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1 **LncRNA analyses reveal increased levels of non-coding centromeric transcripts in**  
2 **hepatocellular carcinoma**

3  
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31 **Abstract**

32 The search for new biomarkers and drug targets for hepatocellular carcinoma (HCC) has  
33 spurred an interest in long non-coding RNAs (lncRNAs), often proposed as oncogenes or  
34 tumor suppressors. Furthermore, lncRNA expression patterns can bring insights into the  
35 global de-regulation of cellular machineries in tumors. Here, we examine lncRNAs in a large  
36 HCC cohort, comprising RNA-seq data from paired tumor and adjacent tissue biopsies from  
37 114 patients. We find that numerous lncRNAs are differentially expressed between tumors  
38 and adjacent tissues and between tumor progression stages. Although we find strong  
39 differential expression for most lncRNAs previously associated with HCC, the expression  
40 patterns of several prominent HCC-associated lncRNAs disagree with their previously  
41 proposed roles. We examine the genomic characteristics of HCC-expressed lncRNAs and  
42 reveal an enrichment for repetitive elements among the lncRNAs with the strongest  
43 expression increases in advanced-stage tumors. This enrichment is particularly striking for  
44 lncRNAs that overlap with satellite repeats, a major component of centromeres. Consistently,  
45 we find increased non-coding RNA transcription from centromeres in tumors, in the majority  
46 of patients, suggesting that aberrant centromere activation takes place in HCC.

47 **Introduction**

48 Following the realization that the human genome harbors thousands of non-coding RNA  
49 genes (Carninci *et al*, 2005), many of which have important cellular functions (Mattick &  
50 Makunin, 2006), a great deal of effort has been put into investigating the contributions of non-  
51 coding RNAs to cancer biology (Gutschner & Diederichs, 2012). In particular, the roles of long  
52 non-coding RNAs (lncRNAs) in cancer have been frequently scrutinized in the past decade.  
53 This category of non-coding RNAs (defined simply as RNA molecules that lack protein-coding  
54 capacity, at least 200 nucleotides long) comprises many transcripts with proven functions in  
55 gene expression regulation, genome stability or nuclear architecture (Engreitz *et al*, 2016).  
56 Numerous recent studies showed that lncRNA loci are part of the alterations that occur in  
57 cancer cells (Yan *et al*, 2015). Thus, studying lncRNAs is perceived as a promising path  
58 towards understanding the molecular mechanisms that underlie cancer onset. Ultimately,  
59 lncRNAs may prove to be valuable in the diagnosis process, or serve as therapeutic targets.

60  
61 The search for novel disease biomarkers and drug targets, including lncRNAs, is  
62 understandably intensive for cancer types for which effective therapies are still lacking. This  
63 is the case for hepatocellular carcinoma (HCC), which is a major cause of cancer-related  
64 mortality world-wide (Yang & Roberts, 2010). As HCC is generally detected at late stages of  
65 tumor progression, surgical treatment options are unavailable for the majority of patients  
66 (Hartke *et al*, 2017). Several systemic therapies now exist, but they increase median patient

67 survival by less than 1 year (Finn *et al*, 2020). Thus, developing new treatments for HCC is  
68 still an urgent need. With this aim, there has been extensive research aiming to identify the  
69 genic and non-genic functional elements that are altered in HCC compared to the healthy liver.  
70 Large-scale transcriptomics studies, comparing HCC samples with adjacent non-tumor tissue  
71 or with normal liver samples, identified hundreds of differentially regulated protein-coding  
72 genes and lncRNAs (Cui *et al*, 2017; Yang *et al*, 2017; Li *et al*, 2019; Jin *et al*, 2019; Unfried  
73 *et al*, 2019). Some of the lncRNAs associated with HCC through genome-wide comparative  
74 analyses were subject to further experimental investigations, aiming to elucidate their  
75 mechanisms of action and the consequences of their differential regulation in tumors. For  
76 some lncRNAs, there are now well-supported models for their behavior in HCC. This is the  
77 case for example for *HOTTIP*, a lncRNA that is strongly up-regulated in HCC, and which likely  
78 acts to enhance the expression of the neighboring genes by recruiting transcriptional co-  
79 activators (Quagliata *et al*, 2014; Pradeepa *et al*, 2017; Quagliata *et al*, 2018). However, for  
80 other lncRNAs experimental studies gave rise to conflicting results. For example, the *H19*  
81 lncRNA (known as a parentally imprinted regulator of placenta growth (Keniry *et al*, 2012))  
82 was alternatively proposed to act as a tumor suppressor (Hao *et al*, 1993; Yoshimizu *et al*,  
83 2008; Schultheiss *et al*, 2017) or as an oncogene (Matouk *et al*, 2007; Zhou *et al*, 2019) in  
84 various cancer types including HCC (Tietze & Kessler, 2020). Likewise, *MALAT1*, initially  
85 described as an abundant lncRNA associated with the presence of metastases (Ji *et al*, 2003),  
86 was first thought to promote tumor growth and invasion in breast cancer (Arun *et al*, 2016),  
87 but is now believed to be a tumor suppressor (Kim *et al*, 2018, 1). In HCC, *MALAT1* was  
88 mainly proposed to act as an oncogene (Hou *et al*, 2017, 1; Liu *et al*, 2019, 1; Chen *et al*,  
89 2020), but there is no consensus on its mechanisms of action. This is also the case for most  
90 of the lncRNAs that have been associated with HCC, although experimental data is  
91 accumulating (Lanzafame *et al*, 2018). Thus, overall, the functions of lncRNAs in HCC and  
92 other cancers are still poorly understood.

93

94 Although we are still far from developing therapies that target lncRNAs in HCC, in the more  
95 immediate future, lncRNAs may prove to be useful as disease biomarkers, to help diagnose  
96 HCC at an earlier stage and to better classify molecular subtypes of tumors. For this purpose,  
97 large-scale transcriptomics comparisons that can identify differentially regulated lncRNAs in  
98 tumor tissues are a valid approach, even in the absence of additional functional experiments.  
99 Although such studies are abundant in the lncRNA literature, they are often restricted to small  
100 cohorts of patients, thus potentially failing to reproduce the full extent of the molecular  
101 heterogeneity of HCC (Boyault *et al*, 2007; Hoshida *et al*, 2009). Further work is still needed  
102 to understand what part lncRNAs play in the molecular characteristics of HCC.

103

104 Studying lncRNA expression patterns in HCC and other cancers is also a means to better  
105 understand the de-regulation of essential cellular machineries in tumors. Although many  
106 lncRNAs have important biological roles (Engreitz *et al*, 2016), there is strong evidence that,  
107 out of the tens of thousands of lncRNAs that are detected with sensitive transcriptome  
108 sequencing approaches in human tissues (Pertea *et al*, 2018; Iyer *et al*, 2015), most may be  
109 non-functional. This is indicated by their weak levels of evolutionary conservation (Necsulea  
110 *et al*, 2014; Washietl *et al*, 2014) and by their expression patterns, which are often restricted  
111 to tissues with open chromatin, permissive to spurious transcription (Soumillon *et al*, 2013;  
112 Darbellay & Necsulea, 2020). Other evidence supporting non-functionality, or even a  
113 deleterious effect of lncRNA transcription comes from their typical processing by the cellular  
114 machinery. lncRNA transcripts are generally inefficiently spliced and poly-adenylated, and  
115 are rapidly degraded by the RNA exosome (Melé *et al*, 2017; Schlackow *et al*, 2017). For  
116 certain classes of lncRNAs, transcription is normally tightly repressed by chromatin-modifying  
117 factors, and their de-repression leads to DNA replication stress and subsequently to cellular  
118 senescence, due to an overlap with DNA replication origins (Nojima *et al*, 2018). It is not clear  
119 yet to what extent similar principles apply to HCC and other cancers. However, the presence  
120 of high lncRNA levels in cancer cells may be a sign of a global de-regulation of the molecular  
121 machineries that normally keep deleterious transcription in check, even if individual lncRNAs  
122 are not “oncogenes” *sensu stricto*. This further highlights the need for detailed investigations  
123 of the patterns of lncRNA expression in cancer.

124

125 In this study, we set out to explore the patterns of lncRNA transcription in a large HCC cohort,  
126 comprising paired tumor and adjacent tissue biopsies from 114 patients. Our work stands out  
127 from previous efforts to characterize lncRNAs in HCC in several important ways. First, we take  
128 advantage of an extensive transcriptome resource, which covers a wide range of tumor  
129 progression stages and underlying liver diseases, and thus can provide a comprehensive  
130 overview of transcriptional de-regulation during HCC development. Importantly, our  
131 transcriptome dataset is derived from biopsies rather than tumor resections, and is thus likely  
132 more faithful to the *in vivo* physiological status of the tumors. Second, we perform a meta-  
133 analysis of the current literature on lncRNAs and HCC and we use our transcriptome collection  
134 to critically re-evaluate previous claims regarding lncRNA expression patterns in HCC. We  
135 can thus highlight the poor reproducibility of some prominent lncRNA-HCC associations.  
136 Third, rather than attempting to propose new candidate oncogene or tumor suppressor  
137 lncRNAs, we perform a detailed analysis of the genomic characteristics of de-regulated  
138 lncRNAs. We thus reveal an increase in repetitive-element derived lncRNA expression in  
139 tumor samples. In particular, we uncover a striking up-regulation of non-coding RNAs derived

140 from centromeric satellite repeats. We discuss the functional implications of this apparent  
141 activation of centromeric chromatin in HCC tumors.

## 142 **Results**

### 143 Transcriptome dataset

144 We analyzed the patterns of protein-coding and lncRNA gene expression in a collection of  
145 268 RNA-seq samples, derived from tumor and adjacent tissue biopsies from 114 HCC  
146 patients (Figure 1a, Supplementary Table 1). This cohort comprises patients with different  
147 underlying diseases, including hepatitis B and hepatitis C, alcoholic or non-alcoholic liver  
148 diseases and cirrhosis (Supplementary Table 1). The Edmondson-Steiner differentiation  
149 grade was recorded for each tumor sample (Supplementary Table 1). Biopsies were  
150 performed during the diagnostic work-up of patients before therapy, and in 3 patients with  
151 HCC recurrence after tumor resection (Supplementary Table 1). Our transcriptome data is not  
152 restricted to poly-adenylated RNAs (Methods) and may thus better reflect the behavior of  
153 lncRNA transcripts, which are inefficiently or not at all poly-adenylated (Schlackow *et al*, 2017).  
154 With this dataset, we could analyze the expression patterns of 19,465 protein-coding genes  
155 and 18,866 lncRNAs, including 7,959 lncRNAs detected *de novo* using our RNA-seq data  
156 (Methods, Supplementary Dataset 1).

### 157 Global trends of gene expression variation in HCC tumors and adjacent tissue samples

158 We first aimed to evaluate broad patterns of gene expression variation among tumor and  
159 adjacent tissue samples. To get a glimpse of the cellular composition changes that take place  
160 in cancer tissue, we analyzed the expression patterns of liver cell type markers  
161 (Supplementary Table 2), obtained from single cell transcriptomics data (MacParland *et al*,  
162 2018). As expected, many of these markers display striking differences between tumor and  
163 adjacent tissue samples, as well as among degrees of tumor differentiation (Figure 1b).  
164 Hepatocyte markers (*PCK1*, *BCHE*, *ARG1*, *ALB*) are low in samples derived from  
165 Edmondson-Steiner grade 4 tumors (Figure 1b). Immune cell markers (e.g., T cell markers  
166 *PTPRC*, *NKG7*, *FCGR3A* or macrophage markers *CD52* and *CD68*) are generally expressed  
167 at lower levels in tumor samples than in the adjacent tissue (Figure 1b). Overall, these patterns  
168 confirm that the cellular environment is substantially different in HCC tumors compared to the  
169 adjacent tissue, but also that there is considerable heterogeneity among tumors.

170

171 The molecular heterogeneity of HCC tumors is well illustrated by principal component  
172 analyses (PCA) performed on protein-coding and lncRNA genes (Methods, Figure 1c,d).  
173 However, although there is substantial variation among tumor samples, this gene expression  
174 map is consistent with the histological classification. For both categories of genes the first axis  
175 of the PCA separates samples with the highest Edmondson-Steiner grades and samples from

176 less advanced tumors and adjacent tissues (Figure 1c,d, Supplementary Figure 1a-d). The  
177 second axis forms a gradient from adjacent tissue to the highest Edmondson-Steiner grades  
178 (Figure 1c,d, Supplementary Figure 1a-d). Notably, paired biopsies do not cluster on the first  
179 factorial map of the gene expression PCA, despite their shared genetic background. We  
180 validated the sample pairing by evaluating the presence of shared alleles in exonic single  
181 nucleotide polymorphisms that were reliably detected with our RNA-seq data (Methods). As  
182 expected, samples stemming from the same patient are genetically very similar, in contrast to  
183 samples derived from different patients (Supplementary Figure 1e).

184

185 In HCC, lncRNAs follow previously reported patterns: they are generally weakly expressed  
186 and are thus detected in fewer samples than protein-coding genes (Supplementary Figure 2).  
187 This trend is even stronger for *de novo* annotated lncRNAs (Supplementary Figure 2).

188

#### 189 Differential expression of protein-coding genes and lncRNAs in HCC

190 We next tested for differential expression (DE) between paired tumor and adjacent tissue  
191 biopsies and among tumors with different Edmondson-Steiner grades (Supplementary Table  
192 3, Methods). We selected differentially expressed genes with a minimum fold change of 1.5  
193 and maximum false discovery rate (FDR) of 1%. With these stringent settings, we found that  
194 4,100 (21%) protein-coding genes and 3,315 (18%) lncRNAs were differentially expressed  
195 between tumor and adjacent tissue biopsies. When comparing tumor samples grouped by  
196 Edmondson-Steiner grade (grades 1 and 2 vs. grades 3 and 4), 2,537 (13%) protein-coding  
197 genes and 2,065 (11%) lncRNAs were significantly differentially expressed. The distribution  
198 of expression fold changes differs between the two categories of genes, with stronger positive  
199 fold changes for lncRNAs for the latter analysis (Figure 2). Genes that were up-regulated in  
200 tumors compared to adjacent tissues or in tumor samples with higher Edmondson-Steiner  
201 grades were enriched in processes related to the cell cycle, to chromosome organization but  
202 also to embryonic development (Figure 2, Supplementary Table 4). In contrast, downregulated  
203 genes were enriched in metabolic processes characteristic of the healthy liver (Figure 2,  
204 Supplementary Table 4). In addition, genes involved in immune response and in cell adhesion  
205 are down-regulated in tumor samples compared to the adjacent tissue (Figure 2a,  
206 Supplementary Table 4). There is substantial overlap between the sets of genes that are  
207 differentially expressed in the two comparisons, with consistent directions of change, for both  
208 protein-coding genes and lncRNAs (Supplementary Figure 3a,b).

209

210 As expected given their involvement in essential cell cycle processes, protein-coding genes  
211 that are up-regulated in tumors compared to the adjacent tissue or in the tumors with the

212 highest Edmondson-Steiner grades had significantly higher levels of evolutionary sequence  
213 conservation than down-regulated genes (Wilcoxon rank sum test p-value  $< 1e-10$  for the first  
214 DE analysis, p-value 0.006 for the second DE analysis, Supplementary Figure 3c). For  
215 lncRNAs, the increase in sequence conservation is only observed for those that are up-  
216 regulated in the tumors compared to the adjacent tissue (Wilcoxon rank sum test, p-value  $3.7$   
217  $e-4$ , Supplementary Figure 3d). In contrast, lncRNAs that are up-regulated in tumors with  
218 higher Edmondson-Steiner grades have slightly lower conservation scores than down-  
219 regulated lncRNAs (Wilcoxon rank sum test, p-value 0.03, Supplementary Figure 3d).

220

221 We next verified whether the DE protein-coding genes and lncRNAs were isolated or clustered  
222 in the genome. To do this, for each DE gene (defined as above) we verified the DE status for  
223 neighboring genes, within a 50 kilobases (kb) window (Methods). We find that the proportion  
224 of DE genes that have a DE neighbor with the same expression change direction is  
225 significantly higher than expected by chance, for both protein-coding genes and lncRNAs  
226 (randomization test, p-value  $< 0.01$ , Supplementary Figure 4, Methods). In contrast, pairs of  
227 neighboring genes with opposite DE orientation are significantly less frequent than expected  
228 by chance (randomization test, p-value  $< 0.01$ , Supplementary Figure 4). This pattern is  
229 observed for both protein-coding and lncRNA genes and for both differential expression tests.

230

231 Finally, we also assessed the effect of other factors (namely, underlying liver disease,  
232 presence of cirrhosis, sex of the patients) on gene expression patterns in HCC tumors. In  
233 contrast with the large numbers of DE genes observed for the two comparisons described  
234 above, only between 36 and 509 genes were significantly DE depending on one of these  
235 factors (maximum FDR 0.01, minimum fold expression change 1.5, Supplementary Dataset  
236 3). For the comparison between sexes, 180 genes were significantly DE, with the strongest  
237 fold changes observed for genes located on sex chromosomes (Supplementary Dataset 3).

238

### 239 Expression patterns of prominent HCC-associated lncRNAs

240 We next aimed to evaluate the behavior of the most prominent HCC-associated lncRNAs in  
241 our gene expression dataset. We performed a PubMed search with the key word  
242 “hepatocellular carcinoma” in the article title, and parsed the abstracts of the resulting articles  
243 to retrieve gene names or an unambiguous mention of lncRNAs as a class (Methods). We  
244 found that the proportion of all HCC publications that mention lncRNAs increased rapidly in  
245 the past decade, from 0 in 2010 to 6.3% in 2019 (Supplementary Figure 5a). In total, we could  
246 find unambiguous citations for 262 lncRNAs, 160 (61%) of which were only mentioned in one  
247 article (Supplementary Table 5, Supplementary Figure 5b). Only 29 lncRNAs were associated



248 with HCC in 5 or more articles. Expectedly, at the top of the list of highly-cited lncRNAs can  
249 be found transcripts that are well known from other biological contexts, such as *MALAT1* (Ji  
250 *et al*, 2003, 1), *H19* (Bartolomei *et al*, 1991), *HOTAIR* (Rinn *et al*, 2007) and *NEAT1*  
251 (Hutchinson *et al*, 2007). The 5<sup>th</sup> highest-cited lncRNA is *HULC*, which was initially described  
252 in the HCC context (Panzitt *et al*, 2007). Among the 262 HCC-associated lncRNAs, 98 (37%)  
253 were significantly DE (maximum FDR 0.01 and minimum fold change 1.5) between tumor and  
254 adjacent tissues, and 57 (22%) were significantly DE between tumor samples with  
255 Edmondson-Steiner grades 1 and 2 and tumor samples with Edmondson-Steiner grades 3  
256 and 4. These proportions are significantly higher than those observed for lncRNAs that are  
257 not cited in the literature (17% and 11%, respectively, Chi-square test, p-value < 1e-10). In  
258 total, 128 (49%) of the HCC-associated lncRNAs were significantly DE in at least one of the  
259 tests; this proportion reached 81% with low stringency criteria (maximum FDR 0.1, no  
260 minimum fold change).

261  
262 We next examined the expression patterns of the 29 lncRNAs that were cited at least 5 times  
263 in association with HCC (Figure 3). For this analysis, we set the maximum FDR at 0.01 as  
264 described above, but we did not require a minimum fold expression change, to increase our  
265 sensitivity. The great majority (90%) of these lncRNAs were significantly DE between tumors  
266 and adjacent tissues, and 14 (48%) of them were also significantly DE between highly  
267 differentiated (Edmondson-Steiner grades 1 and 2) and poorly differentiated tumors (grades 3  
268 and 4). However, we observed several unexpected patterns among the best studied lncRNAs.  
269 First, *MALAT1* was not significantly DE in neither one of the two analyses (Figure 3), despite  
270 previous reports indicating its up-regulation in HCC tumors compared to adjacent tissues (Lin  
271 *et al*, 2007; Lai *et al*, 2012). Importantly, this is not due to a lack of statistical power or due to  
272 noisy expression, as *MALAT1* was expressed at high levels in all samples (Figure 3d).  
273 Second, *HOTAIR* was overall very weakly expressed and not significantly DE in neither of the  
274 two tests (FDR 0.046, Edmondson grades 1&2 against 3&4). Third, *NEAT1* was weakly but  
275 significantly down-regulated in tumors compared to adjacent tissues, despite previous  
276 evidence for up-regulation (Kou *et al*, 2020). For *HULC* (Panzitt *et al*, 2007), we confirmed the  
277 previously reported up-regulation in tumor samples, but surprisingly, we found that it displayed  
278 lower expression levels in advanced-stage tumors (Figure 3). In some cases, the results could  
279 be explained by the distribution of tumor differentiation degrees among the tumor samples.  
280 For example, *UCA1* is overall down-regulated in tumors compared to the adjacent tissue,  
281 contrary to what was previously reported (Wang *et al*, 2015), but is expressed at higher levels  
282 in samples with Edmondson-Steiner grades 3 and 4 (Figure 3). Some of the inconsistencies  
283 observed between our DE analyses and previous reports, for the best-studied HCC-  
284 associated lncRNAs, may also come from the distribution of patient characteristics, for

285 example underlying liver diseases, genetic background etc. However, out of the 29 tested  
286 lncRNAs none showed significant expression differences between patients with different  
287 underlying diseases (Supplementary Dataset 3). Only *XIST* was differently expressed  
288 between sexes (Supplementary Dataset 3). We also did not observe any significant difference  
289 between patients with or without cirrhosis (Supplementary Dataset 3).

#### 291 Increased repetitive sequence content in HCC-upregulated lncRNAs

292 We next wanted to assess the genomic features of the lncRNAs that are significantly  
293 differentially expressed in the two analyses described above. It was previously reported that  
294 transposable elements that are repressed in healthy tissues can become active in cancer cells  
295 (Burns, 2017). We thus analyzed the repetitive sequence content of differentially expressed  
296 lncRNAs (Supplementary Table 6, Supplementary Dataset 4, Methods). The fraction of exonic  
297 sequence covered by repeats was significantly higher for lncRNAs that were up-regulated in  
298 tumors compared to adjacent tissues (median value 47%) than for down-regulated lncRNAs  
299 (median value 40%, Wilcoxon rank sum test, p-value < 1e-10, Figure 4a). Likewise, in the DE  
300 analysis comparing tumor samples with different Edmondson-Steiner grades, up-regulated  
301 lncRNAs had significantly higher repetitive sequence content (median 48%) than down-  
302 regulated lncRNAs (median value 43%, Wilcoxon rank sum test, p-value 1e-6, Figure 4a). For  
303 protein-coding genes, the opposite trend was observed, with higher repetitive sequence  
304 contents for down-regulated genes, in both DE analyses (Figure 4a). Among the most  
305 abundant classes of repetitive elements, we found that this pattern was the strongest for  
306 satellite repeats: for both DE analyses, up-regulated lncRNAs overlap significantly more  
307 frequently with satellite repeats than down-regulated lncRNAs (Chi-square test, p-value 1e-4  
308 for the first DE analysis, p-value 8e-5 for the second DE analysis, Figure 4b). Confirming this  
309 observation, we found that lncRNAs that overlapped with satellite repeats had significantly  
310 higher fold expression changes than lncRNAs without satellite repeats, for both DE analyses  
311 (Wilcoxon rank sum test p-value 2e-6 for the first DE analysis, 0.02 for the second DE analysis,  
312 Figure 4c). We also observed significantly higher fractions of exonic overlap with LTR repeats  
313 for lncRNAs that are up-regulated in tumors with high Edmondson-Steiner grades, compared  
314 to down-regulated lncRNAs (Supplementary Figure 6). However, for this repeat class there  
315 was no significant difference between lncRNAs that are up-regulated or down-regulated  
316 between tumors and adjacent tissues (Supplementary Figure 6).

#### 317 Up-regulation of centromeric non-coding RNAs and centromeric proteins in HCC

318 Satellite repeats are a major functional component of centromeres (Hartley & O'Neill, 2019).  
319 Following our observation that lncRNAs that overlap with satellite repeats tend to be  
320 expressed at higher levels in tumors than in normal tissues, and in particular in advanced-

321 stage tumors, we performed a more direct examination of transcription in centromeric regions.  
322 As these highly repetitive sequences can be difficult to capture with next generation  
323 sequencing approaches, we first determined the centromeric regions that are mappable with  
324 our RNA-seq data – that is, to which sequencing reads can be attributed unambiguously  
325 (Methods). With the exception of the Y chromosome, which had a mappable length of 222 kb,  
326 all centromeric regions had mappable lengths comprised between 1.2 Mb and 5.2 Mb  
327 (Supplementary Figure 7a). We found 752 transcribed loci in centromeric regions, all but one  
328 detected *de novo* with our RNA-seq data (Supplementary Dataset 5). In general, we could  
329 detect at most 10 centromeric transcribed loci *per* chromosome (Supplementary Figure 7b).  
330 However, we found large numbers of transcripts on chromosomes 2 and 18 (173 and 395  
331 transcribed loci, respectively), as well as on chromosomes 1 and 19 (31 and 75 transcribed  
332 loci, respectively). With the exception of an Ensembl-annotated pseudogene, these transcripts  
333 were classified as non-coding, but only 243 passed all lncRNA filtering criteria (Supplementary  
334 Dataset 5). The other non-coding transcripts were generally rejected from the lncRNA dataset  
335 because they were too short (38% of the cases), they overlapped with unmappable regions  
336 (14%), they had insufficient read coverage (4%), or because of a combination of these criteria.

337

338 We evaluated the abundance of centromeric transcripts by counting unambiguously mapped  
339 RNA-seq for each chromosome and strand, normalized by dividing by the total unique read  
340 count attributed to genes, for each sample (Methods, Supplementary Dataset 5). Most  
341 centromeric RNA-seq reads were derived from chromosome 2, followed by chromosome 1  
342 and 19 (Figure 5a). Chromosome 2 also stood out with respect to the differences between  
343 tumor and adjacent tissue samples: on the reverse DNA strand, 94 patients (85%) had higher  
344 transcript levels in tumors than in adjacent tissue samples (Figure 5b). We note that  
345 transcription is not restricted to well-defined loci, but covers the entire centromeric region  
346 (Figure 5c).

347

348 The degree of centromere transcript activation in tumor samples compared to adjacent tissue  
349 samples varies considerably among patients (Figure 5b). To evaluate the determinants of  
350 centromeric transcription variation, we analyzed the association between protein-coding gene  
351 differential expression and centromeric transcript differential expression, across patients.  
352 Specifically, for each patient, we computed the difference in TPM levels between tumors and  
353 adjacent tissues, for each protein-coding gene; we also computed the difference in total  
354 centromeric RPKM levels between tumors and adjacent tissues, and we correlated the two  
355 sets of values across patients. Genes involved in mitotic cell cycle processes were often  
356 positively associated with centromeric transcript activation levels (Supplementary Table 7,  
357 gene ontology enrichment analysis presented in Supplementary Dataset 5). Among the genes

358 with the highest positive correlations with centromeric transcript activation levels were several  
359 genes encoding centromeric proteins (*CENPJ*, *CENPF* and *CENPI*), the CENPA chaperone  
360 *HJURP* (Hori *et al*, 2020), the *DNA2* nuclease/helicase that promotes centromeric DNA  
361 replication (Li *et al*, 2018), etc. (Figure 5d, Supplementary Table 7). Interestingly, *CENPC*,  
362 which is thought to repress alpha-satellite RNA levels (Bury *et al*, 2020), was negatively  
363 associated with centromeric transcript activation levels (Figure 5d, Supplementary Table 7).

364

365 In addition to increased levels of centromeric non-coding RNAs in tumors, we also observed  
366 a strong tendency for up-regulation for centromeric proteins (Supplementary Table 8). Out of  
367 25 protein-coding genes annotated in Ensembl as “centromere proteins”, 20 (80%) were up-  
368 regulated in the tumors compared to adjacent tissue and 13 (52%) were up-regulated in  
369 tumors with Edmondson-Steiner grades 3&4 compared to tumors with Edmondson-Steiner  
370 grades 1&2 (maximum FDR 0.01). At the top of the list, the genes coding for the histone variant  
371 *CENPA* and for centromeric protein F (*CENPF*) were more than 4-fold over-expressed in  
372 tumors compared to adjacent tissues (Supplementary Table 8). Confirming our previous  
373 analysis, we also observed that *CENPC* was down-regulated in tumors compared with  
374 adjacent tissues and in advanced-stage tumors compared to early-stage tumors  
375 (Supplementary Table 8).

376

## 377 **Discussion**

### 378 *Protein-coding gene and lncRNA expression patterns in HCC*

379 With this analysis, our first aim was to investigate the gene expression alterations that  
380 characterize HCC tumors. Compared to the numerous transcriptome collections that were  
381 previously published in the HCC field, our dataset has the advantage of including a large  
382 number of paired tumor and adjacent tissue samples, comprising a total of 268 samples from  
383 114 patients. Importantly, the samples analyzed here are derived from biopsies, which are  
384 likely to better reflect the situation *in vivo*, because they are devoid of changes induced by  
385 hypoxia and hypoglycemia that occur in surgical resection specimens as a consequence of  
386 segmental blood vessel occlusions during the operation. Moreover, our data includes both  
387 poly-adenylated and non-poly-adenylated RNA species, which makes it better suited for the  
388 study of inefficiently poly-adenylated lncRNAs (Schlackow *et al*, 2017).

389

390 We first explored the broad patterns of gene expression variation in our tumor and adjacent  
391 tissue samples. By analyzing the expression patterns of molecular markers for the most  
392 common cell types in the healthy liver (MacParland *et al*, 2018), we confirmed that HCC  
393 tumors have very different cellular environments compared to adjacent tissue samples (Figure

394 1). In particular, immune cell populations appear to be diminished in the majority of tumors,  
395 (Figure 1). Although these patterns are evidently better investigated with single-cell RNA-seq  
396 data, these results confirm that our transcriptome collection reflects the cellular composition  
397 changes that define the “tumor microenvironment” (Hanahan & Weinberg, 2011).

398

399 As expected, we found that gene expression patterns are in good agreement with the  
400 histological classification of the tumor samples. For both protein-coding genes and lncRNAs,  
401 tumor samples with Edmondson-Steiner grades 3 and 4 stand out from tumors with lower  
402 grades and from adjacent tissue samples (Figure 1, Supplementary Figures 1). Other factors,  
403 such as the underlying liver disease, the sex or the age of the patients, have comparatively  
404 little effect on the overall gene expression variation. We thus focused on the protein-coding  
405 genes and lncRNAs that are differentially expressed between paired tumor and adjacent  
406 tissue samples, or between poorly differentiated tumors (Edmondson-Steiner grades 3 and 4)  
407 and highly differentiated tumors (Edmondson-Steiner grades 1 and 2). We observed an over-  
408 representation of biological processes associated with the cell cycle among genes that are  
409 up-regulated in the tumors (Figure 2), which is expected given that cancer cells are rapidly  
410 proliferating. Conversely, genes involved in the metabolic processes performed by the healthy  
411 liver or in immune response tend to be down-regulated in the tumors (Figure 2).

412

413 Both protein-coding genes and lncRNAs contribute to the differential gene expression patterns  
414 observed in HCC tumors (Figure 2). Differentially expressed protein-coding genes and  
415 lncRNAs share many characteristics. For example, for both gene categories, we found that  
416 genes that are up-regulated in tumors compared to adjacent tissue samples have significantly  
417 higher levels of evolutionary sequence conservation than genes with the opposite expression  
418 change (Supplementary Figure 3). This observation is consistent with the enrichment of cell  
419 cycle functions among protein-coding genes that are up-regulated in the tumors, as these  
420 genes have essential biological roles and are thus under strong constraint during evolution.  
421 The increase in sequence conservation for lncRNAs that are up-regulated in tumors suggests  
422 that these lncRNAs may also participate in essential cellular functions and contribute to  
423 cellular proliferation. Another shared feature between protein-coding genes and lncRNA is the  
424 presence of spatial clustering: differentially expressed genes are found in close proximity to  
425 other differentially expressed genes with the same expression change direction significantly  
426 more often than expected by chance (Supplementary Figure 4). This observation may be  
427 explained by a tendency for co-regulation of neighboring lncRNA and protein-coding genes,  
428 or may reflect the presence of large-scale structural variations (rearrangements, duplication  
429 and deletions) in cancer cells, which can affect the expression patterns of multiple neighboring  
430 genes (Spielmann *et al*, 2018). This finding also underlines the importance of evaluating the

431 broader genomic context when aiming to select candidate oncogenes, tumor suppressors, or  
432 biomarkers: the most biologically relevant gene may be the neighbor of the gene initially  
433 selected for validation.

#### 434 Limited reproducibility of differential expression patterns for HCC-associated lncRNAs

435 In the past decade, the number of publications that discuss lncRNAs in the context of  
436 hepatocellular carcinoma has increased exponentially (Supplementary Figure 5). lncRNAs  
437 are often proposed as promising oncogenes or tumor suppressors, based on their patterns of  
438 expression in tumors and healthy tissues. However, lncRNAs are weakly expressed and are  
439 generally highly variable among tissues, cell types or individuals (Kornienko *et al*, 2016). Thus,  
440 it is not clear to what extent the lncRNA expression patterns previously reported in the HCC  
441 literature are reproducible with independent datasets. Here, we evaluated the expression  
442 patterns of lncRNAs that were previously associated with HCC in our transcriptome collection.  
443 The majority of these lncRNAs were strongly differentially expressed between paired tumors  
444 and adjacent tissue samples or between groups of tumors with high or low differentiation.  
445 However, we are still far from confirming differential expression patterns for all HCC-  
446 associated lncRNAs, even when lowering the stringency of our criteria. Even when evaluating  
447 the most prominent HCC-associated lncRNAs, which were cited by at least 5 publications, we  
448 could not always recover the previously reported differential expression observations. This  
449 was the case even for the three lncRNAs that were most frequently associated with HCC in  
450 the literature: MALAT1, H19 and HOTAIR. The biological roles of these lncRNAs in cancer  
451 were already controversial. For example, a recent study showed that MALAT1 suppresses  
452 metastasis in breast cancer (Kim *et al*, 2018, 1), contrary to previous reports which proposed  
453 that this lncRNA promotes metastasis (Arun *et al*, 2016). Likewise, H19 was alternatively  
454 proposed as an oncogene (Matouk *et al*, 2007) or as a tumor suppressor (Yoshimizu *et al*,  
455 2008). For HOTAIR, its role as a metastasis-promoting factor appears to be accepted in the  
456 literature (Gupta *et al*, 2010). However, we note that the initially proposed function for this  
457 lncRNA, namely a role in the regulation of *HOXD* genes during embryonic development (Rinn  
458 *et al*, 2007), was refuted *in vivo* (Amândio *et al*, 2016). These examples illustrate the frailty of  
459 some of the claims that are recurrently put forward regarding lncRNA functions, in cancer or  
460 in other biological contexts, and again highlight the caution that should be exercised when  
461 investigating lncRNAs.

#### 462 Activated transcription of centromeric satellite repeats in HCC tumors

463 Transcriptome comparisons in HCC cohorts or in other cancer types generally aim to select  
464 candidate oncogenes, tumor suppressors or biomarkers, that should be further verified  
465 experimentally. As extensive functional validations were outside of the scope of our study, we  
466 chose instead to analyze the genomic characteristics of the lncRNAs that were differentially

467 expressed in HCC tumors. We were thus able to detect an increase in the repetitive sequence  
468 content of lncRNAs that were up-regulated in tumors compared to adjacent tissues, as well as  
469 in poorly differentiated tumors compared to early stage tumors (Figure 4). Repetitive  
470 sequences make up roughly half of the human genome (Lander *et al*, 2001). The high repeat  
471 fraction observed for lncRNA exons, which is more than triple the fraction observed for protein-  
472 coding gene exons (Figure 4), is likely due to the weak selective pressures that act on these  
473 loci (Darbellay & Necsulea, 2020). However, the increase in repetitive sequence content for  
474 tumor-upregulated lncRNAs cannot simply be explained by a lower proportion of functionally  
475 constrained loci; on the contrary, average sequence conservation scores are higher for tumor-  
476 upregulated lncRNAs than for tumor-downregulated lncRNAs (Supplementary Figure 3).  
477 Moreover, we found that the over-representation of repetitive sequences in upregulated  
478 lncRNAs does not affect all classes of repeats, but is strongest for satellite repeats (Figure 5).  
479 This class of repeats is a major functional component of centromeres (Hartley & O'Neill, 2019).

480

481 Although centromeres were initially thought to be transcriptionally inert, it is now known that  
482 they are transcribed into non-coding RNAs, which associate with centromeric chromatin and  
483 potentially participate in kinetochore formation (Talbert & Henikoff, 2018). However, these  
484 non-coding RNAs are generally weakly transcribed, and higher expression levels can lead to  
485 impaired centromeric function (Bouzinba-Segard *et al*, 2006). Overexpression of centromeric  
486 non-coding RNAs was previously reported in pancreatic cancers and in other types of  
487 epithelial cancers (Ting *et al*, 2011). In mouse models of pancreatic cancers, it was shown  
488 that overexpression of centromeric satellite repeats leads to increased DNA damage and  
489 chromosomal instability, thereby accelerating tumor formation (Kishikawa *et al*, 2016, 2018).  
490 Here, we reveal that centromeric non-coding RNAs are also aberrantly overexpressed in HCC.  
491 This finding is supported by several lines of evidence. First, we showed that satellite repeats,  
492 which are characteristic of centromeric regions, are over-represented in the exonic regions of  
493 tumor-upregulated lncRNAs. Second, we directly quantified centromeric transcription, by  
494 evaluating regions to which RNA-seq reads can be unambiguously attributed, despite the  
495 repetitive sequence context. We thus showed that transcription stems from the entire length  
496 of centromeric regions, rather than from well-defined non-coding RNA loci. Interestingly, all  
497 chromosomes are not equal with respect to detectable centromeric transcription. The  
498 centromere of chromosome 2 appears to be transcriptionally active in tumor samples for the  
499 majority of patients (Figure 5). The mechanisms that underlie this over-representation of  
500 chromosome 2 are unclear. This chromosome has a particular evolutionary history: it is  
501 derived from a chromosome fusion event, which occurred after the divergence of human and  
502 chimpanzee and which led to the loss of one of the two ancient centromeres (Chiatante *et al*,  
503 2017). Although we verified that the over-representation of chromosome 2 is not simply due

504 to a better mappability of satellite repeats (Supplementary Figure 7), we cannot exclude other  
505 technical issues that prevent us from detecting these highly repetitive transcripts from other  
506 chromosomes.

507

508 The levels of centromeric non-coding RNA transcription were previously found to vary during  
509 the cell cycle in mouse, with a peak in the G2/M phase (Ferri *et al*, 2009). Thus, our findings  
510 may be partially explained by an over-representation of cells in the G2/M phase in tumor  
511 samples compared to the adjacent tissue, expected given that cancerous cells are rapidly  
512 proliferating. Indeed, our analysis revealed that genes involved in mitotic cell cycle processes  
513 were positively associated with centromeric transcript up-regulation levels, across patients.  
514 This included several genes encoding centromeric proteins (*CENPJ*, *CENPF* and *CENPI*)  
515 (Figure 5d, Supplementary Table 7). Interestingly, the gene encoding centromere protein C  
516 (*CENPC*) was negatively associated with centromeric transcript up-regulation levels across  
517 patients, and was significantly down-regulated in tumors compared to adjacent tissues and in  
518 advanced stage tumors (Figure 5d, Supplementary Tables 7-8). It was recently reported that  
519 this protein acts to repress centromere-derived alpha-satellite RNA levels (Bury *et al*, 2020).  
520 This observation could thus explain the up-regulation of centromeric transcripts in tumor  
521 compared to adjacent tissue samples, which appears to occur in parallel with a down-  
522 regulation of *CENPC* expression.

523

524 We also note that the ability to detect centromeric non-coding RNAs likely depends on the  
525 methods used to generate RNA-seq data. Our transcriptome collection was generated from  
526 ribo-depleted RNA samples, without enrichment for poly-adenylated RNA species (Methods).  
527 Although it was reported that centromeric transcripts are poly-adenylated (Topp *et al*, 2004),  
528 their subsequent processing into smaller RNA molecules (Talbert & Henikoff, 2018) may lead  
529 to the loss of the polyA tail, thus hampering their detection in polyA-enriched RNA-seq data.  
530 Furthermore, our RNA-seq data consists of relatively long reads (126-136 bp), which likely  
531 increases our ability to unambiguously map RNA-seq reads to the genomic regions from which  
532 they stem, even in the case of repetitive sequences.

533

534 To our knowledge, aberrant transcription of centromeric non-coding RNAs had not been  
535 previously reported in HCC. Given that this phenomenon has been associated with tumor  
536 formation in other types of cancer (Kishikawa *et al*, 2018), our observations are highly relevant  
537 for the search for oncogenic factors driving hepatocellular carcinoma, and thus warrant further  
538 investigations.

539

540



## 541 **Methods**

### 542 Biological sample collection

543 The analyses presented in this manuscript were performed on carcinoma and adjacent liver  
544 tissue biopsies obtained from 114 patients. Human tissues were obtained from patients  
545 undergoing diagnostic liver biopsy at the University Hospital Basel. Written informed consent  
546 was obtained from all patients. The study was approved by the ethics committee of the  
547 northwestern part of Switzerland (Protocol Number EKNZ 2014-099). The samples analysed  
548 here were derived from pre-treatment biopsies, with the exception of 3 patients, for which  
549 samples were collected after tumor resection (Supplementary Table 1). We recorded the sex,  
550 age at the time of biopsy and underlying liver diseases for each patient (Supplementary Table  
551 1). We also recorded the percentage of tumor tissue in the biopsies and the Edmondson-  
552 Steiner grades of the tumors (Supplementary Table 1). Multiple tumor and adjacent tissue  
553 biopsies were collected for 26 and 3 patients, respectively. In total, we analysed 268 samples,  
554 corresponding to 117 adjacent tissue and 151 tumor biopsies.

### 555 RNA extraction and library preparation

556 We extracted RNA and DNA from tissue biopsies using the ZR-Duet DNA/RNA MiniPrep Plus  
557 kit (Zymo Research, catalog number D7003). We performed the in-column DNase I treatment  
558 as specified in the kit to remove residual DNA from the RNA fraction. We prepared RNA-seq  
559 libraries using the Illumina TruSeq stranded RNA protocol, without polyA selection. We  
560 depleted ribosomal RNA using the Ribo-Zero Gold kit from Illumina. We generated single-end  
561 reads, 126 or 136 nucleotides (nt) long (Supplementary Table 1).

### 562 RNA-seq data processing

563 We aligned the RNA-seq reads on the genome using HISAT2 (Kim *et al*, 2015, 2) version  
564 2.0.5. We used the primary assembly of the human genome version GRCh38 (hg38),  
565 downloaded from Ensembl (Cunningham *et al*, 2019). We built the HISAT2 genome index  
566 using additional splice site information from Ensembl release 97, as well as from the CHES  
567 (Pertea *et al*, 2018) and MiTranscriptome (Iyer *et al*, 2015) transcript assemblies. We  
568 extracted unambiguously mapped reads based on the NH tag from HISAT2 reported  
569 alignments. To evaluate the prevalence of strand errors during library preparation, we  
570 identified introns with GT-AG and GC-AG splice sites, supported by spliced RNA-seq reads  
571 aligned on at least 8 nucleotides on each neighboring exon and with a maximum mismatch  
572 frequency of 2%. We then compared the strand inferred based on splice site information with  
573 the strand inferred based on the read alignment orientation and on the library type. All libraries  
574 had strand error rates below 2.5% (Supplementary Table 1). The presence of contradictory  
575 strand assignments was used as a red flag in our lncRNA filtering procedure (see below).

576 Single nucleotide polymorphism analysis

577 We verified that samples derived from the same patient were correctly paired by assessing  
578 their genetic similarity, using RNA-seq information alone. To do this, we first scanned the  
579 RNA-seq alignments to detect putative single nucleotide polymorphisms (SNPs). We used a  
580 a pipeline combining tools from GATK (Van der Auwera *et al*, 2013) version 4.1.9.8 and Picard  
581 (<http://broadinstitute.github.io/picard/>) version 2.18.7. Briefly, we analyzed non-duplicated  
582 aligned RNA-seq reads, re-calibrated the alignment quality around known variants from  
583 dbSNP (Sherry *et al*, 2001) release 151 and called variants with a minimum base quality score  
584 threshold of 20. We combined the detected SNPs across all samples and filtered them to keep  
585 only positions found in dbSNP and in exonic regions, excluding repetitive sequences. For all  
586 resulting SNPs, we counted the number of reads supporting each allele using the  
587 ASEReadCounter tool. We kept biallelic SNPs supported by at least 50 reads. To allow for  
588 sequencing or mapping errors, SNPs were considered to be heterozygous if the estimated  
589 allele frequency was between 0.1 and 0.9, and homozygous if the allele frequency was equal  
590 to 0 or 1. For all pairs of samples, we computed the proportion of SNPs with shared alleles  
591 out of all biallelic SNPs. We compared this measure of genetic similarity between pairs of  
592 samples derived from the same patient or from different patients (Supplementary Figure 1).  
593 We also evaluated the proportion of heterozygous SNPs out of all detected SNPs on  
594 autosomes and on sex chromosomes, for each sample. We excluded the pseudo-autosomal  
595 regions from sex chromosomes. For one male patient (identifier 42), we observed high levels  
596 of heterozygosity on the X chromosome and high *Xist* expression levels, for both tumor and  
597 adjacent tissue biopsies. This patient was excluded from differential expression analyses  
598 (Supplementary Table 1).

599 Evaluation of genomic DNA contamination

600 To assess the amount of genomic DNA contamination, we evaluated the RNA-seq read  
601 coverage on repeat-masked intergenic regions, on both forward and reverse strands. As  
602 genuinely transcribed regions are generally strongly biased in favor of one strand, we  
603 computed the number of regions that had relatively symmetric strand distribution, *i.e.* for which  
604 the absolute value of the  $(\text{forward-reverse})/(\text{forward+reverse})$  coverage ratio was below 0.5.  
605 We then computed for each sample the proportion of intergenic regions with symmetric  
606 coverage, out of all intergenic regions with RNA-seq coverage. We considered that samples  
607 with more than 5% symmetrically transcribed intergenic regions had significant DNA  
608 contamination. These samples were excluded from differential expression analyses  
609 (Supplementary Table 1).

610 Identification of “mappable” and “unmappable” genomic regions

611 To determine whether RNA-seq reads can be correctly traced back to their genomic region of  
612 origin, we performed a “mappability” analysis. To do this, we generated single-end sequencing  
613 reads with the same lengths as in our data (126 and 136 nt) from sliding genomic windows  
614 with 5 nt step. Reads were generated with perfect sequence quality and no mismatches. We  
615 aligned these reads on the genome using HISAT2 with the same parameters as for the real  
616 RNA-seq data. Genomic regions to which simulated reads were mapped back unambiguously  
617 and on their entire length were said to be mappable. We defined unmappable regions by  
618 subtracting mappable intervals from full-length chromosomes.

619 Transcript assembly

620 We performed a genome- and transcriptome-guided transcript assembly with StringTie  
621 (Pertea *et al*, 2015) release 2.1.2. We used as an input the unambiguously mapped reads  
622 obtained with HISAT2, combined across all samples. We used annotations from Ensembl  
623 (Cunningham *et al*, 2019) release 99, excluding read-through transcripts, as a guide for the  
624 assembly. We ran StringTie separately for each chromosome and strand; unassembled  
625 contigs and the mitochondrion were excluded. We filtered the StringTie output to discard  
626 artefactual antisense transcripts stemming from library preparation errors. To do this, we  
627 computed the sense and antisense exonic read coverage for each transcript and kept only  
628 those transcripts which had a sense/antisense ratio of at least 5% in at least one sample. We  
629 also removed transcripts that contained splice junctions with contradictory strand assignments  
630 based on the splice site (GT-AG or GC-AG) and on the read alignment and library type. We  
631 combined Ensembl 99 and filtered StringTie transcript annotations by adding to the Ensembl  
632 reference those *de novo* annotated transcripts which had exonic overlap with at most 1  
633 Ensembl-annotated gene. Ensembl-annotated transcripts were not altered, with the exception  
634 of read-through transcripts (defined as transcripts that overlap with more than one multi-exonic  
635 gene), which were discarded. LncRNAs that overlapped with annotated microRNAs were  
636 annotated separately from the miRNA products.

637 Protein-coding potential of newly assembled transcripts

638 We used the PhyloCSF (Mudge *et al*, 2019) codon substitution frequency score to evaluate  
639 the protein-coding potential of newly assembled transcripts. To do this, we overlapped exonic  
640 coordinates with protein-coding regions predicted by PhyloCSF, in all possible reading frames.  
641 Transcripts were said to be potentially protein-coding if they overlapped with a PhyloCSF  
642 protein-coding region on at least 150 nt. Due to the nature of the genetic code, some  
643 substitutions are synonymous on both DNA strands, which can generate artefactually high  
644 PhyloCSF scores on the antisense strand of protein-coding regions. We thus required that the  
645 overlap with PhyloCSF regions be higher on the sense strand than on the antisense strand of

646 the transcripts. We also evaluated the similarity between lncRNA sequences and known  
647 proteins and protein domains, using DIAMOND (Buchfink *et al*, 2015) against SwissProt  
648 (UniProt Consortium, 2019) and Pfam (El-Gebali *et al*, 2019). We retained SwissProt entries  
649 with high confidence scores (1 to 3) and the Pfam-A subset of Pfam. We searched for hits on  
650 repeat-masked cDNA sequences with the “blastx” flavor of DIAMOND and we required a  
651 maximum e-value of 0.01. Transcripts were said to be potentially protein-coding if they had  
652 similarity with a known protein or protein domain on at least 150 nt, with at least 40% sequence  
653 identity. Genes were said to be potentially protein-coding if at least one of their isoforms was  
654 predicted as protein-coding with either method.

#### 655 LncRNA dataset

656 We established a lncRNA dataset by combining lncRNAs annotated in Ensembl (gene biotype  
657 “lncRNA”) and transcribed loci annotated with StringTie that passed several filters: no protein-  
658 coding potential, evaluated as described above; minimum exonic length of 200 nt for multi-  
659 exonic loci and 500 nt for mono-exonic loci; at most 5% exonic length overlap with unmappable  
660 genomic regions; no overlap with Ensembl-annotated protein-coding genes on the same  
661 strand; at least 5000 nt away from protein-coding gene exons; at most 25% exonic length  
662 overlap with RNA repeats; at most 10% exonic length overlap with retrogenes (coordinates  
663 downloaded from the UCSC Genome Browser database (Casper *et al*, 2018)). We also  
664 required transcribed loci to be supported by at least 100 RNA-seq reads. lncRNA annotations  
665 are provided in Supplementary Dataset 1 online.

#### 666 Literature search for HCC-associated lncRNAs

667 We searched for articles in PubMed with the key word “hepatocellular carcinoma” in the article  
668 title. We retrieved the article abstract, title, journal and publication date. We searched for gene  
669 names in the abstract, based on a list of common gene names and synonyms in the Ensembl  
670 database. We excluded gene names that were ambiguous and matched with common terms  
671 in the HCC literature (e.g., MRI, TACE, etc). We also checked if articles contained general  
672 references to lncRNAs as a class, based on the “long non-coding RNA” and “lncRNA”  
673 keywords, with spelling variations (e.g. “noncoding” instead of “non-coding”, “lincRNA” instead  
674 of “lncRNA”, etc.).

#### 675 Gene expression estimation

676 We evaluated gene expression values with Kallisto (Bray *et al*, 2016) release 0.46.1 (patch by  
677 P.V. to correct bootstrap estimates). We obtained effective read counts and transcript *per*  
678 million (TPM) values for each isoform and obtained gene-level TPM values using tximport  
679 (Soneson *et al*, 2015) in R. We performed an additional normalization across samples, with a  
680 previously-proposed median-scaling approach based on the 100 genes that vary least in terms  
681 of expression ranks among samples (Brawand *et al*, 2011). This approach was applied on

682 gene-level TPM values. For most gene expression analyses, we used log<sub>2</sub>-transformed TPM  
683 values, adding an offset of 1 (TPM to log<sub>2</sub>(TPM+1)). As a control, we also evaluated unique  
684 read counts per gene using featureCounts from Rsubread (Liao *et al*, 2019). Expression  
685 estimation analyses were performed on the full set of detected transcribed loci, including  
686 protein-coding genes, lncRNAs and other types of genes. Gene expression levels are  
687 provided in Supplementary Dataset 2 online.

#### 688 Principal component analyses

689 We performed principal component analyses using the dudi.pca function in the ade4 library in  
690 R (Dray & Dufour, 2007). We used log<sub>2</sub>-transformed TPM levels, for all protein-coding and  
691 lncRNA genes or for each gene type separately. We enabled variable centering but not scaling  
692 and kept 5 axes.

#### 693 Differential expression analyses

694 We used DESeq2 release 1.28.0 and txlImport (Soneson *et al*, 2015) release 1.16.1 in R to  
695 assess differential expression, based on Kallisto-estimated effective read counts *per*  
696 transcript. We performed all differential expression analyses on the combined set of protein-  
697 coding and lncRNA genes. Given that the number of biopsies varied among patients, we first  
698 selected one pair of tumor and adjacent tissue samples *per* patient, to ensure patients  
699 contributed equally to DE results. For patients where biopsies were done before and after  
700 tumor resection, we selected the biopsies obtained before resection. For one patient, an  
701 adjacent tissue biopsy was performed before onset of HCC; we excluded it from DE analyses.  
702 For all other cases where multiple biopsies were available, we selected the sample with the  
703 largest number of uniquely mapped reads for each tissue type. We tested for differential  
704 expression between pairs of tumors and adjacent tissues by fitting a model that explains gene  
705 expression variation as a function of two factors, the tissue type and the patient of origin. We  
706 then evaluated the difference between tumors and adjacent tissues with a Wald test  
707 contrasting the two tissue types and estimated the effect size with the “apeglm” shrinkage  
708 method (Zhu *et al*, 2019). We repeated this analysis separately for males and females. We  
709 also tested for differences in gene expression among tumor samples, depending on the patient  
710 sex Edmondson-Steiner grade, presence or absence of hepatitis C, hepatitis B, cirrhosis,  
711 alcoholic liver disease or non-alcoholic liver disease. To do this, we fitted an additive model  
712 including all these factors and then evaluated the effect of each factor by contrasting its levels  
713 with a Wald test, using the “apeglm” shrinkage method to estimate the effect size (Zhu *et al*,  
714 2019). For the Edmondson-Steiner grade, we contrasted grades 1 and 2 against grades 3 and  
715 4. We performed a preliminary test for an age effect, but as there were no significantly DE  
716 genes this factor was not included in the model. Differential expression analyses were

717 performed only on protein-coding and lncRNA genes. Results are provided in Supplementary  
718 Dataset 3 online.

#### 719 Gene ontology analyses

720 We performed gene ontology enrichment analyses with GOrilla (Eden *et al*, 2009), contrasting  
721 the lists of up- or down-regulated protein-coding genes in each test with a background set  
722 consisting of protein-coding genes expressed in those samples. To define the background set,  
723 we evaluated the minimum expression level (DESeq2-normalized read counts) of differentially  
724 expressed genes and selected genes that had higher or equal expression levels. For the  
725 analysis of the association between protein-coding gene expression and centromeric  
726 transcript levels, across patients, we analyzed the gene ontology enrichment in a single  
727 ranked list, comparing genes at the top of the list (with high, positive correlation coefficients)  
728 to genes at the bottom of the list (with low, negative correlation coefficients).

#### 729 Cell type marker analyses

730 We analyzed the expression patterns of common markers for the most frequent cell types in  
731 the liver from a single cell RNA-seq study (MacParland *et al*, 2018). We computed a Z-score  
732 matrix from the log2-transformed normalized TPM values across samples.

#### 733 Sequence conservation analyses

734 We downloaded PhastCons (Siepel *et al*, 2005) sequence conservation scores, computed on  
735 a multiple genome alignment on human and 29 other mammalian species, from the UCSC  
736 Genome Browser (Casper *et al*, 2018). We computed average PhastCons scores on exonic  
737 regions and splice sites. For loci that overlapped with other genes, we also computed average  
738 scores on non-overlapping exonic regions. Results are provided in Supplementary Dataset 4  
739 online.

#### 740 Repetitive sequence analyses

741 We downloaded repetitive element coordinates predicted with RepeatMasker (Smit *et al*,  
742 2003) from the UCSC Genome Browser (Casper *et al*, 2018). We overlapped the exonic  
743 coordinates of all protein-coding and lncRNA loci with repetitive elements and we analyzed  
744 the exonic fraction covered by each repeat class. Results are provided in Supplementary  
745 Dataset 4 online.

#### 746 Centromeric transcription analyses

747 We downloaded centromeric region coordinates from the UCSC Genome Browser (Casper *et*  
748 *al*, 2018). We determined the mappable regions within each centromere as described above,  
749 by discarding regions deemed unmappable for 126 or 136 nt read lengths. We counted the  
750 number of unambiguously mapped reads that could be attributed to each mappable  
751 centromeric region, on each DNA strand, using featureCounts in the Rsubread R package

752 (Liao *et al*, 2019). We computed normalized expression values (RPKM) by dividing the read  
753 counts by the mappable region length (expressed in kilobases) and by the number of million  
754 mapped reads, counted on the gene models annotated in Ensembl or detected with StringTie.  
755 We extracted centromeric proteins based on the “centromere” keyword in the Ensembl gene  
756 description. We used the same set of samples selected for differential expression analyses.  
757 Results are provided in Supplementary Dataset 5 online. Centromeric transcription read  
758 coverage tracks are available online for all patients: [http://pbil.univ-](http://pbil.univ-lyon1.fr/members/necsulea/MERIC_IncRNAs/)  
759 [lyon1.fr/members/necsulea/MERIC\\_IncRNAs/](http://pbil.univ-lyon1.fr/members/necsulea/MERIC_IncRNAs/) .

#### 760 *Co-expression between centromeric transcript levels and protein-coding gene expression*

761 To evaluate the determinants of centromeric transcription variation, we estimated the  
762 correlation between protein-coding gene expression and centromeric transcript levels across  
763 patients. Specifically, for each patient we estimated the difference in centromeric RPKM  
764 between tumor and adjacent tissue samples, using the samples selected for differential  
765 expression analyses. In parallel, we computed the difference in gene TPM between tumor and  
766 adjacent tissue samples, for all protein-coding genes. We then computed Spearman’s  
767 correlation coefficients for each protein-coding gene, using the values described above for all  
768 patients. Results are presented in Supplementary Table 7 and in Supplementary Dataset 5  
769 online.

#### 770 *Data and code availability*

771 The sequencing data used in this project was submitted to the European Genome-Phenome  
772 Archive under the accession number EGAS00001004976. Supplementary datasets  
773 containing all the information needed to reproduce the results are available at the address:  
774 [http://pbil.univ-lyon1.fr/members/necsulea/MERIC\\_IncRNAs/](http://pbil.univ-lyon1.fr/members/necsulea/MERIC_IncRNAs/) . Scripts are available in GitLab:  
775 <https://gitlab.in2p3.fr/anamaria.necsulea/meric> .

776

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785 Cloud.

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791 **Figure legends**

792 Figure 1. Global expression patterns in HCC tumors and adjacent tissue samples.

793 a. Numbers of patients and RNA-seq samples included in our study. All samples are derived  
794 from pre-treatment biopsies.

795 b. Heatmap representing relative expression levels (log<sub>2</sub>-transformed TPM values, divided by  
796 the maximum value across samples), for 36 markers of the most common cell types in the  
797 healthy liver (MacParland *et al*, 2018).

798 c. Scatter plot representing the first factorial map of a principal component analysis, performed  
799 on log<sub>2</sub>-transformed TPM values for protein-coding genes. Each dot represents one sample.  
800 Colors represent sample types (adjacent tissue in grey, tumor samples colored according to  
801 the Edmonson-Steiner grade).

802 d. Same as c, for lncRNAs.

803

804 Figure 2. Differentially expressed genes between HCC tumors and adjacent tissue samples.

805 a. Density plot of the log<sub>2</sub> fold expression change, for genes that are significantly differentially  
806 expressed (maximum FDR 0.01) between paired tumor and adjacent tissue samples  
807 (Methods). Red: protein-coding genes; blue: lncRNAs. The dotted vertical lines mark an  
808 expression change threshold of 1.5. The numbers of genes that pass the FDR and minimum  
809 fold change thresholds are shown at the top of the plot. The main enriched gene ontology  
810 categories for up-regulated and down-regulated genes are shown below the plot (Methods).

811 b. Same as a, for the analysis comparing tumor samples with different stages (Edmondson-  
812 Steiner grades 1 and 2 vs. Edmondson-Steiner groups 3 and 4).

813

814 Figure 3. Differential expression patterns for prominent HCC-associated lncRNAs.

815 a. Distribution of patient characteristics for the 151 tumor samples analyzed in this study. ALD:  
816 alcoholic liver disease.

817 b. Distribution of the difference in expression levels between tumor and adjacent tissue  
818 samples, across patients, for the 29 lncRNAs that are cited in at least 5 HCC publications. The  
819 black line shows a density plot of the ratio (TPM tumor – TPM adjacent tissue)/(TPM tumor +  
820 TPM adjacent tissue), computed for each patient. Only samples used for the differential  
821 expression analyses were considered. The vertical red line represents the median value.

822 c. Presence/absence and direction of significant expression changes between paired tumor  
823 and adjacent tissue biopsies. Upward arrows indicate up-regulation in tumor samples,  
824 downward arrows indicate down-regulation in tumor samples, with a maximum false FDR of  
825 0.01 (no fold change requirement). Gray arrows represent marginally significant changes  
826 (FDR < 0.1, no fold change requirement).

827 d. Expression levels (log<sub>2</sub>-transformed TPM) for tumor samples, for the 29 lncRNAs that are  
828 cited in at least 5 HCC publications. Samples are colored depending on the Edmondson-  
829 Steiner grade.

830 e. Same as c, for the differential expression analysis comparing tumor samples with  
831 Edmondson-Steiner grades 3 and 4, *versus* tumor samples with Edmondson-Steiner grades  
832 1 and 2.

833

834 Figure 4. Over-representation of satellite repeats among tumor-upregulated lncRNAs.

835 a. Boxplots of the percentage of exonic sequences covered by repetitive sequences, for  
836 protein-coding genes (red) and lncRNAs (blue). We display separately genes that are  
837 differentially expressed (maximum FDR 0.01, minimum fold expression change 1.5) in tumors  
838 compared to adjacent tissues, and in tumors with Edmondson-Steiner grades 3 and 4  
839 compared to tumors with Edmondson-Steiner grades 1 and 2. Horizontal segments represent  
840 median values; notches represent 95% confidence intervals for the median; dashed segments  
841 extend to 1.5 times the inter-quartile range.

842 b. Percentage of genes that have exonic overlap with satellite repeats, for protein coding  
843 genes (red) and lncRNAs (blue). As in a, we display separately genes that show significant  
844 expression differences in our two main DE analyses.

845 c. Distribution of the log<sub>2</sub> fold expression changes in our two main DE analyses, for lncRNAs  
846 that overlap with satellite repeats (dark blue) or not (light blue). Only lncRNAs that are show  
847 significant differences (maximum FDR 0.01, no minimum fold change requirement) are shown.

848

849 Figure 5. Increased centromeric transcription in tumors compared to adjacent tissue samples.

850 a. Dot chart representing the median normalized expression levels (RPKM) for centromeric  
851 regions, across samples, for each chromosome and strand. Red: transcripts on the forward  
852 DNA strand, blue: reverse strand. The bars represent the 95% confidence intervals.

853 b. Density plot of the RPKM difference between tumor and adjacent tissue, across patients,  
854 for the three chromosome/strand combinations with highest RPKM levels (chromosome 2  
855 reverse, 1 reverse and 19 forward strand).

856 c. Top: representation of the regions considered to be unambiguously mappable (Methods),  
857 for the chromosome 2 centromere. Next panels: unique read coverage distribution on the  
858 chromosome 2 centromere, forward and reverse strands, for one patient (identifier 13). The  
859 read coverage was normalized for each sample based on the number of million mapped reads  
860 attributed to genes.

861 d. Boxplots representing the distribution of gene TPM differences between tumor and adjacent  
862 tissues, for three classes of patients defined based on the degree of centromeric transcript  
863 “activation” in tumors compared to adjacent tissues. The first class comprises 23 patients for

864 which the difference in RPKM values for total centromeric transcripts between tumors and  
865 adjacent tissues is below 0 RPKM; the second class comprises 61 patients for which the  
866 difference is between 0 and 50 RPKM; the third class comprises 26 patients for which the  
867 difference is above 50 RPKM. We display the 6 genes mentioned in the text: *CENPJ*, *HJURP*,  
868 *CENPF*, *DNA2*, *CENPI*, *CENPC*. P-values correspond to Kruskal-Wallis non-parametric tests,  
869 for differences among the three classes of patients.  
870

871 **Supplementary figure legends**

872 Supplementary Figure 1. Sample clustering based on gene expression and genetic similarity.

873 a. Boxplots representing the distribution of sample coordinates on principal component 1, for  
874 the PCA performed on protein-coding genes (displayed in figure 1). Samples are grouped  
875 depending on tissue types. Gray: adjacent tissue samples; yellow to red: tumors grouped by  
876 Edmondson-Steiner grade. Horizontal segments represent median values; notches represent  
877 95% confidence intervals for the median; dashed segments extend to 1.5 times the inter-  
878 quartile range.

879 b. Same as a, for principal component 2.

880 c. Same as a, for the PCA performed on lncRNAs (displayed in figure 1).

881 d. Same as c, for principal component 2.

882 e. Distribution of the proportion of shared alleles for pairs of samples, for single nucleotide  
883 polymorphisms detected with our RNA-seq data (Methods). Red: distribution observed for  
884 pairs of samples derived from different patients; black: distribution observed from pairs of  
885 samples derived from the same patient.

886

887 Supplementary Figure 2. Expression patterns of protein-coding genes and lncRNAs in HCC  
888 samples.

889 a. Distribution of the maximum expression level (log<sub>2</sub>-transformed TPM, maximum observed  
890 across samples) for protein-coding genes (red), previously known lncRNAs (dark blue) and  
891 newly annotated lncRNAs (light blue). The dotted vertical line represents the TPM = 1  
892 threshold. Numbers of genes above the threshold are shown in the figure legend.

893 b. Histogram of the number of samples in which the expression level is above the TPM = 1  
894 threshold, for the three categories of genes described in a.

895

896 Supplementary Figure 3. Differential expression patterns in HCC samples.

897 a. Comparison between the log<sub>2</sub> expression fold changes observed for our two main  
898 differential expression analyses (tumors vs. adjacent tissue samples, tumors with  
899 Edmondson-Steiner grades 3 and 4 vs. tumors with Edmondson-Steiner grades 1 and 2), for  
900 protein-coding genes. We show only genes that were significantly DE with a maximum FDR  
901 of 0.01 and a fold expression change above 1.5 in at least one of the two analyses. Green:  
902 genes with consistent expression changes in the both analyses; red: genes with opposite  
903 expression changes; orange: genes that are significantly DE only in the first DE analysis;  
904 purple: genes that are significantly DE only in the second DE analysis.

905 b. Same as a, for lncRNAs.

906 c. Distribution of sequence conservation scores for exonic regions (Methods), for protein-  
907 coding genes. Genes that are up-regulated or down-regulated in our two main DE analyses

908 are shown separately. The dot represents the median conservation score, the vertical  
909 segments represents the 95% confidence interval for the median.

910 d. Same as c, for lncRNAs.

911

912 Supplementary Figure 4. Genomic clustering of differentially expressed genes.

913 a. Proportion of differentially expressed genes (maximum FDR 0.01, minimum fold expression  
914 change 1.5), in the comparison between paired tumors and adjacent samples, that have  
915 another differentially expressed gene within a 50kb distance. Red dots represent the values  
916 observed for protein-coding genes, blue dots for lncRNAs. The gray dots and vertical intervals  
917 represent the average and the 95% confidence intervals for the random expectation, obtained  
918 through simulations (Methods). The direction of the expression change required for the focus  
919 gene and the neighboring gene is displayed below the plot.

920 b. Same as a, for the comparison between tumors with Edmondson-Steiner grades 3 and 4  
921 vs. tumors with Edmondson-Steiner grades 1 and 2.

922

923 Supplementary Figure 5. Growing interest for lncRNAs in the HCC field.

924 a. Bar plot of the fraction of publications that mention lncRNAs and HCC, from 2009 to 2019.  
925 The bars represent the percentage of publications that mention lncRNAs, out of the total  
926 number of HCC publications. The numbers of publications that mention lncRNAs are shown  
927 above the bars.

928 b. Histogram of the number of publications that cite each lncRNA in the context of HCC.  
929 lncRNAs that are cited in 5 or more publications are indicated in the plot.

930

931 Supplementary Figure 6. Increased repetitive sequence content in tumor-upregulated  
932 lncRNAs.

933 Percentage of genes that have exonic overlap with major classes of repeats, for protein coding  
934 genes (red) and lncRNAs (blue). We display separately genes that show significant expression  
935 differences in our two main DE analyses. Significantly different proportions (Chi-square test,  
936 p-value <0.05) are marked by an asterisk.

937

938 Supplementary Figure 7. Centromeric transcription characteristics.

939 a. Bar plot representing the total mappable length of centromeric regions, for each  
940 chromosome (Methods).

941 b. Bar plot representing the number of transcribed loci found in centromeric regions, annotated  
942 with our RNA-seq data.

943

944 **Supplementary tables**

945 Supplementary Table 1. Description of the 268 RNA-seq samples in our transcriptome  
946 collection.

947 Supplementary Table 2. List of cell type-specific markers for the most abundant cells in the  
948 healthy liver.

949 Supplementary Table 3. Results of our two main differential expression analyses for protein-  
950 coding genes and lncRNAs.

951 Supplementary Table 4. Gene ontology enrichment for differentially expressed protein-coding  
952 genes.

953 Supplementary Table 5. Number of HCC-related articles that mention each protein-coding and  
954 lncRNA genes.

955 Supplementary Table 6. Statistics for the overlap with different classes of repetitive elements.

956 Supplementary Table 7. Correlation between protein-coding gene expression and centromeric  
957 transcript levels across patients.

958 Supplementary Table 8. Results of our two main differential expression analyses for protein-  
959 coding genes involved in centromere functions.

960

961 **Supplementary datasets**

962 Supplementary Dataset 1. Genome annotation used in this analysis, obtained by combining  
963 annotations from Ensembl 99 and gene models detected *de novo* with our RNA-seq data.

964 Supplementary Dataset 2. Gene expression data.

965 Supplementary Dataset 3. Full results of the differential expression analyses.

966 Supplementary Dataset 4. Evolutionary sequence conservation and repetitive element overlap  
967 statistics.

968 Supplementary Dataset 5. Mappable region coordinates and expression estimates for  
969 centromeric regions.

970

971

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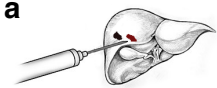
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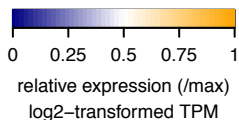
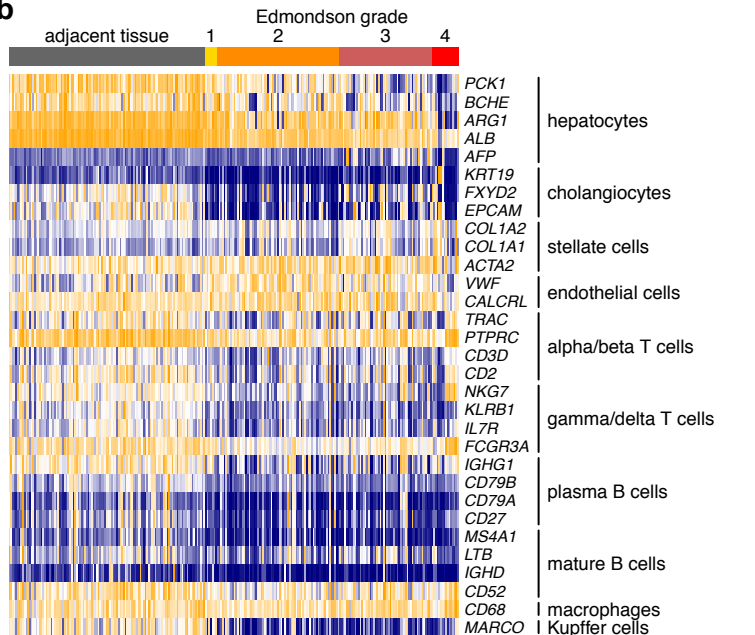
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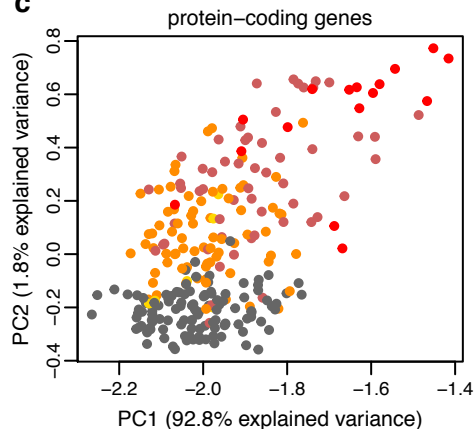
114 patients  
151 tumor biopsies  
117 adjacent tissue biopsies

**b**

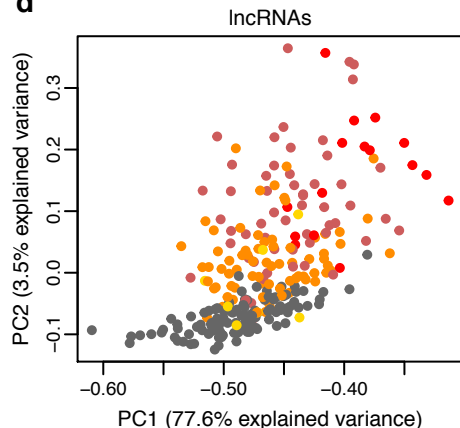


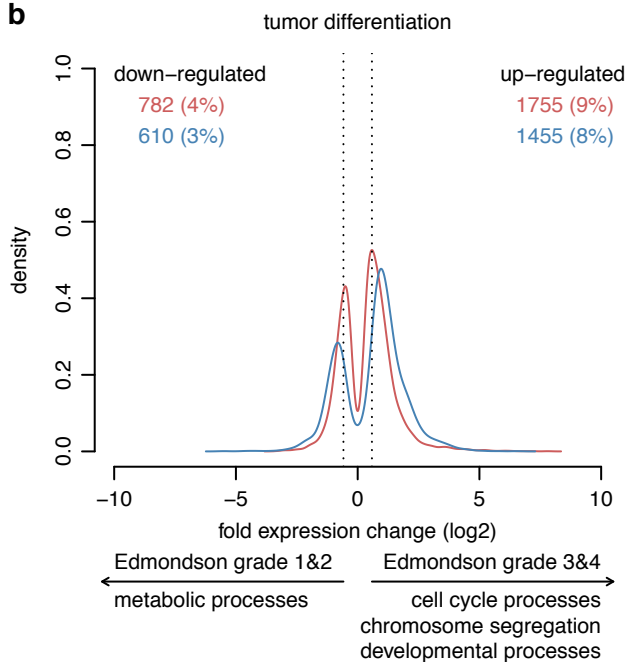
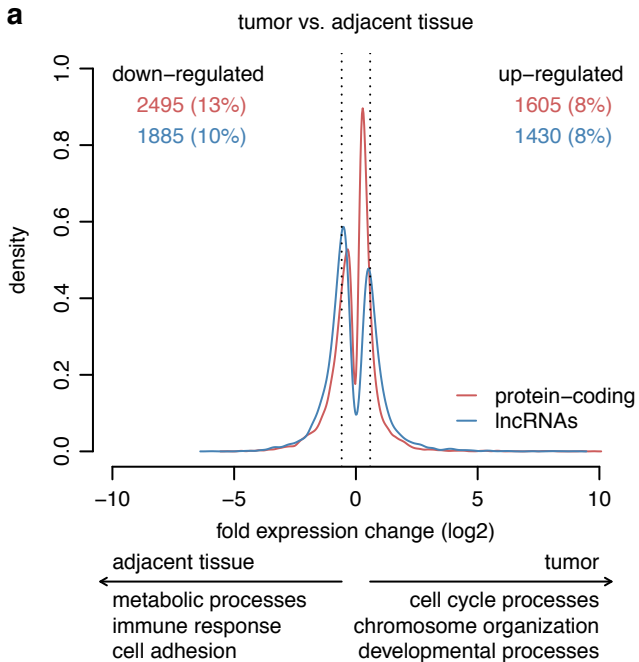
- adjacent tissue (n=117)
- Edmondson grade 1 (n=7)
- Edmondson grade 2 (n=73)
- Edmondson grade 3 (n=55)
- Edmondson grade 4 (n=16)

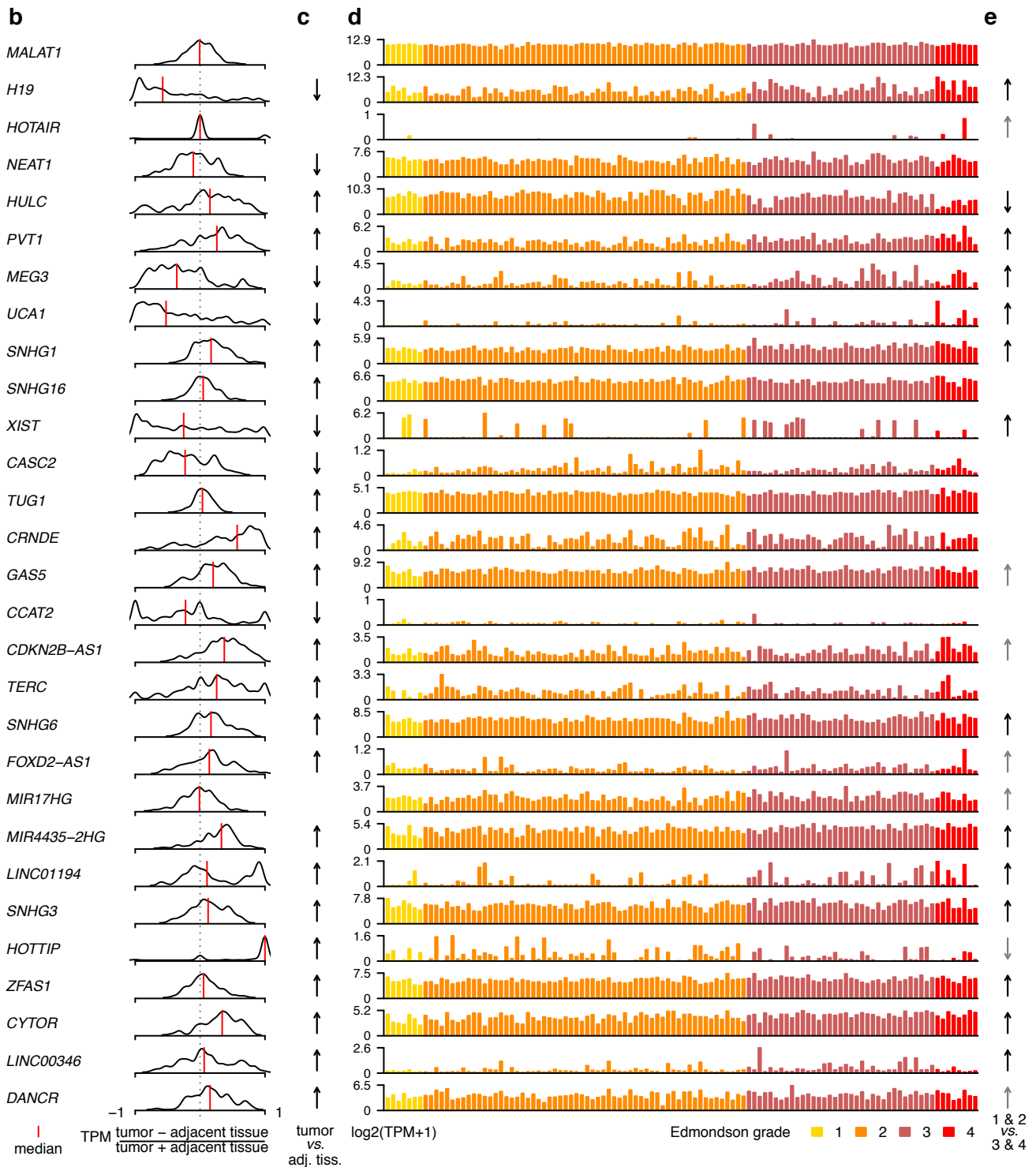
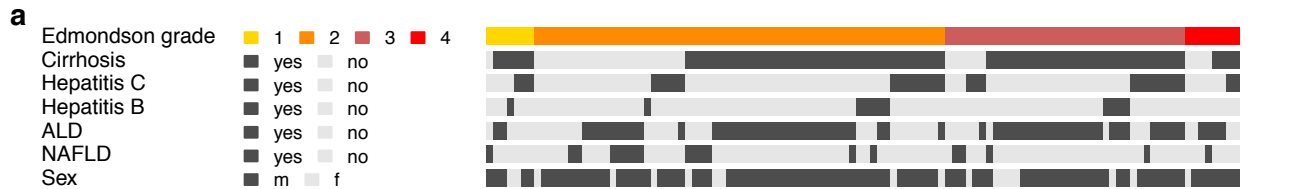
**c**

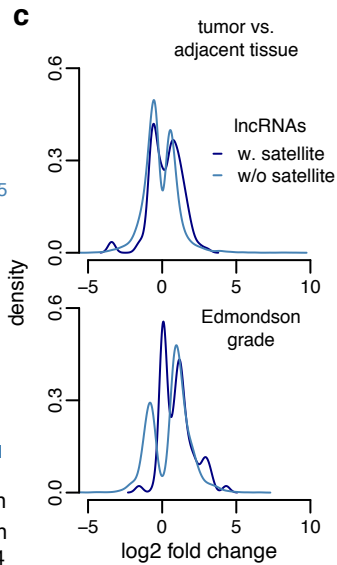
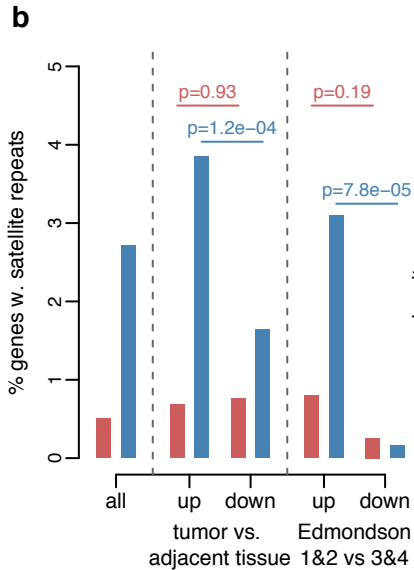
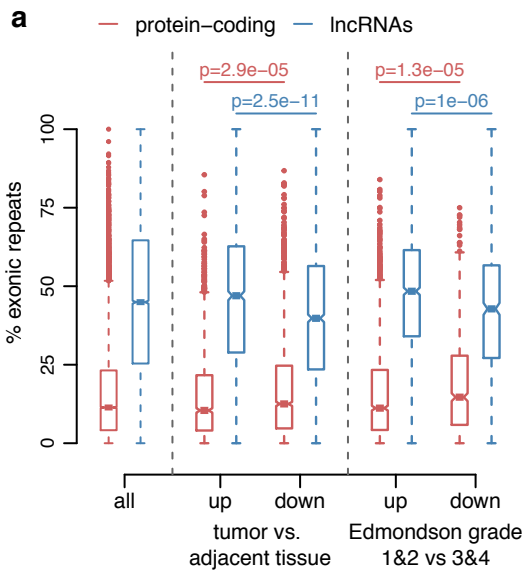


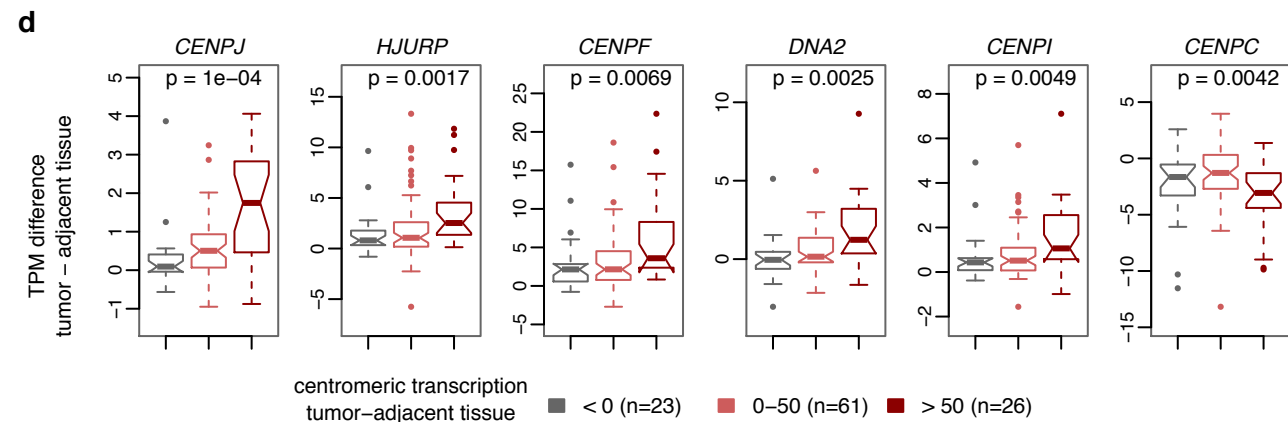
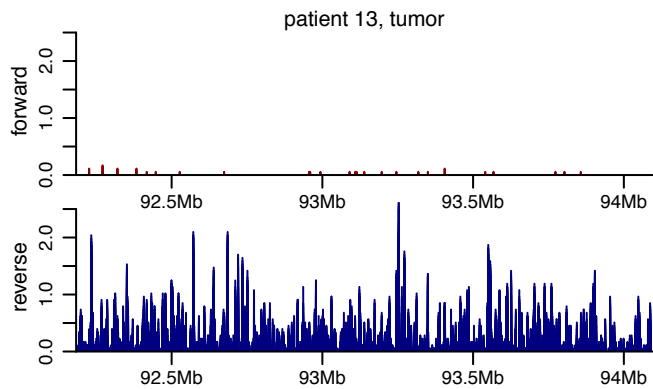
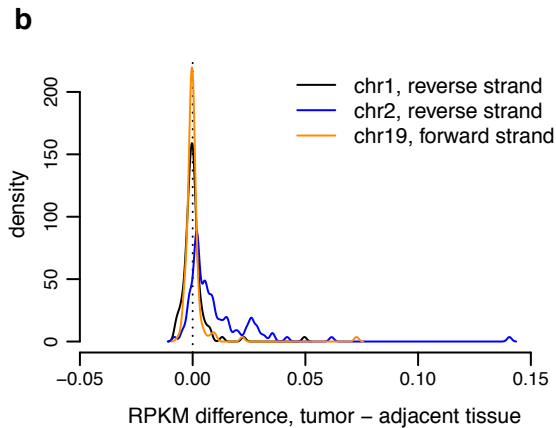
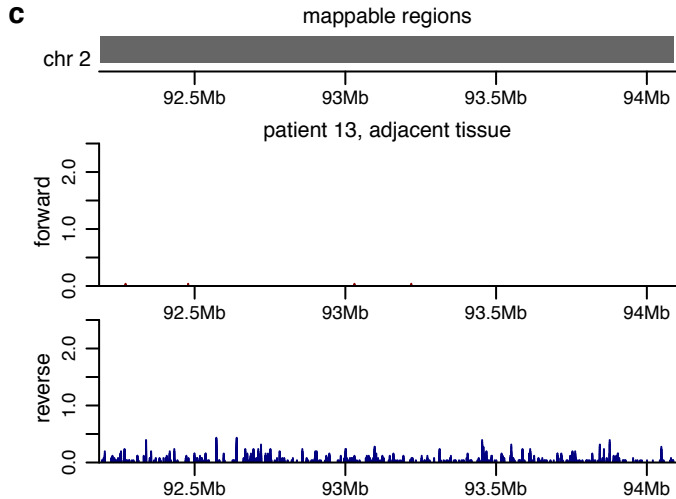
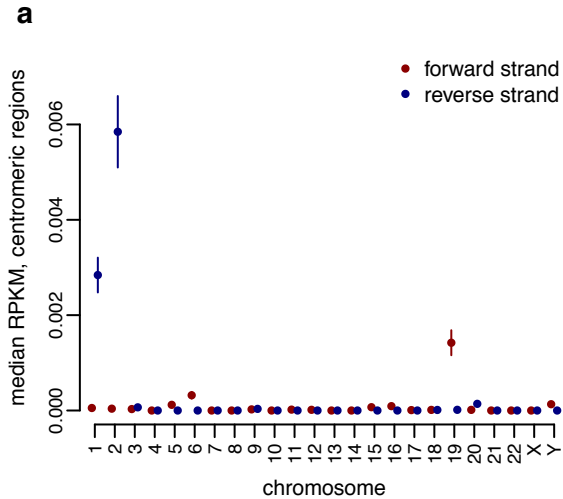
**d**



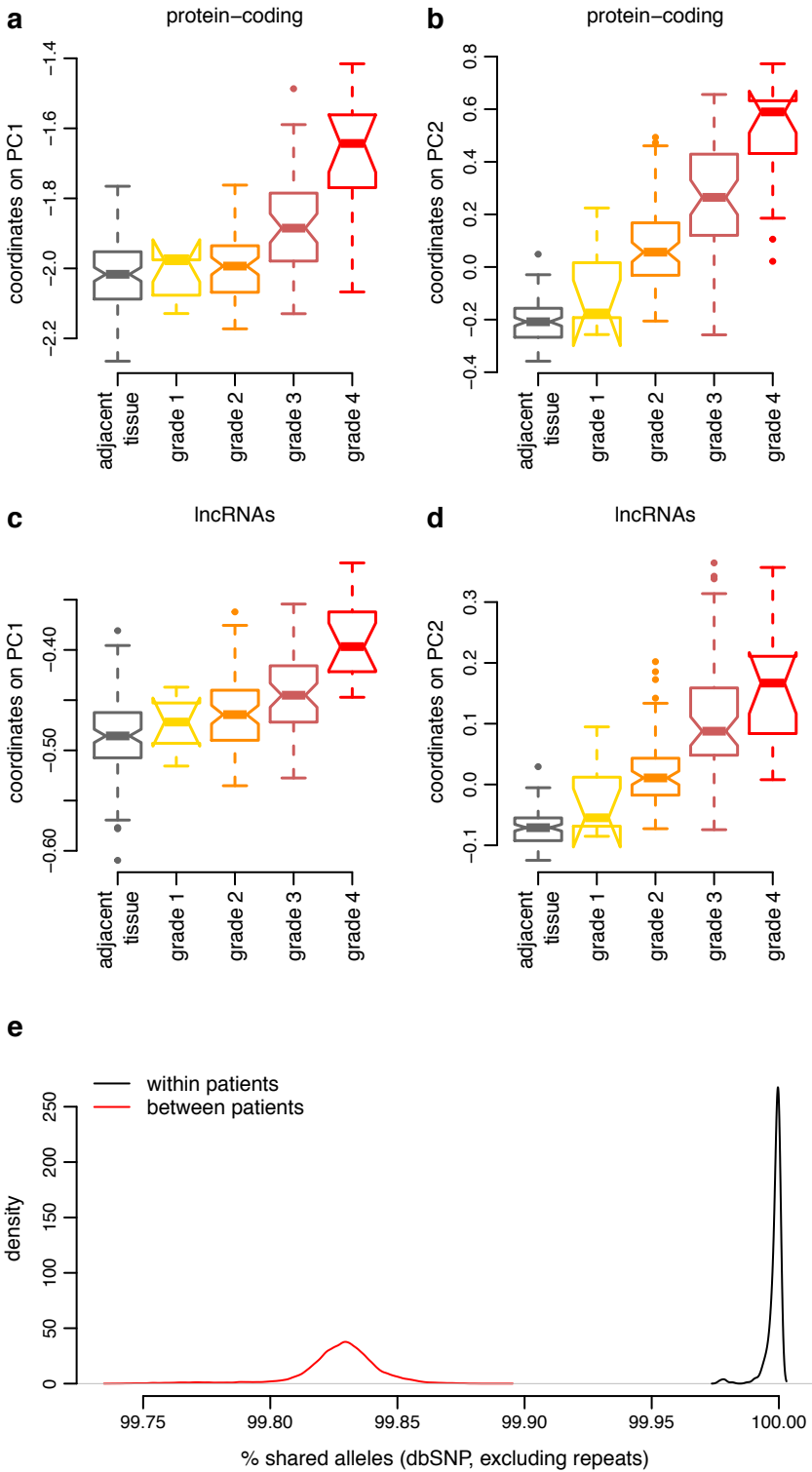


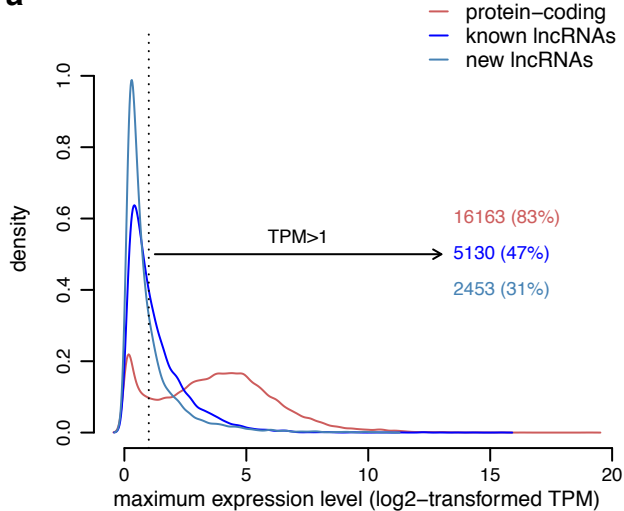
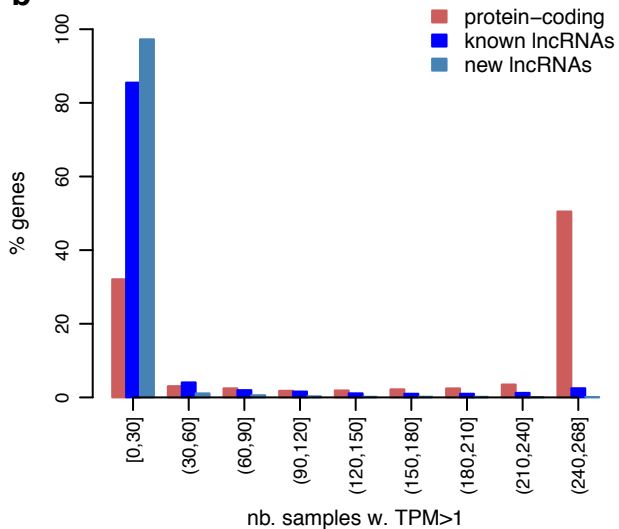


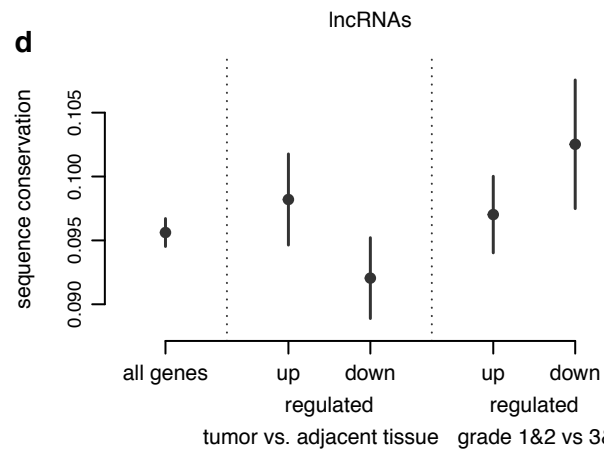
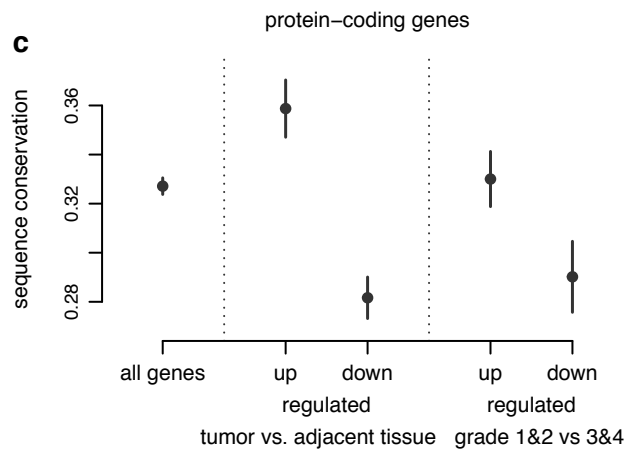
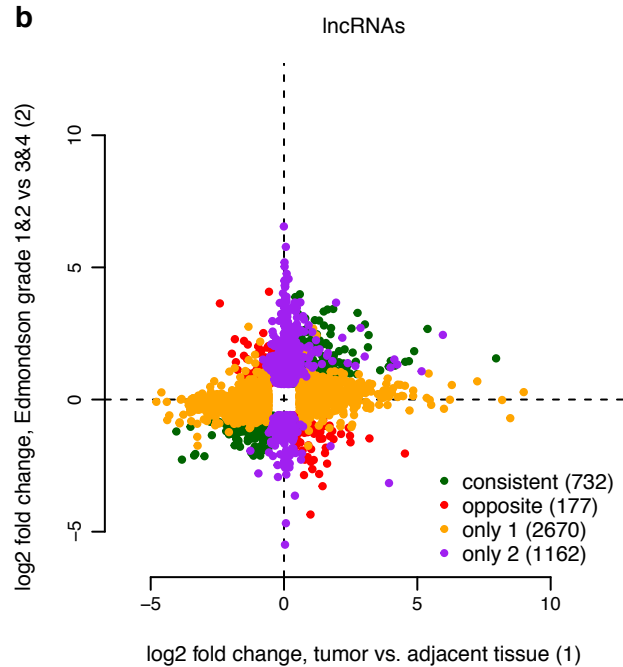
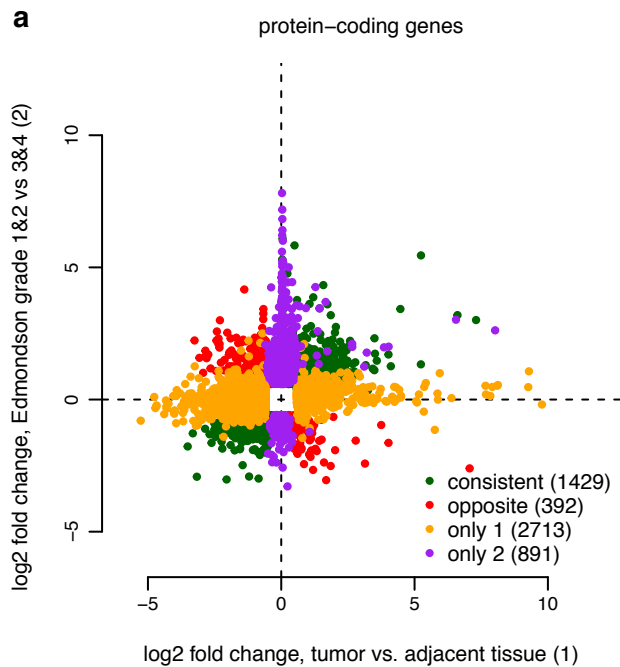


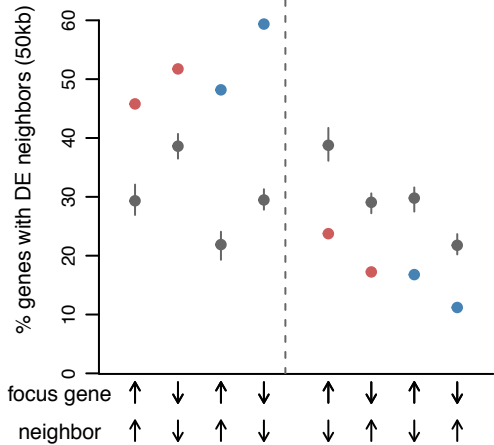
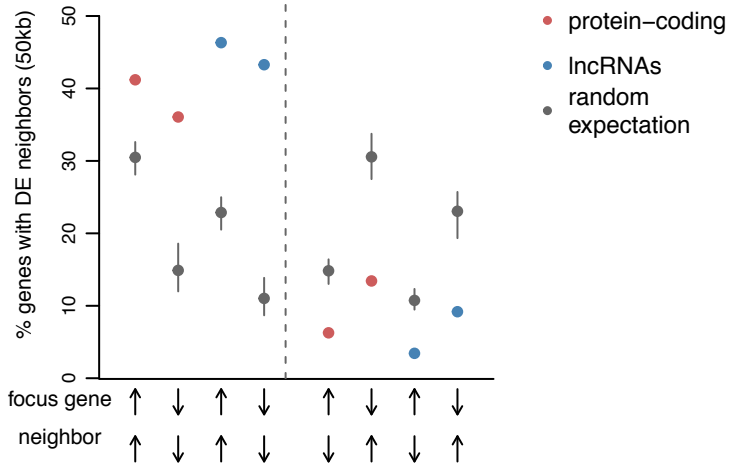


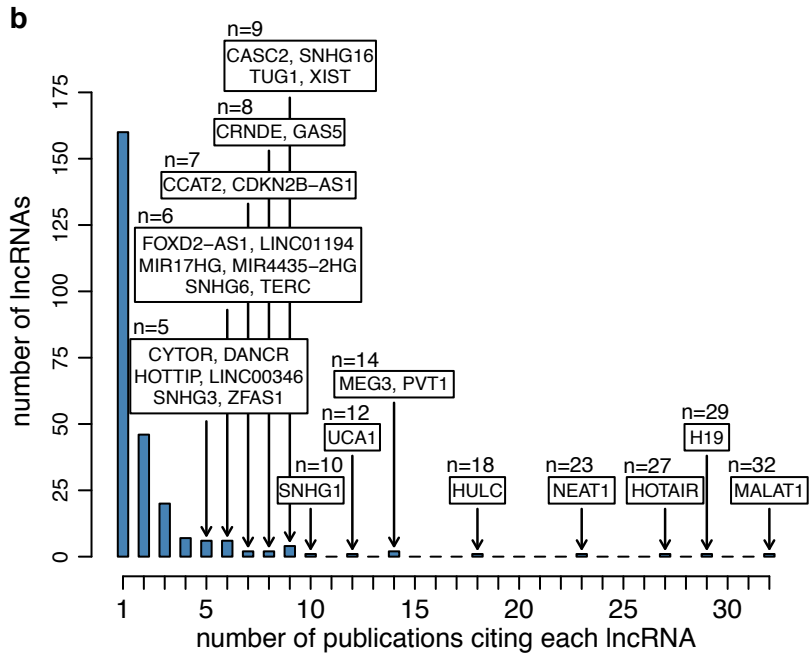
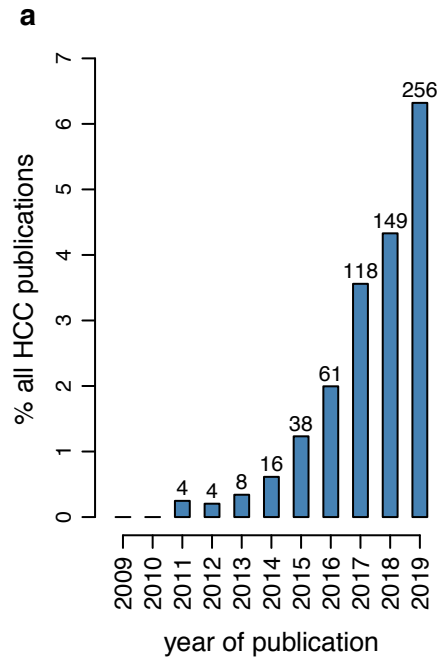


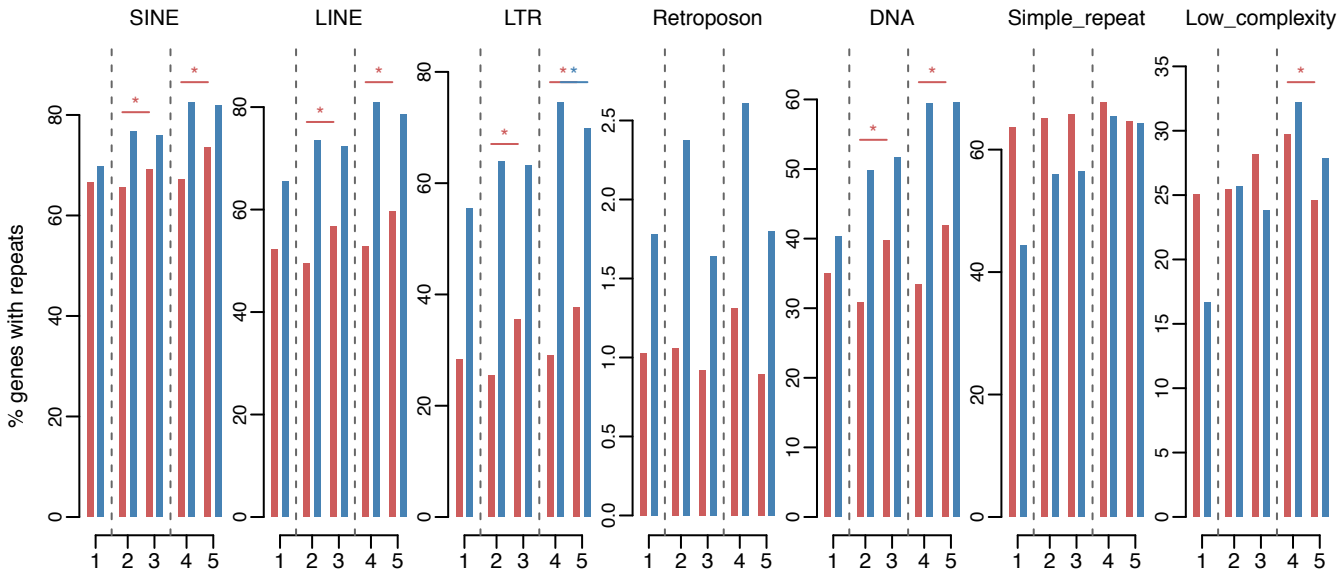


**a****b**



**a** tumor vs. adjacent tissue**b** Edmondson grade 1&2 vs. 3&4

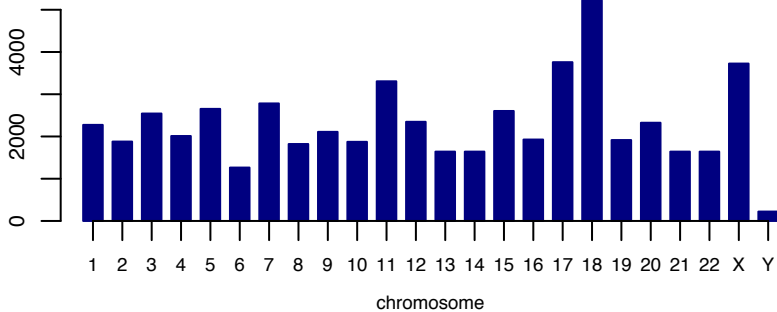




1: all genes 2: up-regulated tumor vs. adjacent tissue 3: down-regulated tumor vs. adjacent tissue

4: up-regulated grade 1&2 vs 3&4 5: down-regulated grade 1&2 vs 3&4

■ protein-coding  
■ lncRNAs

**a**  
mappable centromeric length (kb)**b**

nb. centromeric transcripts

