7. BIOLOGY OF RAS IN THYROID CELLS

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INTRODUCTION

Ras is an almost universal component of signaling pathways in vertebrates, invertebrates and yeast where it plays critical roles in development, proliferation, differentiation and survival. In the twenty years since the first identification of mutated Ras genes in human tumors, intensive effort has been devoted to understanding how Ras promotes neoplastic transformation. What has become clear is that Ras promotes transformation in multiple ways. The effects of Ras are diverse due to the significant complexity of Ras-mediated signaling pathways. Mammalian cells express multiple Ras proteins, which localize to discrete membrane microdomains and exhibit differential affinities towards downstream signaling molecules. The existence of a large number of Ras effectors, many of which are members of multi-gene families, together with extensive sites of crosstalk between Ras and other intracellular signaling pathways, further increases the complexity of Ras-mediated signaling.

Mutations in all three cellular Ras genes (H-, K- and N-Ras) have been identified in benign and malignant thyroid tumors. This has generated immense interest in elucidating the cellular consequences of Ras activation in thyroid cells. The recent discovery of B-Raf mutations in thyroid tumors reaffirms the important contribution of Ras-mediated signaling pathways to thyroid cell transformation. Interestingly, the effects of Ras in thyroid cells are unusual in several respects. Unlike primary fibroblasts where expression of activated Ras induces growth arrest, Ras stimulates proliferation in primary human thyrocytes. Recent data suggests that this may be a consequence of

cell type specific effects on cell cycle regulatory proteins. Thyroid cells are one of few cellular models where proliferation is positively regulated by cAMP. As discussed below, crosstalk between cAMP and Ras markedly influences the signaling pathways activated by Ras. Indeed, TSH has been shown to modulate the effects of Ras on differentiation, proliferation and survival. The focus of this chapter is on the effects of Ras activation in thyroid cells, including the role of cellular Ras in TSH driven proliferation and the contribution of sustained Ras activity to thyroid cell transformation.

RAS REGULATION AND SIGNALING

Ras proteins are 21kDa GTP-binding proteins that function as molecular switches, cycling between active GTP- and inactive GDP-bound states. Cellular Ras activity is regulated by the opposing action of guanine nucleotide exchange factors (GEFs) that catalyze GDP dissociation, and GTPase activating proteins (GAPs) that stimulate intrinsic GTPase activity. Multiple RasGEFs and RasGAPs co-exist in most cells, increasing the diversity of signals that regulate Ras activity. Ras proteins are localized to the plasma membrane where they are poised to respond to signals initiated by the activation of cell surface receptors. Cellular Ras activity is maintained at very low levels. In response to signals such as those elicited by growth factors and hormones, Ras becomes activated in a transient manner. For cell surface receptors with tyrosine kinase activity, receptor dimerization induces tyrosine phosphorylation, thereby creating docking sites for signaling molecules such as Grb-2 and Shc, adaptor proteins comprised of SH2 and SH3 domains. Grb-2 is associated with the RasGEF SOS in the cytosol. Recruitment of Grb-2 to the activated receptor localizes SOS to the plasma membrane in close proximity to Ras, facilitating its activation. For G protein-coupled receptors, Ras is activated through second messengers such as diacylglycerol, calcium, and possibly cAMP (Busca et al., 2000; Pak et al., 2002), as well as through heterotrimeric G protein β/γ subunit- and src-mediated pathways.

In its active conformation, Ras binds to a variety of effectors. Effectors are defined as proteins that interact selectively with the GTP-bound form of Ras, and become activated as a consequence of this interaction. Three downstream effector pathways have been characterized in the most detail (Figure 1). They include members of the Raf, PI3K and RalGDS families (reviewed in Reuther et al., 2000; Shields et al.,

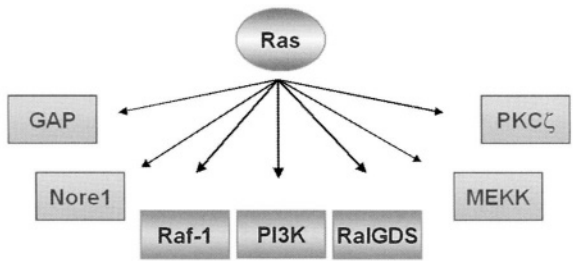

Figure 1. Ras signals through multiple downstream effectors including, but not limited to those illustrated here. In thyroid cells, Ras has been shown to signal through Raf-1, PI3K and RalGDS (shown in bold).

2000). Interaction between GTP-Ras and its first identified target, Raf-1, induces a conformational change that unmasks phosphorylation sites and anchors Raf-1 to the plasma membrane. Once this occurs, Raf-1 activity becomes Ras-independent. Active Raf binds to and phosphorylates MEK1/2 proteins, stimulating their kinase activity. MEK proteins are dual specificity serine/threonine and tyrosine protein kinases that phosphorylate and activate MAPK1/2 (also referred to as ERK1/2), protein kinases that play important roles in many cellular processes including the regulation of gene expression. In a similar fashion, binding of GTP-Ras to the p110 catalytic subunit of PI3K stimulates lipid kinase activity, increasing the production of second messenger phosphoinositide (3,4) P_2 (PIP2) and phosphoinositide (3,4,5) P_3 (PIP3). PIP3 promotes the activation of a kinase cascade that includes PDK-1, Akt (or PKB) and p70 ribosomal S6 protein kinase (p70s6k). These kinases phosphorylate numerous protein substrates with diverse roles in protein synthesis, cell proliferation and cell survival. PI3K also regulates survival through activation of Rac GTPases. Binding of GTP-Ras to RalGDS stimulates GEF activity towards the Ras-related proteins, Ral A and B. Downstream targets of Ral include phospholipase D, Rho, and Rac- and Cdc42-selective GAPs. There are a number of additional putative Ras effectors, including RasGAPs, MEKK, AF6, PKCζ and Nore1. To date, activated Ras has been shown to signal through MAPK, PI3K and RalGDS in thyroid cells, effects that are markedly influenced by cAMP, an important regulator of thyroid cell function and proliferation (Figure 2).

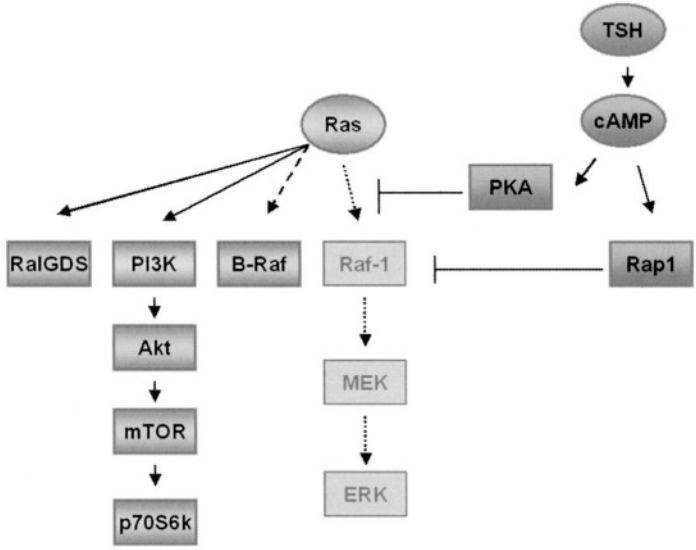

Figure 2. Ras signaling pathways are altered by TSH. TSH elevates cAMP, which activates PKA and Rap1 in thyroid cells. PKA has been reported to disrupt signaling from Ras to Raf-1 by phosphorylating the N-terminus of Raf-1, which decreases the affinity of Raf-1 for Ras. Activated Rap 1 binds to Raf-1, but does not stimulate its activity. It is not yet know whether Ras signals to B-Raf in thyroid cell (dashed line).

RAS MUTATIONS IN THYROID CANCER

A metastatic tumor is the end result of a complex series of steps involving multiple gene products. Work performed over the past decade has identified a number of gene products with putative roles in the initiation and progression of thyroid tumorigenesis. Mutations in Gsα (gsp) and the TSH receptor have been identified in hyperfunctioning adenomas. Ras mutations are prevalent in follicular carcinomas (see below). Mutations in ret, trk and met were identified in papillary carcinomas. Aberrant DNA methylation, leading to loss of expression of the p16 tumor suppressor gene, has been described in both types of cancer. Finally, mutations in p53 appear to play a role in the final dedifferentiation process. The reader is referred to several excellent recent reviews regarding the molecular basis of thyroid cancer (Jhiang, 2000; Gimm, 2001; Puxeddu et al., 2001; Fagin, 2002).

Early reports revealed that Ras mutations were particularly prevalent in benign follicular adenomas and follicular carcinomas, where estimates ranged as high as 50% (Lemoine et al., 1990; Namba et al., 1990; Suarez et al., 1990; Shi et al., 1991; Farid, 1994). The frequency of Ras mutations was initially reported to be similar in benign adenomas and follicular carcinomas, suggesting that Ras played an early role in thyroid transformation. However, more recent studies suggest that Ras mutations are less frequent than was first reported, occurring with an overall frequency of 16–19% (Esapa et al., 1999; Vasko et al., 2003). These studies also revealed a higher frequency of Ras mutations in follicular carcinomas versus adenomas, consistent with a role for Ras in malignant progression. According to recent data, mutations in codon 61 of N-Ras are the most frequent Ras mutation found in thyroid tumors (Nikiforova et al., 2003; Vasko et al., 2003). Besides Ras mutations, a significant proportion of follicular carcinomas exhibit a specific chromosomal translocation that fuses the coding regions for the paired and homeobox binding domains of the Pax-8 transcription factor to the DNA and ligand binding, dimerization and transactivation domains of PPARγ1 (Martelli et al., 2002). Interestingly, follicular carcinomas harboring both Ras mutations and the Pax-8/PPARγ translocation are extremely rare. This indicates either that both changes activate similar signaling pathways or that follicular carcinomas are comprised of at least two distinct tumor types that arise by different mechanisms (Nikiforova et al., 2003).

Although Ras mutations are infrequent in papillary thyroid carcinomas, somatic mutations in B-Raf were recently identified in these tumors (Kimura et al., 2003; Cohen et al., 2003). B-Raf mutations were discovered in a wide range of human tumors only last year (Davies et al., 2002; Rajagopalan et al., 2002). Intriguingly, mutations in B-Raf were found in cancers that typically harbor Ras mutations, such as malignant melanomas, colorectal tumors and ovarian cancers. The most frequent B-raf mutation (V599E) results in the insertion of an acidic residue close to a site of activating phosphorylation in the kinase domain. Recombinant B-RafV559E exhibits increased kinase activity, suggesting constitutive activation of signaling pathways similar to those activated by Ras. Moreover, unlike activated Raf-1 mutants that stimulate transformation through an autocrine mechanism involving Ras, the effects of

B-RafV559E on cell transformation were Ras-independent (Davies et al., 2002). The same B-Raf mutation has now been shown to be the most common genetic change in papillary thyroid carcinomas (Kimura et al., 2003). These results are striking for several reasons. First, there are three mammalian Raf proteins: Raf-1, A-Raf and B-Raf. While Raf-1 is ubiquitously expressed, A- and B-Raf exhibit a more restricted pattern of expression. Intriguingly, B-Raf is expressed in neuronal, neuroendocrine and endocrine cells. Of further interest with regard to thyroid cells, Raf proteins are important sites of integration between signals activated by Ras and cAMP. Cyclic AMP impairs the activation of Raf-1 by serum growth factors and Ras (Figure 2). In contrast, cAMP stimulates B-Raf activity (Erhardt et al., 1995; Vossler et al., 1997; Busca et al., 2000). Therefore, it is perhaps not surprising that melanoma cells harbor B-Raf mutations given their regulation by α-melanocyte stimulating hormone and related proopiomelanocortin-derived peptides that upregulate intracellular cAMP. Similarly, the identification of B-Raf mutations in thyroid tumors is particularly interesting given the growth promoting effects of chronic TSH stimulation. Despite the high frequency of Ras mutations in colorectal cancers, B-Raf mutations were found only in tumors without Ras mutations. In agreement with these results, no overlap was seen between mutations in Ras and B-Raf in papillary thyroid carcinomas (Kimura et al., 2003). These results provide strong support for the notion that B-Raf and Ras mutations are equivalent in their tumorigenic effects. Finally, RET/PTC oncogenes also signal partly through Ras (Barone et al., 2001; Castellone et al., 2003) and possibly through PDK-1 (Kim et al., 2003), a protein kinase that is also activated downstream from Ras. Strikingly, there appears to be no overlap between papillary carcinomas harboring RET/PTC, B-Raf and Ras mutations, which together account for two thirds of all papillary carcinomas. These findings underscore the significant contribution of Ras-mediated signaling pathways to thyroid tumorigenesis. In the following sections, I review what is known regarding the role of endogenous Ras, and the consequences of sustained Ras activity in thyroid cells.

ROLE OF CELLULAR RAS IN THYROID CELLS

TSH regulates the function and proliferation of thyroid follicular cells, highly specialized epithelial cells that synthesize, store and secrete thyroid hormones. Thyroid hormone biosynthesis requires the expression of cell type specific gene products, including the TSH receptor, thyroperoxidase, thyroglobulin and the sodium/iodide symporter (Damante et al., 1994). TSH regulates the expression of these genes in part through effects on thyroid-specific transcription factors such as TTF-1, TTF-2 and Pax-8 (Missero et al., 1998). The proliferation of thyroid cells is TSH-dependent (for recent reviews, see Medina et al., 2000; Kimura et al., 2001). However, for the most part, TSH acts together with insulin or IGF-I and serum to stimulate sustained proliferation. The effects of TSH on function and proliferation are reproduced by cAMP elevating agents and analogs. Positive growth regulation by cAMP is one of the unique features of thyroid cells and stands in marked contrast to the growth inhibitory effects of cAMP in many other cell types (Cook et al., 1993; Sevetson et al., 1993; Wu et al., 1993).

Compared to the effects of ectopic expression of constitutively active Ras, much less is known regarding the roles of endogenous Ras in thyroid cells. TSH-stimulated DNA synthesis was repressed following expression of dominant negative mutant Ras (Ras17N) in Wistar rat thyroid (WRT) (Kupperman et al., 1993) and FRTL-5 cells (Medina et al., 2000; Ciullo et al., 2001). Interference with Rac (Cass et al., 1999) or RhoA (Medina et al., 2002) also impaired TSH-stimulated DNA synthesis and mitogenesis, respectively. RhoA inhibition induced G1 phase cell cycle arrest in FRTL-5 cells (Hirai et al., 1997). Together, these data indicate that Ras family members play an integral role in the proliferation of rat thyroid cells. Whether Ras is required downstream from TSH/cAMP (Medina et al., 2000; Medina et al., 2000; Tsygankova et al., 2000), in addition to functioning downstream from insulin/IGF-I, is controversial (Van Keymeulen et al., 2000).

Although Ras stimulates proliferation through the Raf-1/MAPK cascade in many cells, this effector pathway does not play a major role in TSH signaling. Lamy *et al.* first reported that TSH failed to stimulate MAPK activity in canine thyroid cells (Lamy et al., 1993), in agreement with numerous studies in other cell types where cAMP inhibits growth factor stimulated-MAPK activity. TSH inhibited serum-stimulated MAPK activity in WRT cells (Miller et al., 1997) and treatment with the MEK1 inhibitor PD98059 had no effect on TSH-stimulated DNA synthesis in FRTL-5 cells (Medina et al., 2000). In addition to indicating that TSH does not signal through this cascade, these findings also suggested that Ras is unlikely to signal through the Raf-1/MAPK cascade in the presence of TSH (Figure 2), results that were later confirmed (see below). TSH stimulates p70s6k activity in WRT (Cass et al., 1998) and FRTL-5 cells (Medina et al., 2000), effects that were observed in the absence of insulin. Interference with mTOR, an upstream activator of p70s6k, impaired TSH-stimulated proliferation (Cass et al., 1998; Cass et al., 1999; Coulonval et al., 2000). As p70s6k can be activated downstream from PI3K, the role of PI3K in thyroid cell proliferation was investigated. Treatment with cell permeable PI3K inhibitors, microinjection of a dominant negative PI3K mutant, or injection of a p110-specific antibody inhibited TSH/cAMP stimulated DNA synthesis (Cass et al., 1999). Similarly, expression of dominant negative PI3K induced G1 phase cell cycle arrest in FRTL-5 cells and PI3K inhibitors blocked the stimulatory effects of TSH on cyclin E expression, a molecular marker of G1 phase cell cycle progression (Medina et al., 2000). Wortmannin and LY294002, cell permeable PI3K inhibitors, impaired TSH and insulin-stimulated DNA synthesis in canine thyroid cells (Coulonval et al., 2000) and IGF-I-stimulated proliferation in FRTL-5 cells (Saito et al., 2001). The role of PI3K in thyroid cell proliferation gained further support with the discovery that expression of PTEN, a negative regulator of PI3K, was decreased in a significant proportion of follicular neoplasms (Bruni et al., 2000), and that Akt activity is increased in follicular carcinomas (Ringel et al., 2001).

The acute effects of Ras activation in thyroid cells were first examined following microinjection of purified Ras protein into WRT cells (Kupperman et al., 1993). Microinjection of cellular or activated H-Ras protein was sufficient to stimulate DNA synthesis in quiescent cells. The ability of Ras to stimulate DNA synthesis in starved

cells was impaired by co-injection of a Raf-1 antibody or of a dominant negative MEK1 protein (Al-Alawi et al., 1995), invoking a role for the Raf/MAPK cascade in growth stimulation by Ras. When injected into cells that were treated with TSH or 8BrcAMP, however, co-injection of the Raf-1 antibody or of the dominant negative MEK1 protein failed to impair Ras-stimulated DNA synthesis. These results provided the first indication that crosstalk between TSH/cAMP and Ras influences which effector pathways are activated by Ras in thyroid cells (Figure 2). The ability of TSH to influence Ras signaling received additional strong support from Ciullo *et al.* These authors confirmed that Ras and PI3K are required for TSH-stimulated cell cycle progression in FRTL-5 cells (Ciullo et al., 2001). Importantly, they demonstrated that TSH stimulated complex formation between endogenous Ras and PI3K, whereas it impaired association between Ras and Raf-1.

SUSTAINED RAS ACTIVITY AND THYROID CELL PROLIFERATION

The identification of activating Ras mutations in thyroid tumors prompted studies of the consequences of sustained Ras activity in thyroid cells. Early gene transfer studies revealed the oncogenic potential of Ras in thyroid cells *in vitro.* Stable expression of activated H- or K-Ras in FRTL-5 cells was fully transforming. Ras-expressing cells exhibited hormone-independent proliferation, anchorage-independent growth and formed tumors in nude mice (Fusco et al., 1987). In contrast to these results, rat thyroid PC-CL3 cells were only partially transformed by Ras. *In vivo* studies demonstrated that Ras activation was insufficient for tumor formation. When injected into adult Fischer rats, a K-Ras retrovirus induced the formation of differentiated thyroid carcinomas only in goitrogen-treated animals (Portella et al., 1989). Similarly, targeted expression of activated K-Ras to the thyroid gland stimulated hyperplasia and adenoma formation, but only in rare instances tumor formation, which required goitrogen treatment (Santelli et al., 1993). In seeming contrast to these findings, expression of activated H-Ras in the thyroid gland stimulated hyperplasia and papillary thyroid tumors (Rochefort et al., 1996; Feunteun et al., 1997).

The effects of Ras on the proliferation of human thyroid cells are of enormous interest. Using retroviruses, Lemoine *et al.* demonstrated that activated H-Ras stimulates the sustained proliferation of primary human thyroid cells (Lemoine et al., 1990; Bond et al., 1994; Jones et al., 2000). The mitogenic effects of Ras were also seen following acute introduction of activated Ras protein by scrape loading or microinjection (Gire et al., 1999). The ability of Ras to stimulate proliferation in primary thyroid cells is very different from its effects in primary fibroblasts where expression of activated Ras stimulated growth arrest (Serrano et al., 1997; Olson et al., 1998). Ras-expressing human thyroid cells were morphologically transformed and exhibited anchorage-independent growth, but not tumor formation. Strikingly, after 15–25 population doublings, Ras-expressing cells ceased to proliferate and became senescent, results quite similar to the limited growth potential of benign follicular adenomas harboring Ras mutations. These data strongly support the idea that human thyroid cells harbor a cell-intrinsic mechanism that prevents unchecked proliferation, even in the face of sustained expression of activated Ras.

In human cells, Ras-stimulated proliferation was impaired by inhibition of MAPK and PI3K activity. Although both pathways were required, activation of either signaling cascade alone was insufficient to stimulate proliferation (Gire et al., 1999; Gire et al., 2000). These findings are not dissimilar from those observed in rat thyroid cells, where Ras stimulated DNA synthesis through the Raf/MAPK cascade in the absence of TSH (Al-Alawi et al., 1995).

SUSTAINED RAS ACTIVITY AND THYROID DIFFERENTIATION

Ras transformation suppressed differentiated gene expression in rat thyroid cells (Avvedimento et al., 1985; Fusco et al., 1987; Francis-Lang et al., 1992). Intriguingly, H- and K-Ras impaired differentiation in different ways. H-Ras transformation was associated with the loss of Pax-8 and TTF-2 expression, and inactivation of TTF-1 possibly through decreased phosphorylation (Francis-Lang et al., 1992; Velasco et al., 1998). In contrast, TTF-1 expression was abolished in K-Ras transformed cells (Francis-Lang et al., 1992). K-Ras has also been shown to impair the nuclear localization of PKA, thereby preventing PKA-mediated phosphorylation of nuclear transcription factors (Gallo et al., 1995). Ras-transformed human cells retain their differentiated phenotype (Lemoine et al., 1990; Gire et al., 2000), a finding consistent with the occurrence of Ras mutations in differentiated thyroid tumors. It should be noted that de-differentiation is not an obligate response of rat thyroid cells to activated Ras. Using Ras effector domain mutants that signal through discrete downstream effectors (White et al., 1995), Miller *et al.* demonstrated that Ras signaling through the Raf/MAPK cascade impaired thyroglobulin expression in WRT cells, while Ras signaling to RalGDS (Miller et al., 1998) or PI3K did not (Cass et al., 2000). Stable expression of activated MEK-1, a downstream target of Raf, failed to de-differentiate FRTL-5 cells, perhaps due to its relatively modest effects on MAPK activity (Cobellis et al., 1998). Indeed, further studies by the same authors revealed that transient expression of RasS35, the effector domain mutant that signals selectively through Raf-1, or of activated Raf-1 impaired TTF-1 activity (Missero et al., 2000). Furthermore, MEK inhibitors partially blocked the inhibitory effects of Ras on TTF-1 activity. These data indicate that activation of the MAPK cascade impairs differentiated gene expression in rat thyroid cells, and that there are likely to be additional signals through which Ras suppresses thyroid differentiation. Different effects of RhoA on differentiated gene expression have been reported. Stable expression of activated RhoA impaired thyroglobulin and TTF-1 mRNA and protein levels (Medina et al., 2002), however transient expression of activated RhoA failed to repress TTF-1 promoter activity (Missero et al., 2000). Given the inhibitory effects of Ras signaling to MAPK on thyroid differentiation, the ability of TSH to direct Ras signals to alternate effectors such as PI3K and RalGDS would allow TSH to stimulate proliferation through Ras in cells that retain their differentiated character.

RAS AND THYROID CELL SURVIVAL

Although stable expression of activated Ras in thyroid cells confers hormone and anchorage-independent proliferation, it also renders cells more sensitive to apoptosis.

Serum withdrawal induced apoptosis in H- and K-Ras-transformed, but not parental FRTL-5 cells (DiJeso et al., 1995). H-Ras-transformed WRT cells exhibit an enhanced sensitivity to apoptosis in response to deprivation of adhesion or treatment with MAPK and PI3K inhibitors (Cheng et al., 2001). Inducible expression of activated Ras in PC-CL3 cells stimulated proliferation followed by apoptosis (Shirokawa et al., 2000). Intriguingly, apoptosis was strictly dependent upon TSH or cAMP elevation; in the absence of TSH/cAMP, Ras stimulated proliferation. Although epithelial cells typically perish by anoikis following detachment, Ras stimulated apoptosis in adherent PC-CL3 cells. Interference with either MAPK or JNK impaired apoptosis, invoking a role for these signaling pathways in Ras-mediated apoptosis. Acute infection of WRT, FRTL-5 and PC-CL3 cells with an adenovirus expressing activated H-Ras also stimulated apoptosis, although in this instance cell death occurred in the presence or absence of TSH (Cheng et al., 2003).

The effects of Ras on apoptosis are not limited to rat thyroid cells. Human thyroid cells immortalized by temperature-sensitive SV40 large T antigen underwent rapid cell death following expression of activated Ras at the restrictive temperature (Burns et al., 1993). Exposure to the phorbol ester tumor promoter TPA stimulated apoptosis in human thyroid cells expressing activated H-Ras (Hall-Jackson et al., 1998). Moreover, inhibition of PI3K activity induced apoptosis in H-Ras-expressing human thyroid cells, suggesting that clonal expansion induced by Ras requires PI3K activity to suppress apoptosis (Gire et al., 2000). Together these findings indicate that apoptosis is a conserved response to acute expression of activated Ras in thyroid cells. The relative ease with which rat and human thyroid cells are selected to survive stable expression of activated Ras also indicates that secondary changes that allow for the survival of Ras-expressing thyroid cells are frequent. The elucidation of the factors that dictate whether Ras stimulates transient or sustained proliferation, growth arrest or apoptosis is an exciting area for exploration.

RAS AND GENOMIC INSTABILITY

One of the consequences of Ras mutations in human tumors is destabilization of the karyotype. Expression of activated Ras in a variety of established cell lines and tumor cells induces chromosomal aberrations including an enhanced frequency of gene amplification (Smith et al., 1995), chromosome losses and gains, aberrant chromosome segregation and centrosome amplification (Saavedra et al., 1999). Inducible expression of activated H-Ras in PC-CL3 cells stimulated the formation of micronuclei containing chromosomes and chromosome fragments (Saavedra et al., 2000). Micronuclei with whole chromosomes arise as a consequence of spindle disruption; micronuclei containing chromosome fragments are typically generated by double strand DNA breaks. Although the effects of Ras on micronuclei formation were rapid, they were observed in only a small proportion of cells, perhaps due to the presence of wildtype p53 in these cells. This raises the interesting possibility that Ras predisposes thyroid cells to the acquisition of additional mutations by inducing genomic instability. Over time, cells harboring Ras mutations would acquire additional genetic and epigenetic changes that contribute to their full transformation. In this regard, it is interesting that Ras

mutations occur at a higher frequency in follicular versus papillary carcinomas, given the higher degree of aneuploidy observed in follicular carcinomas (Fagin, 2002). It will be interesting to assess whether papillary tumors bearing B-Raf mutations exhibit similar chromosomal aberrations. Whether Ras induces genomic instability in primary human thyroid cells, which exhibit a more stable karyotype than do rodent cells, has not yet been determined.

While chromosomal instability could provide the mechanism for Ras-stimulated apoptosis, the low frequency of micronuclei formation versus the high frequency of apoptosis argues that these events are distinct (Shirokawa et al., 2000). Apoptosis was TSH-dependent, while micronuclei formation was insensitive to cAMP levels. Furthermore, the signaling pathways through which Ras stimulated micronuclei formation and apoptosis were distinct. Therefore, acute expression of activated Ras stimulates multiple signals leading to DNA damage and chromosomal instability in a small proportion of cells and apoptosis in most cells. The changes that occur in the small population of surviving cells that contribute to their survival in the presence of activated Ras remain to be identified.

CELL CYCLE DEREGULATION AND APOPTOSIS

Acute expression of activated Ras stimulated aberrant cell cycle progression followed by apoptosis in WRT cells (Cheng et al., 2003). Infection of quiescent cells with an adenovirus expressing activated H-Ras induced cell cycle progression into S phase. Rather than completing the cell cycle, the majority of Ras-expressing cells exhibited a protracted S phase and ultimately perished by apoptosis. The effects of Ras on cell cycle regulatory proteins were very different from the mitogens, TSH, insulin, and serum. Unlike mitogen treatment, which increased the expression of cyclins D1 and B, Ras rapidly decreased cyclin D1 expression, and failed to increase cyclin B expression. Excessive mitogenic signaling, for example through overexpression of E2F (Nahle et al., 2002), is a potent inducer of apoptosis, as is delayed cell cycle progression (Meikrantz et al., 1995). These findings suggest that the acute effects of Ras on thyroid cell cycle progression are aberrant, and induce an apoptotic response. Similar effects on cell cycle progression and apoptosis have been reported following expression of high mobility group (HMG) proteins in thyroid cells. Impaired expression of HMGA in PC-CL3 cells blocked Ras transformation, suggesting a role for HMGA downstream from Ras (Fedele et al., 2001). When stably overexpressed, HMGA induced aberrant cell cycle progression characterized by premature entry into S phase and delayed transition into G2/M. These aberrant effects on cell cycle progression were accompanied by apoptosis. These data support the idea that conflict between mitogenic pressure and the inability to proceed through the cell cycle generates an apoptotic signal in rat thyroid cells. The high frequency of cell death in Ras-expressing human thyroid cells at the restrictive temperature for SV40 large T (Burns et al., 1993) may reflect a similar mechanism. The deletion of thyroid cells harboring Ras mutations by apoptosis might explain why Ras mutations are not found in a higher proportion of thyroid tumors.

The effects of activated Ras on the expression of p16, p21 and p27, cyclin-dependent kinase inhibitors, in human thyrocytes (Jones et al., 2000) differed from those reported

in human fibroblasts (Serrano et al., 1997; Olson et al, 1998). In thyroid cells, acute expression of Ras decreased p27 expression, and failed to increase expression of either p16 or p21. These changes would be expected to increase cyclin-dependent kinase activity, in particular cdk-2 activity, which is potently inhibited by p21 and p27 (reviewed in Sherr et al., 1999). These alterations could contribute to the mitogenic activity of Ras in human thyroid cells. In fibroblasts, Ras stimulated p16 and p21 expression, resulting in G1 phase cell cycle arrest. Cessation of proliferation in human thyroid cells was accompanied by re-expression of p27 and *de novo* expression of p16, changes expected to impair cdk activity (Jones et al., 2000). Interestingly, when HPV E7 was expressed in these cells to inactivate growth suppression by p16 and p27, senescence was bypassed. Although the cells continued to proliferate, proliferation was accompanied by increasing cell death. Therefore, in human and rat thyroid cells, proliferative pressure in the face of signals that impair growth induces apoptosis. This would impose selective pressure for cells in which apoptosis is circumvented, for example through the increased expression of positive regulators of cell cycle progression, decreased expression of growth inhibitors or alterations in cell survival signals. Indeed, unlike acute expression of activated Ras, which abolished the expression of cyclin D1, WRT cells selected to survive constitutive expression of activated Ras exhibited increased levels of cyclin D1 (Cheng et al., 2003). The expression of cyclins Dl (Wang et al., 1998; Muro-Cacho et al., 1999) and E (Lazzereschi et al., 1998; Wang et al., 1998) are frequently increased in thyroid tumors. Decreased expression of p27 in carcinomas versus adenomas has been reported (Erickson et al., 1998; Wang et al., 1998). Methylation of the p16 promoter, resulting in reduced p16 expression, has been reported in many differentiated thyroid tumors (Ivan et al., 1996; Jones et al., 1996). Together these findings suggest that alterations in the expression of cell cycle regulatory proteins may contribute to oncogenic transformation by Ras.

CONCLUSIONS AND PERSPECTIVES

While the frequency of Ras mutations in human tumors is only 20% overall, constitutive signaling through Ras is a conserved feature of a much higher proportion of human tumors. Mutations giving rise to increased production of growth factors or sustained activation of growth factor receptors are frequent events in human tumors. Over the past year, mutations in a Ras effector, B-Raf, have been identified in several types of human cancer (Davies et al., 2002; Rajagopalan et al., 2002). Together, mutations that give rise to constitutive signaling through Ras-mediated pathways comprise a significant proportion of human tumors. The fundamental role of Ras in tumorigenesis is particularly evident in the thyroid gland where mutations in Ras, B-Raf and RET/PTC have now been identified. Expression of activated Ras in thyroid cells *in vitro* elicits morphological transformation, sustained proliferation, apoptosis and genomic instability, hallmarks of human tumor cells (Figure 3). Despite the significant advances that have been made in identifying regulators and targets of Ras, these recent advances highlight how little we know regarding the signaling pathways activated by Ras, as well as the consequences of sustained Ras activity in the thyroid cell.

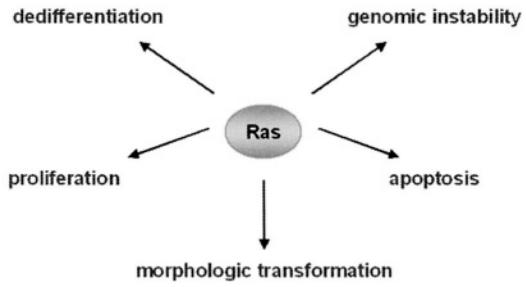

Figure 3. Ras elicits multiple effects in thyroid cells.

Overexpression studies have relied extensively on H- and K- rather than N-Ras, which appears to be a more frequent target for mutation in thyroid cancer. With accumulating evidence that individual Ras proteins localize to different cellular microdomains (Prior et al., 2001; Chiu et al., 2002; Matallanas et al., 2003) and signal in distinct ways (Maher et al., 1995; Villalonga et al., 2001; Walsh et al., 2001), it will be important to assess the specific consequences of N-*ras* activity in thyroid cells. It is interesting that N-Ras provides a survival signal in fibroblasts (Wolfman et al., 2002).

The recent discovery of B-Raf mutations in thyroid tumors paves the way for studies of the contribution of this specific Raf isoform to tumorigenesis. Although cAMP impairs Ras signaling to Raf-1, its effects on B-Raf activity in thyroid cells are largely unknown. Moreover, cAMP itself stimulates several signaling pathways with the potential to regulate B-Raf activity. Thyroid cells are a rich source of Epac, a Rap1-specific GEF (DeRooij et al., 1998; Kawasaki et al., 1998). TSH and cAMP activate endogenous Rap1 in rat (Tsygankova et al., 2001) and canine (Dremier et al., 1997) thyroid cells, effects that are PKA-independent. In other cells, active Rap1 binds to Raf-1 and B-Raf, but with differing consequences. Association between Rap1 and B-Raf stimulates B-Raf activity, whereas Rap-1:Raf-1 complexes are inactive. B-Raf is a substrate for protein kinase A, the other arm of the cAMP signaling pathway. This raises interesting avenues for regulation of B-Raf by cAMP and PKA. The signaling pathways activated by B-Raf in thyroid cells are unknown. While B-Raf binds and activates MEK1/2, it remains to be determined whether this pathway is active in TSH-treated cells.

Rap1 has been linked to TSH effects on differentiated gene expression (Tsygankova et al., 2001) and proliferation (Ribeiro-Neto et al., 2002), although the mechanism through which it elicits these effects is not clear. A limited mutational analysis failed to reveal mutations in Rap 1 or Epac in follicular adenomas (Vanvooren et al., 2001). Nonetheless, the contribution of Rap 1 to thyroid cell biology is important to pursue based on its ability to signal through B-Raf and to affect Ras-mediated signaling. Rap1 was initially isolated as K-rev1, an inhibitor of K-Ras transformation (Kitayama et al., 1989). Although Rap 1 clearly functions in Ras-independent pathways, crosstalk between Ras and Rap 1 has been shown to modulate the ability of Ras to activate

discrete effector pathways. Competition between Rap1 and Ras for downstream signaling molecules may provide a mechanism for balancing the activities of these two signaling molecules and for channeling their effects to discrete effector pathways.

The notion that Ras stimulates genomic instability, predisposing thyroid cells to the acquisition of additional mutations, promises to provide further insight into the molecular mechanisms through which Ras contributes to thyroid cell transformation. Thyroid cancer cell lines and tumors have been shown to exhibit mitotic checkpoint dysfunction, however the genetic and/or epigenetic changes responsible for this have not been identified. A recent analysis failed to reveal mutations in the candidate checkpoint genes, BUB1 or BUBR1 (Ouyang et al., 2002). The relationship between tumors harboring mutations in Ras or B-Raf to DNA damage and effects on p53 deserves further attention given genetic evidence for the acquisition of p53 mutations secondary to mutations in N-Ras in thyroid tumors (Asakawa et al., 2002). In experimental models, stable expression of activated Ras has been shown to induce p53 mutations (Chen et al., 1998). The effects of Ras on cell cycle regulatory proteins needs to be examined in more detail given the unusual effects of Ras on these molecules in human and rat thyroid cells, together with the identification of mutations in cyclins and cyclin-dependent kinase inhibitors in thyroid tumors. It is noteworthy that overexpression of cyclins D1 (Lung et al., 2002) and E (Spruck et al., 1999) has also been linked to genomic instability.

The TSH-dependent nature and relative ease with which rat thyroid cells can be manipulated *in vitro* affords an important cell model for future studies. Transgenic and knock out animal models for discrete Ras and Raf isoforms hold enormous promise for understanding the contributions of these signaling molecules to thyroid tumorigenesis. Whatever is learned from the rodent model systems must be validated in human thyroid cells. With increasing evidence that Ras signals through discrete pathways in rodent versus human cells (Hamad et al., 2002), studies of the signal transduction mechanisms and consequences of Ras and B-Raf activity in human thyroid cells are essential. Finally, while numerous studies have examined the cellular consequences that arise following transient or stable expression of activated Ras, few studies have attempted to model both the primary and secondary adaptive changes that occur in response to sustained Ras activity in the same cells.

REFERENCES

1. Al-Alawi, N., Rose, D. W., Buckmaster, C., Ahn, N., Rapp, U., Meinkoth, J. and Feramisco, J. R., 1995, Thyrotropin-induced mitogenesis is Ras dependent but appears to bypass the Raf-dependent cytoplasmic kinase cascade, *Mol Cell Biol.* **15:** 1162–8.
2. Asakawa, H. and Kobayashi, T., 2002, Multistep carcinogenesis in anaplastic thyroid carcinoma: a case report, *Pathology.* **34:** 94–7.
3. Avvedimento, E. V., Monticelli, A., Tramontano, D., Polistina, C., Nitsch, L. and Di Lauro, R., 1985, Differential expression of thyroglobulin gene in normal and transformed thyroid cells, *EurJ Biochem.* **149:** 467–472.
4. Barone, M. V., Sepe, L., Melillo, R. M., Mineo, A., Santelli, G., Monaco, C., Castellone, M. D., Tramontano, D., Fusco, A. and Santoro, M., 2001, RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho, *Oncogene.* **20:** 6973–82.
5. Bond, J. A., Wyllie, F. S., Rowson, J., Radulescu, A. and Wynford-Thomas, D., 1994, In vitro reconstruction of tumour initiation in a human epithelium, *Oncogene.* **9:** 281–290.

6. Bruni, P., Boccia, A., Baldassarre, G., Trapasso, F., Santoro, M., Chiappetta, G., Fusco, A. and Viglietto, G., 2000, PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing activity in thyroid cancer cells is mediated by p27kip1, *Oncogene.* **19:** 3146–3155.
7. Burns, J., Barton, C., Wynford-Thomas, D. and Lemoine, N., 1993, In vitro transformation of epithelial cells by ras oncogenes, *Epithelial Cell Biol.* **2:** 26–43.
8. Busca, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaux, C., Eychene, A., Ortonne, J. P. and Ballotti, R., 2000, Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes, *EMBO J.* **19:** 2900–10.
9. Cass, L. A. and Meinkoth, J. L., 1998, Differential effects of cyclic adenosine 3′, 5′-monophosphate on p70 ribosomal S6 kinase, *Endocrinol.* **139:** 1991–1998.
10. Cass, L. A. and Meinkoth, J. L., 2000, Ras signaling through PI3K confers hormone-independent proliferation that is compatible with differentiation, *Oncogene.* **19:** 924–32.
11. Cass, L. A., Summers, S. A., Prendergast, G. V., Backer, J. M., Birnbaum, M. J. and Meinkoth, J. L., 1999, Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation, *Mol Cell Biol.* **19:** 5882–91.
12. Castellone, M. D., Cirafici, A. M., De Vita, G., De Falco, V., Malorni, L., Tallini, G., Fagin, J. A., Fusco, A., Melillo, R. M. and Santoro, M., 2003, Ras-mediated apoptosis of PC CL 3 rat thyroid cells induced by RET/PTC oncogenes, *Oncogene.* **22:** 246–55.
13. Chen, C.-Y., Liou, J., Forman, L. W. and Faller, D. V., 1998, Differential regulation of discrete apoptotic pathways by Ras, *J Biol Chem.* **273:** 16700–16709.
14. Cheng, G., Lewis, A. E. and Meinkoth, J. L., 2003, Ras stimulates aberrant cell cycle progression and apoptosis in rat thyroid cells, *Mol Endocrinol.* **17:** 450–9.
15. Cheng, G. and Meinkoth, J. L., 2001, Enhanced sensitivity to apoptosis in Ras-transformed thyroid cells, *Oncogene.* **20:** 7334–41.
16. Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., 2nd, Cox. A. D. and Philips, M. R., 2002, Ras signalling on the endoplasmic reticulum and the Golgi, *Nat Cell Biol.* **4:** 343–50.
17. Ciullo, I., Diez-Roux, G., Di Domenico, M., Migliaccio, A. and Avvedimento, E. V., 2001, cAMP signaling selectively influences Ras effectors pathways, *Oncogene.* **20:** 1186–92.
18. Cobellis, G., Missero, C. and Di Lauro, R., 1998, Concomitant activation of MEK-1 and Rac-1 increases the proliferative potential of thyroid epithelial cells, without affecting their differentiation, *Oncogene.* **17:** 2047–57.
19. Cohen, Y., Xing, M., Mambo, E., Guo, Z., Wu, G., Trink, B., Beller, U, Westra, W. H., Ladenson, P. W and Sidransky, D., 2003, BRAF mutation in papillary thyroid carcinoma, *J. Null. Cane. Inst.* **95:** 625–627.
20. Cook, S. J. and McCormick, F., 1993, Inhibition by cAMP of Ras-dependent activation of Raf, *Science.* **262:** 1059–1062.
21. Coulonval, K., Vandeput, F., Stem, R. C., Kozma, S. C., Lamy, F. and Dumont, J. E., 2000, Phosphatidylinositol 3-kinase, protein kinase B and ribosomal S6 kinases in the stimulation of thyroid epithelial cell proliferation by cAMP and growth factors in the presence of insulin, *Biochem J.* **348:** 351–8.
22. Damante, G. and Di Lauro, R., 1994, Thyroid-specific gene expression, *Biochim Biophys Acta.* **1218:** 255–266.
23. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C.J., Wooster, R., Stratton, M. R. and Futreal, P. A., 2002, Mutations of the BRAF gene in human cancer, *Nature.* **417:** 949–54.
24. DeRooij, J., Zwartkruis, F. J. T., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., Wittinghofer, A. and Bos, J. L., 1998, Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP, *Nature.* **396:** 474–477.
25. Di Jeso, B., Ulianich, L., Racioppi, L., D'Armiento, F., Feliciello, A., Pacifico, F., Consiglio, E. and Formisano, S., 1995, Serum withdrawal induces apoptotic cell death in Ki-ras transformed but not in normal differentiated thyroid cells, *Biochem Biophys Res Commun.* **214:** 819–24.
26. Dremier, S., Pohl, V., Poteet-Smith, C., Roger, P., Corbin, J., Doskeland, S. O., Dumont, J. E. and Maenhaut, C., 1997, Activation of cyclic AMP-dependent protein kinase is required but may not be

sufficient to mimic cyclic AMP-dependent DNA synthesis and thyroglobulin expression in dog thyroid cells, *Mol. Cell. Biol.* **17:** 6717–6726.
27. Erhardt, P., Troppmair, J., Rapp, U. and Cooper, G. M., 1995, Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells, *Mol Cell Biol.* **15:** 5524–5530.
28. Erickson, L. A., Jin, L., Wollan, P. C., Thompson, G. B., van Heerden, J. and Lloyd, R. V., 1998, Expression of p27kip1 and Ki-67 in benign and malignant thyroid tumors, *Mod Pathol.* **11:** 169–74.
29. Esapa, C. T., Johnson, S. J., Kendall-Taylor, P., Lennard, T. W. and Harris, P. E., 1999, Prevalence of Ras mutations in thyroid neoplasia, *Clin Endocrinol* **50:** 529–35.
30. Fagin, J. A., 2002, Minireview: branded from the start-distinct oncogenic initiating events may determine tumor fate in the thyroid, *Mol Endocrinol.* **16:** 903–11.
31. Fagin, J. A., 2002, Perspective: lessons learned from molecular genetic studies of thyroid cancer–insights into pathogenesis and tumor-specific therapeutic targets, *Endocrinology.* **143:** 2025–8.
32. Farid, N. R., Shi, Y., and Zou, M., 1994, Molecular basis of thyroid cancer, *Endocrine Reviews.* **15:** 202–232.
33. Fedele, M., Pierantoni, G. M., Berlingieri, M. T., Battista, S., Baldassarre, G., Munshi, N., Dentice, M., Thanos, D., Santoro, M., Viglietto, G. and Fusco, A., 2001, Overexpression of proteins HMGA1 induces cell cycle deregulation and apoptosis in normal rat thyroid cells, *Cancer Res.* **61:** 4583–90.
34. Feunteun, J., Michiels, F., Rochefort, P., Caillou, B., Talbot, M., Fournes, B., Mercken, L., Schlumberger, M. and Monier, R., 1997, Targeted oncogenesis in the thyroid of transgenic mice, *Horm Res.* **47:** 137–9.
35. Francis-Lang, H., Zannini, M., De Felice, M., Berlingieri, M. T., Fusco, A. and Di Lauro, R., 1992, Multiple mechanisms of interference between transformation and differentiation in thyroid cells, *Mol Cell Biol.* **12:** 5793–800.
36. Fusco, A., Berlingieri, M. T., Di Fiore, P. P., Portella, G., Grieco, M. and Vecchio, G., 1987, One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes, *Mol Cell Biol.* **7:** 3365–70.
37. Gallo, A., Feliciello, A., Varrone, A., Cerillo, R., Gottesman, M. E. and Avvedimento, E. V., 1995, Ki-ras oncogene interferes with the expression of cycliin AMP-dependent promoters, *Cell Growth and Difftn.* **6:** 91–95.
38. Gimm, O., 2001, Thyroid cancer, *Cancer Lett.* **163:** 143–56.
39. Gire, V., Marshall, C. and Wynford-Thomas, D., 2000, PI-3-kinase is an essential anti-apoptotic effector in the proliferative response of primary human epithelial cells to mutant RAS, *Oncogene.* **19:** 2269–76.
40. Gire, V., Marshall, C. J. and Wynford-Thomas, D., 1999, Activation of mitogen-activated protein kinase is necessary but not sufficient for proliferation of human thyroid epithelial cells induced by mutant Ras, *Oncogene.* **18:** 4819–32.
41. Gire, V. and Wynford-Thomas, D., 2000, RAS oncogene activation induces proliferation in normal human thyroid epithelial cells without loss of differentiation, *Oncogene.* **19:** 737–44.
42. Hall-Jackson, C. A., Jones, T, Eccles, N. G., Dawson, T. P., Bond, J. A., Gescher, A. and Wynford-Thomas, D., 1998, Induction of cell death by stimulation of protein kinase C in human epithelial cells expressing a mutant ras oncogene: a potential therapeutic target, *Br J Cancer.* **78:** 641–51.
43. Hamad, N. M., Elconin, J. H., Karnoub, A. E., Bai, W., Rich, J. N., Abraham, R. T., Der, C. J. and Counter, C. M., 2002, Distinct requirements for Ras oncogenesis in human versus mouse cells, *Genes Dev.* **16:** 2045–57.
44. Hirai, A., Nakamura, S., Noguchi, Y., Yasuda, T., Kitagawa, M., Tatsuno, I., Oeda, T., Tahara, K., Terano, T., Narumiya, S., Kohn, L. D. and Saito, Y., 1997, Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27Kip1 and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells, *J Biol Chem.* **272:** 13–6.
45. Ivan, M., Wynford-Thomas, D. and Jones, C. J., 1996, Abnormalities of the p16INK4a gene in thyroid cancer cell lines, *Eur J. Canc.* **32:** 2369–2370.
46. Jhiang, S. M., 2000, The RET proto-oncogene in human cancers, *Oncogene.* **19:** 5590–7.
47. Jones, C. J., Kipling, D., Morris, M., Hepburn, P., Skinner, J., Bounacer, A., Wyllie, F. S., Ivan, M., Bartek, J., Wynford-Thomas, D. and Bond, J. A., 2000, Evidence for a telomere-independent "clock" limiting RAS oncogenc-driven proliferation of human thyroid epithelial cells, *Mol Cell Biol.* **20:** 5690–9.
48. Jones, C. J., Shaw, J. J., Wyllie, F. S., Gaillard, M., Schlumberger, M. and Wynford-Thomas, D., 1996, High frequency deletion of the tumour suppressor gene p16INK4a (MTS1) in human thyroid cancer cell lines, *Mol Cell Endocrinol* **116:** 115–119.

49. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, A. M., 1998, A family of cAMP-binding proteins that directly activate Rap1, *Science.* **282:** 2275–2279.
50. Kim, D. W., Hwang, J. H., Suh, J. M., Kim, H., Song, J. H., Hwang, E. S., Hwang, I. Y., Park, K. C., Chung, H. K., Kim, J. M., Park, J., Hemmings, B. A. and Shong, M., 2003, RET/PTC (Rearranged in transformation/papillary thyroid carcinomas) tyrosine kinase phosphorylates and activates phosphoinositide-dependent kinate 1 (PDK1): An alternative phosphatidylinositol 3-kinase-independent pathway to activate PDK1, *Mol Endo* **17:** 1382–1394.
51. Kimura, E. T., Nikiforova, M. N., Zhu, Z., Knauf, J. A., Nikiforov, Y. E. and Fagin, J. A., 2003, High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma, *Cancer Res.* **63:** 1454–7.
52. Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J. E. and Roger, P. P., 2001, Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models, *Endocrine Reviews.* **23:** 631–656.
53. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M., 1989, A ras-related gene with transformation suppressor activity, *Cell.* **56:** 77–84.
54. Kupperman, E., Wen, W. and Meinkoth, J. L., 1993, Inhibition of thyrotropin-stimulated DNA synthesis by microinjection of inhibitors of cellular Ras and cyclic AMP-dependent protein kinase, *Mol Cell Biol.* **13:** 4477–84.
55. Lamy, F., Wilkin, F., Baptist, M., Posada, J., Roger, P. P. and Dumont, J. E., 1993, Phosphorylation of mitogen-activated protein kinases is involved in the epidermal growth factor and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway, *J Biol Chem.* **268:** 8398–401.
56. Lazzereschi, D., Sambuco, L., Carnovale Scalzo, C., Ranieri, A., Mincione, G., Nardi, F. and Colletta, G., 1998, Cyclin D1 and Cyclin E expression in malignant thyroid cells and in human thyroid carcinomas, *IntJ Cancer.* **76:** 806–11.
57. Lemoine, N. R., Staddon, S., Bond, J., Wyllie, F. S., Shaw, J. J. and Wynford-Thomas, D., 1990, Partial transformation of human thyroid epithelial cells by mutant Ha-ras oncogene, *Oncogene.* **5:** 1833–7.
58. Lung, J. C., Chu, J. S., Yu, J. C., Yue, C. T., Lo, Y. L., Shen, C. Y. and Wu, C. W., 2002, Aberrant expression of cell-cycle regulator cyclin D1 in breast cancer is related to chromosomal genomic instability, *Genes Chromosomes Cancer.* **34:** 276–84.
59. Maher, J., Baker, D. A., Manning, M., Dibb, N. J. and Roberts, I. A. G., 1995, Evidence for cell-specific differences in transformation by N-, H- and K-ras, *Oncogene.* **11:** 1639–1647.
60. Martelli, M. L., Juliano, R., LePera, I., Sama, I., Monaco, C., Cammarota, S., Kroll, T., Chiariotti, L., Santoro, M. and Fusco, A., 2002, Inhibitory effects of peroxisome proliferator-activated receptor gamma on thyroid carcinoma cell growth, *J Clin Endocrinol Metab.* **87:** 4728–4735.
61. Matallanas, D., Arozarena, I., Berciano, M. T., Aaronson, D. S., Pellicer, A., Lafarga, M. and Crespo, P., 2003, Differences on the inhibitory specificities of H-Ras, K-Ras and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization, *J Biol Chem.* **278:** 4572–4581.
62. Medina, D. L., Rivas, M., Cruz, P., Barroso, I., Regadera, J. and Santisteban, P., 2002, RhoA activation promotes transformation and loss of thyroid cell differentiation interfering with thyroid transcription factor-1 activity, *Mol Endocrinol.* **16:** 33–44.
63. Medina, D. L. and Santisteban, P., 2000, Thyrotropin-dependent proliferation of in vitro rat thyroid cell systems, *Eur J Endocrinol.* **143:** 161–78.
64. Medina, D. L., Toro, M. J. and Santisteban, P., 2000, Somatostatin interferes with thyrotropin-induced G1-S transition mediated by cAMP-dependent protein kinase and phosphatidylinositol 3-kinase. Involvement of RhoA and cyclin E/cyclin-dependent kinase 2 complexes, *J Biol Chem.* **275:** 15549–56.
65. Meikrantz, W. and Schlegel, R., 1995, Apoptosis and the cell cycle, *J Cell Biochetm.* **58:** 160–74.
66. Miller, M. J., Prigent, S., Kupperman, E., Rioux, L., Park, S. H., Feramisco, J. R., White, M. A., Rutkowski, J. L. and Meinkoth, J. L., 1997, RalGDS functions in Ras- and cAMP-mediated growth stimulation, *J Biol Chem.* **272:** 5600–5.
67. Miller, M. J., Rioux, L., Prendergast, G. V., Cannon, S., White, M. A. and Meinkoth, J. L., 1998, Differential effects of protein kinase A on Ras effector pathways, *Mol Cell Biol.* **18:** 3718–26.
68. Missero, C., Cobellis, G., De Felice, M. and Di Lauro, R., 1998, Molecular events involved in differentiation of thyroid follicular cells, *Mol Cell Endocrinol.* **140:** 37–43.
69. Missero, C., Pirro, M. T. and Di Lauro, R., 2000, Multiple ras downstream pathways mediate functional repression of the homeobox gene product TTF-1, *Mol Cell Biol.* **20:** 2783–93.

70. Muro-Cacho, C. A., Holt, T., Klotch, D., Mora, L., Livingston, S. and Futran, N., 1999, Cyclin D1 expression as a prognostic parameter in papillary carcinoma of the thyroid, *Otolaryngol Head Neck Surg.* **120:** 200–7.
71. Nahle, Z., Polakoff, J., Davuluri, R. V, McCurrach, M. E., Jacobson, M. D., Narita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D. and Lowe, S. W., 2002, Direct coupling of the cell cycle and cell death machinery by E2F, *Nat Cell Biol.* **4:** 859–64.
72. Namba, H., Rubin, S. A. and Fagin, J. A., 1990, Point mutations of Ras oncogenes are an early event in thyroid tumorigenesis, *Mol. Endocrinol.* **4:** 1474–1479.
73. Nikiforova, M. N., Lynch, R. A., Biddinger, P. W., Alexander, E. K., Dorn, G. W., .2nd, Tallini, G., Kroll, T. G. and Nikiforov, Y. E., 2003, RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma, *J Clin Endocrinol Metab.* **88:** 2318–26.
74. Olson, M. F., Paterson, H. F. and Marshall, C. J., 1998, Signals from Ras and Rho GTPases interact to regulate expression of p21 Waf1/Cip1, *Nature.* **394:** 220–221.
75. Ouyang, B., Knauf, J. A., Ain, K., Nacev, B. and Fagin, J. A., 2002, Mechanisms of aneuploidy in thyroid cancer cell lines and tissues: evidence for mitotic checkpoint dysfunction without mutations in BUB1 and BUBR1, *Clin Endocrinol* **56:** 341–50.
76. Pak, Y., Pham, N. and Rotin, D., 2002, Direct binding of the Bl adrenergic receptor to the cyclic AMP-dependent guanine nucleotide exchange factor CNrasGEF leads to Ras activation, *Mol Cell Biol.* **22:** 7942–7952.
77. Portella, G., Ferulano, G., Santoro, M., Grieco, M., Fusco, A. and Vecchio, G., 1989, The Kirsten murine sarcoma virus induces rat thyroid carcinomas in vivo, *Oncogene.* **4:** 181–8.
78. Prior, I. A. and Hancock, J. F., 2001, Compartmentalization of Ras proteins, *J. Cell. Sci.* **114:** 1603–1608.
79. Puxeddu, E. and Fagin, J. A., 2001, Genetic markers in thyroid neoplasia, *Endocrinol Metab Clin North Am.* **30:** 493–513.
80. Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K. W., Vogelstein, B. and Velculescu, V. E., 2002, RAF/RAS oncogenes and mismatch-repair status, *Nature.* **418:** 934.
81. Reuther, G. W. and Der, C. J., 2000, The Ras branch of small GTPases: Ras family members don't fall far from the tree, *Curr Opin Cell Biol.* **12:** 157–165.
82. Ribeiro-Neto, F., Urbani, J., Lemee, N., Lou, L. and Altschuler, D. L., 2002, On the mitogenic properties of Rap1b: cAMP-induced G1/S entry requires activated and phosphorylated Rap1b, *Proc. Natl. Acad. Sci. USA.* **99:** 5418–5423.
83. Ringel, M. D., Hayre, N., Saito, J., Saunier, B., Schuppert, F., Burch, H., Bernet, V., Burman, K. D., Kohn, L. D. and Saji, M., 2001, Overexpression and overactivation of Akt in thyroid carcinoma, *Cancer Res.* **61:** 6105–11.
84. Rochefort, P., Caillou, B., Michiels, F. M., Ledent, C., Talbot, M., Schlumberger, M., Lavelle, F., Monier, R. and Feunteun, J., 1996, Thyroid pathologies in transgenic mice expressing a human activated Ras gene driven by a thyroglobulin promoter, *Oncogene.* **12:** 111–8.
85. Saavedra, H. I., Fukasawa, K., Conn, C. W and Stambrook, P.J., 1999, MAPK mediates RAS-induced chromosome instability, *J Biol Chem.* **274:** 38083–90.
86. Saavedra, H. I., Knauf, J. A., Shirokawa, J. M., Wang, J., Ouyang, B., Elisei, R., Stambrook, P.J. and Fagin, J. A., 2000, The RAS oncogene induces genomic instability in thyroid PCCL3 cells via the MAPK pathway, *Oncogene.* **19:** 3948–54.
87. Saito, J., Kohn, A. D., Roth, R. A., Noguchi, Y., Tatsumo, I., Hirai, A., Suzuki, K., Kohn, L. D., Saji, M. and Ringel, M. D., 2001, Regulation of FRTL-5 thyroid cell growth by phosphatidylinositol (OH) 3 kinase-dependent Akt-mediated signaling, *Thyroid.* **11:** 339–351.
88. Santelli, G., de Franciscis, V, Portella, G., Chiappetta, G., D'Alessio, A., Califano, D., Rosati, R., Mineo, A., Monaco, C., Manzo, G. and et al., 1993, Production of transgenic mice expressing the Ki-ras oncogene under the control of a thyroglobulin promoter, *Cancer Res.* **53:** 5523–7.
89. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. and Lowe, S. W., 1997, Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a, *Cell.* **88:** 593–602.
90. Sevetson, B. R., Kong, X. and Lawrence, J. C., 1993, Increasing cAMP attenuates activation of mitogen-activated protein kinase, *Proc. Natl. Acad. Sci. USA.* **90:** 10305–10309.
91. Sherr, C. J. and Roberts, J. M., 1999, CDK inhibitors: positive and negative regulators of Gl-phase progression, *Genes Dev.* **13:** 1501–12.
92. Shi, Y.F., Zou, M.J., Schmidt, H., Juhasz, F., Stenzky, V., Robb, D., and Farid, N.R., 1991, *Canc Res* **51:** 2690–2693.

93. Shields, J. M., Pruitt, K., McFall, A., Shaub, A. and Der, C. J., 2000, Understanding Ras: "it ain't over 'till it's over", *Trends in Cell Biol.* **10:** 147–153.
94. Shirokawa, J. M., Elisei, R., Knauf, J. A., Hara, T., Wang, J., Saavedra, H. I. and Fagin, J. A., 2000, Conditional apoptosis induced by oncogenic ras in thyroid cells, *Mol Endocrinol.* **14:** 1725–38.
95. Smith, K. A., Agarwal, K. L., Chernov, M. V., Chernova, O. B., Deguchi, Y., Ishizaka, Y., Patterson, Y. E., Poupon, M. F. and Stark, G. R., 1995, Regulation and mechanisms of gene amplification, *Philos Trans R Soc Lond B Biol* Sci. **347:** 49–56.
96. Spruck, C. H., Won, K. A. and Reed, S. I., 1999, Deregulated cyclin E induces chromosome instability, *Nature.* **401:** 297–300.
97. Suarez, H. G., du Villard, J. A., Severino, M., Cailou, B., Schlumberger, M., M., T., Parmentier, C. and Monier, R., 1990, Presence of mutations in all three ras genes in human thyroid tumors, *Oncogene.* **5:** 565–570.
98. Tsygankova, O. M., Kupperman, E., Wen, W. and Meinkoth, J. L., 2000, Cyclic AMP activates Ras, *Oncogene.* **19:** 3609–15.
99. Tsygankova, O. M., Saavedra, A., Rebhun, J. F., Quilliam, L. A. and Meinkoth, J. L., 2001, Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A, *Mol Cell Biol.* **21:** 1921–1929.
100. Van Keymeulen, A., Roger, P. P., Dumont, J. E. and Dremier, S., 2000, TSH and cAMP do not signal mitogenesis through Ras activation, *Biochem Biophys Res Commun.* **273:** 154158.
101. Vanvooren, V., Allgeier, A., Nguyen, M., Massart, C., Parma, J., Dumont, J. E. and Van Sande, J., 2001, Mutation analysis of the Epac–Rap1 signaling pathway in cold thyroid follicular adenomas, *Eur J Endocrinol.* **144:** 605–10.
102. Vasko, V., Ferrand, M., Di Cristofaro, J., Carayon, P., Henry, J. F. and de Micco, C., 2003, Specific-pattern of RAS oncogene mutations in follicular thyroid tumors, *J Clin Endocrinol Metab.* **88:** 2745–52.
103. Velasco, J. A., Acebron, A., Zannini, M., Martin-Perez, J., Di Lauro, R. and Santisteban, P., 1998, Ha-ras interference with thyroid cell differentiation is associated with a down-regulation of thyroid transcription factor-1 phosphorylation, *Endocrinology.* **139:** 2796–802.
104. Villalonga, P., Lopez-Alcala, C., Bosch, M., Chiloeches, A., Rocamora, N., Marais, R., Marshall, C. J., Bachs, O. and Agell, N., 2001, Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates downstream signaling, *Mol. Cell. Biol.* **21:** 7345–7354.
105. Vossler, M. R., Yao, H., York, R. D., Pan, M.-G., Rim, C. S. and Stork, P. J., 1997, cAMP activates MAP kinase and elk-1 through a B-Raf- and Rap1-dependent pathway, *Cell.* **89:** 73–82.
106. Walsh, A. B. and Bar-Sagi, D., 2001, Differential activation of the Rac pathway by Ha-Ras and K-Ras, *J Biol Chem.* **276:** 15609–15615.
107. Wang, S., Wu, J., Savas, L., Patwardhan, N. and Khan, A., 1998, The role of cell cycle regulatory proteins, cyclin D1, cyclin E, and p27 in thyroid carcinogenesis, *Hum Pathol.* **29:** 1304–9.
108. White, M. A., Nicolette, C., Minden, A., Polverino, A., Aelst, L. V., Karin, M. and Wigler, M. H., 1995, Multiple Ras functions can contribute to mammalian cell transformation, *Cell* **80:** 533–541.
109. Wolfman, J. C., Palmby, T., Der, C. J. and Wolfman, A., 2002, Cellular N-Ras promotes cell survival by downregulation of Jun N-terminal protein kinase and p38, *Mol Cell Biol.* **22:** 1589–1606.
110. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. and Sturgill, T. A., 1993, Inhibition of EGF-activated MAP kinase signaling pathway by adenosine 3′5′-monophosphate, *Science.* **262:** 1065–1068.