Whole-Exome Sequencing Characterizes the Landscape of Somatic Mutations and Copy Number Alterations in Adrenocortical Carcinoma

C. Christofer Juhlin, Gerald Goh, James M. Healy, Annabelle L. Fonseca, Ute I. Scholl, Adam Stenman, John W. Kunstman, Taylor C. Brown, John D. Overton, Shrikant M. Mane, Carol Nelson-Williams, Martin Bäckdahl, Anna-Carinna Suttorp, Matthias Haase, Murim Choi, Joseph Schlessinger, David L. Rimm, Anders Höög, Manju L. Prasad, Reju Korah, Catharina Larsson, Richard P. Lifton, and Tobias Carling

Context: Adrenocortical carcinoma (ACC) is a rare and lethal malignancy with a poorly defined etiology, and the molecular genetics of ACC are incompletely understood.

Objective: To utilize whole-exome sequencing for genetic characterization of the underlying somatic mutations and copy number alterations present in ACC.

Design: Screening for somatic mutation events and copy number alterations (CNAs) was performed by comparative analysis of tumors and matched normal samples from 41 patients with ACC.

Results: In total, 966 nonsynonymous somatic mutations were detected, including 40 tumors with a mean of 16 mutations per sample and one tumor with 314 mutations. Somatic mutations in ACC-associated genes included *TP53* (8/41 tumors, 19.5%) and *CTNNB1* (4/41, 9.8%). Genes with potential disease-causing mutations included *GNAS*, *NF2*, and *RB1*, and recurrently mutated genes with unknown roles in tumorigenesis comprised *CDC27*, *SCN7A*, and *SDK1*. Recurrent CNAs included amplification at 5p15.33 including *TERT* (6/41, 14.6%) and homozygous deletion at 22q12.1 including the Wnt repressors *ZNRF3* and *KREMEN1* (4/41 9.8% and 3/41, 7.3%, respectively). Somatic mutations in ACC-established genes and recurrent *ZNRF3* and *TERT* loci CNAs were mutually exclusive in the majority of cases. Moreover, gene ontology identified Wnt signaling as the most frequently mutated pathway in ACCs.

Conclusions: These findings highlight the importance of Wnt pathway dysregulation in ACC and corroborate the finding of homozygous deletion of Wnt repressors *ZNRF3* and *KREMEN1*. Overall, mutations in either *TP53* or *CTNNB1* as well as focal CNAs at the *ZNRF3* or *TERT* loci denote mutually exclusive events, suggesting separate mechanisms underlying the development of these tumors. **(*J Clin Endocrinol Metab* 100: E493–E502, 2015)**

Adrenocortical carcinoma (ACC) is a rare and highly aggressive disease, with a reported annual incidence of 0.5–2.0 cases per million (1). ACC can be part of rare hereditary syndromes including Beckwith-Wiedemann syndrome and Li-Fraumeni syndrome (LFS), but most cases of ACC are sporadic (1). The median age of diagnosis in the adult population is between 46 and 55 years, and women are more often affected (2). The 5-year survival rate for patients with ACC is 16–38% (3). Patients with ACC typically present because of hormone excess (40–60%) or due to loco-regional symptoms in cases with large tumors (2). As such, ACC is usually diagnosed with an endocrine workup along with cross-sectional imaging. The histopathological diagnosis of adrenal tumors re-

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* C.C.J., G.G., and J.M.H. contributed equally to the study.

Abbreviations: ACC, adrenocortical carcinoma; CNA, copy number alteration; COSMIC, Catalogue of Somatic Mutations in Cancer; DAPPLE, Disease Association Protein-Protein Link Evaluator; ENSAT, European Network for the Study of Adrenal Tumors; GISTIC, Genomic Identification of Significant Targets in Cancer; LFS, Li-Fraumeni syndrome; LOH, loss of heterozygosity; SNV, single nucleotide variant.

mains challenging, and the Weiss system that is most commonly used in assessment is subject to high interobserver variability (4).

The genetics underlying sporadic ACC are not clearly understood. Previously, somatic alterations of *TP53* have been described in 16–33% of all ACCs (5, 6), and loss of heterozygosity (LOH) at 17p13, where *TP53* is located, is frequent (up to 85%) in ACCs (6). Furthermore, ACC is prevalent among individuals with LFS, caused by germline mutations in *TP53*. The Wnt/β-catenin pathway is known to be important in adrenal cortex development (7), and activating mutations in *CTNNB1* have been identified in both adrenocortical adenomas and ACC (5, 7). Activating mutations in exon 3 (including S45 and T41) of β-catenin (*CTNNB1*) have been identified in 22–27% of ACAs and 16–31% of ACCs (8–10); however, abnormal cytoplasmic and/or nuclear β-catenin expression has been demonstrated in 30–85% of ACC specimens (8, 10). Furthermore, recurrent C228T *TERT* promoter mutations were recently reported in subsets of ACCs (11).

Studies of copy number alterations (CNAs) in ACC have identified large amplifications across chromosomes 5, 7, 12, 16q, and 20, and large deletions across chromosomes 1, 3p, 10q, 11, 14q, 15q, 17, and 22q (12). A recent study of ACC identified similar CNAs, and also highlighted deletions of *ZNRF3* and amplifications of *TERT* as common events (5). Using whole-exome sequencing, the current study demonstrates recurrent homozygous deletions of *ZNRF3* and *KREMEN1*, *TERT* amplifications, and confirmed *TP53* and *CTNNB1* as the most recurrently mutated genes across the ACC exome.

Table 1. Overview of Clinical Features of Patients with Adrenocortical Carcinoma Undergoing Whole-Exome Sequencing

Characteristic	No. of Cases	Proportion
Total number	n = 41	
Age, y, mean (range)	52.9 (13–77)	–
Sex		
Male	15	36.6%
Female	26	63.4%
Cohort		
Yale	19	46.3%
Karolinska	14	34.1%
Dusseldorf	8	19.5%
Ethnicity		
Caucasian	37	90.2%
Black	2	4.9%
Hispanic	2	4.9%
ENSAT 2008 stage		
I	1	2.4%
II	16	39.0%
III	13	31.7%
IV	11	26.8%
Tumor diameter, cm, mean (range)	11 (2.8–21.0)	–
Metastatic at presentation	11	26.8%
Outcomes		
Alive, no recurrence	10	24.4%
Alive, recurrent	6	14.6%
Death from recurrence	18	43.9%
Death, cause unknown or other	3	7.3%
Lost to followup/current status unknown	4	9.8%
Hormonal hypersecretion		
Nonhyperfunctional	14	34.1%
Cortisol producing	12	29.3%
Androgen producing	5	12.2%
Cortisol and aldosterone	3	7.3%
Cortisol and androgen	2	4.9%
Aldosterone producing	2	4.9%
17-OH progesterone	1	2.4%
No information available	2	4.9%

Abbreviation: ENSAT, European Network for the Study of Adrenal Tumors.

Materials and Methods

Cases and samples

In total, 41 matched pairs of ACCs and nontumor samples were included from three different institutions (Table 1, Supplemental Table 1). Following approval by the Yale University Institutional Review Board, and after obtaining written informed consent from patients, sporadic cases of histologically confirmed ACC were included in the study. Samples were selected from archived formalin-fixed paraffin-embedded tissue from Yale Pathology Tissue Services. Two additional cohorts of sporadic ACC were obtained from Karolinska University Hospital in Stockholm, Sweden (frozen tissue), and Düsseldorf, Germany (formalin-fixed paraffin-embedded tissue) following ethical approval for genetic studies from local ethics committees. All specimens were examined by experienced endocrine pathologists before nucleic acid extraction. None of the included patients were exposed to neoadjuvant treatment (chemotherapy or radiation) prior to surgical excision of their primary tumor.

Exome capture, massively parallel sequencing, and analysis

Genomic DNA samples generating adequate high-quality libraries were subjected to exome capture and sequencing as previously described (13), and the complete methodology regarding

Yale Endocrine Neoplasia Laboratory (C.C.J., J.M.H., A.L.F., J.W.K., T.C.B., R.K., T.C.), Yale School of Medicine, New Haven, Connecticut 06520; Department of Surgery (C.C.J., J.M.H., A.L.F., J.W.K., T.C.B., R.K., T.C.), Yale School of Medicine, New Haven, Connecticut, 06520; Department of Genetics (G.G., C.N.W., M.C., R.P.L.), Yale School of Medicine and Howard Hughes Medical Institute, New Haven, Connecticut, 06520; Department of Oncology-Pathology (C.C.J., A.S., A.H., C.L.), Karolinska Institutet, Karolinska University Hospital, CCK, SE-171 76 Stockholm, Sweden; Yale Center for Genome Analysis (JDO, SMM), Orange, Connecticut, 06477; Department of Pathology (D.L.R., M.L.P.), Yale School of Medicine, New Haven, Connecticut, 06520; Department of Pharmacology (J.S.), Yale School of Medicine, New Haven, Connecticut 06520; Department of Molecular Medicine and Surgery (M.B.), Karolinska Institutet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden; Division of Nephrology (U.I.S.), University Hospital Düsseldorf, 40225 Düsseldorf, Germany; Department of Pathology (A.C.S.), University Hospital Düsseldorf, 40225 Düsseldorf, Germany; and Division of Endocrinology and Diabetology (M.H.), University Hospital Düsseldorf, 40225 Düsseldorf, Germany

whole-exome sequencing, copy number alteration (CNA) analysis, sequence validation, ontology analyses, statistics, and expressional analyses are detailed in the Supplemental Materials and Methods.

Results

Study cohort

This study included 41 patients with ACC (Table 1, Supplemental Table 1). These samples were obtained from 15 males and 26 females with a mean age at diagnosis of 52.9 years (range, 13–77 y). According to the staging of the European Network for the Study of Adrenal Tumors (ENSAT), one patient (2.4%) presented with stage I, 16 patients (39.0%) with stage II, 13 patients (31.7%) with stage III, and 11 patients (26.8%) with stage IV tumors. Tumors exhibited a mean size of 11 cm (range, 2.8–21 cm). In total, 25 of the tumors (61.0%) produced an excess of one or more hormones, with 17 of these (68.0%) being cortisol-hypersecreting (cortisol alone [n = 12]) or combined with aldosterone/androgens (n = 5).

Whole-exome sequencing

Whole-exome sequencing was performed on DNA from 41 tumors along with matched normal DNA, and all tumor/normal pairs were successfully matched by the exome analysis. Each targeted base in tumor and normal samples was sequenced an average of 243-fold and 124-fold, respectively (Supplemental Table 2); more than 90% of the targeted bases were covered with at least 20 reads. Mean tumor purity for all cases where this information was available was 62% (range, 27–92%; Supplemental Table 2).

Somatic mutations were called based on significant increases in nonreference alleles present in the tumor, compared with the matched normal sample. The results identified an average of 23.6 protein-altering and 7.6 silent somatic mutations per tumor. The mean somatic mutation rate per base was 1.19×10^{-6}. Overall, 966 nonsynonymous single nucleotide variants (SNVs) were identified (Supplemental Table 3). An overview of the whole-exome sequencing results (Figure 1A) and the algorithm-generated arm-length copy number alterations (Figure 1B) are shown in Figure 1. Each gene with a nonsynonymous SNV was reviewed against known mutations identified in prior studies and subjected to MutSig analysis (14).

Adrenocortical carcinoma with hypermutator phenotype

One tumor, sample 545, exhibited a significantly higher number of SNVs compared with others (314 SNVs compared with an average of 16 SNVs in the other 40 samples; range, 1–80; $P = 1.07 \times 10^{-8}$, χ^2). The tumor harbored somatic mutations in *MSH6* (Glu288*) and *POLE* (Arg742His), genes previously associated with hypermutator phenotypes (15, 16) (Supplemental Table 3). However, the sample did not have markedly aberrant patterns of CNA. Because of the skewing effect of the large number of mutations in this tumor, it was excluded from subsequent statistical analyses of mutation burden. Genes of interest distributed among the samples were selected to undergo Sanger sequencing to validate identified mutations. Of the selections that were able to generate adequate sequence, 54 of 55 somatic mutations were confirmed present (98%).

ACC-related gene mutations

Among the somatic coding mutations in the remaining 40 matched tumor-normal pairs excluding the hypermutator case 545, only one gene, *CTNNB1*, encoding β-catenin, had a recurrent mutation (Thr41Ala) observed in two tumors (Table 2, Supplemental Table 3). This is a known gain of function mutation in exon 3 of *CTNNB1* (9). Two additional tumors had somatic mutations in *CTNNB1*: Ser45Pro, also in exon 3 and known to be an activating mutation (9), and Leu513Phe, which is located in the ARM9 (armadillo repeat 9) domain of β-catenin. The Leu513Phe variant has not been described previously as a Catalogue of Somatic Mutations in Cancer (COSMIC) mutation and the pathogenicity remains unknown. However, the leucine at this position is highly conserved among species, and damage prediction analysis using PolyPhen2 version 2.2.2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) suggests the mutation is "probably damaging" with a score of 1.000. Collectively, *CTNNB1* mutations were observed in 4/40 (10%) of the ACCs studied (Table 2).

To determine which genes had more somatic mutations than expected by chance, MutSigCV (14) was applied. Only one gene, *TP53*, was identified as significantly mutated (q-value = 7.68×10^{-2}). Eight somatic mutations in *TP53* were identified in 7/40 tumors (17.5%), all of which were protein altering and in regions of LOH, and 6/8 had read distributions consistent with homozygous variants, whereas 2/8 seemed to be subclonal (Table 2, Supplemental Table 4). A *TP53* mutation was also identified in the hypermutator sample, for a total of 8/41 (19.5%). A germline mutation in *TP53* (Arg156His) was identified in one patient (sample 504) who carried an additional somatic mutation. This germline mutation was previously reported in an individual with LFS (17). Of note, all patients with identified *TP53* mutations in this study were females ($P = .018$, Fisher's exact test.)

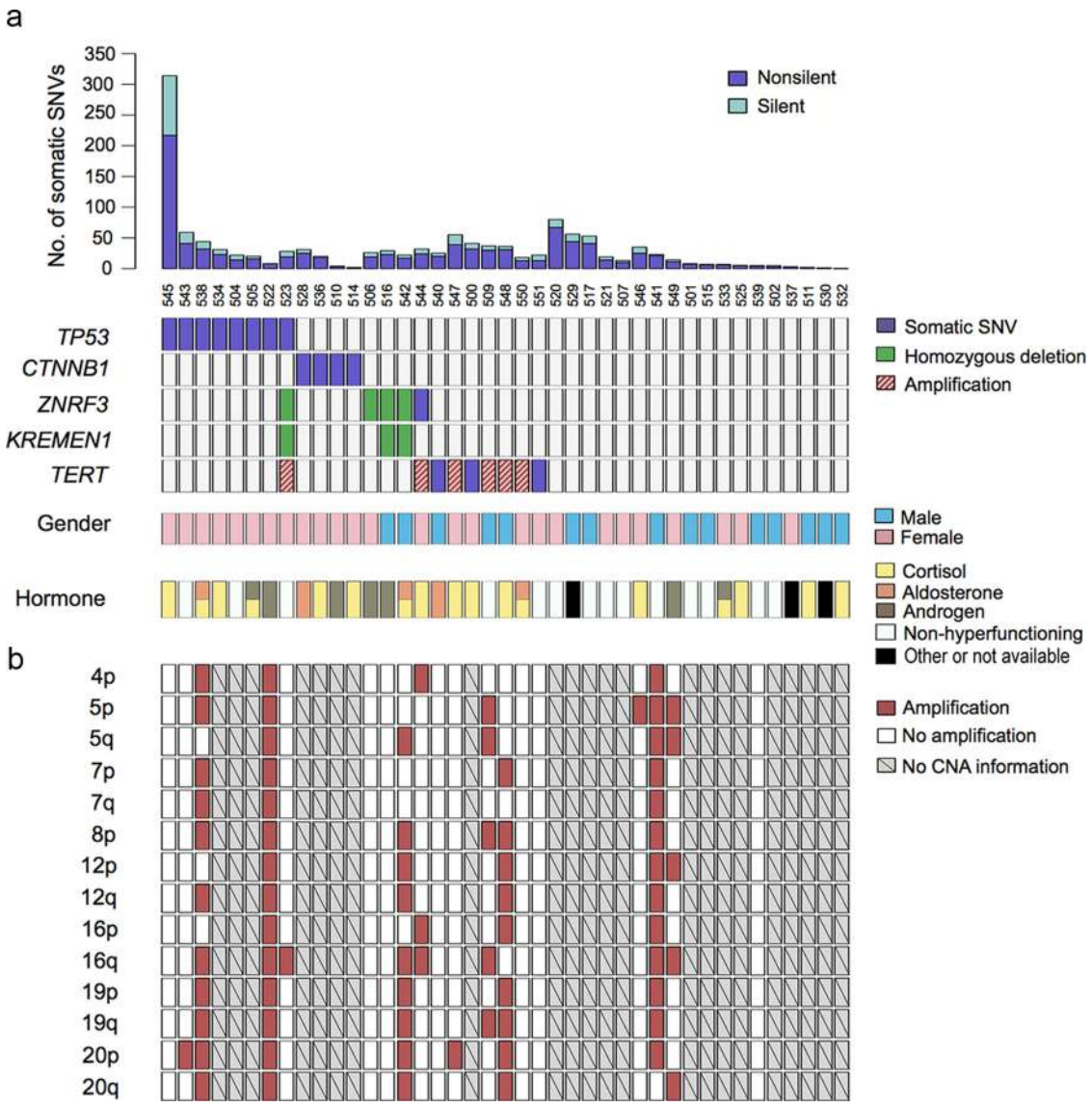

Figure 1. Landscape of somatic mutations and copy number alterations in adrenocortical carcinoma. A, Distribution of SNVs in each tumor (labeled between 500 and 551) is shown. Recurrently altered genes are shown in the middle panel and hormone status and sex are indicated below. B, Significant arm-level amplifications and regions without amplification are shown, by sample. Samples without discernable CNA information are indicated. Each panel corresponds to the tumor numbers shown in panel A.

Identifying rare but functionally relevant mutations in ACC

When conducting genetic analysis of rare tumors such as in this study, it is possible that genes harboring mutations relevant to pathogenesis may not reach statistical significance. Alternatively, relevant genes in the cohort may be recognized based on prior knowledge of their biological function or their established roles in tumorigenesis (18). To perform an analysis in a systematic fashion, all mutations in ACC were compared with those a) previously reported in the COSMIC database, and b) mutations that occurred at positions where recurrent (>1) mutations were previously reported. Applying this criteria, mutations in *RB1* (Leu665Arg) and *GNAS* (Arg201His), both genes with established roles in cancer, were identified in the ACC cohort (Supplemental Table 3). An additional known tumor suppressor, *NF2*, contained truncating mutations (Gln415* and Arg300*) in two separate samples. Overlooking the above-mentioned criteria for genes excluded based on the limited knowledge of biological functions identified additional recurrently mutated COSMIC genes (≥ 3 nonsynonymous mutations in the cohort) with potential roles for tumor development, namely *CDC27*, *SCN7A*, and *SDK1* (Table 2, Supplemental Table 3).

Copy number alteration analysis

Among the 41 ACCs in this study, 19 had discernible CNAs (Figure 1B, Figure 2, and Supplemental Figure 1). Figure 2A illustrates the overall landscape of gains and losses in this cohort across the whole exome. To determine

Table 2. Overview of the Somatic Nonsynonymous Mutations Observed in the ACC Cohort

Gene Name	Location (Chromosome)	No. of Mutated Cases	Mutation(s) Observed	Mutation Type	No. with LOH
Genes with recurrent mutations					
CTNNB1	3p21	2	Thr41Ala	Missense	LOH (1/2)
Recurrently mutated genes (mutations ≥ 3 samples)					
TP53	17p13.1	8	Various	Missense/nonsense	LOH (8/8)
CTNNB1	3p21	4	Various	Missense	LOH (2/4)
CDC27	17q21.32	3	Various	Missense	LOH (2/3)
SCN7A	2q21-q23	3	Various	Missense	LOH (1/3)
SDK1	7p22.2	3	Various	Missense	LOH (1/3)
Recurrently mutated genes with damaging mutations + LOH					
TP53	17p13.1	3	Various	Nonsense	LOH (3/3)
NF2	22q12.2	2	Various	Nonsense	LOH (2/2)

Abbreviation: LOH, loss of heterozygosity.

which regions were significantly gained or deleted more often than expected by chance, Genomic Identification of Significant Targets in Cancer (GISTIC) was applied (19). GISTIC takes into account the frequency of occurrence and amplitude of a chromosomal aberration, as well as the background rate of CNAs, to establish the statistical significance of each CNA observed. This approach identified two recurrent focal CNAs (Figure 2B)—gains at 5p15.33 (q-value = 7.1×10^{-2}), and deletions across 22q12.1 (q-value = 1.6×10^{-3}). The 5p15.33 gain was present in 6/19 tumors with discernible CNAs (31.6%). This segment contains *TERT*, which encodes for telomerase reverse transcriptase. Mutations in the promoter region of the *TERT* gene as well as amplification of this locus have been identified in various human cancers (20, 21). A previous study identified recurrent C228T *TERT* promoter mutations in 3/41 ACCs used in this study (Figure 1), and *TERT* gene expression was demonstrated in all mutated tumors (11). Of note, tumors with *TERT* aberrations were larger in size compared with ACCs with wild-type *TERT*, with mean sizes of 14.6 cm and 10.0 cm, respectively (P = .007).

The 22q12.1 deletion contains the gene *ZNRF3*, and was identified in four tumors with discernible CNA data (Supplemental Figure 2). Further, one additional case (sample 544) harbored a somatic mutation (Ala346Val) in *ZNRF3*. These data suggest *ZNRF3* gene alteration in 5/41 (12.2%) of ACC cases. *ZNRF3* was previously reported to act as a tumor suppressor by regulating the Wnt signaling pathway (5, 22). In addition, the 22q12.1 deletion extended to include all or part of another Wnt repressor, *KREMEN1* in three tumors (Figure 1A).

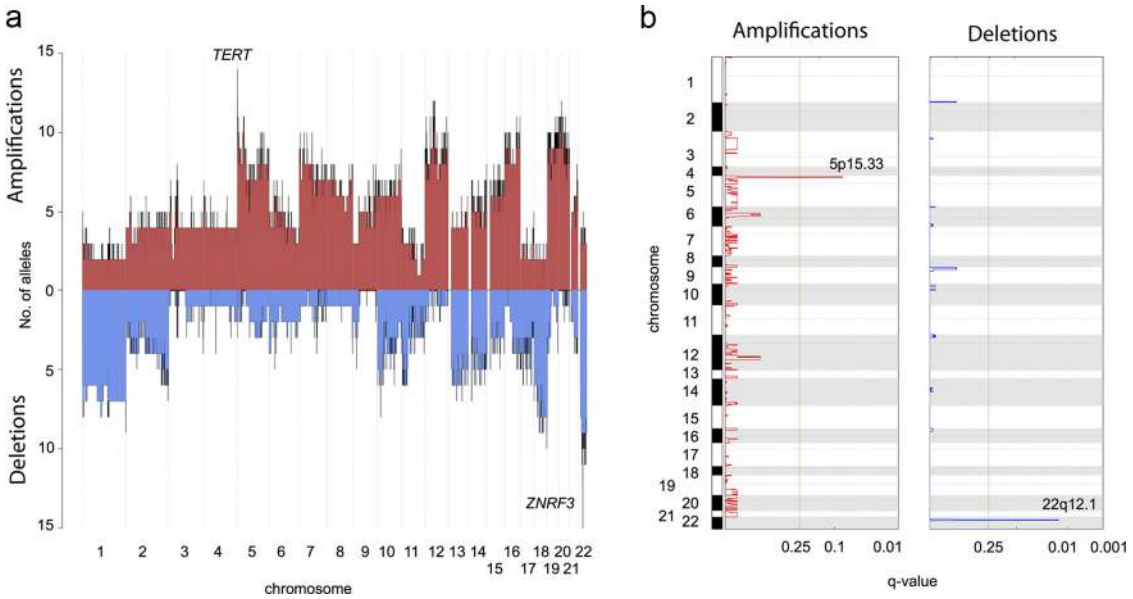

Figure 2. Somatic copy number alteration in adrenocortical carcinoma. A, Overview of copy number alterations identified across the 19 ACCs with discernible CNAs. Chromosome numbers are annotated on the x-axis and amplifications (red) and deletions (blue) are plotted on the y-axis. B, Plot of significant GISTIC peaks (red, amplifications; blue, deletion) identified. CNAs in 5p15.33 (left panel) and 22q12.1 (right panel) represent statistically significant recurrent peaks.

GISTIC further identified significant (q-value <0.1) arm-level amplifications across chromosomes 4p, 5, 7, 8p, 12, 16, 19, and 20 (Figure 1B). These results were generally concordant with other studies on CNAs in ACC (5, 12).

Loss of heterozygosity analyses

The overall loss of heterozygosity (LOH) profile of the 41 cases is presented in Supplemental Figure 3. Extensive LOH events were detected, and the LOH profile is consistent with the CNA profile as seen in Figure 2A.

Survival analyses

Because mutations in either *TP53* or *CTNNB1* as well as focal CNAs at the *ZNRF3* or *TERT* loci were mutually exclusive events in our cohort, Kaplan Meier curves plotting cohort patients with these genetic aberrancies against overall survival were generated (Supplemental Figure 4). Patients with any of the above-described aberrations (*ZNRF3*, *TP53*, *CTNNB1*, and *TERT*) were first compared, and subsequently these patients were combined into a single group and compared vs all remaining patients. Although not statistically significant (log rank $P = .97$ and $P = .72$ for Supplemental Figure 4 A and B, respectively), a trend toward decreased overall survival was noted for patients with *ZNRF3* deletions and *TP53* mutations.

Gene ontology and pathway analyses

Using four gene ontology software programs, an unbiased and significant aggregation of somatic coding mutations in Wnt pathway genes was observed. The Wnt pathway remained one of the highest enriched canonical pathways among this mutated gene set in three of four different gene ontology software programs, and this observation persisted after removal of the case with a hypermutator phenotype. NextBio allows a comparison with the MutSig database of significantly mutated genes, which revealed highly significant enrichments of gene sets involved in metabolism of lipids, developmental biology, and transmembrane transport, in addition to the Wnt signaling pathway. When only listing somatic mutations with damaging properties (nonsense mutations with concurrent LOH), significant associations with the Wnt (NextBio; $P = 4.7 \times 10^{-3}$) and TP53-associated pathways (Genomatix; $P = 1.34 \times 10^{-4}$) were observed (Figure 3). Disease Association Protein-Protein Link Evaluator (DAPPLE) analyses of protein–protein interaction among mutated genes in the ACC cohort further suggest a significant association of Wnt pathway proteins, particularly for the central Wnt effectors β-catenin and CREBBP (Figure 4). These genes were highlighted as having numerous confident interactions with other mutated gene products.

Immunohistochemistry

Expression of β-catenin was studied in three cases with homozygous *ZNRF3/KREMEN1* deletions (516, 523, and 542), as well as the sole case with a *ZNRF3* missense mutation (544). None of these four ACCs displayed nuclear β-catenin localization (Supplemental Figure 5). In addition, case 528 exhibiting a Leu513Phe *CTNNB1* mu-

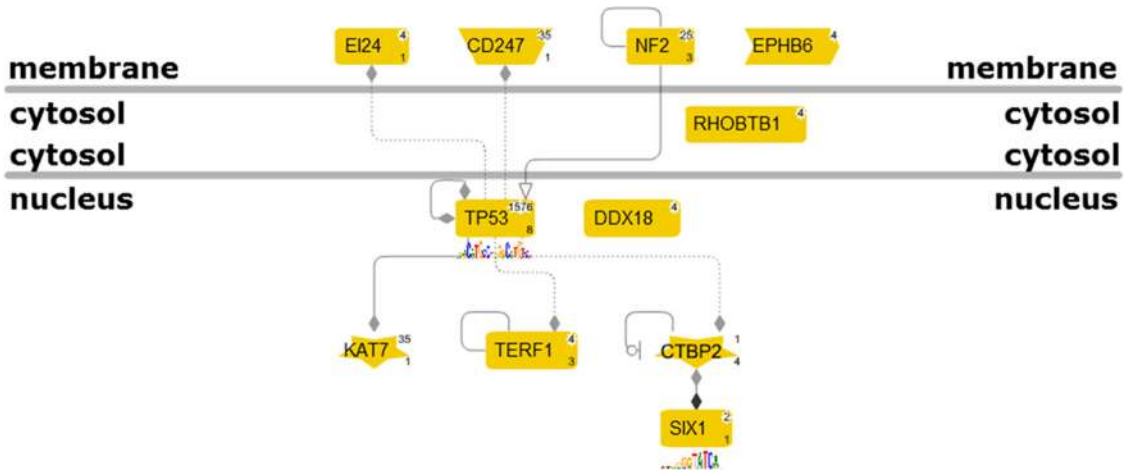

Figure 3. Network of damaging mutations in ACC associates to the TP53 pathway. Genomatix analysis output of genes displaying damaging mutations (nonsense, frameshift) with concurrent LOH of the remaining allele. The aggregation of genes were intimately associated to the TP53 pathway, highlighting the importance of this pathway in ACC tumorigenesis. Gene products are depicted as rounded rectangles, snip-sided rectangles signify proteins kinases and star-shaped symbols symbolize cofactors. The number of sources for a chemical association to established molecules (for pharmaceutic intervention purposes) is suggested in the top right corner of each symbol, whereas the lower right number denotes the number of recognized interactions within the signaling network in addition to the depicted association. Filled lines with arrows represent an activating effect; filled lines with stop line and circle designate inhibitory effect. Dotted lines symbolize evidence at the level of cocitation, as opposed to filled lines, which designate evidence at expert-curation level. Colored letter matrices suggest DNA-binding properties (known transcription factor).

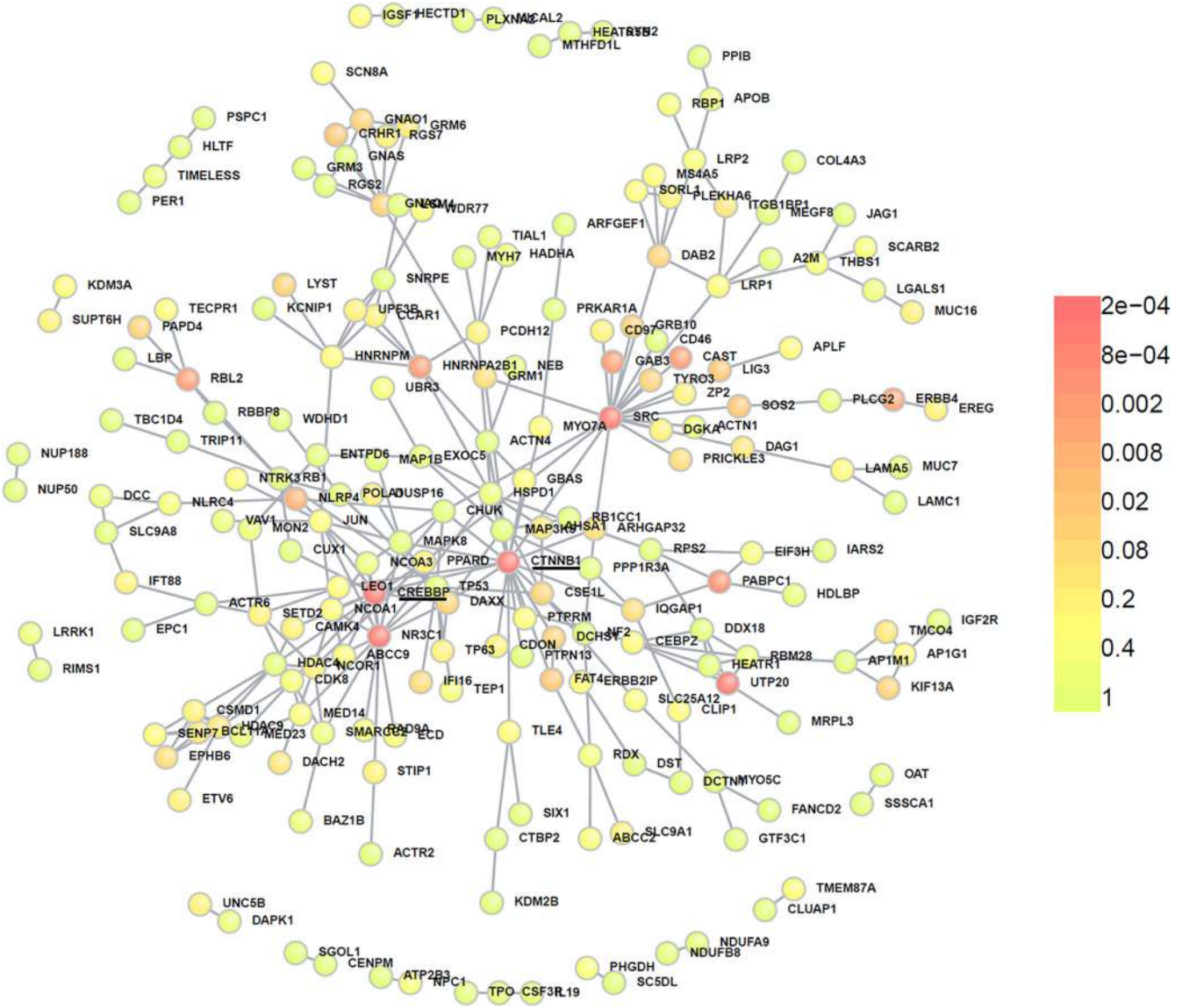

Figure 4. DAPPLE analysis of all somatic coding mutations. DAPPLE output exhibits physical interactions among proteins in which the corresponding gene exhibited a coding mutation in the studied cohort. The level of significance of the associations between mutated gene products is specified by the color of each circle, where red circles suggest a highly significant interaction as reported in the literature. The bar represents each p-value and its assigned color. Among the genes mutated in ACCs, *CTNNB1*/β-catenin and *CREBBP* are central components (underlined in black), as visualized by multiple confident interactions with other mutated gene products.

tation with unknown effect on β-catenin stabilization was investigated by immunohistochemistry using antibodies targeting β-catenin, c-[ital]myc, and cyclin D1 (Supplemental Figure 6). β-catenin was only visualized in the cytoplasm of the tumor cells, and no nuclear accumulation was noted. An identical pattern was seen for -[ital]myc, whereas cyclin D1 stained strongly in 90% of tumor cell nuclei.

Discussion

This study reports on the whole-exome sequencing of ACC, characterizing the landscape of somatic mutations and copy number alterations in these heterogeneous tumors. The findings highlight genes with increased mutation burden and recurrent mutations, including genes previously implicated in ACC pathogenesis (*CTNNB1* and *TP53*) as well as genes not previously known to exhibit mutations in ACCs (*GNAS* and *NF2*). Furthermore, these results corroborate recently uncovered recurrent amplifications of *TERT* and deletions of 22q12.1, including *ZNRF3* (5), and identify COSMIC genes such as *CDC27*, *SCN7A*, and *SDK1* as recurrently mutated in our cohort. Moreover, the finding of mutually exclusive *ZNRF3*/*KREMEN1* and *TERT* loci CNAs as well as *TP53* and *CTNNB1* mutations in this study suggests divergent

pathogenic mechanisms possibly influencing ACC development, although the observed phenomenon might be biased by the amount of ACC cases included in this study.

CNA analysis of ACC identified a recurrent gain of *TERT* in six tumors. *TERT* promoter mutations have been identified in various human cancers, and this cohort includes three tumors with previously demonstrated recurrent C228T mutations, which were previously shown to correlate with increased *TERT* mRNA expression (11). Similar focal gains at 5p15.33, containing *TERT*, have also been identified in lung cancer, oral squamous cell carcinoma, and neuroblastoma (23–25). *TERT* encodes telomerase reverse transcriptase, the catalytic subunit of the enzyme telomerase, which extends telomeres and prevents replicative senescence. In many cancers, telomerase activity correlates with proliferative ability of cancer cells and activation of *TERT* enables replicative immortality (24). There is also evidence for noncanonical functions of TERT, including transcriptional regulation of pathways involved in cancer such as nuclear factor κB and Wnt/β-catenin signaling (26).

Somatic and homozygous deletions at 22q12.1, each including the *ZNRF3* locus, were identified in four of the ACCs. *ZNRF3* has been shown to act as a tumor suppressor, promoting Wnt receptor turnover. Inhibition of *ZNRF3* enhances Wnt/β-catenin signaling, and simultaneous deletion of *ZNRF3* and its related homolog *RNF43* induced rapidly growing adenomas in the intestinal epithelium of mice (27). Down-regulation of *ZNRF3* was also observed in gastric adenocarcinoma tissue compared with adjacent normal tissue, and overexpression of *ZNRF3* in a gastric cancer cell induced apoptosis and suppressed proliferation (22). *KREMEN1* is a high-affinity transmembrane receptor for *DKK1*, which forms a ternary complex with *KREMEN1* in the presence of high levels of *LRP5/6*, resulting in inhibition of Wnt signaling (28). The close proximity of these two Wnt repressors in a region frequently deleted in ACC suggests the potential for a synergistic effect when these genes are concomitantly deleted, and it has been hypothesized that loss of *ZNRF3* and *KREMEN1* together could result in increased accumulation of nuclear β-catenin (29). In our study, all three ACCs with homozygous *ZNRF3/KREMEN1* deletions displayed absence of nuclear accumulation of β-catenin, which would imply that the effect of *ZNRF3* loss of function might stem from noncanonical Wnt pathway activation. Indeed, *ZNRF3* has also been coupled to the noncanonical planar cell polarity pathway, the latter being a β-catenin independent pathway with tumorigenic properties (30).

The observed *KREMEN1* deletions might represent a passenger event, signified by case 506 in which the homozygous deletion only encompassed *ZNRF3*. Indeed, the isolated *ZNRF3* deletion in case 506 was mutually exclusive from mutations in *TP53* and *CTNNB1* (Figure 1). Interestingly, because *ZNRF3* and *KREMEN1* are both upstream components of the Wnt pathway, they represent a potential new target for therapeutics in subsets of ACCs.

A trend toward shorter survival for patients with *ZNRF3* deletions and *TP53* mutations compared with patients with tumors exhibiting *CTNNB1* mutations and *TERT* locus amplifications was seen. Indeed, *TP53* mutations and aberrant p53 expression have previously been linked to adverse prognosis in adrenocortical carcinoma (31), and the similar patterns obtained between *TP53* and *ZNRF3* aberrations suggest that *ZNRF3* deletions could be of some prognostic significance.

The high frequency of mutations in *TP53*, observed in 8/41 (19.5%) of cases, is in the same range as prior reports of *TP53* in ACC (6, 31). Aberrant p53 expression has been previously associated with decreased disease-free survival (31) and patients with *TP53* mutations showed a trend toward association with disease recurrence ($P = .07$). The number of tumors with LOH at 17p13, where *TP53* is located, is much higher (30/40, 75%), and is concordant with previous findings (6). This observation, together with the high percentage of tumors with 17p13 LOH, but no *TP53* mutation, raises the possibility of other genes in the same chromosomal region contributing to ACC pathogenesis.

CTNNB1 mutations were identified in four tumors, three of which were well known gain-of-function mutations in exon 3 (9), as well as a Leu513Phe mutation. The exon 3 mutations lead to stabilization of β-catenin, with accumulation of the transcription factor in the nucleus and downstream activation of the Wnt pathway (32). The Wnt pathway plays an important role in adrenal cell proliferation during development, and *CTNNB1*-activating somatic mutations are frequent in adrenal tumors (8). However, these mutations are present in both adenomas and carcinomas (8), and further investigation is necessary to determine the factors that lead a subset of *CTNNB1* mutant tumors to progress to cancer. Case 528 displaying the *CTNNB1* mutation Leu513Phe was negative for nuclear β-catenin and nuclear c-[ital]myc expression, which implies that the detected *CTNNB1* mutation in this case either represents a passenger event, or alternatively, it affects other β-catenin functions besides the classical canonical Wnt effector properties. The *CTNNB1* mutations in our cohort were mutually exclusive from *TP53* mutations (Figure 1A), an observation that has been previously reported in ACC (33), suggesting perturbation of either pathway could lead to activation of similar targets, contributing to tumorigenesis.

Potentially disease-causing mutations in *GNAS*, *RB1*, and *NF2* were also identified. The *GNAS* Arg201His mutation is a widely recurrent mutation present in pituitary, kidney, pancreas, and colon cancers (34–36). *GNAS* is moreover a recurrently mutated gene in adrenocortical tumors (37), and the Arg201His mutation has been previously described in cortisol-producing adrenocortical tumors, and is known to activate cAMP signaling, leading to increased cortisol production (38). The patient harboring this mutation (sample 533) had a tumor with cortisol and androgen hypersecretion. The mutations identified in *RB1* and *NF2* in this cohort also overlap regions of LOH, consistent with their known tumor suppressor roles (39–40). In addition, recurrently mutated genes included *CDC27*, *SCN7A*, and *SDK1*, three COSMIC genes that could merit further attention in future studies. Overall, gene ontology analyses suggest a substantial accumulation of coding somatic mutations within the Wnt pathway, and mutations in Wnt-associated genes were identified in 27 of 41 cases (66%), reinforcing the relationship between deregulated Wnt signaling and ACC development. This finding implies that molecular aberrancies of the Wnt pathway are potential major contributors to the development of adrenocortical cancer.

To conclude, the current findings help define the genomic landscape of ACC and identify specific pathways that are frequently altered, providing direction for research of targeted therapies against these tumors.

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Address all correspondence and requests for reprints to: Tobias Carling, Yale School of Medicine, 333 Cedar Street, FMB130A, Box 208062, New Haven, CT 06520. E-mail: tobias.carling@yale.edu.

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References

1. **Lebastchi AH, Kunstman JW, Carling T.** Adrenocortical carcinoma: Current therapeutic state-of-the-art. *J Oncol*. 2012;2012:234726.
2. **Else T, Kim AC, Sabolch A, et al.** Adrenocortical carcinoma. *Endocr Rev*. 2014;35:282–326.
3. **Berthon A, Martinez A, Bertherat J, Val P.** Wnt/β-catenin signalling in adrenal physiology and tumour development. *Mol Cell Endocrinol*. 2012;351:87–95.
4. **Fassnacht M, Kroiss M, Allolio B.** Update in adrenocortical carcinoma. *J Clin Endocrinol Metab*. 2013;98:4551–4564.
5. **Assié G, Letouzé E, Fassnacht M, et al.** Integrated genomic characterization of adrenocortical carcinoma. *Nat Genet*. 2014;46:607–612.
6. **Libé R, Groussin L, Tissier F, et al.** Somatic TP53 mutations are relatively rare among adrenocortical cancers with the frequent 17p13 loss of heterozygosity. *Clin Cancer Res*. 2007;13:844–850.
7. **Lerario AM, Moraitis A, Hammer GD.** Genetics and epigenetics of adrenocortical tumors. *Mol Cell Endocrinol*. 2014;386:67–84.
8. **Tissier F, Cavard C, Groussin L, et al.** Mutations of beta-catenin in adrenocortical tumors: Activation of the Wnt signaling pathway is a frequent event in both benign and malignant adrenocortical tumors. *Cancer Res*. 2005;65:7622–7627.
9. **Gaujoux S, Grabar S, Fassnacht M, et al.** β-catenin activation is associated with specific clinical and pathologic characteristics and a poor outcome in adrenocortical carcinoma. *Clin Cancer Res*. 2011;17:328–336.
10. **Heaton JH, Wood MA, Kim AC, et al.** Progression to adrenocortical tumorigenesis in mice and humans through insulin-like growth factor 2 and β-catenin. *Am J Pathol*. 2012;181:1017–1033.
11. **Liu T, Brown TC, Juhlin CC, et al.** The activating TERT promoter mutation C228T is recurrent in subsets of adrenal tumors. *Endocr Relat Cancer*. 2014;21:427–434.
12. **Stephan EA, Chung TH, Grant CS, et al.** Adrenocortical carcinoma survival rates correlated to genomic copy number variants. *Mol Cancer Ther*. 2008;7:425–431.
13. **Scholl UI, Goh G, Stölting Get al.** Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet*. 2013;45:1050–1054.
14. **Lawrence MS, Stojanov P, Polak P, et al.** Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. 2013;499:214–218.
15. **Loeb LA.** Human cancers express mutator phenotypes: Origin, consequences and targeting. *Nat Rev Cancer*. 2011;11:450–457.
16. **Yoshida R, Miyashita K, Inoue M, et al.** Concurrent genetic alterations in DNA polymerase proofreading and mismatch repair in human colorectal cancer. *Eur J Hum Genet*. 2011;19:320–325.
17. **Quesnel S, Verselis S, Portwine C, et al.** p53 compound heterozygosity in a severely affected child with Li-Fraumeni syndrome. *Oncogene*. 1999;18:3970–3978.
18. **Lohr JG, Stojanov P, Lawrence MS, et al.** Discovery and prioritization of somatic mutations in diffuse large b-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A*. 2012;109:3879–3884.
19. **Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G.** GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol*. 2011;12:R41.
20. **Zhang A, Zheng C, Lindvall C, et al.** Frequent amplification of the telomerase reverse transcriptase gene in human tumors. *Cancer Res*. 2000;60:6230–6235.
21. **Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA.**

Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013;339:957–959.
22. **Zhou Y, Lan J, Wang W, et al.** ZNRF3 acts as a tumour suppressor by the Wnt signalling pathway in human gastric adenocarcinoma. *J Mol Histol*. 2013;44:555–563.
23. **Cobrinik D, Ostrovnaya I, Hassimi M, Tickoo SK, Cheung IY, Cheung NK.** Recurrent pre-existing and acquired DNA copy number alterations, including focal TERT gains, in neuroblastoma central nervous system metastases. *Genes Chromosomes Cancer*. 2013; 52:1150–1166.
24. **Freier K, Pungs S, Flechtenmacher C, et al.** Frequent high telomerase reverse transcriptase expression in primary oral squamous cell carcinoma. *J Oral Pathol Med*. 2007;36:267–272.
25. **Kang JU, Koo SH, Kwon KC, Park JW, Kim JM.** Gain at chromosomal region 5p15.33, containing TERT, is the most frequent genetic event in early stages of non-small cell lung cancer. *Cancer Genet Cytogenet*. 2008;182:1–11.
26. **Low KC, Tergaonkar V.** Telomerase: Central regulator of all of the hallmarks of cancer. *Trends Biochem Sci*. 2013;38:426–434.
27. **Koo BK, Spit M, Jordens I, et al.** Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature*. 2012;488:665–669.
28. **Wang K, Zhang Y, Li X, et al.** Characterization of the Kremen-binding site on Dkk1 and elucidation of the role of Kremen in Dkk-mediated Wnt antagonism. *J Biol Chem*. 2008;283:23371–23375.
29. **Nord KH, Nilsson J, Arbajian E, et al.** Recurrent chromosome 22 deletions in osteoblastoma affect inhibitors of the Wnt/beta-catenin signaling pathway. *PLoS One*. 2013;8:e80725-e80725.
30. **Hao HX, Xie Y, Zhang Y, et al.** ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature*. 2012;485:195–200.
31. **Waldmann J, Patsalis N, Fendrich V, et al.** Clinical impact of TP53 alterations in adrenocortical carcinomas. *Langenbecks Arch Surg*. 2012;397:209–216.
32. **Giles RH, van Es JH, Clevers H.** Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta*. 2003;1653:1–24.
33. **Ragazzon B, Libé R, Gaujoux S, et al.** Transcriptome analysis reveals that p53 and {beta}-catenin alterations occur in a group of aggressive adrenocortical cancers. *Cancer Res*. 2010;70:8276–8281.
34. **Dhanasekaran N, Heasley LE, Johnson GL.** G protein-coupled receptor systems involved in cell growth and oncogenesis. *Endocr Rev*. 1995;16:259–270.
35. **Fecteau RE, Lutterbaugh J, Markowitz SD, Willis J, Guda K.** GNAS mutations identify a set of right-sided, RAS mutant, villous colon cancers. *PLoS One*. 2014;9:e87966.
36. **Wu J, Matthaei H, Maitra A, et al.** Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med*. 2011;3:92ra66.
37. **Almeida MQ, Azevedo MF, Xekouki P, et al.** Activation of cyclic AMP signaling leads to different pathway alterations in lesions of the adrenal cortex caused by germline PRKAR1A defects versus those due to somatic GNAS mutations. *J Clin Endocrinol Metab*. 2012; 97:E687–693.
38. **Goh G, Scholl UI, Healy JM, et al.** Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nat Genet*. 2014; 46:613–617.
39. **Di Fiore R, D'Anneo A, Tesoriere G, Vento R.** RB1 in cancer: Different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. *J Cell Physiol*. 2013;228:1676–1687.
40. **Harvey KF, Zhang X, Thomas DM.** The Hippo pathway and human cancer. *Nat Rev Cancer*. 2013;13:246–257.