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RAS And PKA pathways in cancer: new insight from transcriptional analysis

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1. ABSTRACT

Through its ability to regulate the activity of a large number of transcription factors, the Ras pathway is able to control several transcriptional programs leading to proliferation, differentiation, metabolism, cytoskeletal reorganization and immune response. Cyclic AMP (cAMP) is a ubiquitous intracellular second messenger whose major intracellular target in eukaryotes is protein kinase A (PKA). Wide evidence for cross talk between the Ras and cAMP-PKA pathways is available. After reviewing some features of Ras and PKA signalling that are relevant for cancer biology, we re-analyze available genome-wide expression data for genes encoding proteins of the downstream branch of the PKA pathway in human tumor cell lines as a function of the mutational state of the Ras pathway. The observed Ras-dependent pattern of regulation of the analyzed genes may contribute to explain how the cAMP-PKA axis is involved in oncogenic processes induced by Ras.

2. INTRODUCTION

Ras proteins belong to the large superfamily of small GTPases, which are activated in response to various extracellular stimuli. For instance, Ras proteins are activated in response to growth factor stimulation and subsequently bind numerous effector proteins leading to the activation of signalling cascades. The activation of Ras proteins is tightly controlled in normal cells, while defects in Ras signalling may result in malignant transformation. In fact, activating mutations of one of the three major Ras family members (*H-ras*, *N-ras*, and *K-ras*) are found in 30% of all human tumors (1). Upon activation, Ras proteins engage multiple downstream effectors through which they control cellular signalling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival and differentiation. Aberrant regulation of effectors, brought upon by oncogenic Ras proteins mediates several key aspects of malignant transformation, including deregulated cell growth and evasion from apoptosis (2, 3). However,

Table 1. Mutational analysis of Ras related cancers

Cancer type	H-Ras %	K-Ras %	N-Ras %
Biliary tract	0	33	1
Breast	1	5	1
Cervix	9	8	1
Endometriun	1	14	1
Haematopoietic and Lymphoid tissue	0	5	12
Large intestine	0	32	11
Liver	0	7	4
Lung	1	18	1
Ovary	0	15	4
Pancreas	0	60	2
Prostate	6	8	1
Skin	5	2	19
Small Intestine	0	20	0
Soft Tissue	7	12	6
Stomach	4	6	2
Thyroid	4	3	7
Urinary tract	12	4	3

Percentage of mutated form of Ras proteins in several cancer types. The mutation data were obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer. Web Site indicated in text.

the precise role of individual Ras effectors in transformation and tumorigenesis is still poorly understood (4).

The Ras signalling cascade modulates various cellular processes and crosstalks to several signalling pathways. Here we review recent evidence pointing to a crosstalk between the Ras pathway and transcriptional regulation of genes encoding proteins of the downstream branch (*i.e.* from adenylyl cyclase to PKA itself) of the cAMP-PKA pathway (5-7). Such crosstalk appears to be relevant for the tumorigenic processes, since deregulation of the cAMP-PKA pathway has been strongly linked to onset of endocrine tumors and, to a lesser extent, of non endocrine tumors. We will not focus on the signal transduction connections between the two pathways that have been recently reviewed by other authors (8-10).

3. *RAS* GENES AND PROTEINS IN MAMMALIAN CELLS

The mammalian *H-ras*, *N-ras* and *K-ras* genes code for closely related small guanine triphosphate hydrolases [(GTP)ases] acting as critical components of signalling pathways involved in the control of cellular proliferation, survival or differentiation. The transforming potential of *ras* genes was first identified in rats more than 40 years ago (4); since then, *ras* activating mutations have been identified in about 30% of human cancers (11). Ras proteins switch between an inactive GDP-bound state and an active GTP-bound state. Conformational changes caused by GTP binding increase the affinity for the effectors that is reversed by GTP hydrolysis. Guanine nucleotide exchange factors (GEF) stimulate GDP dissociation, allowing rapid GDP replacement by GTP, whose intracellular concentration is higher than that of GDP while GTPase activating proteins (GAPs) stimulate intrinsic GTPase activity of Ras up to 10.000-fold (12). Oncogenic Ras mutations may interfere with the ability of GAPs to interact with Ras, or make GDP/GTP exchange active in the absence of GEF, resulting in constitutively active (*i.e.*, GTP-bound) Ras proteins (13, 14). The presence of multiple Ras activators (15-17) and inhibitors (18, 19) allows to fine tune Ras activation in response to a wealth of signals (20-22). Notably, most GEFs are modular, multi-domain proteins able to activate other GTPases beside Ras thus effectively allowing integrative regulation of GTPase function (23-25).

3.1. Tissue specificity of the three Ras isoforms

The three *ras* genes appear to be ubiquitously expressed in mammals, although differences in expression levels are reported for each gene depending on the tissue and/or developmental stage under consideration (26, 27). Ras isoforms were initially assumed to be functionally redundant due to their high degree of sequence homology; however, many studies pointed out specific roles for each isoform. Indeed, preferential activation of specific Ras isoforms in particular malignancies (1), the different transforming potential of transfected *ras* genes depending on the recipient cell line (28-30), or the distinct sensitivities exhibited by different Ras family members for inhibition by GAPs (31, 32), activation by GEFs (33, 34), or for interaction with various downstream effectors (35-37), suggested otherwise. Early evidence for isoform specific roles came from the analysis of the mutation rates of Ras isoforms associated with different types of cancer. In fact, a strong bias in favour of mutation of only one of the three major *ras* genes in tumors of different cellular origins exists (Table 1) (Catalogue Of Somatic Mutations In Cancer, http://www.sanger.ac.uk/genetics/CGP/cosmic/ and (38)). Activating mutations of individual Ras isoforms are strongly linked to certain cancers; for example, almost 90% of pancreatic and 50% of colon cancers have mutated K-Ras, whilst acute leukemias often possess activated N-Ras (1). Moreover N-Ras mutations occur in approximately 20% of human melanomas, whereas H-Ras and K-Ras mutations are rare. K-Ras mutations have been found preferentially in adenocarcinomas and are common in cholangiocarcinomas, colorectal malignancies and in the adenocarcinoma of the lung (39), whereas H-Ras and N-Ras mutations are rare. H-Ras mutations can be found in cutaneous squamous cell carcinomas and in squamous head and neck tumors. Finally, N-Ras mutations are found most frequently in acute leukemias (mainly of the myeloblastic cell type) and in the myelodysplastic syndromes (40). Activated *ras* genes have been detected in pre-malignant lesions as well, suggesting a potential role in tumor initiation (41-44).

More compelling evidence comes from knockout mice, which revealed that H-, N- and double N-, H-homozygous *ras* knockouts apparently develop normally with no detrimental impact on long-term survival (45, 46). Similarly, the K(A)-*ras* splice variant is dispensable for mouse development (47); in contrast, K(B)-*ras* knockout mice died during embryogenesis between days 12 and 14 (48, 49). These results indicate that only K(B)-*ras* is required during embryogenesis and that there must be redundancy in signalling between the other isoforms during this period.

More information about cell specific activity of the three Ras isoforms has been suggested by recent discovery of germline mutations in the K-*ras* and H-*ras* genes in individuals diagnosed with Noonan and cardio–facio–cutaneous (CFC) and Costello syndromes respectively. Indeed it has now become clear that features of such syndromes (short stature, relative macrocephaly, facial anomalies, learning difficulties) that are found in these three related disorders, are a result of constitutive activation of the Ras–Raf–extracellular signal-regulated and mitogen-activated protein kinase pathway and that the different expression patterns of the various Ras isoforms can influence clinical phenotypes when these isoforms are mutated in the germline cells, underlining a critical role of Ras signalling in human tissue-specific development (50).

An important feature of Ras isoforms, confirmed by several reports, is their differential localization due to several post-translational modifications. Indeed the long-held view that Ras only operates at the plasma membrane has been challenged by several studies that point to Ras signalling from the surface of the Golgi, endosomes, mitochondria and Endoplasmic Reticulum (51-54). Briefly we can state that H-Ras and K(B)-Ras represent the two most surface-localized isoforms in most cells, whereas in many cell types, N-Ras is a prominent endomembranous component.

4. ONCOGENIC RAS PROTEINS INDUCE TRANSFORMATION BY ACTIVATION OF SIGNALING PATHWAYS AND TRANSCRIPTIONAL RESPONSES

Oncogenic Ras proteins have been associated with the onset of several types of tumors and they achieve their transforming capacity by uncontrolled activation of downstream targets. Until now more than ten distinct functional classes of proteins have been implicated as effectors of the small GTPase Ras, but the best studied are Raf kinases, type I phosphoinositide (PI) 3-kinases, Ral-guanine nucleotide exchange factors (Ral-GEFs), the Rac exchange factor Tiam1, and phospholipase C (3) (Figure 1A). These proteins are involved in several physiological cellular processes (proliferation, differentiation, survival, metabolism) and they have been found mutated in several tumors (2, 4).

Raf and phosphatidylinositol 3-kinase (PI3K) were the first two identified Ras effectors and the main focus of research investigating Ras function (55). Raf promotes cell proliferation and differentiation through the MAP kinase (MAPK) pathway (56), at the same time as PI3K generates anti-apoptotic signalling, directly or through Akt pathway activation (57, 58). Both signalling pathways can activate two different signals distinct for their answer timing. Indeed both MAPK and PI3K are able to activate phosphorylation cascades that lead, as primary effect, to post-translational modification of several substrates (membrane targets, cytosolic targets, cytoskeletal targets and nuclear targets), which rapidly activate functional processes. Early response to Ras signalling is quite fast: for instance in resting cells stimulated with mitogens, Ras-GTP level increases within 2 minutes from stimulation with serum (59). Raf-1 undergoes transient activation within 2-3 minutes, and rapidly activates the mitogen-activated protein kinase (MAPK) cascade whose most downstream component, ERK, rapidly moves into the nucleus where it phosphorylates nuclear proteins notably transcription factors (60, 61) whose activity can be controlled by regulating their sub-cellular localization, expression, stability, ability to bind to other components of transcriptional complexes and to DNA, and their ability to remodel chromatin structure (62). Transcription factors that are under the control of MAPK pathway include members of the ETS family (i.e. Ets-1, Ets-2, PU-1), MADS box family (i.e. MEF2A, MEF2C, Sp1), Zinc Finger family (i.e. GATA-2 and GATA-4), bZip family (i.e. Fra-1, c-Jun, JunB, JunD, ATF-2, c-Fos and CREB), bHLH family (i.e. c-Myc, MITF), Nuclear Hormone Receptor (i.e. PR, GR and ER) as well as other transcription factors (i.e., SMAD1, STAT1) and coregulatory proteins (i.e., CBP, p300) (60, 61, 63).

Like ERKs, Akt and other targets of PI3K signalling can phosphorylate and activate transcription factors (57, 64). Akt protein can control several transcription factors directly or indirectly. Direct targets are the forkhead box proteins, FOXO, and the cell cycle inhibitor, MIZ1, both inhibited upon AKT-mediated phosphorylation. AKT-dependent regulation of p53, nuclear factor B (NFkB), c-MYC, activator protein 1 (AP1) and beta -catenin is indirect (65).

It is commonly assumed that developmental and oncogenic signalling pathways achieve their phenotypic effects primarily by directly regulating the transcriptional profile of cells. The PI3K-Akt pathway, however, has a direct effect also on the translational efficiency of specific existing mRNA species. In fact, several authors have shown that this mechanism of protein production control provides a highly specific, robust, and rapid response to oncogenic and developmental stimuli (66-72). The mRNAs so affected, identified as target of this pathway, encode proteins involved in cell-cell interaction, signal transduction, and growth control. Furthermore, a large number of transcription factors are controlled at this level as well. To what degree this translational control is either necessary or sufficient for tumor formation or maintenance remains to be determined.

The relative importance of each effector pathway in Ras-induced tumor formation has been initially studied in cell cultures expressing partial loss-of-function mutant Ras proteins that lose ability to interact with specific effectors (73). The physiological significance of these studies may be limited by the difficulty of using exogenously expressed, artificially activated constructs in cultured cells to mimic the amplitude and duration of Ras effector activation in naturally occurring human tumors. More recent studies took advantage of mouse models of carcinogenesis (39, 74-85) and suggested that while multiple arms of oncogenic Ras signalling (*i.e.*, MAPK, RalGEF and PI3K) are required for tumor initiation, oncogenic activation of the downstream PI3K/Akt pathway

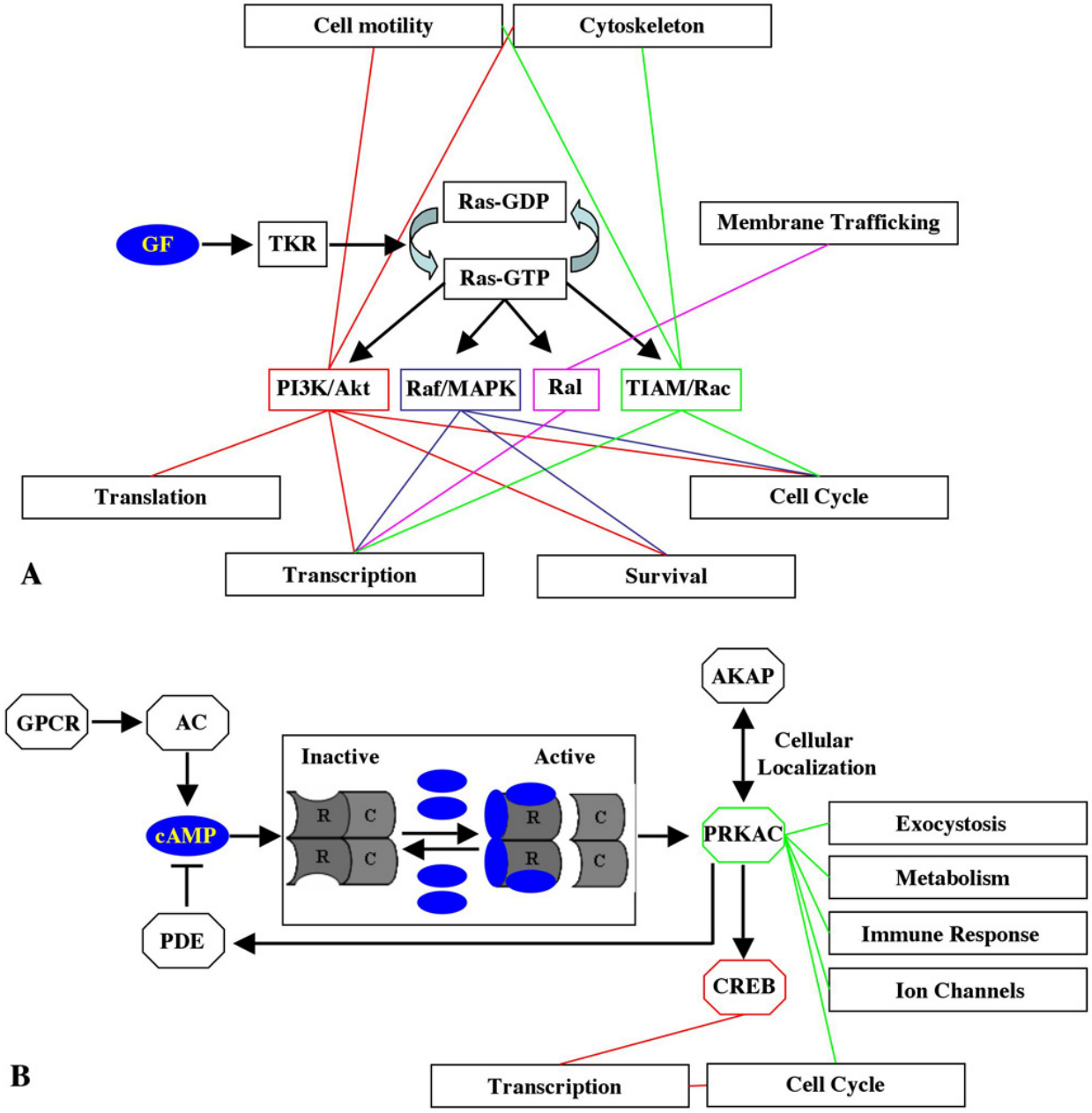

Figure 1. The Ras and PKA pathways. **A**, Ras-GDP proteins, following activation of tyrosine kinase receptors (TRK) by growth factors (GF), are activated through GDP/GTP exchange. In such an active form they are able to bind and regulate many effectors proteins. In the figure are represented the main routes so far identified and the cellular processes in which each effector is involved. The effectors and the processes in which they are involved, are colour-coded. **B**, Diagrammatic scheme of PKA pathway. G protein-coupled receptors (GPCR), following stimulation by several stimuli, activate adenylyl cyclase (AC), which will convert ATP in cAMP increasing its intracellular concentration. cAMP acting as second messenger, is able to turn on PKA, in different cellular compartments, depending of localization of ACs or/and by the sequestering activity of AKAP proteins. The catalytic subunits of PKA, through its phosphorylation activity, will trigger several cellular processes (green line) and in particular will activate the transcription factor CREB (red line), that ultimately will participate to a transcriptional remodelling able to influence several cellular processes (*i.e.* cell cycle).

is sufficient for tumor maintenance. Indeed once a tumor is formed, the activation of the PI3K pathway maintains tumor growth also in the absence of oncogenic Ras. In a similar manner, activation of the MAPK and RalGEF pathways is required for tumor maintenance and, once a tumor is established, it has been found that cells depend upon these two pathways for continued tumor growth (86-88).

5. PKA PATHWAY AND CANCER

Cyclic AMP (cAMP) is an ubiquitous intracellular messenger that regulates numerous cellular functions. Its synthesis is stimulated by G-protein-coupled receptors (GPCRs). Indeed upon binding of their extracellular ligands, each of these receptors activates one or more types of heterotrimeric G protein, so named because of its three distinct subunits (Gα, Gβ and Gγ), the first of which binds either GDP or GTP. The active state of Gα is achieved when it binds GTP. Once stimulated by GPCRs, the Gα subunit dissociates from its two partners, Gβ and Gγ, and activates a number of cytoplasmic enzymes, among which there are adenylyl cyclases (AC) which convert ATP into cAMP. cAMP breakdown is catalyzed by phosphodiesterases (PDEs) belonging to a superfamily of at least 22 members in mammalian cells (PDE1 to PDE11 and their isoforms). Intracellular concentration of cAMP therefore results from the fine balance between the activities of cyclases and PDEs. A major function of cAMP in eukaryotes is activation of cAMP-dependent protein kinase (PKA), a tetramer composed of two catalytic PRKAC (C) and two pseudo-substrate regulatory PRKAR (R) subunits that upon cAMP binding dissociate from the C subunits that are so activated (reviewed in (89-91)). Different subunit isoforms (PRKAR1A, PRKAR1B, PRKAR2A, and PRKAR2B) have different affinity for cAMP thus originating holoenzymes (PKA type I ($R1_2C_2$/$R1_2C_2$) or PKA type II ($R2_2C_2$/$R2_2C_2$) with different subunit composition and affinity for cAMP and thus get activated at either low or high local concentrations of cAMP in the cell. When PRKAR1 subunits are up-regulated, cAMP sensitivity of PKA increases and thereby lowers the threshold for activation of cAMP-mediated downstream effects. The four types of regulatory subunits have different expression patterns in mammals. While PRKAR1A has ubiquitous distribution, PRKAR1B is expressed primarily in brain, testis and B- and T-lymphocytes (92, 93). Similarly, PRKAR2A has ubiquitous distribution, while PRKAR2B is expressed in brain, adipose, and some endocrine tissues (89, 94, 95). PKA types I and II are differentially targeted in the cell through binding to the A kinase-anchoring proteins (AKAP, reviewed in (96)). Recent evidence suggests that PKA type I is associated with growth and proliferation whereas PKA type II is associated with increased differentiation and decreased proliferation (97, 98) (Figure 1B).

Increase in cAMP leads to dissociation of enough PRKAC to allow the translocation of free catalytic subunit into the nucleus and phosphorylation of cAMP response element binding protein (CREB). Such phosphorylation event induces cellular gene expression by activating the transcription factor CREB. The importance of CREB for several physiological events has been confirmed by the high number of targets since now identified: up to 4000 genes involved in several cellular processes (99).

The cAMP/PKA pathway has been reported to stimulate cell growth in many cell types while inhibiting it in others (reviewed in (8, 9)). Data collected from literature about some PKA-related genes, indicate an involvement of PKA in neoplastic transformation and tumor growth, especially in the onset and maintenance of endocrine tumors (hormone-responsive tissues), mainly of the corticotroph axis (pituitary and adrenal cortex) and the thyroid (100-102).

A considerable number of authors have identified specific correlations between G protein-coupled receptors and several tumors (endocrine and non-endocrine). For example, a recent examination of publicly available gene expression data identified a variety of types of GPCRs that are overexpressed in diverse types of cancer tissues (103). Causal relationships have been established by the discovery of the transforming abilities of certain GPCRs (104) and heterotrimeric G-proteins (105-107). Such receptors are able to influence several characteristics of tumor phenotype (protein translation, survival, cell proliferation, angiogenesis) by interfering with signal transduction and specifically by activation of Ras-related pathways (reviewed in (108)).

5. 1. Adenylyl cyclases and Phosphodiesterases

Adenylyl Cyclases (AC) are central molecules that dictate the compartmentalization of the cAMP message through their ability to produce the second messenger cAMP in discrete domains of the cell with specific local consequences. Several authors suggested an increased activity of AC during malignant transformation of human endocrine tumours (106, 109, 110).

PDEs have been extensively reviewed recently (111-113), but their pathophysiological regulation remains an open research field. Many Authors showed an increased activity and/or expression of PDE, notably PDE3, 4 and 11 in several cancer cell line (114-119). Such observations suggested the use of PDEs inhibitors for cancer therapy. Indeed, it has been observed that elevation of cAMP by nonspecific PDE inhibitors delays growth in several human prostatic cancer cell lines and induces terminal differentiation in some of the lines (120, 121). Treatments with specific or nonspecific PDE inhibitors result in growth inhibition in small-cell lung carcinoma (122), acute promyelocytic leukemia (123) and malignant glioma cells (124). PDE inhibitors induce apoptosis in BCLL cells (125, 126), inhibit colon cancer motility (127). On the contrary a positive effect on the proliferation of human malignant melanoma cells has been shown (128).

5. 2. Protein kinase A

The PRKAR1A protein and mRNA have been found to be up-regulated in a series of cell lines and human and rodent neoplasms, suggesting its involvement in tumorigenesis and its potential role in cell cycle regulation, growth, and/or proliferation (reviewed in (6, 129)). However, mice with increased levels of PRKAR1A protein, due to a compensation effect (130, 131) such as the PRKAR1B–/–, PRKAR2A–/–, and PRKAR2B–/– mice, did not show an increased frequency of tumors; it has to be noted that such null mice, showed respectively no change (PRKAR1B) and decreased (PRKAR2A, PRKAR2B) levels of PKA activity (132-135), which can be the cause of

absence of tumors. More recently, using conditional and antisense transgene mouse models for PRKAR1A (the null mice are embryonic lethal), it has been shown that haplo-insufficiency of PRKAR1A leads to an increase in kinase activity associated with multiple tumor appearance (in endocrine and other tissues) and tendency to carcinogenesis (136-138). PRKAR1A-inactivating mutations (germline or somatic mutations) have been found to cause primary pigmented nodular adrenocortical disease, the Carney complex, a multiple neoplasia syndrome, and sporadic endocrine tumors (139-144). Moreover PRKAR1A null mouse embryonic fibroblasts (MEFs), showing a constitutive PKA activation, became immortalized in correlation with up-regulation of D-type cyclins (145) and showed a decreased autophagy, a mechanism that, in some cases, has been associated with transformation inhibition (146).

As reviewed in (147), in many cancer cell lines and tissues an altered ratio - as compared to normal counterpart - between PRKAR1 and PRKAR2 regulatory subunits has been identified at the level of mRNA or protein or activity. Although until now it is not possible to define PRKAR1A subunit as a tumor suppressor or an oncogene, its selective targeting in therapeutic and antitumour strategies, based on the up-regulation of PRKAR1A in several cancers, has become very attractive. Indeed several studies indicated that inhibition of PRKAR1A expression through antisense oligonucleotides resulted in growth arrest of several tumor cell lines (148, 149). On the contrary, overexpression of PRKAR2B inhibits cancer cell growth and induces a reverted phenotype in various cancer cell lines (150, 151). Thus, uncontrolled proliferation and malignant transformation have been associated with mainly altered PRKAR1 expression or changes in the ratio of PKA-I and –II (131, 134, 149, 152-159). PRKACA protein has been shown to be a direct transcriptional target of c-MYC, and proposed to be a crucial component of the program by which constitutive c-MYC expression contributes to cell transformation (160).

5. 3. Protein kinase A anchoring proteins

AKAPs are a group of structurally diverse proteins, which have the common function of binding to the regulatory subunit of PKA and confining the holoenzyme to discrete locations within the cell. An interesting correlation between AKAP proteins and oncogenesis has been found analyzing polymorphic variation of two AKAPs, 10 and 13, associated with increased familial breast cancer risk (161, 162). Both proteins are involved in targeting regulatory subunits of protein kinase A to specific cell sites, and specifically AKAP 10 to the mitochondria and AKAP13 to the plasma membrane. Moreover AKAP13 has been shown to be involved as upstream effector of RhoA signalling. The authors suggested that polymorphic AKAP13 variant could stimulate a constitutive activation of such signalling that could favour the malignant progression, while polymorphism in AKAP 10 influences the binding to PKA in an isoform-specific manner, favouring the binding and the activity of PRKARIA. A correlation has also been found between AKAP3 mRNA levels and poor prognosis in epithelial ovarian cancer. Indeed in all the patients analyzed an increased level of its mRNA has been showed, compared to normal tissues, and its high expression is associated with worse survival (163).

5. 4. CREB

Several lines of evidence obtained from the study of leukemia, fusion oncoproteins, viral oncoproteins, endocrine tumors and CREB signalling support the notion that CREB is involved in oncogenesis (reviewed in (7)). Constitutive activation of CREB transcriptional activity has been shown in Peutz–Jeghers syndrome (PJS), an autosomal dominant disorder due in 80% of the patients to a mutation in the Lkb1 protein and characterized by predisposition to malignancies of the epithelial tissues. The absence of Lkb1 leads to constitutive activation of CREB, suggesting such activation in these cells or tissues as highly relevant to cancer predisposition in PJS (164). Together, the observations reported above demonstrate that PKA pathway has a relevant role also in non-endocrine tumors and that oncogenic alterations of Ras pathways may regulate the transcription of PKA-related genes and modify at post-translational level the PKA-related proteins, inhibiting or activating specific answers linked to tumor progression and persistence.

6. PKA- RAS CROSS-TALK

Although a full description of the mechanisms underlying Ras and cAMP crosstalk relating to signal transduction is outside the scope of this review, we have to point out that the Ras and cAMP pathways are involved in several different cellular processes and have been shown both *in vitro* and *in vivo*, depending on the cell contest and the kind and duration of stimuli, that they may crosstalk for specific activity of both pathways.

G protein-coupled receptors regulate the Ras pathway, both in a PKA-dependent way - as described above - and in a PKA-independent way. As extensively reviewed in (165), this receptor family is able to cross-talk with the Ras pathways inducing both proliferation and other cellular processes (cytoskeletal reorganization, transcriptional activation) through regulation of RhoGEF (guanine nucleotide exchange factor for Rho), PKC, RasGRP and RasGRF (guanine nucleotide exchange factors for Ras), PI3K and Src proteins.

While no literature data have been found for a transcriptional effect of Ras proteins on family members of the AC family, conflicting results have been published on the ability of oncogenic and viral Ras proteins to either stimulate (166-171) or inhibit (172-174) AC activity in different cell lines (thyroid, epithelial, kidney, fibroblast).
Little information is available about the effect of mitogenic stimuli on PDEs mRNA expression. An involvement of MAPK or PI3K pathways in the regulation of PDE activity has been reported, suggesting that mitogenic stimulation may regulate PDE4s expression directly (175) or inducing cAMP elevation which ultimately regulate PDE4s expression (176) and that Erk2 (p42(MAPK) phosphorylation activity has a relevant role in their regulation (177).

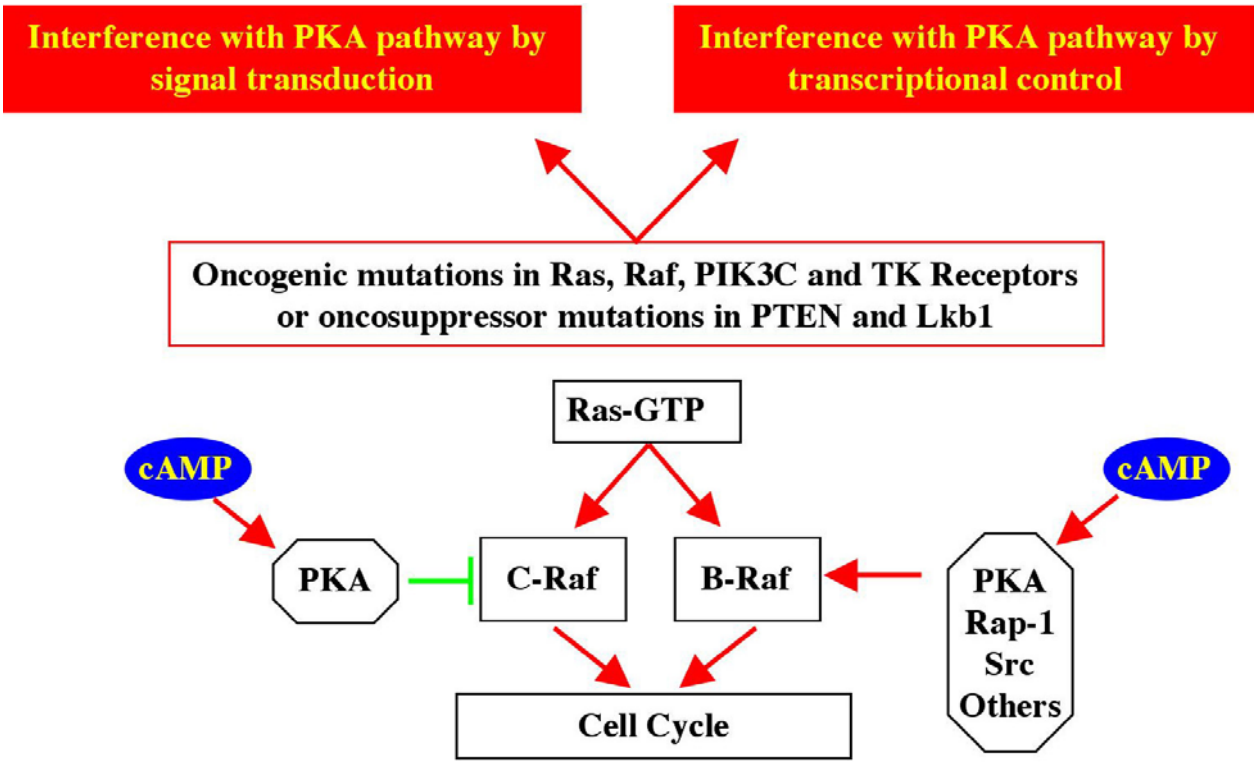

Figure 2. Ras and PKA pathway crosstalk. Activated Ras and PKA proteins control proliferation by regulating the activity of Raf family members (C-Raf and B-Raf). While Ras always induces cell proliferation by binding to Raf proteins, PKA can inhibit or stimulate proliferation depending of the stimuli and the cellular contest. Indeed stimuli inducing cell proliferation arrest and differentiation will inhibit the cAMP-PKA-C-Raf axis (green line), while stimuli inducing cell proliferation will activate the cAMP-PKA-B-Raf axis (red line). Latter axis has been shown to be controlled directly by PKA activity or through other proteins as Src and Rap-1. Oncogenic mutations in proteins of Ras pathway, appear able to interfere with the capacity of PKA to control both roads, influencing the PKA-pathway related genes expression level or/and interfering with the signal transduction regulate by PKA.

Evidence collected by analyzing normal or immortalized cell lines indicates that PKA is able to uncouple Ras activation from C-Raf activation, thus leading to inhibition of cell proliferation (9). On the other hand, a convincing model for cAMP stimulation of proliferation (or differentiation) is still missing. cAMP has been reported to induce proliferation through several proteins (B-Raf, Rap1, Src and others): among these a major role has been ascribed to B-Raf (Figure 2). cAMP is reported to induce proliferation rather than growth inhibition, in several tumors where oncogenic activation of B-Raf has been identified (i.e. melanoma and thyroid cancer).

Searching in the catalogue of human somatic mutations for PRKAR1A in non endocrine tissues, no mutation was identified for this gene. However, in normal human lymphocytes, with PRKAR1A inactivation, an increased proliferation and decreased apoptosis, both associated with B-Raf, MAPK/ERK kinase 1/2 and c-Myc activation and c-Raf inhibition, have been shown (178), suggesting that wild type B-Raf is a target mediating proliferative effect of the PKA pathway (see previous paragraph). Notably, strategies to control proliferation of cancer cells by inhibition of PRKAR1A appear particularly effective in cell lines carrying a mutated ras gene (HL-60, MDA-MB-468, MDA-MB-231, HCT-15), pointing out to a fundamental role of such subunit in Ras-dependent transformation.

AKAP12, also known as Gravin, has been shown to be down-regulated by oncogenic forms of Src and Ras, and its overexpression is able to induce the attenuation of critical Src and Ras-induced proliferative and pro-angiogenic gene expression, acting as a tumor suppressor (179, 180). The expression of AKAP12/Gravin is often lost in tumor cells; it is likely that Gravin normally provide gating functions critical for the regulation of mitogen- and cytoskeletal signalling during contact inhibition. AKAP12 is tyrosine phosphorylated in vivo in response to short-term treatment with EGF, PDGF or serum (181, 182).

A direct link between CREB activation and Ras is the Raf/Erk, pathway one of the main pathways stimulated by Ras. Although the CREB transcription factors are not directly phosphorylated by Erk1/2, they are phosphorylated and transactivated by the Erk1/2-activated Rsk protein (183, 184). Another link between Ras pathway and regulation of CREB activation has also been suggested through another target of Ras, PI3K-Akt pathway. Without entering in

Table 2. NCI60 cell lines with predicted active pathways by mutational analysis

Tumor Type	Ras pathway related mutations	PI3K pathway related mutations	Other pathway related mutations	Not analyzed mutations
Breast	HS578T, MDA-MB231, MD-MB435	MCF7, T47,	-	MDA-N, BT549
CNS	-	SF295, SF359, SNB19, U251	SF268, SNB75	-
Colon	HCC2998, HCT116, HCT15, SW620, COLO205, HT29	KM12	-	-
Leukemia	CCRF-CEM, RPMI-8226, HL60, MOLT4, K562	-	-	SR
Melanoma	SK-MEL2, LOXIMVI, M14, MALME-3M, SK-MEL28, SK-MEL5, UACC257, UACC62	-	-	-
Lung	A549, HOP62, NCI-H23, NCI-H460	-	EKVX, NCI-H226, NCI-H322, NCI-H522	HOP92
Ovarian	OVCAR5, OVCAR8	SKOW3, IGROV1	OVCAR3, OVCAR4	-
Prostate	-	PC3, DU145	-	-
Renal	-	786-0, RXF-393	A498, ACHN, CAKI-1, SNC12C, TK10, U031	-
Unknown	ADR-RES	-	-	-

The 60 cell lines reorganized in 4 categories, as described in the text, on the basis of the most representative mutation of each cell line: Cell lines carrying mutations able to interfere with Ras-Raf-MAPK pathway (Ras pathway related mutations -Ras, Raf, ERBB2 and PDGFRA-); Cell lines carrying mutations able to interfere with PI3K-Akt pathway (PI3K pathway related mutations - PI3KC, PTEN and Lkb1-); Cell lines carrying no somatic mutations interfering with the two above pathways (Other pathway related mutations); Cell lines for which no somatic mutations interfering with the two above pathways have been searched (Not analyzed mutations).

the details of such regulation, we can state that Lkb1 regulates negatively CREB activation and the TOR pathway through a phosphorylation cascade (185), while the same TOR pathway is positively regulated by PI3K-Akt proteins (186), effect associated with human cancer. Moreover several Authors showed that activation of Akt is able to prevent activation of AMPK by Lkb1 and therefore that a competitive effect between these two pathways for signalling cascade exists (187, 188). Such observations may indicate that PI3K-Akt pathway plays a role as activator of CREB as well as happens in Lkb1 deficient cells. Furthermore, another target inhibited by Akt, GSK-3 kinase, has been shown to be able to phosphorylate CREB, enhancing CRE-mediated transcription that again suggests a role of such PI3K-Akt both in positive and negative control of CREB activity (189, 190).

7. REGULATION OF GENE TRANSCRIPTION BY RAS PATHWAYS: THE cAMP-PKA PATHWAY

In the past, multiple strategies have been used to check the functionality of Ras proteins in cells. The development of genomic and proteomic analysis tools has opened the way to more exhaustive, genome-wide studies aimed at characterizing transcriptional networks associated to the function of specific Ras proteins both in physiological and pathological conditions. So far, most studies on Ras-related genomic profiling have been performed to characterize cell lines transformed by various oncogenic Ras forms (191-203) and although most work has been focused on K-Ras, a comparison of isoform-specific regulation of gene expression revealed an almost complete functional overlap between the Ras isoforms. However, the physiological significance of these studies may be limited by the difficulty of using exogenously expressed, artificially activated constructs in cultured cells to mimic the amplitude and duration of Ras effector activation in naturally occurring human tumors.

7.1. 60 human cancer cell lines (NCI60) as a tool to study PKA pathway transcriptional regulation by oncogenic Ras

The amazing volume of molecular data resulting from the rapid spread of post-genomic techniques has highlighted the importance of computational analysis as a key link between data generation and the formulation of new hypotheses (204). Gene-expression profiling has been applied extensively in cancer research. As a first step to identify regulatory mechanisms underlining gene-expression profiles it is required to extract, filter, cross-reference and structure information from cancer-related data sets.

The NCI60 cell collection is composed of 60 human cancer cell lines that are most commonly used in cancer research and drug screening. They have been employed by several Authors and for different purposes to analyze and identify transcriptional profiles regulated in cancer cells and such results are available in public databases (12, 205, 206).

The NCI60 cell collection includes cell lines derived from colorectal, renal, ovarian, breast, prostate, lung and central nervous system cancers, as well as leukemias and melanomas. Comparative analysis, by transcriptional profiles, between these cell lines and their tumor of origin has indicated that for 51 of 59 cell lines there is a good correlation (206, 207). Since they represent a more physiological model to study gene profiles in cancer cells, being stabilized cell lines with features strongly similar to cancer tissues, we reviewed the information present in public databases about the 60 cell lines, to identify transcriptional signatures for PKA-related genes due to oncogenic mutations of the Ras proteins and their effectors. The 60 cell lines were sorted according to mutational status, using the information provided by Catalogue Of Somatic Mutations In Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/) (Table 2), and divided in four main categories based on percentage of incidence of selected mutations:

Table 3. Gene expression profiling datasets on NCI60 cell lines and normal tissues analyzed in this study

Reference	Samples	Sample Size	URL for Data Downloading
207	NCI60 cell lines	-	http://discover.nci.nih.gov/
260, 261	Breast	3	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
260, 261	CNS	16	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
261	Colon	2	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
260, 261	Blood	1	http://www.biotechnologycenter.org/hio/
260, 261	Lung	8	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
260, 261	Skin	3	http://genet.cchmc.org/
260, 261	Ovary	7	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
260, 261	Prostate	9	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/
260, 261	Kidney	8	http://www.ncbi.nlm.nih.gov/ http://www.biotechnologycenter.org/hio/

To generate a comparison between NCI60 cell lines and normal tissues, the gene expression profiles from several public Microarray Database (as indicated in the last column) were downloaded. For each expression profile it has been indicated the tissue of origin (Samples) and the number of transcriptional profiles (Sample Size) of each tissue used for the comparisons with the NCI60 datasets. Experiments are described in the literature referenced on the left column (Reference).

1. Cell lines carrying mutations able to interfere with Ras-Raf-MAPK pathway (*i.e.*, mutations in genes encoding Ras, B-Raf, ERBB2, PDGFRA), 29 cell lines.

2. Cell lines carrying mutations able to interfere with PI3K-Akt pathway (*i.e.*, mutations in genes encoding PI3KCA, PTEN and Lkb1), 13 cell lines.

3. Cell lines carrying no somatic mutations interfering with the two above pathways (*i.e.*, mutations in genes encoding for CDKN2A, p53), 14 cell lines.

4. Cell lines for which no somatic mutations interfering with the two above pathways have been searched (i.e., no mutational analysis was performed), 4 cell lines.

To survey the transcriptional profile of PKA pathway-related genes (adenylyl cyclases, phosphodiesterases, A-kinase anchor proteins, PKA catalytic subunits and PKA regulatory subunits) in such cellular collection, we identified and gathered the transcriptional profile for 41 genes belonging to the PKA pathway and compared them to the expression profiles of primary normal tissues, collected from several databases (Table 3 and unpublished data). Such analysis permitted the identification of 23 cancer-specific regulated transcripts that were de-regulated in the vast majority of the 60 cancer cell lines (Figure 3). Moreover our analysis allowed the recognition of a different capacity of the 3 main categories above described (first, second and third) to regulate the expression of some PKA related genes. Indeed considering all regulated genes (both up-and down-regulated) in all the cell lines for each category, we observed that 73% and 27% were up-regulated and down-regulated respectively by Ras pathway; 60% and 40% were up-regulated and down-regulated respectively by PI3K pathway and conversely, only 13% were up-regulated and 87% down-regulated for the third category. Such striking result seemed to confirm a capacity of Ras pathway and to some extent of PI3K pathway to up-regulate some of the PKA pathway related genes, in contrast to what we observed in cell lines with the other mutations that clearly down-regulate the same pathway. Moreover comparisons with similar gene expression profile screenings done by other Authors through over-expression of oncogenic Ras proteins in cell and organism models (191-203), confirmed some of our findings and indicated the necessity of a high number of samples to better define transcriptional signatures.

7.1.2. Data mining for PKA pathway-related gene promoters

To go beyond from individual genes to biological processes, as the case of the relation between oncogenic Ras proteins and PKA pathway gene regulation, several recent methods have been applied (208-210). Such methods use the gene profiles as the basic building blocks for further analysis. These methods aim to collect a higher-order and more interpretable characterization of transcriptional changes. Moreover, by considering coherent changes in expression in larger modules, they can identify patterns that are too subtle to discern when considering expression profiles of individual genes in isolation.

Cellular processes are regulated by a variety of mechanisms, occurring at every step in the process of going from DNA to functional proteins. Transcriptional regulation, directly observed in gene-expression data, controls the production of mRNA transcripts.

The Ras pathway regulates transcription by multiple mechanisms involving a variety of post-translational modifications of transcription factors, dynamic assembly of nucleoprotein complexes and re-localization of transcription regulatory proteins in target cells. Understanding how the regulation of gene networks is orchestrated is an important challenge for characterizing complex biological processes.

Important components in this process are *cis*-regulatory elements in a target gene's promoter region, *trans*-acting factors that bind to these DNA motifs and signaling molecules that modulate this process based on exogenous and endogenous signals. Genes expressed in the same tissue under similar conditions often share a common

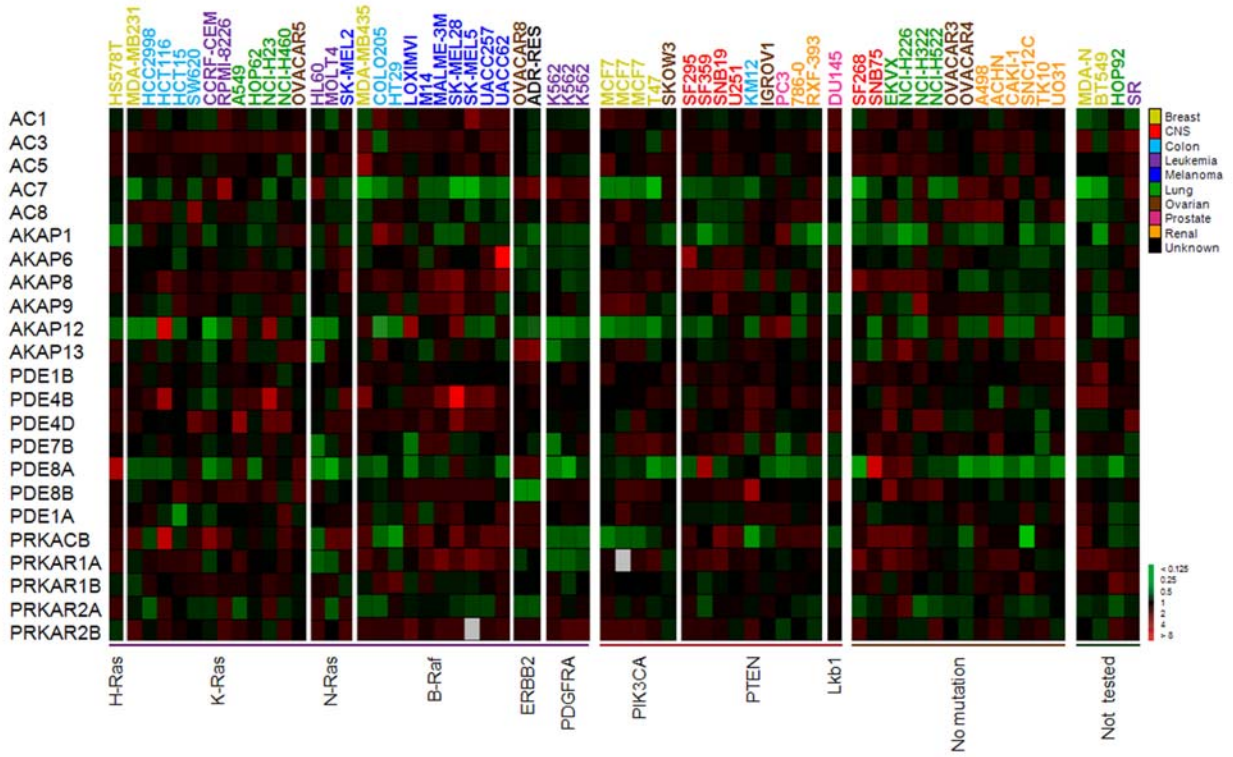

Figure 3. Coloured representation of the 23 cancer-specific regulated transcripts that have been found de-regulated in the vast majority of the 60 cancer cell lines. The colours of the dendrogram represent the Signal Log Ratio (SLR) (Fold Change is equal = 2^{SLR}) of each gene as calculated by Authors, with the rows (genes) and the columns (cell lines). For each mRNA the SLRs for the 60 cancer cells are showed. Data from 64 hybridizations were used, one for each cell line plus two additional independent representations of each of the cell lines K562 and MCF7. These two cell lines are represented in triplicate. The data have been organized on the basis of the most representative mutation of each cell line in way to identify 4 categories (see text for details): Cell lines carrying mutations able to interfere with Ras-Raf-MAPK pathway (Ras, Raf, ERBB2 and PDGFRA) (—); Cell lines carrying mutations able to interfere with PI3K-Akt pathway (PI3KCA, PTEN and Lkb1) (—); Cell lines carrying no somatic mutations interfering with above two pathways (—); Cell lines for which no somatic mutations interfering with the two above pathways have been searched (—). The different cell lines are coloured in agreement with their tissue of origin as represented in the legend on the right of the Figure. The colour scale used to represent the SLR value is shown.

organization of at least some of these regulatory binding elements. In this way the organization of promoter motifs represents a "footprint" of the transcriptional regulatory mechanisms at work in a specific biological context and thus provides information about signal and tissue specific control of expression. As previously described, many transcription factors are linked to the activation of Ras pathways, for this reason we interrogated several databases and we searched in the literature to identify studies about PKA pathways gene-related promoters. Direct evidences, experimentally verified by different molecular approaches, have been found for 13 genes belonging to the PKA pathway: PRKAR1A, PRKAR1B, PRKACA, AKAP1, AKAP9, PDE4D, PDE5A, PDE6A, PDE6B, PDE7A, PDE8A, AC8 and CREB.

PKA type I regulatory subunit A (PRKAR1A) expression has been studied in different cell models by analyzing its mRNA expression and by using its putative promoter region. In its promoter, binding sites for activator protein-1 and 2 (AP-1 and AP-2) and Sp1 (211) have been identified. Mostly these transcription factors are involved in growth-related signal transduction pathways, among which Ras is a main actor, and their over-expression can have positive or negative effects on proliferation (212-215). Moreover, a more recent work showed a direct activity of FOX family (FOXC2, D1 and D2) transcriptional factors members in the regulation of PRKAR1A expression both at transcriptional and at post-transcriptional levels (216, 217). This activity has been shown to be positively regulated by Akt protein, a known Ras target protein.

The promoter of PRKAR1B has been identified and studied in human and mouse: binding sites for Jun and p53 (human) and Oct-1, Egr1 and Pax1 (mouse) have been found. These binding sites have been experimentally verified by Electrophoretic Mobility Shift Assay, functional analysis and Northern blot (218, 219). The direct involvement of members of AP-1 family, as Jun, in growth factor-dependent transcriptional regulation and its relation with Ras pathways has been described by several authors (215, 220). Egr-1 is an early responsive gene linked to

mitogenic stimulation directly regulated by MAPK pathway (221-224). A weaker correlation has been found between Oct-1, Pax1 and Ras pathway.

PRKAR2B promoter has been studied in particular in Sertoli cells (human). Some reports identified binding sites for Sp1 transcription factor, NF-1, Myc, C/EBPbeta, able to induce the PRKAR2B promoter, USF1 and USF2. Interestingly, overexpression of USF2, but not USF1, led to inhibition of both cAMP- and C/EBPbeta-mediated induction of PRKAR2B (225-227). The USF family members participate to several gene regulation networks, including stress and immune responses, cell cycle and proliferation, lipid and glucid metabolism, but no clear evidence associates them to Ras pathway. On the contrary, for Sp1 (228-230), Myc (231, 232), C/EBPbeta (233) and NF-1 (234) a large amount of data about their correlation with Ras pathway have been reported.

The promoter of Protein kinase, cAMP-dependent, catalytic, alpha (PRKACA) has been identified both in human and mouse, but only few information have been produced for human promoter. Indeed, one paper describes the presence of binding sites for USF1 and USF2 transcription factors (235).

AKAP1, AKAP9 and AKAP10 promoters contain binding sites for c-Myc as shown by computational analysis and ChIP experiments in several human cell lines (236, 237). C-Myc regulation by Ras pathways has been reported above. Moreover, a single study indicates the presence in the promoter of AKAP12 of binding sites for Serum Response Factor transcriptional factors (238).

Several promoters of Phosphodiesterase protein family have been isolated and to some extent studied. All the studies have been performed on sequences of human promoters and in particular the PDE5A, PDE6A, PDE6B and PDE7A promoters have been better characterized. In the PDE5A promoter binding sites for Jun and AP-2 have been found (239, 240); in PDE6A and PDE6B promoters binding sites for Sp1 (241) and Sp4 (242, 243) respectively and in PDE7A promoter, Ets2 and NFKB1 binding sites (244). We already discussed the importance of Ras pathway in the activation of Jun and AP-2 transcription factors. Sp family has been shown to be regulated by post-translational mechanisms by Ras pathway (245, 246) as well as Ets2 (247, 248) and NFkB (249-251), and each of these transcriptional factors has been associated with several cellular responses (proliferation, survival, apoptosis) and transformation.

The cyclic AMP response element (CRE)-binding protein CREB promoter has been identified in human, mouse and rat. Analysis done on human promoter, experimentally confirmed, identified binding site for c-Myc (237) and Sp1 (252). Further information about such promoter has been produced in mouse and rat cells which allowed the identification of binding site for NFkB (253).

An important regulative mechanism of the PKA pathway is the feedback control. Indeed as well as the cAMP produced by Adenylyl Cyclases activate PKA kinase activity, PKA is able to inhibit the pathway, activating by phosphorylation the Phosphodiesterases, which ultimately induce hydrolysis of cAMP switching off the pathway. Moreover a huge amount of data have been published regarding the capacity of PKA to activate specific transcription factors by phosphorylation: cyclic AMP response element (CRE)-binding protein CREB, the cAMP response element modulator (CREM), the activating transcription factor 1 (ATF-1) and a repressor, ICER (inducible cAMP early repressor) (99), that, to a certain extent, has been shown to regulate PKA pathway-related genes transcription. Some of the promoters, already discussed above, have been shown to have CRE binding sites. Moreover, two interesting recent publications, have identified and characterized in different cellular contexts and by several approaches, through a genome-wide approach, target genes that are regulated by CREB (254, 255). The authors have identified and proofed by ChiP analysis (PRKAR1A, PDE7B) the presence of CRE site in PRKAR1A, in PDE7B, AKAP8, PDE4C and AC8. In the latter case they did not observe binding by Chip analysis, but another report has shown that its activation is mediated specifically via the canonical CRE site (256). Binding sites for CREB1 have been found in PDE7A (244), PDE4D (257) and experimentally confirmed. Moreover analysis of the promoter of CREB gene showed the presence of several CRE binding sites (258, 259).

An important role in the activation of these CREB family transcription factors play the stimuli able to induce their phosphorylation and consequently their activation. In fact as reviewed in (99) not only the protein kinase A is involved in this function but also several growth factors (NGF, FGF, IGF-I, PDGF, EGF), survival signals and hypoxia that often activate the Ras pathway, pointing out an essential role of the latter pathway also in gene transcriptional regulation by CREB family transcription factors of PKA-related genes.

8. CONCLUSIONS AND PERSPECTIVES

The NCI60 cancer lines have proved increasingly popular in recent years as a convenient tool for the application of genome-wide techniques that are often impractical on human biopsies. The pattern of gene expression identified in the NCI60 cell lines, as described by several Authors, provides basic information necessary for a better understanding of the differences that underlie cancer phenotypes. At the same time, more and more animal models are becoming available. Most of the resulting data may remain largely unused because genome-wide data are often intrinsically noisy and because of heterogeneity of expression between individual tumor cell lines. This requires both validation of data at the experimental level and the development of computational methods able to de-noise, filter and structure “crude” data revealing hidden information. By way of example, and as summarized in Figure 4, we could show that sorting of the NCI60 cell lines in sub-groups related to somatic mutational activation of genes encoding component of the Ras pathway (*i.e. Ras*, *Raf* and *PI3KCA*) allowed to

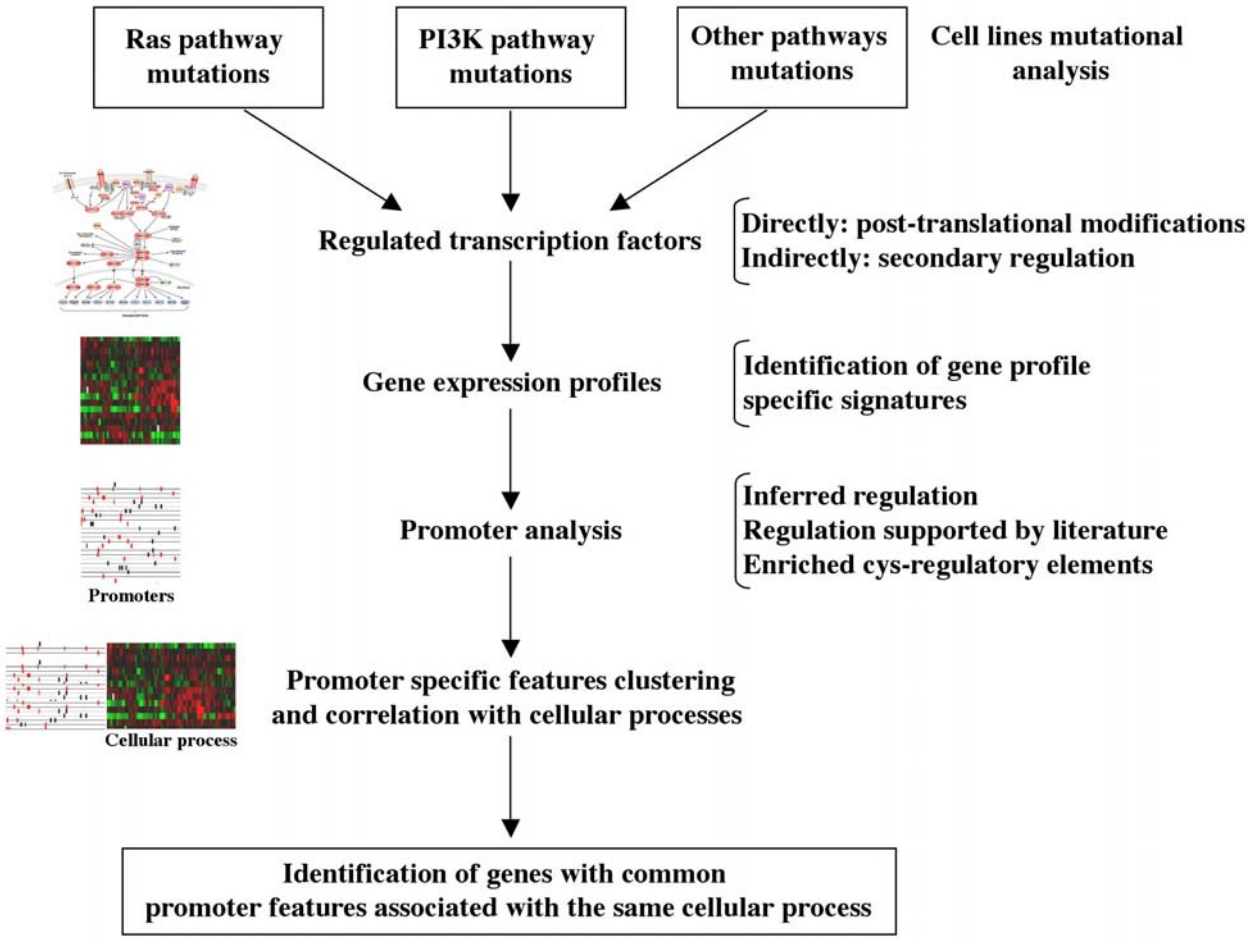

Figure 4. Understanding the oncogenic Ras pathway cancer biology and its cross-talks by multilevel analysis. A scheme of the workflow that allowed uncovering of an observed Ras-dependent pattern of regulation of genes encoding components of the cAMP-PKA pathway (see text for details).

uncover a hitherto unrecognized Ras-dependent pattern of regulation of genes of cAMP/PKA pathway. It is expected that deeper computational integration of transcriptional data with other genome-wide techniques, including - but not limited to - proteomics, interactomics and metabolomics, will allow better and better extraction of hidden information. Ultimately, development of such computational methods and their recursive integration with genome-wide and hypothesis-driven investigations should reconcile experiments from cell cultures, animal models and human tumor samples and contribute to explain at an integrative, systems level how the cAMP-PKA axis is involved in oncogenic processes induced by oncogenic forms of Ras.

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