Cell

Deterministic evolutionary trajectories influence primary tumor growth, TRACERx Renal

--Manuscript Draft--

Manuscript Number:	CELL-D-17-02186R3
Full Title:	Deterministic evolutionary trajectories influence primary tumor growth, TRACERx Renal
Article Type:	Research Article
Keywords:	renal cell cancer, cancer evolution, intratumour heterogeneity, deterministic evolution, evolutionary contingency, evolutionary subtypes, metastasis, chromosome instability
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Abstract:	The evolutionary features of clear-cell renal cell carcinoma (ccRCC) have not been systematically studied to date. We analysed 1206 primary tumour regions from 101 patients recruited into the multi-centre prospective study, TRACERx Renal. We observe up to 30 driver events per tumour, and show that subclonal diversification is associated with known prognostic parameters. By resolving the patterns of driver event ordering, co-occurrence and mutual exclusivity at clone level, we show the deterministic nature of clonal evolution. ccRCC can be grouped into seven evolutionary subtypes, ranging from tumours characterised by early fixation of multiple mutational and copy number drivers, and rapid metastases; to highly branched tumours with >10 subclonal drivers and extensive parallel evolution, associated with attenuated metastases. We identify genetic diversity and chromosomal complexity as determinants of patient outcome. Our insights reconcile the variable clinical behaviour of ccRCC, and suggest evolutionary potential as a biomarker for both intervention and surveillance.
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9th March 2018

Dr Yiaying Tan

Senior Editor

Cell

Dear Dr Tan,

TRACERx Renal: Deterministic evolutionary trajectories govern primary tumour growth

I am pleased to enclose our updated manuscript with the following changes to comply with production requierments:

1. The total character count including the title, author list and affiliations, Summary, Introduction, Results, Discussion, Author Contributions, Acknowledgements, References, and main figure legends but excludes STAR Methods text and supplemental item legends is 68,863.
2. The total number of Supplemnetray items is 14 of which 9 are supplementary figures, and 5 are supplementary tables. Please note that on fo the supplemnetray figures (a PDF) has multiple pages.
3. Consortia section is included.
4. Our figures meet the Cell criteria.
5. Graphical abstract is included.
6. DOI is included.
7. Key Resources Table is included.
8. Highlights are included separately with ETOC blurb

I hope that the manuscript is now appropriate for publication but please let me know fi anything else is required.

Thank you for your consideration

Yours sincerely,

Charles Swanton

On behalf of the TRACERx Renal consortium

Response to Reviewers

The previous version of this paper was not sent out for peer review.

Deterministic evolutionary trajectories influence primary tumour growth, TRACERx Renal

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Summary

The evolutionary features of clear-cell renal cell carcinoma (ccRCC) have not been systematically studied to date. We analysed 1206 primary tumour regions from 101 patients recruited into the multi-centre prospective study, TRACERx Renal. We observe up to 30 driver events per tumour, and show that subclonal diversification is associated with known prognostic parameters. By resolving the patterns of driver event ordering, co-occurrence and mutual exclusivity at clone level, we show the deterministic nature of clonal evolution. ccRCC can be grouped into seven evolutionary subtypes, ranging from tumours characterised by early fixation of multiple mutational and copy number drivers, and rapid metastases; to highly branched tumours with >10 subclonal drivers and extensive parallel evolution, associated with attenuated metastases. We identify genetic diversity and chromosomal complexity as determinants of patient outcome. Our insights reconcile the variable clinical behaviour of ccRCC, and suggest evolutionary potential as a biomarker for both intervention and surveillance.

Keywords: renal cell cancer, cancer evolution, intratumour heterogeneity, deterministic evolution, evolutionary contingency, evolutionary subtypes, metastasis, chromosome instability

Introduction

Renal cell carcinoma (RCC) is the 7th most frequently diagnosed malignancy (Znaor et al., 2015), with a rising incidence in the developed world (Smittenaar et al., 2016). The most common histological subtype, clear cell (ccRCC), is associated with a wide range of clinical outcomes. Around one third of patients with localised ccRCC relapse following surgery, with tumour size, grade and necrosis (Leibovich et al., 2003), the presence of vascular and/or fat invasion (da Costa et al., 2012) and sarcomatoid differentiation (Zhang et al., 2015) shown to impact the risk of recurrence. While these parameters are useful for patient counselling and stratification into follow-up and adjuvant studies, their predictive accuracy is inexact. Solitary metastasis at relapse may be amenable to surgery (metastasectomy) or local therapy (e.g. ablation) on case-by-case basis (Bex et al., 2016). Patients relapsing with multiple but low volume, slow-growing metastases can be observed initially, but the risk of deferring systemic therapy remains unclear (Rini et al., 2016). Up to 30% of patients present with metastatic disease from the outset. In select cases, primary surgery is used with cytoreductive, or, if combined with complete metastasectomy, with curative intent. Patient selection for such interventions remains under intense debate, as does the management of small renal masses (SRMs, renal lesions <4 cm in size). Increasing use of abdominal cross-sectional imaging has led to incidental discovery of SRMs, majority of which have favourable natural history, leading to concerns about over-treatment (Welch, 2017). At present, molecular profiling does not impact decision making in any of these clinical scenarios.

The molecular landscape of ccRCC was elucidated by a number of next generation sequencing studies (Cancer Genome Atlas Research, 2013; Dalgliesh et al., 2010; Sato et al., 2013; Scelo

et al., 2014; Varela et al., 2011) which revealed frequent inactivation of the *VHL* tumour suppressor gene, alterations in the SWI/SNF complex (Varela et al., 2011), histone-modifying genes (Dalgliesh et al., 2010) and the PI3K/AKT/mTOR pathway (Cancer Genome Atlas Research, 2013; Sato et al., 2013; Scelo et al., 2014). Recurrent arm level or focal losses are observed on chromosomes 1p, 3p, 4q, 6q, 8p, 9p, and 14q, and gains on chromosomes 1q, 2q, 5q, 7q, 8q, 12p and 20q (Beroukhim et al., 2009; Cancer Genome Atlas Research, 2013). We previously reported significant mutational and SCNA intratumour heterogeneity (ITH) in ten cases of advanced ccRCC (Gerlinger et al., 2014; Martinez et al., 2013), showing that single biopsy analyses may miss important alterations or misclassify them as clonal due to the "illusion of clonality". This hinders our understanding of tumour evolution as well the validation of biomarkers and therapeutic targets. To date, attempts to molecularly classify ccRCC have included single biopsy analyses of mutations (Hakimi et al., 2013; Kapur et al., 2013; Sato et al., 2013) or global gene expression and methylation (Cancer Genome Atlas Research, 2013; Chen et al., 2016).

To aid an evolutionary classification of RCC we established a multi-centre prospective longitudinal cohort study, TRAcking renal cell Cancer Evolution through Therapy (TRACERx Renal, clinical trials no NCT03226886), with a protocol-specified endpoint of examining the association of ITH with disease stage and clinical outcomes through multi-region genomic profiling of primary tumours (Turajlic and Swanton, 2017). The TRACERx Renal program began recruitment in July 2012, enrolling patients undergoing nephrectomy (with curative or cytoreductive intent) for suspected or confirmed renal cell carcinoma (**STAR Methods**), with a target accrual of 320 patients with ccRCC. We report our interim findings of the patterns of

ITH, clonal evolution, and tumour progression in the first 101 patients with the diagnosis of clear cell non-familial RCC (for full inclusion criteria for this cohort see **STAR methods**).

Results

Intratumour heterogeneity of driver events in primary ccRCC

Clinical annotation of the 101 patients under study is provided in **Table S1**. Demographic and stage distribution were consistent with the referral patterns of the participating centres. All the samples were profiled using a bespoke sequencing panel targeting ~110 putative ccRCC driver genes (**STAR Methods**: Driver Panel; **Figure S1A:** CONSORT diagram). This approach enabled us to maximise the sequencing depth, a critical factor for correctly inferring evolutionary trajectories (Noorbakhsh and Chuang, 2017). Single nucleotide variants (SNVs), dinucleotides variants (DNVs), small insertion and deletions (INDELs) and somatic copy number alterations (SCNAs) were successfully derived from 1206 regions across 106 primary tumours (median 7 (range 3-75) regions per tumour) from 101 patients, as five patients donated pairs of primary tumours. Within the same cohort, 107 regions from 17 tumours were profiled by whole exome sequencing (WES), 81 regions from 27 tumours by whole genome sequencing (WGS), with six further tumours from the broader TRACERx Renal cohort also profiled by WGS (**Figure S1B**).

Median sequencing coverage across 1206 tumour regions profiled by the Driver Panel was 612x (range 105-1520x). We identified a total of 740 somatic mutations including 538 SNVs (440 non-synonymous SNVs), 7 DNVs and 195 INDELs (**Table S2**). We specifically considered non-silent mutations in high-confidence ccRCC driver genes (termed *driver mutations*, annotated in **Figure 1A; STAR Methods**). The median number of driver mutations was 3, range 0-15 per tumour (**Figure 1A**). *VHL* mutations were the only consistently clonal event, present

in 77/106 tumours (**Figure 1A)**. *VHL* was methylated in 17 additional tumours (**Figure 1A, Figure S2**). One tumour harboured a mutation in the *TCEB1* gene, a part of the *VHL* complex (Hakimi et al., 2015) (**Figure 1A**), thus 90% (95/106) of the tumours harboured clonal disruption of the *VHL* pathway. 4/11 *VHL* wild type tumours (K206, K228, K427 and K446, **Figure 1A**) had evidence of sarcomatoid differentiation (**Table S1A**), a feature reported to be associated with a lower frequency of *VHL* mutations (Malouf et al., 2016; Wang et al., 2017). K255, another *VHL* wild-type tumour, had evidence of both clear cell and papillary histology, and we observed SCNAs specific to both, including gains of 5q and 16 (**Figure S3**). We observed no mutations in the known ccRCC driver genes in K110 (**Figure 1A**), but the copy number profile, involving whole chromosome losses on 1, 6, 10 and 17, was consistent with chromophobe RCC (Davis et al., 2014). Additional pathology review confirmed chromophobe histology and K110 was removed from all subsequent analyses.

The overall frequency of driver mutations was higher in our cohort compared to the published single biopsy studies (Cancer Genome Atlas Research, 2013; Sato et al., 2013; Scelo et al., 2014) (**Figure 1B**). Notably, the frequency of *VHL* mutations in our and Scelo studies was higher than that reported in the TCGA and Sato studies, potentially due to the higher overall number of *VHL* INDELs called (**Figure 1B).** *VHL* INDELS in the TRACERx Renal cohort were confirmed by Sanger sequencing (**Figure S2**). The higher frequency of mutations in other driver genes was due to the detection of subclonal events through multi-region profiling in our cohort (**Figure 1B**).

An important goal of the TRACERx Renal study is to determine the contribution of SCNAs to clonal evolution. Recurrent SCNAs occur at a limited number of genomic sites in ccRCC (Beroukhim et al., 2009; Cancer Genome Atlas Research, 2013), usually as whole chromosome

or chromosome arm events; and the rate of genome doubling in ccRCC is low (Zack et al., 2013). Therefore, recurrent SCNAs can be reliably detected by the Driver Panel, as shown by the high level of concordance with WGS results (**Table S2**). We measured the fraction of the tumour genome affected by SCNAs using the weighted genome instability index (wGII) (Endesfelder et al., 2014), taking the maximum observed wGII score across all regions per tumour. Maximum values were utilised in order to capture the highest risk, and hence most clinically relevant, subclones in each tumour (**STAR Methods**). Median wGII in the TRACERx Renal cohort was 32.8% (range 4.7% - 97.4%). All SCNAs were annotated using previously defined cytobands (Beroukhim et al., 2009) to quantify *driver* SCNAs (**Figure 1A, STAR Methods**). In total, we detected 751 driver SCNAs; median 7, range 1-14 per tumour (**Figure 1A**).

Loss of chromosome 3p, which is pathognomonic with ccRCC and encompasses four commonly mutated genes (*VHL, PBRM1*, *SETD2* and *BAP1*), was observed in all but five tumours (K021, K375, K354, K255, K114R; **Figure 1A**). Three had clonal 3p copy neutral allelic imbalance (CNAI) (**STAR methods**) (K021, K375, K354, **Figure S4**), consistent with biallelic inactivation of mutated 3p driver genes. Driver SCNA 3p25.3 (which contains the *VHL* locus) was subclonal in five tumours: one with a *VHL* mutation (K252, **Figure 1A),** one with *VHL* methylation (K070, **Figure 1A**); one *VHL* wild type with a bi-allelic *SETD2* mutation (K427, **Figure 1A**); and two with no mutations in any of the 3p genes (K169, K446, **Figure 1A**).

The overall frequency of driver SCNAs was higher compared to the published single biopsy studies (Cancer Genome Atlas Research, 2013; Sato et al., 2013; Scelo et al., 2014) due to the detection of subclonal SCNAs in our cohort (**Figures 1C**). Notably, the frequency of SCNAs with reported prognostic significance, such as loss of chromosomes 14q and 9p, and gain of

chromosomes 8q and 12p is markedly underestimated in single biopsy studies (Cancer Genome Atlas Research, 2013). Overall ITH was measured as an index (ITH index = # subclonal drivers / # clonal drivers, where "drivers" include all driver mutations and driver SCNAs shown in **Figure 1A** (**STAR methods**)). Median ITH index value was 1, with a high variability across the cohort (range 0-13.5; standard deviation = 2.16).

Clonal evolution and clinical variables in ccRCC

ccRCC prognostic variables include primary tumour size, overall tumour stage (TNM), Fuhrman grade and the presence of necrosis. Overall, the number of driver events was significantly associated with all of these parameters, with the associations specific to subclonal, and not clonal events (**Figure S5**). Similarly, higher ITH index values were associated with advanced tumour size, stage and grade (**Figure S5)**. Clonal ordering techniques (see **STAR methods**) were used to infer clonal structures and driver phylogenetic trees (**Figure 2, Figure S3**). The median number of clones detected was 4 per tumour (range 1-23). Clone number increased with tumour stage and grade (**Figure S5**), but showed a non-linear association with tumour size, initially increasing in line with tumour dimensions but then plateauing at ~10cm beyond which clone number began to marginally reduce with increasing size (**Figure S6**). In conclusion, known prognostic parameters are associated with an increasing repertoire of driver alterations and subclonal driver diversification in ccRCC.

Convergent Evolution

We profiled three patients with synchronous bilateral ccRCCs and two patients with multifocal ccRCCs, with no family history of ccRCC, or germline mutations in the known ccRCC

predisposition genes (**Table S1A**). All five tumour pairs evolved independently, but converged on the VHL pathway. K265, K352, and K334 harboured distinct mutations in *VHL* and 3p loss events in each of the tumours (**Figure 1A, Figure S7).** The right-sided K097 tumour harboured a *VHL* mutation and *VHL* was methylated in the left tumour (**Figure 1A, Figure S2**). Left K114 tumour harboured a *VHL* mutation and 3p loss, while in the right tumour we detected a *TCEB1* mutation with the loss of 8q21.11, encompassing the *TCEB1* locus (**Figure 1A, Figure S7).** K150 tumour was presumed to be a contralateral renal metastasis from a previously resected left high-risk ccRCC. However, the two tumours had distinct *VHL* mutations (**Figure S2**) implying a case of bilateral metachronous ccRCCs. Our findings illustrate the importance of molecular profiling of patients presenting with multiple renal tumours to guide appropriate clinical management.

Parallel evolution

We and others have reported parallel evolution of mutations in the same genes or pathways within distinct tumour subclones in ccRCCs (Brastianos et al., 2015; Gerlinger et al., 2014). In the TRACERx Renal cohort, 13% of untreated primary tumours had evidence of parallel evolution, with *SETD2, BAP1* and *PTEN* (all $p<0.05$, False Discovery Rate (FDR) < 0.1, **Figure 3**) significantly enriched for parallel evolution, corrected for the number of profiled regions. Certain tumours were notable for the number of parallel events they harboured, for example K243 had 10 distinct *SETD2* mutations (**Figure 3**). In tumour K448, we observed 5 distinct *BAP1* mutations, and 3 *SETD2* mutations, but *BAP1* and *SETD2* mutations never co-occurred within the same clone.

We recently identified parallel evolution of SCNAs in non-small cell lung cancer (Jamal-Hanjani et al., 2017) through mirrored subclonal allelic imbalance (MSAI, **Figure S4**). We analysed the incidence of MSAI in a subset of TRACERx Renal patients where whole genome or exome sequencing data were available (n=41) (**STAR Methods**) and observed MSAI events in 15/41 tumours (**Figure S4**, **STAR Methods)**, a subset of which were validated by an orthogonal method (**Figure S4).** Parallel loss of chromosome 14q was the most common event (4 patients, **Figure S4**), encompassing the ccRCC tumour suppressor *HIF1A* locus (Shen et al., 2011).

Identification of conserved ccRCC evolutionary features

To understand the constraints of ccRCC evolution we analysed conserved patterns of driver event co-occurrence, mutual exclusivity and timing to identify statistically significant patterns. We utilised the clonal/phylogenetic hierarchy determined for each case (**STAR methods**), in order to accurately place driver events within the same tumour subclone, and establish the relative ordering of driver events across the evolutionary path of each tumour.

In our analyses of event co-occurrences at the clone level (**STAR methods**) we observe an enrichment for mutual exclusivity between *BAP1* and *SETD2/PBRM1* mutations (**Figure 4A**). However, at a patient level these events were found to co-occur (**Figure 1A**), often in separate spatially distinct major tumour subclones (e.g. K153, **Figure S3**). *BAP1* had a propensity for being a lone additional mutational driver event in *VHL*-mutant clones, whilst *PBRM1* and *SETD2* were enriched for mutual clonal co-occurrence. Due to limited sample size these patterns did not reach formal significance, however we note the results are in agreement with previously published patient-level meta-analysis (Pena-Llopis et al., 2013). Of all the driver mutations, *BAP1* was associated with the highest number of driver SCNAs in the same clone

(**Figure 4A, Figure S8**, p=0.032 for *BAP1* versus no mutational drivers), consistent with its role in chromosomal stability (Peng et al., 2015). Overall, the strongest evidence for co-occurrence was found for the following pairs of driver SCNAs: 14q loss with 4q loss, 14q loss with 9p loss and 4q loss with 9p loss (**Figure 4A,** all p<0.05). These pairs of events were all found to co-occur ≥ 1.8 x more frequently than expected by chance. We validated these observations in the TCGA ccRCC data (all p<0.05, **Figure S8**), showing that the specific event pairings co-occurred together beyond the general expected correlation between SCNAs (*e.g.* for 14q loss, the most common partner event genome wide was 9p loss, **Figure S8**). We note that these SCNAs harbour well-known tumour suppressors 14q31.1- *HIF1A* (Shen et al., 2011), 9p21.3- *CDKN2A* (Beroukhim et al., 2009) and 4q- *CXXC4* (Kojima et al., 2009).

In our previous report of ten ccRCC tumours (Gerlinger et al., 2014) mutations in *VHL* and loss of 3p were consistently clonal, and *PBRM1* was an additional clonal driver mutation in three cases. In our current prospective cohort, we observed a subset of cases that harboured two or more additional clonal driver mutations, aside from *VHL*. Simulated models of tumour growth (Reiter et al., 2013) suggest that just one additional driver will significantly increase the growth rate, and we utilised WGS molecular clock timing data (see companion paper Mitchell et al. 2018) to test this hypothesis in our data. Time to presentation was calculated as the time elapsed from the emergence of the most recent common ancestor (MRCA) to clinical diagnosis. The median time to presentation from the emergence of the MRCA for cases with *VHL* as the only clonal driver mutation, (n=14 cases, 48% of the WGS cohort) was 28 [4 - 49] years. The addition of one further clonal driver mutation (n=13 cases) was associated with a shortening of time to diagnosis, to 5 [1 - 34] years, while the addition of two further clonal driver mutations (n=2 cases) shortened the time to diagnosis to 5 [4 - 7] years

(p=0.007, **Figure 4B**). Despite the shortened time of tumour growth, tumour size was found to be comparable across all the groups (**Figure 4C**), and we observed no difference in the mode of presentation (incidental versus symptomatic) across the three groups, suggesting there was no lead-time bias. Overall, the groups had the same total median number (n=3) of driver mutations (considering clonal and subclonal events). Assessment of proliferation by multiregional Ki67 immunohistochemistry (IHC) staining (**STAR Methods**) showed elevated proliferation index in cases with additional clonal driver mutations (p=0.034, **Figure 4D, Table S3**), consistent with the timing analysis.

Order of Events During ccRCC Evolution

The order in which driver events are acquired can have prognostic and therapeutic implications, as shown by Ortmann and colleagues with respect to the order of *JAK2* and *TET2* mutations in myeloproliferative neoplasms (Ortmann et al., 2015). We considered the ordering of driver events in ccRCC, assessing for recurrent patterns of driver events preceding or following one another. To conduct this analysis, we traced all possible evolutionary trajectories, starting at the base of each driver tree and tracing the path through to each terminal subclone, considering all possible sequential paths between events (**Figure 4E**). Due to the dense spatial sampling in this cohort the driver tree ordering was typically robust, with evidence of sequential waves of clonal expansion between events usually confirmed across multiple biopsy regions. In order to reduce the risks of multiple testing we limited further analyses to those trajectories containing the most frequent ccRCC driver events: *VHL*, *PBRM1, SETD2, BAP1,* PI3K/AKT/mTOR pathway mutations or driver SCNAs (**Figure 1B**). Event combinations which we observed in ten or more cases were then tested for significance in

the ordering pattern (**STAR Methods**). Six significantly conserved patterns were detected (all FDR<0.05), the first three of which confirmed *VHL* as a universally preceding event, as expected. In addition, *PBRM1* mutations were found to consistently precede PI3K pathway mutations, *SETD2* mutations and driver SCNA events (**Figure 4E**). In many of these cases the event sequences were observed exclusively in one direction, *i.e. PBRM1* precedes *SETD2* in 11 separate cases, but the opposite was never observed.

Evolutionary Subtypes

A pertinent question is whether conserved patterns of ccRCC evolution relate to distinct clinical or biological phenotypes; to investigate this in an exploratory context we classified all the tumours under study according to the patterns observed in the evolutionary order, timing and co-occurrence analyses (**Figure 4**). Seven evolutionary subtypes were defined (**Figure 5A**) using a rule based classification system (**STAR Methods**), which was validated by unsupervised clustering (**Figure S9**). Subtypes were compared across different genomic and clinical metrics (**STAR Methods**) including levels of wGII, percentage of cells positive for Ki67, ITH index, clonal structure and clinical parameters including stage, percentage of tumours that are Fuhrman grade 4 (%G4) or presence of microvascular invasion (%MVI) (**Figure 5**). The first subtype consisted of tumours with "multiple clonal drivers" (defined as ≥ two *BAP1*, *PBRM1*, *SETD2* or *PTEN* clonal mutations), and was characterised by high levels of wGII (9 out of 12 cases with wGII > cohort wide median value), enrichment for late stage disease (all cases were stage III+) and a high level of %MVI / %G4 / %Ki67. These tumours harboured a smaller number of clones (average = 5, range (1-14)) and had little evidence of ITH (1 out of 12 cases had ITH > cohort wide median value) (**Figure 5**, **STAR Methods)**. This pattern would be

consistent with sufficient selective fitness being achieved within the dominant clone through fixation of multiple driver mutations and SCNAs causing a clonal sweep at an early stage of tumourigenesis.

A second and related subtype comprised "*BAP1* driven" cases characterised by tumour clones with *BAP1* as a lone mutational driver in addition to *VHL* (**Figure 5**). Where the tumours harboured other driver mutations, they were never found in the same subclone as the *BAP1* mutation (K448, K252, K153, K136, **Figure 1** and **Figure S3**). This group was enriched for tumours with elevated wGII (8 out of 12 > median), fewer clones and a higher tumour grade (%G4). This pattern suggests that *BAP1* mutations coupled with SCNAs afford a fitness advantage such that no additional driver events become fixed making them terminal drivers within individual clones. The third subtype consisted of "*VHL* wildtype" tumours, characterised by high ki67% (highest across all groups), elevated levels of wGII, potentially compensating for a lack of driver mutations, and additional phenotypic differences such as frequent presence of sarcomatoid differentiation.

The fourth subtype were "*PBRM1→SETD2"* driven, a group characterised by highly branched trees (>10 clones per tumour; range (3-23)), the highest mean ITH score in the whole cohort, lower ki67%, frequent parallel evolution events and advanced disease stage (**Figure 5**). This pattern would be consistent with the notion of slower branched growth, with early *PBRM1* mutations followed by strong and repeated selection for *SETD2* mutations. Supporting this notion was the average time to progression (defined as time to progression following cytoreductive nephrectomy, or the time to relapse following nephrectomy with curative intent) in this group (11.7 months), which was more than twice as long as that for "multiple

clonal driver", "*BAP1* driven" and "*VHL* wildtype" tumours (4.7, 5.9 and 4.5 months respectively, not formally significant). Critically, the observed features of this subtype were independent of tumour size, with no significant difference between the highly branched "*PBRM1→SETD2*" (mean tumour size 105mm, **Table S1B**) and the more monoclonal "multiple clonal driver" subtype (mean tumour size 107mm, **Table S1B**). The fifth and sixth subtypes were "*PBRM1→*PI3K*" and "PBRM1→*SCNA", characterised by early *PBRM1* mutation followed by mutational activation of the PI3K/AKT/mTOR pathway or subclonal SCNAs, respectively, and enriched for lower grade tumours.

The final evolutionary subtype consisted of the "*VHL* mono-driver" tumours, which displayed limited branching and a monoclonal structure, with no additional driver mutations, and low wGII. The majority of tumours in this group presented at an early stage (mean tumour size 45mm) suggesting they may be an early evolutionary ancestor of the more complex subtypes described above. Small renal masses (SRMs) without evidence of vascular or fat invasion (T1a) are an increasingly common clinical entity, which can potentially be managed by active surveillance (Jewett et al., 2011). We note that the only ≤4 cm tumour that was upstaged due to the presence of renal vein invasion (K021) was in the "multiple clonal driver" category, consistent with this evolutionary path enhancing vascular invasion independent of tumour size.

Specific evolutionary subtype cases could not be assigned in 37 cases from a wide distribution of disease stages (stage I=12, II=2, III=16, IV=7). These tumours are likely to be driven by rarer evolutionary patterns not yet identifiable with current sample sizes. Several appeared to exhibit precursor subtype features, for example clonal *VHL* mutation, followed by *PBRM1*

mutation in a major subclone, that may have continued to evolve if they remained *in situ*. Further elucidation of the genomic and non-genomic drivers of evolutionary subtypes in larger datasets will be of major interest.

ITH index and saturation of ccRCC driver events

While pervasive ITH has been described in multiple tumour types, only one prospective study of multiregional tumour profiling has been reported to date (Jamal-Hanjani et al., 2017). TRACERx Renal, with 1206 primary tumour biopsies profiled across 101 ccRCC cases, affords an unprecedented opportunity to systematically explore the ITH extent. In a subset of tumours (n=15) which underwent extensive sampling (≥20 biopsies), we considered driver event (mutation and SCNA) *saturation*, measured as the proportion of events discovered with each additional tumour region profiled. Our analysis revealed a wide spectrum of saturation gradients (**Figure 6A**), highlighting the challenge of attempting to establish a biopsy count reliably applicable to all ccRCCs. Accepting this caveat, and considering all the tumours with ≥15 biopsies (n=20) we calculated the stepwise change in driver event discovery when using between 1 to 15 biopsies (**Figure 6B**). On average, two biopsies were required to detect ≥ 50% of all variants and seven for ≥ 75% variants (**Figure 6B**). As expected, these values changed markedly based on tumour ITH, with homogenous tumours (≤median ITH index) achieving ≥ 0.75 detection within four biopsies, as opposed to eight biopsies required for heterogeneous tumours (>median ITH) (**Figure 6B**). Splitting instead by evolutionary subtype, fewest biopsies were needed to reach 0.75 driver detection in the "multiple clonal driver" and "*VHL* monodriver" groups, and largest number for "*PBRM1-->SETD2*" tumours (**Figure 6C**).

We considered the utility of a radiologically guided two-site biopsy approach, for primary tumours which present as an SRM, or larger tumours without (M0) or with metastases (M1).

We down-sampled our dataset to two biopsies per tumour (**STAR methods**), and considered the mean results across all possible combinations to simulate how many subclonal driver events would be missed and how many subclonal events would be misclassified as clonal ("illusion of clonality"). For the SRM group 11/15 tumours had an average of ≤1 driver event missed and ≤1 driver event misclassified as clonal with a paired biopsy approach (**Figure 6D, panel 1)**. For larger tumours, whether metastatic or not, performance was less favourable, with the majority suffering from multiple missed subclonal drivers and/or events misclassified as clonal (**Figure 6D, panels 2&3**). For these tumours, our data suggests that a range of four to eight biopsies is required to capture the majority of events (≥75% detection), although this approach may still miss some important drivers.

Clonal evolution and clinical significance

Association of the ITH index and disease progression was a pre-defined endpoint of the TRACERx Renal study (Turajlic and Swanton, 2017). Patients whose tumours had high ITH index (>median value) had significantly reduced progression free survival (PFS), compared to those with low ITH index (p=0.0160 log-rank, hazard ratio (95% CI) HR = 2.4 [1.1 – 5.2]). Due to the small sample size the association was not significant when adjusted for known prognostic variables in a Cox proportional hazards model (p=0.4800 adjusted) (**Figure 7A**, **STAR Methods**). As elevated wGII was consistently enriched in the high risk evolutionary subtypes, we also considered its association with PFS. Patients in our cohort whose tumours had high wGII (>median value) had a non-significant trend towards shorter PFS compared to those with low wGII (p=0.0717 log-rank HR = 1.9 [0.9 - 4.0], p=0.9400 adjusted, **Figure 7A**). We further investigated ITH and wGII metrics in the larger and more robustly powered TCGA

KIRC cohort, and found both measures to be significantly associated with PFS (p=0.0021 HR = 1.9 [1.2 – 2.8] and p=0.0004 HR = 2.1 [1.4 - 3.3] respectively, log-rank). This association remained independently significant after adjusting for stage and grade (p=0.05 HR = 1.5 [1.0 - 2.3] and p=0.02 HR = 1.7 [1.1 - 2.6] respectively, adjusted, **Figure 7A**), and in addition both measures were found to be significantly associated with overall survival (OS) in an adjusted analysis (p=0.04 HR = 1.7 [1.0 - 2.7] and p=0.04 HR = 1.7 [1.0 - 2.8] respectively, adjusted, **Table S4**). We note that the single biopsy approach is likely to have reduced the sensitivity to detect ITH and subclonal SCNAs in the TCGA cohort.

Next, we considered ITH and wGII measures in combination, to ascertain if a low score in one measure but high in the other was sufficient on its own to be associated with increased patient risk. Significantly reduced survival was observed in all groups compared to “Low ITH and Low wGII”, suggesting that either driver event intratumour heterogeneity, or a homogeneous profile with high wGII (e.g. "Multiple Clonal Driver" evolutionary subtype), were the underlying factors associated with poor prognosis (TRACERx Renal 100: p=0.0019 log-rank, p=0.7500 adjusted, TCGA PFS: p=0.0025 log-rank, p=0.0041 adjusted, **Figure 7A,** TCGA OS: p=0.0001 log-rank, p=0.0040 adjusted, see **Table S4** for full TCGA Cox model results).

We finally considered whether ITH and wGII measures associated with the pattern of metastatic progression. Within our cohort, 37 patients had metastatic disease and we classified their disease progression (following cytoreductive or curative intent nephrectomy) into “rapid” or attenuated” (**Table S1B, STAR Methods**). 67% (n=9) of "Low ITH, High wGII" patients had rapid progression, as compared to 18% (n=28) in the other three groups

(p=0.0106, Fisher's exact) (**Figure 7B**). Although limited by a small number of events (n=14), overall cancer-specific survival analysis (as opposed to PFS) in our cohort also demonstrated an association between ITH / wGII metrics and patient survival (p=0.0065 log-rank). The shortest survival time was observed in the "Low ITH, High wGII" group, further highlighting the aggressive nature of homogeneous tumours with high clonal wGII, a measure reflecting early fixation of chromosomal complexity (**Figure 7C**).

Discussion

We used clonal event co-occurrence, mutual exclusivity and temporal ordering to reveal deterministic features of ccRCC evolution and infer seven evolutionary subtypes. The "multiple clonal drivers" subtype was characterised by clonal co-occurrence of drivers that are usually mutually exclusive (*BAP1* and *PBRM1*; *BAP1* and *SETD2*), pointing to their combination being both tolerated and advantageous in certain contexts. These tumours had high wGII and low diversity, suggesting high clonal fitness with limited ongoing selection or a recent clonal sweep. Despite being the largest tumours in the whole cohort they had the shortest time from the most recent common ancestor to diagnosis, consistent with a recent clonal sweep and accelerated tumour growth, due presumably to the presence of additional drivers as shown in simulated models of tumour growth (Reiter et al., 2013). We note that our findings are in keeping with the observation of an aggressive subgroup of ccRCC with the concurrent loss of expression of *PBRM1* and *BAP1*, a likely surrogate for the "multiple clonal drivers" subtype (Joseph et al., 2016). The "*BAP1* driven" subtype confirmed the tendency to mutual exclusivity between *BAP1* and *PBRM1* (Pena-Llopis et al., 2013) mutations at the clone level. The majority of these tumours had no other detectable mutational drivers, suggesting that *BAP1* mutations combined with SCNAs drive a robust clonal expansion. Accordingly, in a

recently published mouse model of ccRCC co-targeting of *VHL* and *BAP1* resulted in high grade tumours with short latency (Gu et al., 2017).

At the other end of the evolutionary spectrum the "*PBRM1→SETD2*" tumours had extensive branching, high ITH and preponderance for parallel evolution. The conserved ordering of *SETD2* and *PBRM1* mutations and the strong repeated selection of *SETD2* mutant subclones that induce a limited clonal expansion raise interesting biological questions. It is possible that this sequence of events cannot achieve broader clonal growth due to a narrow selective fitness or because it occurs after the primary tumour bulk is established. The spatial clustering of parallel *SETD2* mutations suggests a potential role for niche-specific selection, or even niche construction by the *SETD2* mutant subclones.

PBRM1 mutations are highly enriched as an early event in ccRCC, evidenced by their being clonal in 74% of cases, but also by the "*PBRM1*→PI3K" and "*PBRM1*→SCNA" evolutionary subtypes. In a mouse model of ccRCC (Gu et al., 2017) co-targeting of *VHL* and *PBRM1* led to low grade ccRCC tumours which arose late, while an aggressive phenotype was triggered by the additional disruption of *TSC1,* a component of the PI3K pathway. Thus, although *PBRM1* is frequently selected early on it appears to have a strong necessity for later subsequent driver events.

The "*VHL* wildtype" tumours were characterised by high wGII of cryptic aetiology, and were enriched for sarcomatoid differentiation, while the "*VHL* monodriver" tumours had few driver events and low wGII, and were enriched for SRMs.

The evolutionary subtype group sizes were too small for formal survival analysis and assessment in the full TRACERx Renal study cohort (target n=320) will be of significant interest. Nevertheless, the features critical in distinguishing the evolutionary subtypes,

diversity (ITH) and chromosomal complexity (wGII), were prognostic in our and the TCGA KIRC cohort. Low diversity, high wGII tumours were more likely to progress rapidly and widely, suggesting the presence of occult metastases at presentation, while heterogeneous tumours (high ITH) with or without high wGII, were more likely to have an attenuated progression pattern, often with solitary metastasis. Thus, cytoreductive nephrectomy, metastasectomy or deferral of systemic therapy may be detrimental in the low diversity/high wGII cases, and ongoing investigations will determine if the proposed classification could help to optimise the benefit from these interventions.

An increasingly important area of clinical management are SRMs, which account for almost one-half of all newly diagnosed renal masses (Kane et al., 2008). There is an ongoing debate about their treatment due the low rate of progression observed during active surveillance (Jewett et al., 2011). The majority of SRMs in our cohort had low ITH and low wGII, consistent with high cure rates achieved with early surgical intervention. The *VHL* monodriver subtype, characterised by low ITH and low wGII had a median time from MRCA to diagnosis of 30 years suggesting that such tumours have limited growth potential and could be amenable to observation. However, some SRMs in our cohort were characterised by high ITH or wGII and given the adverse molecular features these tumours potentially would progress in the absence of surgical intervention. Therefore, evolutionary classification could aid an active surveillance strategy in the context of SRMs.

The number of driver events required for tumour initiation, maintenance and progression is subject of active debate and study (Tomasetti et al., 2015). We observed an extensive repertoire of disease drivers, with up to 30 mutational and SCNA driver events detectable in a single tumour. The question remains how many biopsies are required to determine the

panoply of disease drivers. While it appears that the gain in driver detection per additional biopsy begins to decline after ~8 biopsies, in some tumours, especially the *PBRM1-->SETD2* subtype, a large number of driver events would still be missed if only ~8 biopsies are taken. Without taking into account the spatial arrangement of the tumour biopsies we note a two-site biopsy approach recovers nearly all subclonal driver events in the majority of SRMs with a moderate risk of illusion of clonality. For larger tumours, our data suggests a biopsy number in the range of four to eight is required to capture the majority of events. We recognise that in the setting of clinical practice molecular profiling of multiple biopsies will not be practical, and that alternative approaches are needed.

Our data account for a number of clinical and experimental observations in ccRCC and highlight important evolutionary principles. Clonal co-occurrence of multiple drivers resulting in a clonal sweep is consistent with the hypothesis of punctuated evolution, proposed as an alternative to phyletic gradualism by Gould and Eldredge (Eldredge and Gould, 1997); while the contribution of chromosomal complexity to an aggressive phenotype has parallels with Goldschmidt's view of macroevolution, in Material Basis of Evolution (Goldschmidt, 1940). We note however, that both micro and macro evolution influenced the clinical outcomes in our cohort, and we also acknowledge the likely contribution of non-genetic diversity. Finally, while evolutionary contingency was clearly evident in patients with multiple independent primary tumours, the deterministic nature of ccRCC evolution was illustrated by the highly conserved sequence of driver events. We conclude that an understanding of the clonal dynamics and the evolutionary potential of a tumour provide biological insight as well as a potential rationale for clinical decision making.

Consortia

TRACERx Renal Consortium

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Acknowledgments

ST and HX are funded by Cancer Research UK (CRUK) (C50947/A18176). ST, TC, JL and MG are funded by the National Institute for Health Research (NIHR) Biomedical Research Centre (BRC) at the Royal Marsden Hospital and Institute of Cancer Research (A109). JIL is funded by the Ministerio de Economía y Competitividad (MINECO, SAF2016-79847-R). MS, AS, JoL, RF, LA and LS are funded by the Royal Marsden Cancer Charity. KL is funded by UK Medical Research Council (MR/P014712/1). TBKW is funded by the European Union Seventh Framework Programme (FP7-People-2013-ITN). CS is funded by CRUK, the Rosetrees Trust, EU FP7 (259303), the Prostate Cancer Foundation, the Breast Cancer Research Foundation, the European Research Council (THESEUS), and NIHR University College London Hospitals BRC. CS, OP, DR, IC, ZS are funded by NovoNordisk Foundation (ID 16584). OP, DR, IC are funded by the National Research, Development and Innovation Office of Hungary (NVKP_16-1-2016-0004). PC is funded by the Wellcome Trust (WT088340MA). High performance computing was supported by eMedLab. We thank Eleanor Carlyle, Aida Murra, Justine Korteweg, Kim

Edmonds, Karla Lingard, Karen O'Meara, Helen Breeze, Sarah Sarker, Leonora Conneely, Naheed Shaikh, Jeremy Tai, Lesley Cooper, Linda Shephard, Susie Slater and Catherine Rogers for study support. We thank the patients and their families.

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Figure Legends

Figure 1 - Overview

Panel A is an overview of driver alterations, including SNVs, DNVs, INDELs and SCNAs, detected in 101 TRACERx Renal patients. Rectangles and triangles indicate clonal and subclonal mutations respectively. Parallel evolution mutations are annotated in orange with a split indicating >2 events. Five bilateral or multi-focal cases are shown on the right, with distinct *VHL* mutations within pairs indicated with an asterisk. Panel B shows mutational frequency in 14 key driver genes in the TRACERx Renal cohort and three single biopsy ccRCC studies (TCGA KIRC, Sato, and Scelo). Clonal mutations are shown in the darker shade, subclonal in lighter. Panel C shows the frequency of SCNAs in the TRACERx Renal cohort. Copy number gains and losses are indicated in red and blue respectively, with clonal SCNAs darker and subclonal in lighter shade. Putative driver copy number altered regions are annotated. The dotted line indicates the frequency of the same SCNAs in the TCGA cohort.

Figure 2 – Driver phylogenetic trees

Driver phylogenetic trees for each tumour (or multiple tumours from the same patient) are shown ordered by overall tumours stage. The founding clone is indicated in light blue, with subsequent sub clones shown in distinct colours. The size of each node represents the number of SCNAs detected within that subclone. Length of lines connecting tumor subclones does not contain information.

Figure 3 – Parallel Evolution

Table shows driver gene events with >10 subclonal mutations across the cohort. These genes were tested for evidence of parallel evolution using a permutation model accounting for overall gene mutation frequency and the number of biopsies per tumour (see STAR METHODS). *BAP1, SETD2* and *PTEN* were found to show significant evidence of parallel evolution ($p<0.05$, FDR<0.1). Example driver trees and accompanying tumour sampling images are presented for each significant gene: *BAP1, PTEN* and *SETD2.* Parallel events are marked on the driver trees and clone colour is matched from the tree to corresponding sampled region.

Figure 4 – Conserved features of ccRCC evolution

Panel A shows event co-occurrence analysis, with red indicating enrichment for co-occurrence and blue for mutual exclusivity. Values are $\log_2$(observed no. of co-occurrences / expected no. of co-occurrences, STAR METHODS), with significant patterns marked according to the legend. Data is shown for event co-occurrence / mutually exclusivity, in first truncal clones only per case (bottom left) and second all terminal subclones (top right) such that all clonal and subclonal interactions are considered, see STAR METHODS. P-values are calculated under a probabilistic model, as implemented in R package 'co-occur', with only interactions significant in both 'clonal' and 'clonal + subclonal' analyses are considered significant. Panel

B (first boxplot) shows molecular clock timing analysis from the whole genome sequenced cohort, with time from the most recent common ancestor (MRCA) to tumour diagnosis plotted on the x-axis. On the y-axis are cases split into three groups, based on having one, two or three clonal driver events. *VHL* wild type cases (n=2) are excluded on account of their distinct aetiological and phenotypic profile. P-value is based on Kruskal-Wallis test. (Second boxplot) shows the same y-axis patient groups but plotted on the x-axis is tumour size (mm). P-value is based on Kruskal-Wallis test. (Third boxplot) shows on the y-axis all cases from the 100-patient cohort, again *VHL* wild type cases were then excluded, and remaining cases were split into three groups based on one, two or three clonal driver mutations. Multi-region data on % of cells staining positive for proliferation marker ki67 is shown on the x-axis. P-value is based on a linear mixed effect model, to account for non-independence of multiple observations per tumour. Panel C (left) shows an illustrative schematic tree to demonstrate the method used to trace each tumour's evolutionary paths. (Right) shows results from the event ordering analysis for all pairs of events with n=10 or more observations. Plotted are the counts of instances where: event 1 was found to precede event 2, and event 1 was found to follow event 2. Significance was tested via Fisher's exact test with p-values shown after correction for multiple testing using Benjamini–Hochberg procedure.

Figure 5 – Evolutionary subtypes

Figure shows cases grouped by evolutionary subtype, with the following parameters also annotated: presence of clonal wGII (blue > median, white ≤ median), presence of subclonal wGII (blue > median, white ≤ median), ITH index score (red > median, white ≤ median) and tumour size (mm) (range [18-180], white = low, black = high). Occurrences of parallel evolution are denoted in the heatmap with "P". Plotted next is the distribution of stages per subtype, followed by grade, coloured as per the legend, and then a further six metrics are summarised as the average values for each group: i) mean number of tumour clones, ii) % of patients with grade 4 disease, iii) % of patients with microvascular invasion, iv) mean % of cells staining positive for Ki67 proliferation index (mean calculated first per cass, and then across the cohort), v) % of patients with disease relapse/progression, vi) relapse/progression time. Shown next are relapse/progression free survival plots per group and lastly shown are three example driver phylogenetic trees from each group.

Figure 6 – Intratumour heterogeneity index and saturation analysis

Panel A shows the number of tumour biopsies profiled (X-axis) versus the number of driver events (i.e. all gene mutations and SCNAs shown in Figure 1A) discovered (Y-axis) for densely sampled (20+ biopsies) cases. Panel B shows saturation curves for all cases with ≥ 15 biopsies, with biopsy number plotted on x-axis and proportion of the total driver events detected (from all biopsies) on y-axis, increasing with each additional biopsy taken. Data is shown for all cases and tumours split based on low and high ITH (above/below median). Panel C shows a boxplot summary of the absolute number (top) and proportion (bottom) of biopsies needed to detect ≥ 0.75 of driver events for tumours grouped by evolutionary subtype. Panel D illustrates the potential errors arising from a two-site biopsy approach: considering all pairs of biopsies, plotted on the X-axis is the mean number of subclonal driver events misidentified as clonal

(illusion of clonality), on Y-axis is the number of subclonal driver events missed entirely. Data is shown for three clinical scenarios left: Small Renal Masses (size < 4cm), middle: tumours treated by nephrectomy with curative intent and right: tumours treated by cytoreductive nephrectomy. The size of points within a panel is proportional to the number of biopsies available for that tumour and colours vary only to distinguish overlapping points.

Figure 7 - Clinical endpoints

Panel A Kaplan-Meier plots for progression free survival (PFS) in the TRACERx Renal cohort (three plots in top row) and for overall survival (OS) in TCGA KIRC cohort (three plots in bottom row). Three groupings are plotted for each cohort. Left: high (>median) versus low ITH index: middle: high (>median) versus low wGII; and right: four group high/low combination groupings of the two metrics. Log-rank and adjusted (for stage and grade as covariates in a Cox proportional hazard model) p-values are stated. Panel B shows the proportion of cases, within each of the high/low four groups, that progressed to disseminated versus solitary metastases, based on each patient's first progression event. Counts in the highest group "low ITH, high wGII", were compared to all other groups through Fisher's exact test. Panel C shows cancer related deaths OS analysis (as opposed to PFS shown in panel A) for the TRACERx Renal cohort, with patients grouped using the four-category high/low ITH/wGII system. Log-rank and adjusted (for stage and grade as covariates in a Cox proportional hazard model) p-values are stated.

Figure S1 - Consort diagram
Panel A shows the Consort diagram for the filtering steps leading to the reported cohort; Panel B shows the summary of Driver Panel, Whole Exome and Whole Genome Sequencing in the TRACERx Renal 101 Cohort Related to STAR Methods.

Figure S2 - TRACERx Renal cohort: VHL summary.
VHL mutations as confirmed by Sanger sequencing, and *VHL* methylation results. Related to Figure 1 and Star methods.

Figure S3 - Mutation heatmap and driver trees for a subset of TRACERx Renal cohort.
Mutation heatmap and driver trees, for individual cases as referenced in the results section. Related to Figure 1, Figure 2.

Figure S4 - Copy neutral allelic imbalance, MSAI and SCNA profiles for bilateral and multifocal tumours.
Page 1 shows copy neutral allelic imbalance data. Pages 2-17 shows case level MSAI results. Page 18 shows a summary of MSAI results. Pages 19-20 shows MSAI validation results. Page 21 shows SCNA profiles for bilateral and multifocal cases, with each row represents an individual tumour region; blue represents copy number loss, red represents copy number gain. Related to Figure 1, Figure 3 and Star methods.

Figure S5 - TRACERx Renal cohort: Correlation of driver events versus clinical variables and tumour size versus number of clones.

Shown on page 1 are boxplots illustrating comparison of number of variants, ITH score or number of clones classified variously by Tumour Size (in cm), Overall Stage, Grade and Tumour Necrosis status. Drivers refer to "driver events", i.e. non-synonymous SNVs, DNVs, or small INDELS (Muts) in "Driver Genes" or driver copy number events (SCNA). Clonal/Subclonal Drivers refer to events detected in all/not-all primary regions respectively. Driver ITH refers to the ITH index (# of subclonal variants/# of clonal variants) restricted to driver events. P-values refer to pairwise comparisons of groups as indicated and were performed using a Wilcoxon test in all cases. n values indicate the size of the baseline group when no p value is presented, or the comparison group when associated with a p value. Non-significant p values are included for completeness. Shown on page 2 are local polynomial curve fits (using the locpoly function in the R package KernSmooth) and display the results: rows correspond to the fitting of curves of fixed degree, 1 (i.e. linear) in the first row and increasing by 1 until degree 5 (i.e. quintic) curves are applied in the bottom row. Columns correspond to fixed fit bandwidths, which is to say, the size of the window across which the curve is fit, with window sizes of 1, 5, 10, 15 and 20 applied. These local fits are illustrated with the green curve, while a red, global linear least squares fit is also displayed for comparison. Related to Figure 1 and Star methods.

Figure S6 - SCNAs co-occurring with mutational driver events.
Panel A shows SCNAs co-occurring with mutational driver events in TRACERx Renal cohort. Panel B shows SCNA co-occurrence in TCGA KIRC cohort. Panel C shows 14q loss co-occurring with the other SCNAs. 14q loss is shown on X-axis and on Y-axis is log(p-value) for co-occurrence. Panel D shows a null distribution of 1000 random gene sets, which were simulated and tested for an association with shortened time from MRCA to tumour diagnosis. The same method as Figure 4 panel B were used, i.e. regression model with adjustment term included for overall clonal mutation burden per tumour. No evidence of inflation was found in the test statistic (λ=0.96). Panel E shows the analysis of event ordering including VHL as positive control. Panel F shows observed versus expected co-occurrence frequencies. Related to Figure 4.

Figure S7 - TRACERx Renal cohort unsupervised clustering analysis of evolutionary features.
On the x-axis are the rule based evolutionary subtype groups, and on the y-axis are group assignments based on unsupervised clustering. Shown below the x-axis is the percentage of members, from each evolutionary subtype, which are assigned to the same unsupervised cluster. Colours have no meaning except to denote different groups. Related to Figure 5 and Star methods.

Table S1 - TRACERx Renal cohort: clinical characteristics, evolutionary subtypes, progression patterns and survival outcomes.
Related to Figure 1

Table S2 - TRACERx Renal Driver Panels and detected somatic alternations.
Related to Figure 1 and Star Methods.

Table S3 - TRACERx Renal Cohort: Multiregional Ki67 immunohistochemistry (IHC) staining analysis.

Related to Figure 4

Table S4 - TCGA KIRC cohort: multivariate survival analysis and processing notes.
Related to Figure 7 and Star methods.

Table S5 - **Comparison of clone numbers detectable from driver panel and whole exome sequencing in the same cases.**
The left panel shows the raw clone numbers for each case, per sequencing data type.
The right panel shows the correlation of number of tumour clones identified using Renal Driver panel (x-axis) and whole exome sequencing (y-axis). The shaded area represents the confidence interval. Related to Star methods.

STAR Methods

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Charles Swanton (Charles.swanton@crick.ac.uk).

Experimental model and subject details

Patients were recruited into TRACERx Renal Renal, an ethically approved prospective cohort study (National Health Service Research Ethics Committee approval 11/LO/1996). The study sponsor is the Royal Marsden NHS Foundation Trust. The study is coordinated by the Renal Unit at the Royal Marsden Hospital NHS Foundation Trust. The study is open to recruitment at the following sites: Royal Marsden Hospital NHS Foundation Trust, Guy's and St Thomas' Hospital NHS Foundation Trust, Royal Free Hospital NHS Foundation Trust and Western General Hospital (NHS Lothian). Patients were recruited into the study according to the following eligibility criteria:

Inclusion criteria

- Age 18- years or older
- Patients with histologically confirmed renal cell carcinoma, or suspected renal cell carcinoma, proceeding to nephrectomy/metastectomy
- Medical and/or surgical management in accordance with national and/or local guidelines
- Written informed consent (permitting fresh tissue sampling and blood collection; access to archived diagnostic material and anonymised clinical data)

Exclusion criteria

- Any concomitant medical or psychiatric problems which, in the opinion of the investigator, would prevent completion of treatment or follow-up
- Lack of adequate tissue

Further eligibility criteria were applied to the cohort presented in this paper (it therefore follows that these patients do not have consecutive study ID numbers from 001 to 100):

- Confirmed histological diagnosis of clear cell renal cell carcinoma.
- No documented germline renal cell carcinoma predisposition syndrome (including *VHL*).
- At least three primary tumour regions available for analysis.

The cohort was representative of patients eligible for curative or cytoreductive nephrectomy. Full clinical characteristics are provided in **Table S1A**. Demographic data include: Sex, Age and Ethnicity. Clinical data include: Presenting symptoms, Smoking status, BMI, History of Previous RCC, Family History of RCC, Bilateral or Multi-focal RCC, Neoadjuvant therapy (6 patients received systemic therapy prior to nephrectomy). Histology data include: overall TNM Stage (based on Version 7 classification), Location of nephrectomy, Number of harvested and involved lymph nodes, presence of Microvascular Invasion, presence of Renal Vein Invasion, presence of IVC tumour thrombus, Size of primary tumour, Leibovich score, Fuhrman Grade, Time to nephrectomy (days). Clinical status of patients included: Relapse free survival (months), Total follow up (months), Survival Outcome. 16 patients were lost to follow-up: 8 were stage I, 5 stage III and 3 stage IV. For clinical parameter correlation and outcome analyses for cases with multiple tumours (K114, K324, K354, K097, k265) we used the higher stage (or if stage was equal, then the larger of the two tumours, namely: K114_L, K334_R, K352_1, K097_L, K265_1.

Classification of disease progression pattern for metastatic cases. Patterns of disease progression (Table S1B) were classified as follows (1) Rapid- disease progression with multiple new lesions or cancer-specific death within 6 months of surgery (2) Attenuated- no disease progression (for example completely resected metastases at presentation, remains

disease-free); disease progression with a single new lesion within 6 months of surgery (for example a solitary bone, brain or lung deposit) OR disease progression after >6 months of surgery.

Method details

Sample collection

All surgically resected specimens were reviewed macroscopically by a pathologist to guide multi-region sampling for this study and to avoid compromising diagnostic requirements. Tumour measurements were recorded and the specimen were photographed before and after sampling. Primary tumours were dissected along the longest axes and spatially separated regions sampled from the "tumour slice" using a 6 mm punch biopsy needle. The punch was changed between samples to avoid contamination. The total number of samples obtained reflects the tumour size with a minimum of 3 biopsies that are non-overlapping and equally spaced. However, areas which are obviously fibrotic or haemorrhagic are avoided during sampling and every attempt is made to reflect macroscopically heterogeneous tumour areas. Primary tumour regions are labelled as R1, R2, R3… Rn and locations are recorded. Normal kidney tissue was sampled from areas distant to the primary tumour and labelled N1. Each biopsy was split into two for snap freezing and formalin fixing respectively, such that the fresh frozen sample has its mirror image in the formalin-fixed sample which is subsequently paraffin embedded. Fresh samples were placed in a 1.8 ml cryotube and immediately snap frozen in liquid nitrogen for >30 seconds and transferred to -80 C for storage. Peripheral blood was collected at the time of surgery and processed to separate buffy coat.

Nucleic acid isolation from tissue and blood (TRACERx Renal cohort)

DNA and RNA were co-purified using the AllPrep DNA/RNA mini kit. (Qiagen). Briefly, a $2mm^3$ piece of tissue was added to 900ul of lysis buffer and homogenised for five seconds using the TissueRaptor (Qiagen) with a fresh homogenisation probe being used for each preparation. Each lysate was applied to a QiaShredder (Qiagen) and then sequentially purified using the DNA and RNA columns according to the manufacturer's protocol. Germline control DNA was isolated from whole blood using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturers protocol. DNA quality and yield was measured and accessed using the TapeStation (Agilent) and Qubit Fluorometric quantification. (ThermoFisher Scientific)

Detection of VHL mutations by Sanger sequencing

Validation of the patient *VHL* mutations was carried using PCR followed by Big Dye Terminator Sanger sequencing on the ABI 3700. 20ng of patient DNA was amplified for each VHL exon. PCR conditions involved 35 cycles of denaturation at 95^0C, followed by oligonucleotide primer annealing at 55^oC and sequence extension at 72^0C using Qiagen Taq polymerase and reagents. See **Figure S2** for Oligonucleotide sequences

Methylation specific PCR

Methylation of the *VHL* promoter was detected after bisulphite treatment of 500ng of patient DNA using the EZ DNA Methylation-Direct kit (Zymo Research). Bisulphite treated DNA was amplified in the PCR using methylation specific oligonucleotides followed by Big Dye terminator Sanger sequencing. Methylation was confirmed by comparing and contrasting patient tumour and normal renal tissue for methylation protected CpG sequences. See **Figure S2** for oligonucleotide sequences

Independent pathology review of individual tumour regions

Where available, (median of 7 regions per patient (range: 1-63) from 79 patients) histological sections of each region in each case were evaluated by the same pathologist (JIL). Tumor type was assigned to each case following current classification of the International Society of Urologic Pathology (ISUP) (Srigley et al., 2013). Four main histological types were considered based only on hematoxylin-eosin sections: clear cell renal cell carcinoma, papillary renal cell carcinoma, chromophobe renal cell carcinoma and renal oncocytoma. Atypical cases, including unclassified and tumours with mixed histology, were specifically annotated. Tumor architecture was also considered. The presence of rhabdoid and syncytial (Przybycin et al., 2014; Williamson et al., 2014) cells in any region of tumours were also considered, since both are related to a more aggressive clinical course. Tumour grading was performed according to the most up to date ISUP classification (Delahunt et al., 2013) and the presence of necrosis sarcomatoid changes and microvascular invasion was noted. Percentage of viable tumour cells was also estimated in every sample to provide an approximate percentage of tumour content.

Regional staining by Immunohistochemistry and Digital Image Analysis of Ki67

Tissue sections of 4µm were mounted on slides and immunohistochemical staining for Ki67 was performed using a fully automated immunohistochemistry (IHC) system and ready-to-use optimized reagents according to the manufacturer´s recommendations (Ventana Discovery Ultra, Ventana, Arizona, USA). Primary antibody used was rabbit anti-Ki67 (AB16667, Abcam, Cambridge, UK) and secondary antibody was Discovery Omnimap anti-

rabbit HRP RUO (760-4311, Roche, Rotkreuz, Switzerland). DAB kit was Discovery Chromomap DAB RUO (760-4311, Roche). After IHC procedure, slides were first evaluated for Ki67 staining quality using mouse intestine tissue as positive control. Regions containing tumor tissue were identified and marked by a pathologist and subsequently scanned in brightfield at 20x magnification using Zeiss Axio Scan.Z1 and ZEN lite imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany). Digital images were then subjected to automated image analysis using StrataQuest version 5 (TissueGnostics, Vienna, Austria) for Ki67 quantification. Three different gates were set to quantify low, medium and high intensity DAB staining which corresponded to Ki67 expression levels. Results were depicted as total percentage of Ki67-positive nuclei.

Flow Cytometry Determination of DNA Content (FACS)

Fresh frozen tumour tissue samples, approximately 4mm^3 in size, were mechanically disrupted and incubated in 2ml of 0.5% pepsin solution (Sigma, UK) at 37 ºC for 40 minutes to create a suspension of nuclei. The nuclei were washed with phosphate-buffered saline (PBS) and then fixed with 70% ethanol for a minimum of 90 minutes. The nuclei were washed again with PBS and stained with 200µl of propidium iodide (50µg/ml) overnight. Flow cytometric analysis of DNA content was performed using the LSR Fortessa Cell Analyzer (Becton Dickinson, San Jose, USA), BD Facs Diva™ software and FlowJo software (FlowJo LLC, Oregon, USA. A minimum of 10,000 events were recorded (typically up to 20,000 and up to 100,000 in complex samples). Analysis was performed using methods derived from the European Society for Analytical Cellular Pathology DNA Consensus in Flow Cytometry guidelines and following discussions with Derek Davies (Head of Flow Cytometry Facility, The

Francis Crick Institute). Gating of forward and side scatter was applied to exclude debris and cell clumping. Samples with <7,500 events after gating were excluded from further analysis. The coefficient of variation (CV) was measured on each G1 peak. Samples with a CV>10% were excluded from further analysis. Each tumour sample was assumed to contain normal cells to act as internal standard. Where possible the position of the diploid peak was calculated with reference to the peak of diploid cells in a case matched normal tissue sample. The DNA index (DI) of any aneuploid peak present was calculated by dividing the G1 peak of the aneuploid population by the G1 peak of the normal diploid cells. Diploid samples were defined as having DI of 1.00. Any additional peak was defined as aneuploid. A tetraploid peak was defined as having a DI of 1.90-2.10 and containing >15% of total events unless a second peak corresponding to G2 was clear on the histogram. Similarly, aneuploid peaks near to G1 (DI 0.90-1.10) were only considered if there was a clear second peak containing >15% of total events.

Targeted Driver Panel (DP) design and validation

Driver gene panels (Panel_v3, Panel_v5 and Panel_v6) were used in this study. Panel_v3 was designed in 2014, including 110 putative driver genes. Panel_v5 and Panel_v6 were designed in 2015, including 119 and 130 putative driver genes respectively. Driver genes were selected from genes that were frequently mutated in TCGA (accessed in April 2015) or highlighted in relevant studies (Arai et al., 2014; Sato et al., 2013; Scelo et al., 2014). Only alterations in driver genes represented in all three panels were considered in the overall driver mutation analyses. All panels targeted potential driver SCNA regions. To prevent inter-patient samples

swaps, we included the 24 SNPs that were previously identified by Pengelly et al in Panel_v5 and Panel_v6. Details of the 3 panels can be found in Supplementary table (**Table S2**).

Renal Driver Panel Library Construction and Targeted Sequencing

Following isolated gDNA QC, depending on the available yield, samples were normalised to either 1-3 µg or 200 ng for the Agilent SureSelectXT Target Enrichment Library Protocol; standard or low input sample preparation respectively. Samples were normalised using a 1X Low TE Buffer. Samples were sheared to 150-200bp using a Covaris E220 (Covaris, Woburn, MA, USA), following the run parameters outlined in the Agilent SureSelectXT standard 3 µg and low input 200 ng DNA protocols. Library construction of samples was then performed following the SureSelectXT protocols, using 6 pre-capture PCR cycles for the standard input samples and 10 pre-capture PCR cycles for the 200 ng low input samples. Hybridisation and capture were performed for each individual sample using the Agilent custom Renal Driver Panel target-specific capture library (versions 3, 5 & 6). The same version of the capture library being used for all samples from the same patient case. Captured SureSelect-enriched DNA libraries were PCR amplified using 14 post-capture PCR cycles in PCR reactions that included the appropriate indexing primer for each sample. Amplified, captured, indexed libraries passing final QC on the TapeStation 4200 were normalised to 2nM and pooled, ensuring that unique indexes were allocated to all final libraries (up to 96 single indexes available) in the pool. QC of the final library pools was performed using the Agilent Bioanalyzer High Sensitivity DNA Assay. Library pool QC results were used to denature and dilute samples in preparation for sequencing on the Illumina HiSeq 2500 and NextSeq 500 sequencing platforms. The final libraries were sequenced 101bp paired-end multiplexed on

the Illumina HiSeq 2500 and 151bp paired-end multiplexed on the NextSeq 500, at the Advanced Sequencing Facility at the Francis Crick Institute. Equivalent sequencing metrics, including per sample coverage, was observed between platforms.

Whole Exome Library Construction and Sequencing

gDNA isolated from each sample were normalized to 1-3 µg. Libraries were prepared from using the Agilent SureSelectXT Target Enrichment Library protocol and Agilent SureSelectXT Human All Exon v4 enrichment capture library. The libraries were prepared using 6 pre-capture and 12 post-capture PCR cycles. Captured Whole Exome final libraries passing the final QC step were normalised to 2nM and pooled for sequencing on the HiSeq 2500 instrument. Dual HiSeq SBS v4 runs at 101bp paired-end reads generated the data for analysis. Target coverage was 400-500x for the tumour regions and 100-200x for the associated normal.

SNV, and INDEL calling from multi-region DP and multi-region WE sequencing

Paired-end reads (2x100bp) in FastQ format sequenced by Hiseq or NextSeq were aligned to the reference human genome (build hg19), using the Burrows-Wheeler Aligner (BWA) v0.7.15. with seed recurrences (-c flag) set to 10000 (Li and Durbin, 2009). Intermediate processing of Sam/Bam files was performed using Samtools v1.3.1 and deduplication was performed using Picard 1.81 (http://broadinstitute.github.io/picard/) (Li and Durbin, 2009). Single Nucleotide Variant (SNV) calling was performed using Mutect v1.1.7 and small scale insetion/deletions (INDELs) were called running VarScan v2.4.1 in somatic mode with a minimum variant frequency (--min-var-freq) of 0.005, a tumour purity estimate (--tumor-

purity) of 0.75 and then validated using Scalpel v0.5.3 (scalpel-discovery in - -somatic mode) (intersection between two callers taken)(Cibulskis et al., 2013; Fang et al., 2016; Koboldt et al., 2009). SNVs called by Mutect were further filtered using the following criteria: i) ≤5 alternative reads supporting the variant and variant allele frequency (VAF) ≤ 1% in the corresponding germline sample, ii) variants that falling into mitochondrial chromosome, haplotype chromosome, HLA genes or any intergenic region were not considered, iii) presence of both forward and reverse strand reads supporting the variant, iv) >5 reads supporting the variant in at least one tumour region of a patient, v) variants were required to have cancer cell fraction (CCF)>0.5 in at least one tumour region (see **Subclonal deconstruction of mutations** section for details of CCF calculation) , vi) variants were required to have CCF>0.1 to be called as present in a tumour region, vii) sequencing depth in each region need to be >=50 and ≤3000. Finally, suspected artefact variants, based on inconsistent allelic frequencies between regions, were reviewed manually on the Integrated Genomics Viewer (IGV), and variants with poorly aligned reads were removed. Dinucleotide substitutions (DNV) were identified when two adjacent SNVs were called and their VAFs were consistently balanced (based on proportion test, *P*>=0.05). In such cases the start and stop positions were corrected to represent a DNV and frequency related values were recalculated to represent the mean of the SNVs. Variants were annotated using Annovar (Wang et al., 2010). Deleterious mutations were defined if two out of three algorithms - SIFT, PolyPhen2 and MutationTaster - predicted the mutation as deleterious. Individual tumour biopsy regions were judged to have failed quality control and excluded from analysis based on the following criteria: i) sequencing coverage depth below 100X, ii) low tumour purity such that copy number calling failed. Mutations detected in high-confidence driver genes (*VHL, PBRM1,*

SETD2, PIK3CA, MTOR, PTEN, KDM5C, CSMD3, BAP1, TP53, TSC1, TSC2, ARID1A, TCEB1) were defined as *driver mutations*. As *TSC1* and *TSC2* were not targeted in Panel v5, to check the mutation status in these two genes, patients were sequenced using Panel v5 were re-sequenced with Panel v6 and no new mutations were detected.

SCNA calling from multi-region DP and multi-region WE sequencing

To estimate somatic copy number alterations, CNVkit v0.7.3 was performed with default parameter on paired tumour-normal sequencing data (Talevich et al., 2016). Outliers of the derived log2-ratio (logR) calls from CNVkit were detected and modified using Median Absolute Deviation Winsorization before case-specific joint segmentation to identify genomic segments of constant logR (Nilsen et al., 2012). Tumour sample purity, ploidy and absolute copy number per segment were estimated using ABSOLUTE v1.0.6 (Carter et al., 2012). In line with recommended best practice all ABSOLUTE solutions were reviewed by 3 researchers, with solutions selected based on majority vote. Copy number alterations were then called as losses or gains relative to overall sample wide estimated ploidy. Arm gain or loss was called when >50% of the chromosomal have copy number gain or loss. Driver copy number was identified by overlapping the called somatic copy number segments with putative driver copy number regions identified by Beroukhim and colleagues (Beroukhim et al., 2009). We compared SCNA calls between targeted panel and WGS datasets, and SCNA concordance was 87% (**Table S5**). The average proportion of the genome with aberrant copy number, weighted on each of the 22 autosomal chromosomes, was estimated as the weighted genome instability index (wGII).

TCGA WES data analysis

To compare mutation frequency detected in TRACERx Renal cohort with public data (**Figure 1B and 1C**), event calls from 451 TCGA KIRC patients were retrieved from cBioportal (http://www.cbioportal.org/) on 2017/07/21. To investigate the clonality of mutations in TCGA KIRC cohort, we obtained the next generation sequencing data for matched tumour and normal/blood from 338 cases in BAM format from TCGA, which were then converted into FASTQ format files using bam2fastq in bedtools package (Quinlan and Hall, 2010). SNVs, INDELs and SCNAs were called using the same methods as TRACERx Renal data (**STAR Methods:** SNV, and INDEL calling from multi-region DP and multi-region WE sequencing, SCNA calling from multi-region DP and multi-region WE sequencing). 20 cases were excluded from the study as the ABSOLUTE v1.0.6 algorithm failed to find a stable SCNA solution, further details can be found in **Table S4**. Clonality of SNVs and SCNAs were estimated using ABSOLUTE v1.0.6. Cancer cell fraction for INDELs were calculated using method described in **STAR Methods:** Subclonal deconstruction of mutations. INDELs with CCF>0.5 were called clonal. ITH index for each patient was calculated as the measure of intratumour heterogeneity (ITH index = # subclonal drivers / # clonal drivers). However, due to the limitation of single biopsy, intratumour heterogeneity was found to underestimated (ITH index range 0-3, median=0.0, sd=0.41).

Quantification and statistical analysis

R 3.3.2 was used for all statistical analyses.

Saturation Analysis and Phenotypic Correlations

For saturation analysis, the mean number of variants observed for each subset of biopsies of a given size was calculated by exhaustive consideration of all such subsets when the total number of such subsets was less than 18 million and by consideration of a random collection of 18 million subsets, with possible repetition, when the total number of possibilities was greater. For phenotypic correlations, comparisons were performed using the Fisher's Exact test for 2x2 tables and the "non-parametric 2-way anova" Freidman test for n x m tables where at least one of n and m is greater than 2. P-values were corrected for multiple testing using the Benjamini–Hochberg procedure.

Subclonal deconstruction of mutations

To estimate the clonality of a mutation in a region, we used the following formula:

$$vaf = \frac{CN_{mut} * CCF * p}{CN_n * (1-p) + CN_t * p}$$

where vaf is the variant allele frequency at the mutation base; p is estimated tumour purity; CN_t and CN_n are the tumour locus specific copy number and the normal locus specific copy number which was assumed to be 2 for autosomal chromosomes; and CCF is the fraction of tumour cells carrying the mutation. Consider CN_{mut} is the number of chromosomal copies that carry the mutation, the possible CN_{mut} is ranging from 1 to CN_t (integer number). We then assigned CCF with one of the possible value: 0.01, 0.02, ..., 1, together with every possible CN_{mut} to find the best fit cancer cell fraction of the mutation. Since we focused on driver genes in this study and the accuracy of the estimated CCF is limited by the size of the panel, we call mutations with CCF>0.5 as clonal mutations, mutations with CCF≤0.5 and CCF>0.1 are subclonal. To determine the clonality of a mutation in a tumour, we ask for the mutation to be clonal in all regions in a tumour. Exceptions were made for long INDELs that affect >6 bp of the genome, due to VAF under estimation. If a long INDEL is present in all

regions of a tumour, we called it as clonal. To determine the clonality of a SCNA in a tumour, we ask for the SCNA to be presence in all tumour regions, otherwise it is called subclonal.

Driver tree reconstruction

A matrix with presence and absence of nonsynonymous and synonymous point mutations, DNVs, INDELs and arm level SCNAs was created for each tumour, and all the events were clustered based on the following rule: a valid cluster has to have at least two arm level SCNAs or one non-synonymous mutation. The driver events clusters were then ordered into a clonal hierarchy using TRONCO and presented as driver trees (De Sano et al., 2016).

Clustering was performed on multi-region whole exome sequencing using PyClone Dirichlet process clustering (Roth et al., 2014). For each mutation, the observed variant count was used and reference count was set such that the VAF was equal to half the pre-clustering CCF. Given that copy number and purity had already been calculated, we set the major allele copy numbers to 2 and minor allele copy numbers to 0 and purity to 0.5; allowing clustering to simply group clonal and subclonal mutations based on their pre-clustering CCF estimates. PyClone was with 10,000 iterations and a burn-in of 1000, and default parameters, with the exception of --var_prior set to 'BB' and –ref_prior set to 'normal'.

In terms of limitations, we recognise that our Driver Panel phylogenies are based on fewer clonal markers, as compared to whole exome or genome derived phylogenetic trees. As a consequence some tumour clones are based on only a limited number of genomic markers, however three contingency measures are in place to mitigate against phylogenetic misconstruction: i) ultra-deep 500x sequencing coverage, which ensures stably derived cancer cell fraction estimates, ii) a bespoke gene panel which is enriched for driver events,

increasing the likelihood that mutational markers are driving genuine clonal expansion, iii) cross-capture validation with tree structures in >10 cases confirmed using exome sequencing data (**Table S5**). Furthermore, the panel sequencing strategy has allowed extensive tumour sampling, with >1,200 biopsies sequenced, enabling robustness in terms of spatial sampling.

Parallel evolution significance testing

All genes with ≥ 10 subclonal mutations across the cohort were tested for evidence of parallel evolution (qualifying genes: *BAP1, CSMD3, KDM5C, MUC16, MTOR, PBRM1, PTEN, SETD2. TSC1, TP53*). For each gene the observed number of parallel mutations across the 100 case cohort was compared to a null distribution of the expected number of subclonal mutations co-arising in different tumour regions within the same case due to chance. To simulate the null distribution the mutation frequency of each gene per biopsy region was calculated, based on total number of unique subclonal mutations for that gene (cohort wide) divided by the total number of biopsies sequenced (cohort wide). This probability was then used in a simple Bernoulli trials model simulated for each patient, with the number of trials based on the number of biopsy regions sequenced per case. This model allows for the fact that cases with a large number of sampled regions have high chance of co-arising mutations in different biopsy regions by chance rather than due to parallel evolution. The total count of co-arising mutations by chance was calculated across the 100 case cohort (using the specific number of biopsy regions per case) and then compared to the observed number parallel events. Significance was determined through 1000 permutations per gene, with resulting p-values corrected for multiple testing using the Benjamini–Hochberg procedure.

Detection of allelic imbalance

Heterozygous SNPs called using germline variants were identified using VarScan v2.4.1 in mpileup2snp mode. SNPs used must be called in all regions of the tumour and have a B-allele frequency (BAF, total variant base / total reference bases at a position) of between 0.35 and 0.65 in the germline sample. Mean absolute deviation (MAD) from 0.5 calculated for all heterozygous SNPs on each arm in all samples: mean (abs(arm_hz_BAF – 0.5)). The germline MAD was then subtracted from all tumour region MADs for each patient's disease for all chromosome arms. Copy neutral allelic imbalance was then called if: 1) There is no copy number event (gain or loss) associated with the chromosome arm in a sample but there is a MAD of >= 0.1. 2) There is no copy number event associated with the chromosome arm in a sample but its MAD is >= the median MAD of gain/loss events in this sample and is also >= 0.05. 3) If a patient's disease has the same chromosome arm exhibiting copy neutral allelic imbalance in 2 or more regions by the above the two criteria, the same chromosome arm in the other regions is re-examined using the lowest quartile MAD of gain/loss events in each region as a cut off and has a MAD of >=0.05.

Calculating clonality of copy neutral allelic imbalance (CNAI): Only regions with at least one chromosome arm exhibiting a MAD score of greater than 0.05 were considered for this analysis. Regions with no MAD score greater than 0.05 are marked on the patient specific supplementary figures "low purity" (**Figure S4**). Copy neutral allelic imbalance calls are shown as diamonds in the patient specific copy number plots attached in this email. The CNAI occurrences in each patient were then grouped into the following categories: Clonal CNAI – All regions of the tumour have no copy number gains or losses associated with this

chromosome arm but all have been classified as exhibiting CNAI. Clonal loss and CNAI – All regions of the patient's disease have either a loss being called or exhibit CNAI for this chromosome arm.

Detection of mirrored subclonal allelic imbalance (MSAI)

In order to detect mirrored subclonal allelic imbalance (MSAI) allele counts were generated using AlleleCounter (github.com/cancerit/alleleCount) (see companion paper Mitchell et al 2018). The counts from whole exome sequenced samples were analysed using ASCAT (Van Loo et al., 2010) to generate copy number calls. Whole-genome samples were analysed using Battenberg (Nik-Zainal et al., 2012) to generate copy number calls (see companion paper, Mitchell et al 2018). Heterozygous SNPs among the 1000 genomes positions (Genomes Project et al., 2010) used as input for ASCAT/Battenberg analyses were identified by isolating those which had a B-allele frequency (BAF) of between 0.3 and 0.7 (calculated by variant reads over total reads) in the germline sample for each patient. The BAFs of these heterozygous SNPs were then used with the segmentation and copy number calls produced for each region by either ASCAT or Battenberg analyses to detect MSAI events for each patient's disease using the method outlined previously (Jamal-Hanjani et al., 2017).

Using the heterozygous SNPs present in the targeted regions detected by Driver Panel sequencing we identified allelic imbalance (AI) at the level of chromosome arms. In some cases the AI was not associated with a copy number gain or loss relative to the sample's ploidy and was classified as copy neutral allelic imbalance (CNAI) (**STAR methods**). In total, we identified 18 cases where one or more chromosome arms demonstrated clonal CNAIs (34 events total) and 8 patients where, at least one chromosome arm was always affected by

either loss relative to ploidy or CNAI (13 events total). 5 of these 8 patients also demonstrated instances of ubiquitous arm level CNAI in all regions.

Validation of MSAI

Validation of MSAI was achieved using Polymorphic microsatellite markers specific to the chromosome and chromosome region being investigated. Once a polymorphic marker is identified, patient DNA is amplified in the PCR, incorporating a fluorescent primer into the PCR fragment that can be accurately measured for size and fluorescent intensity. Measurement of Fluorescent units under each allele peak can be used to compare and contrast variation between alleles within and between different tumour regions and the normal sample using the formula (At/Bt)/(An/Bn).

Co-occurrence testing

Co-occurrence of driver events in each tumour was conducted based on the driver tree clones as determined above. Analysis was conducted on the most frequent driver mutational events (*BAP1, PBRM1, SETD2, VHL*, **Figure 1B***)*, the most frequent SCNAs (3p loss, 5q gain) and SCNA events with established clinically prognostic value (loss 4q, loss 9p, loss 14q and gain 8q) (Ito et al., 2016; Kojima et al., 2009; La Rochelle et al., 2010; Monzon et al., 2011; Perrino et al., 2015). For each event pairing tumour clones were assessed to determine if the given two events were found to co-occur together in the same clone. Analysis was first conducted using only the "MRCA" clone per case (n=100), to ensure independence of observations at the patient level (for bilateral/multi-focal cases the first/left tumour was taken in each case). Analysis was then repeated using "truncal plus subclonal" clones (total n=306 across all

tumours, with the set of subclones defined as unique terminal tree nodes). R package 'cooccur' (Griffith A, 2016)was used to compare observed event co-occurrence frequencies to those expected by chance under a probabilistic model. The distribution of observed and expected values is shown in **Figure S8**. Values were plotted as enrichment scores calculated as $\log_2$(observed count/expected count). Only patterns found to be significant in both the "truncal" and "truncal plus subclonal" were considered significant overall. Correction for multiple testing was conducted using the Benjamini–Hochberg procedure.

Most recent common ancestor (MRCA) and ki67 analysis

The estimated time of MRCA was calculated using multi-region whole genome sequencing data as detailed in the companion paper by Mitchell et al., (Cell 2018). From the total n=33 cases with WGS data, MRCA timing analysis was successful in n=31 cases, from which known *VHL* wildtype cases (n=2) were excluded on account of their distinct aetiological and phenotypic profile. Of the n=29 cases analysed, n=23 overlapped with the renal TRACERx Renal 101 cohort cohort presented here, and n=6 were additional ccRCC patients recruited to the TRACERx Renal Renal study. The association between time from MRCA to tumour diagnosis and number of clonal driver events was assessed using a linear model, adjusting for the total clonal mutation burden per tumour. The association between tumour region ki67 % of cells stained as positive and number of clonal driver events was assessed using a linear mixed effect (LME) model, to account for the non-independence of multiple samples from individual patients, using all cases with available data in the TRACERx Renal 101 cohort after exclusion of known *VHL* wildtype tumours.

Event ordering analysis

The ordering of driver events was based on the clonal hierarchy of each tumour, as determined by driver tree reconstruction method detailed above. Due to dense spatial sampling (median 7 biopsies per tumour, range [3-75]) the driver tree ordering was typically robust, with evidence of sequential waves of clonal expansion between events usually confirmed across multiple biopsy regions. The set of sequential event paths (*i.e.* event A > event B > event C) for each tumour was captured starting with the events in the MRCA clone. For each MRCA event, evolutionary sequences were traced through each node of the tree until a terminal clone was reached. All possible sequential paths (trajectories) between MRCA and terminal clone events were recorded. To reduce risk of multiple testing we limited further analyses to those trajectories containing the most frequent ("core") ccRCC driver events: *VHL, PBRM1, BAP1, SETD2,* PI3K/AKT/mTOR pathway mutations or driver SCNAs. The list of trajectories was further reduced to ensure pairings of events were counted only once per case, (e.g. in the case of K243 where a single *PBRM1* mutation precedes 10 *SETD2* mutations, this is counted only once) and PI3K/AKT/mTOR pathway mutations interacting with SCNAs were not considered due to the nonspecific many-to-many relationship. The final list of trajectories was analysed using R package Trajectory Miner (Gabadinho et al., 2011) to identify recurrent patterns of event pairs enriched for occurrence is a consistent direction. Event pairings observed in ten or more cases were then tested for significance in a specific ordering direction using a Binomial test, with null expected p=0.5, to reflect an equally balanced 50%:50% distribution of event ordering by random chance. As expected, *VHL* was found to be significantly enriched as an early event preceding all other alterations, consistent

with its known timing as a universally clonal event (data not shown in figure). All p-values were corrected for multiple testing using the Benjamini–Hochberg procedure.

Evolutionary subtype classification

Based on the evolutionary analysis in **Figures 4A-C** a rule based classification was devised in order to assign cases into subgroups and allow for comparison against phenotypic and clinical outcomes. Cases were assigned to groups based on the following series of rules (applied in a hierarchical manner in the order listed): i) presence of ≥ 2 *BAP1, PBRM1, SETD2* or *PTEN* clonal mutational events meant assignment to "multiple clonal driver" group (the selection of these four genes is based on the timing results observed in **Figure 4B**) , ii) presence of a tumour clone/subclone with a *BAP1* mutational driver event, and no other "core" mutational driver events aside from *VHL* in that same clone/subclone, meant assignment to the "*BAP1* driven" group, iii) presence of a tumour clone/subclone with *PBRM1* mutation followed by a *SETD2* mutation, meant assignment to the "*PBRM1*->SETD2" group, iv) presence of a tumour clone/subclone with *PBRM1* mutation followed by a PI3K pathway mutation, meant assignment to the "*PBRM1*->PI3K" group, v) presence of a tumour clone/subclone with *PBRM1* mutation followed by a driver SCNA event, meant assignment to the "*PBRM1*->SCNA" group, vi) absence of *VHL* mutation or methylation meant assignment to "VHL wildtype" group, vii) presence of *VHL* as the only "core" mutational driver event meant assignment to the "*VHL* monodriver" group. For bilateral/multi-focal cases the evolutionary subtype was assigned based on the first/left tumour in each case. To test the stability and validity of the rule based classification an unsupervised clustering analysis was additionally performed, using R function daisy, with the distance matrix computed using Gower's formula on account

of the mixture of continuous and binary data types. Clustering was conducted based on the following measures: wGII (minimum and maximum regional values per tumour), tumour size (mm), clone number, ITH index, number of clonal driver events and presence/absence of the six observed evolutionary patterns (*BAP1* lone driver clone/subclone, *PBRM1->SETD2* clone/subclone, *PBRM1*->PI3K clone/subclone, *PBRM1*->SCNA clone/subclone, *VHL* mutational status, *VHL* as the only "core" mutational driver event). Clustering was performed using a partitioning around medoid method, with cluster number from 2 to 15 considered, and a 10 cluster solution resulting as the optimal solution. Overall high concordance in cluster assignment was observed between the rule based and unsupervised methods (**Figure S9**), and in the unsupervised method three additional subgroups were identified (**Figure S9**, the groups are referred to just by cluster number due to currently unclear evolutionary aetiology): cluster 5 which was characterised by low clone number (median=2) and small size (mean=6.7cm), cluster 7 which exhibited high wGII, and cluster 9 with branched structure (median 11 clones) and large size (mean=10.9cm).

Survival analysis

Survival analysis was conducted using the Kaplan-Meier method, with p-value determined by a log-rank test. Progression free survival (PFS) was defined as the time to recurrence or relapse, or if a patient had died without recurrence, the time to death. In the TRACERx cohort, overall survival (OS) was measured as cancer specific death. For the TCGA cohort, all death events were included in the PFS/OS analyses (consistent with the original author's analysis of the data, on account of a lack of cause of death data). Hazard ratio and multivariate analysis adjusting for clinical parameters was determined through a Cox proportional hazards model.

Downsampling simulation

Empirical error rates were determined by exhaustive consideration of all pairs of biopsies from a given tumour sample and, for each pair, comparing the number of variants detected in one or more of the full set of biopsies not found in either member of that pair ("False negative") or determined to be subclonal in the full set but detected in both samples in that pair ("illusion of clonality"). Each tumour is then represented by the mean value of each of these estimates across all pairs.

Data and Software availability

Sequencing data that supports this study will been deposited at the European Genome-phenome Archive (EGA), which is hosted by the European Bioinformatics Institute (EBI); accession numbers EGAS00001002793.

Additional resources

Clinical trial registry number: https://clinicaltrials.gov/ct2/show/NCT03226886

TRACERx Renal Renal study website, detailing investigators, sponsors and collaborators:

http://TRACERx Renal.co.uk/studies/renal/

Declaration of Interests

S.T., H.X., K.L. and C.S. have a patent on renal cell carcinoma biomarkers pending.

Dr. Swanton reports grant support from Cancer Research UK, UCLH Biomedical Research Council, and Rosetrees Trust, AstraZeneca; personal fees from Boehringer Ingelheim, Novartis, Eli Lilly, Roche, GlaxoSmithKline, Pfizer and Celgene.

Dr Swanton also reports stock options in GRAIL, APOGEN Biotechnologies and EPIC Bioscience, and has stock options and is co-founder of Achilles Therapeutics.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Critical Commercial Assays		
Deposited Data		
Multi-region sequencing data on TRACERx renal 101 patient cohort.	This study.	EGAS00001002793
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		

Oligonucleotides		
Oligonucleotide sequences for VHL exon amplification and methylation specific PCR see Figure S2.	This paper	
Recombinant DNA		
Software and Algorithms		
Burrows-Wheeler Aligner (BWA) v0.7.15	Li and Durbin, 2009	http://bio-bwa.sourceforge.net/
Samtools v1.3.1	Li et al., 2009	http://samtools.sourceforge.net/
Picard 1.81		http://broadinstitute.github.io/picard/
Mutect v1.1.7	Cibulskis et al., 2013	http://archive.broadinstitute.org/cancer/cga/mutect
VarScan v2.4.1	Koboldt et al., 2009	http://varscan.sourceforge.net/
Scalpel v0.5.3	Fang et al., 2016	https://github.com/hanfang/scalpel-protocol
Annovar	Wang et al., 2010	http://annovar.openbioinformatics.org/en/latest/
CNVkit v0.7.3	Talevich et al., 2016	https://cnvkit.readthedocs.io/en/stable/cp
R package 'Copynumber'	Nilsen et al., 2012	http://bioconductor.org/packages/release/bioc/html/copynumber.html
ABSOLUTE v1.0.6	Carter et al., 2012	http://archive.broadinstitute.org/cancer/cga/absolute
bedtools package	Quinlan and Hall, 2010	http://bedtools.readthedocs.io/en/latest/

R package 'TRONCO'	De Sano et al., 2016	http://www.bioconductor.org/packages/release/bioc/html/TRONCO.html
PyClone	Roth et al., 2014	https://bitbucket.org/aroth85/pyclone/wiki/Home
AlleleCounter		github.com/cancerit/alleleCount
ASCAT	Van Loo et al., 2010	https://github.com/Crick-CancerGenomics/ascat
Battenberg	Nik-Zainal et al., 2012	https://github.com/cancerit/cgpBattenberg
R package 'cooccur'	Griffith A, 2016	https://cran.r-project.org/web/packages/cooccur/index.html
R package 'Trajectory Miner'	Gabadinho et al., 2011	http://traminer.unige.ch/

Figure 1

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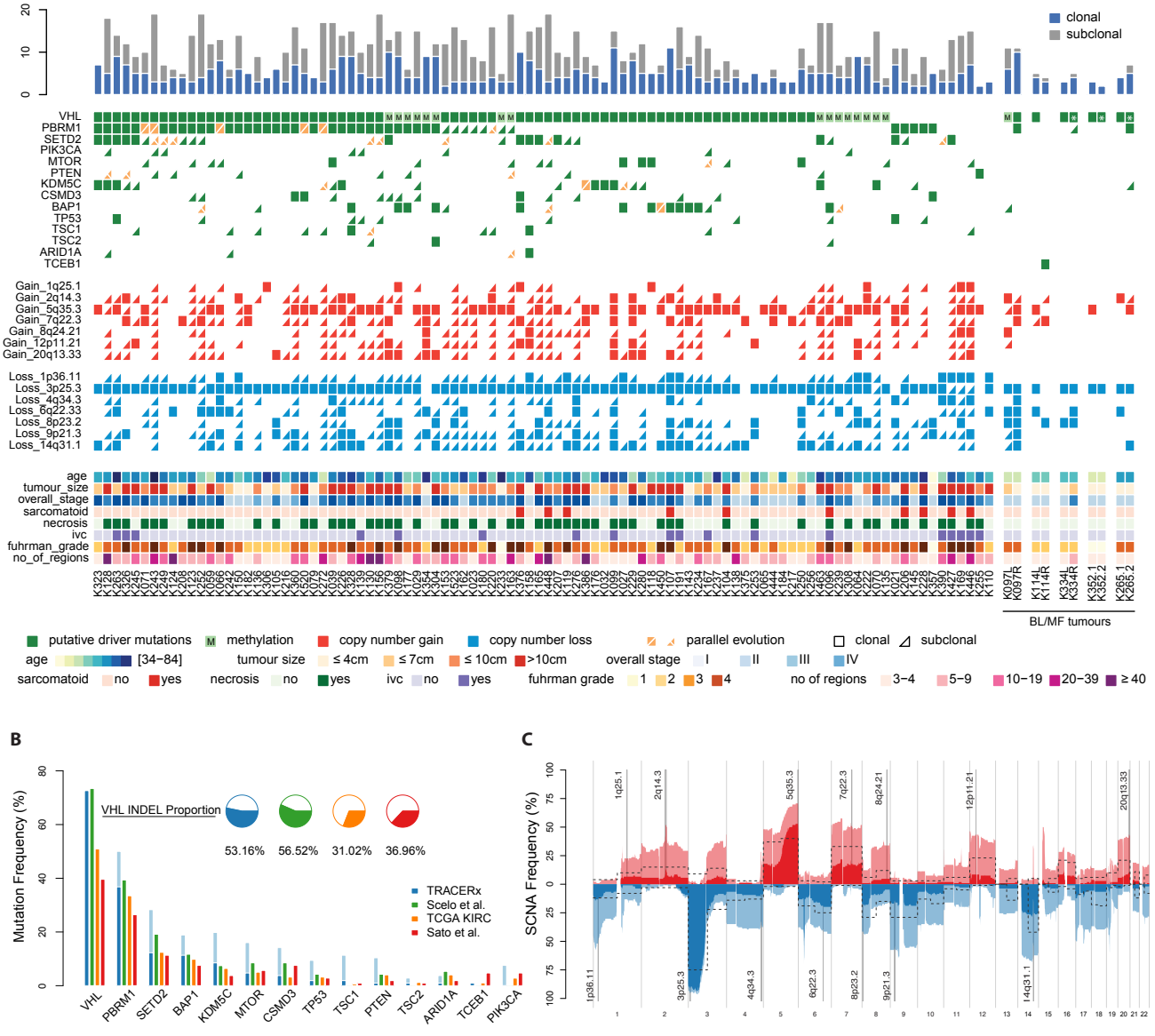

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Figure 2

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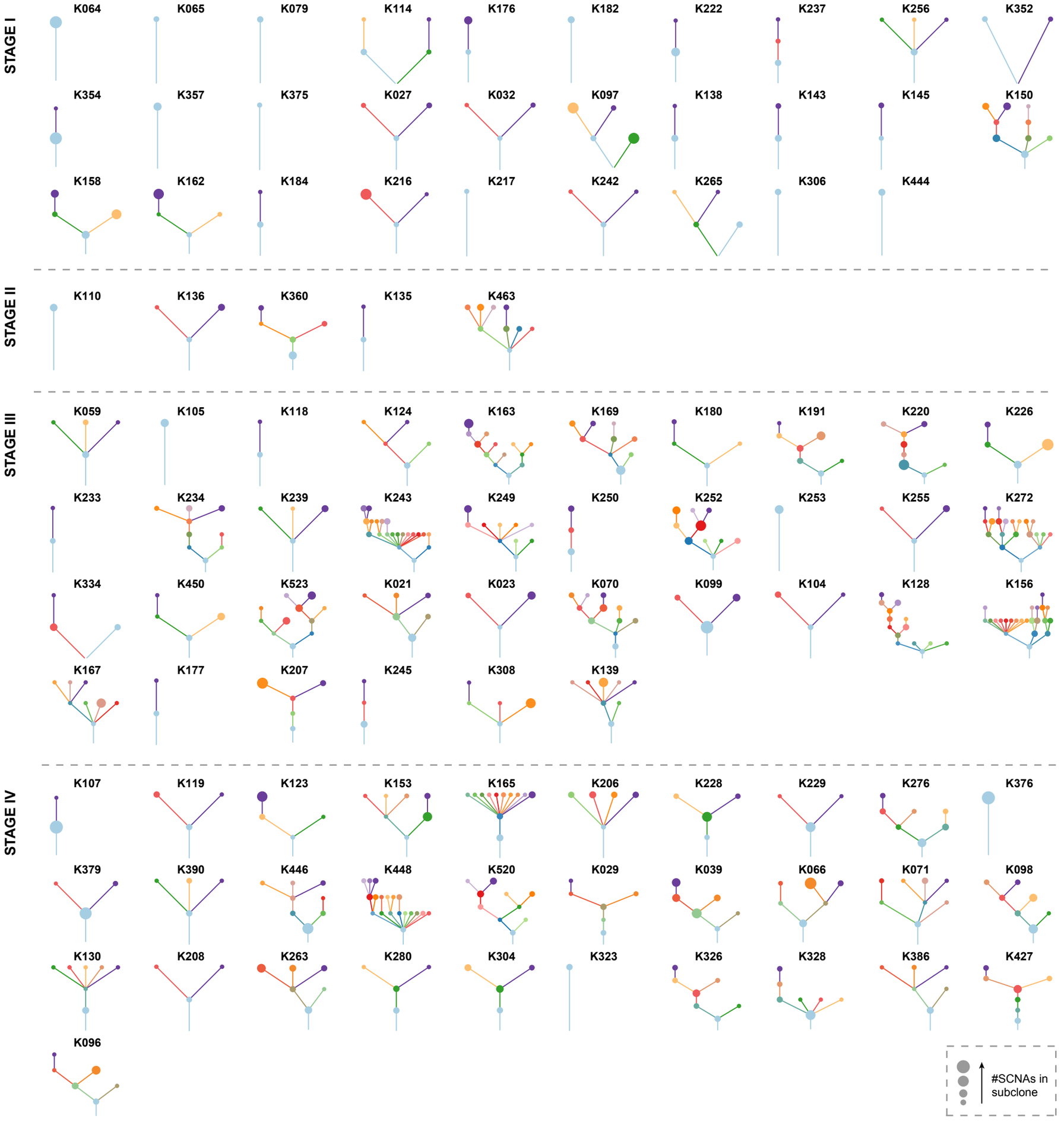

Figure 3

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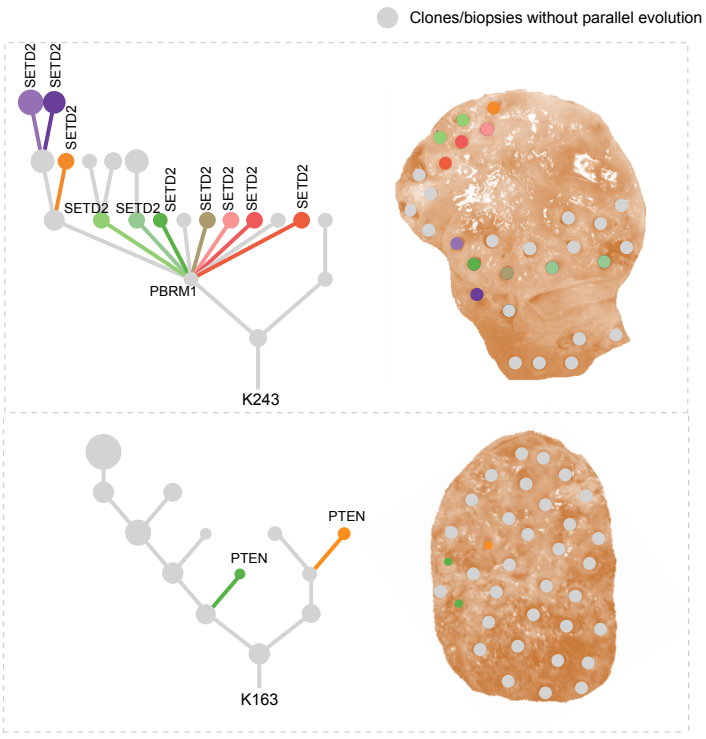
Gene	No. Unique Subclonal Events	No. Parallel Events	$P_{parallel}$	$P_{parallel\_adjs}$	
SETD2	53	36	0.021	0.1	*
BAP1	22	12	0.030	0.1	*
PTEN	15	8	0.030	0.1	*



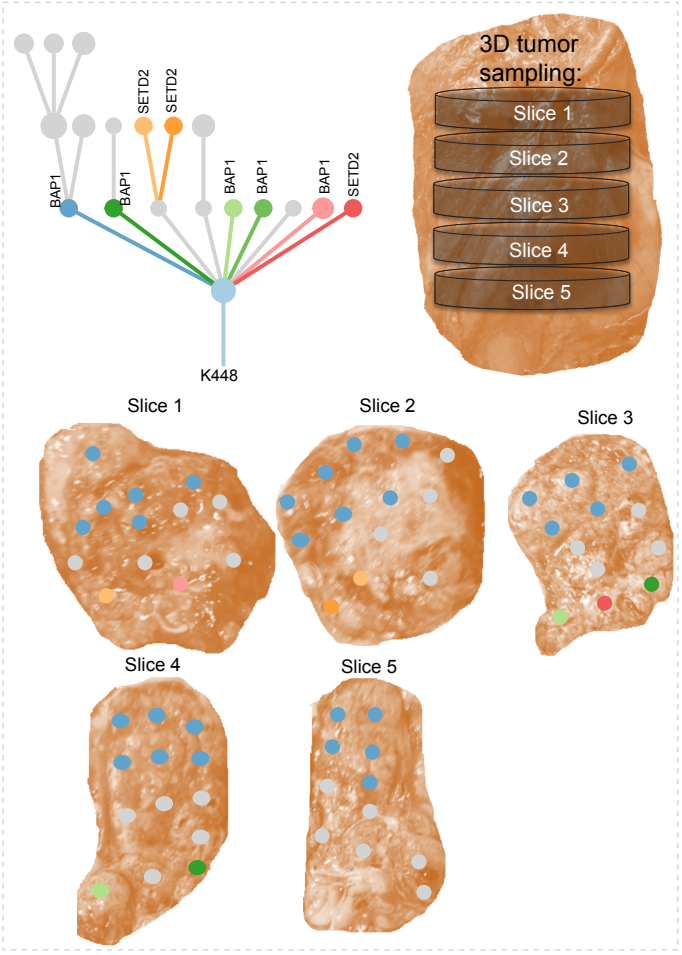


Figure 4

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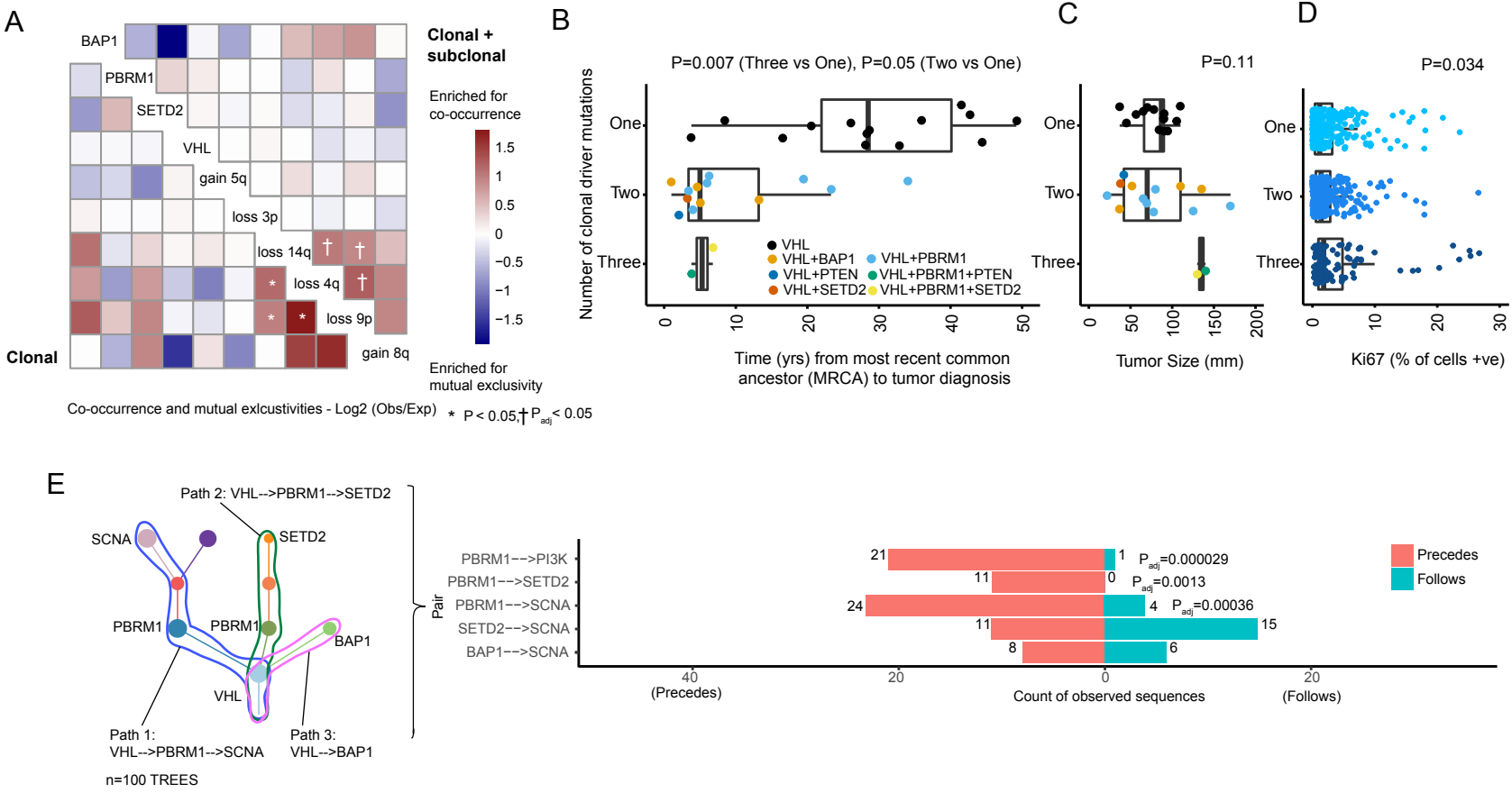


Figure 5

Click here to download Figure Fig_5_updated6.pdf

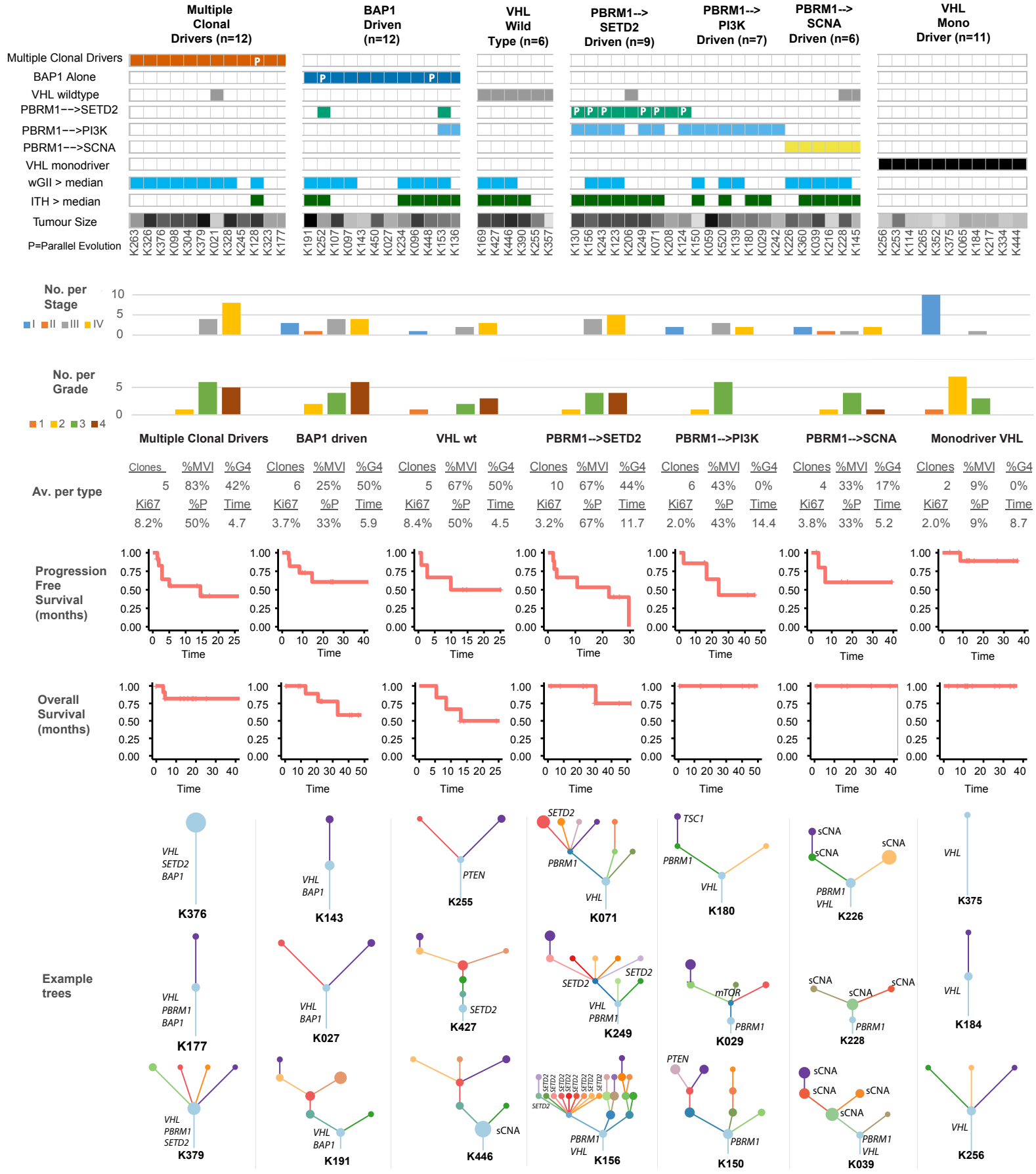


Figure 6

Click here to download Figure Fig_6_updated_final_2.pdf

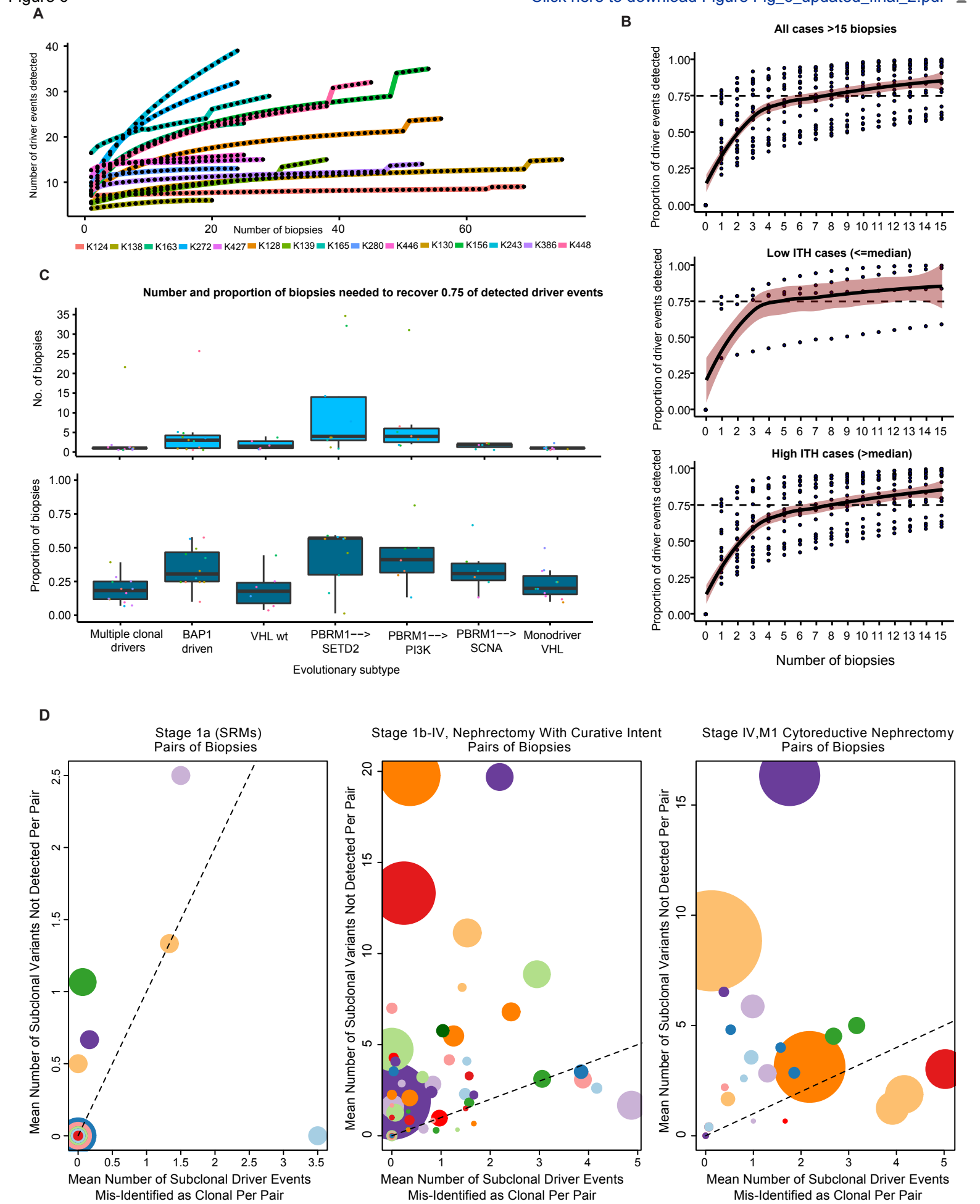
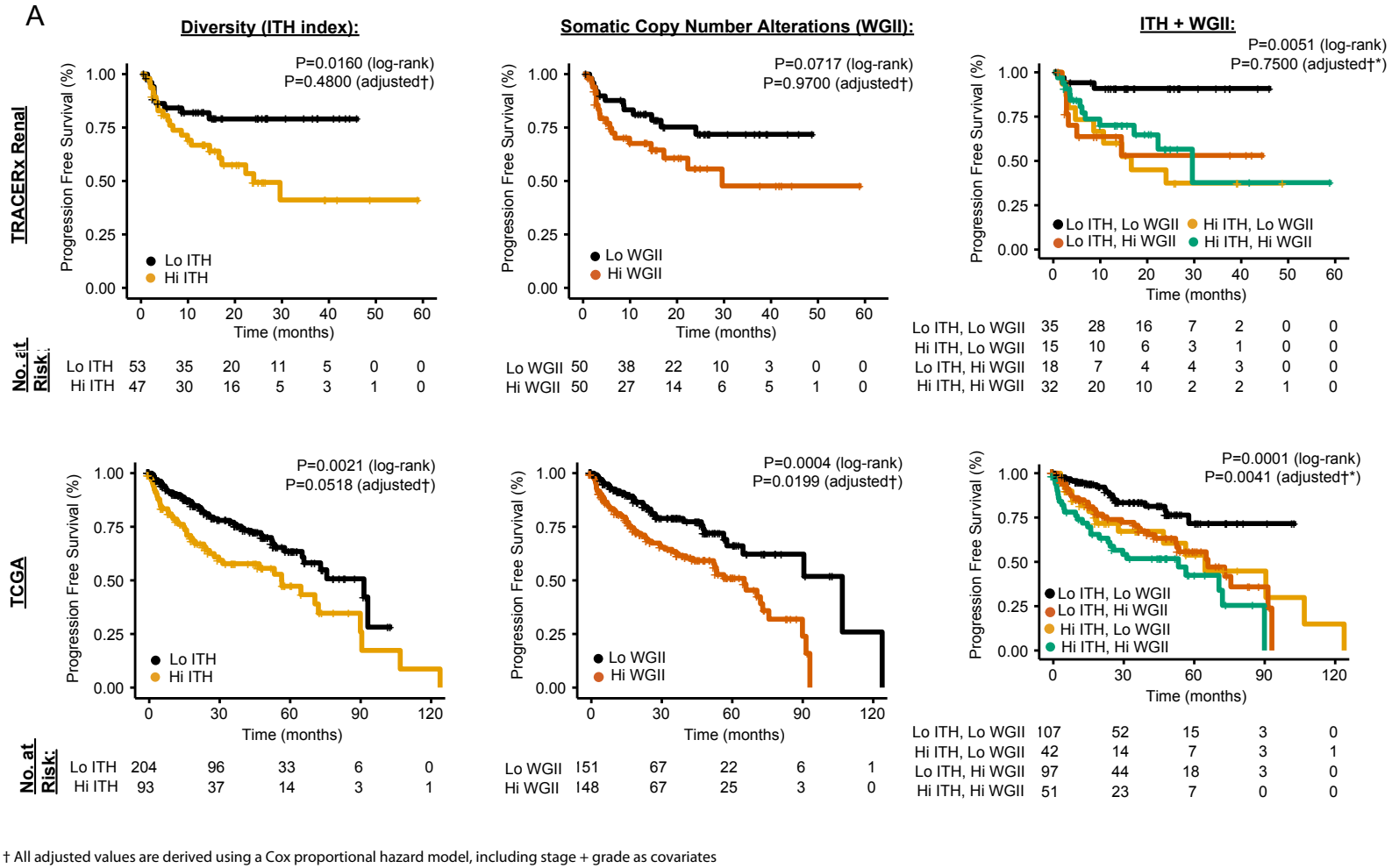


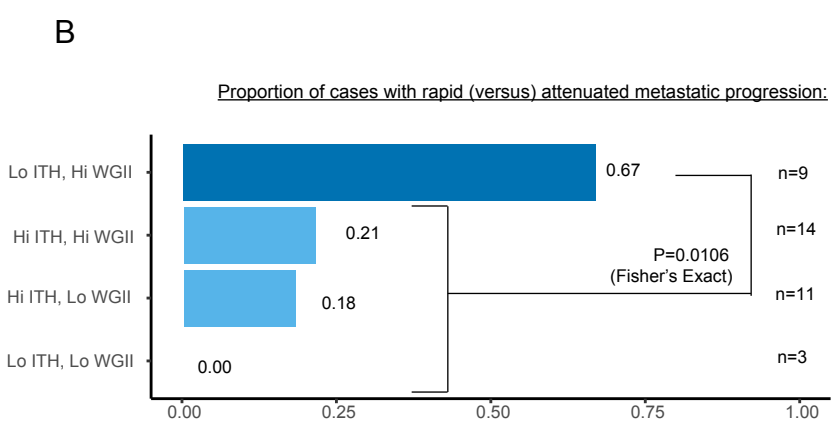
Figure 7

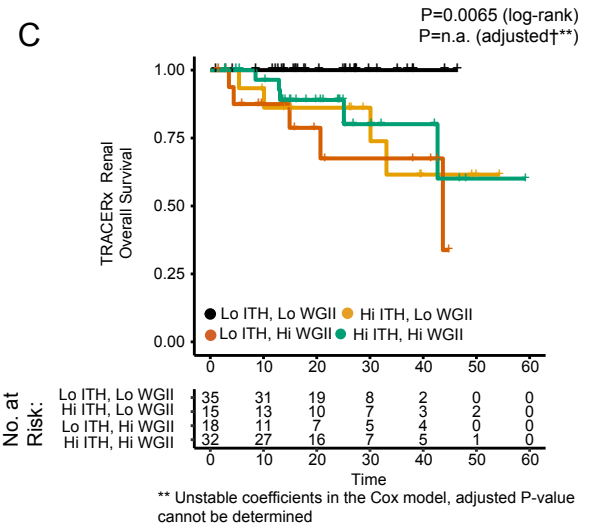
Click here to download Figure Fig_7_KM_curves_final_v3.pdf



† All adjusted values are derived using a Cox proportional hazard model, including stage + grade as covariates

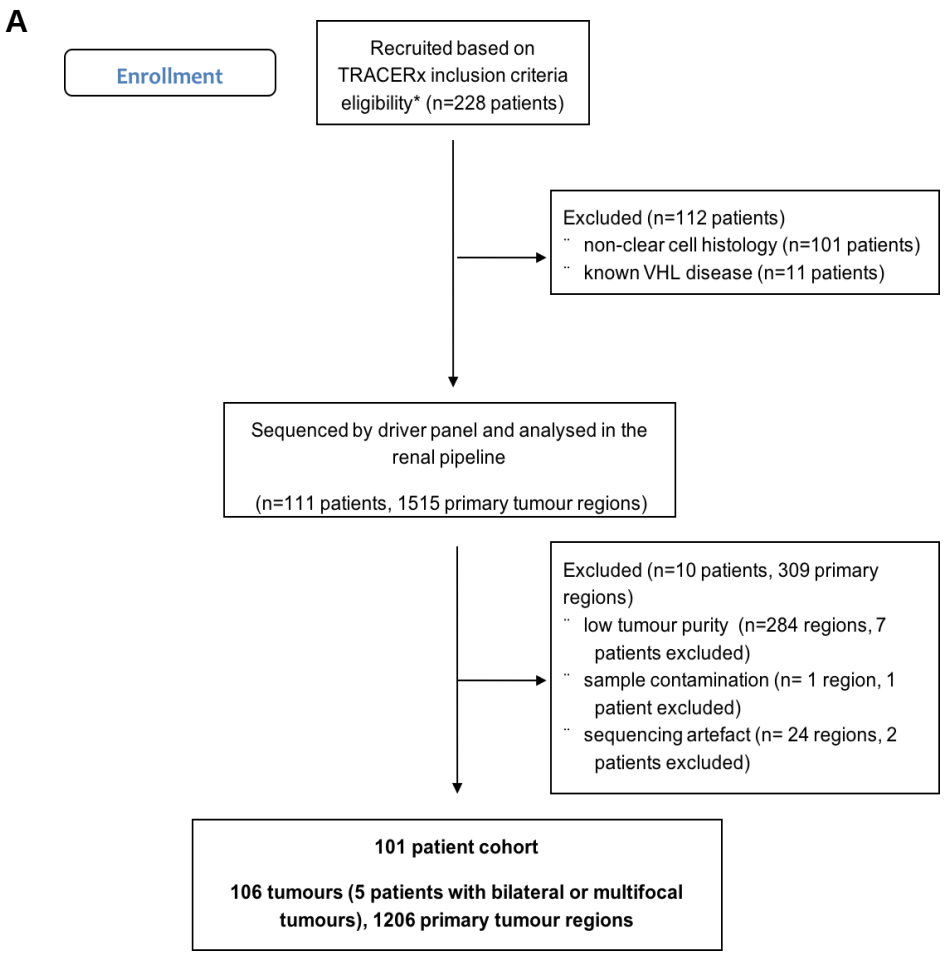
* P value for "Hi ITH, Hi WGII" vs "Lo ITH, Lo WGII" (the most significantly different groups for PFS in adjusted analysis)





** Unstable coefficients in the Cox model, adjusted P-value cannot be determined

Supplemental Figure 1 Click here to download Supplemental Figure Figure S1.pdf



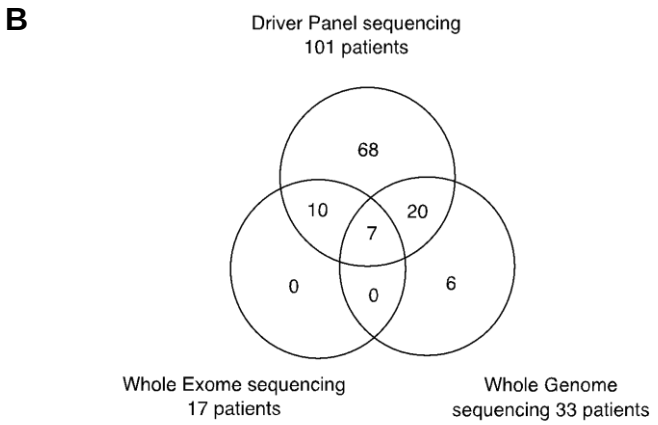
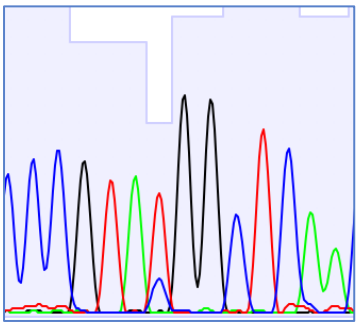


Figure S2: TRACERx Renal cohort: VHL summary. Related to Figure 1 and Star methods.

VHL validation by Sanger sequencing



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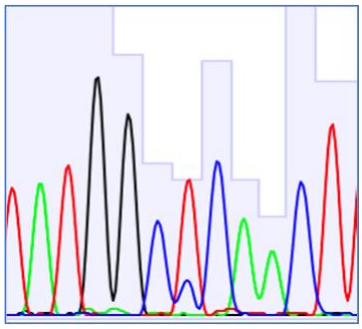

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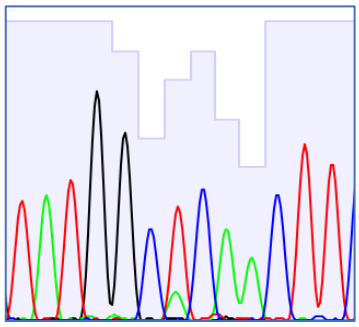

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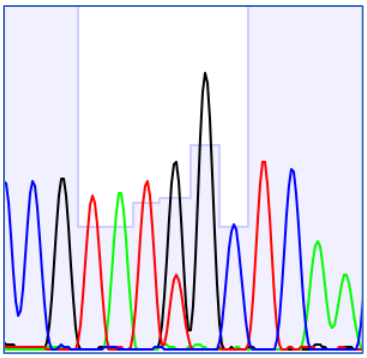

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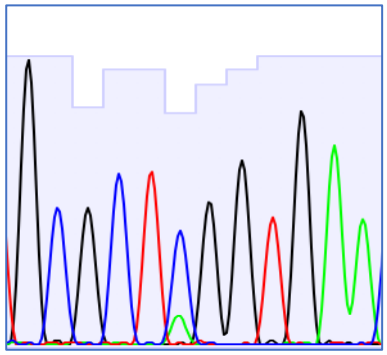

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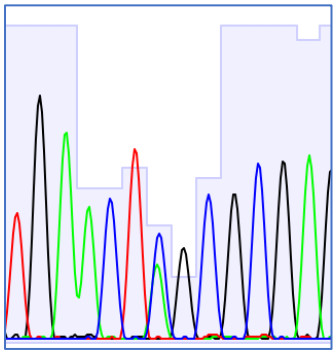

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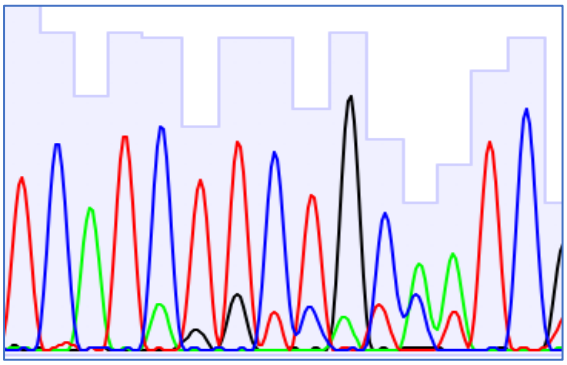

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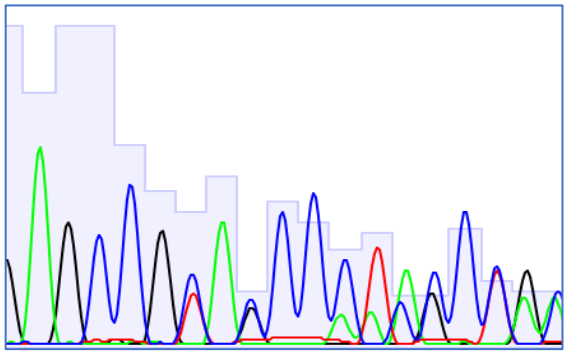

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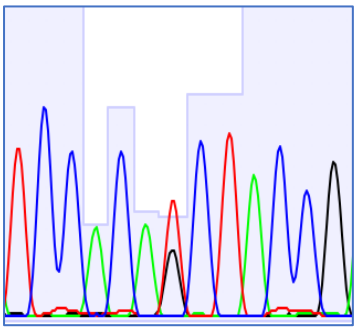

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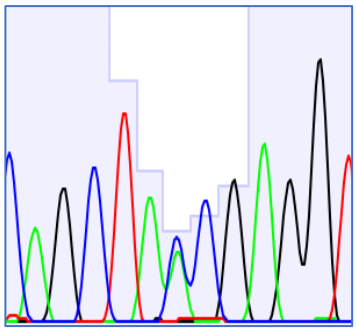

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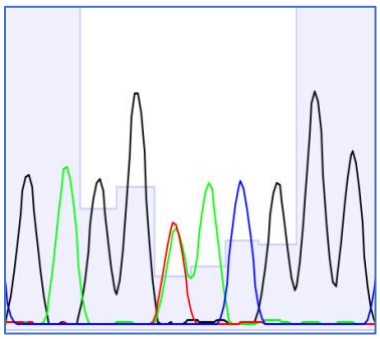

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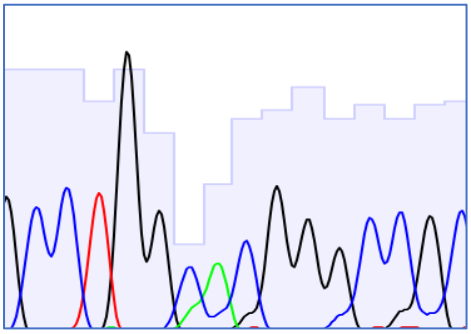

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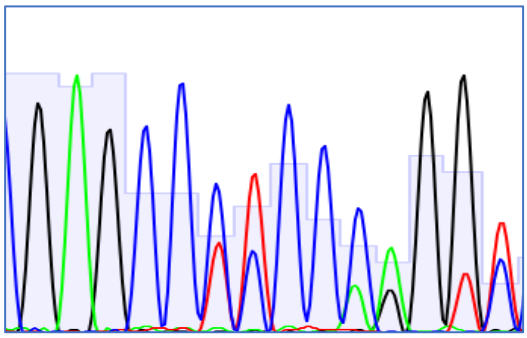

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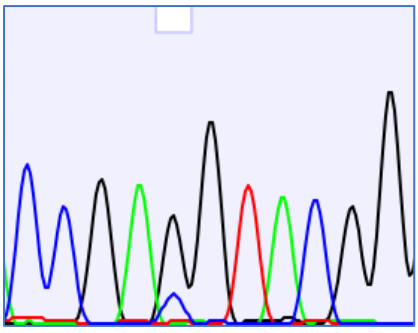

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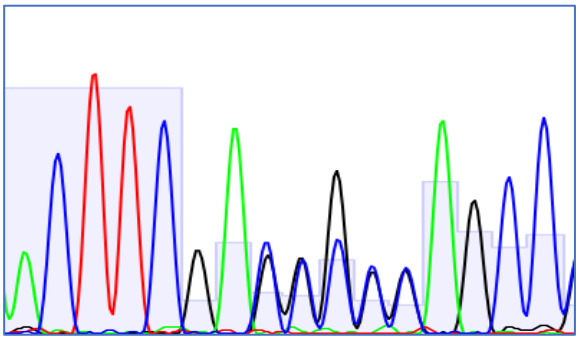

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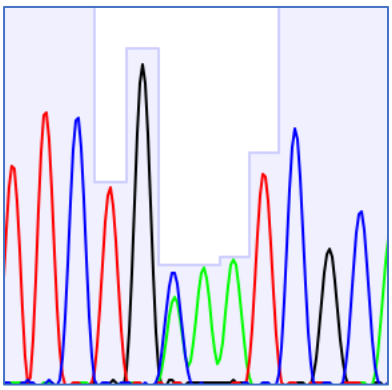

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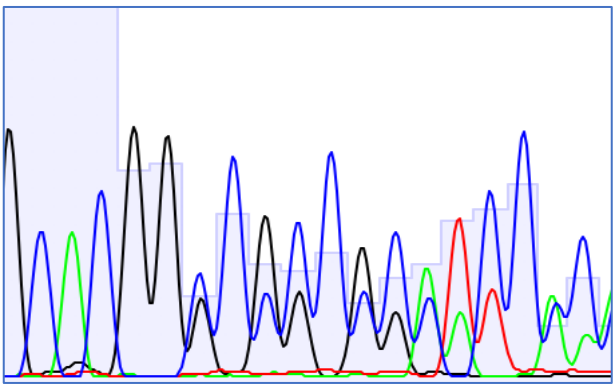

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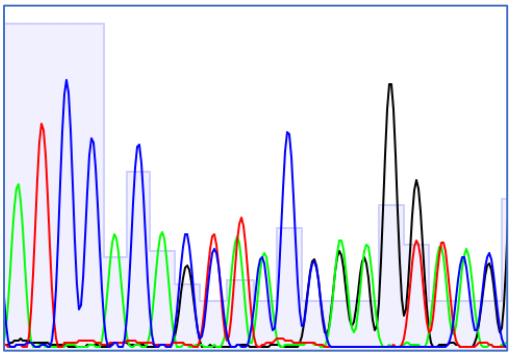

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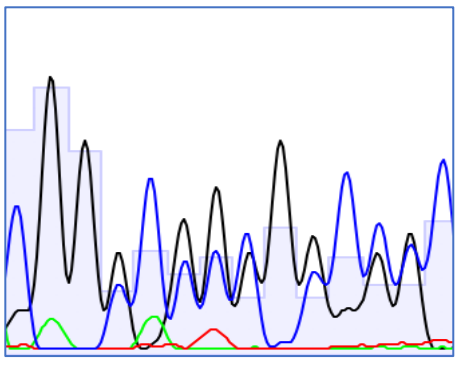

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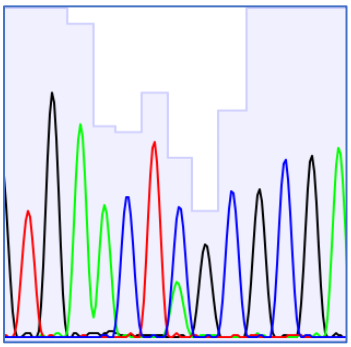

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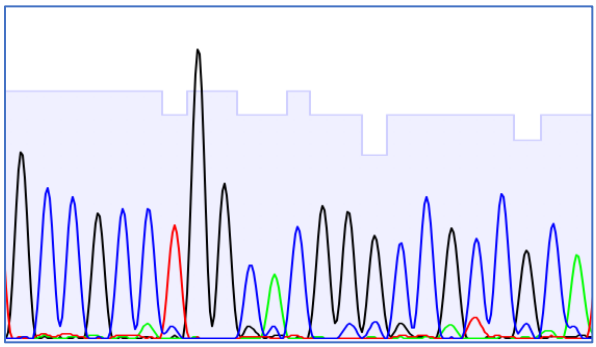

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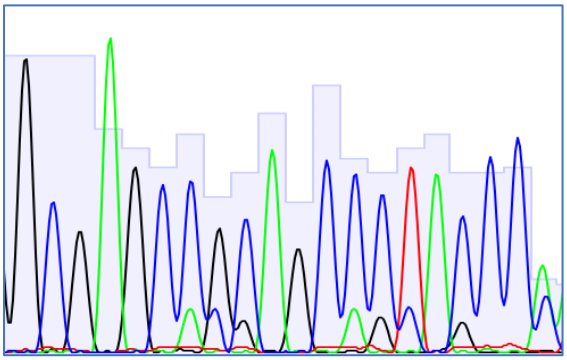

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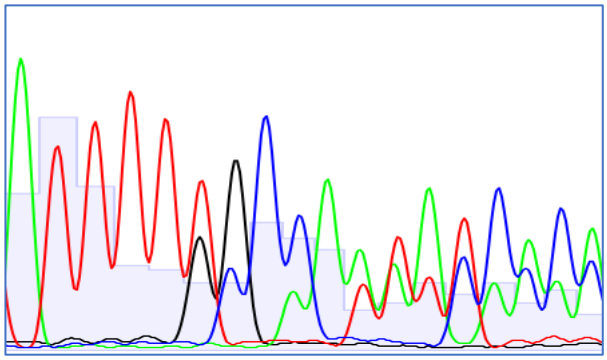

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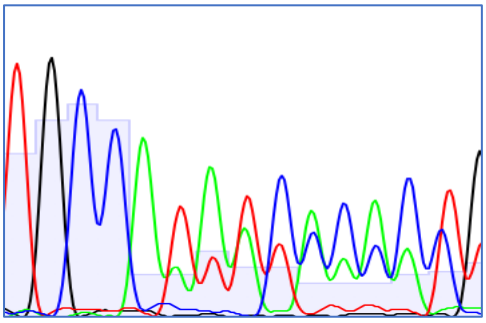

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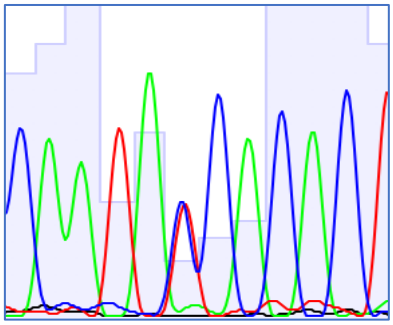

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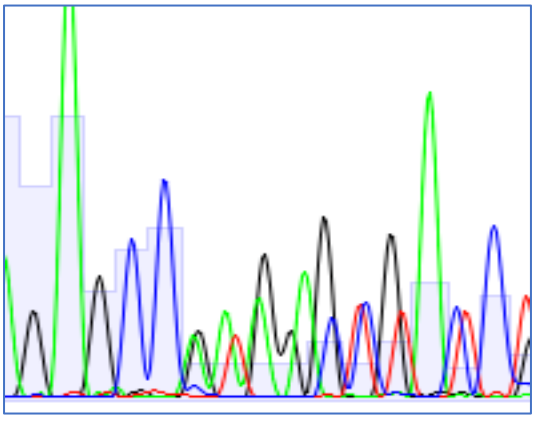

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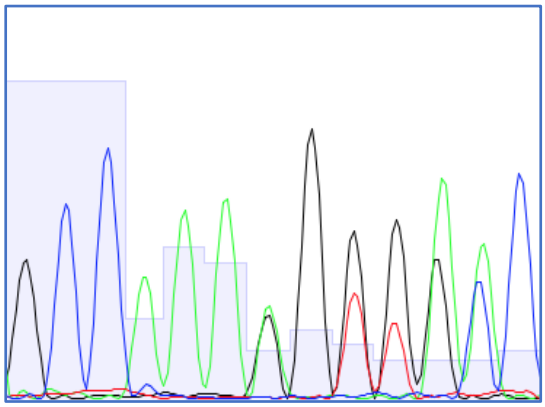

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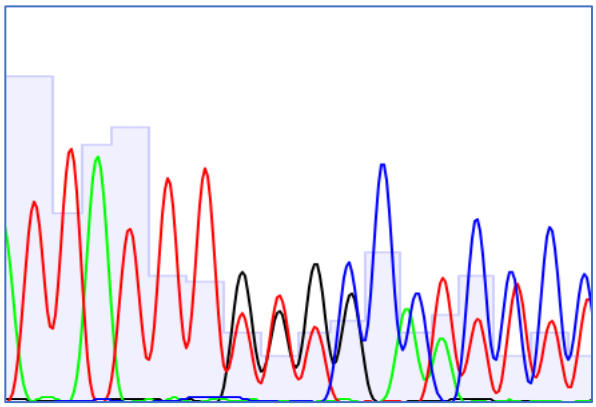

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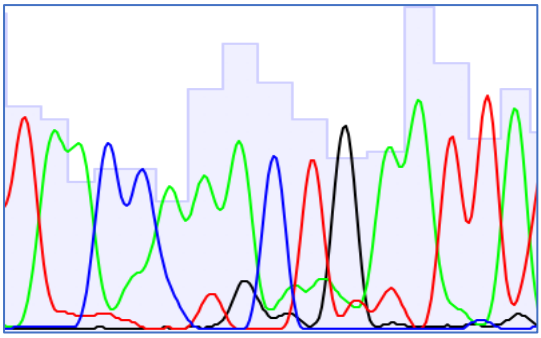

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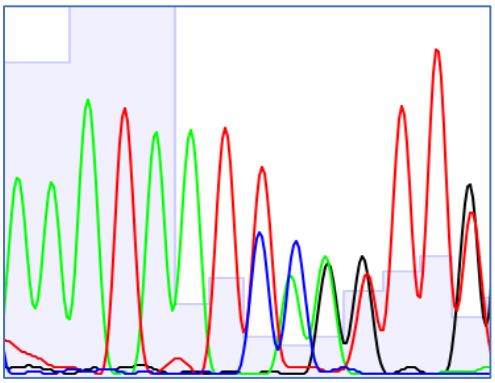

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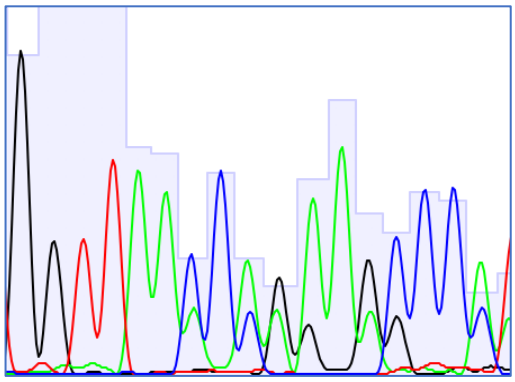

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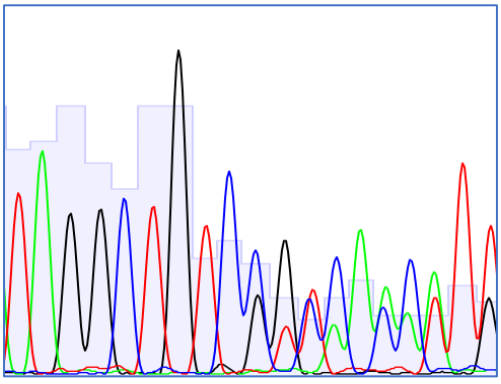

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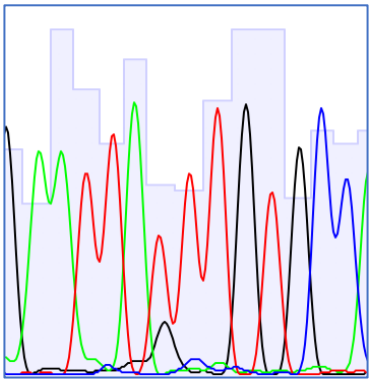

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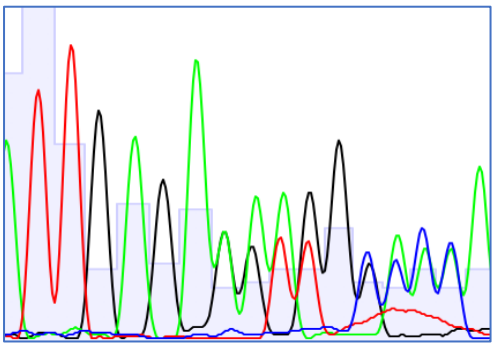

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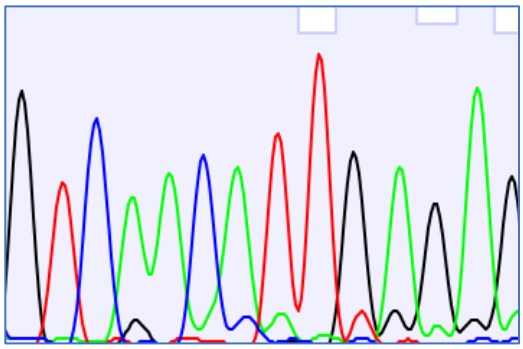

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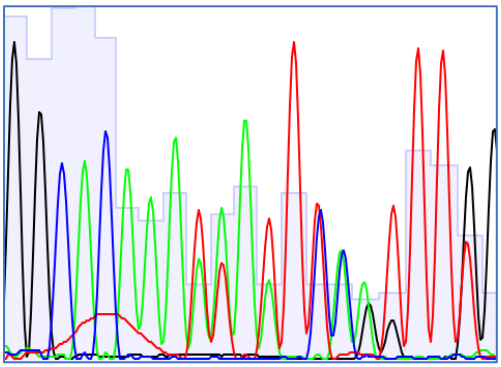

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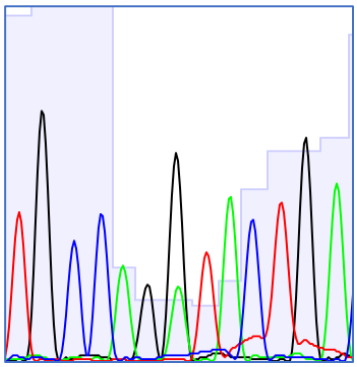

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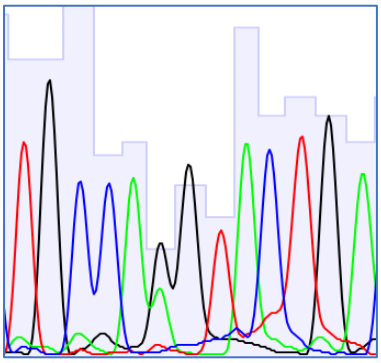

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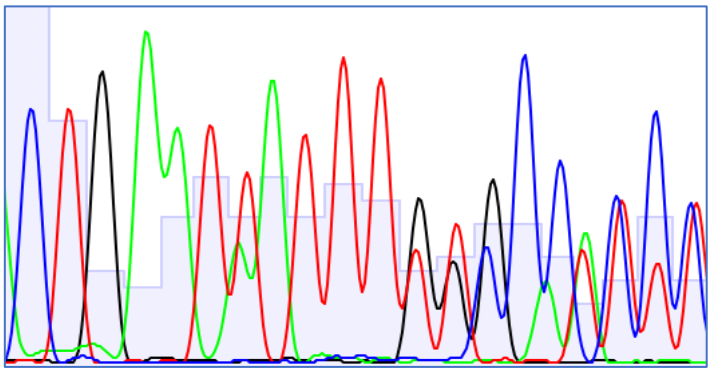

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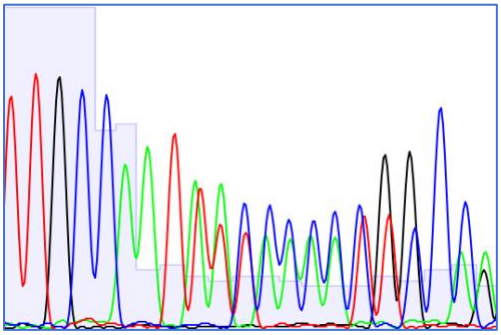

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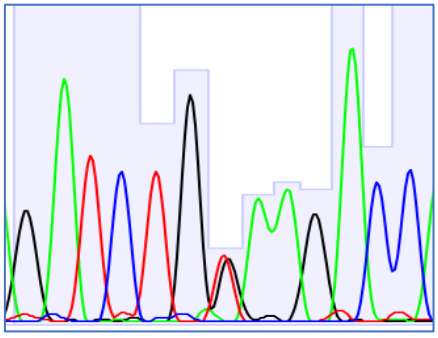

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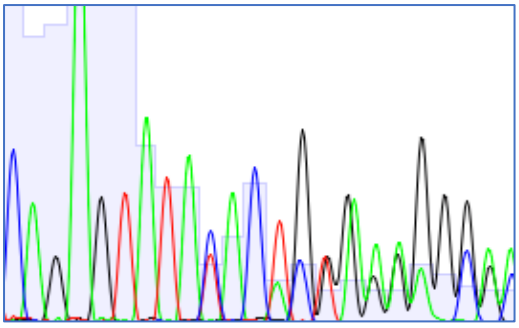

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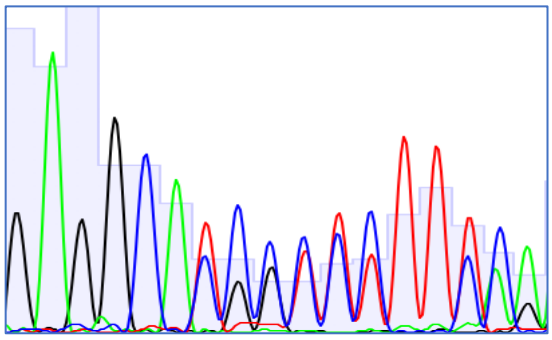

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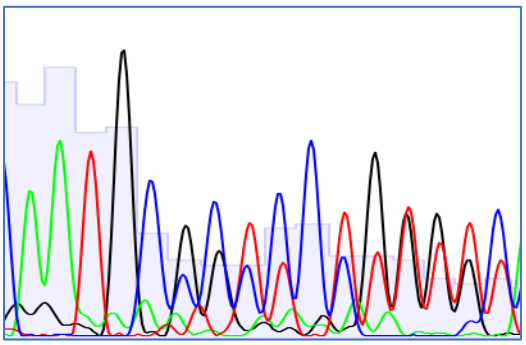

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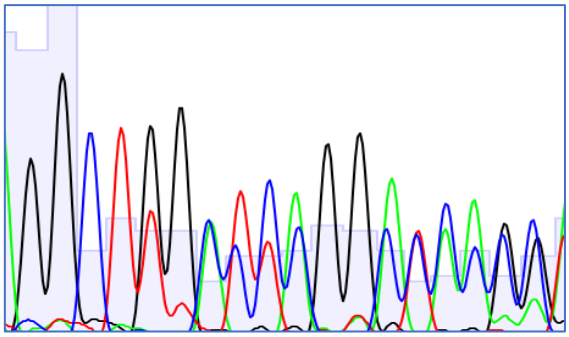

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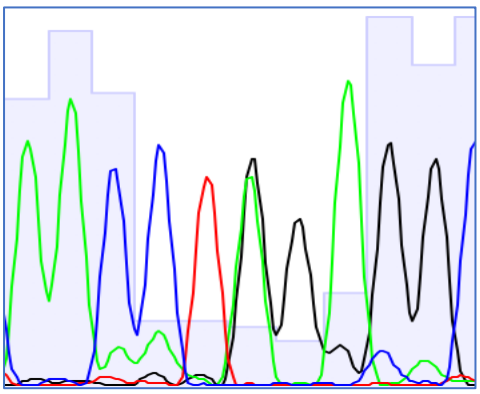
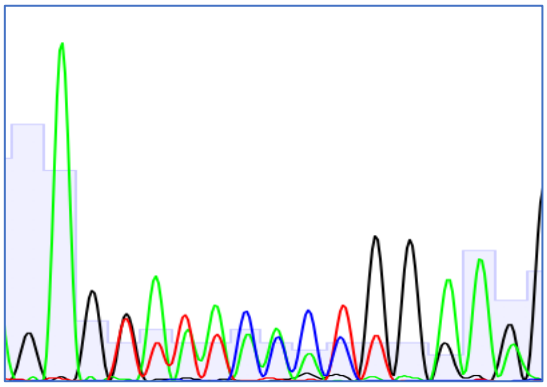
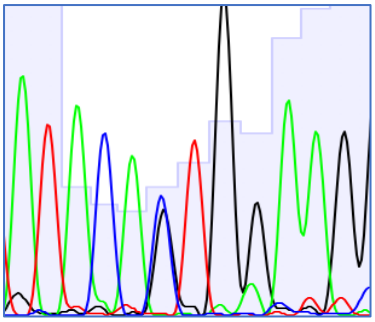


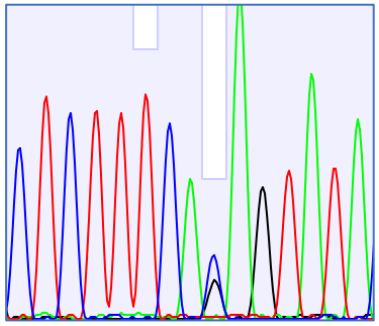
K252 VHL_3:10191497:C:T



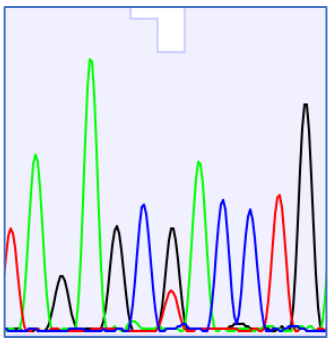
K352 VHL_3:10191476:10191476:-:C



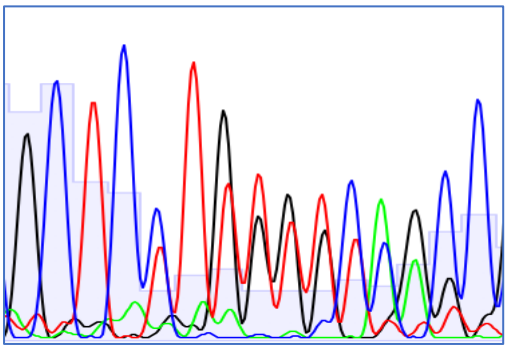
K360 VHL_3:10191470:G:C



K520 VHL_3:10191479:C:G



K523 VHL_3:10191555:C:A



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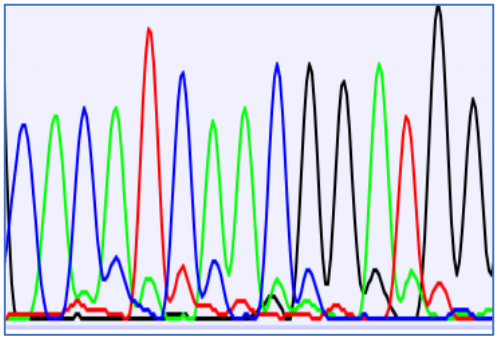

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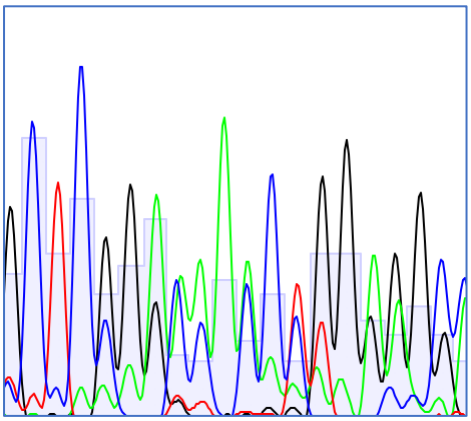
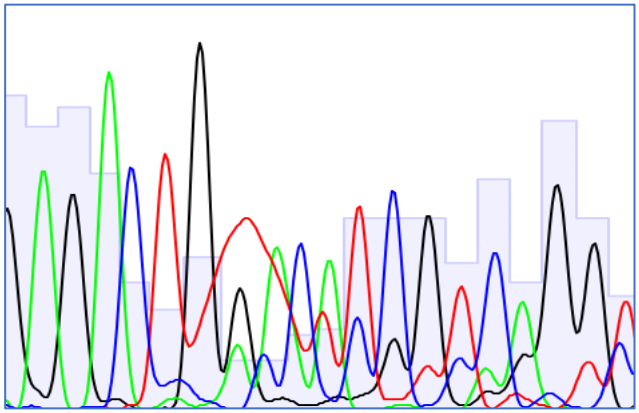
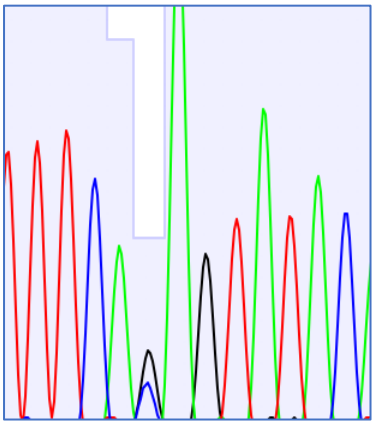


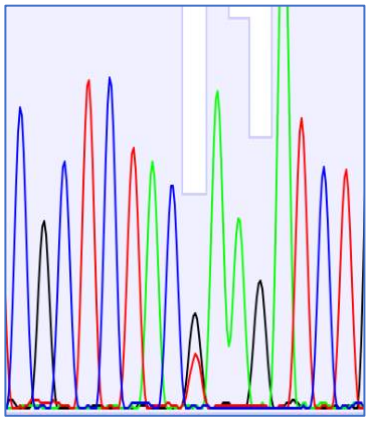
K099 VHL_3:10191507:10191507:G:-



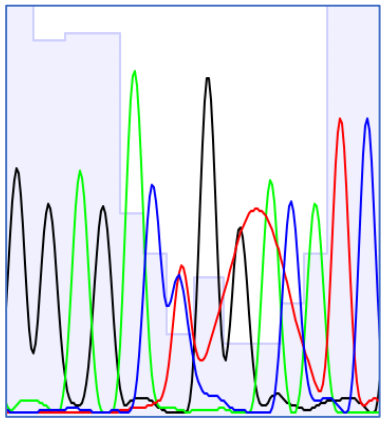
K128 VHL_3:10191541:10191541:G:-



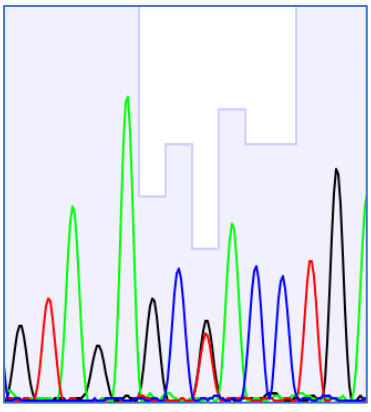
K167 VHL_3:10191479:C:G



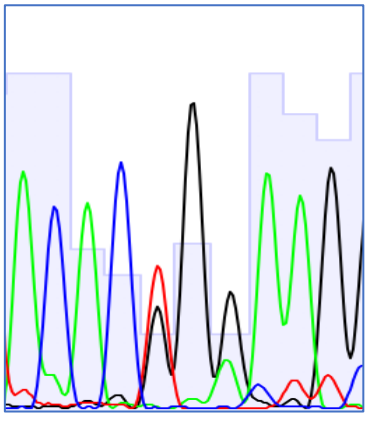
K207 VHL_3:10191563:G:T



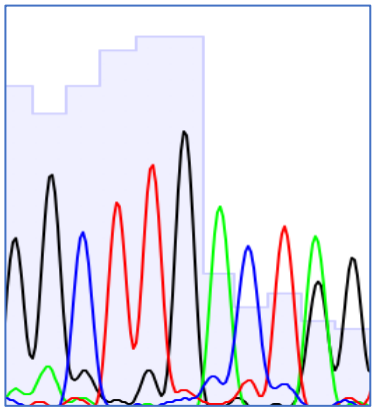
K208 VHL_3:10191540:T:C



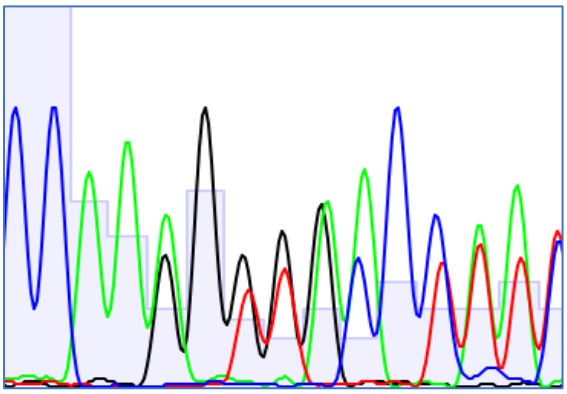
K242 VHL_3:10191555:C:A



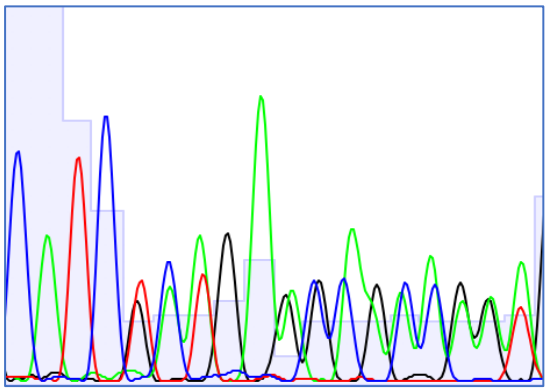
K245 VHL_3:10191469:A:C



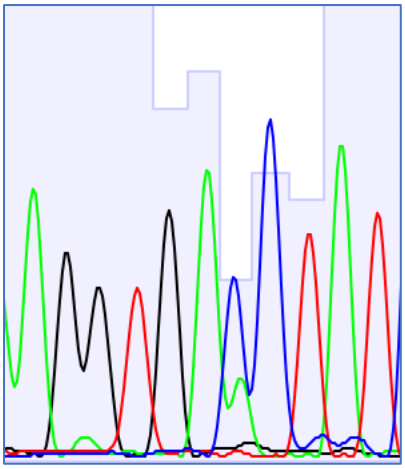
K265 VHL_3:10191513:T:C



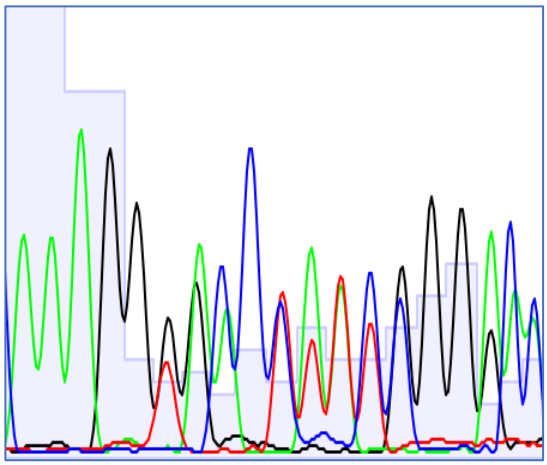
K256 VHL_3:10188204:10188204:T:-



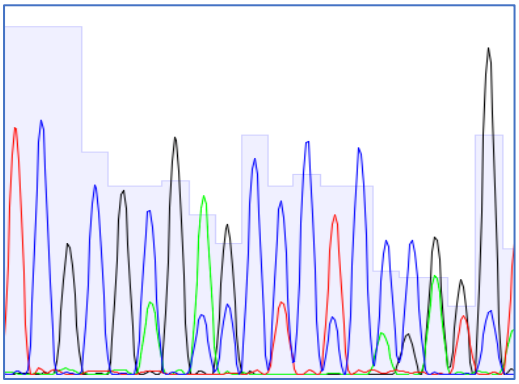
K334 VHL_3:10188215:10188217:AGA:-



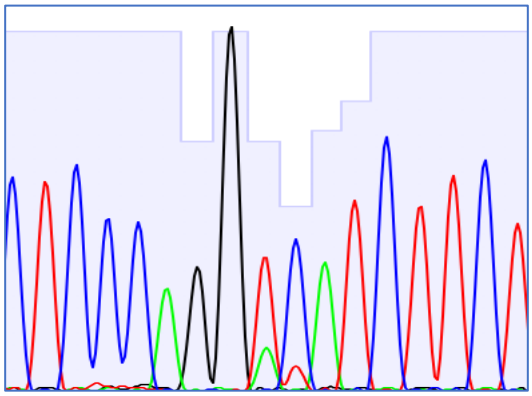
K352 VHL_3:10188198:G:T



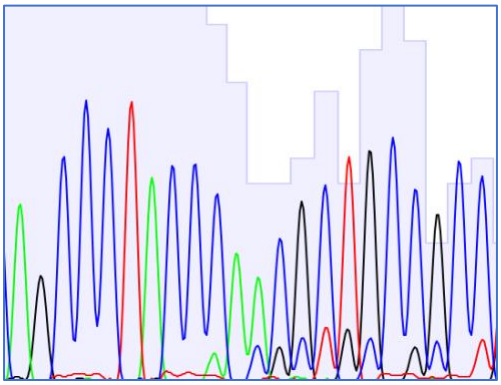
K375 VHL_3:10188201:10188201:A:-



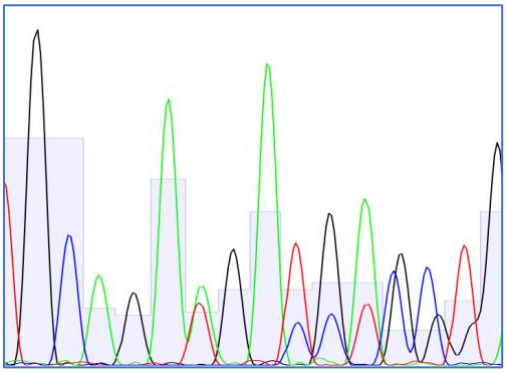
K039 VHL_3:10183734:10183735:CG:-



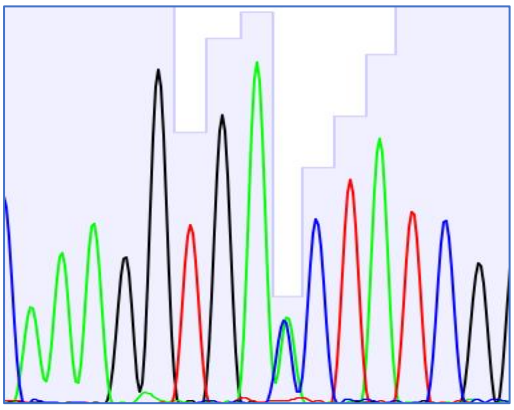
K156 VHL_3:10183752:10183753:TC:AT



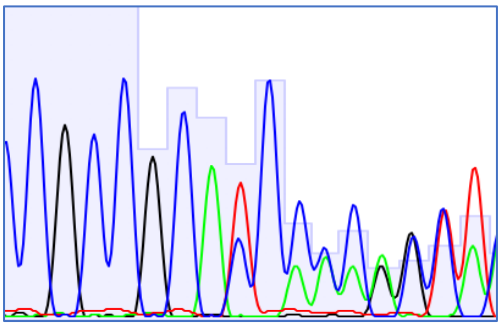
K326 VHL_3:10183825:10183825:C:-



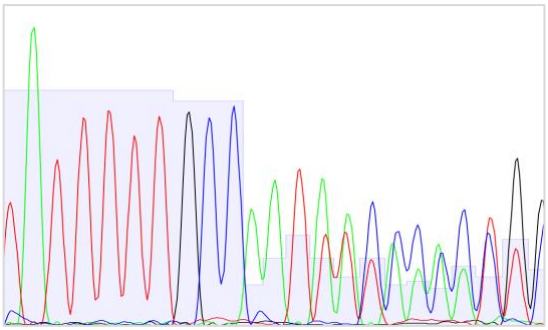
K184 VHL_3:10183755:10183757:TCT:-



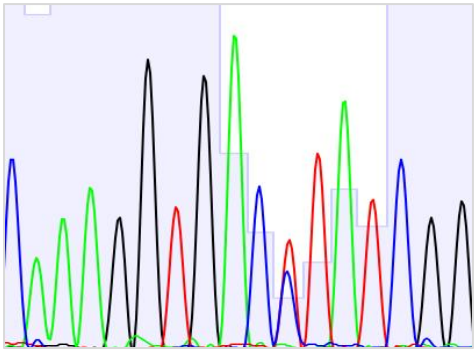
K216 VHL_3:10188198:G:T



K217 VHL_3:10183857:10183857:T:-

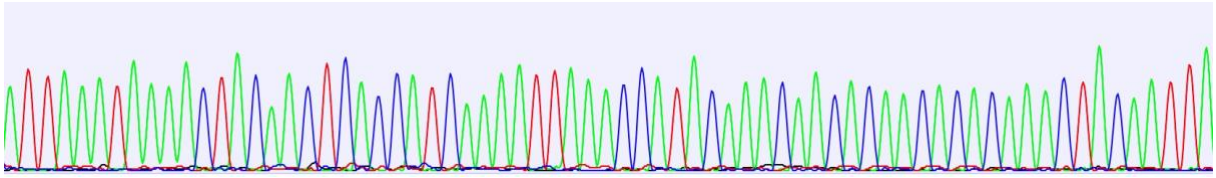


K386 VHL_3:10188306:10188306:-:T

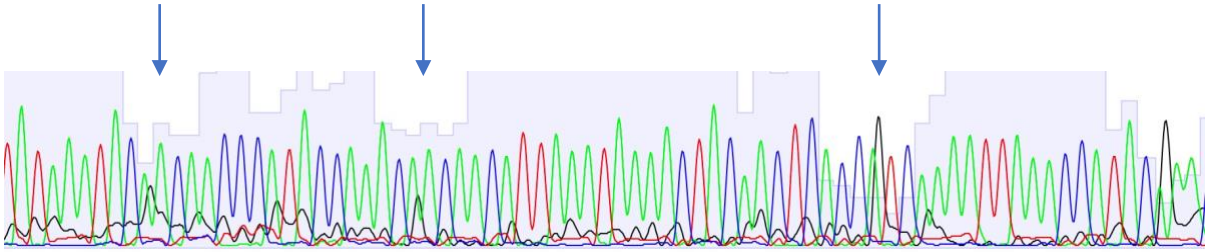


K118 VHL_3:10188197:G:A

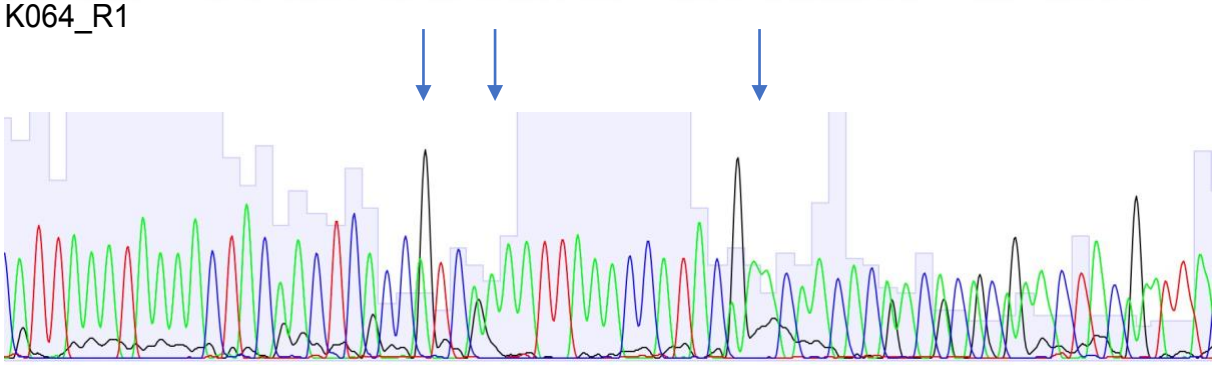
VHL methylation Profiles



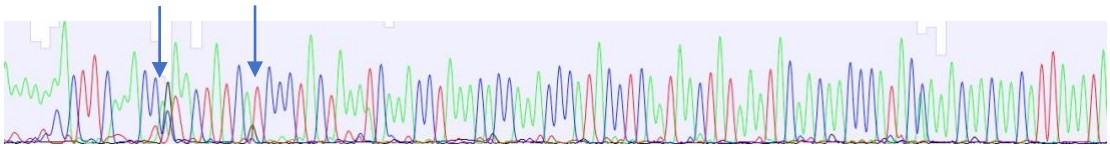
K064_N



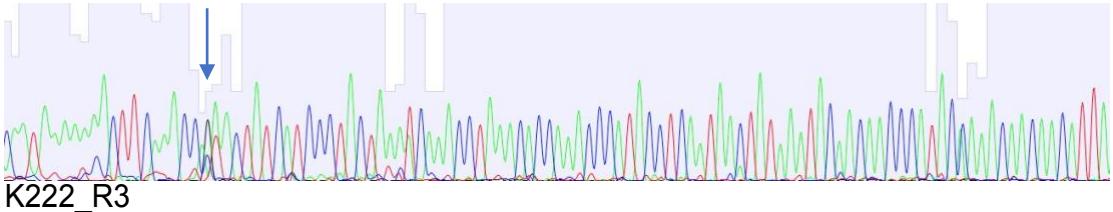
K064_R1



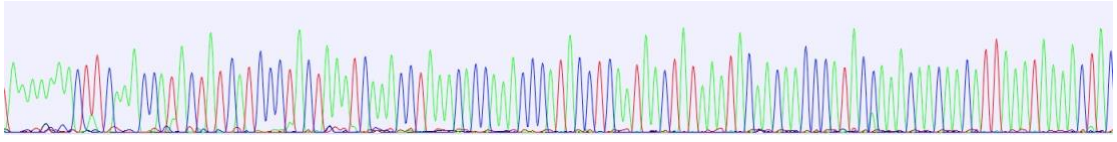
K064_R2



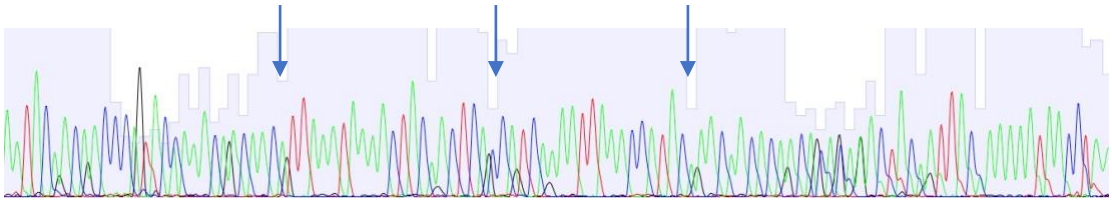
K222_R2



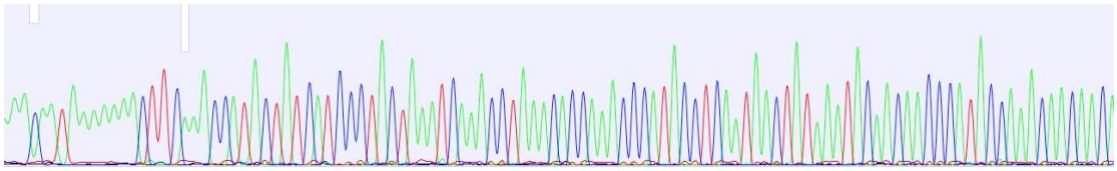
K222_R3



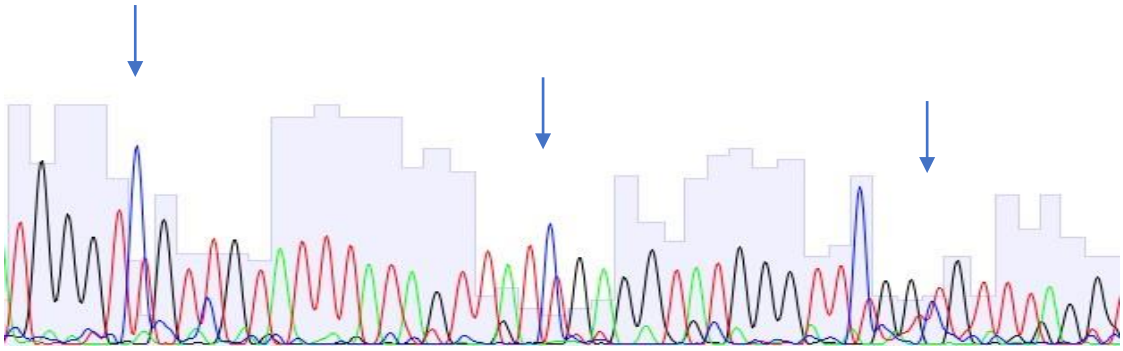
K222_N



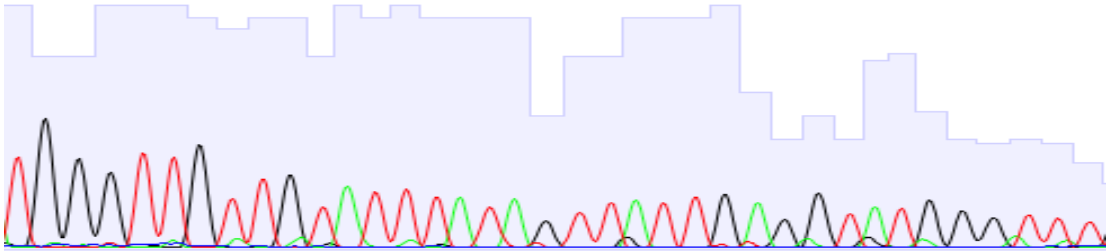
K233_R3



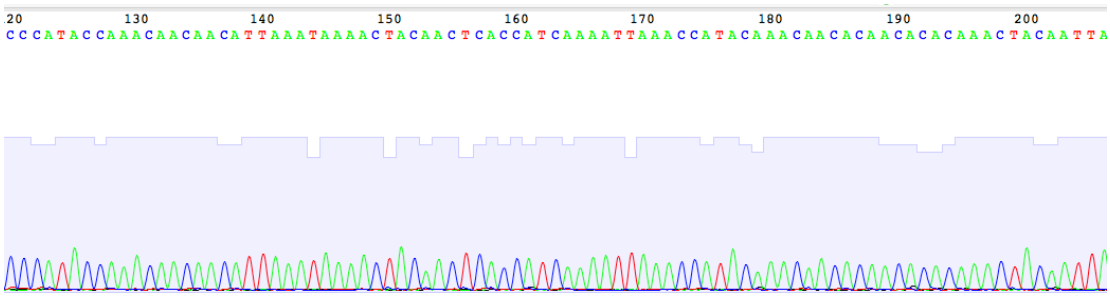
K233_N



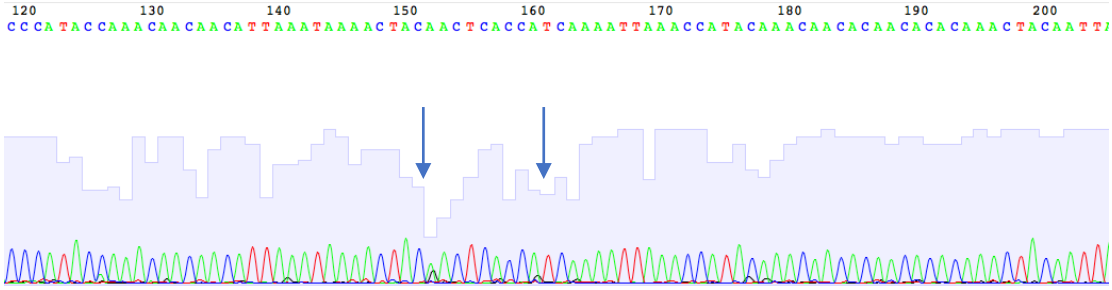
K239_R3



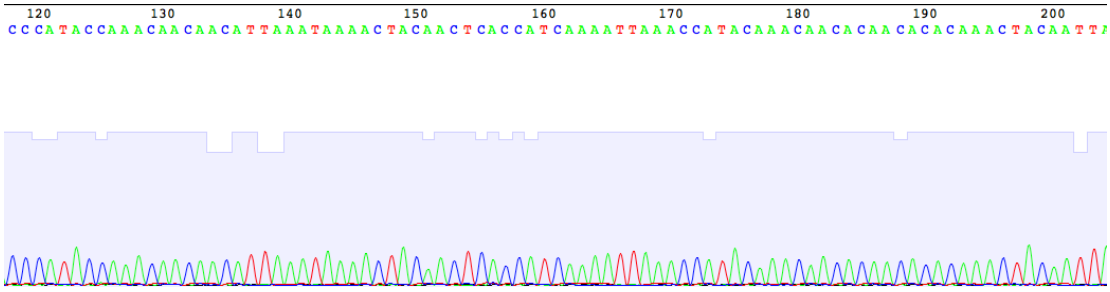
K239_N



K271_R1

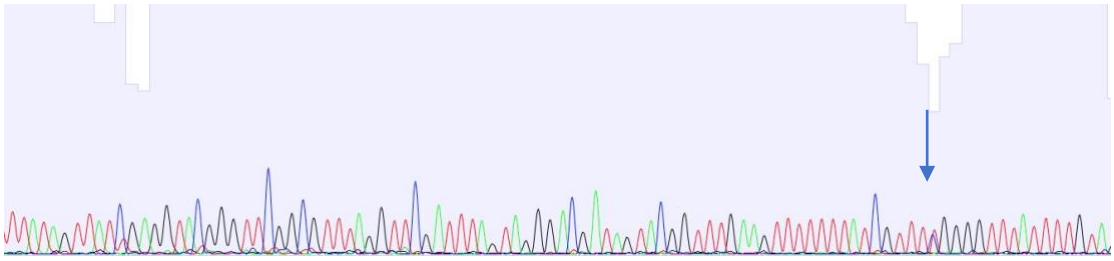


K271_R2

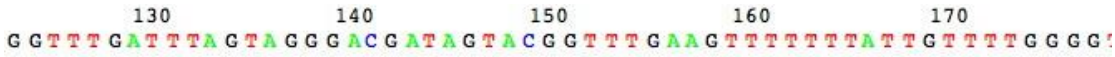


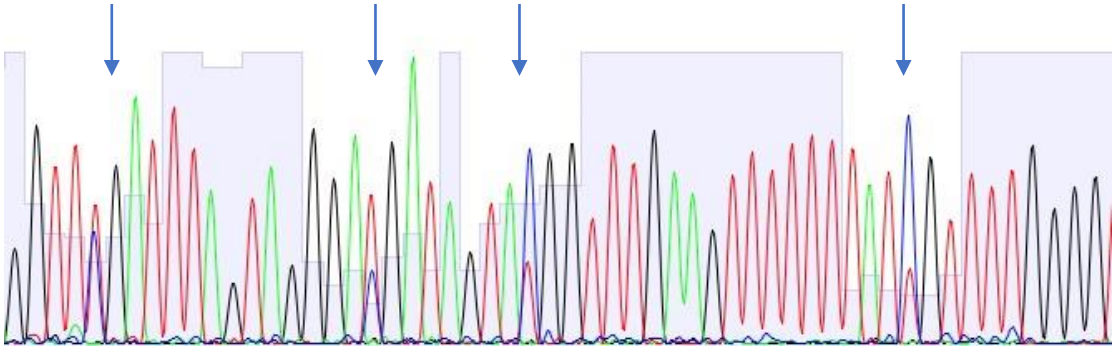
K271_N

K354_R4

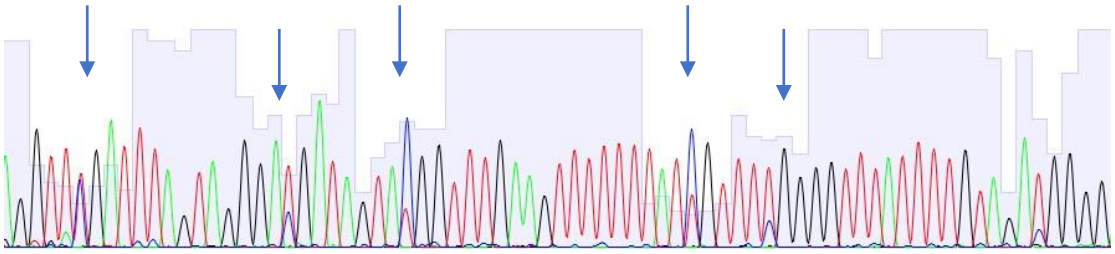


K354_R3

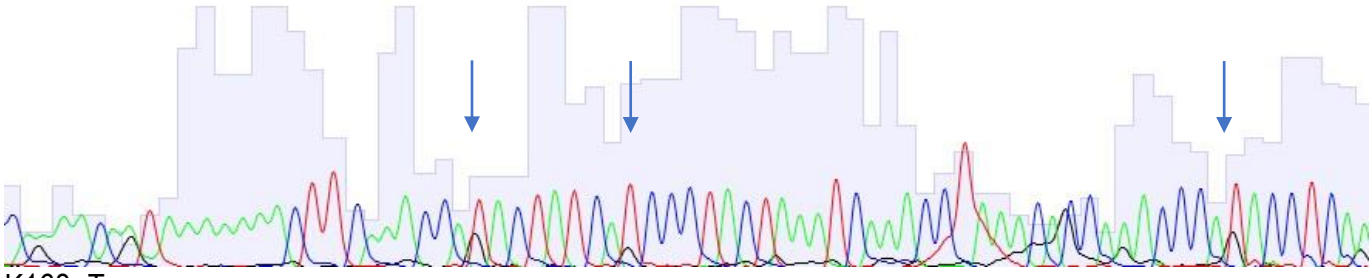




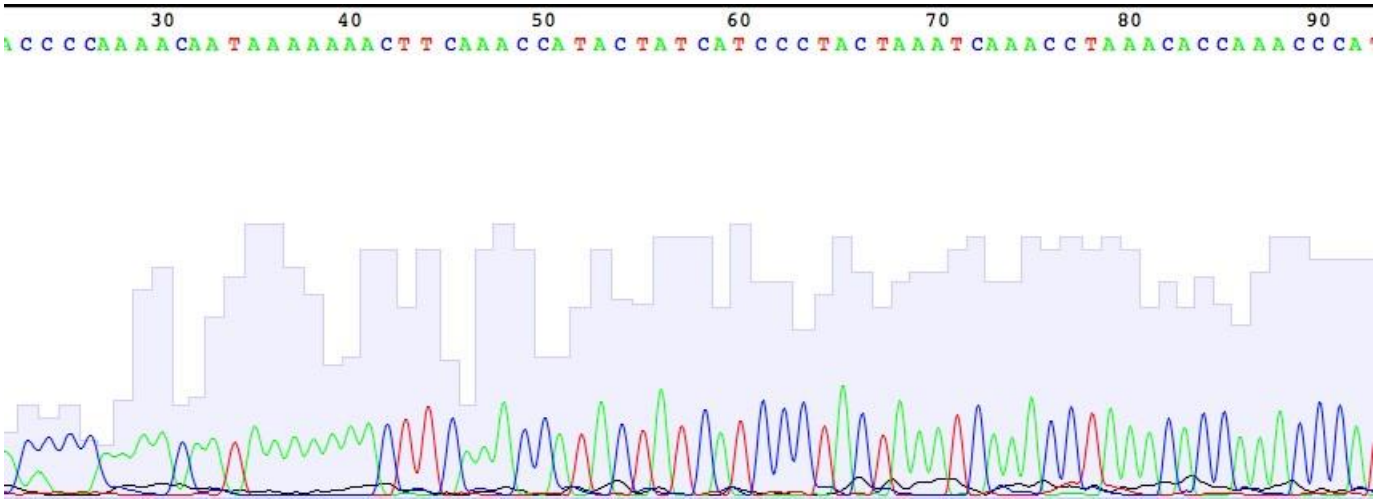
K379_R22



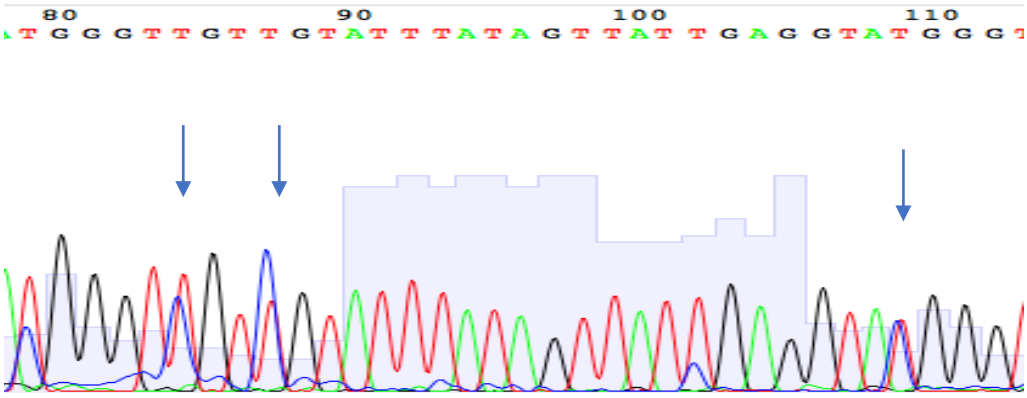
K379_R23



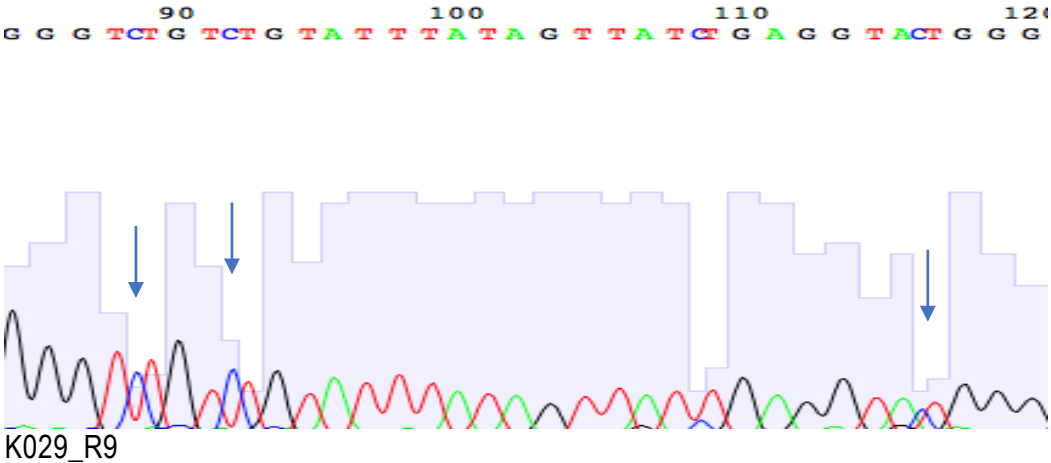
K163_Tumour



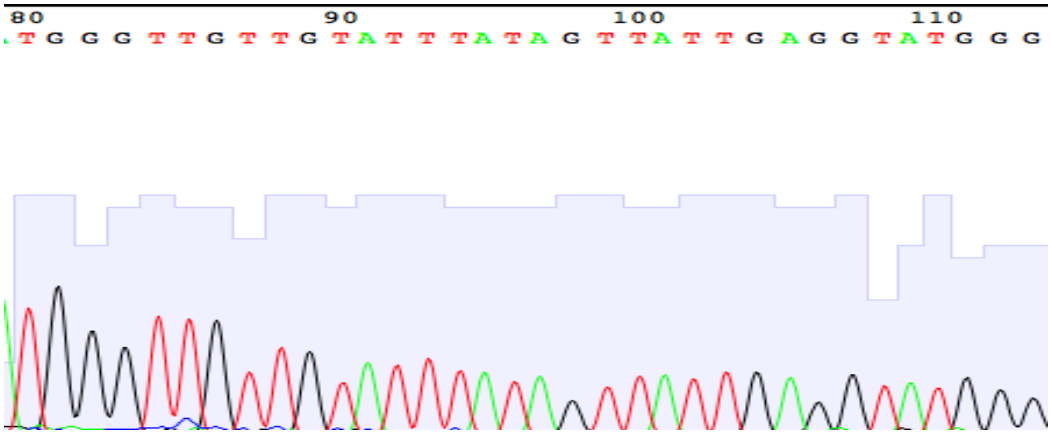
K163_N



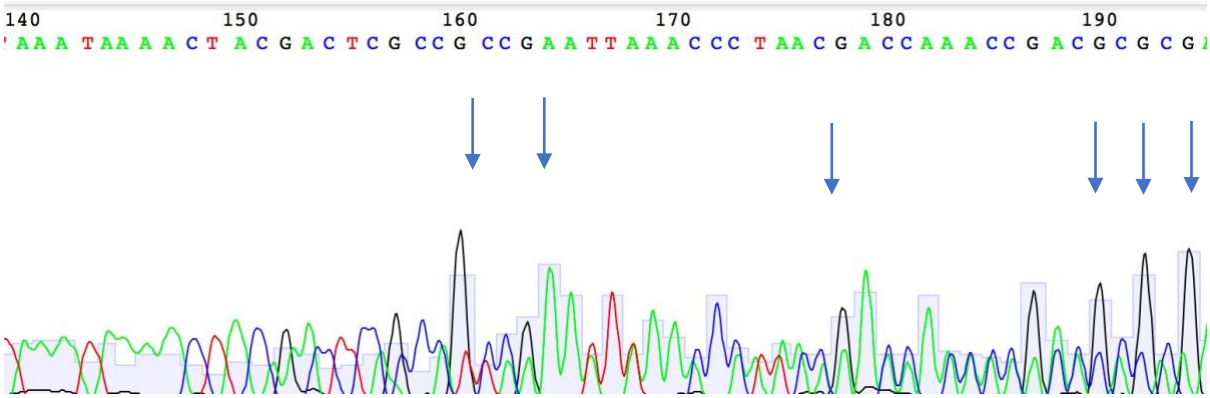
K029_R3



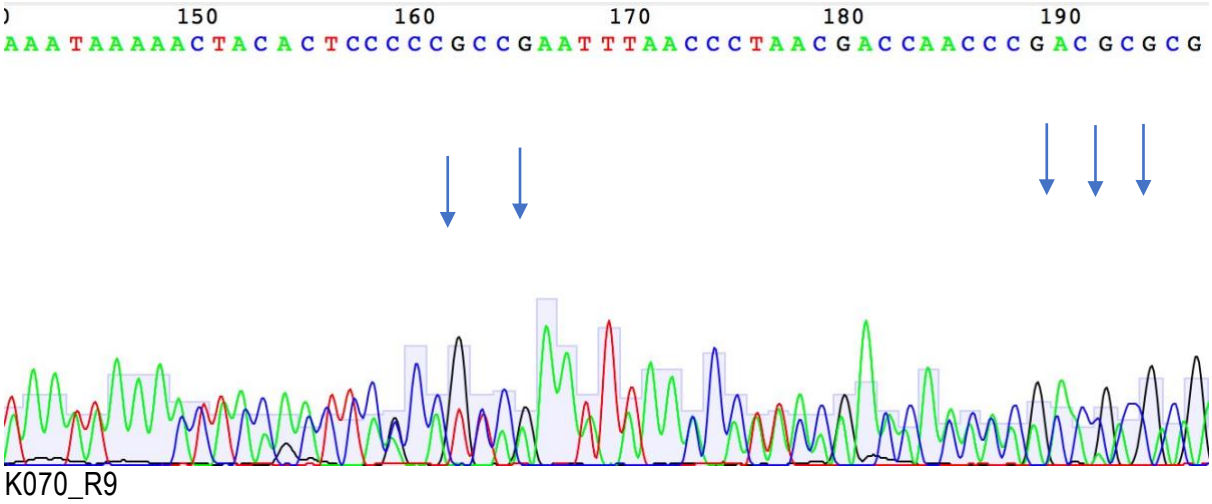
K029_R9



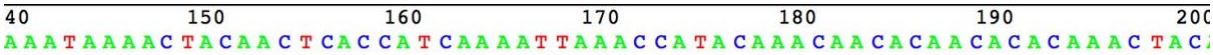
K029_N

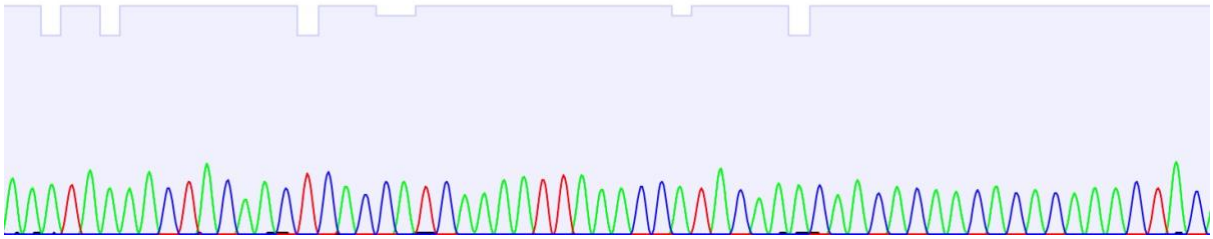


K070_R4

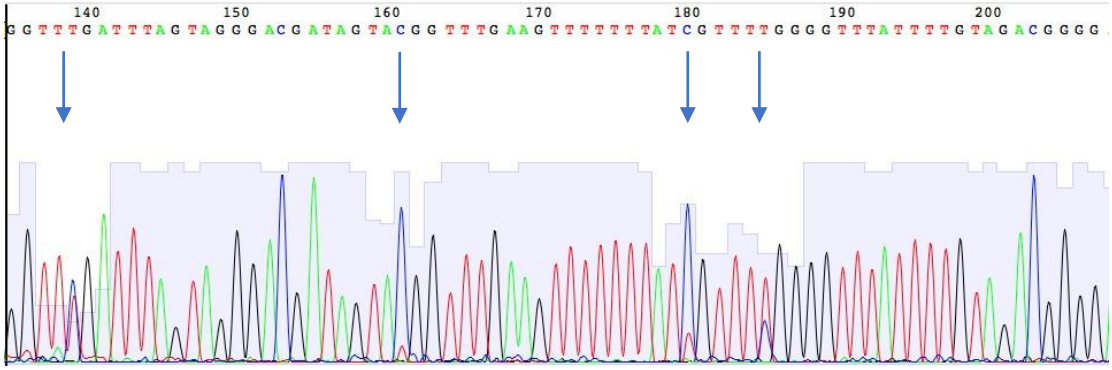


K070_R9

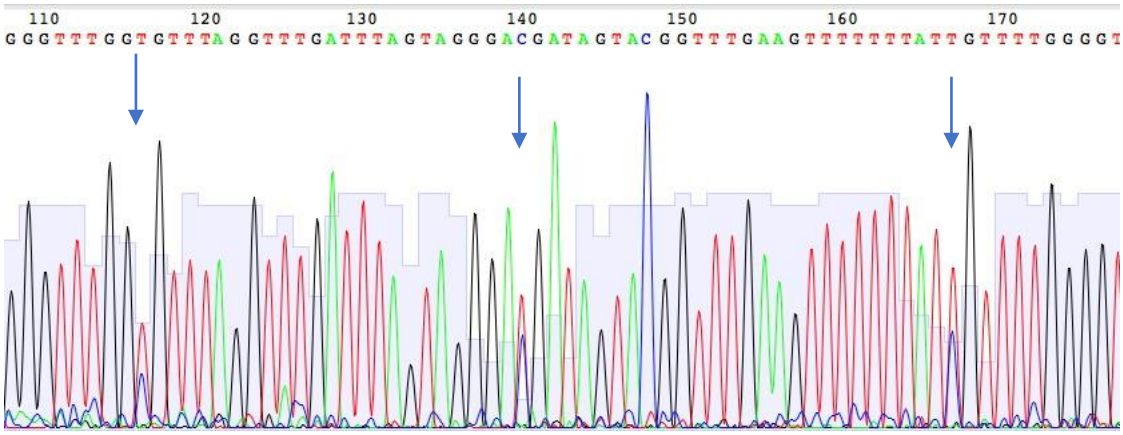




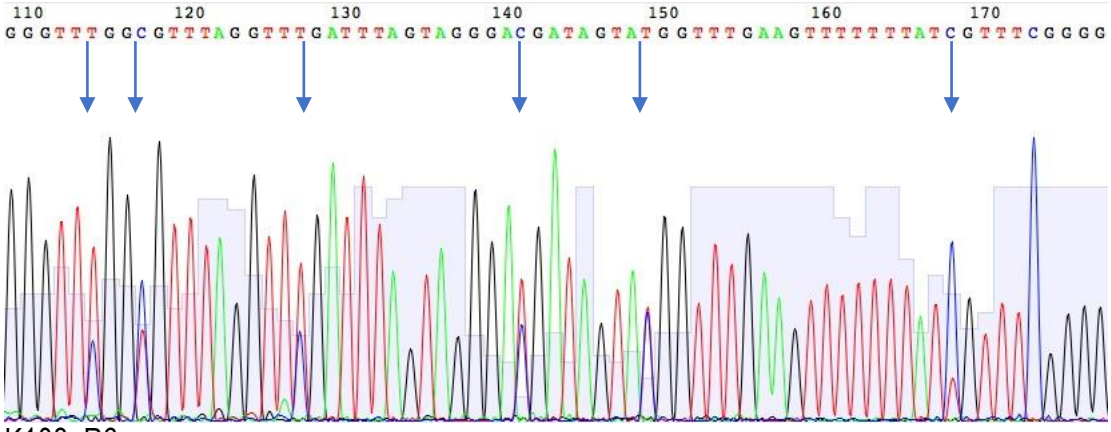
K070_N



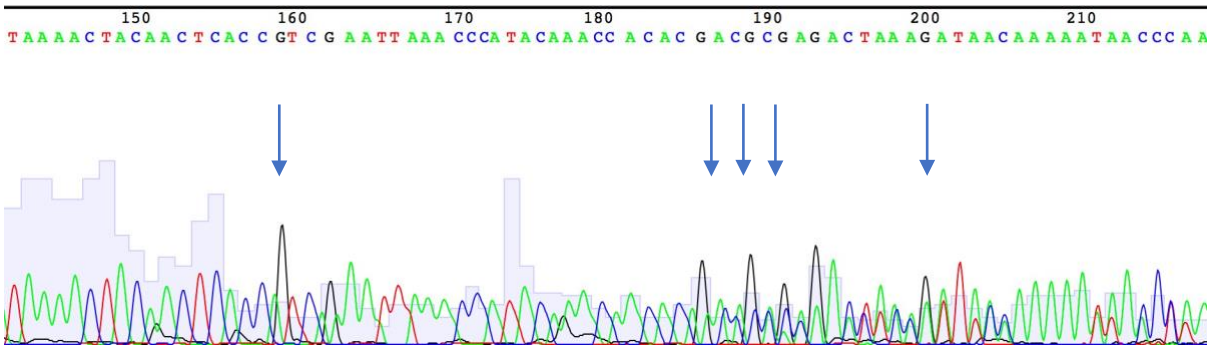
K098_R1



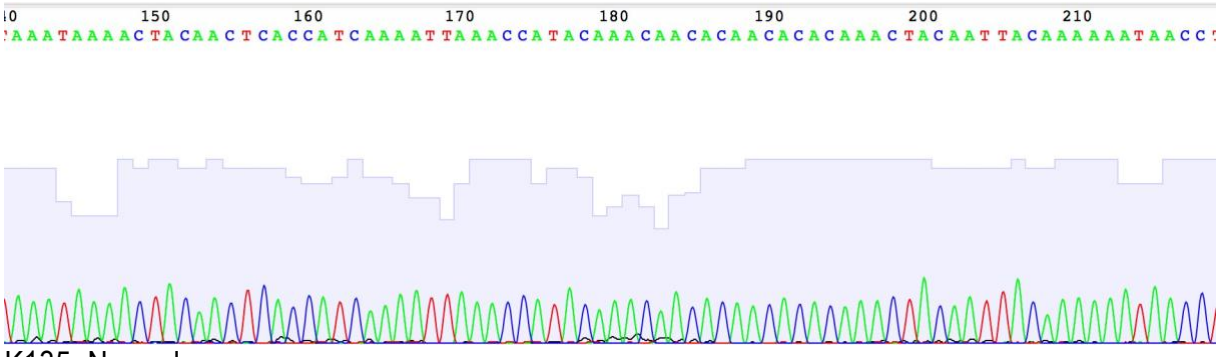
K108_R3



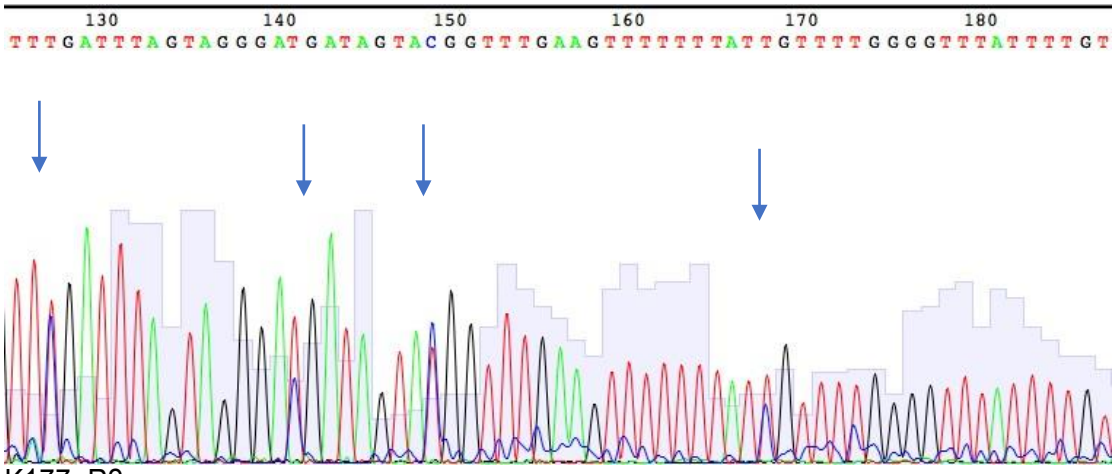
K108_R6



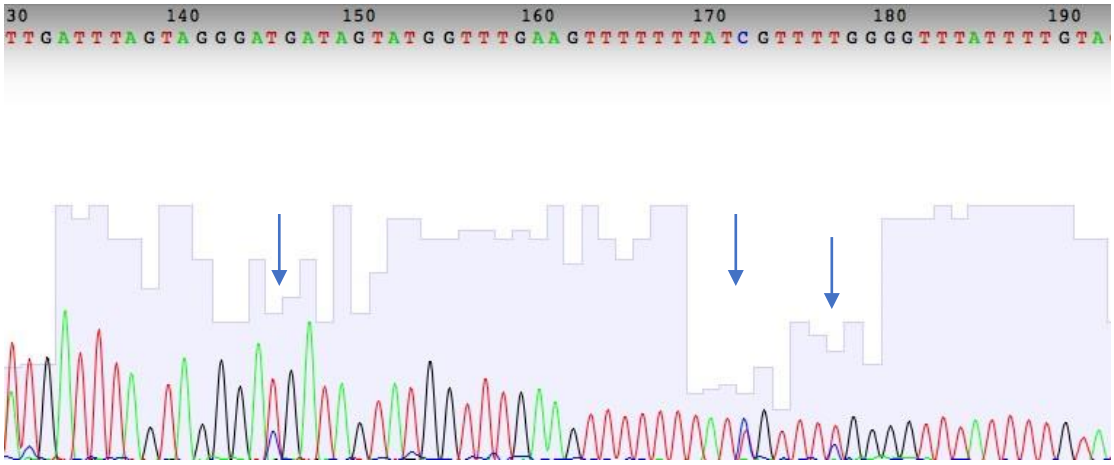
K135_Tumour



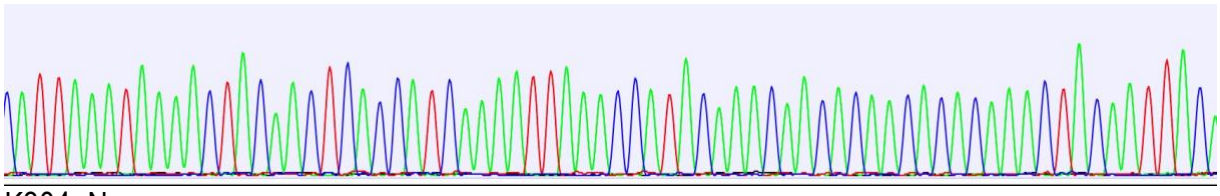
K135_Normal



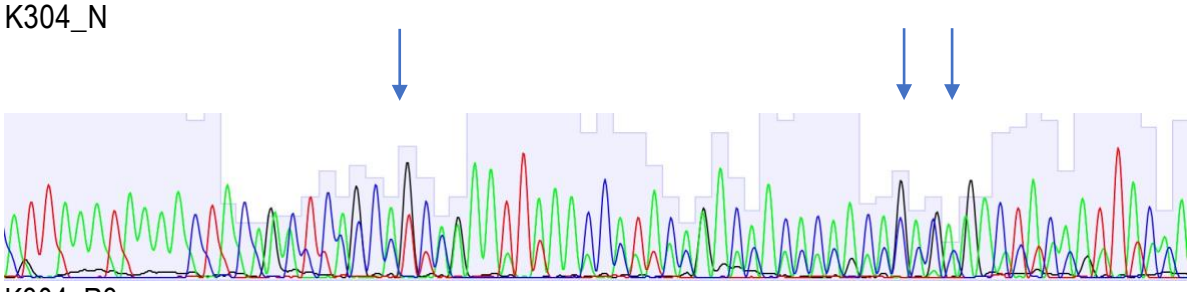
K177_R2



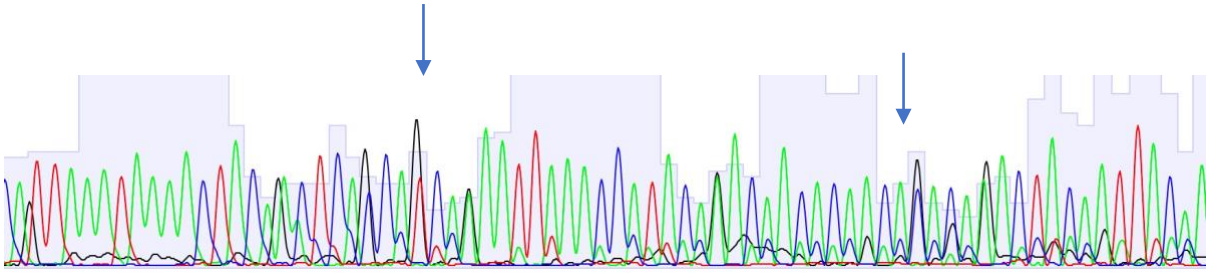
K177_R4



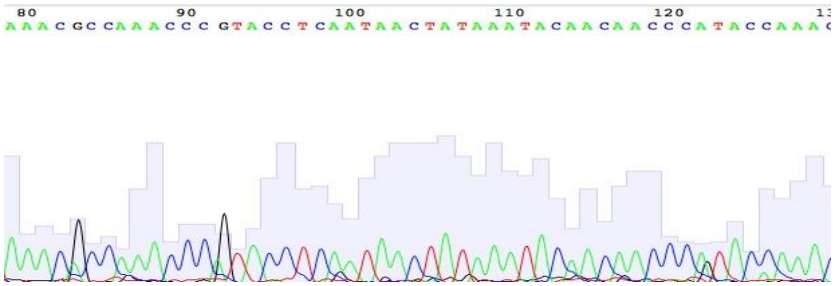
K304_N



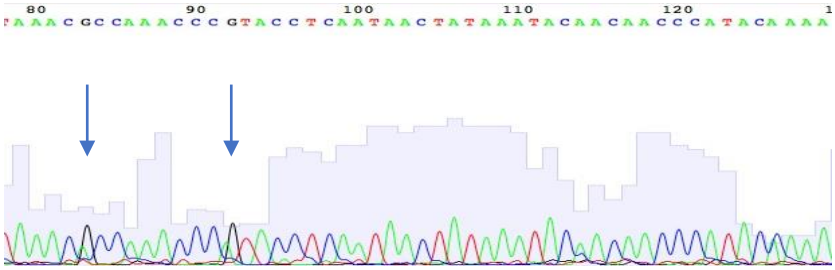
K304_R3



K304_R4

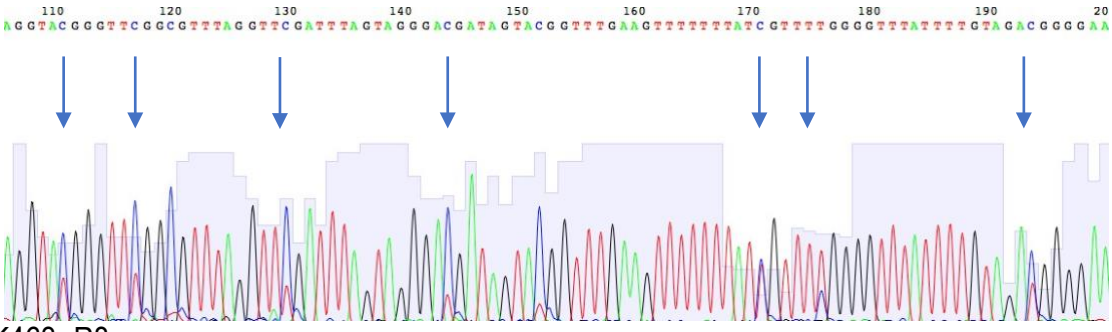


K308_R2

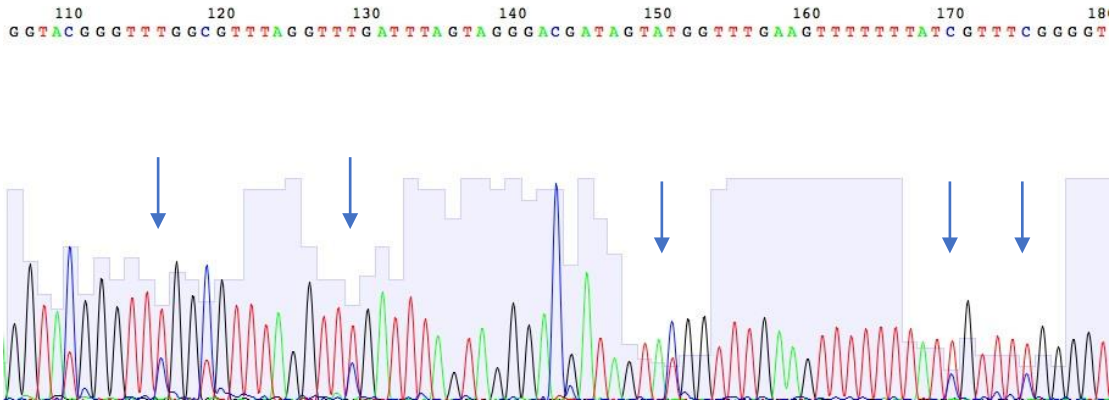


K308_R3

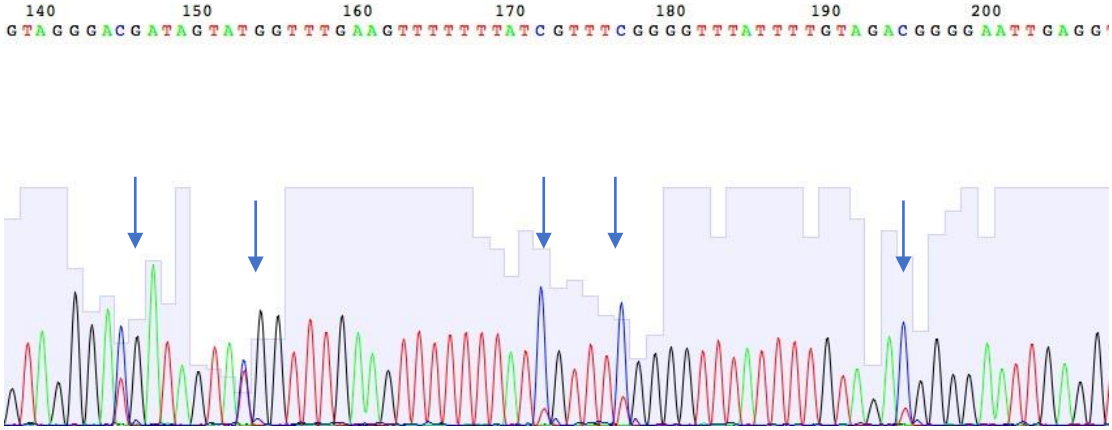
K308_N



K463_R8

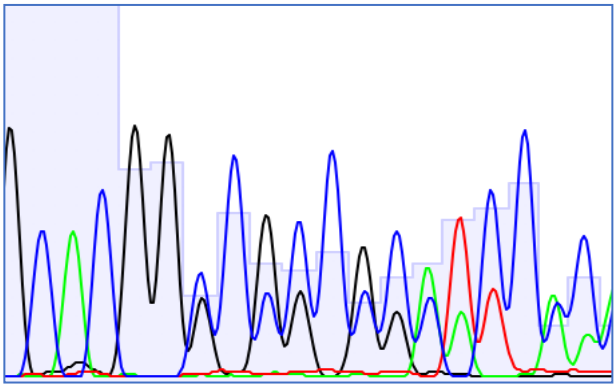


K463_R17

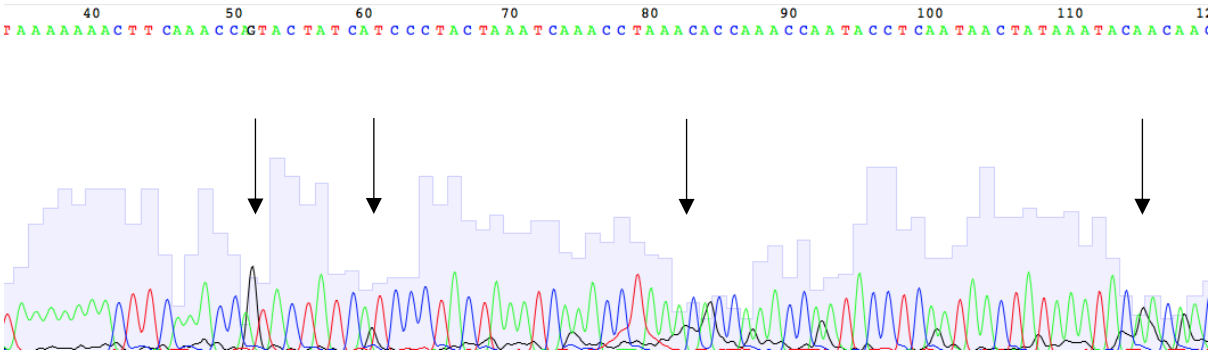


K463_R3

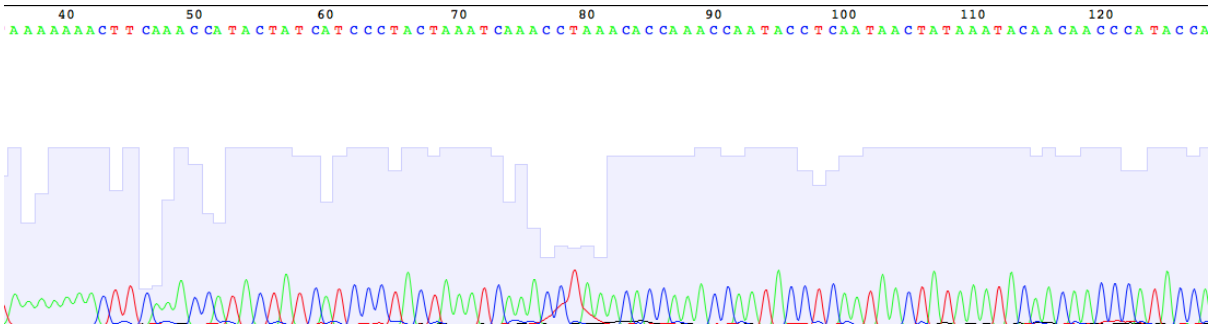
Sanger sequence trace of the VHL INDEL identified in the right kidney tumour of patient K097 and VHL methylation in the left kidney tumour.



K097_Right Kidney VHL_3:10183846:10183846:G:-

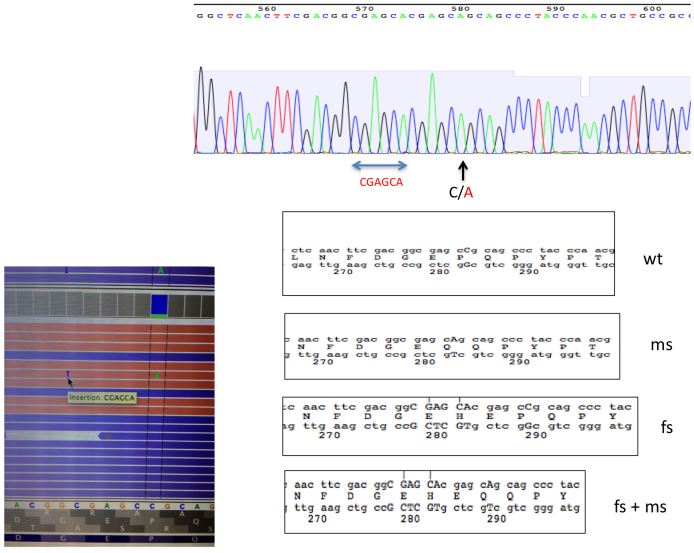


K097_Tumour_methylation – Left Kidney



RK97_Normal Kidney Tissue

VHL mutations in left K150 tumours (historical)

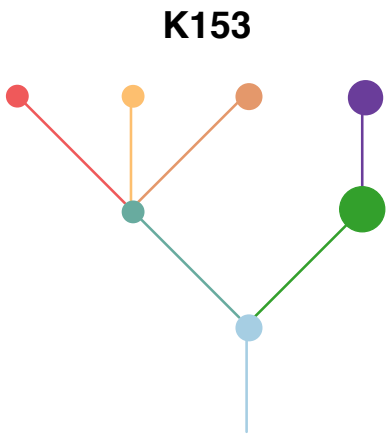


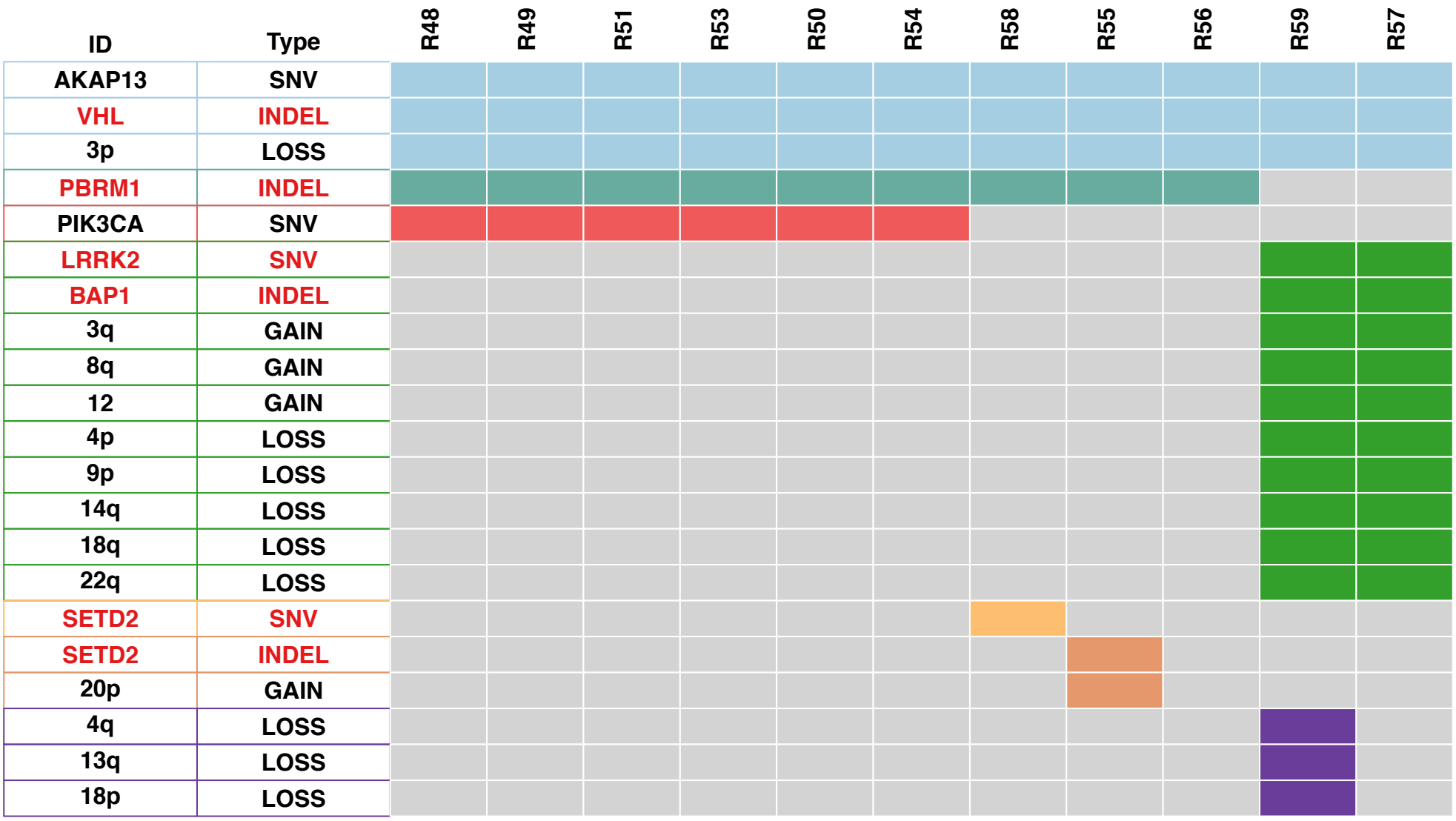
Oligonucleotide sequences for VHL exon amplification and methylation specific PCR

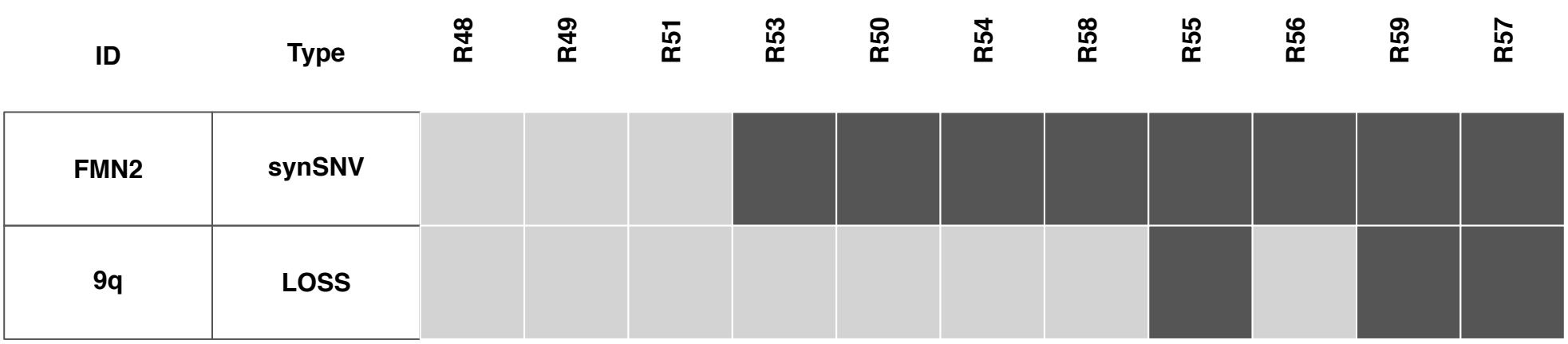
Oligonucleotide name	Oligonucleotide Sequence 5'-3'
VHL_X1_F	gtggaaatacagtaacgagttgg
VHL_X1_R	caggcaaaaattgagaactgg
VHL_X2_F	aacctttgcttgtcccgata
VHL_X2_R	caggcaaaaattgagaactgg
VHL_X3a_F	ctgccactgaggatttggtt
VHL_X3a_R	ccatcaaaagctgagatgaaa
VHL_methylation_1F	gagtttttttaggttatttttgtaat
VHL_methylation_1R,	tcaccctaaatatatatcctacctcaaaa
VHL_methylation_2F	cccctctaaaatttaatattttt
VHL_methylation_2R	ggttaaggttgtagtgagttaagtt

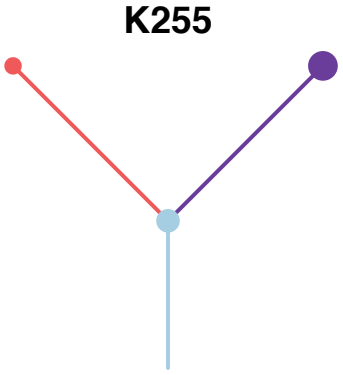
Figure S3: Mutation heatmap and driver trees for a subset of TRACERx Renal cohort. Related to Figure 1, Figure 2.

Presence and absence of mutational events for each tumour region are presented in heatmap (light gray means absence), with clonal events are presented as colored box and subclonal events are presented as colored triangles. Driver phylogenetic trees were build based on the presence or absence of mutational events (Please see Star Methods).



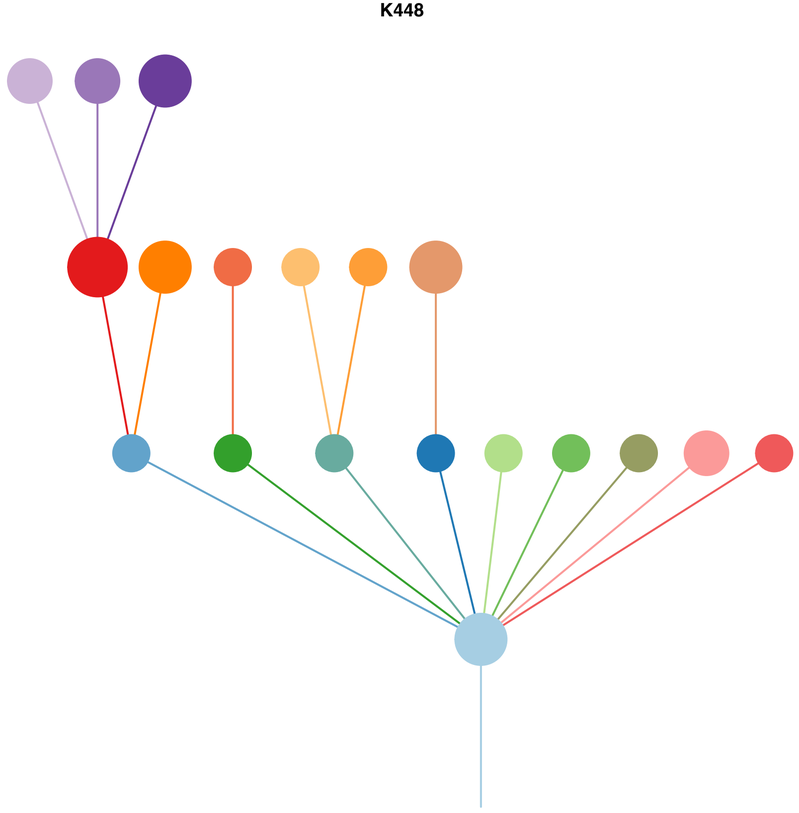






ID	Type	R7	R6	R10	R12	R11	R13	R3
PTEN	SNV							
SYNE2	synSNV							
5	GAIN							
16p	GAIN							
AKT2	SNV							
3p	LOSS							
10p	LOSS							
19q	LOSS							
22q	LOSS							

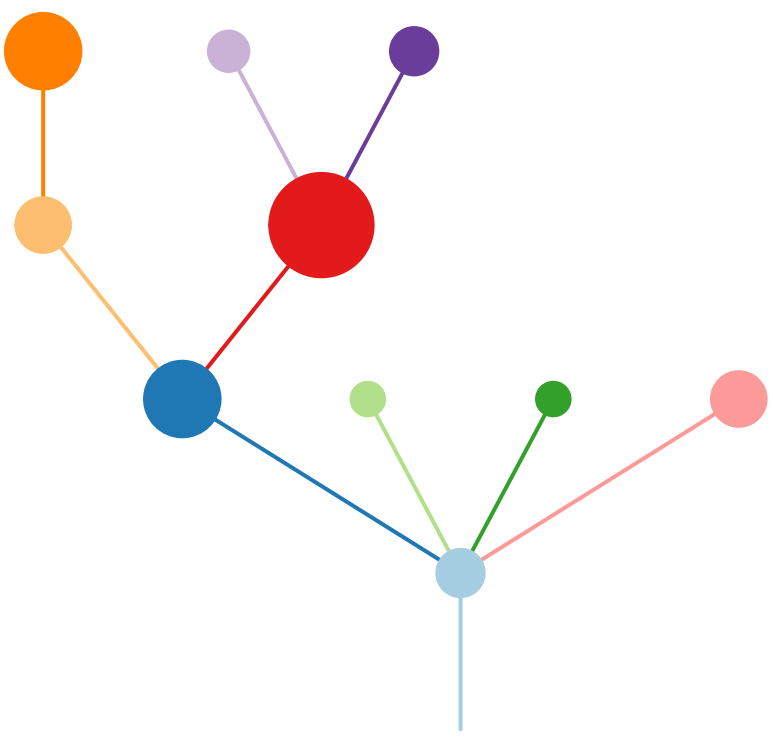
ID	Type	R7	R6	R1	R1	R1	R1	R3
17q	GAIN							
17p	GAIN							
16q	GAIN							
14q	LOSS							
KDM5D	synSNV							



ID	Type	R8	R1	R38	R20	R37	R52	R40	R28	R41	R29	R4	R3	R14	R42	R7	R2	R54	R53	R27	R19	R39	R59	R43	R55	R57	R47	R48	R58	R31	R44	R46	R10	R23	R24	R34	R51	R18	R5	R49	R32	R50	R12	R33	R6	R36
CUBN	SNV																																													
VHL	SNV																																													
3p	LOSS																																													
6q	LOSS																																													
BAP1	INDEL																																													
1	GAIN																																													
19q	GAIN																																													
20	GAIN																																													
COL11A1	SNV																																													
TCHH	synSNV																																													
MDN1	DNV																																													
OBSCN	SNV																																													
BAP1	INDEL																																													
BAP1	SNV																																													
BAP1	INDEL																																													
ATM	SNV																																													
15q	GAIN																																													
TP53	SNV																																													
MTOR	SNV																																													
BAP1	INDEL																																													
17q	LOSS																																													
MED12	SNV																																													
SETD2	SNV																																													
FYB	SNV																																													
15q	LOSS																																													
CSMD3	SNV																																													
SETD2	INDEL																																													
10q	GAIN																																													
19p	GAIN																																													
4p	LOSS																																													
10	LOSS																																													
8p	LOSS																																													
20p	LOSS																																													

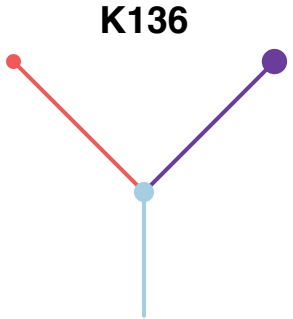
ID	Type	R8	R1	R38	R20	R37	R52	R40	R28	R41	R29	R4	R3	R14	R42	R7	R2	R54	R53	R27	R19	R39	R59	R43	R55	R57	R47	R48	R58	R31	R44	R46	R10	R23	R24	R34	R51	R18	R5	R49	R32	R50	R12	R33	R6	R36
14q	LOSS																																													
9p	LOSS																																													
7q	GAIN																																													
12	GAIN																																													
6p	GAIN																																													
7p	GAIN																																													
13q	LOSS																																													
17p	GAIN																																													
11p	GAIN																																													
17q	GAIN																																													
11q	GAIN																																													
5q	LOSS																																													
9q	LOSS																																													
21q	GAIN																																													
5p	LOSS																																													
18	GAIN																																													
22q	LOSS																																													
2p	GAIN																																													
3q	GAIN																																													
16p	GAIN																																													
2q	GAIN																																													
8	GAIN																																													
16q	GAIN																																													
4q	LOSS																																													
18	LOSS																																													
19p	LOSS																																													
5p	GAIN																																													
16p	LOSS																																													

K252



ID	Type	R1	R5	R10	R9	R4	R3	R8
VHL	SNV							
PBRM1	SNV							
7	GAIN							
6	LOSS							
4	LOSS							
10q	LOSS							
11	LOSS							
14q	LOSS							
17p	LOSS							
18p	LOSS							
DNAH10	synSNV							
TLN2	SNV							
TP53	SNV							
LRP2	SNV							
BAP1	SNV							
PLEC	SNV							
3p	GAIN							
8q	GAIN							
9	GAIN							
10p	GAIN							
12p	GAIN							
1p	LOSS							
8p	LOSS							
13q	LOSS							
18q	LOSS							
20p	LOSS							
2p	GAIN							
19q	GAIN							
20q	GAIN							
USH2A	SNV							
OBSCN	SNV							
BAP1	INDEL							
SYNE1	SNV							
BAP1	INDEL							
21q	GAIN							
9	LOSS							
17q	LOSS							
CSMD3	SNV							
SETD2	INDEL							
2q	GAIN							
17q	GAIN							
20p	GAIN							
10p	LOSS							
21q	LOSS							
22q	LOSS							
DNAH3	INDEL							
22q	GAIN							
12q	GAIN							
19q	LOSS							

ID	Type	R1	R5	R10	R9	R4	R3	R8
3	LOSS							
5	GAIN							
19p	LOSS							



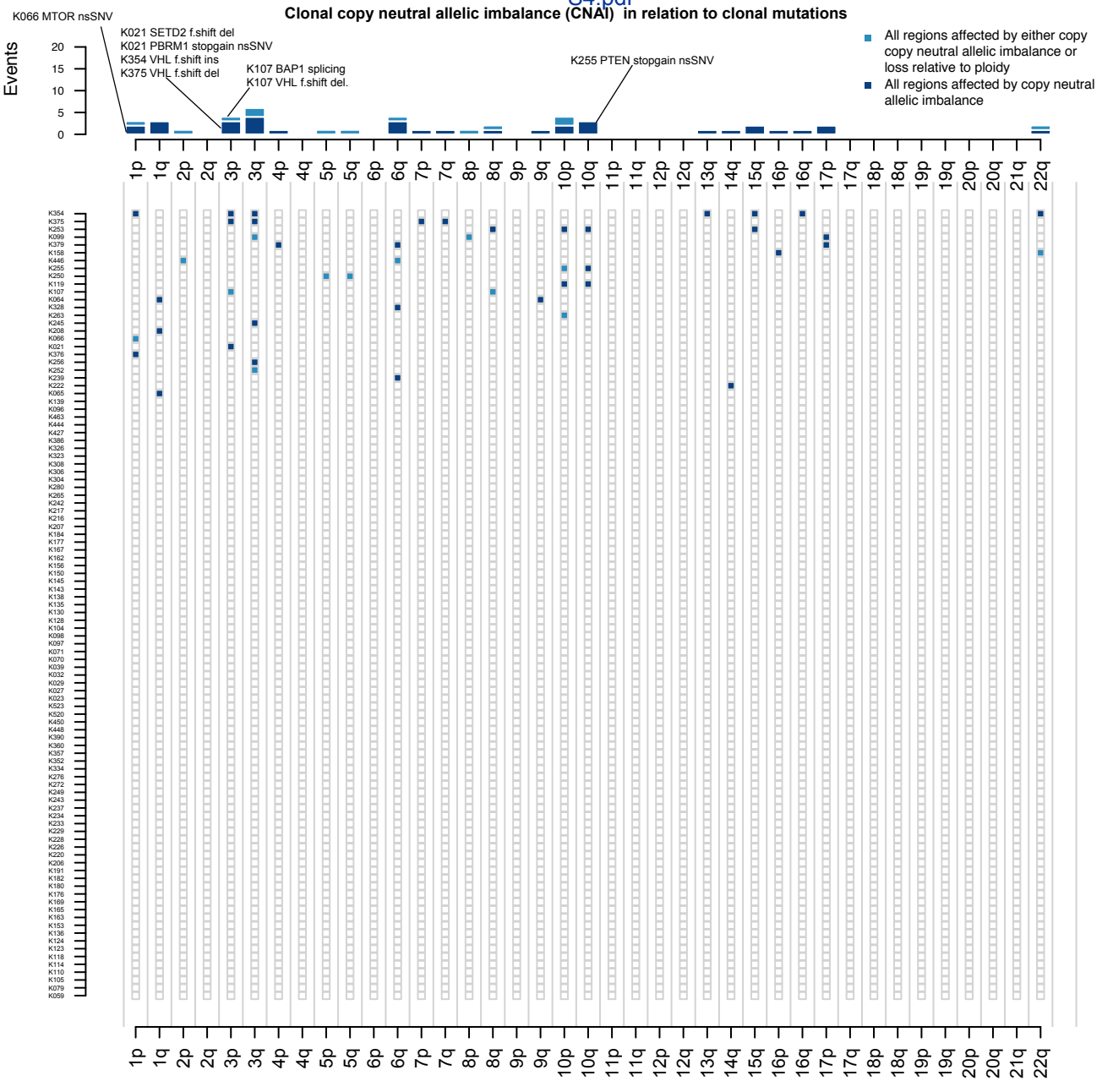
ID	Type	R1	R7	R3	R4	R8	R2	R6	R5
CD27	SNV								
VHL	INDEL								
PBRM1	INDEL								
3p	LOSS								
20p	LOSS								
MUC16	SNV								
BAP1	SNV								
TSC1	SNV								
4	LOSS								
9	LOSS								
14q	LOSS								
18	LOSS								

ID	Type	R1	R7	R3	R4	R8	R2	R6	R5
19p	LOSS								

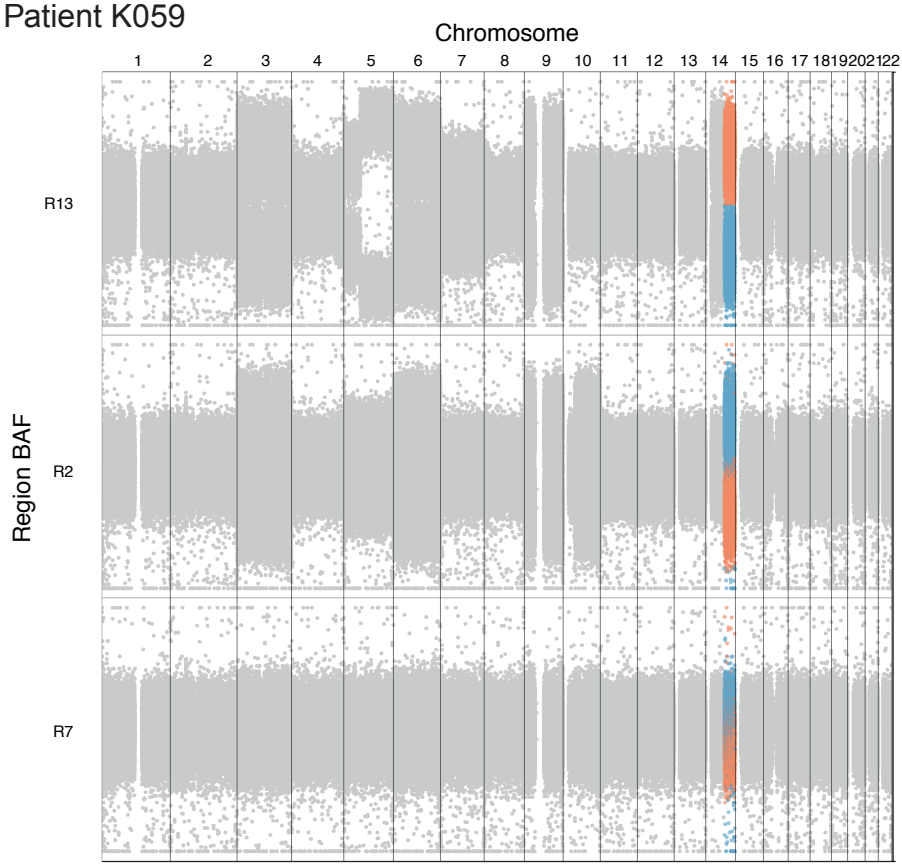
Figure S4: Copy neutral allelic imbalance and MSAI. Related to Figure 1, Figure 3 and Star methods.

Supplemental Figure 4

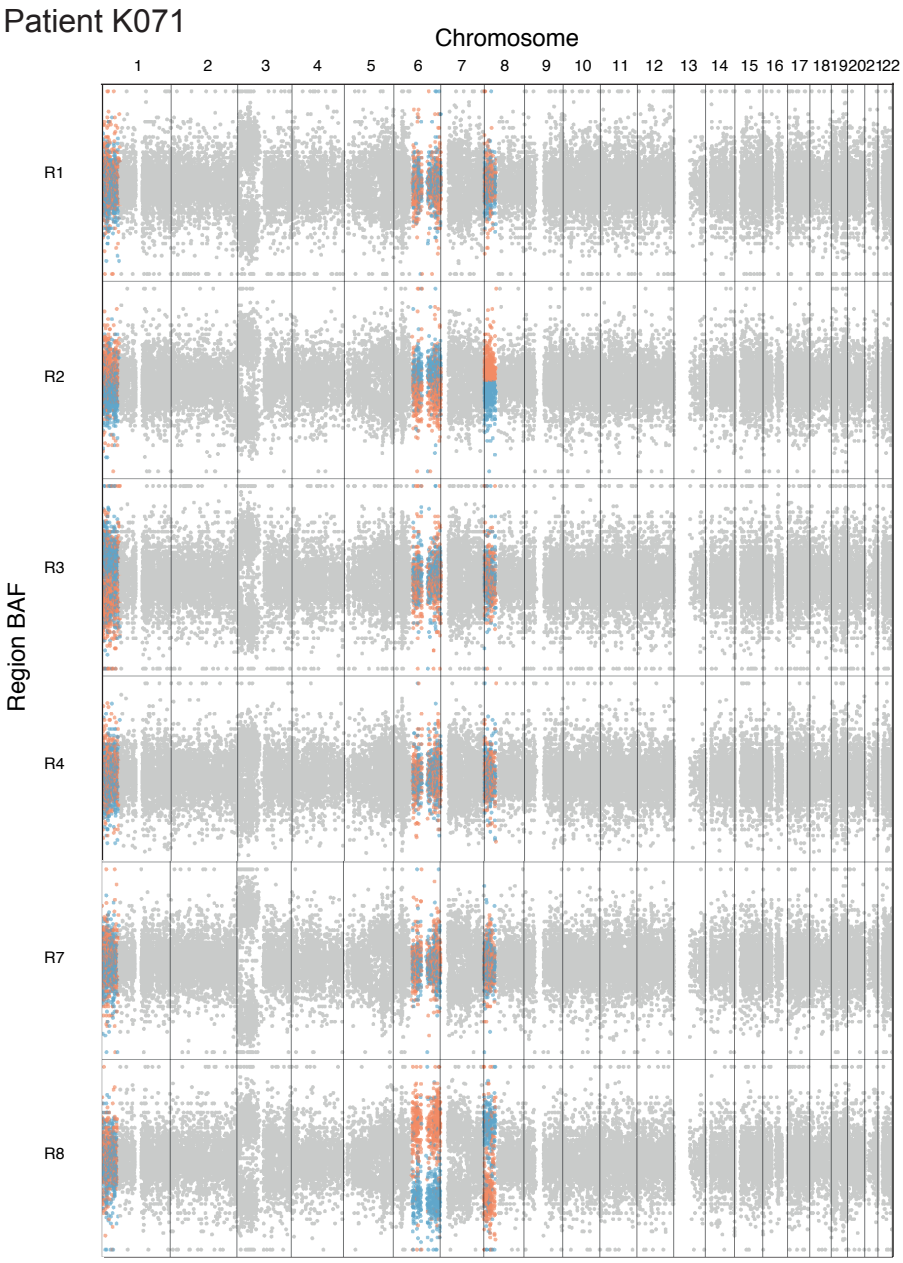
Click here to download Supplemental Figure Figure S4.pdf

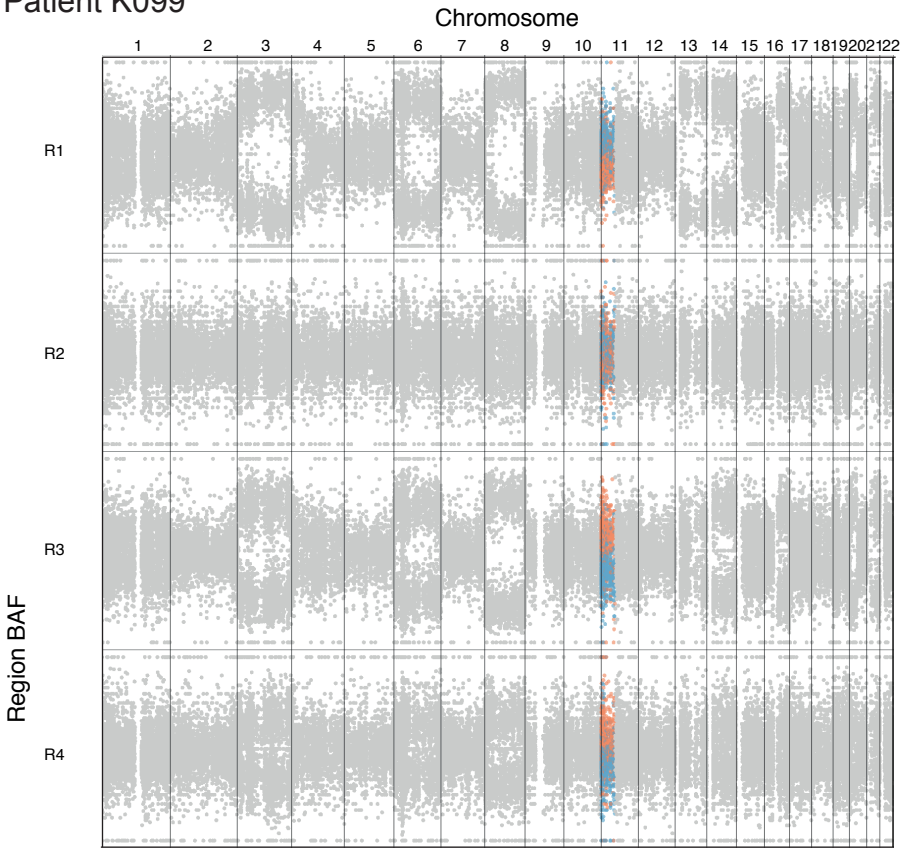


The top panel of this figure is bar chart depicting the number of ubiquitous (present in all regions sequenced for a patient) copy neutral allelic imbalance (CNAI) events in dark blue and the number of ubiquitous CNAI/loss in the cohort for all chromosome arms is indicated in light blue. Patients with co-occurring clonal mutations on these chromosomal arms are shown by lines to the corresponding chromosome arms. The bottom panel is a heatmap indicating which patients' disease from the cohort have either ubiquitous CNAI events (dark blue) or ubiquitous CNAI/loss events (light blue).

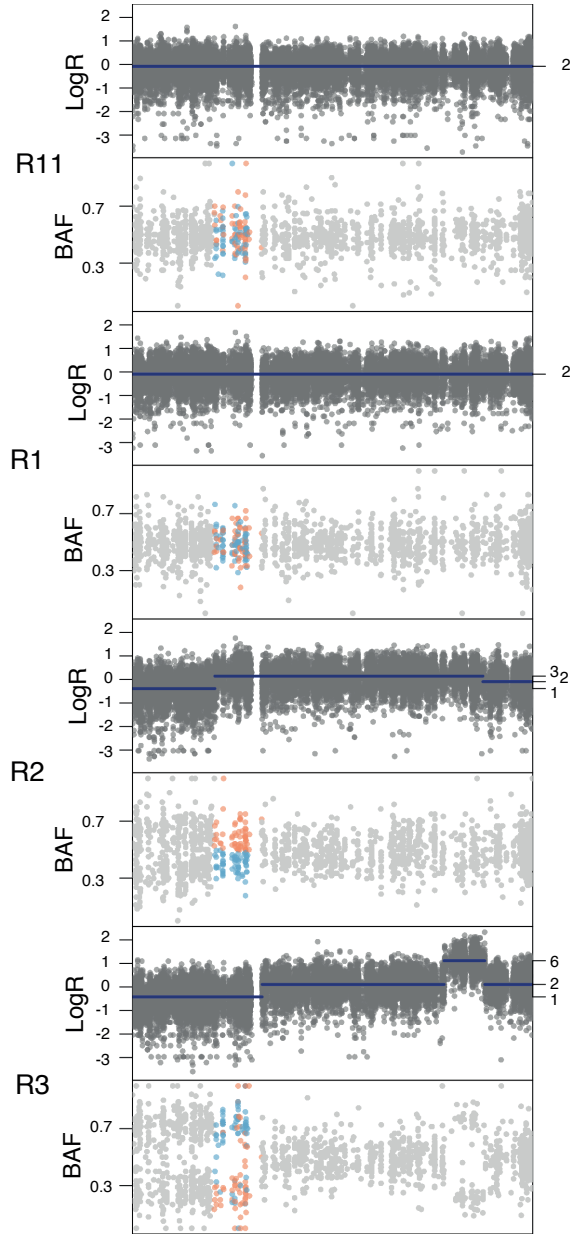


B-allele frequency profile of heterozygous SNPs across the genome (chromosomes 1-22) from all regions obtained from the multi-region whole genome sequencing from the patient's disease. Sections of BAF in regions that have mirrored subclonal allelic imbalance are highlighted in blue or orange. This legend applies to the ther patient specific figures of the same format in this document.


Patient K071
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
R1
R2
R3
R4
R7
R8
Region BAF

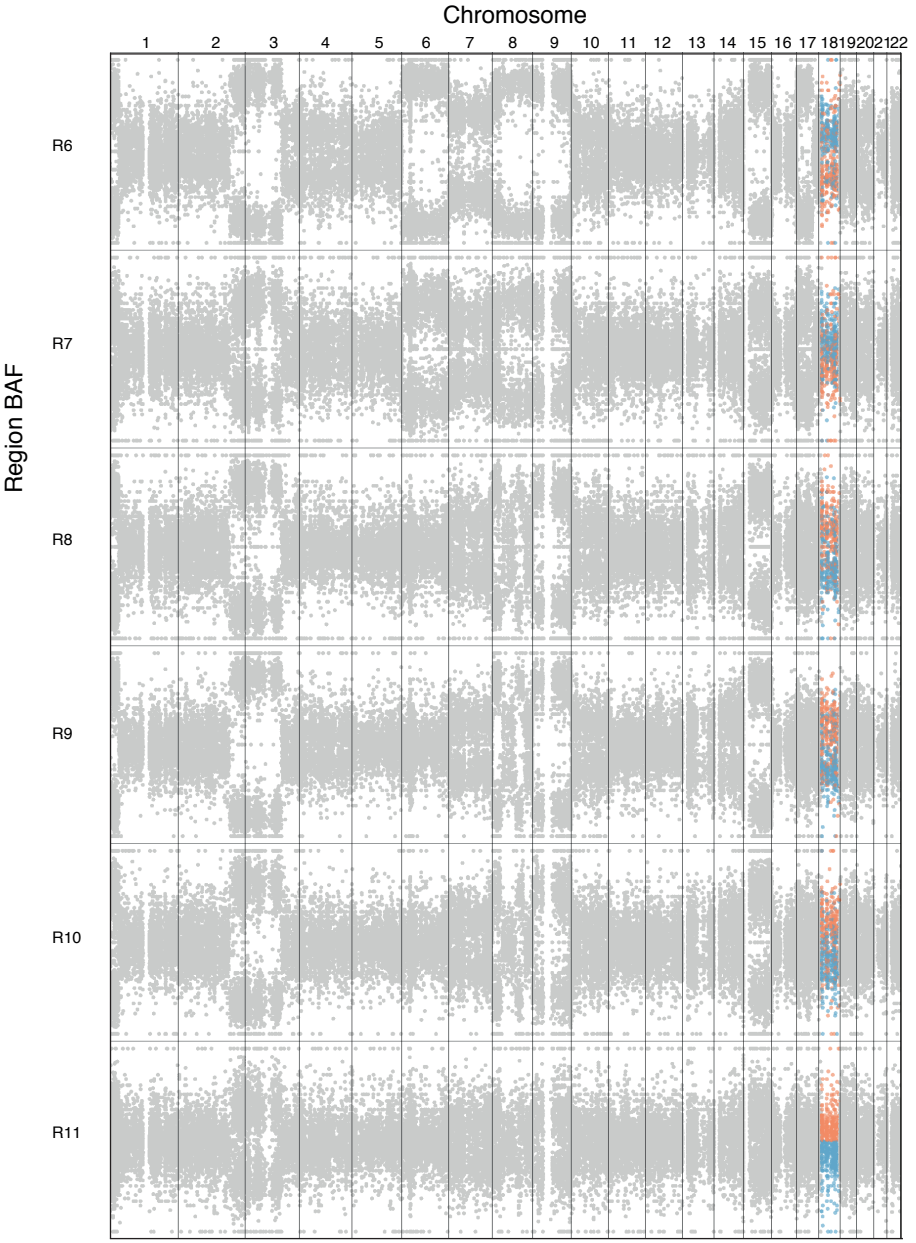

Patient K099
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
R1
R2
R3
R4
Region BAF

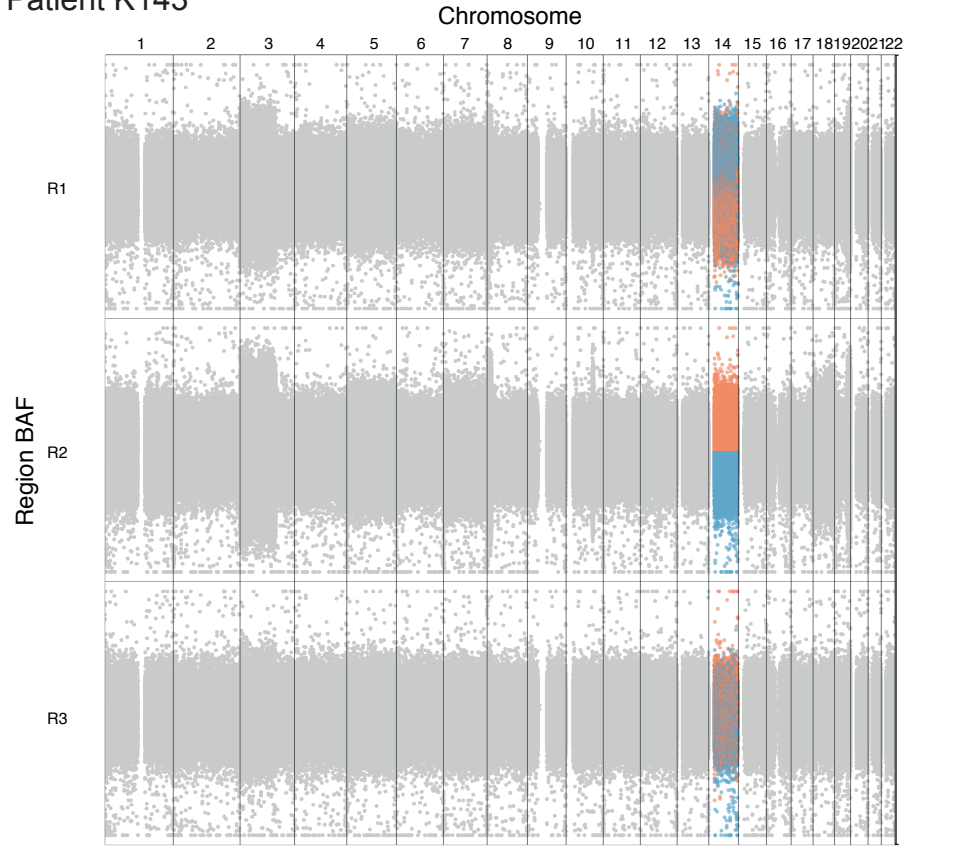
Patient K104 focal
chr8:30242747-47499801

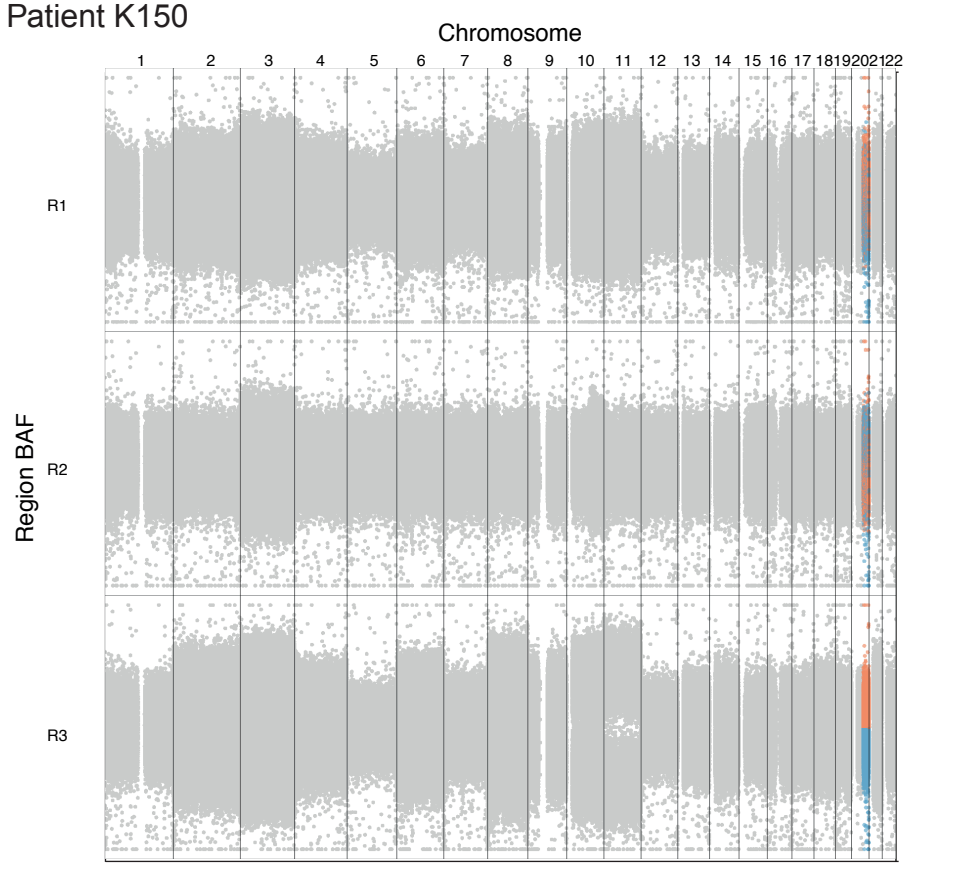


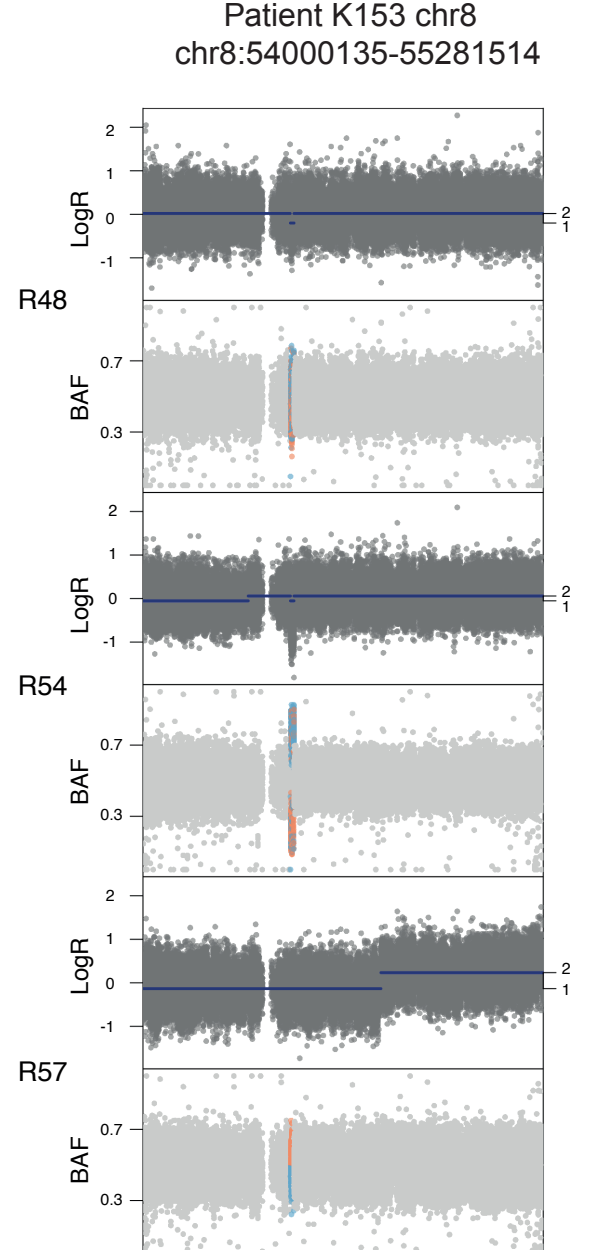
Shows the BAF and LogR (log ratio of the relative levels of sequencing coverage in the tumor region versus the germline normal) profile across part of chromosome 8 for all regions of the tumor samples that underwent whole exome sequencing from patient's disease. Each region has a plot of LogR below with the total copy number assigned to each segment on right of the plot, it also has a BAF plot below. The BAF in regions that have mirrored subclonal allelic imbalance are highlighted in blue or orange. This legend applies to the ther patient specific figures of the same format in this document.

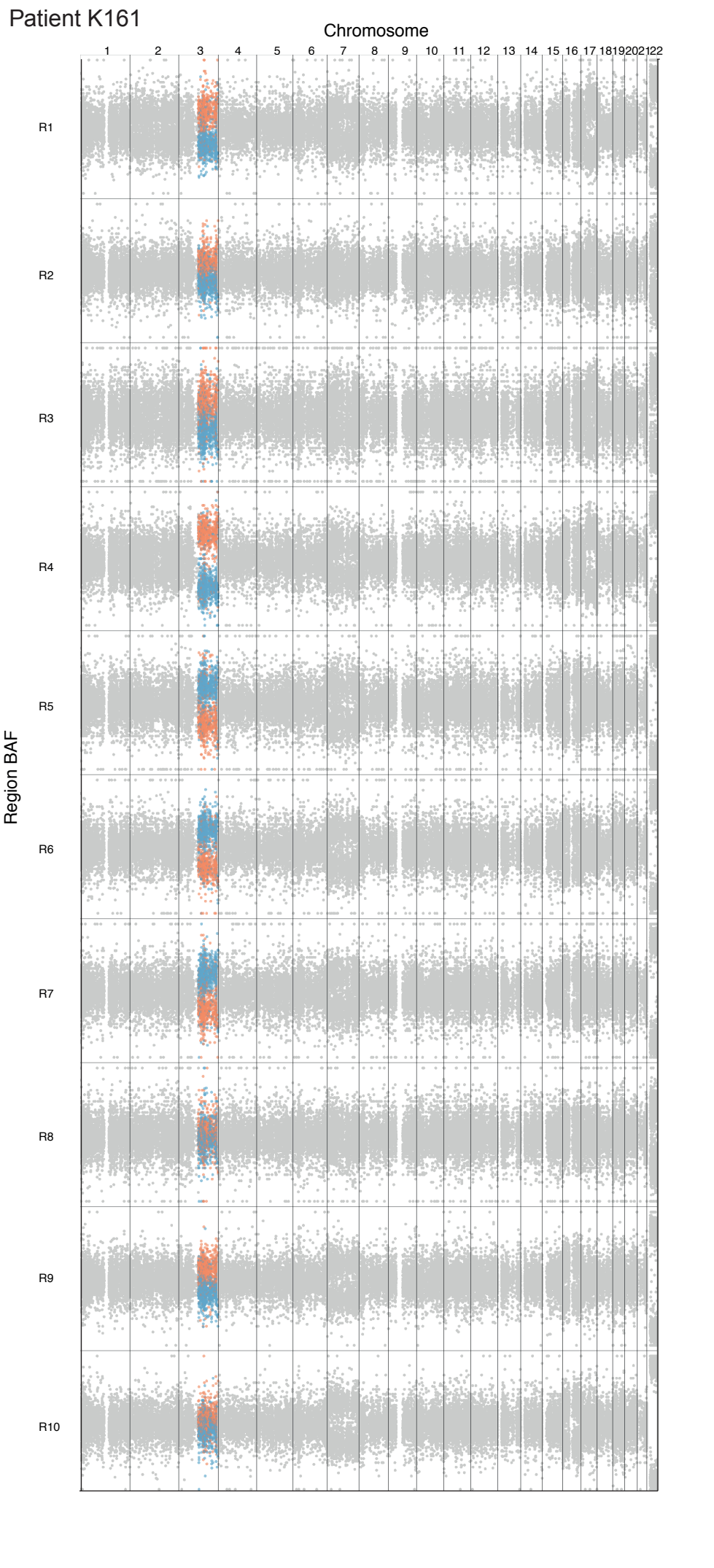
Patient K107

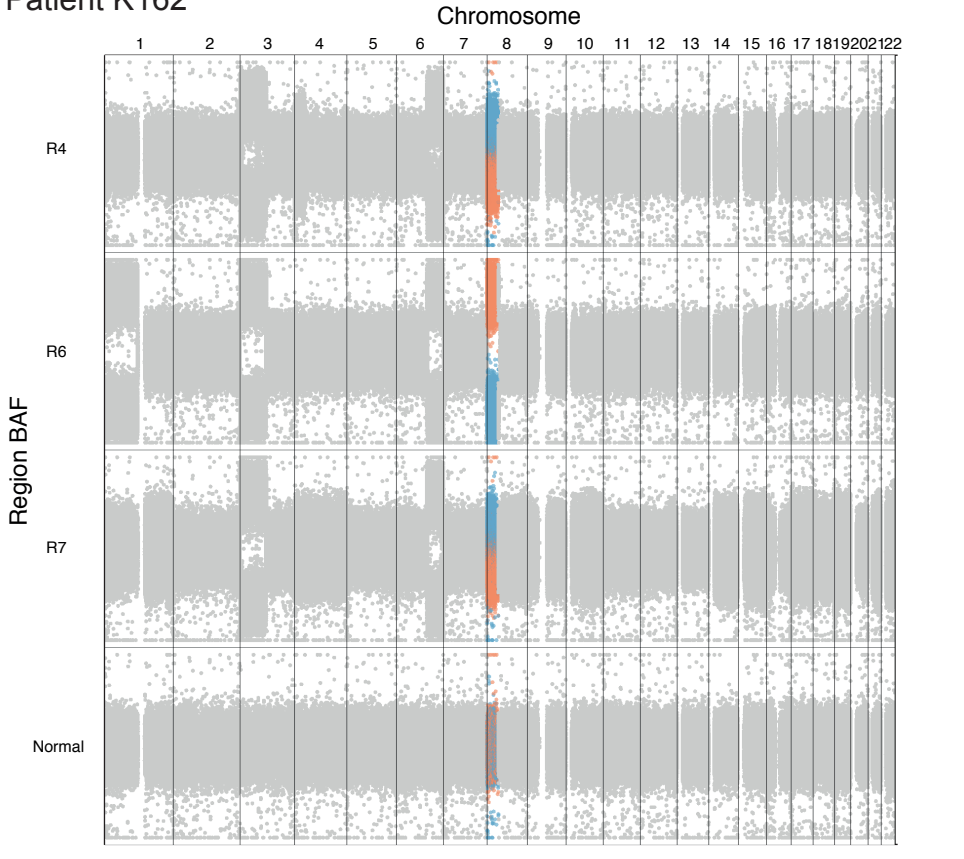


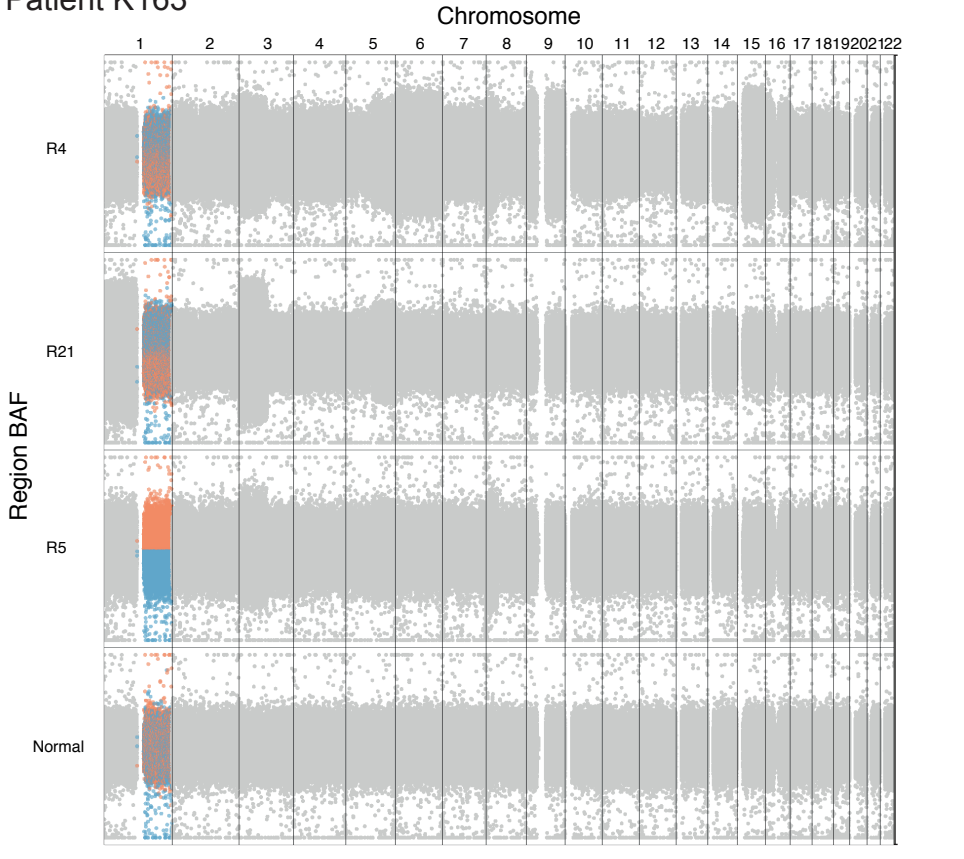

Patient K143
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Region BAF
R1
R2
R3

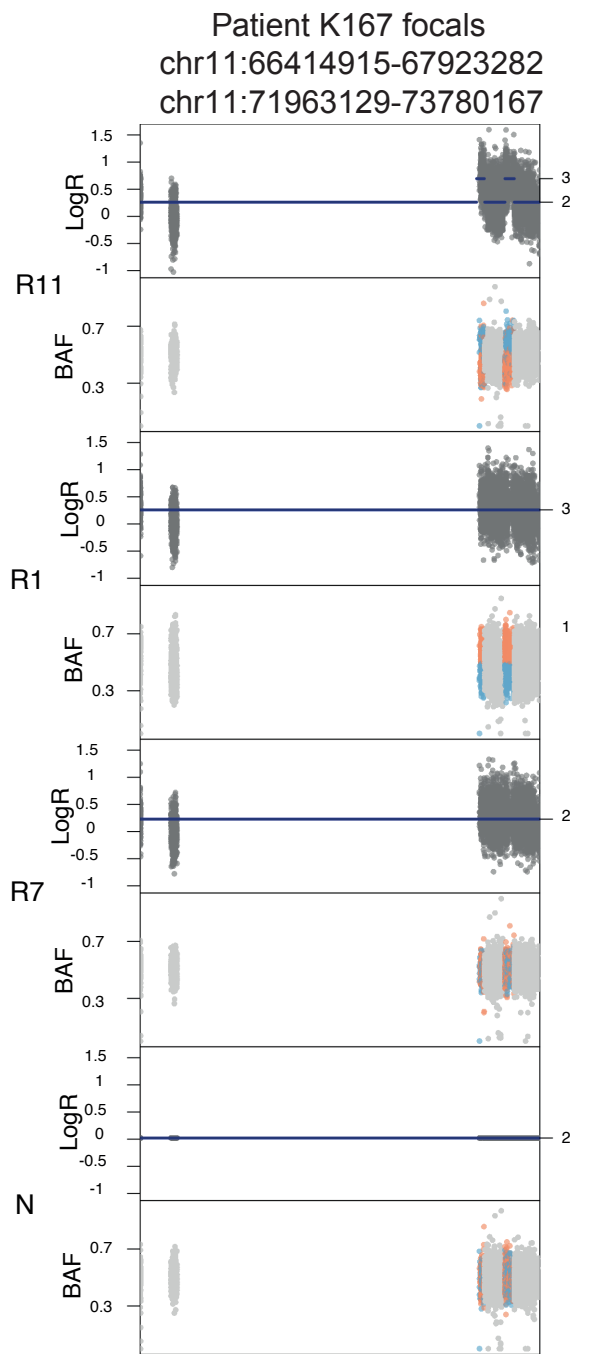

Patient K150
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
R1
R2
R3
Region BAF


Patient K153 chr8
chr8:54000135-55281514
LogR
BAF
R48
R54
R57
2
1
0
-1
0.7
0.3

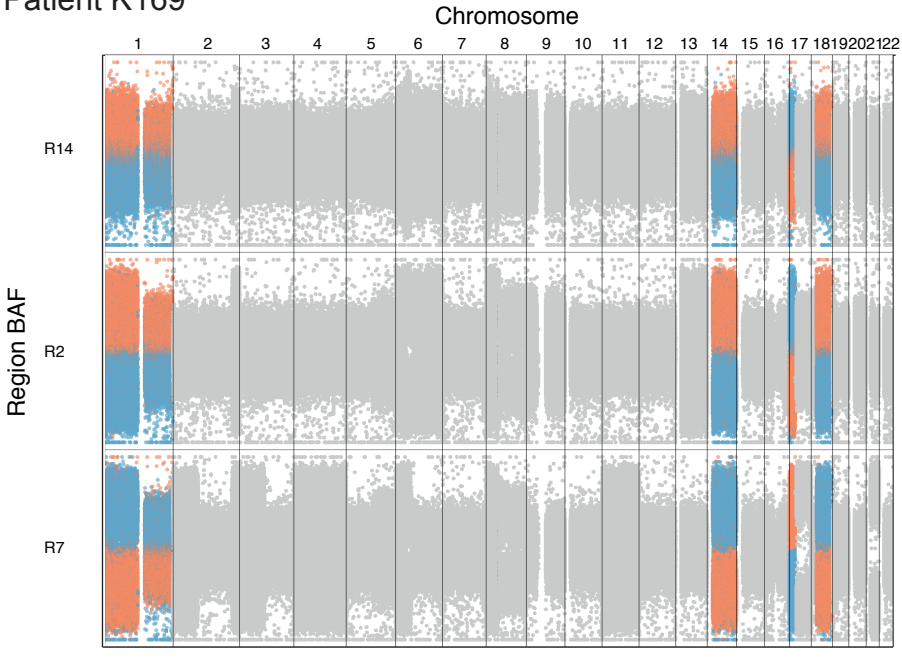

Patient K161
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
Region BAF

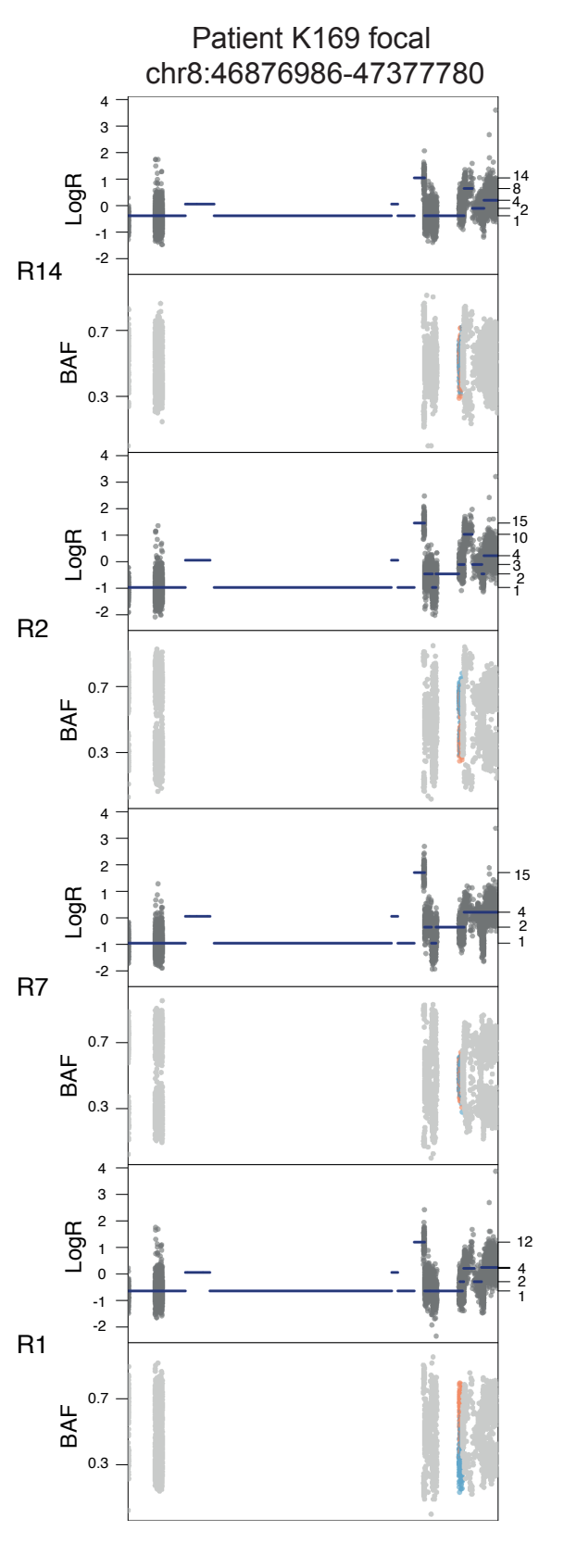

Patient K162
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Region BAF
R4
R6
R7
Normal

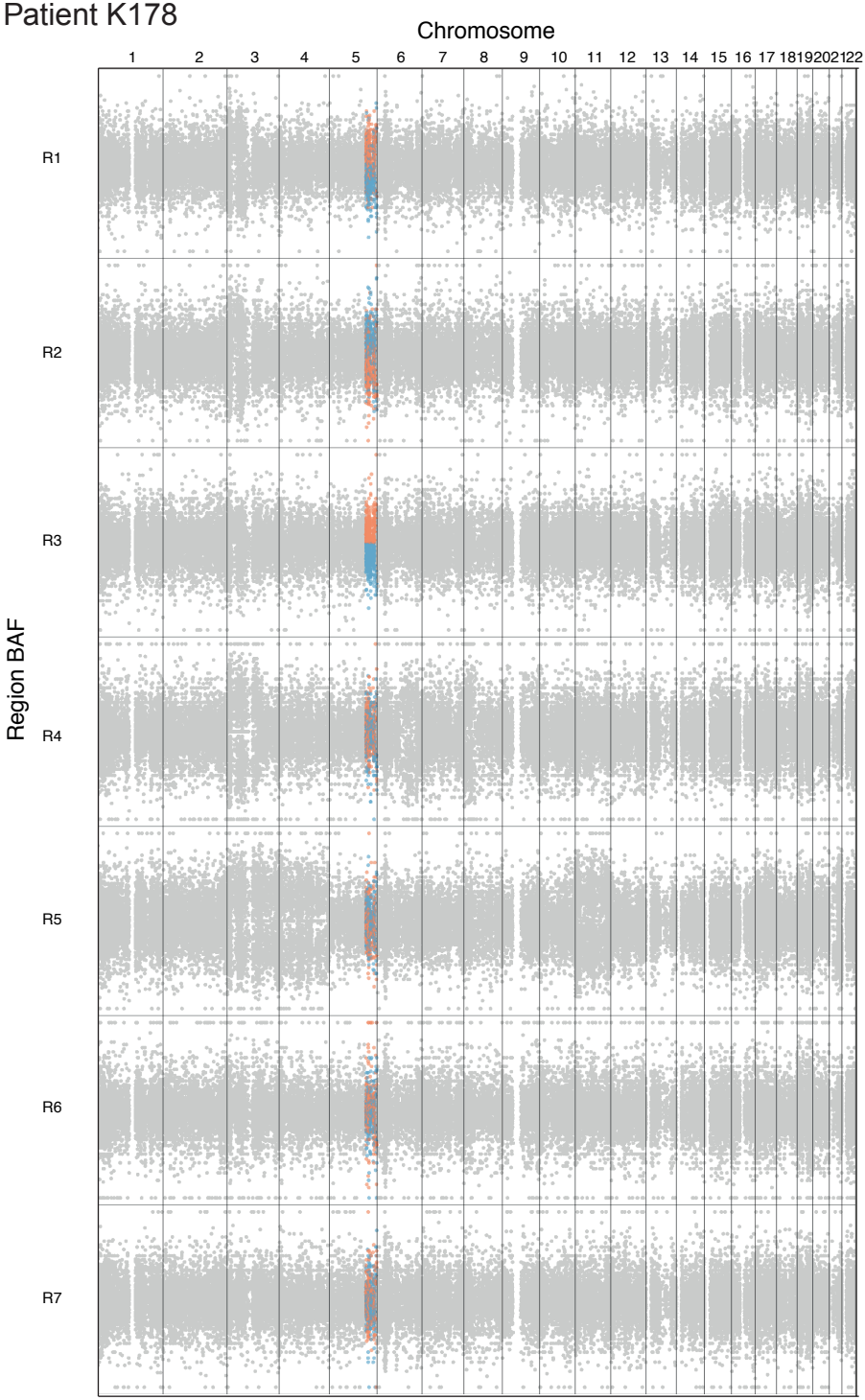

Patient K163
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Region BAF
R4
R21
R5
Normal


Patient K167 focals
chr11:66414915-67923282
chr11:71963129-73780167
R11
R1
R7
N
LogR
BAF
1.5
1
0.5
0
-0.5
-1
0.7
0.3
3
2
1

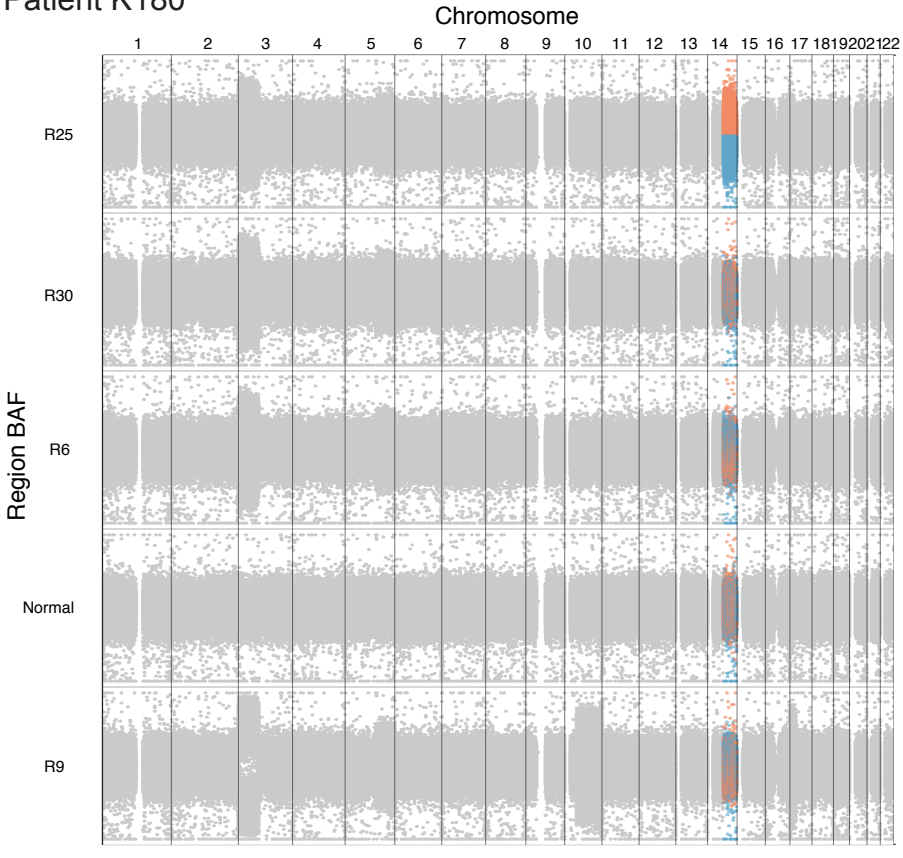
Patient K169



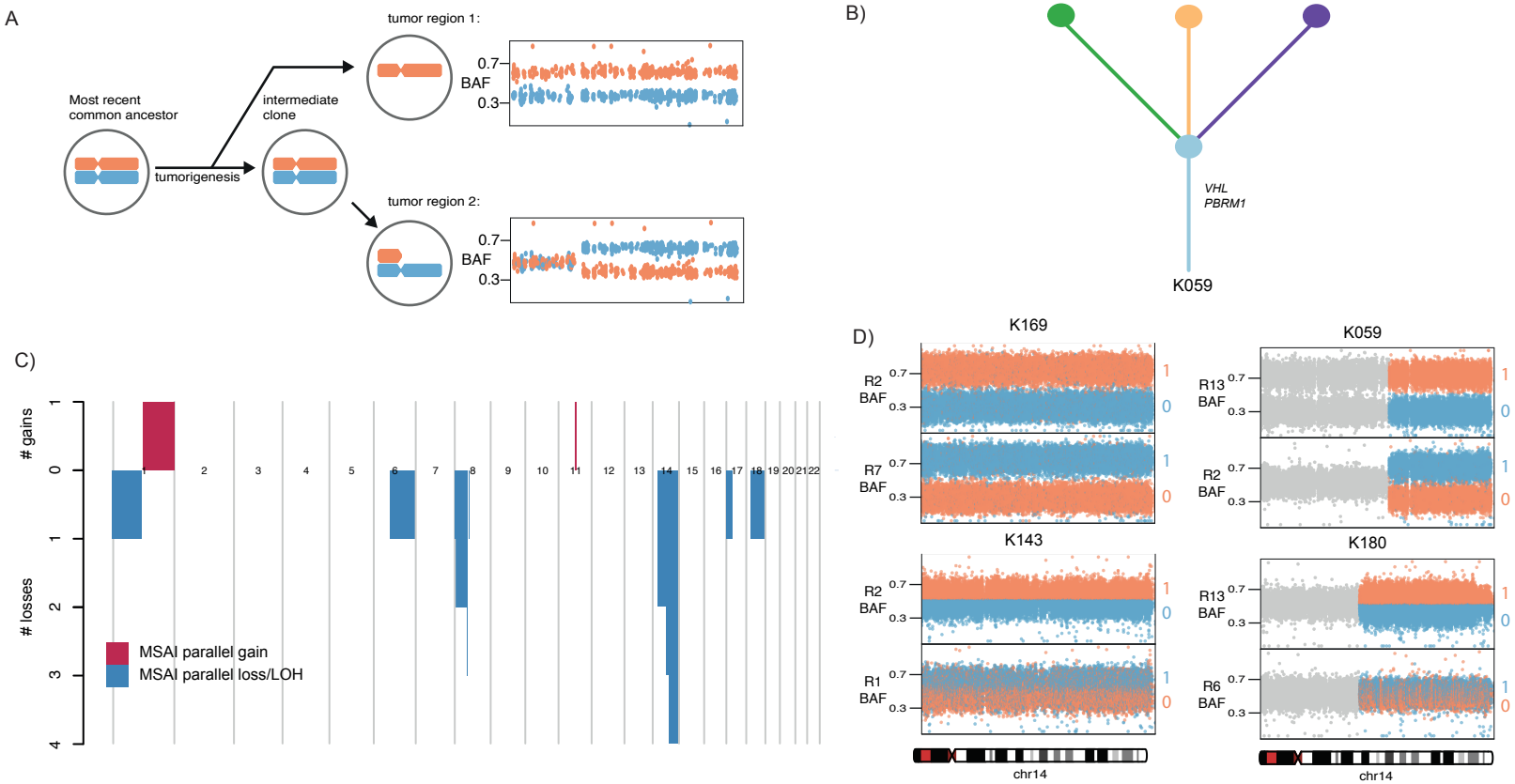

Patient K169 focal
chr8:46876986-47377780
R14
LogR
BAF
14
8
4
2
1
R2
15
10
4
3
2
1
R7
15
4
2
1
R1
12
4
2
1


Patient K178
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Region BAF
R1
R2
R3
R4
R5
R6
R7

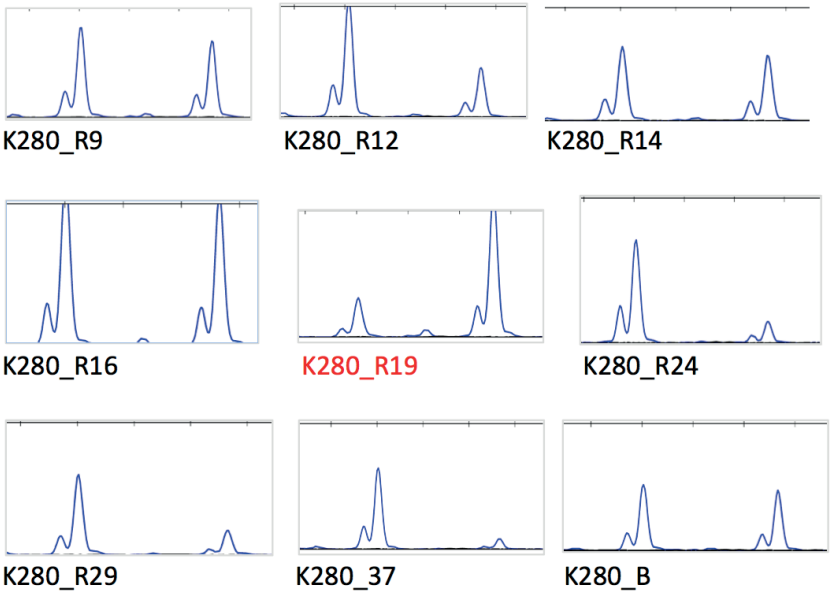
Patient K180



MSAI Summary

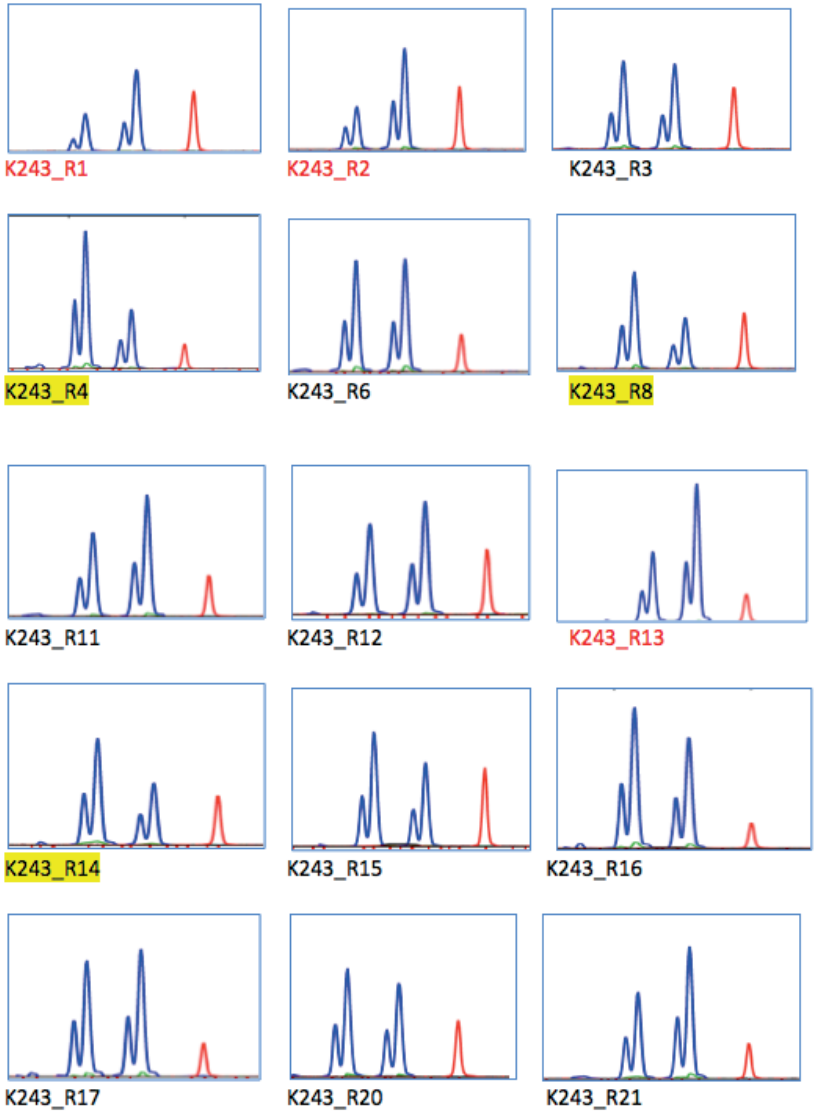


A) Shows a schematic of how a parallel copy number losses could result in mirrored subclonal allelic imbalance (MSAI). This occurs when the maternal allele is gained or lost in a subclone in one region and the paternal allele is gained or lost in a different subclone in another region. In the schematic the entire paternal (blue) chromosome is lost in R1 leading to the allelic imbalance seen in B-allele frequency (BAF) plot with the heterozygous SNPs' from the paternal (blue) chromosome having higher BAF values. In R2 the q arm of the maternal (orange) chromosome is lost leading to isolated allelic imbalance in the plot to the right with the heterozygous SNPs originating from q arm paternal q arm having higher BAF values. B) Phylogenetic tree indicating the subclonal loss of 14q from different alleles in the three regions that underwent whole genome sequencing. C) Shows the genomic position and size of all MSAI events found in the multi-region exome and multi-region whole genome sequencing performed in this study. This includes MSAI events that result from heterogeneous gains and losses relative to ploidy in same patient's disease. MSAI parallel loss of 14q is the most common event. D) shows the B-allele frequency (BAF) plots of two regions from each patient's disease that demonstrates MSAI through parallel loss relative to ploidy and loss of heterozygosity in chromosome 14.



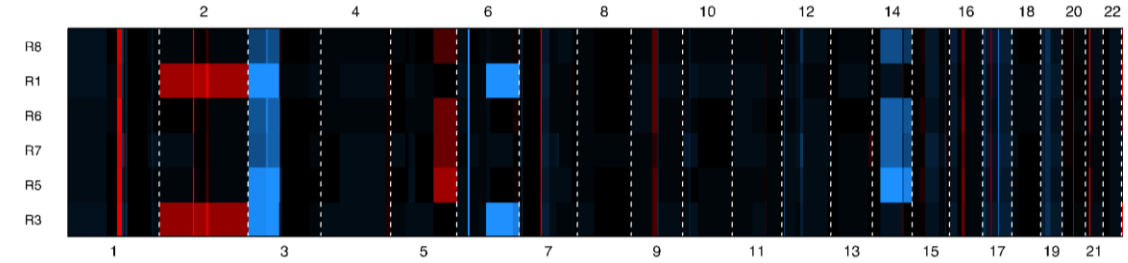
PCR fragment analysis using microsatellite marker D14S306 validating subclonal loss and MSAI (R19) in patient K280. Each number following the patient identifier indicates a tumor region and the germline is indicated by a 'B'.

MSAI validation

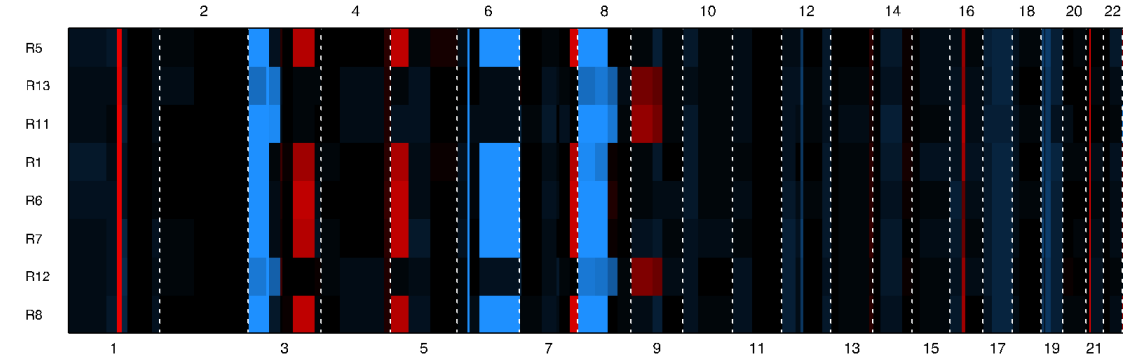


PCR fragment analysis using microsatellite marker D14S306 validating subclonal loss and MSAI (R4, R8 & R14) in patient K243.

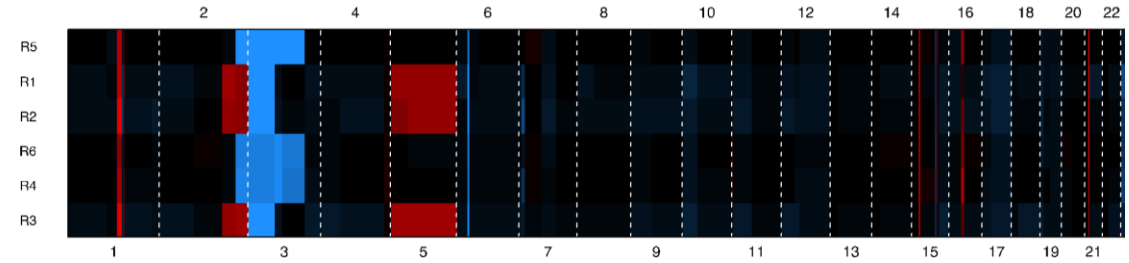
K265 (Primary tumour 1: R1, R3; Primary tumour 2: R5 - R8)



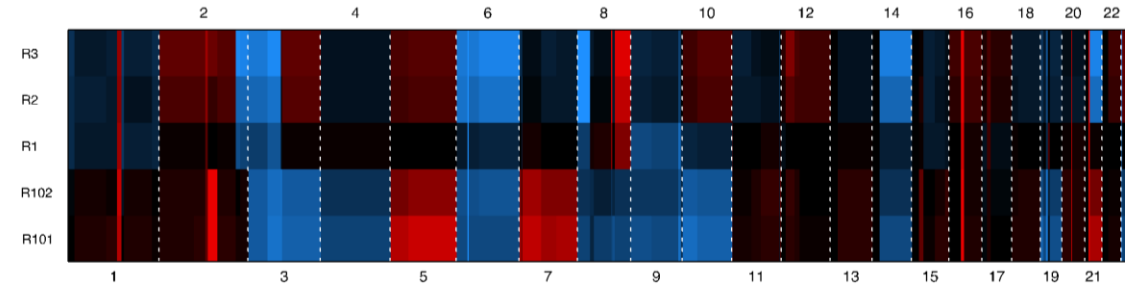
K334 (Left primary tumour: R11 - R13; Right primary tumour: R1, R5 - R8)



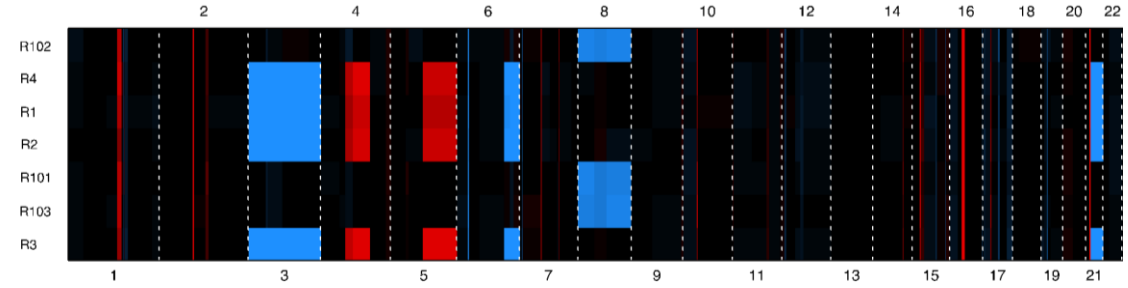
K352 (Primary tumour 1: R1, R2, R3; Primary tumour 2: R4, R5, R6)



K097 (Left primary tumour: R1-R3; Right primary tumour: R101, R102)



K114 (Left primary tumour: R1 – R4; Right primary tumour: R101 – R103)



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Supplemental Figure 5

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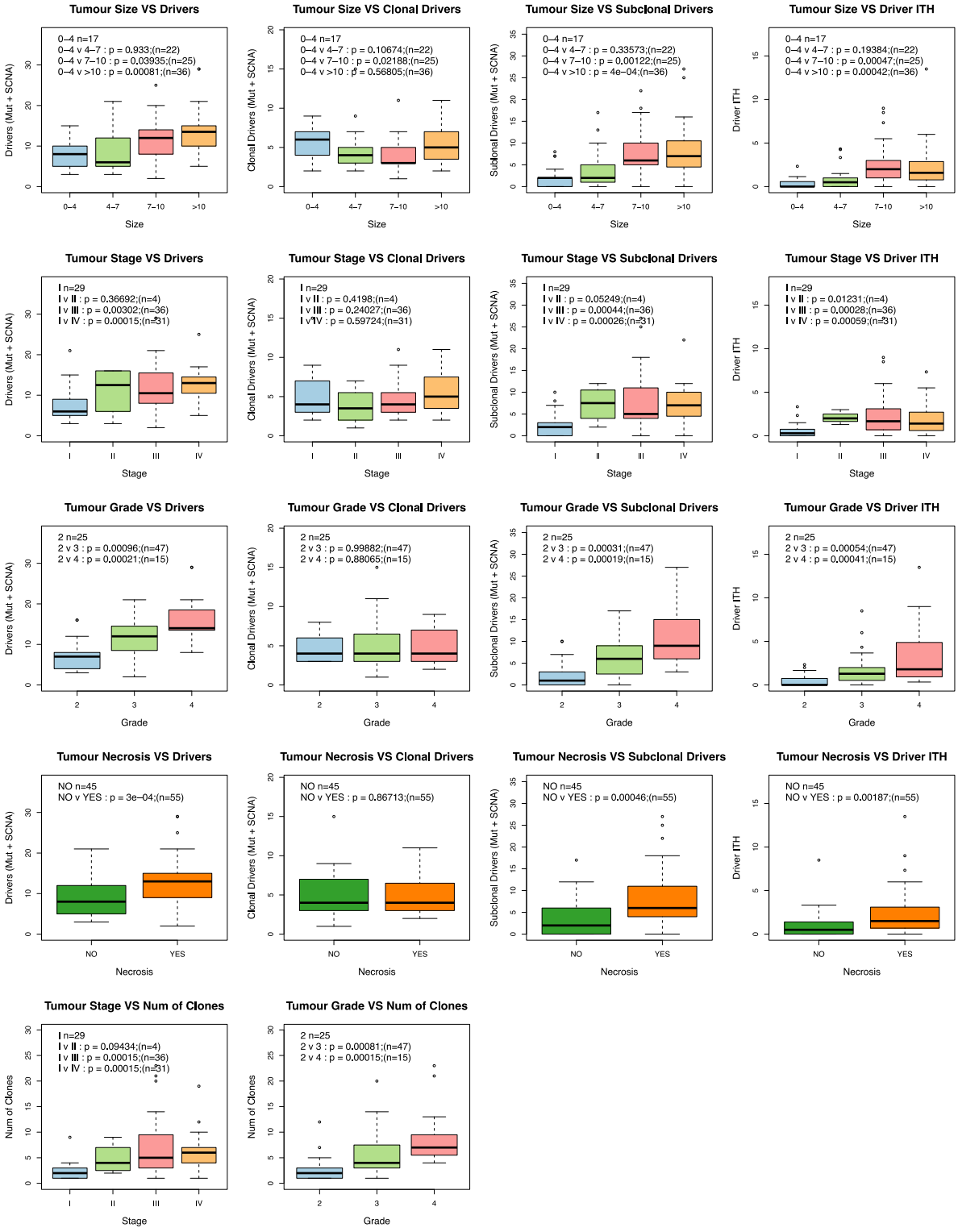

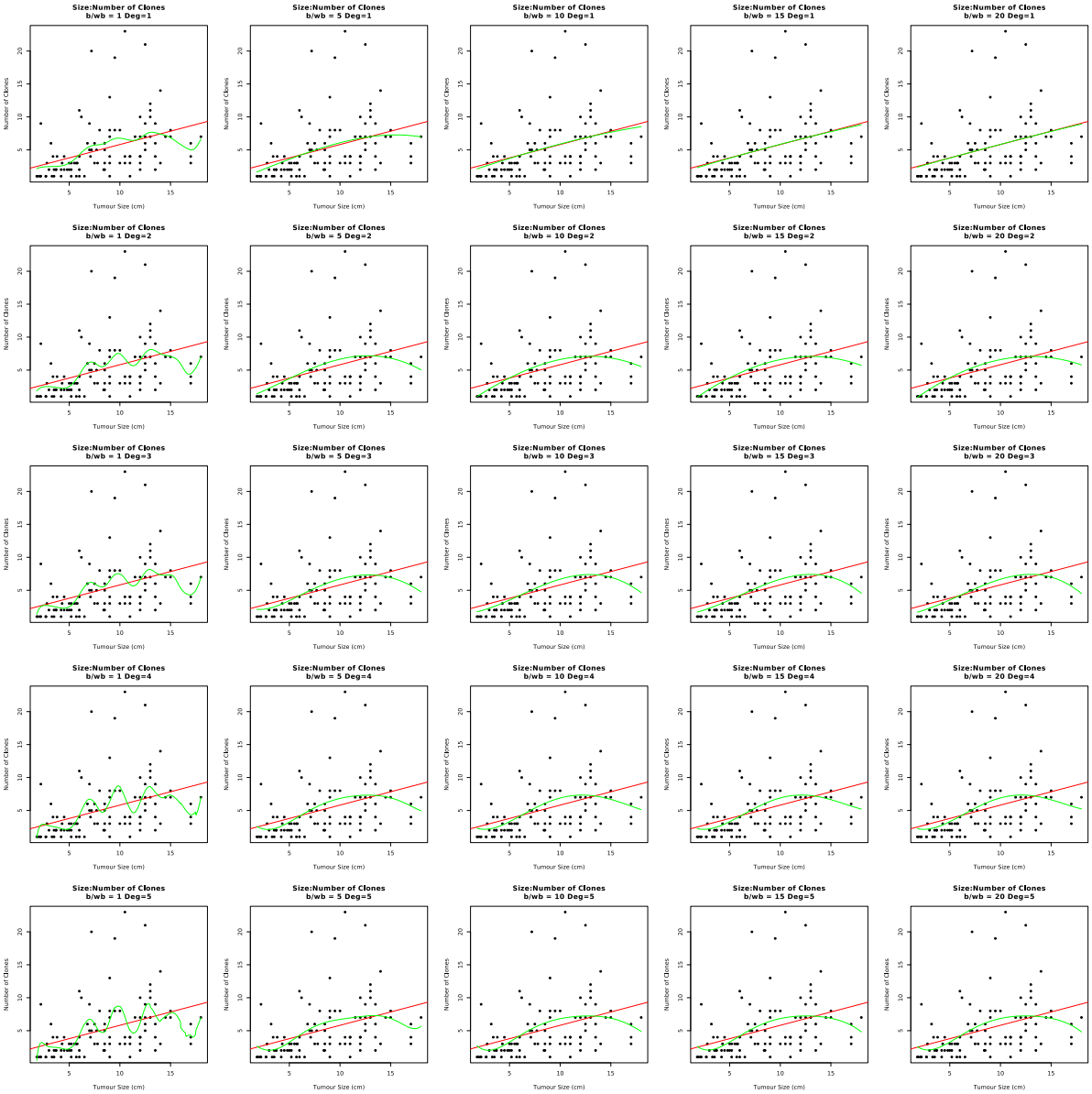
Size:Number of Clones
b/wb = 1 Deg=1
Size:Number of Clones
b/wb = 5 Deg=1
Size:Number of Clones
b/wb = 10 Deg=1
Size:Number of Clones
b/wb = 15 Deg=1
Size:Number of Clones
b/wb = 20 Deg=1
Size:Number of Clones
b/wb = 1 Deg=2
Size:Number of Clones
b/wb = 5 Deg=2
Size:Number of Clones
b/wb = 10 Deg=2
Size:Number of Clones
b/wb = 15 Deg=2
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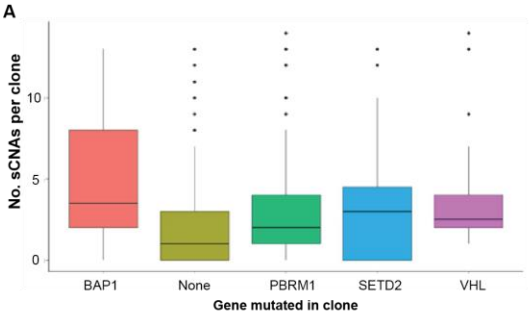
Size:Number of Clones
b/wb = 5 Deg=5
Size:Number of Clones
b/wb = 10 Deg=5
Size:Number of Clones
b/wb = 15 Deg=5
Size:Number of Clones
b/wb = 20 Deg=5
Number of Clones
Tumour Size (cm)

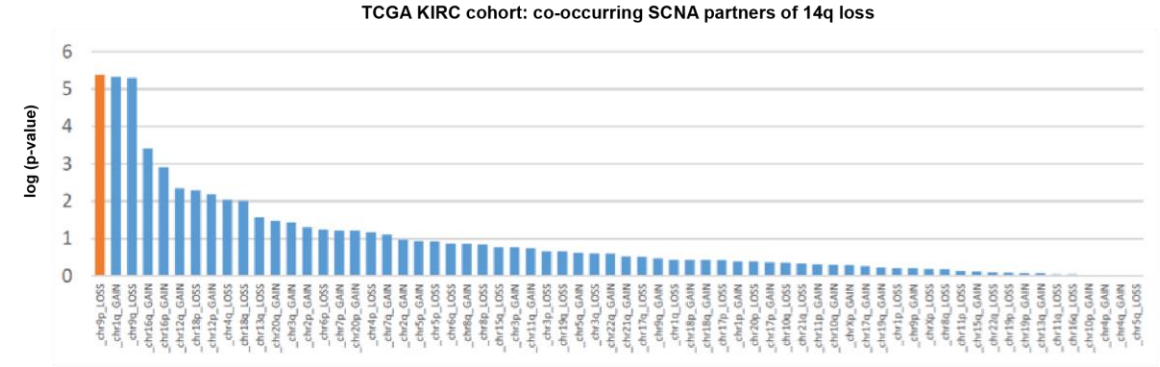
A



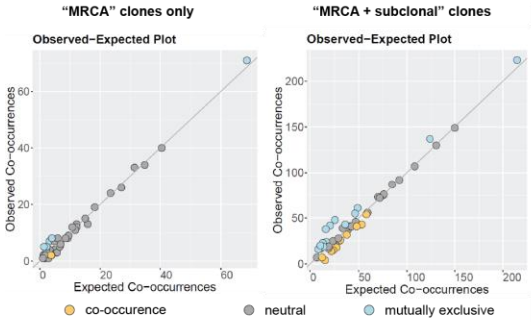
B

Event1	Event2	Count of Co-occurrence	Expected Co-occurrence	%Event1_ Overall	%Event1_on_Event2_Background	P-Value
chr4q_LOSS	chr9p_LOSS	15	5	5.1%	16%	0.000002
chr14q_LOSS	chr9p_LOSS	53	28	31.3%	58%	0.000004
8q_GAIN	chr9p_LOSS	14	7	7.4%	15%	0.004995
chr14q_LOSS	chr4q_LOSS	16	8	31.3%	59%	0.009228
5q_GAIN	8q_GAIN	19	13	33.0%	49%	0.086374
8q_GAIN	chr14q_LOSS	17	12	7.4%	10%	0.168042
5q_GAIN	chr14q_LOSS	63	54	33.0%	38%	0.242138
5q_GAIN	chr9p_LOSS	36	30	33.0%	40%	0.272323
8q_GAIN	chr4q_LOSS	3	2	7.4%	11%	0.476380
5q_GAIN	chr4q_LOSS	10	9	33.0%	37%	0.711733
Any_sCNA	Any_sCNA	425	424	89.6%	90%	0.950772

C



E



Event 1	Event 2	Observed cooccurrence	Expected cooccurrence
BAP1_3	PBRM1_3	18	27
BAP1_3	SETD2_3	4	15
BAP1_3	VHL_3	37	39
BAP1_3	gain_5	15	24
BAP1_3	loss_3	46	47
BAP1_3	loss_14	24	17
BAP1_3	loss_4	17	11
BAP1_3	loss_9	23	13
BAP1_3	gain_8	7	7
PBRM1_3	SETD2_3	61	49
PBRM1_3	VHL_3	137	124
PBRM1_3	gain_5	75	76
PBRM1_3	loss_3	149	150
PBRM1_3	loss_14	43	53
PBRM1_3	loss_4	39	34
PBRM1_3	loss_9	41	42
PBRM1_3	gain_8	14	22
SETD2_3	VHL_3	73	70
SETD2_3	gain_5	41	43
SETD2_3	loss_3	87	85
SETD2_3	loss_14	25	30
SETD2_3	loss_4	17	19
SETD2_3	loss_9	25	24
SETD2_3	gain_8	7	12

Event 1	Event 2	Observed cooccurrence	Expected cooccurrence
VHL_3	gain_5	107	108
VHL_3	loss_3	223	215
VHL_3	loss_14	76	76
VHL_3	loss_4	41	48
VHL_3	loss_9	56	59
VHL_3	gain_8	26	31
gain_5	loss_3	130	131
gain_5	loss_14	55	46
gain_5	loss_4	28	29
gain_5	loss_9	43	36
gain_5	gain_8	19	19
loss_3	loss_14	92	92
loss_3	loss_4	54	58
loss_3	loss_9	72	72
loss_3	gain_8	32	38
loss_14	loss_4	42	21
loss_14	loss_9	48	26
loss_14	gain_8	19	13
loss_4	loss_9	38	16
loss_4	gain_8	16	8
loss_9	gain_8	20	11

Table: "MRCA + subclonal" clone events counts

Supplemental Figure 7

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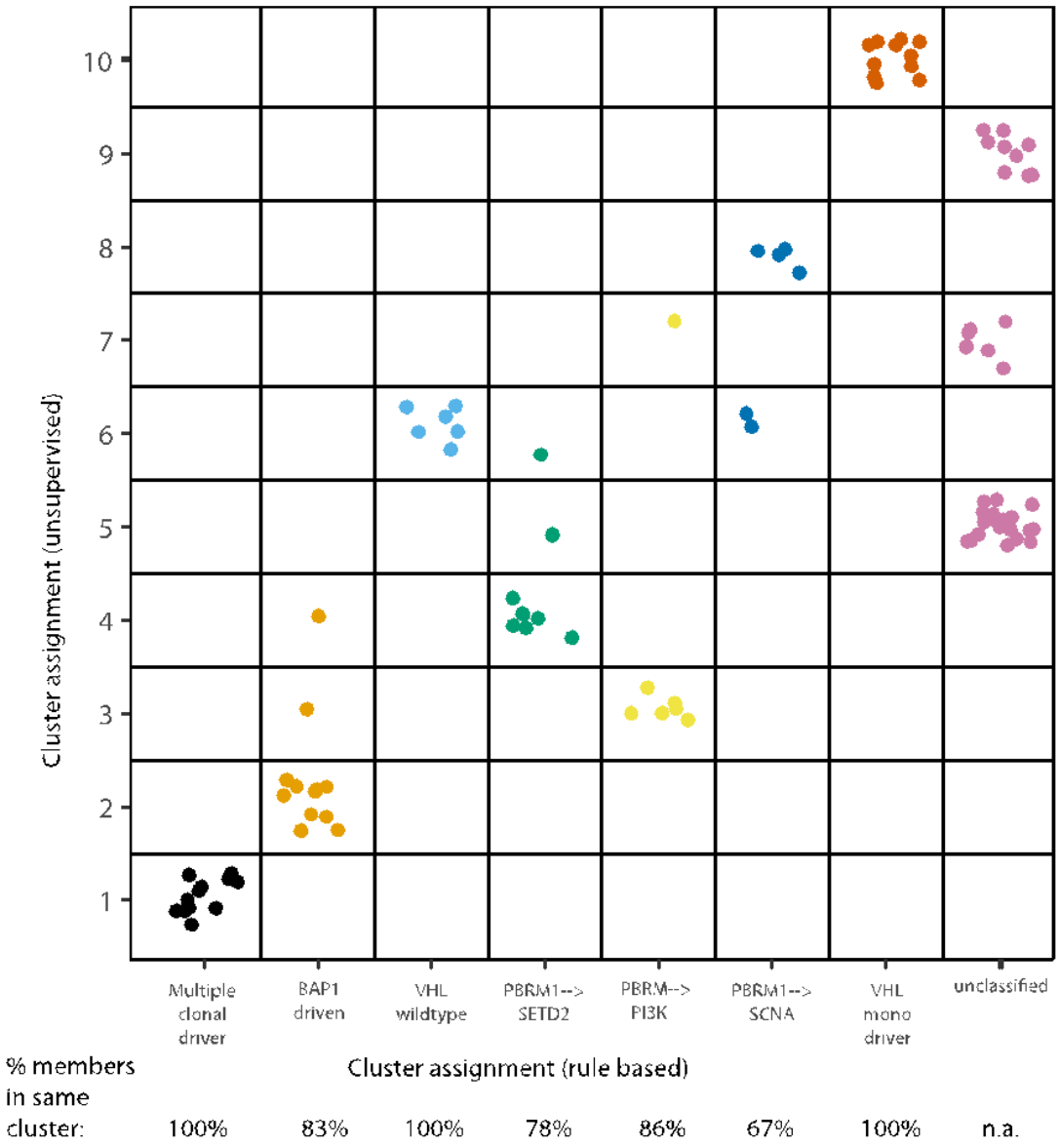

% members in same cluster:	100%	83%	100%	78%	86%	67%	100%	n.a.

Table S1

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Table S1_v3.xlsx

Table S2

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Table S3

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Table S5