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Title: Identification of a novel *RASD1* somatic mutation in a *USP8*-mutated corticotroph adenoma.

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ABSTRACT

Cushing's Disease is caused by pituitary corticotroph adenomas that secrete excess adrenocorticotropic hormone. In these tumors, somatic mutations in the gene *USP8* have been identified as recurrent and pathogenic and are the sole known molecular driver for Cushing's Disease. Although other somatic mutations were reported in these studies, their contribution to the pathogenesis of Cushing's Disease remains unexplored. No molecular drivers have been established for a large proportion of Cushing's Disease cases and tumor heterogeneity has not yet been investigated using genomics methods. Also, even in *USP8*-mutant tumors, a possibility may exist of additional contributing mutations, following a paradigm from other neoplasm types where multiple somatic alterations contribute to neoplastic transformation. The current study utilizes whole exome discovery sequencing on the Illumina platform, followed by targeted amplicon validation sequencing on the Pacific Biosciences platform, to interrogate the somatic mutation landscape in a corticotroph adenoma resected from a Cushing's Disease patient. In this *USP8*-mutated tumor, we identified an interesting somatic mutation in the gene *RASD1*, which is a component of the corticotropin-releasing hormone receptor signaling system. This finding may provide insight into a novel mechanism involving loss of feedback control to the corticotropin-releasing hormone receptor and subsequent deregulation of adrenocorticotropic hormone production in corticotroph tumors.

INTRODUCTION

Cushing's syndrome is caused by chronic exposure to elevated glucocorticoids via exogenous and endogenous sources. The typical clinical features of Cushing's syndrome are related to hypercortolism and include accumulation of central fat, moon facies, neuromuscular weakness, osteoporosis or bone fractures, metabolic complications, and mood changes. It is associated with increased morbidity and mortality especially due to cardiovascular disease (Lacroix et al. 2015; Sharma et al. 2015). Adrenocorticotropic hormone (ACTH)-dependent Cushing's accounts for 80% of endogenous cases, and among these, pituitary corticotroph adenomas are the most common cause (Lacroix et al. 2015). This is known as Cushing's Disease (CD). Corticotroph adenomas account for approximately 10% of pituitary adenomas and the vast majority of them are benign. The incidence of CD ranges from 1.2 to 2.4 per million population per year in Europe

and up to 8 per million population per year in the U.S.A. (Broder et al. 2015; Etxabe and Vazquez 1994; Feelders et al. 2012; Lindholm et al. 2001).

Genetic factors involved in corticotroph tumorigenesis are largely unknown. ACTH-secreting adenomas as a result of germline mutations can rarely arise in the context of familial disorders, such as multiple endocrine neoplasia type 1 (MEN1), familial isolated pituitary adenomas (associated with aryl-hydrocarbon receptor-interacting protein (gene *AIP*) mutations), and MEN4 (associated with cyclin-dependent kinase inhibitors) (Dworakowska and Grossman 2012; Perez-Rivas and Reincke 2016). Rare somatic mutations in the TP53 gene (Kawashima et al. 2009) and in the glucocorticoid receptor (gene *NR3C1*) and related proteins have also been reported (Lacroix et al. 2015; Ma et al. 2015; Reincke et al. 2015).

The identification of recurrent somatic mutations that lead to CD has been elusive until the recent discovery of somatic mutations in the ubiquitin-specific peptidase 8 gene (*USP8*) in 35-62% of CD-causing corticotroph adenomas (Perez-Rivas and Reincke 2016). The *USP8* gene encodes an enzyme with deubiquitinase activity. To date, 22 different *USP8* mutations have been identified in 129 ACTH-secreting adenomas from 271 patients across three studies (Ma et al. 2015; Perez-Rivas et al. 2015; Reincke et al. 2015) (reviewed in (Perez-Rivas and Reincke 2016)), with confirmation of *USP8* mutation prevalence in later studies (Hayashi et al. 2016; Song et al. 2016). All of these mutations were located in exon 14 in a mutation hotspot region that overlaps with the sequence that codes for the 14-3-3 binding motif which is highly conserved among different species. This mutation constitutively activates *USP8*, leading to enhanced recycling of the EGF receptor (gene *EGFR*) to the plasma membrane, resulting in sustained signaling and increased ACTH synthesis (Perez-Rivas and Reincke 2016).

The distinction between a tumor's monoclonal origin (where all tumor cells are descendants of a single cell where a driver mutation occurred) versus polyclonal origin (where tumor cells are a mixture of multiple clonal expansions, possibly containing different driver mutations) is important to understanding the disease mechanism. The clonal origin of a tumor can be determined by X-chromosomal inactivation analysis in female patients with heterozygous alleles at various X-linked loci (Levy 2001). Previous studies using this class of techniques have shown that all (Biller et al. 1992; Gicquel et al. 1992) or most (Herman et al. 1990; Schulte et al. 1991) corticotroph adenomas are monoclonal. However, arguments for polyclonality in a non-negligible fraction of corticotroph and other pituitary adenomas have been proposed (Clayton et al. 2000; Clayton and Farrell 2004; Clayton and Farrell 2001; Levy 2000; Levy 2001). Analysis of allelic fractions of somatic mutations and germline variants in tumor genomic sequencing data can potentially shed light on the genetic heterogeneity and clonal origin of corticotroph adenomas, but such analysis has not been carried out in previous studies where such data were available (Ma et al. 2015; Reincke et al. 2015).

Genome- and exome-wide analyses on DNA from tumors and patient-matched normal controls have been instrumental in identifying driver genes in many neoplasm types. In this study we performed whole exome sequencing (WES) on a tumor sample from a patient with CD, and on blood samples from the patient and her two healthy sisters (identical triplets; data from the two healthy sisters was

not used in this case report). We have identified a novel mutation in the GTP-binding site of the gene *RASD1* that we hypothesize contributes to the pathogenesis of CD in this patient due to the involvement of *RASD1* in regulation of ACTH production by glucocorticoid feedback. Additionally, based on the allelic fractions of the mutations, these tumor cells exhibit either sub-clones or polyclonal origin. These findings challenge the current model that corticotroph adenomas are genetically homogeneous.

RESULTS

Clinical presentation and family history

A 32-year-old woman with no known family history of pituitary diseases, who was one of identical triplet sisters, presented with a history of recently diagnosed Type 2 Diabetes Mellitus and weight gain, easy bruising, and subjective plethora. On review of systems she endorsed occasional acne, moodiness prior to her menstrual period, and chronic insomnia. She denied neuromuscular weakness, significant mood changes, difficulty concentrating, or hirsutism. Evaluation confirmed CD: 24-hour urinary free cortisol (UFC) levels were 154 and 90.4 µg (nl < 50 µg); midnight salivary cortisol (MSC) levels were 0.118, 0.142, and 0.917 µg/dL (nl < 0.112 µg/dL); serum cortisol was 4.1 µg/dL after 8 mg dexamethasone (nl < 1.8 µg/dL); random morning plasma ACTH level was 50 pg/mL; and pituitary MRI showed a clearly defined right-sided 4 mm lesion (Figure 1). She was also found to have dyslipidemia and fatty liver on MRI of the abdomen. She was diagnosed with CD and underwent transnasal transsphenoidal adenomectomy without complication. Immunohistochemistry confirmed a corticotroph adenoma (Figure 2) with a low MIB1 index. Pathologic examination of tissue slices estimated 40-50% tumor cellularity.

Genomic analyses

WES of the blood-derived normal DNA and FFPE-derived tumor DNA from the patient was carried out on the Illumina HiSeq 2500 platform, yielding mean sequencing depth of 145X and 315X, respectively, that was usable for variant calling (Table 1). Germline (constitutional) variants and somatic mutations were called; supporting BAM read alignments for each somatic mutation were manually reviewed in IGV (Robinson et al. 2011; Thorvaldsdóttir et al. 2013), yielding 36 passing calls whose class, type, and trinucleotide context are depicted in Figure 3 (the variant genomic coordinates are provided as Supplementary File 1). C>T transitions were the most common single-nucleotide variant (SNV) type, as is common in many cancer mutation signatures (Alexandrov et al. 2013) (though importantly, (Alexandrov et al. 2013) did not include mutations outside protein coding exons, which are included in Figure 3). Notably, the mutations were dominated by deletions, only 2 of which were in protein-coding regions. No insertions were observed (Table 2).

After variant annotation, 13 of 36 somatic mutation calls were predicted to alter the amino acid sequence of a protein isoform (Table 2). This count of 13

protein-altering mutations is consistent with the low counts previously reported in other WES studies of corticotroph adenomas (median 7, range 3-23 in (Reincke et al. 2015); median 5, range 1-9 in (Ma et al. 2015)). The difference with (Ma et al. 2015) can be explained by the higher sequencing depth in our study and differences in variant calling procedure; in (Ma et al. 2015), calls with allelic fraction of <20% were discarded, which would have eliminated most of our calls (Table 2). Validation was carried out on 12 of the 13 mutations via targeted amplicon sequencing on a second NGS platform (Pacific Biosciences RSII, PacBio), confirming 100% of the attempted mutations as somatic (present in tumor, absent in normal) and also obtaining similar allelic fractions to the original Illumina-based calls. Following the heuristic from (Alexandrov et al. 2013) that WES interrogates approximately 30 megabases (Mb) of protein-coding exons in the human genome, we obtained a somatic mutation rate of 0.43 mutations/Mb for protein-altering mutations and 0.53 mutations/Mb for mutations in coding exons (13 protein-altering, 3 synonymous). When the latter is compared to somatic mutation rates across many cancer types in (Alexandrov et al. 2013), the somatic mutation rate in our patient was low, a finding consistent with previous WES studies of corticotroph adenomas (Ma et al. 2015; Reincke et al. 2015).

The allelic fractions of somatic mutations ranged from 3% to 26%. Under the hypothesis that one of these mutations initiates or precedes neoplastic transformation of a single cell that then undergoes clonal expansion, the tumor purity of the sequenced specimen may therefore be less than 52%, consistent with the estimate of 40-50% from pathologic examination. Although the presence of copy-number alterations (CNAs) can confound such an estimate, we did not observe aneuploidy or CNA events that confound the allelic fractions in Table 2 (using the SAAS-CNV tool (Zhang and Hao 2015) as before (Uzilov et al. 2016); data not shown).

We cross-referenced our somatic mutation calls with the COSMIC database (Forbes et al. 2015) to determine if any had previously been observed in a tumor; no exact matches were found and one approximate match is noted in Table 2. We also cross-referenced these calls with ClinVar (Landrum et al. 2016), as some variants are known to occur as both somatic driver mutations and germline variants in inherited/familial neoplasm syndromes. We then reviewed the gene annotations in the list of 13 protein-altering mutations in the context of potential involvement in the molecular pathways implicated in CD or in corticotroph tissues in general. Notably, our patient's adenoma had the *USP8* p.P720R mutation that has previously been described as involved in the pathogenesis of CD (Ma et al. 2015; Reincke et al. 2015), occurring in 35-62% of CD-causing corticotroph adenomas causing CD (Perez-Rivas and Reincke 2016); this was also the sole mutation identified in the ClinVar cross-reference. The high allelic fraction of this mutation (20-22%, depending on sequencing platform; 3rd highest in the list) is consistent with the hypothesis that it is a driver mutation present early in the clonal expansion of the tumor.

A novel mutation, p.K34M, in the GTP-binding region of *RASD1*, was identified in this tumor, at an allelic fraction (3%) indicative of a subclone with respect to cells containing *USP8* p.P720R. *RASD1* was originally discovered as an

inducible gene in dexamethasone-stimulated AtT-20 mouse corticotroph cells (Kemppainen 1998). Based on this connection to the cell type under study, further computational analysis of the functional significance of this mutation was conducted. Alignment of several related small GTPases, including the well-studied oncogenes *KRAS*, *NRAS*, and *HRAS*, revealed that p.K34M is found within the G1 motif and is likely involved in binding to GTP (Figure 4). However, the precise contacts between the enzyme and substrate are not clear given that the G1 motif diverges in the RASD family from the other small GTPases for which substrate-bound crystal structures are available. Other genes in Table 2 were reviewed for possible connections to CD or corticotroph biology, but were not judged to have a direct connection like *USP8* or *RASD1*, hence these two genes are the focus of this report.

Treatment outcomes

Consistent with successful removal of the patient's ACTH-secreting tumor, her post-operative day 1 serum cortisol level was 3.4 μg/dL. She was discharged on physiologic oral hydrocortisone replacement and subsequently tapered off after four months. Following discontinuation of oral hydrocortisone, urinary free cortisol, 8 mg dexamethasone suppressed serum cortisol, and midnight salivary cortisol concentrations were all within the normal range, consistent with CD remission. CD symptoms and comorbidities, including blood glucose levels, also improved. She remains in remission 3.3 years after surgery.

DISCUSSION

Tumor genetic heterogeneity models

The current case identifies a novel *RASD1* mutation in a *USP8*-positive corticotroph adenoma. The different allelic fractions between the *USP8* and *RASD1* somatic mutations in the studied tumor cells suggest that this ACTH-secreting tumor is genetically heterogeneous. Two models for heterogeneity are proposed as follows. In Model A (Figure 5a), the *USP8* and *RASD1* mutations may be synergistic, with the *USP8* mutation occurring early in tumorigenesis, leading to abnormal proliferation of ACTH-secreting cells. At a later time point in the pathogenesis of the tumor, one of these *USP8*-mutant cells acquires a *RASD1* mutation, resulting in a subclone of *RASD1*-mutant/*USP8*-mutant cells. In Model B (Figure 5b), the *USP8* and *RASD1* mutations are mutually exclusive, giving rise to subclones of cells with different mutation combinations (i.e. *USP8*-mutant/*RASD1*-wildtype versus *USP8*-wildtype/*RASD1*-mutant). In both models, the tumor is genetically heterogeneous. Our hypothesis is that under both these models, *RASD1* is a contributor to cell proliferation and ACTH secretion, but occurs in a small sub-population of the tumor cells. While these findings do not clearly distinguish between monoclonal versus polyclonal origin of the tumor, they nevertheless indicate that the tumor is genetically heterogeneous and suggest further studies into the interplay between multiple possible drivers.

Rationale for *RASD1* as a contributor to pathogenesis

This study identified a mutation in *RASD1* that may alter binding to GTP on the basis of *RASD1* structural homology to well-studied, oncogenic small GTPases *KRAS*, *NRAS*, and *HRAS*. It is appealing to speculate that *RASD1* p.K34M will have a reduced affinity to GTP and will therefore be less active (decreased capacity to interact with downstream proteins) versus wildtype. However, due to the low allelic fraction (presumed subclonal nature) of this mutation, we cannot conclusively determine whether the mutation is homozygous or heterozygous. Several studies have defined a role for *RASD1* in inhibition of $G\alpha_s$ signaling (Graham et al. 2004; Graham et al. 2001). This may occur through an interaction with $G\alpha_i$ (Cismowski et al. 2000). Normal feedback regulation within the hypothalamic-pituitary-adrenal axis involves glucocorticoid induction of genes associated with suppression of corticotropin-releasing hormone receptor (CRHR) signaling. *RASD1* was identified by its virtue of being strongly induced by dexamethasone in mouse corticotroph cell lines and pituitaries (Brogan et al. 2001; Kemppainen 1998; Tu and Wu 1999). Taken together, *RASD1* may be a transcriptionally-inducible negative regulator of CRHR-$G\alpha_s$ signaling in corticotrophs. In cells with non-functional *RASD1*, this loop is no longer intact and CRHR signaling may become insensitive to negative feedback from glucocorticoids, thereby allowing for continued ACTH secretion. This alteration within corticotroph signaling and regulation may occur within the population of *USP8* mutant cells or in a separate subclone. Separate molecular signaling schemes for these situations are depicted in Figure 6, illustrating the effect on the promoter of the gene *POMC* (whose protein product is processed to become ACTH, which is then secreted). It is intriguing to consider the possibility that loss of *RASD1* function and mutation of *USP8* may be additive or synergistic in relation to the pathophysiology of CD (Figure 6d). However, since we did not carry out experimental validation of *RASD1* p.K34M function for this study, we must caution that our claims regarding its involvement in disease biology are only hypothetical and based mainly on what is known about *RASD1* biology from prior studies. Further studies should investigate the functional significance of this novel *RASD1* mutation in the pathogenesis of ACTH tumors.

METHODS

Whole exome sequencing (WES) and targeted validation

Paired-end (2x100) WES on Illumina HiSeq 2500 (Illumina, San Diego, CA) and targeted amplicon validation on PacBio RSII (Pacific Biosciences, Menlo Park, CA) was carried as previously described (Uzilov et al. 2016), with the following modifications. For WES, the SureSelect Human All Exon V5 hybridization capture system (Agilent, Santa Clara, CA) was used. Libraries from 3 tumor and 9 normal samples were multiplexed in a 2:1 tumor:normal ratio and sequenced on all 8 lanes of a High Output flowcell; only 2 of these samples are presented in this work (others to be published in a future work).

Variant calling

WES FASTQ files from the normal and tumor sample were combined into a patient-specific "cohort" and run through an in-house pipeline (Linderman et al. 2014) to yield BAM and VCF files with germline and somatic variant calls (SNVs and small indels). Briefly, this in-house pipeline implements Genome Analysis Toolkit (GATK) (McKenna et al. 2010) version 3.2 best practices for alignment, base quality recalibration, variant calling (using HaplotypeCaller), and variant quality score recalibration (VQSR) (Van der Auwera et al. 2013; DePristo et al. 2011). For read alignment, the hg19 human genome reference from UCSC (Rosenbloom et al. 2015) was used. VQSR was set to 99.5% sensitivity. Read pairs whose 5' coordinates were identical were marked (except for one read pair) as duplicates by the Picard software (http://broadinstitute.github.io/picard) and were not used for variant calling, per the above best practices, to ensure that evidence for each variant was derived from distinct DNA molecules, thus avoiding over-counting possibly over-amplified or over-sampled DNA. A GATK genomic interval list was created from the design file from the WES hybridization-capture kit manufacturer; sequencing depth (Table 1) was computed only within these genomic intervals, whereas variant calling was done within these genomic intervals padded by 100nt on both sides. For somatic variant calling, MuTect (Cibulskis et al. 2013) (version 1.1.6-10b1ba92, HC+PON mode with default settings, using COSMIC (Forbes et al. 2015) version 68, dbSNP (Sherry et al. 2001) version 138, and using variant calls from patient-matched normal control as the "panel of normals" setting) and Varscan2 (Koboldt et al. 2012) (version 2.3.5, with flags --tumor-purity 0.5 and --min-var-freq 0.07, then filtered using VS_SPVAL threshold of 20) were used.

SNV calls from Mutect (N=158) and indel calls from Varscan2 (N=97) were loaded into a custom MySQL (Percona MySQL Server Community Edition 5.6.14-rel62.0.483.rhel6) database schema using in-house scripts and annotated using RVS (Hakenberg et al. 2016) (SnpEff 4.0b (Cingolani et al. 2012) using the Ensembl (Aken et al. 2016) version 75 / GRCh37 resource bundle). Somatic calls whose population allele frequency in ExAC (Lek et al. 2016) exceeded 1% were discarded on the presumption that they are any combination of: contamination, a variant present but missed in normal sample, a low-level artifact, could not be pathogenic because it was too common in general population. All remaining SNV (N=152) and indel (N=64) calls were manually reviewed in IGV (Robinson et al. 2011; Thorvaldsdóttir et al. 2013) and the UCSC Genome Browser (Rosenbloom et al. 2015) to inspect supporting alignment quality in the BAM files and mapability of the genomic region in the hg19 human genome assembly, paying attention to whether a variant call was located in a short tandem repeat or a low-complexity sequence region (Benson 1999), a region with self-homology/duplication in the reference genome, or a region of low alignability according to the GEM track from ENCODE/CRG (Derrien et al. 2012). Uncertain calls, many of which were due to a low-level C>A substitution artifact also present in the normal or due to artifacts in padding regions, were manually rejected at this step, resulting in a final list of 25 SNV and 11 indel calls which are shown in Figure 3, the protein-altering subset of which is shown in Table 2.

Protein sequences and multiple sequence alignment

Protein sequences are from UniProt (Bateman et al. 2015) (retrieved 2016-09-23); only human sequences selected by UniProtKB curators as canonical protein isoforms were used. HGNC gene symbols, UniProt accessions/isoform identifiers, and RefSeq accessions are as follows:
RASD1, Q9Y272,-1 NP_057168.1
RASD2, Q96D21-1, NP_055125.2
DIRAS1, O95057-1, NP_660156.1
DIRAS2, Q96HU8-1, NP_060064.2
NRAS, P01111-1, NP_002515.1
HRAS, P01112-1, NP_005334.1 and NP_001123914.1
KRAS, P01116-1, NP_203524.1

The multiple sequence alignment for Figure 4 was made using the EMBL-EBI Clustal Omega web tool (Goujon et al. 2010; Sievers et al. 2011) (http://www.ebi.ac.uk/Tools/msa/clustalo/, used 2016-09-24, default settings) and edited using Unipro UGENE v1.24.2 (Okonechnikov et al. 2012). Protein sequence identity of the RASD subfamily was determined by *blastp* of *RASD1* and *RASD2* against all human proteins in the RefSeq protein database (Altschul et al. 2005; Altschul et al. 1997) (http://blast.ncbi.nlm.nih.gov, used 2016-09-25).

ADDITIONAL INFORMATION

Data Deposition and Access

All somatic mutation calls passing manual review (including those predicted to not alter protein) are provided as Supplementary File 1 and have been submitted (COSP42647) to the COSMIC database (Forbes et al. 2015). Consent could not be obtained for public release of raw sequencing data.

Ethics Statement

The study was approved by the Institutional Review Board at the Mount Sinai Medical Center. The patient gave written informed consent before participation, including permission to publish the results.

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Author Contributions

AVU, MYF, CP, and ASM carried out the data processing and analysis. AVU, KCC, MYF, and EBG wrote the manuscript. KCC, YL, CYL, MZ, and AG carried out clinical and research coordination. LCN and GD carried out the PacBio validation

work. MH and MF carried out the histopathology work. YKi and MJD carried out tissue processing at the ISMMS Biorepository. KDP carried out surgical resection. YKa, MM, and RS directed the sequencing operations. DS, EES, RS, RC, and EBG directed the study. All authors read and approved the manuscript.

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Disclosure

Yelena Lalazar is presently an employee of Novartis Pharmaceuticals Corporation. She was employed by Mount Sinai during the course of the work for this manuscript.

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FIGURE AND TABLE LEGENDS

Figure 1

Pituitary MRI coronal image confirmed a right sided sellar hypointensity consistent with a 4 mm pituitary adenoma.

Figure 2

Histologic sections revealed a monotonous tumor composed of medium-sized cells with finely stippled chromatin (A,B). Tumor cells were strongly positive for ACTH by immunohistochemistry (C), while a reticulin stain (D) showed effacement of the fibrovascular septae. (A) H&E 100X; (B) H&E 400X; (C) ACTH immunostain, 100X; (D) reticulin, 100X.

Table 1

Sequencing and alignment statistics for Illumina WES of the patient's normal/tumor pair, as given by GATK v3.2 (Van der Auwera et al. 2013; DePristo et al. 2011; McKenna et al. 2010) and Picard (http://broadinstitute.github.io/picard) pipelines. Picard terminology is as defined on https://broadinstitute.github.io/picard/picard-metric-definitions.html, with the Picard output field name given in parentheses. "Duplication" includes both optical/sequencing and PCR duplicates according to Picard and is computed on the entire genome (i.e. not just in exome target regions). "Usable" read bases are bases aligned to exome target regions and not in duplicate reads.

Figure 3

(A) Distribution of the 36 somatic mutation calls passing manual review by type and class. All passing calls were included regardless of predicted impact (i.e. synonymous, intronic, and intergenic calls are included). For SNVs, type is given by the pyrimidine of the mutated base pair as per previous conventions (Alexandrov et al. 2013). (B) SNVs from (A) were further categorized into 96 trinucleotide classes defined by the immediately flanking bases (as previously described (Alexandrov et al. 2013)).

Table 2

Somatic mutations in the patient's tumor that were predicted to alter the protein sequence (SNVs: missense, nonsense, affecting canonical splice site; indels: affecting coding exon, affecting canonical splice site), ordered by decreasing tumor allelic fraction of the alternate (non-reference) allele in the Illumina WES data (ILMN). Read depth statistics are also shown for validation of mutations by targeted amplicon sequencing on PacBio (PB). Some amplicons yielded inconclusive evidence during the first PB sequencing run, and thus starred (*) data are from a second run where multiplexing was adjusted to yield higher depth for the given amplicons. Gene symbols and names are from HGNC (Gray et al. 2015) (retrieved 2016-05-25). Gene IDs are from NCBI Gene (http://www.ncbi.nlm.nih.gov/gene, retrieved 2016-05-25). Mutations are given according to HGVS nomenclature (den Dunnen et al. 2016) version 15.11. Unless otherwise noted, amino acid numbering is from all canonical isoforms based on review of all RefSeq (Pruitt et al. 2014) and UniProtKB (Bateman et al. 2015) isoforms at the given genomic location in the UCSC Genome Browser (Kent et al. 2002) (retrieved 2016-05-25). Predicted amino acid change and effect are from SnpEff version 4.0b (Cingolani et al. 2012) using Sequence Ontology terms (Cunningham et al. 2015). For ILMN data, read counts for total reads and reads supporting alternate allele are taken directly from the respective variant caller (MuTect (Cibulskis et al. 2013) for SNVs, Varscan2 (Koboldt et al. 2012) for indels).

Figure 4

Multiple sequence alignment of select proteins in the Ras family of small monomeric GTPases to which *RASD1* belongs (Wennerberg et al. 2005). Indicated motifs (taken from (Wennerberg et al. 2005) and (Bourne et al. 1991), then verified via UniProt (Bateman et al. 2015), retrieved 2016-09-24): GDP/GTP-binding G-box motifs (G1 through G5); effector region; C*aa*X N-terminal motif that undergoes post-translational modification (*a* denotes any aliphatic amino acid). *RASD2*, encoding the protein Rhes, is shown because it is the closest human homolog to *RASD1* (63% protein sequence identity); the two form the RASD subfamily that is distinct from other Ras family proteins (<45% protein sequence identity). *DIRAS1* and *DIRAS2* are shown because they are the next closest homologs to RASD family proteins. *NRAS*, *HRAS*, and *KRAS* are shown because they are well-characterized oncogenes. Functional impact of *RASD1* mutations in red has been demonstrated experimentally: p.G31V (Cismowski et al. 2000; Cismowski et al. 1999; Vaidyanathan et al. 2004), p.G36V (Cismowski et al. 1999), p.A178V (Graham et al.

2001), p.C278S (Graham et al. 2001; Vaidyanathan et al. 2004). Mutations in *NRAS*, *HRAS*, and *KRAS* (in red) are widely known oncogenic mutations and are also recurrent somatic mutations across multiple neoplasm types in COSMIC (Forbes et al. 2015) (accessed 2016-09-25), except for *HRAS* amino acid A146 (no mutations of any type in COSMIC, although p.A146V results in constitutive activation (Feig and Cooper 1988) and may be germline pathogenic in Costello syndrome, ClinVar accession RCV000013445.18) and *NRAS* amino acid K117 (no mutations of any type in COSMIC and no published evidence on any K117 mutation). Amino acid ranges are given in parentheses next to gene symbols. Amino acids are color-coded according to biochemical class (yellow: nonpolar; green: polar; blue: basic; pink: acidic). For positions where one of several amino acids is possible, the possibilities are given in brackets. X: any amino acid; LOF: loss of function; GOF: gain of function.

Figure 5

Two models that explain the observed allelic fractions of the *USP8* and *RASD1* somatic mutations. (A) Mutation *RASD1* p.K34M occurs in a single cell derived from the clonal expansion of *USP8*-mutant cells. (B) Mutation *RASD1* p.K34M occurs in an independent, *USP8*-wildtype cell and undergoes clonal expansion separate from the *USP8*-mutant clonal expansion.

Figure 6

Hypothesized altered feedback control of the *POMC* gene promoter in cells having *USP8* or *RASD1* mutations (mut) versus wildtype (wt). Contributing flux through pathway components and the effect of *POMC* transcription are shown in cartoon form as small/medium/large arrow thicknesses. Pathway diagram is based on (Jenks 2009). (A) Signaling through the CRHR in the context of intact feedback inhibition as indicated by active *RASD1* and $G\alpha_i$ (red) allows for coordinated biosynthesis and secretion of ACTH. (B) *USP8* mutation (green) allows for enhanced activity of the EGFR-recycling apparatus and thereby triggers stronger positive regulation of ACTH production. (C) Signaling through the CRHR in the context of disrupted feedback inhibition as indicated by *RASD1* and $G\alpha_i$ (white and disconnected from $G\alpha_s$) may allow for dysregulated and increased secretion of ACTH. (D) Signaling schematic in the context of both *USP8* and *RASD1* mutation, showing the possible additive or synergistic effects downstream of ERK. AC: adenylyl cyclase; Ca^{2+}: calcium; CaMKII: calmodulin-dependent protein kinase II; cAMP: cyclic adenosine monophosphate; CRH: corticotropin releasing hormone; CRHR: corticotropin releasing hormone receptor; ERK: extracellular signal–regulated kinase; PKA: protein kinase A; *POMC*: pro-opiomelanocortin gene; VDCC: voltage-dependent calcium channel.

FIGURES AND TABLES

Figure 1

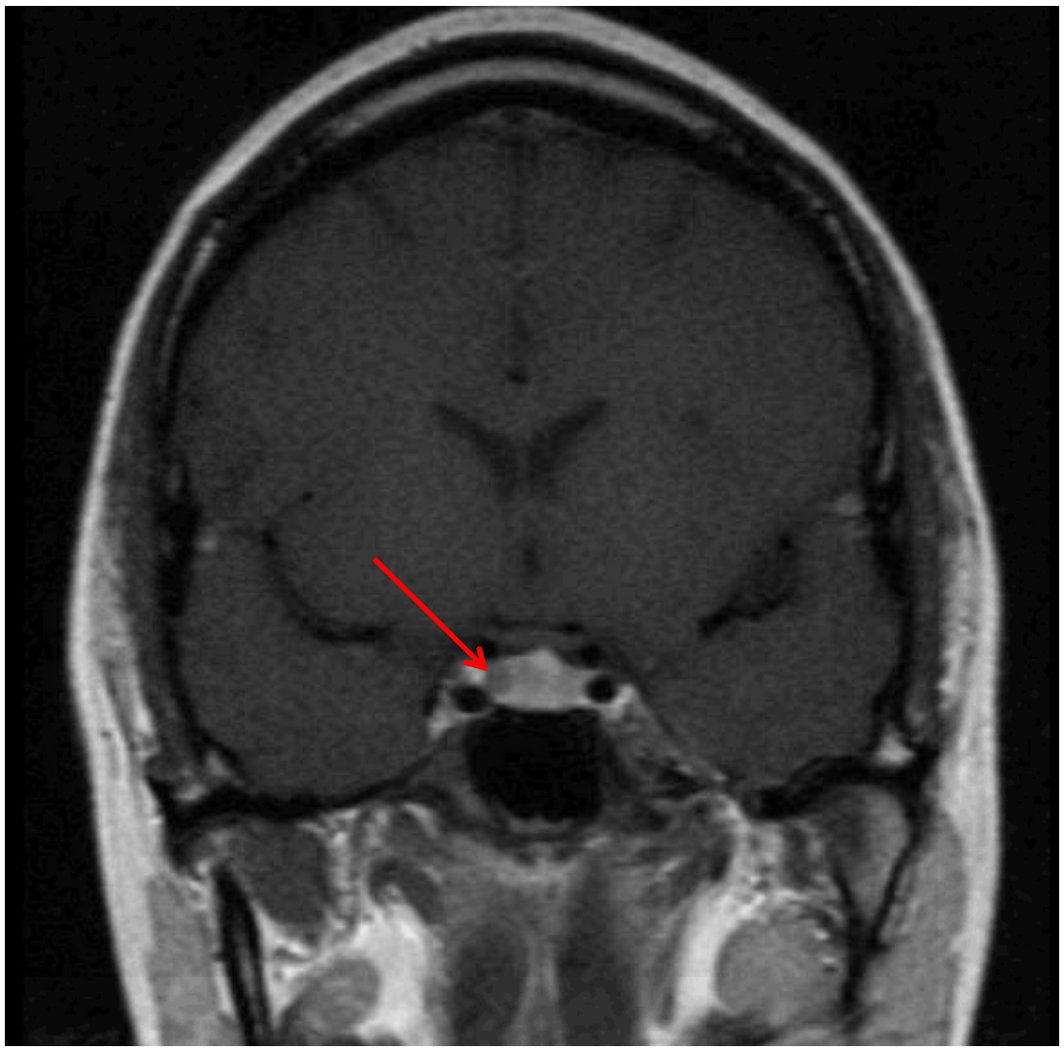
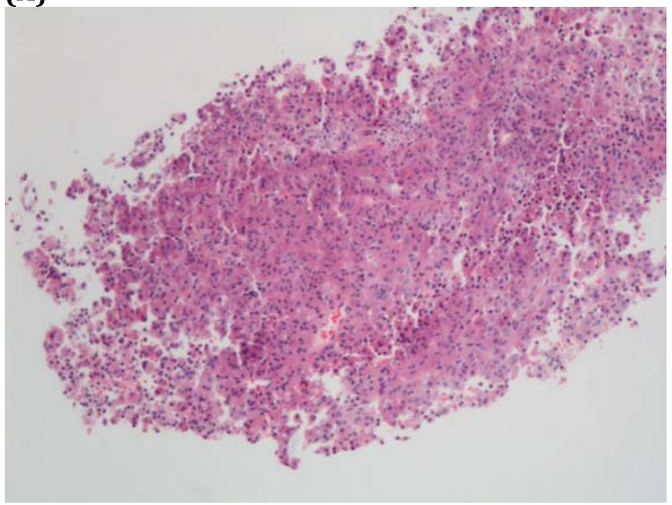


Figure 2

(A)



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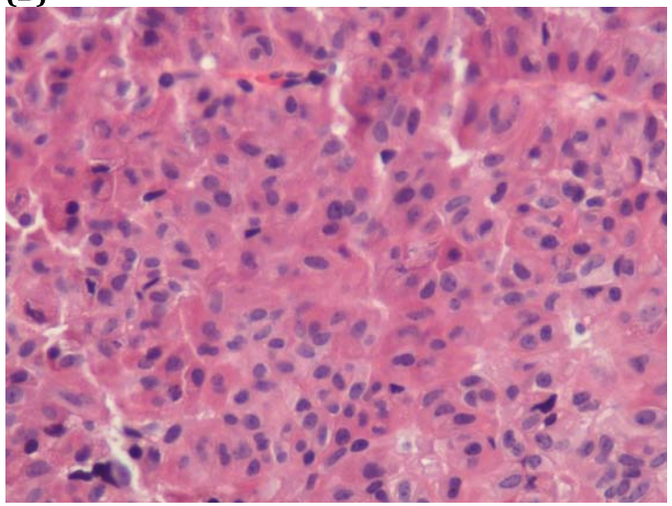

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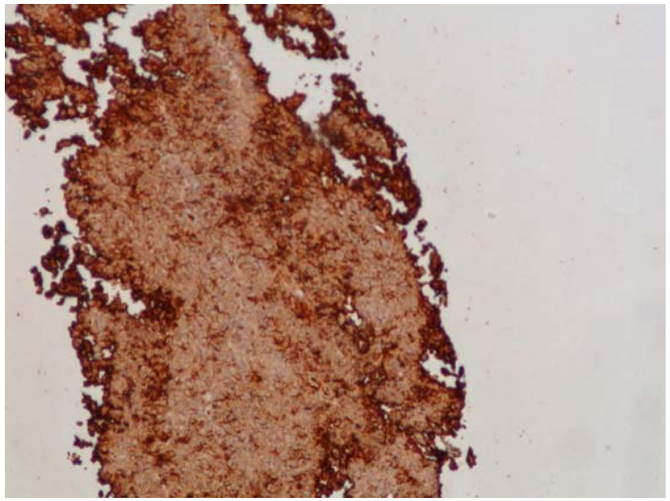


(D)

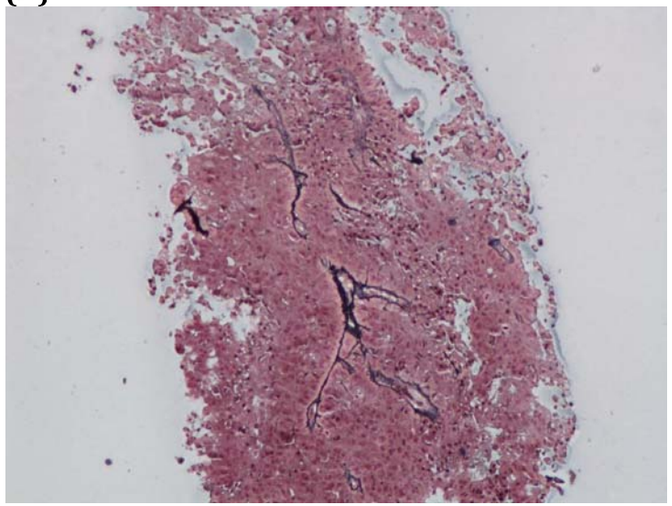


Table 1

Sample	**Number of clusters yielding "pass filter" read pairs** (PF_READS/2)	**Mean usable sequencing depth**	**Percent target bases with >30X usable sequencing depth**	**Percent usable bases out of all "pass filter" read bases** (PCT_USABLE_BASES_ON_TARGET)	**Percent duplication** (PERCENT_DUPLICATION)
normal	77×10^6	145X	96	74	10
tumor	335×10^6	315X	98	37	54

Figure 3

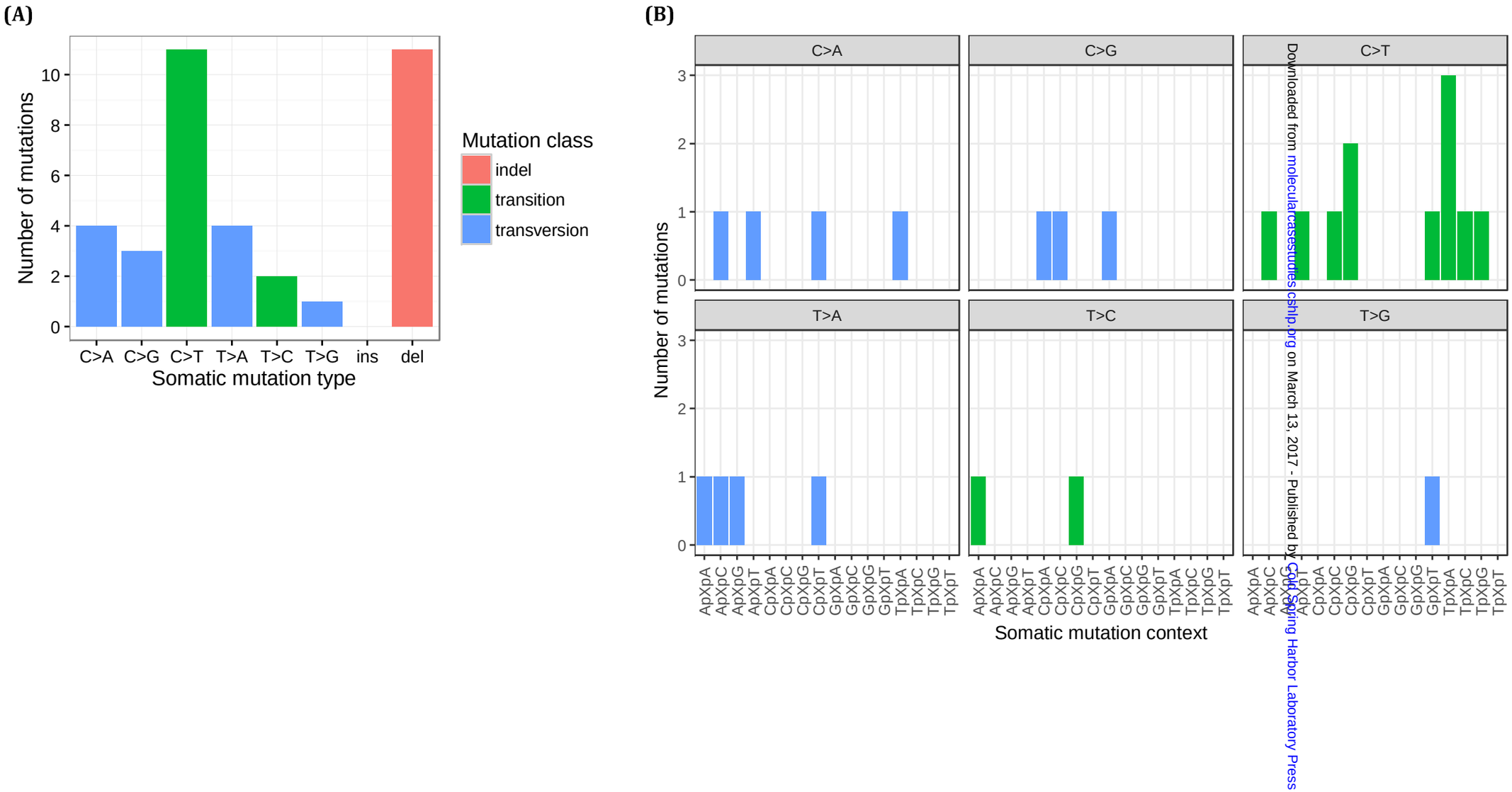


Table 2

									Tumor						Normal				
									Alt Allele Fraction (%)		ILMN		PB		ILMN		PB		
Gene Symbol	Gene Name	Gene ID	Chromosome	Mutation in DNA	Mutation in Protein	Variant Type	Predicted Effect	dbSNP ID	ILMN	PB	Total Reads	Alt Allele Reads	Total Reads	Alt Allele Reads	Total Reads	Alt Allele Reads	Total Reads	Alt Allele Reads	Comments
AIG1	androgen-induced 1	51390	6	NC_000006.11:g.143656205A>T	p.(Met239Leu)	SNV	missense_variant		26	21*	338	88	8,182*	1,730*	148	0	953	0	protein is predicted to change only in transcript isoform ENST00000275235 (intronic in all Refseq and Uniprot isoforms)
ATP8A2	ATPase phospholipid transporting 8A2	51761	13	NC_000013.10:g.26138117A>T	p.(Asp434Val), p.(Asp474Val)	SNV	missense_variant		24	23*	649	158	8,158*	1,874*	333	0	709	1	
USP8	ubiquitin specific peptidase 8	9101	15	NC_000015.9:g.50782647C>G	p.(Pro720Arg), p.(Pro614Arg)	SNV	missense_variant	rs672601311	22	20*	333	72	7,044*	1,417*	141	0	694	0	pathogenic mechanism previously described (Ma et al. 2015; Reincke et al. 2015); ClinVar accession RCV000149420.1
FRYL	FRY like transcription coactivator	285527	4	NC_000004.11:g.48529993G>C	p.(Pro2379Ala)	SNV	missense_variant		20	5	81	16	757	38	29	0	523	0	
PRPF18	pre-mRNA processing factor 18	8559	10	NC_000010.10:g.13642266A>G	p.(Gln56Arg)	SNV	missense_variant		19	15	471	89	1,288	190	169	0	1,090	1	
LGI3	leucine-rich repeat LGI family member 3	203190	8	NC_000008.10:g.22006350G>C	p.(Gln324Glu)	SNV	missense_variant		9	9	235	20	411	38	90	0	464	0	
MINK1	misshapen like kinase 1	50488	17	NC_000017.10:g.4795451AGAG>A	p.(Arg671del)	deletion	inframe_deletion		8	8	310	26	4,602	367	131	1	459	2	
PPFIBP2	PPFIA binding protein 2	8495	11	NC_000011.9:g.7661089C>A	p.(Leu455Met)	SNV	missense_variant		7	2	154	11	518	9	120	0	563	0	
SLFN12	schlafen family member 12	55106	17	NC_000017.10:g.33749940CA>C	p.(Leu36Argfs*6)	deletion	frameshift_variant		6	nd	501	28	nd	nd	385	1	nd	nd	did not attempt PB validation
PPEF1	protein phosphatase with EF-hand domain 1	5475	X	NC_000023.10:g.18800487T>A	p.(Tyr243*)	SNV	stop_gained		5	7	152	7	667	45	87	0	415	0	region undergoes copy loss based on CNA analysis; gene has an intronic somatic SNV (NC_000023.10:g.18752019G>A) with alt allele fraction of 17%
MMP26	matrix metallopeptidase 26	56547	11	NC_000011.9:g.5012679G>A	p.(Gly183Glu)	SNV	missense_variant		4	3*	185	8	6,025*	188*	117	0	148	0	
RASD1	ras related dexamethasone induced 1	51655	17	NC_000017.10:g.17399395T>A	p.(Lys34Met)	SNV	missense_variant		3	3*	406	12	32,529*	923*	175	0	1,790	1	a different somatic mutation (p.K34R) has been observed at this position (COSMIC database mutation ID: COSM5385794)
DCHS2	dachsous cadherin-related 2	54798	4	NC_000004.11:g.155219314G>A	p.(Ser1596Leu)	SNV	missense_variant	rs747828053	3	20	557	19	576	118	430	0	588	0	

Figure 4

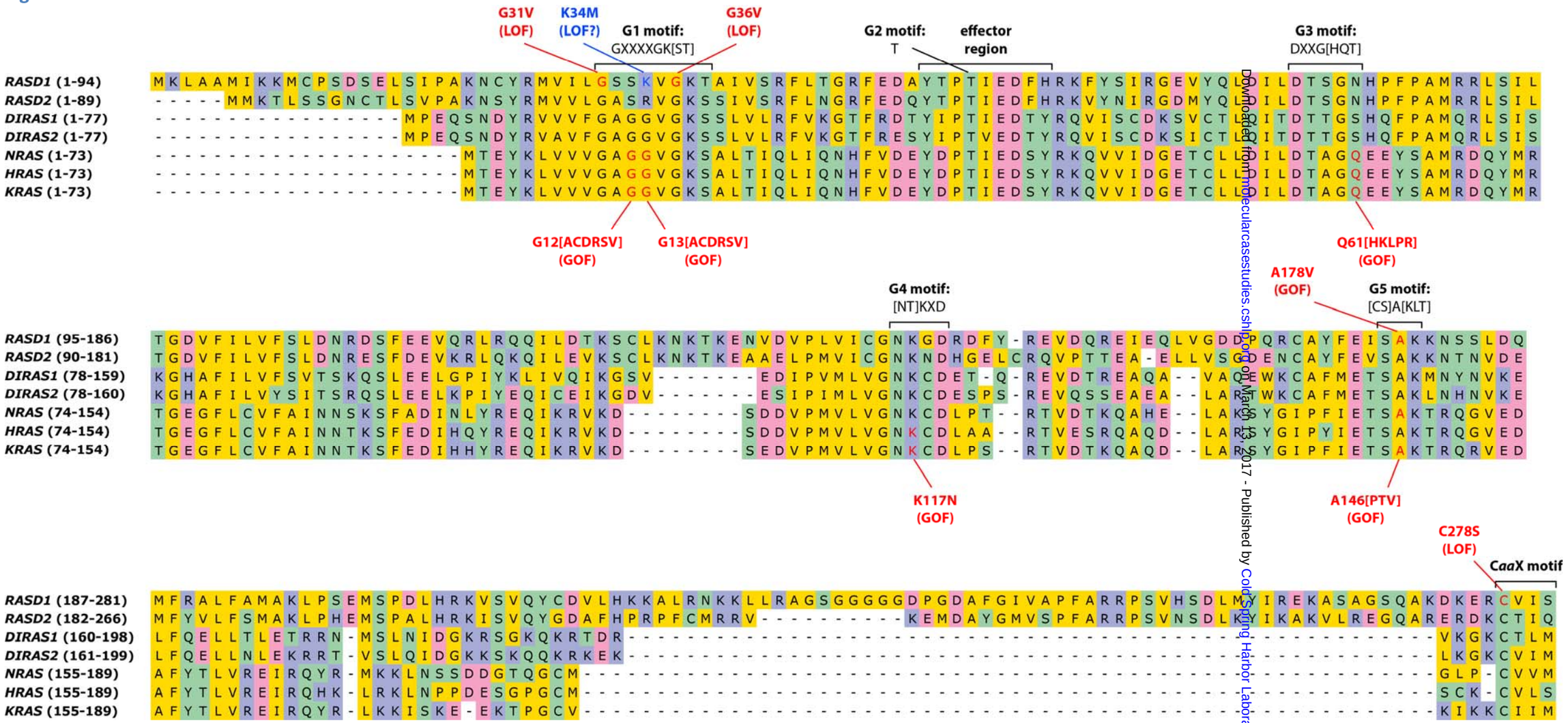


Figure 5

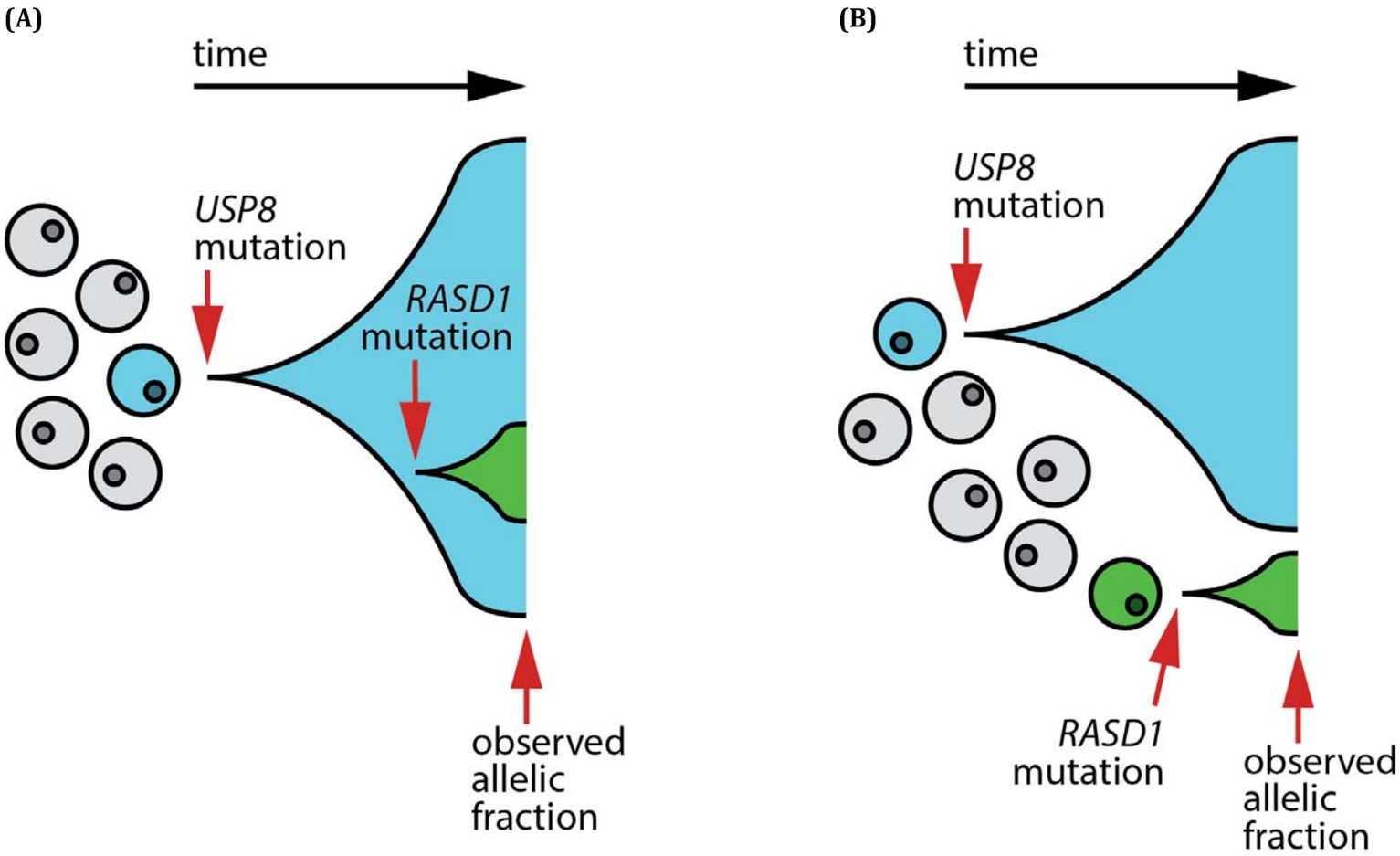
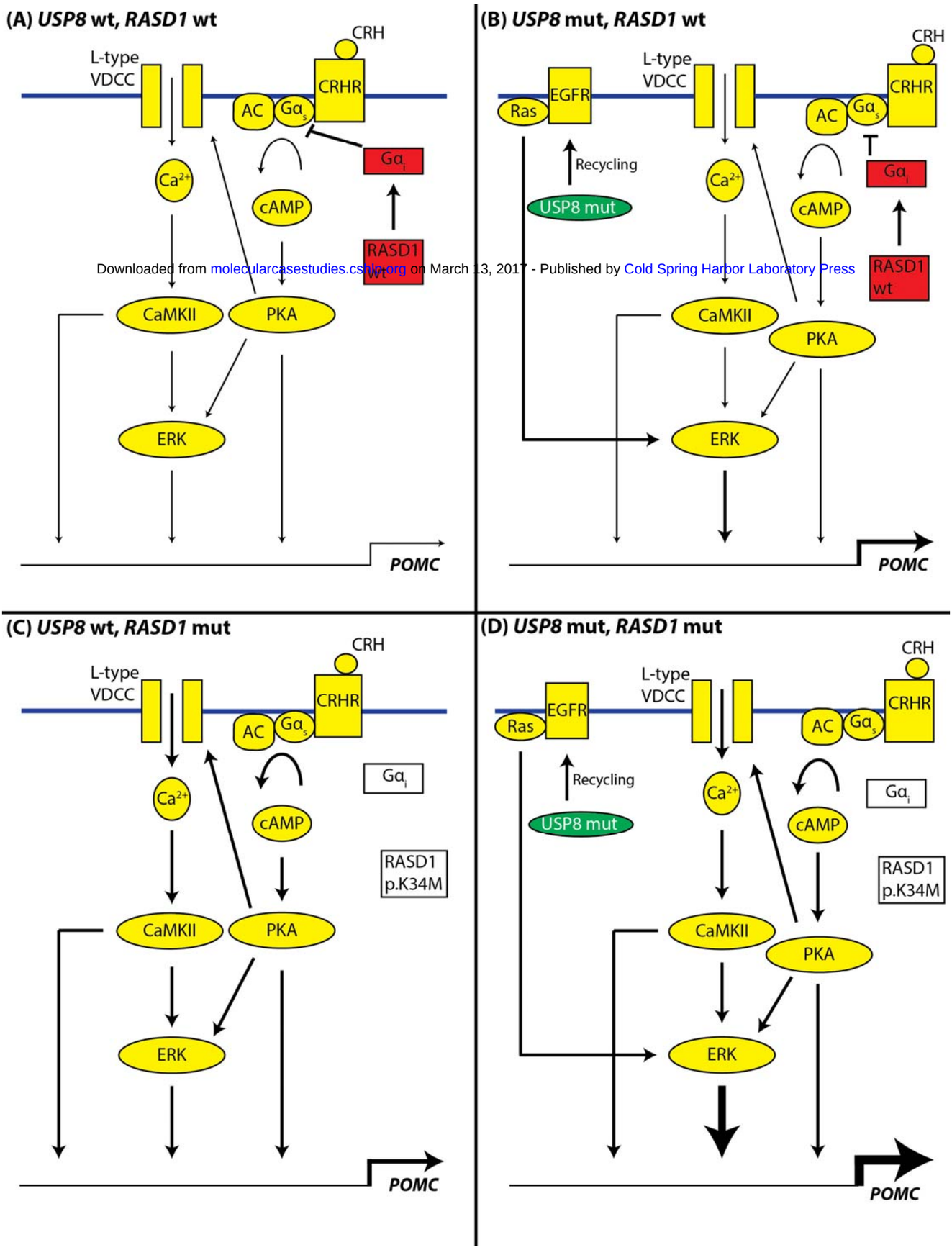


Figure 6



Supplementary Material

Supplementary File 1

Variant Call Format (VCF) file containing all 36 somatic mutation calls passing manual review. Column "ID" contains the reversible, globally unique identifier ("vkey") for the alternate allele, as used by RVS (Hakenberg et al. 2016) and DIVAS (Cheng et al. 2015) databases.

Identification of a novel RASD1 somatic mutation in a USP8-mutated corticotroph adenoma

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