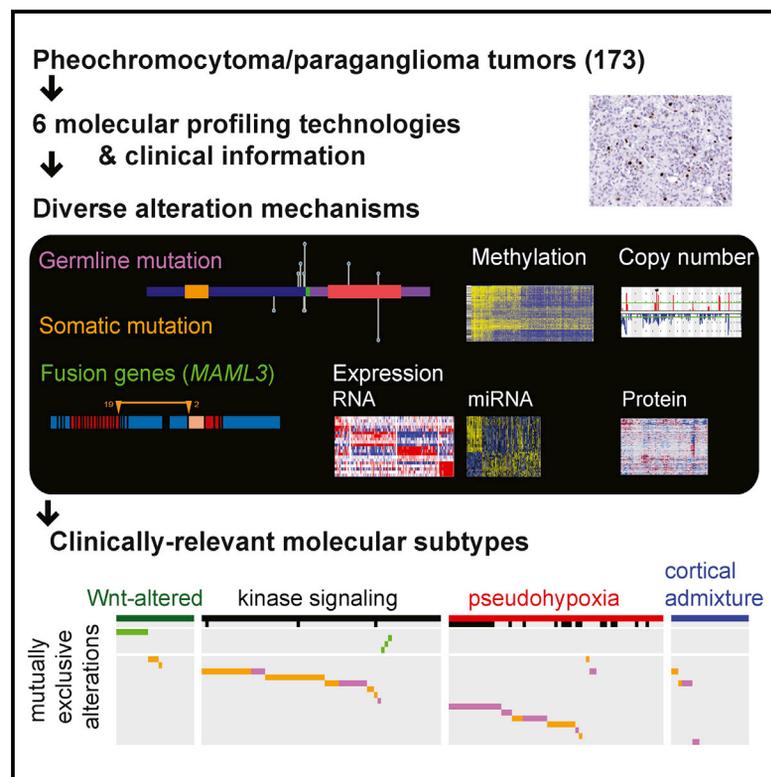


# Comprehensive Molecular Characterization of Pheochromocytoma and Paraganglioma

## Graphical Abstract



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## In Brief

Fishbein et al. show that neuroendocrine tumors, pheochromocytomas and paragangliomas, have a low genome alteration rate but diverse driver alterations, which coalesce into four molecular subtypes. The Wnt-altered subtype, driven by *MAML3* fusions and *CSDE1* somatic mutations, correlates with poor clinical outcome.

## Highlights

- Comprehensive molecular profiling of 173 pheochromocytoma and paraganglioma tumors
- Single drivers in tumors by germline mutation, somatic mutation, or fusion gene
- *MAML3* fusion gene and *CSDE1* somatic mutation define a Wnt-altered subtype
- Prognostic markers of metastatic disease include the *MAML3* fusion gene



# Comprehensive Molecular Characterization of Pheochromocytoma and Paraganglioma

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## SUMMARY

We report a comprehensive molecular characterization of pheochromocytomas and paragangliomas (PCCs/PGLs), a rare tumor type. Multi-platform integration revealed that PCCs/PGLs are driven by diverse alterations affecting multiple genes and pathways. Pathogenic germline mutations occurred in eight PCC/PGL susceptibility genes. We identified *CSDE1* as a somatically mutated driver gene, complementing four known drivers (*HRAS*, *RET*, *EPAS1*, and *NF1*). We also discovered fusion genes in PCCs/PGLs, involving *MAML3*, *BRAF*, *NGFR*, and *NF1*. Integrated analysis classified PCCs/PGLs into four molecularly defined groups: a kinase signaling subtype, a pseudohypoxia subtype, a Wnt-altered subtype, driven by *MAML3* and *CSDE1*, and a cortical admixture subtype. Correlates of metastatic PCCs/PGLs included the *MAML3* fusion gene. This integrated molecular characterization provides a comprehensive foundation for developing PCC/PGL precision medicine.

### Significance

Pheochromocytomas and paragangliomas (PCCs/PGLs) are rare neuroendocrine tumors with a unique genetic background and few options for treating metastatic disease. Comprehensive molecular analysis revealed that PCCs/PGLs have a low genome alteration rate with a remarkable diversity of driver alterations including germline and somatic mutations, and somatic fusion genes. This diversity coalesced into molecular subtypes, including the discovery of a Wnt-altered subtype driven by a *MAML3* fusion gene and *CSDE1* somatic mutation. This subtype correlates with poor clinical outcome, providing opportunities for molecular diagnosis and prognosis in patients. The diversity of single drivers among PCCs/PGLs makes these tumors a model for future targeted therapy and pan-cancer molecular etiology research.

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## INTRODUCTION

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neuroendocrine tumors that originate from chromaffin cells and occur in the adrenal medulla (PCCs) and in sympathetic or parasympathetic ganglia (PGLs). Most present as benign, yet show high morbidity and mortality due to excessive catecholamine production, leading to hypertension, arrhythmia, and stroke. Up to 25% are malignant (Ayala-Ramirez et al., 2011), as defined by distant metastases to non-chromaffin tissues. Patients with metastatic PCCs/PGLs have limited treatment options and poor prognosis, often with less than 50% surviving at 5 years (Hescot et al., 2013). Despite a low incidence (0.8 per 100,000 for PCCs) (Beard et al., 1983), over one-third of PCCs/PGLs are associated with inherited cancer susceptibility syndromes, which is the highest rate among all tumor types (Dahia, 2014). Inherited mutations have been identified in more than 15 well characterized genes, most commonly in *VHL*, *SDHB*, *SDHD*, *NF1*, and *RET* (Favier et al., 2015). Markers of metastatic disease are limited, including germline *SDHB* mutations, extra-adrenal location, tumor size, and elevated plasma methoxytyramine levels (Amar et al., 2005; Ayala-Ramirez et al., 2011; Eisenhofer et al., 2012).

Although the inherited basis of PCCs/PGLs has been well characterized, somatic profiles have not been well delineated. To date, limited somatic profiling has identified mutations at various frequencies in several genes including *EPAS1* (*HIF2 $\alpha$* ), *RET*, *VHL*, *RAS*, *NF1*, and *ATRX* (Burnichon et al., 2011, 2012; Cho et al., 2005; Comino-Mendez et al., 2013; Crona et al., 2013; Fishbein et al., 2015; Hrascan et al., 2008; Komminoth et al., 1994; Toledo et al., 2016; Zhuang et al., 2012), and has identified recurrent somatic copy-number alterations (Flynn et al., 2015a). Nevertheless, there is still a substantial fraction of PCCs/PGLs for which the etiology of tumorigenesis is not well understood. As part of The Cancer Genome Atlas (TCGA), we aimed to generate a comprehensive genomic characterization of PCCs/PGLs.

## RESULTS

### Patient Cohort and Molecular Analysis Strategy

Through the TCGA, we collected and analyzed a cohort of PCCs/PGLs from 173 patients (Table S1). Fifty-seven percent of patients were female and 43% were male. The mean age at

initial diagnosis of PCC/PGL was 47 years, with a range of 19–83 years. Eleven patients (6%) had distant metastatic events. In total, 16 patients (9%) had aggressive disease events defined by having distant metastatic events, positive local lymph nodes, or local recurrence. Plasma or urine biochemical testing results were available for 144 patients (83%). Clinical genetic testing results were available for 116 patients (67%).

To identify and characterize PCC/PGL genome alterations, tissue specimens were analyzed by multiple genomic assays (Table 1). Matched normal tissue and tumor specimens were analyzed by whole-exome sequencing for mutations and SNP arrays for copy number analysis. Tumor specimens were also analyzed by mRNA sequencing, microRNA (miRNA) sequencing, DNA methylation arrays, and reverse-phase protein arrays for targeted proteome analysis. Our analysis strategy involved a systematic interrogation by platform to identify genomic alterations in PCCs/PGLs, including germline mutations, somatic mutations, fusion genes, and copy number alterations. Multi-platform integration and computational analysis was then performed to: (1) characterize the broad molecular correlates of prominent driver alterations; (2) identify a PCC/PGL molecular subtype classification; (3) identify disrupted pathways; and (4) identify molecular discriminants of metastatic disease. The integrated clinical and genomic datasets are available through the NCI's Genomic Data Commons.

### Germline and Somatic Mutations

Because susceptibility gene mutations are prevalent in patients with PCCs/PGLs, we first analyzed DNA exome sequencing of normal specimens to identify germline mutations in the cohort. Pathogenic germline mutations were detected within eight previously reported PCC/PGL susceptibility genes in 46 patients (27% of the cohort) (Figure 1; Table S2). Our germline mutation calls agreed with available clinical testing results. *SDHB* (9%), *RET* (6%), *VHL* (4%), and *NF1* (3%) exhibited the highest rates of germline mutation. Germline mutations in *SDHD*, *MAX*, *EGLN1* (PHD2), and *TMEM127* were rare at  $\leq 2\%$  each, consistent with prior studies containing cohorts of predominantly PCCs (Dahia, 2014; Favier et al., 2015).

Turning to somatic mutations, PCCs/PGLs exhibited a low somatic sequence mutation rate (mean 0.67 mutations per megabase) relative to other cancer types (Lawrence et al., 2013). Analyzing somatic mutations for recurrent, statistically significant driver genes identified five genes: *HRAS*, *NF1*, *EPAS1*,

**Table 1. Summary of Data Types**

Data Type	Platforms	Cases (n)	Data Access
TCGA Core Sample Set (n = 173 total cases)			
Whole-exome DNA sequencing	Illumina; SureSelect v2	173	controlled: BAM files open: somatic mutations
DNA copy number	Affymetrix SNP 6.0	173	controlled: CEL files open: copy number
mRNA sequencing	Illumina	173	controlled: BAM files open: expression
miRNA sequencing	Illumina	173	controlled: BAM files open: expression
CpG DNA methylation	Illumina Infinium HM 450	173	open
Reverse-phase protein array	Aushon Biosystems 2470; Canoscan 9000F	76	open

*RET*, and *CSDE1* (MutSig2 [Lawrence et al., 2013]  $q < 0.05$ ; Figure 1; Table S3). The majority of these mutations were clonal (Carter et al., 2012). Somatic *HRAS* mutations clustered at the Q61 hotspot, known to activate the mitogen-activated protein kinase (MAPK) signaling pathway (Crona et al., 2013). Somatic *EPAS1* (HIF2 $\alpha$ ) mutations occurred at hotspots (A530, P531, and Y532) associated with increased HIF stabilization and transcriptional activity (Zhuang et al., 2012). Analysis for known hotspots and cancer-relevant genes identified *BRAF* (p.G469A), *IDH1* (p.R132C), *FGFR1*, *VHL*, *ATRX*, *TP53*, *SETD2*, and *ARNT* mutations.

We observed that three genes with pathogenic germline mutations also had somatic mutations in the cohort: *RET*, *NF1*, and *VHL*. *RET* mutations occurred in distinct protein coding regions with germline mutations clustered at codon C634 in the extracellular domain and somatic mutations clustered at codon M918 in the intracellular tyrosine kinase domain ( $p < 0.001$ ; Figure 2A), similar to the pattern seen in medullary thyroid carcinoma (Figlioli et al., 2013). *RET* was significantly overexpressed in mutated tumors, both germline and somatic, relative to wild-type tumors ( $p < 0.003$ , Figure 2B). In contrast, *NF1* and *VHL* mutations did not display positional tendencies based on somatic or germline origin.

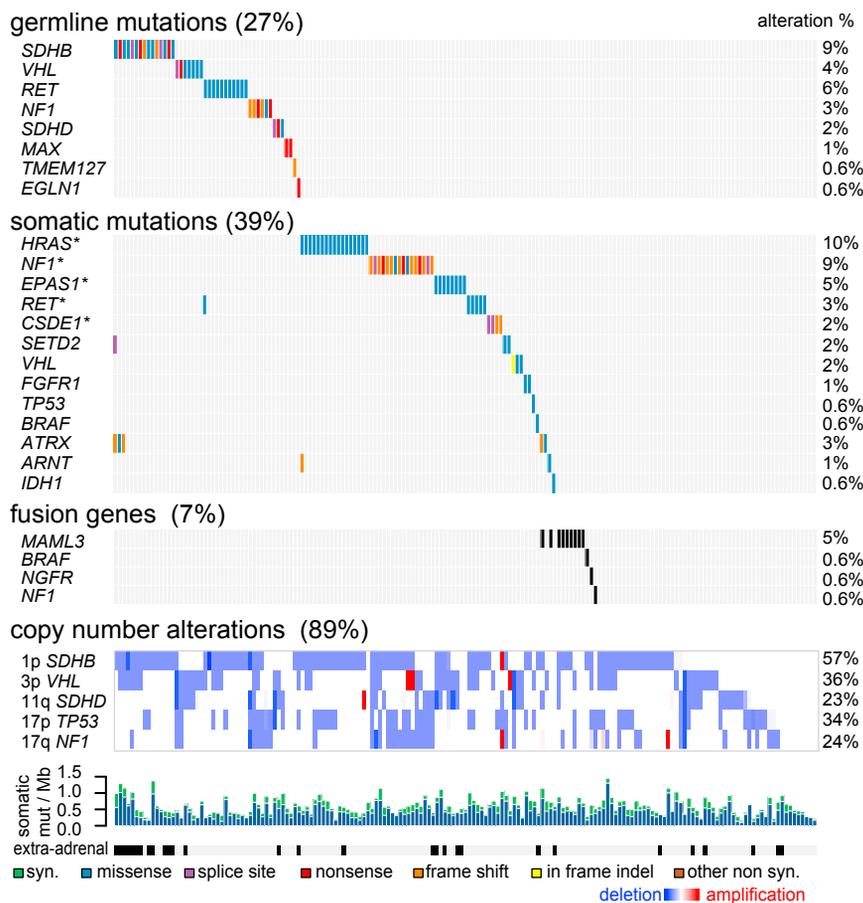
Considering the genes with pathogenic germline mutations or significant somatic mutations (i.e., the 18 genes in Figure 1), there was striking mutual exclusivity among mutations ( $p < 1 \times 10^{-4}$ ), indicating that tumors typically have at most one mutation in this gene set. Only four tumors had both germline and somatic mutations in these genes: three tumors had both *SDHB* germline and *ATRX* somatic mutations, a previously reported association (Fishbein et al., 2015), and one tumor had both *RET* germline (p.V804M) and somatic (p.M918T) mutations. Germline-mutated PCCs/PGLs also possessed somatic copy-number events, indicating that these tumors are clonally derived (Figure 1). As expected, germline mutation in *NF1*, *SDHB*, or *VHL* tended to co-occur with somatic copy number deletion of the respective locus.

This study found that the *Cold Shock Domain-containing E1* gene (*CSDE1*) is significantly mutated in PCCs/PGLs. *CSDE1*,

formerly known as *UNR*, is required developmentally and affects translation initiation, RNA stability, cell-type-specific apoptosis, differentiation, and neuronal development (Kobayashi et al., 2013; Mihailovich et al., 2010). Four tumors contained *CSDE1* mutations, two frameshift, and two splice-site mutations, which clustered proximally within the gene. Analysis of tumor mRNA sequencing (Wilkerson et al., 2014) confirmed that both tumors with splice-site mutations had distinctive splicing alterations (Figure 2C). One tumor had an acceptor site mutation that resulted in intron retention and a truncated protein sequence (593 versus 844 amino acids) (right purple triangle in Figures 2C and S1A). An additional tumor had a mutation in an intron donor site, resulting in upstream exon skipping (left purple triangle in Figures 2C and S1A) and a transcript with a small, in-frame protein sequence truncation (789 versus 844 amino acids). Multi-platform integration revealed that *CSDE1*-mutated tumors had marked genomic deletion and underexpression of the gene, supporting a loss-of-function role (Figure 2D). Located at 1p13.2, *CSDE1* provides a possible target of the broad 1p deletion observed in PCCs/PGLs in addition to *SDHB* loss. Finally, comparing the expression profiles of *CSDE1* mutant PCCs/PGLs with published microarrays of *Csde1* knockout in mouse embryonic stem cells (Dormoy-Raclet et al., 2007; Elatmani et al., 2011) revealed significant correlation, supporting the functional role of *CSDE1* mutations in PCCs/PGLs (Figure S1B).

### Fusion Gene Discovery and Integrated Characterization

We then analyzed DNA focal copy number in PCCs/PGLs using GISTIC2 (Mermel et al., 2011). Many focal deletion peaks ( $n = 27$ ) were detected, including *NF1* (Figure 3A), as reported previously (Burnichon et al., 2012). In contrast, focal amplification peaks were sparse ( $n = 7$ ). Further analysis of the focal amplifications led to a striking finding. Ten of 16 primary tumors with focal 4q31.1 amplification also had 17q21.31 focally amplified ( $p < 3 \times 10^{-8}$ ; Figures 3B and S2A). Analysis of fusion transcripts from RNA sequencing (Wang et al., 2010) revealed that seven of these ten co-amplified tumors ( $p < 2 \times 10^{-9}$ ) possessed in-frame RNA fusion transcripts spanning the 5' portion of *UBTF* (*Upstream Binding Transcription Factor*) on 17q21.31 and the 3' portion of *MAML3* (a member of the Mastermind-like family of transcriptional co-activators [McElhinny et al., 2008]) on 4q31.1. *UBTF-MAML3* fusion-positive tumors expressed one of two mutually exclusive fusion isoforms, with the mRNA transcript starting at either exon 17 or 19 of *UBTF*, suggesting two different introns for the DNA breakpoints (Figure 3B; Table S4). RNA sequencing also identified one *TCF4-MAML3* fusion transcript, which occurred in a tumor with focal DNA co-amplification of *TCF4* on 18q21.2 and *MAML3* (Figures 3B and S2A). Using the ABRA program (Mose et al., 2014) to reassemble DNA exome sequencing, chimeric DNA of the *UBTF-MAML3* translocation was found in two of the mRNA fusion-positive tumors and in two additional tumors, including a primary/metastasis pair with the same DNA translocation breakpoint (Figures 3B and S2A–S2C). The sole adjacent normal tissue specimen from a *MAML3* fusion-positive case did not contain the fusion, supporting tumor specificity. Providing further validation, RT-PCR of tumor RNA for the product spanning the *UBTF-MAML3* fusion breakpoint confirmed both *UBTF-MAML3* isoforms (Figures S2D and S2E). In all, ten tumors were positive for a *MAML3* fusion



### Figure 1. Germline and Somatic Genome Alterations

Genomic features in rows and primary tumors (n = 173) in columns; shading indicates the effect of a mutation on protein sequence. Significant somatically mutated genes (MutSig2,  $q < 0.05$ ) indicated by an asterisk (\*). See also Tables S1, S2, and S3.

tumor type with exon 1-deleted *MAML3* fusion genes (Wang et al., 2014). *MAML3* fusion-positive tumors were not distinctive histologically. Searching for correlated molecular alterations that might point to *MAML3* fusion gene functional consequences in PCCs/PGLs, we found fusion-positive tumors to have a unique and expansive methylation profile relative to *MAML3* fusion-negative tumors (4,229 significant probes) (Figure 4A). The number of differentially methylated probes was far greater than expected by chance (352 probes;  $p < 0.002$ ; Figure S3B). The predominant effect was hypomethylation of fusion-positive compared with fusion-negative tumors. Among these probes, increasing hypomethylation was positively correlated with mRNA overexpression of corresponding target genes ( $p < 4 \times 10^{-10}$ ; Figure S3C). Analyzing *MAML3* fusion-

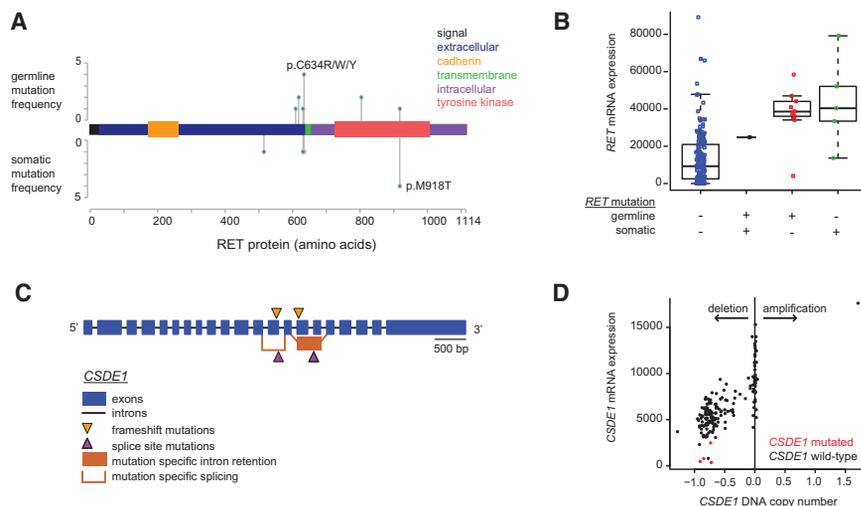
positive tumors by pathway analysis, we found that genes in developmental pathways, Wnt receptor signaling and Hedgehog signaling, were significantly overexpressed (Figures S3D and 4B), several of which were also hypomethylated (Figure S3C). By miRNA analysis, the strongest marker of the fusion-positive tumors was an underexpression of miR-375, a negative regulator of the Wnt-signaling pathway member FZD8 (Miao et al., 2015) (Figures S3E and 4B). Finally, reverse-phase protein arrays (RPPA) analysis showed that Wnt pathway members  $\beta$ -catenin, DVL3, and GSK3 were overexpressed in *MAML3* fusion-positive tumors (Figure 4B). This non-canonical association of *MAML3* with increased signaling through the Wnt pathway is also supported by a study of MAML proteins in colon cancer cell lines (Alves-Guerra et al., 2007), describing TCF target gene activation via  $\beta$ -catenin.

gene. The boundaries of *UBTF* DNA copy number amplification typically aligned with the fusion transcript location at exon 17 (Figure S2A) and the latter half of *UBTF* is not amplified, suggesting that DNA translocation preceded DNA amplification of the fusion gene. In other tumors, we identified an overexpressed *KIAA1737-NGFR* fusion gene (*NGFR* 3.0-fold overexpression versus cohort mean), an overexpressed *RUNDC1-BRAF* fusion gene (*BRAF* 5.2-fold overexpression), and an underexpressed *NF1-RAB11FIP4* fusion gene (*NF1* 9.9-fold underexpression). Interestingly, all but one of these fusion genes had a breakpoint localized to 17q (Figure 3C).

The *MAML3* fusion gene appears to be a gain-of-function event in PCCs/PGLs as fusion-positive tumors substantially overexpressed *MAML3* compared with fusion-negative tumors (2.7-fold overexpression,  $p < 5 \times 10^{-6}$ ). Furthermore, the expression pattern across the native exons suggests that the promoter of *UBTF* or *TCF4* drives overexpression of *MAML3*, with the 5' exons in *UBTF* or *TCF4* and the 3' exons of *MAML3*, overexpressed relative to exons not in the fusion product (Figure 3D).

Although *MAML3* is conventionally known as a NOTCH transcriptional co-activator, the PCC/PGL *MAML3* fusion genes do not contain the NOTCH binding site and PCCs/PGLs with *MAML3* fusion genes do not consistently overexpress NOTCH target genes (Figure S3A). These results suggest that altered NOTCH signaling is not the primary consequence of *MAML3* fusion genes, similar to another study examining a different solid

Recently, Heynen et al. (2016) found that *MAML3* overexpression plays a role in retinoic acid resistance in neuroblastoma, a developmentally related tumor type. These authors reported an 828 gene expression signature of *MAML3* activation derived from a neuroblastoma cell line transfected with an exon 1-deleted *MAML3* overexpression vector compared with the untransfected parental cell line. This truncated *MAML3* is similar to the PCC/PGL *MAML3* fusion gene. The Heynen et al. signature was highly overexpressed in *MAML3* fusion-positive PCCs/PGLs compared with PCCs/PGLs without the fusion ( $p < 3 \times 10^{-6}$ ; Figure 4C). In particular, analysis of the Heynen et al. signature revealed that many Wnt receptor and Hedgehog



**Figure 2. Integrated Alterations in *RET* and in *CSDE1***

(A) Location of somatic and germline *RET* mutations within the protein sequence.

(B) *RET* mRNA expression of mutation-positive (+) and mutation-negative (-) tumors. Boxplot horizontal lines indicate the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles, whiskers extend to the furthest point less than or equal to 1.5 times the interquartile range. Points indicate primary tumors, with horizontal jitter added to aid visualization.

(C) Mutations within the *CSDE1* gene structure.

(D) Association of *CSDE1* mRNA expression versus *CSDE1* DNA copy number; points represent primary tumors. See also Figure S1.

signaling genes, such as *WNT4*, *WNT11*, *WNT5A*, *NKD1*, and *GLI2*, were overexpressed after truncated *MAML3* activation, whereas NOTCH targets were not recurrently overexpressed. Wnt and Hedgehog signaling, thus, appear to be consequences of *MAML3* activation. Finally, Heynen et al. (2016) demonstrated that the overexpressed *MAML3* caused greater proliferation rates in the cell line model, suggesting that the *MAML3* fusion gene may be associated with an increased growth rate in PCCs/PGLs.

### Molecular Classification

To derive a molecular classification for PCCs/PGLs, we performed unsupervised consensus clustering of tumor mRNA expression profiles (Wilkerson and Hayes, 2010), detecting four statistically significant expression subtypes (SigClust [Liu et al., 2008]  $p < 0.001$ ; Figures S4A and S4B). To validate our findings, we re-analyzed an independent cohort of PCCs/PGLs (Burnichon et al., 2011) and found the same four expression subtypes, indicating that the subtypes are reproducible (Figures S4C and S4D). Next, we compared the expression subtypes by the other five genomic platforms and identified many subtype-specific molecular alterations (Figures 5A and S5). We designated the subtypes “kinase signaling,” “pseudohypoxia,” “Wnt-altered,” and “cortical admixture.” Subtypes detected from clustering analyses of other platforms (methylation, copy number, miRNA, and RPPA) were each significantly associated with the expression subtypes (Figures 5 and S5).

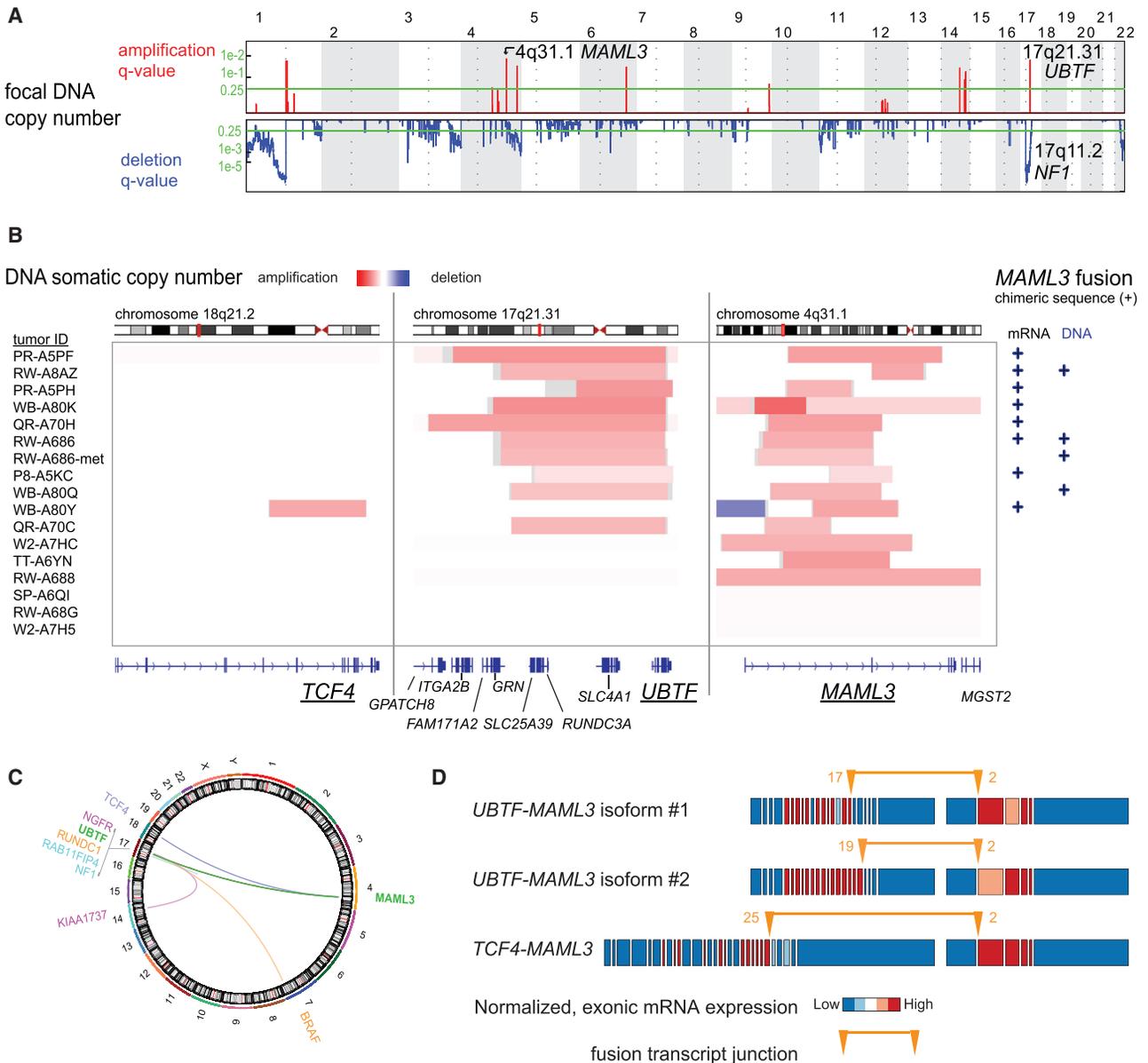
The Wnt-altered subtype consisted of adrenal PCCs and overexpressed genes in the Wnt and Hedgehog signaling pathways, such as *WNT4* and *DVL3*. This subtype also had the highest overexpression of *CHGA* ( $p < 0.002$ ), a gene relevant to chromaffin cell function. The *CHGA* product, chromogranin A, is a clinical marker of neuroendocrine tumors and is known to correlate with the presence of PCC/PGL and, to a certain degree, with the presence of metastatic disease (Bilek et al., 2008). Strikingly, this subtype contained all tumors having *MAML3* fusion genes ( $p < 4 \times 10^{-9}$ ) and three of four with *CSDE1* mutations ( $p < 0.01$ ). The strong associations of these alterations with an unsupervised expression subtype are consistent with *MAML3* fusion genes and *CSDE1* somatic mutations being important driver

events. Alterations in these genes appear to be two independent routes to activate Wnt and Hedgehog signaling in PCCs/PGLs.

As no mutations in germline susceptibility genes were observed within these tumors, the Wnt-altered subtype was specific to sporadic PCC.

The kinase signaling subtype was observed predominantly in PCCs and had the highest expression of *PNMT*, which encodes the enzyme that converts norepinephrine to epinephrine. *PNMT* expression is associated with the adrenergic phenotype of specific hereditary PCCs/PGLs (Eisenhofer et al., 2011). This subtype had somatic and germline mutations in *NF1*, *RET*, *TMEM127*, and *HRAS*, as reported previously (Burnichon et al., 2011; Castro-Vega et al., 2015), and rarer events affecting kinase signaling, including fusion genes involving *NF1*, *BRAF*, and *NGFR*. This subtype, particularly the *HRAS*-mutated subset, was enriched within protein expression (RPPA) cluster 3, which had increased expression of the RAS-MAPK signaling pathway and reduced expression of the DNA damage pathway (Figure S5A). This subtype was also enriched with DNA copy number cluster 2, as defined by 1p, 3q, and 17q deletions (Figure S5B). Nearly all (95%) *NF1* germline or somatically mutated tumors also had 17q11.2 focal deletions, the vast majority (86%) of which occurred in the kinase signaling subtype.

The pseudohypoxia subtype consisted of both PCCs (57%) and PGLs (43%), and typically had negative epinephrine and metanephrine secretion. Germline mutations in *SDHB*, *SDHD*, and *VHL*, and somatic mutations in *VHL* and *EPAS1* were completely specific to this subtype (Figure 5A), consistent with earlier studies (Burnichon et al., 2011; Dahia et al., 2005; Weller et al., 2014). In addition to this mutational profile, the pseudohypoxia subtype displayed distinctive molecular profiles on several other platforms. Most genome-doubled tumors (74%), in which nearly all chromosomes are amplified, occurred in the pseudohypoxia subtype, mostly in conjunction with *EPAS1* or *VHL* mutations (Figures 5A, 5B, and S5B). The pseudohypoxia subtype also contained two of the three unsupervised clusters of DNA methylation (hypermethylated and intermediate), confirming earlier reports (Letouze et al., 2013), with most *SDHB* and *SDHD* germline mutations occurring in the hypermethylated subtype and nearly all *VHL* and *EPAS1* mutations in the intermediate subtype. Lastly, miRNA cluster 3 was tightly associated with the pseudohypoxia subtype (Figure S5C) and



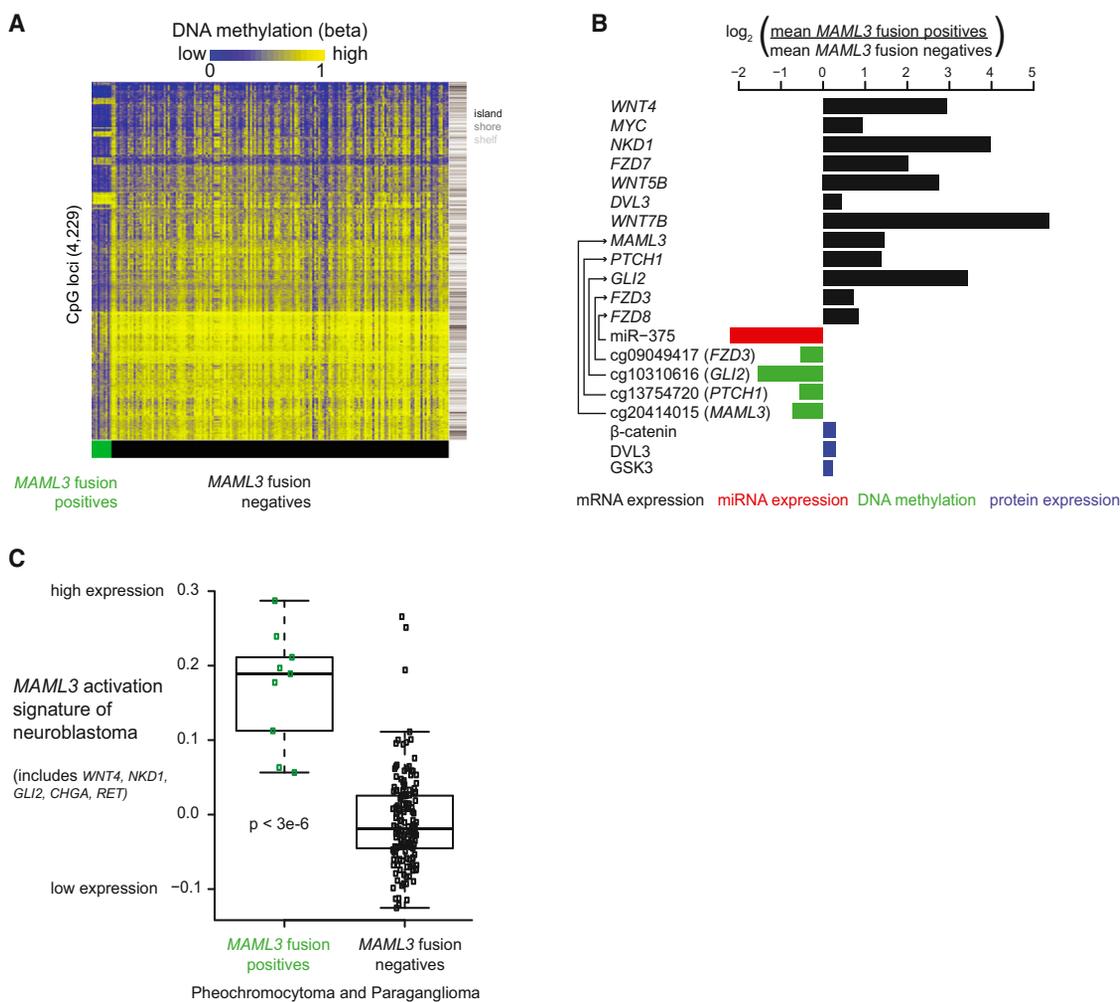
**Figure 3. Detection of Fusion Genes**

(A) Focal copy number amplifications and deletions from GISTIC2 analysis.  
 (B) DNA copy number alterations at the *TCF4*, *UBTF*, and *MAML3* loci for tumors with *MAML3* amplification; rectangles indicate DNA breakpoints with shading proportional to DNA copy number. mRNA or DNA fusion sequence positivity indicated by “+”.  
 (C) Circos diagram of mRNA fusion genes. Color denotes fusion mates.  
 (D) Exon expression diagrams for representative tumors from each *MAML3* fusion gene species. Colors indicate relative differential expression across exons. Orange arrows indicate fusion breakpoints and exon number. See also [Tables S2 and S4](#) and [Figure S2](#).

displayed overexpression of mir-210, a marker of tumor hypoxia (Huang et al., 2009).

Finally, the cortical admixture subtype overexpressed known adrenal cortex markers (*CYP11B2*, *CYP21A2*, and *STAR*) (Figure 5A). Given this, our expert pathologist re-analyzed all tumors to determine the presence of any cortical cells within the sample. A significant ( $p < 5 \times 10^{-5}$ ) association with the presence of cortical cells was found with this expression subtype (Figure S6A), but no other histological features differentiated the

expression subtypes. Tumors in this subtype had reduced tumor purity determined from DNA analysis (Carter et al., 2012), and elevated leukocyte infiltration determined from DNA methylation profiles (Figure 5A). Thus, these findings suggest the possibility of impure tumor sampling. Next, we evaluated the possible similarity of this subtype to adrenal cortex tissue by performing unsupervised mRNA and DNA methylation analysis on the pool of PCC/PGL tumors, available adjacent normal tissue specimens of the adrenal cortex, and TCGA



#### Figure 4. Molecular Correlates of MAML3 Fusion

(A) Differentially methylated probes among tumors by MAML3 fusion status.

(B) Log<sub>2</sub> ratios for select mRNA, miRNA, and DNA methylation markers (false-discovery rate [FDR] < 0.05). Log<sub>2</sub> ratios for select RPPA markers (Kruskal-Wallis tests:  $\beta$ -catenin,  $p < 0.022$ ; GSK3,  $p < 0.14$ ; DVL3,  $p < 0.18$ ). GSK3 refers to both GSK3 $\alpha$  and GSK3 $\beta$  because the antibody used interacts with both. For display, RPPA expression were increased by the minimum value of each marker to provide positive values for the log<sub>2</sub> ratio calculation. Log<sub>2</sub> ratios calculated using primary tumors. Arrows indicate regulatory relationships, i.e., methylation within a particular gene region or a miRNA binding partner.

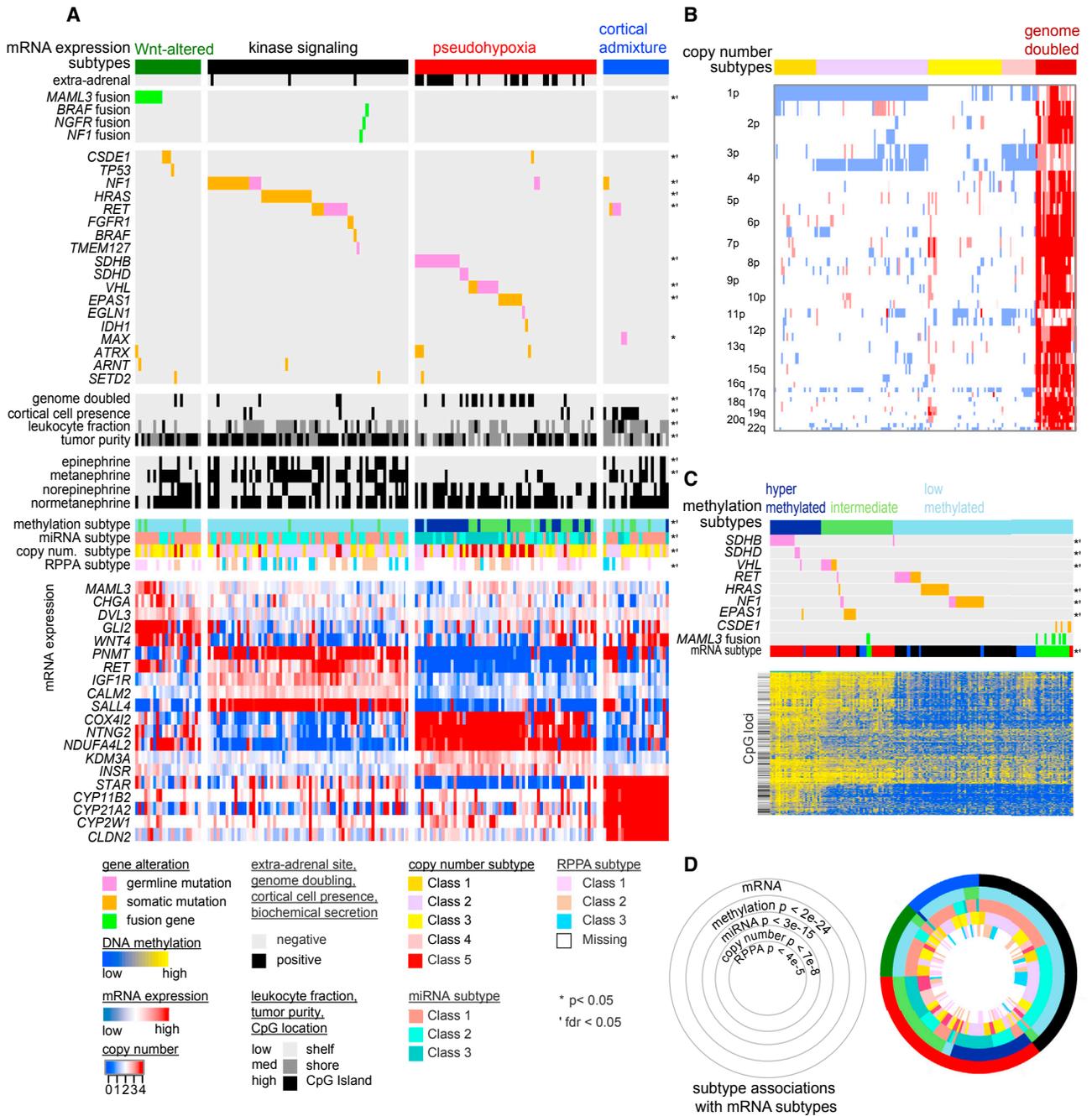
(C) Expression scores based on published MAML3 signature (Heynen et al., 2016). See Supplemental Experimental Procedures. Boxplot horizontal lines indicate the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles, whiskers extend to furthest point less than or equal to 1.5 times the interquartile range. Points indicate primary tumor values, with horizontal jitter added to aid visualization. See also Figure S3.

adrenocortical carcinomas (Zheng et al., 2016). By mRNA analysis, the cortical admixture subtype tumors overexpressed both PCC/PGL markers and adrenal cortex markers (Figure S6B). By DNA methylation analysis, cortical admixture subtype tumors typically exhibited the PCC/PGL methylation profile and not the normal adrenal cortex or adrenocortical carcinoma profiles (Figure S6C). Thus, the cortical admixture tumors have molecular features of PCCs/PGLs and are not merely defined by adrenal cortex molecular features alone. In addition, both germline mutations in MAX occurred in the cortical admixture subtype ( $p < 0.032$ ), supporting a distinct underlying biology. MAX mutation-associated PCCs have been reported to contain multiple tumor foci within one adrenal gland (Burnichon et al., 2012). We hypothesize that multi-focal disease associated with MAX

mutations also may explain the presence of interspersed cortical cells in the cortical admixture subtype.

#### Pathway Analysis

Careful manual review of the somatic and germline alterations identified signaling pathways that were disrupted in PCCs/PGLs: the kinase and hypoxia signaling pathways, Krebs cycle/electron transport, and Wnt signaling (Figure 6). The kinase signaling pathway contained alterations in NF1, HRAS, and RET, as reported previously (Burnichon et al., 2011), and we now expand this set with alterations in BRAF, FGFR, NGFR, and subunits of cAMP-dependent protein kinase A (PKA). Mutations in the subunits of PKA have been implicated in other adrenal pathologies; notable examples are PRKAR1A in Carney complex



**Figure 5. Integrated Molecular Subtypes**

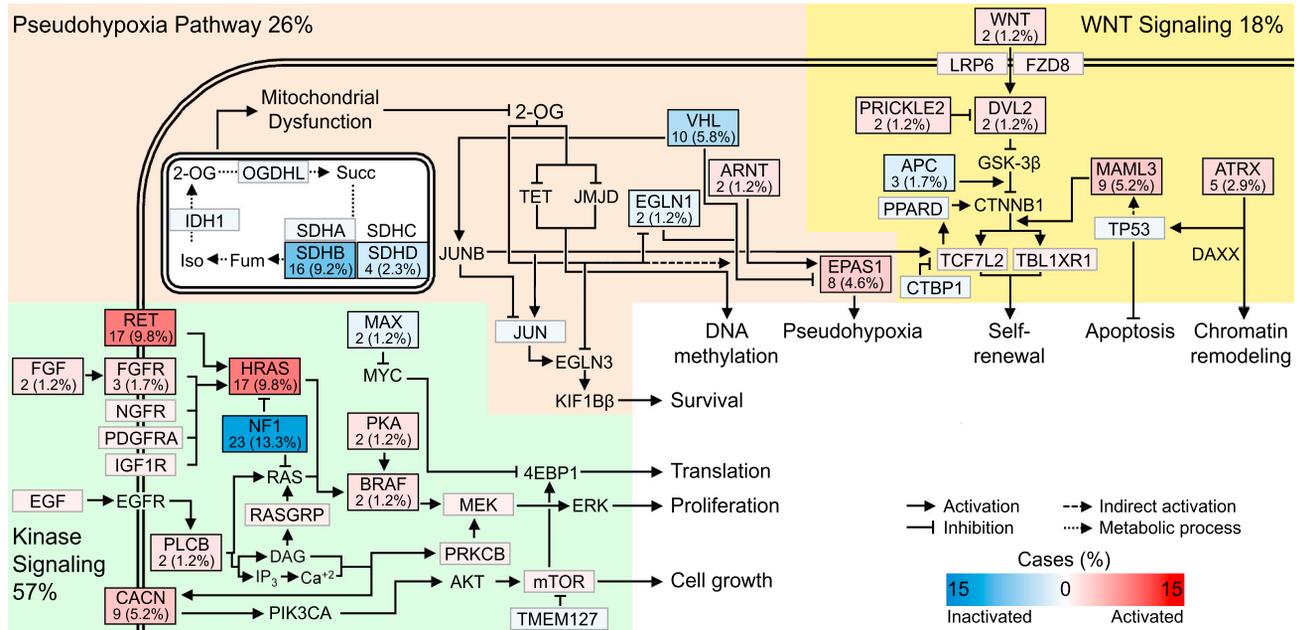
(A) mRNA subtypes. Primary tumors ( $n = 173$ ) appear in columns, and clinical and genomic features are displayed in rows. Categorical features analyzed using Fisher's exact tests; continuous features were analyzed using Kruskal-Wallis tests. Select differentially expressed genes are displayed below each subtype.

(B) DNA copy number (Carter et al., 2012) clustering. Primary tumors appear in columns ( $n = 173$ ).

(C) DNA methylation clustering. Primary tumors ( $n = 173$ ) appear in columns. Features tested for association with methylation subtypes by same method as in (A). (D) Ring plot displaying cross-platform subtype association.  $p$  prefers to chi-square tests on platform subtype versus mRNA expression subtype. See also Table S2 and Figures S4–S6.

and *PRKACA* in adrenocortical carcinoma (Berthon et al., 2015). Within the hypoxia signaling pathway, we found mutually exclusive mutations in interacting proteins, including *VHL*, *ANRT* (*HIF1 $\beta$* ), *EPAS1* (*HIF2 $\alpha$* ), and *EGLN1* (*PHD2*). Disruption of the hypoxia signaling pathway leads to a state of pseudohypoxia

that drives cell proliferation. Many tumors had mutations in the Krebs cycle genes, *SDHB* and *SDHD*, as expected, and one had an *IDH1* mutation. The *SDHx* and *IDH* mutations are predicted to impair glucose consumption and metabolism, leading to inhibition of 2-oxoglutarate-dependent histone and DNA



**Figure 6. Recurrently Altered Pathways**

Selected pathways recurrently altered by germline mutations, non-silent somatic variants, and somatic fusion genes. Pathway heading percentages reflect alteration rates in the cohort ( $n = 173$ ). Box shading reflects the alteration rate: red, activating; blue, inactivating. Protein alteration frequencies and percentages are displayed within the respective boxes. Gray boxes have alteration rates  $\leq 1\%$ . Metabolites are abbreviated (Succ - succinate, Iso - isocitrate, Fum - fumarate, 2OG - 2-oxoglutarate).

demethylase enzymes, resulting in epigenetic silencing (Yang and Pollard, 2013). Several genes in the Wnt signaling pathway were altered, with *MAML3* being the most common (Alves-Guerra et al., 2007). We also found mutations in *ATRX*, often with concurrent *SDHB* mutations. *ATRX* mutations have been reported previously in conjunction with mutations in *IDH1* (Jiao et al., 2012) and *SDHB* (Fishbein et al., 2015), suggesting that they are synergistic in tumor development.

### Clinical Outcome Associations

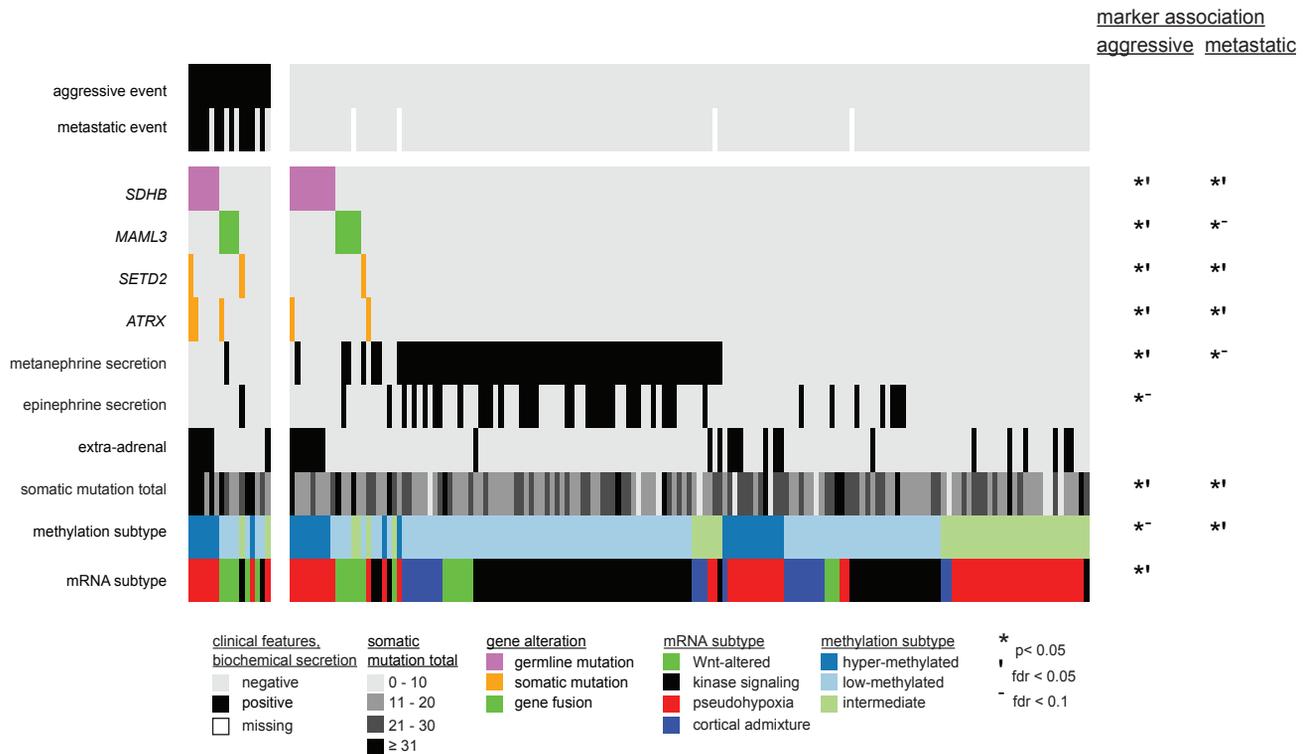
As it is both clinically important and challenging to distinguish malignant from benign PCCs/PGLs, we sought to identify molecular features associated with negative clinical events. Aggressive disease-free survival (ADFS), the time until the occurrence of distant metastases, local recurrence, or positive regional lymph nodes, was significantly associated with nine molecular markers (Figure 7). Markers associated with poor ADFS included *MAML3* fusion gene, *SDHB* germline mutation, somatic mutation in *SETD2* or *ATRX*, high somatic mutation total, mRNA subtype (Wnt-altered), and methylation subtype (hypermethylated). In contrast, plasma and/or urine metanephrine and epinephrine positivity and the kinase signaling expression subtype were associated with longer ADFS. Analysis of metastatic-free survival, the time until occurrence of distant metastases, resulted in significant associations for seven of the nine prior markers, all except mRNA subtype and epinephrine positivity, and no additional markers. Analysis of Ki-67 protein expression by immunohistochemistry in a subset of PCCs/PGLs ( $n = 62$ ) was found to positively correlate with metastatic disease (Figures S7A–S7C). Interestingly, the tumor with the highest Ki-67 expression was *MAML3*

fusion-positive (Figure S7D). In summary, our analysis confirmed *SDHB* germline mutations, *ATRX* somatic mutations, and Ki-67 expression as clinical outcome markers (Ayala-Ramirez et al., 2011; Dahia, 2014; Fishbein et al., 2015), and identified seven additional molecular markers for clinical outcome, including the *MAML3* fusion gene.

### DISCUSSION

We report a comprehensive molecular profiling with six platforms to characterize the molecular basis of PCCs/PGLs. We identified a driver mutation, fusion gene, or copy number alteration in a majority of PCCs/PGLs (95%), thus explaining the molecular etiology of most of the cohort. We report several additional driver alterations in PCCs/PGLs, including *CSDE1* mutations and *MAML3* fusion genes. In particular, this study identified recurrent DNA translocation and fusion genes as a component of PCC/PGL tumorigenesis. The mechanisms underlying PCCs/PGLs are astonishingly diverse, with both inherited and somatic drivers influencing tumorigenesis through a broad range of biological pathways. This heterogeneity is elegantly captured in the four expression subtypes, with the Wnt-altered and cortical admixture subtypes extending previous classifications. Finally, our analysis expanded markers of aggressive disease, including *MAML3* fusion genes.

Based on our results, *MAML3* fusion genes are an important molecular alteration in PCC/PGL tumorigenesis. The tumors with *MAML3* fusions lacked other driving alterations and were associated with a specific expression subtype, Wnt pathway activation, DNA hypomethylation, and poor clinical outcome.



**Figure 7. Molecular Discriminants of Clinical Outcome**

Primary tumors appear in columns (n = 173). Molecular and clinical features appear in rows. Somatic mutation total is the number of somatic mutations in a tumor. Marker and outcome associations were determined by log rank tests (p). See also [Figure S7](#).

The DNA hypomethylation profile may be a consequence of widespread, aberrant *MAML3* binding to the genome and promotion of gene expression. This hypothesis is supported by the inverse correlation of methylation and gene expression of these tumors at the same loci. Although our data support that *MAML3* is the target of the fusion event, we cannot exclude tumorigenic properties specifically conferred by the upstream fusion partners, *UBTF* or *TCF4*.

Fusion genes involving mastermind family members have been reported in other tumor types ([Amelio et al., 2014](#); [Enlund et al., 2004](#); [Wang et al., 2014](#)). In biphenotypic sinonasal sarcoma, *PAX3-MAML3* fusion genes had the same *MAML3* breakpoint as in PCC/PGL and were not associated with increased expression of NOTCH target genes ([Wang et al., 2014](#)). On the other hand, the *CRTC1-MAML2* fusion gene in mucoepidermoid carcinomas did affect NOTCH signaling ([Enlund et al., 2004](#)) and also had a gain-of-function interaction with Myc ([Amelio et al., 2014](#)), consistent with *MYC* overexpression in *MAML3* fusion-positive PCCs/PGLs. Future work may illuminate whether mastermind fusions in other tumor types lead to Wnt signaling pathway upregulation.

Truncating mutations in *CSDE1* emerged as a driver of PCC/PGL tumorigenesis, with integrated analysis indicating a tumor suppressor role. To our knowledge, *CSDE1* has not been described as a driver gene in any cancer type nor been previously associated with the Wnt signaling pathway. Querying a cancer mutation database ([Cerami et al., 2012](#)) for *CSDE1* returned rare truncating mutations in other tumor types, suggesting that *CSDE1* may have a driver role in other cancers.

Our results provide significant, clinically relevant information by confirming and identifying several molecular markers (including germline mutations in *SDHB*, somatic mutations in *ATRX*, and fusions involving *MAML3*) that were associated with an increased risk of aggressive and metastatic disease. The molecular alterations described herein also may serve as potential drug targets. For example, *SDH*-mutant tumors have high levels of glutamine, and glutaminase inhibitors ([Gross et al., 2014](#)) are currently being evaluated in NCT02071862. As the *MAML3* fusion genes activate Wnt signaling, downstream inhibitors, such as antagonists of  $\beta$ -catenin (PRI-274) ([Lenz and Kahn, 2014](#)) and STAT3 (BB1608), merit investigation. Cancers with alternative lengthening of telomeres associated with loss of *ATRX* have been shown to be sensitive to ATR inhibitors ([Flynn et al., 2015b](#)). Finally, US Food and Drug Administration-approved targeted therapies are available for patients whose tumors carry *VHL*, *RET*, *BRAF*, *EPAS1*, and *FGFR1* mutations. In summary, our comprehensive characterization significantly advances the molecular understanding of PCCs/PGLs and enables the advancement of precision medicine for these rare diseases.

## EXPERIMENTAL PROCEDURES

### Samples and Clinical Data

PCC/PGL tumor tissue, normal tissue, and blood samples were obtained from patients with informed consent and with approval from local institutional review boards at tissue source sites (see [Supplemental Experimental Procedures](#)). Cases with neoadjuvant treatment were excluded. Head and neck PGLs were not included because such tumors are often embolized prior to surgery

leaving excessive necrotic tumor tissue that is insufficient for molecular analysis. Adjacent normal tissues were at least 2 cm away from the tumor, mostly in the adrenal cortex. An expert endocrine pathologist (A.S.T.) reviewed frozen sections to confirm PCC/PGL diagnosis and to determine the presence of any cortical cells in the tissue.

Clinical records were analyzed by an expert subcommittee to assign clinical outcome events by consensus definitions. Tissue source sites were contacted to clarify ambiguities when needed. Metastatic events were defined as the occurrence of distant metastases in anatomical locations where chromaffin tissue is not normally present, as per WHO definition (DeLellis et al., 2004). Aggressive disease events were defined by the occurrence of distant metastases, positive regional lymph nodes, or local recurrence. In total, 16 cases were clinically aggressive, with 11 cases having distant metastases.

### Molecular Analysis

DNA and RNA were collected from tissue specimens using the AllPrep Kit (QIAGEN). Tissue specimens were assayed by DNA exome sequencing, mRNA sequencing, DNA methylation microarrays, miRNA sequencing, DNA copy number microarrays, and RPPA. Analysis details are described in the Supplemental Experimental Procedures section. Data are available at <https://gdc.cancer.gov/> and [https://tcga-data.nci.nih.gov/docs/publications/pcpg\\_2016](https://tcga-data.nci.nih.gov/docs/publications/pcpg_2016).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2017.01.001>.

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M.D.W., K.P., and K.L.N. jointly led the PCPG Analysis Working Group. M.D.W. coordinated overall analysis. L.F. served as data coordinator. T.M.L. coordinated clinical data. T.J.G., S.L.A., and A.S.T. performed the pathological analysis. V.W., S.R.J., and M.D.W. coordinated RNA sequencing analysis. A.R.J., A.L.A., L.M., and M.D.W. performed *MAML3* fusion gene validation analysis. I.L. and M.D.W. coordinated the DNA sequencing analyses. K.L.N., L.F., I.L., T.E., and M.D.W. performed the germline mutation analysis. I.L. and B.A.M. coordinated copy number analysis. A.G.R. coordinated miRNA sequencing analysis. L.D. coordinated DNA methylation analysis. S.L. coordinated RPPA analysis. L.F., A.A.d.C., B.W., and W.K.R. coordinated pathway analysis. K.L.N., L.F., K.P., A.S.T., H.K.G., T.E., W.K.R., T.J.G., S.L.A., A.P.G.R., and E.K. contributed clinical analysis. K.L.N., M.D.W., and L.F. wrote the manuscript, which all authors reviewed.

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