

# The Effect of Compound Kushen Injection on Cancer Cells: Integrated Identification of Candidate Molecular Mechanisms.

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## Abstract

**Background:** Because Traditional Chinese Medicine (TCM) preparations are often combinations of multiple herbs containing hundreds of compounds, they have been difficult to study. Compound Kushen Injection (CKI) is a complex mixture cancer treatment used in Chinese hospitals for over twenty years.

**Purpose:** To demonstrate that a systematic analysis of molecular changes resulting from complex mixtures of bioactives from TCM can identify a core set of differentially expressed (DE) genes and a reproducible set of candidate pathways.

**Study Design:** We used a cancer cell culture model to measure the effect of CKI on cell cycle phases, apoptosis and correlate those phenotypes with CKI induced changes in gene expression.

**Methods:** We treated cancer cells with CKI in order to generate and analyse high-throughput transcriptome data from two cancer cell lines. We integrated these differential gene expression results with previously reported results.

**Results:** CKI induced cell-cycle arrest and apoptosis and altered the expression of 363 core candidate genes associated with cell cycle, apoptosis, DNA replication/repair and various cancer

pathways. Of these, 7 are clinically relevant to cancer diagnosis or therapy and 14 are cell cycle regulators, and most of these 21 candidates are downregulated by CKI. Comparison of our core candidate genes to a database of plant medicinal compounds and their effects on gene expression identified one-to-one, one-to-many and many-to-many regulatory relationships between compounds in CKI and DE genes.

**Conclusions:** By identifying promising candidate pathways and genes associated with CKI based on our transcriptome-based analysis, we have shown this approach is useful for the systematic analysis of molecular changes resulting from complex mixtures of bioactives.

**Keywords:** Compound Kushen Injection, cancer cell, transcriptome, multiple targets, cell cycle, apoptosis

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## Abbreviations:

DE, differentially expressed; TCM, traditional Chinese medicine; CKI, compound Kushen injection; GO, Gene Ontology; DO, Disease Ontology; KEGG, Kyoto Encyclopedia of Gene and Genomes; PI, propidium iodide.

## 1 Introduction

2 The treatments of choice for cancer are often radiotherapy and/or chemotherapy, and while  
3 these can be effective, they can cause quite serious side-effects, including death. These side-effects  
4 have driven the search for adjuvant therapies to both mitigate side-effects and/or potentiate the  
5 effectiveness of existing therapies. Traditional Chinese Medicine (TCM) is one of the options for  
6 adjuvant therapies, particularly in China, but increasingly so in the West. While clinical trial data  
7 on the effectiveness of TCM is currently limited, it remains an attractive option because of its long  
8 history and because its potential effectiveness is believed to result from the cumulative effects of  
9 multiple compounds on multiple targets [13]. Because TCM often has not been subjected to  
10 rigorous evidence-based assessment and because it is based on an alternative theoretical system  
11 compared to Western medicine, adoption of its plant derived therapeutics has been slow.

12 In this report, we continue to characterize the molecular effects of Compound Kushen Injection

13 (CKI) on cancer cells. CKI has been approved by the State Food and Drug Administration  
14 (SFDA) of China for clinical use since 1995 [31] (State medical license no. Z14021231). CKI is an  
15 herbal extract from two TCM plants, Kushen (*Sophora flavescens*) and Baituling (*Smilax Glabra*)  
16 and contains more than 200 different chemical compounds. These compounds include alkaloids  
17 and flavonoids such as matrine, oxymatrine and kurarinol that have been reported to have  
18 anti-cancer activities [31, 47, 35, 48]. Some of these activities have been shown to influence the  
19 expression of TP53, BAX, BCL2 and other key genes known to be important in cancer cell growth  
20 and survival [45, 38, 25, 17].

21 We have previously characterized the effect of CKI on the transcriptome of MCF-7 breast  
22 carcinoma cells and in this report, we extend our previous results to two additional human cancer  
23 cell lines (MDA-MB-231, breast carcinoma and Hep G2, hepatocellular carcinoma). Both cell lines  
24 have also been shown to undergo apoptosis in response to the ingredients of CKI [35, 48, 38, 44].  
25 Hep G2 is one of the most sensitive cancer cell lines with respect to exposure to CKI [39] and CKI  
26 is often used in conjunction with Western chemotherapy drugs for the treatment of liver cancer  
27 patients in China. While the specific mechanism of action of CKI is unknown, several recent  
28 studies have reported that CKI or its primary compounds affect the regulation/expression of  
29 oncogene products including  $\beta$ -catenin, TP53, STAT3 and AKT [31, 35, 22, 18, 41].

30 However, these and other reports did not evaluate the entire range of molecular changes from  
31 treatment with a multi-component mixture such as CKI [10, 8]. Whilst several research databases  
32 and tools for TCM research have been developed [32, 34, 5], they are limited by the fact that most  
33 of the studies that contribute to the corpus of these databases are from different experimental  
34 systems, use single compounds or measure effects based on one or a handful of genes/gene  
35 products.

36 In contrast to previous studies, our strategy was to carry out comprehensive transcriptome  
37 profiling and network reconstruction from cancer cells treated with CKI. Instead of focusing on  
38 specific genes or pathways in order to design experiments, we have linked phenotypic assessment  
39 and RNA-seq analysis to CKI treatment. This allows us to present an unbiased, comprehensive  
40 analysis of CKI specific responses of biological networks associated with cancer. Our results  
41 indicate that different cancer cell lines that undergo apoptosis in response to CKI treatment can

42 exhibit different CKI induced gene expression profiles that nonetheless implicate similar core genes  
43 and pathways in multiple cell lines.

44 The current study presents the effects of CKI on gene expression in cancer cells with an aim to  
45 identify candidate pathways and regulatory networks that may be perturbed by CKI *in vivo*. To  
46 this end we primarily use concentrations of CKI higher than used *in vivo* in order to be able to  
47 detect effects in the short time frames available to tissue culture experiments. We also combine  
48 our current analysis with previously published data to focus on a shared, much smaller set of  
49 candidate genes and pathways.

## 50 Material and Methods

### 51 *Cell culture and reagents*

52 CKI (total alkaloids concentration of 25 mg/ml) in 5 ml ampoules was provided by Zhendong  
53 Pharmaceutical Co. Ltd. (Beijing, China). Chemotherapeutic agent, Fluorouracil (5-FU) was  
54 purchased from Sigma-Aldrich (MO, USA). A human breast adenocarcinoma cell line,  
55 MDA-MB-231 and a hepatocellular carcinoma cell line Hep G2 were purchased from American  
56 Type Culture Collection (ATCC, VA, USA). The cells were cultured in Dulbecco's Modified Eagle  
57 Medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum  
58 (Thermo Fisher Scientific). Both cell lines were cultured at 37°C with 5% CO<sub>2</sub>.

59 For all *in vitro* assays, 4×10<sup>5</sup> cells were seeded in 6-well trays and cultured overnight before  
60 being treated with either CKI (at 1 mg/ml and 2 mg/ml of total alkaloids) or 5-FU (150 µg/ml for  
61 Hep G2 and 20 µg/ml for MDA-MB-231). As a negative control, cells were treated with medium  
62 only and labelled as “untreated”. After 24 and 48 hours of treatment, cells were harvested and  
63 subjected to the downstream experiments.

### 64 *Cell cycle and apoptosis assay*

65 The assay was performed as previously described [28]. For each cell line, three operators  
66 replicated the assay twice in order to ensure reproducibility of the observations. The results were  
67 obtained by flow cytometry using either FACScanto or LSRII (BD Biosciences, NJ, US).

68 *RNA isolation and sequencing*

69 The treated cells were harvested, and the cell pellets were snap frozen with liquid nitrogen and  
70 stored at -80°C. Total RNA was isolated with PureLink™ RNA Mini Kit (Thermo Fisher  
71 Scientific) according to the manufacturer's protocol. After quantified using a NanoDrop  
72 Spectrophotometer ND-1000 (Thermo Fisher Scientific), the quality of the total RNA was verified  
73 on a Bioanalyzer by Cancer Genome Facility (SA, Australia) ensuring all samples had RINs>7.0.

74 For both cell lines, the sequencing was performed in Ramaciotti Centre for Genomics (NSW,  
75 Australia). The sample preparation for each cell line was TruSeq Stranded mRNA-seq with dual  
76 indexed, on the NextSeq500 v2 platform. The parameter was 75bp paired-end High Output. The  
77 fastq files were generated and trimmed through Basespace with application FASTQ Generation  
78 *v1.0.0*.

79 *Bioinformatics analysis of RNA sequencing*

80 The clean Hep G2 reads were aligned to reference genome (hg38) using STAR v2.5.1 with  
81 following parameters: –outFilterMultimapNmax 20 –outFilterMismatchNmax 10 –outSAMtype  
82 BAM SortedByCoordinate –outSAMstrandField intronMotif [6]. The clean MDA-MB-231 reads  
83 were aligned to reference genome (hg19) using TopHat2 v2.1.1 with following parameters:  
84 –read-gap-length 2 –read-edit-dist 2 [15]. Differential expression analysis for reference genes was  
85 performed with edgeR and differentially expressed (DE) genes were selected with a False Discovery  
86 Rate<0.05 [29].

87 The DE genes in common for both Hep G2 and MDA-MB-231 cell lines at 24 hours and 48  
88 hours after CKI treatment were selected as “shared” genes. These shared genes were utilized to  
89 describe the major anti-cancer functions and principal mechanisms of CKI.

90 Gene Ontology (GO), and Kyoto Encyclopedia of Gene and Genomes (KEGG)  
91 over-representation analyses of both cell lines were carried out using the online database system  
92 ConsensusPathDB [14] with the following settings: “Biological process” at third level (for GO); q  
93 values (<0.01) were corrected for multiple testing with the system default settings. Disease  
94 ontology (DO) over-representation analyses of both cell lines were performed by using the  
95 Bioconductor R package clusterProfiler v3.5.1 [40]. For the function analyses of shared/core genes,

96 the method was as similar as our previous study [28] using ClueGO app 2.2.5 in Cytoscape v3.6.0.  
97 We enriched our GO terms in the biological process category level 3 and KEGG pathways,  
98 showing only terms/pathways with  $p$  values less than 0.01. Specific Over-represented  
99 terms/pathways and gene expression status mapping in KEGG pathways were visualised with the  
100 R package “Pathview” [21].

101 *Gene expression-based investigation of bioactive components in CKI*

102 To integrate with previous data from MCF-7 cells [28], all the shared DE genes regulated by  
103 CKI identified in all three cell lines using edgeR were mapped to the BATMAN-TCM database  
104 [19]. The pharmacophore modelling method [16] was used to generate the interaction network  
105 between the key genes and TCM components using R package igraph [4].

106 *Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

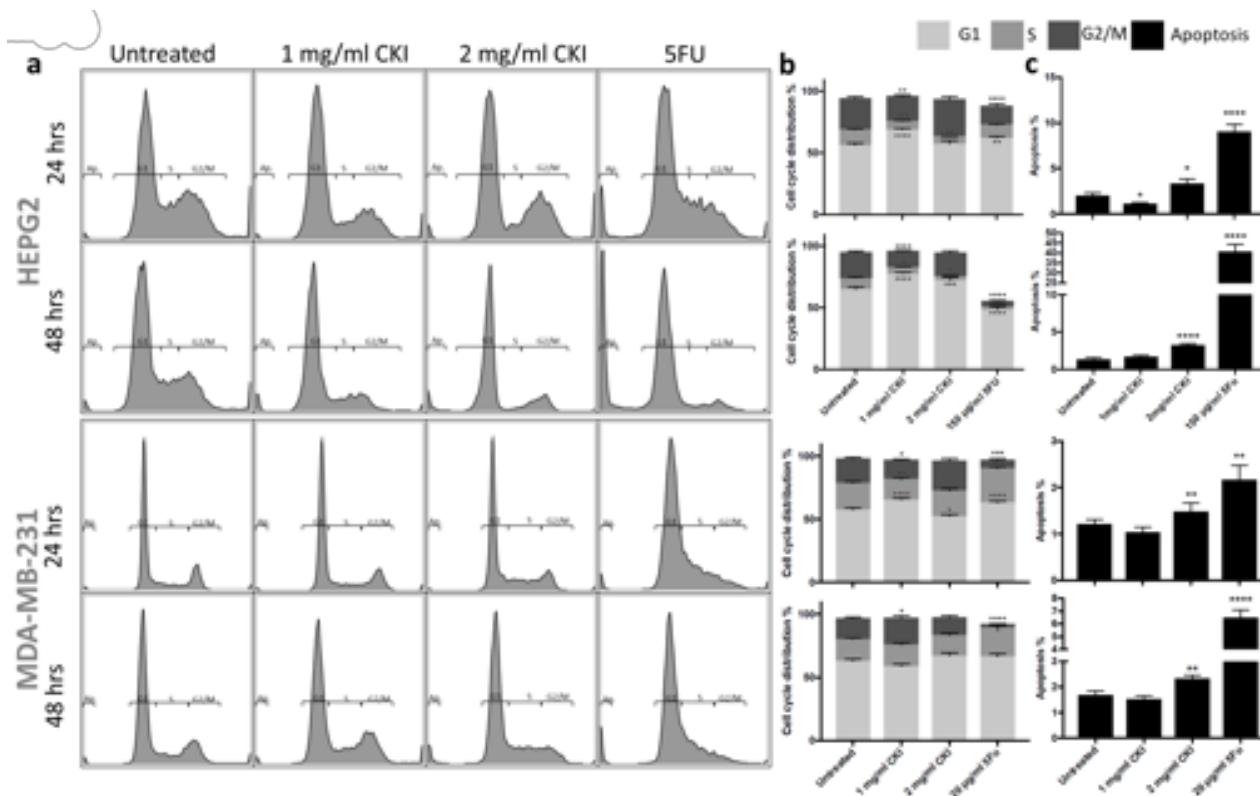
107 RT-qPCR was performed as previously described [28]. The list of target genes selected for this  
108 study and the sequences of all primers are shown in Additional file 1: Table S1.

109 **Results**

110 *Effect of CKI on the cell cycle and apoptosis*

111 In our previous study, CKI significantly perturbed/suppressed cancer cell target genes/networks.  
112 In the current study we present results that confirm and generalize our previous work. We  
113 observed in the MCF-7 study, low concentrations of CKI in our short-term cell assay showed  
114 no/little phenotypic effect within 48 hours, and very high doses resulted in excessive cell death at  
115 48 hours precluding the isolation of sufficient RNA for transcriptome analysis [28]. Therefore, in  
116 our current study with the two additional cell lines, to ensure consistency, we also selected 1  
117 mg/ml and 2 mg/ml total alkaloid concentrations of CKI for our assays because they generated  
118 reproducible and significant phenotypic effects in our cell culture assay.

119 We used flow cytometric analysis of propidium iodide stained cells to assess both CKI induced  
120 alterations to the cell cycle and apoptosis. In Hep G2 cells, CKI treatment resulted in an overall



**Figure 1. Effects of different treatment on cell cycle and apoptosis of Hep G2 and MDA-MB-231 cells.** A) The apoptosis and cell cycle distribution of each cell line after 24- and 48-hour treatments with CKI or 5-Fu assessed PI staining. B) Percentages of cells in different phases of cell cycle resulting from treatment. C) Percentage of apoptotic cells after treatment. Results shown are mean  $\pm$  SEM (n=6). Statistically significant differences from untreated control were identified using two-way ANOVA (\*p<0.05, \*\*p<0.01, \*\*\*p<0.0001).

121 increase in the proportion of cells in G1 phase and decrease in S phase (Fig. 1a and b). Similarly,  
122 in MDA-MB-231 cells, although a consistent increase in G1 phase was not observed, CKI caused a  
123 decrease in S phase particularly at the 24-hour time point (Fig. 1a and b) indicating possible  
124 incidence of cell cycle arrest at G1 phase. Furthermore, at 2 mg/ml of total alkaloids, CKI  
125 consistently induced significantly higher level of apoptosis in both cell lines at both time points  
126 compared to untreated controls (Fig. 1c). These data together suggest that CKI has effects on the  
127 cell cycle by interfering with the transition between G1 to S phase as well as by acting on the  
128 apoptosis pathway and promoting cell death.

#### 129 CKI perturbation of gene expression

130 In order to elucidate the molecular mechanisms of action of CKI on these cancer cells,  
131 transcriptome analysis of CKI treated cells was performed. As mentioned above, RNA samples  
132 from two cell lines were sequenced with 2 $\times$ 75 bp paired-end reads. We had previously sequenced

133 transcriptomes from CKI treated MCF-7 cells [28] and have included those results for comparison  
134 below. The samples from each cell line contained 7 groups at 3 time points (Fig. 2a), in triplicate  
135 for every group. In the multidimensional scaling (MDS) analysis, each cell line clustered  
136 independently and generally, within the cell line clusters, untreated cells clustered apart from  
137 treated cells (Additional file 2: Fig. S1).

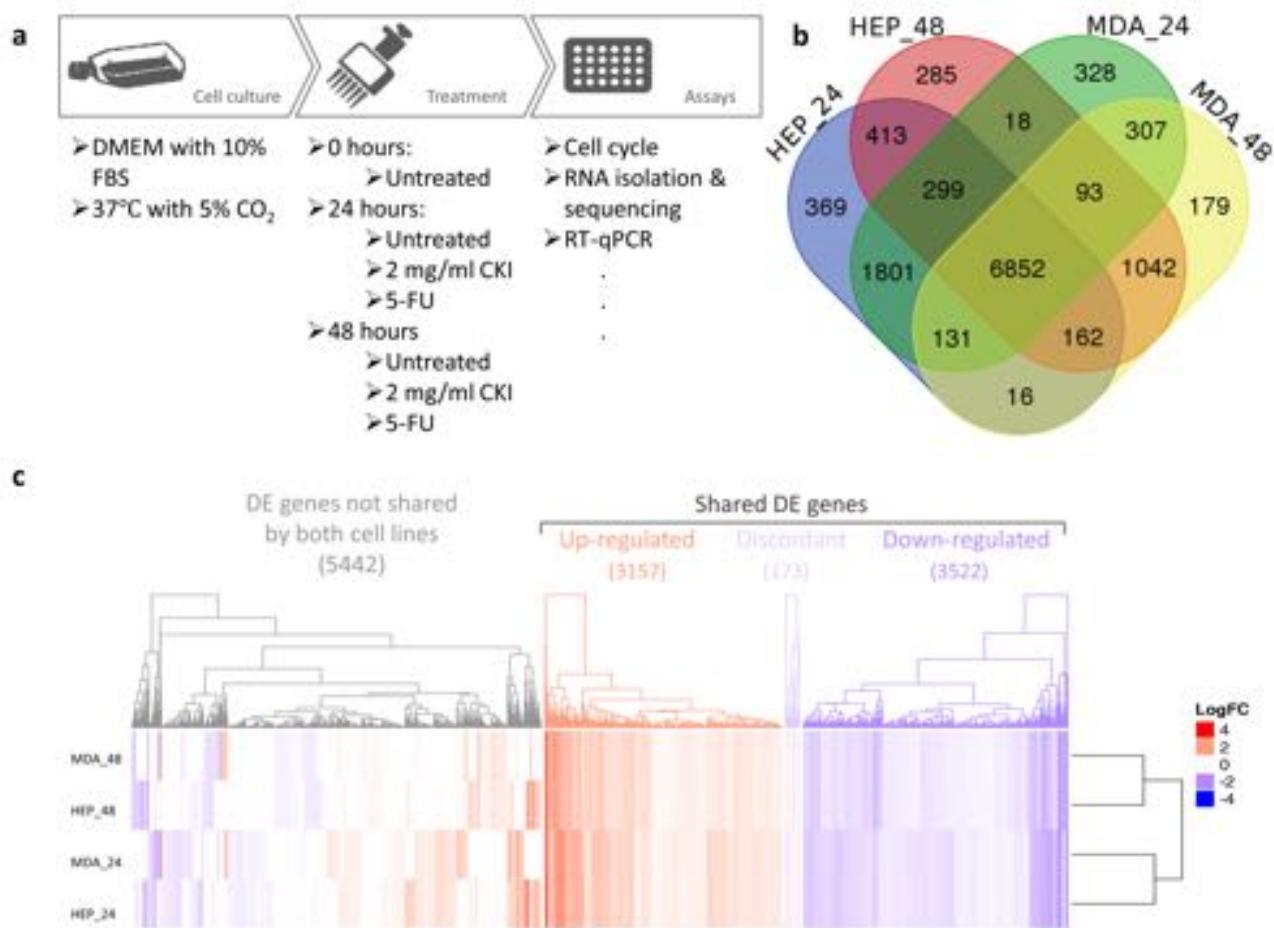
138 With the mapping rate were around 90% (Additional file 3: Table S2), a *p*-value based ranked  
139 list of DE genes (compared to untreated from each time point) was generated for both cell lines  
140 (Additional file 4: Table S3, sheet 1-4). This list was used to select the shared DE genes. This  
141 analysis generated thousands of DE genes (Additional file 4: Table S3, sheet 5) across Hep G2 and  
142 MDA-MB-231 cell lines.

143 Because for each cell line the respective treatment groups clustered together on the MDS plot,  
144 there were large numbers of shared genes between them. As a result, we identified a set of 6852  
145 shared DE genes by identifying common DE genes from Hep G2 and MDA-MB-231 cell lines, at 24  
146 hours and 48 hours (Fig. 2b). These shared genes might predict a common molecular signature for  
147 CKI's activity. However, there were still a large number of DE genes that were not shared by both  
148 cell lines, as seen in the heatmap in Fig. 2c. The expression of the shared gene set in both Hep G2  
149 and MDA-MB-231 is highly consistent. Interestingly, this consistency is with respect to treatment  
150 time, rather than with respect to cell line.

### 151 *RT-qPCR validation and dose response of gene expression to CKI*

152 Based on our previous results [28], and analysis below, we selected the 4 top ranked DE genes  
153 expressed in G1-S phase of the cell cycle (TP53 and CCND1 for expression level validation and  
154 E2F2 and PCNA for low dose response), as well as the proliferation and differentiation relevant ras  
155 subfamily encoding gene (RAP1GAP1) for low dose response. We also selected a prominently  
156 expressed gene (CYP1A1) for validation because of its sensitivity to CKI treatment. CYP1A1,  
157 TP53 and CCND1 expression changes were validated with RT-qPCR with all three genes showing  
158 similar patterns of expression in the transcriptome data and RT-qPCR (Fig. 3a).

159 Because low dose treatment with CKI did not cause significant gross phenotypic effects in either  
160 cell line, we decided to use gene expression as a more sensitive measure of phenotype to look at



**Figure 2. DE genes shared in both cell lines at both time points.** A) Work flow diagram showing experimental design and sample collection. B) Venn diagram showing the number of shared DE genes between Hep G2 and MDA-MB-231. C) Heatmap presenting the overall gene expression pattern in both cell lines treated with CKI. Heatmap is split into four parts based on gene content and expression pattern: 5442 differentially regulated genes with expression not shared between the two cell lines, 3157 upregulated genes shared between both cell lines, 3522 down-regulated genes shared between both cell lines, and 173 discordantly regulated genes with differential expression shared between both cell lines.

161 the effect of lower doses of CKI. We used 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml  
162 concentrations to look for dose dependency of gene expression. Our results showed an obvious  
163 dose-dependent expression trend (Fig. 3b) in both cell lines. Because the 0.125 mg/ml  
164 concentration of CKI is equivalent to what cancer patients are treated with, our results are  
165 potentially clinically relevant.

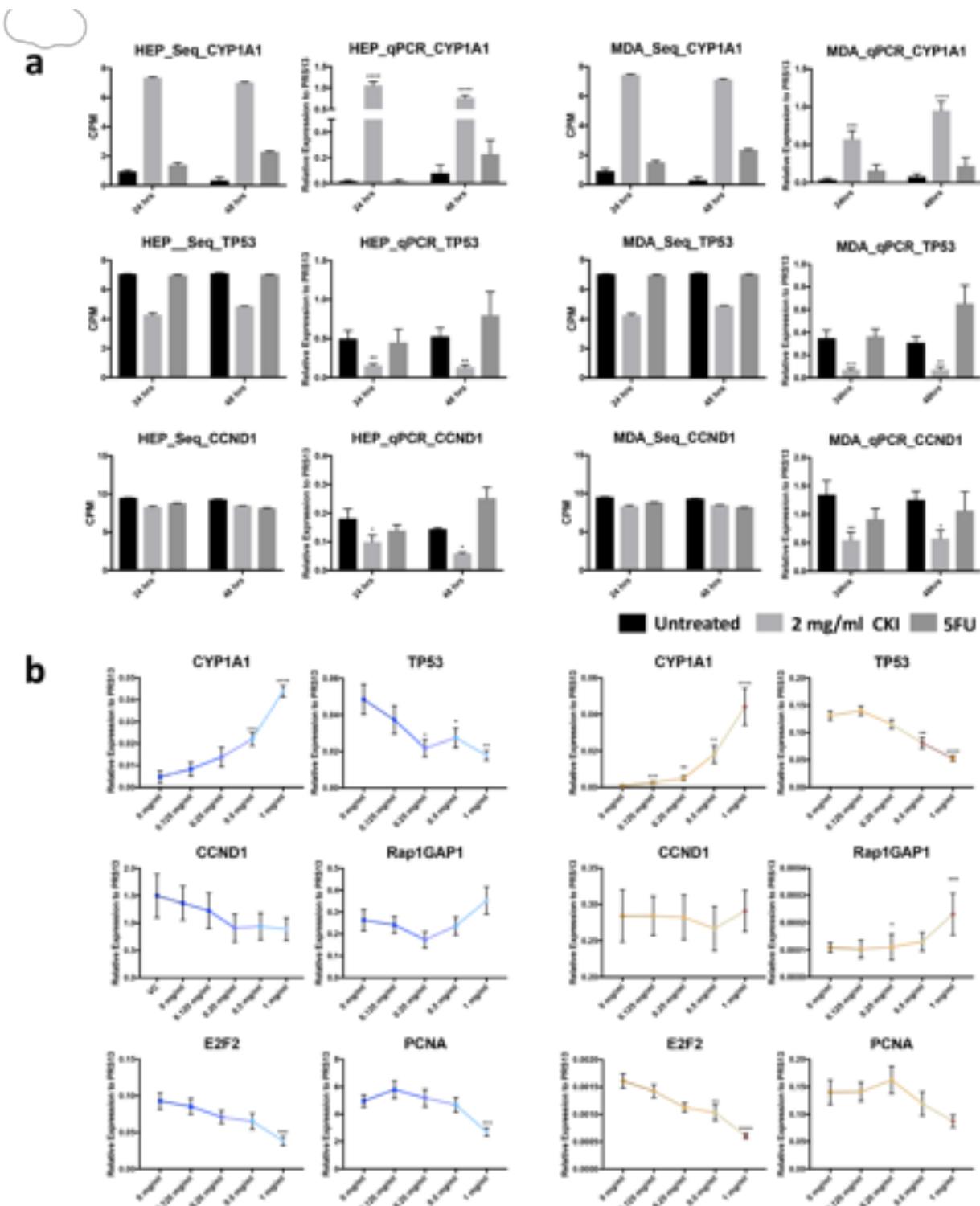
166 *Function enrichment analysis*

167 To identify candidate mechanisms of action of CKI, we carried out functional enrichment  
168 analysis. We used ConsensusPathDB [14] and Clusterprofiler [16] along with GO and KEGG  
169 pathways for over-representation analysis, along with disease ontology (DO) [30] enrichment.

170 GO over-representation test was determined based on Biological Process level 3 and q value  
171 <0.01. The results for both cell lines at both time points were summarized and visualized based  
172 on semantic analysis of terms in Fig. 4a. From this result, it was obvious that there were a large  
173 proportion of enriched GO terms relating to cell cycle, such as “cell cycle checkpoint”,  
174 “negative/positive regulation of cell cycle process” and so on prominently featured for all data sets  
175 (Additional file 5: Fig. S2, Additional file 6: Table S4, sheet 1-4).

176 We then used KEGG pathways to determine the specific pathways altered by CKI in cancer.  
177 The most regulated over-representative KEGG pathways are summarized according to KEGG  
178 Orthology (KO) (Fig. 4b). Cell cycle related pathways such as “cell cycle”, “DNA replication”,  
179 and “apoptosis” were also consistently seen in the KEGG enrichment results (Additional file 6:  
180 Table S4, sheet5-8) at both 24 and 48 hours. Moreover, in addition to the cell cycle relevant  
181 pathways, some cancer related pathways were also observed, such as “prostate cancer” and  
182 “chronic myeloid leukaemia”, and a large number of DE genes (283) from the two cell lines were  
183 relevant in “pathways in cancer”.

184 Because the KEGG enrichment revealed many pathways relating to diseases, most of which  
185 were cancers, we decided to explore the enrichment of DE genes with respect to DO terms (Fig.  
186 4c). In the DO list (Additional file 6: Table S4, sheet 9-12), all top ranked terms listed are cancers.  
187 Interestingly, most cancer types listed are from the lower abdomen, for example “ovarian cancer”,  
188 “urinary bladder cancer “and “prostate cancer” etc. occurring in genitourinary organs (Additional



**Figure 3. Validation of gene expression and effects of low dose CKI using RT-qPCR.** A) Comparison of DE genes between RNA-seq results (left) and RT-qPCR validation (right) for each cell line at 2 time-points. Three DE genes (CYP1A1, TP53 and CCND1) were chosen for validation. Gene expression was generally consistent between transcriptome data and qPCR data. B) Dose response of CKI using a subset of genes with conserved expression in Hep G2 (left), and MDA-MB-231 (right) from 0 mg/ml to 1 mg/ml of total alkaloids. Six genes (CYP1A1, TP53, CCND1, Rap1GAP1, E2F2 and PCNA) were selected based on their relevance to important pathways perturbed by CKI. RT-qPCR results are presented as expression relative to RPS13. Data are represented as mean  $\pm$  SEM (n>3). A t-test was used to compare CKI doses with “untreated” (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).

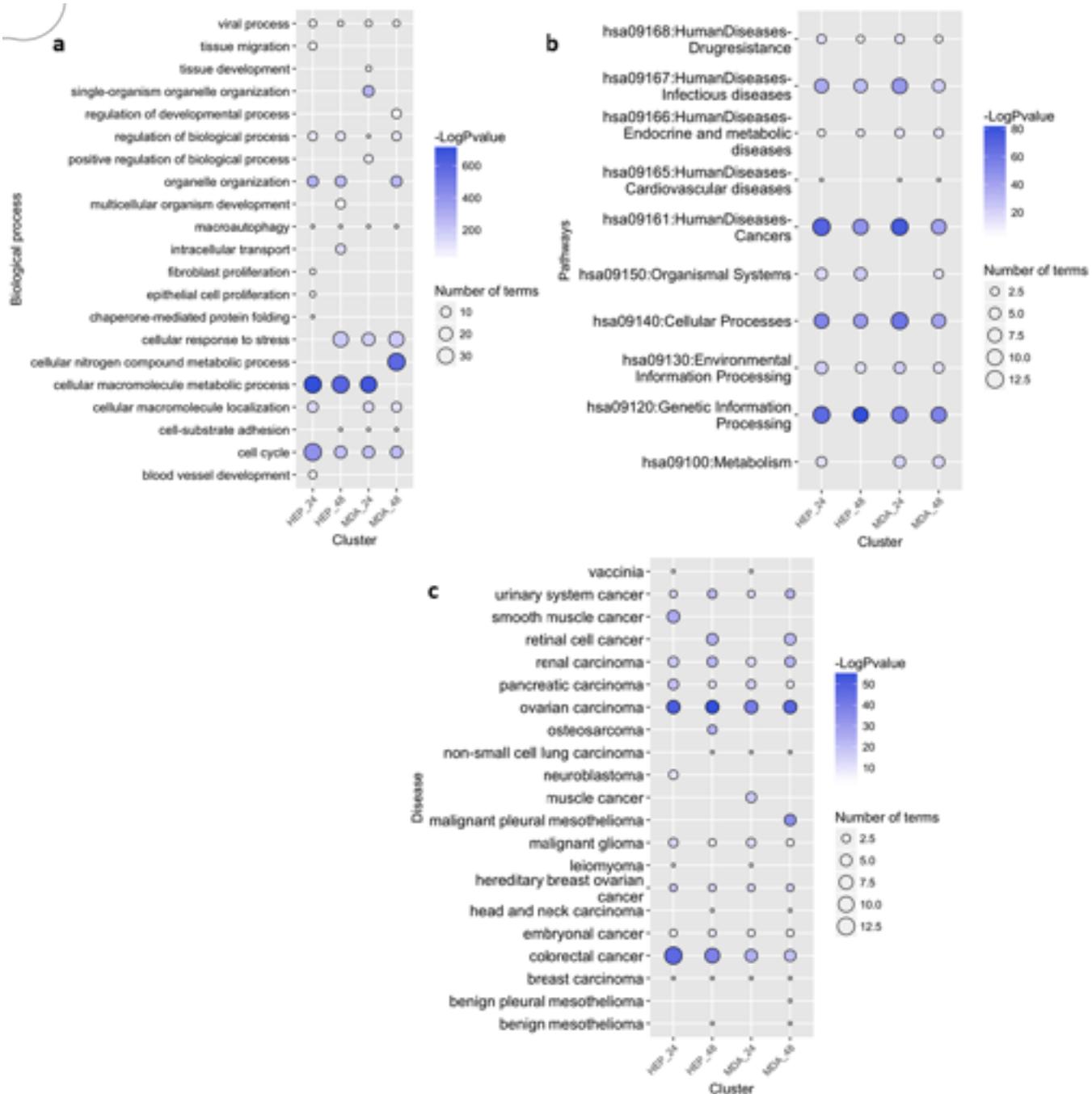
189 file 6: Table S4, sheet 9-12). For both KEGG pathway and DO enrichment, the effects of CKI on  
190 both cell lines were similar.

191 In addition to cell line specific functional enrichment of DE genes, we also analyzed the  
192 over-represented GO terms for shared DE genes (Fig. 5a). The most significant clusters were  
193 highly relevant to metabolic process, such as “cellular macromolecule metabolic process”, as well as  
194 the corresponding positive/negative regulatory biological process (Additional file 6: Table S4, sheet  
195 13). Moreover, various signaling pathways, though not forming a large cluster, were also significant,  
196 for example, “regulation of signal transduction” and “intracellular receptor signaling pathway”.  
197 Finally, some “cell cycle” related terms constituted relatively large sub-clusters, including “cell  
198 division” and “mitotic cell cycle process”. The enriched GO analysis was consistent with the cell  
199 line specific enriched results, and with our previous analysis of MCF-7 cells [28]. It is worth noting  
200 that for “cell cycle” related terms, most of the participating genes were down-regulated by CKI.

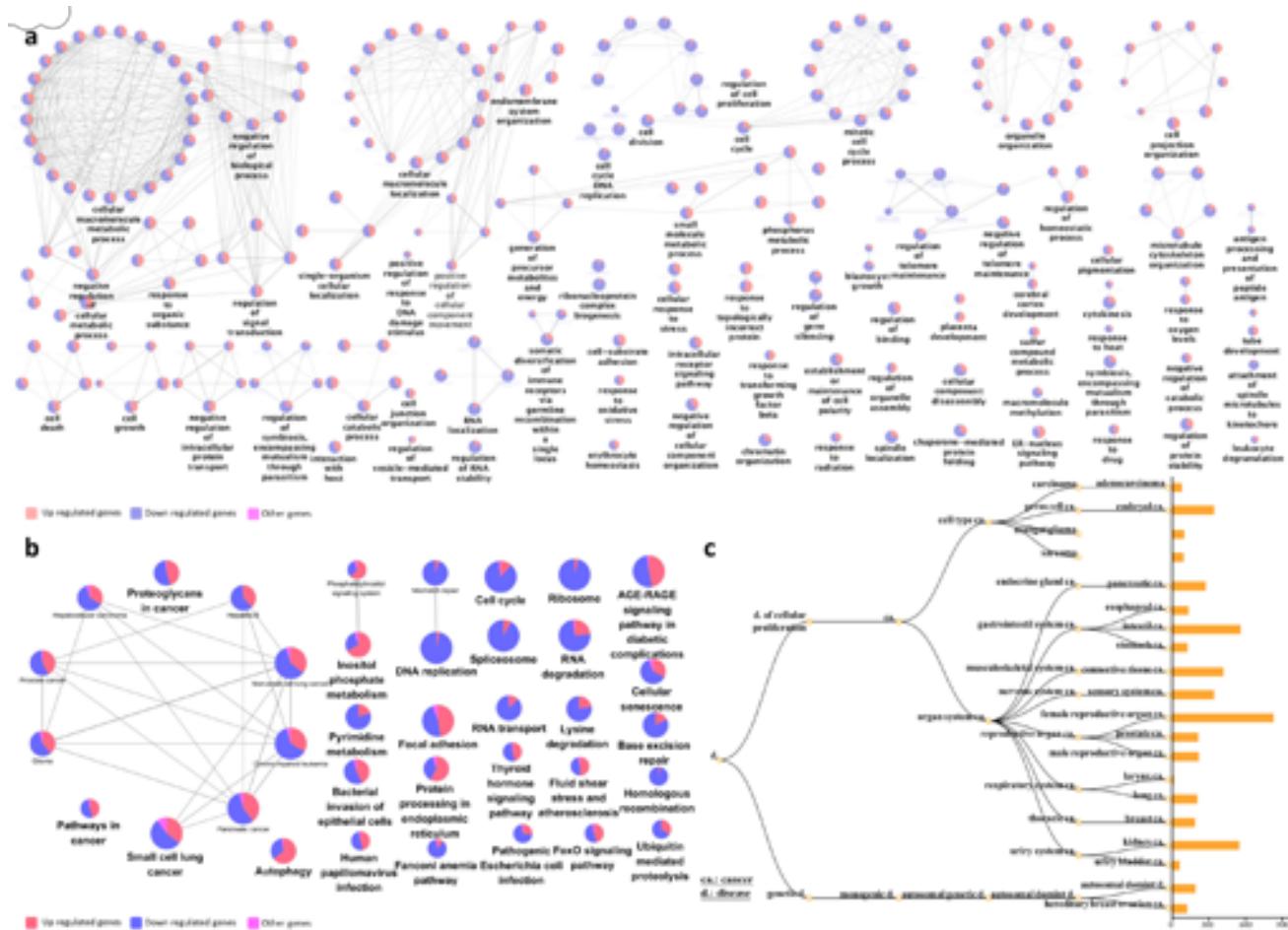
201 Similar results were observed from KEGG analysis (Fig. 5b, and Additional file 6: Table S4,  
202 sheet 14) of shared genes. Various pathways related to cancer, formed a large cluster. Pathways  
203 such as “DNA replication”, “Ribosome” and “cell cycle” were mostly down-regulated, while  
204 up-regulated pathways included “inositol phosphate metabolism” and “protein processing in  
205 endoplasmic reticulum”.

206 We also carried out over-representation analysis of DO terms (Fig. 5c) for all shared DE genes.  
207 The analysis results were consistent with the single cell line DO term analysis with mostly cancer  
208 related terms; in particular genitourinary or breast cancer terms. While this was also partially  
209 similar to the KEGG results for shared DE genes, there were some differences in the KEGG  
210 results for disease pathways compared to the DO results, such as “bacterial invasion of epithelial  
211 cells”, “Fanconi anemia pathway” and “AGE-RAGE pathway in diabetic complications”.

212 Specific to the therapeutic potential of CKI for cancer treatment, we applied our data set  
213 mapping to KEGG cancer pathways: pathways in cancer - homo sapiens (Additional file 7: Fig.  
214 S3). The R package Pathview [21] was used to integrate log fold change values of all the gene  
215 expression patterns into these target pathways. Within the 21 pathways in cancer, the “cell cycle”  
216 still featured prominently (Fig. 6a). The expression of almost every gene in the cell cycle pathway  
217 was affected by CKI, with most of them suppressed. We did not observe this kind of overall



**Figure 4. Functional annotation of DE genes for each cell line as a result of CKI treatment.** Summary of over-represented A) GO terms for Biological Process, B) KEGG pathways and C) DO terms for DE genes as a result of CKI treatment in each cell line at two time points. For GO semantic and enrichment analysis, Lin's algorithm was applied to cluster and summarize similar functions based on GO terms found in every treatment. Similarly, by back-tracing the upstream categories in the KEGG Ontology, we were able to obtain a more generalized summary of KEGG pathways for each treatment. The size of each bubble represents the number of GO terms/pathways, and the colour shows the statistical significance of the relevant function or pathways. The DO summary for each treatment was determined by back-tracing to parent terms.



**Figure 5. Functional annotation of DE genes with shared expression in both cell lines as a result of CKI treatment.** Over-representation analysis was performed to determine A) GO terms for Biological Process, B) KEGG pathways, and C) DO terms for DE genes shared in both cell lines. In nodes for both GO terms and KEGG pathways, node size is proportional to the statistical significance of over-representation. For DO terms, all the enriched terms are statistically significant ( $p < 1 \times 10^{-5}$ ) in each category, and the bar length represents the number of expressed genes that map to the term.

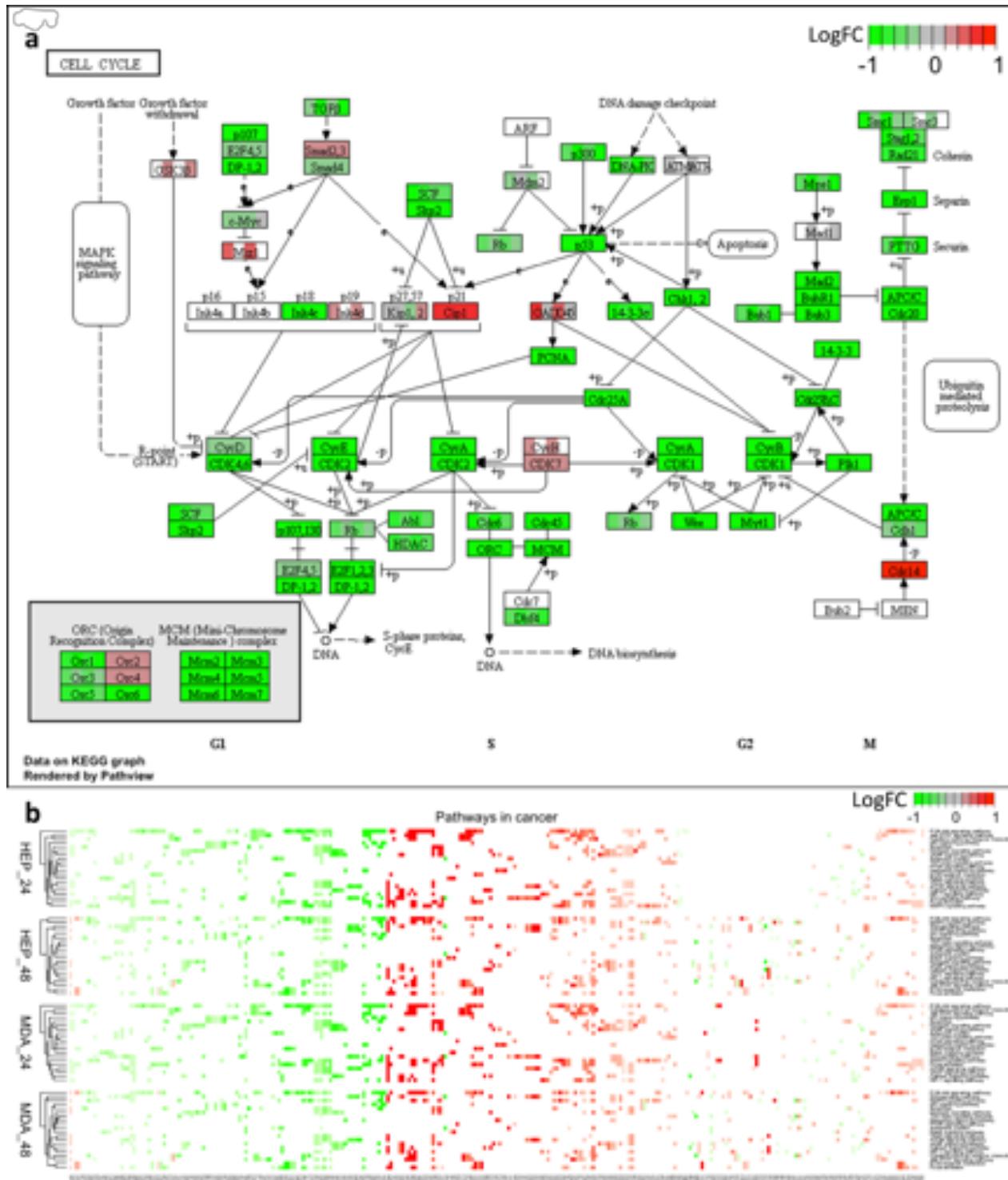
218 pathway suppression in any of the other pathways. We have displayed the summaries for the  
219 remaining 20 pathways in the heatmap in Fig. 6b. Although all the pathways were all perturbed  
220 by CKI, they include both over and under expressed genes in roughly equal proportions.

221 Collectively, these results suggest a direct anti-cancer effect of CKI, and implicate specific  
222 candidate mechanisms of action based on the perturbed molecular networks. The most obvious  
223 example is the cell cycle, where G1-S phase is significantly altered, resulting in the induction of  
224 apoptosis. The downstream process triggered by CKI is the suppression of gene expression of cell  
225 cycle regulators, including TP53 and CCND1. The other perturbed cancer pathways provide  
226 additional candidate mechanisms of action for CKI. In the following section we integrate these  
227 results with previous results reported in the literature to refine the core set of genes and pathways  
228 perturbed by CKI.

## 229 Discussion

230 Although Hep G2 (liver cancer – mesodermal tissue origin) and MDA-MB-231 (mammary  
231 epithelial adenocarcinoma – ectodermal tissue origin) are different cancer types, they shared a  
232 large number of CKI DE genes with similar expression profiles, presumably these shared genes  
233 include CKI response genes that are essential to the apoptotic response triggered by CKI.  
234 However, the number of shared CKI DE genes is too high to allow straight forward identification  
235 of genes critical to the CKI response. We therefore decided to combine these data with previously  
236 reported CKI DE genes from MCF-7 cells [28] in order to reduce the number of core CKI response  
237 genes. The intersection of MCF-7 CKI DE genes with the shared CKI DE genes yielded 363 core  
238 CKI DE genes (Additional file 8: Fig. S4).

239 Among the 363 core CKI DE genes, cytochrome P450 family 1 subfamily A member 1  
240 (CYP1A1) gene is the most over-expressed. This gene is consistently up-regulated by CKI in all  
241 three cell lines, and showed significant dose response. In liver cancer cells, over-expression of  
242 CYP1A1 induced by plant natural products has been associated with Aryl-hydrocarbon Receptor  
243 transformation [2, 49]. Furthermore, as a steroid-metabolizing enzyme, CYP1A1 is part of cancer  
244 metabolic processes relevant to steroid hormone responsive tumors, such as breast cancer, ovarian  
245 cancer and prostate cancer [24, 26, 23, 27]. Therefore, CYP1A1 may be of particular interest for



**Figure 6. Comparison of shared genes expression in specific pathways across two cell lines.** A) Cell cycle pathways, where each coloured box is separated into 4 parts, from left to right representing 24 hour CKI treated Hep G2, 48 hour CKI treated Hep G2, 24 hour CKI treated MDA-MB-231 as well as 48 hour CKI treated MDA-MB-231. B) Heatmap of pathways in cancer. The top two heatmaps summarise the effects of CKI on Hep G2 cells for two time-points, and the bottom two heatmaps show the effects of CKI on MDA-MB-231 cells. In addition to the cell cycle pathway, there were 21 associated pathways in cancer that were perturbed by CKI. The effects of CKI on both cell lines were similar, with changes in TARGET database genes indicated by arrows. Compared to other pathways in cancer, the effects of CKI on the cell cycle pathway showed overall down-regulation.

246 understanding the mechanism of action of CKI on cancer cells.

247 Comparison of the 363 core genes to the 135 Tumor Alterations Relevant for Genomics-driven  
248 Therapy (TARGET) genes (version 3) from The Broad Institute (<https://www.broadinstitute.org/cancer/cga/target>) identified 7 DE genes that were shared across the three cell lines and two time  
249 points (Fig. 7a). Of these seven genes, six (TP53, CCND1, MYD88 (Myeloid differentiation  
250 primary response gene 88) , EWSR1, TMPRSS2 and IDH1 (isocitrate dehydrogenase 1) were  
251 similarly regulated (either always over-expressed or under-expressed), while CCND3 was  
252 over-expressed in all three cell lines at both time points except at 48 hours in MCF-7 cells, where  
253 it was under-expressed.  
254

255 The TP53 gene encodes a tumor suppressor protein, that can induce apoptosis [11]. However,  
256 in all cell lines TP53 was down-regulated, and all cell lines showed increased apoptosis. This  
257 suggests that CKI induced apoptosis was not TP53-dependent. Support for this comes from the  
258 fact that transcripts for PCNA (proliferating cell nuclear antigen), and a group of transcription  
259 factors: MCM (mini-chromosome maintenance) complex and the E2F family are down-regulated.  
260 The E2F transcription factors regulate the cell cycle and TP53-dependent and -independent  
261 apoptosis [37, 12, 42, 33]. In addition, other core genes present in the TARGET database have  
262 also been shown to induce apoptosis. For example, inhibition of MYD88 induces apoptosis in both  
263 triple negative breast cancer and bladder cancer [3, 43]. The increased expression of IDH1 may be  
264 important, as IDH1 is frequently mutated in cancers [16] and when mutated, it causes loss of  
265  $\alpha$ -ketoglutarate production and may be important for the Warburg effect. TMPRSS2  
266 (transmembrane protease, serine 2) has also been shown to regulate apoptosis in cancer [1].  
267 Therefore, CKI may induce apoptosis through a variety of means.

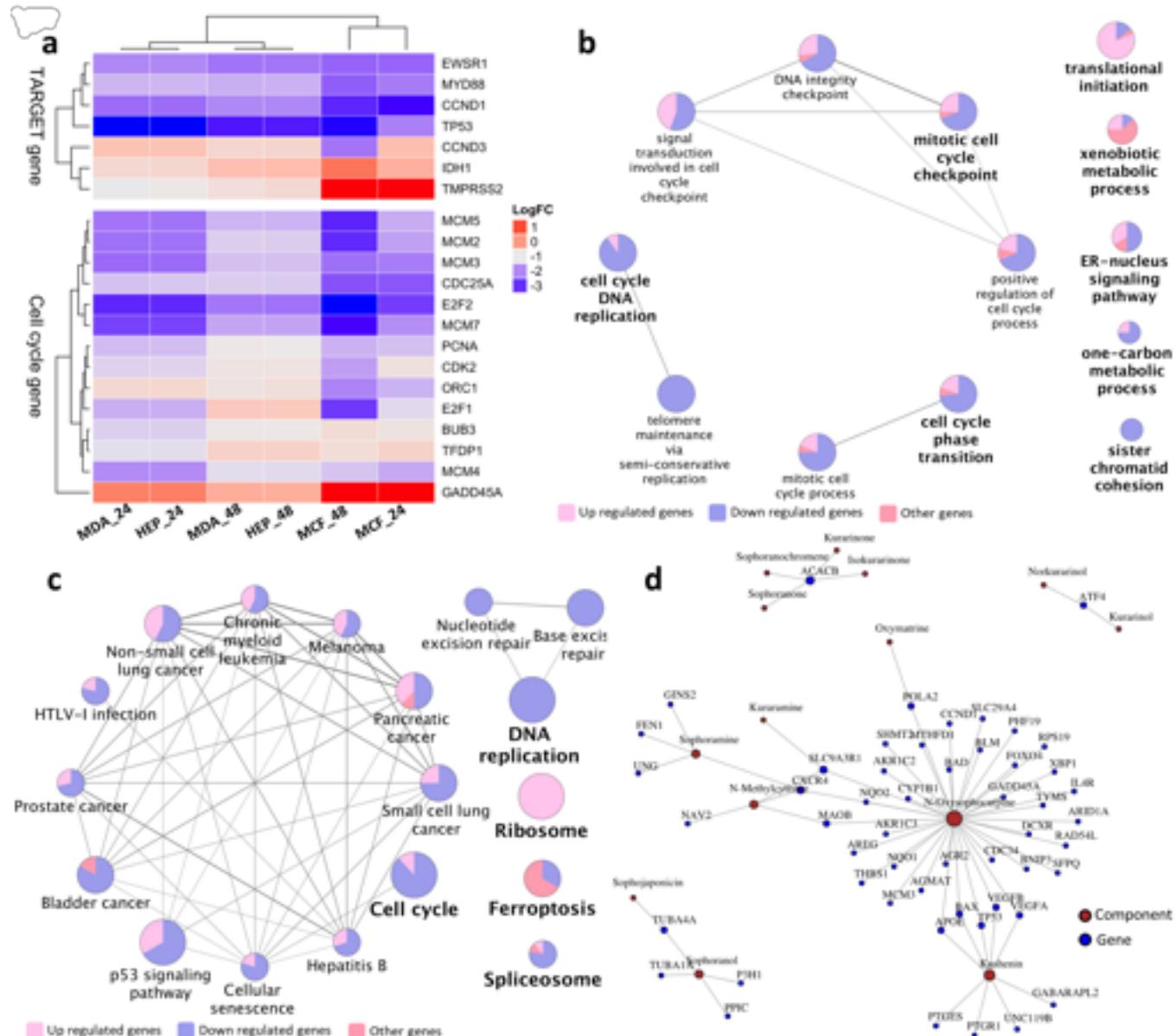
268 In the GO (Fig. 7b) and KEGG (Fig. 7c) over-representation analysis of the 363 core genes  
269 yielded enrichment for cell cycle and cancer pathways. In the GO enriched genes, cell cycle and  
270 related pathways accounted for the majority of functional sub-clusters. In the KEGG enriched  
271 pathways, cell cycle and cancer pathways predominated in a single cluster. Most of the core genes  
272 in GO and KEGG clusters were down-regulated by CKI. In addition to the cell cycle, CKI  
273 treatment also caused enrichment for terms or pathways related to cancer progression, such as  
274 “focal adhesion” and “blood vessel development”. (Additional file 6: Table S4, sheet 5-8). These

275 developmental processes contribute to tumorigenesis and metastasis [20, 36]. It is tempting to  
276 speculate that CKI may alter these functions *in vivo*, possibly altering angiogenesis which is  
277 critical for tumors [9]. In addition, there were metabolic pathways and terms that were also  
278 identified as perturbed by CKI. Effects on many targets/pathways is one of the expected features  
279 of TCM drugs which likely hit multiple targets [7].

280 We have examined the effect of a complex mixture of plant natural products (CKI) on different  
281 cancer cell lines and have identified specific, consistent effects on gene expression resulting from  
282 this mixture. However, the complexity of CKI makes it difficult to determine the mechanism of  
283 action of individual components, and often testing of individual components has resulted in either  
284 no effect or contradictory results in the research literature. In spite of this complexity, it is possible  
285 to map our results on to a pre-existing corpus of work that links individual natural compounds to  
286 changes in gene expression. We have used BATMAN [19], an online TCM database of curated  
287 links between compounds and gene expression. Based on this resource, we have identified 14  
288 components of CKI that have been linked to the regulation of 52 of our core genes (Fig. 7d). We  
289 can see from the network diagram in Fig. 7d that one to one, one to many and many to many  
290 relationships exist between CKI components and genes which is consistent with previous studies  
291 [38, 18, 46]. As more information becomes available for individual components, we will be able to  
292 construct a more comprehensive model of CKI mechanism based on network analysis.

## 293 Conclusion

294 Our systematic analysis of gene expression changes in cancer cells caused by a complex herbal  
295 extract used in TCM has proven to be effective at identifying candidate molecular pathways. CKI  
296 has consistent and specific effects on gene expression across multiple cancer cell lines and it also  
297 consistently induces apoptosis *in vitro*. These effects show that CKI can suppress the expression of  
298 cell cycle regulatory genes and other well characterized cancer related genes and pathways.  
299 Validation of a subset of DE genes at lower doses of CKI has shown a dose-response relationship  
300 that suggests that CKI may have similar effects *in vivo* at clinically relevant concentrations. Our  
301 results provide a molecular basis for further investigation of the mechanism of action of CKI.



**Figure 7. Analysis of CKI regulated core genes from this report combined with previous available data.** A) Fold changes of TARGET and cell cycle regulatory gene expression in MDA-MB-231, Hep G2 and MCF-7 [28] cell lines 24 and 48 hours after CKI treatment. Only seven TARGET genes are affected by CKI in all three cell lines. Most of the 14 cell cycle regulatory genes differentially expressed in all three cell lines are down-regulated. B) GO term enrichment analysis of 363 core genes from MDA-MB-231, Hep G2 and MCF-7 cell lines. C) KEGG pathway enrichment of 363 core genes from MDA-MB-231, Hep G2 and MCF-7 cell lines. D) Some individual compounds present in CKI linked to genes they regulate that are also found in this report and our previous study [28]. Node size is proportional to the number of related components/genes.

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304 **Conflict of Interest**

305 We wish to draw the attention of the Editor to the following facts which may be considered as  
306 potential conflicts of interest and to significant financial contributions to this work. While a  
307 generous donation was used to set up the Zhendong Centre by Shanxi Zhendong Pharmaceutical  
308 Co Ltd, they did not determine the research direction for this work or influence the analysis of the  
309 data. JC: no competing interests, ZQ: no competing interests, YHL: no competing interests, HS:  
310 no competing interests, TNA: no competing interests, WW: is an employee of Zhendong Pharma  
311 secondeed to Zhendong Centre to learn bioinformatics methods, RDK: no competing interests, DLA:  
312 Director of the Zhendong Centre which was set up with a generous donation from the Zhendong  
313 Pharmaceutical Co Ltd. Zhendong Pharmaceutical has had no control over these experiments,  
314 their design or analysis and have not exercised any editorial control over the manuscript.

315 **Author contributions statement**

316 JC experimental design, carried out experiments, analyzed data, wrote paper, ZQ experimental  
317 design, assisted with experiments, assisted with data analysis, wrote paper, YHL experimental  
318 design, assisted with experiments, assisted with data analysis, wrote paper, HS assisted with  
319 experiments, TNA assisted with experiments, WW assisted with experimental design, assisted  
320 with experiments, RDK experimental design, and DLA supervised the research, acquired funding  
321 for the experiments, experimental design, wrote paper.

322 **Data Availability**

323 All RNA-seq data raw and processed data were deposited at the Gene Expression Omnibus  
324 (GEO) data repository (XXXXXXX).

325 **Role of the funding source**

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327 **References**

328 **References**

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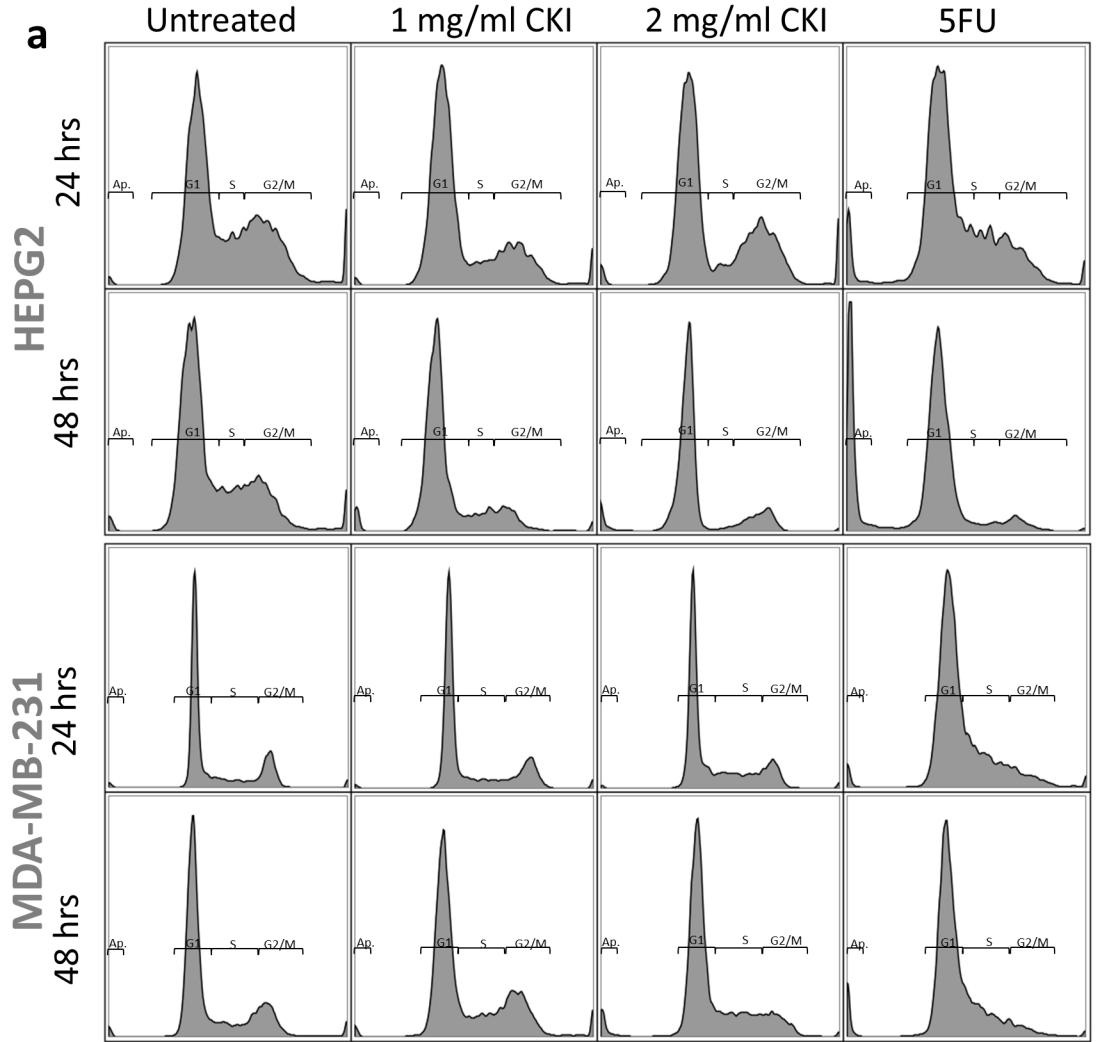
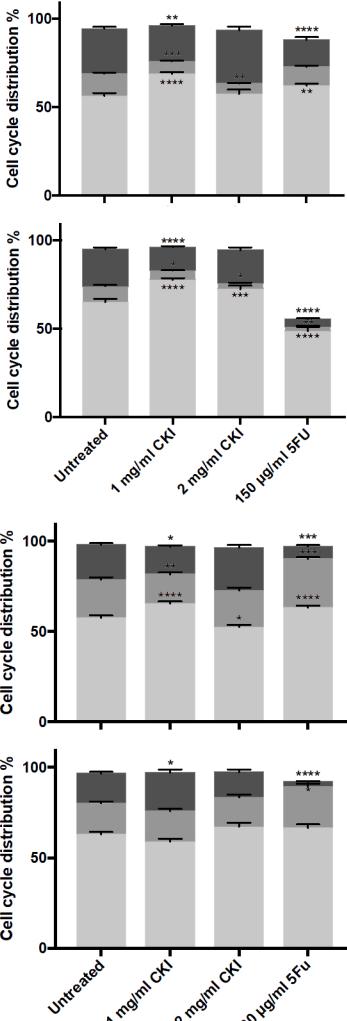
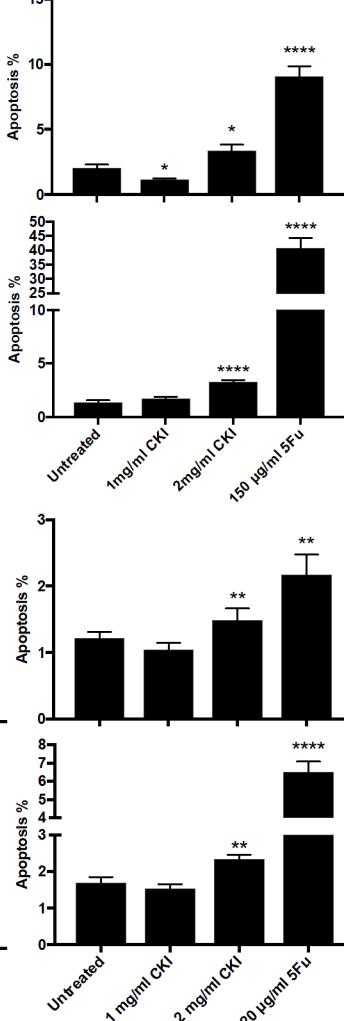
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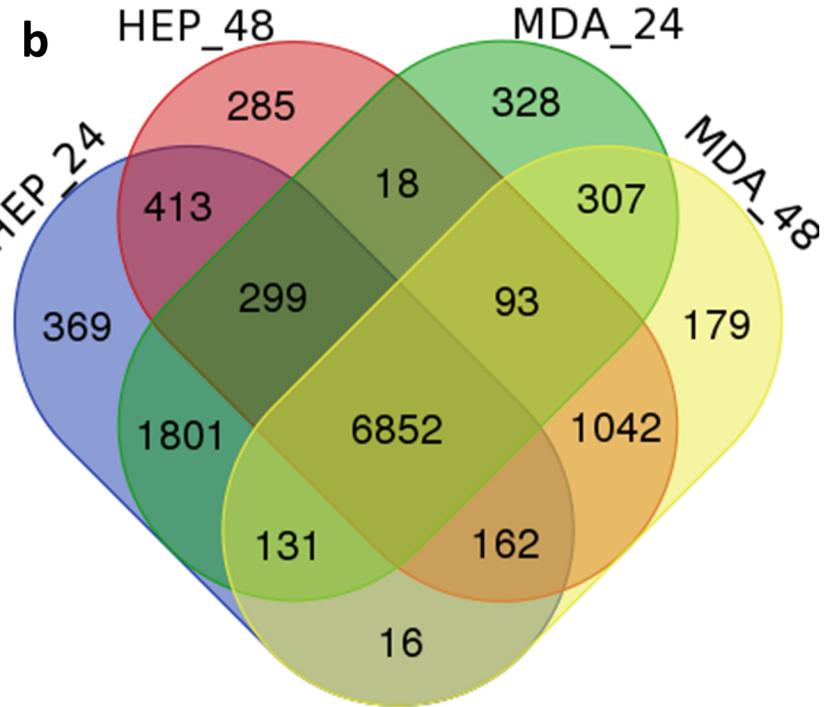
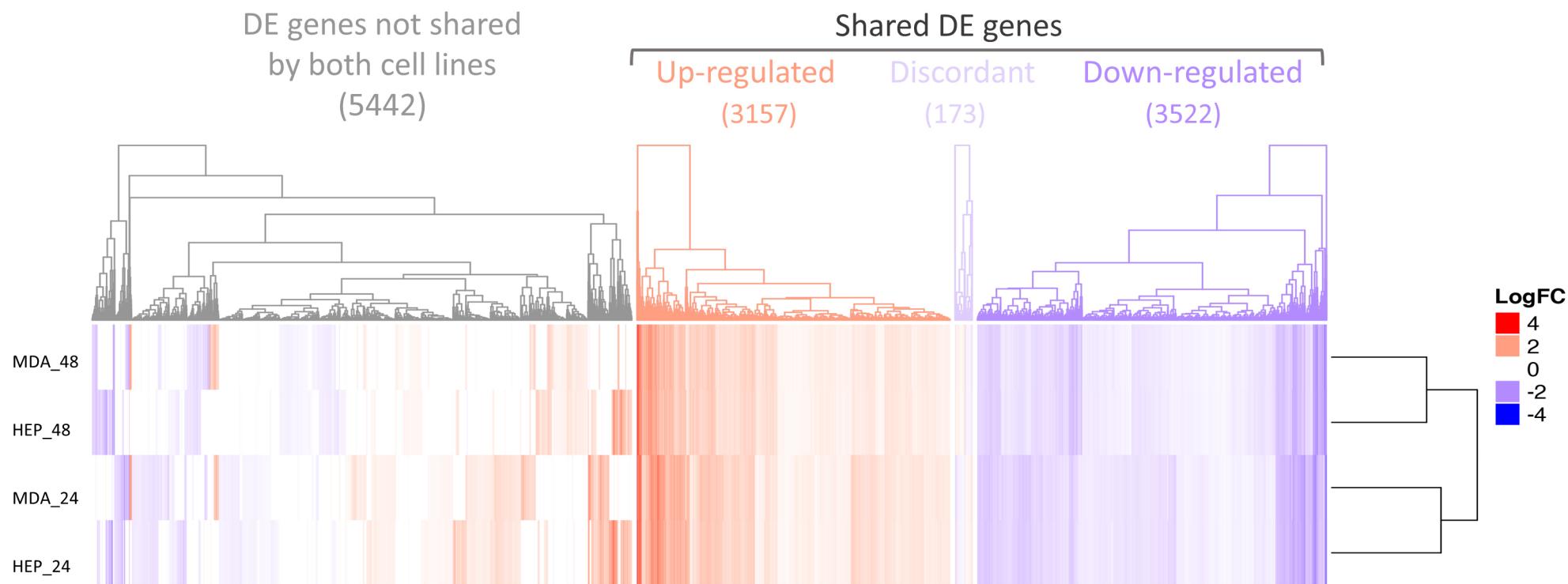
**a****b****c**

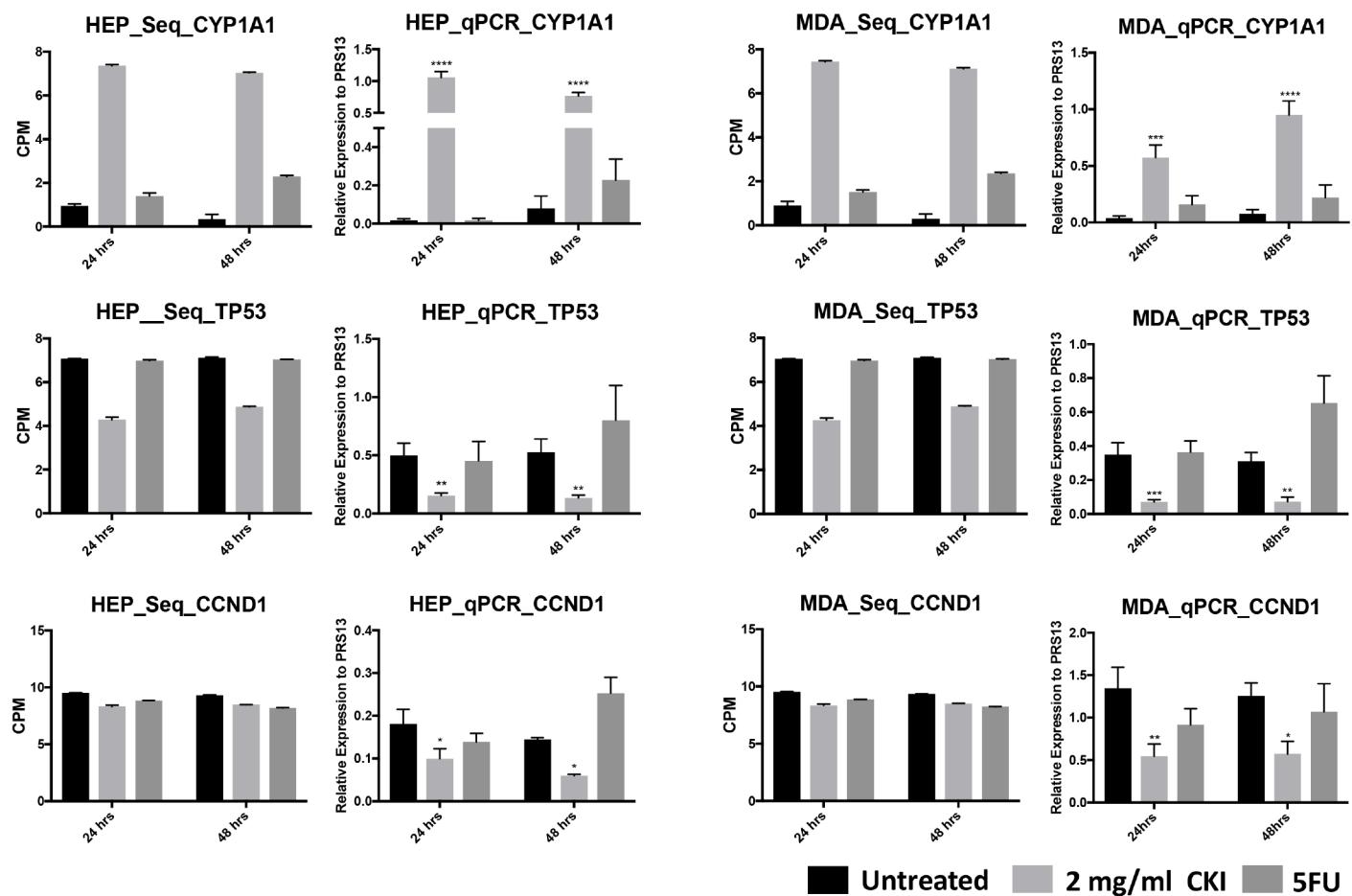
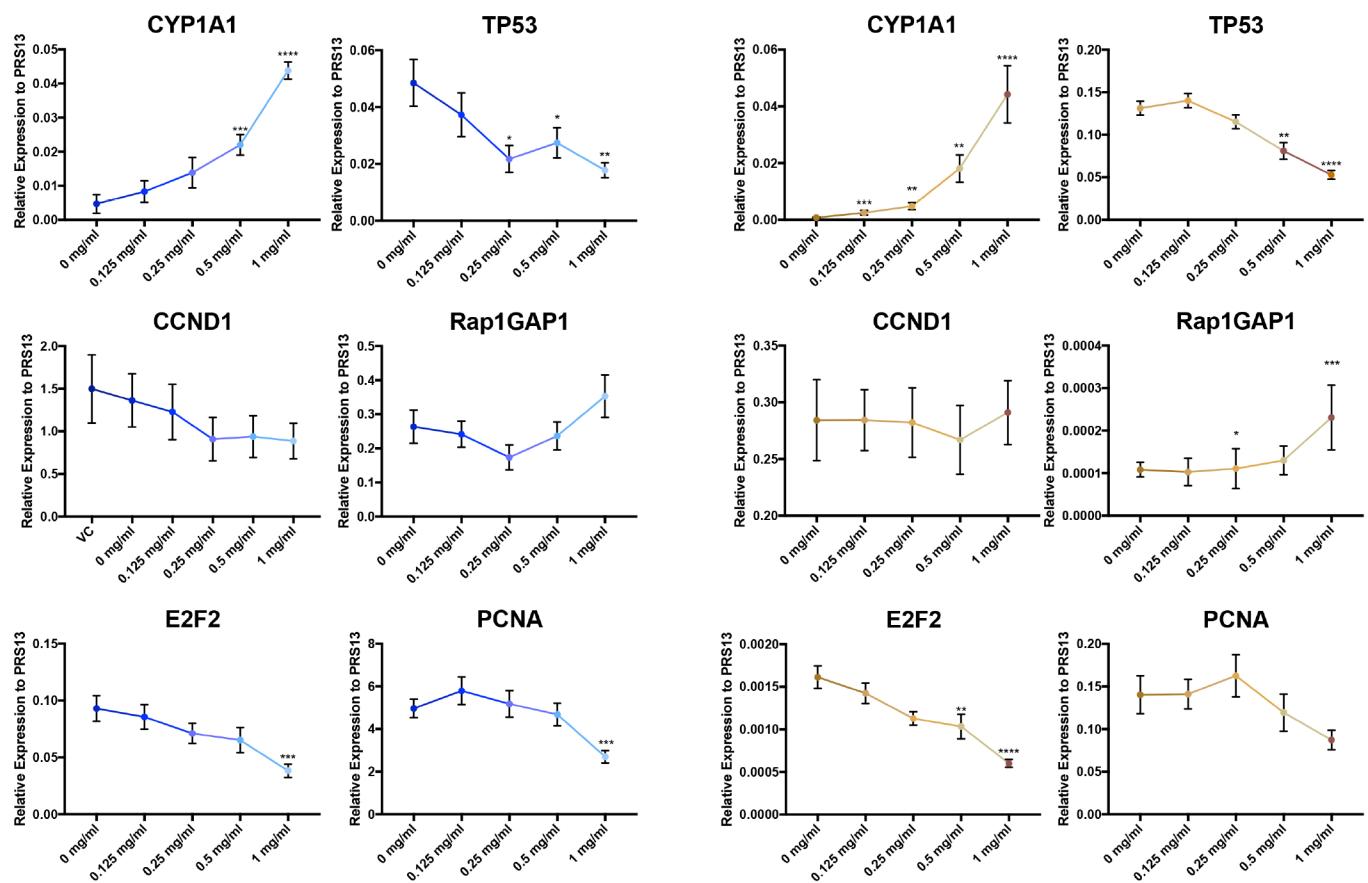
G1    S    G2/M    Apoptosis

**a**

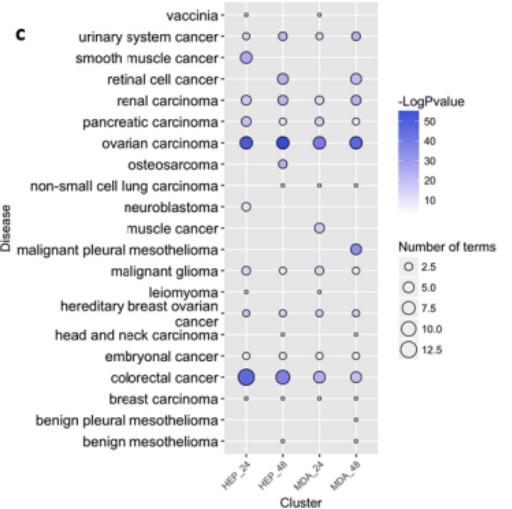
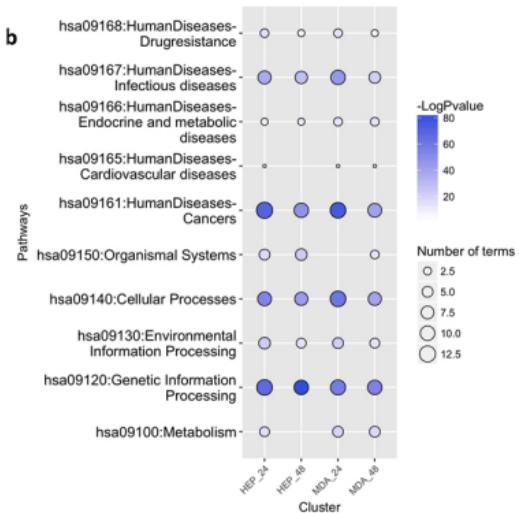
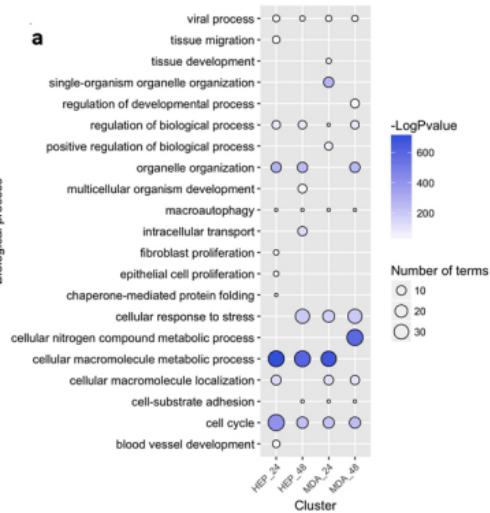
- DMEM with 10% FBS
- 37°C with 5% CO<sub>2</sub>
- 0 hours:
  - Untreated
  - 24 hours:
    - Untreated
    - 2 mg/ml CKI
    - 5-FU
  - 48 hours:
    - Untreated
    - 2 mg/ml CKI
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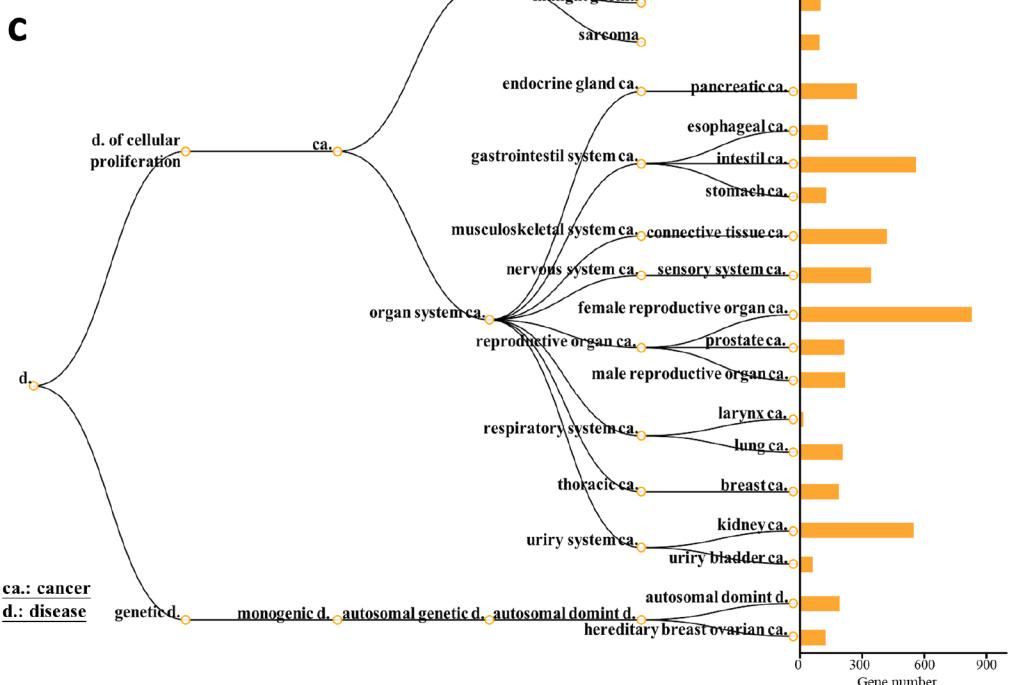
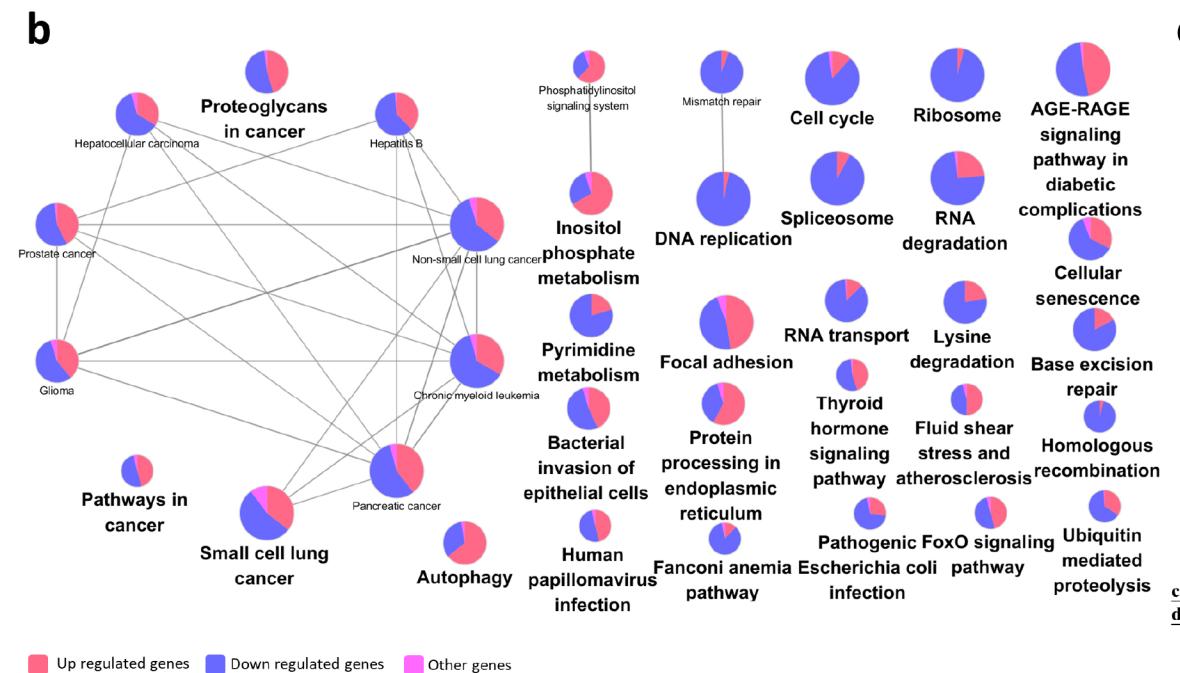
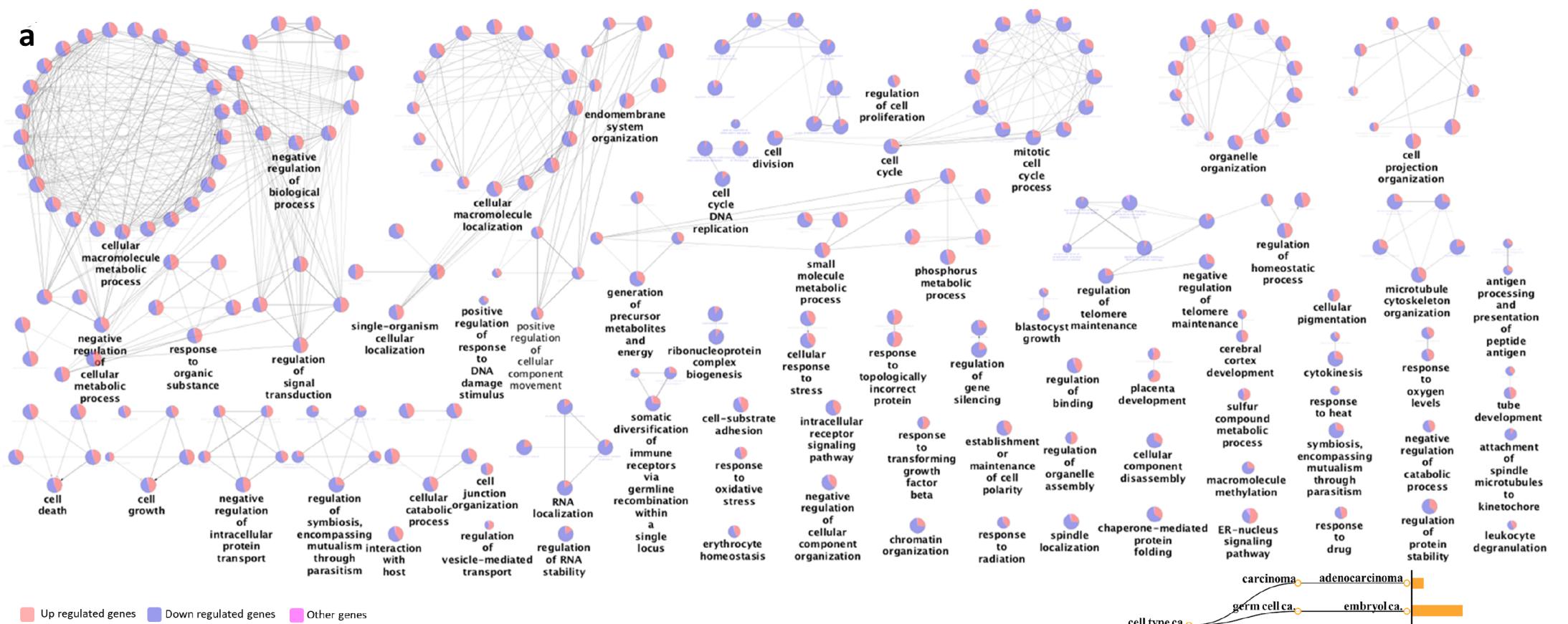
- Cell cycle
- RNA isolation & sequencing
- RT-qPCR
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- .

**c**

**a****b**

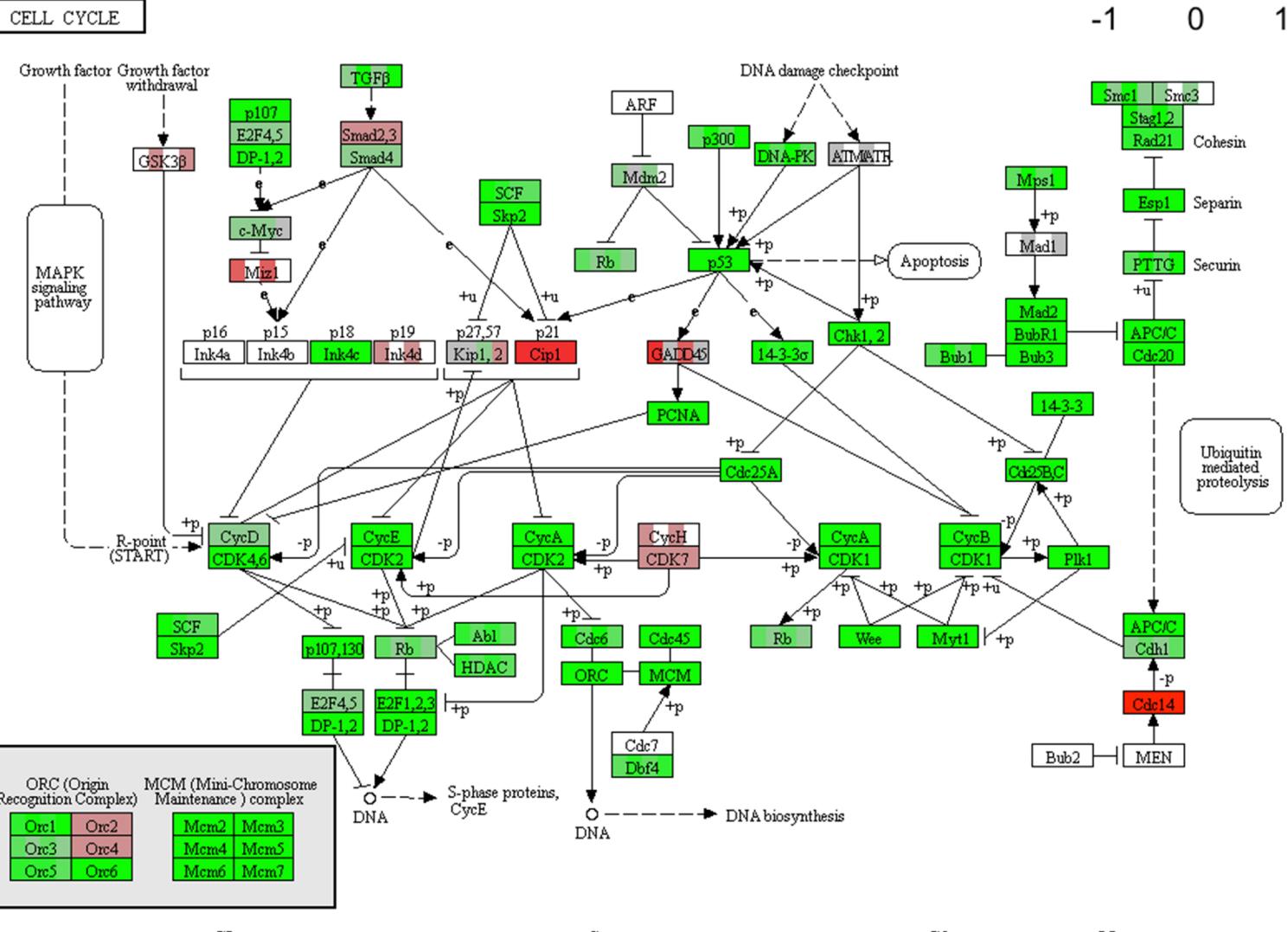
## Biological process





a

LogFC



## Data on KEGG graph Rendered by Pathview

b

