The landscape of selection in 551 Esophageal Adenocarcinomas defines genomic biomarkers for the clinic

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Abstract:

Esophageal Adenocarcinoma (EAC) is a poor prognosis cancer type with rapidly rising incidence. Our understanding of genetic events which drive EAC development is limited and there are few molecular biomarkers for prognostication or therapeutics. We have accumulated a cohort of 551 genomically characterised EACs (73% WGS and 27% WES) with clinical annotation and matched RNA-seq. Using a variety of driver gene detection methods we discover 65 EAC drivers (66% novel) and describe mutation and CNV types with specific functional impact. We identify a mean of 3.7 driver events per case derived almost equally from copy number events and mutations. We compare driver mutation rates to the exome-wide mutational excess calculated using Non-synonymous vs Synonymous mutation rates (dNdS). We see mutual exclusivity or co-occurrence of events within and between a number of EAC pathways (GATA factors, Core Cell cycle genes, TP53 regulators and the SWI/SNF complex) suggestive of important functional relationships. These driver variants correlate with tumour differentiation, sex and prognosis. Poor prognostic indicators (SMAD4, GATA4) are verified in independent cohorts with significant predictive value. Over 50% of EACs contain sensitising events for

28 CDK4/6 inhibitors which are highly correlated with clinically relevant sensitivity in a panel EAC cell
29 lines.

30

31 ## Introduction

32 Esophageal cancer is the eighth most common form of cancer world-wide and the sixth most
33 common cause of cancer related death[1]. Esophageal Adenocarcinoma (EAC) is the predominant
34 subtype in the west, including the UK and the US. The incidence of EAC in such countries has been
35 rapidly rising, with a seven-fold increase in incidence over the last 35 years in the US[2]. EAC is a highly
36 aggressive neoplasm, usually presenting at a late stage and is generally resistant to chemotherapy,
37 leading to five-year survival rates below 15%[3]. It is characterised by very high mutation rates in
38 comparison to other cancer types[4] but also, paradoxically, there is a paucity of recurrently mutated
39 genes. EACs also display dramatic chromosomal instability and thus may be classified as a C-type
40 neoplasm which may be driven mainly by structural variation rather than mutations[5,6]. Currently our
41 understanding of precisely which genetic events drive the development of EAC is highly limited and
42 consequentially there is a paucity of molecular biomarkers for prognosis or targeted therapeutics
43 available in the clinic.

44 Driver events undergoing positive selection during cancer evolution are a small proportion
45 of total number of genetic events that occur in each tumour[7]. Methods to differentiate driver
46 mutations from passenger mutations use features associated with known driver events to detect
47 regions of the genome, often genes, in which mutations are enriched for these features[8]. The
48 simplest of these features is the tendency of a mutation to co-occur with other mutations in the
49 same gene at a high frequency, as detected by MutsigCV[9]. MutsigCV has been applied on several
50 occasions to EAC cohorts[6,10,11] and has identified ten known cancer genes as high confidence EAC
51 drivers (TP53, CDKN2A, SMAD4, ARID1A, ERBB2, KRAS, PIK3CA, SMARCA4, CTNNB1 and FBXW7).

52 However these analyses leave most EAC cases with only one known driver mutation, usually TP53,
53 due to the low frequency at which other drivers occur. Equivalent analyses in other cancer types
54 have identified three or four drivers per case[12,13]. Similarly, detection of copy number driver events
55 in EAC has relied on identifying regions of the genome recurrently deleted or amplified, as detected
56 by GISTIC[10,14-17]. However, GISTIC identifies relatively large regions of the genome, containing
57 hundreds of genes, with little indication of which specific gene-copy number aberrations (CNAs) may
58 actually confer a selective advantage. There are also several non-selection based mechanisms which
59 can cause recurrent CNAs, such as fragile sites where a low density of DNA replication origins causes
60 frequent structural events at a particular loci. These have not been differentiated properly from
61 selection based recurrent CNAs[18].

62 Without proper annotation of the genomic variants which drive the biology of EAC tumours
63 we are left with a very large number of events, most of which are likely to be inconsequential,
64 making it extremely difficult to detect statistical associations between genomic variants and various
65 biological and clinical parameters. To address these issues, we have accumulated a cohort of 551
66 genomically characterised EACs using our esophageal ICGC project, which have high quality clinical
67 annotation, associated whole genome sequencing (WGS) and RNA-seq on cases with sufficient
68 material. We have augmented our ICGC WGS cohort with publically available whole exome[19] and
69 whole genome sequencing[20] data. We have applied a number of complementary driver detection
70 tools to this cohort, using a range of driver associated features combined with analyses of RNA
71 expression to produce a comprehensive assessment and characterisation of mutations and CNAs
72 under selection in EAC. We then use these events to define functional cell processes that have been
73 selectively dysregulated in EAC and identify novel, clinically relevant biomarkers for prognostication,
74 which we have verified in independent cohorts. Finally, we have used this compendium of EAC
75 driver variants to provide an evidence base for targeted therapeutics, which we have tested *in vitro*.

76

77 # Results

78 **A Compendium of EAC driver events and their functional effects**

79 In 551 EACs we called a total of 11,813,333 single nucleotide variants (SNVs) and small insertions or
80 deletions (Indels), with a median of 6.4 such mutations / Mb (supplementary figure 1), and 286,965
81 copy number aberrations (CNAs). We also identified 134,697 structural variants (SVs) in WGS cases.
82 Mutations or copy number variants under selection were detected using specific driver associated-
83 mutation features (Fig 1A). We use several complementary driver detection tools to detect each
84 feature, and each tool underwent quality control to ensure reliability of results (see methods). These
85 features include highly recurrent mutations within a gene (dNdScv[21] and MutsigCV2[9]), high
86 functional impact mutations within a gene (OncodriveFM[22]), mutation clustering (OncodriveClust[23],
87 eDriver[24] and eDriver3D[25]) and recurrent amplification or deletion of genes (GISTIC[14]) undergoing
88 concurrent over or under-expression (see methods) (Fig 1A)[8].

89 These complementary methods produced highly significant agreement in calling EAC driver
90 genes, particularly within the same feature-type (Supplementary Figure 2) and on average more
91 than half of the genes identified by one feature were also identified by other features (Fig 1B). In
92 total sixty five EAC driver genes were discovered, 64% of which have not been detected in EAC
93 previously[10,11,15-17,19]. Of the sixty five gene identified, 82% are known drivers in pan-cancer analyses
94 giving confidence in our methods[21,26,27].

95 EAC is notable among cancer types for harbouring a high degree of chromosomal
96 instability[20]. Using GISTIC we identified 126 recurrently deleted or amplified loci across the genome
97 (Fig 2A). To determine which genes within these loci confer a selective advantage when they
98 undergo CNAs we use a subset of 119 cases with matched RNA-seq to detect genes within these loci
99 in which homozygous deletion or amplification causes a significant under or over-expression
100 respectively, a prerequisite for selection of CNAs. The majority of genes in these regions showed no

101 CN associated expression change (74%). We observed highly significant expression changes in 17
102 known cancer genes within GISTIC peaks such as ERBB2, KRAS and SMAD4 which we designate high-
103 confidence EAC drivers. We also found five tumour suppressor genes where copy number loss was
104 not necessarily associated with expression modulation but tightly associated with presence of
105 mutations leading to LOH, for example ARID1A and CDH11. To determine whether copy number
106 changes in genes not previously associated with cancer may contribute to oncogenesis we searched
107 for genes with similar expression-CN profile as most of our high-confidence drivers (see methods).
108 We found 74 such cases which we designated "candidate copy number (CN) drivers" (supplementary
109 tables 1 and 2). Several GISTIC loci contained only one candidate driver such as ZNF131, PRKCI and
110 MYBL2 which are promising candidates for further study.

111 In a subset of GISTIC loci, we observed extremely high copy number amplification,
112 commonly greater than 100 copies, and these loci were highly correlated with presence of CN-
113 drivers (Wilcox test, $p<Ex10^{-6}$) (Supplementary Figure 3). To discern a mechanism for these ultra-high
114 amplifications we assessed structural variants (SVs) associated with these events and the copy
115 number steps surrounding them. For many of these events the extreme amplification was produced
116 largely from a single copy number step the edges of which were supported by structural variants
117 with ultra-high read support. Two examples are shown in Fig2B. In the first example an inversion has
118 been followed by circularisation and amplification KRAS and in the second circularisation and
119 amplification initially occurred around MYC but subsequently incorporated ERBB2 from an entirely
120 different chromosome. A pattern of extrachromosomal amplification via double minutes has been
121 previously noted in EAC[20], and hence we refer to this amplification class with ultra-high amplification
122 (Ploidy adjusted Copy number >10) as 'extrachromosomal-like'. Several deletion loci co-align with
123 fragile sites (Fig 2A). Most deletion loci were dominated by heterozygous deletions while a small
124 subset had a far higher percentage of homozygous deletions including CDKN2A and several
125 associated with fragile site loci (Fig. 2A). For some cases we may have been unable to identify drivers
126 in loci simply because the aberrations do not occur in the smaller RNA-seq matched cohort.

127 We found extrachromosomal-like amplifications had an extreme and highly penetrant
128 effects on expression while moderate amplification (ploidy adjusted copy number > 2) and
129 homozygous deletion had highly significant (Wilcox test, $p<Ex10^{-4}$ and $p<Ex10^{-3}$ respectively) but less
130 dramatic effects on expression with a lower penetrance (Fig 2C). This lack of penetrance was
131 associated with low cellularity (fisher's exact test, expression cut off = 2.5 normalised FPKM, $p<0.01$)
132 in amplified cases but also likely reflects that genetic mechanisms other than gene-dosage can
133 modulate expression in a rearranged genome. We also detected several cases of over expression or
134 complete expression loss without associated CN changes which may reflect non-genetic mechanisms
135 for driver dysregulation. For example, one case overexpressed ERBB2 at 28-fold median expression
136 however had entirely diploid CN in and surrounding ERBB2 and a second case contained almost
137 complete loss of SMAD4 expression (0.008-fold median expression) despite possessing 5 copies of
138 SMAD4.

139

140 **Landscape of driver Events in EAC**

141 The overall landscape of driver gene mutations and copy number alterations per case is depicted in
142 Figure 3A. These comprise both oncogenes and tumour suppressor genes activated or repressed via
143 different mechanisms. Occasionally different types of events are selected for in the same gene, such
144 as KRAS which harbours both activating mutations and amplifications in 19% of cases. Passenger
145 mutations occur by chance in most driver genes. To quantify this we have used the
146 observed:expected mutation ratios (calculated by dNdScv) to estimate the percentage of driver
147 mutations in each gene and in different mutation classes. For many genes, only specific mutation
148 classes appear to be under selection. Many tumour suppressor genes; ARID2, RNF43, ARID1B for
149 example, are only under selection for truncating mutations; ie splice site, nonsense and frameshift
150 Indel mutations, but not missense mutations which are passengers. However, oncogenes, like
151 ERBB2, only contain missense drivers which form clusters to activate gene function in a specific

152 manner. Where a mutation class is <100% driver mutations, mutational clustering can help us define
153 the driver vs passenger status of a mutation (supplementary figure 4). Clusters of mutations
154 occurring in EAC or mutations on amino acids which are mutation hotspots in other cancer types[28]
155 (supplementary table 3) are indicated in figure 3A. Novel EAC drivers of particular interest include
156 B2M, a core component of the MHC class I complex and resistance marker for Immunotherapy[29],
157 MUC6 a secreted glycoprotein involved in gastric acid resistance and ABCB1 a channel pump protein
158 which is associated with multiple instances of drug resistance[30]. Lollipop plots showing primary
159 sequence distribution of mutations in these genes are provided (supplementary data).

160 The identification of driver events provides a rich information about the molecular history of
161 each EAC tumour. We detect a median of four events in driver genes per tumour (IQR = 3-6, Mean =
162 5.1) and only a very small fraction of cases have no such events detected (11 cases, 2%). When we
163 remove the predicted percentage of passenger mutations using dnds ratios we find a mean of 3.7
164 true driver events per case which derive quite evenly from both copy number events and mutations
165 (Fig 3B). dNdScv, one of the driver gene detection methods used, also analyses the genome-wide
166 excess of non-synonymous mutations based on expected mutation rates to assess the total number
167 of driver mutations across the exome which is calculated at 4.8 (95% CIs: 3.7-5.9) in comparison to
168 2.1 driver mutations which we calculate in our gene-centric analysis after passenger removal. This
169 suggests low frequency driver genes may be prevalent in the EAC mutational landscape (see
170 discussion). Further analysis suggests these missing mutations are mostly missense mutations and
171 our gene-centric analysis captures almost all predicted splice and nonsense drivers (Supplementary
172 Figure 5). Some of our methods use enrichment of nonsense and splice mutations as a marker of
173 driver genes and hence have a higher sensitivity for these mutations.

174 To better understand the functional impact of driver mutations we analysed expression of
175 driver genes with different mutation types and compared their expression to normal tissue RNA,
176 which was sequenced alongside our tumour samples (Figure 3C). Since surrounding squamous

177 epithelium is a fundamentally different tissue, from which EAC does not directly arise, we have used
178 duodenum and gastric cardia samples as gastrointestinal phenotype controls, likely to be similar to
179 the, as yet unconfirmed, tissue of origin in EAC. A large number of driver genes have upregulated
180 expression in comparison to normal controls, for example TP53 has upregulated RNA expression in
181 WT tumour tissue and in cases with missense (non-truncating) mutations but RNA expression is lost
182 upon gene truncation. In depth analysis of different TP53 mutation types reveals significant
183 heterogeneity within non-truncating mutations, for example R175H mutations correlate with low
184 RNA expression (supplementary figure 6). Normal tissue expression of CDKN2A suggests that
185 CDKN2A is generally activated in EAC and returns to physiologically normal levels when deleted.
186 Heterogeneous expression in WT CDKN2A cases suggest a different mechanism of inhibition such as
187 methylation in some cases. Overexpression of other genes in wild type tumours, such as SIN3A, may
188 be a confer selective advantage due to their oncogenic properties, in this case cooperating with
189 MYC, which is also overexpressed in EACs (Fig 3C). A smaller number of driver genes are
190 downregulated in EAC tissue- 3/4 of these (GATA4, GATA6 and MUC6) are involved in the
191 differentiated phenotype of gastrointestinal tissues and may be lost with tumour de-differentiation.
192 Driving alterations in these genes have been observed in other GI cancers[13,31,32] however their
193 oncogenic mechanism is unknown. In most genes we did not observe expression loss at the RNA
194 level with truncation, for instance ARID1A (supplementary figure 7).

195

196 **Dysregulation of specific pathways and processes in EAC**

197 It is known that selection preferentially dysregulates certain functionally related groups of genes and
198 biological pathways in cancer[33]. This phenomenon is highly evident in EAC, as shown in Figure 4
199 which depicts the functional relationships between EAC drivers. This provides greater functional
200 homogeneity to the landscape of driver events.

201 While TP53 is the dominant driver in EAC, 30% of cases remain TP53 wildtype. MDM2 is a E3
202 ubiquitin ligase that targets TP53 for degradation. Its selective amplification and overexpression is
203 mutually exclusive with TP53 mutation suggesting it can functionally substitute the effect of TP53
204 mutation via its degradation. Similar mutually exclusive relationships are observed between; KRAS
205 and ERBB2, GATA4 and GATA6 and Cyclin genes (CCNE1, CCND1 and CCND3). Activation of the Wnt
206 pathway occurs in 19% of cases either by mutation of phospho-residues at the N terminus of β-
207 catenin, which prevent degradation, or loss of Wnt destruction complex components like APC. Many
208 different chromatin modifying genes, often belonging to the SWI/SNF complex, are also selectively
209 mutated (31% of cases). In contrast SWI/SNF genes are co-mutated significantly more often than we
210 would expect by chance (fisher's exact test, $p<0.01$ see methods), suggesting an increased advantage
211 to further mutations once one has been acquired. We also assessed mutual exclusivity and co-
212 occurrence in genes in different pathways and between pathways themselves (Figure 4B). Of
213 particular note are co-occurring relationships between TP53 and MYC, GATA6 and SMAD4, Wnt and
214 Immune pathways as well as mutually exclusive relationships between ARID1A and MYC,
215 gastrointestinal (GI) differentiation and RTK pathways and SWI-SNF and DNA-Damage response
216 pathways. We were able to confirm some of these relationships in independent cohorts in different
217 cancer types (supplementary table 4) suggesting some of these may be pan-cancer phenomenon. As
218 shown in figure 3, all of these pathways interact to stimulate the G1 to S phase transition of the cell
219 cycle via promoting phosphorylation of Rb, although many of these pathways have multiple
220 oncogenic or tumour suppressive functions.

221 A number of other driver genes have highly related functional roles including core
222 transcriptional components (TAF1 and POLQ), drivers of immune escape (JAK1 and B2M[29]), cell
223 adhesion receptors (CDH1, CHDL and PCDH17), core ribosome components (ELF3 and RPL22), core
224 RNA processing components (GPATCH8 and COIL), ion channels (KCNQ3 and TRPA1) and Ephrin
225 type-A receptors (EPHA2 and EPHA3).

226 **Clinical significance of driver variants**

227 Events undergoing selection during cancer evolution influence tumour biology and thus impact
228 tumour aggressiveness, response to treatment and patient prognosis as well as other clinical
229 parameters. Clinical-genomic correlations can provide useful biomarkers but also give insights into
230 the biology of these events.

231 Univariate Cox regression was performed for events in each driver gene with driver events
232 occurring in greater than 5% of EACs (ie after removal of predicted passengers, 16 genes) to detect
233 prognostic biomarkers (Fig 5A). Events in two genes conferred significantly poorer prognosis after
234 multiple hypothesis correction, GATA4 (HR : 0.54 , 95% CI : 0.38 – 0.78, *P* value = 0.0008) and SMAD4
235 (HR : 0.60 , 95% CI : 0.42 – 0.84, *P* value = 0.003). Both genes remained significant in multivariate Cox
236 regression including pathological tumour stage (GATA4 = HR adjusted : 0.63, 95% CIs adjusted : 0.40
237 - 0.98, *P* value = 0.042 and SMAD4 = HR adjusted : 0.63, 95% CI adjusted : 0.41 – 0.97, *P* value =
238 0.038). 31% of EACs contain either SMAD4 mutation or homozygous deletion or GATA4 amplification
239 and cases with both genes altered had a poorer prognosis (figure 5B). We validated the poor
240 prognostic impact of SMAD4 events in an independent TCGA gastroesophageal cohort (HR = 0.58,
241 95% CI = 0.37 – 0.90, *P* value =0.014) (Fig 5C) and we also found GATA4 amplifications were
242 prognostic in a cohort of TCGA pancreatic cancers (HR = 0.38 95% CI: 0.18 – 0.80, *P* value = 0.011)
243 (Fig 5D), the only available cohort containing a feasible number of GATA4 amplifications. The
244 prognostic impact of GATA4 has been suggested in previously published independent EAC cohort[16]
245 although it did not reach statistical significance after FDR correction and SMAD4 expression loss has
246 been previously linked to poor prognosis in EAC[34]. We also noted stark survival differences between
247 cases with SMAD4 events and cases in which TGFβ receptors were mutated (Fig 5E, HR = 5.6, 95% CI
248 : 1.7 – 18.2, *P* value = 0.005) in keeping with the biology of the TGFβ pathway where non-SMAD
249 TGFβ signalling is known to be oncogenic[35].

250 In additional to survival analyses we also assessed driver gene events for correlation with
251 various other clinical factors including differentiation status, sex, age and treatment response. We
252 found Wnt pathway mutations had a strong association with well differentiated tumours (p=0.001,
253 OR = 2.9, fisher's test, see methods, Fig 5F). We noted interesting differences between female
254 (n=81) and male (n=470) cases. Female cases were enriched for KRAS mutation (p = 0.001, fisher's
255 exact test) and TP53 wildtype status (p = 0.006, fisher's exact test) (Fig 5G). This is of particular
256 interest given the male predominance of EAC[3].

257

258 **Targeted therapeutics using EAC driver events.**

259 The biological distinctions between normal and cancer cells provided by driver events can be used to
260 derive clinical strategies for selective cancer cell killing. To investigate whether the driver events in
261 particular genes and/or pathways might sensitise EAC cells to certain targeted therapeutic agents
262 we used the Cancer Biomarkers database[36]. We calculated the percentage of our cases which
263 contain EAC-driver biomarkers of response to each drug class in the database (summary shown Fig
264 6A, and full data supplementary table 5). Aside from TP53, which has been problematic to target
265 clinically so far, we found a number of drugs with predicted sensitivity in >10% of EACs including
266 EZH2 inhibitors for SWI/SNF mutant cancers (23% and 33% including other SWI/SNF EAC
267 drivers), and BET inhibitors which target KRAS activated and MYC amplified cases (25%). However,
268 by far the most significantly effective drug was predicted to be CDK4/6 inhibitors where >50% of
269 cases harboured sensitivity causing events in the receptor tyrosine kinase (RTK) and core cell cycle
270 pathways (eg in CCND1, CCND3 and KRAS).

271 To verify that these driver events would also sensitise EAC tumours to such inhibitors we
272 used a panel of eight EAC cell lines which have undergone whole genome sequencing[37] and assessed
273 them for presence of EAC driver events (Figure 6B). The mutational landscape of these lines was
274 broadly representative of EAC tumours. We found that the presence of cell cycle and or RTK

275 activating driver events was highly correlated with response to two FDA approved CDK4/6 inhibitors,
276 Ribociclib and Palbociclib and several cell lines were sensitive below maximum tolerated blood
277 concentrations in humans (Figure 6B, supplementary table 6, Supplementary Figure 8)[38]. Such EAC
278 cell lines had comparable sensitivity to T47D which is derived from an ER +ve breast cancer where
279 CDK4/6is have been FDA approved. We noted three cell lines without sensitising events which were
280 highly resistant, with little drug effect even at 4000nM concentrations, similar to a known Rb mutant
281 resistant line breast cancer cell line (MDA-MB-468). Two of these three cell lines harbour
282 amplification of CCNE1 which is known to drive resistance to CDK4/6i by bypassing CDK4/6 and
283 causing Rb phosphorylation via CDK2 activation[39].

284

285 **Discussion**

286 We present here a detailed catalogue of events that have been selected for during the evolution of
287 esophageal adenocarcinoma. These events have been characterised in terms of their relative impact,
288 related functions, mutual exclusivity and co-occurrence and expression in comparison to normal
289 tissues, producing insights into EAC biology. We have used this set of biologically important gene
290 alterations to identify prognostic biomarkers and actionable genomic events for personalised
291 medicine.

292 While clinical annotation and matched RNA data is a strength of this study, in some cases we
293 may have been unable to assess selected variants for survival associations or expression changes
294 which were detected in the full 551 cohort, due to lack of representation in clinically annotated or
295 RNA matched sub cohorts. Despite rigorous analyses to detect selected events, assessment of the
296 global excess of mutations by dNdScv suggests we are unable to detect all events selected in EAC,
297 similar to many other cancer types[21]. All driver gene detection methods which we have used rely on
298 driver mutation re-occurrence in a gene to some degree. Many of these undetected driver

299 mutations are hence likely to be spread across a large number of genes whereby each is mutated at
300 low frequency across EAC patients. This tendency for low frequency EAC drivers may be responsible
301 for the low yield of MutsigCV in previous cohorts and may suggests that C-type cancers such as EAC,
302 are not less 'mutation-driven' than M-type cancers but rather that their mutational drivers are
303 spread across a larger number of genes[5]. The identification of these very low frequency mutations
304 will require substantially different detection techniques to those which are currently in wide spread
305 use and such methods are in development[40] although they require validation. Undoubtedly many
306 copy drivers are also left undiscovered and validation of candidates identified here is an important
307 avenue of future work.

308 While a number of previous reports have attempted to detect EAC drivers, they have had a
309 limited yield per case for a variety of reasons. The first such study[19] used methods which, despite
310 being well regarded at the time, were subsequently discredited[9]. Hence a number of known false
311 positive genes (EYS, SYNE1 and CNTTAP5) were erroneously reported as drivers, along with an
312 additional unknown number of genes. Since then a number of reports, including our own, on
313 medium and large cohort sizes using MutsigCV[10,11,17] were only able to detect a small number of
314 mutational driver genes (7, 5 and 15 in each study). By using both a large cohort and more
315 comprehensive methodologies we have significantly increased this figure to 52 mutational driver
316 genes (excluding CN drivers). Detection of driver CNAs has previously relied on GISTIC to detect
317 recurrently mutated regions[10,14-17] but no analyses have been performed to evidence which genes in
318 these large regions are true drivers. Many of the genes annotated by such papers are unlikely to be
319 CN drivers from this analysis due to their lack of expression modulation with CNAs (eg YEATS4 and
320 MCL1), the role of recurrent heterozygous losses to drive LOH in some mutational drivers (ARID1A
321 and CDH11) or their association with fragile sites (PDE4D, WWOX, FHIT). Conversely, we have been
322 able to identify novel EAC copy number drivers (eg CCND3, AXIN1 and APC).

323 A number of discoveries made in this work require further investigation. Functional
324 characterisation of many of the driver genes described is needed to understand why they are
325 advantageous to EAC tumours and how they modify EAC biology. Particularly interesting are the GI
326 specific genes GATA4/6 and MUC6 which modulate prognosis and have expression loss during the
327 transition from normal to tumour tissue. Biological pathways and processes that are selectively
328 dysregulated deserve particular attention in this regard as do the gene pairs or groups with mutually
329 exclusive or co-occurring relationships such as MYC and TP53 or SWI/SNF factors, suggestive of
330 particular functional relationships. Prospective clinical work to verify and implement SMAD4 and
331 GATA4 biomarkers in this study would be worthwhile. While whole genome or whole exome
332 sequencing may be impractical for use in the clinic, targeted NGS panels to detect mutations and
333 copy number alterations have been implemented to detect genomic biomarkers in a cost effective
334 and sensitive manner for some cancer types[41]. In EAC development of a customised panel is likely to
335 be required on the basis of this analysis. A number of targeted therapeutics may provide clinic
336 benefit to EAC cases based on their individual genomic profile. In particular CDK4/6 inhibitors
337 deserve considerable attention as an option for EAC treatment as they are, by a significant margin,
338 the treatment to which the most EACs harbour sensitivity-causing driver events, excluding TP53 as
339 an unlikely therapeutic biomarker. The in vitro validation of these biomarkers for CDK4/6 inhibitors
340 in EAC is also persuasive of possible clinical benefit using a targeted approach.
341 In summary this work provides a detailed compendium of mutations and copy number
342 alterations undergoing selection in EAC which have functional and clinical impact on tumour
343 behaviour. This comprehensive study provides us with useful insights into the nature of EAC tumours
344 and should pave the way for evidence based clinical trials in this poor prognosis disease.

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346

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401

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403 RCF and AMF conceived the overall study. AMF and SJ analysed the data and performed statistical
404 analyses. RCF and AMF designed the experiments. AMF and JM performed the experiments. GC
405 contributed to the Structural variant analysis and data visualisation. SK helped compile the clinical
406 data and aided statistical analyses. JP and SA produced and QC'ed the RNA-seq data. SM and NG
407 coordinated the clinical centres and were responsible for sample collections. MO led the
408 pathological sample QC for sequencing. LB and GD ran variant calling pipelines. RCF and ST
409 supervised the research. RCF and ST obtained funding. AMF and RCF wrote the manuscript. All
410 authors approved the manuscript.

411

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413

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417 of this data allowed augmentation of our ICGC cohort and a greater sensitivity for the detection of
418 EAC driver genes.

419

420 # Figure Legends:

421 **Figure 1 Detection of EAC driver Genes. a.** Types of driver-associated features used to detect
422 positive selection in mutations and copy number events with examples of genes containing such
423 features **b.** Driver genes identified and their driver-associated features.

424

425 **Figure 2. Copy number variation under positive selection. a.** Recurrent copy number changes across
426 the genome identified by GISTIC. Frequency of different CNV types are indicated as well as the position
427 of CNV high confidence driver genes and candidate driver genes. The q value for expression correlation
428 with amplification and homozygous deletion is shown for each gene within each amplification and
429 deletion peaks respectively and occasions of significant association between LOH and mutation are
430 indicated in green. Purple deletion peaks indicate fragile sites. **b.** Examples of Extrachromosomal-like
431 amplifications suggested by very high read support SVs at the boundaries of highly amplified regions
432 produced from a single copy number step. In the first example (bi) two populations of
433 extrachromosomal DNA are apparent (biii), one amplifying only MYC and the second also
434 incorporating ERBB2 from a different chromosome. In the second example (bii) an inversion has
435 occurred before circularization and amplification around KRAS (biv). **c.** Relationship between copy
436 number and expression in CN driver genes.

437

438 **Figure 3. The driver gene landscape of Esophageal Adenocarcinoma. a.** Driver mutations or CNVs are
439 shown for each patient. Amplification is defined as >2 Copy number adjusted ploidy (2 x ploidy) of that
440 case and extrachromosomal amplification as >10Copy number adjusted ploidy (10 x ploidy) for that
441 case. Driver associated features for each driver gene are displayed to the left. On the right the
442 percentages of different mutation and copy number changes are displayed, differentiating between
443 driver and passenger mutations using dNdScv, and the % of predicted drivers by mutation type is
444 shown. Above the plot are the number of driver mutations per sample with an indication of the mean

445 (red line). **b.** Assessment of driver event types per case and comparison to exome-wide excess of
446 mutations generated by dNdScv. **c.** Expression changes in EAC driver genes in comparison to normal
447 intestinal tissues. Genes with expression changes of note are shown**.**
448
449 **Figure 4. Biological pathways undergoing selective dysregulation in EAC. a.** Biological Pathways
450 dysregulated by driver gene mutation and/or CNVs. WT cases for a pathway are not shown. Inter
451 and intra-pathway interactions are described and mutual exclusivities and/or associations between
452 genes in a pathway are annotated. GATA4/6 amplifications have a mutually exclusive relationship
453 although this does not reach statistical significance (fisher's exact test p=0.07 OR =0.52). **b.** Pairwise
454 assessment of mutual exclusivity and association in EAC driver genes and pathways.
455
456 **Figure 5. Clinical significance of Driver events in EAC. a.** Hazard rations and 95% confidence
457 intervals for Cox regression analysis across all drivers genes with at least a 5% frequency of driver
458 alterations * = q < 0.05 after BH adjustment. **b.** Kaplan-Meier curves for EACs with different status of
459 significant prognostic indicators (GATA4 and SMAD4). **c.** Kaplan-Meier curves for different
460 alterations in the TGFbeta pathway. **d.** Kaplan-Meier curves showing verification GATA4 prognostic
461 value in GI cancers using a pancreatic TCGA cohort**. e.** Kaplan-Meier curves showing verification
462 SMAD4 prognostic value in Gastroesophageal cancers using a gastroesophageal TCGA cohort. **f.**
463 Differentiation bias in tumours containing events in Wnt pathway driver genes**. g.** Relative frequency
464 of KRAS mutations and TP53 mutations driver gene events in females vs males (fishers exact test).
465
466 **Figure 6. CDK4/6 inhibitors utility in EAC. a.** Drug classes for which sensitivity is indicated by EAC
467 driver genes with data from the Cancer Biomarkers database[36]**. b.** Area under the curve (AUC) of
468 sensitivity is shown in a panel of 8 EAC cell lines with associated WGS and driver event, based in
469 tumour analysis, in these cell lines indicated. Also AUC is shown for two control lines T47D, an ER

+ve breast cancer line (+ve control) and MDA-MB-468 a Rb negative breast cancer (-ve control). *CCNE1 is a known marker of resistance to CDK4/6is due to its regulation of Rb downstream of CDK4/6 hence bypassing the need for CDK4/6 activity (see figure 5).

Supplementary figure legends

Supplementary figure 1. Distribution of small scale mutations (SNVs and Indels) across the 551 EAC cohort. Red line indicates the median mutations per case (6.4)

Supplementary Figure 2. Concordance between driver gene detection methods. A. Hierarchical clustering between tools based on gene identified**. B** Genes identified by each tool**.**

S**upplementary Figure 3.** Frequency of Extrachromosomal like events (CN adjusted Ploidy >10) in GISTIC amplification peaks and presence of high confidence drivers in those peaks indicated**.**

Supplementary Figure 4. A scheme demonstrating how to use mutational clustering along with dnds ratios to estimate the probability of a particular mutation being a driver. In this case the dnds ratio suggests 2/3 of missense mutations are drivers hence 10/15. 7 missense mutation lie in a mutational cluster, in this case of known significance in the N-terminal of B-Catenin, making it likely that these are drivers and a most (5/7) other mutations are passengers. Similarly, mutations on amino acids known to be hyper mutated in other cancer types (see Supplementary table 3, for instance if we found a single KRAS G12 mutation) can be considered likely drivers.

492 **Supplementary Figure 5.** A detailed breakdown of mutation and copy number types per case and a
493 breakdown of exome wide dnds excess for different mutation types (note that exome wide indel
494 cannot be calculated excess as they have no synonymous mutation equivalent, although a null
495 model is used in the per gene dnds method to use them to detect driver genes).

496

497 **Supplementary Figure 6.** TP53 expression in different TP53 mutation types in comparison to TP53
498 WT tumours and normal duodenum and gastric cardia tissues.

499

500 **Supplementary Figure 7.** Expression of all EAC driver genes across different genomic states for the
501 gene in question in 119 EAC tumours, and in comparison to duodenum and gastric cardia tissues.

502

503 **Supplementary Figure 8.** Growth inhibition responses of EAC cell lines and control lines to CDK4/6
504 inhibitors Palbociclib and Ribociclib.

505

506 Methods

507 **Cohort, sequencing and calling of genomic events**

508 380 cases (69%) of our EAC cohort were derived from the esophageal adenocarcinoma WGS ICGC
509 study, for which samples are collected through the UK wide OCCAMS (Oesophageal Cancer
510 Classification and Molecular Stratification) consortium. The procedures for obtaining the samples,
511 quality control processes, extractions and whole genome sequencing are as previously described[17].
512 Strict pathology consensus review was observed for these samples with a 70% cellularity
513 requirement before inclusion. Comprehensive clinical information was available for the ICGC-

514 OCCAMS cases. In addition, previously published samples were included in the analysis from Dulak
515 et al 2013[19] – 139 WES and 10 WGS (total 27%) and Nones et al 2014[20] with 22 WGS samples (4%) to
516 total 551 genome characterised EACs. RNA-seq data was available from our ICGC WGS samples
517 (119/380). BAM files for all samples (include those from Dulak et al 2013 and Nones et al 2014) were
518 run through our alignment (BWA-MEM), mutation (Strelka) and copy number (ASCAT) and structural
519 variant (Manta) calling pipelines, as previously described[17]. Our methods were benchmarked against
520 various other available methods and have among the best sensitivity and specificity for variant
521 calling (ICGC benchmarking excerise[42]). Mutation and copy number calling on cell lines was
522 performed as previously described[37].

523 Total RNA was extracted using All Prep DNA/RNA kit from Qiagen and the quality was checked on
524 Agilent 2100 Bioanalyzer using RNA 6000 nano kit (Agilent). Qubit High sensitivity RNA assay kit from
525 thermo fisher was used for quantification. Libraries were prepared from 250ng RNA, using TruSeq
526 Stranded Total RNA Library Prep Gold (Ribo-zero) kit and ribosomal RNA (nuclear, cytoplasmic and
527 mitochondrial rRNA) was depleted, whereby biotinylated probes selectively bind to ribosomal RNA
528 molecules forming probe-rRNA hybrids. These hybrids were pulled down using magnetic beads and
529 rRNA depleted total RNA was reverse transcribed. The libraries were prepared according to Illumina
530 protocol[43]. Paired end 75bp sequencing on HiSeq4000 generated the paired end reads.

531

532 **Analysing EAC mutations for selection**

533 To detect positively selected mutations in our EAC cohort, a multi-tool approach across various
534 selection related 'Features' (Recurrance, Functional impact, Clustering) was implemented in order to
535 provide a comprehensive analysis. This is broadly similar to several previous approaches[8,44].
536 dNdScv[21], MutsigCV[9], e-Driver[24] and e-Driver3D[25] were run using the default parameters. To run
537 OncodriverFM[22], Polyphen[45] and SIFT[46] were used to score the functional impact of each missense
538 non-synonomous mutation (from 0, non-impactful to 1 highly impactful), synonymous mutation

539 were given a score of 0 impact and truncating mutations (Non-sense and frameshift mutations) were
540 given a score of 1. Any gene with less than 7 mutations, unlikely to contain detectable drivers using
541 this method, was not considered to decrease the false discovery rate. OncodriveClust was run using
542 a minimum cluster distance of 3, minimum number of mutations for a gene to be considered of 7
543 and with a stringent probability cut off to find cluster seeds of $p = Ex10^{-13}$ to prevent infiltration of
544 large numbers of, likely, false positive genes. For all tool outputs we undertook quality control
545 including Q-Q plots to ensure no tool produces inflated q-values and each tool produced at least
546 30% known cancer genes. Two tools were removed from the analysis due to failure for both of these
547 parameters at quality control (Activedriver[47] and Hotspot[28]). For three of the QC-approved tools
548 (dNdScv, OncodriveFM, MutsigCV) where this was possible we also undertook an additional fdr
549 reducing analysis by re-calculating q values based on analysis of known cancer genes only[21,26,27] as
550 has been previously implemented[21,48]. Tool outputs were then put through various filters to remove
551 any further possible false positive genes. Specifically, genes where <50% of EAC cases had no
552 expression (TPM<0.1) in our matched RNA-seq cohort were removed and, using dNdScv, genes with
553 no significant mutation excess (observed: expected ratio > 2:1) of any single mutation type were also
554 removed (8 genes). We also removed two (MT-MD2, MT-MD4) mitochondrial genes which were
555 highly enriched for truncating mutations and were frequently called in OncodriveFM as well as other
556 tools. This is may be due to the different mutational dynamics, caused by ROS from the
557 mitochondrial electron transport chain, and the high number of mitochondrial genomes per cell
558 which enables significantly more heterogeneity. These factors prevent the tools used from
559 calculating an accurate null model for these genes however they may be worthy of functional
560 investigation.

561

562

563

564 **Detecting selection in CNVs**

565 ASCAT raw CN values were used to detected frequently deleted or amplified regions of the genome
566 using GISTIC2.0[14]. To determine which genes in these regions confer a selective advantage, CNVs
567 from each gene within a GISTIC identified loci were correlated with FPKM from matched RNA-seq in
568 a sub-cohort of 119 samples and with mutations across all 551 samples. To call copy number in
569 genes which spanned multiple copy number segments in ASCAT we considered the total number of
570 full copies of the gene (ie the lowest total copy number). Occasionally ASCAT is unable to confidently
571 call the copy number in a highly aberrant genomic regions. We found that the expression of genes in
572 such regions matched well what we would expect given the surrounding copy number and hence we
573 used the mean of the two adjacent copy number fragments to call copy number in the gene in
574 question. We found amplification peak regions identified by GISTIC2.0 varied significantly in precise
575 location both in analysis of different sub-cohorts and when comparing to published GISTIC data from
576 EACs[10,15,16]. A peak would often sit next to but not overlapping a well characterised oncogene or
577 tumour suppressor. To account for this, we widened the amplification peak sizes upstream and
578 downstream by twice the size of each peak to ensure we captured all possible drivers. Our
579 expression analysis allows us to then remove false positives from this wider region and called drivers
580 were still highly enriched for genes closer to the centre of GISTIC peak regions.

581 To detect genes in which amplification correlated with increased expression we compared
582 expression of samples with a high CN for that gene (top 25% percentile of CN) with those which have
583 a normal CN (median +/- 1) using the Wilcox rank-sum test and using the specific alternative
584 hypothesis that high CN would lead to increased expression. Q-values were then generated based on
585 Benjamini & Hochberg method, not considering genes without significant expression in amplified
586 samples (at least 80% amplified samples with FPKM > 1) and considering q<0.001 as significant. We
587 also included an additional known driver gene only FDR reduction analysis as previously described
588 for mutational drivers with q<0.05 considered as significant given the additional evidence for these

589 genes in other cancer types. We took the same approach to detect genes in which homozygous
590 deletion correlated with expression loss. Expression modulation was a highly specific marker for
591 known CN driver genes and was not a widespread feature in most recurrently copy number variant
592 genes. However, while expression modulation is a requirement for selection of CNV only drivers, it is
593 not sufficient evidence alone and hence we grouped such genes into those which have been
594 characterised as drivers previously in other cancer types (high confidence EAC CN drivers) and other
595 genes (Candidate EAC CN drivers) which await functional validation. We used fragile site regions
596 detected in Wala et al 2017[49]. We also defined regions which may be recurrently heterozygous
597 deleted, without any significant expression modulations, to allow LOH of tumour suppressor gene
598 mutations. To do this we analyses genes with at least 5 mutations in the matched RNA cohort for
599 association between LOH (ASCAT minor allele = 0) and mutation using fisher's exact test and
600 generated q values using the Benjamini & Hochberg method. The analysis was repeated on known
601 cancer genes only for reduced FDR and $q < 0.05$ considered significant for both analyses. For those
602 high confidence drivers we chose to define amplification as CN/ploidy (referred to as Ploidy adjusted
603 copy number) this produces superior correlation with expression. We chose a cut off for
604 amplification at CN/ploidy = 2 as has been previously used, and as causes a highly significant
605 increase in expression in our CN-driver genes.

606

607 **Pathways and relative distributions of genomic events**

608 The relative distribution of driver events in each pathway was analysed using a fisher's exact test in
609 the case of pair-wise comparisons including WT cases. In the case of multi-gene comparisons such as
610 the Cyclins we calculate the p value and odds ratio for each pair in the group by fisher's exact test
611 and combine p values using the Fisher method, Genes without comparable Odds ratios to the rest of
612 the genes in question were removed. For this analysis we also remove highly mutated cases (>500
613 exonic mutations, 41/551) as they bias distribution of genes towards co-occurrence. We repeated

614 this analyses across all pairs of driver genes using BH multiple hypothesis correction. We validated
615 these relationships in independent TGCA cohorts of other GI cancers where we could find cohorts
616 with reasonable numbers of the genomic events in question (not possible for GATA4/6 for instance)
617 using the cBioportal web interface tool[50].

618

619 **Correlating genomics with the clinical phenotype**

620 To find genomic markers for prognosis we undertook univariate Cox regression for those driver
621 genes present in >5% of cases (16) along with Benjamini & Hochberg false discovery correction. We
622 considered only these genes to reduce our false discover rate and because other genes were unlikely
623 to impact on clinical practise given their low frequency in EAC. We validated SMAD4, in the TCGA
624 gastroesophageal cohort which had a comparable frequency of these events, but notably is
625 composed mainly of gastric cancers, and GATA4 in the TCGA pancreatic cohort using the cBioportal
626 web interface tool. We also validated these markers as independent predictors of survival both in
627 respect of each other and stage using a multivariate Cox regression in our 551 case cohort. When
628 assessing for genomic correlates with differentiation phenotypes we found only very few cases with
629 well differentiated phenotypes (<5% cases) and hence for statistical analyses we collapse these cases
630 with moderate differentiation to allow a binary fisher's exact test to compare poorly differentiated
631 with well-moderate differentiated phenotypes.

632

633 **Therapeutics**

634 The cancer biomarker database was filtered for drugs linked to biomarkers found in EAC drivers and
635 supplementary table 6 constructed using the cohort frequencies of EAC biomarkers. 8 EAC cell lines
636 with WGS data[37] were used in proliferation assays to determine drug sensitivity to CDK4/6 inhibitors,
637 Palbociclib (Biovision) and Ribociclib (Selleckchem). Cell lines were grown in their normal growth

638 media (methods table 1). Proliferation was measured using the Incucyte live cell analysis system
639 (Incucyte ZOOM Essen biosciences). Each cell line was plated at a starting confluency of 10% and
640 growth rate measured across 4-7 days depending on basal proliferation rate. For each cell-line drug
641 combination concentrations of 16, 64, 250, 1000 and 4000nM were used each in 0.3% DMSO and
642 compared to 0.3% DMSO only. Each condition was performed in at least triplicate. The time period
643 of the exponential growth phase in the untreated (0.3% DMSO) condition was used to calculate GI50
644 and AUC. Accurate GI50s could not be calculated in cases where a cell line had >50% proliferation
645 inhibition even with the highest drug concentration and hence AUC was used to compare cell line
646 sensitivity. T47D had a highly similar GI50 for Palbociclib to that previously calculated in other
647 studies (112 nM vs 127 nM)[51].

648

649 # References

650 **1.** Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide:
651 sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* Mar 1
652 2015;136(5):E359-386.
653 **2.** Coleman HG, Xie SH, Lagergren J. The Epidemiology of Esophageal Adenocarcinoma.
654 *Gastroenterology.* Jan 2018;154(2):390-405.
655 **3.** Smyth EC, Lagergren J, Fitzgerald RC, et al. Oesophageal cancer. *Nat Rev Dis Primers.* Jul 27
656 2017;3:17048.
657 **4.** Campbell PJ, Getz G, Stuart JM, Korbel JO, Stein LD. Pan-cancer analysis of whole genomes.
658 *bioRxiv.* 2017.
659 **5.** Ciriello G, Miller ML, Aksoy BA, Senbabaoglu Y, Schultz N, Sander C. Emerging landscape of
660 oncogenic signatures across human cancers. *Nat Genet.* Oct 2013;45(10):1127-1133.
661 **6.** Secrier M, Li X, de Silva N, et al. Mutational signatures in esophageal adenocarcinoma define
662 etiologically distinct subgroups with therapeutic relevance. *Nat Genet.* Oct
663 2016;48(10):1131-1141.
664 **7.** Stratton MR, Futreal PA. Cancer: understanding the target. *Nature.* Jul 1 2004;430(6995):30.
665 **8.** Tamborero D, Gonzalez-Perez A, Perez-Llamas C, et al. Comprehensive identification of
666 mutational cancer driver genes across 12 tumor types. *Sci Rep.* Oct 2 2013;3:2650.
667 **9.** Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search
668 for new cancer-associated genes. *Nature.* Jul 11 2013;499(7457):214-218.
669 **10.** Integrated genomic characterization of oesophageal carcinoma. *Nature.* Jan 12
670 2017;541(7636):169-175.
671 **11.** Lin DC, Dinh HQ, Xie JJ, et al. Identification of distinct mutational patterns and new driver
672 genes in oesophageal squamous cell carcinomas and adenocarcinomas. *Gut.* Aug 31 2017.

673 **12.** Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature.* Mar 20
674 2014;507(7492):315-322.
675 **13.** Comprehensive molecular characterization of gastric adenocarcinoma. *Nature.* Sep 11
676 2014;513(7517):202-209.
677 **14.** Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G. GISTIC2.0 facilitates
678 sensitive and confident localization of the targets of focal somatic copy-number alteration in
679 human cancers. *Genome Biol.* 2011;12(4):R41.
680 **15.** Dulak AM, Schumacher SE, van Lieshout J, et al. Gastrointestinal adenocarcinomas of the
681 esophagus, stomach, and colon exhibit distinct patterns of genome instability and
682 oncogenesis. *Cancer Res.* Sep 1 2012;72(17):4383-4393.
683 **16.** Frankel A, Armour N, Nancarrow D, et al. Genome-wide analysis of esophageal
684 adenocarcinoma yields specific copy number aberrations that correlate with prognosis.
685 *Genes Chromosomes Cancer.* Apr 2014;53(4):324-338.
686 **17.** Secrier M, Fitzgerald RC. Signatures of Mutational Processes and Associated Risk Factors in
687 Esophageal Squamous Cell Carcinoma: A Geographically Independent Stratification Strategy?
688 *Gastroenterology.* May 2016;150(5):1080-1083.
689 **18.** Zack TI, Schumacher SE, Carter SL, et al. Pan-cancer patterns of somatic copy number
690 alteration. *Nat Genet.* Oct 2013;45(10):1134-1140.
691 **19.** Dulak AM, Stojanov P, Peng S, et al. Exome and whole-genome sequencing of esophageal
692 adenocarcinoma identifies recurrent driver events and mutational complexity. *Nat Genet.*
693 May 2013;45(5):478-486.
694 **20.** Nones K, Waddell N, Wayte N, et al. Genomic catastrophes frequently arise in esophageal
695 adenocarcinoma and drive tumorigenesis. *Nat Commun.* Oct 29 2014;5:5224.
696 **21.** Martincorena I, Raine KM, Gerstung M, et al. Universal Patterns of Selection in Cancer and
697 Somatic Tissues. *Cell.* Nov 16 2017;171(5):1029-1041 e1021.
698 **22.** Gonzalez-Perez A, Lopez-Bigas N. Functional impact bias reveals cancer drivers. *Nucleic Acids*
699 *Res.* Nov 2012;40(21):e169.
700 **23.** Tamborero D, Gonzalez-Perez A, Lopez-Bigas N. OncodriveCLUST: exploiting the positional
701 clustering of somatic mutations to identify cancer genes. *Bioinformatics.* Sep 15
702 2013;29(18):2238-2244.
703 **24.** Porta-Pardo E, Godzik A. e-Driver: a novel method to identify protein regions driving cancer.
704 *Bioinformatics.* Nov 1 2014;30(21):3109-3114.
705 **25.** Porta-Pardo E, Hrabe T, Godzik A. Cancer3D: understanding cancer mutations through
706 protein structures. *Nucleic Acids Res.* Jan 2015;43(Database issue):D968-973.
707 **26.** Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. *Nat Rev Cancer.* Mar
708 2004;4(3):177-183.
709 **27.** Kandoth C, McLellan MD, Vandin F, et al. Mutational landscape and significance across 12
710 major cancer types. *Nature.* Oct 17 2013;502(7471):333-339.
711 **28.** Chang MT, Asthana S, Gao SP, et al. Identifying recurrent mutations in cancer reveals
712 widespread lineage diversity and mutational specificity. *Nat Biotechnol.* Feb 2016;34(2):155-
713 163.
714 **29.** Zaretsky JM, Garcia-Diaz A, Shin DS, et al. Mutations Associated with Acquired Resistance to
715 PD-1 Blockade in Melanoma. *N Engl J Med.* Sep 1 2016;375(9):819-829.
716 **30.** Chen Z, Shi T, Zhang L, et al. Mammalian drug efflux transporters of the ATP binding cassette
717 (ABC) family in multidrug resistance: A review of the past decade. *Cancer Lett.* Jan 1
718 2016;370(1):153-164.
719 **31.** Comprehensive molecular characterization of human colon and rectal cancer. *Nature.* Jul 18
720 2012;487(7407):330-337.
721 **32.** Waddell N, Pajic M, Patch AM, et al. Whole genomes redefine the mutational landscape of
722 pancreatic cancer. *Nature.* Feb 26 2015;518(7540):495-501.

723 **33.** Leiserson MD, Vandin F, Wu HT, et al. Pan-cancer network analysis identifies combinations
724 of rare somatic mutations across pathways and protein complexes. *Nat Genet.* Feb
725 2015;47(2):106-114.
726 **34.** Singhi AD, Foxwell TJ, Nason K, et al. Smad4 loss in esophageal adenocarcinoma is associated
727 with an increased propensity for disease recurrence and poor survival. *Am J Surg Pathol.* Apr
728 2015;39(4):487-495.
729 **35.** Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in
730 human cancer. *Cytokine Growth Factor Rev.* Feb-Apr 2006;17(1-2):41-58.
731 **36.** Tamborero D, Rubio-Perez C, Deu-Pons J, et al. Cancer Genome Interpreter Annotates The
732 Biological And Clinical Relevance Of Tumor Alterations. *bioRxiv.* 2017.
733 **37.** Contino G, Eldridge MD, Secrier M, et al. Whole-genome sequencing of nine esophageal
734 adenocarcinoma cell lines. *F1000Res.* 2016;5:1336.
735 **38.** Liston DR, Davis M. Clinically Relevant Concentrations of Anticancer Drugs: A Guide for
736 Nonclinical Studies. *Clin Cancer Res.* Jul 15 2017;23(14):3489-3498.
737 **39.** Herrera-Abreu MT, Palafox M, Asghar U, et al. Early Adaptation and Acquired Resistance to
738 CDK4/6 Inhibition in Estrogen Receptor-Positive Breast Cancer. *Cancer Res.* Apr 15
739 2016;76(8):2301-2313.
740 **40.** D'Antonio M, Ciccarelli FD. Integrated analysis of recurrent properties of cancer genes to
741 identify novel drivers. *Genome Biol.* May 29 2013;14(5):R52.
742 **41.** Zehir A, Benayed R, Shah RH, et al. Mutational landscape of metastatic cancer revealed from
743 prospective clinical sequencing of 10,000 patients. *Nat Med.* Jun 2017;23(6):703-713.
744 **42.** Ding J, McConechy MK, Horlings HM, et al. Systematic analysis of somatic mutations
745 impacting gene expression in 12 tumour types. *Nat Commun.* Oct 5 2015;6:8554.
746 **43.** Nagai K, Kohno K, Chiba M, et al. Differential expression profiles of sense and antisense
747 transcripts between HCV-associated hepatocellular carcinoma and corresponding non-
748 cancerous liver tissue. *Int J Oncol.* Jun 2012;40(6):1813-1820.
749 **44.** Rheinbay E, Nielsen MM, Abascal F, et al. Discovery and characterization of coding and non-
750 coding driver mutations in more than 2,500 whole cancer genomes. *bioRxiv.* 2017.
751 **45.** Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense
752 mutations using PolyPhen-2. *Curr Protoc Hum Genet.* Jan 2013;Chapter 7:Unit7 20.
753 **46.** Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function.
754 *Annu Rev Genomics Hum Genet.* 2006;7:61-80.
755 **47.** Reimand J, Wagih O, Bader GD. The mutational landscape of phosphorylation signaling in
756 cancer. *Sci Rep.* Oct 2 2013;3:2651.
757 **48.** Northcott PA, Buchhalter I, Morrissy AS, et al. The whole-genome landscape of
758 medulloblastoma subtypes. *Nature.* Jul 19 2017;547(7663):311-317.
759 **49.** Wala JA, Shapira O, Li Y, et al. Selective and mechanistic sources of recurrent
760 rearrangements across the cancer genome. *bioRxiv.* 2017.
761 **50.** Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and
762 clinical profiles using the cBioPortal. *Sci Signal.* Apr 2 2013;6(269):pl1.
763 **51.** Finn RS, Dering J, Conklin D, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor,
764 preferentially inhibits proliferation of luminal estrogen receptor-positive human breast
765 cancer cell lines in vitro. *Breast Cancer Res.* 2009;11(5):R77.

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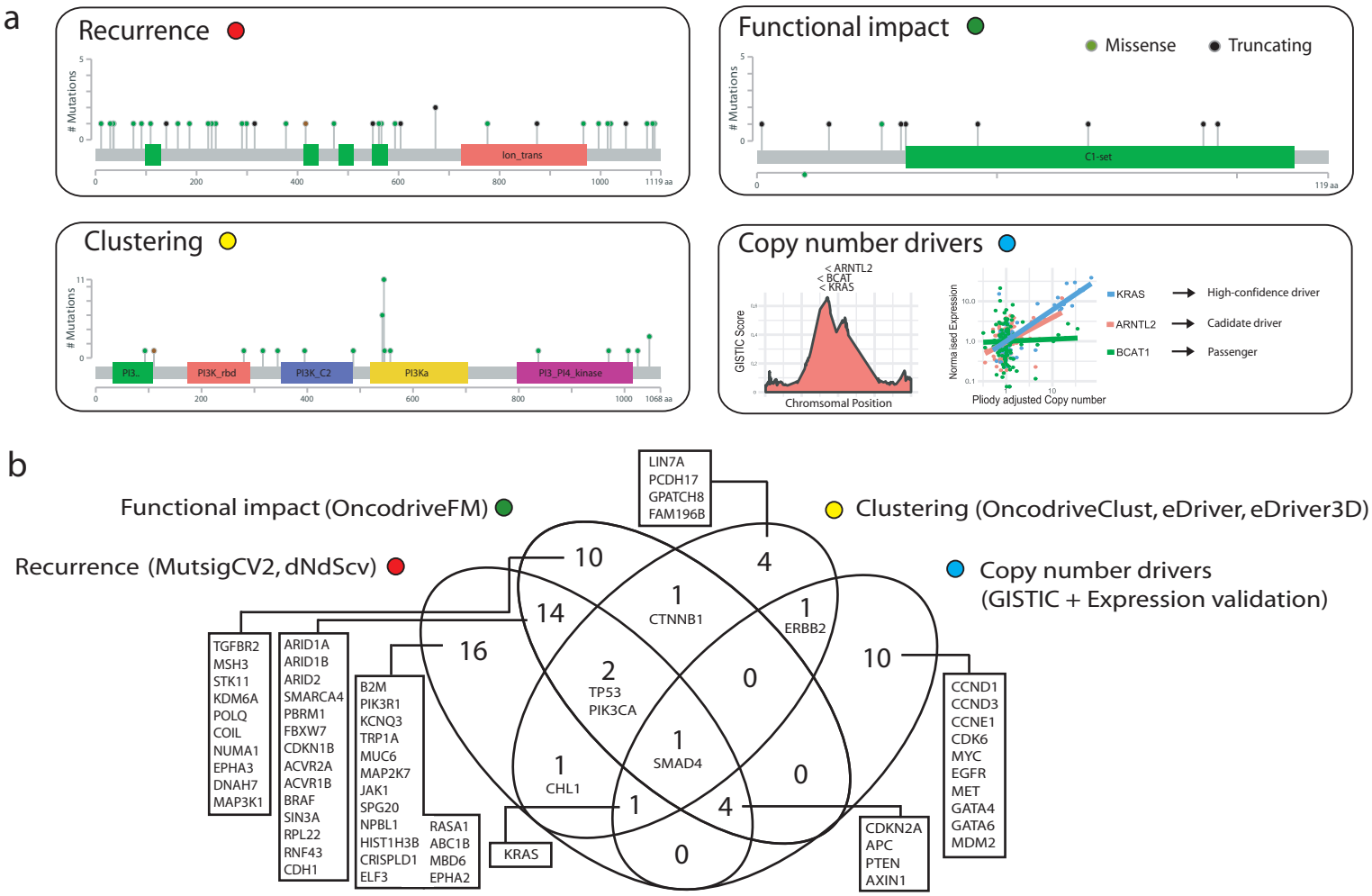
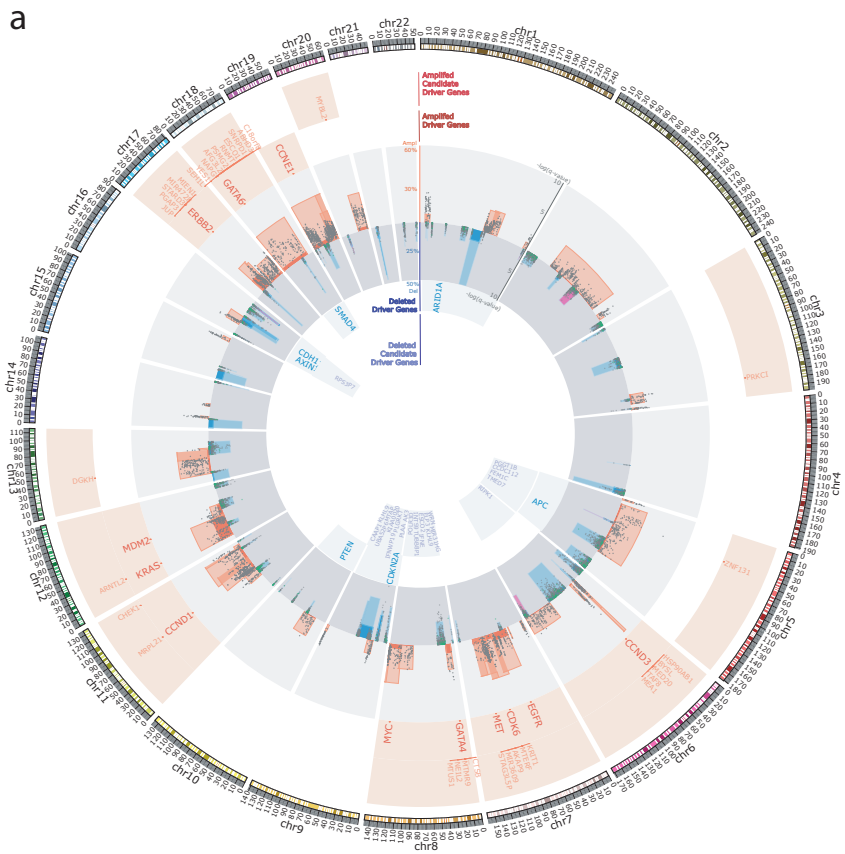
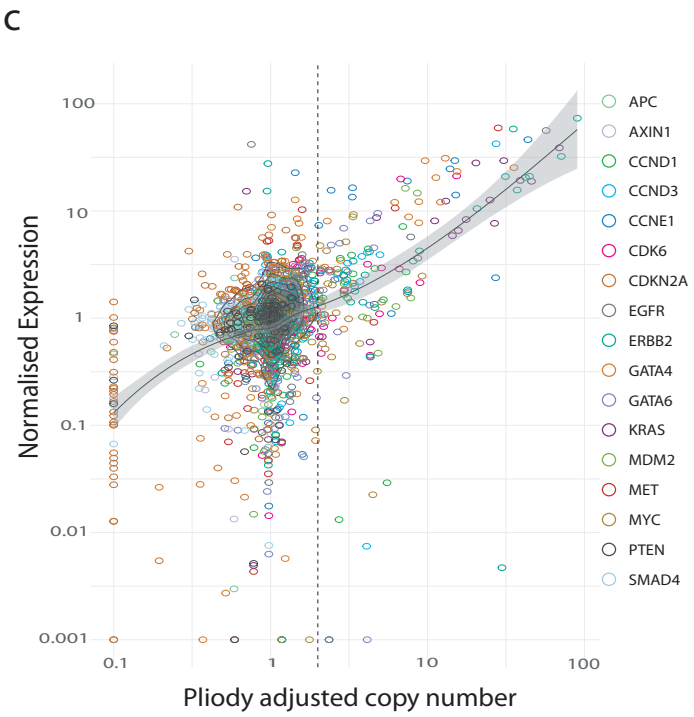
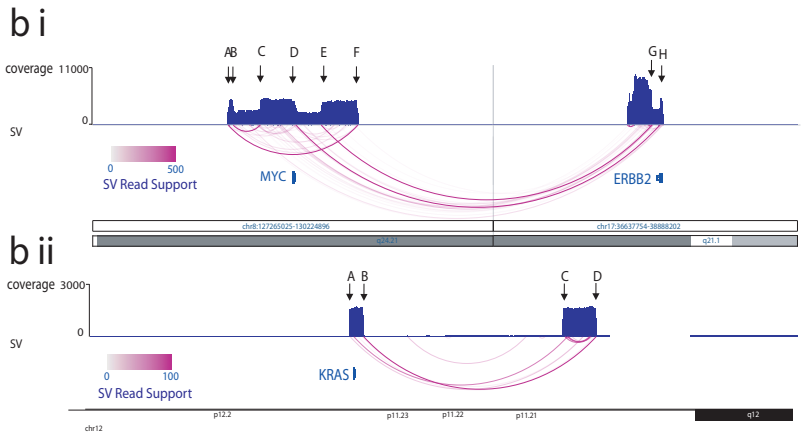


Figure 1 Detection of EAC driver Genes. a. Types of driver-associated features used to detect positive selection in mutations and copy number events with examples of genes containing such features **b.** Driver genes identified and their driver-associated features.







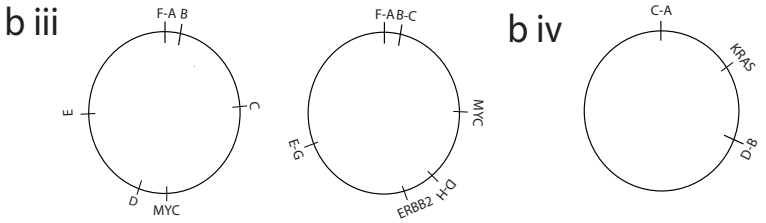


Figure 2. Copy number variation under positive selection. **a.** Recurrent copy number changes across the genome identified by GISTIC. Frequency of different CNV types are indicated as well as the position of CNV high confidence driver genes and candidate driver genes. The q value for expression correlation with amplification and homozygous deletion is shown for each gene within each amplification and deletion peaks respectively and occasions of significant association between LOH and mutation are indicated in green. Purple deletion peaks indicate fragile sites. **b.** Examples of Extrachromosomal-like amplifications suggested by very high read support SVs at the boundaries of highly amplified regions produced from a single copy number step. In the first example (bi) two populations of extrachromosomal DNA are apparent (biii), one amplifying only MYC and the second also incorporating ERBB2 from a different chromosome. In the second example (bii) an inversion has occurred before circularization and amplification around KRAS (biv). **c.** Relationship between copy number and expression in CN driver genes.

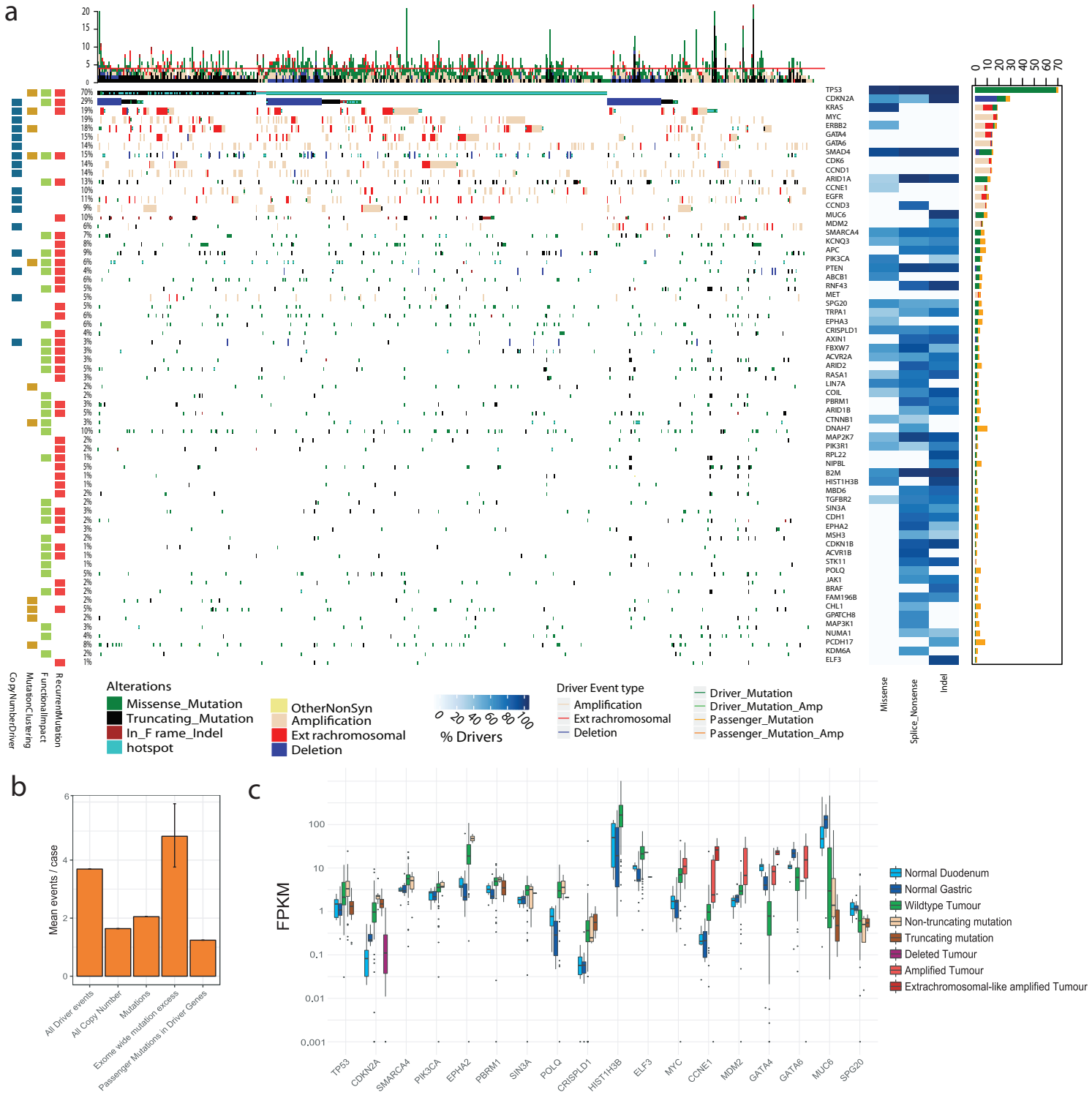
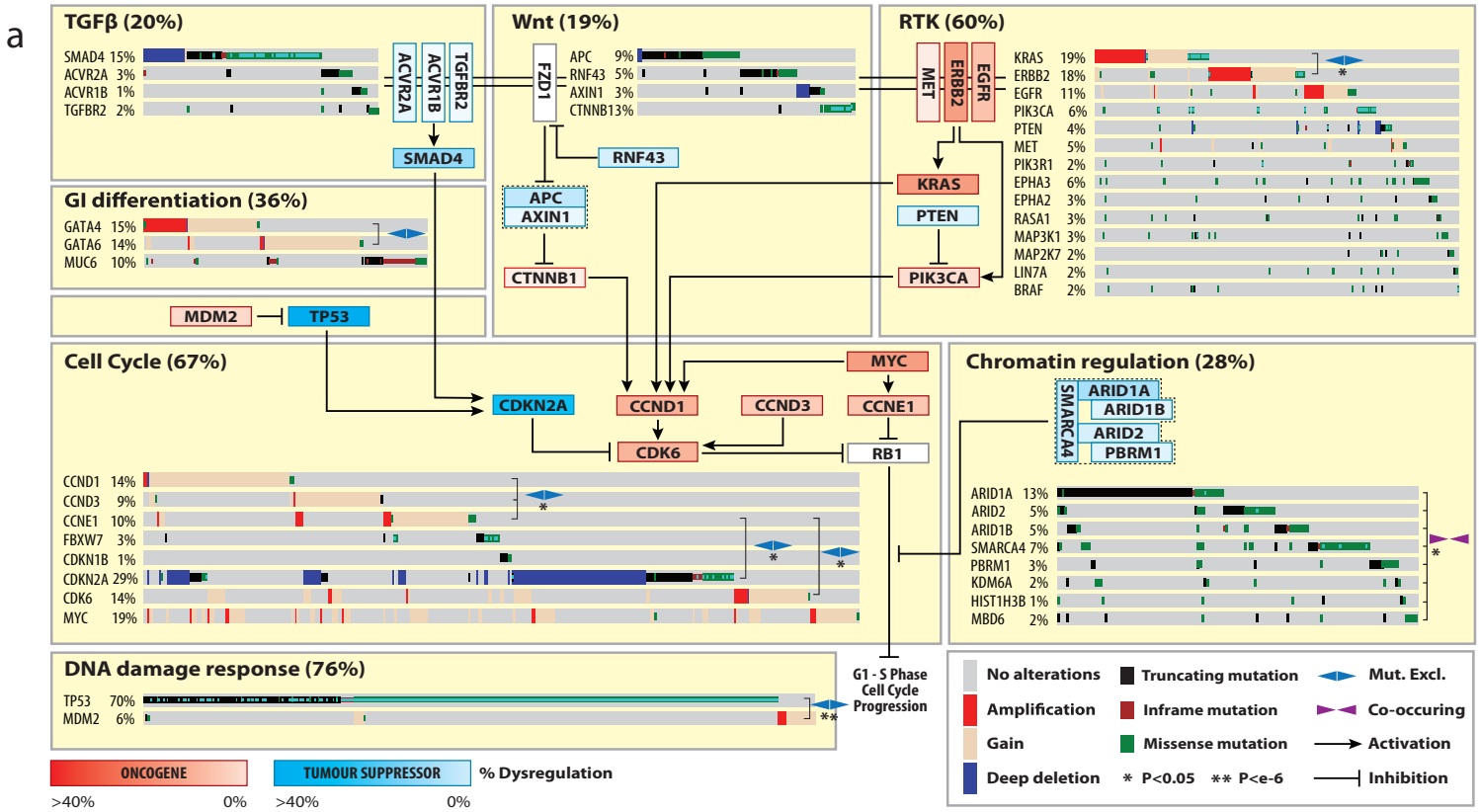


Figure 3. The driver gene landscape of Esophageal Adenocarcinoma. a Driver mutations or CNVs are shown for each patient. Amplification is defined as >2 Copy number adjusted ploidy (2 x ploidy of that case) and extrachromosomal amplification as >10 Copy number adjusted ploidy (10 x ploidy for that case). Driver associated features for each driver gene are displayed to the left. On the right the percentages of different mutation and copy number changes are displayed, differentiating between driver and passenger mutations using dNdScv, and the % of predicted drivers by mutation type is shown. Above the plot are the number of driver mutations per sample with an indication of the median (red line = 4). **b**. Assessment of driver event types per case and comparison to exome-wide excess of mutations generated by dNdScv. **c**. Expression changes in EAC driver genes in comparison to normal intestinal tissues. Only genes with signifcant expression changes of note are shown.



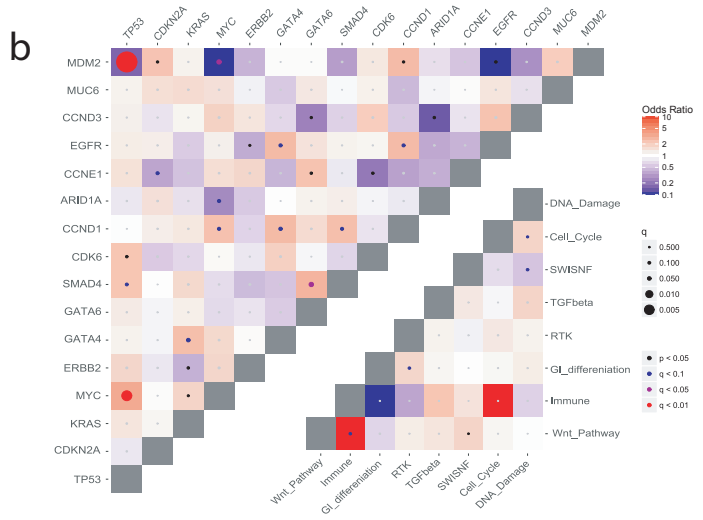


Figure 4. Biological pathways undergoing selective dysregulation in EAC. a. Biological Pathways dysregulated by driver gene mutation and/or CNVs. WT cases for a pathway are not shown. Mutual exclusivities and/or associations between genes in a pathway are annotated. GATA4/6 amplifications have a mutually exclusive relationship (ie GATA4 ampification is more common in GATA6 WT cases) although this does not reach statistical significance (fisher's exact test p=0.07 OR =0.52). **b.** Pairwise assessment of mutual exclusivity and association in EAC driver genes and pathways.

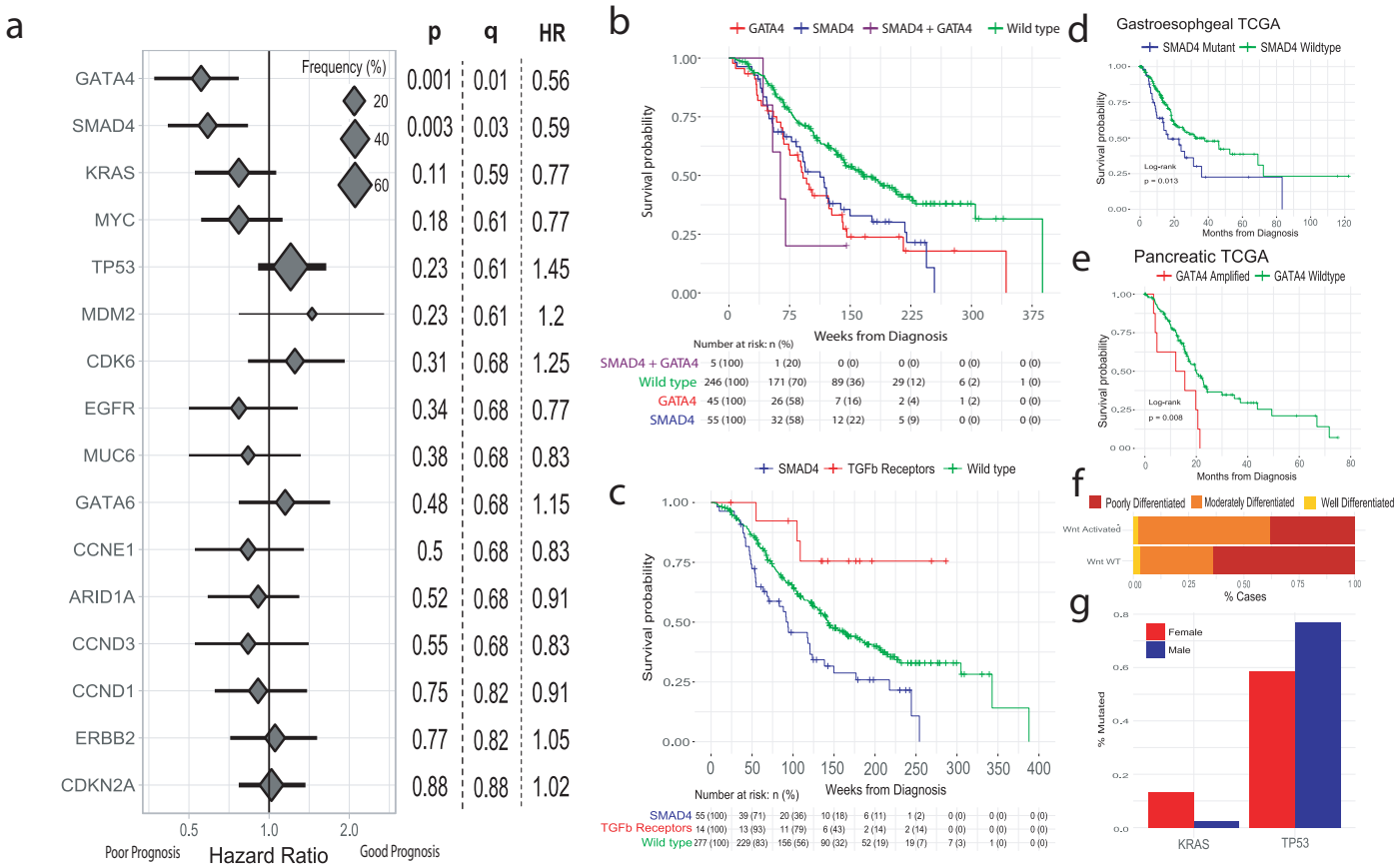


Figure 5. Clinical significance of Driver events in EAC. a. Hazard rations and 95% confidence intervals for Cox regression analysis across all drivers genes with at least a 5% frequency of driver alterations. P values are generated from the wald test and q values generated using BH correction. **b.** Kaplan-Meier curves for EACs with different status of significant prognostic indicators (GATA4 and SMAD4). **c.** Kaplan-Meier curves for different alterations in the TGFbeta pathway. **d.** Kaplan-Meier curves showing verification GATA4 prognostic value in GI cancers using a pancreatic TCGA cohort. **e.** Kaplan-Meier curves showing verification SMAD4 prognostic value in Gastroesophageal cancers using a gastroesophageal TCGA cohort. **f.** Differentiation bias in tumours containing events in Wnt pathway driver genes. **g.** Relative frequency of KRAS mutations and TP53 mutations driver gene events in females vs males (fishers exact test).

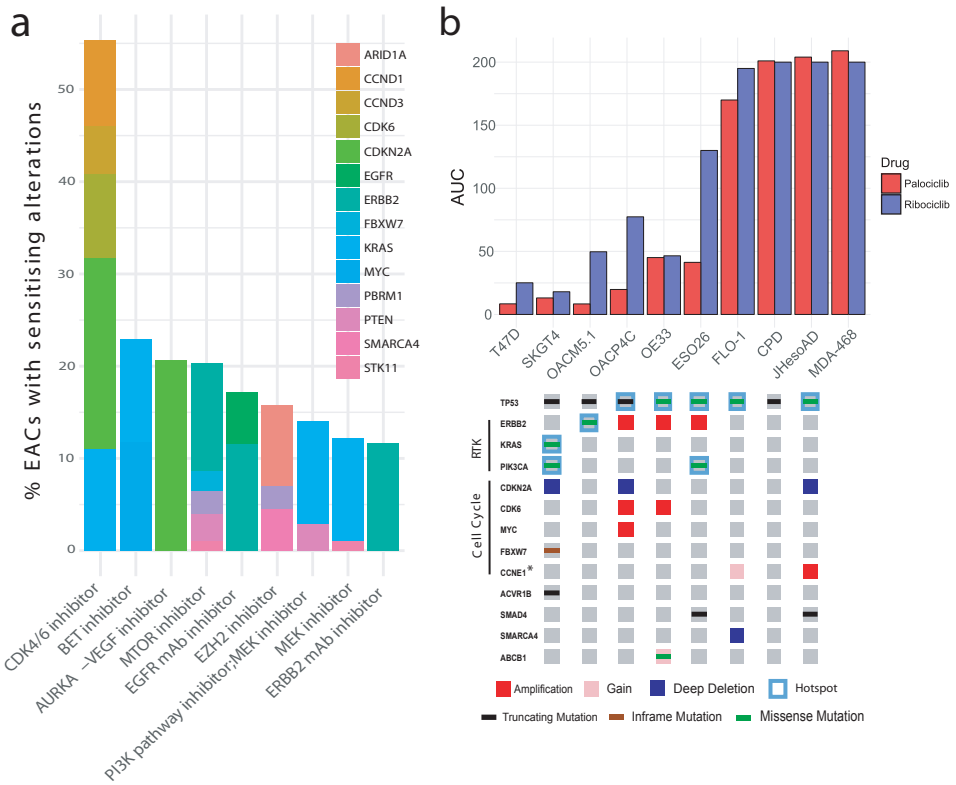


Figure 6. CDK4/6 inhibitors utility in EAC. a. Drug classes for which sensitivity is indicated by EAC driver genes with data from the Cancer Biomarkers database36. **b.** Area under the curve (AUC) of sensitivity is shown in a panel of 8 EAC cell lines with associated WGS and driver event, based in tumour analysis, in these cell lines indicated. Also AUC is shown for two control lines T47D, an ER +ve breast cancer line (+ve control) and MDA-MB-468 a Rb negative breast cancer (-ve control). *CCNE1 is a known marker of resistance to CDK4/6is due to its regulation of Rb downstream of CDK4/6 hence bypassing the need for CDK4/6 activity (see figure 4).