Published in final edited form as:

Mol Carcinog. 2016 August ; 55(8): 1243–1250. doi:10.1002/mc.22366.

Transcriptional and Post-translational Modifications of B-Raf in Quinol-Thioether Induced Tuberous Sclerosis Renal Cell Carcinoma

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Abstract

Increased activity of B-Raf has been identified in approximately 7% of human cancers. Treatment of Eker rats (*Tsc-2*^{EK/+}), bearing a mutation in one allele of the tuberous sclerosis-2 (*Tsc-2*) gene, with the nephrocarcinogen 2,3,5-*tris*-(glutathion-S-yl)hydroquinone (TGHQ) results in loss of the wild-type allele of *Tsc-2* in renal preneoplastic lesions and tumors. These tumors have increased protein expression of B-Raf, C-Raf (Raf-1), and increased expression and activity of ERK kinase. Similar changes are observed in Raf kinases following TGHQ-mediated transformation of primary renal epithelial cells derived from *Tsc-2*^{EK/+} rats (QTRRE cells), cells that are also null for tuberin. Herein, we utilized LC-MS/MS to identify constitutive phosphorylation of S345 and S483 in both 100- and 95-kDa forms of B-Raf in QTRRE cells. Using microRotofor liquid-phase isoelectric focusing, we identified four fractions of B-Raf that contain different post-translational modification profiles in QTRRE cells. Amplification of the kinase domain of B-Raf from QTRRE cells, outer-stripe of the outer medulla of 8-month TGHQ- or vehicle-treated *Tsc-2*^{+/+} and *Tsc-2*^{EK/+} rats, as well as tumors excised from 8-month TGHQ-treated *Tsc-2*^{EK/+} rats revealed three splice variants of B-Raf within the kinase domain. These splice variants differed by approximately 340, 544, and 600 bp; confirmed by sequencing. No point mutations within the kinase domain of B-Raf were identified. In addition, B-Raf/Raf-1/14-3-3 complex formation in the QTRRE cells was decreased by sorafenib, with concomitant selective decreases in p-ERK levels. Transcriptional and post-translational characterization of critical kinases, such as B-Raf, may contribute to the progression of tuberous sclerosis RCC. (246/250)

Keywords

B-Raf; Raf-1; MAPK; renal cell carcinoma; Quinol-thioether

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Introduction

The Raf-A (68 kDa), -B (75-100 kDa), and -C (72-74 kDa) proteins contain three conserved regions (CR); including the N-terminal CR1 and CR2 regions, and the C-terminal CR3 kinase domain [1]. *B-Raf* mRNA gives rise to a range of proteins, from 75 to 100 kDa as a consequence of alternative splicing within a number of different domains [2]. The expression patterns of the Rafs vary greatly; Raf-1 is ubiquitously expressed, but A-Raf and B-Raf have restricted expression [1]. Limited data exist on the tissue specific expression levels of A-Raf and B-Raf protein and mRNA. *A-Raf* mRNA is highly expressed in urogenital organs, whereas *B-Raf* mRNA and protein is predominately expressed in neuronal tissues, with significantly lower levels detected in a wide range of other tissues [2,3].

Activating mutations in B-Raf, especially V600E, have been identified in a number of cancers, such as melanoma, colorectal cancer, thyroid cancer, and lung carcinomas [4-11]. We reported a significant induction of B-Raf protein in tuberous sclerosis renal tumors in the Eker rat [12]. The Eker rat (*Tsc-2^{EK/+}*), carrying an inactive allele of the *Tsc-2* gene, is a useful experimental animal model to enhance our knowledge of the pathology and signal transduction cascades involved in kidney tumorigenesis. Treatment of wild-type (*Tsc-2^{+/+}*) and mutant (*Tsc-2^{EK/+}*) Eker rats with 2,3,5-*tris*-(glutathion-*S*-yl)hydroquinone (TGHQ), a potent nephrotoxic [13] and nephrocarcinogenic [14] metabolite of hydroquinone, induces preneoplastic lesions, including toxic tubular dysplasias, and increases the incidence of renal tumors in animals carrying the mutant *Tsc-2^{EK/+}* allele [14]. Kidney tumors formed following treatment of *Tsc-2^{EK/+}* rats with TGHQ exhibit increased protein expression of B-Raf, Raf-1, and pERK [12], and increased ERK activity [15]. Additionally, TGHQ is mutagenic, and transforms primary renal epithelial cells isolated from Eker (*Tsc-2^{EK/+}*) rats *in vitro*, giving rise to the quinol-thioether-transformed rat renal epithelial (QTRRE) cell line [16]. QTRRE cells are null for tuberlin [16], and give rise to tumors when subcutaneously injected into athymic nude mice [17]. QTRRE cells express constitutively high levels of fully activated ERK, B-Raf and Raf-1 [18,19]. Transient transfection of *Tsc-2* cDNA in QTRRE cells results in a substantial decrease in both ERK and B-Raf kinase activity, with a less substantial decrease in Raf-1 activity [18].

Transcriptional and post-translational modulation of B-Raf is not well characterized in kidney tissues or renal tumors. In this study, we examined post-translational phosphorylation of B-Raf, isolation and sequencing of B-Raf specific splice variants, and protein-protein interactions of Raf isoforms in tumorigenic QTRRE cells and/or TGHQ treated *Tsc-2^{+/+}* and *Tsc-2^{EK/+}* rats.

Materials and Methods

Cell culture

The tuberlin-negative cell line QTRRE was established from primary renal epithelial cells [16]. QTRRE and HK2 cells were grown in DMEM/F12 (1:1) (Invitrogen, Carlsbad, CA) with 10% FBS. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

Animal treatment and tissue preparation

Male Eker rats (wild-type; *Tsc-2^{+/+}*, and mutant; *Tsc-2^{EK/+}*), 8 weeks old, were obtained from the University of Texas MD Anderson Cancer Center (Smithville, TX). The animals were housed according to a 12:12-h light-dark cycle and allowed food and water *ad libitum*. TGHQ was synthesized as previously described and used at >98% purity, as determined by high performance liquid chromatography [20]. The rats were divided into four subgroups: 1) *Tsc-2^{EK/+}* control, 2) *Tsc-2^{EK/+}* TGHQ-treated, 3) *Tsc-2^{+/+}* control and 4) *Tsc-2^{+/+}* TGHQ-treated. The rats were administered TGHQ [2.5 μ mol/kg in 0.5 ml of 1x phosphate-buffered saline (PBS), i.p.] 5 days a week for 4 months; followed by treatment with 3.5 μ mol/kg for an additional 4 months, according to a previously established protocol [14]. Control rats were administered PBS only. The TGHQ dosing solution was prepared fresh in 1xPBS daily. The animals were euthanized by CO₂ asphyxiation. For biochemical assays, the outer-stripe of the outer medulla (OSOM), cortex, and renal tumors were excised, frozen immediately in liquid nitrogen, and stored at -80°C.

Mass spectrometry

QTRRE cell lysates were generated using Cell Lysis Buffer 10X (Cell Signaling Technologies, Danvers, MA) containing 1 mM Pefabloc SC, Complete protease inhibitor cocktail tablet and phosphatase inhibitor cocktail tablet (Roche, South San Francisco, CA). Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA). Protein samples were separated on 7.5 % Criterion™ XT Bis-Tris Gels (Bio Rad, Hercules, CA). The gel was silver-stained and the appropriate bands were excised. Samples were trypsin-digested and the peptides were subjected to LC-MS/MS analysis. Peptides were separated through a in-house C18 column at 300 nL/min. An LCQ-DECA XP PLUS quadrupole ion trap mass spectrometer equipped with a nanoESI source and a nanoflow HPLC system (Thermo, Waltham, MA) was used for analysis. Raw data was analyzed using X!Tandem. The modified peptides were manually validated by the program, Ionen, which generates theoretical b- and y-ions from a user specified peptide sequence and adjusts the masses for specified adductions.

Liquid isoelectric focusing for separation of B-Raf protein

Protein (500 μ g) from QTRRE cell lysate was diluted to a final volume of 2.75 mL with a focusing solution (7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTT and 4% Bio-Lyte 3/10), which was then loaded into the focusing chamber. 6 mL 0.5 M acetic acid was added to the anode electrolyte chamber and 6 mL 0.1 M NaOH was added to the cathode electrolyte chamber. The MicroRotor for Liquid-Phase IEF Cell (Bio-Rad, Hercules, CA) was run at a constant 1 W until both the voltage and amps came to a plateau (~50 min). Fractions were removed from the focusing chamber and the pH was determined for each fraction. Fractions were run on a 7% SDS-PAGE gel, transferred to PVDF membrane, and probed by Western blot for B-Raf. Blots were visualized with Amersham ECLTM Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK).

PCR amplification of B-Raf kinase domain

Total RNA from QTRRE cells, 8-month TGHQ- or vehicle-treated Tsc-2^{+/+} and Tsc-2^{EK/+} rats, or tumors excised from 8-month TGHQ- Tsc-2^{EK/+} rats were isolated with TRIReagent (Sigma, St. Louis, MO) utilizing the manufacturer's protocol. Total RNA (4.5 µg), in a 20 µl total reaction volume, was reverse transcribed using the First Strand cDNA Synthesis kit (Fermentas, Pittsburgh, PA) according to the manufacturer's protocol. PCR products were generated using the Advantage cDNA PCR kit (Clontech, Mountain View, CA) according to manufacturer's protocol. B-Raf primers used were as follows: 5'-GGC TGA AAG CTT CAG CAC CCA CAC CTC AGC-3' (fwd), and 5'-AT CTG GAT CCT GTT GTT GAT GTT TGA ATA AGG-3' (rev). The thermocycler conditions for B-Raf PCR were as follows: 95°C for 5 minutes; then 28 cycles of 95°C for 30 seconds, 59°C for 40 seconds, and 72°C for 60 seconds, followed by a 10 minute 72°C final extension. PCR products were separated on 2% ethidium bromide stained agarose gel; alongside a 1 kb ladder (New England Biolabs, Ipswich, MA). Bands were excised and DNA was isolated from the gel using Ambion Spin Column and Tubes (Ambion, Grand Island, NY), according to manufacturer's protocol, and sent for sequencing at the University of Arizona Sequencing Core Facility. RNA isolation and PCR amplification were repeated n=4.

Immunoprecipitation

Each phospho-immune complex (2-4 µg of B-Raf, Raf-1, A-Raf, and 14-3-3 isoforms; Cell Signaling Technologies, Danvers, MA) were added to microcentrifuge tubes containing 5 mg of QTRRE tissue lysates and protein-A/G sepharose beads (Amersham, Pittsburgh, PA), and incubated with rotation for 18 h at 4°C. Microcentrifuge tubes were centrifuged at 7500 *g* for 2 min at 4°C, supernatant was removed, and beads were washed three times with ice-cold cell lysis buffer. Proteins were separated from protein-antibody bead complex with 4x XT sample loading buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol. Samples were run on 7% SDS-PAGE gel, transferred to PVDF membrane, and analyzed via Western blot.

Western blot analysis

Whole cell lysates were generated using Cell Lysis Buffer 10X (Cell Signaling Technologies, Danvers, Massachusetts) containing 1 mM Pefabloc SC, Complete protease inhibitor cocktail tablet and phosphatase inhibitor cocktail tablet (Roche, South San Francisco, California). Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, California). Protein was subjected to SDS-PAGE, followed by electrophoretic transfer to PVDF membranes. Primary antibodies used were p-ERK, total ERK, B-Raf, Raf-1, total 14-3-3 (Cell Signaling Technologies, Danvers, Massachusetts) and GAPDH (Abcam, Cambridge, Massachusetts). Secondary anti-mouse and anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Cell Signaling Technologies, Danvers, Massachusetts) were used at a 1:3000 dilution in 5% milk/TBS-T. The blots were visualized with ECL Western Blotting Detection Reagent (Thermo, Waltham, Massachusetts) using the ChemiDoc XRS+ Imager (Bio-Rad).

Results

B-Raf is constitutively phosphorylated at serine residues S345 and S483 in QTRRE cells

The activation of Raf kinases is highly dependent on the phosphorylation state at a number of crucial sites within all three domains. Human B-Raf is phosphorylated on a number of sites: S365, S428, and T439 (inhibitory); S445, T598, S601, and S728 (activating). In QTRRE cells, B-Raf was found to have elevated kinase activity [18]. The kinase activity of B-Raf is known to be associated with phosphorylation of T598 and S601 within the kinase domain (CR3). In order to identify other constitutively phosphorylated sites on B-Raf in QTRRE cells, we isolated B-Raf by immunoprecipitation (IP), excised two bands at 100- and 95-kDa from a silver stained gel, and performed LC-MS/MS analysis on the samples, (Fig 1). Peptides SSSAPNVHINTIEPVNIDDLIR (residues 345-386) and RDSSDDWEIPDGQITVGQR (residues 481-499) were identified by LC-MS/MS as having an 80 kDa mass addition, indicative of phosphorylation, at S345 and S483 (italicized) in both 100- and 95-kDa forms of B-Raf (Fig 1). These sites are equivalent to human S365 and S445.

Fractionation of B-Raf modification groups

Distinct patterns of post-translational modifications influence a protein's activation state, which can be distinctly different depending on the combination of modifications [21]. To determine whether different combinations of B-Raf modifications exist, we utilized in-solution isoelectric focusing (IEF) to fractionate B-Raf modification groups [22]. The fractionation of QTRRE protein lysate by IEF in solution allows for isolation of proteins in their native state by their isoelectric point (pI) [22]. The theoretical pI of B-Raf is approximately 7.85, reflective of B-Raf in its native state. Within the focusing chamber, the QTRRE protein lysate was fractionated into 10 compartments, each with a different pH. The pH of the lysate from each fraction was measured, run on SDS-PAGE, and then probed by Western blot for B-Raf. Fractionation groups of B-Raf predominantly exist at pH 6.46, 7.14, 7.81, and 8.45, corresponding to approximately 1 to 7 phosphorylated residues (Fig 2).

Splice variants within the kinase domain of B-Raf in rats

Since we identified two constitutively phosphorylated isoforms of B-Raf around 95- and 100-kDa, we amplified the kinase domain of B-Raf to determine if any splice variants exist in our rat *in vitro* (QTRRE cells) and *in vivo* (*Tsc-2^{EK/+}* rat) models. RNA isolated from QTRRE cells were reverse transcribed into cDNA, and 544 bp of B-Raf were PCR amplified with primers (see methods section) that flanked the kinase domain of B-Raf [TKVS-activation segment; threonine (T), lysine (K), valine (V), serine (S)]. The gel resolved three distinct bands of B-Raf within the kinase domain (Fig 3A, lane 2) around 340, 544, and 600 bp; and the negative control (Fig 3A, lane 3) had a single clear band of primer dimer of equivalent size to the band in lane 2. All three DNA bands were excised from the gel, purified, and submitted for sequencing. With a similar method, RNA was isolated from the OSOM of 8-month TGHQ- or vehicle-treated *Tsc-2^{EK/+}* and *Tsc-2^{+/+}* rats, as well as tumors from TGHQ treated *Tsc-2^{EK/+}* rats; and the B-Raf PCR DNA products were excised from the gel, purified, and submitted for sequencing (Fig 3B). Analogous to the amplification of B-Raf in the QTRRE cells, all of the tissue samples from both *Tsc-2^{EK/+}* and *Tsc-2^{+/+}* rats

exhibited three distinct bands of B-Raf within the kinase domain (Fig 3B), approximately 340, 544, and 600 bp in size. The 544 bp band from Fig 3A and Fig 3B were sequenced and contained no point mutations (Fig 3C; primer sequence in blue, TKVS-activation segment of B-Raf in red). The 340 and 600 bp bands from both QTRRE cells and rat tissues were identified to have some sequence similarity to B-Raf, but due to the low resolution of the band sequencing, and inconsistency between replicate samples, we are not able to report sequences. Amplification and purification were repeated an additional three times, with no improvement in sequencing results for the 340 or 600 bp bands.

B-Raf/Raf-1 heterodimerization in QTRRE cells

A number of studies have revealed that Raf isoform heterodimers possess increased cellular activity [23-25]. To assess Raf heterodimer formation in QTRRE cells, B-Raf, Raf-1, and 14-3-3 isoforms were immunoprecipitated from QTRRE cell lysates. Each IP and total cell lysate (TCL) from QTRRE cells were immunoblotted for B-Raf (Fig 4A), Raf-1 (Fig 4B), or 14-3-3 isoforms (Fig 4C) protein expression. The data suggests that B-Raf/Raf-1 form a dimer that complexes with 14-3-3 in QTRRE cells (Fig 4).

Sorafenib modulates B-Raf/14-3-3 protein interaction in QTRRE cells

Sorafenib is a first-line therapy for advanced renal cell carcinoma that targets Raf, and other kinases, to inhibit tumor growth. In QTRRE cells, we determined the effect of sorafenib on B-Raf/14-3-3 protein interaction as a contributor to anti-tumor drug efficacy. QTRRE cells were treated with vehicle or sorafenib (1h) and TCL was collected and immunoblotted for p-ERK, total ERK, B-Raf, Raf-1, total 14-3-3, and GAPDH. Sorafenib treatment showed a pronounced decrease in p-ERK signaling with no modulation of total B-Raf, Raf-1, or total 14-3-3. (Fig. 5A) In separate experiments, cell lysates were subjected to 14-3-3 protein immunoprecipitation followed by Western blot for total 14-3-3, B-Raf, and GAPDH. The blots revealed a marked decrease in protein interaction between B-Raf and 14-3-3 compared to vehicle-treated cells (Fig. 5B).

Discussion

B-Raf isoforms exist between 69-75 kDa and 79-100 kDa, depending on species [2,26,27]. Isoforms of B-Raf have frequently exhibited similar activating post-translational modifications. The different isoelectric points observed for B-Raf identified by fractionation by IEF (Fig. 2) may be isoforms, splice variants or combinations of post translational modifications to B-Raf, which result in an alteration of the native pI. In QTRRE cells, B-Raf is constitutively expressed and activated at a higher level than Raf-1, as determined by Raf-1 and B-Raf kinase assays [18]. The kinase assays confirm that B-Raf is fully activated and phosphorylated within its kinase domain at T598 and S601. In this study, we utilized LC-MS/MS to identify other constitutively phosphorylated sites on B-Raf. We identified an 80 Da addition, consistent with a phosphorylation, on S345 and S483 in both 100- and 95-kDa forms of B-Raf in QTRRE cells (Fig 1). These sites correlate with human S365 and S445. B-Raf S365 is phosphorylated by serum and glucocorticoid-inducible kinase (SGK) [28]. Phosphorylation on this site, and others, is associated with complex formation of B-Raf with 14-3-3 adapter proteins [29,30].

14-3-3 binding at specific phosphorylated sites modulate B-Raf/Raf-1 heterodimerization [23-25]. In our tumorigenic QTRRE cells, we observed B-Raf/Raf-1/14-3-3 complex formation (Fig 4). Compared to the formation of Raf homodimers or monomers, isolated B-Raf/Raf-1 heterodimers exhibit amplified kinase activity [23]. Phosphorylation of Raf-1 within its activation domain facilitates 14-3-3 binding and subsequent activation by B-Raf [23,24]. Therefore, B-Raf activation in tuberous sclerosis RCC may be modulated through the formation of B-Raf/Raf-1 heterodimers [23-25]. Moreover, the expression of B-Raf may alter which Raf isoform predominately activates ERK. When B-Raf is expressed and activated, it has a higher affinity for MEK1/2 than Raf-1 [24,31], which may contribute to the higher level of B-Raf kinase activity compared to Raf-1 in QTRRE cells [18].

To assess the role of Raf activation in Raf/14-3-3 complex formation, we treated QTRRE cells with the Raf inhibitor, sorafenib, and probed for total protein levels of B-Raf, Raf-1, ERK, pERK, or total 14-3-3 by Western blot analysis (Fig. 5A), and immunoprecipitated total 14-3-3 to determine the impact on complex formation (Fig. 5B). Western analysis revealed that sorafenib effectively decreased MAPK signaling of p-ERK through inhibition of Raf activity, without affecting total protein levels of B-Raf, Raf-1, or 14-3-3 (Fig. 5A). QTRRE cells treated with sorafenib were immunoprecipitated with total 14-3-3 antibody and Western analysis was performed for complex formation of total 14-3-3 with B-Raf. In QTRRE cells, sorafenib treatment effectively disrupted the interaction of 14-3-3 with B-Raf, concordant with disruption of downstream p-ERK signaling (Fig. 5B). When sorafenib is bound to B-Raf, it locks the kinase in an inactive conformational state, likely preventing B-Raf activation, and inhibiting the binding of 14-3-3 [32]. Though the upstream regulating kinase for 14-3-3 binding site phosphorylation of B-Raf is not well defined, there is evidence for both auto-phosphorylation and other kinases as the upstream regulator [30,32,33]. The critical requirement for S483 in determining B-Raf activity may ultimately determine the autophosphorylation of residues necessary for 14-3-3 binding. Additionally, B-Raf/14-3-3 may be indirectly decreased through reduced dimerization with sorafenib-inhibited Raf-1, since sorafenib binds to both isoforms of Raf. Either way, 14-3-3 in complex with B-Raf is a contributor to its active conformation, and thus a key contributor in downstream tumor growth. Further studies are required to more precisely determine the role of site-specific phosphorylation in the regulation of Raf activity, and the exact mechanism of sorafenib-induced disruption of the Raf/14-3-3 complex.

In melanoma, B-Raf is commonly mutated within the kinase domain to yield a constitutively active B-Raf [34]. We sought to determine if B-Raf activation in QTRRE cells was, in part, due to point mutations within the kinase domain. Amplification of the kinase domain of B-Raf in QTRRE cells, OSOM of 8-month TGHQ- or vehicle-treated *Tsc-2^{+/+}* and *Tsc-2^{EK/+}* rats, or tumors excised from 8-month TGHQ- *Tsc-2^{EK/+}* rats did not reveal any point mutations within the 544 bp sequence of the kinase domain of B-Raf. Interestingly, the data revealed three possible splice variants of B-Raf within the kinase domain in all samples (*in vitro* and *in vivo*). Since the variants were displayed in all samples, we suspect the variants are native to Long Evans rats and not an effect of TGHQ or loss of tuberlin in TGHQ induced tuberous sclerosis renal cell carcinoma. These data confirm one clinical study, where eleven human RCC biopsies were sequenced for point mutations in the kinase domain of B-Raf, but none were identified [35].

Signal transduction pathways regulating renal tumor growth are quite complex, and our previous work has established B-Raf as a critical kinase regulating ERK/MAPK tumor growth in tuberous sclerosis RCC [12,19]. We showed that B-Raf is overexpressed in renal tumors [12], and that modulation of B-Raf kinase activity directly alters p27 protein levels, and promotes the cytoplasmic relocation of p27 during TGHQ-mediated tuberous sclerosis renal cell carcinoma [19]. Therefore, gaining a greater understanding of the transcriptional and post-translational changes to B-Raf may further our understanding of the role Raf kinases in tuberous sclerosis renal tumor growth and progression.

Acknowledgment

This work was supported by grants from the National Institutes of Health [grant number GM039338 to SSL], National Institutes of Environmental Health Sciences Training Grant [grant number T32ES007091 to JDC]. Mass spectrometric data was acquired by the Arizona Proteomics Consortium supported by NIEHS grant P30ES06694 to the Southwest Environmental Health Sciences Center, NIH/NCI grant P30CA023074 to the Arizona Cancer Center and by the BIO5 Institute of the University of Arizona. We thank Dr. Owen Kinsky for his assistance with preparation of figures.

Abbreviations

CR	conserved regions
ERK	extracellular signal-regulated kinases
HK2	human kidney 2 proximal tubular cell
IEF	isoelectric focusing
IP	immunoprecipitation
MAPK	mitogen-activated protein kinase
MEK1/2	mitogen-activated protein kinase kinase 1/2
OSOM	outer-stripe of the outer medulla
PBS	phosphate-buffered saline
pI	isoelectric point
QTRRE	quinol-thioether rat renal epithelial cells
Raf	rapidly accelerated fibrosarcoma
RCC	renal cell carcinoma
SGK	serum and glucocorticoid-inducible kinase
TCL	total cell lysate
TGHQ	2,3,5-tris-(glutathion-S-yl)hydroquinone
TKVS	threonine (T), lysine (K), valine (V), serine (S)
TSC	tuberous sclerosis complex

Tsc-2	tuberous sclerosis-2
Tsc-2^{EK/+}	Eker rats
Tsc-2^{+/+}	wild-type Eker rats

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Figure 1. B-Raf is constitutively phosphorylated at serine residues S345 and S483 in QTRRE cells

(A) Silver stained gel of B-Raf immunoprecipitations (5 replicates were pooled) from QTRRE cells. Both the 100 kDa and 95 kDa species were identified as B-Raf by LC-MS/MS analysis. Both species have constitutive phosphorylations on peptides described in (B) and (C). (B) Spectrum of peptide 345-366 from B-Raf. This peptide was identified by X! Tandem from a band excised from a gel corresponding to the molecular weight of B-Raf. Serine-345 was found to have a mass addition of 80, corresponding to a phosphorylation; this finding was confirmed by manual validation. The spectrum was magnified to a maximum of 20% relative abundance to account for the proline effect seen with the y9 and b13 ions [36]. (C) Spectrum of peptide 481-499 from B-Raf. Serine-483 was found to have a mass addition of 80, corresponding to a phosphorylation; this finding was confirmed by manual validation.

Figure 2. Fractionation of B-Raf modification groups in QTRRE cells

Protein lysate from QTRRE cells were fractionated using a Liquid-Phase IEF Cell run at 1 W constant until both the voltage and amps came to a plateau (~50 min). The pH was determined for each fraction, and then run on separate wells of a 7% SDS-PAGE gel. Following transfer to a PVDF membrane, the fractions were probed by Western blot for B-Raf.

Figure 3. Splice variants within the kinase domain of B-Raf in QTRRE cells, Tsc-2^{EK/+} and Tsc-2^{+/+} TGHQ- and vehicle-treated rats

RNA was isolated from (A) QTRRE cells, or (B) 8-month TGHQ- or vehicle-treated Tsc-2^{EK/+} and Tsc-2^{+/+} rats. PCR amplification of 544 bp of the kinase domain of B-Raf was performed as described in the Methods. B-Raf PCR products were run on a 2% agarose gel. (A) Lane 1 is the ladder, lane 2 is the B-Raf PCR amplification product of QTRRE cells, and lane 3 is PCR amplification control. Bands below 400 bp are primer dimer. (B) Lane 1 is DNA ladder, lane 2-3 vehicle treated rats, lane 4-5 TGHQ treated rats, and lane 6 is tumor excised from a TGHQ-treated Tsc-2^{EK/+} rat. (C) The 544 bp sequence amplified within the kinase domain of B-Raf. The highlighted blue text represents the forward and reverse primers, and the red text is the TKVS-activation segment of B-Raf.

Figure 4. B-Raf/Raf-1 heterodimerization in QTRRE cells

B-Raf, Raf-1 and 14-3-3 isoforms were immunoprecipitated (IP) from QTRRE lysates. Each IP and total cell lysate from QTRRE cells were immunoblotted for (A) B-Raf, (B) Raf-1 protein expression, and (C) 14-3-3 isoforms. QTRRE lysate was incubated with beads with no antibody (Control).

Figure 5. Sorafenib modulates B-Raf/14-3-3 protein interaction in QTRRE cells

QTRRE cells were treated with sorafenib (10 or 50µM, 1h). TCL was (A) visualized by western blot analysis for p-ERK, total ERK, B-Raf, Raf-1, and total 14-3-3 or (B) immunoprecipitated for total 14-3-3 and visualized by western blot analysis for total 14-3-3 and B-Raf. GAPDH was used as a loading control.