SAPIENZA
UNIVERSITÀ DI ROMA

FACOLTÀ DI MEDICINA E PSICOLOGIA

Dottorato in Oncologia Digestiva e Metodologia della Ricerca Oncologica

Curriculum: Oncologia Digestiva

XXVII Ciclo

Study of adaptive responses to drug treatments in pancreatic cancer cells

Candidata:

Ilaria Passacantilli

Docente guida/Tutor:

Prof. Claudio Sette

Coordinatore del Curriculum:

Prof. Gianfranco Delle Fave

Anno Accademico 2014-2015

INDEX

Introduction to the Thesis

One of the most important and unsolved problems in the therapy of pancreatic tumors is the development of resistance to pharmacological treatments. Inefficacy of therapies leads to the progression of these cancers, worsening the quality of life and shortening the lifespan.

Tumors that interest the pancreas are quite different from each other, depending on the cell type by which it origins and on the alterations of pancreatic functionality. They may be classified in two groups: endocrine tumors, which arise from pancreatic endocrine cells, and carcinomas, which derive from cells with exocrine functions.

Cancers derived from these two groups are very different in terms of prognosis. Indeed, pancreatic endocrine tumors are defined as “indolent”, due to their slow rate of growth and to the long survival of patients in most of the cases. On the contrary, pancreatic ductal carcinoma is considered one of the most lethal tumors, due to the inefficacy of chemotherapeutic treatment and by the short survival of patient.

In both cases, tumors are incurable in most patients. The pharmacological approaches used for the treatment of endocrine or ductal tumors are different: in endocrine tumors, which have a slow progression rate, inhibitors of cell growth are used in the majority of cases, while in ductal carcinomas, which have a faster progression, chemotherapy with agents that impair replication of cancerous cells is preferred.

The aim of the work presented in this thesis was to investigate the mechanisms at the basis of drug resistance in pancreatic tumors. The first part of the work is focused on the study of endocrine tumors and it proposes a novel strategy to counteract the problem of resistance of pharmacological treatment with Everolimus, using *in vitro* models. The second part of the work concerns the investigation of the molecular features of pancreatic ductal cancer cells, and how these mechanisms may be involved in the malignity of cancer cells and in the acquisition of resistance to chemotherapy.

CHAPTER I

Pancreatic Endocrine Tumors

Pancreatic Endocrine tumors (PETs) are a rare class of heterogeneous neoplasms, which represent only 1-2 % of all pancreatic malignancies (Fraenkel *et al*., 2012). PETs arise from the endocrine cells which take part of the diffuse neuroendrocrin system of pancreas, whose main function is to produce several hormones, as insulin and glucagon. Despite their low incidence, PETs display 10% prevalence among patients affected by pancreatic tumors (Fitzgerald *et al*., 2008). The high percentage of prevalence is due to the "indolent" behaviour of PETs, which permits a long survival of patients. However, two thirds of patients display metastasis at time of diagnosis, becoming not amenable for surgery (Sadaria *et al*., 2013).

Although the long survival of patients and the slow rate of growth of these tumors, the therapeutic approaches currently in use are not effective for the cure of PETs, highlighting the needing of novel therapy for the management of these tumors.

1.1 Classification and symptoms of PETs

PETs are typically classified in two groups:

- Functioning tumors
- Non functioning tumors

This subdivision is based on the ability of the tumor to secret hormones. Moreover, the classification of functioning tumors depends from the hormones produced. Most functioning endocrine tumors are considered "silent", due to the low amount of hormones produced or for the release of inert precursors of hormones (Metz and Jensen, 2008).

1.1.1 Functioning tumors

The most common functioning tumors are insulinomas, gastrinomas, glucagonomas, VIPomas and somatostatinomas.

- ***Insulinoma***: it is the most common among PETs and it is characterized by the over-production of insulin, which causes hypoglycemic symptoms. Insulinomas are present in 10% of patient affected by Multiple Endocrine Neoplasia 1 (MEN1) syndrome, an authosomal recessive disease characterised by the onset of several tumors in endocrine organs. This kind of tumor, which may arise

within the entire body of pancreas, in 90% of cases is benign and characterized by small size (0,5-2 cm). Since the high percentage of benign features of insulinomas, patients have a long term-survival. (Grant *et al*., 2005; Fendrich *et al*., 2009)

- ***Gastrinoma:*** it is responsible of Zollinger-Ellison syndrome, a complex disease characterised by the secretion of gastrin, which leads to an over-production of gastric acids. Zollinger-Ellison syndrome is the second most frequent PET, and in 80% of cases it is a sporadic tumor, while in 20% of patients it is associated to MEN1 syndrome. Gastrinomas may occur in duodenum or in pancreas and they are characterised by slow growth, albeit in 60-70% of cases are malignant and most patients present with metastases at time of diagnosis. Time of survival of patients depends on the presence of liver metastasis. Indeed, in the absence of metastasis, survival of 95% of patients is grater than 20 years (Yu *et al*., 1999; Krampitz and Norton, 2013).
- ***Glucagonoma:*** this disease is characterized by an hyper-production of glucagon. Therefore, patients affected by glucanoma present a complex medical spectrum. This disease is in the majority of the cases malignant, and patients often present as metastatic at time of diagnosis, becoming not eligible for surgery (Bornman et *al.*, 2001; Doherty *et al*., 2005).
- ***VIPoma:*** this tumor, arising in the tail of pancreas, is characterised by the production of high level of Vasoactive intestinal peptide (VIP). VIP is able to activate endothelial cells of intestine, thus inducing the up-take of electrolytes and water (Laburthe *et al*., 2002). For this reason, VIPomas are associated to watery diarrhea. 80% of patients with VIPomas, which may be associated to MEN1 syndromes, present with metastases at time of diagnosis (Ito *et al*., 2012; Krampitz and Norton, 2013).
- ***Somatostatinoma:*** is characterised by the production of high amount of somatostatin that is released at high levels in blood stream. Diagnosis of this kind of tumors often occurs lately, due to the complexity of the medical case. Indeed high levels of somatostatin induce inhibition of pancreatic exocrine activities and of the release of gastrin, which is important for the production of hypocloridric acid in stomach. In two thirds of somatostatinomas, metastases are present at time of diagnosis (Ito *et al*., 2012; Krampitz and Norton, 2013).

1.1.2 Non Functioning tumors

Non functioning PETs (NF-PETs) do not cause syndromes associated with over-production of hormones, albeit NF-PET may produce other substances that do not lead to symptoms. The most common hormones produced by NF-tumors are chromogranin A, teurotensin and pancreatic enzymes. These tumors may also produce low levels of insulin, glucagon and other pancreatic hormones.

Due to the absence of symptoms, 60% of patients with NF-PETs present liver metastases (Metz and Jensen, 2008), thus influencing long-term survival, which is 20-30% after 5 years from initial presentation (Akerström and Hellman, 2007, Fendrich *et al*., 2009).

1.2 Diagnosis

Imaging techniques have an important role in the diagnosis of PETs, which are required also for the localization, staging and monitoring of diseases. The most important problem during the diagnosis is the small dimension of this type of tumors. Computed tomography (CT) is used for the detection of PETs with a diameter inferior to 2 cm. Magnetic resonance imaging (MRI) is able to detect tumors smaller than 2 cm and metastases, whereas endoscopic ultrasonography (EUS), can detect very small tumors (< 1 cm), undetectable by MRI and CT. Moreover, EUS can guide biopsy of tumors and provide histological diagnosis. Somatostatin receptor scintigraphy (Octreoscan, SRS) is used to detect functioning and non-functioning tumors expressing high level of somatostatin receptors through radiolabeled somatostatin analogs. However, SRS is not able to detect insulinomas or non-functioning tumors that express low level of somatostatin receptors.

Moreover, diagnosis requires the analysis of the levels of hormones in serum of patients. This kind of analysis allows a better identification of the features of tumor (Kramptiz and Norton, 2013).

1.3 Therapy

Surgical resection is the first line treatment for PETs and it guarantees good prognosis in patients with no evidence of metastatic disease. Indeed, surgical approach depends on the location of the tumor, size and the presence of metastases. In patients with no evidence of hepatic or linfonodal metastases, surgery is recommended to

resolve symptoms of tumors and to increase 5-year survival from prognosis (Yalcin *et al.*, 2011).

Surgical therapy is supported by non-surgical approach, in order to monitor both tumor size and symptoms. Chemoterapy is not used as first line treatment for PETs, due to the low rate of tumor growth, albeit several trials have been employed for advanced metastatic PETs.

In the last years, novel target therapies have been considered for the treatment of PETs. Indeed, molecular characterization of these neoplasms has permitted to identify novel molecular targets and to develop target therapies for the treatment of PETs. PETs show a strong expression of vascular endothelial growth factor receptors (VEGFR) and they are characterized by strong vascularisation and induction of angiogenesis (Corbo *et al.*, 2012). For this reason, novel inhibitors that target angiogenesis, as Sunitinib, have been employed for PET treatments. Sunitinib was approved by the Food and Drug Administration (FDA) for the treatment of advanced metastatic PanNET, due to the improvements in prolongation of life (Blumenthal *et al*., 2012). It targets several kinases involved in angiogenesis, such as VEGFR, PDGFR, c-KIT and RET (Mendel *et al*., 2003). However, beside its demonstrated antitumor activity, clinical trials have shown that Sunitinib also exerts side effects in patients.

Studies on tissues and cell lines derived from PETs patients demonstrated that the PI3K/AKT/mTOR pathway, which regulates protein synthesis and cell proliferation, is frequently de-regulated in this type of tumors (Missiaglia *et al*., 2010, Di Florio *et al*., 2007, 2011; Yao *et al*., 2011; de Wilde *et al*., 2012). For this reason, the use of inhibitors specific for this pathway, such as Everolimus, has been considered for the treatment of PETs. Everolimus, was approved for the treatment of advanced PETs by FDA in 2011 (Yao *et al*., 2011), and after preclinical studies, which demonstrated the positive effect of Everolimus on survival (Chiu *et al*., 2010), this drug was employed for advanced PETs. Notably, Everolimus showed a significant effect on progression-free survival of patients (Yao *et al*., 2011), thus providing a novel therapeutic approach for this class of tumors.

1.4 Molecular characterization of PETs

Some evidences of genetics and/or molecular alterations in PETs have been reported. Most of these heterogeneous diseases arise from patients affected by different genetic syndromes, while the pathogenesis of sporadic tumors depends from mutations

or aberrant expression of oncogenes and alteration in pathways that regulate cell proliferation and cell metabolism (Capurso *et al.*, 2012).

The most frequent inherited genetic syndromes involved in PETs are Multiple Endocrine Neoplasia Type 1, Von Hippel Lindau Disease, Tuberous sclerosis complex and Neurofibromatosis Type 1. However, most of the mutations responsible for genetic syndromes associated with PETs arise also in sporadic cases of this class of tumors, in addition with other mutations that are not typical of genetically linked PETs.

1.4.1 Genetic Diseases associated with PETs

Multiple Endocrine Neoplasia Type 1 (MEN1)

MEN1 is an autosomal dominat disease characterised by the appearance of several neuroendocrine tumors in gastro-entero-pancreatic organs (Lemos and Takkher, 2008). The disorder is caused by mutations in the *MEN1* gene, located in chromosome 11q13, which inactivates the function of the protein encoded by the gene: Menin (Metz and Jensen, 2008). It was reported that 10% of patients affected by PETs have MEN1 syndrome (Metz and Jensen, 2008). Menin is important for the modulation of the expression of genes involved in the regulation of cell cycle at the trancritptional level, such as *p27* and *p18* genes (Karnik *et al*., 2005), by promoting their methylation (Wu *et al.*, 2011). Indeed, Menin takes part to the mixed lineage leukemia (MLL) histone methyl transferase complex, and it is required for the H3K4 methyltrasferase activity of the MLL complex (Murai *et al*., 2011; Karnik *et al*., 2005). Mutations in the *MEN1* gene often impair protein nuclear localization (Corbo *et al.*, 2010), thus causing its down-regulation. Moreover, Menin down-regulation also depends on the activation of PI3K/AKT pathway. In fact, activation of this pathway promotes FOXO1 expression, which in turns binds the *MEN1* promoter and suppresses its expression (Zhang *et al*., 2012).

Loss of Heterozigosity (LOH) for *MEN1* or mutations in both alleles of *MEN1* are frequent also in sporadic cases of PETs, and it was estimated that mutations in this gene affects about 44% of cases of sporadic PETs (Jao *et al*., 2011). The key role of the *MEN1* gene in PET development was confirmed also in a mouse murine model of the disease, where homozygous deletion of the *Men1* gene is embryonic lethal whereas its heterozygous deletion causes the onset of endocrine tumors, thus recapitulating the effect of *MEN1* loss in human tumors (Crabtree *et al*., 2001).

All these observations enforce the key role played by loss of Menin function in the oncogenic process leading to PET development.

Von Hippel Lindau Disease

Von Hippel Lindau disease is an autosomal recessive disorder characterised by multiple tumors, including PETs (Libutti *et al*., 2000). This disorder is caused by mutations in the *VHL* gene, which encodes for two proteins derived from the splicing of VHL mRNA. Both VHL isoforms regulates the Hipoxia Inducible Factor a (HIF1α). In particular, VHL proteins form a α-ubiquitinase complex that interacts with HIF1α and leads to its degradation. Depletion of VHL induces HIF1α stabilization, which activates the transcription of genes involved in angiogenesis, a key feature of PETs (Lonser *et al*., 2003).

PETs arising from Von Hippel Lindau Disease represent a small fraction of tumors associated with this disorder (Mukhopadhyay *et al*., 2002), and they are often small and non-functioning tumors. Moreover, mutations or depletion of the *VHL* gene occurs with low frequency in sporadic tumors, albeit tumors arising from *VHL* mutations often show malignant features (Schmitt *et al*., 2009).

Tuberous sclerosis complex (TSC1/TSC2)

Tuberous sclerosis complex is a recessive autosomal disease associated with PETs. The rate of patients affected by this genetic disease is very low (Curatolo *et al*., 2008). The genes mutated in tuberous sclerosis complex are TSC1 and TSC2, two important negative regulators of mTORC1 activity. Therefore, mutated TSC1/2 proteins were found also in sporadic PETs and in immortalized cell lines used as *in vitro* model for PETs (Missiaglia *et al*., 2010). Association of the genetic disorder of TSC to PETs is a strong indication of the role of the mTOR pathway on the progression of tumor transformation in PETs, and alterations of protein expression of TSC2 were found also in sporadic cases of PETs (Missiaglia *et al*., 2010).

Neurofibromatosis Type 1

Neurofibromatosis Type 1 is an autosomal disorder characterised by the occurrence of several tumors, including PETs. It derives from mutation in the *NF1* gene, which encodes for neurofibromin, a GTPase protein involved in the regulation of the RAS and mTOR pathway. LOH or puntiform mutations inactivating the protein

function have been described in PETs (McClatchey *et al*., 2007), highlighting its involvement in progression of PETs.

1.4.2 Sporadic Mutations associated with PETs pathogenesis

The characterization of the genetic profiles of PETs and the association of these tumors with specific genetic disorders underline the recurrence of mutations as an important factor for the development of these tumors. In particular, mutations in genes associated with loss of proliferation control and angiogenesis are associated with PET. As mentioned above, the most frequent genes mutated in sporadic tumors are those associated with genetic syndromes. However, additional genes have been found mutated in PET patients, such as *DAXX*, *ATRX*, *VEGF*, *PDGF*, *KIT*,, Src family kinases, and components of the PI3K/AKT/mTOR pathway (discussed in a separated session).

DAXX/ATRX

The *DAXX* gene encodes for a histone chaperone that, in cooperation with ATRX, is involved in the regulation of telomere lengthening, and was found mutated in 43% of PETs (Jiao *et al*., 2011). Thus, mutations in *DAXX* and *ATRX* affect genome stability and their impairement may provide the ability of undergo to unlimited cell cycling to cancer cells. Moreover, DAXX protein is also involved in regulation of p53 activity. Indeed, DAXX promotes p53 stabilization, which in turn activates the G1/S check point. Hence, the loss of DAXX gene function leads to a loss of p53 activation and promotes tumor progression (Taguchi *et al.*, 2011), thus suggesting a direct role in PET tumorigenesis. Nevertheless, in spite of their high frequency of incidence, mutations in DAXX and ATRX are not related with poor prognosis in PETs, as they are preferentially associated with well- or moderate-differentiated tumors (Jiao *et al*., 2011).

VEGF/PDGF/c-kit

PETs are characterised by extensive vascularisation, and deregulation of pathways involved in angiogenesis has a great relevance for the acquisition of this feature. One of the most important molecules required for angiogenesis in normal pancreatic cells is vascular endothelial growth factor (VEGF), whose expression is deregulated in many tumors. Increased angiogenesis is among the most critical features observed also in the *Rip-Tag2* mouse model of endocrine tumors (Hanahan *et al*., 1985; Hanahan and Folkman, 1996). In light of these observations, several pathways involved

in angiogenesis have been analyzed in PETs. Receptors for molecules required for the formation of novel blood vessels, like VEGFRs, PDGFR and c-kit, were found over-expressed in specimens of PETs (Fjallskog *et al*., 2003; Fjallskog *et al*., 2007). However, it was also found that the expression of VEGF was higher in well-differentiated endocrine tumors with respect to low-differentiated tumors (Couvelard e*t al*., 2005), together with higher presence of blood vessels respect with less differentiated tumors (Marion-Audibert, 2003). These data correlates with an higher level of HIF1-α protein in high grade-tumors (Couvelardet *et al*., 2005), which may provides advantages in terms of proliferation and survival to cancer cells. Thus, angiogenesis, which is a specific feature of PETs, may guide tumor progression trough the expression of specific pro-angiogenic factors.

Src family tyrosine kinases

Recently, a novel role for the oncogenic Src tyrosine kinase in PETs has been proposed. Src is the prototype of a large family of nine membrane associated-tyrosine kinases (Thomas and Brugge, 1997), the Src-family kinases (SFKs), which are involved in the transduction of extracellular stimuli. Src drives several cellular processes related to cancer, including cell metabolism, cell cycle control and migration (Thomas and Brugge, 1997). The involvement of SFKs in PETs was found initially on the increased level of Lck (a member of SFKs) in specimens of PETs (Capurso *et al*., 2006). More recently, it was found that Src protein is over-expressed in specimens of PETs with respect to normal tissue (Di Florio *et al*., 2007) and that Src controls PET cell adhesion and protein synthesis through the crosstalk with the mTOR pathway (Di Florio *et al*., 2007; 2011). Collectively, these observations underline the relevance of Src activity in tumorigenic features of PETs.

References

Akerström G, Hellman P. Surgery on neuroendocrine tumours. Best Pract Res Clin Endocrinol Metab. 2007 Mar;21(1):87-109

Bornman PC, Beckingham IJ. ABC of diseases of liver, pancreas, and biliary system. Pancreatic tumours. BMJ. 2001 Mar;322(7288):721-3.

Blumenthal GM, Cortazar P, Zhang JJ, Tang S, Sridhara R, Murgo A, Justice R, Pazdur R.. FDA approval summary: sunitinib for the treatment of progressive well-differentiated locally advanced or metastatic pancreatic neuroendocrine tumors. Oncologist. 2012;17(8):1108–13.

Capurso G, Lattimore S, Crnogorac-Jurcevic T, Panzuto F, Milione, M, Bhakta V, Campanini N, Swift SM, Bordi C, Delle Fave G, Lemoine NR. Gene expression profiles of progressive pancreatic endocrine tumours and their liver metastases reveal potential novel markers and therapeutic targets. Endocr Relat Cancer. 2006 Jun; (13); 541–58.

Chiu CW, Nozawa H, Hanahan D. Survival benefit with proapoptotic molecular and pathologic responses from dual targeting of mammalian target of rapamycin and epidermal growth factor receptor in a preclinical model of pancreatic neuroendocrine carcinogenesis. J Clin Oncol. 2010 Oct 10;28(29):4425–33.

Corbo V, Dalai I, Scardoni M, Barbi S, Beghelli S, Bersani S, Albarello L, Doglioni C, Schott C, Capelli P, Chilosi M, Boninsegna L, Becker KF, Falconi M, Scarpa A.. MEN1 in pancreatic endocrine tumors: analysis of gene and protein status in 169 sporadic neoplasms reveals alterations in the vast majority of cases. Endocr Relat Cancer. 2010 Aug 16; 17(3):771–83.

Corbo V, Beghelli S, Bersani S, Antonello D, Talamini G, Brunelli M, Capelli P, Falconi M, Scarpa A. Pancreatic endocrine tumours: Mutational and immunohistochemical survey of protein kinases reveals alterations in targetable kinases in cancer cell lines and rare primaries. Ann. Oncol. 2012 Jan; 23(1): 127–34.

Couvelard A, O'Toole D, Turley H, Leek R, Sauvanet A, Degott C, Ruszniewski P, Belghiti J, Harris AL, Gatter K, Pezzella F. Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. Br J Cancer. 2005 Jan 17; 92(1):94–101.

Crabtree JS, Scacheri PC, Ward JM, Garrett-Beal L, Emmert-Buck MR, Edgemon KA, Lorang D, Libutti SK, Chandrasekharappa SC, Marx SJ, Spiegel AM, Collins FS.. A mouse model of multiple endocrine neoplasia, type 1, develops multiple endocrine tumors. Proc Natl Acad Sci U S A. 2001 Jan 30 ;98(3):1118–23.

Curatolo P, Bombardieri R, Jozwiak S. Tuberous sclerosis. Lancet. 2008 Aug 23; 372(9639):657–68.

de Wilde RF, Edil BH, Hruban RH, Maitra A. Well-differentiated pancreatic neuroendocrine tumors: from genetics to therapy. Nat Rev Gastroenterol Hepatol. 2012 Feb 7;9(4):199–208.

Di Florio A, Capurso G, Milione M, Panzuto F, Geremia R, Delle Fave G, Sette C. Src family kinase activity regulates adhesion, spreading and migration of pancreatic endocrine tumor cells. End Relat Cancer. 2007 Mar; 14(1): 111-24.

Di Florio A, Adesso L, Pedrotti S, Capurso G, Pilozzi E, Corbo V, Scarpa A, Geremia R, Delle Fave G, Sette C. Src kinase activity coordinates cell adhesion and spreading with the activation of mammalian target of rapamycin in pancreatic endocrine tumor cells. Endocr Rel Cancer. 2011 Aug 30; 8 (5): 541-54.

Doherty GM. Rare endocrine tumours of the GI tract. Best Practice & Research. Clinical Gastroenterology. 2005 Oct; 19: 807–17

Fendrich V, Waldmann J, Bartsch DK, Langer P. Surgical management of pancreatic endocrine tumors. Nat Rev Clin Oncol. 2009 Jul;6(7):419–28.

Fitzgerald TL, Hickner ZJ, Schmitz M, EJ. Changing incidence of pancreatic neoplasms. A 16-year review of statewide tumor registry. Pancreas. 2008 Aug; (37):134–8.

Fjallskog ML, Lejonklou MH, Oberg KE, Eriksson BK, Janson ET. Expression of molecular targets for tyrosine kinase receptor antagonists in malignant endocrine pancreatic tumors. Clin Cancer Res. 2003 Apr; 9(4):1469–73.

Fjallskog ML, Hessman O, Eriksson B, Janson ET. Upregulated expression of PDGF receptor beta in endocrine pancreatic tumors and metastases compared to normal endocrine pancreas. Acta Oncol. 2007;46(6):741–6.

Fraenkel M, Kim MK, Faggiano A, Valk GD Epidemiology of gastroenteropancreatic neuroendocrine tumours. Best Pract Res Clin Gastroenterol. 2012 Dec; 26(6):691-703.

Grant CS. Insulinoma. Best Pract Res Clin Gastroenterol. 2005 Oct; 19(5):783-98.

Hananah D. Heritable formation of pancreatic b- cells tumors in transgenic mice expressing recombinant insulin /simian virus 40 oncogenes. Nature. 1985 May 9-15; 315(6015):115-22.

Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996 Aug 9; 86(3):353–64.

Ito T, Igarashi H, Jensen RT. Pancreatic neuroendocrine tumors: clinical features, diagnosis and medical treatment: advances. Best Pract Res Clin Gastroenterol. 2012 Dec; 26(6):737-53.

Jiao Y, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfgang CL, Choti MA, Velculescu VE, Diaz LA Jr, Vogelstein B, Kinzler KW,Hruban RH, Papadopoulos N. DAXX/ATRX, MEN1, and mTOR pathway genes

are frequently altered in pancreatic neuroendocrine tumors. Science. 2011 Mar 4; 331(6021):1199–203.

Karnik SK, Hughes CM, Gu X, Rozenblatt-Rosen O, McLean GW, Xiong Y, Meyerson M, Kim SK. Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c. Proc Natl Acad Sci U S A. 2005 Oct 11; 102(41):14659-64.

Krampitz GW, Norton JA. Pancreatic neuroendocrine tumors. Curr Probl Surg. 2013 Nov; 50(11):509-45.

Laburthe M, Couvineau A. Molecular pharmacology and structure of VPAC Receptors for VIP and PACAP. Regul Pept. 2002 Oct 15; 108(2-3):165-73.

Lemos MC, Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. Hum Mutat. 2008 Jan; 29(1):22-32.

Libutti SK, Choyke PL, Alexander HR, Glenn G, Bartlett DL, Zbar B, Lubensky I, McKee SA, Maher ER, Linehan WM, Walther MM.. Clinical and genetic analysis of patients with pancreatic neuroendocrine tumors associated with von Hippel- Lindau disease. Surgery. 2000 Dec; 128(6):1022–27.

Lonser RR, Glenn GM, Walther M, Chew EY, Libutti SK, Linehan WM, Oldfield EH. von Hippel-Lindau disease. Lancet. 2003 Jun 14;361(9374):2059-67.

Marion-Audibert AM, Barel C, Gouysse G, Dumortier J, Pilleul F, Pourreyron C, Hervieu V, Poncet G, Lombard-Bohas C, Chayvialle JA, Partensky C, Scoazec JY. Low microvessel density is an unfavorable histoprognostic factor in pancreatic endocrine tumors. Gastroenterology. 2003 Oct;125(4):1094–104

McClatchey AI. Neurofibromatosis. Annu Rev Pathol. 2007; 2:191-216.

Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, Murray LJ, Carver J, Chan E, Moss KG, Haznedar JO,Sukbuntherng J, Blake RA, Sun L, Tang C, Miller T, Shirazian S, McMahon G, Cherrington JM.. *In vivo* antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: Determination of a pharmacokinetic/pharmacodynamic relationship. Clin. Cancer Res. 2003 Jan; 9(1): 327–37.

Metz DC, Jensen RT . Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. Gastroenterology. 2008 Nov; 135(5):1469-92.

Missiaglia E, Dalai I, Barbi S Beghelli S, Falconi M, Della Peruta M, Piemonti L, Capurso G, Di Florio A, Delle Fave G, Pederzoli P, Croce CM, Scarpa A. Pancreatic Endocrine Tumors: Expression profiling evidences a role of PI3K-Akt pathway . J Clin Oncol. 2010 Jan 10; 28(2): 245-55.

Mukhopadhyay B, Sahdev A, Monson JP, Besser GM, Reznek RH, Chew SL. Pancreatic lesions in von Hippel–Lindau disease. Clinical Endocrinology (Oxf). 2002 Nov; 57(5):603-8.

Murai MJ, Chruszcz M, Reddy G, Grembecka J, Cierpicki T et al. Crystal structure of menin reveals binding site for mixed lineage leukemia (MLL) protein. J Biol Chem. 2011 Sept 9; 286(36):31742–8.

Sadaria MR, Hruban RH, Edil BH. Advancements in pancreatic neuroendocrine tumors. Expert Rev Gastroenterol Hepatol. 2013 Jul;7(5):477-90.

Schmitt AM, Schmid S, Rudolph T, Anlauf M, Prinz C, Klöppel G, Moch H, Heitz PU, Komminoth P, Perren A 2009 VHL inactivation is an important pathway for the development of malignant sporadic pancreatic endocrine tumors. Endocr Relat Cancer. 2009 Dec; 16(4):1219-27.

Taguchi R, Yamada M, Horiguchi K, Tomaru T, Ozawa A, Shibusawa N, Hashimoto K, Okada S, Satoh T, Mori M.. Haploinsufficient and predominant expression of multiple endocrine neoplasia type 1 (MEN1)-related genes, MLL, p27Kip1 and p18Ink4C in endocrine organs. Biochem Biophys Res Commun. 2011 Nov 18; 415(2):378–83.

Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol. 1997 ; (13): 513–609.

Wu T, Hua X. Menin represses tumorigenesis via repressing cell proliferation. Am J Cancer Res. 2011;1(6):726–39.

Yalcin S. Advances in the systemic treatment of pancreatic neuroendocrine tumors. Cancer Treat Rev. 2011 Apr;37(2):127-32.

Yao JC, Shah MH, Ito T, Lombard Bohas C, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevilla J, de Vires EGE, Tomassetti P, Pavel ME, Hoosen S, Haas T, lincy J, Lebwohl D, et al. Everolimus for Advance Pancreatic Neuroendocrine tumors. N Engl J Med. 2011 Feb 10; 364(6): 514-23.

Yu F, Venzon DJ, Serrano J, Goebel SU, Doppman JL, Gibril F, Jensen RT. Prospective study of the clinical course, prognostic factors, causes of death, and survival in patients with long-standing Zollinger-Ellison syndrome. J Clin Oncol. 1999 Feb; 17(2):615-30.

Zhang H, Li W, Wang Q, Wang X, Li F, Zhang C, Wu L, Long H, Liu Y, Li X, Luo M, Li G, Ning G. Glucose-mediated repression of menin promotes pancreatic β-cell proliferation. Endocrinology. 2012 Feb; 153(2):602-11.

CHAPTER II

Role of the PI3K/AKT/mTOR pathway in PETs

Regulation of cell metabolism is important for the activation or inhibition of several cellular functions. Cells must regulate their homeostasis through anabolic and catabolic reactions, which cooperate to control cellular growth, mRNA translation, proliferation and energy balance. The most important pathway involved in this regulation is the PI3K/AKT/mTOR one.

This pathway is coordinated by the three kinases, Phosphatidylinositol-3-kinase (PI3K), Protein Kinase B (also known as AKT), and mammalian target of rapamycin (mTOR), which in turn take part of two different protein complexes, mTORC1 and mTORC2 (Figure 2.1).

The extensive crosstalk between these kinases activates or inhibits cell growth in response to extracellular stimuli or various intra-cellular cues.

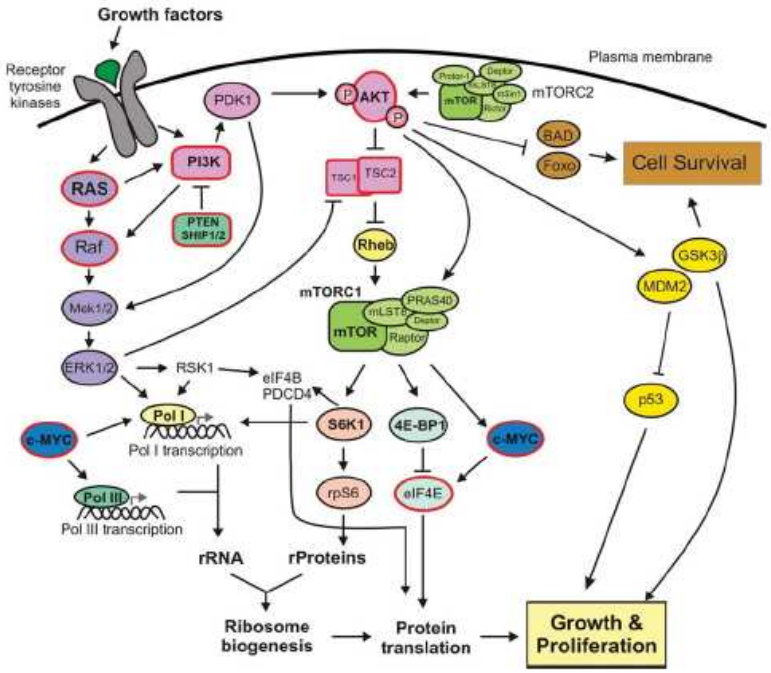


Figure 2.1. Representative scheme of the PI3K/AKT/mTOR pathway. The signalling network coordinated by the pathway regulates cell growth, proliferation, ribosome biogenesis and protein translation (From Hannan *et al*., 2011).

2.1 The PI3K/AKT/mTOR pathway

2.1.1 PI3K

PI3K is a lipid kinase situated upstream of the entire pathway. It is involved in the transduction of external stimuli triggered by the activation of a large class of receptor tyrosine kinases (Hannan *et al.*, 2011). The most important molecule that conveys the extracellular stimuli is phosphatidylinositol-3,4,5-trisphosphate (PIP_3), whose phosphorylation sets in motion the downstream signalling cascade. Indeed, PI3K initiates the intracellular signal by phosphorylating phosphatidylinositol-4,5-diphosphate (PIP_2), a class of lipid enriched into the plasma membrane, in PIP_3. Once produced, PIP_3 binds proteins containing a PIP3-binding-motif, such as Pleckstrin Homology (PH) domain, thereby regulating protein relocalization to the plasma membrane, vescicle trafficking, cell proliferation and survival (Fruman *et al*., 2014).

There are three classes of PI3K enzymes (Fruman *et al*., 2014), known as Classes I, II and III. Only the class I is able to phosphorylate PIP_2 into PIP_3, thus participating to the transduction of the signal. Indeed, Class II phosphorylates only phosphatydilinsositol or phospatydilinostitol-4-phospate, whereas Class III acts only on Phosphatydilinsositol.

The activity of PI3K is determined by the release of the p110 catalityc subunit from the regulatory p85 subunit or by the activation mediated by Rat sarcoma (RAS) protein. RAS, which have a GTPase activity, is another factor involved in the transduction of extracellular and intracellular stimuli. It activates several pathway, the most important is the mitogen-activated protein (MAP) kinase pathway. However, RAS is able to converge signals into the PI3K/AKT/mTOR pathway, through the activation of PI3K. Several isoforms of PI3K catalytic and regulatory subunits have been characterized. They are classified in Class IA (P110α, β, δ and p85α, β, δ), which are typically activated by receptors with tyrosine kinase activity, and Class IB (p110γ and regulatory subunit p101), which are activated by G protein associated receptors (McNamara and Degterev, 2011).

In line with the relevance of the signalling triggered by PIP3, PI3K activity is finely tuned in the cell. A major layer of regulation is comprised by protein-protein interactions and post-translational modifications of the subunits of the PI3K enzymes. For instance, the regulatory subunit of PI3K, through the interaction with receptors tyrosine kinase, controls activation and subcellular localization of catalytic subunit of

PI3K (Hay *et al*., 2005). In addition, activation of the PI3K/AKT/mTOR pathway is subject to a tight control by another protein, the phosphatase tensin homologue (PTEN), which is able to convert PIP3 to PIP2, thus switching off the signalling activated by PI3K (Song *et al*., 2012)

2.1.2 AKT Kinase

AKT kinases are the most important effectors of the PI3K pathway. AKTs crosstalk with several crucial pathways, that ultimately control cell proliferation and survival. Three AKT kinases (AKT1, AKT2 and AKT3) have been characterized and their phosphorylation in specific residues is required for full activation of the downstream pathway. AKT is indirectly activated by PI3K. Activation of PI3K determines an increase of PIP_3 levels in the plasma membrane, which recruits and activates the 3'- phosphoinositide-dependent kinase 1 (PDK1). PDK1, in turn, recruits and phosphorylates AKT in Threonine 308 (T308), thus leading to partial activation of the kinase. However, a second phosphorylation event is required for the full activation of AKT. Indeed, a second residue, Serine 473 (S473), is targeted by another important complex, mTORC2, which leads to full activation of AKT (Manning and Cantley, 2007).

Several pathways are influenced by activity of AKT. For instance, it has been extensively documented how AKT regulates cell death, cell growth, proliferation and metabolism (Manley and Cantley, 2007). AKT is involved in the regulation of apoptosis by phosphorylating Caspase-3 and BAD proteins and inhibiting cell death (Datta *et al*., 1997). AKT also phosphorylates the transcription factor FOXO, repressing transcription of pro-apoptotic genes (Van Der Heide *et al*., 2004). Furthermore, phosphorylation of the p53 binding protein MDM2 induces its translocation into the nucleus, where it binds p53, leading to degradation of p53 and promoting cell survival (Marine *et al*., 2010, Zhou *et al*., 2001). AKT is also known to regulate cell proliferation by phosphorylating GSK3β, which in turn regulates several processes. For instance, GSK3b regulates the stability of transcription factors involved in cell proliferation and survival such as, the activator protein 1 (AP-1), nuclear factor kappa B (NFκB), c-Myc and Snail (Buss *et al*., 2004; Yada *et al*., 2004, Yook *et al*., 2006).

Importantly, AKT indirectly regulates the activation of the mTORC1 complex, which is crucial for protein synthesis. In particular, AKT interacts with and phosphorylates and inhibits TSC2, a component of the TSC complex, composed by

TSC1 and TSC2. TSC complex, which act as a GTP-ase activating protein (GAP) interacts with the Ras homolog enriched brain (Rheb), that activates mTOR when it is bound to GTP (Tee *et al*., 2003). The GTP-bound form of Rheb is inactivated when interacts with TSC complex, which converts Rheb to the GDP-bound form, thus inhibiting mTORC1 functions (Inoki *et al*., 2003).

All these observations indicate that AKT has pro-survival functions and strongly suggest that its deregulation may favour pro-oncogenic properties.

2.1.3 The mTOR protein, complexes and effectors

Mammalian Target of Rapamycin, or mTOR, is a large protein that owes its name to the drug impairing its activity in yeast (Cafferkey *et al*., 1993; Kunz *et al*., 1993) and in mammalian cells (Brown *et al*., 1994; Sabatini *et al*., 1994). Later, it was found that mTOR controls fundamental cellular process, such as ribosome biogenesis, protein synthesis and cell growth. Several years after its discovery, mTOR was found to be part of two protein complexes, involved in different activities, but cooperating within the same pathway (Figure 2.2). In the cell, mTOR is present in the mTORC1 complex (known as rapamycin-sensitive complex) (Kim *et al*., 2002; Loewith *et al.*, 2002) and in the mTORC2 complex (rapamycin-insensitive complex) (Sarbassov *et al*., 2004; Jacinto *et al*., 2004). As mentioned above, mTORC1 is indirectly regulated by AKT, thus its activity is controlled by the PI3K/AKT axis and mTOR is generally situated downstream of these kinases in the pathway. The mTORC2 complex is another important regulator of PI3K/AKT/mTOR pathway. Indeed, it normally responds to growth factors and participates in the regulation of cell survival, metabolism, and cytoskeleton organization and its most known function is to promote the full activation of AKT by the phosphorylation in S473.

The two complexes, mTORC1 and mTORC2, containing the mTOR protein differ for the interacting proteins (Figure 2.2). Indeed, in the mTORC1 complex mTOR interacts with RAPTOR and PRAS40, whereas the mTORC2 complex contains RICTOR, mSIN1 and PROTOR (only in higher eukaryotes) (Guertin and Sabatini, 2007). Both complexes contain other proteins, such as DEPTOR, mLST8, tt1 and tel2. In the mTORC1 complex, RAPTOR is the activator of mTOR and it is antagonized by PRAS40, while, in the mTORC2 complex, both RICTOR and mSIN1 cooperates to activitate mTOR.

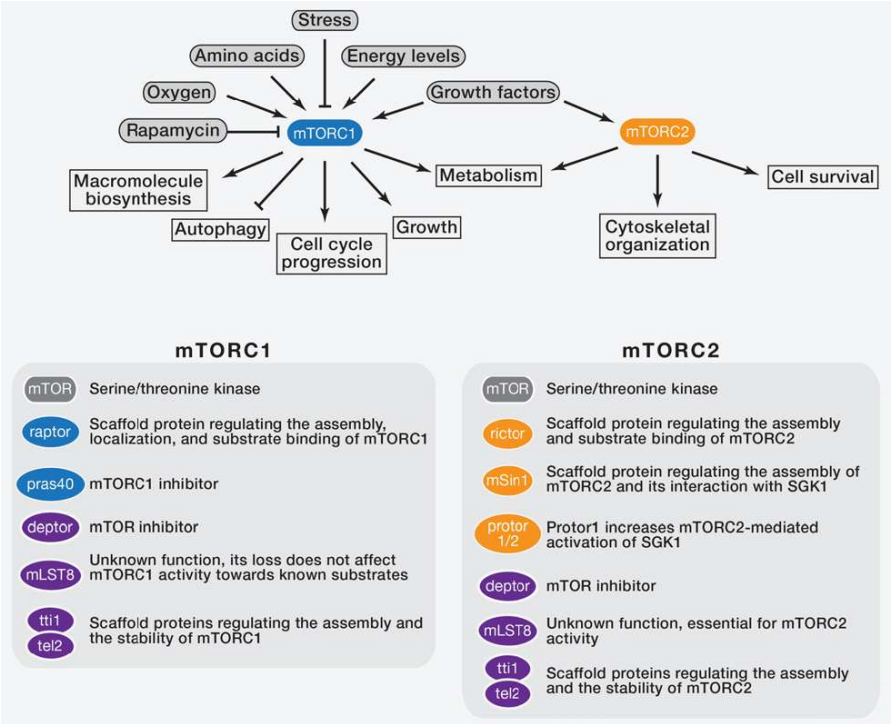


Figure 2.2 Scheme of cellular functions regulates by mTORC1 and mTORC2 complexes, and list of proteins contained in both complexes (modified from Laplante and Sabatini, 2012)

The most studied complex for its role in the regulation of cell growth and metabolism is the mTORC1 complex. The mTORC1 complex is finely regulated by the upstream PI3K/AKT axis in response to several types of stimuli, such as growth factors or the energy status of the cell. As described above, AKT indirectly activates the mTORC1 complex by phosphorylating the TSC1/TSC2 complex, which inhibits the small GTPase Rheb. Thus, AKT removes the negative influence of TSC1/TSC2 and promotes Rheb-dependent activation of mTORC1. Moreover, AKT directly activates mTORC1 by phosphorylating mPRS40, which negatively regulates the complex (Haar *et al.*, 2007).

The multilayer nature of mTORC1 regulation highlights the important need for the cell to keep the activity of this complex under tight control. mTORC1 mainly phosphorylates effectors involved in protein synthesis and ribosome biogenesis, processes that require high energy consumption and have to be kept in balance with the availability of nutrients and the requirement of cells to grow in size and proliferate. The most studied function of mTORC1 is regulation of protein translation. mTORC1 phosphorylates the eIF4E-Bindig protein 1 (4E-BP1) and the ribosomal protein S6

kinase 1 (S6K1). Phosphorylation of 4E-BP1 is required to disrupt the binding of 4E-BP1 with eIF4E, the factor able to bind the 5'UTR of messenger mRNA and that promotes the assembly of the ribosome for the translation (Mamane *et al.*, 2009). S6K1 promotes protein synthesis through the activation of the scaffold protein eIF3, which binds messenger RNAs and leads to the assembly of ribosomal subunit 40S (Sonenberg and Hinnebusch, 2009).

The activity of mTORC1 downstream effectors may, in turn, regulate upstream components of the PI3K/AKT pathway. For instance, when mTORC1 is active, S6K1 exerts a negative feedback on the pathway by phosphorylating and the Insulin receptor substrate 1 (IRS-1) (Shah and Turner, 2006). In this way, IRS-1 is degraded and PI3K signalling is attenuated. When mTORC1 activity in blocked for nutrient deprivation or pharmacological inhibition, the pathway can be stimulated through reactivation of IRS-1 and RTK signalling, which both turn on PI3K/AKT axis (Hay *et al*., 2005).

Due to its central role in regulation of protein synthesis, cell growth and many other important cellular functions (Laplante and Sabatini, 2012), mTORC1 was extensively studied in tumorigenesis, and it represents one of the most important targets for the pharmacological treatments of human cancers.

2.2 Deregulation of PI3K/AKT/mTOR in PETs

The PI3K/AKT/mTOR pathway was found deregulated in a high percentage of PETs. Notably, mutations in negative or positive regulators of the pathway, or alterations of the key components of the axis were associated to this class of diseases.

For instance, the expression and localization of the main negative regulator of the PI3K/AKT/mTOR pathway, PTEN, was found deregulated in primary samples of PETs (Missiaglia *et al*., 2010). Furthermore LOH in *PTEN* was associated to malignant features of PETs (Perren et al., 2000; Han *et al*., 2013), indicating its involvement in the oncogenesis of these endocrine tumors.

Other negative regulators of the PI3K/AKT/mTOR pathway were found mutated in primary samples of PETs. One of the most known negative regulators whose mutation is associated with these tumors is TSC2. Indeed, mutations in genes of the TSC complex are known to cause the genetic syndrome of Tuberous Sclerosis, which is also associated with PETs (Larson *et al*., 2012; Arva *et al.*, 2012). Furthermore, in PETs that are not associated with these genetic syndromes, TSC2 expression was down-

regulated and its low expression correlated with poor prognosis (Missiaglia *et al*., 2010).

The activity of AKT has been extensively studied in PETs, due to its involvement in the activation of pro-survival pathways. AKT was found up-regulated in over 60% of PETs (Ghayouri *et al*., 2010), indicating a positive correlation with the disease. Moreover, AKT activation is able to inhibit the expression of MEN-1 gene (Zang *et al.*, 2012), and at the same time, depletion of MEN-1 promotes the activation of AKT downstream signals (Wang *et al*., 2011).

mTOR protein activity and its effectors are also frequently deregulated in PETs. For instance, high levels of p-mTOR were found in PETs samples (Zhou *et al*., 2011, Komori *et al.*, 2014). Moreover, the expression of the mTOR effectors is aberrant in PET patients. For instance, higher levels of phosphorylated 4E-BP1 (p-4EBP1) were correlated with poor outcome (Di Florio *et al*., 2011) and deregulation of the mTOR pathway was also observed in PET cell lines (Missiaglia *et al*., 2010; Di Florio *et al*., 2011).

Collectively, these data underline the importance of perturbation of the PI3K/AKT/mTOR pathway in PETs and suggest that this pathway is a suitable therapeutic target to counteract progression of PET cancer cells.

2.3 Inhibitors of PI3K/AKT/mTOR in PETs

Preclinical studies on pharmacological inhibition of mTOR in neuroendocrine tumors (Chiu *et al*., 2010; Moreno *et al*., 2008) encouraged the research of novel therapeutic approaches acting on this important kinase. Preliminary studies on patients affected by PETs, demonstrated that pharmacological inhibition of mTOR exerted a significant effect on survival (Yao *et al*., 2008; Yao *et al*., 2010). The drug used for the studies was Everolimus (RAD001, Novartis), which was approved by Food and Drug Administration for the treatment of advanced endocrine tumors (Yao *et al*., 2011). Everolimus, as well as other molecules derived from rapamycin (rapalogs), acts as an allosteric inhibitor by binding and inactivating FKBP12, a protein that in turn activates mTOR in the mTORC1 pathway (Faivre *et al*., 2006) (Figure 2.3).

However, despite improvements in prolongation of life, some patients display a primary resistance to the drug and do not respond to Everolimus (Capurso *et al*., 2015). To date, the causes of primary resistance to the treatment are not known. Moreover, a quote of patients treated with Everolimus acquires a secondary resistance to treatments,

indicating that cancer cells can develop the ability to grow in presence of mTORC1 inhibition during chronic therapeutic treatments.

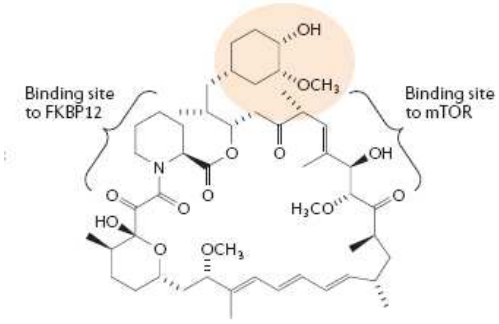


Figure 2.3. Molecular structure of Rapamycin. The figure shows the binding site of FKP12 and mTOR. In pink it was evidenced the portion of the molecule that has been modified to convert rapamycin in other affine molecules (rapalogs) (Modified from Faivre *et a*l., 2006)

Pharmacological inhibition of mTOR using rapamycin analogues such as Everolimus, causes a feedback reactivation of PI3K/AKT pathway (Laplante and Sabatini 2012; Meric-Bernstam *et a*l., 2012), leading tumor cells to escape from mTOR block. In particular, increased phosphorylation of AKT promotes prosurvival responses that may allow cells to withstand treatment with Everolimus and other rapalogs. .

These observations suggest that novel therapeutic approaches are required to counteract primary and acquired resistance to Everolimus in PET patients. Since feedback activation of AKT is likey involved in such resistance (Meric-Bernstam *et al*., 2012) treatment with inhibitors of PI3K was thought to possibly prevent the activation of such pro-survival pathways in patients treated with Everolimus.

Three novel inhibitors of PI3K have been proposed for the treatment of PETs, and recently tested in pre-clinical and clinical studies as anti-cancer treatments (Capurso *et al*., 2012) (Figure 2.4):

- BEZ235: this inhibitor is classified as dual inhibitor, as it inactivates all the PI3K class I isoforms but it also binds to the catalytic site of mTOR, thus potentially blocking both mTORC1 and mTORC2 (Maira *et al*., 2008);
- BKM120: This inhibitor is specific for the class I PI3K isoforms (Koul *et al*., 2012)
- BYL719: This inhibitor is specific for the p100α isoform of PI3K (Furet *et al*., 2013).

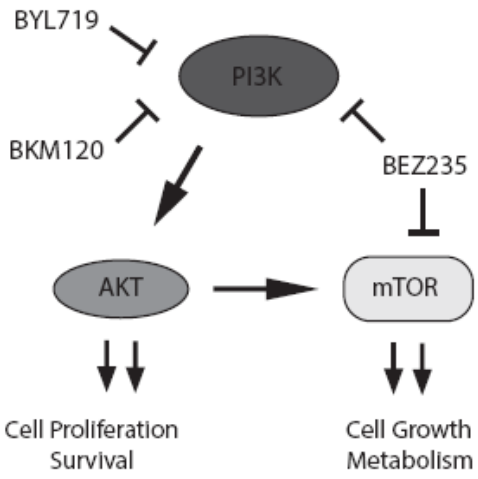


Figure 2.4. Schematic representation of the targets of the inhibitors BEZ235, BKM120 and BYL719.

The use of these novel PI3K inhibitors, either alone or in combination with Everolimus, has been considered a potentially valuable tool to avoid the mechanisms of resistance to mTORC1 inhibition, in order to avoid resistance to Everolimus treatment.

References

Arva NC, Pappas JG, Bhatla T, Raetz EA, Macari M, Ginsburg HB, Hajdu CH. et al. Well-differentiated pancreatic neuroendocrine carcinoma in tuberous sclerosis—case report and review of the literature. Am J Surg Pathol. 2012 Jan; 36(1):149–53.

Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, and Schreiber, SL. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature. 1994 Jun 30; 369(6483): 756–8.

Buss H, Dorrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M. Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity. J Biol Chem 2004 Nov 26; 279(48):49571-4.

Cafferkey R, YoungPR, McLaughlin MM, Bergsma DJ, Koltin Y, Sathe GM, Faucette L, Eng WK, Johnson RK, Livi GP. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol Cell Biol. 1993 Oct; (13):6012–23.

Capurso G, Fendrich V, Rinzivillo M, Panzuto F, Bartsch DK, Delle Fave G. Novel Molecular Targets for the Treatment of Gastroenteropancreatic Endocrine Tumors: Answers and Unsolved Problems. Int J Mol Sci. 2012 Dec 20;14(1):30-45.

Capurso G, Archibugi L, Delle Fave G. Molecular pathogenesis and targeted therapy of sporadic pancreatic neuroendocrine tumors. J Hepatobiliary Pancreat Sci. 2015 Jan 25.

Chiu CW, Nozawa H, Hanahan D. Survival benefit with proapoptotic molecular and pathologic responses from dual targeting of mammalian target of rapamycin and epidermal growth factor receptor in a preclinical model of pancreatic neuroendocrine carcinogenesis. J Clin Oncol. 2010 Oct 10;28(29):4425–33.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cellintrinsic death machinery. Cell. 1997 Oct 17; 91(2):231–41.

Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov. 2006 Aug; 5(8):671-88.

Fruman DA , Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014 Feb;13(2):140-56.

Furet P, Guagnano V, Fairhurst RA, Imbach-Weese P, Bruce I, Knapp M, Fritsch C, Blasco F, Blanz J, Aichholz R, Hamon J, Fabbro D, Caravatti G. Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 kinase alpha inhibitor selected for clinical evaluation. Bioorg Med Chem Lett. 2013 Jul 1;23(13):3741-8.

Ghayouri M, Boulware D, Nasir A, et al. Activation of the serine/theronine protein kinase Akt in enteropancreatic neuroendocrine tumors. Anticancer Res. 2010 Dec; 30(12):5063–7.

Haar EV, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. 2007 Mar; 9(3), 316–23.

Han X, Ji Y, Zhao J, Xu X, Lou W. Expression of PTEN and mTOR in pancreatic neuroendocrine tumors. Tumour Biol. 2013 Oct;34(5):2871-79.

Hannan KM, Sanij E, Hein N, Hannan RD, Pearson RB. Signaling to the ribosome in cancer--It is more than just mTORC1. IUBMB Life. 2011 Feb; 63(2):79-85.

Hay N. The Akt-mTOR tango and its relevance to cancer. Cancer Cell. 2005 Sept ;8(3):179-83.

Inoki K, Li Y, Xu T, Guan, KL . Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003; 17(15):1829–1834.

Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004 Nov; 6(11), 1122–8.

Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002 Jul 26; 110(2):163-75.

Komori Y, Yada K, Ohta M, Uchida H, Iwashita Y, Fukuzawa K, Kashima K, Yokoyama S, Inomata M, Kitano S. Mammalian target of rapamycin signaling activation patterns in pancreatic neuroendocrine tumours. J Hepatobiliary Pancreat Sci. 2014 Apr; 21(4);288–95.

Koul D, Fu J, Shen R, LaFortune TA, Wang S, Tiao N, Kim YW, Liu JL, Ramnarian D, Yuan Y, Garcia-Echevrria C, Maira SM, Yung WK. Antitumor activity of NVP-BKM120--a selective pan class I PI3 kinase inhibitor showed differential forms of cell death based on p53 status of glioma cells. Clin Cancer Res. 2012 Jan 1;18(1):184-95.

Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell. 1993 May; 73(3): 585–96.

Larson AM, Hedgire SS, Deshpande V, Stemmer-Rachamimov AO, Harisinghani MG, Ferrone CR, Shah U, Thiele EA.Pancreatic neuroendocrine tumors in patients with tuberous sclerosis complex. Clin Genet. 2012 Dec; 82(6):558–63.

Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN . Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell. 2002 Sept; 10(3):457–68.

Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol-3-kinase/mammalian target of rapamycin inhibitor with poten in vivo antitumor activity. Mol. Cancer Ther. 2008 Jul; 7(7): 1851-63.

Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW, Sonenberg N. eIF4E-from translation to transformation. Oncogene. 2004 Apr 19; 23(18):3172-9.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007 Jun 29; 129(7):1261–74.

Marine JC, Lozano G. Mdm2-mediated ubiquitylation: p53 and beyond. Cell Death Differ. 2010 Jan; 17(1):93–102.

McNamara CR, Degterev A. Small-molecule inhibitors of the PI3K signaling network. Future Med Chem. 2011 Apr;3(5):549-65.

Meric-Bernstam F,Akcakanat A,Chen H, Do KA, Sangai T, Adkins F, Gonzalez-Angulo AM, Rashid A, Crosby K, Dong M, Phan AT, Wolff RA, Gupta S, Mills GB, Yao J. PIK3CA/PTEN mutations and Akt activation as markers of sensitivity to allosteric mTOR inhibitors. Clin Cancer Res. 2012 Mar 15; 18(6):1777–89.

Missiaglia E, Dalai I, Barbi S Beghelli S, Falconi M, Della Peruta M, Piemonti L, Capurso G, Di Florio A, Delle Fave G, Pederzoli P, Croce CM, Scarpa A. Pancreatic Endocrine Tumors: Expression profiling evidences a role of PI3K-Akt pathway . J Clin Oncol. 2010 Jan 10; 28(2): 245-55.

Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC, Meric-Bernstam F. Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. Endocr Relat Cancer. 2008 Mar; 15(1):257-66.

Nair VD, Olanow CW. Differential modulation of Akt/glycogen synthase kinase-3β pathwayregulates apoptotic and cytoprotective signaling responses. J Biol Chem. 2008 May 30; 283(22):15469–78.

Perren A, Komminoth P, Saremaslani P, Matter C, Feurer S, Lees JA, Heitz PU, Eng C. Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine pancreatic tumors compared to normal islet cells. Am J Pathol. 2000 Oct; 157(4): 1097–103.

Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin dependent fashion and is homologous to yeast TORs. Cell. 1994 Jul 15; 78(1):35–43.

Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004 Jul 27; 14(14): 1296–302.

Shah OJ, Hunter T. Turnover of the active fraction of IRS1 involves raptor-mTOR- and S6K1-dependent serine phosphorylation in cell culture models of tuberous sclerosis. Mol Cell Biol. 2006 Sep; 26(17):6425-34.

Sonenberg N, Hinnebusch AG. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. Cell. 2009 Feb 20; 136(4):731-45.

Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN_mour suppressor. Nat Rev Mol Cell Biol. 2012 Apr 4; 13(5):283-96.

Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003 Aug 5; 13(15), 1259–68.

Van Der Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J. 2004 Jun 1; 380(Pt. 2):297–309.

Wang Y, Ozawa A, Zaman S, Prasad NB, Chandrasekharappa SC, Agarwal SK, Marx SJ. The tumor suppressor protein menin inhibits AKT activation by regulating its cellular localization. Cancer Res. 2011 Jan 15; 71(2): 371–82.

Yada M, Hatakeyama S, Kamura T, Nishiyama M, Tsunematsu R, Imaki H, Ishida N, Okumura F, Nakayama K, Nakayama KI: Phosphorylationdependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J. 2004 May 19; 23(10):2116-25.

Yao JC, Phan AT, Chang DZ, Wolff RA, Hess K, Gupta S, Jacobs C, Mares JE, Landgraf AN, Rashid A, Meric-Bernstam F.. Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low- to intermediate- grade neuroendocrine tumors: results of a phase II study. J Clin Oncol. 2008 Sep 10; 26:4311-8.

Yao JC, Lombard-Bohas C, Baudin E, Kvols LK, Rougier P, Ruszniewski P, Hoosen S, St Peter J, Haas T, Lebwohl D, Van Cutsem E, Kulke MH, Hobday TJ, O'Dorisio TM, Shah MH, Cadiot G, Luppi G, Posey JA, Wiedenmann B. Daily oral Everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. J Clin Oncol. 2010 Jan 1; 28(1):69-76.

Yao JC, Shah MH, Ito T, Lombard Bohas C, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevilla J, de Vires EGE, Tomassetti P, Pavel ME, Hoosen S, Haas T, lincy J, Lebwohl D, et al. Everolimus for Advance Pancreatic Neuroendocrine tumors. N Engl J Med. 2011 Feb 10; 364(6): 514-23.

Yook JI, Li XY, Ota I, Hu C, Kim HS, Kim NH, Cha SY, Ryu JK, Choi YJ, Kim J, et al.: A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. Nat Cell Biol. 2006 Dec; 8(12):1398-406.

Zhang H, Li W, Wang Q, Wang X, Li F, Zhang C, Wu L, Long H, Liu Y, Li X, Luo M, Li G, Ning G.. Glucose-mediated repression of menin promotes pancreatic beta-cell proliferation. Endocrinology. 2012 Feb; 153(2):602–11.

Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Aktmediated MDM2 phosphorylation. Nat Cell Biol. 2001 Nov; (3):973–82.

Zhou CF, Ji J, Yuan F, Shi M, Zhang J, Liu BY, Zhu ZG. mTOR activation in well differentiated pancreatic neuroendocrine tumors: a retrospective study on 4 cases. Hepatogastroenterology. 2011 Nov-Dec; 58(112):2140-3.

CHAPTER III

Combined treatment with RAD001 and BEZ235 overcomes resistance of PET immortalized cell lines to mTOR inhibition

Pancreatic endocrine tumors (PETs) are characterised by an indolent behaviour in terms of tumor growth. However, at time of diagnosis most patients present with metastatic disease and they are not eligible for surgical treatment. Molecular evidence suggests that the PI3K/AKT/mTOR pathway is deregulated in PETs. In light of this, Everolimus (RAD001), which inhibits specifically mTOR, is used as pharmacological approach in PET therapy. However not all patients respond to the treatments, and most patients that initially respond eventlually develop resistance to Everolimus.

Chronic inhibition of mTOR may lead to a feedback re-activation of PI3K activity, thus restoring the PI3K/AKT axis functionality and providing an escape route to cancer cells. For this reason, PI3K became a novel important target in order to counteract the resistance to mTOR inhibition, and its specific inhibition has been considered as potential therapeutic approach for PETs.

In our work, we tested three novel PI3K inhibitors (BEZ235, BKM120 and BYL719) in three cell lines used as a model for PETs tumors. In particular we tested the inhibitors in cells that respond to Everolimus (BON-1), cells unresponsive to the drug (QGP-1) and cells that acquired resistance to Everolimus after a chronic treatments of 8 weeks (BON-1 RR). We found that BEZ235 was the most efficient in term of inhibition of cell proliferation in all cell lines analyzed. Moreover, combined treatment with BEZ235 and RAD001 exerted a synergic effect on inhibition of cell proliferation with respect to treatment with single agents alone. Analysis of the PI3K/AKT/ mTOR pathway demonstrated that combined treatment significantly affects the phosphorylation of 4EBP1, indicating that the effect on cell proliferation might rely on the inhibition of the translation machinery. Indeed, we found that combined treatment affects the assembly of the translation initiation complex and, consequently, it significantly impairs protein synthesis as compared with treatment by single agents.

Our results suggest a novel approach in the treatment of PETs. Indeed, our study documents that targeting of mTOR with Everolimus and BEZ235 could overcome resistance to mTOR inhibition, providing a novel suitable therapy to counteract primary and secondary resistance to Everolimus in PETs.

Combined therapy with RAD001 e BEZ235 overcomes resistance of PET immortalized cell lines to mTOR inhibition

Ilaria Passacantilli[1,2], Gabriele Capurso[2], Livia Archibugi[2], Sara Calabretta[1,2], Sara Caldarola[3], Fabrizio Loreni[3], Gianfranco Delle Fave[2] and Claudio Sette[1,4]

[1] Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy

[2] Digestive & Liver Disease Unit, University of La Sapienza, Rome, Italy

[3] Department of Biology, University of Rome Tor Vergata, Rome, Italy

[4] Laboratory of Neuroembryology, Fondazione Santa Lucia, Rome, Italy

Correspondence to: *Claudio Sette,* ***email***: *claudio.sette@uniroma2.it*

Gianfranco Delle Fave, ***email***: *gianfranco.dellefave@uniroma1.it*

Keywords: PETs; mTORC1 chemical inhibitors; RAD001; BEZ235; adaptation to chronic treatment; combined treatment

Received: *March 28, 2014* ***Accepted***: *June 17, 2014* ***Published***: *June 18, 2014*

ABSTRACT

Pancreatic endocrine tumors (PETs) are characterised by an indolent behaviour in terms of tumor growth. However, most patients display metastasis at diagnosis and no cure is currently available. Since the PI3K/AKT/mTOR axis is deregulated in PETs, the mTOR inhibitor RAD001 represents the first line treatment. Nevertheless, some patients do not respond to treatments and most acquire resistance. Inhibition of mTOR leads to feedback re-activation of PI3K activity, which may promote resistance to RAD001. Thus, PI3K represents a novel potential target for PETs. We tested the impact of three novel PI3K inhibitors (BEZ235, BKM120 and BYL719) on proliferation of PET cells that are responsive (BON-1) or unresponsive (QGP-1) to RAD001. BEZ235 was the most efficient in inhibiting proliferation in PET cells. Furthermore, combined treatment with BEZ235 and RAD001 exhibited synergic effects and was also effective in BON-1 that acquired resistance to RAD001 (BON-1 RR). Analysis of PI3K/AKT/mTOR pathway showed that RAD001 and BEZ235 only partially inhibited mTOR-dependent phosphorylation of 4EBP1. By contrast, combined therapy with the two inhibitors strongly inhibited phosphorylation of 4EBP1, assembly of the translational initiation complex and protein synthesis. Thus, combined treatment with BEZ235 may represent suitable therapy to counteract primary and acquired resistance to RAD001 in PETs.

INTRODUCTION

Pancreatic endocrine tumors (PETs) are rare neoplasms that represent 1-2% of all pancreatic cancers [1,2]. They are heterogeneous in terms of clinical presentation, histological features, tumor grading and staging at time of diagnosis [3,4]. Despite PETs are considered to have an "indolent" behaviour, most patients display metastases at the time of diagnosis, being not eligible for surgery [3]. Furthermore, as PETs are characterised by a low proliferation rate, chemotherapy is chosen as first line treatment only for a subgroup of patients with more aggressive features, whereas treatment of the majority of PETs has generally employed somatostatin analogues [4]. Nevertheless, these therapeutic approaches offer limited clinical benefits for patients.

In the past few years, this therapeutic scenario has dramatically changed. Two novel targeted therapies, with the selective inhibitor for the serine-threonine kinase mTOR (Everolimus or RAD001) and the multi-target tyrosine-kinase inhibitor sunitinib, have been approved for advanced progressive pancreatic neuroendocrine tumors (pNETs) [5,6]. In particular, the rationale for the use of RAD001 in PETs is sustained by solid preclinical data with human samples and in vitro models that highlighted the relevance of the PI3K-mTOR pathway in PETs [7-10]. For instance, low expression levels of negative regulators of mTOR, such as PTEN and the TSC1/2 complex, are

associated with worse prognosis in PET patients [7]. Moreover, a number of studies have confirmed the efficacy of RAD001 in models of neuroendocrine tumours in vitro [11,12]. However, despite the strong rationale and the specific mechanism of action of RAD001, not all patients respond to the treatment. Indeed, one third of patients display primary insensitivity [13], whereas others initially experience disease stabilization but they eventually develop resistance to the drug and undergo disease progression [5].

The PI3K/AKT/mTOR axis is involved in the regulation of cell survival, proliferation and motility [14]. mTOR is assembled in two main complexes named mTORC1, which regulates mRNA translation and protein synthesis in response to nutrients [15], and mTORC2, which is mainly involved in cytoskeleton remodelling and cell survival [16]. Notably, RAD001 specifically targets the mTORC1 complex whereas mTORC2 is insensitive to it, thus leaving some mTOR functions unaltered upon treatment [15,16]. Importantly, mTORC1 participates to a negative feedback that keeps the activity of PI3K under tight control [17]. As a consequence, mTORC1 inhibition can lead to activation of PI3K and of the pro-survival kinase AKT [18]. Thus, novel therapeutic strategies that avoid instauration of feedback activation of the PI3K/AKT axis might be beneficial in long-term treatments of PET patients with mTORC1 inhibitors.

In this work, we aimed at evaluating the response of PET cell lines to three novel PI3K inhibitors, the dual PI3K-mTOR inhibitor BEZ235 [19], the pan-PI3K inhibitor BKM120 [20] and the PI3Kα inhibitor BYL719 [21]. BEZ235 was the most efficient among the PI3K inhibitors in limiting PET cell growth. Notably, although BEZ235 alone did not provide an advantage with respect to RAD001, combined treatment with these inhibitors could overcome the resistance of PET cells to RAD001 and it significantly lowered the dose required to exert anti-proliferative effects. The synergic effect relied on more efficient inhibition of 4EBP1 phosphorylation, consequent impairment of the assembly of the translation initiation complex eIF4F and strong inhibition of protein synthesis. Thus, our results demonstrate *in vitro* the efficacy of combined treatment with RAD001 and BEZ235 in PET cells, providing the basis for studies using *in vivo* models of PET.

RESULTS

Establishment of a PET cell model of acquired resistance to RAD001

Clinical data indicate that a subset of PET patients respond to RAD001 treatment with tumor regression or stabilization, whereas others display primary resistance. In addition, the majority of patients that initially respond to the treatment then develop secondary resistance within 1 year [13]. We aimed at developing cell models representing these clinical situations to test the effect of three novel PI3K inhibitors in PETs. The PET cell lines BON-1 and QGP-1 exhibit a different sensitivity to RAD001 in terms of proliferation, with BON-1 cells being highly sensitive to the inhibitor and QGP-1 rather resistant [7,10]. To determine whether RAD001-sensitive cells could acquire resistance to the drug, we treated BON-1 cells with RAD001 for 8 consecutive weeks. RAD001 (10 nM) was supplied every 48 hours together with fresh medium (Figure 1A). Treatment with RAD001 almost completely blocked proliferation of BON-1 cells in the first week (Supplementary Figure 1A). However, after 10-15 days of treatment cells started to grow slowly and by the end of the treatment they exhibited a proliferation rate in the presence of RAD001 that was comparable to that of parental BON-1 cells in the absence of the drug (Supplementary Figure 1B). These cells, which we named BON-1 RR (RAD001 Resistant) for their acquired phenotype, displayed a more elongated shape and fewer cell-cell contacts with respect to the morphology of parental cells (Figure 1A). Although changes in elongated shape are often a hallmark of epithelial-to-mesenchymal transition in cancer cells, as exemplified by the MCF-7 and MDA-MB-231 breast cancer cells (Figure 1B), we found that this is not the case for BON-1 cells. Indeed, parental BON-1 cells express mixed markers of both epithelial and mesenchymal phenotype and their expression levels are not significantly changed in BON-1 RR cells (Figure 1B).

To validate the differential sensitivity of PET cell lines to RAD001, we performed colony formation assays, which measure the ability of cells seeded at clonal dilutions to form new colonies [22]. As expected, parental BON-1 cells were highly sensitive to RAD001, with approximately 75-90% inhibition of colony formation at 1-10 nM concentrations (Figure 1C). QGP-1 cells were substantially resistant to the drug, which caused a 20-35% reduction in number of colonies (Figure 1C). Strikingly, BON-1 RR cells were strongly resistant to RAD001, with approximately 10% reduction in colony formation at the highest dose (Figure 1C). These results suggest that PET cells that are sensitive to mTORC1 inhibition can develop resistance to RAD001 treatment, similarly to what observed in patients [5,13].

PI3K inhibitors display different efficacy in the inhibition of PET cell growth.

In various cancer cell lines, inhibition of mTORC1 activity causes a feedback activation of PI3K and phosphorylation of AKT, resulting in a pro-survival response [18]. To test whether such feedback control is also active in PET cells, we treated BON-1 and QGP1

cells with different doses of RAD001. Notably, RAD001 induced sustained (4-24 hours) phosphorylation of AKT in Thr 308 and Ser 473 in both PET cell lines (Supplementary Figure 2). These results suggest that the prosurvival PI3K/AKT pathway is activated in both RAD001-sensitive and -resistant PET cells.

BEZ235 is a dual inhibitor that inhibits the catalytic activity of mTOR and of all class I PI3K isoforms by targeting their ATP binding site [19]. BKM120 acts on all class I PI3K isoforms [20], whereas BYL719 specifically inhibits the activity of the p110α catalytic isoform [21]. To evaluate the activity of these compounds in PET cells, we initially tested the minimal dose of each drug required to inhibit AKT phosphorylation and mTORC1 activity in BON-1 and QGP-1 cells (Supplementary Figure 3). Phosphorylation of AKT in Thr 308, which is mediated by PDK1, was evaluated as marker of PI3K activity, phosphorylation of AKT in Ser 473 was evaluated as target of mTORC2 activity, whereas phosphorylation of rpS6 and of 4EBP1 were evaluated as downstream targets of mTORC1 (see Figure 2A for pathway representation).

In both cell lines, BEZ235 inhibited mTORC1 activity at 10 nM, as indicated by reduced phosphorylation of rpS6 and the shift to faster electrophoretic mobility of 4EBP1 (α isoform in Figure 2B). However, at 100-250 nM BEZ235 also impaired PI3K and mTORC2 activities, as shown by reduced phosphorylation of AKT in Thr 308 and Ser 473, respectively (Figure 2B). By contrast, the other two drugs were less effective in inhibiting these pathways and required much higher dosage (Supplementary Figure 3A,B). BKM120 inhibited PI3K (AKT Ser308) and partially mTORC1/2 activities at 250-500 nM, whereas BYL719 was active at concentrations in the micromolar range (1-10 μM) (Figure 2B and Supplementary Figure 3A,B). Specific inhibition of mTORC1 activity by BEZ235 was also tested by monitoring phosphorylation of mTOR in Ser 2448, a substrate of the ribosomal S6 kinase (23) that is activated by the mTORC1 complex (15). As expected, we found that increasing doses of BEZ235 reduced mTOR phosphorylation, whereas the PI3K-specific inhibitors were ineffective (BKM120) or exerted a partial effect only at high doses in the RAD001-sensitive BON-1 cells 10 μM BYL719) (Supplementary Figure 3C,D).

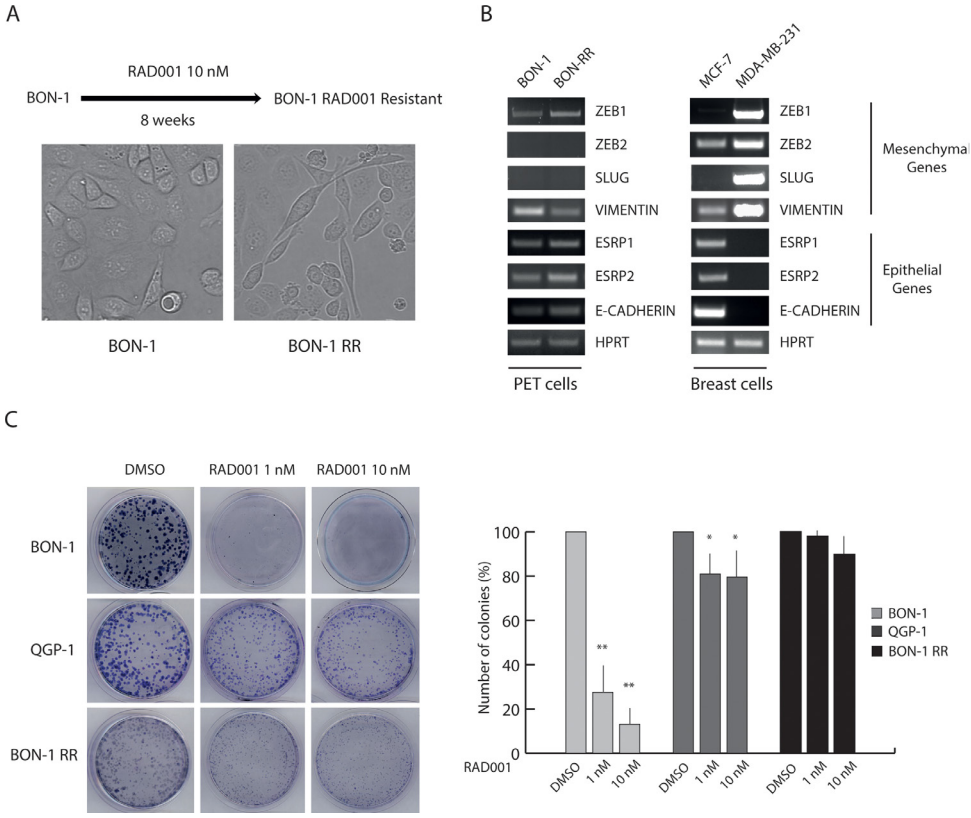


Figure 1: Chronic treatment selects RAD001-resistant BON-1 cells. (A) Scheme of the protocol used to select a RAD001-Resistant BON-1 cell line (BON-1 RR). Representative images of parental and RAD001-resistant BON-1 cells. BON-1-RR show a more elongated shape and fewer cell-cell contacts with respect to the morphology of parental cell (40X magnification). (B) RT-PCR analysis of the expression of mesenchymal and epithelial genes in BON-1 and BON-1 RR cells. MCF-7 and MDA-MB-231 breast cancer cells were used as positive control of epithelial and mesenchymal phenotype, respectively. (C) Representative images of colony formation assay performed with BON-1, QGP-1 and BON-1 RR treated with 1 or 10 nM RAD001. Histograms represent the percentage of inhibition of colony formation in comparison to control cells from three experiments (mean ± s.d.). Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

In order to evaluate the effect of the PI3K inhibitors on PET cell proliferation and survival, we performed colony formation assays. In BON-1 cells, BEZ235 did not confer an advantage with respect to RAD001 and suppressed colony formation at 3-10 nM concentration (Figure 2C), a dose at which this inhibitor affected mTORC1 activity but not PI3K activity (Figure 2B). By contrast, in QGP-1 cells, which are rather resistant to RAD001 and to doses of BEZ235 that inhibit only mTORC1, increasing doses of BEZ235 significantly inhibited colony formation and growth with respect to the effect of RAD001 (Figure 2C). This result might indicate that inhibition of PI3K activity overcomes RAD001 resistance in PET cell lines. However, BKM120 and

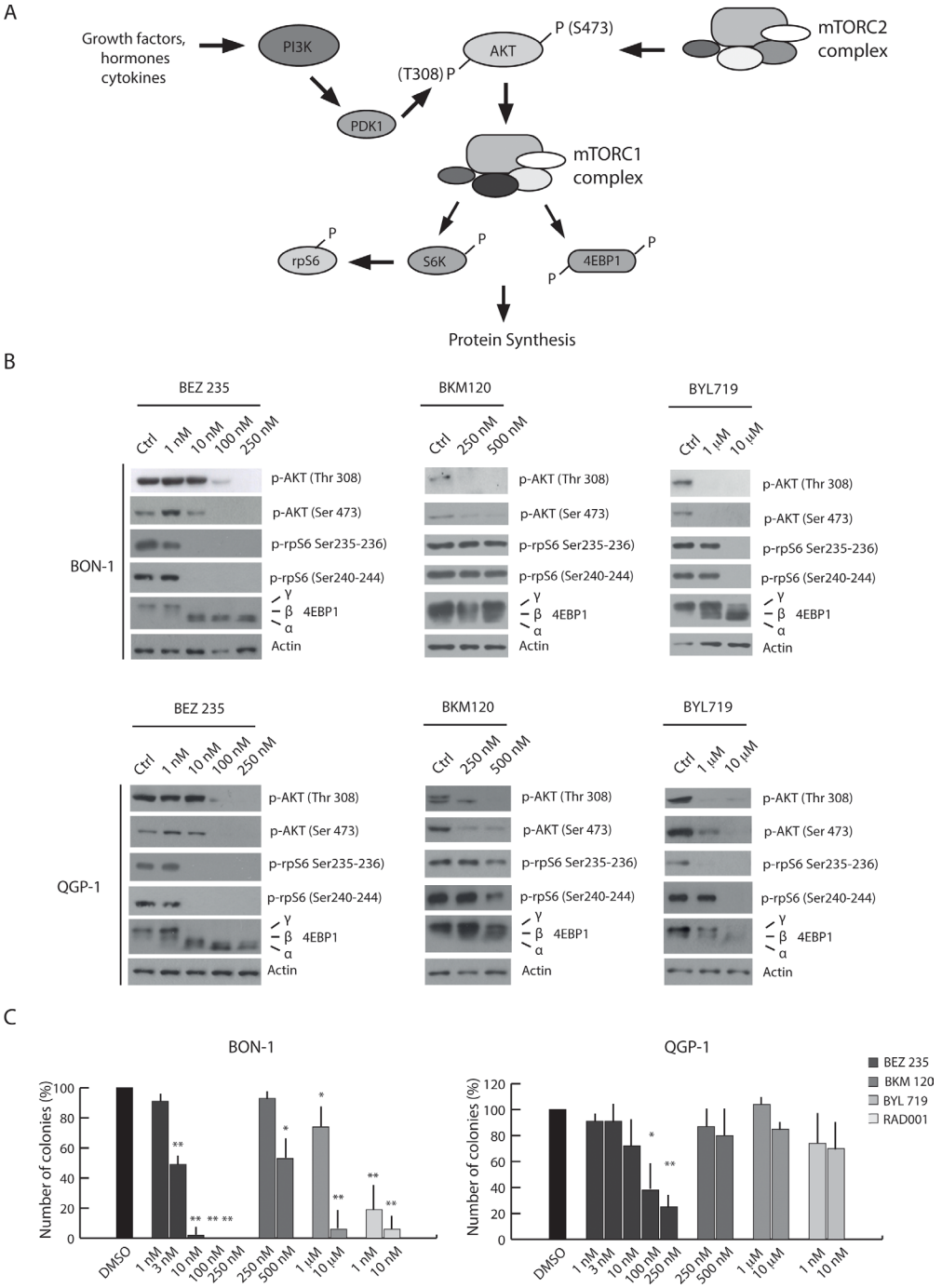


Figure 2: PI3K inhibitors display different efficacy in the inhibition of PET cell growth. (A) Schematic representation of the PI3K/Akt/mTOR pathway. (B) Western blot analysis of 4EBP1, p- AKT Ser 473, p-AKT Thr 308, p-rpS6 Ser 240-244 and p-rpS6 Ser 235-236 in BON-1 and QGP-1 treated with the PI3K inhibitors BEZ235, BKM120 and BYL719 for 4 hours. Actin was used as loading control. (C) Colony formation assays performed in BON-1 and QGP-1 treated with the PI3K inhibitors and RAD001. Histograms show the percentage of inhibition of colony formation in comparison to control cells from three experiments (mean ± s.d.). Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

BYL719 did not suppress QGP-1 colony formation even at doses that efficiently inhibited PI3K activity, whereas they were more efficacious in BON-1 cells (Figure 2C). Thus, these observations suggest that the concomitant inhibition of mTORC1 and PI3K/mTORC2 exerted by high doses of BEZ235 is beneficial to limit growth of RAD001-resistant PET cells.

Combined treatment with BEZ235 and RAD001 overcomes resistance of PET cells to RAD001

To test whether combined treatment with BEZ235 and RAD001 provides an advantage with respect to single treatment with each drug, we evaluated their effect in PET cells that are resistant to RAD001 alone (QGP-1 and BON-1 RR cells) by colony formation assays. As shown above (Figure 2C), QGP-1 cells were rather resistant to BEZ235 at concentrations (1-10 nM) that selectively inhibited mTORC1, whereas their growth was strongly inhibited at concentrations (100-250 nM) that also suppress PI3K and mTORC2 activity (Figure 3A). A similar result was observed for BON-1-RR cells (Figure 3B). However, when low doses of BEZ235 (1-10 nM) were administered in combination with 1 nM RAD001, growth was significantly inhibited in both QGP-1 (~25-65% inhibition, Figure 3A) and BON-1-RR (~50-80% inhibition, Figure 3B) cells. Importantly, increasing the dose of RAD001 to 10 nM did not provide a significant amelioration of the effect of BEZ235 (Figure 3A,B). Direct measurement of cell proliferation and cell death indicated that the combined treatment mainly affected proliferation of PET cells (Supplementary Figure 4A,B). Notably, co-treatment of cells with RAD001 and BKM120, which inhibits only PI3K, did not exert a synergic effect on PET cell proliferation (Supplementary Figure 5). These data indicate that combined treatment of PET cells with RAD001 and BEZ235 is more effective with respect to the action of each drug alone. Furthermore, the combined treatment allows lowering tenfold the minimal concentration of both drugs required to exert significant inhibition of PET cell growth.

Combined treatment with BEZ235 and RAD001 efficiently suppresses phosphorylation of AKT and 4EBP1 in RAD001-resistant PET cells

To investigate the molecular mechanism(s) underlying the synergic effect of BEZ235 and RAD001 in PET cells, we performed Western blot analyses of relevant targets of the PI3K and mTORC1 pathway. We found that in both QGP-1 (Figure 4A) and BON-1 RR (Figure 4B), treatment with 10 nM RAD001 or 10 nM BEZ235 alone did not completely block mTORC1 activity, as indicated by the substantial amount of high molecular weight forms (β and γ isoforms) of its direct substrate 4EBP1. In addition, under these conditions AKT remained

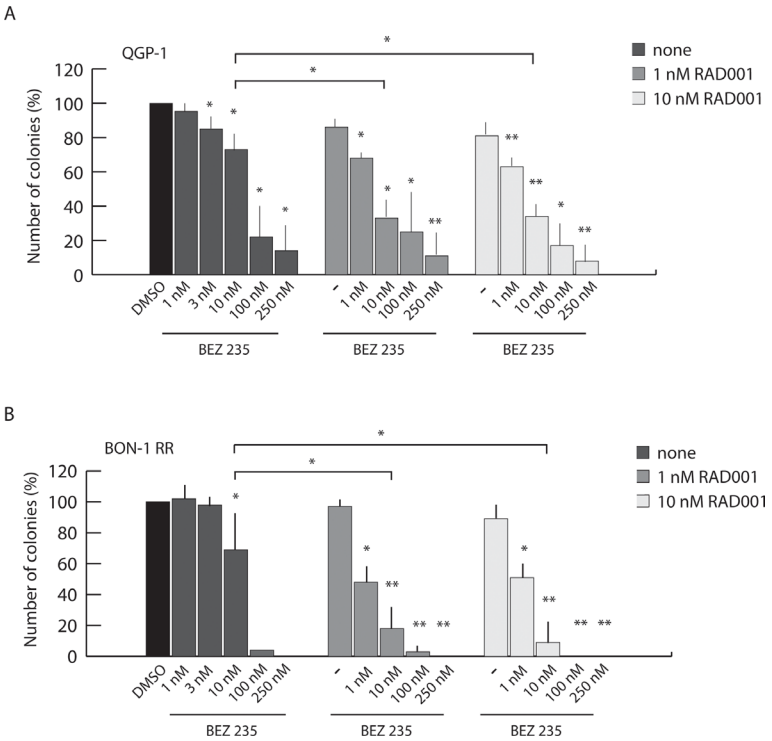


Figure 3: Combined treatment with BEZ235 and RAD001 overcomes resistance of PET cells to RAD001. Colony assay performed in QGP-1 (A) and BON1-RR (B) treated with BEZ235, RAD001 or both inhibitors as indicated. Histograms show the percentage of inhibition of colony formation in comparison to control cells from three experiments (mean ± s.d.). Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

phosphorylated in Thr 308 and Ser 473. By contrast, when RAD001 and BEZ235 were administered together (10 nM each), 4EBP1 phosphorylation was completely suppressed, whereas AKT phosphorylation was attenuated in QGP-1 cells (Figure 4A) and abolished in BON-1-RR cells (Figure 4B). Similarly to what observed with the colony formation assay, concomitant inhibition of PI3K/mTORC2 and mTORC1 pathways could be obtained by raising the concentration of BEZ235 alone to 100 nM. These results suggest that the synergism between RAD001 and BEZ235 is due to more efficient inhibition of both the PI3K/mTORC2 and the mTORC1 pathways elicited by the combined treatment (see Discussion).

Combined treatment with BEZ235 and RAD001 is required to inhibit protein synthesis in RAD001-resistant PET cells

A key function of the mTORC1 complex is the regulation of mRNA translation initiation complex [24].

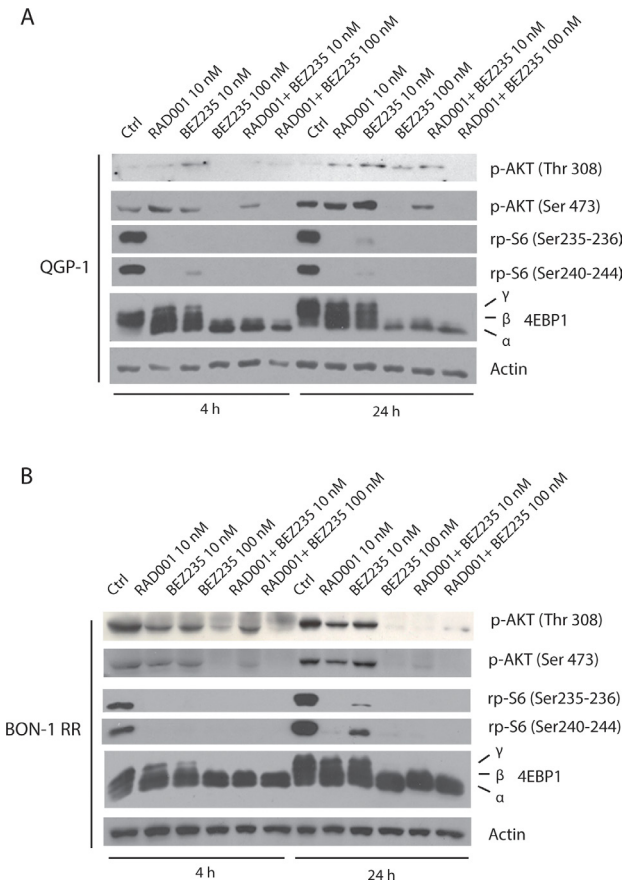


Figure 4: Combined treatment with BEZ235 and RAD001 efficiently suppresses phosphorylation of AKT and 4EBP1 in RAD001-resistant PET cells. 4EBP1, p-AKT Ser 473, p-AKT Thr 308, p-rpS6 Ser 240-244 and p-rpS6 Ser 235-236 in QGP-1 (A) and BON-1 RR (B) treated with the BEZ235 (indicated dose), RAD001 (10 nM) or both inhibitors. Actin was used as loading control.

Phosphorylation of 4EBP1 by mTORC1 inhibits its interaction with the eukaryotic initiation factor eIF4E. This allows recruitment of the scaffold protein eIF4G and the RNA helicase eIF4A by eIF4E to form the active eIF4F translation initiation complex at the 5'-cap of mRNAs and triggers translation initiation [24]. To test whether combined treatment with RAD001 and BEZ235 affected translation initiation, we performed 5'-methyl-cap assays to pull down eIF4E and its associated proteins [25]. In the absence of treatment, eIF4E was associated with substantial amount of eIF4G and eIF4A in both QGP-1 (Figure 5A, right panel) and BON-1-RR (Figure 5B, right panel) cells, indicating efficient assembly of eIF4F. Treatment with RAD001 or BEZ235 alone did not significantly reduce eIF4F formation, even though a partial de-phosphorylation of 4EBP1 was observed in the cell extracts (Figure 5A,B, left panels). By contrast, concomitant treatment with RAD001 and BEZ235 completely suppressed 4EBP1 phosphorylation and promoted its strong association with eIF4E and disassembly of eIF4F, as demonstrated by the strong reduction in eIF4G and eIF4A bound to eIF4E (Figure 5A,B).

The results illustrated above suggest that the effect of combined treatment with RAD001 and BEZ235 on PET cell growth correlates with the inhibition of translation initiation and consequent reduction in protein synthesis. To directly test this hypothesis, we performed metabolic labelling of PET cells with a mix containing ^{35}S-labeled aminoacids to measure the effect of RAD001 and BEZ235 on protein synthesis. We found that RAD001 or BEZ235 alone had mild (QGP-1 cells, Figure 5C) or no effect (BON-1 RR cells, Figure 5D) on protein synthesis. By contrast, when the two inhibitors were administered in combination, protein synthesis was strongly reduced in both QGP-1 cells (70%, Figure 5C) and BON-1 RR cells (55%, Figure 5D), confirming the results on the effect on eIF4F assembly. Notably, although in QGP-1 cells each inhibitor could slightly reduce protein synthesis, combined treatment exerted a significantly stronger effect than each single agent (brackets in Figure 5C), similarly to what observed on cell growth and eIF4F assembly.

Collectively, these observations strongly indicate that combined treatment with RAD001 and BEZ235 exerts a synergic effect on PET cell growth through enhanced inhibition of the PI3K/AKT/mTOR axis, impairment of eIF4F complex assembly and consequent reduction of protein synthesis.

DISCUSSION

Everolimus (RAD001) is currently employed as first line agent for advanced, progressive PETs [5,13,26,27]. The molecular target of RAD001 is the PI3K/AKT/mTORC1 axis, which is often deregulated in PET patients [13,26] and in other types of human cancers [24,28].

Unfortunately, however, a relevant number of PET patients show a primary resistance to treatment with RAD001 or acquire a secondary resistance after chronic exposure [13,26]. Furthermore, clinical trials have demonstrated that RAD001 delays but does not block PET progression [27]. PET cell models showing sensitivity to RAD001 (BON-1) or primary resistance to the drug (QGP-1) have been already described [7,10]. Herein, we have established a new PET cell model for the study of acquired resistance to RAD001. BON-1 cells were exposed to the drug chronically for 8 weeks, until their growth rate became similar to that of parental cells in the absence of RAD001 (BON-1-RR cells). Direct examination of the sensitivity of BON-1 RR to RAD001 confirmed their increased resistance to mTORC1 inhibition. Thus, we employed BON-1, QGP-1 and BON-1-RR cells as in vitro models of the clinical cases of PET patients to test the efficacy of drugs that target the PI3K/AKT/mTORC1 axis at different levels.

RAD001 is a synthetic analogue (rapalog) of rapamycin, which functions as an allosteric inhibitor of the mTORC1 complex [29]. The sensitivity to rapalogs has been associated to low eIF4E/4EBP1 ratios, whereas increased eIF4E expression and/or decreased 4EBP1 expression confer resistance in cancer cells [30]. However, we found that the eIF4E/4EBP1 ratio was not significantly different in RAD001-sensitive (BON-1) and insensitive (QGP-1 and BON-1 RR) PET cells (Supplementary Figure 6), suggesting that another mechanism is involved.

Adaptation of cancer cells to chronic treatment with rapalogs has been attributed to feedback activation of the PI3K/AKT pathway and to the consequent induction of prosurvival responses in cancer cells [17,30]. Moreover, for unknown reasons rapalogs efficiently block mTORC1-dependent S6 kinase activation but poorly suppress mTORC1-dependent phosphorylation of 4EBPs [30]. This weakness can be, however, overcome by new generation mTOR inhibitors that directly target the catalytic activity of the kinase [31,32]. Herein, we aimed at determining whether inhibition of PI3K activity could be beneficial to counteract growth of PET cell lines that are, or become, resistant to rapalogs. Among the inhibitors tested, BEZ235 resulted the most efficient in terms of inhibition of the PI3K/AKT/mTORC1 pathway and cell proliferation. Interestingly, BEZ235 is a dual inhibitor that targets the catalytic activity of PI3K, mTORC1 and mTORC2 [19]. However, we found that BEZ235 apparently inhibits these kinases at different concentrations in PET cells, with mTORC1 activity being suppressed at 1-10 nM while higher doses (100-250 nM) were required to inhibit PI3K and mTORC2. Notably, BON-1 RR and QGP-1, which show resistance to RAD001, do not respond to BEZ235 at doses that impair solely mTORC1 activity, but are sensitive to higher doses at which PI3K and mTORC2

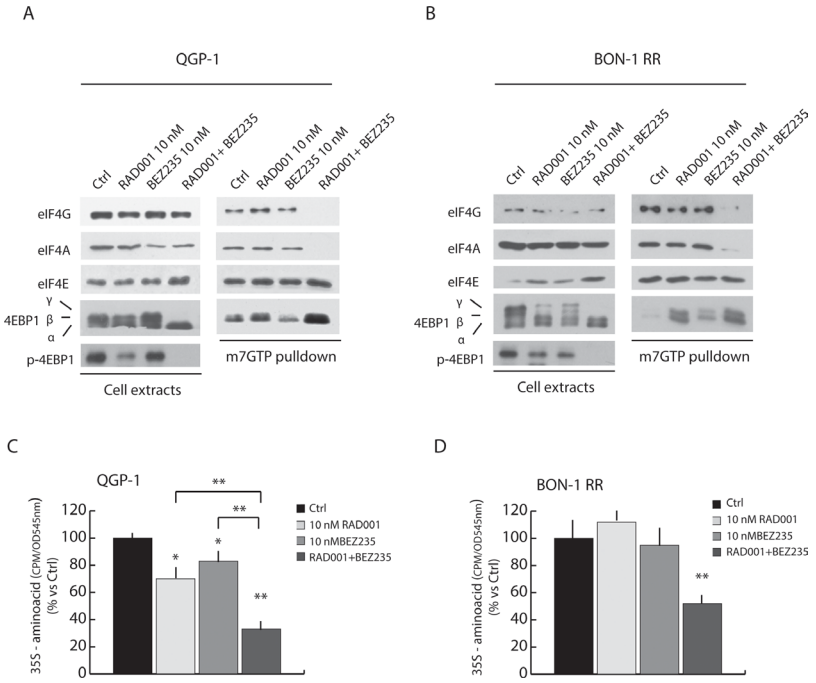


Figure 5: Combined treatment with BEZ235 and RAD001 efficiently suppress assembly of the translation initiation complex eIF4F and protein synthesis in RAD001-resistant PET cells. 7-methyl-GTP Sepharose Assay in QGP-1 (A) and BON-1 RR (B) treated with RAD001 (10 nM), BEZ235 (10 nM) or both inhibitors (10 nM each). The proteins absorbed to 7-methyl-GTP-Sepharose beads were analyzed in Western blot with antibody for eIF4G, eIF4A and 4EBP1. (C-D) Protein synthesis was measured by ^{35}S-aminoacids incorporation in QGP-1 (C) and BON-1 (D) cell lines. Cells were treated for 72 hours with inhibitors as indicated in the legend. The inhibitors were supplied at T=0 hours and at T= 36 hours together with fresh medium. ^{35}S-aminoacid mix was added in the last 30 min of the culture. Results of ^{35}S-aminoacid incorporation are expressed as mean ± s.d. of three experiments. Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

are also blocked. Inhibition of PI3K activity *per se* does not explain the sensitivity of PET cells to high doses of BEZ235, as the other two PI3K inhibitors tested (BKM120 and BYL719) had marginal effects on cell growth despite their ability to suppress PI3K-dependent phosphorylation of AKT. Thus, the higher activity of BEZ235 resides in its ability to concomitantly suppress PI3K and mTOR activity at higher doses.

The clinical use of BEZ235 is currently being evaluated in a variety of solid tumors [33]. A limitation to its employment at high doses might potentially be represented by development of toxicity. On the other hand, the clinical limitation of RAD001 is represented by development of resistance in patients [13,26,27]. To determine whether a combined treatment with RAD001 and BEZ235 could circumvent these problems, we exposed PET cells to both drugs. Our study revealed two important findings. First, we found that co-treatment with the two drugs exerted a synergic effect on PET cell proliferation, with up to 80% inhibition obtained with doses of each compound that caused marginal effects when supplied alone. Moreover, we found that the synergic effect of BEZ235 was elicited at doses that efficiently inhibited mTORC1 but not PI3K activity. This effect might be due to the different nature of mTOR inhibition by RAD001 and BEZ235, with the former acting allosterically by binding to FKB12 and the latter directly on the catalytic activity of the kinase [32]. It should also be reminded that although at the 10 nM concentration BEZ235 appears to selectively block mTORC1 in PET cells, recent evidence suggest that extensive crosstalk between the mTORC1 and mTORC2 complexes exist [34] and may enhance the effect of this drug in vivo. Moreover, since 10 nM overcomes the IC50 of BEZ235 on PI3Ks (19), it is also possible that, although we do not detect an inhibition of AKT phosphorylation, PI3K activity might be partially inhibited and this could affect PET cell proliferation. Another possibility is that BEZ235 exerts its synergic effect by inhibiting another kinase beside PI3K and mTOR. However, although we cannot completely rule out this possibility, it remains unlikely, as in vitro studies demonstrated an extreme specificity of BEZ235, with all other kinases tested being inhibited at doses much higher than dose employed in our study [19]. Thus, our results suggest that targeting the mTORC1 complex with allosteric and catalytic inhibitors synergistically impairs PET cell proliferation. In this regard, BEZ235 may present several advantages with respect to other catalytic inhibitors of mTOR [30,35], because the clinical use of BEZ235 is already in a more advanced stage (see below) and this drug can concomitantly inhibit the PI3K pathway, thus possibly reinforcing growth inhibition.

Previous studies in other cancer cell types indicated that limited or absent inhibition of phosphorylation of 4EBPs was the main cause of resistance to rapalogs and that this resistance could be eliminated by new generation inhibitors targeting the kinase activity of mTOR [30,35]. However, our results indicate that combined treatment with RAD001 and BEZ235 results in much stronger growth inhibition than either drug alone in PET cells, suggesting that the effect is not simply due to stronger efficacy of catalytic inhibitors with respect to rapalogs. Other studies also suggest that combined treatment with RAD001 and BEZ235 may exert a synergic effect on cell survival and proliferation in other cancer types [36,37], although the mechanism underlying this effect was not addressed. Here, we found that the anti-proliferative effect of the combined therapy directly correlates with the ability to suppress 4EBP1 phosphorylation and to interfere with the assembly of the translation initiation complex in PET cells. Indeed, our data show that RAD001 and BEZ235 used as single agents only partially reduced phosphorylation of 4EBP1 and interaction of eIF4E with eIF4G and eIF4A, whereas they abolished 4EBP1 phosphorylation and eIF4F assembly when administered together. This effect is biologically relevant, as it was reflected in a much stronger inhibition of protein synthesis in BON-1 RR and QGP-1 cells with respect to cells treated with RAD001 or BEZ235 alone. Thus, concomitant allosteric and catalytic inhibition of mTORC1 is necessary to efficiently circumvent primary and secondary resistance to RAD001 in PET cell lines.

BEZ235 is currently under evaluation in a clinical trial that investigates its use in patients with advanced PETs after failure of RAD001 (ClinicalTrials.gov Identifier:NCT01658436). In this regard, we found that BEZ235 had minor effects on RAD001-resistant PET cells at doses at which it completely suppressed mTORC1-dependent S6K activity. This result suggests that acquired resistance to rapalogs in PET cells cannot be overcome by treatment with catalytic inhibitors of mTORC1. By contrast, increasing the dose of BEZ235 to 100 nM, at which PI3K and mTORC2 activities are also inhibited, strongly suppressed PET cell growth. Thus, BEZ235 might be beneficial as second line agent but it is likely that the high dosage required for cell growth inhibition will cause unwanted responses in patients. Nevertheless, our findings suggest that addition of low doses of RAD001 may strongly sensitize PET cells to BEZ235 and potentially limit the onset of adverse responses in patients, as the combined therapy reduced the dose of each drug required to obtain efficient tumor growth inhibition in vitro. Notably, such strategy is under evaluation in different solid tumors [33], but not in PETs to the best of our knowledge.

In conclusion, our study suggests that combined therapy with RAD001 and BEZ235 might be particularly beneficial to patients that become insensitive to RAD001 treatment and provide the basis to test this hypothesis in a preclinical model of PET *in vivo*.

MATERIAL AND METHODS

Inhibitors

RAD001 and the PI3K inhibitors (BEZ235, BKM120 and BYL719) were generously provided by Novartis Oncology (Basel, Swiss). Inhibitors were dissolved in dymethil sulfoxide (DMSO, Sigma- Aldrich), and the stock solutions were diluted to final concentrations in medium.

Cell cultures, treatments and extracts preparation

BON-1 and QGP-1 cells were cultured as previously described [10,38]. BON-1 RR (RAD001-Resistant) cells were obtained after chronic treatment with RAD001 for eight weeks. During treatment, 10 nM RAD001 was added to the cell culture every 48 hours.

For western blot analysis, cells were seeded at 70% of confluence. After 24h medium was changed and inhibitors was added to the cultures. After incubation, cells were washed with ice-cold phosphate buffered saline (PBS) and resuspended in lysis buffer (100mM NaCl, 15mM MgCl2, 30mM Tris-HCl pH 7.5, 1mM dithiothreitol, 2mM Na-ortovanadate, Protease-Inhibitor Cocktail (Sigma-Aldrich) and 1% Triton X-100). Cells were incubated on ice for 10 minutes and protein extracts were separated by centrifugation at 12000*g*, resuspended in SDS-page sample buffer and boiled for 5 minutes before using them for SDS-PAGE analysis.

RT-PCR Analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions and 1μg was used for retrotranscribed (RT) using M-MLV reverse transcriptase (Invitrogen). Five percent of the RT reaction was used as template for PCR analysis (GoTaq, Promega). The sequences of all primers used are listed in Supplemental Table 1.

SDS- PAGE and Western blot analyses

Protein extracts were separated by SDS-PAGE and analysed by Western blot as previously described [38]. The primary antibodies used are: rabbit anti-actin (1:1000, Sigma-Aldrich); rabbit anti-4E-BP1 (1:1000), rabbit anti-4EBP1 pSer65 (1:500), rabbit anti-eIF4E (1:1000), rabbit anti-eIF4G (1:1000), rabbit anti-AKT pThr308 (1:200), rabbit anti-rpS6 pSer240-244 (1:1000), rabbit anti-rpS6 pSer235-236 (1:1000) (all from Cell Signalling Technology); rabbit anti-pSer473 AKT (1:1000) (from BioSource); rabbit anti-eIF4A (1:1000) (Abcam). Actin was used as loading control to normalize the samples. Secondary IgGs conjugated with horseradish peroxidise (1:10 000; Amersham Bioscience) were incubated for 1 hour and signals were detected by enhanced chemioluminescence (Santa Cruz Biotechnology).

Colony Formation Assay

Single-cell suspensions were plated in 35mm plates at low density (1000 cells/plate) [22]. After 1 day, cells were treated with inhibitors as indicated in the figures. The medium was changed every 48 hours and inhibitors were added at every change of medium. After 10 days, cells were fixed in methanol for 10 minutes and stained overnight with 5% Giemsa. Plates were then washed twice with PBS and dried. Pictures were acquired using digital camera to count the colonies. Results represent the mean ± s.d. of three experiments.

Cell Count and viability Assay

Cell count was used to monitor cell proliferation. BON-1 RR and QGP-1 were seeded at 40000 cells/plate in 24-well plates and treated for 72 hours as described. At the end of treatments, cells were washed in PBS, trypsinized and counted using the Thoma's chamber.

For apoptosis assays, cells were seeded at 80 000 cells/plate in 12-well plates and treated with inhibitors as indicated. Cells were fixed for 10 minutes in 4% paraforlmaldeyde (PFA) and permeabilized with 0,1% Triton X-100 for 10 minutes. After 1 hour with PBS with 3% BSA, cells processed for immunofluorescence analysis using the anti-cleaved caspase-3 antibody (1:400 dilution, Sigma-Aldrich) for 1 hour and 30 minutes. Cells were incubated with secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratories). Five random fields were chosen and at least 100 cells/field were counted. Results represent the mean ± s.d. of three experiment performed in triplicate.

7-Methyl-GTP-Sepharose Chromatography

Assembly of eIF4F complex was evaluated essentially as previously described [10,25]. Briefly, PET cells were resuspended in lysis buffer (see above) containing 0,5% Triton X-100 and 30 U/mL RNasin (Promega). Cell extracts were incubated for 10 minutes on ice and centrifuged at 12000*g* for 10 minutes at 4°C. The supernatant fractions were pre-precleared for 1 hour on Sepharose beads (Sigma-Aldrich) and then centrifuged at 1000*g* for 1 minute. Pre-cleared supernatants were then incubated with 7-Methyl-GTP Sepharose (Amersham Bioscience) for 90 minutes at 4 °C under constant shaking. After three washes in lysis buffer, bound protein were

eluted in SDS-PAGE sample buffer and analysed by Western blot

Protein Synthesis Assay.

For the measurement of protein synthesis rate, $2x10^5$ cells were plated in 35 mm dishes in presence of inhibitors as indicated. In the last 30 minutes [^{35}S]-cell labelling mix (Perkin Elmer EasyTag ™ Express35S35S, 1000 Ci/nmol) was added to final concentration of 10 μCi/mL. Cells were lysed in High Salt Buffer (HSB) (Tris HCl pH 7.5 50 mM, NaCl 350 mM, MgCl2 1 mM, EDTA 0,5 mM, EGTA 0,1 mM) with 1% NP-40) and proteins were precipitated in 10% trichloroacetic acid. After three washes with 5% cold thricloroacetic acid, the insoluble materials was collected on GFC filters (Whatman) and the incorporated radioactivity was measured in scintillation fluid. Results represent mean ± s.d. of three experiments.

ACKNOWLEDGEMENTS

We wish to thank Novartis Oncology to provide the PI3K inhibitors used for the experiments. This work was supported by grants provided by Associazione Italiana Ricerca sul Cancro (AIRC IG-14581), Association for International Cancer Research (AICR 12-0150), and Italian Association for Neuroendocrine Tumor (It.A.NET).

REFERENCES

1. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: Pancreatic endocrine tumors. Gastroenterology. 2008; 135(5): 1469–1492.
2. Fitzgerald TL , Hickner ZJ , Schmitz M, Kort EJ. Changing incidence of pancreatic neoplasms. A 16-year review of statewide tumor registry . Pancreas. 2008; 37(2) : 134 – 8.
3. Panzuto F, Boninsegna L, Fazio N, Campana D, Brizzi MP, Capurso G, Scarpa A, De Braud F, Dogliotti L, Tomassetti P, Delle Fave G, Falconi M. Metastatic and locally advanced pancreatic endocrine carcinomas: analysis offactors associated with disease progression. J Clin Oncol. 2011; 29(17): 2372–2377.
4. Pavel M, Baudin E, Couvelard A, Krenning E, Öberg K, Steinmüller T, Anlauf M, Wiedenmann B, Salazar R; Barcelona Consensus Conferences partecipants. ENET Consensus Guidelines for the management of patients with liver and other distant metastases from neuroendocrine neoplasms of foregut, midgut, hindgut, and unknown primary. Neuroendocrinology. 2012; 95(2): 157-176.
5. Yao JC, Shah MH, Ito T, Lombard Bohas C, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevilla J, de Vires EGE, Tomassetti P, Pavel ME, Hoosen S, Haas T, lincy J, Lebwohl D, et al. Everolimus for Advance Pancreatic Neuroendocrine tumors. N Engl J Med. 2011; 364(6): 514-523.
6. Raymond E, Dahan L, Raoul JL, Bang YJ, Borbath I, Lombard Bohas C, Valle J, Metrakos P, Smith D, Vinik A, Chen JS, Hörsch D, Hammel P, Wiedenmann B, Van Cutsem E, Patyna S, et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. N Engl J Med. 2011; 364(6): 501-513.
7. Missiaglia E, Dalai I, Barbi S Beghelli S, Falconi M, Della Peruta M, Piemonti L, Capurso G, Di Florio A, Delle Fave G, Pederzoli P, Croce CM, Scarpa A. Pancreatic Endocrine Tumors: Expression profiling evidences a role of PI3K-Akt pathway . J Clin Oncol. 2010; 28(2): 245-255.
8. Jiao Y, Shi C, Edil BH, de Wilde R, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfang CL, Choti MA, Velculescu VE, Diaz Jr LA, Vogelstein B, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science. 2011; 331(6021): 1199–1203.
9. Zhou CF, Ji J, Yuan F, Shi M, Zhang J, Liu BY, Zhu ZG. mTOR activation in well differentiated pancreatic neuroendocrine tumors: a retrospective study on 34 cases. Hepato-Gastroenterology, 2011; 58(112): 2140–2143.
10. Di Florio A, Adesso L, Pedrotti S, Capurso G, Pilozzi E, Corbo V, Scarpa A, Geremia R, Delle Fave G, Sette C. Src kinase activity coordinates cell adhesion and spreading with the activation of mammalian target of rapamycin in pancreatic endocrine tumor cells. Endocr Rel Cancer. 2011; 18 (35): 541-554.
11. Zatelli MC, Minoia M, Martini C, Tagliati F, Ambrosio MR, Schiavon M, Buratto M, Calabrese F, Gentilin E, Cavallesco G, Berdondini L, Rea F , Degli Uberti EC. Everolimus as a new potential antiproliferative agent in aggressive human bronchial carcinoids. Endocr Rel Cancer. 2010; 17(3): 719-729.
12. Bollard J, Couderc C, Blanc M, Poncet G, Lepinasse F, Hervieu V, Gouysse G, Ferraro-Pevret C, Benslama N, Walter T, Scoazec JY, Roche C. Antitumor effect of everolimus in preclinical models of high-grade gastroenteropancreatic neuroendocrine carcinomas. Neuroendocrinology. 2013; 97(4): 331-340.
13. Capurso G, Fendrich V, Rinzivillo M, Panzuto F, Bartsch D, Delle Fave G. Novel Molecular Targets for the Treatment of Gastroenteropancreatic Endocrine Tumors: Answers and Unsolved Problems. Int J Mol Sci. 2013; 14(1): 30-45.
14. Meric-Bernstam F, Gonzalez- Angulo AM. Targeting the mTOR signalling network for cancer therapy. J Clin Oncol. 2009; 27(13): 2278–87.
15. Proud CG. mTORC1 regulates the efficiency and cellular capacity for protein synthesis. Biochem Soc Trans. 2013;41(4):923-926.
16. Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. Cell Cycle. 2011; 10(14):2305-2316.
17. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga

J, Rosen N. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 2006; 66(3): 1500–1508.

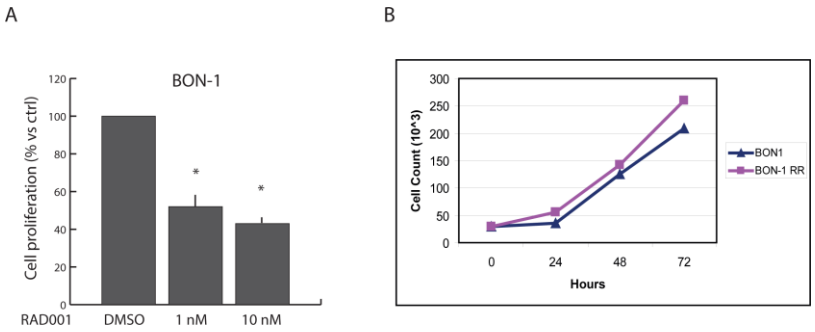
18. Manning BD, Cantely LC. AKT/PKB signalling: navigating downstream. Cell. 2007; 129(7): 1261-1274.
19. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol-3-kinase/mammalian target of rapamycin inhibitor with poten in vivo antitumor activity. Mol. Cancer Ther. 2008; 7(7): 1851-1863.
20. Koul D, Fu J , Shen R, LaFortune TA, Wang S, Tiao N, Kim YW, Liu JL, Ramnarian D, YaunY, Garcia-Echevrria C, Maira SM, Yung WK. Antitumor activity of NVP-BKM120 – A selective Pan Class I PI3 kinase inhibitor showed differential forms of cell death based on p53 status of glioma cells. Clin Cancer Res. 2012; 18(1): 184-195.
21. Furet P, Guagnano V, Fairhurst RA, Imbach-weese P, Bruce I, Knapp M, Fritsch C, Blasco F, Blanz J, Aichholz R, Hamon J, Fabbro D, Caravatti G. Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 Kinase alpha inhibitor selected for clinical evaluation. Bioorg Med Chem Lett. 2013; 23(13): 3741-3748.
22. Adesso L, Calabretta S, Barbagallo F, Capurso G, Pilozzi E, Geremia R, Delle Fave G, Sette C. Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNKs/eiF4E pathway. Oncogene. 2013; 32(23): 2848-2857.
23. Chiang GG, Abraham RT. Phosphorilation of mammalian target of rapamycin (mTOR) at Ser2448 is mediated by p70S6 kinase. J Biol Chem. 2005; 280(27); 25485-25490
24. Laplante M, Sabatini DM. mTOR signalling in growth control and disease. Cell. 2012; 149(2): 274- 293.
25. Bianchini A, Loiarro M, Bielli P, Busà R, Paronetto MP, Loreni F, Geremia R, Sette C. (2008) Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. Carcinogenesis, 29, 2279-2288.
26. Zhang J, Francois R, Iyer R, Seshadri M, Zajac-Kaye M, Hochwald SN. Current understanding of the molecular biology of pancreatic neuroendocrine tumors. J Natl Cancer Inst. 2013; 105(14): 1005-1017.
27. Yao JC, Phan AT, Jehl V, Shah G, Meric-Bernstam F. Everolimus in Advanced Pancreatic Neuroendocrine tumors: the clinical experience. Cancer Res. 2013; 73(5): 1449-1453.
28. Faivre S, Kroemer g, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov. 2006; 5(8): 671-688.
29. Alain T, Morita M, Fonseca BD, Yanagiya A, Siddiqui N, Bhat M, Zammit D, Marcus V, Metrakos P, Voyer LA, Gandin V, Liu Y, Topisirovic I, Sonenberg N. eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies. Cancer Res. 2012; 72(24):6468-6476.
30. Gilley R, Balmanno K, Cope CL, Cook SJ. Adaptation to chronic mTOR inhibition in cancer and aging. Biochem Soc Trans. 2013; 41(4): 956-961.
31. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D, Shokat KM. Active-Site inhibitors of mTOR target rapamycin-resistant Outputs of mTORC1 and mTORC2. PLoS Biol. 2009; 7(2): 371-383.
32. Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, Shi EY, Stumpf CR, Christensen C, Bonham MJ, Wang S, Ren P, Martin M,Jessen K, Feldman ME, Weissman JS, et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature. 2012; 485 (7396): 55-61.
33. Salkeni MA, Rixe O, Karim NA, Ogara S, Feiler M, Moorthy G, Mercer CA, Thomas H, Desai PB, Fathallah H, Kozma S, Thomas G, Morris JC. BEZ235 in combination with everolimus for advanced solid malignancies: Preliminary results of a phase Ib dose-escalation study. J Clin Oncol. 2013; 31 (suppl; abstr e13518).
34. Xie J, Proud CG. Crosstalk between mTOR complexes. Nat Cell Biol. 2013; 15(11):1263-1265.
35. Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, Meyuhas O, Shokat KM, Ruggero D. Genetic Dissection of the Oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP1-eIF4E. Cancer Cell. 2010; 17(3): 249-261.
36. Xu CX, Li Y, Yue P, Owonikoko TK, Ramalingam SS, Khuri FR, Sun SY. The combination of RAD001 and NVP-BEZ235 exerts synergistic anticancer activity against Non-Small cell lung cancer in vitro and in vivo. PLoS One. 2011; 6(6): 1-8.
37. Nyfeler B, Chen Y, Li X, Pinzon-Ortiz M, Wang Z, Reddy A, Pradhan E, Das R, Lehár J, Schlegel R, Finan PM, Cao ZA, Murphy LO, Huang A. RAD001 enhances the potency of BEZ235 to inhibit mTOR signalling and tumor growth. PLoS One. 2012; 7(11): 1-9.
38. Di Florio A, Capurso G, Milione M, Panzuto F, Geremia R, Delle Fave G, Sette C. Src family kinase activity regulates adhesion, spreading and migration of pancreatic endocrine tumor cells. End Relat Cancer. 2007; 14(1): 111-124.

Combined therapy with RAD001 e BEZ235 overcomes resistance of PET immortalized cell lines to mTOR inhibition

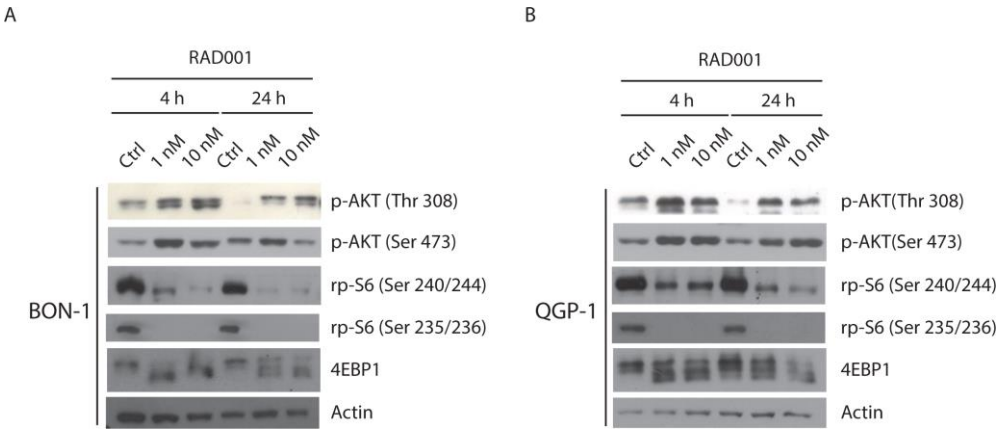
Supplementary Information

Supplemental Table 1. List of primers used for PCR analyses in this work.

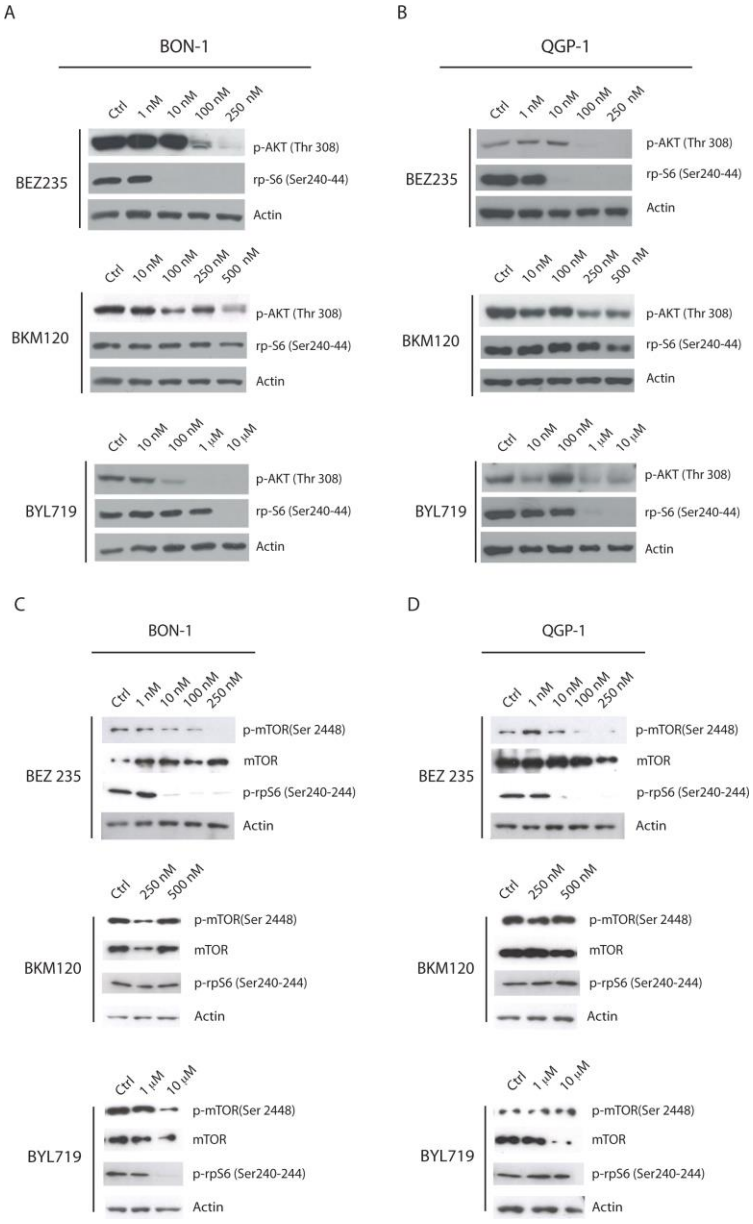
Primer	Sequence
ZEB1 Forward	CATTGCTGACCAGAACAGTGTTCC
ZEB1 Reverse	CGGTCAGCCCTGCAGTCCAAG
ZEB2 Forward	GAGGCGCGCGAGAAAGG
ZEB2 Reverse	GCCCAGCTTCCCGTAGCC
SLUGForward	AGTCCAAGCTTTCAGACCCCCATGCCATTG
SLUG Reverse	TTCTCCCCCGTGTGAGTTCTA
VIMENTIN Forward	AGACACTATTGGCCGCCTGCAGGATG
VIMENTIN Reverse	GAAGAGGCAGAGAAATCCTGCTCTCCTCGCCTTCCA
E-CADHERIN Forward	CACCCCCTGTTGGTGTCTTT
E-CADHERIN Reverse	TGGATCCTCAACTGCATTCCC
ESRP1 Forward	GGCTCGGATGAGAAGGAGTTGAT
ESRP1 Reverse	GAAGGAAGTCCCTACTCCAAT
ESRP2 Forward	ACGCTGCACAAATCGCTGGTT
ESRP2 Reverse	GTGCAGGACCTGTCGCAAT
HPRT Forward	TGACCAGTCAACAGGGGACA
HPRT Reverse	TTCGGGGGTCCTTTTCACC



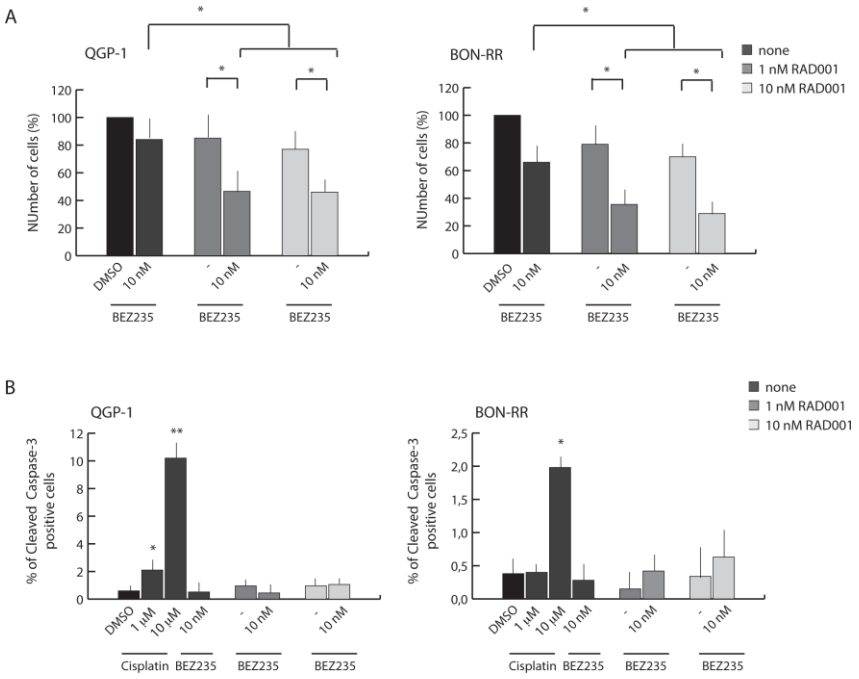
Supplementary Figure 1: Effect of RAD001 on cell proliferation in BON-1 and BON-RR. (A) Proliferation of BON-1 was assayed by MTS assay after 72 hours in presence of different doses of RAD001, as indicated. (B) The graph represents the cell count of BON-1 (without RAD001) and BON-1 RR (in presence of 10 nM RAD001) at different times, as indicated.



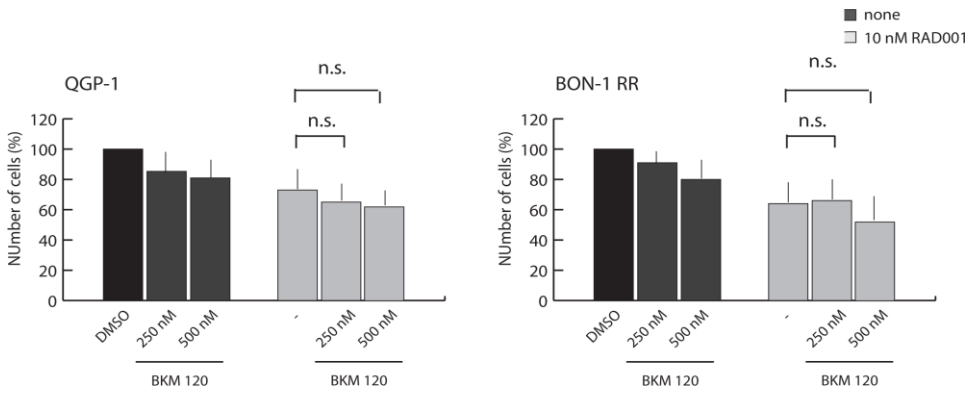
Supplementary Figure 2: RAD001 treatment induces phosphorylation of AKT in PET cell lines. Western Blot analysis of p-AKT in Thr 308 and Ser 473, p-rpS6 in Ser 235-235 and Ser 240-244, and 4EBP1 during treatment with different doses of RAD001 in BON-1 (A) and QGP-1 (B).



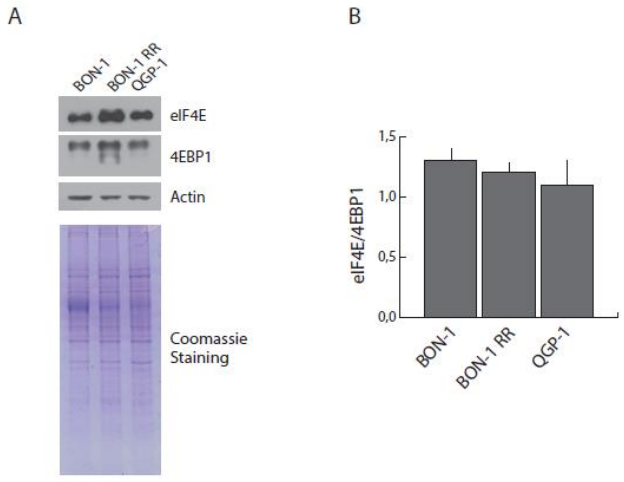
Supplementary Figure 3: Effect of PI3K inhibitors on the PI3K/AKT/mTOR pathway in PET cell lines. Western Blot analysis of p-AKT Thr 308 and p-rpS6 Ser 240-244 in BON-1 (A) and QGP-1 (B) and of mTOR, p-mTOR Ser 2448 and p-rpS6 Ser 240-244 in BON-1 (C) and QGP-1 (D) cells. PET cells were treated for 4 hours with increasing doses of PI3K inhibitors as indicated in the figure.



Supplementary Figure 4: Combined treatment with BEZ235 and RAD001 exerts a synergic effect on cell proliferation. (A) Cell proliferation was assayed by cell count after 72 hours in QGP-1 and BON-1 RR cells treated with BEZ235, RAD001 or both inhibitors as indicated. Histograms show the percentage of inhibition of number of cells in comparison to control cells from three experiments (mean ± s.d.). **(B)** Cell death was detected by immunofluorescence analysis of the cleaved form of caspase-3 after 72 hours in QGP-1 and BON-1 RR cells treated with Cisplatin, BEZ235, RAD001 or both inhibitors as indicated. Histograms show the percentage of cleaved caspase-3 positive cells from three experiments (mean ± s.d.). Statistical analysis was performed by the paired Student's t-test; * $P \leq 0.05$, ** $P \leq 0.01$.



Supplementary Figure 5: Combined treatment with BKM120 and RAD001 does not exert a synergic effect on cell proliferation and cell viability. Cell proliferation was assayed by cell count after 72 hours in QGP-1 and BON-1 RR treated with BKM120, RAD001 or both inhibitors as indicated. Histograms show the percentage of inhibition of number of cells in comparison to control cells from three experiments (mean ± s.d.).Statistical analysis was performed by the paired Student's t-test; * $P \leq 0.05$, ** $P \leq 0.01$.



Supplementary Figure 6: RAD001-sensitive and insensitive PET cells display similar eIF4E/4EBP1 ratios. (A) Representative image of Western Blot analysis of eIF4E, 4EBP1 and Actin in PET cell lines. Actin and Coomassie Staining were used as loading control. (B) Densitometric analysis of eIF4E and 4EBP1, after each was normalized with respect to Actin levels. Results are the mean + standard deviation of three independent experiments.

CHAPTER IV

Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most aggressive and lethal solid tumors, characterized by a 5 years survival-rate of 6% and of an incidence rate almost equal to the mortality rate (Ferlay *et al*., 2010). PDAC represents 95% of pancreatic tumors, while the rest 5% is represented by endocrine tumors.

The incidence of PDAC is only 4% of all tumors, although it represents the fourth cause of cancer-related death in the western-world (Ferlay *et al*., 2010; Hariharan *et al*., 2008). The high incidence of mortality for this cancer is due to late diagnosis, caused by the absence of symptoms in the early stages of cancer. Moreover, most patients present with metastasis at time of diagnosis, and only a small fraction of cases is eligible for surgery treatment, which remains the only treatment that prolongs survival. Indeed, PDAC is characterized also by poor response to chemotherapy and radiotherapy, which determines a low median survival of patients affected by this aggressive disease (Cunningham *et al*., 2009; Stathis and Moore, 2010). These data strongly emphasize the urgent need for improvements in diagnosis and treatment of PDAC, which remains, to date, incurable.

4.1 Risk factors and symptoms

Genetic and environmental risk factors were extensively studied for their association with PDAC. Among the established risks, advanced age and sex were linked to this disease. Indeed, most patients are in advanced age (50-70 years) at time of diagnosis, and the disease is more common in man than women, with a ratio between two sexes of 1.3/1 (Ferlay *et al*, 2010).

Among the risk factors studied for their association to PDAC, the most important is smoke, which is associated with PDAC in 20-30% of cases, and it increases the risk of insurgence of PDAC by 74%. Moreover, dietary factors are included among risk factors, as consumption of red meat and alcohol (with a 20-30% of increased risk of PDAC), obesity and presence of diabetes. Other risk factors related to PDAC are also chronic infection by HCV and HBV and chronic pancreatitis (Wörmann and Algül, 2013).

Genetic traits have also been associated with PDAC. Up to 10% of PDAC cases show family history and are linked to inherited genetics factors associated with inherited syndormes. Among these, PDAC is associated with hereditary cancer syndromes, such

as the Peutz–Jeghers syndrome, Familial-atypical multiple melanoma syndrome, Li–Fraumeni syndrome, Hereditary non-polyposis colorectal cancer, Familial adenomatosis polyposis and Ataxia teleangiectasia (Wörmann and Algül, 2013).

In the early stages of PDAC, most symptoms are not specific of the pathology and do not allow an early diagnosis. The most diffused symptoms are abdominal pain, weight loss, and jaundice, caused by the infiltration of tumor in the common bile duct. Hence, most of PDACs are diagnosed in the late phase of the disease, when symptoms are related to local or metastatic spreading of tumor.

4.2 Diagnosis and Staging

Symptoms of PDAC are often related to aberrant functionality of pancreas and liver. For this reason, blood analysis is initially used to detect dysfunctions of these organs. If PDAC is suspected, the approach used to detect it is a radiological diagnosis. In order to visualize a suspected tumor, Multidetector Computed Tomography (MDCT), Magnetic Resonance (MRI) and Endoscopic Ultrasound (EUS) techniques are used. During EUS, samples of tissue can be taken in order to allow morphological and molecular inspection of the pancreatic lesions. Moreover, MRI and Computed Tomography (CT) are employed for the staging of the disease. In this way, the three-dimensional reconstruction of the neoplasm provides more information about the position of tumor with respect to neighbouring vessels, in order to classify tumors as surgically resectable. Position Emission Tomography (PET) is also employed to define the staging of the disease and to detect distal metastases (Vincent *et al*., 2011).

Radiological techniques used for diagnosis provide information required for the concomitant staging of PDAC. The TNM classification, which is largely used for PDAC, describes tumors for size, presence of lymph nodes invasion, distal metastases and the resectability of primary tumor. (Varadhachary *et al*., 2006).

On the basis of TNM classification, PDAC is classified in four stages:

- Stage I: Tumor is localized in pancreas and resectable.
- Stage II: Tumor is spread locally without invasion of other organs or limph nodes, and it is resectable.
- Stage III: tumor spread to lymph nodes and invaded the pancreatic duct, only a fraction of cancers is resectable
- Stage IV: tumor is spread to distal organs as lungs, liver and colon, and the disease at this stage is defined as metastatic.

The survival rate related to different stages is variable, albeit survival at five years from diagnosis remains extremely low, thus suggesting the need to improve early detection of PDAC.

4.3 Treatment

Treatment of PDAC depends on the stage at time of diagnosis. If the tumor is confined to pancreatic tissues, surgical resection is the first line treatment. Furthermore, rurgical resection is followed by adjuvant therapy with chemotherapy, recommended in patients that choose surgery with curative intent. When patients have a borderline resectable disease, chemotherapy is applied in order to reduce the size of the primary tumor before surgical resection (neoadjuvant treatment) (Vincent *et al*., 2011).

In advanced metastatic disease, chemotherapy is used as a palliative treatment, in order to improve the quality of life. The chemotherapeutic drug used for PDAC is Gemcitabine, which slightly increases overall survival of patients. Gemcitabine is an analogue of cytidine, and during DNA replication is incorporated in neo-replicated molecules of DNA (Burris *et al*., 1997), leading cells exposed to this genotoxic stress to the activation of apoptosis.

Gemcitabine was selected for treatment of PDAC for increased benefits in terms of prolongation of life with respect to 5-Fluorouracile (5-FU) (Burris *et al*., 1997). It is currently used in monotherapy or in combination with other drugs. For instance, combined treatment with gemcitabine and nab-paclitaxel was shown to increase the overall survival compared to gemcitabine alone (Von Hoff *et al*., 2013). Indeed nab-paxlitaxel (nanoparticle albumine-bound paxlitaxel), penetrates more efficiency through the stroma present around the PDAC lesions, which is thought to represent a physical barrier for chemotherapeutic drugs. However, after cycles of gemcitabine treatment, patients invariably become unresponsive to chemotherapy, suggesting that PDAC cells are able to activate pro-survival pathways and to acquire a resistant phenotype.

Another chemotherapeutic approach proposed for the treatment of PDAC is FOLFIRINOX (5-FU, leucovorin, irinotecan and oxaliplatin), which increases survival of patients with PDAC, albeit it does not improve their quality of life due to side effects during the therapy (Conroy *et al*., 2011).

Despite the novel therapies proposed for the treatment of advanced PDAC, no significant results have been obtained in terms of survival. This is due primarily to the

the acquisition of chemoresistance, the main feature of the malignancy of PDAC, which must be overcome to improve the efficiency of therapies

4.4 Molecular characterization of PDAC

PDAC arises from neoplastic lesions in ductal cells. Among the lesions, the most common are the Pancreatic Intra-epithelial Neoplasia (PanIN) (Hruban *et al*., 2001), which are classified into three groups, based on the grade of dysplasia and morphological transformation of the cells: PanIN-1, PanIN-2 and PanIN-3. In PanIN-1, ductal cells show a low grade of displasia. This gradually increases until the PanIN-3 grade, which is defined also as carcinoma *in situ*. All the PanIN lesions are confined within the basement membrane, without invasion of other tissues. With the increase of cellular dysplasia, the neoplastic lesions become invasive PDAC.

The morphological changes of pancreatic ductal cells during tumorigenesis are accompanied by defined mutations and genetic alterations (Figure 4.1), which have been characterized for the first time through a genetic global analysis of PDAC tumor samples (Jones *et al*., 2008). Mutations in the oncogene *KRAS2* was found in 20% of PanIN-1, and the percentage of mutation in this gene increases up to 90% in PanIN-3 (Kanda *et al*., 2012). Another important gene mutated in early stages of PDAC is in the gene encoding the oncosuppressor and cell cycle regulator CDKN2A/p16, which was found mutated in 50% of PanIN-1 and in 95% of PDAC patients (Wilentz *et al*., 1998; Otthenof *et al*., 2011).

During the progression of PDAC tumorigenesis other important genes, involved in several important cellular functions, were found mutated, such as *TP53*, *CCND1*, *BRCA2, SMAD4* , with a concomitantly increase of Ki-67. Deregulation of these genes during the last stages of PanIN promotes the progression of neoplastic lesions to PDAC, where the percentage of most of these mutations increases over 50% (Otthenof *et al*., 2011).

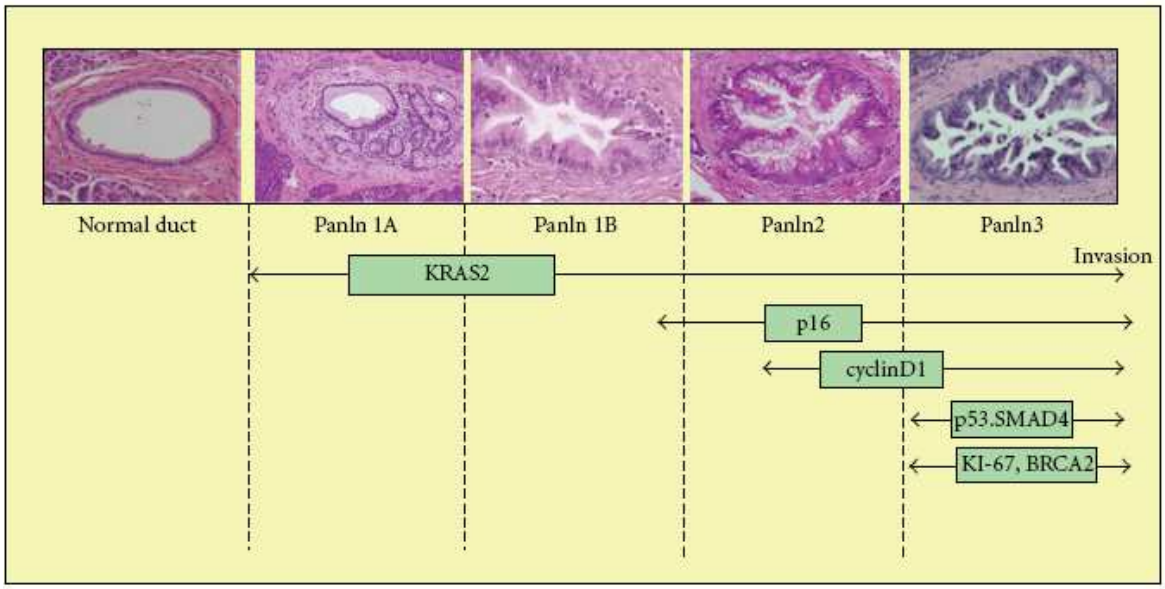


Figure 4.1. Schematic representation of genetic alterations during PanIN progression (Otthenof *et al*., 2011).

High-throughput analyses of PDAC samples contributed to a global dissection of the pathways deregulated in this disease, inclusing those regulating cell cycle progression, apoptosis, DNA damage repair, cell adhesion and invasion and important signalling pathways, such as the MAPK and TGFβ ones (Otthenof *et al*., 2011). A more recent study provided an even deeper understanding of genetic alterations in PDAC tumors, and illustrated the correlation between mutations and response to therapies (Waddel *et al*., 2015). Importantly, alterations in chromosomal structures were also found in PDAC tumors, indicating that also chromosomal rearrangement play an important role in PDAC progression (Waddel *et al*., 2015).

The recent evidences of genetic alterations in PDAC underline a large heterogeneity, which may be the basis of differences in progression and response to chemotherapeutic treatment. Thus, it would be important defines the genetic pattern of the desease in order to identify a specific therapy for each case of PDAC.

References

Burris, H. 3rd, Moore, M., Andersen, J., Green, MR., Rothenberg, ML., Modiano, MR. Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA,Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-Line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997 Jun; 15(6): 2403-13.

Conroy T, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer;PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011 May 12; 364(19):1817-25.

Cunningham D, Chau I, Stocken DD, Valle JW, Smith D, Steward W, Harper PG, Dunn J, Tudur-Smith C, West J, Falk S, Crellin A, Adab F, Thompson J,Leonard P, Ostrowski J, Eatock M, Scheithauer W, Herrmann R, Neoptolemos JP, Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol. 2009 Nov 27; 27(33):5513-8.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. M. Int J Cancer. 2010 Dec 15; 127(12):2893-917.

Hariharan D, Saied A, Kocher HM. Analysis of mortality rates for pancreatic cancer across the world. HPB (Oxford). 2008; 10(1):58-62.

Hruban RH, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN Kern SE, Klimstra DS, Klöppel G, Longnecker DS, Lüttges J, Offerhaus GJ. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol 2001 May; 2(5)5:579–86.

Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008 Sep 26; 321(5897):1801-6.

Kanda M, Matthaei H, Wu J, Hong SM, Yu J, Borges M, Hruban RH, Maitra A, Kinzler K, Vogelstein B, Goggins M. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. Gastroenterology. 2012 Apr; 142(4):730-733.

Ottenhof NA, de Wilde RF, Maitra A, Hruban RH, Offerhaus GJ. Molecular characteristics of pancreatic ductal adenocarcinoma. Patholog Res Int. 2011 Mar 27: 620601.

Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. Nat Rev Clin Oncol. 2010 Mar; 7(3):163-72.

Varadhachary GR, Tamm EP, Abbruzzese JL, Xiong HQ, Crane CH, Wang H, Lee JE, Pisters PW, Evans DB, Wolff RA. Borderline resectable pancreatic cancer: definitions, management, and role of preoperative therapy. Ann Surg Oncol. 2006 Aug; 13(8): 1035–46.

Vincent , Herman J, Schulick R, Hruban RH, Goggins M Pancreatic cancer. Lancet. 2011 Aug; 378(9791):607-20.

Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N,Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013 Oct; 369(18):1691-703.

Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, Johns AL, Miller D, Nones K, Quek K, Quinn MC, Robertson AJ, Fadlullah MZ, Bruxner TJ,Christ AN, Harliwong I, Idrisoglu S, Manning S, Nourse C, Nourbakhsh E, Wani S Wilson PJ, Markham E, Cloonan N, Anderson MJ, Fink JL, Holmes O,Kazakoff SH, Leonard C, Newell F, Poudel B, Song S, Taylor D, Waddell N, Wood S, Xu Q, Wu J, Pinese M, Cowley MJ, Lee HC, Jones MD, Nagrial AM,Humphris J, Chantrill LA, Chin V, Steinmann AM, Mawson A, Humphrey ES, Colvin EK, Chou A, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Pettitt JA, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Jamieson NB, Graham JS, Niclou SP, Bjerkvig R, Grützmann R, Aust D,Hruban RH, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Falconi M, Zamboni G, Tortora G, Tempero MA;Australian Pancreatic Cancer Genome Initiative, Gill AJ, Eshleman JR, Pilarsky C, Scarpa A, Musgrove EA, Pearson JV, Biankin AV, Grimmond SM. Whole genomes redefine the mutational landscape of pancreatic cancer. Nature. 2015 Feb 26; 518(7540):495-501.

Wilentz RE, Geradts J, Maynard R, Offerhaus GJ, Kang M, Goggins M, Yeo CJ, Kern SE, Hruban RH. Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression. Cancer Res. 1998 Oct 15;58(20):4740-4.

Wörmann SM, Algül H. Risk Factors and Therapeutic Targets in Pancreatic Cancer. Front Oncol. 2013 Nov 18;3: 282.

CHAPTER V

Role of Epithelial to Mesenchymal Transition (EMT) in PDAC

Epithelial to Mesenchymal Transition (EMT) is a physiological process that determines morphological changes in epithelial cells, allowing the acquisition of fibroblastic-like shape and the ability to lose cell-to-cell contacts and to migrate (Figure 5.1). EMT is characterised by a profound reprogramming of gene expression, which drives transformation of cells from an epithelial phenotype to a mesenchymal one.

EMT occurs normally during embryonic development and it is required for the process of gastrulation, where cells must acquire the ability to migrate in order to form new tissues and organs. However, this process may be also activated in differentiated cells, and in this case it may promote or accompany neoplastic transformation. Indeed, in carcinoma, the ability of epithelial transformed cells to migrate and reach metastatic sites depends on the dissolution of intercellular contacts and on the acquisition of motility (Thiery *et al*., 2009).

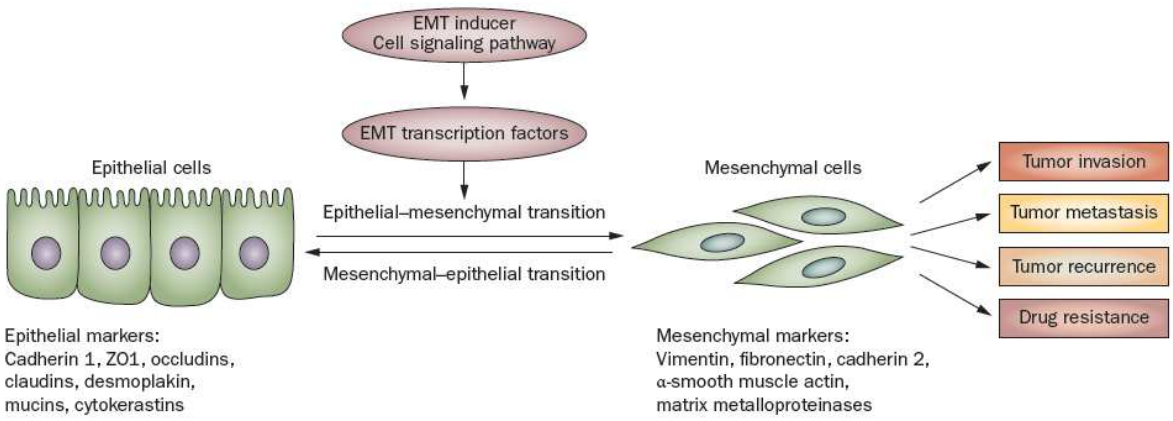


Figure 5.1. EMT in cancer progression. Differentiated cells that undergo EMT, acquire a fibroblastike-like morphology and acquire cancer features, such as drug resistance, ability to migrate and to form metastasis (from Wang *et al*., 2011).

The induction of EMT in cancer can be influenced by extracellular context, and often by the interaction between epithelial cells and tumor environment, such as

fibroblasts, cytokines or other factors (TGFβ and EGF), that are normally present in the tumor microenvironment (Thiery and Sleeman, 2006);

Molecular events (including mutations in oncogenes or oncosuppressor) affecting different steps in key biological processes are required to induce EMT and the acquisition of invasivity and motility. Moreover, EMT can be a transient event that is reverted (Mesenchymal to epithelial transition, MET). In particular, MET is important during the extravasation of metastatic cells from blood stream, in order to colonize the host organs and to form metastasis. All these observations indicate that EMT is a dynamic process, finely regulated in a cell specific context (De Craine and Berx, 2013).

In several tumors, EMT has been related to specific features of cancer cells, for instance resistance to chemotherapy, resistance to anoikia, alteration in DNA repair invasion and ability to forms metastasis. Furthermore, EMT has been associated to the acquisition of stem cell-like phenotypes, suggesting a correlation between cell remodelling and re-programming of cellular functions (Visvader *et al.*, 2012).

During the last years, several studies stressed the importance of EMT in the acquisition of metastatic potential by PDAC cells. For instance, a study using murine models suggested that EMT occurs in the first stages of PanIN, promoting stemness and motility of cancer cells soon after the formation of the primary tumor (Rhim *et al*., 2012). Moreover, there are evidences of the implication of EMT in another important feature of PDAC: the acquisition of resistance to chemotherapy, which is responsible for the failure of the treatment in advanced cancers (Wang *et al*., 2011, Arumugam *et al*., 2009)

5.1 Regulation of EMT in cancer

At the molecular level, EMT requires widespread reprogramming of gene expression that occurs through regulation of transcription, alternative splicing, expression of non-coding RNAs and translation (Figure 5.2).

Regulation of transcription is one of the events that drive strong reprogramming in cells undergoing EMT (Puisieux *et al*., 2014). The first transcription factors identified for the induction of EMT were those involved in the down regulation of E-cadherin, one of the most important gene expressed in epithelial cells. The most relevant transcription factors identified for their action on E-cadherin transcription were SNAI1, known also as SNAIL (Batlle *et al*., 2000; Cano *et al*. 2000), SLUG (Hajra *et al*., 2006), ZEB1 (Eger *et al*., 2002) and ZEB2 (Comijn *et al*., 2001). Over-expression of EMT-related

transcription factors also leads to repression of other genes encoding for proteins required for cell-to-cell contact, such as occludins and claudins (De Craene *et al*., 2005; Vandewalle *et al*., 2005). As a consequence, these transcription factors trigger a general reprogramming of cell shape and promote the expression of mesenchymal genes, such as vimentin, fibronectin and N-cadherin. Other transcription factors have been included among the inductors of EMT. The most relevant is TWIST, which is essential for metastatic potential in cancer cells (Yang *et al*., 2004).

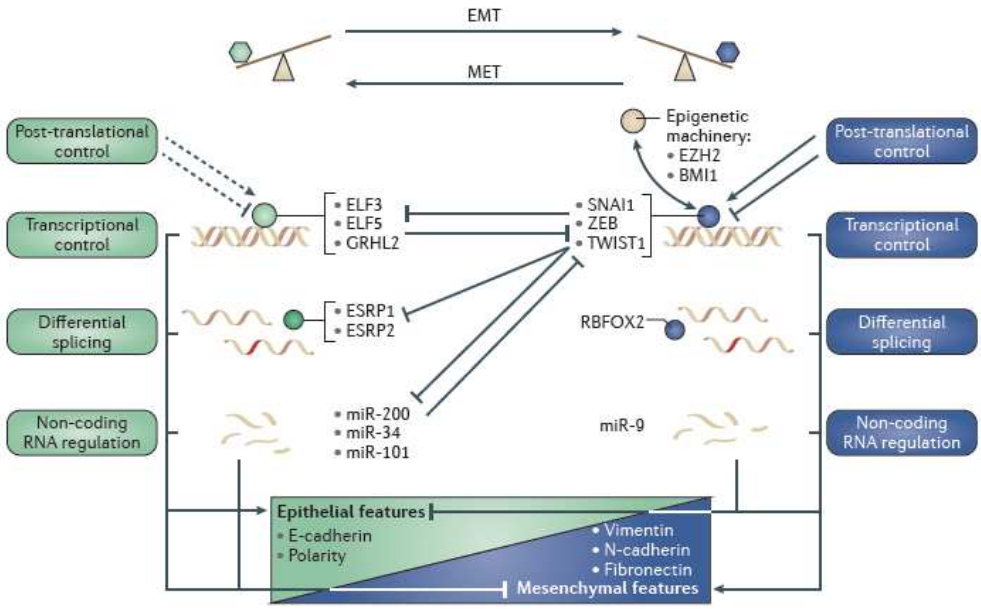


Figure 5.2. Scheme of the principal rnetworks of the regulation of gene expression involved in the activation of EMT program (De Craene and Berx, 2013).

EMT-related transcription factors involved in the repression of epithelial genes act through epigenetic mechanisms. Indeed, several histon-deacetylases (HDACs) and DNA methyl-transferases were associate with EMT transcription factors. For instance, SNAI1 exerts repression of the E-cadherin promoter through the recruitment of HDACs (Peinado *et al*., 2004) or histone methyl transferases (Herranz *et al*., 2008; Dong *et al*., 2013). In the same way, ZEB1 repress E-cadherin expression through epigenetic modifications (Byles *et al*., 2012; Sanchez-Tillo *et al*., 2010), whereas TWIST is able to suppress E-cadherin expression by cooperating with an important chromatin remodelling factor: Bmi1 (Yang *et al*., 2010). The expression of all these factors and histone-remodelling proteins were found deregulated in many types of cancers (De

Craene and Berx, 2013), suggesting their important role in cell reprogramming and tumorigenesis.

EMT is regulated also at the post-transcriptional level. On important step of regulation is the alternative splicing. Several differential splicing events are determinant for the epithelial or mesenchymal phenotype. For instance, two splicing factors, ESRP1 and ESRP2, were found specifically expressed in epithelial cells, and they are important for the acquisition of epithelial features, by regulating the splicing of genes like *MENA*, *CD44*, *CTNND1* and *FGFR2* (Horiguchi *et al*., 2012). Therefore, overexpression of ESRP1/2 is able to induce radical changes of the splicing pattern in mesenchymal cells, reverting their phenotype, whereas down-regulation of ESRP1/2 in epithelial cells leads to up-regulation of mesenchymal genes (Warzeca *et al*., 2010). Moreover, the expression of ESRP1/2 is regulated by EMT related transcription factors, like ZEB1 and SNAIL (Horiguchi *et al*., 2012; Reinke *et al.*,2012).

Another example of alternative splicing event involved in EMT is the exclusion of exon 11 of tyrosine kinase receptor RON, regulated by the first splicing factor known to have pro-oncogenic functions, SRSF1 (Ghigna *et al*., 2005; Karni *et al*., 2007). Skipping of exon 11 confers constitutive activity and pro- invasive properties to RON, determining EMT. All these observations indicate that alternative splicing represents an important post-trancriptional event that influences cancer progression and EMT.

Another layer of regulation of gene expression that impacts on EMT is represented by miRNAs, which are crucial for the balance of different networks of EMT. For instance, the miRNA-200 family is required for the determination of the epithelial phenotype, and it is also a negative regulator of ZEB1 and ZEB2 expression (Park *et al*., 2008). Moreover, ZEB1 is able to inhibit the transcription of members of the miRNA-200 family, thus creating a negative loop, whose balance may be critical for the choice of epithelial or mesenchymal fate (Wellner *et al*., 2009; Brabletz and Brabletz, 2010). Like the miRNA-200 family, other miRNAs , such as miR-205 and miR-34, were linked to determination of the epithelial phenotype. MiR-205 and mi-R34, in turn, can be transcriptionally regulated by EMT related transcription factors (Gregory *et al*., 2008; Siemens *et al*., 2011; Burk *et al*., 2008), and they were found down regulated in several kind of cancer, as breast, prostate and pancreatic carcinomas (Wellner *et al*., 2009; Kong *et al*., 2010; Tellez *et al*., 2011; Hur *et al*., 2013).

Post-transcriptional regulation events, such as the regulation of mRNA translation, also play a key role in EMT. For instance, over-expression of the RNA

binding protein YB1 promotes cap-independent translation of SNAI1 and other EMT transcription factors (Evdokimova *et al*., 2009). Moreover, the stability of EMT-related transcription factors is regulated by post-translational modifications that regulate the protein turnover, and these processes are often aberrant in cancer cells (De Craene and Berx, 2013).

In conclusion, transition of epithelial cells to a mesenchymal morphology is the result of cooperation of several pathways, which interact with each other in order to alter cell fate determination, underlines the complexity of the network that regulates this biological process.

5.2 Role of ZEB1 in PDAC and genotoxic stress response

5.2.1 ZEB1: structure and function

Among the factors involved in EMT, ZEB1 is the most studied in PDAC, and several observations suggest the strong impact that ZEB1 exerts on resistance to chemotherapy, invasiveness and stemness-like properties of PDAC cells (Arumungam *et al*., 2009; Rhim *et al*., 2012).

ZEB1 is a transcription factor that belongs to a small family of zing finger proteins, the ZEB family, composed by ZEB1 and ZEB2. It is characterised by the presence of two clusters of C2H2-type zinc finger domain separated by a homodomain (Figure 5.3). The zinc finger domains are required for the recognition and the binding of promoters of genes containing Z-boxes and E-boxes motif sequences (Remacle *et al*., 1999).

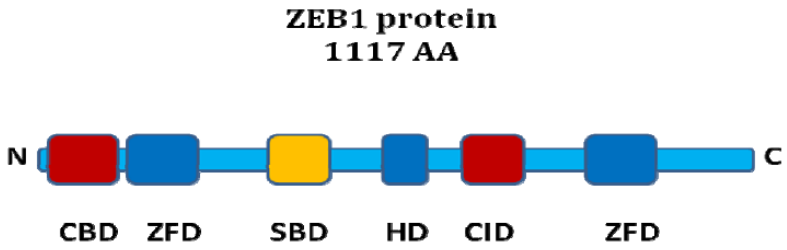


Figure 5.3. Schematic representation of ZEB1 structure and domain: Co-activator binding domain (CBP); Zing finger domain (ZNF); Smad binding domain (SBD); Homeodomain (HD); CtBP interaction domain (CID) (From Wellner *et al*., 2010).

ZEB1 activity is regulated by the interaction with several factors, which modulate its ability to repress or activate gene transcription. For instance, ZEB1 induces

the activation of transcription of the ATPase1 gene in MDCK cells but it represses it in rat fibroblasts, suggesting a bivalent behaviour of ZEB1, which may depends on cellular context (Watanabe *et al.*, 1993). The activity of ZEB1 can be activated by the TGFβ pathway, which causes activation of R-SMADs and their binding to the SMAD-binding domain (SMD) of ZEB1. Thus, the ZEB1-R-SMADs complex promotes repression of the E-cadherin promoter (Shirakihara *et al.*, 2007). It has been proposed that the repressive activity of the ZEB1-SMAD complex depends on other ZEB1 co-interactors, such as p300/pCAF (Postigo *et al.*, 2009).

Activity of ZEB1 is regulated also at the post-translational level. For instance, SUMOylation is a frequent modification that occur in ZEB1, thus modulating its activity (Long *et al*., 2005). Furthermore, the interaction of CtBP1 and CtBP2 with the CID domain of ZEB1 favours its repressive activity on target genes, for instance E-cadherin, through the recruitment of histone deacetylase, HDAC1 and HDAC2 (Furusawa *et al*., 1999; Shi *et al*., 2003).

These observations underline the fine regulation of ZEB1 activity, which is mediated by interaction with other binding factors, post-translational modifications and cellular context.

5.2.2 ZEB1 in PDAC and genotoxic stress response

Expression of ZEB1 is related with poor prognosis in several tumors, especially in PDAC. Indeed, ZEB1 is expressed in PDAC tumor tissues and, importantly, its expression correlates with EMT and acquisition of resistance to gemcitabine treatment and other kind of genotoxic stresses in PDAC cell lines (Arumugan *et al*., 2009). Moreover, silencing of ZEB1 in PDAC cell lines that over-expresses it restore sensitivity to chemotherapeutic treatment (Arumungam *et al*., 2009; Wellner *et al*., 2009).

Since there is a correlation between ZEB1 expression and chemoresistance, ZEB1 expression could favour the selection of resistant cells population during stress condition, such as prolonged gemcitabine treatment. Indeed, it has been demonstrated that PDAC cells with acquired gemcitabine resistance over-expressed ZEB1 and show an induction of the EMT program mediated by the Notch-2 pathway (Wang *et al*., 2009).

Expression of ZEB1 correlates with EMT and its expression is regulated by the miRNA 200 family in PDAC (Wellner *et al*., 2009). The regulatory loop between

ZEB1/ZEB2 and miR200 was confirmed also in vivo (Kent *et al*., 2009). Moreover, the direct correlation between expression of miRNAs 200 and E-cadherin was found also in samples of patients with PDAC, where patients with a better prognosis have high expression of miRNAs belonging to miRNA-200 family (Yu *et al*., 2010).

Expression of ZEB1 is also required for acquisition of cell motility and the ability to reach distal organs, and its correlates with acquisition of stemness in cancer cells *in vitro* and *in vivo* (Rihm *et al*., 2012; Wellner *et al*., 2009). Furthermore, silencing of ZEB1 in PDAC cell lines reduces their tumorigenic potential, determining a decrease of tumor size derived from PDAC cells injected in a xenograft mouse model (Wellner *et al*., 2009).

These data support the concept that ZEB1 and EMT are important for tumor transformation and progression in PDAC, promoting the acquisition of cellular features, such as chemoresistance and invasiveness, which are responsible for the poor prognosis in patients.

References

Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. Cancer Res. 2009 Jul 15;69(4): 5820–28.

Batlle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J, García De Herreros A.The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol. 2000 Feb;2(2):84-9.

Brabletz S, Brabletz T. The ZEB/miR-200 feedback loop--a motor of cellular plasticity in development and cancer? EMBO Rep. 2010 Sep;11(9):670-7.

Byles V, Zhu L, Lovaas JD, Chmilewski LK, Wang J, Faller DV, Dai Y. SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. Oncogene. 2012 Oct;31(43):4619-29.

Burk_U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion ncancer cells. EMBO Rep. 2008 Jun;9(6):582-9.

Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000 Feb; 2(2):76-83.

Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell. 2001 Jun; 7(6):1267-78.

De Craene, B. Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G. The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. Cancer Res. 2005 Jul 15; 65(14): 6237–44.

De Craene B, Berx G.Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer. 2013 Feb; 13(2):97-110.

Dong C, Wu Y, Wang Y, Wang C, Kang T, Rychahou PG, Chi YI, Evers BM, Zhou BP. Interaction with Suv39H1 is critical for Snail-mediated E-cadherin repression in breast cancer. Oncogene. 2013 Mar 14; 32(11):1351-62.

Eger, A. Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H, Foisner R. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene, 2005 Mar 31;24(14): 2375–85.

Evdokimova V, Tognon C, Ng T, Ruzanov P, Melnyk N, Fink D, Sorokin A, Ovchinnikov LP, Davicioni E, Triche TJ, Sorensen PH. Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. Cancer Cell. 2009 May 5; 15(5):402-15.

Furusawa T, Moribe H, Kondoh H, Higashi Y. Identification of ctbp1 and ctbp2 ascorepressors of zinc finger-homeodomain factor deltaef1. Mol Cell Biol. 1999 Dec;19(2): 8581–90.

Ghigna C, Giordano S, Shen H, Benvenuto F, Castiglioni F, Comoglio PM, Green MR, Riva S, Biamonti G. Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. Mol Cell. 2005 Dec 22;20(6):881-90.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008 May; 10(5):593-601.

Hajra KM, Chen DY, Fearon ER .The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res. 2002 Mar 15; 62(6):1613–8.

Herranz N, Pasini D, Díaz VM, Francí C, Gutierrez A, Dave N, Escrivà M, Hernandez-Muñoz I, Di Croce L, Helin K, García de Herreros A, Peiró S. Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. Mol Cell Biol. 2008 Aug; 28(15):4772-81.

Horiguchi K, Sakamoto K, Koinuma D, Semba K, Inoue A, Inoue S, Fujii H, Yamaguchi A, Miyazawa K, Miyazono K, Saitoh M. TGF-β drives epithelial-mesenchymal transition through δEF1-mediated downregulation of ESRP. Oncogene. 2012 Jun 28; 31(26):3190-3201.

Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR, Goel A. MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. Gut. 2013 Sep;62(9):1315-26.

Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nat Struct Mol Biol. 2007 Mar; 14(3):185-93

Kent OA,Mullendore M, Wentzel EA, Lopez-Romero P, Tan AC, Alvarez H, West K, Ochs MF, Hidalgo M, Arking DE, Maitra A, Mendell JT. A resource for analysis of microrna expression and function in pancreatic ductal adenocarcinoma cells. Cancer Biol. Ther. 2009 Nov; 8(21):2013–24.

Kong D, Banerjee S, Ahmad A, Li Y, Wang Z, Sethi S, Sarkar FH. Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. PLoS One. 2010 Aug 27; 5(8):e12445.

Long J, Zuo D, Park M. Pc2-mediated sumoylation of smad-interacting protein 1 attenuates transcriptional repression of e-cadherin. J. Biol. Chem. 2005 Oct 21; 280(42), 35477–89.

Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev.2008 Apr 1; 22(7): 894–907.

Peinado H, Ballestar E, Esteller M, Cano A. Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. Mol Cell Biol. 2004 Jan; 24(1): 306–19.

Postigo, A.A. Opposing functions of zeb proteins in the regulation of the tgfbeta/bmp signalling pathway. EMBO J. 2003 May; 22: 2443–52.

Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. Nat Cell Biol. 2014 Jun; 16(6):488-94.

Reinke LM, Xu Y, Cheng C. Snail represses the splicing regulator epithelial splicing regulatory protein 1 to promote epithelial–mesenchymal transition. J. Biol. Chem. 2012 Oct; 287(43), 36435–42.

Remacle JE, Kraft H, Lerchner W, Wuytens G, Collart C, Verschueren K, Smith JC, Huylebroeck D. New mode of DNA binding of multi-zinc finger transcription factors: Deltaef1 family members bind with two hands to two target sites. EMBO J. 1999 Sep 15; 18(18): 5073–84.

Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. EMT and dissemination precede pancreatic tumor formation. Cell. 2012 Jan 20; 148(1-2):349-61.

Sánchez-Tilló E, Lázaro A, Torrent R, Cuatrecasas M, Vaquero EC, Castells A, Engel P, Postigo A. ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. Oncogene. 2010 Jun 17; 29(24):3490-500.

Shi Y, Sawada J, Sui G, Affar el B, Whetstine JR, Lan F, Ogawa H, Luke MP, Nakatani Y, Shi Y. Coordinated histone modifications mediated by a ctbp co-repressor complex. Nature. 2003 Apr 17; 422(17):735–8.

Shirakihara T, Saitoh M, Miyazono K. Differential regulation of epithelial and mesenchymal markers by dEF1 proteins in epithelial mesenchymaltransition induced by TGF-b. Mol Biol Cell. 2007 Sep; 18(9): 3533-44.

Siemens H, Jackstadt R, Hünten S, Kaller M, Menssen A, Götz U, Hermeking H. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. Cell Cycle. 2011 Dec 15; 10(24):4256-71.

Tellez CS, Juri DE, Do K, Bernauer AM, Thomas CL, Damiani LA, Tessema M, Leng S, Belinsky SA. EMT and stem cell-like properties associated with miR-205 and miR-200 epigenetic silencing are earlymanifestations during carcinogen-induced transformation of human lung epithelial cells. Cancer Res. 2011 Apr 15; 71(8):3087-97.

Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol. 2006 Feb; 7(2):131-42.

Thiery JP, Acloque H, Huang RY, Nieto MA Epithelial-mesenchymal transitions in development and disease Cell. 2009 Nov 25; 139(5):871-90.

Vandewalle, C. Comijn J, De Craene B, Vermassen P, Bruyneel E, Andersen H, Tulchinsky E, Van Roy F, Berx G. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell–cell junctions. Nucleic Acids Res. 2005 Nov 24; 33(20): 6566–78.

Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell. 2012 Jun 14; 10(6):717-28.

Wang Z, Li Y, Ahmad A, Banerjee S, Azmi AS, Kong D, Sarkar FH. Pancreatic cancer: understanding and overcoming chemoresistance. Nat Rev Gastroenterol Hepatol. 2011 Jan; 8(1):27-33.

Wang Z Li Y, Kong D, Banerjee S, Ahmad A, Azmi AS, Ali S, Abbruzzese JL, Gallick GE, Sarkar FH. Acquisition of epithelial mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. Cancer Res. 2009 Mar 15; 69(6):2400-7.

Warzecha CC, Jiang P, Amirikian K, Dittmar KA, Lu H, Shen S, Guo W, Xing Y, Carstens RP. An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. EMBO J. 2010 Oct 6;29(19):3286-300.

Watanabe Y, Kawakami K, Hirayama Y, Nagando K. Transcription factors positively and negatively regulating the Na,K-ATPase alpha 1 subunit gene. J Biochem. 1993 Dec;114(6):849– 55.

Wellner_U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, Waldvogel B, Vannier C, Darling D, zur Hausen A, Brunton VG, Morton J, Sansom O, Schüler J, Stemmler MP, Herzberger C, Hopt U, Keck T, Brabletz S, Brabletz T. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol. 2009 Dec; 11(12):1487-95.

Wellner U, Brabletz T, Keck T. ZEB1 in Pancreatic Cancer. Cancers (Basel). 2010 Aug 18; 2(3):1617-28.

Yang, J. Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004 Jun 25; 117(7), 927–39.

Yang MH, Hsu DS, Wang HW, Wang HJ, Lan HY, Yang WH, Huang CH, Kao SY, Tzeng CH, Tai SK, Chang SY, Lee OK, Wu KJ. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell Biol. 2010 Oct;12(10):982-92.

Yu J, Ohuchida K, Mizumoto, K, Sato N, Kayashima T, Fujita H, Nakata K, Tanaka M. Microrna, hsa-mir-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. Mol Cancer. 2010 Jun 28; 9: 169.

CHAPTER VI

Genotoxic stress and DNA Damage Response (DDR) in PDAC

Continuous replication in cancer cells is one of the most common features in tumors. Chemotherapy takes advantage of this hallmark, which is important to target cancer cells and to have less impact on normal cells, which normally do not proliferate in adult tissues.

The chemotherapeutic agents that target replication of cancer cells are classified in:

- alkylating agents, which bind covalently DNA and cause DNA damage during the replication;
- topoisomerase inhibitors, which inhibit the activity of the enzymes topoisomerase I and II during the initiation of replication of DNA, thereby imposing a physical stress and causing single strand- and double strand-breaks (SSB and DSB);
- antimetabolite agents that are analogues of purynes and pyrimidines, which impair DNA and RNA synthesis.

Chemotherapic drug used for PDAC treatment is gemcitabine, which is a cytidine analogue that is incorporated during S-phase into replicating DNA. When gemcitabine penetrates into the cells, it is phosphorylated by deoxycytidine kinase (DCK). Then, gemcitabine is converted by cytidine monophosphate kinase into cytidine diphosphate (dFdCDP) and then in triphosphate (dFdCTP) (Mini *et al*., 2006; Nakano *et al*., 2007). Both compounds inhibit the DNA synthesis, exerting an antiproliferative effect (Plunkett *et al*., 1995; Galmarini *et al*., 2002).

6.1 The DDR pathway

The incorporation of gemcitabine induces steric hindrance during the replication of DNA, causing the replication fork stalling, single strand breaks and the subsequent collapse of the replication fork (Huang *et al*., 1991). To prevent the collapse of the forks and induction of apoptosis, cells activate a program of stabilization of the fork in order to repair the damage and to recover DNA integrity (Lopes *et al.*, 2001). Thus, gemcitabine triggers genotoxic stress in cancer cells, inducing the activation of pathways involved in the repair of DNA Damage.

The DNA damage response (DDR) pathways that are involved in the response to chemotherapeutic treatments rely on the Ataxia-Telangectasia Mutated (ATM) - Checkpoint Kinase 2 (CHK2) and the Ataxia-Telangectasia and Rad3 related (ATR) - Checkpoint Kinase 1 (CHK1) axes (Figure 6.1). Their action depends on the different DNA aberrant structures that form during DNA damage: ATM-CHK2 responds to DNA double strand breaks (DSBs) (Lee and Paull, 2007), whereas ATR-CHK1 responds to single strand breaks (SSBs) and stalled replication forks (Zou and Elledge, 2003). Moreover, factors that are recruited on the damage sites following ATM and ATR activation are different. Indeed, ATM recruits on DSBs the NMR complex, composed by NSB1, MRE11 and Rad50 (Lee and Paull, 2005), while ATR is recruited on DNA by Replication protein A, which binds ssDNA, and in turn interacts with HUS1-RAD9-RAD1 complex (Delacroix *et al.*, 2007). In both cases, ATM and ATR kinases have the role to signal the sites of the damage by mediating phosphorylation of Histone H2AX on Serine 139, also known as γH2AX (Fernandez-Capetillo *et al.*, 2004, Ward and Chen, 2001).

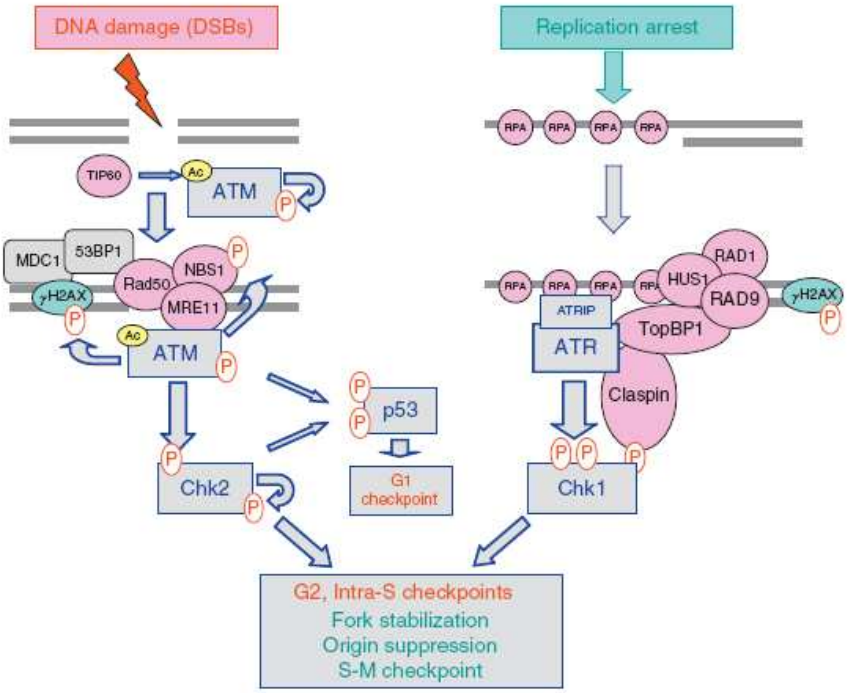


Figure 6.1. Schematic representation of ATM and ATR pathway in DNA damage response (from Smith *et al.*, 2010).

The main effectors of ATM and ATR are, respectively, the kinases CHK2 and CHK1, albeit both ATM and ATR also phosphorylates other substrates that are important for the regulation of the DDR, such as, p53BP, MDM2 and BRAC1 (Smith *et al.*, 2010).

CHK2 is activated through the phosphorylation mediated by ATM. Once activated, CHK2 is dispersed into the nucleus and regulates several cellular of DDR, such as apoptosis, cell cycle progression and gene expression. For instance, CHK2 regulates, togheter with CHK1, progression of cell cycle by phosphorylating members of the Cdc25 phosphatase family (Blasina *et al*., 1999). CHK2 influences cell cycle progression also by inhibiting p53 activity and by phosphorylating MDMX, a p53 regulator (Chehab *et al*., 2000; Chen *et al*., 2005). Furthermore, CHK2 controls the activation the repair of DNA damage activating BRCA1 (Lee *et al.*, 2000), acting in cooperation with BRCA2.

CHK1, once activated by ATR, also controls cell cycle progression in response to DNA damage. Indeed, it phosphorylates and inactivates Cdc25 phosphatases, thus blocking cell cycle progression (Falck *et al*., 2002; Blasina *et al*., 1999). Inactivation of Cdc25 family phosphatases is required to repress the activity of Cdk2, thus blocking the formation of novel replication forks and preventing the collapse of the forks. Therefore, CHK1 is considered the main regulator of G1/S checkpoint during the inhibition of DNA synthesis, when cells must prevent the formation of novel replication origins (Branzei and Foiani, 2009). Moreover, upon activation of the DDR, CHK1 phosphorylates BRCA2 and RAD51, and blocks transcription through the phosphorylation of histone H3 (Sorensen *et al*., 2005; Bahassi *et al.*, 2008; Shimada *et al*., 2008).

However, the ATM-CHK2 and ATR-CHK1 pathways are not completely independent and extensive crosstalk has been demonstrated between the two signalling cascades (Smith *et al*., 2010). For instance, when SSBs or replication forks are stalled, this defect leads to the induction of DSBs, allowing the activation of ATM and CHK2 signalling, and both kisases cooperates to activates the repair or DNA Damage (Bahassi *et al*., 2008). Moreover, as mentioned before, CHK1 and CHK2 share the same substrates for the regulation of cell cycle progression as the members of Cdc25 family. Furthermore, more recently it has been proposed that ATM could enhance DDR stabilizing CHK1 protein (Zhang *et al*., 2014) Thus, ATM and ATR often co-participate to the DDR triggered by DNA damaging agents.

6.2 DDR in PDAC and during gemcitabine treatment

Since the nucleoside analogs, like gemcitabine, mainly interfere with progression of the replication forks during S-phase, the kinase that is immediately activated is CHK1 (Ewald *et al*., 2008). Accordingly, CHK1 has been demonstrated to be important for the recovery of DNA damage induced by gemcitabine treatment. Indeed, gemcitabine induces a strong activation of γH2AX, which was enhanced by depletion or pharmacological inhibition of CHK1, thus increasing the percentage of cell death (Edwald *et al*., 2007). It has been proposed that inhibition of CHK1 could be used in concomitance with inhibition of EGFR. Indeed, EGFR is up-regulated in up to 90% of PDAC (Tobita *et al*., 2003), and inhibition of CHK1 activity in combination with gemcitabine and EGFR inhibitor decreases tumor burden in xenograft model mice injected with PDAC cells (Al-Ejeh *et al*., 2014). In this regard, it has been proposed that inhibition of CHK1 sensitizes PDAC cells to gemcitabine treatment by interfering with formation of RAD51 foci, which are required for the recovery of stalled forks, but without blocking the progression through S-phase (Parsels *et al*., 2009).

Prolonged exposure of cells to gemcitabine also induces formation of DSBs and activation of the ATM pathway. Prolonged stalling of the replication forks leads to the persistence of BRAC1 and RAD51 foci, which in turn may induce cell death (Jones *et al*., 2014). Furthermore, it has been observed that prolonged treatment on PDAC cells with gemcitabine induces phosphorylation of CHK2, which is required for cell survival and to prevent ROS-mediated stress (Duong *et al*., 2013).

All these observations suggest the importance to counteract the ability of cells to repair DNA damage induced by chemotherapeutic treatment, which, concomitantly with the loss of cell cycle checkpoints, prevents the induction of apoptosis and cell death after DNA damage.

References

Al-Ejeh F, Pajic M, Shi W, Kalimutho M, Miranda M, Nagrial AM, Chou A, Biankin AV, Grimmond SM; Australian Pancreatic Cancer Genome Initiative, Brown MP, Khanna KK. Gemcitabine and CHK1 inhibition potentiate EGFR-directed radioimmunotherapy against pancreatic ductaladenocarcinoma. Clin Cancer Res. 2014 Jun 15; 20(12):3187-97.

Bahassi EM, Ewald B, Sampath D, Plunkett. Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene. 2008 Oct 27; 27(50):6522-37.

Blasina A, de Weyer IV, Laus MC, Luyten WH, Parker AE, McGowan CH. A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr Biol. 1999 Jan 14; 9(1):1-10.

Branzei D, Foiani M. The checkpoint response to replication stress. DNA Repair (Amst). 2009 Sep 2; 8(9):1038-46.

Chehab NH, Malikzay A, Appel M, Halazonetis TD. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. 2000 Feb 1; 14(3):278-88.

Chen L, Gilkes DM, Pan Y, Lane WS, Chen J. ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. EMBO J. 2005 Oct 5;24(19):3411-22.

Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, Karnitz LM. The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signalling via TopBP1. Genes Dev. 2007 Jun 15; 21(12):1472-7.

Duong HQ, Hong YB, Kim JS, Lee HS, Yi YW, Kim YJ, Wang A, Zhao W, Cho CH, Seong YS, Bae I. Inhibition of checkpoint kinase 2 (CHK2) enhances sensitivity of pancreatic adenocarcinoma cells to gemcitabine. J Cell Mol Med. 2013 Oct; 17(10):1261-70.

Ewald B, Sampath D, Plunkett W H2AX phosphorylation marks gemcitabine-induced stalled replication forks and their collapse upon S-phase checkpoint abrogation. Mol Cancer Ther. 2007 Apr;6(4):1239-48. Epub 2007 Apr 3.

Ewald B, Sampath D, Plunkett W.ATM and the Mre11-Rad50-Nbs1 complex respond to nucleoside analogue-induced stalled replication forks and contribute to drug resistance. Cancer Res. 2008 Oct 1; 68(19):7947-55.

Falck J, Petrini JH, Williams BR, Lukas J, Bartek J. The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat Genet. 2002 Mar; 30(3):290-4.

Fernandez-Capetillo O, Lee A, Nussenzweig M,Nussenzweig A. H2AX: the histone guardian of the genome. DNA Repair (Amst). 2004 Aug-Sep; 3(8-9):959967

Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol. 2002 Jul; 3:415–24.

Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer Res. 1991 Nov 15; 51(22):6110-7.

Jones RM, Kotsantis P, Stewart GS, Groth P, Petermann E. BRCA2 and RAD51 promote double-strand break formation and cell death in response to gemcitabine. Mol Cancer Ther. 2014 Oct;13(10):2412-21.

Lee J, Kumagai A, Dunphy WG. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. Mol Biol Cell. 2001 Mar; 12(3):551-63.

Lee JH , Paull TT. Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. Oncogene. 2007 Dec 10; 26(56): 7741–8.

Lee, JH, Paull TT. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science. 2005 Apr 22; 308(5721), 551–4.

Lopes M, Cotta-Ramusino C, Pellicioli A, Liberi G, Plevani P, Muzi-Falconi M, Newlon CS, Foiani M. The DNA replication checkpoint response stabilizes stalled replication forks.Nature. 2001 Aug 2; 412(6846):557-61.

Mini E, Nobili S, Caciagli B, Landini I, Mazzei T. Cellular pharmacology of gemcitabine. Ann Oncol. 2006 May; 17 Suppl 5:v7–v12.

Nakano Y, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, Izawa T, Mizukami Y, Okumura T, Kohgo Y. Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. Br J Cancer. 2007 Feb 12; 96:457–63.

Parsels LA, Morgan MA, Tanska DM, Parsels JD, Palmer BD, Booth RJ, Denny WA, Canman CE, Kraker AJ, Lawrence TS, Maybaum J. Gemcitabine_sensitization by checkpoint kinase 1 inhibition correlates with inhibition of a Rad51 DNA damage response in_pancreatic cancer_cells. Mol Cancer Ther. 2009 Jan;8(1):45-54.

Plunkett W, Huang P, Xu YZ, Heinemann V, Grunewald R, Gandhi V. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. Semin Oncol. 1995 Aug; 22 (4 Suppl 11):3–10.

Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M. Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. Cell. 2008 Jan 25; 132(2):221-32.

Sørensen CS, Hansen LT, Dziegielewski J, Syljuåsen RG, Lundin C, Bartek J, Helleday T. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nat Cell Biol. 2005 Feb; 7(2):195-201.

Tobita K, Kijima H, Dowaki S, Kashiwagi H, Ohtani Y, Oida Y, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S, Makuuchi H Epidermal growth factor

receptor expression in human pancreatic cancer: Significance for liver metastasis. Int J Mol Med. 2003 Mar; 11(3):305-9.

Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem. 2001 Dec 21; 276(51):47759-62.

Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, Yuan J, Wang M, Chen D, Sun Y, Woodward WA, Liu Y, Dean DC, Liang H, Hu Y, Ang KK, Hung MC, Chen J, Ma L. ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. Nat Cell Biol. 2014 Sep; 16(9):864-75.

Zou L,Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003 Jun 6; 300(5625): 1542–8.

CHAPTER VII

ZEB1 contributes to gemcitabine resistance and DNA damage response in PDAC cell lines.

Pancreatic adenocarcinoma (PDAC) is one of the most aggressive cancers in the western world, characterised by limited response to conventional chemotherapeutic treatments. At molecular level, mounting evidence suggests that resistance to chemotherapy in PDAC relies on a population of cells that undergoes a transition from an epithelial to mesenchymal phenotype. However, the mechanisms that confer resistance to gemcitabine, the standard chemotherapeutic approach for PDAC treatment, are not clearly understood.

We found that the ability of PDAC cells to resist to gemcitabine treatment is correlated to their mesenchymal phenotype. Moreover, among the transcription factors involved in the acquisition of the mesenchymal phenotype, only ZEB1 clearly segregates resistant cells from sensitive cells. Silencing of ZEB1 in resistant cell lines impairs cell viability, even in absence of chemotherapeutic treatments. Furthermore, silencing of ZEB1 induces a higher number of dead cells in the ZEB1-silenced population. Our results suggest the involvement of ZEB1 in the ability to respond to DNA damage caused by gemcitabine treatment. In particular silencing of ZEB1 impairs the activation of phosphorylation of CHK2, and it might play a role also in the maintenance of genome stability in cells that express higher level of this transcription factors.

Thus, our data strongly indicate a novel role for ZEB1 in the mechanisms of resistance to gemcitabine treatments in PDAC cells, which may be suitable to overcome resistance to chemotherapy in PDAC.

ZEB1 contributes to gemcitabine resistance and DNA damage response in PDAC cell lines.

Ilaria Passacantilli[1,2], Sara Calabretta[1,2], Gabriele Capurso[1], Gianfranco Delle Fave[1], and Claudio Sette[2,3].

[1] *Department of science medical/chirurgic and translational medicine, University of Rome "Sapienza", Rome, Italy;*
[2]*Department of Biomedicine and Prevention, Section of Anatomy, University of Rome "Tor Vergata", Rome, Italy;*
[3]*Laboratry of Neuroembryology, Fondazione Santa Lucia IRCCS, Rome, Italy;*

Keywords: ZEB1, PDAC, gemcitabine, DNA damage, apoptosis.

Running title: ZEB1 contributes to drug resistance

Corresponding Authors:

Gianfranco Delle Fave
Digestive and liver Disease Unit
S.Andrea Hospital
University of Rome "La Sapienza"
Via di Grottarossa, 1035
00189 Rome, Italy
Telephone: +39 06 33775691
Email: gianfranco.dellefave@uniroma1.it

Claudio Sette
Dept. Biomedicine and Prevention
University of Rome "Tor Vergata"
Via Montpellier, 1
00133 Rome, Italy
Telephone: +39 06 72596260
Fax: +39 06 72596268
Email: claudio.sette@uniroma2.it

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a common cause of death among solid cancers in the western world. Despite improvements in surgical techniques and new chemotherapeutic agents, outcome for patients remains extremely poor, displaying a median survival of less than one year from diagnosis and overall 5-year survival lower than 5% (Kern *et al*., 2011). Gemcitabine, the current standard first-line treatment, offers only marginal benefits to patients in terms of symptom control and prolongation of life.

To date, no clear molecular characterization of the drug resistant phenotype of pancreatic cancer cells is available. At molecular level, recent evidence suggests that resistance to chemotherapeutic treatment in PDAC cells is associated with an increased migratory and invasive phenotype. This process, also known as Epithelial-to-mesenchymal transition (EMT), occurs in differentiated epithelial cells during tumor transformation, allowing tumor cells to increase their ability to migrate and invade distal tissues (De Craine and Berx, 2013). A number of observations indicate that EMT contributes to the malignant phenotype and drug resistance in PDAC cells, suggesting that this event plays a key role in the acquisition of chemoresistance (Arumugam *et al.*, 2009; Wellner et *al.*, 2009). EMT is characterised by profound changes in gene expression, which involve transcriptional and post-transcriptional events. In particular, at the transcriptional level, EMT is orchestrated by several transcription factors, that repress the expression of epithelial genes. Among all the transcription factors involved in the induction of EMT in PDAC, expression of ZEB1 plays an important role in the acquisition of chemoresistance to several agents (Arumugam *et al*., 2009, Wellner *et al*., 2009). Moreover, expression of ZEB1 in early EMT events in PanIN (Pancreatic Intraepithelial neoplasia) determines dissemination of metastatic cells, which precedes the formation of primary tumors (Rhim *et al*., 2012). To date, although expression of ZEB1 and induction of EMT have been correlated with chemoresistance of cancer cells, how ZEB1 induces this phenotype is still largely unknown. Recently, a role for ZEB1 in the DNA damage response (DDR) has been proposed. ZEB1 was shown to interact with and stabilize CHK1, a protein kinase involved in DNA repair by homologous recombination, thereby enhancing the resistance to radiotherapy of breast cancer cells (Zhang *et al*., 2014). Indeed, enhancement of the DDR pathway is one of the features of cancer cells, which determines acquisition of drug-resistance.

In the present work, we investigated the mechanisms by which ZEB1 determines gemcitabine resistance in PDAC cell lines. We found that PDAC cells that are able to respond to gemcitabine treatment show a mesenchymal phenotype, whereas sensitive cell lines express epithelial markers. Moreover, we found that silencing of ZEB1 affects basal cell viability and increases number of dead cells in the ZEB1-silenced population. The restoration of sensitivity to gemcitabine, however, is not related to reversion of the mesenchymal phenotype to an epithelial one, suggesting that maintenance of a mesenchymal phenotype is not sufficient for gemcitabine resistance. However, we found that silencing of ZEB1 affects phosphorylation of Checkpoint Kinase 2 (CHK2) during gemcitabine treatment, suggesting that ZEB1 may be important for response to genotoxic stress by sustaining a robust DDR. Thus, our results suggest a novel scenario, in which ZEB1 is required to enhance response to genotoxic stress and progression of cell cycle in aggressive PDAC cells, highlighting novel mechanisms that are required for gemcitabine resistance.

RESULTS

PDAC cell lines show different sensitivity to gemcitabine treatment

In order to investigate the molecular mechanisms that underline gemcitabine resistance in PDAC, we tested cell viability in a panel of PDAC cell lines in presence of increasing doses of gemcitabine. We selected a non tumoral cell line derived from immortalized pancreatic ductal cells, the HPDE cell line, as control, and other cell lines derived from primary PDAC tumors, the HPAF-II, MiaPaCa-2 and Pt45P1 cell lines. Their sensitivity to gemcitabine treatment was tested by performing clonogenic assays (Figure 1A). We found that the control line HPDE is the most sensitive to gemcitabine, while tumoral cell lines are more resistant. However, tumor cell lines display different sensitivity to the drug. HPAF-II cells show the highest sensitivity, while Pt45P1 and MiaPaCa-2 cells are more resistant. However, MiaPaCa-2 results the most resistant cell line, showing a higher percentage of colony numbers with respect to Pt45P1. To confirm these results by a different approach, we performed western blot analysis of the cleaved form of the pro-apoptotic protein PARP (Figure 1B). In line with the clonogenic assay, HPDE and HPAF-II cells show an increased ratio of cleaved PARP/full length PARP at higher doses of gemcitabine (10 uM-1 mM) than Pt45P1 and MiaPaCa-2 cells.

It is known that PDAC cells with higher sensitivity to multidrug treatments have an epithelial phenotype (Arumungam et al., 2009). To investigate if sensitivity of cells analyzed is related to the expression of epithelial or mesenchymal markers, we analyzed the expression of E-cadherin (for the epithelial phenotype) and vimentin (for the mesenchymal phenotype) through RT-PCR (Figure 1C) and western blot (Figure 1D). Our results show cell lines sensitive to gemcitabine treatment (HPDE and HPAF-II) express E-cadherin, whereas resistant cell lines (MiaPaCa-II and Pt45P1) express Vimentin.

Altogether, our results indicate that the mesenchymal phenotype of PDAC cells is associated with increased resistance to gemcitabine.

ZEB1 is expressed in resistant PDAC cell lines

During PDAC development, cancer cells may undergo EMT, which confers to cancer cells the ability to detach from the primary tumor and invade other organs (Rhim *et al*., 2012). At the molecular level, several transcription factors are crucial for the induction of the transition. In order to investigate if the resistance of mesenchymal

PDAC cell lines depends on the expression of specific transcription factors, we analyzed the mRNA expression of a subset of transcription factors involved in the EMT (SNAIL, SLUG and ZEB1). Analysis of mRNA expression shows that, among all the transcription factors analyzed, only ZEB1 clearly segregates resistant and mesenchymal cells from sensitive and epithelial cells (Figure 2A). These results were also confirmed by the western blot analysis (Figure 2B).

Thus, our results suggest that ZEB1 expression is correlated to mesenchymal phenotype and gemcitabine resistance in PDAC cell lines.

ZEB1 affects cell viability in PDAC cell lines

Expression of ZEB1 has been related to resistance to multidrug treatment, including gemcitabine, in PDAC cell lines (Arumugam *et al*., 2009). Moreover, it has also been shown that ZEB1 plays a role in the acquisition of pro-metastatic behaviour of PDAC cells, and that genes involved in EMT can be involved in "oncogene addiction" and cell viability in mesenchymal cells (Singh *et al*., 2009)

In order to investigate if ZEB1 is involved in PDAC cells viability, we silenced it transiently in cell lines expressing high levels of ZEB1 (MiaPaCa-2 and Pt45P1). Notably, we found that ZEB1 knockdown impairs cell proliferation and cell viability under basal conditions (Figure 3A-B), indicating that ZEB1 is involved in the control of these processes in PDAC cells. Furthermore, treatment with gemcitabine showed higher number of dead cells in the ZEB1-silenced population with respect to cells silenced with a control siRNA (Figure 3B). However, this effect was not accompanied by increased sensitivity to the drug, but it likely reflected the increased basal levels of death observed in the cells silenced for ZEB1.

EMT has been previously proposed as a mechanism to acquire drug resistance in PDAC cells. However, transient silencing of ZEB1 in PDAC cells did not affect expression of vimentin nor it increased the expression of E-cadherin (Figure 4A-B). Moreover, ZEB1 knockdown did not induce significant changes in cellular shape and morphology (Figure 4C-D), suggesting that the increase in cell death and the inhibition of cell proliferation is not correlated to transition from mesenchymal to epithelial-phenotype in the PDAC cells analyzed.

Thus, our results suggest that silencing of ZEB1 impairs proliferation in PDAC cells and increases the number of dead cells, both under both basal conditions and in

response to gemcitabine treatment, without triggering a mesenchymal-to-epithelial transition (MET).

Different activation of the DDR response in epithelial and mesenchymal PDACs cell lines

In order to understand the mechanisms by which ZEB1 regulates response to gemcitabine treatment, we analyzed the DDR pathway. Recently, it was found that ZEB1 is involved in the response to radiation therapy in breast cancer (Zhang *et al.*, 2014). In particular, expression of ZEB1 stabilizes the Checkpoint Kinase 1 (CHK1) protein and enhances the ability of cells to repair DNA damage induced by radiation.

We analyzed the induction of the DDR by gemcitabine treatment in two cell lines that are sensitive and resistant to drug treatment. We selected HPAF-II and MiaPaCa-II cell lines, and we tested increasing doses of gemcitabine (0,1 μM - 1 mM). Both HPAF-II and MiaPaCa-2 readily activated phosphorylation of Histone H2AX in Serine 139 (γH2AX), starting from the dose of 10 μM of the drug (Figure 5A). Concomitantly, gemcitabine induces the activation of Ataxia-telancgectasica mutated (ATM), through the activation of its phosphorylation on Ser 1981 (Figure 5A). These data suggest that both cell lines readily induce the DDR at the dose of 10 μM.

In order to understand if the expression of ZEB1 affects the activation of CHK1 and CHK2, the downstream effectors of DDR response, we performed a time course of gemcitabine treatment at the dose of 10 μM. In both HPAF-II, which does not express ZEB1, and MiaPaCa-2, that express ZEB1, gemcitabine induces an early activation of the phosphorylation of CHK1 (Figure 5B). However, CHK1 activation persists in MiaPaCa-2 until 72 hours, while it is inhibited at 48 hours in HPAF-II. Moreover gemcitabine induces a sustained activation of CHK2 phosphorylation in MiaPaCa-2, whereas its activation was not detected in HPAF-II.

These data suggest a different activation of the downstream effectors of the DDR in HPAF-II and MiaPaCa-2 cells, which may underline their different sensitivity to gemcitabine treatment.

ZEB1 regulates the activation of p-ATM and CHK2 in PDAC cell lines

In order to understand if ZEB1 is involved in the stronger activation of CHK1 and CHK2 in MiaPaCa2 cells, we silenced it and evaluated the DDR pathway during treatment with gemcitabine (Figure 6). Notably, we found that silencing of ZEB1 does

not impair the activation of CHK1. On the contrary, silencing of ZEB1 almost completely abolished activation of CHK2 and induction of ATM phosphorylation (Figure 6).

These results suggest that ZEB1 regulates the activation of CHK2 during gemcitabine treatment, providing a possible mechanism for the involvement of ZEB1 in the response to gemcitabine treatment.

DISCUSSION

Understanding the mechanisms involved in the acquisition of chemoresistance in PDAC is one of the most important steps to improve the prognosis for this disease, which remains still incurable for advanced stages.

In our work we have investigated the mechanism(s) that PDAC cells set in motion in order to resist to gemcitabine treatment. We tested the ability three PDACs cell lines, HPAF-II, Pt45P1 and MiaPACa-2, to respond to gemcitabine, in comparison to a non-tumor cell line, HPDE. We found that MiaPaCa-2 and Pt45P1 were more resistant than HPAF-II to treatments, and higher resistance was correlated with their mesenchymal phenotype. Moreover, among the transcription factors known to be involved in the determination of the mesenchymal phenotype, only ZEB1 expression specifically correlated with the mesenchymal phenotype and gemcitabine resistance of PDAC cell lines. Silencing of ZEB1 impaired cell proliferation and enhanced basal cell death, affecting in turn also viability in response to gemcitabine.

It is known that ZEB1 plays a role in the acquisition of multidrug chemoresistance in PDAC cell lines (Arumungam *et al*., 2009), and that silencing of ZEB1 is important to reduce the metastatic potential also in vivo in PDAC (Wellner *et al*., 2009). Furthermore, expression of ZEB1 in pancreatic cells may occur in the third stage of Pancreatic intra-neoplasia (PanIN-3), before the formation of primary tumor and promoting the detachment of metastatic cells (Rhim *et al*., 2012). ZEB1 plays a key role in drug resistance and in tumoral potential also in other cancers, such as breast cancer, melanoma and glioblastoma (Chaffer *et al*., 2013, Caramel *et al*., 2013, Siebzehnrubl *et al*., 2013). Thus, these data suggests that ZEB1 potentially takes part of cell mechanisms which impact on tumor transformation. Nevertheless, the mechanisms by which ZEB1 regulates drug-resistance and malignancy of tumors cells are still unknown.

Notably, we found that silencing of ZEB1 is able to impair basal cell viability in MiaPaCa-2 cell lines. It has been demonstrated that EMT factors may be important in the regulation of cell survival in PDAC and lung cell lines which display a KRAS-independence for cell viability (Singh *et al*., 2009). Thus, our results suggest a possible addiction of mesenchymal PDAC cells to ZEB1 for cell survival, and highlight the key role of ZEB1 in cancer cells.

Our results show that silencing of ZEB1 increase the percentage of cell death in presence of gemcitabine, even in the absence of reversal to an epithelial phenotype.

Therefore, our data suggest that mechanisms other than loss of a mesenchymal phenotype are involved in gemcitabine resistance of PDAC cells. Recently, it has been proposed a novel role for ZEB1 in the protection to DNA damage induced by radiotherapy (Zhang *et al*., 2014). ZEB1 was shown to enhance the ability of cancer cells to recover DNA damage after exposure to radiotherapy, through stabilization of CHK1. This effect was mediated by phosphorylation of ZEB1 by ATM. Hence, the crosstalk beetwen ZEB1 and ATM appears to be important for the acquisition of resistance to radiotherapy. In order to understand if ZEB1 regulates the response to DNA damage induced by gemcitabine, we analyzed the activation of the DDR in MiaPaCa-2 and HPAF-II, which respectively express or not ZEB1. Gemcitabine induced the same activation o γH2AX and p-ATM in both cell lines, starting from the 10 μM dose. However, the activation of the downstream effectors of the DDR, CHK1 and CHK2, was different in HPAF-II and MiaPaCa-2. Indeed, phosphorylation of p-CHK1 is maintained until 72 hours in MiaPaCa-2 cells, while in HPAF-II cells is inhibited at 48 hours. Moreover, phosphorylation of CHK2 is activated in MiaPaCa-2 cells, whereas is not induced in HPAF-II cells. In order to understand if ZEB1 is involved in the activation of CHK1 or CHK2 we silenced ZEB1 in MiaPaCa-2 cells. Notably, the absence of ZEB1 affected the activation of CHK2 but not CHK1. Intriguingly, silencing of ZEB1 affected also the activation of p-ATM, suggesting that ZEB1 may affect CHK2 phosphorylation by impairing ATM activation. Previous data indicated that silencing of ATM in tumor cells affected response to gemcitabine treatment (Karnitz *et al*., 2005). Moreover, fibroblasts depleted of ATM are sensitized to gemcitabine and their repopulation capability was impaired after drug exposure (Ewald *et al*., 2008). These data suggests that ZEB1 could regulate the DDR by modulationg ATM activation.

However, CHK2 activation downstream of ATM may also be fundamental for DDR in response to gemcitabine. Indeed, inhibition of CHK1 and CHK2 sensitizes PDAC and other cancer cells to gemcitabine treatment (Karnitz *et al*., 2005; Matthwes *et al*., 2007). In line with this observation, depletion of CHK2 or pharmacological inhibition of CHK2 in MiaPaCa2 cells sensitized them to gemcitabine treatment, promoting formation of ROS-agents which increased cell death. Thus, activation of the ATM/CHK2 pathway is required for PDAC cells to improve the response to gemcitabine treatment.

In conclusion, our work proposes a novel role for ZEB1 in the acquisition of gemcitabine resistance through the regulation of the ATM-CHK2 pathway. Thus counteracting this DDR pathway or ZEB1 expression in cells resistant to gemcitabine may enhance the effect of chemotherapy on treatment of advanced PDAC.

MATERIALS AND METHODS

Cell Culture and treatments

HPDE, HPAF-II and Pt45P1 were cultured in RPMI 1640 medium (Lonza), MiaPaCa-2 was cultured in DMEM medium (Lonza). Both media were supplemented with 10% fetal bovine serum (Gibco), penicillin and streptomycin. Cells were growth in a 37°C humidified atmosphere of 5% CO2. Gemcitabine (Eli Lilly & Company, Indianapolis, IN, USA) was dissolved in water and stored at -20 °C.

Cell transfections

For RNA interference, cells at ~50-60% confluence were transfected with siRNAs (Sigma- Aldrich) using Lipofectamine RNAiMAX (Invitrogen) and OptiMEM medium (Invitrogen), according to the manufacturer's instructions. Briefly, PDAC cells were transfected with 50 nM of ZEB1 or control siRNA. At the end of transfection, cells were tripsinized and seeded for Trypan Blue Count, MTS assay, immunofluorescence and for protein extraction. Sequences of ZEB1 and control siRNA are listed in Table 1.

RT-PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After digestion with RNase free DNase (Ambion), RNA was resuspended in RNase free water (Sigma Aldrich); 1μg of total RNA was retrotranscribed using M-MLV reverse transcriptase (Promega). Five percent of the retrotranscription reaction was used as template for PCR analysis (GoTaq, Promega). Primers used are listed in Table1.

Protein extraction and Western blot analysis

For protein extraction, cells were resuspended in lysis buffer (100mM NaCl, 15mM $MgCl_2$, 30mM Tris-HCl pH 7.5, 1mM dithiothreitol, 2mM Na-ortovanadate, Protease-Inhibitor Cocktail (Sigma-Aldrich) and 1% Triton X-100). After 10 min of incubation in ice, the extracts were centrifuged for 10 min at 12,000 rpm at 4°C and the supernatants were resuspended in SDS-page sample buffer, and boiled for 5 minutes. Western blot analysis was performed as previously described (Di Florio et al., 2007).

Following primary antibodies (overnight at 4°C) were used: rabbit anti-Actin (1:1000, Sigma Aldrich), mouse anti-GAPDH (1:1000, Santa Cruz Biotechnology), rabbit anti-E-cadherin (1:1000, Santa Cruz Biotechnology), mouse anti-Vimentin (1:1000, Santa Cruz Biotechnology), rabbit anti-ZEB1 (1:1000, Sigma-Aldrich), mouse anti-pATM Serine 1981 (1:1000, Cell Signalling Technology), rabbit anti p-CHK2 Threonine 68 (1:1000, Cell Signalling Technology), rabbit anti p-CHK1 Serine 296 (1:1000, Cell Signalling Technology), rabbit anti p-H2AX Serine 139 (1:1000, Cell Signalling Technology). Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated for 1 h at RT (1:10000). Immunostained bands were detected by chemiluminescence method (Santa Cruz Biotechnology).

Coomassie staining

After separation on 10% SDS-PAGE, protein bands were visualized by placing gels in a solution of 40% distilled water, 10% acetic acid, and 50% methanol with the addition of 0.25% by weight Coomassie Brilliant Blue R-250 (Sigma-Aldrich). Gels were incubated 2 hours at room temperature and then washed in a mixture of 40% distilled water, 10% acetic acid, and 50% methanol by shaking for 1 hour. The washing mixture was replaced with fresh rinse mixture until the excess dye has been removed.

Colony formation assay

Single-cell suspensions were plated in 35mm plates (500 cells/plate for Pt45P1 and MiaPaCa-2; 700 cells/plate for HPDE and HPAF-II). After 1 day, cells were treated for 24 h with gemcitabine. At the end of the incubation, the medium was replaced every 48 h. After 10 days, cells were fixed in methanol for 10 min, stained overnight with 5% Giemsa (Sigma Aldrich), washed twice in PBS and dried. Pictures were taken using a digital camera to count and measure the colonies. Results represent the mean of at least 3 experiments ±s.d.

Trypan Blue and viability assay

For Trypan Blue Assay, PDAC cells were seeded at ~70% confluence in 24-well plate and treated as described in the text for 72 h, washed in PBS and trypsinized. Cellular suspension was incubated with of 0.4% Trypan Blue Stain (Invitrogen) and cells were counted using Cell Countess II System (Invitrogen). Cell viability was measured by the MTS Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay

(Promega) according to manufacturer's instructions by plating 2000 cells (MiaPaCa2) or 3000 cells (PT45P1) per well. Results of Trypan blue and cell viability assays represent mean±s.d. of three experiments.

Immunofluorescence analysis

For phalloidin analysis, PDAC cells were fixed in 4% paraformaldehyde and washed three times with PBS. Cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated for 1 h in 3% BSA. Cells were washed three times with PBS and incubated for 1 h at room temperature with antibodies (anti-phalloidin 1:400, Sigma-Aldrich). Slides were a mounting solution with Hoechst dye (Invitrogen). At least 200 cells for each experiment were acquired.

Table 1: List of oligos used in this study

Oligo name	**Sequence 5'➔3'**	**Reference**
E-Cadherin FW	AGTTTTCCACCAAAGTCACGC	This study
E-Cadherin RV	AGGAGTTGGGAAATGTGAGCA	This study
Vimentin FW	AGACACTATTGGCCGCCTGCAGGATG	This study
Vimentin RV	GAAGAGGCAGAGAAATCCTGCTCTCCTCGCCTTCCA	This study
ZEB1 FW	CATTGCTGACCAGAACAGTGTTCC	This study
ZEB1 RV	TGGGCGGTGTAGAATCAGAGTCAT	This study
SNAIL FW	CACTATGCCGCGCTCTTTC	Evdokimova, 2009
SNAIL RV	GCTGGAAGGTAAACTCTGGATTAGA	Evdokimova, 2009
SLUG FW	AGTCCAAGCTTTCAGACCCCCATGCCATTG	Valacca, 2010
SLUG RV	TTCTCCCCCGTGTGAGTTCTA	Valacca, 2010
HPRT FW	TGACCAGTCAACAGGGGACA	This study
HPRT RV	TTCGTGGGGTCCTTTTCACC	This study
Si CTRL	AGACGAACAAGUCACCGAC	This study
Si ZEB1	AGAUGAUGAAUGCGAGUCG	Wellner, 2009

References

Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. Cancer Res. 2009 Jul 15;69(4): 5820–8.

Caramel J, Papadogeorgakis E, Hill L, Browne GJ, Richard G, Wierinckx A, Saldanha G, Osborne J, Hutchinson P, Tse G, Lachuer J, Puisieux A, Pringle JH,Ansieau S, Tulchinsky E. A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. Cancer Cell. 2013 Oct 14;24(4):466-80.

Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, D'Alessio AC, Young RA, Weinberg RA. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. Cell. 2013 Jul 3; 154(1):61-74.

Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene. 2007 Feb 1; 26(5):711-24.

De Craene B, Berx G.Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer. 2013 Feb; 13(2):97-110.

Di Florio A, Capurso G, Milione M, Panzuto F, Geremia R, Delle Fave G, Sette C. Src family kinase activity regulates adhesion, spreading and migration of pancreatic endocrine tumor cells. End Relat Cancer. 2007 Mar; 14(1): 111-24.

Duong HQ, Hong YB, Kim JS, Lee HS, Yi YW, Kim YJ, Wang A, Zhao W, Cho CH, Seong YS, Bae I. Inhibition of checkpoint kinase 2 (CHK2) enhances sensitivity of pancreatic adenocarcinoma cells to gemcitabine. J Cell Mol Med. 2013 Oct; 17(10):1261-70.

Evdokimova V, Tognon C, Ng T, Ruzanov P, Melnyk N, Fink D, Sorokin A, Ovchinnikov LP, Davicioni E, Triche TJ, Sorensen PH. Translational activation of snail1 and other developmentally regulated transcription factors by B-1 promotesan epithelial-mesenchymal transition. Cancer Cell. 2009 May 5; 15(5):402-15.

Ewald B, Sampath D, Plunkett W.ATM and the Mre11-Rad50-Nbs1 complex respond to nucleoside analogue-induced stalled replication forks and contribute to drug resistance. Cancer Res. 2008 Oct 1; 68(19):7947-55.

Karnitz LM, Flatten KS, Wagner JM, Loegering D, Hackbarth JS, Arlander SJ, Vroman BT, Thomas MB, Baek YU, Hopkins KM, Lieberman HB, Chen J, Cliby WA, Kaufmann SH. Gemcitabine-induced activation of checkpoint signaling pathways that affect tumor cell survival. Mol Pharmacol. 2005 Dec; 68(6):1636-44.

Kern SE, Shi C, Hruban RH. The complexity of pancreatic ductal cancers and multidimensional strategies for therapeutic targeting. *J Pathol* 2011; 223(2): 295–306

Matthews DJ, Yakes FM, Chen J, Tadano M, Bornheim L, Clary DO, Tai A, Wagner JM, Miller N, Kim YD, Robertson S, Murray L, Karnitz LM. Pharmacological abrogation of S-phase checkpoint enhances the anti-tumor activity of gemcitabine in vivo. Cell Cycle. 2007 Jan 1; 6(1):104-10.

Rhim AD, Mirek ET,Aiello NM,Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. EMT and dissemination precede pancreatic tumor formation. Cell. 2012 Jan 20; 148(1-2):349-61.

Siebzehnrubl FA, Silver DJ, Tugertimur B, Deleyrolle LP, Siebzehnrubl D, Sarkisian MR, Devers KG, Yachnis AT, Kupper MD, Neal D, Nabilsi NH, Kladde MP, Suslov O, Brabletz S, Brabletz T, Reynolds BA, Steindler DA. The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. EMBO Mol Med. 2013 Aug; 5(8):1196-212.

Singh A, Greninger P, Rhodes D, Koopman L, Violette S, Bardeesy N, Settleman J.A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cellsurvival. Cancer Cell. 2009 Jun 2; 15(6):489-500.

Valacca C, Bonomi S, Buratti E, Pedrotti S, Baralle FE, Sette C, Ghigna C, Biamonti G. Sam68 regulates EMT through alternative splicing-activated nonsense-mediated mRNA decay of the SF2/ASF proto-oncogene. J Cell Biol. 2010 Oct 4; 191(1):87-99.

Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, Yuan J, Wang M, Chen D, Sun Y, Woodward WA, Liu Y, Dean DC, Liang H, Hu Y, Ang KK, Hung MC, Chen J, Ma L. ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. Nat Cell Biol. 2014 Sep; 16(9):864-75.

FIGURE LEGENDS

Figure 1. PDAC cell lines show different sensitivity to gemcitabine treatment. A) Western Blot analysis of the total protein PARP and the cleaved-isoform in PDAC cells treated with different doses of gemcitabine for 72 hours. Actin was used as loading control. B) Histograms represent the percentage of inhibition of colony formation in comparison to control cells from three experiments (mean ± s.d.). Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. C) RT-PCR analysis in PDAC cell lines of E-cadherin and Vimentin genes. HPRT was used as loading control. D) Western Blot analysis of E-cadherin and Vimentin Protein in pDAC cell lines. Coomassie Staining was used as loading control.

Figure 2. ZEB1 is expressed in resistant PDAC cell lines. A) RT-PCR analysis of EMT transcription factors genes ZEB1, SLUG and SNAIL. HPRT was used as loading control. B) Western blot analysis of ZEB1 in PDAC cell lines. Coomassie staining was used as loading control.

Figure 3. ZEB1 affects cell viability in PDAC cell lines. A) Histograms represent MTS analysis of MiaPaCa-2 and Pt45P1 cells transfected with a control or ZEB1 siRNA. B) Trypan Blue analysis of cells transfected as in (A) treated or not with the indicated doses of gemcitabine. Statistical analysis was performed by the paired Student's t-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Figure 4. Transient silencing of ZEB1 does not revert mesenchymal phenotype. A-B) Western Blot analysis of ZEB1, E-Cadherin and Vimentin protein level. GAPDH was used as loading control. C-D) Representative images of PDAC cells transfected with a control or ZEB1 siRNA in bright field (C) or stained with phalloidin (D) (40X magnifications).

Figure 5. PDACs cell lines display a different activation of DDR response. A) Western Blot analysis in HPAF-II and MiaPaCa-2 for p-ATM and γH2AX in presence of gemcitabine, as indicated in figure. B) Western Blot analysis in HPAF-II and MiaPaCa-2 for p-ATM, p-CHK2, p-CHK1 and γH2AX in presence of gemcitabine 10 μM at the times indicated in figure. Actin and GAPDH were used as loading control for the western blot analysis.

Figure 6. ZEB1 regulates the activation of p-ATM and CHK2 in PDAC cell lines. Western Blot analysis MiaPaCa-2 transfected with control or ZEB1 si RNAs for p-ATM, p-CHK2, p-CHK1 and γH2AX in presence of gemcitabine 10 μM at the times indicated in figure. GAPDH was used as loading control for all the western blot analysis.

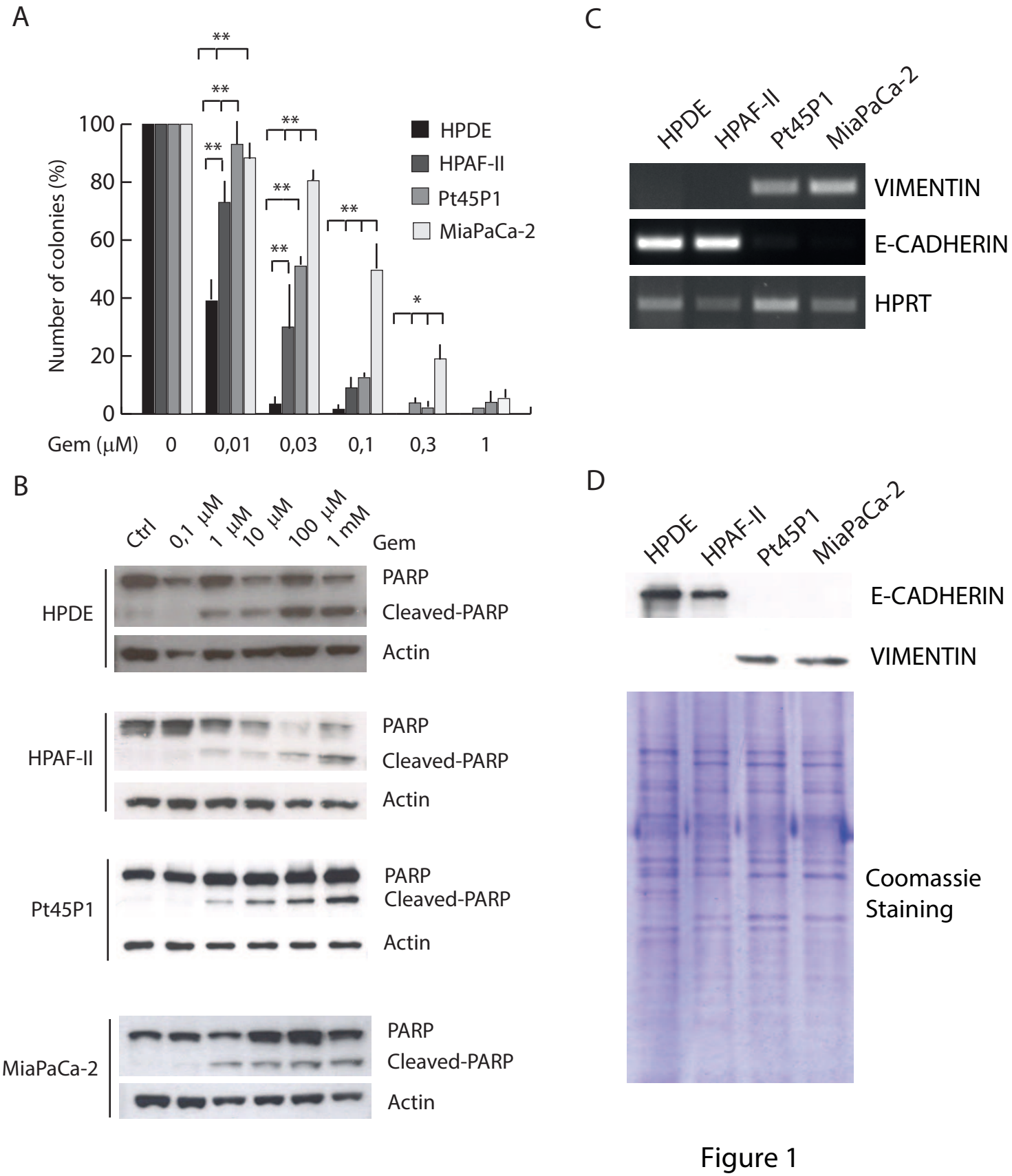
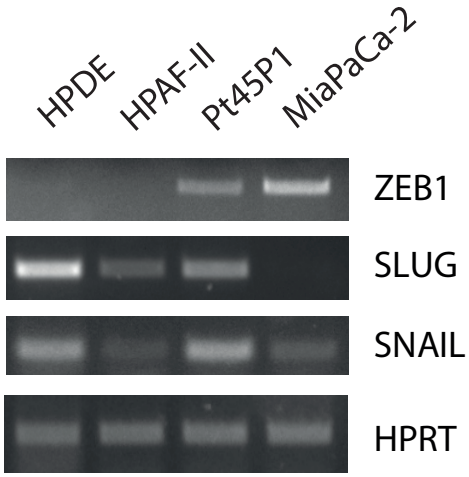

A
Number of colonies (%)
100
80
60
40
20
0
HPDE
HPAF-II
Pt45P1
MiaPaCa-2
Gem (μM) 0 0,01 0,03 0,1 0,3 1
B
Ctrl
0,1 μM
1 μM
10 μM
100 μM
1 mM
Gem
HPDE
HPAF-II
Pt45P1
MiaPaCa-2
PARP
Cleaved-PARP
Actin
C
HPDE
HPAF-II
Pt45P1
MiaPaCa-2
VIMENTIN
E-CADHERIN
HPRT
D
HPDE
HPAF-II
Pt45P1
MiaPaCa-2
E-CADHERIN
VIMENTIN
Coomassie Staining

Figure 1

A


HPDE
HPAF-II
Pt45P1
MiaPaCa-2
ZEB1
SLUG
SNAIL
HPRT

B

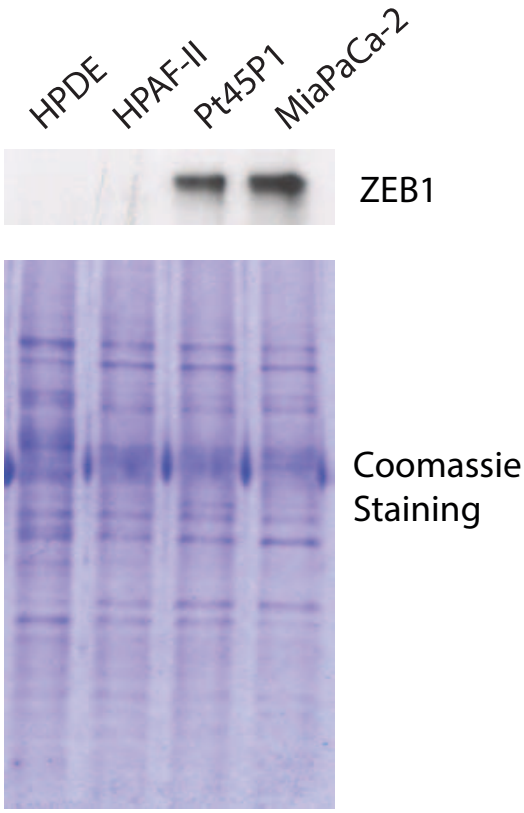
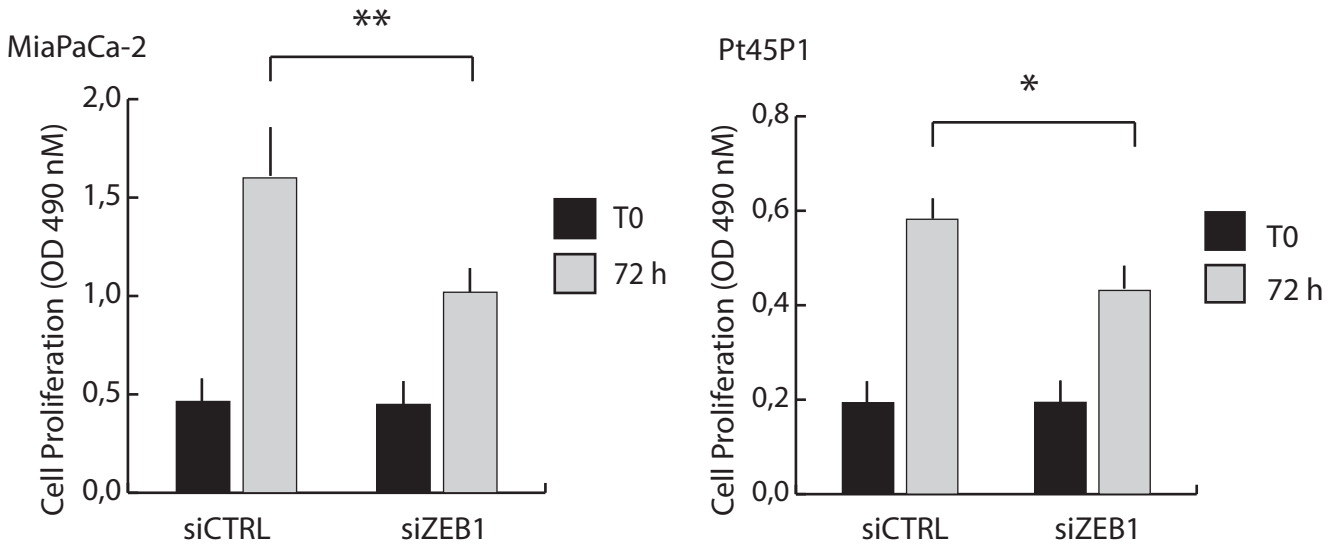

HPDE
HPAF-II
Pt45P1
MiaPaCa-2
ZEB1
Coomassie
Staining

Figure 2

A


MiaPaCa-2
**
Cell Proliferation (OD 490 nM)
2,0
1,5
1,0
0,5
0,0
T0
72 h
siCTRL
siZEB1
Pt45P1
*
0,8
0,6
0,4
0,2
0,0
T0
72 h
siCTRL
siZEB1

B

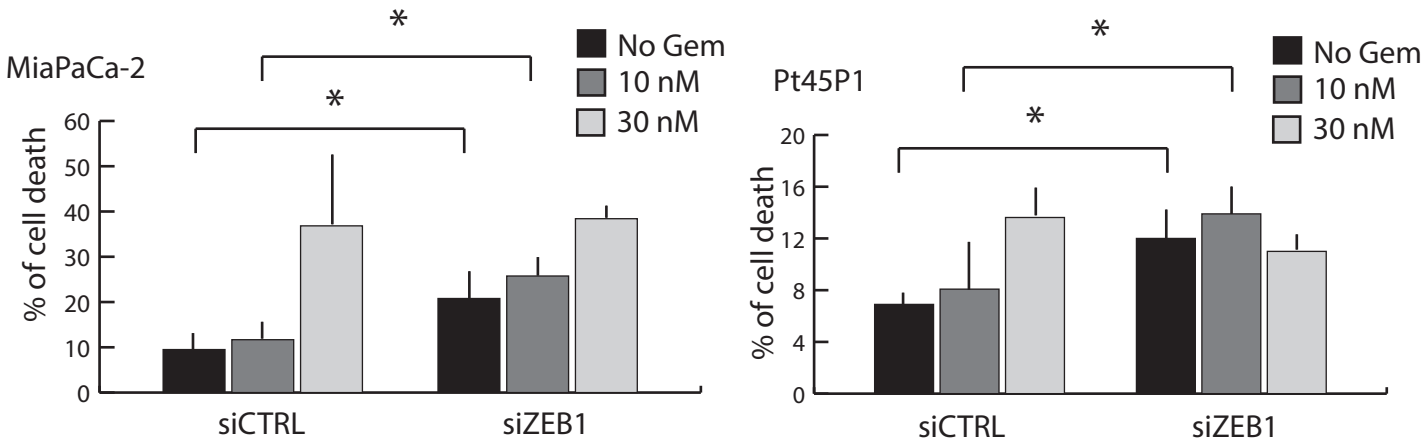

MiaPaCa-2
*
*
No Gem
10 nM
30 nM
% of cell death
60
50
40
30
20
10
0
siCTRL
siZEB1
Pt45P1
*
*
No Gem
10 nM
30 nM
% of cell death
20
16
12
8
4
0
siCTRL
siZEB1

Figure 3

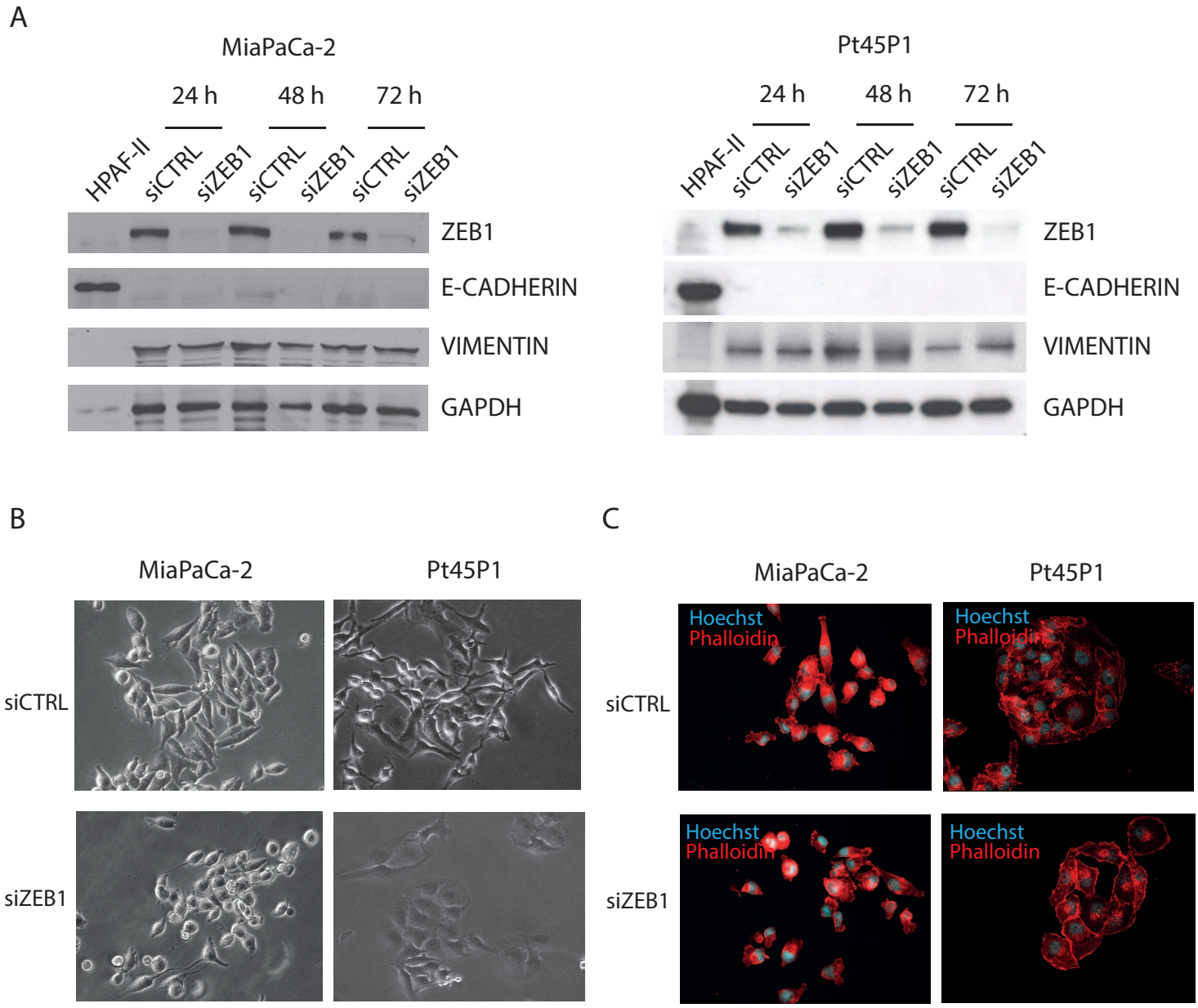

A
MiaPaCa-2
24 h
48 h
72 h
HPAF-II
siCTRL
siZEB1
siCTRL
siZEB1
siCTRL
siZEB1
ZEB1
E-CADHERIN
VIMENTIN
GAPDH
Pt45P1
24 h
48 h
72 h
HPAF-II
siCTRL
siZEB1
siCTRL
siZEB1
siCTRL
siZEB1
ZEB1
E-CADHERIN
VIMENTIN
GAPDH
B
MiaPaCa-2
Pt45P1
siCTRL
siZEB1
C
MiaPaCa-2
Pt45P1
siCTRL
siZEB1
Hoechst
Phalloidin

Figure 4

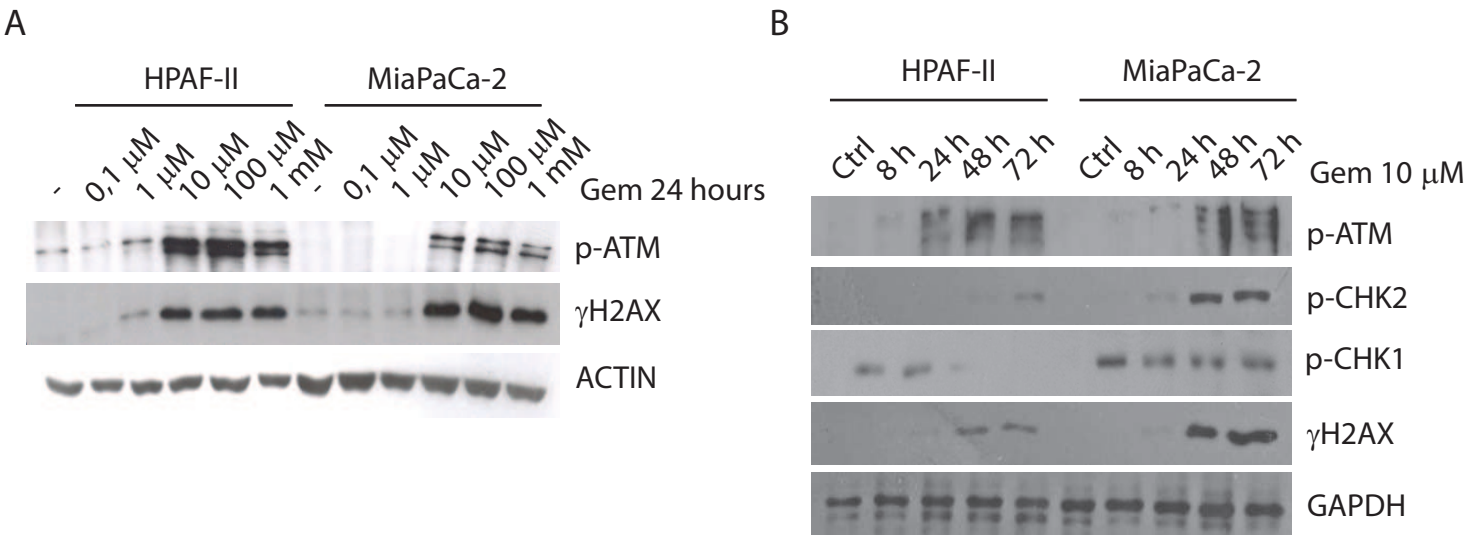

A
HPAF-II
MiaPaCa-2
-
0,1 μM
1 μM
10 μM
100 μM
1 mM
-
0,1 μM
1 μM
10 μM
100 μM
1 mM
Gem 24 hours
p-ATM
γH2AX
ACTIN
B
HPAF-II
MiaPaCa-2
Ctrl
8 h
24 h
48 h
72 h
Ctrl
8 h
24 h
48 h
72 h
Gem 10 μM
p-ATM
p-CHK2
p-CHK1
γH2AX
GAPDH

Figure 5

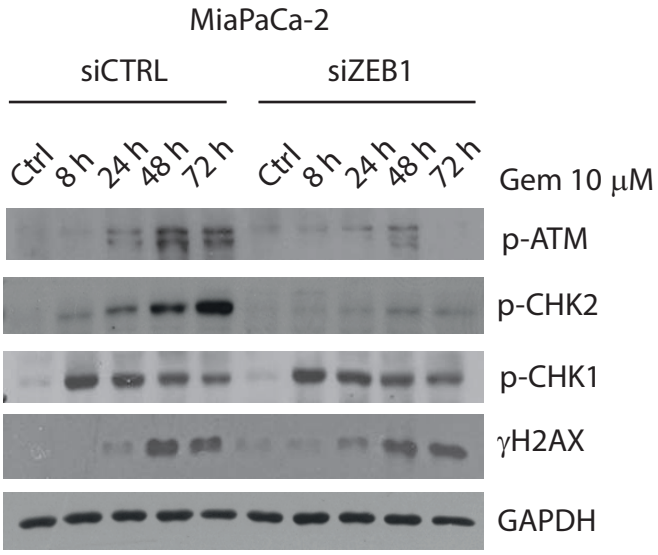

MiaPaCa-2
siCTRL
siZEB1
Ctrl
8 h
24 h
48 h
72 h
Ctrl
8 h
24 h
48 h
72 h
Gem 10 μM
p-ATM
p-CHK2
p-CHK1
γH2AX
GAPDH

Figure 6

APPENDIX

Modulation of *PKM* alternative splicing by PTBP1 promotes gemcitabine resistance in pancreatic cancer cells.

Acquisition of resistance to gemcitabine treatment in PDAC represents one of the most important causes of the poor prognosis for this disease. In the present work we have investigated the possible mechanisms which underline resistance to chemotherapy in PDAC. Our work highlights the role of alternative splicing and its deregulation in cancer progression and drug resistance.

We established a subpopulation of cells that are resistant to drug treatment through the chronic exposure of PDAC cell lines to gemcitabine. We obtained a drug-resistant sub-population (DR-PDAC cells) with higher ristance to gemcitabine and cisplatin with respect to the parental cell lines (PCL-PDAC cells). In order to investigate the mechanisms responsible for the gemcitabine resistance, we analyzed a subset of AS events known to be important in cancer progression. We found that the AS of the pyruvate kinase gene, *PKM,* is differentially regulated in DR-cells with respect to PCL cells. Indeed, the PKM2 splicing isoform is favoured in DR-cells, and PKM2 expression correlates with poor prognosis in tumor samples of PDAC. Moreover, by reverting the splicing of PKM2 to the PKM1isoform using splicing-specific antisense oligonucleotides (ASO), we restored the sensitivity of DR-cells to gemcitabine and cisplatin, suggesting a role of PKM2 in the acquisition of resistance to chemotherapeutic treatment. In order to understand the mechanisms responsible for the selection of PKM2 variant respect with PKM1, we analyzed the expression of splicing factors known to be involved in the regulation of *PKM* splicing. We found that expression of the polypiridine-tract binding protein 1 (PTBP1) correlates with the PKM2 splicing variant. Importantly, we found that PTB1 is recruited on PKM pre-mRNA with higher efficiency in DR-cells respect with PCL-cells, and silencing of PTBP1 in DR-cells causes a decrease of the recruitment of PTB1 on PKM pre-mRNA. Notably, silencing of PTBP1 causes the switching of PKM splicing in DR-cells, determining an increase of PKM1 isoform, concomitantly with a restoration of gemcitabine sensitivity.

In conclusion, our study demonstrated that chronic treatment with gemcitabine in PDAC cell lines determines the selection of cells with a drug-resistant phenotype, which may be selected by the deregulation of *PKM* AS. Thus, our study demonstrated that the PKM2 variant and the splicing factor PTBP1 may represent targetable molecular events to prevent the acquisition of the chemoresistant phenotype and to improve the prognosis in PDAC.

This work has been accepted for publication in Oncogene on June 8th, 2015.

Modulation of *PKM* alternative splicing by PTBP1 promotes gemcitabine resistance in pancreatic cancer cells

Sara Calabretta [1,2], Pamela Bielli [3], Ilaria Passacantilli [1,2], Emanuela Pilozzi [4], Volker Fendrich [5], Gabriele Capurso [2], Gianfranco Delle Fave [2] and Claudio Sette [1,3]

1 Department of biomedicine and prevention, University of Rome Tor Vergata, Rome, Italy

2 Department of science medical/chirurgic and translational medicine, University of Rome La Sapienza, Rome, Italy

3 Laboratory of neuroembriology, Fondazione Santa Lucia, Rome, Italy

4 Department of clinic and molecular medicine, University of Rome La Sapienza, Rome, Italy

5 Department of surgery, Philipps-University Marburg, Marburg, Germany

***Running title**:* Role of PKM2 and PTBP1 in PDAC drug resistance

Keywords: PKM2, PTBP1, gemcitabine, drug resistance, pancreatic adenocarcinoma

Corresponding Authors: Claudio Sette
Dept. of Biomedicine and Prevention
University of Rome "Tor Vergata"
Via Montpellier, 1
00133, Rome, Italy
Telephone: 3906 72596260
Fax: 3906 72596268
Email: claudio.sette@uniroma2.it

Gianfranco Delle Fave
Dept. of science medical/chirurgic and translational medicine
University of Rome, "La Sapienza"
Via di Grottarossa, 00189 Rome, Italy
Telephone : 3906 33775691
Fax: 3906 33775526
Email: gianfranco.dellefave@uniroma1.it

Conflict of Interest: the authors declare no conflict of interests
Grant Support: Association for International Cancer Research (AICR) [grant #12-0150]; Associazione Italiana Ricerca sul Cancro (AIRC) [grant #14581]; Fondazione Santa Lucia Ricerca Corrente.
Author' contribution: S.C., P.B., I.P., performed the experiments and analyzed the data; E.P. and V.F. performed IHC experiments and score analysis; G.C. performed statistical analysis of patients; S.C., G.D.F., G.C. and C.S. wrote the manuscript; S.C. and C.S. designed the study.
Word count: 4500 words

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and incurable disease. Poor prognosis is due to multiple reasons, including acquisition of resistance to gemcitabine, the first line chemotherapeutic approach. Thus, there is a strong need for novel therapies, targeting more directly the molecular aberrations of this disease. We found that chronic exposure of PDAC cells to gemcitabine selected a subpopulation of cells that are drug-resistant (DR-PDAC cells). Importantly, alternative splicing of the pyruvate kinase gene (*PKM*) was differentially modulated in DR-PDAC cells, resulting in promotion of the cancer-related PKM2 isoform, whose high expression also correlated with shorter recurrence free survival in PDAC patients. Switching *PKM* splicing by antisense oligonucleotides to favour the alternative PKM1 variant rescued sensitivity of DR-PDAC cells to gemcitabine and cisplatin, suggesting that PKM2 expression is required to withstand drug-induced genotoxic stress. Mechanistically, up-regulation of the polypyrimidine-tract binding protein (PTBP1), a key modulator of *PKM* splicing, correlated with PKM2 expression in DR-PDAC cell lines. PTBP1 was recruited more efficiently to *PKM* pre-mRNA in DR- than in parental PDAC cells. Accordingly, knockdown of PTBP1 in DR-PDAC cells reduced its recruitment to the *PKM* pre-mRNA, promoted splicing of the PKM1 variant and abolished drug resistance. Thus, chronic exposure to gemcitabine leads to up-regulation of PTBP1 and modulation of *PKM* alternative splicing in PDAC cells, conferring resistance to the drug. These findings point to PKM2 and PTBP1 as new potential therapeutic targets to improve response of PDAC to chemotherapy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers, being characterized by very low 5-year survival rate (1). Lack of early symptoms and late diagnosis contribute to poor prognosis, with most patients presenting with metastasis. When surgical resection is unfeasible, chemotherapy with gemcitabine, administered either alone or in combination with other compounds, represents the clinical option for PDAC. Nevertheless, relapse always occurs with more aggressive features and insensitivity to chemotherapy, contributing to high lethality (2,3). Thus, identification of new diagnostic markers and elucidation of the molecular pathways involved in acquisition of drug resistance represent clinical priorities for PDAC (2,4).

Adaptation to variable stresses is a key feature of neoplastic cells. Recent evidence highlighted how cancer cells can flexibly modulate gene expression at the level of alternative splicing (AS) to withstand hostile conditions (5-8). In this regard, changes in expression of some splicing factors have been directly linked to expression of oncogenic splice variants that confer various advantages to cancer cells (9-14). Moreover, genotoxic stress was shown to modulate splicing regulation (15), in some cases by affecting the localization or activity of specific splicing factors, such as SAM68 (16) or EWS (17). In the case of PDAC cells, it was previously shown that increased expression of the serine/arginine (SR)-rich protein kinase SRPK1, a prototypic splicing factor kinase, confers resistance to treatment with gemcitabine (18). Notably, since SRPK1 modulates the activity of several SR protein splicing factors with implication in cancer (19), including SRSF1 (20), it is likely that up-regulation of this kinase contributes to the expression of oncogenic splice variants expressed in PDAC cells (21).

Herein, we aimed at investigating the role of AS and splicing factors in the acquisition of a drug-resistant (DR) phenotype in PDAC cells. We observed that chronic treatment with gemcitabine promoted the formation of DR subpopulations highly resistant to drug-induced genotoxic stress. In order to understand the contribution of AS to the DR phenotype, we analyzed a

group of cancer-related splice variants involved in oncogenic features (5-8). We found that DR-PDAC cells exhibited a switch in *PKM* AS, a gene encoding two alternative splice variants, PKM1 and PKM2, through usage of mutually exclusive exons. PKM2 is typically expressed in cancer cells where it confers oncogenic features (22- 24). We show that splicing of PKM2 is favoured in DR-PDAC cells with respect to the parental cells and promotes drug resistance, as interference with this splicing event in DR-PDAC cells restored sensitivity to gemcitabine and cisplatin. Mechanistically, we demonstrate that the polypyrimidine-tract binding protein PTBP1 is up-regulated in DR-PDAC cells and that its increased recruitment to the *PKM* pre-mRNA promotes PKM2 splicing. Knockdown of PTBP1 in DR-PDAC cells reduces its binding to *PKM* pre-mRNA, favours the expression of PKM1 and rescues drug sensitivity. Hence, our results indicate a positive role for PTBP1 and PKM2 in the acquisition of drug resistance, suggesting that this regulatory pathway represents a novel potential therapeutic target for PDAC.

Results

Isolation of drug-resistant (DR)-PDAC cells

To isolate drug-resistant (DR) PDAC cell sub-populations, we exposed to chronic treatment with gemcitabine (10 μM) two cell lines: Pt45P1, which displays higher sensitivity to the drug, and PANC-1, which is more resistant to treatment (Supplementary Figure 1A). As expected, gemcitabine caused massive cell death in both cell lines in the seven days of treatment. However, 15 days after removal of the drug, few viable clones were visible in the plates of both cell lines. Clones were pooled, amplified and cultured by exposing them to a 24 hour-pulse of gemcitabine every other week to maintain selection of the DR populations (Figure 1A,B).

To confirm that DR-PDAC cells were indeed more resistant to drug treatment than the parental cell line (PCL), we analyzed cell survival by colony formation assays. PCL- and DR-PDAC cells were cultured for 24 hours with sub-optimal doses of gemcitabine and then allowed to grow in complete medium until they formed visible colonies (Figure 1C,D). Treatment with gemcitabine reduced the number of colonies in a dose dependent-manner in PCL cells, whereas DR cells were resistant to the lower dose of gemcitabine and less sensitive to the higher dose (Figure 1C,D). Analysis of cell death by trypan blue cell count or by immunofluorescence analysis of the cleaved/activated form of caspase-3 confirmed that gemcitabine was more cytotoxic for PCL- than DR-PDAC cells (Supplementary Figure 1B,C). Collectively, these results indicate that the selected cell populations have acquired a drug-resistant phenotype.

PKM splicing is regulated in DR-PDAC cells

Recent evidence suggests a key role for mis-regulation of AS in the acquisition of oncogenic features and drug-resistance by human cancer cells (5-8). Thus, we tested whether PCL- and DR-PDAC cells display changes in splice variants of a subset of cancer-relevant genes. We selected a group of genes whose AS was reported to promote oncogenic features in cancer cells, such as the apoptotic genes *CASP9* (25), *CASP2* (26), *BCL-X* (27), *BIM* (28) and *FAS* (29) (Figure 2A and

Supplementary Figure 2A), genes involved in DNA repair and drug resistance, such as *USP5* (30) and *MKNK2* (31,32) (Figure 2B and Supplementary Figure 2B), genes affecting basal metabolism, such as *PKM* (22) (Figure 2C), genes involved in cell migration and invasion, such as *RON* (10), *CD44* (5), and c-*MET* (33) (Figure 2D and Supplementary Figure 2C) or the cell cycle gene *CCND1* (34) (Figure 2E). RT-PCR analysis showed that AS of most of these genes was either unchanged between PCL- and DR-PDAC cells (*CASP2*, *CCND1*, *c-MET*, *USP5*, *MKNK2* and *RON*) or not modulated in the same direction in DR-PDAC cell lines (*CASP9*, *BCL-X*, *BIM*, *CD44* and *FAS*) (Figure 2 and Supplementary Figure 2). On the contrary, splicing of the PKM2 variant was favoured with respect to PKM1 in both DR-PDAC cell lines (Figure 2C), suggesting that modulation of *PKM* AS correlated with acquisition of drug resistance in PDAC cells.

PKM2 protein is up-regulated in DR-PDAC cells and correlates with relapse free survival in PDAC patients

We focused on the regulation of *PKM* AS because growing evidence supports a key role for this splicing event in tumorigenesis (22,35). The PKM2 splice variant is prevalently expressed in cancer cells (22,36), where it regulates processes spanning from cell metabolism (22,24), to transcription (23), cell cycle (37) and cell death (38,39). Differential expression of PKM1 and PKM2 in DR-PDAC cells was confirmed by RT-PCR analysis using primers positioned either in exon 9 (PKM1) or 10 (PKM2) to amplify each splice variant (Figure 3A). Furthermore, the switch in *PKM* splice variants was confirmed at the protein level, as DR-PDAC cells expressed higher levels of PKM2, whereas PKM1 was almost undetectable with respect to PCL-PDAC cells (Figure 3B). Notably, PANC-1 cells, which are more resistant to gemcitabine (Supplementary Figure 1A), also express higher levels of PKM2 and lower levels of PKM1 than the more sensitive Pt45P1 cells (Figure 3C). These observations indicate that the DR phenotype of PDAC cells correlates with increased expression of PKM2.

To assess the relevance of PKM2 *in vivo*, we investigated its expression levels by immunohistochemistry in a cohort of 42 patients diagnosed with primary PDAC in the absence of metastases, who received radical surgery and subsequent gemcitabine-based adjuvant treatment. Our hypothesis was that patients expressing high levels of PKM2 could be more resistant to gemcitabine and display worse clinical outcome. The anti-PKM2 antibody was validated by immunofluorescence and Western blot analyses of PANC-1 cells silenced for PKM2 and with mouse tissues expressing (embryonic) or not (adult) PKM2 (Supplementary Figure 3A-C). Titration analysis established 1: 1600 as the optimal dilution for immunohistochemistry (Supplementary Figure 3D). The neoplastic lesions of all 42 samples (100%) showed cytoplasmic PKM2 staining (Figure 3D), whereas non-neoplastic pancreatic tissue occasionally displayed very weak PKM2 staining in normal ductal and acinar cells (Supplementary Figure 3D). A linear score of staining (range 0-5) was assigned to each sample (see Materials and Methods) and patients were subdivided in two groups: the "low PKM2" group comprised 16 samples characterized by weak PKM2 staining (i.e. $\leq$3) (Figure 3D, upper panels), whereas the "high PKM2" group comprised 26 samples displaying stronger PKM2 staining (i.e. >3) (Figure 3D, lower panels). No differences regarding age, sex and pathological features (mean tumour size, grade, stage and resection margins) were found between the two groups (Supplementary Table 1). However, the recurrence free survival (RFS), defined as the time elapsing from surgery to disease recurrence, was significantly shorter in patients with "high PKM2" (mean 11.6 months) as compared with the "low PKM2" group (mean 19.8 months; p=0.04; Supplementary Table 1). Accordingly, RFS estimated by Kaplan-Meier curve was significantly shorter in the first group (Figure 3E), and PKM2 was the only risk factor significantly associated with shorter RFS at a Cox proportional-hazards regression (HR 1.12; 95% CI 1-4.4; p=0.04). These data suggest that tumors with higher PKM2 basal expression display more aggressive behavior and worse response to chemotherapy.

Modulation of *PKM* splicing impairs drug resistance of DR-PDAC cells

AS can be modulated in live cells by antisense short oligonucleotides (ASOs) directed against a specific regulatory region (40). In the case of *PKM*, an ASO targeting exon 10 could efficiently induce splicing of PKM1 at the expense of PKM2 (38). We used this tool to modulate *PKM* splicing in PDAC cells and to evaluate the contribution of PKM2 to the DR phenotype. RT-PCR and western blot analyses indicated that AS of endogenous *PKM* could be efficiently modulated by transfection of the ASO in PDAC cells (Figure 4A,B). Analysis of cell death by immunofluorescence for the cleaved/activated form of caspase-3 (Figure 4C), showed that ASO-mediated switching of PKM AS in favour of PKM1 increased the sensitivity of DR-PDAC cells to gemcitabine without affecting the basal level of cell death (Figure 4C). Furthermore, overexpression of PKM2 in PCL-PDAC cells protected them from gemcitabine-induced cell death (Supplementary Figure 4A,B). These results indicate that PKM2 expression in DR-PDAC cells is required to maintain gemcitabine resistance.

PTBP1 is up-regulated in DR-PDAC cells

Three hnRNPs (hnRNPI/PTBP1, hnRNPA2/B1 and hnRNPA1) were shown to cooperate to suppress exon 9 inclusions in the *PKM* transcript, leading to exon 10 inclusion and expression of the PKM2 variant (35). Notably, these splicing factors were up-regulated in brain tumors and their expression strongly correlated with that of PKM2 (35). Thus, we investigated if the expression of these hnRNPs was altered in DR-PDAC cells with respect to PCL cells. We found that only PTBP1 was markedly up-regulated in both DR-Pt45P1 and DR-PANC-1 cells (Figure 5A,B). The highly homologous PTBP2 protein was not detected in PDAC cells (Figure 5A,B). By contrast, hnRNPA2/B1 levels were unchanged in PCL- and DR-PDAC cells, whereas hnRNPA1 was up-regulated in DR-PANC-1 (Figure 5B) but slightly reduced in DR-Pt45P1 (Figure 5A). Furthermore, PTBP1 expression correlated with sensitivity of PDAC cells to gemcitabine, as it was higher in PANC-1 cells than in Pt45P1 cells (Supplementary Figure 5A). The correlation between PTBP1 expression and PKM2 splicing in both DR-PDAC cell lines was specific, as demonstrated by

western blot analysis of other cancer-related SR proteins and hnRNPs in PCL- and DR- PDAC cells, which showed either marginal or inconstant alterations. For instance, up-regulation of SRSF1 was detected in DR-Pt45P1 cells but not in DR-PANC-1 cells (Supplementary Figure 5B), possibly because PANC-1 cells are more resistant to drug treatment and express higher basal levels of SRSF1. By contrast, SRSF6 was strongly up-regulated in DR-PANC-1 but slightly reduced in DR-Pt45P1 (Supplementary Figure 5B). Thus, up-regulation of PTBP1 appears to specifically correlate with regulation of PKM2 splicing in DR-PDAC cells.

PTBP1 binds *in vivo PKM* intron 8 and its downregulation impairs PKM2 expression and sensitizes DR-PDAC cells to drug-induced cell death

To test whether PTBP1 was recruited to the *PKM* transcript more efficiently in DR-PDAC cells, we analyzed *in vivo* binding by CLIP assays in PCL- and DR -Pt45P1 cells, silenced or not for PTBP1 (Figure 6A). Binding of PTBP1 in intron 8 of *PKM* favours skipping of exon 9 in the mature transcript (35), thereby generating the PKM2 isoform. Thus, we analyzed two regions in intron 8, named A and B (Figure 6A), which were identified as high PTBP1-bound sequences by CLIP-seq analysis (41). CLIP assays showed that PTBP1 was recruited more efficiently to *PKM* intron 8 in DR-Pt45P1 cells with respect to PCL-Pt45P1 cells (Figure 6A). Increased binding was specific and likely dependent on the higher expression of PTBP1 in DR cells, as it was suppressed by knockdown of the protein to levels comparable with those expressed in PCL cells (Figure 6A). Thus, up-regulation of PTBP1 in DR-PDAC cells leads to increased recruitment of this splicing factor to *PKM* intron 8.

To evaluate the contribution of PTBP1 to the regulation of the PKM2 variant, we analyzed *PKM* AS in DR-PDAC cells knocked down for PTBP1. Knockdown of endogenous PTBP1 increased PKM1 splicing in DR-PDAC cells, resulting in a switch in PKM1 and PKM2 protein levels (Figure 6B). These results confirm that up-regulation of PTBP1 promotes PKM2 splicing in DR-PDAC cells. To test whether PTBP1 affected additional splicing events in DR- PDAC cells, we

analyzed a subset of genes whose AS is regulated by this splicing factor in other cell types (41,42,43). RT-PCR analysis indicated that only the FGFR2 IIIc variant correlated with the higher expression of PTBP1 in both DR-PDAC cell lines (Supplementary Figure 6A). However, silencing of PTBP1 did not affect this splicing event (Supplementary Figure 6E), indicating that expression of FGFR2 IIIc correlates with but is not dependent on high PTBP1 expression in DR-PDAC cells. By contrast, splicing of *EZH2*, *CTTN*, *RASSF8*, *MINK1*, *EIF4G2*, *FAM38A*, *CCDC138* and *TPM1* was either similar in PCL- and DR-PDAC cells or altered in one of the two DR cell lines (Supplementary Figure 6A-C). These findings indicate that splicing of *PKM* is specifically modulated by PTBP1 over-expression in DR-PDAC cells.

To investigate whether PTBP1 expression is required for the resistance of DR-PDAC to chemotherapeutic treatments, we analyzed gemcitabine-induced cell death in PTBP1-depleted DR-PDAC cells. Down-regulation of PTBP1 expression significantly rescued the sensitivity of DR-PDAC cells to treatment with gemcitabine, reaching levels of cell death similar to those of PCL-PDAC cells (Figure 6C,D). We also tested resistance to cisplatin as prototype of a class of drugs currently used in clinical trials of combined chemotherapy for advanced PDAC (44). Cell death analysis showed that DR-PDAC cells were more resistant to cisplatin treatment than PCL-PDAC cells (Supplementary Figure 7A). However, switching *PKM* splicing by either ASO transfection (Supplementary Figure 7B) or knockdown of PTBP1 (Supplementary Figure 6C) rescued sensitivity to cisplatin, suggesting that the PTBP1/PKM2 axis is involved in PDAC cell survival to multiple cytotoxic drugs.

Collectively, these results indicate that high PTBP1 expression levels are required for maintenance of the DR phenotype of PDAC cells and suggest that this splicing factor mainly confers drug resistance to PDAC cells through the promotion of PKM2 splicing.

Discussion

PDAC is a human cancer characterized by very poor prognosis. Chemotherapeutic approaches are largely ineffective and treatment with the elective agent gemcitabine slightly improves survival in patients with advanced disease, but does not represent a cure (1). For this reason, understanding the biology of PDAC cells might shed light on novel therapeutic strategies for the management of advanced PDAC. In this work, we show that chronic gemcitabine treatment leads to isolation of DR-PDAC cells that display higher resistance not only to gemcitabine, but also to cisplatin, a prototype of cytotoxic drugs largely used in human cancer therapy, including PDAC (44). These findings suggest that hostile stimuli promote the adaptive capabilities of PDAC cells, thus favouring the selection of drug- resistant populations.

In order to elucidate the molecular mechanisms involved in the acquisition of the DR phenotype by PDAC cells, we focused on AS regulation because this process is emerging as a key determinant of eukaryotic cell plasticity (45) that is often altered in human cancers (5-8). Furthermore, genotoxic stresses, like those imposed by chemotherapeutic treatments, can finely tune the expression of splice variants that protect cancer cells (15). In this regard, our study identifies *PKM* splicing as a novel contributor to drug resistance acquired by PDAC cells during chronic chemotherapeutic treatment. We found that promotion of PKM2 is the AS event that correlates more closely with drug resistance among a subset of cancer-relevant splicing events analyzed. Importantly, PKM2 splicing and expression are functionally relevant for the resistance to chemotherapeutic treatment, as switching splicing toward PKM1 by ASO transfection restored sensitivity of DR-PDAC cells to both gemcitabine and cisplatin. These results point to PKM2 as a new potential prognostic marker and therapeutic target for PDAC. In support of this hypothesis, we also found that high PKM2 expression was the only risk factor significantly associated with shorter RFS in patients receiving radical surgery and adjuvant chemotherapy with gemcitabine. These results suggest that increased PKM2 expression might be responsible for lower response of residual cancer cells to chemotherapy. Although we did not find a significant correlation between PKM2

expression and overall survival, the observed trend suggests that patients expressing higher PKM2 levels also have a shorter survival rate (Supplementary Table 1). Studies with a larger cohort of patients will be required to further assess whether or not PKM2 can be used as marker for prediction of severity of the disease and response to treatments.

The role of PKM2 in cancer is not fully elucidated yet. Nevertheless, several observations pointed out that this splice variant is expressed at higher levels in cancer tissues *versus* their normal counterparts (22,35). PKM2 protein was proposed as a potential molecular marker of PDAC, as immune reactivity toward this isoform was elevated in blood from patients and positively correlated with metastatic disease (46). We now show that PKM2 is barely detectable in areas of pancreas with normal glands, whereas its expression is increased in neoplastic lesions. Moreover, our findings document that up-regulation of PKM2 in DR-PDAC cells is required for survival in the presence of gemcitabine or cisplatin. Notably, depletion of PKM2 in several human cancer cell lines caused apoptosis even in the absence of chemotherapeutic treatments (38,39). This effect was cancer-specific, as depletion of PKM2 in non-cancerous cells did not affect their viability (39). In the case of DR-PDAC cells, however, depletion of endogenous PKM2 *per se* does not trigger cell death, indicating that PDAC cells are somewhat less dependent on this enzyme for viability. Nevertheless, PKM2 was strictly necessary to withstand genotoxic stress in DR-PDAC cells. Importantly, PKM2 expression has been linked to response to chemotherapy also in lung cancer, as its depletion sensitized to apoptosis triggered by chemotherapeutic treatment in mouse xenograft models (47). Our work also supports this scenario and suggests that modulation of *PKM* splicing by ASO treatment is a potential therapeutic tool to increase the efficacy of standard chemotherapy in advanced PDAC. This strategy might represent a promising approach, as ASOs are already in clinical trials for other splicing-caused diseases and improvement of their design and administration protocols might insure their use in cancer therapy in the near future (40).

Aberrant expression of several splicing factors correlates with cancer onset, progression and/or response to therapeutic treatments (48). Our findings indicate that the switch in *PKM* AS

correlates with the up-regulation of PTBP1 in DR-PDAC cells. A role for PTBP1 in *PKM* splicing was already shown in glioblastoma, where this splicing factor acted in concert with hnRNP A1 and A2/B1 to promote PKM2 splicing (35). However, we found that neither of these hnRNPs was consistently modulated in DR-PDAC cells. Moreover, knockdown of PTBP1 to mimic the levels observed in PCL-PDAC cells was sufficient to raise PKM1 levels to those present in parental cells. Thus, PTBP1 is the main player in the regulation of *PKM* AS during the acquisition of the DR phenotype by PDAC cells. Importantly, the effect of PTBP1 on PKM2 splicing in PDAC cells appears to be direct, as it correlates with the extent of PTBP1 recruitment to *PKM* intron 8. Furthermore, its effect on *PKM* AS can account for the acquired resistance to genotoxic drugs, as DR-PDAC cells knocked down for PTBP1 switched AS in favour of PKM1 and became sensitive to gemcitabine and cisplatin like PCL-PDAC cells. Although the pro-survival effect of PTBP1 up-regulation in PDAC cells might also involve other targets of this splicing factor, our observations suggest that splicing of PKM2 represents the main player. Indeed, by monitoring a group of splice variants previously shown to be target of PTBP1 in other cellular systems (41,42,43), we did not observe striking and consistent changes correlating with the DR-PDAC phenotype. The only relevant change observed was promotion of the FGFR2 IIIc variant, which, however, was unaffected by knockdown of PTBP1 in DR-PDAC cells. Furthermore, selectively restoring the PCL pattern of *PKM* splicing by ASO transfection almost completely rescued the sensitivity of DR-PDAC cells to genotoxic stresses. Thus, our results suggest that *PKM* AS is particularly sensitive to changes in the expression levels of PTBP1 in PDAC cells and that this splicing event represent a key resource for these cells to acquire drug resistance.

In conclusion, our work characterizes a novel PTBP1/PKM2 pro-survival pathway triggered by chronic treatment of PDAC cells with gemcitabine. Interfering with this axis by repressing PKM2 splicing or PTPB1 expression can restore sensitivity of DR-PDAC cells to drug treatment. Since development of therapeutic ASOs is already a clinical approach for other human diseases (40),

these findings might represent a promising strategy to improve therapeutic approaches for PDAC and to impact the resistance of cancer cells to current treatments.

Materials and methods

Cell culture, treatments and transfections

Pt45P1 and PANC-1 cells were obtained from the Centre for Molecular Oncology, Barts Cancer Institute (London, UK) in 2004 and authenticated in 2012. DR cells were obtained by treating PCL with 10 μM gemcitabine continuously for 7 days (medium replaced every 72 hours) and then released in normal medium for 15 days. Resistant clones were pooled, amplified and cultured by performing a 24 hour-pulse of 10 μM gemcitabine every other week to maintain selection. PCL and DR cells were maintained in RPMI 1640 (Lonza, Switzerland) supplemented with 10% FBS, gentamycin, penicillin and streptomycin. Gemcitabine (Eli Lilly & Company, IN, USA) and cisplatin (Sigma-Aldrich, MO, USA) were dissolved in water. For ASO transfection, cells were transduced by scraping delivery according to manufacturer's instructions (Gene Tools, Oregon, USA) with PKM2 or control ASO (10 μM for DR-PANC-1 cells, 15 μM for DR-Pt45P1 cells). For RNA interference, cells were transfected twice with 30 nM PTBP1 siRNAs (On target plus human PTBP1 5725 siRNA, Dharmacon, CO, USA) using Lipofectamine RNAiMAX and Opti-MEM medium (Life Technologies, California, USA) according to manufacturer's instructions.

Colony formation assay and cell death analyses

Single cell suspensions were plated in 6-well plates (500 cells/plate for Pt45P1, 750 cells/plate for PANC-1). After 1 day, cells were treated for 24 hours with gemcitabine. Fresh medium was replaced every 48h. After 10-12 days, cells were fixed in methanol for 10 min, stained overnight with 5% Giemsa (Sigma-Aldrich), washed in PBS and dried. Pictures were taken using a digital camera and colonies were counted. For cell death analyses, cells were seeded at 70% confluence and treated as described for 72 h. Cells were then washed in PBS and either trypsinized and incubated with 0.4% Trypan Blue Stain (Sigma-Aldrich) or processed for caspase 3 immunofluorescence using anti-cleaved caspase-3 antibody (1:500; Sigma-Aldrich) as previously described (31,49). Positive cells were then counted using the Thoma's chamber (trypan blue) or

fluorescence microscopy (caspase 3). Five random fields were chosen for each treatment and at least 200 cells/field were counted.

Polymerase chain reaction (PCR) analyses

RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. After digestion with RNase-free DNase (Life Technologies), 1 μg of total RNA was retrotranscribed using M-MLV reverse transcriptase (Promega, WI, USA), used as template for conventional PCR reactions (GoTaq, Promega). Products were analysed on agarose or acrylamide gels. RT-PCR images were collected with Biorad Universal Hood II using Image Lab software. Real-time quantitative PCR (qPCR) analysis was performed using LightCycler 480 SYBR Green I Master and the LightCycler 480 System (Roche, Germany), according to the manufacturer's instructions. Primers used are listed in Supplementary Table 2.

Protein extracts and western blot analysis

Cells were resuspended in RIPA buffer: 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM dithiothreitol, 0.5 mM NaVO4, and protease inhibitor cocktail (Sigma-Aldrich). After 10 min on ice, extracts were centrifuged for 10 min at 12,000 g ,supernatants were collected and used for western blot as described (49). Primary antibody incubation (1:1000) was carried out with the following antibodies: PKM1, hnRNPA1, hnRNPA2/B1, hnRNPC1/C2 (Sigma-Aldrich); PKM2 (Cell Signaling Technology, MA, USA); PTBP1, SRSF1, SRP20, SRp40/p55/p75 (Santa Cruz Biotechnology, CA, USA); hnRNPF/H (Abcam, UK). PTBP2 antibody was a generous gift of Professor Douglas L. Black (UCLA, CA). Images of the western blot were acquired as TIFF files.

Immunohistochemistry analysis

Immunohistochemistry (IHC) was performed as previously described (31). Briefly, formalin-fixed, paraffin-embedded tissue samples obtained from 42 primary non metastatic PDAC patients receiving surgery with radical intent were investigated for PKM2 expression, upon informed consent. All patients received gemcitabine-based adjuvant therapy after surgery. Clinical and histopathological data, time of tumor recurrence and survival for each patient were recorded. IHC was performed on 4μm-thick sections. Antigen retrieval was carried out with EDTA at pH8 (60 min at room temperature). Staining was carried out using anti PKM2 antibody (1:1600, Cell Signaling), visualized by Envision-Flex (Dako, Denmark). Staining of PKM2 in neoplastic cells was scored based on distribution and intensity. Distribution was scored as 0 (0%), 1 (1-50%) and 2 (51-100%). Intensity was scored as 0 (no signal), 1 (mild), 2 (intermediate), 3 (strong). Values were summed in a total score from 0 to 5. Samples were classified as "low PKM2" expression (score $\leq$3) and as "high PKM2" expression (score >3). Statistical analysis was performed by MedCalc® 9.6 (www.medcalc.be). Differences for continuous variables were evaluated by t-test and for categorical variables by Fisher's test. Analysis of recurrence-free survival (RFS) and of overall survival was performed by Kaplan-Meier method and analysed by Log-rank test. Univariate and multivariate analyses for risk factors affecting survival were performed by Cox-proportional hazards regression model test; a p value <0.05 was considered as statistically significant (Supplementary Table 1). Images were taken from a Zeiss axioskop 2 plus and elaborate with software Zeiss axiovision.

UV-crosslinked and RNA immunoprecipitation (CLIP) assays

For CLIP assays, cell extract were performed as previously described (50,51). Half extract (1mg) was treated with Proteinase K for 30 min at 37°C and RNA was purified (input). The remaining half (1 mg) was diluted to 1 ml with lysis buffer and immunoprecipitated by using anti-PTBP1 (Santa Cruz Biotechnology) antibody or IgGs (negative control), in presence of protein-G magnetic dynabeads (Life Technologies). 10 μl/ml of RNaseI 1:1000 (Life Technologies) were added. After

immunoprecipitation and washes (50,51), an aliquot (10%) of the sample was kept as control of immunoprecipitation while the rest was treated with 50μg of Proteinase K and incubated for 1hr at 55°C. RNA was then isolated.

Image acquisition and manipulation

Images in Figure 1B were taken from an inverted microscope (IX70; Olympus) using an LCA ch 40×/0.60 objective. Adobe Photoshop and Illustrator were used for composing the panels.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We wish to thank Andrea D'Ascenzo and Enrico Duranti for help with immunohistochemistry, Dr. Chiara Naro for helpful suggestions throughout the study and Dr. Vittoria Pagliarini for critical reading of the manuscript. This work was supported by Association for International Cancer Research (AICR) [grant #12-0150]; Associazione Italiana Ricerca sul Cancro (AIRC) [grant #14581]; Fondazione Santa Lucia Ricerca Corrente.

References

1. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol*. 2010;**7(3)**:163-72

2. Michl P, Gress TM. Current concepts and novel targets in advanced pancreatic cancer. *Gut*. 2013;**62(2)**:317-26

3. Hidalgo M Pancreatic cancer. *N Engl J Med*. 2010;**362(17)**:1605-17

4. Tang SC, Chen YC. Novel therapeutic targets for pancreatic cancer. *World J Gastroenterol*. 2014;**20(31)**:10825-10844.

5. David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev*. 2010;**24(21)**:2343-64.

6. Zhang J, Manley JL. Misregulation of pre-mRNA alternative splicing in cancer. *Cancer Discov*. 2013;**3(11)**:1228-37.

7. Bonomi S, Gallo S, Catillo M, Pignataro D, Biamonti G, Ghigna C. Oncogenic alternative splicing switches: role in cancer progression and prospects for therapy. *Int J Cell Biol*. 2013;**2013**:962038.

8. Sette C, Ladomery M, Ghigna C. Alternative splicing: role in cancer development and progression. *Int J Cell Bio*l. 2013;**2013**:421606.

9. Cooper TA, Wan L, Dreyfuss G. RNA and disease. *Cell*. 2009;**136(4)**:777-93.

10. Ghigna C, Giordano S, Shen H, Benvenuto F, Castiglioni F, Comoglio PM, *et al*. Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. *Mol Cell*. 2005;**20(6)**:881-90.

11. Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol*. 2007;**14(3)**:185-93.

12. Venables JP, Klinck R, Koh C, Gervais-Bird J, Bramard A, Inkel L, *et al*. Cancer-associated regulation of alternative splicing. *Nat Struct Mol Biol*. 2009;**16(6)**:670-6.

13. Paronetto MP, Cappellari M, Busà R, Pedrotti S, Vitali R, Comstock C, *et al*. Alternative splicing of the cyclin D1 proto-oncogene is regulated by the RNA-binding protein Sam68. *Cancer Res*. 2010;**70(1)**:229-39.

14. Zhou X, Li X, Cheng Y, Wu W, Xie Z, Xi Q, *et al*., BCLAF1 and its splicing regulator SRSF10 regulate the tumorigenic potential of colon cancer cells. *Nat Commun*. 2014 5;**5**:4581.

15. Dutertre M, Sanchez G, Barbier J, Corcos L, Auboeuf D. The emerging role of pre-messenger RNA splicing in stress responses: sending alternative messages and silent messengers. *RNA Biol*. 2011;**8(5)**:740-7.

16. Busà R, Geremia R, Sette C. Genotoxic stress causes the accumulation of the splicing regulator Sam68 in nuclear foci of transcriptionally active chromatin. *Nucleic Acids Res*. 2010;**38(9)**:3005-18.

17. Paronetto MP, Miñana B, Valcárcel J. The Ewing sarcoma protein regulates DNA damage-induced alternative splicing. *Mol Cell*. 2011;**43(3)**:353-68.

18. Hayes GM, Carrigan PE, Miller LJ. Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas. *Cancer Res*. 2007;**67(5)**:2072-80.

19. Naro C, Sette C. Phosphorylation-mediated regulation of alternative splicing in cancer. *Int J Cell Biol.* 2013;**2013**:151839.

20. Amin EM, Oltean S, Hua J, Gammons MV, Hamdollah-Zadeh M, Welsh GI, *et al*. WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. *Cancer Cell*. 2011;**20(6)**:768-80.

21. Omenn GS, Yocum AK, Menon R. Alternative splice variants, a new class of protein cancer biomarker candidates: findings in pancreatic cancer and breast cancer with systems biology implications. *Dis Markers*. 2010;**28(4)**:241-51.

22. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, *et al*. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*. 2008;**452(7184)**:230-3.

23. Yang W, Xia Y, Hawke D, Li X, Liang J, Xing D, *et al*. PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell*. 2012;**150(4)**:685-96.

24. Yang W, Xia Y, Cao Y, Zheng Y, Bu W, Zhang L, *et al*. EGFR-induced and PKCε monoubiquitylation-dependent NF-κB activation upregulates PKM2 expression and promotes tumorigenesis. *Mol Cell*. 2012;**48(5)**:771-84.

25. Shultz JC, Goehe RW, Murudkar CS, Wijesinghe DS, Mayton EK, Massiello A, *et al*. SRSF1 regulates the alternative splicing of caspase 9 via a novel intronic splicing enhancer affecting the chemotherapeutic sensitivity of non-small cell lung cancer cells. *Mol Cancer Res*. 2011;**9(7)**:889-900.

26. Droin N, Rébé C, Bichat F, Hammann A, Bertrand R, Solary E. Modulation of apoptosis by procaspase-2 short isoform: selective inhibition of chromatin condensation, apoptotic body formation and phosphatidylserine externalization. *Oncogene*. 2001;**20(2)**:260-9.

27. Mercatante DR, Mohler JL, Kole R. Cellular response to an antisense-mediated shift of Bcl-x pre-mRNA splicing and antineoplastic agents. *J Biol Chem*. 2002;**277(51)**:49374-82.

28. Anczuków O, Rosenberg AZ, Akerman M, Das S, Zhan L, Karni R, *et al*. The splicing factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. *Nat Struct Mol Biol*. 2012;**19(2)**:220-8

29. Proussakova OV, Rabaya NA, Moshnikova AB, Telegina ES, Turanov A, Nanazashvili MG, *et al*. Oligomerization of soluble Fas antigen induces its cytotoxicity. *J Biol Chem*. 2003 Sep;**278(38)**:36236-41.

30. Nakajima S, Lan L, Wei L, Hsieh CL, Rapić-Otrin V, Yasui A, *et al*. Ubiquitin-specific protease 5 is required for the efficient repair of DNA double-strand breaks. *PLoS One*. 2014;**9(1)**:e84899.

31. Adesso L, Calabretta S, Barbagallo F, Capurso G, Pilozzi E, Geremia R, *et al*. Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway. *Oncogene*. 2013;**32(23)**:2848-57

32. Maimon A, Mogilevsky M, Shilo A, Golan-Gerstl R, Obiedat A, Ben-Hur V, *et al*. Mnk2 alternative splicing modulates the p38-MAPK pathway and impacts Ras-induced transformation. *Cell Rep*. 2014;**7(2)**:501-13

33. Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer*. 2006;**6(8)**:637-45.

34. Sawa H, Ohshima TA, Ukita H, Murakami H, Chiba Y, Kamada H, *et al*. Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. *Oncogene.* 1998;**16(13)**:1701-12.

35. David CJ, Chen M, Assanah M, Canoll P, Manley JL. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature*. 2010;**463(7279)**:364-8.

36. Tamada M, Suematsu M, Saya H. Pyruvate kinase M2: multiple faces for conferring benefits on cancer cells. *Clin Cancer Res*. 2012;**18(20)**:5554-61.

37. Jiang Y, Li X, Yang W, Hawke DH, Zheng Y, Xia Y, *et al*. PKM2 regulates chromosome segregation and mitosis progression of tumor cells. *Mol Cell*. 2014;**53(1)**:75-87.

38. Wang Z, Jeon HY, Rigo F, Bennett CF, Krainer AR. Manipulation of PK-M mutually exclusive alternative splicing by antisense oligonucleotides.*Open Biol*. 2012;**2(10)**:120133.

39. Goldberg MS, Sharp PA. Pyruvate kinase M2-specific siRNA induces apoptosis and tumor regression. *J Exp Med*. 2012;**209(2)**:217-24.

40. Kole R, Krainer AR, Altman S. RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat Rev Drug Discov*. 2012;**11(2)**:125-40.

41. Xue Y, Zhou Y, Wu T, Zhu T, Ji X, Kwon YS, *et al*. Genome-wide analysis of PTB-RNA interactions reveals a strategy used by the general splicing repressor to modulate exon inclusion or skipping. *Mol Cell*. 2009;**36(6)**:996-1006.

42. Carstens RP, Wagner EJ, Garcia-Blanco MA. An intronic splicing silencer causes skipping of the IIIb exon of fibroblast growth factor receptor 2 through involvement of polypyrimidine tract binding protein. *Mol Cell Biol*. 2000;**20(19)**:7388-400

43. Llorian M, Schwartz S, Clark TA, Hollander D, Tan LY, Spellman R, *et al*. Position-dependent alternative splicing activity revealed by global profiling of alternative splicing events regulated by PTB. *Nat Struct Mol Biol*. 2010;**17(9)**:1114-23.

44. Costello BA, Borad MJ, Qi Y, Kim GP, Northfelt DW, Erlichman C, *et al*. Phase I trial of everolimus, gemcitabine and cisplatin in patients with solid tumors. *Invest New Drugs*. 2014;**32(4)**:710-6.

45. Braunschweig U, Gueroussov S, Plocik AM, Graveley BR, Blencowe BJ. Dynamic integration of splicing within gene regulatory pathways. *Cell*. 2013;**152(6)**:1252-69.

46. Cerwenka H, Aigner R, Bacher H, Werkgartner G, el-Shabrawi A, Quehenberger F, *et al*. TUM2-PK (pyruvate kinase type tumor M2), CA19-9 and CEA in patients with benign, malignant and metastasizing pancreatic lesions. *Anticancer Res*. 1999;**19(1B)**:849-51.

47. Shi HS, Li D, Zhang J, Wang YS, Yang L, Zhang HL, *et al*. Silencing of pkm2 increases the efficacy of docetaxel in human lung cancer xenografts in mice. *Cancer Sci*. 2010;**101(6)**:1447-53.

48. Grosso AR, Martins S, Carmo-Fonseca M. The emerging role of splicing factors in cancer. *EMBO Rep*. 2008;**9(11)**:1087-93.

49. Paronetto MP, Achsel T, Massiello A, Chalfant CE, Sette C. The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *J Cell Biol*. 2007;**176(7)**:929-39

50. Bielli P, Busà R, Di Stasi SM, Munoz MJ, Botti F, Kornblihtt AR, *et al*. The transcription factor FBI-1 inhibits SAM68-mediated BCL-X alternative splicing and apoptosis. *EMBO Rep*. 2014;**15(4)**:419-27.

51. Bielli P, Bordi M, Di BiasioV, Sette C. Regulation of BCL-X splicing reveals a role for the Polypyrimidine-tract binding protein (PTBP1/hnRNP I) in alternative 5 'splice site selection. *Nucleic Acids Res*.2014;**42(19)**:12070-81

Figure legends:

Figure 1: Chronic treatment with gemcitabine selects DR-PDAC cells

(A) Schematic representation of the protocol used to obtain drug-resistant (DR) PDAC cells from parental PDAC cells (PCL). **(B)** Representative phase contrast images of PCL- and DR-Pt45P1 (left panels) or PANC-1 (right panels) cells (40x magnification). **(C-D)** Representative images of the colony assay (upper panels) performed in PCL- and DR-Pt45P1 **(C)** or PANC-1 cells **(D)**. **(C-D)**. Bar graphs (bottom panels) show the percentage of survival with respect to untreated cells from three experiments (mean ± SD), as assessed by colony formation. Brackets indicate statistical comparison of the indicated samples. Statistical analyses were performed by the paired Student's t-test. ** $p \leq 0.01$.

Figure 2: The PKM2 splice variant is promoted in DR-PDAC cells

(A-E) RT-PCR analysis in PCL- and DR-Pt45P1 or PANC-1 cells of splice variants encoded by the indicated cancer-related genes. Schematic representation of the cancer-related AS events analyzed is shown in the upper panels. Exons (boxes) and introns (lines) are indicated. Black arrows indicate primers used for the RT-PCR analysis (bottom panels). **(C)** RT-PCRs of *PKM* gene were followed by PstI digestion in order to distinguish the amplicons. Bar graphs represent the percentage of the indicated AS variants, as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's t-test comparing PCL- and DR-PDAC cells values (mean ± SD, n=3, **$p<0.01$, ns: not significant). **(F)** HPRT was used as loading control for RT-PCR analyses in panels A-E.

Figure 3: PKM2 protein expression in PDAC cells and PDAC tissues

RT-PCR **(A)** and Western blot **(B)** analyses of PKM1 and PKM2 splicing variants in PCL- and DR-PDAC cells. Schematic representation of the *PKM* gene is shown in the upper panel, black arrows

indicate the specific primers used to amplify the PKM1 and PKM2 in PCL- and DR-PDAC cells. HPRT and *PKM* exon 5-6 region were used as loading control **(A)**. Coomassie staining was used as loading control **(B)**. **(C)** Western blot analysis of PKM1 and PKM2 protein in PCL-PDAC cells. Coomassie staining was used as loading control. **(D)** Representative images of PKM2 immunohistochemistry in PDAC tissues (10X magnification). Upper panels show neoplastic glands with weak staining (low PKM2 group; score ≤3), bottom panels show neoplastic glands with strong staining (high PKM2; group score >3). **(E)** Analysis of recurrence free survival (RFS) of PDAC patients. Low PKM2 group comprised 16 patients (continuous line) while high PKM2 group comprised 26 patients (dotted line); p=0.04 at log-rank test.

Figure 4: Modulation of *PKM* splicing enhances gemcitabine-induced cell death in DR-PDAC cells

(A-B) RT-PCR **(A)** and western blot **(B)** analyses of *PKM* splicing variants performed in DR-Pt45P1 (left panels) and DR-PANC-1 (right panels) cells transduced with a control ASO (CTRL ASO) or with a specific ASO used to revert *PKM* splicing (PKM2 ASO) in favour of PKM1 (see Supplementary Table 1). **(A)** Bar graphs represent the percentage of PKM2 variant, as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's t-test comparing DR-PDAC cells values with those obtained in PCL-PDAC cells while brackets indicate statistical comparison of the indicated samples (** $p \leq 0.01$; ns: not significant). **(B)** Coomassie staining was used as protein loading control. **(C)** Bar graphs show the percentage of cell death from three experiments (mean ± SD) as assessed by immunofluorescence analysis of the cleaved form of caspase-3 in PCL- and DR-PDAC cells transduced with CTRL or PKM2 ASO and treated as indicated. Statistical analyses were performed by the paired Student's t-test, comparing DR-PDAC cell values with those obtained in PCL-PDAC cells treated with gemcitabine, while brackets indicate statistical comparison of the indicated samples (* $p \leq 0.05$, ** $p \leq 0.01$, ns: not significant).

Figure 5: PTBP1 is up-regulated in DR-PDAC cells

(A-B) Western blot analysis of PTBP1, PTBP2, hnRNPA1 and hnRNPA2/B1 protein expression in PCL- and DR-Pt45P1 **(A)** or PANC-1 cells **(B)**. Coomassie staining was used as loading control.

Figure 6: PTPB1 up-regulation is required for PKM2 splicing and gemcitabine resistance in DR-PDAC cells

(A) UV crosslink immunoprecipitation (CLIP) of PTBP1 performed in PCL- or DR-Pt45P1 PDAC cells transfected with either a control (si-ctrl) or PTBP1 (si-PTBP1) siRNAs, in presence of RNaseI (1:1000). Associated *PKM* pre-mRNA was quantified by qPCR, black arrows indicate primers used to amplify A and B regions (upper panels; see Supplementary Table 1). Data are represented as percentage of input (bottom panels; mean ± SD; n=3). Statistical analyses were performed by the paired Student's t-test (** $p \leq 0.01$, ns: not significant). PTBP1 silencing in DR-Pt45P1 cells and IP efficiency were assessed by western blot analysis. **(B**) RT-PCR and western blot analyses to evaluate PKM1 and PKM2 expression in DR-PDAC cells transfected with either ctrl or PTBP1 siRNAs. Bar graphs represent the percentage of PKM2 variant, as assessed by densitometric analysis of the bands. Statistical analyses were performed by the paired Student's t-test comparing the values of DR- PDAC cells transfected with si-ctrl with those obtained in DR-PDAC cell transfected with si-PTBP1 siRNA (** $p \leq 0.01$; (upper panels, mean ± SD, n = 3, ** $p \leq 0.01$). **(B)** PTBP1 silencing was assessed by western blot analysis. Coomassie staining was used as loading control. **(C-D)** Western blot analyses assessing PTBP1 expression levels in PCL- and DR-Pt45P1 or PANC-1 PDAC cells transfected with ctrl or PTBP1 siRNAs. Coomassie staining was used as loading control. **(C-D)** Bar graphs show the percentage of cell death from three experiments (mean ± SD) as assessed by immunofluorescence analysis of the cleaved form of caspase-3 in PCL-, DR-PDAC cells described in panel C-D and treated as indicated for 72 hours. Statistical analyses were performed by the paired Student's t-test comparing DR-PDAC cell values with those obtained in

PCL-PDAC cells treated with gemcitabine, while brackets indicate statistical comparison of the indicated samples (** $p \leq 0.01$, ns: not significant).

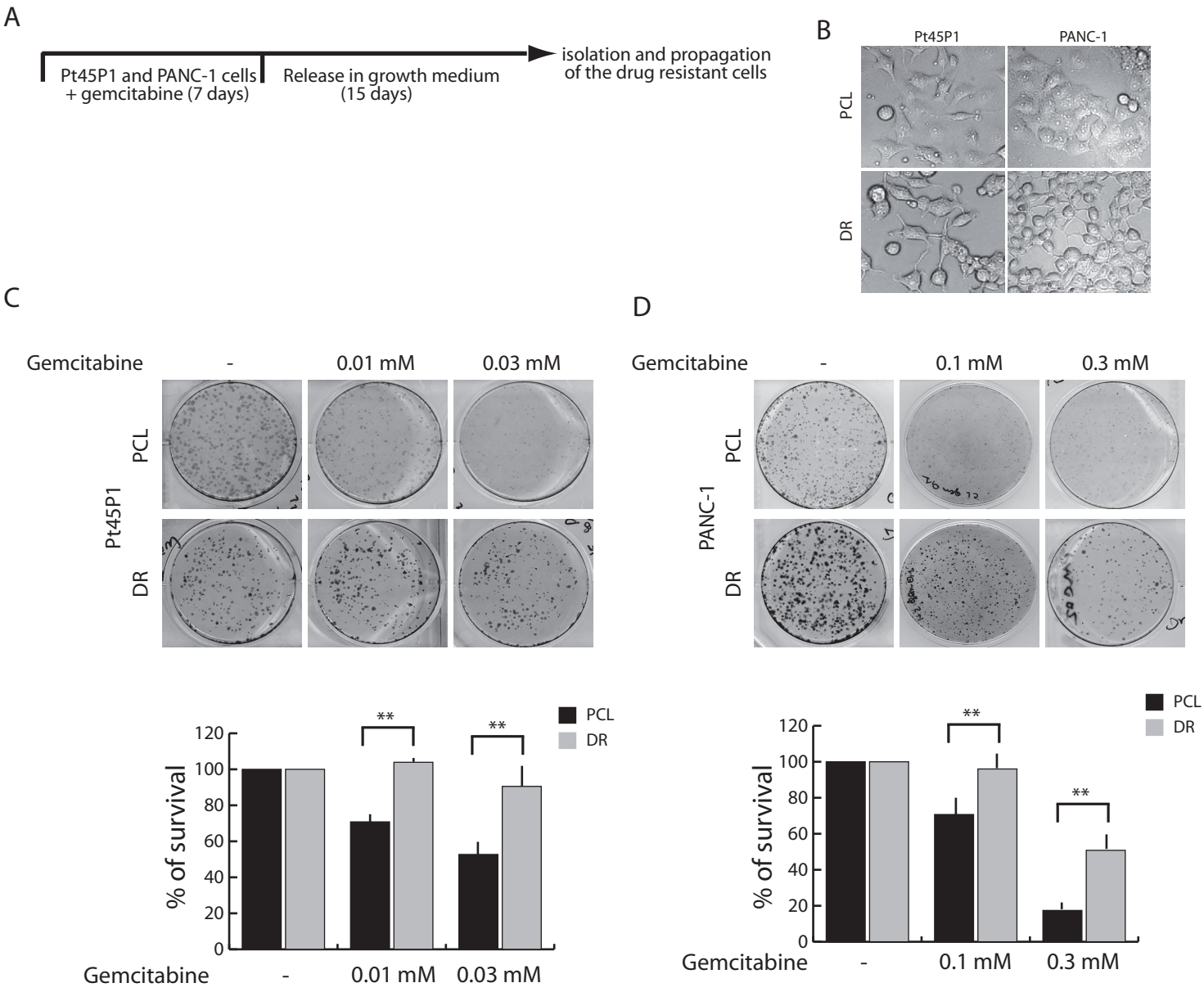

A
Pt45P1 and PANC-1 cells
+ gemcitabine (7 days)
Release in growth medium
(15 days)
isolation and propagation
of the drug resistant cells
B
Pt45P1
PANC-1
PCL
DR
C
Gemcitabine
-
0.01 mM
0.03 mM
Pt45P1
PCL
DR
% of survival
120
100
80
60
40
20
0
**
**
PCL
DR
Gemcitabine
-
0.01 mM
0.03 mM
D
Gemcitabine
-
0.1 mM
0.3 mM
PANC-1
PCL
DR
% of survival
120
100
80
60
40
20
0
**
**
PCL
DR
Gemcitabine
-
0.1 mM
0.3 mM

Figure 1

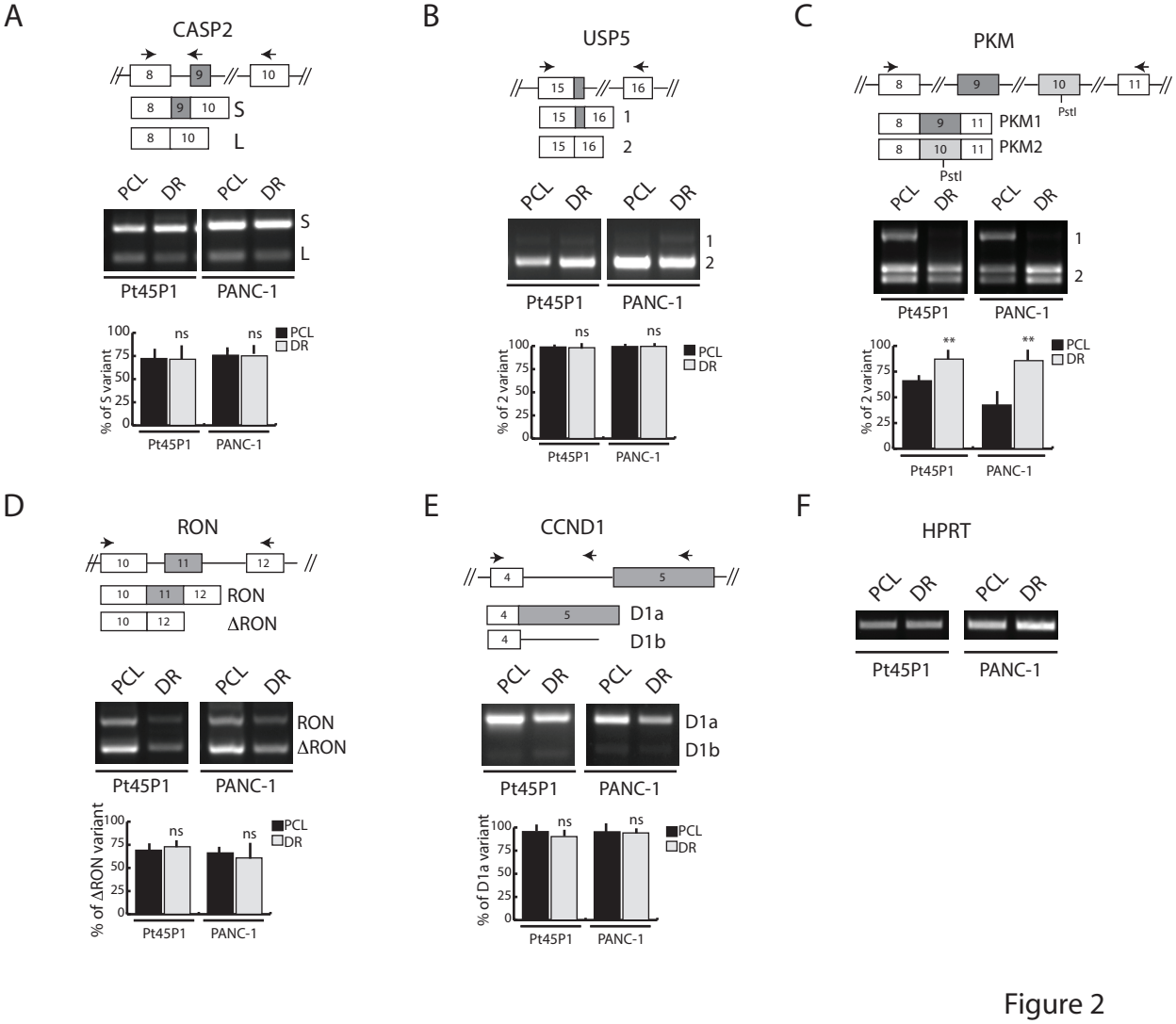

A
CASP2
S
L
PCL
DR
Pt45P1
PANC-1
% of S variant
ns
PCL
DR
B
USP5
1
2
% of 2 variant
C
PKM
PKM1
PKM2
PstI
**
D
RON
ΔRON
% of ΔRON variant
E
CCND1
D1a
D1b
% of D1a variant
F
HPRT

Figure 2

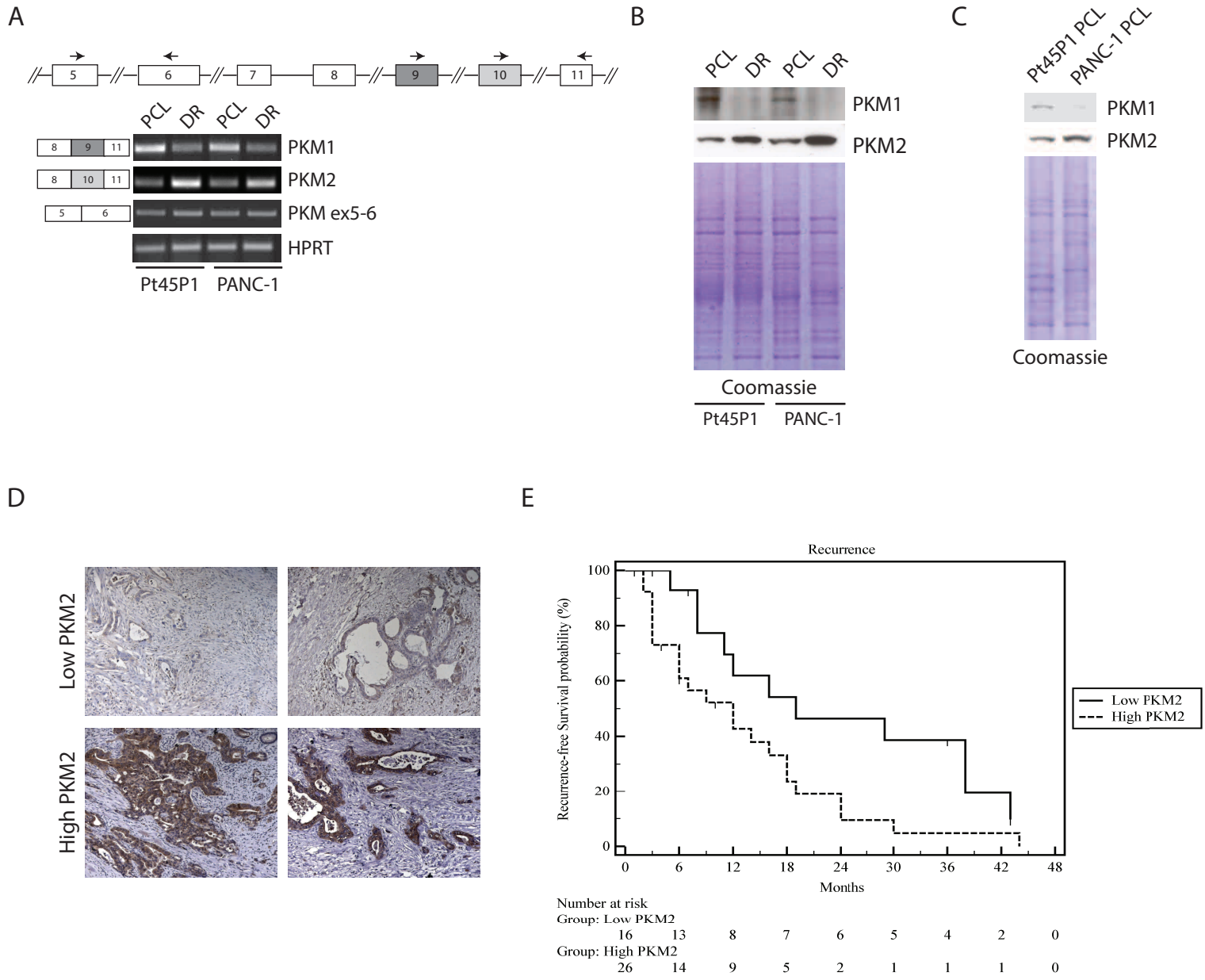
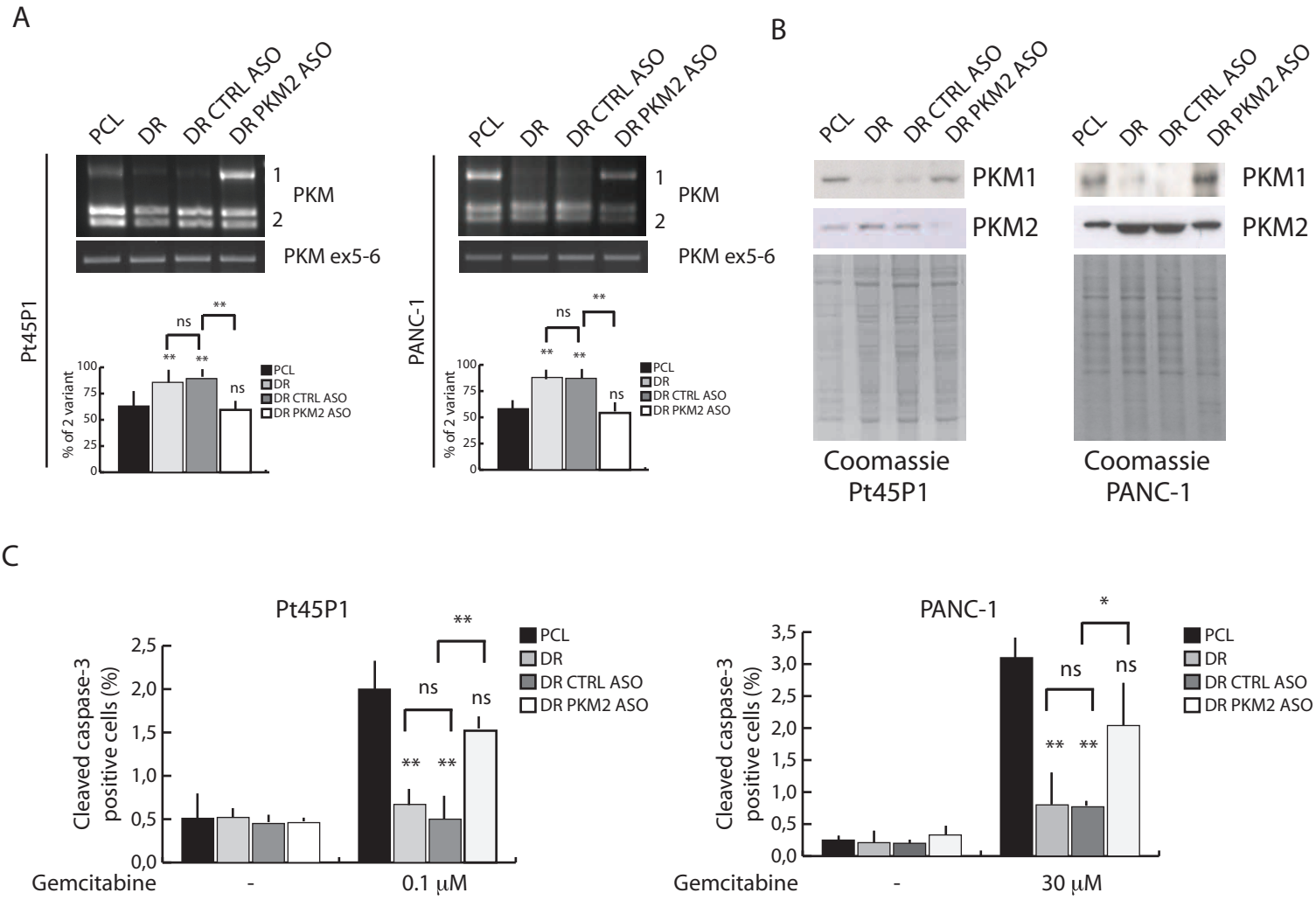

A
5
6
7
8
9
10
11
PCL
DR
PCL
DR
8 9 11
PKM1
8 10 11
PKM2
5 6
PKM ex5-6
HPRT
Pt45P1
PANC-1
B
PCL
DR
PCL
DR
PKM1
PKM2
Coomassie
Pt45P1
PANC-1
C
Pt45P1 PCL
PANC-1 PCL
PKM1
PKM2
Coomassie
D
Low PKM2
High PKM2
E
Recurrence
Recurrence-free Survival probability (%)
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Months
Low PKM2
High PKM2
Number at risk
Group: Low PKM2
16 13 8 7 6 5 4 2 0
Group: High PKM2
26 14 9 5 2 1 1 1 0

Figure 3


A
PCL
DR
DR CTRL ASO
DR PKM2 ASO
1
2
PKM
PKM ex5-6
Pt45P1
PANC-1
% of 2 variant
ns
**
PCL
DR
DR CTRL ASO
DR PKM2 ASO
B
PKM1
PKM2
Coomassie
Pt45P1
Coomassie
PANC-1

C
Pt45P1
PANC-1
Cleaved caspase-3
positive cells (%)
Gemcitabine
-
0.1 μM
30 μM
ns
*
**
PCL
DR
DR CTRL ASO
DR PKM2 ASO

Figure 4

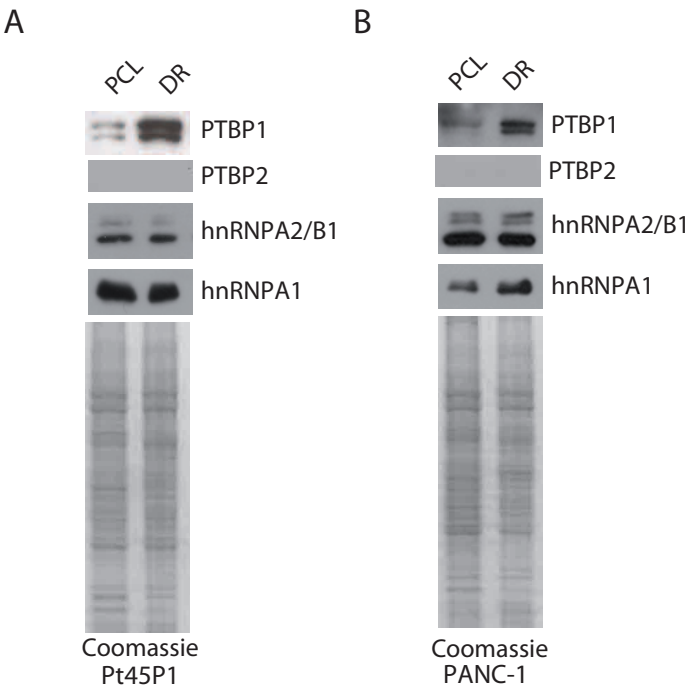

A
B
PCL
DR
PTBP1
PTBP2
hnRNPA2/B1
hnRNPA1
Coomassie
Pt45P1
PCL
DR
PTBP1
PTBP2
hnRNPA2/B1
hnRNPA1
Coomassie
PANC-1

Figure 5

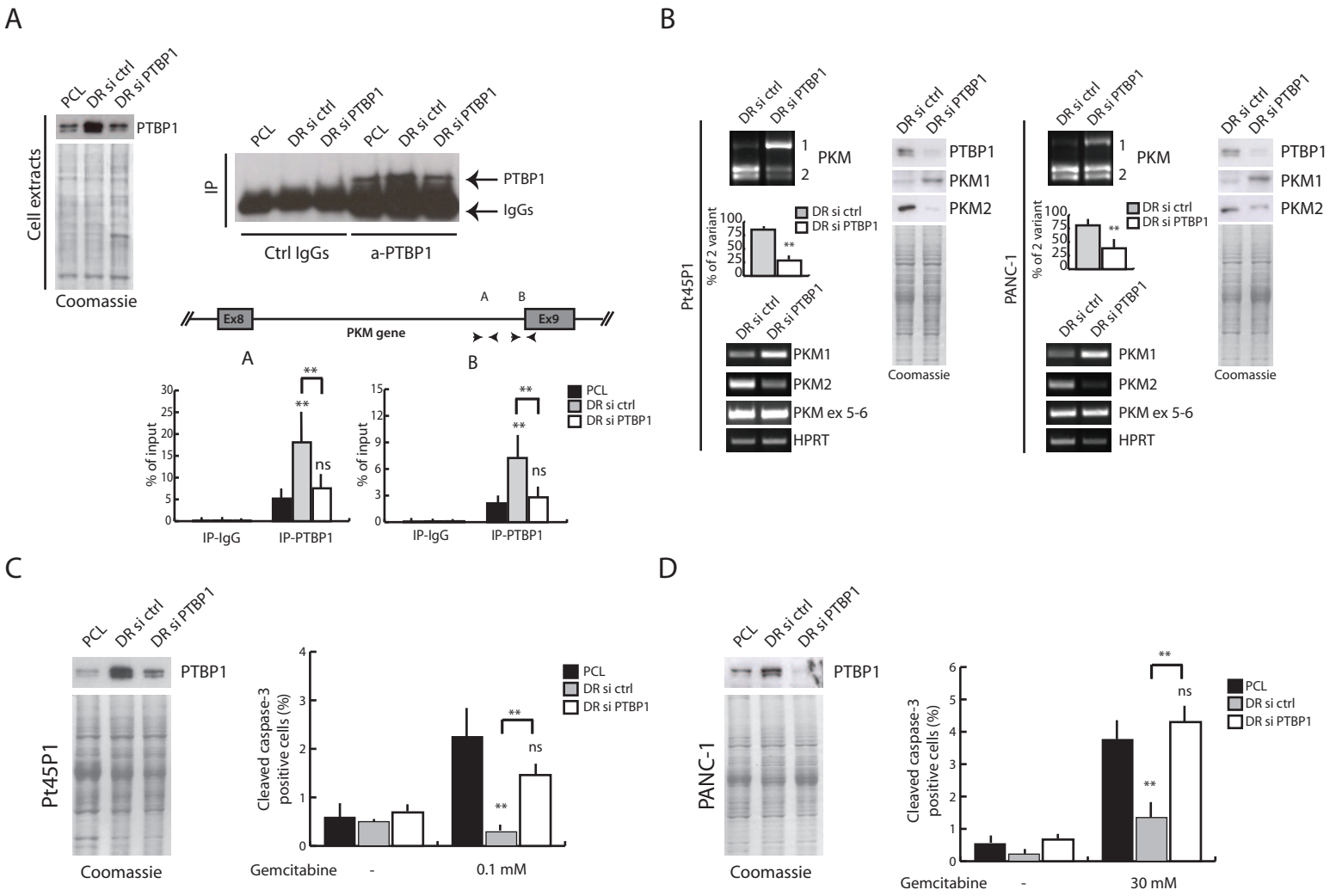

A
PCL
DR si ctrl
DR si PTBP1
PTBP1
Cell extracts
Coomassie
IP
PTBP1
IgGs
Ctrl IgGs
a-PTBP1
Ex8
Ex9
PKM gene
A
B
% of input
IP-IgG
IP-PTBP1
ns
B
Pt45P1
PKM
1
2
% of 2 variant
PKM1
PKM2
PKM ex 5-6
HPRT
PANC-1
C
D
Cleaved caspase-3 positive cells (%)
Gemcitabine
0.1 mM
30 mM

Figure 6