, 2014).

Moreover, CRC organoids have been derived from murine *APC*-deficient intestinal adenomas (Barker et al., 2009; Onuma et al., 2013). *APC* can also be deleted *in vitro* in organoids by shRNA knockdown or CRISPR/Cas9 editing (Li et al., 2014; Schwank et al., 2013). The adenoma-carcinoma sequence in human CRC has been modeled by isogenic human organoids obtained after the CRISPR/Cas9-mediated editing of *APC*, followed by deletion of *SMAD4*, and *TP53*, and introduction of activating mutations in *KRAS* and *PIK3CA* (Drost et al., 2015; Matano et al., 2015).

The Clevers lab has developed libraries of organoids grown from different tissues and tumors, specially from human CRCs and the adjacent normal colon. Thanks to this platform, they are able to conduct *in vitro* drug testing and select therapeutic agents, with efficacy in the tumor organoid that are not toxic to the normal organoid, for further study (van de Wetering et al., 2015). Thus, organoid culture of malignant and normal colorectal tissue is a novel platform for translating tumor genetic data into personalized therapy (Golovko et al., 2015).

AIM of the STUDY

Colorectal cancer (CRC) is one of the most common neoplastic disease in the worldwide and approximately 20% of patients already show a metastatic stage at the time of diagnosis. The development of metastatic CRC is relatively well understood, and originates from the progressive accumulation of genetic and epigenetic alterations that drive the transformation of healthy colon epithelial cells into an aberrant phenotype. Specific mutations can activate intracellular signaling pathways involving in colorectal carcinogenesis, as RAS/MAPK and PI3K/AKT pathways. Indeed, both these two pathways are frequently altered in this tumor. In particular, KRAS is a proto-oncogene mutated in about 40% of CRC cases.

At present, anticancer therapies for CRC include surgery, radiation, chemotherapy, and anti-VEGF or anti-EGFR monoclonal antibodies. However, all of the clinical studies conducted to date have shown that these treatment modalities are not curative in patients with metastatic CRC, especially in those whose tumors express mutant KRAS. For these reasons, one possible successful therapeutic strategy could be to selectively target molecular pathways active in colon cancer cells, like KRAS and its effectors. Unfortunately, for the past few decades, efforts to target RAS directly have been unsuccessful in generating drug therapies. Therefore, there is an urgent need to identify new therapeutic strategies for targeting mutated KRAS and its related activated downstream effectors in CRC.

3-phosphoinositide-dependent kinase 1 (PDK1) is a serine/threonine kinase discovered as the kinase able to phosphorylate and activate AKT. During the past years several studies have pointed the attention on this protein mainly in breast cancer, where it is frequently found amplified and overexpressed. PDK1 targeting has been proven to be a tool to counteract tumor growth, invasion and metastatization of this type of cancer, even in the presence of KRAS mutations (Gagliardi et al., 2012). Notably, PDK1 has also been found to be amplified in prostate cancer and iperactivated in melanoma. Moreover, it has been shown that pharmacological inhibition of PDK1 blocked the growth of KRAS-driven pancreatic cancer in transgenic mice (Eser et al., 2013). However, PDK1 still remains an underestimated therapeutic target in cancer and only few studies have pointed the attention at targeting it in colorectal cancer.

Based on the data reported in literature and that obtained in our previous research, we hypothesized that PDK1 could be involved in KRAS-driven tumorigenesis. We decided to test our hypothesis by studying the efficacy of PDK1 targeting on CRC, where KRAS is frequently mutated and PDK1 role has never been investigated. Therefore, the present study is aimed at investigating PDK1 role in CRC growth, survival and tumor progression by observing the effects of its inhibition by small molecules or silencing.

As a starting approach, we decided to study the behavior of KRAS-mutated colon cancer (CRC) cell lines. We inhibited PDK1 in these cells and we decided to investigate the possibility that the blockade of PDK1 kinase activity could hamper the ability of CRC to grow in the absence of anchorage, a condition that correlates with the ability of cells to survive in a hostile environment and to form metastasis.

The second aim of our research has been to understand the molecular mechanism involved in PDK1 signaling transduction downstream of mutated KRAS. To achieve this aim, we focused our attention on Akt and RSK, which are direct substrates of PDK1 and are involved in PI3K and KRAS/MAPK signaling pathways, respectively. Upon our analysis, we decided to exclude the possible involvement of Akt, and we focused on RSK which is necessary for the growth in the absence of adhesion of all CRC cell lines. Therefore, to demonstrate the role of RSK as PDK1 downstream effector in tumor cells, we designed and cloned a specific RSK phosphomimetic mutant, whose activation was independent from PDK1, to rescue the PDK1 inhibition. To our surprise, we discovered that this mutant was inactive. We therefore performed a molecular dynamic simulation of the N-terminal kinase domain (NTKD) structure of both wild type and mutated RSK2, in order to identify the chemical interactions responsible for the maintenance of the proper configuration of the wild type protein and that established instead in the phosphomimetic protein. The interactions established in the mutant were different and destabilized the structure of the NTKD making it inactive. Thus, we demonstrated that it is not possible to decouple RSK activation from phosphorylation mediated by PDK1.

While cell lines represent a powerful tool for research, they lack a few important features when compared to more realistic models, notably the three-dimensional (3D) morphology and the ability to preserve stem cell hierarchies. To fully consider these important features, we established a more realistic model of CRC, i.e. a 3D *in vitro* culture of mutated KRAS metastatic CRC (mCRC) patient-derived organoids (PDOs), starting from mCRC patient-derived xenografts (PDXs), and we investigated the effects of PDK1 inhibition on their

viability, growth and morphology. Specifically, our aim was to test the small molecules inhibiting PDK1-kinase activity on a selected panel of KRAS-mutated PDOs, on which performing an accurate analysis of their size, metabolic activity, proliferation, apoptosis and morphology.

The results gathered in this project may lead to the definition of new therapeutic opportunities in more advanced CRC cases, where conventional treatments usually fail, proposing PDK1 as a new possible target downstream to oncogenic *KRAS*.

Cell cultures

Cell lines were handled using sterile conditions to avoid contaminations. Cell lines manipulation was carried out in a laminar flow cabinet in which air is drawn through high efficiency particulate (HEPA) air filters and using sterile supplies. HeLa and 293T cell lines were cultured in DMEM medium supplemented with 10% FCS, penicillin, streptomycin and L-glutamine. Established colorectal cancer (CRC)cell lines were used as research models were provided by ATCC (American Type Culture Collection). Colon cancer cells were cultured as monolayers in 100 mm dishes and fed with different cell cultured media. DLD1, HCT116, SW620 were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FCS), antibiotics (penicillin and streptomycin) and glutamine. SK-CO1, LS513, HCC2998 were cultured in RPMI-1640 Medium supplemented with 10% FCS, penicillin, streptomycin and glutamine. SW48 was cultured in Dulbecco's Modified Eagle Medium(DMEM) supplemented with 10% FCS, penicillin, streptomycin and glutamine. Cells were maintained in humidified cell culture incubators at 37°C and 5% CO_2.

Chemical compounds

PDK1 inhibitors BX795, BX912 and GSK2334470, AKT inhibitor MK2206 and RSK inhibitor BI-D1870 were purchased from Sigma-Aldrich and dissolved in DMSO to a final concentration of 10 mM. Then, the obtained solutions were aliquoted and stored at 20°C.

Plasmid constructs and lentivirus production

Lentivirus vectors were produced as described previously (Gagliardi et al., 2012). In brief, for PDK1 stable silencing, three pLKO.1 lentiviral vectors carrying PDK1 targeting shRNA called, respectively, shPDK1 #79 (TRCN0000039779; Sigma-Aldrich), shPDK1#81 (TRCN0000039781) and shPDK1#82 (TRCN0000039782) were used. A vector leading the expression of a scrambled not targeting shRNA, called shScr, Addgene plasmid 1864 (Sarbassov et al., 2005), was used as a negative control. Cell infection was performed with an MOI equal to 1, in the presence of 8 µg/ml Polybrene (H-9268; Sigma-Aldrich).
The procedure to obtain the lentivirus expressing the KRAS WT and KRAS G13D mutation has been described elsewhere (Di Nicolantonio et al., 2008).

To mutate RSK1 and RSK2, the following primers were designed:
RSK1S221D FW 5'-CCACGAGAAGAAGGCCTATGATTTCTGCGGGACAGTGG-3';
RSK1 S221D RE 5'-CCACTGTCCCGCAGAAATCATAGGCCTTCTTCTCGTGG-3';
RSK1 K210A FW 5'-CTGACTTTGGCCTGAGCGCAGAGGCCATTGACCACGA-3';
RSK1 K210A RE 5'-TCGTGGTCAATGGCCTCTGCGCTCAGGCCAAAGTCAG-3';
RSK2 S227D FW 5'-CCATGAAAAGAAGGCATATGATTTTTGTGGAACTGTGG-3';
RSK2 S227D RE 5'-CCACAGTTCCACAAAAATCATATGCCTTCTTTTCATGG-3'.
Then, RSK1WT, RSK1S221, RSK1K210A, RSK2WT, RSK2S227D and RSK2K100A (provided by Professor Ramos JW, (Gawecka et al., 2012) mutants were cloned into p304 plasmid to obtain their overexpression in HeLa cells. These mutants were also cloned in pDONR/zeo using Gateway BP Clonase (Thermo Fisher Scientific). Then, these constructs were transferred through Gateway LR Clonase in the pDEST27 (GST-N-Term Tag, Thermo Fisher Scientific) to obtain fusion proteins.

Western blot

In order to evaluate PDK1 inhibition in CRC cell lines, cells were treated with different conditions: 1) normal medium overnight; 2) normal medium overnight followed by a short stimulation with Epidermal Growth Factor (EGF, 10 ng/ml for 5 min); and 3) normal medium supplemented with the PDK1 inhibitor BX795 (3 μM) overnight, followed by a short stimulation with EGF (10 ng/ml for 5 min). Furthermore, to verify RSK2 regulation and activation, HeLa cells overexpressing or not RSK2WT were treated with following conditions: 1) serum free (SF) medium overnight; 2) SF medium overnight, followed by a short stimulation with EGF (10 ng/ml for 5 min); and 3) SF medium supplemented with the PDK1 inhibitor GSK2334470 (3 μM) overnight, followed by a short stimulation with EGF (10 ng/ml for 5 min). Moreover, to test RSK2 mutants activation, HeLa cells overexpressing or not RSK2WT, RSK2S227D or RSK2K100A were cultured in serum free medium overnight, and then treated or not with phorbol myristate acetate (PMA, 60 ng/ml) or EGF (10 ng/ml) for 5 minutes. Finally, to test RSK1 mutants activation, HeLa cells overexpressing GST, GST-RSK1WT, GST-RSK1S221D, GST-RSK1S72A and GST-RSK1K210A were cultured in serum free medium overnight and then treated or not with EGF (10 ng/ml for 5 min). After treatment, cells were washed twice with PBS 1X and lysed with 300 μl of “Lysis Buffer” (0.5M Tris-HCl pH 6.8, 10% SDS, 10% Glycerol). Lysates were then sonicated for 10 sec, in order to break DNA strand and to reduce viscosity. After that, samples were heated at 95°C for 5 min. 10 μl each sample, were used for BCA

quantification at 562 nm wavelength and a variable volumes (corresponding to 5 or 10μg of proteins), were loaded in 7.5% SDS-PAGE wells. Proteins wererun in polyacrylamide gel at constant voltage (200V) for 35 min. Then, proteins were transferred on a PVDF membrane, using Trans-Blot Turbo BIORAD. The PVDF membranes were let completely dry, and then hydrated with TBS (Tris Buffer Saline) 0.1% Tween for 30 min at room temperature (RT). Afterwards, membranes were incubated with primary antibody (Ab) under gentle agitation overnight at 4°C. Polyclonal Ab rabbit anti-pS241PDK1, monoclonal Ab rabbit anti-pT308AKT,monoclonal Ab rabbit anti-pS473AKT, monoclonal Ab rabbit anti AKT, monoclonal Ab mouse anti-pP44/42 MAPK (T202-Y204), monoclonal Abrabbit anti-P44/42 MAPK (ERK1/2), monoclonal Ab rabbit anti-pT359RSK, monoclonal Ab rabbit anti-pS380RSK, monoclonal Ab rabbit anti-pS102YB1 and monoclonal Ab rabbit anti-YB1 were purchased from Cell Signaling Technology. Polyclonal Ab rabbit anti-pS221/227RSK1/2 was purchase from ThermoFisher Scientific. Polyclonal Ab mouse anti-RSK2, polyclonal Ab rabbit anti-RSK1 and polyclonal Ab goat anti-β-actinwere purchase from Santa Cruz Biotechnology. Each antibody was diluted 1:1000 in TBS, 0.1% Tween, 5% BSA. After primary Ab incubation, the membrane was washed 3 times with TBS 0.1% Tween, then incubated at RT under gentle agitation with secondary Ab (anti-mouse, anti-rabbit or anti-goat), which are conjugated to Horseradish Peroxidase (HRP) enzyme. The secondary antibody was used at 1:10000 dilution in TBS 0.1% Tween. After secondary Ab incubation, the membrane was washed 3 times with TBS 0.1% Tween, and incubated for 1 min in the Western Lightning^{TM} Chemiluminescent Reagent Plus ECL (PerkinElmer), containing H_2O_2 and luminol. The peroxidase linked to secondary antibody oxidizes, in the presence of H_2O_2, the luminol, which decays from an excited state, generating light. Generated light is used to impress a photographic film.Loading control was performed using β-actin immunodetection.

Viability assay

This assay is a method that determines the relative amount of viable cells in culture on the base of the quantification of ATP cell content, which reportsthe presence of metabolically active cells. It consists in cell lysis followed by the generation of a luminescent signal proportional to the amount of ATP cell content.

CRC cells were plated in 96 well plates, each condition in triplicate. In each well 5x10^{3}cells were plated in 200 µl of normal culture medium containing different concentrations of the PDK1 inhibitors BX795 and BX912 (0 – 0.01 – 0.03 – 0.1 – 0.3 - 1 - 3 - 10 µM). 24 hours

after cell seeding, cell viability was evaluated using CellTiter-Glo® Luminescence Cell Viability Assay (Promega Corporation, Madison, USA). 100 µl of medium wereremoved and replaced with 100 µl of reagent mixture. Then, the plate was incubated at RT under gentle agitation for 10 min to stabilize the signal. Luminescent signal was detected at the plate reader SynergyHT BioTec. This assay wasalso performed by plating $2.5x10^3$ cells in each well and evaluating their viability 48 hours after cell seeding.

Patient-derived organoids were dissociated at single cells and plated in 96 well plates, previously coated with a layer of BME, each condition in triplicate. $3x10^4$ cells were plated in 100 µl of their culture medium containing different concentrations of the PDK1 inhibitors BX795 and BX912 (0 - 1 - 3 - 10 µM). Medium was changed every two days for 15 days, in order to feed the organoids and to avoid the metabolization of the inhibitor by them. Images were acquired at the 15th day using 4X magnification at the Cytation 3 Imaging Reader BioTec. Then, cell viability was evaluated using CellTiter-Glo® Luminescence Cell Viability Assay (Promega Corporation, Madison, USA). Medium was completely removed and 100 µl of reagent mixture were added in each well. Then, the plate was incubated for 15 min at RT under gentle agitation to stabilize the signal. Luminescent signal was detected at the plate reader SynergyHT BioTec.

Soft agar assay

Anchorage-independent growth of colon cancer cells was evaluated using soft agar assay. This assay was performed using 12 well plates. All steps have been done sterilely in the following way:

- *Preparation of 1.4% Agar and 2X RPMI*

2.8 g of Difco™ Agar Noble (BD) were dissolved in 200 ml of H_2O. Then, 1.4% Agar was melted in a microwave oven, filtered through 0.22 µm pore membrane and maintained at 45°C in a waterbath to prevent the gelification.

5.2 g of RPMI-1640 Medium and 1 g of $NaHCO_3$ were dissolved in 250 ml of H_2O. Then, RPMI2X was filtered through 0.22 µm pore membrane and maintained and heated to 45°C in a waterbath.

- *Preparation of Base Agar*

Equal volumes of the two solutions were mixed to obtain 0.7% agar in RPMI 1X, and maintained at 45°C in waterbath. 0.4 ml of mixture were added to each well of a 12 well plate and agar were let polymerizefor 1h.

- *Preparation of Top Agar*

1.4% agar and RPMI 2X were mixed 1:1 to obtain 0.7% Agar RPMI 1X and maintained at 45°C in waterbath. Adherent CRC cells were detached with trypsin 0.25%, EDTA 0.02%. In the first experiments, we used $5x10^3$ cells of DLD1, HCT116, SW620, SK-CO1, SW48 and HCC2998, and $1.5x10^4$ cells of LS513. In the subsequent experiments, we instead used 10^4 cellsof DLD1 and LS513, $5x10^3$ cells of HCT116, and $2x10^4$ cells of SK-CO1. After being counted, cells were suspended in 0.6 ml of 1:1 mixture of 0.7% Agar RPMI1X and appropriate culture medium with 10% FCS and added to each well. Then, the plates were maintained 1h at 4°C to allow agar with cell suspension to solidify.

- *Cell culture medium with inhibitors*

After agar polymerization, 1 ml of normal culture medium with 10% FCS was added. After 24h, the medium was replaced with normal culture medium with 10% FCS and added with different inhibitors. Then, the cell culture medium with the inhibitor was changed every 2 days. Specifically, in the first set of experiments we used BX795, BX912, MK2206 or BI-D1870 at the final concentrations of 0.3, 1, and 3 μM. In the second set of experiment, we used BX795 or BX912 at the different final concentrations of 0.250, 0.500, 0.750, 1, and 5 μM. The plates were maintained in a humidified cell culture incubator at 37°C and 5% CO_2. The experiments were followed up to colony formation (15-30 days). Living colonies were stained with Nitrotetrazolium Blue Chloride(NBT) solution (0.05%). NBT wasadded to each well for 1 day. After that, the medium was removed, images of the wells were acquired with ChemiDoc™ Imaging System (Bio-Rad), and the number and size of the stained colonies were determined byusing “analyse particles” function of ImageJ software.

Pull-down and Kinase assay

pDEST27-RSK2WT and pDEST27-RSK2S227D plasmids were transfected in 293T cells with calcium phosphate (Promega). After 24h, cell lysates were extracted in ice using cold lysis buffer (25 mM Hepes, 300 mM $NaCl_2$, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% Triton X-100, 20 mM Na-β-glycerophosphate, 1 mM Na_3VO_4, 0.2 mM EDTA, and 1:1000 protease inhibitor cocktail; Sigma-Aldrich). GST-tagged proteins were isolated through Glutathione Sepharose 4B beads (GE Healthcare). The GST-tagged proteins isolated from 293T bound to glutathione beads were used to pull-down proteins from 293T extracts and they were washed with the same buffer used before but containing 1M $NaCl_2$. The pulled-down proteins were dissociated using a reducing buffer (10mM L-Glutathione Reduced and 50 mM Tri-HCl, ph 8.0) and analyzed by immunoblot.

RSK kinase assay was performed by using Abcam p70 S6K Activity Kit (ab139438), which is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for p70-S6K, but also for p90-S6K (RSK), and a polyclonal antibody that recognizes the phosphorylated form of the substrate. Purified GST-RSK2WT and GST-RSK2S227D were diluted 1:30 in reducing buffer (10 mM L-Glutathione Reduced and 50 mM Tri-HCl, ph 8.0). Then, Kinase Assay Dilution Buffer was added to samples, as reported in the assay procedure, and transferred in appropriate wells of the kit plate. To initiate the kinase reaction 0.5 mM ATP was added in each well and the plate was incubated at 30°C for 1h. Next steps were performed as described in the assay procedure. Finally, the reaction was stopped and absorbance of each sample was measured at the wavelength of 450 nm.

Molecular Dynamics (MD) simulation

The crystal structure of N-terminal RSK2 kinase in complex with 2-amino-7-substituted benzoxazole (PDB[8] ID 4NW5) was taken into account since it shows the lowest *RMSD* value (0.954 Å) compared to active Akt/PKB (PDB[8] ID 1O6K). Moreover, the loop carrying Ser227 was visible in this structure. The Ser227 was modified in a phosphorylated residue (pSer227) or mutated into Asp (Asp227) to obtain the phosphomimetic, using UCSF Chimera software. The most favorable for Asp227 rotamer was considered.

The Mg-ATP bound state was generated by using the coordinates of Mn_2AMP-PNP from the structure of Akt/PKB complexed with a GSK3 peptide (PDB[8] code 1O6K) (Yang et al., 2002).

A 20 ns MD simulation was run on the protein containing pSer227 and on the molecule carrying Asp227Ser mutation using the Yasara package and AMBER14 force field at 1 atm, pH 7.4 and 298 K of temperature. The simulations were set up with 1.25 fs time step under periodic boundary conditions.

Three-dimensional (3D) matrix

Basement Membrane Extract (BME) type II Matrix is solubilized basement membrane (BM) preparation that gels at room temperature toform a reconstituted basement membrane. In particular, BME isrich in extracellular matrix proteins including laminin (a major component), collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. It also contains TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other growth factors.

3D *in vitro* cultures of metastatic colorectal cancer (mCRC) patient-derived organoids (PDOs) on BME

mCRC organoids were obtained from patient-derived xenografts (PDX) of mCRC patients maintained and propagate by Translational Cancer Medicine Laboratory of Candiolo Cancer Institute. Basement Membrane Extract (BME) type II Matrix was thawed overnight at 4°C. After mechanical disaggregation using a scalpel, tumor tissue was washed in PBS 1X, centrifuged, resuspended in BME and spread in a 48- or 24-well plate. Plates were set aside for 10-15 minutes at 37°C, to allow the polymerization of BME. After that, 700 µl of DMEM-F12 medium supplemented with EGF (20 ng/ml), N-2 Supplement (Gibco), B-27 Supplement (Gibco), 0.1 mM N-Acetyl cysteine, antibiotics (penicillin and streptomycin) and 1% L-glutamine were added in each well. Organoids were maintained in humidified cell culture incubators at 37°C and 5% CO_2.

Characterization of the mCRC PDOs: fluorescence staining and confocal microscopy

In order to characterize the mCRC PDOs morphology and to evaluate its changes after the PDK1 inhibition, an immunofluorescence experiment of 3D *in vitro* PDOs culture was performed. Basement Membrane Extract (BME) type II was thawed overnight at 4°C. A coating of BME was performed in each well of a 8-wells Falcon™ Chambered Cell Culture Slide. Cell Culture slides were set aside for 30 min at RT, to allow the BME polymerization. Then, organoids were mechanical disaggregated with a micropipette, in order to maintained their structure (referred to as "preformed organoids"), or dissociated at single cells with trypsin 0.25%, EDTA 0.02%.Preformed organoids were resuspended in their specific medium added with 2% of BME and spread evenly in four wells of the chamber slide. Single cells were instead resuspended in their medium added with 10% FCS to block the trypsin. Then, cells were resuspended in the specific PDOs medium added with 2% of BME, and spread in the other four wells of the chamber slide. PDOs were allowed to grow in a 5% CO_2 humidified incubator at 37°C. Medium was changed in each well of the cell culture slides, in the presence or absence ofdifferent concentrations of the PDK1 inhibitor BX795 (0 – 0.3 – 1 – 3 µM), every two days for 8 days, in order to feed the organoids and to avoid the dilution metabolization of the inhibitor by them.At day 8th, after removal the medium, each well was fixed in 400µl of 4% paraformaldehyde with 0.025% glutaraldehyde at 37°C for 30 min and conserved in PBS1X at 4°C. Organoids were

subsequently permeabilized with TritonX100 and labeled using the Click-iT® Plus EdU Alexa Fluor® 647 Imaging Kit (ThermoFisher), which allows the staining of proliferating cells. Organoids were also stained with phalloidin conjugated with an Alexa Fluor® 488, in order to visualize F-actin, active caspase 3 (a marker of apoptotic cells),and DAPI for nuclei. Finally, PDOs were mounted with Fluorescent Mounting Medium (Agilent Dako, Santa Clara, CA, US) and images were acquired by confocal microscopy Leica SPEII with 20X magnification, using the oil immersion objective.

PDK1 targeting inhibits anchorage-independent growth of mutated KRAS cancer cells

In 2012 our group published a paper in which the role of PDK1 in breast cancer progression was evaluated. As a model of study, they used breast cancer cell lines harbouring either *PIK3CA* or *KRAS* mutations, and they transduced these cells with two specific shRNAs for *PDK1*. Then, they tested the ability of these cells to grow both in the presence and the absence of adhesion. Indeed, the anchorage-independent growth more closely represents the situation when cancer cells, during invasion and metastatization, disseminate in a non-physiological environment. Interestingly, my colleagues observed that the reduced level of PDK1 did not modify the ability of all breast cancer cell lines tested to grow in adhesion, while it reduced their ability to grow in the absence of adhesion. In particular, MDA-MB-231 cell line, harboring a KRAS mutation (G13D), showed a better response to *PDK1* silencing compared to cells with a *PIK3CA* mutation (T47D). Their observation that PDK1 is required for anchorage-independent growth in MDA-MB-231, where *PIK3CA* is normal while *KRAS* is mutated, suggest that PDK1 is not only involved in tumorigenic properties sustained by PI3K/AKT pathway mutations, but also in the case of tumor cells harboring mutation in KRAS/MAPK pathway (Gagliardi et al., 2012).

In order to confirm and validate this hypothesis, we decided to use a synthetic model, expressing wild type (WT) or mutated (G13D) KRAS in the cell line HMEC (Human Mammary Epithelial Cells), which doesn't harbor any mutation in KRAS/MAPK pathway, and we verified their ability to grow in the absence of adhesion. We observed a significant increase of the anchorage-independent growth when cells expressed WT-KRAS, and an even greater increase of cells when they expressed G13D-KRAS, the active form of KRAS (Fig.1A). Then, to evaluate the role of PDK1 in KRAS/MAPK pathway, we blocked the kinase activity of PDK1 with increasing concentrations (0 - 0.03 - 0.1 - 0.3 - 1 - 3 - 10 µM) of an ATP-competitive inhibitor (BX795). HMEC overexpressing both WT and mutated KRAS showed high sensitivity to PDK1 inhibition in the absence of adhesion (Fig.1B). We also verified this effect by downregulating *PDK1*with two specific shRNAs (shPDK1#79 and shPDK1#81) in HMEC overexpressing mutated KRAS. Likewise the inhibition of the kinase activity, silenced cells exhibited a strong reduction of their anchorage-independent

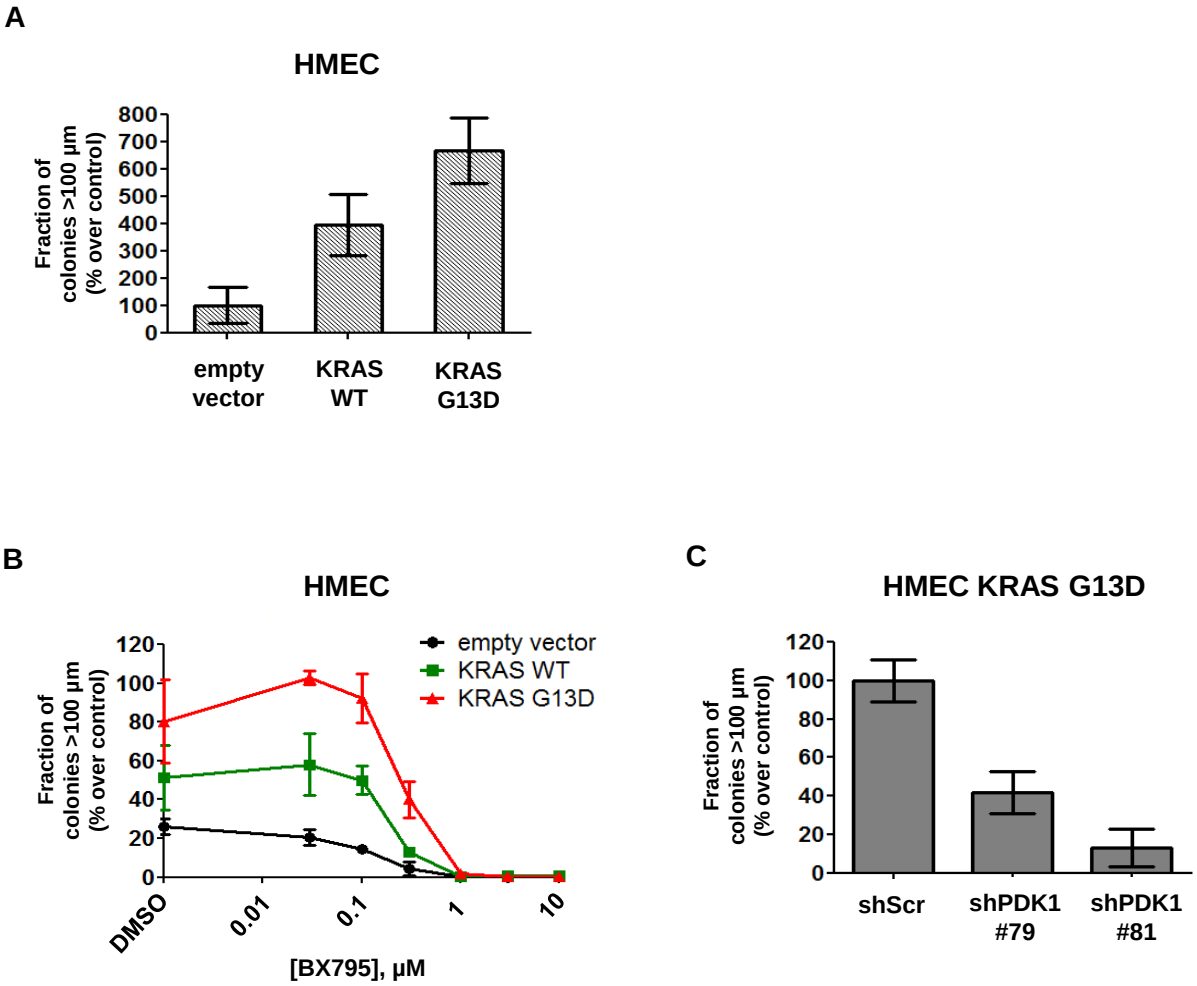

Fig. 1 | PDK1 blockade impairs the anchorage-independent growth induced by oncogenic KRAS in a synthetic model. A. HMEC (Human Mammary Epithelial Cells) overespressing wild type (WT) or mutated (G13D) KRAS, compared to cells expressing an empty vector, were tested for their anchorage-independent growth ability; **B.** Dose-response curves from HMEC overexpressing WT or mutated (G13D) KRAS treated with different concentrations (0 – 0.03 – 0.1 – 0.3 – 1 – 3 – 10 µM) of the PDK1 inhibitor BX795 and grown in the absence of adhesion; **C.** HMEC overespressing KRAS G13D were transduced with a control shRNA (shScr) or shRNAs silencing PDK1 (shPDK1#79 and shPDK1#81 or #82). Then, these cells were assayed for their ability to grow in the absence of adhesion.

Anchorage-independent growth of all these cells was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than 100 µm. Then, the number of colonies grown after PDK1 inhibition or silencing were normalized dividing by the number of colonies grown in their respective control. The resulting percentages are reported. Data are shown as average±S.D.

growth ability (Fig.1C). These observations demonstrate that pro-tumorigenic effect triggered by mutated KRAS in our synthetic model can be intercepted by PDK1 targeting.

PDK1 inhibition impairs anchorage-independent growth of colorectal cancer (CRC) cell lines

Based on these preliminary findings, we hypothesised that PDK1 involvement in *KRAS*-driven tumorigenesis could be a more general phenomenon. Activating mutations of KRAS occur in about 30% of human cancers. They are prevalent in colorectal, pancreatic and lung cancer. In 2013, Eser S. and colleagues (Eser et al., 2013) investigated the role of cell autonomous PDK1 signaling in mutated *KRAS*-driven pancreatic and lung carcinogenesis. They demonstrated that *PDK1* deletion, specifically in the epithelial compartment of the pancreas of floxed*PDK1* and mutated *KRAS* mice, completely blocked acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDAC) formation. In contrast, deletion of *PDK1* in mutated *KRAS*-driven non-small cell lung cancer mice models was unable to block the lung cancer formation. Therefore, they claimed that cell autonomous KRAS/PI3K/PDK1 signaling is a critical and therapeutically tractable axis in pancreatic cancer initiation and maintenance but not in lung cancer formation.

As previously reported, *KRAS* mutations also frequently occur in colorectal cancer, approximately in 40% of cases. However, the role of PDK1 in this tumor has never been investigated. Thus, with the aim of verifying our hypothesis and taking advantage from the large dataset of CRC models availableat the Candiolo Cancer Institute, we decided to explore PDK1 role in colorectal cancer (CRC). We selected seven established cell lines of CRC (SW48, DLD1, HCT116, SW620, LS513, SK-CO1 and HCC2998) that, with the exception of SW48, harbor a somatic mutation in *KRAS* gene (G13D in DLD1 and HCT116; G12D in LS513; G12V in SW620 and SK-CO1; and A146T in HCC2998).First, we investigated the effect of PDK1 kinase activity inhibition on anchorage-independent growth of these cells. We resuspended $5x10^3$ cells per 35 mm well in soft agar. We used this cell density for all the cell lines, except for LS513 cell line, in which an higher density($15x10^4$) was needed to form colonies. We let the cells grow for 24 hours and then we treated them with increasing concentrations (0 - 0.3 - 1 - 3 μM) of the PDK1 inhibitor BX795 for 15-30 days, changing the medium every two days. To quantify the anchorage-independent growth ability, we stained the colonies with the metabolic reporter Nitrotetrazolium blue chloride solution and we acquired images of each well (Fig. 2A).

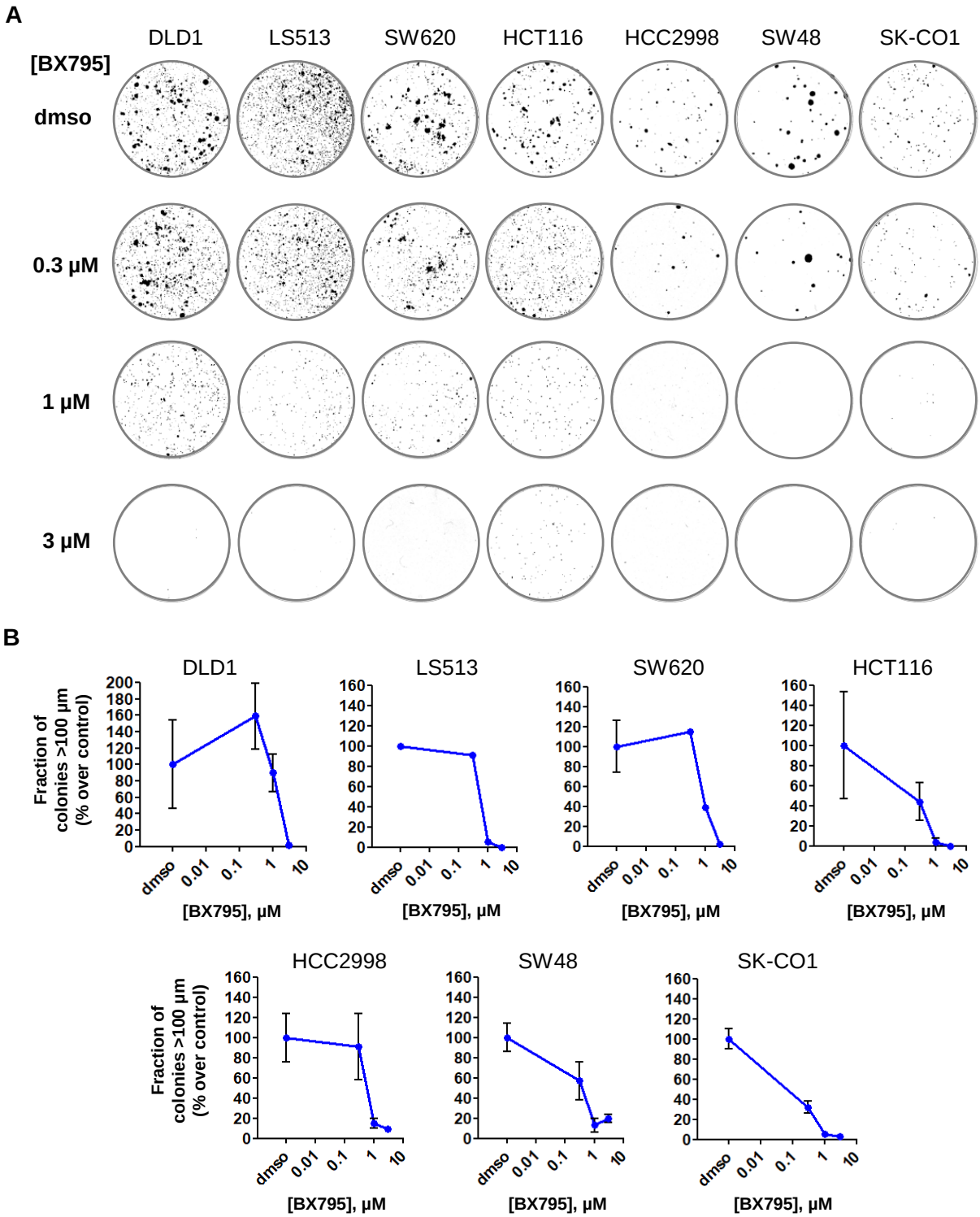

Fig. 2 | BX795 impairs anchorage-independent growth of CRC cell lines. A. Snapshots of representative wells of soft agar assay performed on the different cell lines and using the PDK1 inhibitor BX795. $5x10^3$ cells (DLD1, HCT116, SW620, SK-CO1, SW48, HCC2998) or $1.5x10^4$ cells (LS513) were allowed to grow for 15 - 30 days with medium supplemented with increasing concentrations (0 – 0.3 – 1 – 3 µM) of BX795. Living colonies were stained with Nitrotetrazolium Blue Chloride solution; **B.** Anchorage-independent growth of CRC cell lines in the presence of the PDK1 inhibitor BX795 was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than to 100 µm. Then, the number of colonies grown with BX795 was normalized dividing by the number of colonies grown without the PDK1 inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

Analysing the colonies size, we observed that the growth in the absence of adhesion of all CRC cell lines (excluding DLD1) was significantly reduced after treatment with 1 μM concentration of the inhibitor. Notably, the PDK1 inhibition caused a strong decrease of the growth at 0.3 μM in HCT116, SW48 and SK-CO1 cell lines (Fig. 2B).

BX795 was initially developed as a PDK1inhibitor. However, subsequent study highlighted its bioactivity also as a potent and relatively specific inhibitor of TBK1 and closely related IKKε, with half-maximal inhibitory concentrations (IC50) of 6, 41, and 111 nM for TBK1, IKKε and PDK1 respectively (Clark et al., 2009; Peifer and Alessi, 2008). The ability of BX795 to inhibit the TBK1-catalyzed phosphorylation of IRF3 at Ser-396 decreases as the ATP concentration is increased. This indicates that BX795 is an ATP competitive inhibitor of TBK1, as is the case for PDK1 [6]. Therefore, to confirm the results obtained with BX795, we decided to compare previously obtained results with those obtained with a more specific PDK1 inhibitor, the BX912. This is also a competitive inhibitor of PDK1 activity with respect to its substrate, ATP, suggesting that BX912 binds to the ATP binding pocket of PDK1 [7]. We tested the anchorage-independent growth ability of the seven CRC cell lines in the presence of different concentrations (0 - 0.3 - 1 - 3 μM) ofBX912, performing the experiment in the same conditions used previously for BX795. The results that we obtained with BX912 confirmed those previously obtained with BX795.

Colorectal cancer cell lines showed different sensitivity to the specific PDK1 inhibition. The most sensitive cell lines were SK-CO1, SW48, HCC2998 and HCT116, whose growth was already impaired with 0.3 μM of the inhibitor. In contrast SW620, LS513 and DLD1 cell lines were more resistant to PDK1 inhibition by BX912, as their growth was reduced only with the higher concentration of the inhibitor (Fig. 3A and 3B).

These results suggest that the anchorage-independent growth of CRC is sensitive to PDK1 inhibition, although the effect of the treatment with inhibitors showing different PDK1 specificity is not completely superimposable.

To better understand the differences in dose-response curves among the different cell lines, we decided to increase the resolution of the used concentrations (0 - 0.250 - 0.500 - 0.750 - 1 - 5 μM). For practical reasons we performed the experiments with both BX795 and BX912 on four cell lines, chosen on the base of the previous experiments. Specifically, we usedthe two more resistant (DLD1 and LS513) and the two more sensitive (SK-CO1 and HCT116) mutated *KRAS*CRC cell lines to the PDK1 inhibition. In the attempt to improve the reliability of the experiments, we adjusted the number of seeded cells on the different rates of proliferation and survival, specific for each cell line. We resuspended 10^4

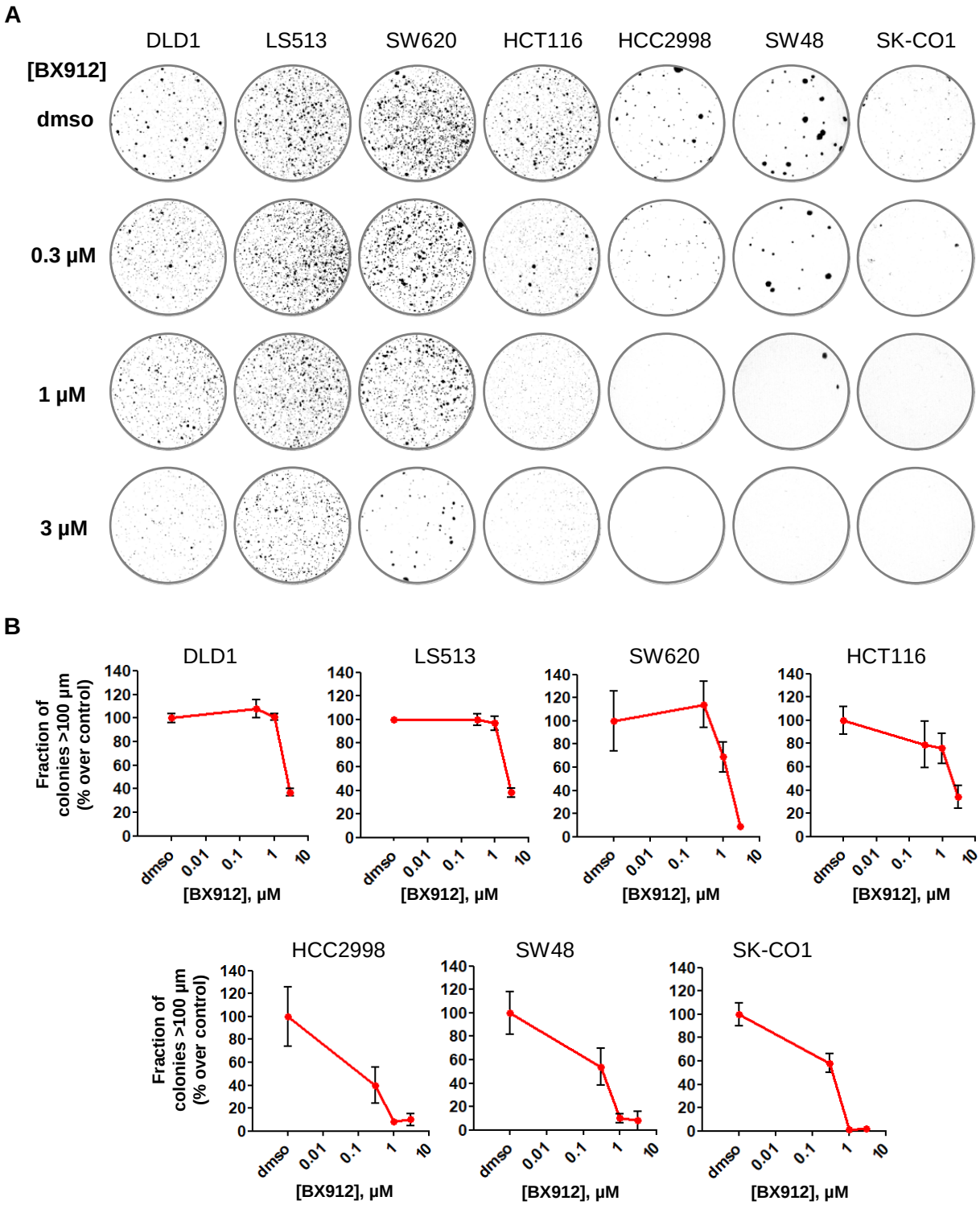

Fig. 3 | BX912 inhibits anchorage-independent growth of CRC cell lines similarly to BX795. A. Snapshots of representative wells of soft agar assay performed on the different cell lines and using the more specific PDK1 inhibitor BX-912. $5x10^3$ cells (DLD1, HCT116, SW620, SK-CO1, SW48, HCC2998) or $1.5x10^4$ cells (LS513) were allowed to grow for 15 - 30 days with medium supplemented with increasing concentrations (0 – 0.3 – 1 – 3 µM) of BX912. Living colonies were stained with Nitrotetrazolium Blue Chloride solution; **B.** Anchorage independent growth of CRC cell lines in the presence of the PDK1 inhibitor BX912 was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than to 100 µm. Then, the number of colonies grown with BX912 was normalized dividing by the number of colonies grown without PDK1 inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

cells of DLD1 and LS513, $5x10^3$ cells of HCT116, and $2x10^4$ of SK-CO1 in a soft agar. We let these cells grow for 24 hours and thenwe treated them for 10 days with the five different concentrations (0 - 0.250 - 0.500 - 0.750 - 1 - 5 µM) of either the BX795 (Fig. 4A and 4B) or the BX912 (Fig. 5A and 5B). The results that we obtained confirmed the previous ones. In addition, the increase accuracy of the experiment allowed us to calculate the IC50 for each cell line. SK-CO1 cells showed high sensitivity to both inhibitors (IC50 BX795=0.276 µM and IC50 BX912=0.376 µM). HCT116 cells, while showing a high sensitivity to BX912 (IC50=0.310 µM), resulted to be less sensitive to the BX795 (IC50=0.672 µM).The ability to grow in the absence of adhesion of LS513 cells was strongly hampered by BX795 with an IC50 of 0.310 µM, while this cell line was poorly sensitive to BX912 (IC50=2.54 µM). Conversely, the less sensitive cells were DLD1, which showed an high IC50 for both the inhibitors (IC50 BX795=2.523 µM and IC50 BX912=2.95 µM) (Fig. 4 and 5).

We therefore confirmed the effect of the PDK1 blockade observed in the previous experiments, despite the different culture conditions adopted. Colorectal cancer cell lines showed a different sensitivity to the inhibition of the PDK1 kinase activity. However, the anchorage-independent growth of the majority of these cell lines was considerably reduced by both the BX795 and the more specific BX912.

PDK1 silencing impairs anchorage-independent growth of CRC cell lines

In order to obtain a further confirmation of the effect of the PDK1 targeting on CRC cell lines ability to grow in the absence of adhesion, we silenced *PDK1* gene with two specific shRNAs (shPDK1#79 and shPDK1#82) by transducing them in the two mutated *KRAS* CRC cell lines which better respond to the PDK1 inhibition (SK-CO1 and HCT116).We used as control the wild type *KRAS* cell line (SW48). Once generated, we performed a soft agar assay with the PDK1 silenced cell lines and we compared them with cells expressing a not-targeting scramble shRNA (shScr). After 15 day, colonies were stained and counted. The anchorage-independent growth of all PDK1 silenced cell lines was notably decreased compared to shScr cells (Fig. 6A and 6B).

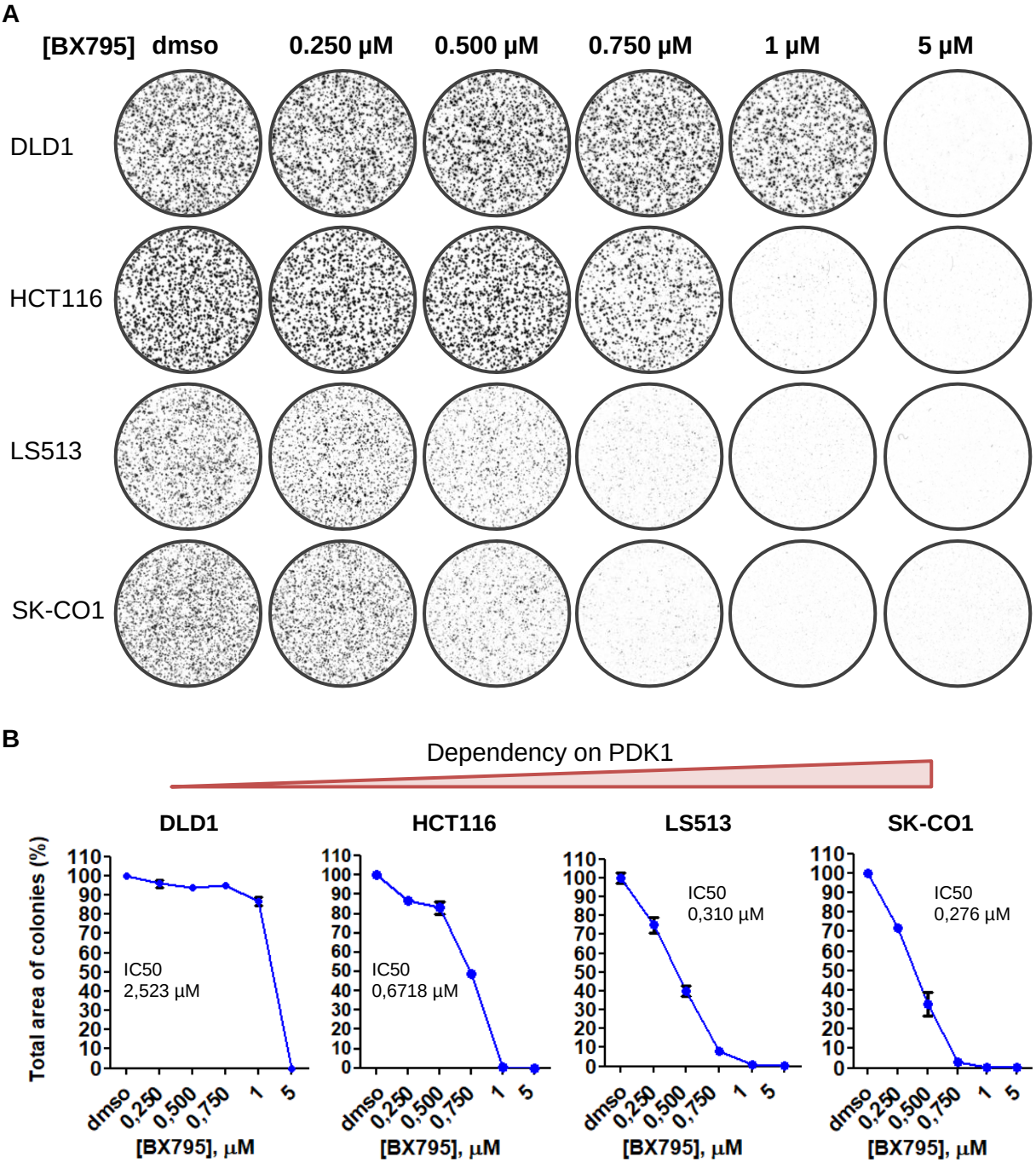

Fig. 4 | PDK1 inhibition with BX795 altered anchorage-independent growth of CRC cell lines. A. Snapshots of representative wells of soft agar assay performed on the different cell lines and using the PDK1 inhibitor BX795. 10^4 cells (DLD1, LS513) or $5x10^3$ cells (HCT116) or $2x10^4$ (SK-CO1) were resuspended in 1:1 mixture of 0.7% Agar RPMI 1X and appropriate culture medium with 10% FCS. Cells were allowed to grow for about 10 days with medium added with different concentrations of BX795 (0 – 0.250 – 0.500 – 0.750 – 1 – 5 µM), which was changed every 2 days. Living colonies were stained with Nitrotetrazolium Blue Chloride solution; **B.** Anchorage-independent growth of CRC cell lines in the presence of PDK1 inhibitor BX795 was quantified using "analyze particles" function of ImageJ software by counting the total area of the colonies. Then, the total area of colonies grown with BX795 was normalized dividing by the total area of colonies grown without the PDK1 inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

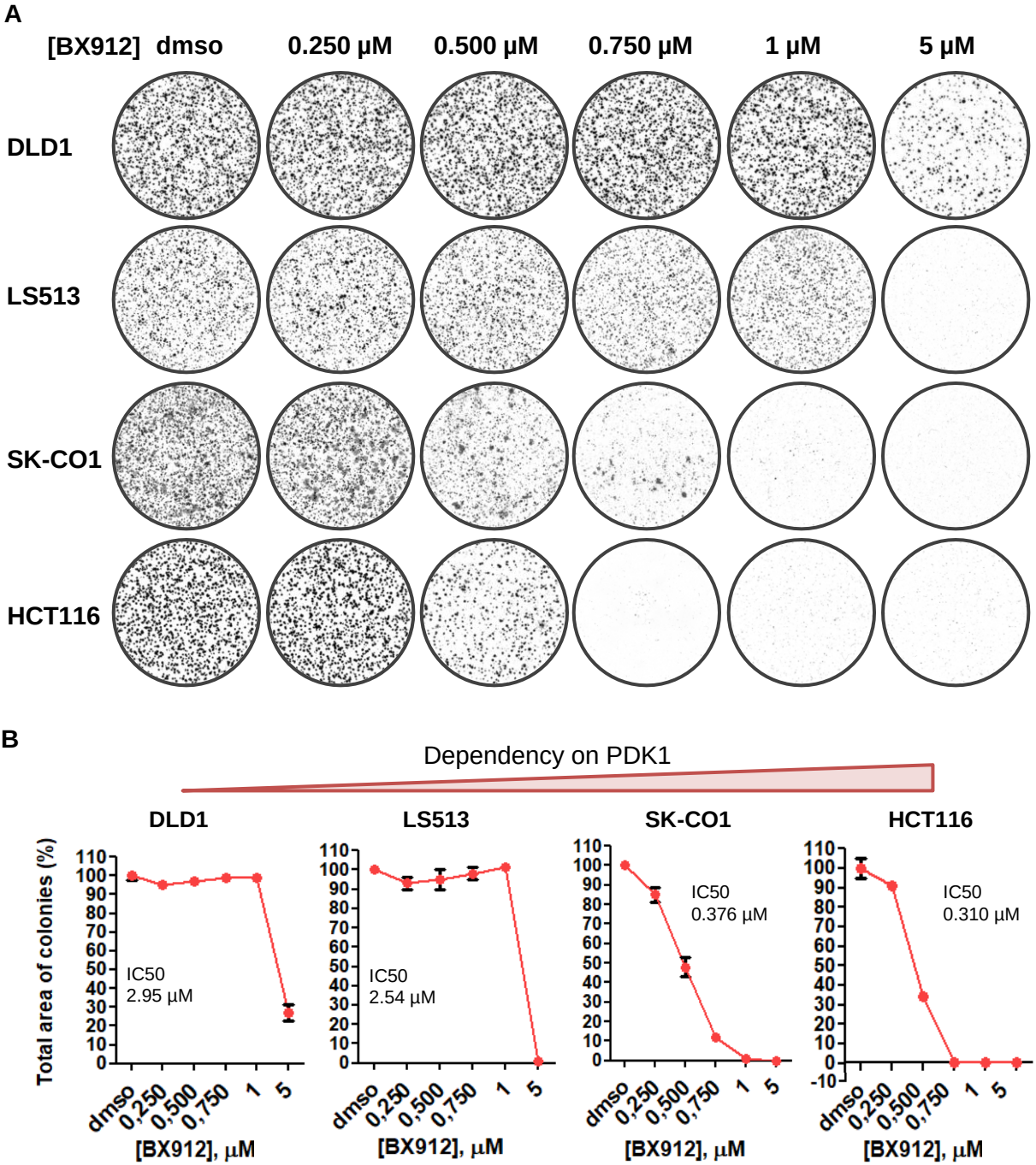

Fig. 5 | CRC cell lines showed different sensitivity to the PDK1 inhibitor BX912 in soft agar. A. Snapshots of representative wells of soft agar assay performed on the different cell lines and using the more specific PDK1 inhibitor BX-912. 10^4 cells (DLD1, LS513) or $5x10^3$ cells (HCT116) or $2x10^4$ (SK-CO1) were resuspended in 1:1 mixture of 0.7% Agar RPMI 1X and appropriate culture medium with 10% FCS. Cells were allowed to grow for about 10 days with medium added with increasing concentrations of BX912 (0 – 0.250 – 0.500 – 0.750 – 1 – 5 µM), which was changed every 2 days. Living colonies were stained with Nitrotetrazolium Blue Chloride solution; **B.** Anchorage-independent growth of CRC cell lines in the presence of the PDK1 inhibitor BX912 was quantified using "analyze particles" function of ImageJ software by counting the total area of the colonies. Then, the total area of colonies grown with BX912 was normalized dividing by the total area of colonies grown without the PDK1 inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

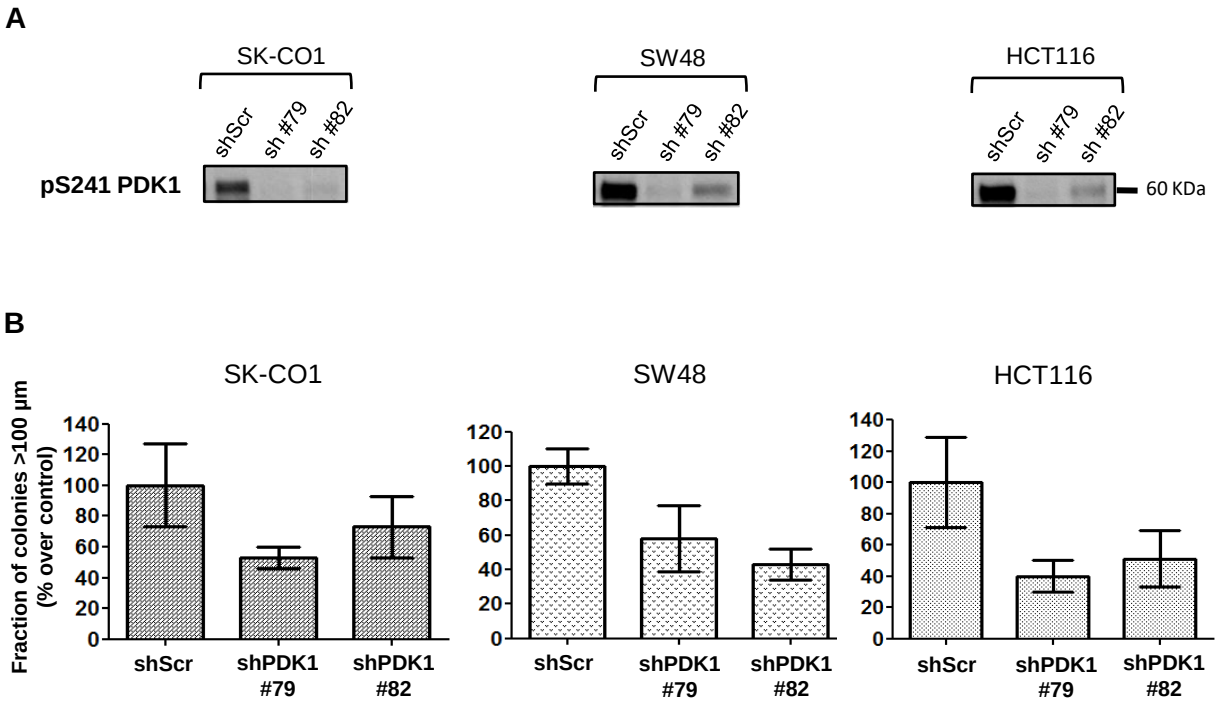

Fig. 6 | *PDK1* silencing impairs anchorage-independent growth of CRC cell lines. A. Mutated KRAS CRC cell lines (HCT116 and SK-CO1) and wild type KRAS CRC cell line (SW48) were transduced with a control shRNA (shScr) or shRNAs silencing PDK1 (shPDK1#79 and shPDK1#82). Then, these cells were lysed and *PDK1* silencing was verified through a western blot analysis, using the pS241 PDK1 antibody; **B.** *PDK1* silenced CRC cell lines were assayed for their ability to grow in absence of adhesion by soft agar assay. Their anchorage-independent growth was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than 100 µm. Then, the number of *PDK1* silenced colonies was normalized dividing by the number of shScr transduced colonies. The resulting percentages are reported. Data are shown as average±S.D.

PDK1 inhibition poorly influences the viability of different CRC cell lines

As mentioned above, in 2012 our group published a paper in which we demonstrated that PDK1 targeting (with either silencing or its inhibition) doesn't have a general effect on viability in adhesion, but only when cells are exposed to the critical conditions of absence of adhesion (Gagliardi et al., 2012). Based on these observations, we wondered if this occurs also in the CRC cell lines. Therefore, we tested the effect of the treatment with BX795 or BX912 on viability of the CRC cell lines in adhesion. We performed the experiments with four cell lines chosen among the most (SK-CO1 and HCT116) or less affected (LS513 and DLD1) by PDK1 inhibition in absence of adhesion. We treated these cells with both PDK1 inhibitors using eight different concentrations (0 - 0.01 - 0.03 - 0.1 - 0.3 - 1 - 3 - 10 μM) for 24 or 48 hours, and then we evaluated the amount of viable cells using a CellTiter-Glo® Luminescence Cell Viability Assay. The treatment with both BX795 and BX912 for 24 hours did not affect the viability of CRC cell lines, excluding the LS513 cells, which showed reduced viability at the highest concentration (10 μM). Only after 48 hours we observed a small decrease in cell viability. In particular, DLD1 and HCT116 cell survival was reduced (about 20-40%) by both PDK1 inhibitors at the intermediate concentrations. SK-CO1 cells viability was instead reduced (about 20-40% over control) at the highest concentrations (Fig. 7A and 7B). In summary, we concluded that PDK1 blockade weakly affected the growth in adhesion of the mutated KRAS CRC cells. Only high inhibitor concentrations and 48 hours of treatment caused reduced viability of CRC cells.

Effects of RSK and Akt inhibition on anchorage-independent growth of CRC cell lines

We identified two PDK1 substrates that can potentially explain the effects of PDK1 inhibition on the anchorage-independent growth of CRC cell lines. AKT, because multiple reports in literature indicate that it is the main mediator of PI3K effects in carcinogenesis (Manning and Cantley, 2007) and RSK, because it is one of the downstream effectors of RAS/MAPK pathway (David et al., 2005; Romeo et al., 2012). Both the two proteins must be phosphorylated by PDK1 to be activated and, therefore, to exert their respective enzymatic effects. To verify whether the inhibition of one of these two PDK1 substrates has the same effect of PDK1 inhibition, we tested the ability of the CRC cells to grow in the

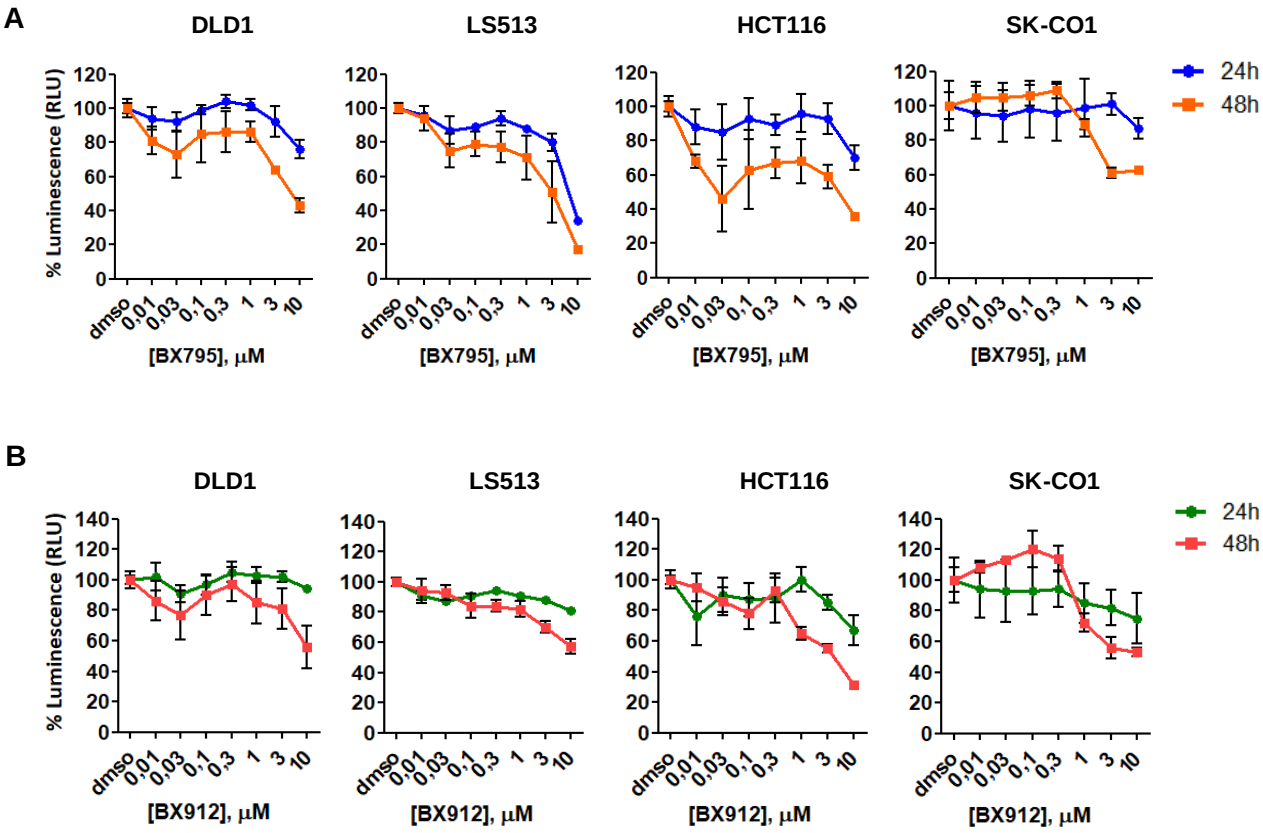

Fig. 7 | PDK1 inhibition poorly influences the viability of different mutated KRAS CRC cell lines. CRC cell lines were plated in normal culture medium. After 24h, medium containing increasing concentrations (0 - 0.01 - 0.03 - 0.1 - 0.3 - 1 - 3 - 10 µM) of BX-795 **(A)** or BX912 **(B)** was added. Cells were allowed to grow for 24 or 48 h. Then, cell viability was evaluated using CellTiter Glo® Luminescence Cell Viability Assay (Promega Corporation, Madison, USA). Luminescence signal was normalized dividing by the cell viability at 0 µM (dmso). The resulting percentages are reported. Data are shown as average±S.D.

absence of adhesion in the presence of MK2206, which inhibits Akt, or in the presence of BI-D1870, which inhibits RSK. The cell lines which showed the highest sensitivity to the AKT inhibition were HCC2998, SK-CO1 and LS513 cells, whose anchorage independent growth was significantly reduced already with MK2206 at 0.3 μM. Moreover, we observed that the AKT inhibition affected the anchorage-independent growth of SW48, HCT116 and DLD1 by impairing the size of colonies and less their overall number. SW620 cells showed the highest resistance to MK2206, being insensitive to all the tested concentration (Fig. 8A). Contrary to MK2206, the RSK inhibitor, BI-D1870, caused impairment of anchorage-independent growth of all colon cancer cell lines at 3 μM concentration. Instead, at 1 μM we observed a more heterogeneous degree of impairment, depending on the cell line (Fig. 9A). To better evaluate the effect of MK2206 and BI-D1870 on the anchorage-independent growth of the CRC cells we quantified the number of colonies having size greater than 100 μm. We obtained that the ability of the cell lines to grow in soft agar in the presence of BX795 didn't match with their grow in the presence of MK-2206 (Fig. 8B). On the contrary, the growth ability in the presence of BX795 roughly recapitulated the growth upon treatment of BI-D1870 (Fig. 9B). These data suggest that, despite the Akt inhibition is able to impair the anchorage-independent growth of the large majority of the tested colorectal cancer cell lines, the molecular mechanisms underpinning PDK1 and Akt inhibition are likely different. On the contrary, the mechanisms of PDK1 and RSK inhibition could lie in the same regulatory pathway. Therefore, we were pushed to investigate RSK as a possible downstream effector of PDK1 in tumors with KRAS mutation.

Biochemical effect of PDK1 inhibition in CRC cell lines

To verify that the PDK1 inhibitor BX795 really affected the kinase activity of PDK1 and its signalling pathway, we analysed PDK1 downstream effectors activation. We treated the seven CRC cell lines with the PDK1 inhibitor BX795 at 3 μM concentrations over-night (about 15 hours), and then we stimulated the cells with 10 ng/ml of epidermal growth factor (EGF) for 5 minutes. EGF stimulation was used to overactivate both PI3K/Akt and RAS/MAPK pathways, since PDK1 is involved in both these two pathways, and thus to increase the phosphorylation status of the PDK1 substrates (Akt and RSK). By western blotting analysis we observed a decrease of the phosphorylation on the residue Thr308 of Akt in all cell lines, except for SW620 cells, which did not show any phosphorylation even in presence of EGF stimulation, and for LS513, which showed a poor Akt activation also after the EGF stimulation. As expected, PDK1 didn't regulate the phosphorylation of

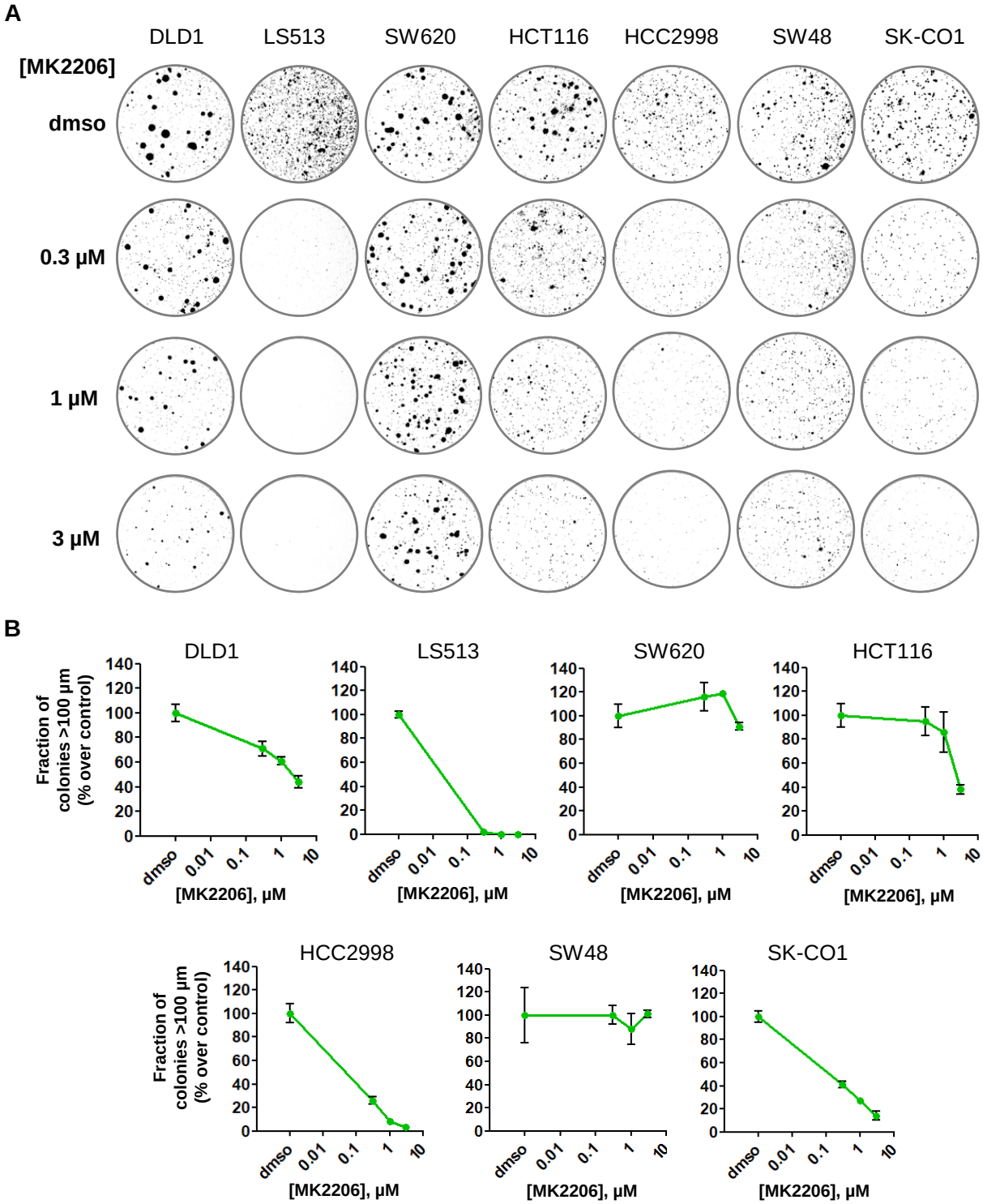

Fig. 8 | CRC cell lines growth in soft agar is reduced in the presence of the Akt inhibitor MK2206. A. Snapshots of representative wells of soft agar assay performed on the different CRC cell lines and using the Akt inhibitor MK2206. $5x10^3$ cells (DLD1, HCT116, SW620, SK-CO1, SW48, HCC2998) or $1.5x10^4$ cells (LS513) were allowed to grow for 15 - 30 days with medium added with increasing concentrations (0 – 0.3 – 1 – 3 µM) of MK2206. Living colonies were stained with Nitrotetrazolium Blue Chloride solution; **B.** Anchorage-independent growth of CRC cell lines in the presence of the Akt inhibitor MK2206 was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than to 100 µm. Then, the number of colonies grown with MK2206 was normalized dividing by the number of colonies grown without the Akt inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

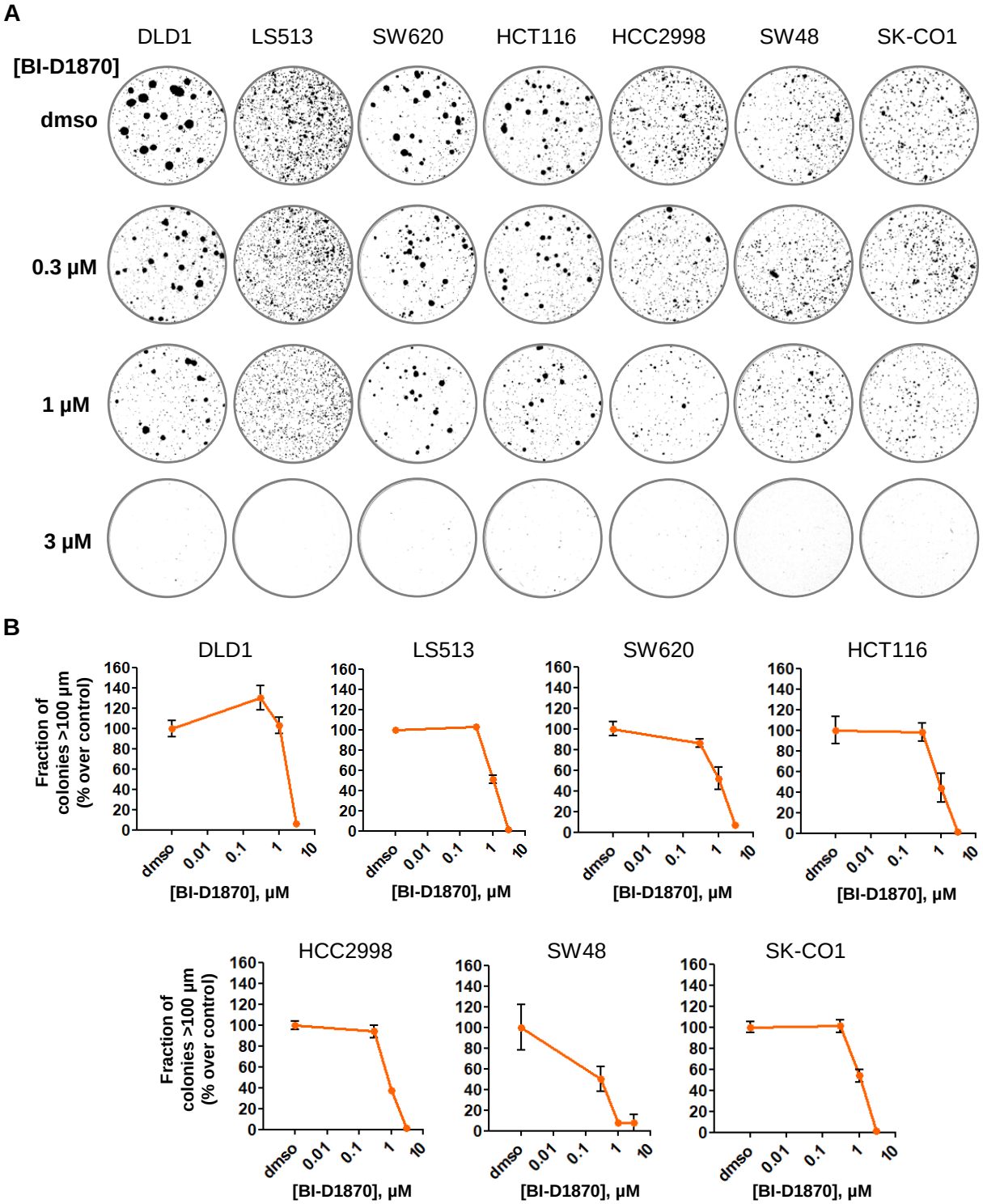

Fig. 9 | The RSK inhibitor BI-D1870 is able to abrogate CRC cell lines growth in the absence of adhesion. A. CRC cell lines were tested for their anchorage-independent growth ability in the presence of increasing concentrations (0 – 0.3 – 1 – 3 µM) of the RSK inhibitor BI-D1870, for 15-30 days. Snapshots of representative wells of the soft agar assay are shown; **B.** Anchorage-independent growth of CRC cell lines in the presence of RSK inhibitor BI-D1870 was quantified using "analyze particles" function of ImageJ software by counting only colonies with size equal or greater than to 100 µm. Then, the number of colonies grown with BI-D1870 were normalized dividing by the number of colonies grown without the RSK inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

ERK1/2. In fact, the EGF-mediated increase of the phosphorylation on the residues Thr202 and Tyr204 of ERK1/2 was not impaired by BX795 in all CRC cell lines. On the contrary, we observed a significant effect of PDK1 inhibition on the phosphorylation of Ser221 of RSK in all the tested cell lines. Moreover, the inhibitor induced a reduction of the phosphorylation on the residue Ser241 of PDK1 in all cell lines. In fact, the phosphorylation of this serine is catalyzed by an auto-trans-phosphorylation reaction (Fig. 10). Thus, we confirmed that the inhibitor BX795 is able to block the PDK1 kinase activity, even under the presence of a strong activating stimulation.

RSK2 kinase activity is impaired by amino acid substitution of serine 227 in the NTKD

To investigate whether the effect of PDK1 in KRAS-mutated cells is mediated by RSK, we performed specific biochemical experiments. As previously reported, RSK to be fully active must be phosphorylated on a serine in the activation loop (AL) of its N-terminal kinase domain (NTKD) by PDK1 (Eser et al., 2013). We verified RSK regulation and activation by overexpressing the wild type protein (RSK2 WT) in HeLa cells. Then, we stimulated or not these cells with EGF (10 ng/ml) and we treated or not them with 3 µM of GSK2334470, the most specific PDK1 inhibitor available on the market (which however can be used for short term biochemical experiments, due to its low stability). As expected, we observed an increase of the phosphorylation on T359, S386 and S227 of RSK2 after stimulation with EGF, as well as a little increased in phosphorylation of its substrate YB1. In presence of the PDK1 inhibitor, the serine 227 (S227) is instead completely dephosphorylated and YB1 phosphorylation was abrogated (Fig. 11A). To confirm these results in a more controlled experiment, we performed an *in vitro* kinase assay to test the kinase activity of RSK2 in the presence or in the absence of the PDK1 inhibitor GSK2334470 (1 µM). The inhibition of PDK1 induced the complete inactivation of RSK2 activity (Fig. 11B).

To study the role of RSK as downstream effector of PDK1 in tumor cells we decided to decouple RSK2 activation from PDK1 by producing an RSK2 mutant whose activation was independent from PDK1. To this aim, we rationally designed a phosphomimetic mutation on S227 of RSK2 by substituting it with an aspartate that mimics the negative charges of the phosphate group added by PDK1 on S227. Moreover, as a negative control, we used a kinase-dead mutant of the NTKD. This mutant (K100A) has an alanine (Ala, A) instead of the lysine (Lys, K) in the ATP-binding site in the NTKD, leading to the inactivation of the kinase activity of the protein. We overexpressed these RSK2 mutants in HeLa cells, and

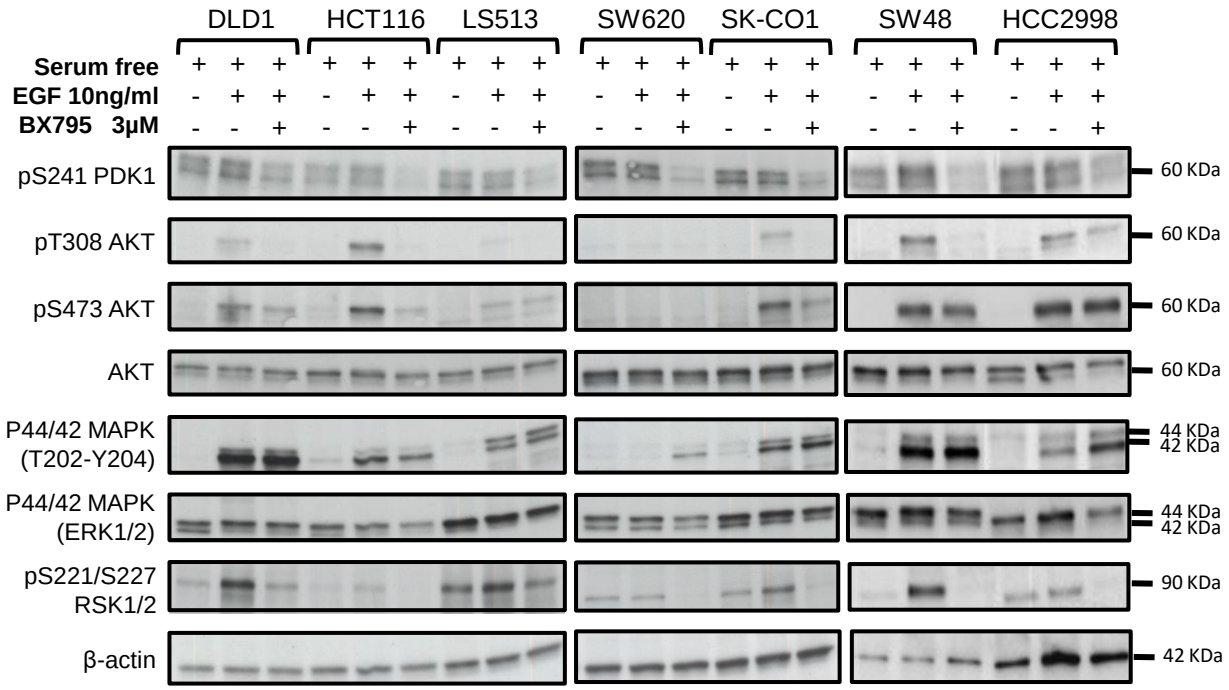

Fig. 10 | BX795 efficiently inhibits PDK1 kinase activity. Mutated or wild type KRAS CRC cell lines were starved overnight with normal culture medium without FCS and with or without the addition of the PDK1 inhibitor BX795 at 3 μM. Then, they were stimulated or not with EGF (10 ng/ml) for 5 min and lysed. Western blot analysis were performed using the indicated primary antibodies.

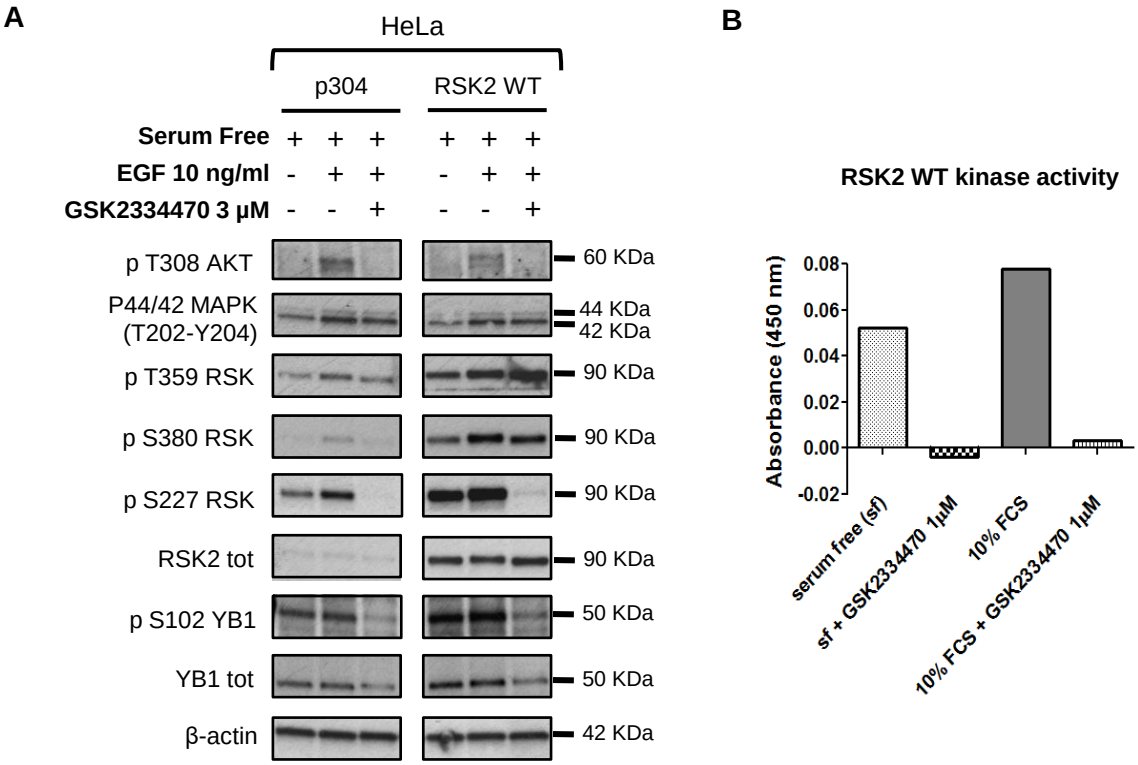

Fig. 11 | RSK2 kinase activity is impaired by PDK1 inhibition. A. HeLa cells were transfected with p304 vector containing or not the RSK2 coding sequence. These cells were starved overnight with normal culture medium without FCS and with or without the addition of the specific PDK1 inhibition GSK2334470 at 3 µM. Than, HeLa cells were stimulated or not with EGF (10 ng/ml) for 5 min and lysed. Western blot analysis were performed using the indicated primary antibodies; **B.** 293T were transfected with pDEST27-RSK2WT plasmid and cultured with or without FCS. Then, these cells were lysed in ice. GST-RSK2WT was isolated from 293T extracts and assayed for the phosphorylation of a specific synthetic peptide as its substrate, in the presence or the absence of the PDK1 inhibitor GSK2334470 at 1 µM.

we stimulated these cells with PMA (phorbol myristate acetate, 60 ng/ml) or with EGF (10 ng/ml), which are both able to activate the MAPK signaling pathway. Phosphorylations of RSK2 at T359, S386 and S227 were well modulated when wild type protein was overexpressed, making the protein fully active. As a consequence, the phosphorylation of YB1 increased after stimulation. Unexpectedly, we observed that phosphomimetic mutant is completely inactive, like the kinase-dead mutant (Fig. 12A). To validate these results, we purified wild type and phosphomimetic proteins and we tested their kinase activity with the *in vitro* kinase assay. In this assay as well, the phosphomimetic mutant showed a lower kinase activity then wild type RSK2 (Fig. 12B). Therefore, we concluded that phosphomimetic mutations failed to recapitulate the phosphate group added to the serine residue.

Computational modeling of RSK2 structure reveals critical interaction between AL phosphate and distant residues, which are not mimicked by the carboxyl group of aspartate

Since phosphomimetic mutants have been widely exploited for scientific research purposes, the unexpected behavior of our RSK2S227D mutant prompted us to investigate the underlying molecular reasons of that failure. The reduced or absent kinase activity of the phosphomimetic mutant in the activation loop (AL) is a phenomenon that has already been described in other elements of AGC kinase family, but never been further explored. Indeed, a similar study has been performed on PAK1 (p21-activated kinase 1), which despite not belonging to AGC kinase family, harbor an AL which is phosphorylated by PDK1 (King et al., 2000). In this case, the substitution of a threonine in the AL of PAK1 with a glutamate determined the inactivation of the protein. This effect was explained by the different chemical bonds established by the phosphorylated threonine or by the glutamate with neighboring residues (Ng et al., 2010).

The active state of kinases is well defined and comprises a closed lobe conformation, a well-structured activation loop suitable for recognition of the substrate, and a firmly anchored αC-helix forming an ion pair with the active site lysine, enabling cofactor binding. By contrast, crystal structures of inactive kinases have revealed a large diversity of conformations, and at least one of the key regulatory elements is often displaced or disordered (Huse and Kuriyan, 2002).

Therefore, based on these considerations, in collaboration with Prof. Giovanna Di Nardo of the University of Torino, we tried to understand whether this molecular explanation could

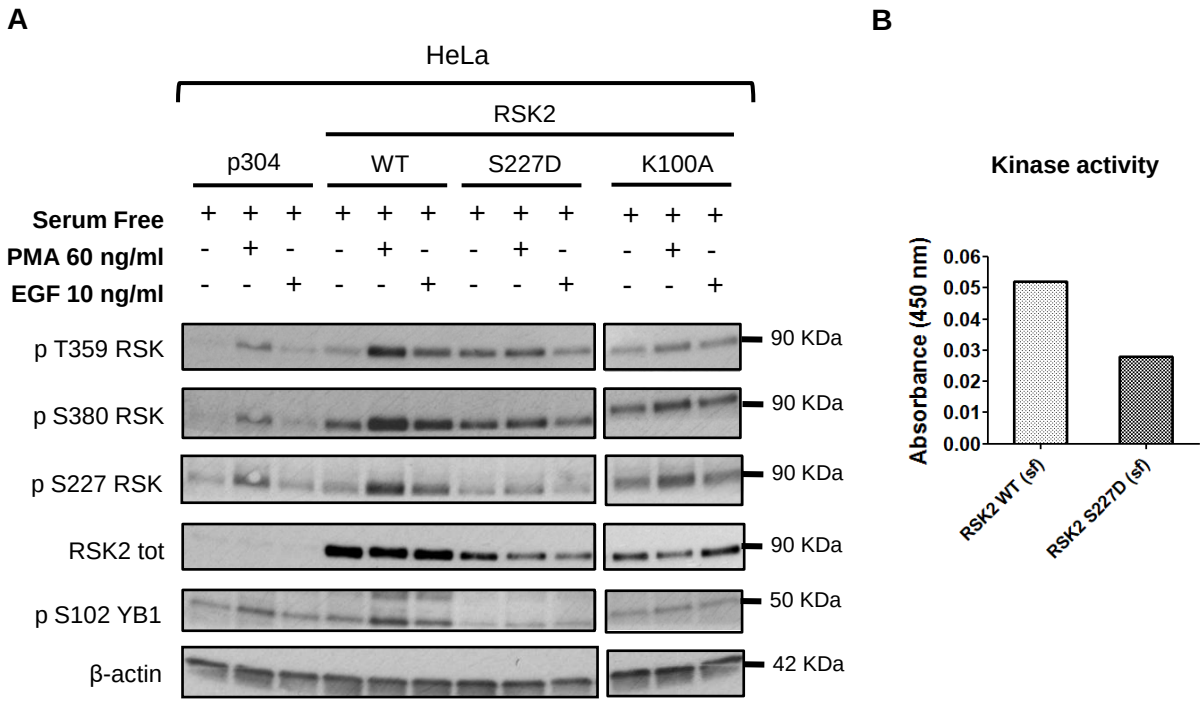

Fig. 12 | RSK2 kinase activity is impaired by amino acid substitution of Serine 227 in the NTKD. A. HeLa cells were transfected with p304 vector containing or not the wild type or mutated (S227D and K100A) RSK2 coding sequence. Then, these cells were starved overnight with normal culture medium without FCS. After that, HeLa cells were stimulated or not with PMA (60 ng/ml) or with EGF (10 ng/ml) for 5 min and lysed. Western blot analysis were performed using the indicated primary antibodies; **B.** 293T were transfected with pDEST27-RSK2WT or pDEST27-RSK2S227D plasmids and cultured without FCS. Then, these cells were lysed in ice. GST-RSK2WT and GST-RSK2S227D were isolated from 293T extracts and assayed for the phosphorylation of a specific synthetic peptide as their substrate.

be extended also to RSK2, as an example of members of AGC kinase family activated by PDK1. To investigate the bonds which arise in the AL of the protein, we analyzed the structural motility, performing molecular dynamics (MD) simulations, of the active wild type RSK2 (with the phosphorylated serine 227) or of RSK2 harboring the mutation S227D, comparing them to the activeAkt/PKB structure. Indeed, MD simulations are used to the study of the motions of macromolecular systems of high complexity, such as biomolecules (Phillips et al., 2005). The two most common measures of structural fluctuations are the Root-Mean-Square-Deviation (*RMSD*) and the Root-Mean-Square-Fluctuations (*RMSF*) (Humphrey et al., 1996). The *RMSD* is the average displacement of the atoms at an instant of the simulation relative to a reference structure, usually the first frame of the simulation or the crystallographic structure. The *RMSF* is a measure of the displacement of a particular atom, or group of atoms, relative to the reference structure, averaged over the number of atoms. The *RMSD* is frequently used to discern whether a structure is stable in the time-scale of the simulations or if it is diverging from the initial coordinates. Most times, the divergence from the initial coordinates is interpreted as a sign that the simulation is not equilibrated. When a simulation is equilibrated, that is, when the structure of interest fluctuates around a stable average conformation, it makes sense to compute the fluctuations of each subset of the structure (each atom, for example) relative to the average structure of the simulation, the RMSF (Martínez, 2015).

The crystal structure of N-terminal RSK2 kinase in complex with 2-amino-7-substituted benzoxazole (PDB[8] ID 4NW5) was taken into account since it shows the lowest *RMSD* value (0.954 Å) compared to active Akt/PKB (PDB[8] ID 1O6K) (Yang et al., 2002). The Ser227 was modified in a phosphorylated residue (pSer227) or mutated into Asp (Asp227) to obtain the phosphomimetic mutant. The starting protein showed that pSer227 is part of a hydrogen bond network, similar to that found in Akt/PKB, which involves Arg114, Arg192 and Lys216 in RSK2 (Fig. 13A) and His196, Arg274 and Lys298 in Akt/PKB (Fig. 13C). Such a network is important to have a well-structured activation loop promoting substrate recognition due to the αC-helix anchored through Arg114 and carrying a conserved acidic residue that forms an ion pair with a conserved lysine in the active site that enables cofactor binding, as previously shown for PAKs (Eswaran et al., 2007). Such an ion pair is conserved in RSK2 and involves Glu118 and Lys110. When Ser227 was replaced by Asp, even if the best rotamer is the most superimposable with pSer227, the number of interactions with the surrounding basic residues decreased and the N atom of Lys216 side chain was 5 Å away from the oxygen atom of Asp side chain. Thus, this residue does not

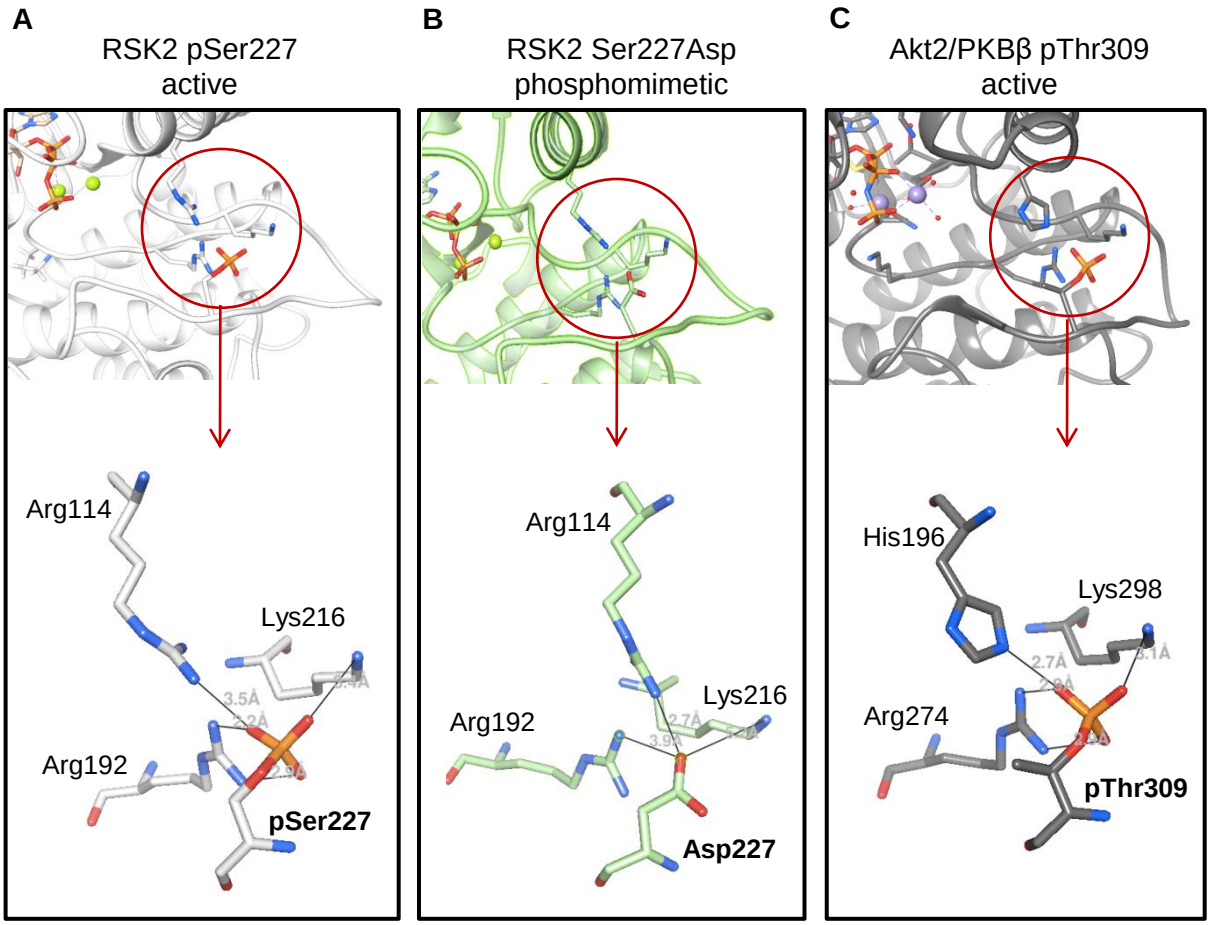

Fig. 13 | Molecular dynamics (MD) simulation of the wild type or mutated RSK2 crystal structures compared to the active Akt2/PKBβ one. The crystal structure of N-terminal wild type **(A)** or mutated **(B)** RSK2 kinase in complex with 2-amino-7-substituted benzoxazole (PDB ID 4NW5) was compared to the stuctute of the active Akt2/PKBβ (PDB ID 1O6K) **(C)**. **A.** The serine 227 (Ser227) of RSK2 was modified in a phosphorylated residue (pSer227) to obtain the active protein and the hydrogen bonds that the pSer227 formed with neighboring residues (Arg114, Arg192 and Lys216) are shown; **B.** The serine 227 of RSK2 was mutated into an aspartate (Asp227) to obtain the phosphomimetic mutant and the hydrogen bonds that it could formed with the neighboring amino acids are shown; **C.** The threonine 309 (Thr309) of Akt2/PKBβ was modified in a phosphorylated residue (pThr309) to obtain the active protein. Then, the hydrogen bonds that the pThr309 formed with neighboring residues (Hys196, Arg274 and Lys298) are shown.

participate in the stabilization of the loop. Moreover, Asp227 can potentially interact with only one oxygen atom with Arg192 even if the distance Asp227-Arg192 is larger (3.9 Å) compared to that of pSer227-Arg192 (3.5 Å), where two interactions are predicted to form (Fig. 13B). Thus, the overall structural stability of the loop seems to be lower when Asp is present in position 227. Moreover, the αC-helix-*RMSD* profile, as a function of time during the MD simulation, showed a different behavior for pSer227 and Asp227 proteins. In both cases, a first rapid increase in the αC-helix-*RMSD* within 4 ns was observed. After this time, the Asp227 simulation showed a plateau, whereas the pSer227 one showed a slowest increase in the overall RMSD (Fig. 14A). The RMSF profiles also showed that the region 119-124 (corresponding to αC-helix) was more flexible for the Asp227 mutant (Fig. 14B). The analysis of the activation loop in the average structure of the MD simulation as well as in the final structure after 20 ns of the simulation showed that the H-bonds network involving pSer227 was still present with distances between the oxygen atoms of the phospho-serine and the nitrogen atoms of lysine and arginine residues ranging from 2.9 to 3.2 Å. Whereas, when the Ser227Asp mutation was present, Arg114 was free to move away (Fig. 14C) triggering a larger shift in the αC-helix, compared to that induced after the serine phosphorylation. The shift in the αC-helix was accompanied in both simulations by the propensity of this element to unfold starting from the C-terminal. Moreover, the ion pair Lys100-Glu118 moved away from the ATP during the simulation, especially for Asp227 mutant, preventing ATP from binding.

Therefore, the MD simulation results allowed us to conclude that, in the case of the phosphomimetic mutant, the decreased stabilization of the activation loop seemed to play a key role in the propensity of the protein conformation to change from the inactive state to the active state.

In summary, our findings support the hypothesis that the presence of a phosphate group on the activation loop of RSK2 is critical for its activation. As a consequence, the mere presence of different negative charged residues in place of the phosphate group is unable to cause the same conformational rearrangements necessary to determine kinase activation. This model can be likely extended to the whole AGC kinase family. Furthermore, our finding strongly supports the idea that is not possible to decouple PDK1 from the activation of its substrates by aminoacidic substitutions in their activation loop.

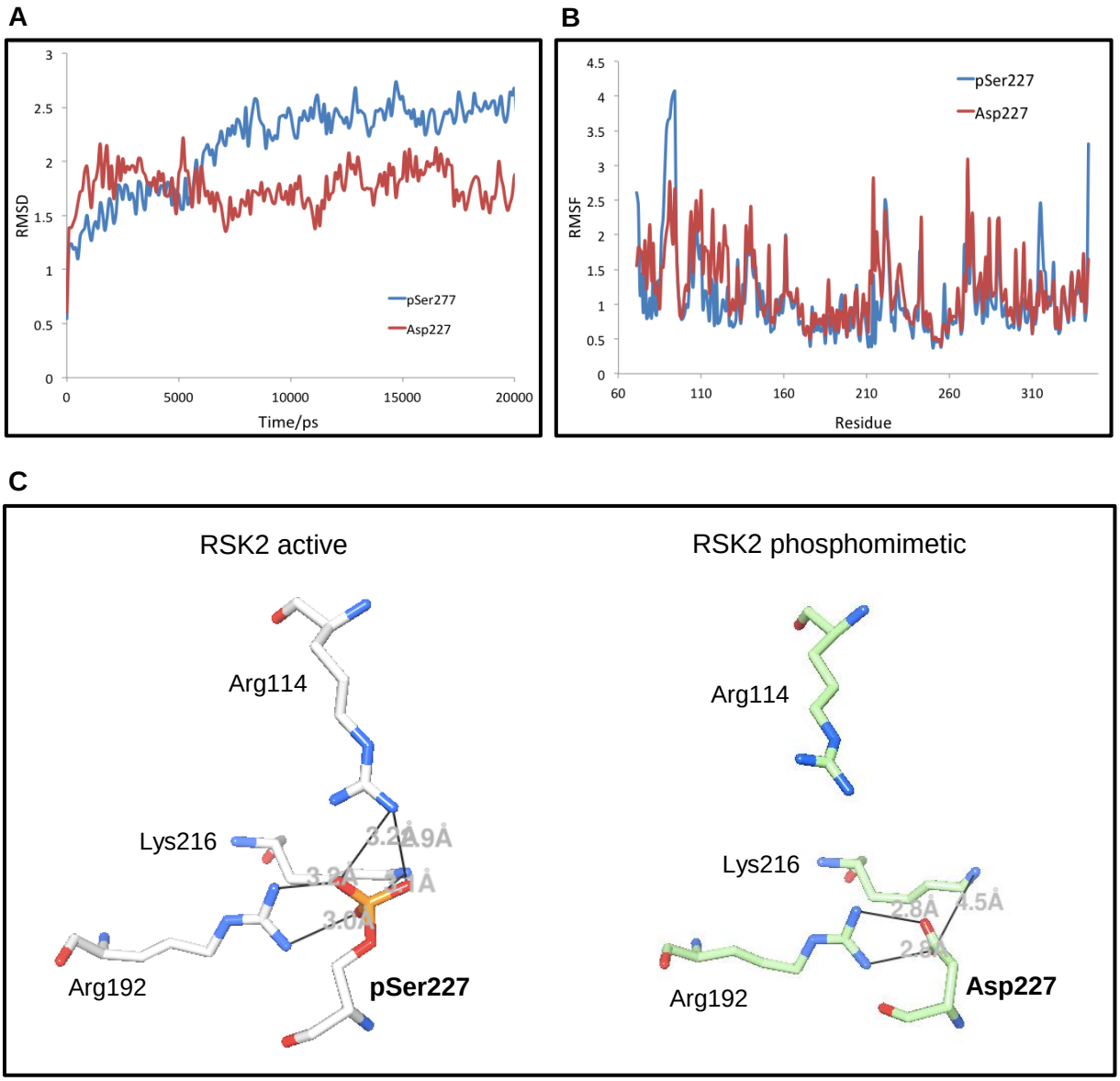

Fig. 14 | The structure of the activation loop in the NTKD of the RKS2 phosphomimetic mutant is more flexible compared to RSK2 WT one. A. The αC-helix-*RMSD* profiles during the MD simulation rapidly increase within 4 ns. Then, phosho-serine 227 (pSer227) simulation showed a slow increase in the overall *RMSD*, while the aspartate 227 (Asp227) simulation presented a plateau; **B.** The *RMSF* profiles showed that αC-helix (residues from 119 to 124) and the region 221-218 were more flexible in the mutant RSK2 S227D than in the active wild type protein (pSer227); **C.** The crystal structures of the amino acids in the activation loop of the wild type (on the left) and the mutant (on the right) RSK2 kinase (PDB ID 4NW5) were compared**.** The serine 227 of RSK2 was modified in a phosphorylated residue (pSer227) to obtain the active protein and the hydrogen bonds that it formed with neighboring residues (Arg114, Lys216 and Arg192) are shown. Then, the pSer227 of RSK2 was mutated into an aspartate (Asp227) to obtain the phosphomimetic mutant and the hydrogen bonds that it could formed with the neighboring amino acids are shown. After MD simulation of both wild type and mutated RSK2, the arginine 114 (Arg114) moved away triggering a larger shift in the αC-helix in the mutant RSK2 S227D, while it formed a hydrogen bond with the pS227 in the active wild type protein (RSK2 pS227) stabilizing the activation loop.

Mutation of a distant residue, predicted to interact with the AL phosphate, phenocopies the substitution with Asp

In order to demonstrate that the binding between the phosphorylated serine in the activation loop of the NTKD of RSK and another aminoacid close to it is essential for the full activation of the protein, we produced a mutant for RSK1 and one for RSK2 in which we substituted one of these other aminoacids with an alanine. According to the MD simulation of the phosphomimetic mutant of RSK2, the distance between aspartate 227 and lysine 210 is longer than the distance required to form a regular hydrogen bond. Therefore, we hypothesized that the inability of aspartate 227 to form a bond with lysine 210 is involved in the failure of the phosphomimatic mutation to constitutively activate the kinase activity. To test this hypothesis, we cloned RSK1 K210A mutant in pDEST27 GST-fused expression vector. We supposed that, if this aminoacid is necessary for the full activation of the protein, after its substitution, the binding with the phosphorylated serine could not be formed and so the protein would be inactive. We overexpressed the wild type protein, the phosphomimetic mutant (RSK1 S221D), and the RSK1 K210A mutant in HeLa cells, and then we stimulated or not these cells with 10 ng/ml of EGF for 15min.Then, cells were lysed and we verified the activation of the overexpressed proteins by western blot. Confirming our hypothesis, the K210A mutant of RSK1 was completely inactive despite the stimulation, like the phosphomimetic mutant. Indeed, we didn't observe the increased phosphorylation of the Ser102 of the RSK substrate (YB1) after the EGF stimulation, as instead happened with the overexpressed wild type protein (Fig. 15). Thus, the lysine 210 is necessary for the stabilization of the activation loop and the conformational change in the active state. We will confirm this result also with the RSK2 mutant (K216A).

Development and characterization of a three-dimensional (3D) *in vitro* culture of metastatic CRC (mCRC) patient-derived organoids (PDOs)

For the past 30 years 2D-cell-based assay models have dominated preclinical cancer drug discovery, but monolayer cell cultures don't recapitulate the tumor complexity. In fact, they lack of the pathological tissue heterogeneity, the 3D tissue architecture and the tumor-stroma interaction. For this reason, recent efforts have been focused on identifying more complex systems, which are more predictive of *in vivo* efficacy. A more reliable model is patient-derived tumor xenograft (PDTX or PDX), which better mimics the original tumor

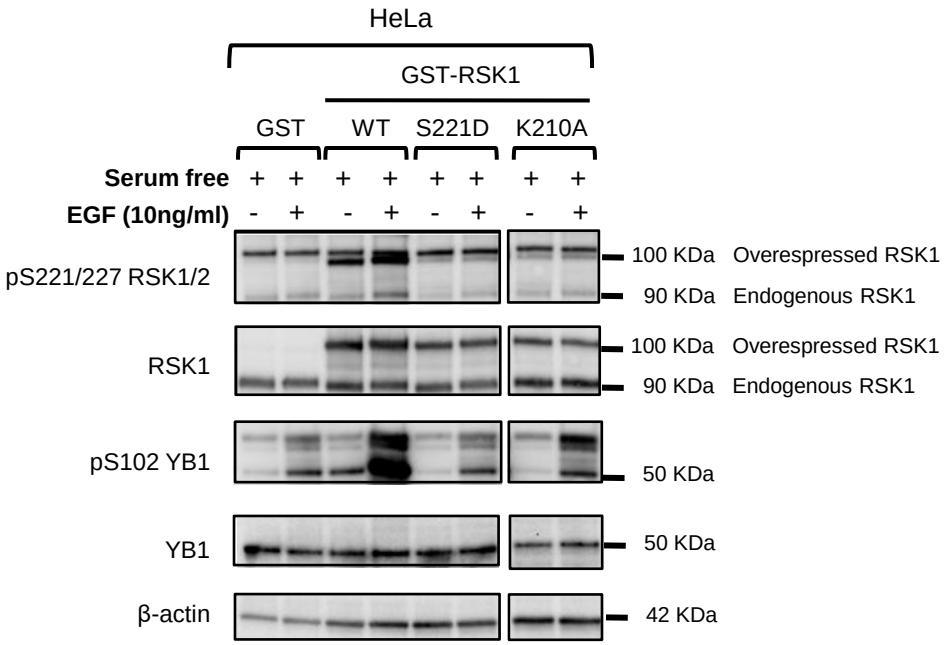

Fig. 15 | RSK1 kinase activity is impaired by amino acid substitution of lysine 210 in the NTKD. HeLa cells were transfected with pDEST27 plasmid containing or not the wild type or mutated (S221D and K210A) RSK1 coding sequence. These cells were starved overnight with normal culture medium without FCS. Than, HeLa cells were stimulated or not with EGF (10 ng/ml) for 5 min and lysed. Western blot analysis were performed using the indicated primary antibodies.

conditions and constitutes a valid preclinical model. In addition to PDXs, it has been recently developed an *in vitro* model more realistic than traditional cell lines: the *in vitro* 3D culture of patient-derived organoids (PDOs)**.** These *in vitro* 3D cultures, sustained by extracellular matrices like Matrigel or Basement Membrane Extract (BME) mimicking the *in vivo* environment, can be directly derived from patient tumor samples or from samples propagated and amplified in mice as PDXs.

By mimicking the environment of intestinal stem cells, Sato and colleagues (Sato et al., 2011a) succeeded in establishing the minimal requirements for the growth of crypt-villus structures without mesenchymal compartment. Intestinal organoids are able to efficiently grow *in vitro*, to self-renew, and to be expanded as long-term cultures while remaining genetically stable. Currently, organoids are also being established from a variety of tumors with colorectal cancer leading the way. They preserve *in vitro* the tumor features present *in vivo.* Therefore, organoids could potentially fill the gap between cancer cell lines and xenografts. This innovative culture approach of tumor cells allows performing more rapid, variegated and translational studies including mid-throughput screenings and multiple drug combinations.

Taking into account these consideration and based on the protocol published by Clevers (Sato et al., 2011a), our group set up PDO culture conditions and generated a platform of PDOs, deriving them from primary CRC samples or from the large metastatic CRC (mCRC) PDXs biobank that has already been generated in Candiolo Cancer Institute by the Translational Cancer Medicine Laboratory. Until now, we successfully kept in culture 80 out of 103organoids (77,66%). We have followed their growth over time in the presence of different EGF concentrations (1 - 2 - 2.5 - 5 ng/ml) added to their culture medium (Fig. 16). All organoids showed a specific and quite different morphology (Fig. 17). Thus, we have characterized them in detail by performing immunofluorescence experiments, to simultaneously observe their structure, proliferation, apoptosis and nuclear polarity. We cultured mCRC PDOs, previously mechanical disaggregated (Fig. 18) or dissociated at single cells (Fig. 19), on BME and we let them grow. After about 8 days of culture, we fixed organoids and stained them with EdU Alexa Fluor® 647 (an intercalating agent marking cells that underwent proliferation during the period of treatment) overnight, and then with active caspase 3 (a protein responsible for apoptosis execution), phalloidin conjugated with Alexa Fuor® 488 (to stain for actin) and DAPI (for nuclei). We observed that mCRC PDOs showed a well-organized structure, which resembled the structure of the normal colon. They presented a simple columnar epithelium with a strong apical-basal polarity:

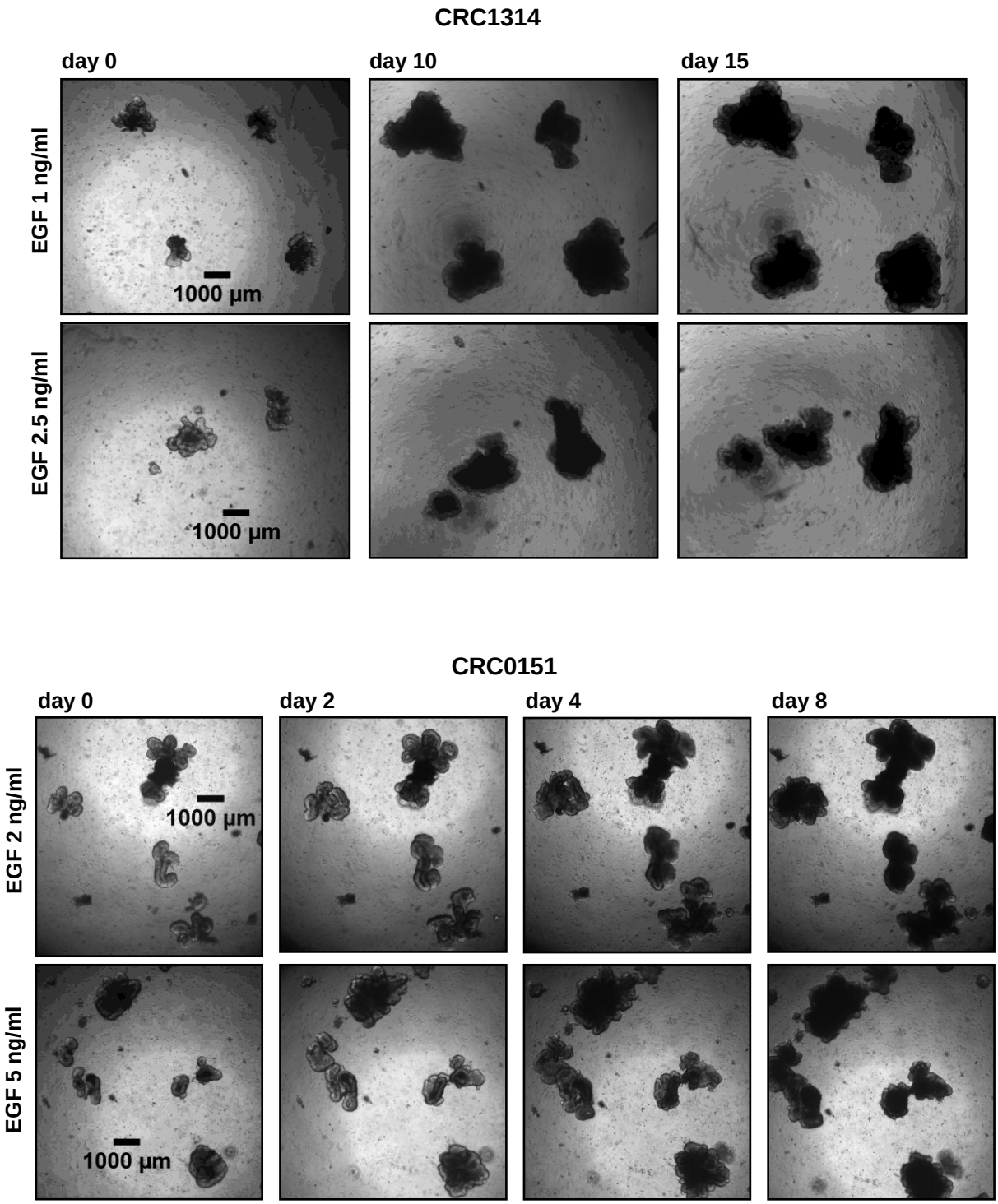

Fig. 16 | A timescale of a three-dimension (3D) *in vitro* culture of mCRC patient-derived organoids (PDOs). A collection of 3D tumor organoids, with original samples isolated from mCRC patient-derived xenografts (PDX) and harboring different genetic lesions, was generated. mCRC PDOs were cultured *in vitro* embedded in a 3D matrix (Cultrex Basement Membrane Extract, BME) with medium added with increasing concentrations of EGF (1 – 2 – 2.5 – 5 ng/ml) and incubated at 37°C and 5% CO_2. Organoids growth was followed over time at day 0, day 10 and day 15; or at day 0, day 2, day 4 and day 8, and images were acquired in brighfield by using 10X objective. Images of two PDOs are shown. Scale bar: 1000 µm.

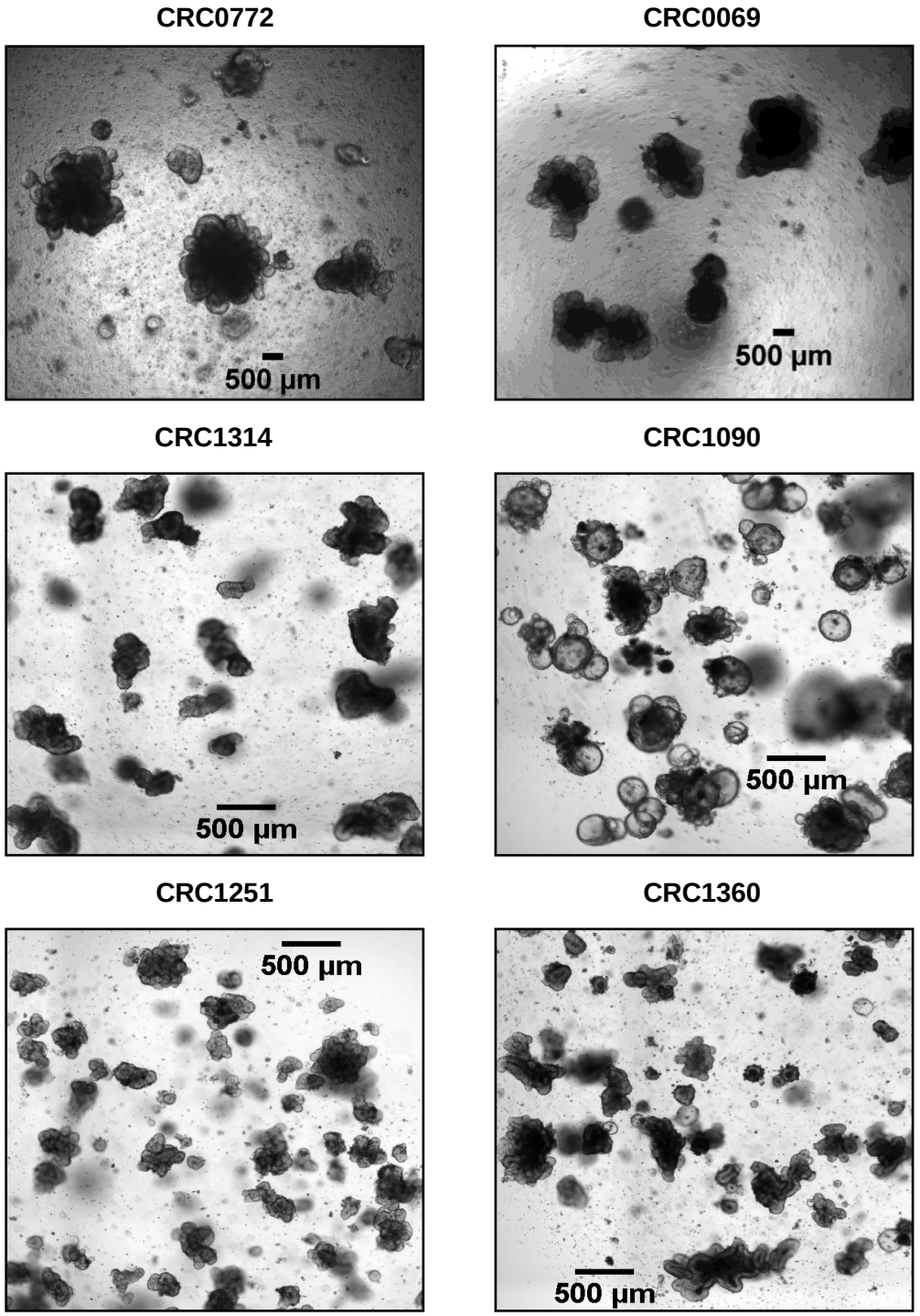

Fig. 17 | Development of a three-dimension (3D) *in vitro* culture of mCRC patient-derived organoids (PDOs). A collection of 3D tumor organoids, with original samples isolated from mCRC patient-derived xenografts (PDX) and harboring different genetic lesion, was generated. mCRC PDOs were cultured *in vitro* embedded in a 3D matrix (Cultrex Basement Membrane Extract, BME) with medium added with EGF (20 ng/ml) and incubated at 37°C and 5% CO_2. Images were acquired in brighfield by using 4X or 10X objective. Images of different mCRC PDOs were acquired in brighfield by using 10X objective. Scale bar: 500 µm.

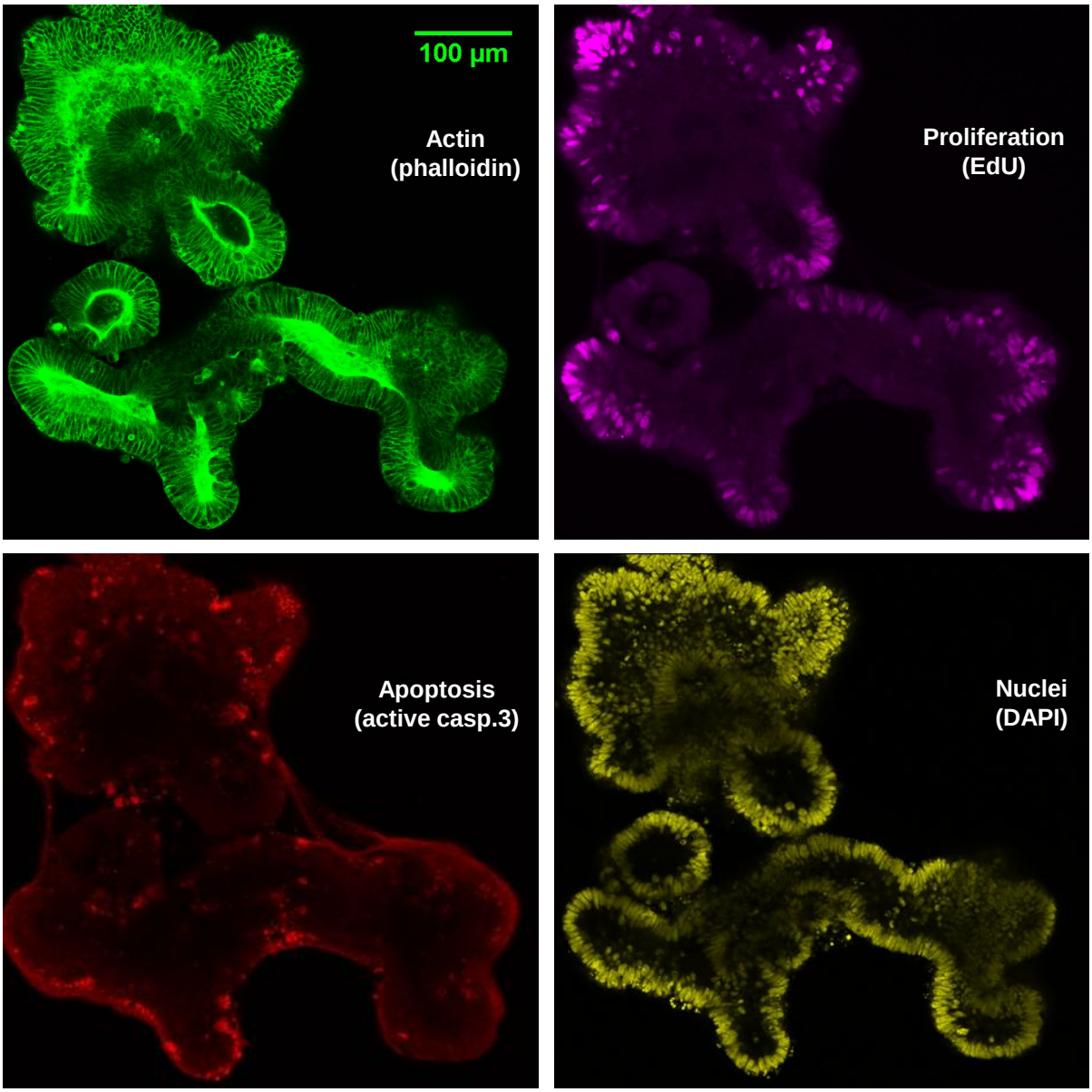

Fig. 18 | Characterization of a three-dimension (3D) *in vitro* culture of mCRC patient-derived organoids (PDOs) by immunofluorescence. A mutated KRAS mCRC PDO (CRC1090) was mechanical disaggregated and cultured *in vitro* on a layer of a matrix (Cultrex Basement Membrane Extract, BME). Medium supplemented with 2% of BME was changed every 2 days. After 8 days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at confocal microscope by using 20X objective. Scale bar: 100 µm.

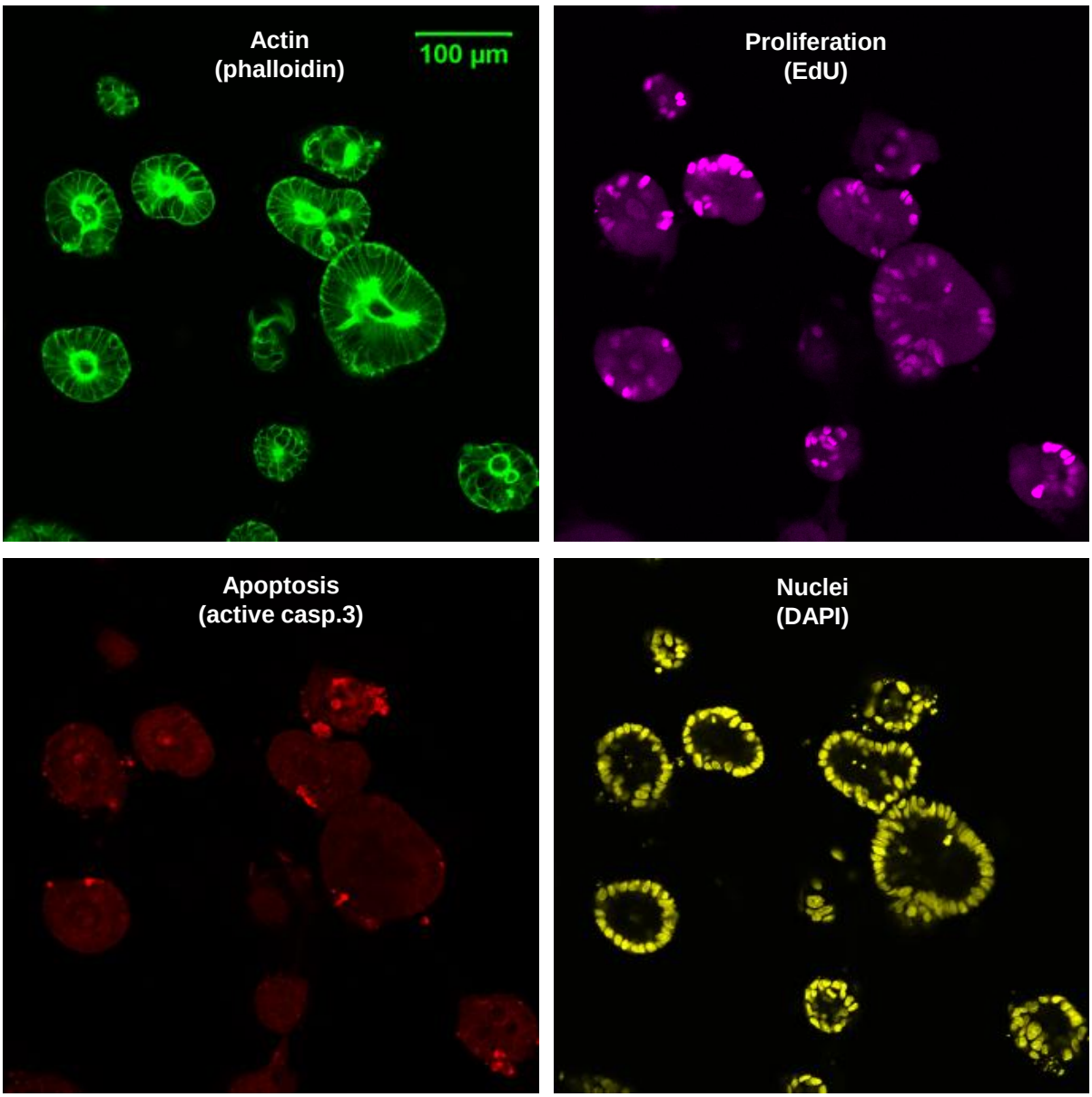

Fig. 19 | Characterization of a three-dimension (3D) *in vitro* culture of mCRC patient-derived organoids (PDOs) by an immunofluorescence. A mutated KRAS mCRC PDO (CRC1360) was dissociated at single cells and cultured *in vitro* on a layer of a matrix (Cultrex Basement Membrane Extract, BME). Medium supplemented with 2% of BME was changed every 2 days. After 8 days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at confocal microscope by using 20X objective. Scale bar: 100 µm.

actin was localized apically toward the lumen where they formed microvilli, while nuclei were localized at the basal side of the cells. Moreover, they formed crypt-like structures with a lot of proliferating cells at their bases (Fig. 18). Furthermore, PDOs were able to restore their original structure and morphology, even starting from single cells. Indeed, they showed a high rate of proliferation, given that the majority of the cells in each organoid was stained with the EdU, while the apoptosis was confined to few cells (Fig. 19).

PDK1 inhibition affects the viability of WT or mutated KRAS mCRC patient-derived organoids (PDOs)

We decided to investigate the involvement of PDK1 in the CRC progression by using the PDO model. Therefore, we expanded in culture a collection of different wild type or KRAS-mutated mCRC PDOs (Table 1). Two of the selected KRAS-mutated PDOs also harbour a mutation in *PIK3CA* (CRC1090 and CRC1588). To verify the effect of PDK1 inhibition on these PDOs growth, after disaggregation at single cells, we cultured them (10^4 cells/well) on a layer of BME and treated them with different concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795, every two days for 15 days. We acquired bright field images at the 15th day. PDK1 blockade induced a remarkable reduction of the growth of all organoids (Fig. 21). We also evaluated the viability of the PDOs grown after the treatment, using the CellTiter-Glo® Luminescence Viability Assay. We observed a strong impairment of the survival of all mCRC PDOs in the presence of BX795, already at the lowest concentration (1 µM) in many cases (Fig. 22).

To confirm these results, we repeated this experiment with the same PDOs but using the more specific PDK1 inhibitor BX912. The eight mutated KRAS mCRC PDOs tested up to now showed a substantial sensitivity to these inhibitor, which exactly corresponds to their sensitivity to the BX795 observed before (Fig. 23 and 24). Therefore, we could conclude that PDK1 is involved in metastatic colorectal cancer progression, and we demonstrated it in a model which is more closely related to the patients.

PDK1 inhibition alters the morphology of KRAS-mutated mCRC patient-derived organoids (PDOs)

Finally, we wanted to understand whether PDK1 inhibition determined either a block of the proliferation or an induction of the cell death of PDOs. Thus, we mechanically disaggregated (Fig. 25 and 26) or dissociated at single cells (Fig. 27 and 28) some

mutated KRAS mCRC PDOs and we cultured them on a layer of BME. Then, we treated these organoids with increasing concentrations (0, 1, 3, and 10 µM) of the BX795, refreshed every 48 hours for eight days. At the end of the experiment we performed an immunofluorescence analysis of proliferation and apoptosis. PDOs were fixed and labeled with EdU Alexa Fluor® 647 (a marker of proliferating cells) overnight, and then with active caspase 3 (a marker of apoptotic cells), phalloidin conjugated with Alexa Fuor® 488 (a marker of actin) and DAPI (for nuclei). At the end of the treatment, PDK1 blockade caused a strong alteration of the PDOs structure and morphology, which were almost entirely disrupted. In fact, treated PDOs were smaller than the untreated ones and the epithelium of PDOs treated with 3 µM of BX795did not show the typical structure of a simple columnar epithelium. Moreover, PDK1 inhibition caused reduction of proliferating cells and at the same time an increase of cell death. In fact, the DAPI staining showed that the DNA of the cells was disorganized and accumulated in the lumen of all organoids, indicating the presence of apoptotic bodies (Fig. 25 and 28).

PDO	KRAS	NRAS	BRAF	PIK3CA
CRC1090	G12V	-	-	Q546K
CRC1588	G12D	-	-	E545A
CRC1307	G12D	-	-	-
CRC0031	G12D	-	-	-
CRC1360	G12C	-	-	-
CRC1251	G12A	-	-	-
CRC1589	G12C	-	-	-
CRC1314	Q61H	-	-	-
CRC1448	A146P	-	-	-
CRC1257	G12A	-	-	-
CRC1245	G13D	-	-	-
CRC0578	-	-	-	-
CRC0124	-	-	-	-
CRC1575	-	-	-	-

Table 1 | A collection of an *in vitro* three-dimension (3D) culture of mCRC patient-derived organoids (PDOs). Tumor organoids, with original samples isolated from mCRC patient-derived xenografts (PDX) and harboring different genetic lesion, was generated. Their specific mutations in *KRAS* and/or *PIK3CA* are reported in the table.

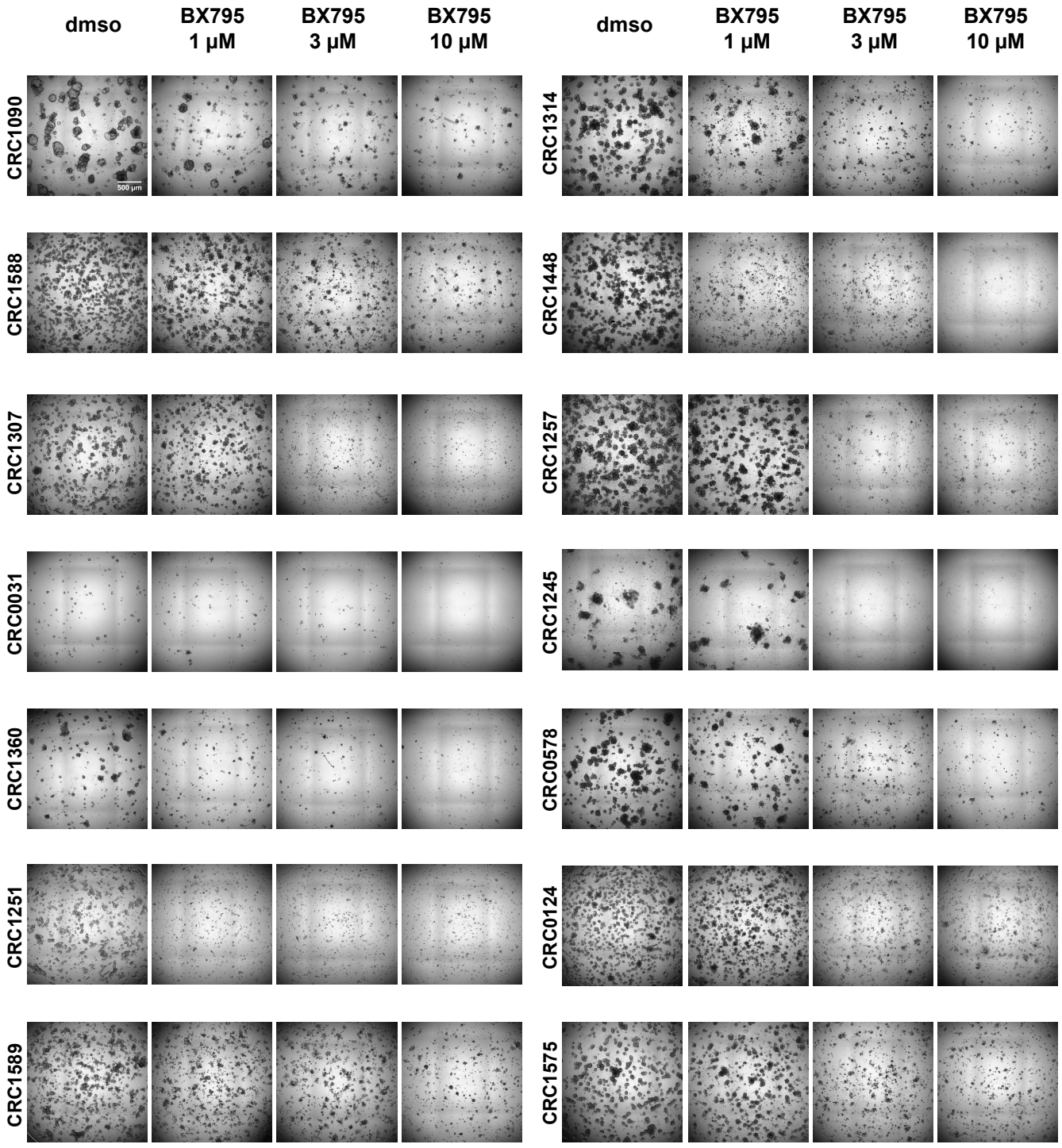

Fig. 21 | mCRC Patient-derived organoids growth is impaired by the inhibition of the PDK1 kinase activity. Wild type (CRC0578, CRC0124 and CRC1575) and mutated (the others) KRAS patient-derived organoids (PDOs) were dissociated at single cells. 10^4 cells of each organoids were cultured on BME (in triplicate). After 48 hours, they were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 added to the normal culture PDOs medium. Medium was changed every two days for 15 days. Images were acquired at the 15th day using a 4X objective. Scale bar 500 µm.

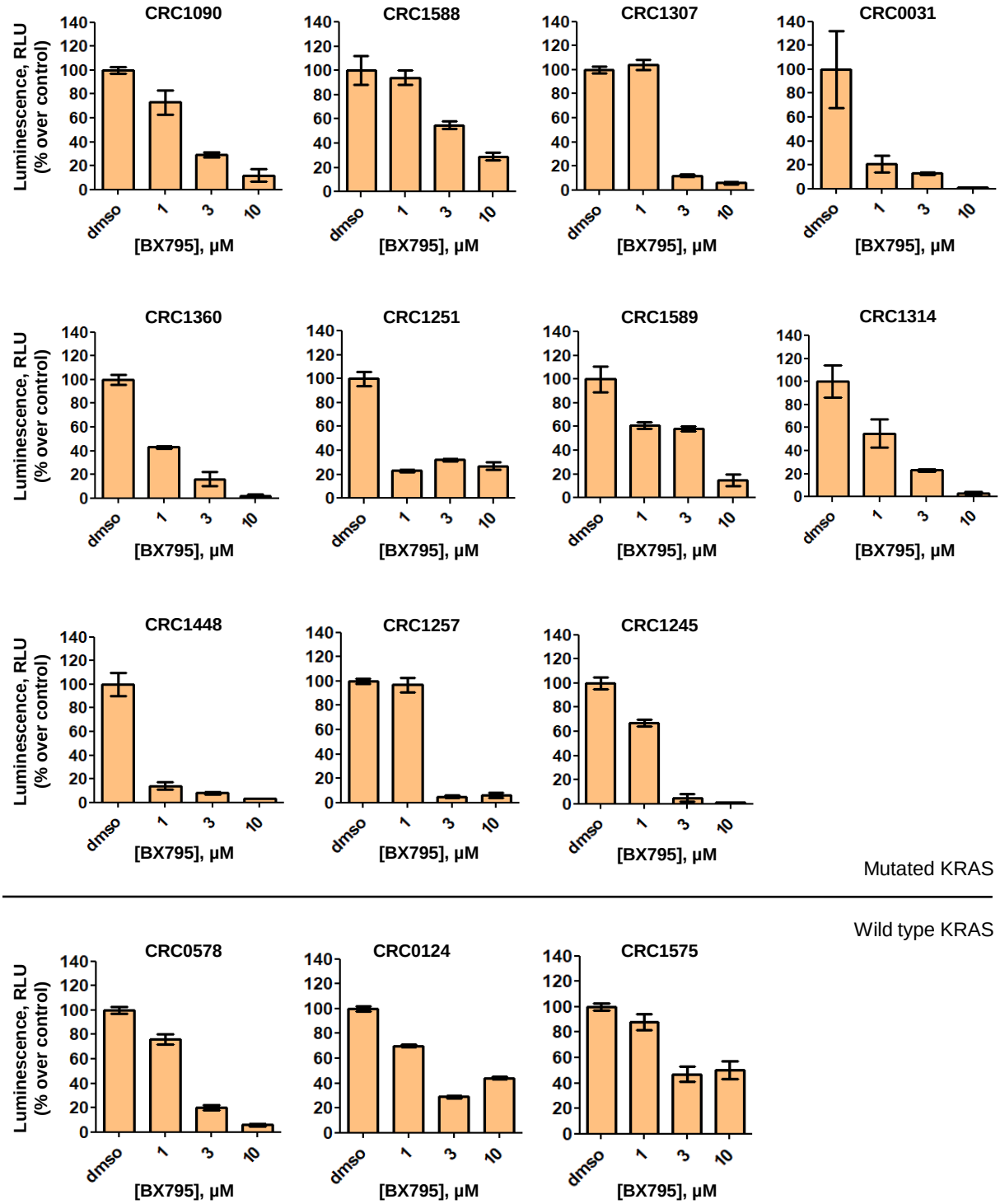

Fig. 22 | mCRC Patient-derived organoids growth is impaired by the inhibition of the PDK1 kinase activity. PDOs were dissociated at single cells. 10^4 cells of each organoids were cultured on BME (in triplicate). After 48 hours, they were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 added to the normal culture PDOs medium. Medium was changed every two days for 15 days. Viability of PDOs was evaluated using CellTiter Glo® Luminescence Cell Viability assay at the 15th day. Then, the viability of organoids grown with BX795 was normalized dividing it by the viability of organoids grown without the inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

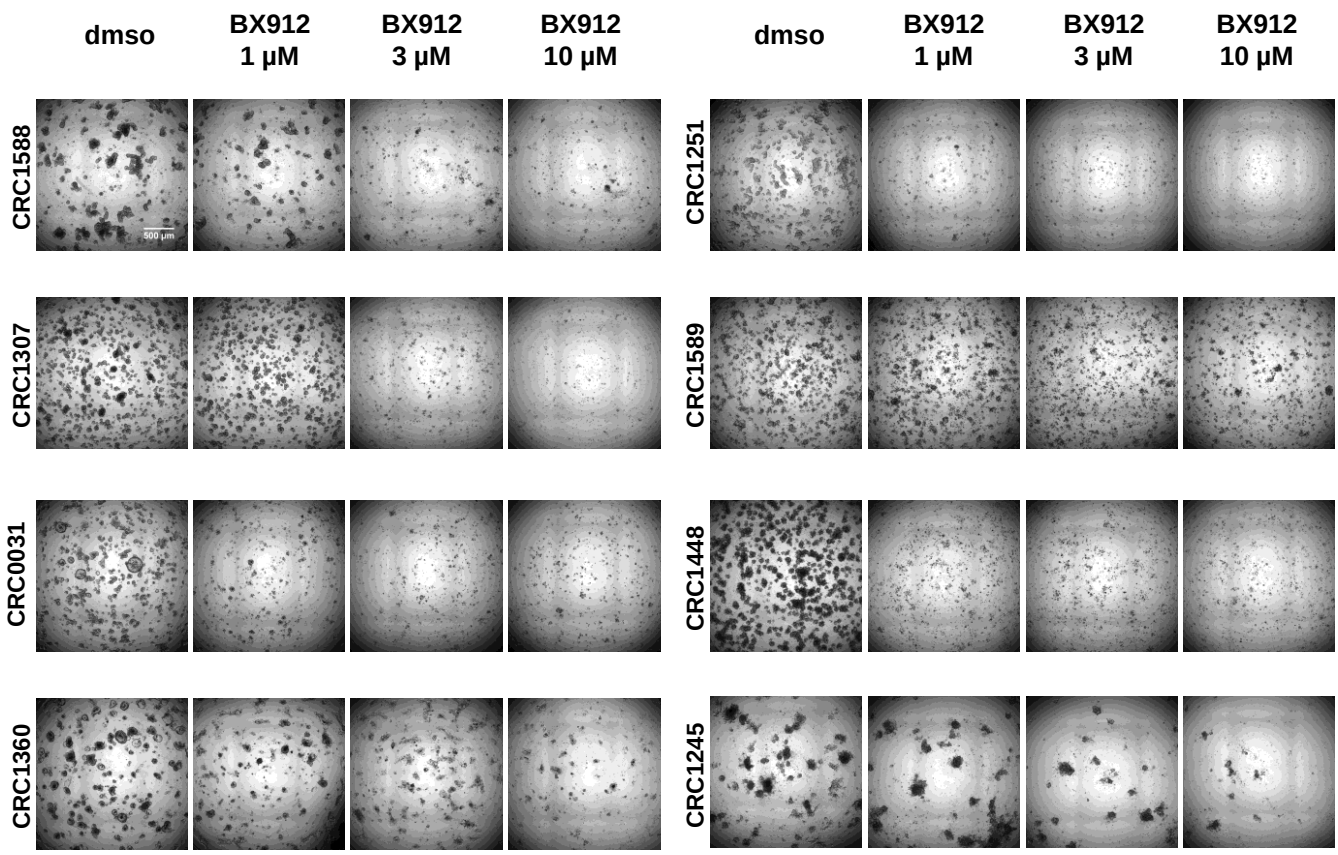

Fig. 23 | mCRC Patient-derived organoids growth is impaired by the specific inhibition of the PDK1 kinase activity. Mutated KRAS patient-derived organoids (PDOs) were dissociated at single cells. 10^4 cells of each organoids were cultured on BME (in triplicate). After 48 hours, they were treated with increasing concentrations (0, 1, 3 and 10 µM) of the specifc PDK1 inhibitor BX912 added to the normal culture PDOs medium. Medium was changed every two days for 15 days. Images were acquired at the 15th day using 4X objective. Scale bar 500 µm.

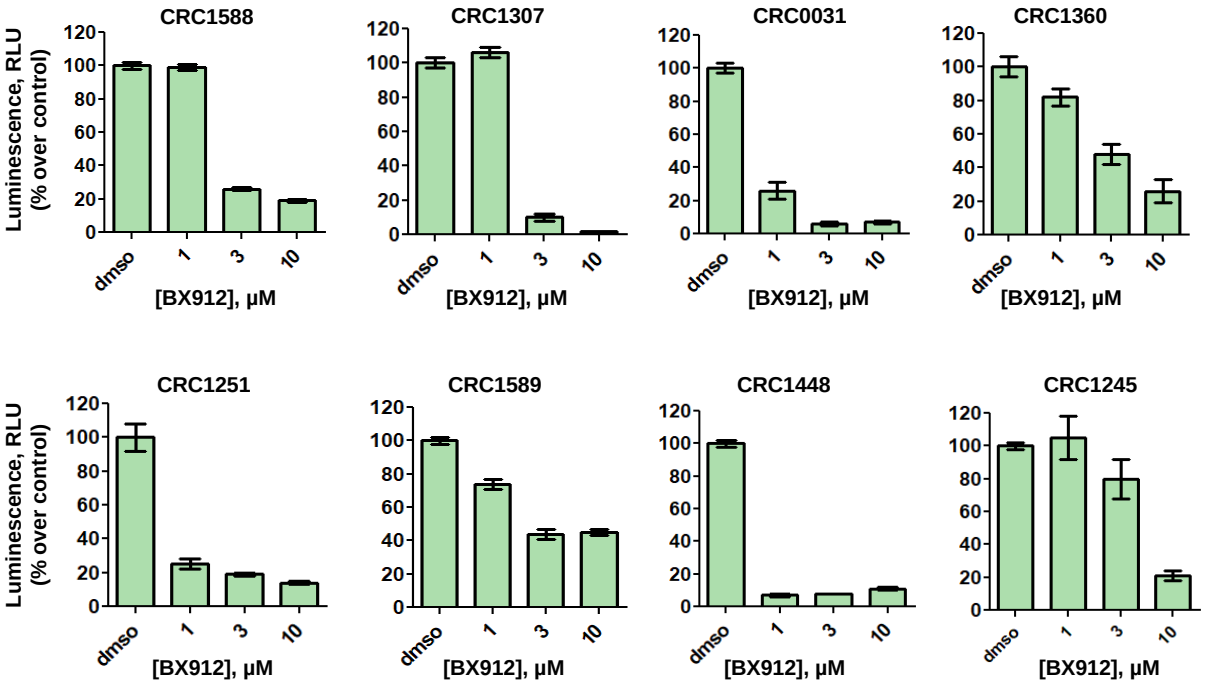

Fig. 24 | mCRC Patient-derived organoids growth is impaired by the specific inhibition of the PDK1 kinase activity. C. PDOs were dissociated at single cells. 10^4 cells of each organoids were cultured on BME (in triplicate). After 48 hours, they were treated with increasing concentrations (0, 1, 3 and 10 µM) of the more specific PDK1 inhibitor BX912 added to the normal culture PDOs medium. Medium was changed every two days for 15 days. Viability of PDOs was evaluated using CellTiter Glo® Luminescence Cell Viability assay at the 15th day. Then, the viability of organoids grown with BX912 was normalized dividing it by the viability of organoids grown without the inhibitor. The resulting percentages are reported. Data are shown as average±S.D.

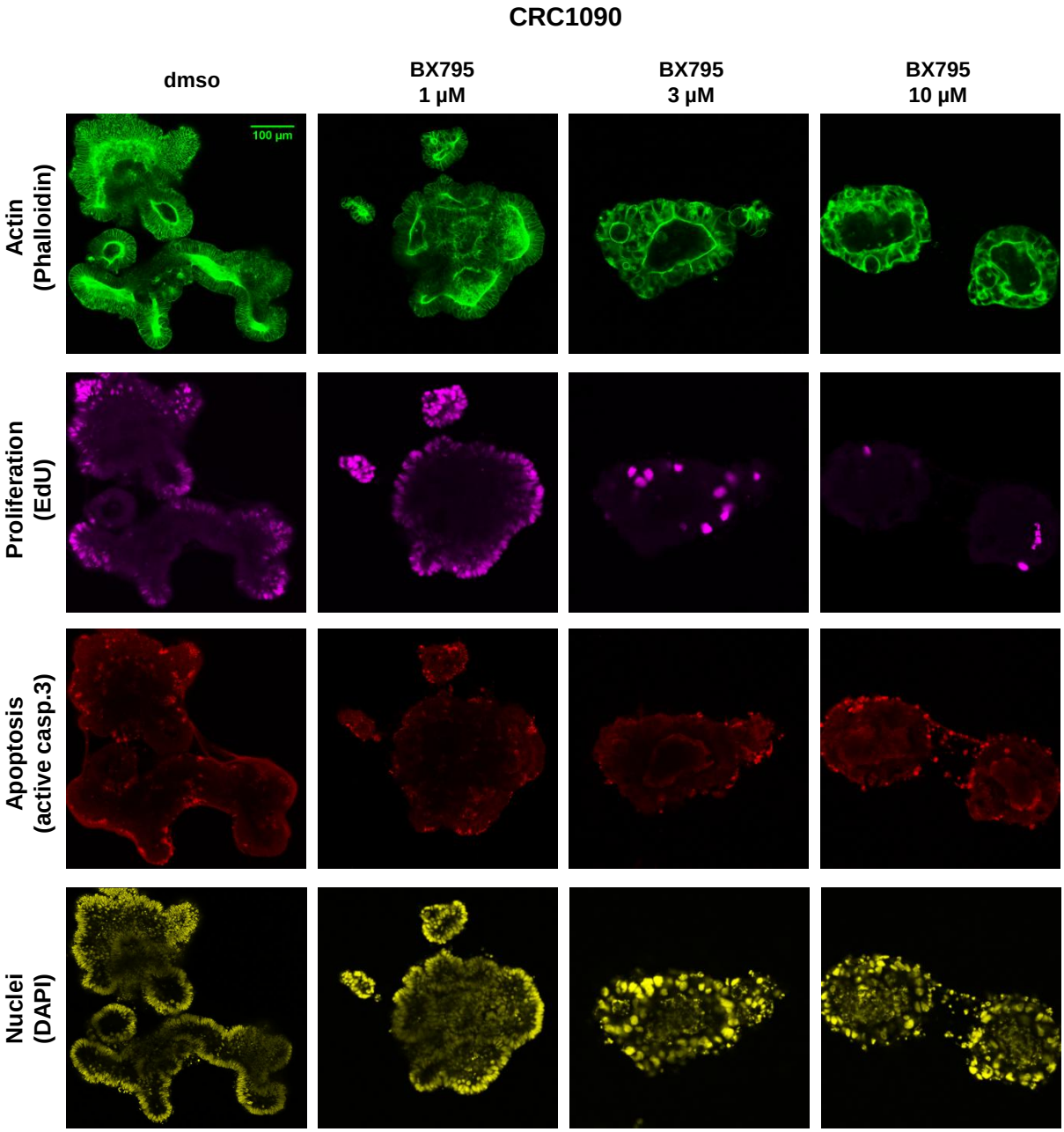

Fig. 25 | PDK1 inhibition alters the morphology of mutated KRAS mCRC patient-derived organoids (PDOs). A mutated KRAS mCRC PDOs (CRC1090) were mechanically disaggregated and cultured *in vitro* on a layer of BME. Then, organoids were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 every 48 hours for 8 days. Medium was also supplemented with 2% of BME (Cultrex Basement Membrane Extract type II). After eight days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at the 8th day by confocal microscopy Leica SPEII with 20X magnification, using the oil immersion objective. Scale bar 100 µm.

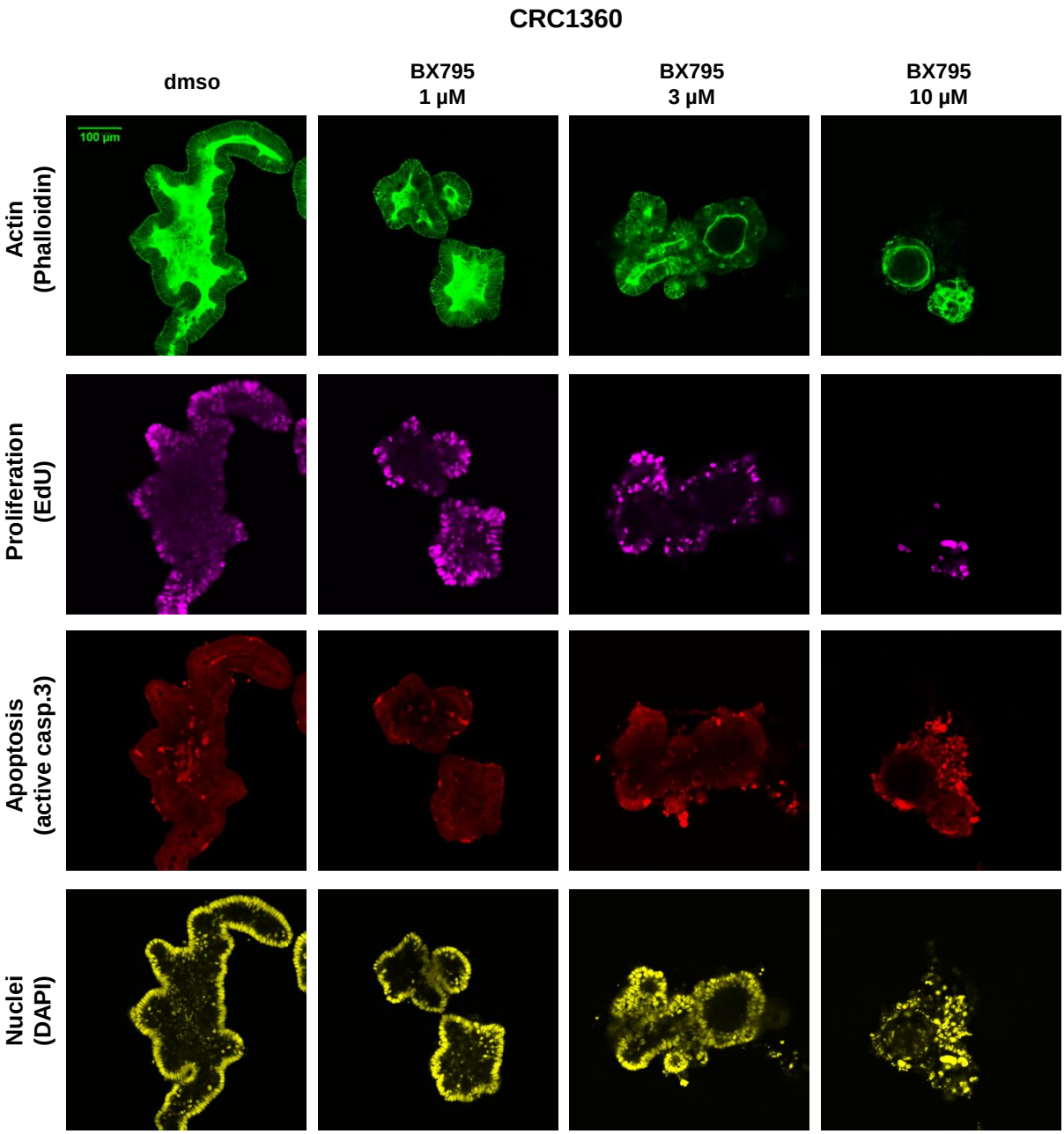

Fig. 26 | PDK1 inhibition alters the morphology of mutated KRAS mCRC patient-derived organoids (PDOs). A mutated KRAS mCRC PDOs (CRC1360) were mechanically disaggregated and cultured *in vitro* on a layer of BME. Then, organoids were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 every 48 hours for 8 days. Medium was also supplemented with 2% of BME (Cultrex Basement Membrane Extract type II). After eight days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at the 8th day by confocal microscopy Leica SPEII with 20X magnification, using the oil immersion objective. Scale bar 100 µm.

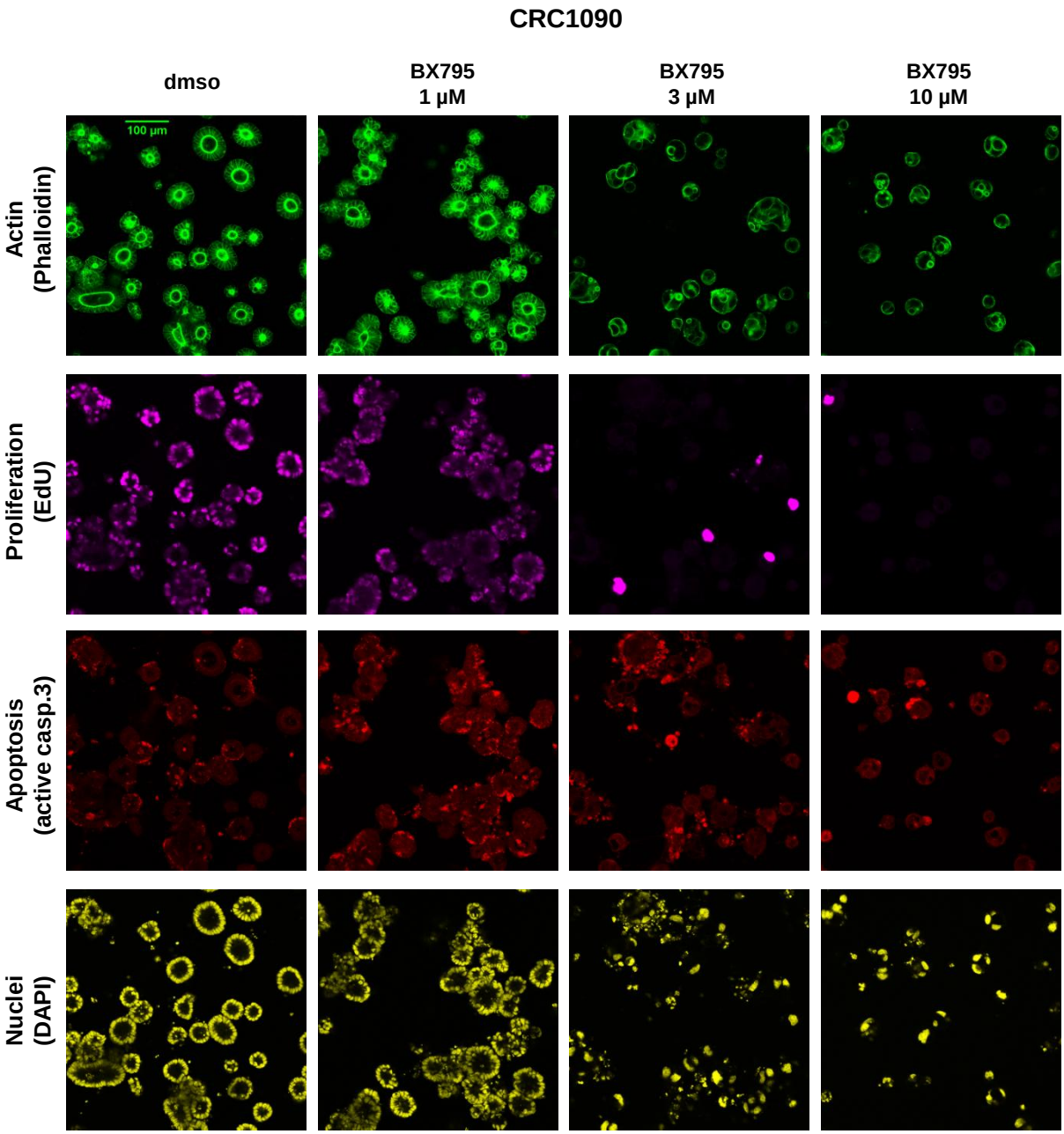

Fig. 27 | PDK1 inhibition alters the morphology of mutated KRAS mCRC patient-derived organoids (PDOs). A mutated KRAS mCRC PDOs (CRC1090) were dissociated at single cells and cultured *in vitro* on a layer of BME. Then, organoids were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 every 48 hours for 8 days. Medium was also supplemented with 2% of BME (Cultrex Basement Membrane Extract type II). After eight days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at the 8th day by confocal microscopy Leica SPEII with 20X magnification, using the oil immersion objective. Scale bar 100 µm.

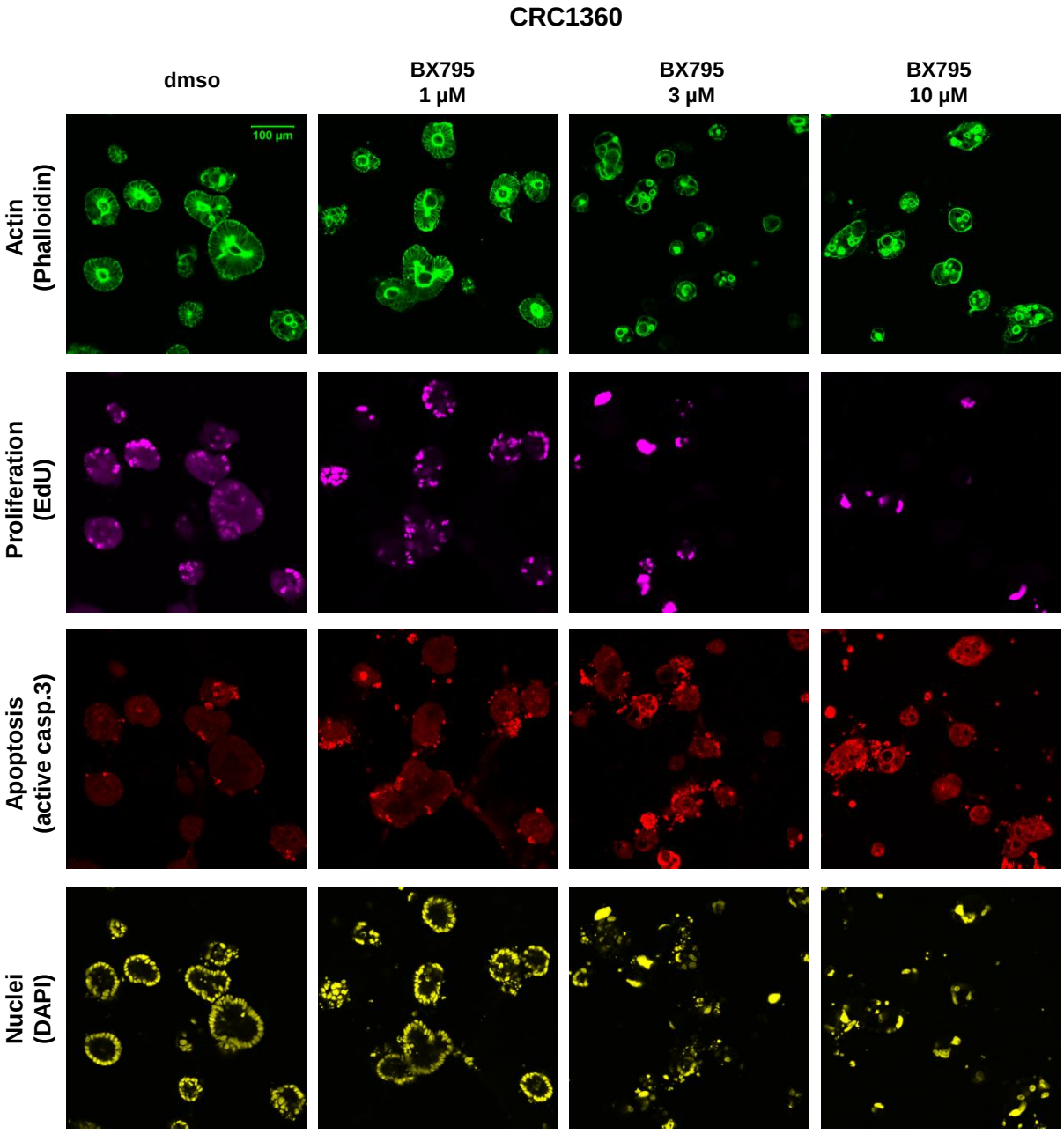

Fig. 28 | PDK1 inhibition alters the morphology of mutated KRAS mCRC patient-derived organoids (PDOs). A mutated KRAS mCRC PDOs (CRC1360) were dissociated at single cells and cultured *in vitro* on a layer of BME. Then, organoids were treated with increasing concentrations (0, 1, 3 and 10 µM) of the PDK1 inhibitor BX795 every 48 hours for 8 days. Medium was also supplemented with 2% of BME (Cultrex Basement Membrane Extract type II). After eight days of culture, organoids were fixed and stained with EdU Alexa Fluor® 647 (a marker of proliferative cells), active caspase 3 (a marker of apoptosis), phalloidin conjugated with Alexa Fluor® 488 (a marker of actin) and DAPI (for nuclei). Images were acquired at the 8th day by confocal microscopy Leica SPEII with 20X magnification, using the oil immersion objective. Scale bar 100 µm.

DISCUSSION and FUTURE PERSPECTIVES

Several studies have unquestionably proved that mutations of *KRAS* gene, which occur in 35% to 40% of colorectal cancer, are associated with worst prognosis and extremely low response rates to current targeted therapies, such as treatment with anti-EGFR antibodies (cetuximab and panitumumab) (De Roock et al., 2009). Furthermore, several retrospective analyses have provided preliminary evidence that mutations of other component of the Ras pathway (NRAS, BRAF and, possibly, PI3KCA) seem to negatively affect sensitivity to EGFR inhibitors (De Roock et al., 2010). As a consequence, KRAS is an attractive therapeutic target for advanced colorectal cancer and for extending the use of cetuximab and panitumumab, which are currently limited to patient with wild type *KRAS* tumors. Unfortunately, 25 years of work on drugs targeting KRAS have proven to be unsuccessful and to date, no effective therapies that specifically targets mutant KRAS are actionable. Because of difficulty of targeting KRAS directly, efforts have been put into targeting the downstream effector pathways, particularly the Raf-MEK-ERK pathway (Sridhar et al., 2005) and PI3K/Akt pathway (Baselga, 2011). KRAS can activate both PI3K and Raf, which lie in different signaling cascades. If only one pathway is inhibited, the cell can circumvent this event by overexpressing the other pathway and thereby creating a resistant phenotype (Knight et al., 2010). Therefore, a better treatment option would be to simultaneously block both pathways.

In a recent published paper, our laboratory showed that 3-phosphoinositide-dependent kinase 1 (PDK1) targeting has negligible effects on normal epithelial cells, while it was effective to counteract tumor growth, invasion and metastatization of breast cancer cells. Notably, these cells, which showed a strong sensitivity to PDK1 inhibition, harbored either PIK3CA or KRAS mutations. In particular, MDA-MB-231 cells, which exhibited the better response to PDK1 inhibition, were mutated in KRAS (G13D) (Gagliardi et al., 2012). Given the non-toxicity of PDK1 blockade in normal cells compared to cancer cells, PDK1 could be a promising target for the treatment of mutated *KRAS* tumors. However, the concept that PDK1 targeting could potentially impair the oncogenic signaling activated by RAS/MAPK pathway still needs clear-cut demonstrations.

Published data showed that reduction of PDK1 and its substrate SGK3 in mutated-BRAF melanoma cells, thus with an activated MAPK pathway, caused an impairment of cell growth and an increase of apoptosis (Scortegagna et al., 2015). Moreover, PI3K and PDK1, but not CRAF, were described to be key effectors of oncogenic KRAS in pancreas,

mediating cell plasticity, acinar-to-ductal metaplasia (ADM), and pancreatic ductal adenocarcinoma (PDAC) formation. Thus, the cell autonomous KRAS/PI3K/PDK1 signaling appears to be a critical and therapeutically tractable axis in pancreatic cancer initiation and maintenance. On the contrary KRAS-driven non-small cell lung cancer, depends on CRAF, but not on PDK1 (Eser et al., 2013). In another study, selective inactivation of PDK1 in the melanocytes of BRAFV600E::Pten-/- or BRAFV600E::Cdkn2a-/-::Pten-/- mice delayed the development of pigmented lesions and melanoma induced by systemic or local administration of 4-hydroxytamoxifen. The authors found that melanoma invasion and metastasis were significantly reduced or completely prevented by *PDK1* deletion, thus proving a role of PDK1, not only in KRAS-driven tumorigenesis, but also in BRAF V600E oncogenic effects (Scortegagna et al., 2014).

In the present thesis, we confirmed, through a synthetic model made with HMEC overexpressing wild type or mutated KRAS, that PDK1 kinase activity is necessary to transduce the signaling downstream the oncogenic KRAS. In particular, HMEC overexpressing mutated KRAS (G13D) were not able to grow in the absence of adhesion after PDK1 blockade, obtained either by treating cells with the PDK1 inhibitor BX795 or by transducing cells with two PDK1-specific shRNAs (shPDK1#79 and shPDK1#81).

Therefore, based on our data and on previously cited literature, we investigated PDK1 role in the advanced stage of KRAS-driven colorectal cancer. We showed that PDK1 kinase activity is required for the anchorage-independent growth of different CRC cell lines. We obtained these results with two different PDK1 kinase inhibitors and by silencing the expression of PDK1. Although the majority of mutated-KRAS CRC cell lines that we tested displayed sensitivity to PDK1 targeting, also wild type KRAS CRC cells (SW48) were responsive, thus suggesting that KRAS mutational status does not provide specific indication for PDK1 sensitivity. Similarly, mutated KRAS CRC cell lines (HCC2998, HCT116 and SK-CO1), which showed the highest sensitivity to PDK1 inhibition, did not display any molecular features that could explain their response. Therefore, it would be interesting to further elucidate the mutational or transcriptional profile that characterizes CRC responsive to PDK1 inhibitor.

Although monolayer cell cultures represent an excellent model for preclinical cancer drug discovery, they present many disadvantages some which have come clear in the recent years. Three of the most important features that 2D cell cultures lack are pathological tissue heterogeneity, 3D tissue architecture and tumor-stroma interactions. In order to obtain a more realistic model that could resemble more closely the *in vivo* tumor, an *in*

vitro 3D culture of patient-derived organoids (PDOs) was developed (Lancaster and Knoblich, 2014). We exploited this model to confirm the role of PDK1 in CRC. In particular, we generated a platform of wild type or mutated KRAS mCRC PDOs, starting from the large mCRC patient-derived xenografts (PDXs) biobank available in Candiolo Cancer Institute and managed by the Translational Cancer Medicine Laboratory.

All PDOs that we tested showed a strong sensitivity to the inhibition of PDK1 kinase activity, with both the inhibitors. Indeed, cell viability was dramatically reduced, already at low concentration of the two inhibitors in the majority of the cases. These results strongly support a potential clinical application of PDK1 inhibitors to mCRC harboring KRAS mutations. Currently, these tumors are not treatable with approved target therapy, such as EGFR inhibitors, because they do not respond to such treatments. Therefore, PDK1 targeting could constitute an important advancement in the treatment of these tumors.

Of course, the already promising results obtained on both cell lines and PDOs will require more preclinical data, for example by PDK1 blockade*in vivo* on PDX mice models, which, among all the available models, better mimic the original tumor conditions. In particular, we would be interested in treating the mCRC PDXs derived from the same patients of the PDOs that we used in our *in vitro* experiments with the PDK1 inhibitors.

Our data show that Akt phosphorylation decreases after PDK1 inhibition in CRC cells and Akt was previously described as the main mediator of PDK1 activity in tumorigenesis (Baxi et al., 2012; Choi et al., 2008; Liu et al., 2009; Maurer et al., 2009). Conversely, the precise mechanism by which PDK1 could interplay with RAS/MAPK signaling pathway has yet to be fully understood. Recent reports showed that PDK1 is involved in cancer by targeting different effectors. For example, an Akt-independent effect of the PDK1 inhibition has been described in breast cancer cells which display impaired soft agar growth and increased apoptosis (Gagliardi et al., 2012). Notably, we have been able to exclude the involvement of Akt in the decrease of the anchorage-independent growth due to PDK1 inhibition, since the effect of the Akt inhibition does not correlate with the effects of the PDK1 inhibition. Consequently, among other PDK1 substrates we hypothesized that RSK isoforms (RSK1, RSK2, RSK3, RSK4) could constitute the link between PDK1 and RAS/MAPK pathway. Indeed, after the activation of MAPK cascade, ERK1/2 binds RSK on a specific consensus sequence at the C-terminal kinase domain (CTKD) and phosphorylates two residues in the hydrophobic motif and one in the activation loop of the CTKD. The activated CTKD autophosphorylates RSK on a serine in the hydrophobic motif, which becomes the docking site for PDK1. PDK1 binds this phosphorylated serine by its

PIF pocket (PDK1-interactin fragment) and it phosphorylates another serine (Ser221 in RSK1 or Ser227 in RSK2) in the activation loop of the N-terminal kinase domain (NTKD), resulting in the complete RSK activation. Once activated RSK is able to phosphorylate and regulate its substrates. NTKD can also phosphorylate a serine in the CTKD of RSK, which results in the dissociation of ERK1/2 from the RSK C-terminal tail and causes RSK inactivation by an internal negative feedback loop (Anjum and Blenis, 2008). We experimentally observed that PDK1 inhibition is able to reduce RSK phosphorylation in all CRC cell lines tested. In order to understand if RSK isoforms are the mediators of PDK1 requirement for CRC cells, we treated our panel of cell lines growing in the absence of adhesion with the specific RSK inhibitor BI-D1870 (Sapkota et al., 2007). We found that the effect of RSK inhibition correlated, better than AKT inhibition, with the effect of PDK1 inhibition. While this result is not the definitive proof that RSK is PDK1 mediator, it does strongly support our hypothesis. Therefore, to confirm RSK role as mediator of PDK1 signaling in mutated KRAS CRC, we cloned RSK1 and RSK2 phosphomimetic mutants (in which the Ser221 for RSK1 and Ser227 for RSK2, phosphorylated by PDK1, are substituted with an aspartate) with the aim to obtain the rescue of the anchorage-independent growth of CRC cell lines treated with the PDK1 inhibitor. Unfortunately, these mutants turned out to be inactive. Thus, we are investigating the chemical bonds which arise in the activation loops of both wild type and mutated RSK2, through the simulation of the molecular dynamics of their structure. Preliminary data show that RSK2 S227D inactivation is due to different electrostatic bonds arising between the Asp227 and neighboring aminoacids, compared to those that are formed in the active wild type protein. The phosphorylated serine in the activation loop of the NTKD of the wild type protein establishes bonds with other residues which maintain the structure of the loop in a fixed and stable conformation. The aspartate in the phosphomimetic mutant is instead not able to recreates the same bonds, thus destabilizes the structure of the loop making the NTKD completely inactive. Asp227 is thus not able to mimic the phosphorylated Ser227. We can conclude that it is not possible to obtain the rescue of PDK1 inhibition and to demonstrate the likely importance of KRAS/PDK1/RSK axis in colorectal cancer progression. Indeed, it is not possible to decouple PDK1 from the activation of its substrates by aminoacidic substitutions in their activation loop.

As future prospective, we would like to investigate other possible PDK1 downstream effectors, which could be involved in the mutated KRAS mCRC tumorigenesis. Among the critical signaling pathways which could be further explored we certainly would try

PDK1/PLK1/Myc pathway (Cunningham and Ruggero, 2013) and Hippo pathway with YAP as the core effector (Fan et al., 2013).

Our discovery points at PDK1 as a crucial signaling transducer in the crossroad of the most commonly altered pathway in solid cancers. In this line, PDK1 is able to transduce its signaling in cancer by different substrates (Akt, RSK, Myc, YAP and so on). Proving which substrate is the most relevant in each tumor type still remains a demanding task. However, our investigation could represent a key point to understand the unexpected dependence of the mutated *KRAS* tumors on PDK1. A full comprehension of PDK1-dependence of tumor cells or patient-derived organoids in the contexts of mutational profile and tumor type contribute to depict a rational therapeutic use of the already developed PDK1 inhibitors (Peifer and Alessi, 2008). In summary, our research highlights the need to enforce investigations on PDK1 targeting to develop new therapeutic opportunities in cancer and, in particular, in metastatic colorectal cancer.

ACKNOWLEDGMENTS

I'm firstly grateful to Luca Primo, Paolo Armando Gagliardi, Laura Di Blasio, Alberto Puliafito, Giulia Chiaverina, Marianela Vara Messler, Miriam Palmiero and Valentina Monica for their precious advices and for their essential support to the preparation and realization of this thesis.

Thanks to every colleagues, who became friends, with whom I shared laughter, discussion, comparisons, valuable advice and the lunchtime.

I'm very grateful to my parents, Donatella and Danilo, for supporting me every moment and for having always believed in me.

A special thanks to my friends, who endured and encouraged me in all these years.

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