Kidney toxicity of the BRAF-kinase inhibitor vemurafenib is driven by off-target ferrochelatase inhibition

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A multitude of disease and therapy related factors drive the frequent development of kidney disorders in cancer patients. Along with chemotherapy, the newer targeted therapeutics can also cause kidney dysfunction through on and off-target mechanisms. Interestingly, among the small molecule inhibitors approved for the treatment of cancers that harbor BRAF-kinase activating mutations, vemurafenib can trigger tubular damage and acute kidney injury. BRAF is a proto-oncogene involved in cell growth. To investigate the underlying mechanisms, we developed cell culture and mouse models of vemurafenib kidney toxicity. At clinically relevant concentrations vemurafenib induces cell-death in transformed and primary mouse and human kidney tubular epithelial cells. In mice, two weeks of daily vemurafenib treatment causes moderate acute kidney injury with histopathological characteristics of kidney tubular epithelial cells injury. Importantly, kidney tubular epithelial cell-specific BRAF gene deletion did not influence kidney function under normal conditions or alter the severity of vemurafenib-associated kidney impairment. Instead, we found that inhibition of ferrochelatase, an enzyme involved in heme biosynthesis contributes to vemurafenib kidney toxicity. Ferrochelatase overexpression protected kidney tubular epithelial cells and conversely ferrochelatase knockdown increased the sensitivity to vemurafenib-induced kidney toxicity. Thus, our studies suggest that vemurafenib-associated kidney tubular epithelial cell dysfunction and kidney toxicity is BRAF-independent and caused, in part, by off-target ferrochelatase inhibition.

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KEYWORDS: acute kidney injury; BRAF kinase; ferrochelatase; onconephrology; protein kinase inhibitors; renal tubular epithelial cells

Translational Statement

BRAF is the most frequently mutated protein kinase and a critical oncogenic driver in human cancers. In melanoma and other cancers with BRAF-activating mutations, BRAF-targeted small-molecule therapeutics, such as vemurafenib and dabrafenib, have shown remarkable clinical benefits. However, recent clinical studies have shown that a significant number of patients who receive vemurafenib develop acute kidney injury through mechanisms that remain unknown. The present study describes the development of novel experimental models of vemurafenib nephrotoxicity and reveals the underlying off-target mechanisms that contribute to renal injury.

A broad array of factors increase the risk of renal dysfunction in cancer patients.[1–3] Although certain malignancies can directly affect kidney function,[4] cancer therapy can also trigger fluid and electrolyte disorders,[5] acute kidney injury (AKI),[6] and chronic kidney disease.[7] Renal disorders jeopardize the continuation of cancer

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therapy and contribute to the development of debilitating short- and long-term adverse sequelae. Along with chemotherapeutics, targeted antibodies[8] and small-molecule inhibitors[9] are also associated with renal impairment. Thus, one of the challenges in the emerging field of onconephrology[10–13] is to determine the mechanisms associated with these toxicities to develop mitigating strategies.

Dysregulation of the mitogen-activated protein kinase pathway is a major driver of oncogenesis.[14] The rapidly accelerated fibrosarcoma family of serine/threonine kinases act as a conduit between upstream Ras signaling and downstream mitogen-activated protein kinase activation, relaying signaling cues from the extracellular environment and directing cell proliferation, differentiation, migration, and survival.[15] Mammals possess 3 RAF proteins: RAF1 (CRAF), ARAF, and BRAF, which play essential and distinct physiological roles. More important, somatic activating mutations in BRAF are frequent in hairy-cell leukemia (100%), melanoma (50%–60%), and thyroid cancer (40%–60%).[15,16] The recent development of orally bioavailable BRAF inhibitors (namely, vemurafenib[17,18] and dabrafenib[19]) has brought exceptional clinical benefits, especially in melanoma patients.

Dermatologic toxicities are the major adverse effects related to vemurafenib treatment.[20] Although renal toxicities were not observed in the clinical trials,[21] a growing body of literature suggests that a significant percentage of patients treated with vemurafenib can develop AKI.[22–26] Acute tubular necrosis, electrolyte disorders, and subnephrotic-range proteinuria have been reported in a subset of vemurafenib-treated patients.[22,25] These studies have shown that patients treated with vemurafenib compared with dabrafenib have a higher incidence of AKI, and analysis of kidney biopsies has established tubular injury as the major histopathologic lesion.[22]

Because of paucity of experimental models, the mechanisms underlying vemurafenib nephrotoxicity remain unclear. Furthermore, it is unknown if BRAF is essential for renal tubular function. It is also unknown whether vemurafenib nephrotoxicity is BRAF dependent. To address these key questions, herein we have developed renal tubule-specific *BRAF* knockout mice and established *in vitro* and *in vivo* models of vemurafenib nephrotoxicity. Our studies suggest that *BRAF* knockout in renal tubules does not trigger renal impairment, and vemurafenib-associated renal tubular epithelial cell (RTEC) dysfunction and AKI are BRAF-independent.

METHODS

Animal experiments

Mice were housed and handled in accordance with the Institutional Animal Care and Use Committee of the Ohio State University and the University of Tennessee Health Science Center. Vemurafenib (20 mg/kg, p.o., twice daily) was administered for 15 to 20 days, and blood urea nitrogen, serum creatinine, *Ngal* expression, and hematoxylin and eosin–based histologic examinations were used to assess the extent of renal injury. Details regarding animal procedures are provided in Supplementary Methods.

Cell culture

BUMPT, HK-2, and primary murine RTECs were cultured according to previously described methods.[27,28] Cell viability and cell death were monitored by trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and caspase activity measurements. Further information regarding the experimental procedures is provided in Supplementary Methods.

Vemurafenib pharmacokinetic analysis

Plasma levels of vemurafenib were measured using previously described methods[29,30] and as detailed in the Supplementary Methods.

Ferrochelatase assay and intracellular heme measurement

Ferrochelatase activity was measured by enzymatic formation of zinc–protoporphyrin IX using a well-established method.[31] For determination of total intracellular heme levels, cells were homogenized in 1% Triton-X100 in triethanolamine-buffered saline, followed by heme quantification based on a previously described method.[32] These procedures are described in detail in the Supplementary Methods.

Protein and gene expression analysis

For protein analysis, whole cell lysates were prepared using a modified radioimmunoprecipitation a buffer supplemented with 1% sodium dodecylsulfate. Invitrogen bis-tris gradient midi-gels were used for Western blot analysis, and the antibodies used are described in the Supplementary Methods. For gene expression analysis, we performed quantitative polymerase chain reaction, as described in our previous work[33] and in the Supplementary Methods.

Statistical analysis

Data are presented as mean with SD. $P < 0.05$ was considered statistically significant. The GraphPad Prism software was used for statistical analysis. Additional details are provided in Supplementary Methods.

RESULTS

Vemurafenib induces cell death in cultured RTECs

We initially sought to determine if vemurafenib could cause direct toxicity in cultured RTECs. To this end, we treated transformed tubular epithelial cells of murine (BUMPT) and human origin (HK-2) with vemurafenib and tested its effect on cellular survival. In these experiments, we also included cisplatin, a well-studied nephrotoxic drug,[34] as well as BRAF inhibitor dabrafenib, and the multikinase and CRAF inhibitor sorafenib.[35] Similar to cisplatin, vemurafenib treatment reduced cellular viability, as measured by trypan blue staining (Figure 1a and b) in both BUMPT and HK-2 cells (50% inhibitory concentration = ∼50 μM). Notably, pharmacokinetic studies in humans have shown that plasma levels of vemurafenib can range from 50 to 100 μM.[21] Confirmatory experiments with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays showed that at 50 μM concentration, vemurafenib can

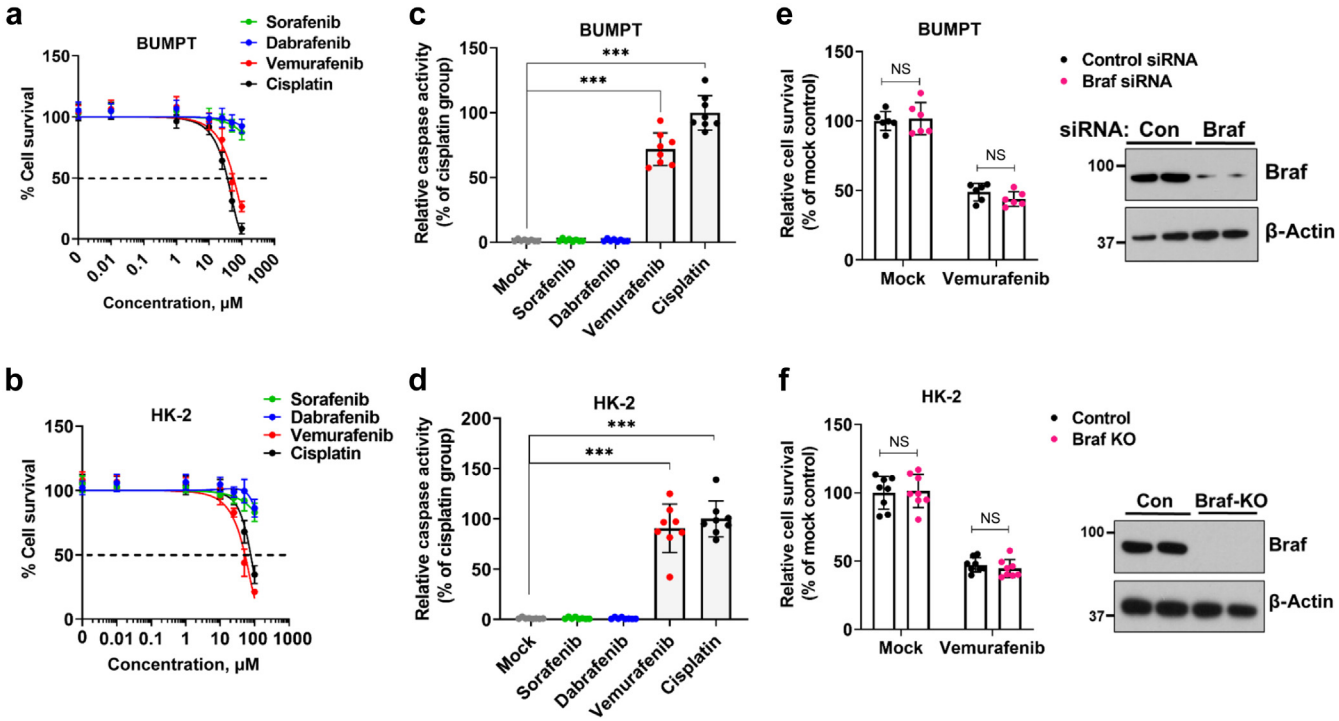

Figure 1 | Vemurafenib induces cell death in murine and human tubular epithelial cell lines. Tubular epithelial cell lines of murine (BUMPT) and human (HK-2) origin were treated with vehicle, cisplatin, or kinase inhibitors, including vemurafenib, followed by assessment of cell viability and cell death. (**a,b**) Dose-response experiments (0–100 μM) and trypan blue–based cellular viability assays at 48 hours after treatment showed that vemurafenib can induce cell death in renal tubular epithelial cell (RTEC) lines with 50% inhibitory concentration values of ≈50 μM. Survival data were normalized to the vehicle group and are presented as mean (n = 5 biologically independent samples), from 1 of 3 independent experiments, all producing similar results. (**c,d**) BUMPT and HK-2 cells were treated with vehicle or indicated drugs at 50 μM concentration, followed by the measurement of caspase activity at 48 hours. The results show that, similar to cisplatin, vemurafenib can reduce RTEC viability. Data are presented as individual data points (n = 8 biologically independent samples from 3 independent experiments). (**e**) RNA interference–mediated *Braf* knockdown in BUMPT cells did not influence vemurafenib-associated cell death (50 μM for 48 hours), as assessed by the trypan blue–based viability assay. Data are presented as individual data points (n = 6 biologically independent samples from 3 independent experiments). A representative immunoblot (right panel) shows the successful knockdown of the *Braf* gene. (**f**) CRISPR/Cas9-mediated Braf knockout (KO) in HK-2 cells did not influence vemurafenib-associated cell death (50 μM for 48 hours), as assessed by trypan blue–based viability assay. Data are presented as individual data points (n = 8 biologically independent samples from 3 independent experiments). A representative immunoblot (right panel) shows the successful knockout of the Braf gene. In all of the graphs (n = 5–8 biologically independent samples), experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Dunnett test, was performed, and statistical significance is indicated. ***$P < 0.001$. Con, control; NS, not significant; siRNA, small, interfering RNA.

cause ∼50% reduction in cellular viability within 48 hours (Supplementary Figure S1A and B), with a parallel increase in caspase activation (Figure 1c and d).

Intriguingly, kinase assays showed that at 50 μM concentration, vemurafenib, dabrafenib, and sorafenib inhibited BRAF-kinase activity to similar levels (Supplementary Figure S1C and D); however, under these conditions, only vemurafenib triggered RTEC cell death. We next questioned whether BRAF kinase is essential for RTEC survival and a causal factor in vemurafenib-induced cell death. To address this, we performed RNA interference–mediated knockdown and CRISPR/Cas9-mediated BRAF knockout in BUMPT and HK-2 cells, respectively (Figure 1e and f). BRAF knockdown or knockout had no impact on cellular viability under normal conditions and did not influence vemurafenib-induced cell death (Figure 1e and f and Supplementary Figure S2). These results suggest that vemurafenib can trigger cell death in cultured RTECs, seemingly through a BRAF-independent mechanism.

Establishment of a mouse model of vemurafenib nephrotoxicity

To establish a murine model of vemurafenib nephrotoxicity, we initially performed a pharmacokinetic study in C57B6/J mice. In concordance with previous work,[36] we found that at 20 mg/kg dose, plasma drug (∼50 μM) concentrations (Supplementary Figure S3) were similar to those observed in humans.[21] We then determined the effect of 20 mg/kg twice daily vemurafenib administration on kidney function (Figure 2a). We noticed a significant increase in blood urea nitrogen (Figure 2b) and serum creatinine (Figure 2c) levels after 2 weeks of twice daily dosing. In concordance with studies in humans, our histologic examination (Figure 2d–f and Supplementary Figure S4) revealed tubular epithelial

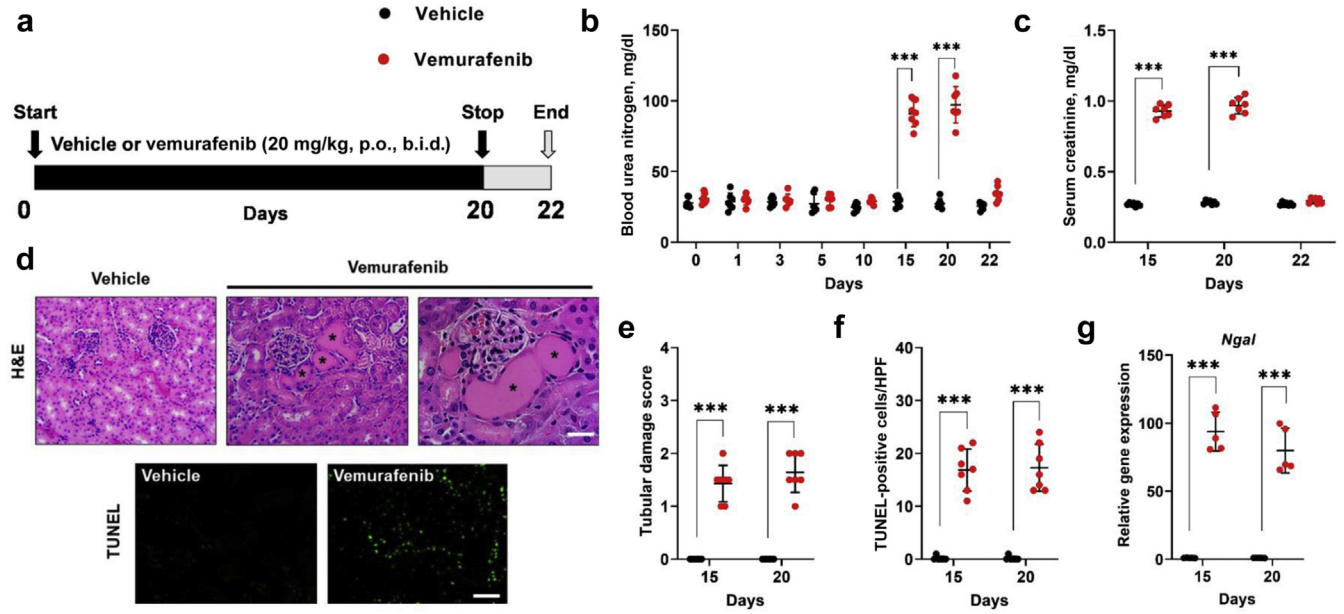

Figure 2 | Development of a mouse model of vemurafenib nephrotoxicity. (**a**) Schematic representation of dosing strategy. Age-matched, 8- to 12-week-old male C57BL/6J mice were treated with either vehicle or 20 mg/kg vemurafenib (p.o., twice daily [b.i.d.]) for 20 days, followed by cessation of drug administration for 2 days and subsequent endpoint analysis of renal function. (**b**) Analysis of blood urea nitrogen (BUN) levels showed that vemurafenib can induce acute kidney injury (AKI) after 2 weeks of continuous treatment, and cessation of drug administration reversed the increase in BUN levels. (**c**) Vemurafenib treatment also resulted in increased serum creatinine levels, indicating significant renal impairment. (**d,e**) Histologic analysis of renal tissues showed that vemurafenib-treated mice had clear tubular epithelial injury and cell death. Representative hematoxylin and eosin (H&E) staining depicted renal tubular damage (indicated by an asterisk) linked with vemurafenib-associated AKI. (**d,f**) Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining of renal tissues revealed significant tubular epithelial cell death in the vemurafenib-treated mice. (**d**) Bar = 100 μm. (**g**) Renal *Ngal* gene expression analysis further confirmed significant renal damage in vemurafenib-treated mice. In all the bar graphs (n = 5–7 biologically independent samples), experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. The Student *t* test or nonparametric Mann-Whitney *U* test was performed, and statistical significance is indicated. ***$P < 0.001$. HPF, high-power field. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

injury and cell death as the major pathologic lesion. In C57B6/J mice, several injury,[37] repair,[38] and inflammatory genes[39–42] are upregulated during ischemia, cisplatin, and rhabdomyolysis-associated AKI.[43] We found a similar increase in injury, repair, and inflammation-related genes during vemurafenib nephrotoxicity (Figure 2g and Supplementary Figure S5). Similar to humans,[22,25] we also found that treatment discontinuation in mice reverses vemurafenib-associated renal impairment (day 20 vs. 22).

Vemurafenib nephrotoxicity is BRAF-independent

To evaluate the RTEC-specific role of BRAF kinase, we generated conditional knockout mice ($BRAF^{PT-/-}$) by crossing the *BRAF* floxed[44] mice with the Ggt1-Cre mice.[45] We found that *BRAF* deficiency (Figure 3a) did not influence normal renal function (Supplementary Figure S6). Moreover, when the control and $BRAF^{PT-/-}$ littermates were challenged with vemurafenib, the extent of renal damage, as evaluated by blood urea nitrogen, serum creatinine, and histologic damage and injury biomarker analysis, was found to be similar in both groups (Figure 3b–f). To corroborate the *in vivo* findings, we isolated primary RTECs from *BRAF* floxed mice and performed *in vitro* Cre-mediated deletion (Supplementary Figure S7A). Vemurafenib treatment induced cell death in primary RTECs, and *BRAF* gene deletion did not influence cell death under these conditions (Supplementary Figure S7B). These findings support the notion that BRAF-kinase inhibition is unlikely to be the underlying cause of vemurafenib-associated AKI.

Identification of molecular targets associated with vemurafenib toxicity

Small-molecule kinase inhibitors target the ATP-binding pocket; however, the high conservation of the ATP binding site within the kinase families poses a significant challenge in developing highly specific kinase inhibitors.[46] Previous studies[47,48] have systematically profiled the promiscuity of kinase inhibitors, including vemurafenib. To ascertain if inhibition of these kinases contributes to vemurafenib cytotoxicity in RTECs, we used a chemical genetics approach.[49] BUMPT cells were transfected with plasmids encoding wild-type or inhibitor-resistant kinase genes, and their effect on cellular survival was monitored (Supplementary Figure S8). Surprisingly, inhibitor-resistant kinase overexpression did not rescue cytotoxicity in BUMPT cells (Supplementary Figure S8). Furthermore, vemurafenib is frequently used in

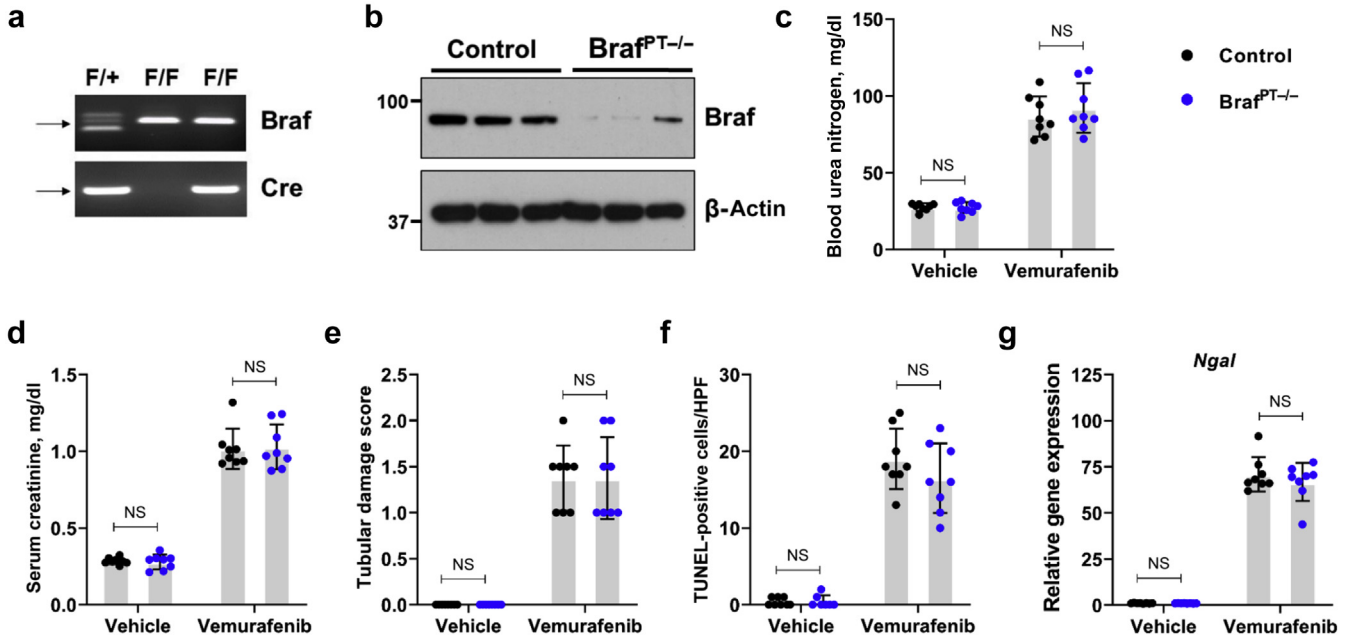

Figure 3 | Vemurafenib nephrotoxicity is not influenced by renal tubular epithelial cell–specific Braf gene deletion. To generate mice with renal tubule-specific *Braf* knockout, *Ggt1-Cre* mice were crossed with *Braf*-floxed mice. (**a,b**) Representative genotyping and immunoblots showing successful knockout in the renal tissues. Littermate control and *Braf* conditional knockout mice (indicated by $Braf^{PT-/-}$) were used to study the role of Braf in vemurafenib nephrotoxicity. Age-matched, 8- to 12-week-old male littermate mice were treated with either vehicle or 20 mg/kg vemurafenib (p.o., twice daily) for 20 days, followed by subsequent endpoint analysis of renal function. (**c**–**e**) The (**c**) blood urea nitrogen, (**d**) serum creatinine, and (**e**) histologic analyses were performed to examine renal function and damage. The extent of functional renal impairment and damage was similar between the control and *Braf*-deficient mice. (**f**) Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining also showed a similar amount of renal epithelial cell death in the control and knockout tissues. (**g**) Renal *Ngal* gene expression analysis. In all of the bar graphs (n = 8 biologically independent samples), experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Tukey multiple-comparison test or nonparametric Mann-Whitney *U* test, was performed, and statistical significance is indicated. HPF, high-power field; NS, not significant.

conjunction with cobimetinib, a mitogen-activated protein kinase kinase inhibitor, and this combination therapy has been suggested[50] to possibly reduce vemurafenib nephrotoxicity. However, our *in vivo* and *in vitro* combination studies at clinically relevant concentrations[51] (Supplementary Figures S9 and S10) showed that concomitant treatment with cobimetinib does not influence the severity or prevalence of vemurafenib nephrotoxicity. These results raised the possibility of the involvement of a non–kinase-related mechanism in vemurafenib nephrotoxicity.

Interestingly, chemical proteomics studies[52,53] have shown that vemurafenib but not dabrafenib can inhibit ferrochelatase (FECH). FECH is a mitochondrial protein that catalyzes the insertion of ferrous iron into protoporphyrin IX, and is the terminal enzyme involved in heme biosynthesis.[54] To ascertain if FECH inhibition causes vemurafenib-associated RTEC cell death, we initially quantified FECH activity in BUMPT and HK-2 cells. We found that vemurafenib significantly inhibited FECH activity in RTEC cell lines (Figure 4a and b) and caused a reduction in intracellular heme levels (Supplementary Figure S11). More important, intracellular heme serves as a prosthetic group for several proteins that constitute the complexes of mitochondrial electron transport chain.[55] Therefore, heme plays a pivotal role in oxidative phosphorylation and oxygen consumption. Using seahorse flux analyzer-based assay, a well-established technique for the measurement of oxygen consumption rate and mitochondrial respiration,[42,56] we found that mitochondrial dysfunction is triggered at early time points after vemurafenib treatment (Supplementary Figure S12). To establish the functional role of FECH in vemurafenib-mediated RTEC cell death, we performed overexpression studies. We found that overexpression of wild-type *FECH* protected BUMPT and HK-2 from vemurafenib-associated cell death (Figure 4c–g). Overexpression of an inactive *FECH* mutant did not influence vemurafenib-associated cell death. These results indicate that FECH inhibition might contribute to RTEC cell death under *in vitro* conditions.

RTEC-specific inhibition of FECH during vemurafenib nephrotoxicity

Because vemurafenib nephrotoxicity is associated with RTEC injury and cell death, we examined FECH expression in these and other renal cells. To this end, analysis of single-cell RNA-sequencing data[57] (Supplementary Figure S13) showed that FECH is expressed in multiple renal cell lines, with a subset of proximal tubular cells showing particularly high expression. Immunofluorescence studies showed that FECH is expressed in the cortical tubular epithelial cells, the major site of

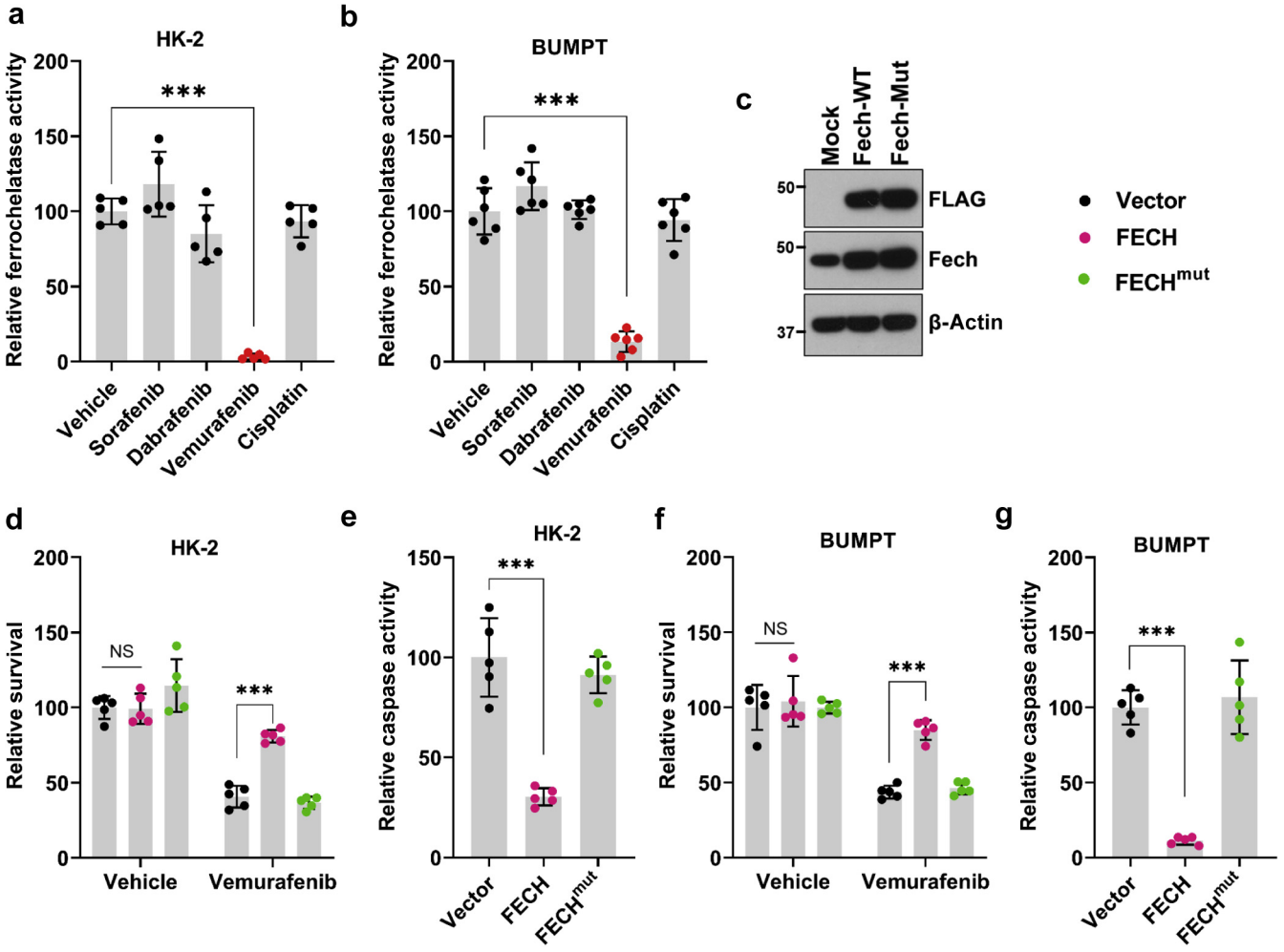

Figure 4 | Ferrochelatase (FECH) inhibition contributes to vemurafenib-mediated renal tubular epithelial cell death. Tubular epithelial cell lines of murine (BUMPT) and human (HK-2) origin were treated with vehicle, cisplatin, or kinase inhibitors, including vemurafenib, at 50 μM concentration, followed by assessment of FECH activity at 24 hours. (**a,b**) FECH activity was inhibited by vemurafenib in BUMPT and HK-2 cells. (**c**) Representative immunoblot, showing overexpression of FLAG-tagged wild-type (WT) and mutant *FECH* ($FECH^{mut}$). Blots are representative of 3 independent experiments. (**d**–**g**) Empty vector, WT *FECH*, or $FECH^{mut}$ (M98K) was overexpressed in BUMPT (transient transfection) and HK-2 (lentiviral transduction) cells, followed by treatment with either vehicle or 50 μM vemurafenib for 48 hours. Trypan blue–based survival assays and caspase assays showed that WT *FECH* overexpression can protect BUMPT and HK-2 cells from vemurafenib-associated cell death. All the bar graphs (n = 5–6 biologically independent samples) are from 1 of 3 independent experiments, all producing similar results, and experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Tukey multiple-comparison test, was performed, and statistical significance is indicated. ***$P < 0.001$. NS, not significant.

nephrotoxic injury (Supplementary Figure S14A). Time-course studies showed that although FECH protein levels remained unaltered, a progressive decline in FECH activity occurred during vemurafenib nephrotoxicity (Supplementary Figure S14B and C). Reduction in FECH activity was vemurafenib specific and not a generalized consequence of AKI (Supplementary Figure S14D). We next investigated whether the decrease in cortical renal FECH activity is RTEC specific. To label and isolate RTECs from murine kidneys, we crossed the $ROSA^{mT/mG}$ strain with the RTEC-specific Ggt1-Cre mice to generate transgenic mice that express membrane-localized green fluorescent protein (GFP) in the tubular epithelial cells (Figure 5a). As described in our recent work,[28,43] we then isolated GFP-positive cells (RTECs) and GFP-negative cells (other cell types) from the kidneys of untreated and vemurafenib-treated mice (Figure 5b). Initial examination of cells from untreated mice showed that FECH protein expression is relatively higher in RTECs compared with other cell types (Figure 5c and d). Similar to C57B6/J mice, on vemurafenib treatment, these reporter mice also exhibited similar extent of nephrotoxicity (Supplementary Figure S15). Remarkably, when we isolated GFP-positive and GFP-negative cells from vehicle- and vemurafenib-treated mice, we found that FECH activity (Figure 5e) and intracellular heme levels (Figure 5f) were specifically reduced in RTECs. Finally, we observed robust caspase activation specifically in RTECs from vemurafenib-treated mice (Figure 5g). These results suggest that RTECs are specifically

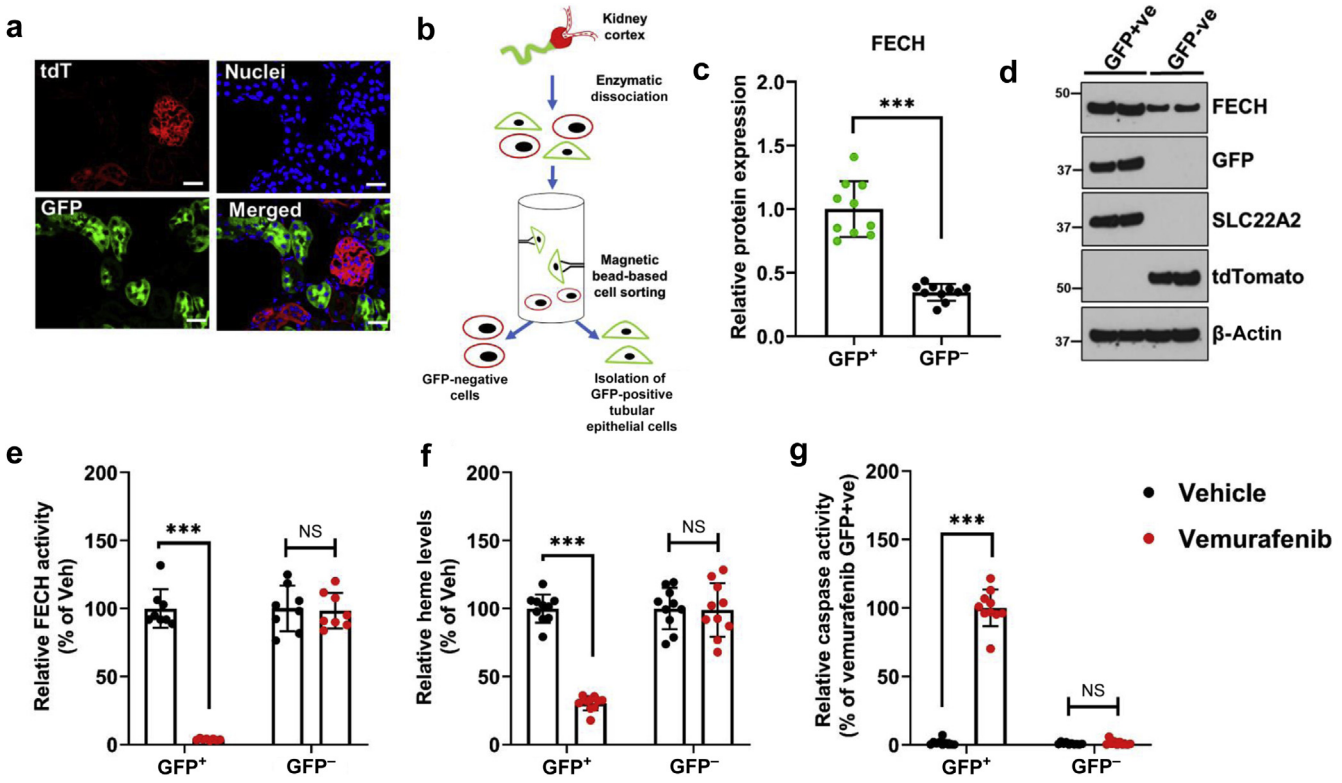

Figure 5 | Ferrochelatase (FECH) expression and inhibition in renal tubular epithelial cells (RTECs) during vemurafenib nephrotoxicity. To label, isolate, and examine RTECs, we crossed the Ggt1-Cre mice with ROSA$^{mT/mG}$ mice. The resulting transgenic mice express membrane-localized enhanced green fluorescent protein (EGFP) in RTECs, whereas the other cell types express membrane-localized tdTomato. (**a**) A representative image showing EGFP expression in renal tubular cells, whereas the cells within the glomerulus are TdTomato-positive. (**b**) Schematic representation of the methods used to isolate EGFP-positive renal epithelial cells. (**c,d**) Kidneys from control mice were used to isolate green fluorescent protein (GFP)–positive cells (RTECs) and negative cells, followed by immunoblot and densitometric analysis. FECH expression was found to be higher in RTECs compared with other cell types. As shown in the representative blot, the effectiveness of the cell isolation method was monitored by immunoblot analysis of RTEC- (GFP and SLC22A2) and non-RTEC–(tdTomato) specific markers. (**e**–**g**) Age-matched, 8- to 12-week-old male transgenic mice were treated with either vehicle (Veh) or 20 mg/kg vemurafenib (p.o., twice daily) for 15 days, followed by isolation of GFP-positive and GFP-negative cells. These isolated cells were then used to examine FECH activity, intracellular heme levels, and caspase activity. The results show that in the vemurafenib-treated mice, there is an RTEC-specific decrease in FECH activity, heme depletion, and caspase activation. All of the bar graphs (n = 8–10 biologically independent samples) are shown from 3 independent experiments, and experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. The Student *t* test or one-way analysis of variance, followed by the Tukey multiple-comparison test, was performed, and statistical significance is indicated. ***$P < 0.001$. NS, not significant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

sensitive to vemurafenib-associated FECH inhibition, heme depletion, and cell death.

In vivo FECH knockdown hastens vemurafenib nephrotoxicity

Given that a progressive decline in FECH activity preceded the development of AKI at 2 weeks, we questioned if *FECH* knockdown would influence vemurafenib nephrotoxicity. Using hydrodynamic small, interfering RNA (siRNA) injection approach,[29,33] we identified a specific siRNA that reduced FECH protein expression by ∼70% (Figure 6a and b). *FECH* knockdown did not influence normal renal function; however, strikingly, mice with *FECH* knockdown developed vemurafenib nephrotoxicity within 3 days in contrast to 2 weeks in control mice (Figure 6c–f). This increased sensitivity was vemurafenib specific because no difference in the extent of renal impairment was observed when the mice were challenged with cisplatin (Supplementary Figure S16). We also performed these *in vivo* siRNA experiments in the reporter mice with RTEC-specific GFP expression. Immunoblot analysis of isolated GFP-positive cells from these mice confirmed siRNA mediated *FECH* knockdown in RTECs (Supplementary Figure 17A and B). Similar to C57B6/J mice, siRNA-mediated *FECH* knockdown also hastened the development of vemurafenib nephrotoxicity in these reporter mice (Supplementary Figure 17C–F). These results suggest that FECH knockdown can remarkably accelerate the development of vemurafenib-associated AKI.

Accelerated development of vemurafenib nephrotoxicity in FECH mutant mice

In humans, mutations associated with reduced ferrochelatase activity can cause erythropoietic protoporphyria, a disease characterized by cutaneous photosensitivity and liver damage. In the mice with homozygous (*fch/fch*) mutations that reduce

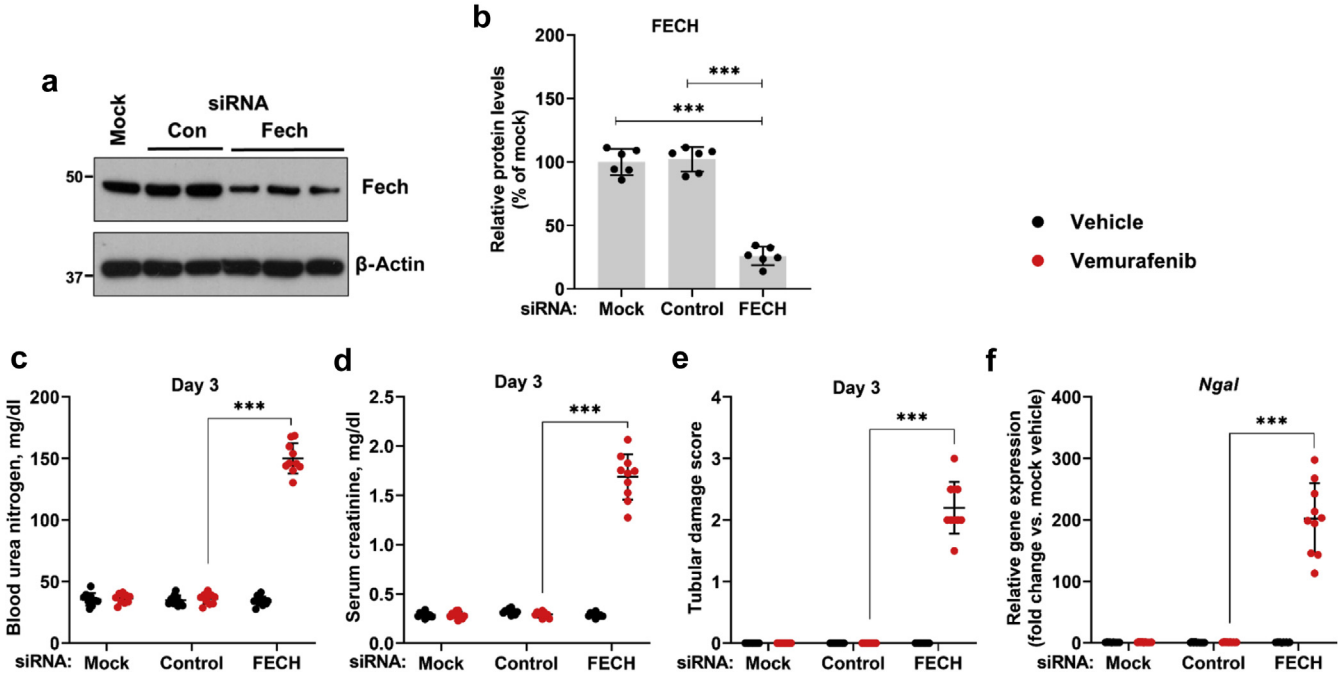

Figure 6 | *In vivo* small, interfering RNA (siRNA)–mediated ferrochelatase (FECH) knockdown hastens the development of vemurafenib nephrotoxicity. Age-matched male (aged 8–12 weeks) C57BL/6 mice were administered with 3 once-daily i.v. injections of control (Con; nonspecific) or *FECH*-targeting siRNAs (25 μg in 0.5 ml of phosphate-buffered saline [PBS]). In 1 group (mock), 0.5 ml of PBS was injected. One day later, mice were treated with either vehicle or 20 mg/kg vemurafenib (p.o., twice daily) for 3 days, followed by endpoint analysis of renal function. (**a,b**) The (**a**) representative blots and (**b**) densitometric analysis showed that the targeted siRNA was able to knock down FECH proteins levels by ≈75%. Blots are representative of 3 independent experiments, all producing similar results. (**c**–**f**) The (**c**) blood urea nitrogen, (**d**) serum creatinine, (**e**) histologic analysis, and (**f**) renal *Ngal* gene expression analysis showed that the FECH knockdown mice developed vemurafenib nephrotoxicity within 3 days of treatment, whereas the Con group demonstrated no obvious renal injury or damage. In all the bar graphs (n = 6–10 biologically independent samples), experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Tukey multiple-comparison test or nonparametric Mann-Whitney *U* test, was performed, and statistical significance is indicated. ***$P < 0.001$.

the FECH activity to 2.7% to 6.3%, photosensitivity and hepatic dysfunction are observed.[54] On the other hand, the heterozygotes (+/*fch*) mice have 45% to 65% of normal FECH activity and do not display any skin or liver damage. Congruent with a previous study,[54] we found that the heterozygous mutant mice had ≈50% reduction in renal FECH activity (Figure 7a). Next, we studied the consequence of reduced FECH activity on the development of vemurafenib nephrotoxicity. In comparison to control littermates, the heterozygous mice developed vemurafenib nephrotoxicity in an accelerated manner. At 7 days of vemurafenib treatment, when the control mice had no apparent renal impairment, blood urea nitrogen, serum creatinine, and histologic and gene expression analysis (Figure 7b–e) showed a precipitous decline in renal function in the heterozygous mice. Given that vemurafenib inhibits FECH activity in RTECs and genetic reduction of FECH activity accelerates the development of AKI, we propose that inhibition of renal FECH function contributes to vemurafenib nephrotoxicity.

CRISPR/Cas9-mediated FECH knockout triggers cell death in HK-2 cells

We questioned if genetic FECH inhibition would be sufficient to trigger cell death in cultured renal tubular epithelial cells. To address this, we utilized a doxycycline-inducible CRISPR/Cas9 system, wherein we generated stable HK-2 cells that express either control or FECH-targeted single-guide RNA, along with doxycycline-inducible Cas9. As shown in Figure 8a and b, doxycycline treatment resulted in decline in FECH protein expression in the stable cells expressing the FECH targeted single-guide RNA. When we further monitored these cells for 24 to 72 hours after doxycycline induction, we noticed a clear decline in cellular viability in the *FECH* knockout cells, as measured by trypan blue staining (Figure 8c). At 72 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and caspase activity assays confirmed reduced viability and increased cell death in the *FECH* knockout cells (Figure 8d and e). The observation that genetic *FECH* deficiency impairs RTEC viability supports the hypothesis that FECH inhibition could trigger RTEC cell death and dysfunction.

DISCUSSION

In the current study, we have established cell culture and murine models of vemurafenib nephrotoxicity that have provided important mechanistic insights. Our studies reveal that vemurafenib triggers RTEC dysfunction and

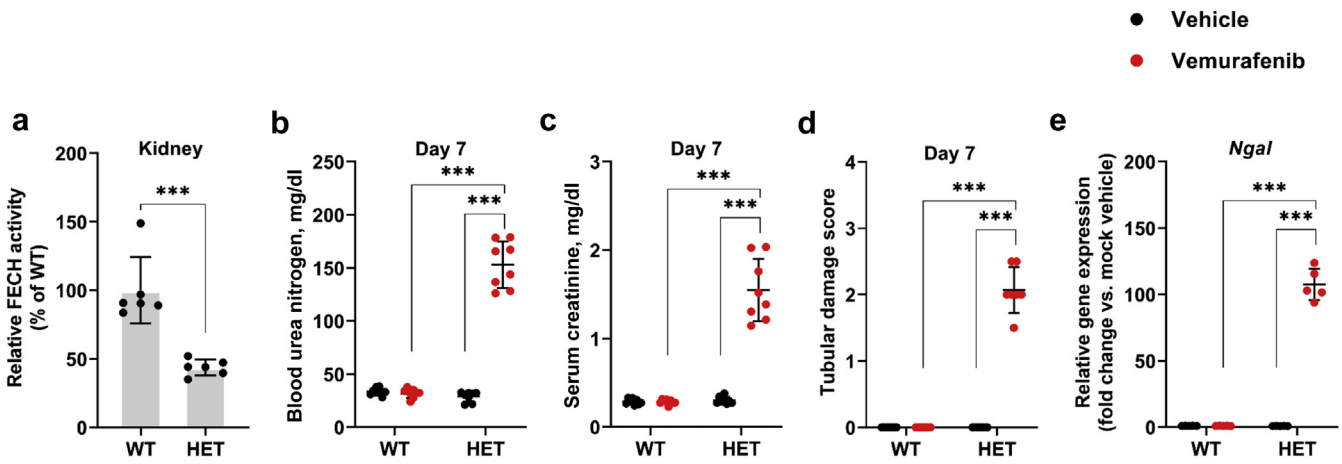

Figure 7 | Accelerated development of vemurafenib nephrotoxicity in ferrochelatase (FECH) mutant mice. (**a**) Renal tissues from wild-type (WT) and heterozygous (HET; *Fch/+*) mutant mice (littermates) were used to evaluate FECH activity using an enzymatic assay. The results show an ≈50% reduction in FECH activity in the HET mice. The WT and HET mice were then challenged with either vehicle or 20 mg/kg vemurafenib (p.o., twice daily) for 7 days, followed by endpoint analysis of renal function. (**b**–**e**) The (**b**) blood urea nitrogen, (**c**) serum creatinine, (**d**) histologic analysis, and (**e**) renal *Ngal* gene expression analysis showed that the FECH HET mutant mice developed vemurafenib nephrotoxicity within 7 days of treatment, at a time point when the WT group demonstrated no obvious renal injury or damage. In all of the bar graphs (n = 6–8 biologically independent samples), experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Tukey multiple-comparison test or nonparametric Mann-Whitney *U* test, was performed, and statistical significance is indicated. ***$P < 0.001$.

nephrotoxicity through a BRAF-independent and ferrochelatase-dependent manner.

Similar to most protein kinase inhibitors, vemurafenib is mainly excreted via feces (94%), with urinary excretion (1%) playing an insignificant role.[21,58] On the basis of our *in vitro* and *in vivo* studies, we propose that vemurafenib causes direct toxicity to tubular epithelial cells. Several lines of evidence suggest that this toxicity is independent of BRAF-kinase inhibition. First, both vemurafenib and dabrafenib treatment resulted in BRAF inhibition; however, only vemurafenib triggered RTEC cell death. Second, *BRAF* deletion in murine and human RTECs did not influence cell survival under normal conditions. *BRAF* deletion or knockdown also did not influence vemurafenib-induced cell death and AKI. This also eliminates the possibility that paradoxical mitogen-activated protein kinase activation, initiated by inhibitor-induced wild-type BRAF dimerization,[59] might contribute to nephrotoxicity. Third, chemical genetic studies with transfection of vemurafenib-resistant *BRAF* gatekeeper mutants did not confer protection from vemurafenib-induced cell death. Although genetic compensation remains a possibility, collectively our results suggest that BRAF-kinase is not an essential gene in RTECs and vemurafenib nephrotoxicity is BRAF-independent. However, it is important to note that AKI is a multifaceted disease[2] that results from a complex interplay between epithelial,[38,60,61] immune,[40] and endothelial[62,63] cells. Thus, notwithstanding our studies with RTECs, further work is required to discern the role of immune and endothelial cells in vemurafenib nephrotoxicity.

We propose that vemurafenib inhibits ferrochelatase, which likely contributes to RTEC cell death and AKI. Ferrochelatase is the terminal enzyme of the heme biosynthesis pathway.[54] FECH is a mitochondrial protein that catalyzes the insertion of the reduced form of iron (Fe^{2+}) into protoporphyrin. In humans, mutations associated with reduced FECH activity are associated with erythropoietic protoporphyria that is characterized by cutaneous photosensitivity and progressive liver damage.[54] Proteomic profiling of leukemia cells previously revealed that vemurafenib, but not dabrafenib, is a potent FECH inhibitor.[52,53] It was thus speculated that FECH inhibition might contribute to photosensitivity and skin toxicities associated with vemurafenib treatment. Our *in vivo* studies show that vemurafenib treatment results in a progressive decline in FECH activity and development of AKI 2 weeks after treatment initiation. More important, RNA interference–mediated knockdown or *FECH* loss-of-function mutations cause a striking acceleration in the development and onset of vemurafenib-associated AKI (3–7 vs. 15 days). Furthermore, *FECH* overexpression in cultured RTECs provided significant protection from vemurafenib-induced cell death.

In contrast, with targeted antiangiogenic therapeutics,[64] where the entire class of drugs cause hypertension due to "on-target" effects, not all BRAF inhibitors seem to cause AKI.[22] This is supported by the observation that patients treated with vemurafenib compared with dabrafenib have a higher incidence of AKI.[22,25] We propose that this is not a consequence of differential BRAF inhibition but is dependent on the unique property of vemurafenib to inhibit FECH. In support, BRAF gene deletion in RTECs did not result in renal impairment *in vivo*. Although it is unknown if BRAF is an essential gene in other renal cell types, it is interesting that BRAF inhibition has been suggested as a therapeutic option to prevent podocyte injury.[65,66] Currently, it is difficult to

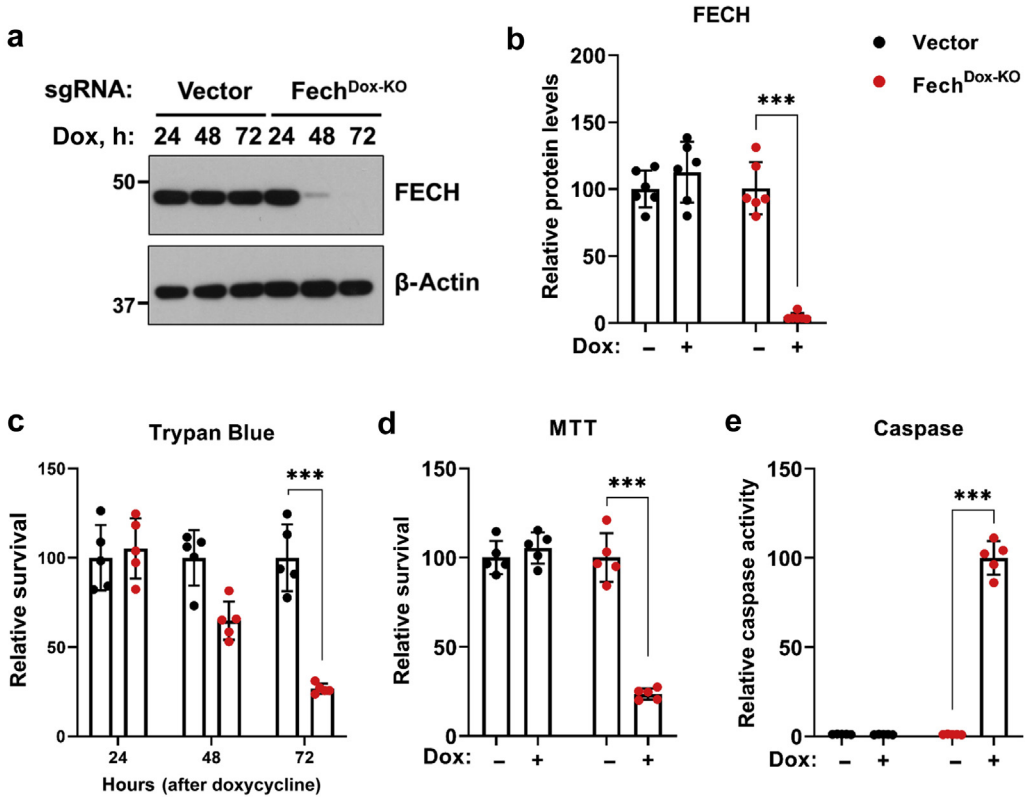

Figure 8 | CRISPR/Cas9-mediated ferrochelatase (*FECH*) knockout induces cell death in human (HK-2) cells. Stable HK-2 cells were generated with either a control or FECH-targeted single-guide RNA (sgRNA). In these stable cell lines, Cas9 expression can be induced by doxycycline (Dox) treatment. (**a**) In the presence of Dox, FECH gene deletion occurs in the cells that express *FECH*-targeted sgRNA. Representative immunoblot depicting successful gene knockout. Blots are representative of 3 independent experiments. (**b**) Densitometric analysis of FECH protein after 72 hours of Dox treatment. (**c**) Control (vector) and FECH sgRNA (FECH$^{Dox\text{-}KO}$) stable cells growing in normal media were treated with Dox, and trypan blue–based survival assays were performed at 24 to 72 hours. The results show that *FECH* knockdown resulted in significant reduction in cell survival. (**d,e**) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and caspase assays at 72 hours confirmed that *FECH* gene depletion results in cell death activation. In all the bar graphs (n = 5–6 biologically independent samples), data are from 1 of 3 independent experiments, all producing similar results, and experimental values are presented as mean ± SD. The height of error bar = 1 SD, and $P < 0.05$ was indicated as statistically significant. One-way analysis of variance, followed by the Dunnett test, was performed, and statistical significance is indicated. ***$P < 0.001$.

predict which patients are more likely to develop vemurafenib nephrotoxicity. It would be interesting to examine if genetic polymorphisms or physiological conditions associated with altered renal FECH activity play a contributing role. Altered iron homeostasis is a known feature of AKI[67–69]; however, the role of FECH in RTEC function and AKI has not been previously explored.

We currently do not know if RTECs in particular proximal tubular segments have differential FECH expression and/or are more sensitive to vemurafenib toxicity. However, our cell labeling experiments clearly show that, compared with other cell types, vemurafenib treatment results in RTEC-specific FECH inhibition, intracellular heme depletion, and cell death activation. So, why are RTECs particularly sensitive to vemurafenib-mediated FECH inhibition and cell death? We propose that this could be attributable to 2 unique functional and physiological properties of RTECs. First, because of the higher expression of transport systems, it is likely that vemurafenib might accumulate at higher levels in RTECs than other cell types. Although future studies are required to identify the transport mechanisms, this differential uptake could explain why FECH inhibition only occurs in RTECs. Second, although liver and the bone marrow are the key sites for systemic heme synthesis, kidneys also have significant heme biosynthetic capacity, which is interestingly localized mainly within the cells of the proximal convoluted tubule.[67,70] Hence, within the kidneys, FECH might have a particularly critical role in heme synthesis and cellular viability in RTECs. Given the critical role of mitochondria in RTEC function[61] and the critical role of heme in mitochondrial function, it is not surprising that FECH is an essential gene in these cells. Indeed, our studies show that both pharmacologic and genetic FECH inhibition in cultured RTECs results in cell death induction. Studies with conditional knockout mice could further clarify if FECH is an essential gene *in vivo* in RTECs and other renal cells.

Why does FECH inhibition result in RTEC cell death? FECH is a mitochondrial enzyme that is involved in biosynthesis of heme, an iron-containing cyclic tetrapyrrole. Heme is a cofactor for protein complexes involved in oxygen

transport, mitochondrial respiration, oxidation-reduction reactions, circadian rhythm, transcription, and translation.[55,71] More important, heme is a cofactor for several electron transport chain components, where it mediates electron transfer reactions that are coupled to formation of the mitochondrial proton gradient. One possible indirect consequence of vemurafenib-mediated FECH inhibition could be the accumulation of by-products of heme biosynthesis[53] within the mitochondria, resulting in increased oxidative stress, mitochondrial dysfunction, and direct cellular toxicity. On the other hand, the direct consequence of vemurafenib-mediated FECH inhibition could be heme depletion, loss of mitochondrial function, and RTEC cell death. In support of these possibilities, our studies show that vemurafenib treatment causes a significant decrease in intracellular heme levels in RTECs, which correlates with a precipitous decline in mitochondrial function and cell death induction. Future in-depth studies are, however, warranted to tease out exactly how vemurafenib-mediated FECH inhibition influences the electron transport chain and triggers mitochondrial dysfunction. In addition, although genetic *FECH* knockout triggers RTEC cell death, it is unlikely that FECH inhibition in RTECs is solely responsible for vemurafenib nephrotoxicity. FECH-dependent and FECH-independent mechanisms in RTECs and other renal cells, including endothelial and immune cells, might play a role in this toxicity.

Because of the availability of BRAF inhibitors, such as dabrafenib, and the moderate nature of vemurafenib nephrotoxicity, currently there is no dire need to develop therapeutic strategies to prevent vemurafenib-associated AKI. However, several approved, experimental, and investigational kinase inhibitors have recently been demonstrated to inhibit FECH activity, through mechanisms that remain elusive.[53] For instance, an anaplastic lymphoma kinase inhibitor, alectinib, can inhibit FECH[52] and is linked with renal dysfunction,[72] although the causality remains to be investigated. Because FECH inhibition is not limited to vemurafenib but has emerged as a relatively common feature of several protein kinase inhibitors, future genetic and pharmacologic studies are required to understand the role of this enzyme in renal function and AKI.

In summary, our work suggests that vemurafenib-associated AKI is an off-target toxicity that can be partly attributed to FECH inhibition in RTECs. By identifying the underlying mechanisms, our study reveals that drugs with FECH-inhibiting ability might cause RTEC dysfunction and nephrotoxicity.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

YB, JYK, BB, LAJ, MG, JAS, VS, KMH, SDB, REC, AS, AB, and NSP acquired and analyzed most of the data. YB, VS, JP, JAS, REC, MSB, KS, KDJ, and NSP performed data analysis. YB, JYK, AB, and NSP conceived the study. NSP approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary References.

Figure S1. Braf inhibition and vemurafenib-induced renal tubular epithelial cell (RTEC) cell death.

Figure S2. Vemurafenib-induced renal tubular epithelial cell (RTEC) cell death is Braf-independent.

Figure S3. Vemurafenib pharmacokinetic analysis.

Figure S4. Histopathologic analysis of vemurafenib nephrotoxicity.

Figure S5. Renal gene expression analysis during vemurafenib nephrotoxicity.

Figure S6. Effect of Braf gene knockout on renal function.

Figure S7. *In vitro* Braf gene deletion does not influence vemurafenib-associated renal tubular epithelial cell (RTEC) cell death.

Figure S8. Chemical genetics approach to evaluate the role of kinase inhibition in vemurafenib-mediated renal tubular epithelial cell (RTEC) cell death.

Figure S9. Mitogen-activated protein kinase (MEK) inhibition does not influence the severity of vemurafenib nephrotoxicity.

Figure S10. Mitogen-activated protein kinase (MEK) inhibition does not influence the severity of vemurafenib-induced renal tubular epithelial cell (RTEC) cell death.

Figure S11. Vemurafenib induces intracellular heme depletion in renal tubular epithelial cells (RTECs).

Figure S12. Vemurafenib induces mitochondrial dysfunction in renal tubular epithelial cells (RTECs).

Figure S13. Fech expression in proximal tubular and other cells of healthy mouse kidneys.

Figure S14. Vemurafenib-associated acute kidney injury (AKI) is associated with renal ferrochelatase inhibition.

Figure S15. Vemurafenib nephrotoxicity in the transgenic reporter mice.

Figure S16. *In vivo* small, interfering RNA (siRNA)–mediated ferrochelatase (FECH) knockdown does not influence the severity of cisplatin nephrotoxicity.

Figure S17. *In vivo* small, interfering RNA (siRNA)–mediated ferrochelatase (FECH) knockdown in reporter mice.

Figure S18. Uncropped images of immunoblot data.

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