Passenger mutations in 2500 cancer genomes: Overall molecular functional impact & its 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. However, the classical model posits that only a small number of these mutations strongly drive tumor progression, and that the remaining mutations (termed "nominal passengers") are considered inconsequential for tumorigenesis. In this study, we leverage the comprehensive variant data from PCAWG to predict the extent of molecular impact of each variant, including nominal passengers, to decipher their overall molecular impact on different coding and noncoding genomic elements. The overall molecular impact distribution of PCAWG SNVs 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, molecular impact relates to the underlying mutational signature and thus different signatures confer different extent of functional impact. Moreover, burdening of variants is non-random in their differential terms of affecting different regulatory subsystems and for different categories of genes. In addition, we find that functional impact varies based on subclonal architecture (i.e. early vs late mutations) and can be also related to survivability of patients. Finally, we speculate on how the differential burdening might be related to the existence of both weak positive and negative selection during tumor evolution.

Style Definition: Normal
Style Definition: Revision
Deleted: ~
Deleted: Classical models of
Formatted: Not Highlight
Deleted: posit
Deleted: variants
Deleted: variants
Deleted: the Pan-Cancer Analysis of Whole Genomes (PCAWG) project to evaluate
Deleted: functional
Deleted: functional
Deleted: functional burdening
Deleted: contribute to the functional burdening to
Deleted: .
Deleted: effecting
Deleted: functional
Formatted: Not Highlight
Deleted: We
Deleted: burdening
Deleted: also further
Formatted: Not Highlight
Deleted: . ... [1]
Formatted: Line spacing: 1.5 lines
Formatted: Font:Times New Roman, Bold, Underline, Pattern: Clear (White)

Introduction

Previous studies have extensively focused on characterizing variants occupying coding regions of cancer genomes \cite{391996}. However, the extensive Pan-cancer Analysis of Whole Genomes (PCAWG) variant dataset, which comprises 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. In addition, 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 INDELs.

Nonetheless, of the 30 million SNVs in the PCAWG data set, few thousands ($< 5/\text{tumor}^1$) \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, and their molecular and fitness consequences are poorly understood. 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, which in the literature have been reported as "mini-drivers" \cite{26456849} and "deleterious passengers" \cite{23388632}, respectively.

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 degree of molecular impact of different variants (**Fig 1**). Previous power analyses \cite{24390350} suggest that, in practice, existing cohort sizes support the identification of the 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 albeit at lower extent. 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). Furthermore, the relevance of molecular functional impact is firmly established for few driver mutations - positively-selected variants promoting tumor growth. However, 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.

Deleted: various

Formatted: Font:Times New Roman, 11 pt, Font color: Black, Pattern: Clear

Deleted: To a first approximation, all clinically significant consequences of genomic variants in cancer are mediated through changes in gene expression or gene activity; i.e. "functional impact." The relevance of functional impact is firmly established for driver mutations - positively-selected variants promoting tumor growth \cite{ 26304545, 23770567}. Nonetheless, of the thousands of variants in a typical tumor, very few of these

Formatted: Indent: First line: 0"

Deleted: to drive

Deleted: variants, which we call

Deleted: represent the overwhelming majority

Deleted: functional

Formatted: Font:Not Italic

Formatted: Font:Times New Roman, 11 pt, Font color: Black, Pattern: Clear

Formatted: Font:Times New Roman, 11 pt, Font color: Black

Deleted: We reason that if any nominal passenger variants do indeed impact tumor cell fitness, this effect should be mediated by their functional impact; therefore, predicted functional impact serves as a good starting point to assess the potential tumor fitness effects of nominal passenger variants.

Deleted: In this work, we explore the functional landscape of passenger variants in various cancer cohorts by leveraging extensive pan-cancer variant calls from PCAWG. More specifically, we build upon existing tools \cite{ 25273974} to annotate and score the predicted functional impact of each variant, including SNVs, INDELs and SVs in the pan-cancer dataset.

Formatted: Indent: First line: 0.5"

Moved down [1]: This systematic annotation effort generates a comprehensive annotation compendium of PCAWG variants, which can serve as a useful resource.

Deleted: Furthermore, integration of annotation and impact score allows for quantification of overall functional impact of variants occupying different genomic elements. We observe that disruption of regulatory elements in the noncoding genome correlates with altered gene expression. Moreover, regulatory elements are differentially affected by mutation process, as elucidated by our signature analys ... [2]

Deleted: fitness effects

Deleted: cells

Deleted: functional

Formatted: Font:Times New Roman, 11 pt, Font color: Black

Deleted:

Deleted: mostly-

Deleted: Some

Deleted: However

Similarly, rapid accumulation of weak and strong deleterious passengers, which undergo negative selection, could adversely affect the fitness of tumor cell \cite{23388632}.

Impactful passenger and their prevalence

In this work, we leverage the exhaustive PCAWG variant data set to perform the most comprehensive investigation to decipher the landscape of molecular and fitness consequences of nominal passenger variants in 37 cancer histological subtypes. More specifically, 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. This systematic annotation effort generates a comprehensive annotation compendium of PCAWG variants, which can serve as a useful resource. Furthermore, 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 any nominal passenger variants do indeed impact tumor cell fitness, their effect should be mediated by their functional impact. Therefore, in order to relate the presence of different categories of nominal passenger SNVs and their role in cancer progression, we surveyed the predicted functional impact distribution of somatic variants in different cancer genomes. The functional impact distribution varies among different cancer types and different genomic elements. For instance, impact score distributions of non-coding variants in different cancer genomes indicate 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 functional impact regime corresponds to what we term *impactful nominal passengers,* which 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 are most definitively established through specialized genetic functional experiments, 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 null distribution is useful for making descriptive statements about the functional properties of the human genome and the functional impact of mutational processes in cancer. A more sophisticated null distribution formed by variant shuffling has the potential to show suggestive evidence of selection and is described in more detail in supplemental Method X.X.

According to a simple 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-

Deleted: moderately

Deleted: and even drive tumor cell population to extinction in certain conditions

Deleted:

Formatted: Line spacing: single

Formatted: Font:Arial

Deleted: In order to substantiate the presence of the above-mentioned continuum among nominal passenger SNVs and their role in cancer progression, we surveyed the

Moved (insertion) [1]

Formatted: Indent: First line: 0.5"

Deleted: The interpretation

Deleted: impact

Deleted: depends on which

Deleted: distribution serves as the comparison

Deleted: Supplemental

Formatted: Font color: Black

Deleted: functional

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 (LoF) SNVs, we examined the fraction of deleterious LoFs affecting genes across four categories of cancer-related functional annotation (**Fig 2d**). Driver LOFs, which are well understood high impact variants, showed significantly high enrichment in each category of cancer-related functional annotation compared to 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 vitro*, our observations suggest that both driver and non-driver LoF mutations exert 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 subtle and less easy to gauge. In this regard, transcription factor binding site (TFBS) variants are somewhat similar to LoFs, as their presence manifest through the creation or destruction of TF binding motifs (gain or loss of motif). In both cases (gain or loss), we observe 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 such as 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 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 highlight genes undergoing 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 a more reduced number of 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 leading to gain and break events in promoter significantly

Deleted: functional

Deleted: Furthermore, based on uniform expectation, we would assume that the fraction of *impactful variant* will remain constant as one accumulate large amount of mutation in certain cancer sample.

Moved down [2]: In contrast, we observe that as we acquire more SNVs in cancer, the fraction of impactful mutations decreases suggesting that the earlier variants tend to be impactful and drive the cancer whereas the later 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).**

Formatted: Font:Bold

Formatted: Indent: First line: 0"

Formatted: Font color: Black

Moved down [3]: One might further expect that nominal passenger variants will contribute uniform functional burden across the genome. Consequently, we comprehensively analyzed the overall mutational burdening of various genomic elements

Formatted: Not Highlight

Deleted: , including TF (

Formatted: Not Highlight

Deleted:) binding motifs in various cancer genomes. The presence of a variant within a TF binding

Formatted: Not Highlight

Formatted: Not Highlight

Deleted: can lead to either

Formatted: Not Highlight

Deleted: function

Formatted: Not Highlight

Deleted: ,

Formatted: Not Highlight

Formatted: Not Highlight

Deleted: observe

Formatted: Not Highlight

Deleted: influencing gene expression by

Deleted: TF

Deleted: For instance, a strong motif creation bias event among ETS family TFs was detected in the TERT promoter region in various cancer cohorts including glioblastoma, medulloblastoma, thyroid adenocarcinoma and oligoastrocytoma.

perturb the overall downstream gene expression (**Fig 3g**). For example, a close inspection of overall expression level of target genes for different TFs undergoing motif breaking events in lung adenocarcinoma cohort, indicate significantly lower expression values compared to instances when there was no loss in those TF motifs. 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 JRF4 and PSIP1 gene display a strong motif creation bias and a significant change in their expression (with p-value JRF4=0.001 and p-value PSIP1=0.019).

Deleted: IRF

Deleted: IRF

Formatted: Indent: First line: 0"

Signature Analysis

The disproportionate functional load on certain TFs in different cancers can be further related to the underlying mutational spectrum (ie signature) of variants influencing their binding sites. For instance, mutation spectrum of motif breaking events observed in SP1 TF binding sites (TFBS) suggest 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 mutation spectrum profiles. Similarly, comparing signature composition of low and high impact SNVs in certain cancer-cohort can help us to distinguish between mutational processes that generate distinct impact classes of variants. For instance, we observed distinct signature distributions for the low and high impact non-coding passengers in the kidney-RCC cohort. While the majority of passengers 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 higher percentage of high impact non-coding passengers (**Fig4c**). Our findings suggest various mutational processes shape and disproportionally burden cancer genomes.

Formatted: Not Highlight

Overall variant impact

One might further expect that nominal passenger variants will contribute uniform functional burden across the genome. Consequently, we comprehensively analyzed the overall mutational burdening of various genomic elements. Based on uniform expectation, we would assume that the fraction of *impactful variants* will remain constant as one accumulate large amount of mutation in certain cancer sample. In contrast, we observe that as we acquire more SNVs in cancer, the fraction of impactful mutations decreases suggesting that the earlier variants tend to be impactful and drive the cancer whereas the later 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**).

Moved (insertion) [3]

Formatted: Not Highlight

Formatted: Indent: First line: 0"

Moved (insertion) [2]

Formatted: Font:Bold

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.

Subclonality and impact score

Moved down [4]: Thus, we annotated and evaluated the impact of large SVs in the entire PCAWG cohort.

Deleted:

Survivability and age of onset

Moved down [5]: 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. ... [3]

Deleted: Simplistically, we would expect majority of SVs to be distributed uniformly across the genome regardless of their extent of overlap with functional elements of the genome. However, our annotation analysis of somatic and germline SVs in PCAWG portrays a different picture. As expected, we observed higher enrichment of somatic SVs compared to germline SVs engulfing or partially overlapping with either coding region or transcription factor peaks, which suggest positive and negative selection of large SVs in somatic and germline cancer genomes, respectively. Similarly, we also observe significant enrichment of large engulfing somatic deletions as well as duplications among pseudogenes, non-coding RNAs, UTRs and ultra-conserved regions of the genome. Moreover, engulfing SVs are highly enriched compared to partially overlapping SVs. The observed enrichment bias of SVs toward certain regions of the genome as well as the extent of their overlap suggest that selection processes play a key in role in emergence of somatic SVs. We quantified the effect of these selection processes by evaluating functional impact of these large deletions and duplications across various cancer-types.

Formatted: Highlight

Deleted: impactful passengers might

Deleted: CAN

Formatted: Font color: Black

Deleted:

Moved (insertion) [4]

Moved (insertion) [5]

Formatted: Highlight

Furthermore, we also explored the role of impactful variants in cancer evolution by integrating their sub-clonality information. Intuitively, one might hypothesize that high impact mutations should either achieve higher prevalence in tumor cells if they are advantageous to the tumor, or a lower prevalence if deleterious. Interestingly, one finds suggestive evidences corroborating this hypothesis. We observe that high functional impact passenger variants in coding regions have higher pervasiveness among parental subclones (**Fig 5a**). More specifically, high impact nominal passenger SNVs in tumor suppressor and apoptotic gene regions show enrichment in early subclones (**Fig 5a**). In contrast, high impact passenger SNVs in oncogenes appear slightly depleted. Similarly, impactful SNVs in DNA repair and cell cycle genes are depleted in early subclones (**Fig 5a**). Furthermore, we also observe lower heterogeneity among higher impact variants suggesting that pervasiveness of high impact variants within a tumor is more uniform compared to lower impact variants. This observation is consistent for both coding and non-coding variants (**Fig 5c**).

Moved down [6]: One interpretation of these findings is that passenger variants in tumor suppressor genes may have weak driver activity and that passenger variants in oncogenes impair oncogenic activity as a detriment to tumor fitness.

Formatted: Font color: Text 1

Deleted:), suggesting that a high impact variant might eventually provide a critical burden for the survival of tumor cell. This observation is consistent with prior studies highlighting role of deleterious passengers inhibiting cancer progression.

Deleted:

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.

Deleted:

Formatted: Pattern: Clear, Highlight

Discussion

There are good *a priori* reasons to think that nominal passenger variants could affect tumor cell fitness. Intuitively, tumor cells must require some minimal set of essential genes in working order to maintain homeostasis. One might imagine then 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{}. Similarly, increased mutation counts in coding genes or regions relevant for splicing increase the antigenic cross-section of tumor cells, making them potentially vulnerable to immune surveillance \cite{}. Conversely, any variants that reduces the energy a cell spends on its organism-supporting functions to optimize cell-division could be expected to have a small but not easily detected positive effect on tumor fitness. Moreover, certain variants through their complex genetic regulatory interactions might moderately increase the expression levels of canonical oncogenes. These weak undiscovered driver variants have been proposed to undergo small positive selection to benefit tumor growth.

In this work, we came across multiple observations that support the notion that some nominal passenger variants affect tumor fitness. First, we observe overall enrichment and depletion of nominal passengers among TSGs and oncogenes, respectively. One interpretation of these findings is that passenger variants in tumor suppressor genes may have weak driver activity and that passenger variants in oncogenes impair oncogenic activity as a detriment to tumor fitness. Similarly, depletion of nominal passengers among DNA repair and cell cycle genes indicate that a high impact variant might eventually provide a critical burden for the survival of tumor cell. Second, the finding that variants at more conserved positions have lower VAFs suggests that impactful passenger variants can encumber the tumor cells they inhabit. Third, in some cancer subtypes, the most mutated tumors have a lower fraction of impactful variants than do less-mutated tumors, suggesting either that the aggregate impact of impactful passenger variants becomes more deleterious at higher mutation loads, or alternatively but equally interestingly, that some fixed number of undiscovered drivers is diluted at higher mutation counts. Finally, our LoF related analysis indicate 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. Furthermore, this putative fitness impact of nominal passenger variants may help explain why patient survival times are correlated with functional impact load in select 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).

Deleted: have

Deleted: effects on tumor cells

Formatted: Font color: Text 1

Moved (insertion) [6]

Formatted: Font color: Text 1

Deleted: First

Formatted: Font color: Text 1, Pattern: Clear

Formatted: Font color: Text 1

Deleted: Second

Formatted: Font color: Text 1

Deleted: Third

Formatted: Font color: Text 1

Deleted: This

Formatted: Font color: Text 1

Formatted: Font color: Text 1, Highlight

Deleted:

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).

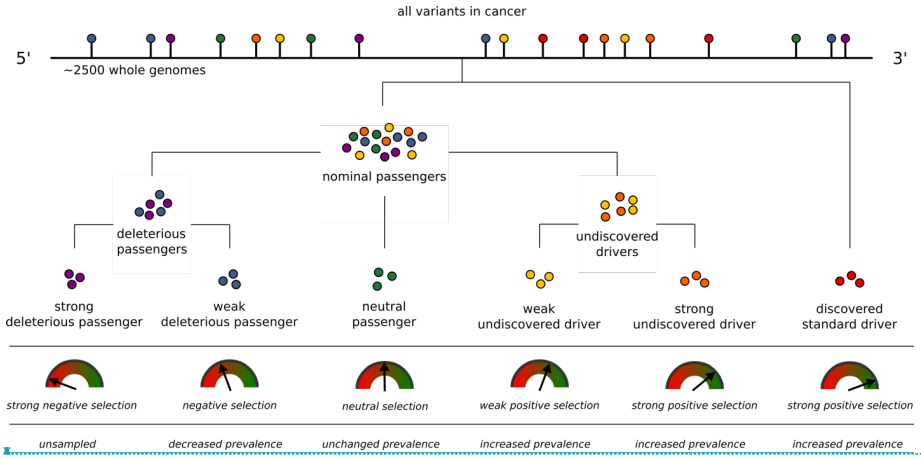


Figure 1. Classification of somatic variants into different categories based on their functional impact and selection characteristics: Both coding and non-coding variants can be classified as drivers and passengers based on their impact and signal of positive selection. Among nominated passengers, true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

Formatted: Font:11 pt

Formatted: Indent: Left: 0", Line spacing: 1.5 lines

Deleted: .. [4]

Formatted: Font:9.5 pt, Font color: R,G,B (34,34,34)

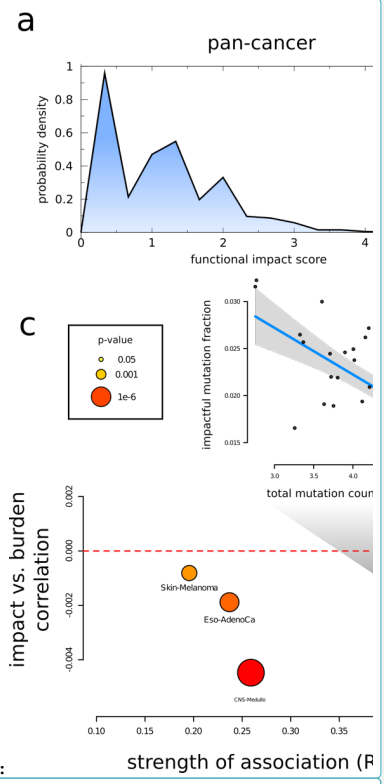
Formatted: Font:(Default) Times New Roman

Formatted: Font:Times New Roman, 11 pt

Formatted: Line spacing: 1.5 lines

Formatted: Font:Times New Roman, 11 pt

Formatted: Line spacing: 1.5 lines



Deleted:

Formatted: Font:9 pt

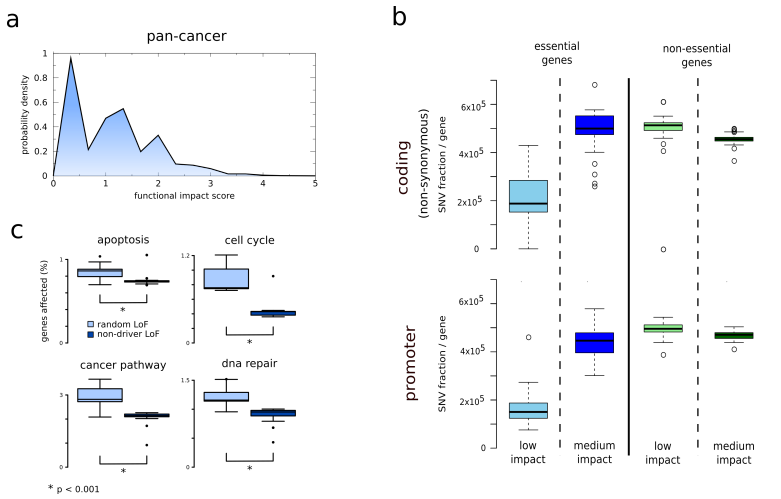


Figure 2: Functional impact scores for PCAWG SNVs: a) Functional impact distribution in noncoding region: three peaks correspond to low, medium and high impact variants. b) Fraction of impactful variants per gene in essential and non-essential gene sets: non-synonymous(top), promoter(middle) and loss-of-function(bottom).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

Formatted: Font:(Default) Times New Roman

Formatted: Font:Times New Roman, Bold

Deleted: Correlation between number

Deleted: impactful and total SNV frequencies for

Deleted: cohorts

Formatted: Font:Times New Roman, 11 pt

Formatted: Line spacing: 1.5 lines

Formatted: Font:Times New Roman, Bold

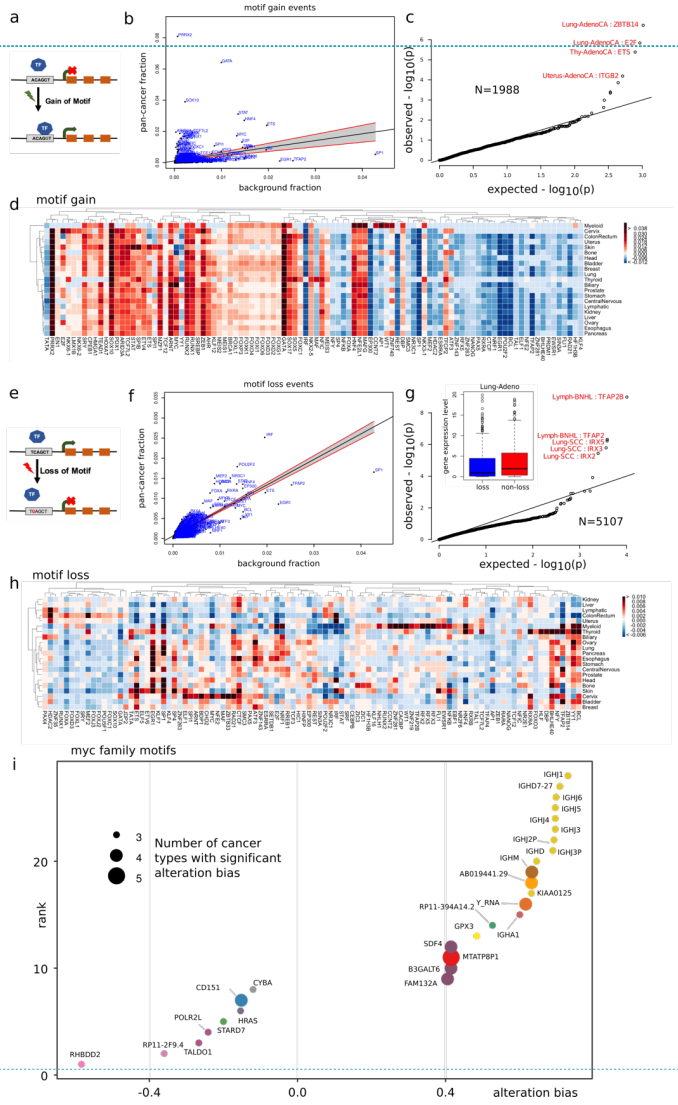


Figure 3: **Overall functional burdening of TF motifs:** ***Pan-cancer overview of TFs burdening****:* scatter plots for b) motif loss and f) motif gain events, ***Heat map presenting differential burdening of various TFs****:* SNVs leading to d) motif breaking and H) motif gain events in different cohorts 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.

Deleted: ... [5]

Formatted: Font:9 pt

Formatted: Font:(Default) Times New Roman

Formatted: Font:Times New Roman, 11 pt, Pattern: Clear (White)

Formatted: Font:Times New Roman, 11 pt, Pattern: Clear (White)

Deleted: **<sp>**

Formatted: Font:9 pt

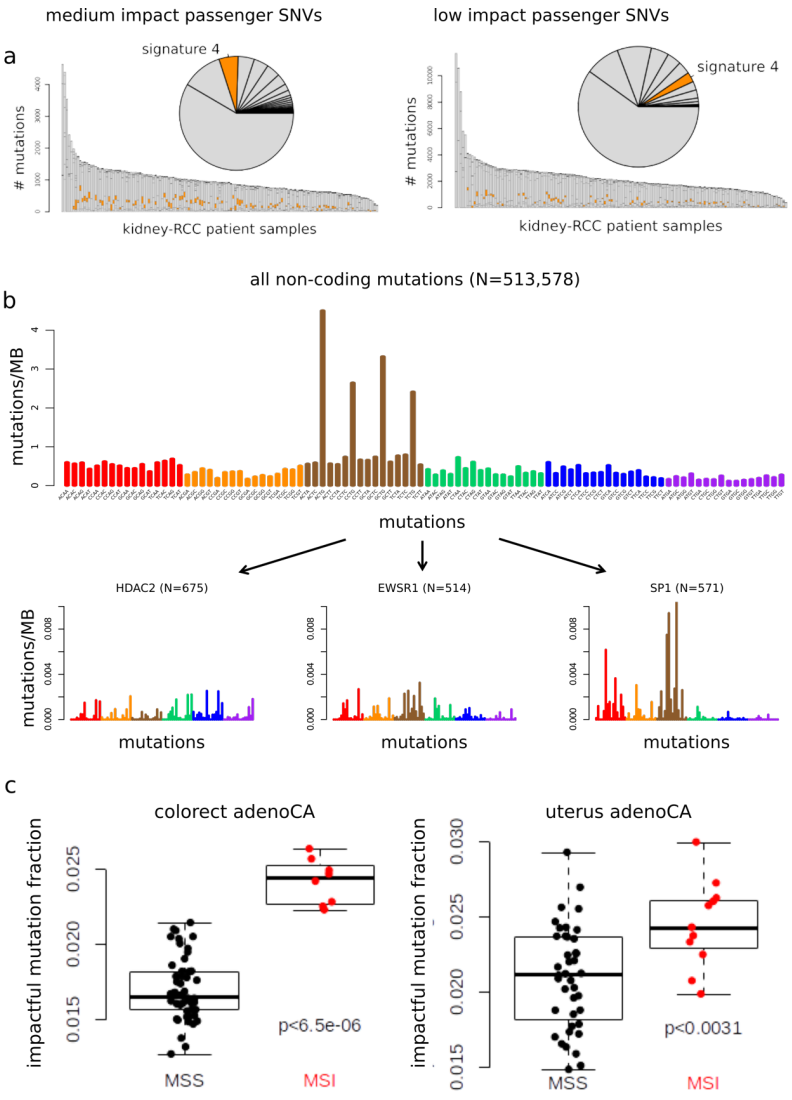


Figure 4: Mutational signatures associated with different categories of impactful variants: a) Distribution of canonical signatures in the kidney-RCC cohort for impactful (left) and low-impact SNVs (right). b) Mutation spectra associated with motif breaking events observed in HDAC2, EWSR1 and SP1 in the kidney-RCC cohort. c) fraction of impactful SNVs in MSI and MSS samples in Colorectal Adenocarcinoma(left) and Uterine Adenocarcinoma (right).

Formatted: Font:9 pt

Formatted: Font:Times New Roman, 11 pt

Formatted: Line spacing: 1.5 lines

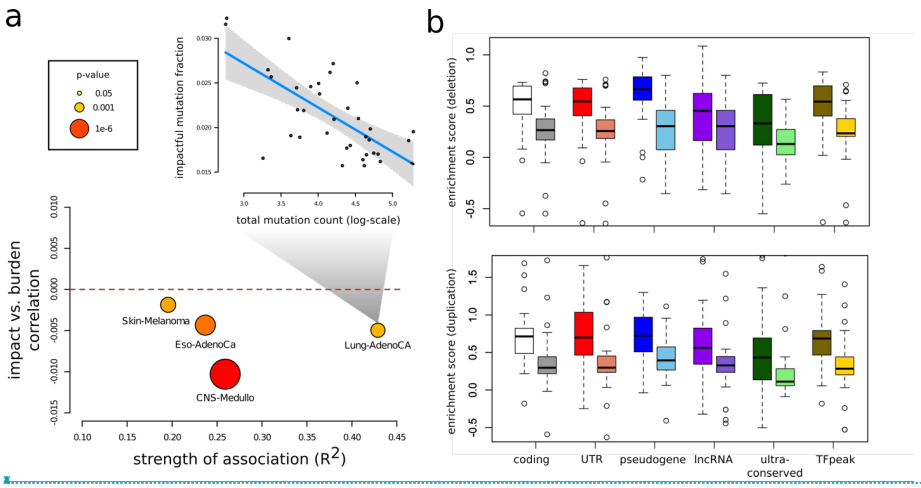


Figure 5: Overall variant impact: a) Correlation between number of impactful and total SNV frequencies for different cohorts. b) Fold enrichment score for somatic large deletions overlapping with different regions of the genome : pair of boxplot for each annotation correspond to enrichment score distribution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

Deleted: . ... [6]

Formatted: Font:9 pt

Formatted: Font:(Default) Times New Roman

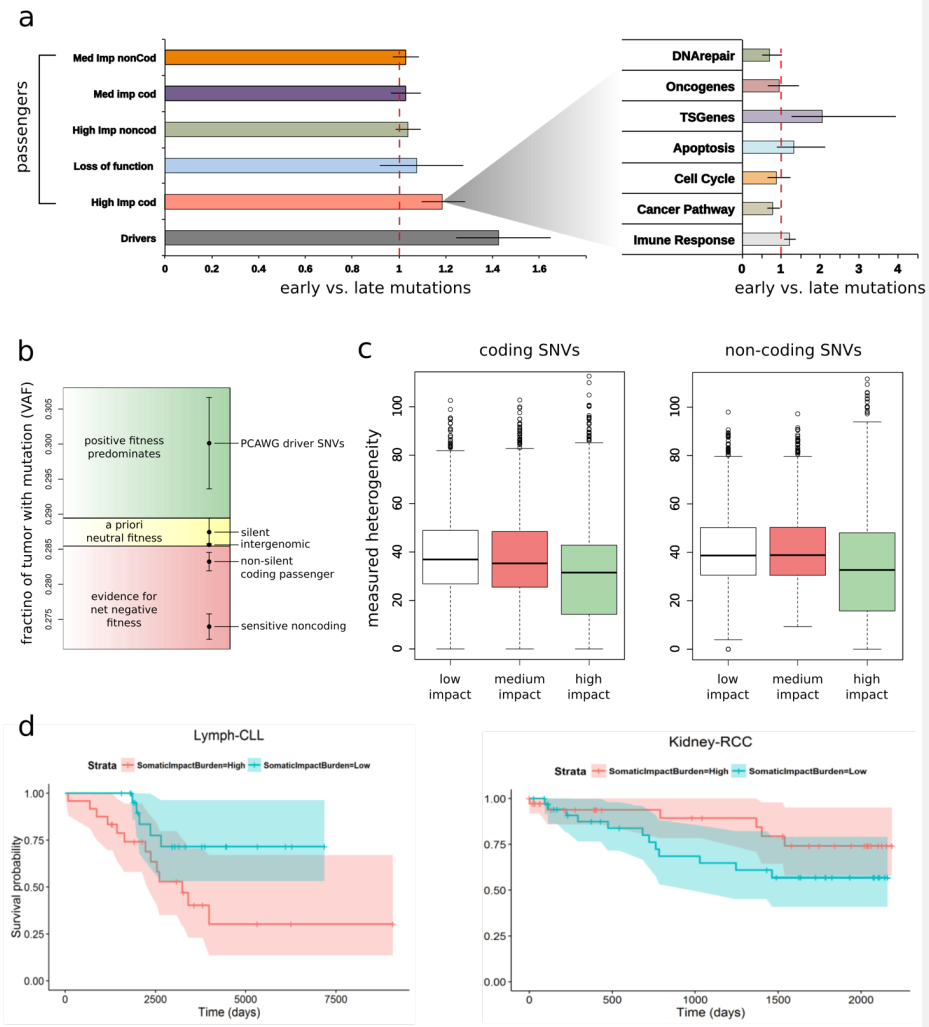


Figure 6: Correlating functional burdening with subclonal information and patient survival: a) Subclonal ratio (early/late) for different categories of SNVs (coding/non-coding) based on their impact score. Subclonal ratio for high impact SNVs occupying distinct gene sets. b) Stratifying SNVs in different 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.

Deleted: .

Formatted: Font:9 pt

Page 1: [1] Deleted	Microsoft Office User	4/2/17 12:25:00 AM

Page 2: [2] Deleted	Microsoft Office User	4/2/17 12:25:00 AM

Furthermore, integration of annotation and impact score allows for quantification of overall functional impact of variants occupying different genomic elements. We observe that disruption of regulatory elements in the noncoding genome correlates with altered gene expression. Moreover, regulatory elements are differentially affected by mutation process, as elucidated by our signature analysis. Furthermore, we found that overall functional impact correlates with age at cancer diagnosis, patient survival, and tumor clonality. Finally, the ensemble of our work provides suggestive evidence that subsets of functionally impactful passenger variants confer weak fitness effects to tumor cells.

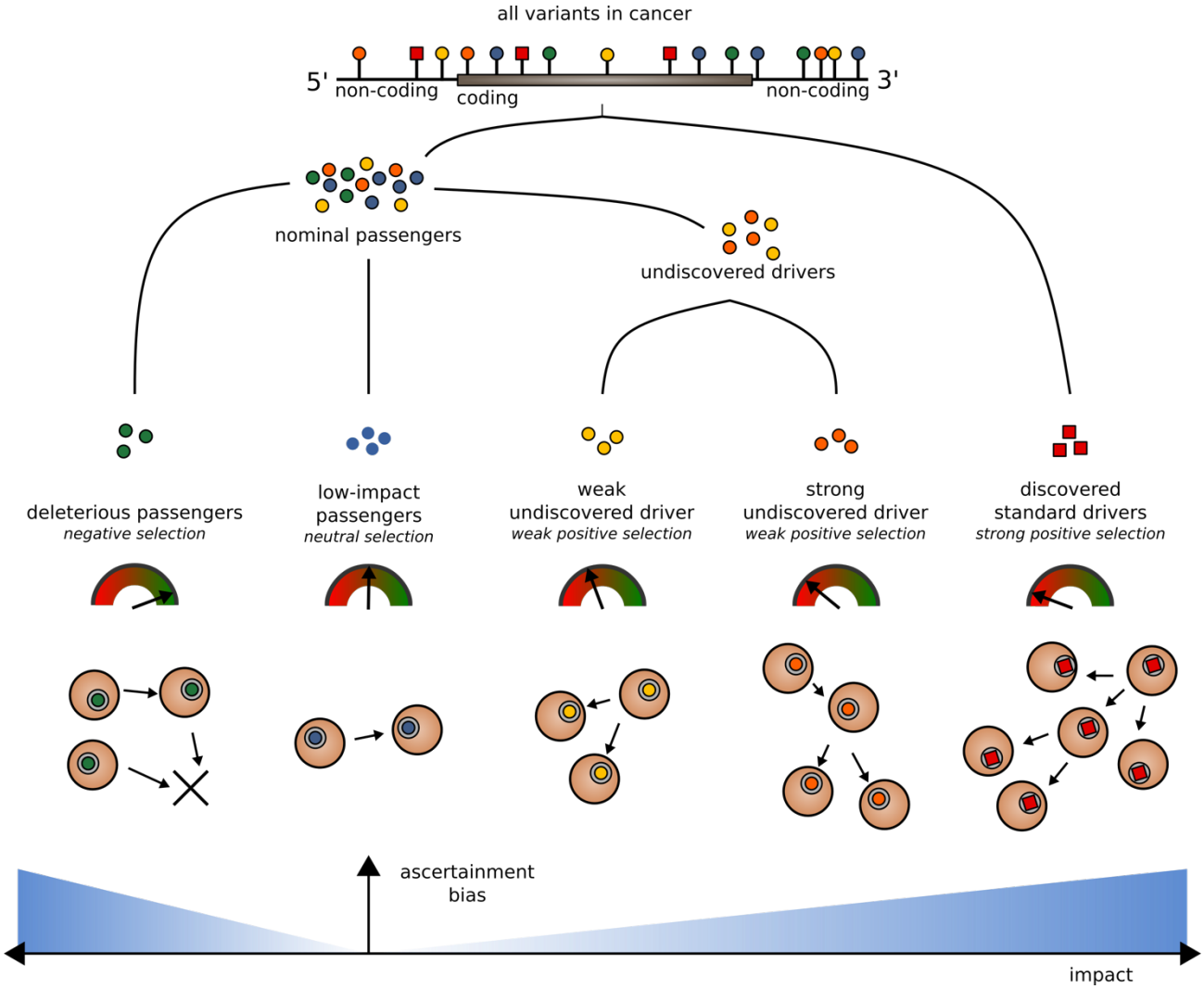
Classifying variants based on impact and underlying selection process

In a continuum model

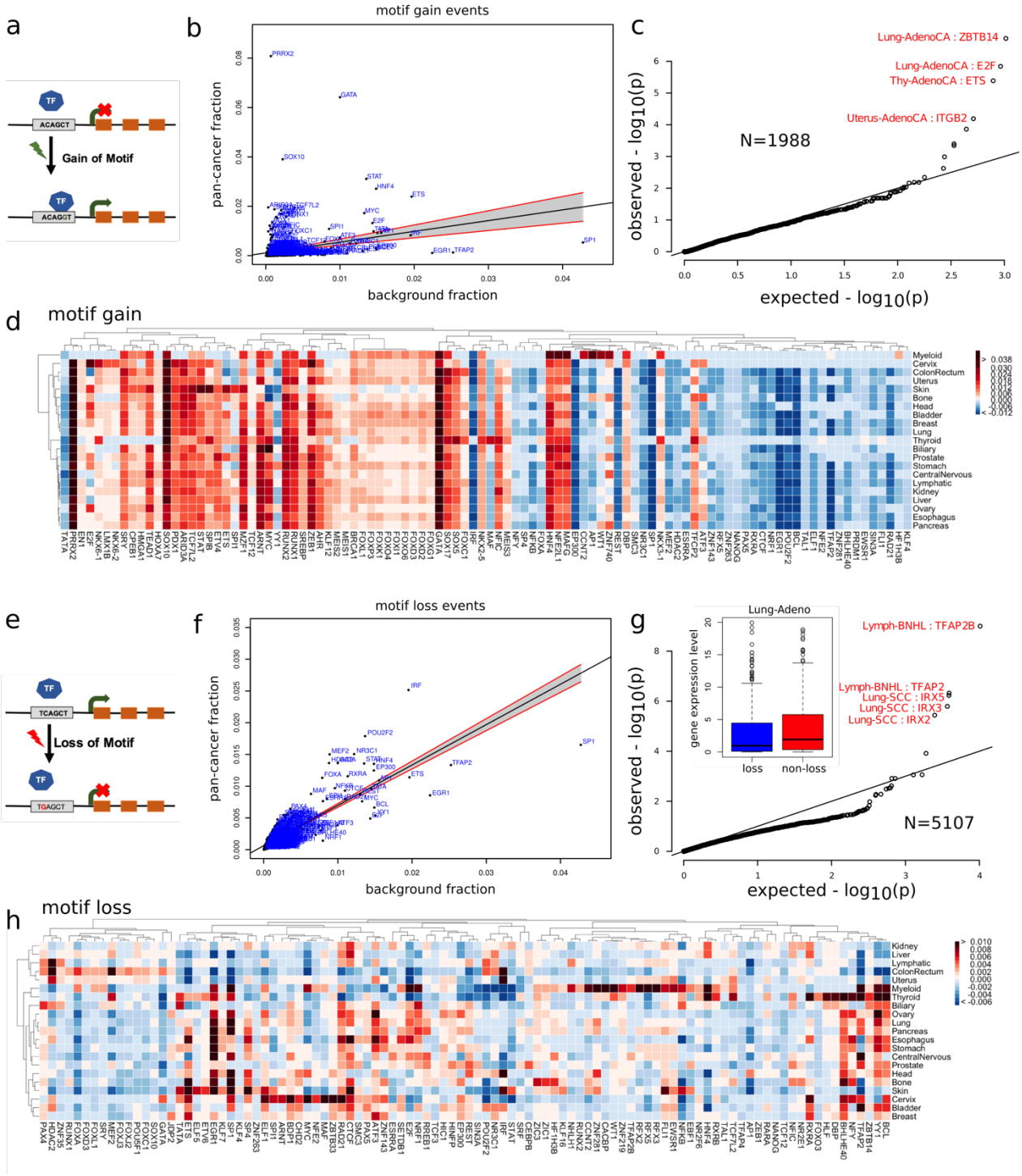
Page 6: [3] Moved to page 6 (Move #5)	Microsoft Office User	4/2/17 12:25:00 AM

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**.**

Page 9: [4] Deleted	Microsoft Office User	4/2/17 12:25:00 AM



Page 11: [5] Deleted	Microsoft Office User	4/2/17 12:25:00 AM


a
TF
ACAGCT
Gain of Motif
TF
ACAGGT
b
motif gain events
pan-cancer fraction
background fraction
PRRX2
GATA
SOX10
STAT
HNF4
ETS
MYC
E2F
SPI1
SP1
EGR1
TFAP2
c
Lung-AdenoCA : ZBTB14
Lung-AdenoCA : E2F
Thy-AdenoCA : ETS
Uterus-AdenoCA : ITGB2
N=1988
observed - log10(p)
expected - log10(p)
d
motif gain
Myeloid
Cervix
ColonRectum
Uterus
Skin
Bone
Head
Bladder
Breast
Lung
Thyroid
Biliary
Prostate
Stomach
CentralNervous
Lymphatic
Kidney
Liver
Ovary
Esophagus
Pancreas
e
TF
TCAGCT
Loss of Motif
TF
TGAGCT
f
motif loss events
pan-cancer fraction
background fraction
IRF
POU2F2
SP1
TFAP2
ETS
EGR1
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
observed - log10(p)
expected - log10(p)
h
motif loss
Kidney
Liver
Lymphatic
ColonRectum
Uterus
Myeloid
Thyroid
Biliary
Ovary
Lung
Pancreas
Esophagus
Stomach
CentralNervous
Prostate
Head
Bone
Skin
Cervix
Bladder
Breast

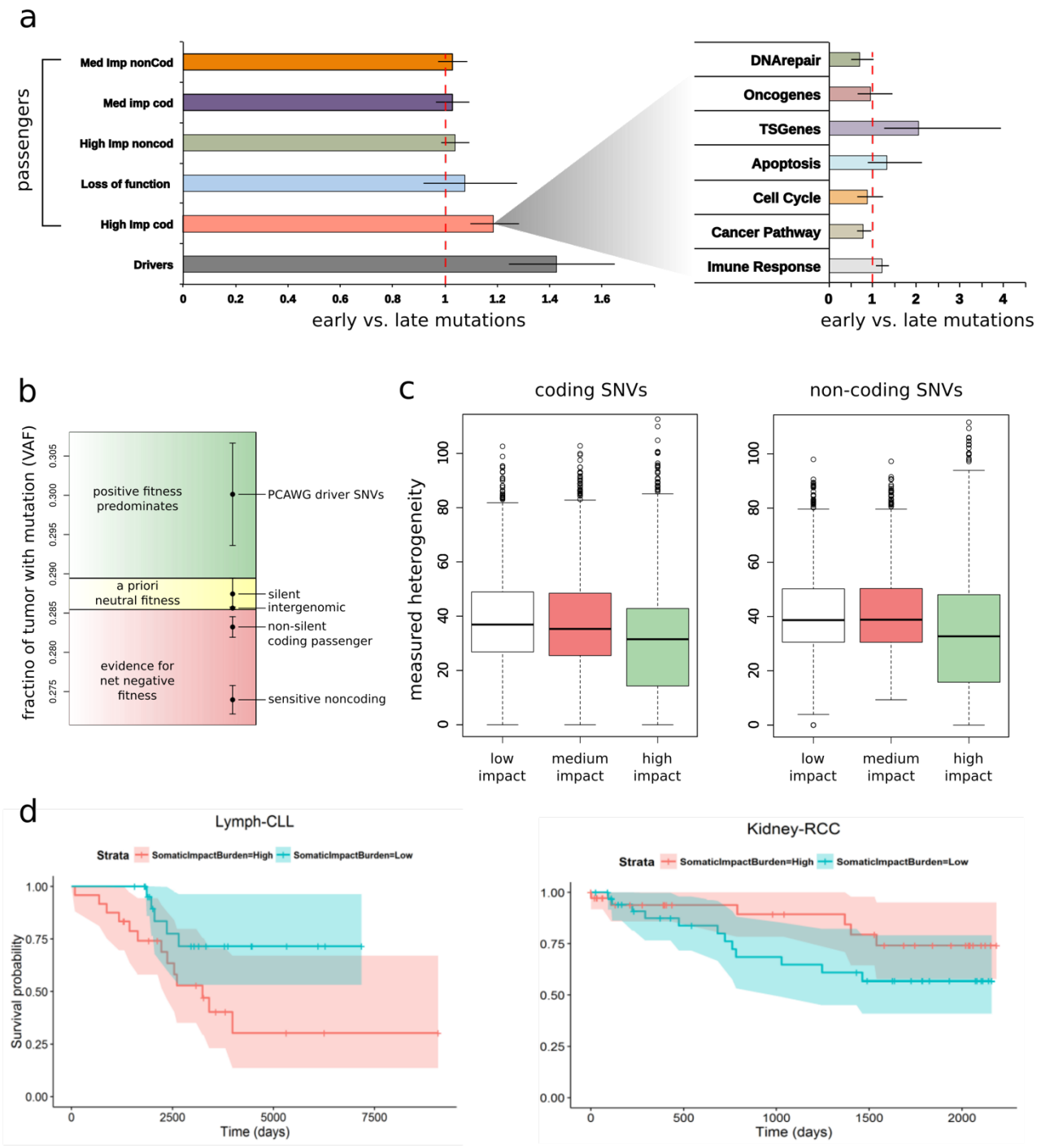

a
passengers
Med Imp nonCod
Med imp cod
High Imp noncod
Loss of function
High Imp cod
Drivers
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
early vs. late mutations
DNArepair
Oncogenes
TSGenes
Apoptosis
Cell Cycle
Cancer Pathway
Imune Response
0
1
2
3
4
early vs. late mutations
b
fractino of tumor with mutation (VAF)
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
Kidney-RCC
Strata
SomaticImpactBurden=High
SomaticImpactBurden=Low
Survival probability
1.00
0.75
0.50
0.25
0.00
0
2500
5000
7500
500
1000
1500
2000
Time (days)

Figure 5