Passenger mutations in 2500 cancer genomes: Overall molecular functional impact and consequences

Abstract

The Pan-cancer Analysis of Whole Genomes (PCAWG) project provides an unprecedented opportunity to comprehensively characterize a vast set of uniformly annotated coding and non-coding mutations present in thousands of cancer genomes. Classical models of cancer progression posit that only a small number of these mutations strongly drive tumor progression, and that remaining mutations (termed "nominal passengers") are considered inconsequential for tumorigenesis. In this study, we leverage the comprehensive variant data from PCAWG to predict the molecular impact of each variant, including nominal passengers. This allows us to decipher their overall molecular impact on different coding and noncoding genomic elements. The overall molecular impact distribution of PCAWG mutations shows that, in addition to high impact drivers and low-impact passengers, there is a group of medium-impact passenger variants predicted to influence gene expression or activity. Furthermore, we find that molecular impact relates to the underlying mutational signature. Thus, different signatures confer different extent of molecular functional impact. Moreover, burdening of variants is non-random in effect on different regulatory subsystems and for different categories of genes. In addition, we find that molecular functional impact varies based on subclonal architecture (i.e. early vs late mutations) and can be also related to patient survival. Finally, we speculate on how the differential burdening might be related to the existence of both weak positive and negative selection during tumor evolution.

Deleted: & its

Deleted: However, the classical model posits

Deleted: the

Deleted: extent of

Deleted: ,

Deleted: SNVs

Deleted: and thus

Deleted: their differential terms of affecting

Deleted: survivability of patients.

Introduction

Previous studies have focused on characterizing variants occupying coding regions of cancer genomes \cite{24071849}. However, the extensive Pan-cancer Analysis of Whole Genomes (PCAWG) dataset, which includes variant calls from ~2500 uniformly processed whole cancer genomes, offers an unparalleled opportunity to investigate the overall molecular functional impact of variants influencing different non-coding genomic elements. Given that the majority of cancer variants lie in non-coding regions \cite{26781813}, this variant dataset serves as a substantially more informative resource than the many existing datasets focused on exomes. Moreover, it also contains a full spectrum of variants, including copy number variants (CNVs) and large structural variants (SVs), in addition to single-nucleotide variants (SNVs) and small insertion & deletions (INDELS).

Of the 30 million SNVs in the PCAWG data set, a few thousand (< 5/tumor[1]) \cite{26559569} can be identified as driver variants – positively selected variants that favor tumor growth. The remaining ~99% of SNVs are termed nominal passenger variants, with poorly understood molecular consequences and fitness effects. Furthermore, the bulk of these nominal passengers fall within noncoding regions of the genome, making these the main product of whole-genome sequencing of tumors. Recent studies have proposed that, among variants that have not been found to be driver variants (i.e. nominal passenger variants), some may weakly affect tumor cell fitness by promoting or inhibiting tumor growth. In prior studies, these variants have been described as "mini-drivers" \cite{26456849} and "deleterious passengers" \cite{23388632}.

Conceptually, variants can be classified into three categories based on their impact on tumor cell fitness: positively-selected driver variants, neutrally-selected neutral passenger variants, and negatively-selected deleterious passenger variants. This broad classification can be further refined by considering ascertainment-bias and the putative molecular impact of different variants (**Fig 1**). Previous power analyses \cite{24390350} suggest that existing cohort sizes support the identification of strong positively-selected driver variants, but that many weaker drivers, and even some moderately strong driver variants would be missed. However, these moderately strong and weak driver variants can also provide potential fitness advantage to tumor cells. As for the functional-impact-based-classification: the philosophy of molecular reductionism holds that any positively or negatively selected variants have some functional impact (i.e. effect on gene expression or activity). The relevance of molecular functional impact is firmly established for driver mutations - positively-selected variants promoting tumor growth. However, rapid accumulation of weak and strong deleterious passengers, which undergo negative selection, could adversely affect the fitness of tumor cell \cite{23388632}. Moreover, majority of low impact and some high functional impact variants may alter tumor gene expression or activity in ways that are not ultimately relevant for tumor fitness; hence, these variants will be under neutral selection.

Deleted: extensively

Deleted: 391996

Deleted: variant

Deleted: comprises

Deleted:

Deleted: In addition

Deleted: INDELs.

Deleted: Nonetheless, of

Deleted: thousands

Deleted: and their

Deleted: and fitness

Deleted: are poorly understood

Deleted: , which in the literature

Deleted: reported

Deleted:

Deleted:

Deleted: }, respectively.

Deleted: degree of

Deleted:

Deleted: , in practice,

Deleted: the

Deleted: albeit at lower extent.

Deleted: The

Deleted: Furthermore, the

Deleted: few

Deleted: , some high functional impact variants may alter tumor gene expression or activity in ways that are not ultimately relevant for tumor fitness; hence, will be under neutral selection. Moreover, all low impact non-functional variants will be neutrally selected. Similarly

Formatted: Font:Bold, Underline

Impactful passengers and their prevalence

In this work, we leveraged the PCAWG variant dataset to assess the molecular consequence of nominal passenger variants in 37 cancer histological subtypes. We build on existing tools \cite{25273974} to annotate and score the predicted molecular impact of each variant, including SNVs, INDELs and SVs in the pan-cancer dataset. The integration of annotation and impact score allows for the quantification of overall molecular functional impact of variants occupying different genomic elements.

One would expect that if a nominal passenger variant does indeed impact tumor cell fitness, its effect should be mediated through its molecular functional impact. Therefore, in order to relate the presence of different categories of nominal passenger SNVs to their role in cancer progression, we surveyed the putative molecular functional impact distribution of somatic variants in different cancer genomes. The molecular functional impact distribution varies across different cancer types and different genomic elements. For instance, impact score distributions of non-coding variants in different cancer genomes demonstrate three distinct peaks. The upper and the lower extremes of this distribution correspond to traditional definitions of high-impact putative driver variants and low impact neutral passengers respectively. In contrast, the middle peak in the intermediate molecular functional impact regime corresponds to what we term *impactful nominal passengers*. This intermediate functional impact category could include undiscovered drivers (strong & weak) as well as potentially deleterious passengers (**Fig 2a**). Conceptually, fitness effects of mutations can be positive or negative for tumor cells. Although fitness effects can be directly established through specialized genetic functional experiments \cite{}, one powerful statistical approach for detecting the fitness effects of variants is to identify discrepancies between observed mutation feature distributions and appropriate null models of neutral mutation. A uniform distribution of mutation across the genome is a useful null model for making descriptive statements about the functional properties of the human genome, and about the functional impact of mutational processes in cancer. A null distribution formed by shuffling the location of variants identified in cancer genomes has the potential to provide suggestive evidence of selection and is described in more detail in supplemental Method X.X.

According to a uniform null expectation, we might assume that the overall burden of variants in a cancer genome will be uniformly distributed across different functional elements and among different gene categories. In contrast, we observe that the molecular impact burden in certain cancers is concentrated in particular gene categories. This is easiest to understand in terms of coding loss-of-function (LoF) variants, where the molecular impact is most intuitive. For instance, as a measure of the molecular impact of both driver and non-driver loss of function SNVs, we examined the fraction of deleterious LoFs affecting genes across four categories of cancer-related functional annotation (**Fig 2d**).

Deleted: passenger

Deleted: leverage

Deleted: exhaustive

Deleted: data set

Deleted: perform

Deleted: most comprehensive investigation to decipher the landscape of

Deleted: and fitness consequences

Deleted: More specifically, we

Deleted:

Deleted: This systematic annotation effort generates a comprehensive annotation compendium of PCAWG variants, which can serve as a useful resource. Furthermore, the

Deleted: any

Deleted: variants do

Deleted: their

Deleted: by their

Deleted: and

Deleted: predicted

Deleted: among

Deleted: indicate

Deleted: ,

Deleted: , which

Deleted: are most definitively

Deleted: ,

Deleted: null

Deleted: A more sophisticated

Deleted: variant

Deleted: show

Deleted:

Deleted: simple

Deleted: (LOF

Deleted: (LoF)

Driver LoF variants, which are well understood high impact variants, showed significant enrichment in each category of cancer-related functional annotation compared to a random (shuffled-variant) control ($p < 0.001$). Conversely, non-driver LoF SNVs displayed depletion in each of these categories ($p < 0.001$). Driver, non-driver, and random loss of function mutations were all enriched in comparison to germline LoF mutations ($p < 0.001$). Given the high selective pressure presumed to act against germline deleterious loss of function mutations *in utero*, our observations suggest that both driver and non-driver LoF mutations exert molecular functional impact. Similarly, compared with the uniform null distribution, we observe that *impactful variants* (nonsynonymous & promoter SNVs) tend to occur in essential genes more often compared to low impact variants (**Fig 2b**). Conversely, low impact passengers constitute larger fractions of variants influencing non-essential genes. This observation is consistent with underlying functional properties of the human genome.

TF binding landscape and overall impact of variants

Similar to LoF variants, we can also quantify the overall burden of the noncoding region of the genome. However, for majority of noncoding variant, functional impact is less easy to gauge. For instance, noncoding and coding variants occupying the terminal region of the gene or undergoing alternatively splicing, will have little functional consequence. In contrast, transcription factor binding site (TFBS) variants are among the noncoding variants with functional impact that is more straightforward to measure. Similar to the effect of a LoF variant on a gene, their molecular impact is clearly manifested through the creation or destruction of TF binding motifs (gain or loss of motif). In both cases (gain or loss), we observed significant differential burdening of TFBS among different cancer cohorts. For instance, we detect significant enrichment of high impact variants creating new motifs in various TFs including GATA, PRRX2 and SOX10 (**Fig 3b**) across major cancer types, compared with uniform expectation. Similarly, high impact variants breaking motifs, were highly enriched in TFs such as IRF, POU2F2, NR3C1and STAT (**Fig 3f**) in the majority of cohorts. This selective enrichment or depletion suggests distinct alteration profiles associated with different components of regulatory networks in various cancers. A gene-centric analysis of these alteration patterns highlights genes with bias towards creation or disruption of specific motifs in their regulatory elements (promoters and enhancers). For instance, TERT shows the largest alteration bias for ETS motif creation across a variety of cancer types (Fig 3d), with other genes (such as NEAT1) showing a similar bias, albeit in fewer cancers. Interestingly, ETS motifs appear to show a systematic bias towards motif creation, whereas MYC-family motif alterations show alteration biases in both directions (Fig. 3d). Furthermore, enrichment of SNVs in selective TF motifs leads to gain and break events in promoter that significantly perturb the overall downstream gene

Deleted: LOFs
Deleted: significantly high
Deleted: *vitro*
Formatted: Pattern: Clear (White)
Formatted: Indent: First line: 0.5"
Deleted: subtle and
Deleted: this regard
Deleted: somewhat similar
Deleted: LoFs, as
Deleted: presence manifest
Deleted: observe
Deleted: such as
Deleted: highlight
Deleted: undergoing
Deleted: a more reduced number of
Deleted: leading

expression (**Fig 3g**). For example, among lung adenocarcinoma patients, target gene expression for TFs undergoing motif breaking events was significantly lower than for target genes without TF motif loss. Moreover, in lung adenocarcinoma, we found gain events in three TFBSs (ZBTB14, E2F and HNF4) that significantly increase downstream expression level (p<5e-7, 3e-6 and 2e-4 respectively) (**Fig 3c**). Similarly, ETS family transcription factor at the regulatory region of IRF4 and PSIP1 gene displayed a strong motif creation bias and a significant change in gene expression (with p-value IRF4=0.001 and p-value PSIP1=0.019).

Deleted: a close inspection of overall
Deleted: level of target genes
Deleted: different
Deleted: in lung adenocarcinoma cohort, indicate
Deleted: expression values compared to instances when there was no
Deleted: in those TF motifs.
Deleted: display
Deleted: their

Signature Analysis

The disproportionate functional load on certain TFs in cancers can be related to an underlying mutational spectrum (signature) influencing their binding sites. For instance, the mutational spectrum of motif breaking events observed in SP1 TF binding sites (TFBS) suggests major contribution from C>T and C>A mutation (**Fig 4b**). In contrast, motif breaking events at TFBS of HDAC2 and EWSR1 have relatively uniform mutational profiles. Similarly, comparing the signature composition of low and high impact SNVs in certain cancer-cohorts can help us to distinguish between mutational processes that generate distinct variant impact classes. For instance, we observed distinct signature distributions for the low and high impact non-coding passengers in the renal cell carcinoma cohort. While the majority of passenger variants can be explained by signature 5, high impact passengers have a higher fraction of SNVs explained by signature 4 (**Fig4a**). Moreover, we observed cancers showing microsatellite instability (MSI) due to failure of DNA mismatch repair, have a higher percentage of high impact non-coding passengers (**Fig4c**). Our findings suggest that various mutational processes shape and disproportionally burden cancer genomes.

Deleted: different
Deleted: further
Deleted: the
Deleted: ie
Deleted: of variants
Deleted: mutation
Deleted: suggest
Deleted:
Deleted: mutation spectrum
Deleted: cohort
Deleted: of variants
Deleted: kidney-RCC
Deleted: passengers

Overall variant impact

One might further expect that nominal passenger variants will be uniformly distributed across the cancer genome. Consequently, we analyzed the overall mutational burdening of various genomic elements. For a uniform mutation distribution, we would expect that the fraction of *impactful variants* will remain constant as one accumulate large number of mutations in a given cancer sample. In contrast, we observed that as we acquire more SNVs in cancer, the fraction of impactful mutations decreases. This suggests that earlier variants tend to be impactful, and drive the cancer progression. Conversely, later variants are more likely to be random, i.e. collateral damage. This trend is particularly strong in CNS medulloblastoma ($p < 4e-8$, Bonferroni's correction), lung adenocarcinoma ($p<3e-4$, Bonferroni's correction), and a few other cancers (**Fig 2c).**

Deleted: contribute uniform functional burden
Deleted: comprehensively
Deleted: Based on
Deleted: expectation
Deleted: assume
Deleted: amount
Deleted: mutation
Deleted: certain
Deleted: observe
Deleted: suggesting
Deleted: the
Deleted: whereas the

Additionally, we sought to examine whether cumulative molecular impact of variants can be associated with tumor initiation and progression. Using a cox proportional hazard model, we used the mean GERP of somatic passenger variants per patient to predict patient survival in the 9 tumor subtypes with 10 or more patient deaths. Mean GERP was used as a measure of overall mutation impact because it was presumed to predict the impact of further mutations after sequencing and because this measure is not directly related to tumor age, which would be an important confounder in sum-based impact scores. Patient age at diagnosis and total number of mutations were used as covariates. We obtained significant results in two subtypes after multiple test correction. In both cases, higher mean GERP predicted better patient survival (p-value 0.00023 in Lymph-CLL, 0.0020 in Ovary-AdenoCA). The prolonged survival of high mean GERP patients in these subtypes is consistent with the possibility that an important subset of mutations at conserved positions are deleterious to tumor cells and benefit the patient. It is not clear why significant results were obtained in only 2 of 9 tumor subtypes. One potential explanation for why high mean GERP appeared protective in Lymph-CLL appeals to the famous indolence of Lymph-CLL. In a slow-growing tumor (which is to say, one with low fitness), hitchhiking deleterious passenger variants could confer the same small absolute fitness impact conferred in other cancer types, and yet this fitness impact could be meaningful relative to the already-low fitness of the affected cancer cells.

In addition to SNVs, we also looked at the annotation and overall impact of structural variants (SVs) in the PCAWG dataset. We compared the pattern of somatic SV enrichment in cancer genomes with those from germline. First, we observed, that as expected, somatic SVs were more enriched among functional regions compared to germline SVs, because the latter ones will be under negative selection for disrupting functional regions. Furthermore, we observed a distinct pattern of enrichment for SVs that split a functional element versus those that engulf it. As has been previously noted, there is greater enrichment of germline SVs that engulf an entire functional element rather than for those break a functional element partially. Furthermore, we observed the same pattern for somatic SVs. This is contrary to what one would expect from a purely randomized model, and suggests some form of selection. Finally, we also quantified the functional impact of somatic SVs across various cancer-types. A close inspection of SV and SNV impact scores suggest that certain cancer subtypes tend to harbor large number of high impact SVs, while others were more burdened with high impact SNVs. Many of these correlations have previously been observed \cite{24071851}. For example, it is known that ovarian cancer tends to be associated with driver SVs, whereas clear cell kidney cancer is often driven by SNVs. However, we also find new associations, such as the predominance of high impact SVs compared to SNVs in the bone leiomyoma cohort.

Deleted: Additionally, we sought to examine whether aggregated molecular functional impact of variants can be associated with tumor initiation and progression. Therefore, we performed survival analysis to see if somatic molecular impact burden –the ranked sum of the impact scores of coding and noncoding variants – predicted patient survival within individual cancer subtypes. These correlations varied substantially in different cancer types. For instance, we observed that somatic mutation burden predicted substantially earlier death in chronic lymphocytic leukemia (CLL) and substantially prolonged survival in renal cell carcinoma (RCC) (**Fig5d**). These observations remained after redefining somatic impact burden in relation to the burdening of corresponding variant-shuffled randomized sets. Furthermore, these patterns remained after adjusting for patient age at diagnosis, low-impact mutation load, and –in the case of CLL, including a covariate for IgVH mutation status. These results lend support to the hypothesis that the aggregate number of impactful passengers is clinically meaningful. More specifically, these results suggest that undiscovered drivers are clinically more important than deleterious passengers in CLL, but that the situation is reversed in RCC. In addition, we observed similar correlation between patient's age at cancer diagnosis with their impactful germline mutation burden. More specifically, we found that patients harboring a larger number of high-impact rare germline alleles were diagnosed with cancer at earlier ages in three cancer subtypes including breast adenocarcinoma, CNS medulloblastoma and pancreatic endocrine cancer. ... [1]

Formatted: Highlight

Subclonality and impact score

Furthermore, we also explored the role of impactful variants in cancer evolution by analyzing variants in the context of their associated tumor sub-clone. One might hypothesize that high impact mutations achieve greater prevalence in tumor cells if they are advantageous to the tumor, and a lower prevalence if deleterious. Interestingly, there is evidence to corroborate this hypothesis. We observed that high impact passenger variants in coding regions have greater prevalence among parental subclones (**Fig 5a**) – an effect driven by high impact nominal passenger SNVs in tumor suppressor and apoptotic genes (**Fig 5a**). In contrast, high impact passenger SNVs in oncogenes appear slightly depleted. Similarly, impactful SNVs in DNA repair genes and cell cycle genes are depleted in early subclones (**Fig 5a**). Furthermore, we also observe low heterogeneity in prevalence among higher impact variants compared to lower impact variants. This observation is consistent for both coding and non-coding variants (**Fig 5c**).

Estimated number of deleterious passenger variants removed by selection

A recent study of the ratio of nonsynonymous mutations to synonymous mutations in cancer exomes found that passenger variants are rarely removed by selection from coding regions, but that their removal is more common in haploid regions of essential genes. We applied conservation measure (GERP score) to perform an analogous measure for noncoding regions of cancer genome. We hypothesized that a subset of somatic variants at positive GERP positions might be deleterious to human cells, including cancer cells. Following the logic of Martincorena *et al.* {cite}, we further hypothesized that this effect would be particularly pronounced when recessively deleterious variants are unmasked by complementary deletions and when the variants occur in the regulators of genes essential to cell survival, especially in strong and confidently-annotated regulators such as promoters. Therefore, we compared the observed fraction of variants having positive GERP scores in noncoding regions with the expected fraction, as derived from nonparametric, patient-matched permutation sets. We excluded regulatory elements associated with known or suspected cancer genes so as to reduce the confounding influence of driver variants.

There were 2% fewer noncoding mutations observed at conserved residues than expected. The most straightforward interpretation of this 2% shortfall is that it represents the removal of 2% of mutations at conserved positions, corresponding to a median of 48 removed noncoding deleterious passenger variants per tumor. As hypothesized, this shortfall was particularly pronounced in the noncoding regulators of essential genes in haploid regions (17%) and most intense at promoters (32%). These figures would tend to underestimate the true number of removed deleterious passengers, because they may be partially offset by latent drivers, which will also tend to occur at positive GERP residues and which are presumed to occur more frequently in the observed mutation sets than in the null sets.

Deleted: integrating

Deleted: clonality information. Intuitively, one

Deleted: should either

Deleted: higher

Deleted: or

Deleted: one finds suggestive evidences corroborating

Deleted: observe

Deleted: functional

Deleted: higher pervasiveness

Deleted:). More specifically,

Deleted: gene regions show enrichment in early subclones

Deleted: lower

Deleted: suggesting that pervasiveness of high impact variants within a tumor is more uniform

Deleted: **Functional impact and variant allele frequ** ... [2]

Detection of retained deleterious passenger variants

In addition to the deleterious passenger variants removed by selection, we were interested in detecting the presence of deleterious passenger variants that are retained in the tumor despite negative fitness impacts. Retained deleterious passengers are missed by dN/dS-like approaches, which focus on removed passengers. We argue that the VAF of a variant represents a continuous albeit noisy measure of the variant's fitness impact: fitness-enhancing variants should tend to get higher VAFs and fitness-impairing variants should tend to get lower VAFs. By comparing GERP with VAF, we detect pervasive, gradated deleteriousness among retained passenger variants. Variants were grouped into seven bins representing increasing GERP scores, and variants associated with known or suspected driver genes and their regulators were excluded. We hypothesized that higher GERP bins, representing disruption of more conserved positions, would tend towards greater deleteriousness and therefore lower VAFs. This hypothesis was supported by the data: each successive GERP bin has a lower mean VAF than the preceding bin. The deleteriousness of retained passenger mutations is especially interesting because, in contrast to removed deleterious passenger variants, retained deleterious passenger variants persist in the tumor and therefore continue to encumber cancer cells.

Discussion

Previous studies \cite{20562875} related to the missing heritability problem in GWAS, indicate that SNVs of low level association with a complex trait, usually cannot be identified through a stringent statistical test. However, the cumulative effect of SNVs can explain the majority of this missing association in a GWAS study. Similar to this, here we investigated the hypothesis that cumulative molecular impact of many weak somatic SNVs can have a meaningful impact on cancer progression. This hypothesis stands in contrast to the classical model of cancer, which holds that a few driver variants promote tumor growth, while the thousands of remaining mutations are of no significance to tumor fitness. Intuitively, tumor cells must maintain function of some minimal set of essential genes in order to achieve homeostasis. It is plausible that the aggregate effect of functionally impactful passenger variants on these essential genes would be deleterious to tumor cells \cite{23388632}. For instance, radiation therapy and some chemotherapies are believed to kill tumor cells by causing DNA damage \cite{PARP inhibitors are a good example...}. Similarly, increased mutation counts in coding genes or regions relevant for splicing increase the antigenic cross-section of tumor cells, potentially making them vulnerable to immune surveillance \cite{}. Conversely, any variant that optimizes cell-division at the expense of organism-supporting functions is expected to have a small positive effect on tumor fitness that may be challenging to detect. Moreover, certain variants through their complex genetic regulatory

Deleted: There are good *a priori* reasons

Deleted: think

Deleted: nominal passenger

Deleted: could affect

Deleted: cell

Deleted: require

Deleted: working

Deleted: maintain

Deleted: One might imagine then

Deleted: {}.

Deleted: making them

Deleted: variants

Deleted: reduces

Deleted: energy a cell spends on its

Deleted: to optimize cell-division could be

Deleted: but not easily detected

Deleted: .

interactions may moderately increase the expression levels of canonical oncogenes. It has been proposed that these weak undiscovered driver variants benefit tumor growth and have a small associated positive selection.

In this work, several observations support the notion that some nominal passenger variants undergo weak selection. First, we observed overall enrichment and depletion of nominal passenger variants among TSGs and oncogenes, respectively. An interpretation of this finding is that passenger variants in tumor suppressor genes have weak driver activity, while passenger variants in oncogenes impair oncogenic activity to the detriment to tumor fitness. Similarly, our finding of depletion of nominal passenger variants among DNA repair and cell cycle genes may indicate that high impact variants affecting these genes decrease tumor cell survival in relation to greater mutational burden. We also found that variants at more conserved positions have lower VAF, suggesting that impactful passenger variants can encumber the tumor cells they inhabit. Consistent with a possible deleterious effect of passenger variants on tumor growth, in some cancer subtypes, the most mutated tumors have a lower fraction of impactful variants. This may relate to the aggregate impact of passenger variants becoming more deleterious at higher mutation loads. Alternatively, a fixed number of undiscovered drivers may become diluted by neutral passengers at higher mutation counts. Our LoF mutation analysis indicates that driver LoF mutations exert a positive selective effect, whereas non-driver LoF mutations apparently exert a net negative selective pressure. This observation is consistent with prior evidence of net negative selective effect among nominal passenger missense mutations \cite{}. The aggregate fitness impact of nominal passenger variants may help explain why patient survival times are correlated with functional impact load in select cancer subtypes. In conclusion, our work highlights that an important subset of somatic variants originally identified as passengers nonetheless show biologically and clinically relevant functional roles across a range of cancers.

References

1. Vogelstein, B. & Kinzler, K. W. The Path to Cancer --Three Strikes and You're Out. *N. Engl. J. Med.* **373,** 1895–8 (2015).
2. Nussinov, R. & Tsai, C. J. 'Latent drivers' expand the cancer mutational landscape. *Current Opinion in Structural Biology* **32,** 25–32 (2015).
3. Castro-Giner, F., Ratcliffe, P. & Tomlinson, I. The mini-driver model of polygenic cancer evolution. *Nat. Rev. Cancer* **15,** 680–685 (2015).
4. McFarland, C. D., Korolev, K. S., Kryukov, G. V, Sunyaev, S. R. & Mirny, L. A. Impact of deleterious passenger mutations on cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 2910–5 (2013).

Deleted: might
Deleted: These
Deleted: have been proposed to undergo small positive selection to
Deleted: we came across multiple
Deleted: that
Deleted: affect tumor fitness.
Deleted: observe
Deleted: passengers
Deleted: One
Deleted: these findings
Deleted: may
Deleted: and that
Deleted: as a
Deleted: passengers
Deleted: a
Deleted: variant might eventually provide a critical burden for the survival of
Deleted: . Second, the finding
Deleted: VAFs suggests
Deleted: Third
Deleted: than do less-mutated tumors, suggesting either that
Deleted: impactful
Deleted: becomes
Deleted: , or alternatively but equally interestingly, that some
Deleted: is
Deleted: Finally, our
Deleted: related
Deleted: indicate
Deleted: . Furthermore, this putative
Deleted:
Formatted: Pattern: Clear (White), Not Highlight

Deleted: . ... [3]
Formatted: Font:(Default) Times New Roman
Formatted: Font:(Default) Times New Roman
Formatted: Font:(Default) Times New Roman
Formatted: Font:9 pt
Formatted: Font:(Default) Times New Roman
Formatted: Font:9 pt

Additionally, we sought to examine whether aggregated molecular functional impact of variants can be associated with tumor initiation and progression. Therefore, we performed survival analysis to see if somatic molecular impact burden –the ranked sum of the impact scores of coding and noncoding variants – predicted patient survival within individual cancer subtypes. These correlations varied substantially in different cancer types. For instance, we observed that somatic mutation burden predicted substantially earlier death in chronic lymphocytic leukemia (CLL) and substantially prolonged survival in renal cell carcinoma (RCC) (**Fig5d**). These observations remained after redefining somatic impact burden in relation to the burdening of corresponding variant-shuffled randomized sets. Furthermore, these patterns remained after adjusting for patient age at diagnosis, low-impact mutation load, and –in the case of CLL, including a covariate for IgVH mutation status. These results lend support to the hypothesis that the aggregate number of impactful passengers is clinically meaningful. More specifically, these results suggest that undiscovered drivers are clinically more important than deleterious passengers in CLL, but that the situation is reversed in RCC. In addition, we observed similar correlation between patient's age at cancer diagnosis with their impactful germline mutation burden. More specifically, we found that patients harboring a larger number of high-impact rare germline alleles were diagnosed with cancer at earlier ages in three cancer subtypes including breast adenocarcinoma, CNS medulloblastoma and pancreatic endocrine cancer.

In addition to SNVs, large structural variations (SVs) also play an important role in cancer progression. Thus, we annotated and evaluated the impact of large SVs in the entire PCAWG cohort. We observe depletion of germline SVs in coding and noncoding regions, which indicate negative selection of large SVs in germline cancer genomes. In contrast, we detect significant enrichment of large somatic deletions as well as duplications among various regulatory elements. Moreover, both somatic and germline SVs prefer to completely engulf compared to partially overlap with various genomic elements. In addition, we also quantified the functional impact of these large somatic SVs across various cancer-types. The functional impact score distribution of SVs for different cancer-types indicate that meta tumor cohorts such as CNS, glioma and sarcoma tend to harbor higher impact large deletions and duplications compared to others. In addition, gene-centric analysis on the pan-cancer level reveals that CDKN2A and TEKT2 genes have the largest observed enrichment of high impact deletions and duplications, respectively**.**

Functional impact and variant allele frequency

Finally, we employed a similar analysis using variant allele frequency (VAF) to explore whether passenger variants with high functional impact also conferred a fitness impact to tumor cells.

We would expect for variants that enhance tumor cell fitness to achieve an overall higher than average mean VAF, while variants that reduce tumor cell fitness to occur at an overall lower mean VAF. Indeed, driver SNVs occur at higher mean VAF, non-silent coding SNVs and noncoding variants in sensitive regions occur at lower mean VAF, and synonymous variants along with variants in inter-genomic regions occur at intermediate mean VAF (**Fig 5b**). This suggest that in aggregate, non-silent passenger variants and noncoding variants in sensitive regions impair cancer cell fitness. Additionally, we generalize our observations among functional classes by correlating their respective variant frequency with the degree of conservation. Highly conserved positions (i.e. those with high GERP) are expected to be important for organismal fitness, as polymorphisms at those positions could hurt cellular function and in other cases because polymorphisms at those positions could promote undue cellular fitness (i.e. cancer) at the cost of organismal fitness. As expected, we observe that in PCAWG driver genes, VAF and GERP have a small but statistically significant positive correlation (with coefficient 0.0040 and p-value 0.0046). Interestingly, VAF and GERP have a correlation of similar magnitude but in opposite direction among variants not in driver genes, with very high significance (coefficient -0.0034, p-value < 2.2e-16). The observed trend for passenger variants at more conserved positions to occur at lower VAF is consistent with the deleterious passenger hypothesis.

Page 9: [3] Deleted	Kumar, Sushant	5/27/17 5:06:00 AM

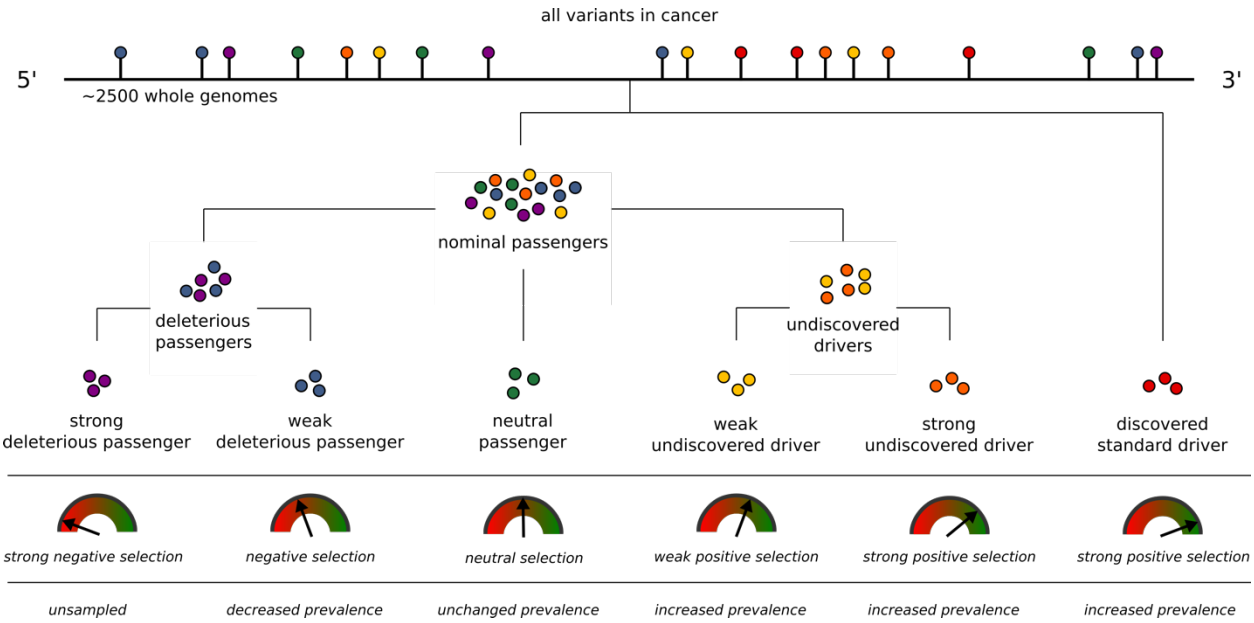


Figure 1. Classification of somatic variants into different categories based on their functional impact and selection characteristics: Both coding and non-coding variants can be classified as drivers and passengers based on their impact and signal of positive selection. Among nominated passengers, true passengers undergo neutral selection and tend to have low

functional impact. Deleterious passengers, latent drivers and mini-drivers represent various categories of higher impact nominal passenger variants, which undergo weak negative or positive sections.

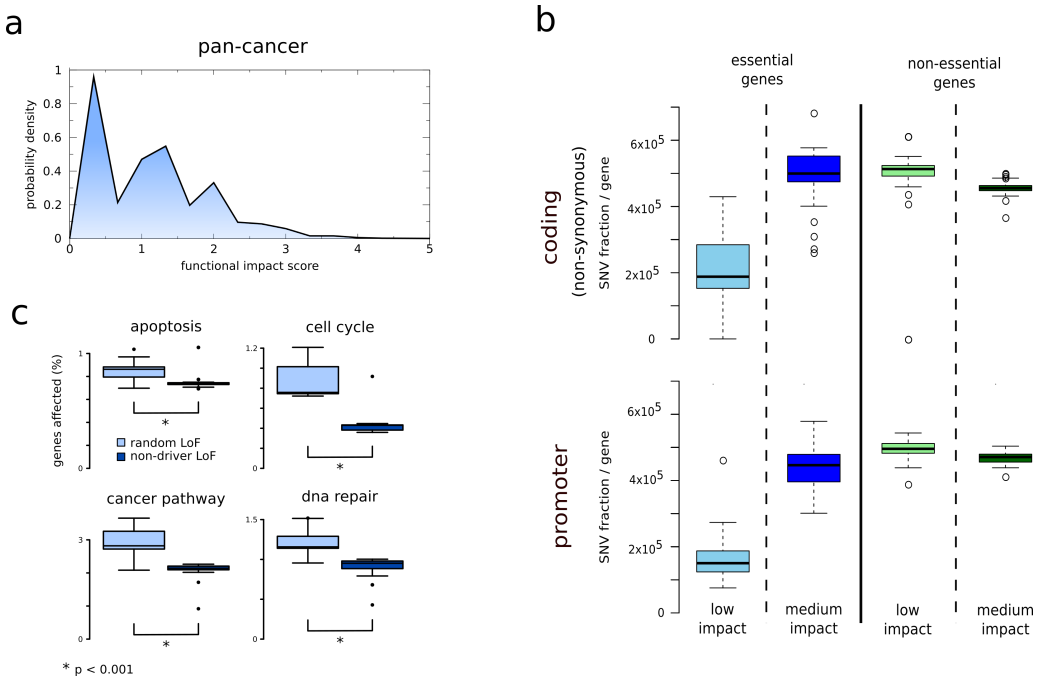


Figure 2: Functional impact scores for PCAWG SNVs: a) Functional impact distribution in noncoding region: three peaks correspond to low, medium and high impact variants. b) Fraction of impactful variants per gene in essential and non-essential gene sets: non-synonymous(top), promoter(middle) and loss-of-function(bottom). c) Percentage of different categories of genes affected by non-drivers LOF SNVs in original and randomized data.

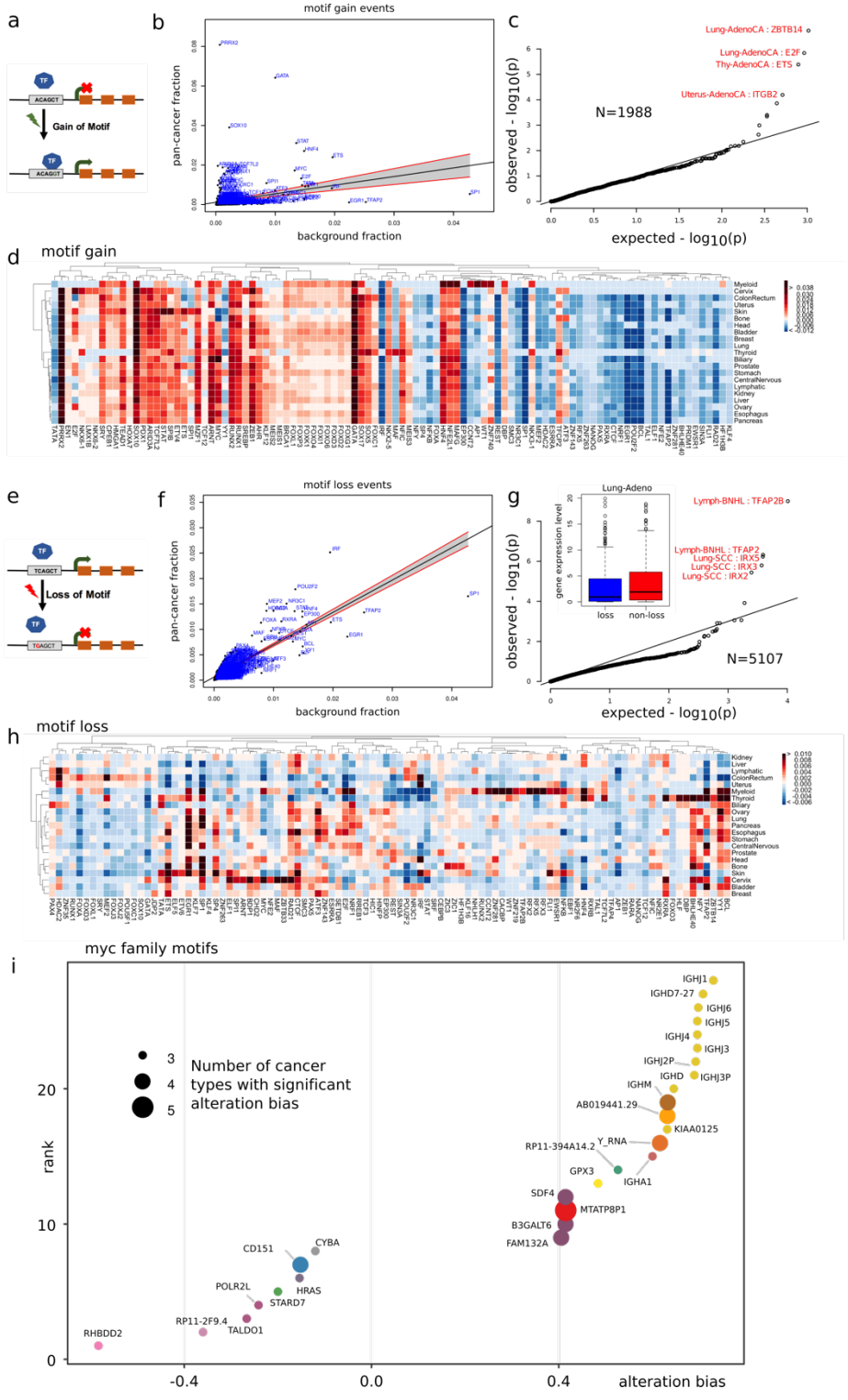

a
b
motif gain events
pan-cancer fraction
background fraction
c
Lung-AdenoCA : ZBTB14
Lung-AdenoCA : E2F
Thy-AdenoCA : ETS
Uterus-AdenoCA : ITGB2
N=1988
observed - log10(p)
expected - log10(p)
d
motif gain
e
f
motif loss events
g
Lung-Adeno
gene expression level
loss
non-loss
Lymph-BNHL : TFAP2B
Lymph-BNHL : TFAP2
Lung-SCC : IRX5
Lung-SCC : IRX3
Lung-SCC : IRX2
N=5107
h
motif loss
i
myc family motifs
Number of cancer types with significant alteration bias
rank
alteration bias
IGHJ1
IGHD7-27
IGHJ6
IGHJ5
IGHJ4
IGHJ3
IGHJ2P
IGHD
IGHJ3P
IGHM
AB019441.29
KIAA0125
RP11-394A14.2
Y_RNA
GPX3
IGHA1
SDF4
MTATP8P1
B3GALT6
FAM132A
CD151
CYBA
POLR2L
HRAS
STARD7
RP11-2F9.4
TALDO1
RHBDD2

Figure 3: **Overall functional burdening of TF motifs:** ***Pan-cancer overview of TFs burdening****:* scatter plots for b) motif loss and f) motif gain events, ***Heat map presenting differential burdening of various TFs****:* SNVs leading to d) motif breaking and H) motif gain events in different cohorts compared to the genomic background. ***Gene expression changes due to motif alteration***: c) gene expression distribution for target genes for motif breaking and non-breaking scenario in Lung-Adenocarcinoma. g) Expression of target genes for TFs undergoing motif gain events.

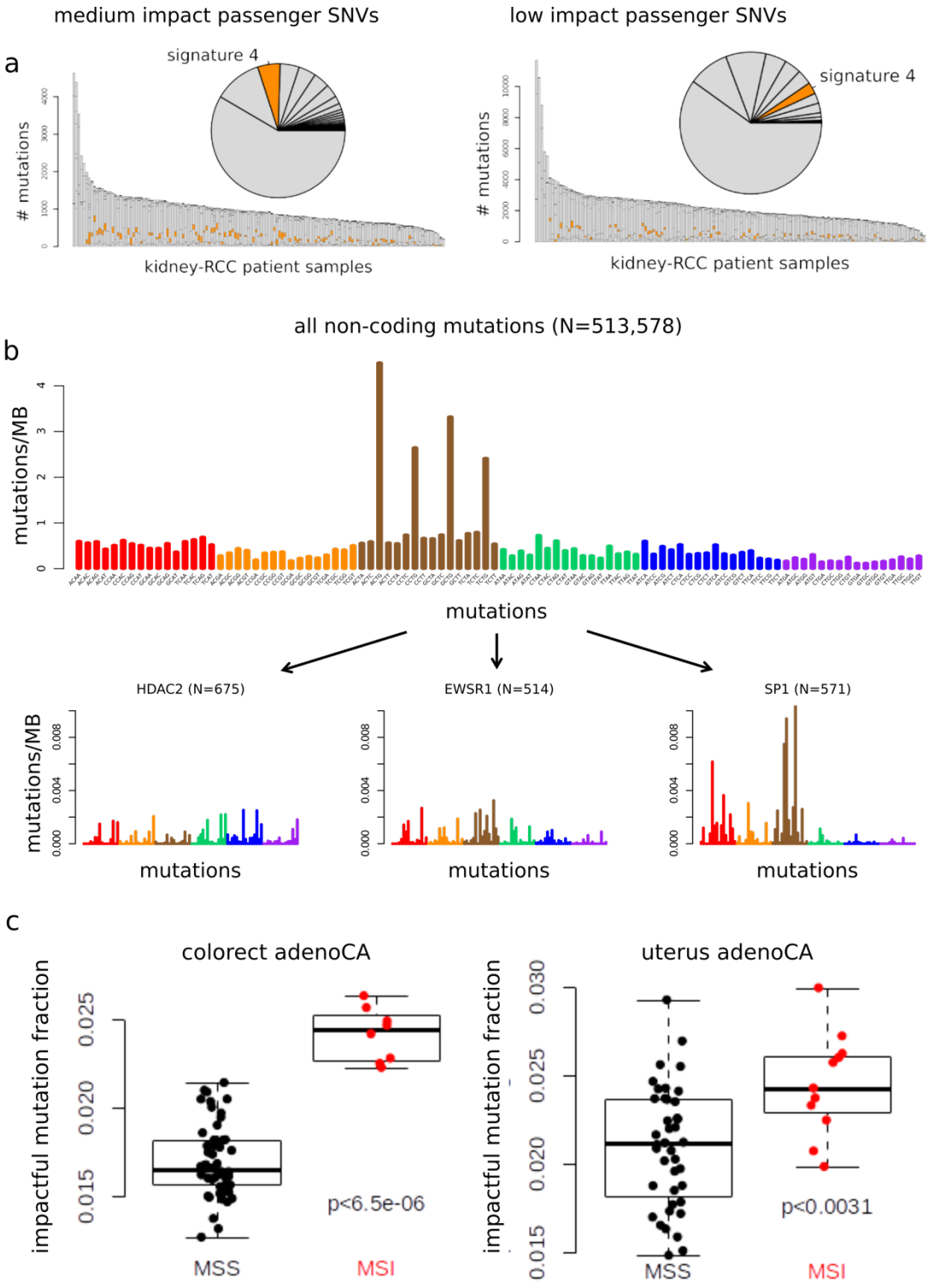


Figure 4: Mutational signatures associated with different categories of impactful variants: a) Distribution of canonical signatures in the kidney-RCC cohort for impactful (left) and low-impact SNVs (right). b) Mutation spectra associated with motif

breaking events observed in HDAC2, EWSR1 and SP1 in the kidney-RCC cohort. c) fraction of impactful SNVs in MSI and MSS samples in Colorectal Adenocarcinoma(left) and Uterine Adenocarcinoma (right).

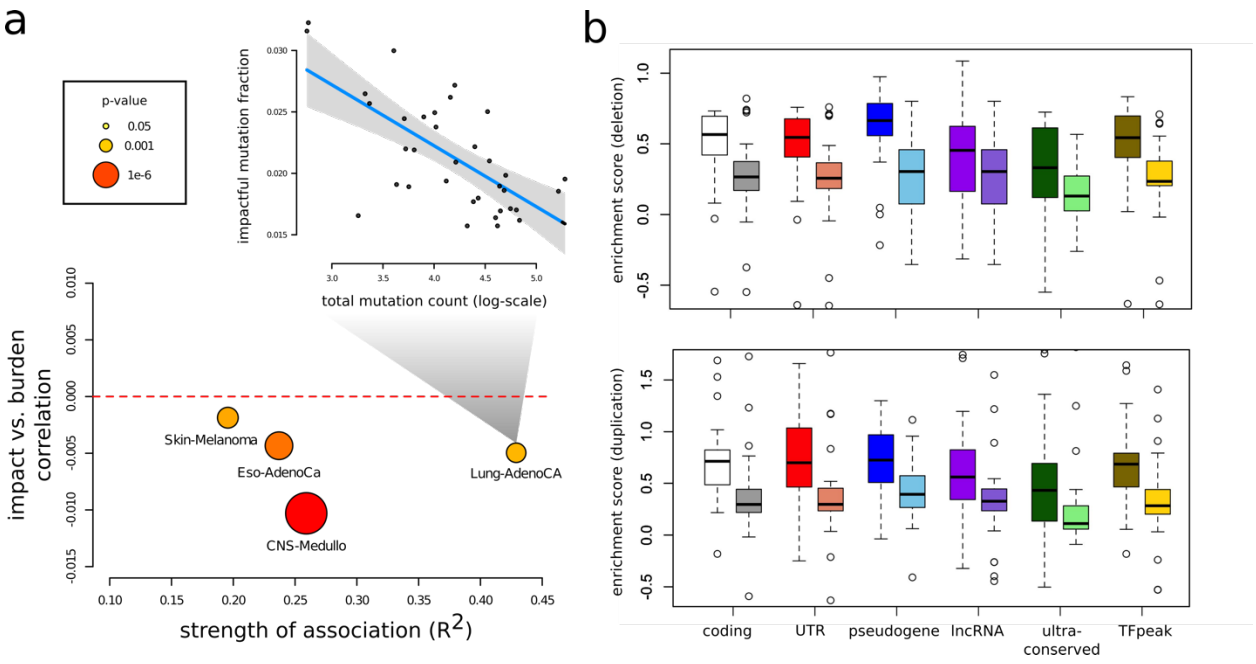
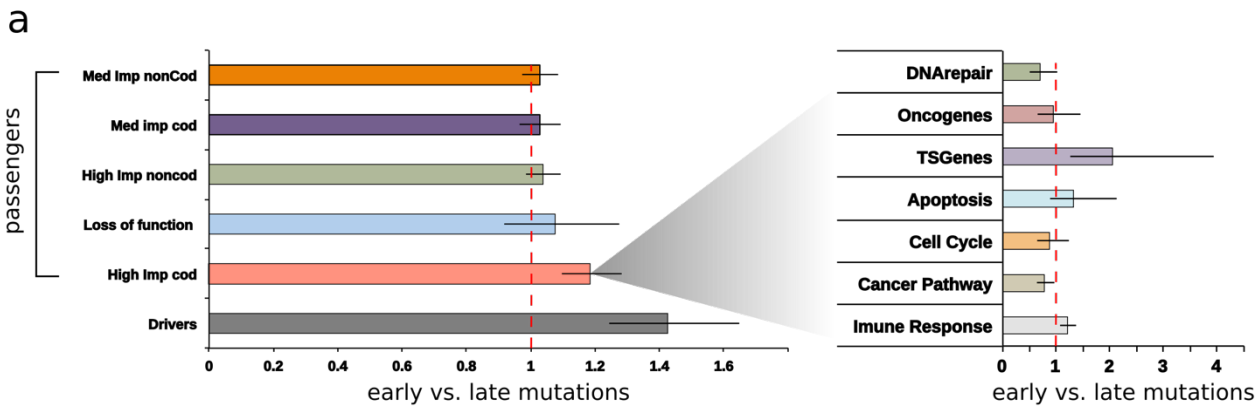
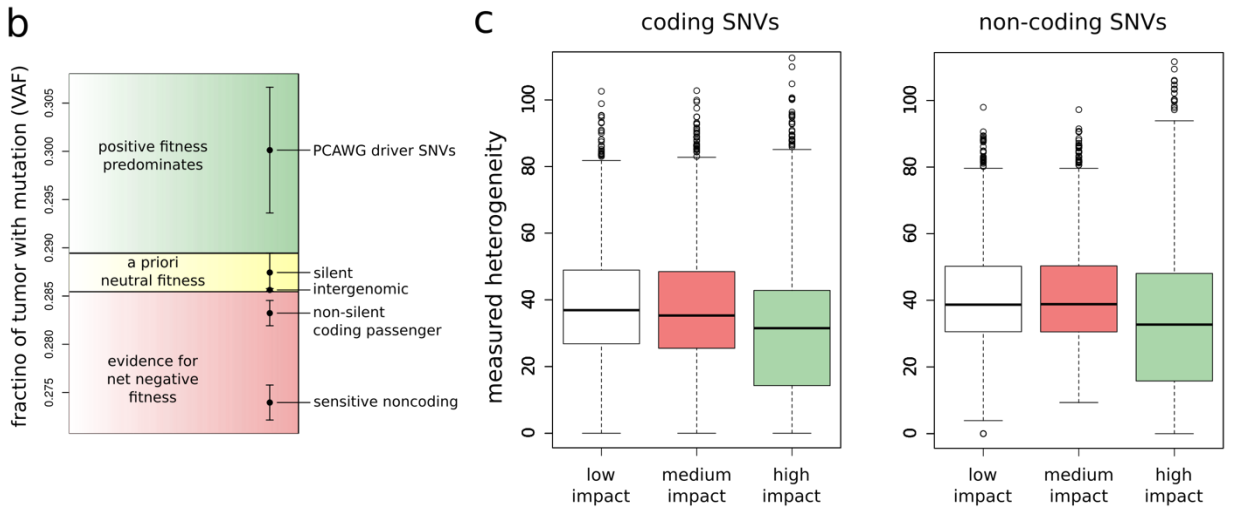
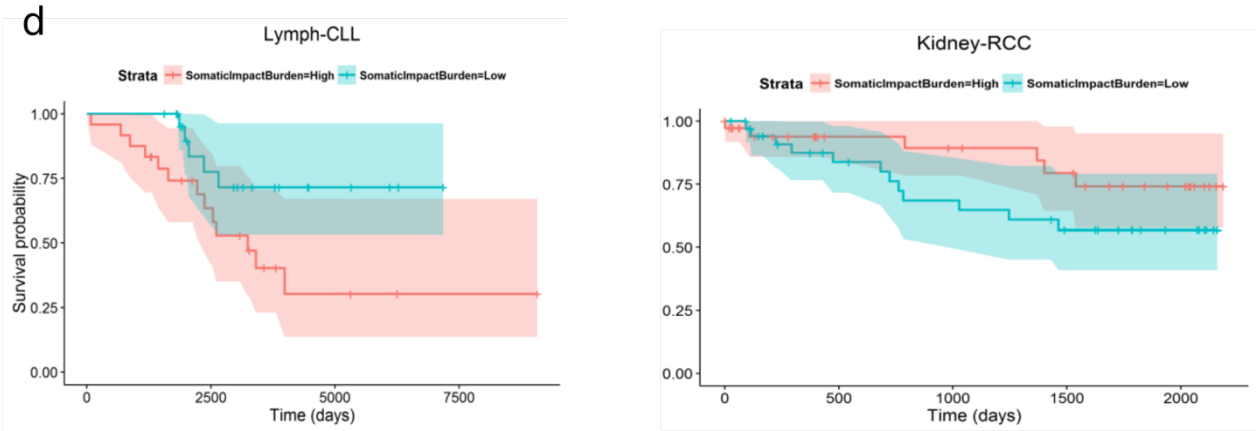


Figure 5: Overall variant impact: a) Correlation between number of impactful and total SNV frequencies for different cohorts. b) Fold enrichment score for somatic large deletions overlapping with different regions of the genome : pair of boxplot for each annotation correspond to enrichment score distribution for the engulfing(left) and partially overlapping (right) large deletions.

Figure 6: Correlating functional burdening with subclonal information and patient survival: a) Subclonal ratio (early/late) for different categories of SNVs (coding/non-coding) based on their impact score. Subclonal ratio for high impact SNVs occupying distinct gene sets. b) Stratifying SNVs in different selection classes based on their pervasiveness measured through mean VAF. c) Mutant tumor allele heterogeneity difference comparison between high, medium and low impact SNVs for coding(left) and non-coding regions(right). d) Survival curves in CLL (*left panel*) and RCC (*right panel*) with 95% confidence intervals, stratified by normalized impact burden.


a
passengers
Med Imp nonCod
Med imp cod
High Imp noncod
Loss of function
High Imp cod
Drivers
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
early vs. late mutations
DNArepair
Oncogenes
TSGenes
Apoptosis
Cell Cycle
Cancer Pathway
Imune Response
0
1
2
3
4
early vs. late mutations


b
fractino of tumor with mutation (VAF)
0.305
0.300
0.295
0.290
0.285
0.280
0.275
positive fitness predominates
a priori neutral fitness
evidence for net negative fitness
PCAWG driver SNVs
silent intergenomic
non-silent coding passenger
sensitive noncoding
c
coding SNVs
non-coding SNVs
measured heterogeneity
0
20
40
60
80
100
low impact
medium impact
high impact


d
Lymph-CLL
Strata
SomaticImpactBurden=High
SomaticImpactBurden=Low
Survival probability
1.00
0.75
0.50
0.25
0.00
0
2500
5000
7500
Time (days)
Kidney-RCC
Strata
SomaticImpactBurden=High
SomaticImpactBurden=Low
1.00
0.75
0.50
0.25
0.00
0
500
1000
1500
2000
Time (days)