Early loss of mitochondrial complex I and rewiring of glutathione metabolism in renal oncocytoma

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Renal oncocytomas are benign tumors characterized by a marked accumulation of mitochondria. We report a combined exome, transcriptome, and metabolome analysis of these tumors. Joint analysis of the nuclear and mitochondrial (mtDNA) genomes reveals loss-of-function mtDNA mutations occurring at high variant allele fractions, consistent with positive selection, in genes encoding complex I as the most frequent genetic events. A subset of these tumors also exhibits chromosome 1 loss and/or cyclin D1 overexpression, suggesting they follow complex I loss. Transcriptome data revealed that many pathways previously reported to be altered in renal oncocytoma were simply differentially expressed in the tumor's cell of origin, the distal nephron, compared with other nephron segments. Using a heuristic approach to account for cell-of-origin bias we uncovered strong expression alterations in the gamma-glutamyl cycle, including glutathione synthesis (increased GCLC) and glutathione degradation. Moreover, the most striking changes in metabolite profiling were elevations in oxidized and reduced glutathione as well as γ-glutamyl-cysteine and cysteinyl-glycine, dipeptide intermediates in glutathione biosynthesis, and recycling, respectively. Biosynthesis of glutathione appears adaptive as blockade of GCLC impairs viability in cells cultured with a complex I inhibitor. Our data suggest that loss-of-function mutations in complex I are a candidate driver event in renal oncocytoma that is followed by frequent loss of chromosome 1, cyclin D1 overexpression, and adaptive up-regulation of glutathione biosynthesis.

Significance

Renal oncocytomas are benign kidney tumors with numerous mitochondria. Here, we analyze the mitochondrial (mtDNA) and nuclear genomes of these tumors. Our analysis finds mtDNA mutations in complex I (the first step in mitochondrial respiration) to be early genetic events that likely contribute to tumor formation. Since mtDNA mutations can lead to severe degenerative disorders, the cellular responses allowing renal oncocytoma cells to grow are important to consider. To properly understand authentic gene expression changes in tumors, we found it important to consider the gene expression pattern of the tumor's cell of origin, the distal nephron. By doing so, we uncover alterations in glutathione synthesis and turnover that likely represent an adaptive metabolic response in renal oncocytoma.

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Nuclear Genomes in RO

To search for somatic alterations in nuclear and mitochondrial genomes, we performed WES on 19 high purity formalin-fixed paraffin-embedded (FFPE) RO samples and adjacent normal kidney (RO MGH). Mean nuclear DNA coverage was 171× in normals and 212× in tumors with a relatively low non-synonymous mutation rate of ∼15 mutations per sample (Dataset S1). MutSig analysis did not reveal any significantly mutated nuclear genes.

We then evaluated tumors for karyotypic alterations in the nuclear DNA. ROs were largely diploid with hemizygous loss of chromosome 1 or 1p in 12/19 patients (Fig. 1A and SI Appendix, Fig. S1A) and no recurrent focal copy number events. As previous studies detected cyclin D1 rearrangements leading to cyclin D1 overexpression (13), we assessed cyclin D1 via immunohistochemistry (IHC) (14), finding strong expression in 3/19, moderate expression in 4/19, and absent expression in 12/19 samples (SI Appendix, Fig. S1B and Dataset S1). Chromosome 1 was lost in 3/7 samples showing cyclin D1 overexpression by IHC (Fig. 1A). Together, 63% of RO tumors show chromosome 1 loss and 37% show cyclin D1 overexpression.

Mitochondrial Genomes in RO

We next analyzed off-target mtDNA reads from WES to identify somatic mtDNA variants in RO MGH (Methods). We obtained

Fig. 1. Analysis of mitochondrial and nuclear genomes in RO. (A) Somatic mutations in mtDNA for RO samples (columns) across two cohorts (RO MGH and RO GEN). Somatic protein-altering mutations in the 13 mtDNA genes are shown with color (red, LoF; black, missense) and shade to indicate VAF (see legend). OXPHOS complexes are indicated (CI/III/IV/V: complex I/III/IV/V, respectively). Chromosome 1 and cyclin D1 status are shown (see legend). (B) Position of mutations on an mtDNA molecule from position 0 (Left) to 16,569 (Right). Top rows show positions of 5 base-pair homopolymeric tracts (gray bars); red bars highlight sites of recurrent mutation (≥2 samples). (Bottom) Shows all somatic mutations as diamonds (see legend) in each RO sample (horizontal lines) from both cohorts (RO MGH and RO GEN) by position. (C) Stacked barplots show the percent of kidney tumors with complex I LoF mutations at ≥40% VAF. LoF, loss-of-function.
mean coverage of the mtDNA in normals and 114× coverage in tumors (Dataset S1). Consistent with a prior pan-cancer analysis of mtDNA across 31 different types of cancer, we observed a G > A and T > C bias on the light strand, suggestive of a replicative mutational process (Dataset S2) (15).

We find that 16/19 tumors harbor protein-truncating mtDNA mutations with variant allele fraction (VAF) ≥ 0.4 (Fig. 1A). Strikingly, these all occur in complex I genes (MT-ND1, MT-ND5, MT-ND4, MT-ND3), with no loss-of-function (LoF) mutations in complexes III, IV, or V at this VAF (Fig. 1A). Importantly, these LoF mutations had high VAF, a surrogate for heteroplasmy (fraction of mutated mtDNA molecules). These LoF mutations were typically short indels occurring at specific mtDNA sites (Fig. 1B). Of the 16 LoF mutations with VAF ≥0.4, one was a nonsense mutation (m.10126; MT-ND3:p.Trp23) and 15 were short indels (1–2 base pairs) typically within 4–6 base-pair homopolymeric DNA tracts often resulting in early frame-shift mutations (Dataset S3). Many mutations were recurrent, including seven mutations at m.3565; MT-ND1:p.Thr87, three mutations at m.10946; MT-ND4:p.Trh63, and two mutations at m.11866; MT-ND4:p.Thr370 (Fig. 1B and SI Appendix, Fig. S2). We note that homopolymeric tracts occur in all mtDNA genes including seven mutations at m.3565; MT-ND1:p.Thr87, three shift mutations (15) were short indels (1–2 base pairs) typically within 4–6 base-pair homopolymeric DNA tracts often resulting in early frame-shift mutations (Dataset S3). Many mutations were recurrent, including seven mutations at m.3565; MT-ND1:p.Thr87, three mutations at m.10946; MT-ND4:p.Trh63, and two mutations at m.11866; MT-ND4:p.Thr370 (Fig. 1B and SI Appendix, Fig. S2). We note that homopolymeric tracts occur in all mtDNA genes including seven mutations at m.3565; MT-ND1:p.Thr87, three shift mutations (15) were short indels (1–2 base pairs) typically within 4–6 base-pair homopolymeric DNA tracts often resulting in early frame-shift mutations (Dataset S3). Many mutations were recurrent, including seven mutations at m.3565; MT-ND1:p.Thr87, three mutations at m.10946; MT-ND4:p.Trh63, and two mutations at m.11866; MT-ND4:p.Thr370 (Fig. 1B and SI Appendix, Fig. S2). We note that homopolymeric tracts occur in all mtDNA genes including seven mutations at m.3565; MT-ND1:p.Thr87, three shift mutations (15) were short indels (1–2 base pairs) typically within 4–6 base-pair homopolymeric DNA tracts often resulting in early frame-shift mutations (Dataset S3). Many mutations were recurrent, including seven mutations at m.3565; MT-ND1:p.Thr87, three mutations at m.10946; MT-ND4:p.Trh63, and two mutations at m.11866; MT-ND4:p.Thr370 (Fig. 1B and SI Appendix, Fig. S2).

Mitochondrial Genomes in RO\textsuperscript{GEN}

The above analysis of RO\textsuperscript{MGH} revealed that 19/19 samples had evidence for selection of disabling complex I mutations, hence making complex I loss a universal feature of these tumors. A large subset, but not all, of these samples also likely harbor deleterious complex I mutations (Dataset S3). One tumor had a complex I LoF event at 0.13 VAF in a sample with only 20% tumor purity by pathology estimate, consistent with likely high heteroplasmy in the tumor (SI Appendix, Fig. S3). The remaining 2/19 samples harbor missense mutations in complex I subunits: MT-ND4, p.E123K at 0.36 VAF and MT-ND1,p.W179M at 0.89 VAF. Both missense mutations were at highly conserved residues (identical in at least 34/44 aligned vertebrates) and based on structural modeling of bacterial complex I (16) are predicted to impair complex I proton transport (MT-ND4:p.E123K) or disrupt an accessory subunit interaction site (MT-ND1.p.W179M) (17).

Mitochondrial Genomes Across RCC

We next asked if mtDNA mutations were specific to RO or if they occurred in RCC variants. We analyzed WES off-target mtDNA reads from 8 cosinophilic chRCC, 19 classic chRCC, and 12 pRCC from Durinck et al. as well as 104 ccRCC from the TCGA (2) (Dataset S2). Specifically, we analyzed the seven complex I genes compared with the six other oxidative phosphorylation (OXPHOS) genes for LoF mutations. This mtDNA analysis revealed a large fraction of complex I LoF mutations in RO\textsuperscript{MGH} (84%), RO\textsuperscript{GEN} (69%), and cosinophilic chRCC (62%)—precisely the renal tumors known to exhibit mitochondrial accumulation (Fig. 1C and Dataset S2). These complex I LoF mutations are likely drivers of pathology because they (i) occur preferentially in complex I compared with other OXPHOS complexes (SI Appendix, Fig. S4A), and (ii) have significantly higher VAF compared with silent or noncoding variants (SI Appendix, Fig. S4B). This contrasts with a recent pan-cancer analysis across 31 different cancers in which LoF mtDNA mutations exhibited lower VAF compared with silent mutations (15). Thus, the analysis of five kidney tumors as well as 1,664 pan-cancer samples shows that RO and cosinophilic chRCC are unique for their high VAF, LoF complex I mtDNA mutations.

As functional alterations in mtDNA can also result from copy number variation, we used quantitative PCR to measure mtDNA copy number in our RO\textsuperscript{MGH} cohort. We found a significant increase in mtDNA copy number in RO (mean ~2,700 copies per cell) compared with normal kidney (mean ~1,900 copies per cell; SI Appendix, Fig. S5A). It was notable the copy number increase was less than twofold considering the numerous mitochondria in these tumors. We found the fraction of WES reads aligning to mtDNA correlated strongly with mtDNA copy number estimated by quantitative PCR (SI Appendix, Fig. S5B). Thus, we extended our analysis using WES reads to other RCC variants, finding no significant differences between tumors and normals except in ccRCC where there was a striking decrease in mtDNA copies (SI Appendix, Fig. S5C). We note that such analyses across RCC subtypes will be influenced by differences in tumor purity, nuclear copy number, and importantly mtDNA copy number in the cell of origin across tumors.

Transcriptional Profiles in RO

We next sought to characterize gene expression changes in RO to relate them back to primary genetic lesions in mtDNA complex I genes. Previous groups have reported significant up-regulation of mitochondrial biogenesis genes, including PPARGC1a and OXPHOS subunits, as well as marked elevation in AMP-activated protein kinase (AMPK) subunits (PRKAB2, PRKAG2) and its targets (ACACA, ACCB) (12, 13). Since AMPK activation may result from complex I dysfunction, we measured the levels of AMPKα Thr172 phosphorylation, a modification required for AMPK activation (18), in tumors and normal kidney.

Although we saw strong AMPK activation in tumors as previously reported (SI Appendix, Fig. S6), on closer examination, the signal was increased compared with normal kidney proximal tubules but comparable to normal distal tubules (SI Appendix, Fig. S6 and Supplementary Materials and Methods for tubule definitions). Since RO derives from the distal nephron (6), we concluded the apparent AMPK phosphorylation enrichment was not due to a tumor versus normal distal tubule difference, rather a difference in distal tubules compared with the other cortical nephron segments (estimated by volume in rat to be roughly 68% proximal tubules, 15% distal tubules, 8% glomeruli and capillaries, and 9% interstitial and extracellular space in the subcapsular cortex; ref. 19). The increased staining in distal tubules relative to proximal tubules was also evident for the OXPHOS subunits NDUF8 (a subunit of complex I) and MT-CO1 (a subunit of complex IV), with subsequent attenuation of only the complex I signal in tumors consistent with their pattern of mtDNA mutation (Fig. 2). These observations raise the possibility that prior reports (12, 13) of differential gene expression in RO may have suffered from extensive cell-of-origin bias.

To investigate if a pervasive cell-of-origin bias besets prior RO gene expression analysis, we developed a heuristic approach to identify transcripts genuinely altered in tumors. Our approach is conceptually simple and is conservative: ROs arise from the distal nephron, whereas the normal cortex consists of mostly proximal tubules (estimated to represent ~60–90% of the cortex;
of origin; shown in blue are genes we infer are reliably reduced in RO and not obviously biased by cell of origin. We substantiated the validity of this heuristic method by revisiting known nephron markers as well as genes argued to be differentially expressed in RO (SI Appendix, Figs. S7 and S8 and Supplementary Text).

What then are genes whose expression is reliably altered in RO compared with normal distal tubules? We focused our attention on the 24 dark red (“reliably up”) and 635 dark blue (“reliably down”) genes whose expression is in the opposite direction to what would be predicted based on cell of origin (Fig. 3A and Dataset S4). We observed reliable up-regulation of the rate-limiting enzyme in glutathione biosynthesis, GCLC (Fig. 3B) (22). This was noteworthy because glutathione was the most up-regulated metabolite detected by metabolome profiling of RO, a finding confirmed by IHC (described below).

Closer inspection revealed up-regulation of the entire transulfuration and glutathione synthesis programs (CBS, CTH, GCLC, GCLM, GSS) even after cell of origin was considered (Fig. 3C). Moreover, there were distinct changes in the glutathione degradation phase of the γ-glutamyl cycle that would favor glutathione accumulation. The γ-glutamyl transferase genes involved in the first step of glutathione degradation showed preferential expression in the proximal tubules (GGT1), down-regulation in tumors (GGT3, GGT4), or ambiguous expression (GGT7) (Fig. 3C). The dipeptidase, DPEP1, involved in the second step of glutathione degradation was down-regulated in tumors despite being a distal tubule gene (23). Furthermore, there was up-regulation of glutathione exporters (ABCC4, CFTR) and the SLC7A11 subunit of the xCT cystine-glutamate antiporter (SI Appendix, Fig. S9). The coordinated up-regulation of intracellular glutathione synthesis alongside the down-regulation of extracellular glutathione degradation demarcates a change in the γ-glutamyl cycle expected to boost glutathione levels in tumors.

Metabolic Profiles in RO
To complement the DNA and RNA analysis, we performed metabolic profiling on an additional cohort of fresh frozen RO (n = 10) and normal kidney (n = 7) samples (Dataset S5), recognizing the results will certainly be impacted by cell-of-origin effects. We used four liquid chromatography-tandem mass spectrometry (LC-MS) methods to interrogate a total of 592 metabolites of confirmed identity, of which 554 were detected in at least 20% of our samples (Dataset S5). Of these 554 metabolites, 184 were differentially abundant between tumors and normals at a nominal P value <0.05 (Dataset S5). These 184 metabolites (75 elevated and 109 reduced in tumors) are shown in Fig. 4A and listed in Dataset S5.

From the distribution of fold change, it was clear that metabolites related to glutathione were among the most altered (Fig. 4A). Both oxidized and reduced glutathione were strongly elevated in tumors relative to normals (Fig. 4B). Reduced glutathione showed a striking 209-fold increase and oxidized glutathione, which was not detected in normals, was reproducibly detected at high levels in tumors. The glutathione result, which is consistent with our RNA analysis (Fig. 3C), is unlikely a cell-of-origin artifact since glutathione levels are actually higher in the proximal compared with distal tubules (24). Using IHC, we validated in our ROGEN cohort that glutathione levels are increased in human proximal relative to distal tubules and that tumors show increased glutathione staining compared with distal tubules (Fig. 4C). Hence, abundant glutathione levels are a genuinely acquired feature of RO.

This marked and reliable up-regulation of glutathione prompted us to examine other metabolites related to glutathione metabolism (Fig. 4B). Cystine, a precursor for cysteine, which is the rate-limiting substrate in glutathione synthesis (22), was one of the most decreased metabolites with an ~18-fold reduction in tumors (Fig. 4A and B). Taurine, a byproduct of cysteine
Fig. 3. Analysis of RO transcriptional profiles with respect to cell of origin. (A) Heuristic method to spotlight genes reliably up or down in RO relative to normal distal tubules using two publicly available datasets. Transcripts from the rat nephron RNA-seq atlas were scored as distal (P=D), ambiguous (P?D), or proximal (P?D). Transcripts from RO versus normal kidney RNA-seq were scored as down in tumor (T<N), ambiguous (T?N), or up in tumor (T>N). Intersecting these datasets yields n = 11,835 transcripts that are common to both datasets and can be organized on a 3 x 3 grid. See legend for interpretation of each box by color. (B) List of 24 genes reliably up (T>N, P=D) in RO. (C) Schematic for glutathione metabolism with transsulfuration, glutathione synthesis, glutathione degradation, and the γ-glutamyl cycle. The γ-glutamyl cycle includes all steps except for the transsulfuration reactions. Each gene symbol is color coded by the scheme in A.

degradation, was decreased in tumors, suggesting the drop in cystine was more likely due to utilization rather than degradation. Glycine, another metabolite involved in glutathione biosynthesis, was also significantly lower in tumors (Fig. 4B).

Based on the transcriptional changes in glutathione synthesis and degradation (increased GCLC and decreased DPEP1; Fig. 3C), we predicted an elevation in the dipeptides γ-glutamyl-cystine (γGluCys, the product of GCLC) and cysteinyl-glycine

Fig. 4. Metabolic profiles of RO. (A) Distribution of fold change of 184 metabolites with significant differential abundance (nominal P value <0.05) in our second RO cohort of fresh frozen tumors (n = 10) versus normal kidney (n = 7). The 10 most elevated and reduced metabolites in tumors are labeled. (B) Relative abundance of metabolites related to glutathione metabolism are shown for normals and tumors. (C) Representative glutathione IHC sample at the interface of tumor with normal kidney (indicated by brackets) from our cohort (n=17,144 genes) (Lee et al. 2005). (D) Transcripts from RO versus normal kidney RNA-seq were scored as down in tumor (T<N), ambiguous (T?N), or up in tumor (T>N) versus normal kidney (n=9,542 genes) (Durinck et al. 2010). Intersecting these datasets yields n = 11,835 transcripts that are common to both datasets and can be organized on a 3 x 3 grid. See legend for interpretation of each box by color. (B) List of 24 genes reliably up (T>N, P=D) in RO. (C) Schematic for glutathione metabolism with transsulfuration, glutathione synthesis, glutathione degradation, and the γ-glutamyl cycle. The γ-glutamyl cycle includes all steps except for the transsulfuration reactions. Each gene symbol is color coded by the scheme in A.

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samples harbor complex I mutations, only a subset harbor disorders (35). Why germ-line, complex I dysfunction leads to a G can act like a tumor suppressor a cycle coordinating Gopal et al. Drosophila γ RO reveals recurrent complex I loss to be an early and likely driver event that is accompanied by frequent loss of chromosome 1, cyclin D1 overexpression, and increased glutathione.

Our work points to complex I loss being the earliest detected genetic alteration that subsequently undergoes positive selection, a feature of a driver event. Supporting this claim are the high prevalence across two independent cohorts of recurrent, high VAF complex I LoF mutations. The high VAF contrasts a pan-cancer analysis showing selection against deleterious mtDNA mutations (15) and argues for positive selection in RO similar to the tissue-specific enrichment of protein-altering mtDNA variants in a more recent pan-cancer analysis (25). The high VAF mutations are unlikely to occur by simple drift, as we do not see such mtDNA mutations in genes encoding subunits of complexes III–V. However, we cannot exclude that disruptive mutations in these downstream complexes may be incompatible with cell viability, while complex I loss may be tolerated as it sits at a branch point for entry into the electron transport chain. Finally, although the disruptive complex I mutations described here have features of candidate driver events, the completely benign nature of RO suggests that these complex I events correlate with a notable lack of aggressive tumor behavior.

The recurrent chromosome 1 loss and cyclin D1 overexpression appear to be later genetic events that may complement complex I loss. Our assertion is supported by the fact that while all 19/19 RO samples harbor complex I mutations, only a subset harbor chromosome 1 and cyclin D1 changes. Moreover, in RO, all samples with chromosome 1 loss occur in complex I mutant samples. In Drosophila, complex I dysfunction leads to a G1–S cell-cycle checkpoint as part of a retrograde response triggered by increased reactive oxygen species (26). Forced expression of cyclin D1 could provide a mechanism to overcome this mitochondrial cell-cycle checkpoint. Loss of chromosome 1 is a frequent event in cancer that is thought to indicate the presence of an important tumor suppressor in this region (27). Therefore, we speculate that alterations in either chromosome 1 or cyclin D1 help to further enable tumor formation following complex I loss.

Collectively, our work suggests that complex I—whose classical role is in energy metabolism—can act like a tumor suppressor (10, 28). Consistent with this notion, cellular models with impaired complex I show potentiation of tumor growth (29) and diminished apoptosis (30). A recent study identified the mitochondrial protein LACTB as a tumor suppressor (31), which is notable since phylogenetic profiling and biochemical studies from our laboratory showed that LACTB is required for complex I activity (32). Although the presence of recurrent inactivating mutations at the same mtDNA position is unusual for a tumor suppressor, such homopolymeric hotspots for indels have been reported in cancer (33). Moreover, homopolymeric frameshifts allow for rapid gene inactivation and adaptation in bacteria, raising the possibility that a similar process occurs in the mtDNA of RO (34). Paradoxically, while the inactivating LoF complex I mutations reported here are enriched in RO, inherited mtDNA missense mutations in complex I can lead to rare, degenerative respiratory chain disorders (35). Why germ-line complex I loss leads to encephalomyopathy while its tissue-specific somatic loss is observed in cancer remains an important and unresolved problem.

The current study, employing RNA-seq and metabolomics, demonstrates marked differential expression for genes and metabolites involved in the γ-glutamyl cycle. By accounting for cell-of-origin effects, we uncovered marked up-regulation of transcripts involved in glutathione synthesis, including the rate-limiting enzyme GCLC, and down-regulation, or maintained absence of expression, of genes involved in glutathione degradation. These gene expression alterations create an imbalance in the γ-glutamyl cycle that would favor glutathione production. This assertion is supported by our metabolomics revealing a striking increase in glutathione, a finding corroborated by an independent report showing a similar rise in glutathione in RO that was published since the submission of this manuscript (36). We utilized IHC to validate that the increased glutathione is not due to a cell-of-origin effect, hence confirming that glutathione metabolism is authentically rewired in RO.

It is notable that the mammalian retrograde response to damaged mitochondria is characterized by increased trans-sulfuration, redirecting homocysteine toward glutathione synthesis (37). The altered flux to glutathione as part of this retrograde response fits with our finding that inhibition of glutathione biosynthesis causes a pronounced growth defect when complex I is disrupted and suggests that this response is likely adaptive. Why increased biosynthesis of glutathione is adaptive in the face of complex I loss is not known. One possibility is that flux through the pathway is critical, while another possibility is that the biosynthetic products aid in defending against oxidative stress (26). Emerging evidence points toward oncogenic pathways, such as activated PI(3)K/AKT, up-regulating glutathione to cope with oxidative stress and confer a growth advantage (38). The increased γGluCys and CysGly highlight a prominent change in the γ-glutamyl cycle—a cycle coordinating the intracellular and extracellular recycling of glutathione and its constituent amino acids (39). While the elevated CysGly levels suggest impaired glutathione degradation, this dipeptide may also provide a source of cysteine, the rate-limiting substrate for glutathione, in tumors (40). Thus, loss of complex I may create a metabolic response, punctuated by a dramatic increase in glutathione, that supports cell growth analogous to TCA cycle mutant tumors (41).

Our study underscores the importance of accounting for cell of origin in molecular analyses of renal tumors. Our current heuristic approach is simple and conservative; however, there are important limitations such as the inclusive grouping of all distal nephron sites together when RO may derive from a particular distal segment. Although new computational methods are now available for deconvoluting gene expression profiles from cellular mixtures, these methods make assumptions about the linearity of gene expression and rely on the fidelity of reference expression profiles (42). To our knowledge, an RNA-seq atlas of human microdissected kidney and matched whole kidney is currently not available. In the future, as atlases of single human cells become richer and the true cell of origin of RO is experimentally verified, it may be possible to perform more principled...
gene expression analysis to expose additional molecular changes authentic to these unusual tumors.

Materials and Methods
We provide a brief description of methods below. Full details are available in SI Appendix, Supplementary Materials and Methods.

Sample Acquisition. Specimens were obtained with Partners Human Research Committee institutional review board (IRB) approval (protocol no. 2011P000023) of RO and matched normal kidney from patients undergoing surgical resection. DNA for sequencing was isolated from n = 19 FFPE RO samples with matched normal kidney (RO–control). A second cohort of fresh frozen specimens of RO with matched normal kidney whenever possible was also obtained under the same IRB approval. All samples from both cohorts have been deidentified.

Whole Exome Sequencing and Analysis. Exomes were sequenced at the Broad Institute using Illumina SureSelect with paired 75 bp base-pair reads. A BAM file was produced using the Picard pipeline (broadinstitute.github.io/picard), which aligns tumor and normal sequences to the hg19 human genome build from raw illumina reads using the BWA aligner. BAM files were uploaded into the Firehose pipeline (archive.broadinstitute.org/cancer/cga/Firehose), which manages input and output files to be executed by GenePattern. Artifacts introduced by DNA oxidation during sequencing or from FFPE were computationally removed using a filter-based method (43). MuTect (version 1.1.6) was applied to identify somatic single-nucleotide variants (44). Arm-level somatic copy number alterations were analyzed by Alleic CapSeq, an updated iteration of HapSeq.

Analysis of mtDNA from Exomes. All WES reads mapping to GRCh37 chromosome MT or 1,049 nuclear sequences of mitochondrial origin (NUMTs) (45) were realigned to mtDNA revised Cambridge Reference Sequence (NC_012920 equivalent to GRCh37 chromosome MT) using GATK version 3.3 (46), BWA version 0.7.10 (47), and Picard Tools version 1.119 (broadinstitute.github.io/picard). Haplotyping variants were detected via the GATK HaplotypeCaller assuming a mixture of 100 chromosomes, which estimated VAF, equivalent to heteroplasmy in normal samples and in 100% pure tumor samples.

mtDNA Copy Number Analysis. Tumor and normal kidney DNA were analyzed by multiplex TaqMan real-time quantitative PCR per protocol (37). WES data were also used to calculate mtDNA copy number by estimating the ratio of reads aligning to the mtDNA and to the entire genome using samtools idxstats (48).

Tumor/Normal RNA-Seq Analysis. Paired-end RNA-seq fastq files for RO from a previously published study were obtained with permission from the European Genome-phenome Archive (12). Only the 12 pairs of matched tumornormal samples for which the mtDNA sequence was also analyzed were studied.

Cell-of-Origin Bias Correction. Genes were defined as being up (T>N) or down (T<N) in tumors if there was at least a twofold difference in expression with a Benjamini–Hochberg adjusted P value <1.0 × 10−5. All other genes were classified as having an unclear difference between tumors and normals (T7N). To account for cell-of-origin effects, an atlas of microdissected rat nephron segments from Lee et al. (21) was analyzed. Proximal tubules were defined as S1 proximal tubule (51), S2 proximal tubule (52), and S3 proximal tubule (33) segments while distal tubules were defined as cortical thick ascending limb, distal convoluted tubule, connecting tubule, and cortical collecting duct segments. A median proximal and distal value per gene was then calculated using the median RPKM values for the above segments. Genes were defined as being proximal (P>D) or distal (P<D) if there was at least a fivefold difference in median between the defined proximal and distal segments. All other genes were designated as having no, or unclear, difference between proximal and distal regions (P7D). The intersection of genes included in both datasets (Durinck et al. and Lee et al.), mapped by gene symbol, were then analyzed to look for reliable gene expression changes that were not due to a cell-of-origin bias.

Metabolite Profiling. Tissue pieces (17–35 mg) from fresh frozen tumor and normal kidney samples were homogenized in 4 μL of water per milligram of sample using a bead mill (TissueLyser II; Qiagen). Metabolite profiles were measured using a combination of four liquid chromatography-tandem mass spectrometry (LC-MS) methods. We applied two polar metabolite profiling methods and a lipid method similar to those described in Mascarenhas et al. (49).

Immunohistochemistry. Primary antibodies were as follows: P-AMPK (Cell Signaling 2535), HNF4α (Cell Signaling 1311), MTCO1 (Abcam ab18405), NDUFB8 (Abcam ab110246), CD117 (Cell marque catalog no. 117R-18), and cyclin D1 (Cell marque catalog no. 241R-18). For cell-of-origin interpretation, proximal tubules (including both the convoluted and straight segments, i.e., S1, S2, and S3 segments) were identified morphologically as tubules lining the simple columnar epithelium with abundant apical cytology, prominent brush borders, and often frothy tubular lumens. Distal tubules were identified by the presence of simple cuboidal epithelium that was variably attenuated.

Cell Growth Assay. HEK293T cells were grown in DMEM (11955; Thermo Fisher) supplemented with 10% FBS (Sigma), penicillin-streptomycin, and 50 μg/mL uridine at 37 °C and 5% CO2. HEK293T cells were plated into six-well plates and grown for 48 h in the presence of vehicle control (DMSO), BSO (100 μM; Sigma), piericidin A (1 μM; Santa Cruz), or a combination of BSO plus piericidin and cell number was then determined using a Coulter Counter.

Statistical Analysis. Data are reported as mean unless otherwise specified. For two-group comparisons, the two-sample Student’s t test was used unless otherwise indicated in the figure.

Data Availability. Somatic variants from exome sequencing, RNA-seq analysis with cell-of-origin interpretation, and metabolite profiling are made fully available in Datasets S1–S5.

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