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PKA and B-Raf mediate ERK activation by TSH

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List of non-standard abbreviations: 4',6-diamidino-2-phenylindole, DAPI; Epidermal growth factor receptor, EGFR; 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate, EcAMP; exchange protein activated by cAMP, Epac; extracellular signal-regulated kinase, ERK; forskolin, Fsk; protein kinase A, PKA; heat-stable PKA inhibitor, PKI; thyroid stimulating hormone, TSH; Wistar rat thyroid, WRT; N^6- monobutyryladenosine- 3', 5'- cyclic monophosphate, 6MB-cAMP

ABSTRACT

TSH regulates thyroid cell proliferation and function through cAMP-mediated signaling pathways that activate PKA and Epac/Rap1. The respective roles of PKA versus Epac/Rap1 in TSH signaling remain unclear. We set out to determine whether PKA and/or Rap1 mediate ERK activation by TSH. Neither blocking Rap1 activity nor silencing the expression of *Rap1* impaired TSH or forskolin-induced ERK activation in Wistar rat thyroid cells. Direct activation of Epac1 failed to stimulate ERK activity in starved cells, suggesting that Epac-induced Rap1 activity is not coupled to ERK activation in rat thyroid cells. By contrast, PKA activity was required for cAMP-stimulated ERK phosphorylation, and sufficient to increase ERK phosphorylation in starved cells. Expression of dominant negative Ras inhibited ERK activation by TSH, forskolin and 6MB-cAMP, a selective activator of PKA. Silencing the expression of *B-Raf* also inhibited ERK activation by TSH, forskolin and 6MB-cAMP, but interestingly, not that stimulated by insulin or serum. Depletion of B-Raf impaired TSH-induced DNA synthesis, indicating a functional role for B-Raf in TSH-regulated proliferation. Collectively, these results position PKA, Ras and B-Raf as upstream regulators of ERK activation, and identify B-Raf as a selective target of cAMP elevating agents in thyroid cells. These data provide the first evidence for a functional role for B-Raf in TSH signaling.

Although the TSH receptor couples to multiple G proteins (Laugwitz et al., 1996), most of the effects of TSH are mediated through Gsα and cAMP (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)). Cyclic AMP activates multiple downstream targets including PKA, guanine nucleotide exchange factors (GEFs) for Rap (de Rooij et al., 1998; Kawasaki et al., 1998) and Ras (Pak et al., 2002; Pham et al., 2000) and certain ion channels. The respective contributions of PKA, Rap1 and Ras to the effects of TSH are unclear. There is general agreement that TSH activates endogenous Rap1 via a PKA-independent mechanism (Dremier et al., 2000; Iacovelli et al., 2001; Tsygankova et al., 2001). Rap1 activation is presumably mediated by Epac1, which is highly expressed in thyroid cells (Dremier et al., 2007; Hochbaum et al., 2008; Iacovelli et al., 2001; Mei et al., 2002). However, the functional significance of Rap1 activation by TSH is controversial. Activation and phosphorylation of Rap1 are required for TSH-stimulated DNA synthesis in rat thyroid PCCL3 cells (Ribeiro-Neto et al., 2002). Roles for both Epac and PKA in TSH-induced DNA synthesis in these cells were reported (Hochbaum et al., 2008). In contrast, studies in canine thyroid cells showed that PKA, and not Epac/Rap1, mediates the effects of TSH, including those on cell proliferation (Dremier et al., 2007).

In cells where cAMP stimulates ERK activity, Rap1 has been shown to induce B-Raf-dependent activation of ERK (reviewed in (Stork and Schmitt, 2002)). As B-Raf is highly expressed in thyroid cells (Mitsutake et al., 2005), we explored whether Rap1 mediates the effects of TSH on ERK activity in Wistar rat thyroid (WRT) cells. Unexpectedly, based on a report that TSH stimulates Rap1-dependent ERK activity in FRTL-5 rat thyroid cells (Iacovelli et al., 2001), our studies identified PKA as the primary mediator of cAMP-stimulated ERK activity. They further revealed that Ras and B-Raf function downstream from PKA in the regulation of ERK activity and cell proliferation. These findings identify Ras and B-Raf as important components of TSH-mediated signaling pathways that converge on ERK activation and cell proliferation.

MATERIALS AND METHODS

Reagents. Phospho-ERK (Thr202/Tyr204), phospho-MEK1/2 (Ser221), phospho-(Ser/Thr) PKA substrate, DYKDDDDK FLAG epitope, Akt and MEK1 antibodies were from Cell Signaling Technology (Beverly, MA). ERK2, B-Raf, Rap1 and Rap1GAP antibodies were from Santa Cruz (Santa Cruz, CA). Rac1 antibody was from Upstate (Lake Placid, NY). HA antibody was kindly provided by Dr. Jeffrey Field (Department of Pharmacology, University of Pennsylvania). Sheep anti-BrdU antibody was from BioDesign (Carmel, NY). AlexaFluor 488 donkey anti-sheep IgG was from Invitrogen (Eugene, OR). Crude bovine TSH and forskolin were from Sigma (St. Louis, MO). Glutathione sepharose beads were from GE Healthcare (Piscataway, NJ). 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate (EcAMP) and N^6- monobutyryladenosine- 3', 5'- cyclic monophosphate (6MB-cAMP) were from Axxora LLC (San Diego, CA). UO126 was purchased from Promega (Madison, WI).

Cell culture. WRT, PCCL3 and FRTL5 rat thyroid cells were cultured in Coon's modified Ham's F12 medium supplemented with calf serum (5%), insulin (10 μg/mL), TSH (1 mU/mL), and transferrin (5 μg/mL), referred to as 3H growth medium. Cells were starved in basal medium (Coon's modified Ham's F12 medium devoid of growth factors and serum) for 48 h prior to stimulation with TSH (1 mU/mL), forskolin (10 μM), insulin (10 μg/mL), EcAMP (100 μM) or 6MB-cAMP (1 mM). Starved cells were pretreated with UO126 (10 μM) for 1 h prior to stimulation.

Viral infection. Rap1GAP adenovirus was constructed as previously described (Tsygankova et al., 2007). The PKI adenovirus was a kind gift from Dr. Mark A. Giembycz (Department of Pharmacology & Therapeutics, University of Calgary). Cells were infected overnight in basal medium, transferred to growth medium for 6 h and starved in basal medium overnight. On day 2 post-infection, cells were stimulated and total cell lysates prepared for western blotting.

Rap activation assay. Rap1 activation was assessed as previously described (Tsygankova et al., 2007) except that lysates were collected in Triton-based lysis buffer (20mM TrisHCl, 100mM NaCl, 10mM $MgCl_2$, 1.0% Triton-X-100, 100 μM pefabloc, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 200 μg/mL Na_3VO_4).

Transient transfection of siRNA. siRNA duplexes were introduced into cells using the Amaxa Nucleofector (Germany) as described previously (Tsygankova et al., 2007). Cells (1.5×10^6) were transfected with siRNA duplexes (200-1000 nM) in suspension, plated overnight in growth medium, transferred to basal medium for 24 h and subsequently stimulated. B-Raf siRNAs were from Invitrogen (Carlsbad, CA). Scrambled siRNAs, Rap1 siRNAs and a second set of B-Raf siRNAs were from Qiagen (Valencia, CA).

Immunoprecipitation. The Flag-RasN17 plasmid was generously provided by Dr. Phillip Stork (Vollum Institute, Oregon Health and Sciences University). The HA-ERK1 plasmid was a kind gift from Dr. Margaret Chou (Department of Pathology and Lab Medicine, Children's Hospital of Pennsylvania). Plasmids (5 μg) were co-transfected using the Amaxa Nucleofector. Cells were starved for 24 h prior to treatment. At 48 h post-transfection, cells were stimulated and then lysed in 20 mM Tris pH 7.8, 100 mM NaCl, 0.5% Triton, 80 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 100 μM pefabloc, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 200 μg/mL Na_3VO_4. Lysates were clarified by centrifugation and the supernatant pre-cleared with protein G agarose beads (Invitrogen, Carlsbad, CA). Proteins (200 μg) were precipitated with HA antibody for 2 h at 4˚C, protein G agarose (100 μL) added for 1 h, and beads collected by centrifugation. After washing 3 times in PBS, 2X Laemmli buffer was added, the samples boiled for 5 min, and subjected to western blotting.

Western blotting. Western blotting was performed as previously described (Tsygankova et al., 2007). Proteins were visualized via chemiluminescence using the FUJI-LAS 3000 system and Multi Gauge V3.0 software (Fuji, Japan).

DNA synthesis. Following transfection with siRNAs and plating overnight, cells were starved for 24 hr in basal medium supplemented with calf serum (0.2%), stimulated with TSH (1mU/mL) or insulin (10μg/mL) for 28 hr and BrdU added for the final 4 hr (24-28 hr). In parallel, starved cells were stimulated with 3H growth medium for 20 hr and labeled with BrdU for 4 hr (16-20 hr). Cells were fixed in 3.7% formaldehyde/PBS, stained with sheep anti-BrdU, AlexaFluor488 anti-sheep IgG and 4',6-diamidino-2-phenylindole (DAPI) (to stain nuclei). At least six fields (>200 cells) were scored in a

blinded fashion. Parallel dishes of cells were harvested and analyzed by western blotting to confirm silencing in each experiment.

Statistics. All experiments were performed at least three times with similar results unless otherwise indicated. Statistical significance was determined using student's t-test.

RESULTS

TSH stimulates MEK1 and ERK activity through cAMP. TSH stimulates rapid and transient activating phosphorylation of ERK in rat thyroid cells (Iacovelli et al., 2001). In WRT cells, ERK phosphorylation was stimulated maximally at 2-3 min and decreased to near basal levels by 15 min after TSH addition (Figure 1A). Forskolin elicited similar effects, demonstrating that increased cAMP is sufficient to activate ERK (Figure 1B). The effects of TSH/cAMP on MEK1 activity have not been reported. TSH and forskolin induced activating phosphorylation of MEK1 with a time course similar to that of ERK phosphorylation. Pretreatment with the MEK inhibitor UO126 blocked ERK activation by cAMP elevating agents (Figure 1C). These data indicate that the transient activation of ERK by TSH and cAMP is a result of stimulatory effects on MEK1, and focused our analysis on upstream regulators of MEK1 activity.

Rap1 is not required for cAMP-stimulated ERK activity. TSH and forskolin activate endogenous Rap1 in canine and rat thyroid cells (Dremier et al., 2000; Iacovelli et al., 2001; Tsygankova et al., 2001). To assess whether Rap mediates cAMP-stimulated ERK activity, Rap activity was inhibited by overexpressing Rap1GAP, a negative regulator of Rap activity. As the transfection efficiency of rat thyroid cells in transient assays is very low, an adenovirus was used to drive efficient expression of Rap1GAP. Preliminary studies were conducted to determine the lowest dose of virus sufficient to block TSH- and forskolin-stimulated Rap1 activity. When infected at 500 particles/cell, Rap1GAP was expressed in virtually all cells (Figure 2A) and completely blocked Rap1 activity stimulated by TSH and forskolin (Figure 2B). Under these conditions, Rap1GAP failed to impair ERK activation by TSH or forskolin (Figure 2C, D). This result was unexpected based on an earlier report that cAMP stimulates Rap1-dependent ERK activation in FRTL-5 rat thyroid cells (Iacovelli et al., 2001). Hence, to further explore the contribution of Rap1 to cAMP-stimulated ERK activation, the effects of silencing *Rap1* expression were investigated. WRT cells were transfected with two sets of Rap1-directed siRNAs that target different regions of the Rap1 message or scrambled siRNAs as a control. Although the expression of Rap1 was markedly reduced in cells transfected with Rap1-directed siRNAs, ERK activation was not

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affected (Figures 3A, B). To assess whether direct activation of Rap by Epac was sufficient to stimulate ERK activity, starved cells were stimulated with the Epac agonist, EcAMP (Enserink, 2002). Similar to the effects of TSH, EcAMP stimulated Rap1 activity (Figure 3C). Nonetheless, EcAMP failed to stimulate ERK phosphorylation (Figure 3D). Collectively, these data exclude a major role for Rap1 in the regulation of ERK activity by cAMP in WRT cells.

Because our studies were conducted in WRT cells and those reported previously in FRTL-5 cells (Iacovelli et al., 2001), we extended our analysis to FRTL-5 and PCCL3 cells, both continuous lines of rat thyroid follicular cells (see (Kimura et al., 2001; Medina and Santisteban, 2000)). Overexpression of Rap1GAP failed to inhibit cAMP-stimulated ERK activity in both rat thyroid cell lines (Figure 4). These data differ from the single published report that analyzed the mechanism through which TSH stimulates ERK and concluded that cAMP stimulates Rap1-dependent ERK activity (Iacovelli et al., 2001).

PKA mediates cAMP-stimulated ERK activation. Having excluded a role for Rap in cAMP-regulated ERK activity, we next investigated the role of PKA. PKI, a highly specific inhibitor of PKA, was employed in these experiments. Overexpression of PKI using an adenovirus markedly reduced PKA activity as determined by western blotting for substrates phosphorylated by PKA using a PKA-phospho-substrate antibody (Figure 5A, middle panel). Under these conditions, PKI significantly impaired cAMP-stimulated ERK activity (Figures 5A, B). To determine whether PKA activity was sufficient to stimulate ERK activity, starved cells were treated with the selective PKA agonist, 6MB-cAMP. 6MB-cAMP stimulated activating phosphorylation of both ERK and MEK1 with a similar time course (Figure 5C). Thus, PKA activity is required for cAMP-stimulated ERK activity and sufficient to increase MEK1 and ERK activity.

Ras is required for cAMP-stimulated ERK activity. Studies were next conducted to elucidate the mechanism through which PKA stimulates ERK activity. Having excluded a role for Rap in the regulation of ERK activity, the contribution of Ras was investigated. Expression vectors encoding FLAG-tagged dominant negative RasN17 and HA-tagged ERK1 were co-transfected into WRT cells and ERK activation assessed in HA immunoprecipitates. This approach circumvented difficulties associated

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with low transfection efficiency by monitoring ERK activity selectively in transfected cells. Forskolin stimulated activating phosphorylation of HA-ERK1, and co-transfection with FLAG-RasN17 but not empty vector impaired ERK activation (Figure 6A). To determine whether the requirement for Ras was upstream or downstream of PKA, the effects of RasN17 on 6MB-cAMP-stimulated ERK activation were examined. Expression of RasN17 inhibited ERK phosphorylation by 6MB-cAMP (Figure 6B), supporting a role for Ras downstream from PKA in the regulation of ERK activity.

ERK activation by cAMP requires B-Raf. WRT cells express Raf-1 (data not shown) and B-Raf (Figure 7). Although B-Raf plays a major role in the regulation of ERK activity in human thyroid tumor cells (Melillo et al., 2005), the role of B-Raf in the regulation of ERK by TSH is unknown. To assess whether B-Raf mediates the effects of TSH on ERK activity, the expression of B-Raf was silenced. Decreasing the expression of B-Raf using two different sets of siRNAs significantly impaired TSH- and forskolin-stimulated ERK phosphorylation (Figures 7A, B, D). Silencing *B-Raf* induced a similar reduction in MEK1 phosphorylation by forskolin and TSH (data not shown). Interestingly, in contrast to the effects observed using cAMP elevating agents, silencing *B-Raf* did not impair insulin-stimulated ERK activation (Figure 7A). Similar results were observed for serum-stimulated ERK activity (data not shown).

To assess whether activation of ERK by PKA required B-Raf, the consequences of silencing *B-Raf* on ERK activation by 6MB-cAMP were examined. As for the cAMP elevating agents, ERK activation by 6MB-cAMP was reduced in cells depleted of B-Raf (Figures 7C, D). These findings indicate that PKA lies upstream from B-Raf in the regulation of ERK activity, and that the requirement for B-Raf in ERK activation appears to be selective to cAMP elevating agents in these cells.

B-Raf is required for TSH-stimulated DNA synthesis. To assess the contribution of B-Raf to the biological effects of TSH, the consequences of silencing B-Raf expression on DNA synthesis were examined. As TSH fails to stimulate proliferation in the absence of cooperating growth factors (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)), DNA synthesis was examined in cells starved in the presence of 0.2% calf serum (Figure 8A). TSH-stimulated DNA synthesis was significantly reduced

in the presence of the MEK1 inhibitor (UO126). Depletion of B-Raf also inhibited TSH-induced DNA synthesis. In contrast, silencing B-Raf did not impair insulin-stimulated DNA synthesis. Thyroid cell proliferation is maximal in growth medium containing TSH, insulin and serum (3H). Importantly, although modest in magnitude, silencing the expression of B-Raf, or treatment with the MEK1 inhibitor significantly decreased 3H-stimulated DNA synthesis (Figure 8B). We did not anticipate a more marked decrease in 3H-stimulated DNA synthesis, as other pathways, most notably those mediated through PI3K and mTOR, make substantial contributions to thyroid cell proliferation *in vitro* (Cass and Meinkoth, 1998; Cass et al., 1999; Kimura et al., 2001; Medina and Santisteban, 2000) and *in vivo* (Miller et al., 2009; Yeager et al., 2008). These data confirm that B-Raf contributes to the proliferation of non-transformed thyroid cells, possibly through the ability of cAMP elevating agents to stimulate B-Raf dependent ERK activation.

DISCUSSION

Given the important role played by Rap1 in the activation of B-Raf (reviewed in (Stork and Schmitt, 2002)), together with the frequent mutational activation of B-Raf in thyroid tumors (reviewed in (Kondo et al., 2006; Xing, 2005), we investigated whether Rap1 contributes to ERK activation by TSH/cAMP. Our findings indicate that PKA, and not Rap1, mediates B-Raf-dependent ERK activation by TSH. A role for endogenous Rap1 in the regulation of ERK by TSH/cAMP was excluded based on two major lines of evidence. Overexpression of Rap1GAP failed to impair TSH/cAMP-stimulated ERK activity, and silencing the expression of *Rap1* had no effect on ERK activation. Additionally, direct activation of Epac with the specific agonist EcAMP (Enserink, 2002) failed to stimulate ERK activity in starved cells. Collectively, these data show that Rap1 activation is neither required for nor sufficient to induce ERK activation in WRT cells. These results differ from the single report published to date that concluded that Rap1 was required for TSH/cAMP-induced ERK activity in FRTL-5 cells (Iacovelli et al., 2001). The reason for these differences remains to be determined. In our hands, overexpression of Rap1GAP had no effect on cAMP-stimulated ERK activity in either FRTL-5 or rat thyroid PCCL3 cells, another widely used model of rat thyroid cells (reviewed in (Kimura et al., 2001; Medina and Santisteban, 2000)). Clearly, further work is required before an accepted model for the regulation of ERK by TSH can be derived.

Our findings indicate that PKA mediates cAMP-stimulated ERK activation. Inhibition of PKA activity using the highly selective inhibitor PKI impaired TSH/cAMP-stimulated ERK activity. Moreover, treatment with the selective PKA analog 6-MB-cAMP was sufficient to stimulate activating phosphorylation of MEK1 and ERK in starved cells. The molecular mechanism through which PKA regulates ERK activity was explored. Silencing the expression of *B-Raf* impaired ERK activation by TSH, forskolin and 6MB-cAMP, documenting a role for B-Raf in the regulation of ERK by cAMP and PKA. Having excluded a role for Rap1 in the regulation of ERK activity, our analysis focused on Ras. TSH activates Ras in rat thyroid cells (Iacovelli et al., 2001; Tsygankova et al., 2000). Expression of dominant negative Ras inhibited TSH-, forskolin- and 6MB-cAMP-stimulated ERK activity. Although

there are precedents for PKA-dependent Ras activation (Ambrosini et al., 2000; Obara et al., 2007; Yang et al., 2003), we reported that TSH activates Ras through a PKA-independent mechanism, experiments that were conducted in thyroid cells overexpressing human H-Ras (Tsygankova et al., 2000). Unfortunately, we have been unable to detect the activation of endogenous Ras in WRT cells, even in response to serum mitogens (Vuchak, unpublished). *Iacovelli et al* reported that TSH activates endogenous Ras in FRTL-5 cells and that Ras is required for ERK activation. Unfortunately, the mechanism through which Ras was activated was not explored (Iacovelli et al., 2001). It is conceivable that PKA-dependent and -independent mechanisms of Ras activation co-exist in thyroid cells, similar to to what has been reported for Rap1 where PKA-dependent and -independent modes of activation have been described. Alternatively, the requirement for Ras in PKA-dependent ERK activation could reflect effects of PKA on Ras signaling. TSH influences downstream events in Ras signaling. TSH transiently impaired ERK activation by RasV12S35, an effector domain mutant that signals preferentially through Raf, whereas it enhanced ERK activation by RasV12G37, a mutant that signals via RalGDS and Ral proteins (Miller et al., 1998). In the absence of TSH, Raf-1 was required for DNA synthesis stimulated by microinjected cellular Ras protein, whereas in the presence of TSH, Ras-stimulated DNA synthesis did not require Raf1 (al-Alawi et al., 1995). *Ciullo et.al.* reported that TSH stimulated the association of Ras with the p85 regulatory subunit of PI3K through PKA-mediated phosphorylation of p85. Moreover, these authors showed that cAMP disrupted Ras/Raf-1 complexes at least partly through PKA (Ciullo et al., 2001; De Gregorio et al., 2007). Collectively, these studies indicate that there are multiple sites of crosstalk between PKA- and Ras-mediated signaling.

Our data is the first to ascribe a functional role to B-Raf in TSH signaling. Silencing the expression of *B-Raf* impaired DNA synthesis stimulated by TSH. Depleting B-Raf also induced a modest decrease in DNA synthesis stimulated by 3H growth medium, which contains TSH, insulin and serum. In that depletion of B-Raf failed to inhibit insulin- or serum-induced ERK activation, these data suggest that the requirement for B-Raf in cell proliferation resides downstream of TSH. This is consistent with previous studies showing that expression of dominant negative Ras (Ciullo et al., 2001; Kupperman et al.,

1993; Medina and Santisteban, 2000) or treatment with MEK1 inhibitors ((Iacovelli et al., 2001); this report) impairs TSH-stimulated DNA synthesis. Even in canine thyroid cells where PKA mediates all or many of the effects of TSH (Dremier et al., 2007), treatment with a MEK1 inhibitor impaired TSH-stimulated DNA synthesis (Vandeput et al., 2003). Other ligands that activate Gs-coupled receptors have been shown to signal through B-Raf. PTH stimulated PKA- and B-Raf-dependent ERK activation in CHO cells expressing the PTH1 receptor (Wang et al., 2008). Silencing the expression of *B-Raf* impaired MSH-induced ERK activation in melanocytes (Dumaz et al., 2006).

In conclusion, our findings highlight important roles for PKA, Ras and B-Raf in TSH signaling to ERK. The requirement for Ras in TSH-induced ERK activation provides further evidence that Ras functions in TSH signaling (al-Alawi et al., 1995; Ciullo et al., 2001; Iacovelli et al., 2001; Kupperman et al., 1993; Medina and Santisteban, 2000; Tsygankova et al., 2000). Mutations in *Ras* and *B-Raf* are prevalent in thyroid tumors. Patients with Carney's syndrome, caused by inactivating mutations in the gene for the PKA regulatory subunit R1α exhibit an increased frequency of thyroid tumors (reviewed in (Boikos and Stratakis, 2007)). Loss of heterozygosity for PRKAR1A and increased PKA activity have been observed in thyroid tumors (Sandrini et al., 2002). Elucidating sites of crosstalk between these important signaling molecules in non-transformed thyroid cells may reveal novel insight into the molecular basis of thyroid cancer.

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FOOTNOTES

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FIGURE LEGENDS

Fig 1. TSH stimulates cAMP-dependent MEK and ERK activation. Starved WRT cells were stimulated with (A) TSH or (B) forskolin (fsk) for the times indicated (min). (C) Cells were pretreated with UO126 (10μM) for 30 min prior to stimulation. Total cell lysates were analyzed for ERK and MEK activation by western blotting for phospho-ERK (ERK-P) and phospho-MEK1/2 (MEK-P). Equal protein loading was confirmed by western blotting for Rac1 or ERK2.

Fig 2. Cyclic AMP stimulates Rap-independent ERK activation. (A) WRT cells infected with Rap1GAP adenovirus (AdRap1GAP, 500 particles/cell) were fixed and stained for HA-Rap1GAP at days 1 and 2 post-infection. Nuclei were stained with DAPI. (B) Cells infected with AdRap1GAP were starved for 24 h, stimulated with forskolin (F) or TSH (T) for 2 min (on day 2 post-infection), subjected to RalGDS-RBD pull down assay and subsequently blotted for activated Rap1 (Rap1GTP). Total cell lysates were subjected to western blotting for Rap1 and Rap1GAP. (C) Cells infected with AdRap1GAP were starved and stimulated with TSH (upper panels) or forskolin (lower panels) and total cell lysates analyzed for ERK phosphorylation and Rap1GAP expression. Equal protein loading was confirmed by western blotting for ERK2. (D) The results from three experiments are summarized.

Fig 3. Rap1 is not coupled to ERK activation. (A) WRT cells transfected with Rap1 siRNAs (Rap#1, #2) were starved, stimulated with forskolin for 2 min and ERK phosphorylation, Rap1 and Akt expression (as a loading control) analyzed. Rap1 expression was reduced by more than 80% following transfection with Rap1-directed siRNAs. (B) The results from three experiments are summarized.   (C) Starved cells were stimulated with EcAMP or TSH for the indicated times (min). Lysates were subjected to RalGDS-RBD pull down assay and blotted for Rap1GTP. Total cell lysates were analyzed for Rap1 expression. (D) Starved cells were stimulated with EcAMP for the indicated times (min) and analyzed for ERK phosphorylation. Western blotting for actin was used to ensure equal protein loading.

Fig 4. ERK activation in FRTL5 and PCCL3 cells does not require Rap activity. FRTL-5 (A) and PCCL3 cells (B) infected with Rap1GAP adenovirus (500 particles/cell) were starved for 24 hr, stimulated with forskolin for the indicated times (min) and subjected to western blotting for ERK phosphorylation,

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Rap1GAP and Akt expression, the latter to confirm equal protein loading. Similar results were obtained

in two experiments in each cell line.

Fig 5. ERK activation is PKA-dependent. (A) WRT cells infected with LacZ or PKI adenoviruses were

starved overnight and stimulated with forskolin for the indicated times (min). ERK and PKA substrate

phosphorylation were analyzed by western blotting. ERK2 expression documented equal protein loading.

(B) PKI significantly reduced forskolin-stimulated ERK activation ($*p<0.05$ at 2 and 5 min). (C) Starved

cells were stimulated with 6MB-cAMP for the indicated times (min), and analyzed by western blotting for

phospho-ERK, phospho-MEK and Rac1 as a loading control. MEK1 activation by 6-MB-cAMP was

analyzed in a single experiment.

Fig 6. Ras activity is required for ERK activation. (A) WRT cells transfected with HA-ERK and FLAG-

RasN17 or empty vector were starved, stimulated with forskolin for 2 min and HA-ERK

immunoprecipitated (IP) using an HA antibody and subjected to western blotting for phospho-ERK and

HA-ERK expression. Whole cell extracts (CE) were analyzed for FLAG-RasN17 expression. (B) Cells

transfected as described in (A) were stimulated with 6MB-cAMP for 2 min. Two experiments using

forskolin and 6-MB-cAMP, and a single experiment using TSH were performed with similar results.

Fig 7. B-Raf is required for cAMP-stimulated ERK activation. (A) WRT cells transfected with

scrambled- (Scr) versus B-Raf-directed siRNAs were starved, stimulated with forskolin (2 min), insulin

(5 min) or (B) TSH (2 min) and subjected to western blotting for phospho-ERK and B-Raf. Equal protein

loading was confirmed by blotting for ERK2 or Akt. Depletion of B-Raf using an independent set of

siRNAs (B-Raf#2) inhibited ERK activation by forskolin and TSH. (C) siRNA-transfected cells were

stimulated with 6MB-cAMP for the times indicated (min) and analyzed for phospho-ERK, B-Raf and Akt

as a loading control. (D) The decrease in ERK activation in B-Raf-depleted cells was statistically

significant ($**p<0.01$).

Fig 8. B-Raf is required for TSH-dependent DNA synthesis. Cells pretreated with UO126 (10uM) for 1

h or depleted of B-Raf were stimulated with (A) TSH, insulin or (B) 3H growth medium and DNA

synthesis analyzed. The % BrdU-positive nuclei (13.2% for TSH, 19.1% for insulin, 52.6% for 3H versus

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2.0% for starved cells) in mitogen-stimulated cells was set to 1. The decrease in DNA synthesis in

UO126-treated (**$p<0.01$ for TSH, **$p<0.01$ for 3H) and B-Raf-depleted (***$p<0.001$ for TSH, *$p<0.05$

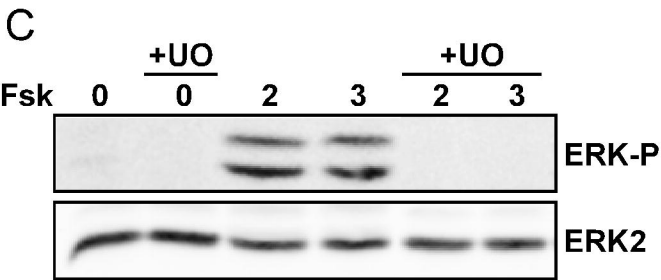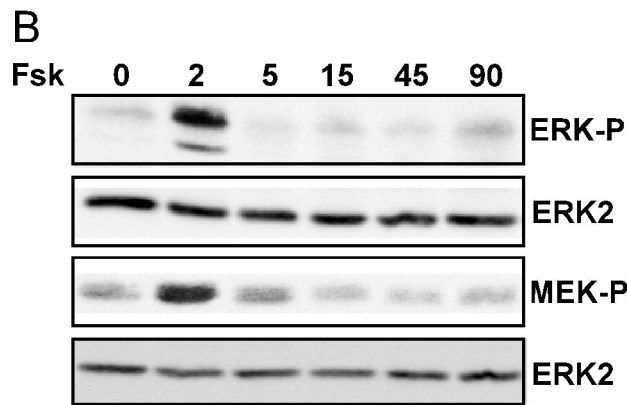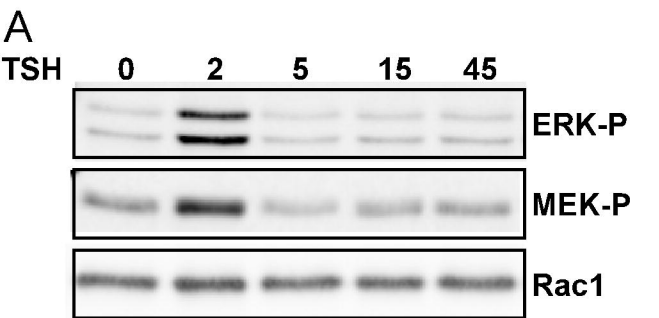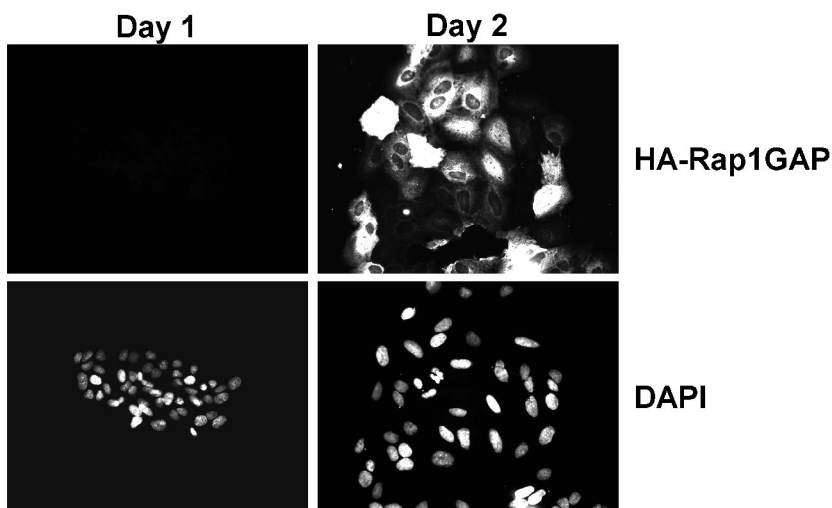for 3H) cells was statistically significant.

Figure 1

Figure 2

Figure 3

Figure 4

A

	Mock			Rap1GAP			FRTL5
Fsk	0	2	5	0	2	5	

ERK-P

Rap1GAP

Akt

B

	Mock			Rap1GAP			PCCL3
Fsk	0	2	5	0	2	5	

ERK-P

Rap1GAP

Akt

Figure 5

A

Fsk 0 2 5 0 2 5

LacZ | PKI

ERK-P

P-PKA substrates

ERK2

B

Fold ERK activation

LacZ
PKI

Fsk (min)

C

6MB 0 2 5 15 45

ERK-P

MEK-P

Rac1

Figure 6

A

Vector	-	+	-
RasN17	+	-	+
Fsk	-	+	+

IP ⎰ ERK-P
HA-ERK ⎱ HA

CE | FLAG

B

Vector	-	+	-
RasN17	+	-	+
6MB	-	+	+

IP ⎰ ERK-P
HA-ERK ⎱ HA

CE | FLAG

Figure 7

Figure 8