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Title: The cornerstone K-ras mutation in pancreatic adenocarcinoma: from cell signaling network, target genes, biological processes to therapeutic targeting

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The cornerstone K-RAS mutation in pancreatic adenocarcinoma: from cell signaling network, target genes, biological processes to therapeutic targeting

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1. Introduction: RAS GTPase and mutations

RAS super family belongs to the small G protein class that includes more than 150 guanosine tri-phosphate hydrolases (GTPase) (1). This highly conserved protein family plays major roles in signal transduction from membrane receptors within the cell (2,3). The family is divided in 5 main sub-families: RAS, Rho, Rab, ARF and Ran (2).

Among them, RAS proteins are mainly involved in the regulation of proliferation, differentiation, metabolism and survival genes. Among this family, the most characterized are H-RAS, N-RAS, and two isoforms K-RAS4A and K-RAS4B that originate from alternate splicing of the exon 4 of the *K-RAS* gene. Mostly, K-RAS designates K-RAS4B which expression is ubiquitous whereas K-RAS4A expression is more variable in the different tissues (4).

The RAS-family proteins are small 188-189 aa-21kDa GTPases with a very similar three dimensional structure. These proteins harbor two domains and a central region (5): The N-terminal G domain (aa 1-165) is highly conserved and includes a P-loop that binds phosphate, an effector binding domain (aa 32-40) and two switch regions (aa 32-38 and aa 59-76) that are responsible for conformation changes (**Figure 1A**). The central region (aa 85-165) displays 85-90 % homology across the RAS GTPase super family. The C-terminal hyper variable region (HVR) (aa 165-185) controls the membrane fixation and acts as auto-inhibitory domain. The CAAX final sequence is farnesylated on the cysteine residue allowing membrane anchoring. In addition, K-RAS4B HVR domain also contains a lysine-rich sequence that stabilizes the protein at the membrane.

In its inactivated state; K-RAS protein is located at the cell membrane internal surface and is bound to a guanosine-di-phosphate (GDP) (**Figure 1B-C**). Activation of a membrane receptor leads to the recruitment of the RAS Guanine Exchange Factor (RASGEF) SOS1 *via* SH2 and SH3 domains of adaptors. This allows SOS1 interaction with RAS and induces GDP dissociation. The free RAS protein then quickly binds to a guanosine-tri-phosphate

(GTP) molecule, propagating the signal in the cell activating K-RAS target genes (6). However, RAS activation is transient because its GTPase activity hydrolyses GTP to GDP and goes back to the inactivated state. The GTP hydrolysis is promoted by a GTPase activating protein (GAP) that allows normal K-RAS activation/inactivation cycle (1,6) (**Figure 1C**). In the GDP-bound state, the catalytic domain of K-Ras4B interacts with HVR maintaining auto-inhibitory state that will be reverted upon activation (7).

RAS is also regulated by its cellular distribution and notably by its clustering in lipid rafts called nanoclusters (8). This modulation of the local phospho-lipid content subsequently alters RAS/RAF/MAPK signaling. According to its activation state, RAS forms nanocluster and allows efficient recruitment of effectors. The number of nanoclusters is proportional to the activation signal intensity. RAS conformation changes during GDP/GTP cycling: GDP-bound K-RAS is associated with the membrane via its HVR domain whereas GTP-bound is partially detached. Furthermore, GTP or GDP-bound RAS associates with different kind of phospholipids and therefore alter lipid content of the nanocluster (9,10). Interestingly, H- and N-RAS isoforms each form nanoclusters that do not non-overlap. This distribution could explain differential activation of signaling pathways despite their high homologies (11). While being mainly localized at the plasma membrane, K-RAS can also be phosphorylated on S181 by PKC leading to its delocalization on the mitochondrial membrane where it interacts with Bcl^{XL} and induces apoptosis (12). This Ser181 phosphorylation is inhibited by calmodulin (13).

2. K-RAS mutation and PDAC: So it begins.

Mutations of *K-RAS* gene are detected in 27% of cancers (COSMICv78 database <http://www.sanger.ac.uk/genetics/CGP/cosmic/>). These mutations (97-99 %) affect glycine12 (G12), glycine13 (G13) or glutamine61 (Q61) residues (**Figure 1D**). G12 residue mutation sterically blocks the orientation of the Q61 that is essential for RAS/GAP interaction, leading

to an accumulation of GTP-bound RAS unable to hydrolyze the GTP and hence maintaining the active conformation (14,15). G13 amino acid mutation enhances the flexibility of GTP binding area and leads to faster GTP/GDP cycle and impaired sustaining of activation (15). The Q61L mutation interferes with the nucleophilic attack on the γ -phosphate of GTP and impairs its hydrolysis (16).

In pancreatic adenocarcinomas (PDAC), one of the most deadly cancers in occidental countries (17), mutations of *K-RAS* gene are nearly systematic and are associated with a bad prognosis (18,19). Analysis of cBioPortal pancreatic adenocarcinoma samples confirmed the alteration of *K-RAS* in 89.8-94.9% of 740 cases out of 4 independent studies (ICGC, UTSW, TCGA and QCMG 2016). Recently, Bailey and colleagues confirmed that *K-RAS* activation occurs in more than 90% of PDAC in a wide transcriptional analysis clustering 96 tumors with high epithelial content (20). Interestingly, genes defined as “upregulated in overexpressing oncogenic *K-RAS* cells” were enriched in the aberrantly differentiated endocrine/exocrine subtype (ADEX) cells whereas squamous cells, previously named quasi-mesenchymal, harbor lesser enrichment of those genes (20,21).

The mouse model developed by D. Tuveson using the Cre-Lox strategy using *Pdx1* (key transcription factor in pancreatic fate) promoter-driven, displays the pancreatic tissue specific expression of a constitutively active mutant K-Ras^{G12D} (22-24). In this model that harbors Cre-recombination in all pancreatic cell lineages, expression of the K-Ras^{G12D} is sufficient to induce occurrence of Pancreatic Intraepithelial Neoplasia (PanINs) highlighting its major role in the initiation of pancreatic carcinogenesis. *Ptf1/P48-Cre* (exocrine Cre expression), *LoxStopLox(LSL)-K-ras^{G12D}* mice develop a similar spectrum of ductal and PanIN lesions (22). These K-ras^{G12D} mice are usually referred as KC mice. Using elastase promoter-driven recombinase Cre (acinar cells), Ji and colleagues showed that an increase above threshold of Ras activity intensity leads to senescence of acinar cells, promotes inflammation and induces fibrosis mimicking histologic features of human chronic pancreatitis (CP), acinar-ductal metaplasia and PanIN lesions (25). Once these lesions appeared, K-RAS remains an

essential actor to their survival and progress although a slow evolution that requires additional genetic events such as Trp53, Ink4a/ARF, Smad4 tumor suppressor mutations to form invasive adenocarcinoma (25-29). Characterization of an inducible oncogenic K-Ras model (*iK-ras p53^{L/+}*) showed that K-Ras^{G12D} is required for PDAC maintenance as K-Ras extinction (by doxycycline removal) led to rapid tumor regression and degeneration of stromal compartment (30). Other genetic events such as GNAS^{R201H} or loss of TIF1γ lead to distinct cancer phenotypes and notably promote the intraductal papillary mucinous neoplasm (IPMN)-to-PDAC progression (31,32). Once pancreatic adenocarcinoma is developed, K-RAS still participates in the tumor cell-proliferation, -survival and -migration as well as in chemoresistance abilities (28,33-35). Signature of K-RAS dependency/addiction genes was determined and was shown to be associated with epithelial differentiation (e.g. integrin-β6) (35) and corresponds to classical subtype described by Collisson (21). On the contrary, K-RAS independent phenotype, occurring in later stages of PDAC, is linked to a metabolic adaptation relying on increase mitochondrial adaptation (36).

Recently, the use of PDAC organoids cultures expanded and allowed new avenues in fundamental and clinical research regarding pancreatic cancer (37). Expression of mutant K-RAS protein in progenitor organoids induces morphological changes such as cystic organization with apically positioned nuclei that are consistent with early pancreatic tumor lesions (38). Therefore, these progenitor organoids are suggested as models to investigate early stages of cancer transformation. Organoids culture also allows study of normal pancreatic cells without immortalization and thus enables comparisons of normal and tumor cells retaining patient-specific traits. Organoids may be used to predict clinical responses and are essential for drug screening (38).

3. K-RAS is central in cellular signaling network

Activated K-RAS mutants participate in a tremendous diversity of biological events by interconnecting regulation of several signaling pathways (39,40) (**Figure 2**). The best characterized direct effectors lead to the activation of the Mitogen-Activated Protein Kinase (MAPK), Phosphoinositide 3-Kinase (PI3K) and RalGEF (Ral Guanidine exchange factor) pathways (41-48). Stress response (p38 and c-Jun N-terminal Kinase (JNK)) and Nuclear Factor-kappa B (NF- κ B) pathways are also induced by K-RAS activation, either by interconnection with other activated signaling pathways or by oncogenic stress signals such as production of reactive oxygen species (5,41,49) (**Figure 2**).

In pancreatic cancer, activation of the MAPK pathway is associated with a bad prognosis (50). Recruitment of RAF effector and subsequent activation of the ERKs pathway by K-RAS promotes proliferation and independent anchorage-growth of pancreatic tumor cells (25,51). ERKs activation is also involved in the survival/apoptosis balance (52) and in migration and invasion properties of pancreatic tumors (53,54). Finally, the activation of ERK1/2 MAPK pathway contributes to tumor cell chemoresistance (55) and to inflammation (56).

The implication of p38-MAPKs pathway is controversial in carcinogenesis. Although, this pathway promotes invasive abilities of pancreatic tumors (57), the detection of its activation is associated with a good prognosis (58). The effect of JNK activation is opposed to that of p38 MAPK pathway. The activation of JNK pathway by K-RAS promotes pancreatic tumor formation and cancer stem cell maintenance (59). The pathway is also involved in autophagic processes increasing survival of tumor cells (60). Finally, JNK pathway is involved in the occurrence of chemoresistance and invasion of pancreatic tumor cells (53,61).

The NF- κ B pathway, which is indirectly activated by K-RAS, is constitutively activated in pancreatic cancer (62). The canonical NF- κ B signaling leads to activation of p65/p50 complex and involves IKK complex (I κ B kinase). This cytoplasmic complex consists in IKK α and IKK β kinases associated with the protein NEMO (=IKK ϵ) (63,64). NF- κ B is mainly

described as activated by stress and pro-inflammatory signals but also displays important interconnections with other pathways. The NF- κ B pathway plays an essential role in carcinogenesis and inflammation through an amplification loop of K-RAS activity (65-67). The NF- κ B is also involved in invasion and metastases properties of the pancreatic tumor cells (68).

PI3Ks are a family of heterodimeric kinases composed of p110 catalytic and regulatory subunits that lead to phosphatidylinositol triphosphate (PIP3) production and activation of downstream signals and biological processes (69). The key downstream effectors of PI3K signaling such as Akt or GSK3 are activated in KC mice (70). PI3K signaling enhances acinar-ductal metaplasia (ADM) that generate duct from acinar cells. These ADM lesions will evolve towards PanIN lesions (71). Pancreas-specific invalidation of p110 α isoform (kinase dead) prevent occurrence of all type of lesions. Interestingly, pancreatic invalidation of the other p110 β isoform did not affect PanIN formation in an activated K-RAS context (72). K-RAS4B isoform can also interact with calcium modulator protein calmodulin via HVR and modulates preferentially PI3K/Akt rather than RAF/MEK/ERK pathway (73).

The RalA/B pathway, initially described as a minor actor in transformation in rodents, is an essential component of RAS transformation in human cells (41,74,75). In pancreatic cancer, Ral GTPases are activated by K-RAS and are involved in anchorage-independent growth and survival (76,77). RalA promotes tumor initiation whereas RalB is essential for invasion and metastasis (76). Ral GTPase pathway is also involved in pancreatic tumor chemo- and radio-resistance (78,79).

Overall, more than 100 proteins containing putative RAS-association (RA) or RAS binding domains (RBDs) were described (41,80) (**Figure 2**): T-lymphoma invasion and metastasis protein-1 (Tiam1) is a Rac-GEF harboring a RBD and activates JNK pathways. RAS association domain family (RASSF) contains a RAS domain lacking catalytic function. RASSFs act as tumor suppressors and are involved in apoptotic processes. Nore1 binds to

RAS-like GTPases and heterodimerizes with RASSFs (81). The RAS inhibition (RINs) GEF links RAS signaling and receptor-mediated endocytosis via the activation of Rab5 (82). RAS was also proposed to allow convergence of signals from PLC, AF6 and PKC ζ (41).

Among pathways undergoing genetic alterations in PDAC, K-RAS was also linked to Wnt and Hippo pathways. K-RAS is able to repress the Frizzled 8 (Frz8) and CaMKII-mediated non-canonical Wnt/Ca²⁺ signaling pathway via direct calmodulin binding (83) thereby relaxing the suppression of the canonical Wnt signaling, which occurs by blocking β -catenin/TCF4 interaction (84,85). Indeed, enhanced Wnt/ β -catenin signaling has been observed in human PDAC tissues. The Hippo/MST1/2 pathway includes the coactivator Yes-associated protein (YAP) which is abundantly expressed in the PanIN lesions of *p48-Cre; LSL-K-ras^{G12D}* mice. The Hippo/MST1/2 pathway plays a crucial role in regulating tissue homeostasis and organ size (86). The oncogenic K-Ras–MAPK pathway induces post-transcriptional modification of YAP that inactivates YAP-mediated signaling (87). However, YAP is also associated with K-Ras^{G12D}-independent tumor maintenance (88).

4. K-RAS regulates a great diversity of biological processes

4.1. Understanding K-RAS and intrinsic biological properties of PDAC cells: the contribution of transcriptomes

As K-RAS mutations control a wide array of cell signaling pathways (**Figure 2**), it was expected that these mutations might regulate a tremendous diversity of genes involved in every biological property that defines hallmarks of cancer (proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis) (89). As previously described, the *K-Ras^{G12D}* mutant mouse model is associated with 100 % occurrence of PanINs highlighting the mandatory role of K-Ras in the initiation of pancreatic carcinogenesis (22). Characterization

of mice expressing reversible iKras^{G12D} indicates that K-Ras is also mandatory for tumor survival and maintenance (27).

Moreover, K-Ras regulates the abilities of tumor cell chemoresistance (90). Indeed, K-Ras is described as central in the proliferation regulation circuit but is also connected with motility and viability regulatory circuits. At this stage, microarray-based transcriptome analysis allowed a global view of K-ras target genes in pancreatic cancer. These genes are involved in functions such as transcription, proteolysis, cell proliferation, cell death, adhesion, cell surface receptor or intracellular signaling (91). In **Table 1**, we present the gene ontology clustering obtained by analyzing three available PDAC expression data sets retrieved from Gene Omnibus Expression database and compared the mutated vs wild type K-ras transcriptomes. PDAC bearing *Pdx1-Cre ; K-ras^{G12D}* mice show a strong enrichment in gene clusters involved in protein glycosylation, inflammatory response, extracellular matrix or protease activity, (GSE53695, (92)). Ablation of K-Ras in mouse pancreas, using a tetracycline-inducible *K-Ras* allele, leads to an important enrichment of intracellular organelle genes and a mild enrichment of genes involved in non-coding or ribosomal RNA processing and protein trafficking (GSE58307, (36)). The succession of K-ras activation-inactivation-reactivation in pancreatic tumor spheres also revealed an enrichment of genes involved in metabolic pathways and notably highlighted an increased mitochondrial activity (36). The surviving cells following K-Ras-ablation harbor an increase oxidative phosphorylation (OXPHOS) similarly to what is observed in patients resistant to MEK or PI3K targeting highlighting the promise of combined K-Ras and mitochondrial respiration targeting (36). Analysis of deregulated genes in K-Ras addicted pancreatic cancer cell lines revealed enrichment of gene expression signature involved in various biological processes such as metal and ion bindings, apical and basal plasma membrane cytoskeletal protein binding, fatty acid metabolic process and vesicular cell systems (GSE15126, (35)). K-Ras dependency signature genes such as SYK, integrin- β 6 (ITGB6) and MST1R are related with both epithelial differentiation state and well- to moderately-differentiated tumor phenotype (35).

Altogether this highlights the intrinsic pivotal role of K-Ras activation in pancreatic cancer cells and how it is linked to tumor growth and metastasis (**Figure 3**).

4.2. Cell metabolism reprogramming

A common feature of cancer cells is described as the Warburg effect which is an increase in glucose uptake and a shift from mitochondrial oxidative phosphorylation to aerobic glycolysis (93). This metabolic shift allows generating energy and nutrients in order to survive in a generally hypoxic and energetic inhospitable environment. Indeed, K-RAS-transformed human cells show glucose and glutamine metabolism alteration (94-96). K-Ras^{G12D} extinction alters multiple metabolic pathways. Notably, K-Ras^{G12D} promotes the glycolytic flux by increasing glucose uptake and lactate production through increase of glucose transporters (*glut1/Slc2a1*), crucial glycolytic enzymes (*Hk1*, *Hk2*, *Pfk1*, *Ldha*), enzyme of the hexosamine pathway (*Gfpt1*) and non-oxidative pentose phosphate pathway (PPP) enzymes (*Rpia* and *Rpe*). These genes are regulated at the transcriptional level *via* MAPK and Myc signaling pathways, both affected by K-Ras activation. Moreover, K-ras^{G12D} activates the hexosamine biosynthesis pathway (HBP) affecting protein O-glycosylation and non-oxxydative PPP generation for ribose production (30). Interestingly, lactate that is produced by glycolytic cells in hypoxic area is used for normoxic cells growth in a paracrine manner (97). PDAC cells harbor an increased expression of lactate transporter MCT4 that reflects glycolytic activity and promotes cell survival and tumorigenic growth (98). K-Ras activation also contributes to stimulating glutamine (Gln) metabolism by transcriptional repression of *glutamate dehydrogenase* (*GLUD1*) and induction of *aspartate aminotransferase* (*GOT1*) expression and thus coordinates the switch to Gln metabolism that is critical to maintain tumor growth and survival probably *via* the redox balance (99). The oncogenic Reactive Oxygen Species (ROS) levels are tightly regulated by Nrf2 transcription factor (100). K-ras^{G12D} drives the increase of *Nrf2* transcription *via* RAF/MEK/ERK/Jun pathway. This Nrf2 increase promotes

the antioxidant program (*Hmox1*, *Nqo1*, *Gclc*, *Gclm*, *Ggt1*) and decreases the intracellular ROS, thus favors a reduced intracellular redox microenvironment. The characterization of *Nrf2*^{KO} mice showed that activation of ROS by K-Ras/Nrf2 axis contributes to pancreatic carcinogenesis initiation (101). K-Ras-activated organoids also exhibit an increased activation of translation machinery. Combined K-Ras activation and loss of Nrf2 led to oxidation of factors involved in eIF4F complex (involved in cap-dependent mRNA translation), altered EGF and Akt signaling pathways and consequently impaired protein synthesis and maintenance of pancreatic tumors (102). This work highlights the promise of synthetic lethality of combined AKT inhibitors with oxidizing agents in PDAC.

K-Ras also stimulates recycling process such as macrospinocytosis or autophagy. Macrospinocytosis is the uptake of nutrients via endocytosis leading to accumulation of catabolic intermediates that stimulates carbon metabolism and sustains cell proliferation (103). Autophagy is a process addressing cytoplasmic constituents to lysosomes for degradation and is critical for the maintenance of late stages of PDAC. *K-ras*^{G12D} mice develop PanIN lesions harboring markers of autophagosomes (LC3 puncta) in an Atg5/Atg7 dependent manner (104). Therapeutic targeting of autophagy by chloroquine could be promising. It was initially debated because homozygous *Trp53* deletion led to paradoxical pro-carcinogenic effect following autophagy inhibition suggesting *p53* status dependency (104-106). However, autophagy inhibition is in fact not affected when using the more clinically relevant model harboring *Trp53* loss of heterozygosity as usually observed in patients (107).

4.3. Tumor microenvironment and immune system

Oncogenic RAS alters the tumor microenvironment by promoting angiogenesis and by modulating immune responses. Immune cell infiltration is classically observed in the tumor stroma of PDAC patients. *LSL-K-Ras*^{G12D} immune compartment was characterized and a

high fibro-inflammatory reaction containing both stromal and immune cells was thus shown to emerge within the tumor. Regulatory T cells infiltrate early in disease progression of pancreatic cancer, notably before invasive stage. Disease progression was also accompanied by infiltration of macrophages (early) and Myeloid-derived Suppressor Cells (MDSC) (late) (108). K-Ras activation was previously shown to foster inflammation through chemokine production (CXCL1/KC, CXCL2/MIP2, CXCL5/LIX and CXCL8/IL8) in lung or breast cancer cells (109,110). IL6 secretion is the most characterized cytokine in PDAC as it is induced both by the tumor cells and the myeloid cells from the surrounding stroma and is associated with tumor survival (111-114). Different fibroblastic and epithelial cell types transfected with oncogenic K-RAS^{G12D} had an increased secretion of IL6 which is required for human tumor cell growth *in vivo* (115). However, among the different cell types, the macrophages were the principal source of IL-6 in PDAC. Paracrine IL6 induces strong phosphorylation of *signal transducer and activator of transcription 3* (STAT3) that promotes PanIN-PDAC progression in *K-Ras*^{G12D} mice (111). In acute pancreatitis, treatment with a RAS inhibitor (farnesylthiosalicylic acid) decreases levels of CXCL1, CXCL2 and IL6 and regulates neutrophil recruitment (116). Zhang and colleagues showed that the K-Ras secretome (CTGF, Cyr61, Cox2, mmp7, IL1A and IL6) of pancreatic cancer cells is regulated by YAP mediated transcriptional activity (87). Its invalidation in *Yap*^{flox/flox} mice leads to a lack of CD45 lymphocyte infiltration, compromises the activation of stromal fibroblasts and their collagen secretion.

PanINs also produce granulocyte-macrophage colony-stimulating factor (GM-CSF) which subsequently leads to recruitment of Gr1(+)CD11b(+) myeloid cells suppressing anti-tumor CD8+ T-cell immunity (117,118). These mice also harbor a high expression of receptor-interacting-1 (RIP1) and RIP3 kinases that are the main components of a macromolecular signaling complex called the necrosomes mediating the programmed necrose (necroptosis). Necroptotic tumor cells release soluble factors that induce peri-tumoral immune suppression. This immune-suppressive microenvironment relies on the RIP1/3 signaling *via* CXCL1 and

the C-type lectin receptor Mincle signaling (activating NF- κ B) to favor tumor progression (119).

Angiogenesis occurs in response to tumor hypoxia and lack of nutrients and is essential for tumor growth. Angiogenesis is defined as the formation of new blood vessel and involves an equilibrium of angiogenic and anti-angiogenic factors (120). VEGF which appears to be critical in pancreatic angiogenesis (121) is correlated with detection of K-RAS mutation and is associated with a poorer prognosis (122). The oncogenic K-RAS^{G12V} induces VEGF, CXC chemokines and COX2 promoting human umbilical vein endothelial cells (HUVEC) invasion and tumor formation *via* MEK/c-jun pathway (123). Similarly, in colorectal cancer cells, disruption of mutant K-RAS led to a reduced VEGF production (124). In addition, chemokine CXCL8/IL8 was also shown to drive the RAS-induced tumor angiogenesis (110).

Oncogenic K-RAS is crucial in the crosstalk with stroma and notably pancreatic stellate cells (PSC). Additionally to K-RAS cell autonomous effect, both cell types set up a reciprocal signaling involving Sonic hedgehog (SHH) mediated activation of PSC and reciprocal signals *via* IGFR1/AXL and Akt activity that leads to increased mitochondrial performance, proliferation and resistance to apoptosis (125).

4.4. Mucins

Mucins belong to a heterogeneous group of large O-glycoproteins (secreted or membrane bound) that are expressed by epithelial cells. The extended structure of mucins extracellular domains confer them a role of molecular sensors and in cell–cell, cell–extracellular matrix interactions and in cell signaling. MUC1 and MUC4 were extensively described as key promoters of pancreatic carcinogenesis (126-129). Mucus-producing cells normally restricted to respiratory and intestinal epithelia are observed in PanINs of the *K-Ras*^{G12D} mouse model (22). Indeed, membrane-bound Muc1 and Muc4 and secreted Muc5ac are aberrantly expressed during pancreatic cancer progression (PanINs, primary tumors and metastases)

(130). Interestingly, mucin expression is correlated with an increase of inflammatory cytokines IFN- γ , CXCL1 and CXCL2. Recently, we also showed that MUC4 is a target of K-Ras^{G12D} mutation *via* both transcriptional (p42/44 MAPK and p65 NF- κ B) and post-transcriptional (RalB) mechanisms (131). Therefore, we hypothesize that mucins, as K-RAS target genes, could be promising therapeutic tools for gene therapy and immunotherapeutic approaches in pancreatic cancer (126).

4.5. miRNA-mediated gene regulation

MicroRNAs (miRNA) are 22-24 nucleotides-long non coding RNAs that regulate expression of mRNA mostly by binding their 3'-untranslated region (UTR) and emerge as major post-transcriptional regulatory mechanisms with potential therapeutic interest. MiRNA expression pattern is profoundly altered in carcinogenesis where they can act as tumor suppressor or oncomiR depending on the panel of their targets (132-135). Laser capture microdissection of PanIN tissues of *K-Ras*^{G12D} mice led to the identification of miR-21, miR-205 and miR-200 as all induced by K-Ras (136). In addition, miR-155, that is also induced by K-Ras, mediates cell proliferation through ROS accumulation (49). On the contrary, the tumor-suppressors miR-29a, miR-330-5p and miR-219-1-3p are decreased in PanIN lesions (137,138). Finally, some miRNA, such as miR-96, miR-217 and Let7 were shown to directly regulate K-Ras expression or alter its associated signaling pathways and could be considered as potential therapeutic strategies (139-141).

5. K-RAS as a therapeutic target

As a central point of pancreatic carcinogenesis, K-RAS is an obvious and bona fide therapeutic target. In the next section, we describe strategies that were designed at various levels: (i) direct targeting of the K-RAS protein, (ii) indirect K-RAS targeting of the protein

location and (iii) targeting K-RAS oncogenic activity by interfering with its downstream effectors (142) (**Figure 4**).

5.1. Direct targeting of K-RAS activity

The first strategy to inhibit K-RAS expression was the development of small antisense nucleotides sequences directly targeting *K-RAS* mRNA. The ISI6957 oligonucleotide showed efficient inhibition of K-RAS expression *in vitro* (143). However, its stability and the strong toxicity did not allow a clinical use.

Despite of this failure, the strategy was optimized and Khvalevsky and colleagues developed a local prolonged siRNA delivery system (Local Drug EluteR, LODER) (144). Use of a biodegradable polymer, allowing RNA protection from degradation, reduces the toxicity. Although encouraging, this strategy needs to be further improved and therefore is still not clinically usable (145).

A specific and irreversible inhibitor of the K-RAS^{G12C} mutant induces an internal steric bulk blocking the conformational change necessary for activation (146,147). However, the G12C mutation remains rare in pancreatic cancer (3 % of K-RAS mutations) and other compounds targeting the G12D and G12V main mutants remain to be developed. The compound ARS-853 reacts with GDP-bound K-RAS^{G12C} isoform, and alters downstream signaling, and cell survival in K-RAS^{G12C} cell lines (148). ARS-853 is thought to impair normal SOS recruitment and strengthens the SOS targeting strategy that was previously developed thanks to the knowledge of well-defined binding pockets (149,150).

Targeting K-RAS-GTP interaction is considered as not feasible because of the high affinity of K-RAS for the GTP (pico molar range) and the strong intracellular concentration of GTP (milli molar range). An alternative strategy was to modify the GTP in order to make it hydrolysable by the mutant. The GTP analog DABP-GTP (Diamino-benzophenone-phosphoroamidate-

GTP) can be hydrolyzed by G12 mutants more effectively than by wild type K-RAS (151). However, no compound derived from this strategy has passed beyond the *in vitro* studies.

5.2. Indirect targeting of K-RAS oncogene by altering its localization

5.2.1. Prenylation inhibitor

Other therapeutic strategies target post-translational modifications of the K-RAS protein, necessary for its membrane location. The first post-translational modification of K-RAS proteins is a modification of its HVR region. The farnesyl transferase enzyme (FTase) mediate farnesylation/prenylation on the cysteine of the terminal CAAX sequence adding a hydrophobic chain allowing membrane stabilization (152). Numerous inhibitors of FTase were developed to target this modification and inhibit K-RAS addressing to the membrane (153,154). The non-peptidomimetic competitive FTI, Tipifarnib (R115777), was tested in phase III trials in pancreatic cancer (155,156) but was ineffective because of an unexpected alternative prenylation mechanism (geranylgeranylation) involving geranylgeranyl transferase (GGTase) (157). GGT-I can recognize the CAAL motif and geranylates K-RAS4B. Combined therapies using inhibitors of FTase and GGT-I display high toxicity precluding their use in clinic (158).

5.2.2. Icmt1 inhibitors

The K-RAS protein undergoes a cleavage of the AAX terminal sequence followed by a methylation of the formed isoprenylcysteine. These two stages are successively catalysed by two enzymes: The endoprotease RAS converting enzyme 1 (Rce1) and Isoprenylcysteine carboxymethyl transferase 1 (Icmt1), respectively. The targeting of these two key enzymes was investigated. Since Rce1 inhibition induces severe cardiomyopathies, the efforts focused on Icmt1 inhibition (159,160). Icmt1 targeting was developed in cancers and in particular in

pancreatic cancer. However, *Idh1* inhibition dramatically accelerated the development of K-Ras-driven pancreatic neoplasia because of its requirement for Notch1 signaling (161).

5.2.3. Deltarasin, the PDE δ inhibitor

K-RAS addressing to the plasma membrane involves the hydrophobic prenylation trafficking process by the prenyl-binding chaperon protein: the phospho diesterase δ (PDE δ / PDE δ). Deltarasin, an inhibitor of the PDE δ , showed promising results. Indeed, Deltarasin blocks PDE δ -K-RAS interaction preventing membrane localization in human K-RAS mutated pancreatic tumor cells, hence inducing a fast decrease of proliferation and increased apoptosis. In vivo, pancreatic tumor cells xenografts showed a dose dependent reduction of tumor growth following Deltarasin regimen (162,163). Clinical use of this recent strategy remains to be proven.

5.3. K-RAS downstream pathway targeting

A large number of inhibitors targeting components of K-RAS oncogene downstream signaling pathways were developed (164-166). Targeted therapies were focused on the MAPK pathway and led to the development of RAF and MEK inhibitors (167,168). In the context of pancreatic cancer, RAF inhibitors were ineffective. This may be related to the paradoxical induction of increased MAPK activity via a decreased feedback regulation as it was demonstrated with PLX4032 in melanoma (169). RAF inhibitors lack of effectiveness could be related to the binary recruitment of RAF by activated K-RAS in nanoclusters (10). MEK kinases were also targeted by compounds such as CI-1040 but induced only partial response in pancreatic cancers independently of the K-ras mutation status (170). Another MEK inhibitor, Selumetinib (AZD6244), led to promising results on preclinical models but eventually failed in the clinical trial as it showed not significant benefit as second line

treatment (171-173). Pimasertib is associated with sensitivity to gemcitabine and is currently under clinical evaluation (174). These failures are probably due to strong interconnections between signaling pathways (166,167). As MAPK and PI3K seem to be the major deregulated signaling pathways, combined MEK-PI3K inhibition is under evaluation in a cellular and mouse models of pancreatic cancer (173,175). PI3K targeting alone by using Evolorimus (mTOR inhibitor) has been evaluated in phase 2 clinical trial but only showed minimal clinical benefit (176). Very recently, Rigosertib, a RAS mimetic that is evaluated in clinical trials for myelodysplastic syndrome associated with acute myeloid leukemia, has been proposed for PDAC treatment. Rigosertib binds to RBD and thus inhibits the interaction of RAS-RAF, Ral and PI3K, blocks the activation of the RAS-RAF-MEK pathway and impairs tumor growth in *K-Ras^{G12D}* mice (177). PKC inhibitor bryostatin-1 blocks K-RAS-S181 phosphorylation by PKC and induces apoptosis by inducing the K-RAS delocalization onto the mitochondrial membrane (178). Inhibition of other pathways, such as JNK and NF- κ B was also investigated. However, most of these treatments are still either in experimental stages and are not yet used routinely in clinics (59,164,168,179-183).

5.4 Synthetic lethal interaction with K-RAS

An alternative strategy that has been developed is to identify new therapeutic target linked to K-RAS status: RNAi screening were performed in cell lines from different organs that were either K-RAS wild-type or K-RAS mutated in order to select genes that specifically impair viability of K-RAS mutated cells (153). Hit-genes involved in different biological processes were identified such as genes involved in survival signals (TAK1, TBK1), transcriptional program (GATA2, SNAIL2), chromosomal stability (Survivin, TPX2, PLK1, APC/C, proteasome) and apoptosis/senescence (WT1, Bcl^{XL}). Future research will be necessary to validate those targets in PDAC. Following the initial screening, the top candidates including GATA2 and PLK1 were further evaluated as new therapeutic targets (184,185). Very few

analyses were focused on pancreatic cancer. 187 genes were identified and were enriched with the immune system, the ETS and the LAIR pathways (186).

6. Conclusions

More than 50 years after the inception of RAS research in 1964 showing that a retroviral isolate induced sarcoma; and the beginning of RAS characterization in the early 80s; the scientific community has produced huge efforts in deciphering RAS structure, biological functions, cell-signaling mechanisms and identifying target genes in patho-physiological conditions (187). K-RAS oncogene has been proven to be a central driver in cancers. K-RAS mutation, as the earliest event in pancreatic cancer, could be seen as the “starting pistol” of carcinogenesis sequence. Targeting K-RAS is an obvious goal but it has been an unfortunate clinic failure despite many different strategies (153) highlighting the urgent need for new approaches. Targeting cell adaptative metabolic mechanisms occurring after K-RAS activation *via* KPM2 or the non-specific OXPHOS inhibitor Metformin is promising (188). Recently, checkpoint inhibitors/immune modulators such as antibodies targeting the programmed cell death protein 1 (PD-1) are promising targets in gastrointestinal cancer, including pancreatic cancer, and are a hot topic in the field (189). Based on this literature review, one can suggest that targeting K-RAS effectors (cell signaling or miRNA) or some crucial target genes may be a potential alternative strategy. Lately, FOLFIRINOX and Nab-Paclitaxel emerge as new standard of care but the overall survival still does not exceed one year (190,191) and pancreatic cancer remains one of the deadliest cancers; Therefore, no option should be excluded.

Conflicts of interest: Authors declare no conflict of interest

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Figure legends

Figure 1: Schematic representation of K-RAS GDP/GTP cycling and its mutations (A)

Schematic K-RAS4B protein. β : β -strand, α : helix- α . HVR: hypervariable region, KKKK: lysine rich region. * indicates the cysteine residue that is farnesylated. CAAX represents a sequence in which C= cysteine, A= aliphatic amino acid (Leu, Ileu or Val), X= Met, Ser, Leu or Gln. (B) 3D structure of GDP-bound Human K-RAS (4OBE) from protein data bank (<http://www.rcsb.org/pdb>) (C) K-RAS GTPase cycle. K-RASG12 mutation induces an accumulation of GTP-bound proteins unable to hydrolyze GTP and maintains the active conformation. (D) Frequency of K-RAS G12, G13, Q61 and other mutations in pancreatic cancer.

Figure 2: K-RAS effectors and signaling pathways

Figure 3: K-RAS mutation and biological alterations of PDAC cells and tumor microenvironment

Figure 4: Therapeutic strategy targeting K-RAS and its post-transcriptional modifications. FTase: Farnesyltransferase, FTI: FTase inhibitors, GGTase: Geranylgeranyltransferase, GGTI: GGTase inhibitors, Me: methyl, DABP-GTP (Diamino-benzophenone-phosphoamidate-GTP), Rce1: RAS converting enzyme 1, Icmt1: Isoprenylcysteine carboxymethyl transferase 1, PDE6 δ : phospho diesterase 6 δ .

Table1: Available PDAC data set retrieved from Gene Omnibus Expression comparing mutated K-RAS vs wild type K-RAS. GSE15126, GSE53659, GSE58307 transcriptome were analysed with GEO2R. Gene ontology clustering was subsequently performed using David Functional Annotation Tool (<https://david.ncifcrf.gov/>).

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Table

Cluster	Gene ontology	Enrichment score
K-RAS dependant vs K-RAS independant PDAC cell lines, GSE15126		
Cluster 1	metal-binding, metal ion binding, cation binding, ion binding, zinc, transition metal ion binding, zinc ion binding, zinc-finger	2.11
Cluster 2	basolateral plasma membrane, apical part of cell, apical plasma membrane	1.8
Cluster 3	actin binding, cytoskeletal protein binding, cytoskeleton	1.55
Cluster 4	icosanoid metabolic process, unsaturated fatty acid metabolic process, fatty acid metabolic process	1.53
Cluster 5	cell fraction, membrane fraction, insoluble fraction, vesicular fraction, microsome, endoplasmic reticulum, endoplasmic reticulum	1.5
K-Ras ablated cells vs 24h K-Ras expressing cells (<i>mus musculus</i>) GSE58307		
Cluster 1	Nucleolus, nuclear lumen, ribosome biogenesis, intracellular organelle lumen, organelle lumen, ribonucleoprotein complex biogenesis, membrane-enclosed lumen, non-membrane-bounded organelle, intracellular non-membrane-bounded organelle	8.6
Cluster 2	ribosome biogenesis, ribonucleoprotein complex biogenesis, ncRNA processing, rRNA processing, rRNA metabolic process, ribosome biogenesis, ncRNA metabolic process, RNA processing, RNA binding	3.95
Cluster 3	ncRNA processing, ncRNA metabolic process, trna processing, tRNA processing, tRNA metabolic process	2.68
Cluster 4	nucleocytoplasmic transport, nuclear transport, protein import, protein targeting, protein import into nucleus, protein localization in organelle, nuclear import, protein localization in nucleus, cellular protein complex assembly, pore complex, protein import into nucleus, docking, Importin-beta, N-terminal, domain: Importin N-terminal, nuclear pore, intracellular transport, protein complex assembly, protein complex biogenesis, Armadillo-like helical, intracellular protein transport, cellular macromolecular complex assembly, protein polymerization, cellular protein localization, cellular macromolecule localization, cellular macromolecular complex subunit organization, macromolecular complex assembly, macromolecular complex subunit organization, protein transporter activity, protein transport, establishment of protein localization, nuclear envelope, protein localization, protein transport, endomembrane system, organelle envelope, envelope	1.71
Cluster 5	Methyltransferase, RNA methyltransferase activity, binding site:S-adenosyl-L-methionine, s-adenosyl-L-methionine	1.41
WT vs bitransgenic Pdx1-cre/K-Ras^{G12D} mice bearing Pancreatic Ductal Adenocarcinoma, GSE53659		
Cluster 1	signal peptide, disulfide bond, glycoprotein, glycosylation site : N-linked (GlcNAc...), disulfide bond, secreted, extracellular region	12.43
Cluster 2	defense response, response to wounding, inflammatory response	4.41
Cluster 3	regulation of cell-substrate adhesion, proteinaceous extracellular matrix, extracellular matrix, regulation of cell adhesion, positive regulation of cell-substrate adhesion, extracellular matrix, positive regulation of cell adhesion, extracellular matrix organization, extracellular structure organization	2.91
Cluster 4	pattern binding, polysaccharide binding, glycosaminoglycan binding, carbohydrate binding, heparin binding, heparin-binding	2.8
Cluster 5	domain: Peptidase S1, Peptidase S1A, chymotrypsin, Peptidase S1/S6, chymotrypsin/Hap, active site, propeptide: Activation peptide, Peptidase S1 and S6, chymotrypsin/Hap, active site: Charge relay system, Tryp_SPc, serine proteinase, serine-type endopeptidase activity, zymogen, serine-type peptidase activity, serine hydrolase activity, PIRSF001135:trypsin, endopeptidase activity, Serine protease, hydrolase, peptidase activity, acting on L-amino acid peptides, peptidase activity, Protease, proteolysis	2.53
Cluster 6	Fibronectin, type III-like fold, Fibronectin, type III, FN3, short sequence motif:Box 1 motif, short sequence motif:WSXWS motif, cytokine binding, domain:Fibronectin type-III 2, cytokine receptor activity, domain:Fibronectin type-III 1, Short hematopoietin receptor, family 1, conserved site, Cytokine-cytokine receptor interaction, domain:Fibronectin type-III 4, domain:Fibronectin type-III 6, domain:Fibronectin type-III 3, Jak-STAT signaling pathway, domain:Fibronectin type-III 5	2.5
Cluster 7	Tumour necrosis factor-like, Complement C1q protein, domain:C1q,	2.15

	hydroxylation, C1Q, Collagen triple helix repeat, collagen, domain: Collagen-like	
Cluster 8	positive regulation of immune system process, negative regulation of lymphocyte activation, immune response, negative regulation of cell activation, negative regulation of leukocyte activation, immunoglobulin mediated immune response, B cell mediated immunity, lymphocyte mediated immunity, negative regulation of immune system process, immune effector process, adaptive immune response, adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, immune response, leukocyte mediated immunity, Systemic lupus erythematosus	2

Table1: Available PDAC data set retrieved from Gene Omnibus Expression comparing mutated K-RAS vs wild type K-RAS. GSE15126, GSE53659, GSE58307 transcriptome were analyzed with GEO2R. Gene ontology clustering was subsequently performed using David Functional Annotation Tool (<https://david.ncifcrf.gov/>).

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Highlights

- The early K-RAS mutation contributes to pancreatic carcinogenesis initiation
- K-RAS is central in cellular signaling network of pancreatic cancer cells
- Oncogenic RAS alters the tumor microenvironment and modulates immune responses
- K-RAS regulates genes that alter multiple metabolic pathways and generate energy
- So far, clinic failure of K-RAS targeting highlights the need of new approaches