

1 **Heritable gene expression variability governs clonal heterogeneity in**  
2 **circadian period**

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9 Short title: heritable basis of clonal circadian period heterogeneity

10

## 11 **ABSTRACT**

12 A ubiquitous feature of circadian clocks across life forms is its organization as a network of  
13 coupled cellular oscillators. Individual cellular oscillators of the network often exhibit a  
14 considerable degree of heterogeneity in their intrinsic periods. While the interaction of coupling  
15 and heterogeneity in circadian clock networks is hypothesized to influence clock's entrainability,  
16 our knowledge of mechanisms governing network heterogeneity remains elusive. In this study,  
17 we aimed to explore the principles that underlie inter-cellular period variation in circadian clock  
18 networks (clonal period-heterogeneity). To this end, we employed a laboratory selection  
19 approach and derived a panel of 25 clonal cell populations exhibiting circadian periods ranging  
20 from 22 h to 28 h. We report that while a single parent clone can produce progeny clones with a  
21 wide distribution of circadian periods, heterogeneity is not entirely stochastically driven but has  
22 a strong heritable component. By quantifying the expression of 20 circadian clock and clock-  
23 associated genes across our panel, we found that inheritance of different expression patterns in  
24 at least three clock genes might govern clonal period-heterogeneity in circadian clock networks.  
25 Furthermore, we provide preliminary evidence suggesting that epigenetic variation might  
26 underlie such gene expression variation.

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## 28 **INTRODUCTION**

29 The majority of life forms on earth exhibit ~24 h (circadian) behavioural and physiological  
30 rhythms generated by endogenous time-keeping mechanisms - circadian clocks. In addition to  
31 driving such endogenous rhythms, circadian clocks facilitate synchronization of organisms'  
32 rhythms to daily and seasonal changes in the environment to enhance their survivability, thereby

33 functioning as an adaptive mechanism (Kumar, 2017). The fundamental basis of circadian  
34 rhythm-generation across all life-forms are cell-autonomous molecular oscillations comprising  
35 evolutionarily conserved auto-regulatory transcription-translation feedback loops (TTFL)  
36 (Dunlap, 1999). In higher organisms, such cell-autonomous clocks often function as a network of  
37 coupled oscillators which in unison drive circadian rhythms (Bell-Pedersen *et al.*, 2005). Welsh  
38 and co-workers first reported that neurons within the suprachiasmatic nucleus (SCN; the master  
39 pacemaker in the hypothalamus of mammals) are surprisingly heterogeneous in their intrinsic  
40 periods of circadian firing pattern (Welsh *et al.*, 1995). Subsequent studies revealed that such  
41 period-heterogeneity is not restricted to the SCN alone, but is also characteristic of mammalian  
42 peripheral clock cells (Nagoshi *et al.*, 2004; Leise *et al.*, 2012) as well as of clock cells in *Drosophila*  
43 (Sabado *et al.*, 2017) and plants (Yakir *et al.*, 2011; Muranaka and Oyama, 2016). The ubiquity of  
44 this network feature suggests that heterogeneity may be functionally relevant for circadian clocks  
45 (Jagota *et al.*, 2000; Schaap *et al.*, 2003; Gonze *et al.*, 2005; Bernard *et al.*, 2007; Inagaki *et al.*,  
46 2007; VanderLeest *et al.*, 2007; Gu *et al.*, 2016, 2019), thus likely being a substrate for natural  
47 selection. Interestingly, the observed period-heterogeneity among circadian clock cells within an  
48 organism cannot be entirely attributed to functionally different cell types as cells of the same  
49 subtype (clonal cells) also exhibit such variation (Nagoshi *et al.*, 2004; Leise *et al.*, 2016). Clonal-  
50 heterogeneity or clonal-phenotypic variability is common in biology and can stem from various  
51 external factors such as stochastic changes in the microenvironment or internal factors like  
52 stochastic partitioning of cellular components during cell-division or stochasticity in gene  
53 expression (Neildez-Nguyen *et al.*, 2008; Brock, Chang and Huang, 2009; Huang, 2009; Altschuler  
54 and Wu, 2010; Geiler-Samerotte *et al.*, 2013; Roberfroid, Vanderleyden and Steenackers, 2016;

55 Evans *et al.*, 2018). In this study, we aimed to explore the possible mechanisms underlying clonal-  
56 heterogeneity of circadian period in human circadian oscillator cells.

57 We hypothesised that clonal period-heterogeneity in mammalian cells is due to a) stochastic  
58 variation (Geva-Zatorsky *et al.*, 2006; Chang *et al.*, 2008; Brock, Chang and Huang, 2009; Frank  
59 and Rosner, 2012) and/or b) heritable variation (Dubnau and Losick, 2006; Gordon *et al.*, 2009,  
60 2013). Since the term ‘stochastic’ is used in the context of both non-heritable (external noise and  
61 gene expression noise) as well as heritable gene expression variation (epigenetic stochasticity),  
62 for the rest of this manuscript we define ‘stochasticity’ as any non-heritable variation (both  
63 internal and external). To test the two hypothesis outlined above, we employed a laboratory  
64 selection approach and derived a panel of 25 clonal cell lines (from a common ancestral/founding  
65 culture) exhibiting a range of periods between 22h and 28h. We observed that the period-  
66 heterogeneity among progeny clones stemming from a single parent cell is not entirely stochastic  
67 but has a substantial heritable component. We then measured expression of 20 clock and clock-  
68 associated genes in our panel and observed that variation in gene expression levels of at least  
69 three clock genes (transcription factors) might underlie clonal period-heterogeneity.  
70 Furthermore, we provide preliminary evidence that epigenetic variation might govern the  
71 observed clonal-variation in gene expression.

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## 76 RESULTS

### 77 Clonal period-heterogeneity is not entirely stochastically driven but is largely inherited

78 Is the variation in period among individual circadian oscillator cells just due to intrinsic and/or  
79 extrinsic stochastic noise? Or is there a heritable component? To test this, we single-cell cloned  
80 a ‘founding culture’ of U-2 OS cells (an established model of peripheral circadian clocks) harboring  
81 a *BMAL1*-luciferase reporter construct (Maier *et al.*, 2009). Upon reaching confluence, the period  
82 of bioluminescence rhythms from these progeny cultures was determined by live-cell  
83 bioluminescence recording. As expected, we observed a distribution of circadian periods (23.5 h  
84 - 27.5 h; Figure 1a top panel). We repeated this protocol for several ‘assay-generations’ by each  
85 time selecting short and long period cultures as ‘parents’ for the next assay-generation (Study  
86 outline in Supplementary Figure S1).

87 Interestingly, by repeating this protocol for several assay-generations we observed a directional  
88 divergence of the progeny period-distributions on either side of the ‘founding culture’s’  
89 distribution (Figure 1a). The mean circadian periods of progeny cultures in every assay-  
90 generation were always very similar to those of their parental cultures (Figure 1a). Over the  
91 course of the selection protocol, the periods of short and long period clonal lines (SCL and LCL)  
92 significantly diverged from each other and from the ‘founding culture’. The periods of both SCLs  
93 and LCLs diverged significantly by assay generation-2 and this divergence reached saturation as  
94 the periods did not diverge further ( $p < 0.001$ ; ANOVA followed by Unequal N HSD; Figure 1b). At  
95 assay generation 4, the circadian periods of LCLs were  $\sim 3.4$  h longer, and those of SCLs were  $\sim 1.7$   
96 shorter than the ‘founding culture’s’ period (Figure 1b; Supplementary Figure S2).

97 As a measure of period-heritability, we regressed mean periods of the progeny cultures on  
98 parental cultures and observed that parental period is a very good predictor of the mean progeny  
99 period ( $R^2 = 0.96$ ; Figure 1c). In addition, even though the divergence of period saturated over  
100 the last three assay generations (Figure 1b), we still observe a distribution of periods even after  
101 three assay generations. We reason that this distribution might be due to non-heritable  
102 stochasticity. Taken together, our results suggest that clonal period-heterogeneity might partly  
103 be stochastically driven but has a significant heritable component.

#### 104 **Inheritance of differential gene expression levels might underlie period heritability**

105 We further aimed to explore the likely basis for heritability of the circadian period underlying  
106 heterogeneity. During the course of our experiments, we observed that the short and long period  
107 clones consistently exhibited low and high bioluminescence intensities/levels respectively  
108 (Supplementary Figure S2a). This encouraged us to test correlation of the period with circadian  
109 rhythm parameters such as amplitude, damping rate and bioluminescence intensity.

110 We observed a positive correlation of bioluminescence intensity (Pearson  $r = 0.65$ ,  $p < 0.0001$ )  
111 with clone-period; the correlation of relative amplitude with period was negative but not  
112 significant (Pearson  $r = -0.08$ ,  $p = 0.19$ ) and damping rate was not significantly correlated with  
113 period either (Spearman  $r = 0.26$ ,  $p = 0.06$ ; Supplementary Figure S3a). We reasoned that mean  
114 bioluminescence intensity can, in-principle serve as a proxy for the average expression level of  
115 the underlying gene (*BMAL1* in this case) and hypothesized that clonal inheritance of average  
116 gene expression might underlie the observed period heritability. This was further supported by  
117 the observation that parental bioluminescence intensity was the best predictor of the respective

118 progeny values ( $R^2 = 0.76$ ; Figure 2a), while relative amplitude ( $R^2 = 0.04$ ; Supplementary Figure  
119 S3b) and damping rate ( $R^2 = 0.40$ ; Supplementary Figure S3c) were only poor predictors.

### 120 **Differential expression of E-Box associated factors may govern clonal period-heterogeneity**

121 To test, whether gene expression correlates with clonal period-heterogeneity, we used the  
122 NanoString multiplex technology to measure the average expression levels of 20 clock and clock-  
123 associated genes (Supplementary Table S1) across our panel of 25 clones. Not surprisingly, we  
124 observed a high degree of cross-correlation in expression of the measured genes (Figure 2b) likely  
125 due to the high interconnectivity in the circadian clock molecular loop. We subjected the dataset  
126 to Principal Component Analysis (PCA) aiming to extract the major features/genes that might  
127 underlie (or is a major contributor to) clonal-period heterogeneity. Based on the Broken-Stick  
128 model (Jolliffe , 2011), we retained the first two Principal Components (PCs) which collectively  
129 explained 70.2% of the circadian period variance (Figure 2c). Interestingly, the first two PCs also  
130 clustered the panel of clones into three categories of short (22.3-23h), intermediate (23.8-26.9h)  
131 and long (27.6-28.2h) period clones (Figure 2d). PC1 clustered the clones into two groups: i)  
132 intermediate periods and ii) the rest including both short and long period clones (non-  
133 intermediate). In contrast, PC2 appeared to be important for the three observed clusters (Figure  
134 2d). Based on the  $\cos^2$  values (a measure of the quality of representation of the genes on a PC;  
135 Figure 2e-f) and contributions of genes to PC2 (Supplementary Figure S4), we shortlisted the top  
136 25% of the candidate genes (*ARNTL2*, *BHLHE40*, *DBP*, *NR1D2*, *PER2*) that we hypothesized to  
137 largely account for the period-variation.

138 We implemented hierarchical clustering on our dataset based on expression of the five  
139 shortlisted candidate genes and observed that clustering of clones was similar (with one  
140 exception) to that by the first two PCs (Figure 2g). The amalgamation schedule suggested a  
141 possibility of three clusters (red, blue and green dashed-rectangles, Figure 2g) which was also in  
142 agreement with the optimal cluster number reported by five different indices (Supplementary  
143 Figure S5).

144 Clustering-based heat map revealed that the expression of *ARNTL2* and *BHLHE40* correlated  
145 positively with the circadian period, *DBP* and *NR1D2* correlated negatively, while *PER2* exhibited  
146 a clear trend (Figure 2g, Supplementary Figure S6a). As a control measure, we also similarly  
147 shortlisted top 25% genes from PC1 (*NR1D1*, *CLOCK*, *CSNK1D*, *CIPC* and *NFIL3*) and, as expected,  
148 we observed that these genes were not sufficient to discriminate the short and long periods  
149 thereby resulting in only two clusters – intermediate and non-intermediate (Figure 2h).  
150 Interestingly, all five genes from PC1 have higher expression in ‘intermediate’ period clones and  
151 their expression reduces as the period deviates from ‘intermediate’ (Figure 2h, Supplementary  
152 Figure S6b). Thus, we reasoned that changes in expression of PC2 genes are likely to drive period  
153 heterogeneity while those from PC1 are likely to be a consequence of period heterogeneity.

154 We hypothesized that if differences in expression of the shortlisted PC2 genes governs period  
155 heterogeneity, then depletion of these genes should result in large period change while depletion  
156 of those from PC1 should not have a significant effect on period. Specifically, based on their  
157 expression patterns (Figure 2g) knockdown of *ARNTL2* and *BHLHE40* should shorten the circadian  
158 period while *DBP* and *NR1D2* knockdown should result in period lengthening. To test this, we  
159 used RNAi mediated silencing to individually knockdown the shortlisted genes in 3-short, 2-



160 intermediate and 3-long period clones (based on clustering in Figure 2g) and studied the effect  
161 on circadian period. Indeed, we observed that knockdown of *NR1D2* resulted in significant period  
162 lengthening across all clones while *BHLHE40* and *ARNTL2* knockdown resulted in significant  
163 period shortening (Mixed model ANOVA followed by Tukey's HSD;  $p < 0.00001$ ; Figure 3a-b).  
164 *NR1D2* knockdown had the largest effect on period, significantly higher compared to all other  
165 genes across both the PCs; followed by *BHLHE40* that was similar to *ARNTL2* and had a  
166 significantly higher effect on period compared to all other genes. Knockdown of none of the other  
167 genes across both PCs resulted in a period change significantly differing either from zero (one  
168 sample t test,  $p > 0.05$ ) or from each other (Mixed model ANOVA followed by Tukey's HSD;  $p >$   
169  $0.05$ ; Figure 3a-b). Accordingly, we observed that the average absolute period change upon  
170 knockdown of PC2 genes was significantly higher than that by PC1 genes (Figure 3c).

171 Taken together, these results suggest that differential expression of *NR1D2*, *BHLHE40* and  
172 *ARNTL2* likely underlies heterogeneity in clonal circadian period.

### 173 **Epigenetic regulation might underlie altered gene expression patterns associated with clonal** 174 **period-heterogeneity**

175 Having observed that clonal period-heterogeneity is associated with altered gene expression  
176 patterns, we next asked 'what causes such altered expression among clonal cells?' We ruled out  
177 the possibility of random mutation accumulation (see Discussion) and hypothesized that  
178 epigenetic variation might account for the observed differences in gene expression patterns  
179 among clonal lines. As a preliminary test, we treated all 25 clonal cell populations in our panel  
180 with the commonly used epigenetic modifier Suberoylanilide Hydroxamic Acid (SAHA) and  
181 studied the effect of the treatment on clone period. SAHA is a Class I and Class II Histone

182 Deacetylase Inhibitor which upregulates gene expression by multiple mechanisms (Marks *et al.*,  
183 2008). We reasoned that if reduction in expression of the identified subset of genes across our  
184 clonal panel is due to epigenetic suppression (in this case, acetylation status), treatment with  
185 SAHA should upregulate the expression of these genes thereby lengthening and shortening the  
186 circadian period in short and/or long period clones respectively.

187 Interestingly, we observed that treatment with SAHA differentially influenced the short,  
188 intermediate and long period clones. SAHA treatment resulted in a significant period shortening  
189 in the long period clones (ANOVA followed by Tukey's HSD,  $p < 0.05$ ; Figure 3d) whereas, the  
190 magnitude of period change in short and intermediate period clones did not differ from each  
191 other (ANOVA followed by Tukey's HSD,  $p = 0.85$ ) or from zero (one sample t test,  $p > 0.05$ ; Figure  
192 3d).

193 Although the possible reasons for the differential effects of SAHA treatment on short and long  
194 period clones will be discussed later, this provides preliminary evidence suggesting that  
195 epigenetically regulated gene expression differences might underlie clonal period-heterogeneity.

## 196 **DISCUSSION**

197 We used human U-2 OS cells to investigate whether period-heterogeneity in circadian clock  
198 network stems from intrinsic/extrinsic non-heritable stochasticity or whether it has a heritable  
199 component. We employed a laboratory selection protocol to select for clonal cell populations  
200 exhibiting short and long circadian periods through which we derived a panel of 25 clonal cell  
201 populations exhibiting circadian periods between 22 h to 28 h.

202 We observed that parental clones always produced progeny with mean periods closely  
203 resembling the former thus resulting in a directional response (divergence of short and long  
204 period clones from the founding culture) to our selection protocol (Figure 1a). Consistently, the  
205 period of parental culture was a very good predictor ( $R^2 = 0.96$ ) of the progeny's mean period  
206 (Figure 1c). Taken together, these results suggest that clonal period-heterogeneity is unlikely to  
207 be stochastically driven and has a strong heritable component. This raises an interesting  
208 question: why would natural selection favour the evolution of heritable mechanisms to drive  
209 period-heterogeneity over entirely stochastically driven heterogeneity? We hypothesize that,  
210 although period heterogeneity can be functionally beneficial (Jagota *et al.*, 2000; Schaap *et al.*,  
211 2003; Gonze *et al.*, 2005; Bernard *et al.*, 2007; Inagaki *et al.*, 2007; VanderLeest *et al.*, 2007; Gu  
212 *et al.*, 2016, 2019), very large heterogeneity can negatively influence clock functionality as well  
213 (Gonze *et al.*, 2005; Bernard *et al.*, 2007; Gu *et al.*, 2016). Stochastic mechanisms can potentially  
214 lead to very large variation in inter-cellular/oscillator period which would be detrimental,  
215 whereas heritable mechanisms may impose phenotypic constraints (Wagner, 2011) within which  
216 period-heterogeneity can be maintained and thus being favoured by natural selection.

217 Over the course of our experiments, we observed that long-period clones often exhibited higher  
218 bioluminescence intensity compared to the short-period clones (Supplementary Figure S2a) and  
219 further analysis revealed that parent bioluminescence intensity was a good predictor of progeny  
220 bioluminescence intensity but this was not the case for either relative amplitude or the damping  
221 rate (Figure 2a, Supplementary Figure S3). We reasoned that bioluminescence intensity could  
222 serve as a proxy measure for *BMAL1* expression and thus hypothesised that period heritability is  
223 likely to be due to the inheritance of gene expression levels from parental clones. To further

224 explore this, we measured average expression of 20 circadian clock and clock-associated genes  
225 (Supplementary Table S1) across all 25 clones in our panel. By employing principal component  
226 analysis, we identified five candidate genes (*ARNTL2*, *BHLHE40*, *DBP*, *NR1D2* and *PER2*) that  
227 grouped the clones into three distinct clusters – short, intermediate and long periods (Figure 2c-  
228 g, Supplementary Figures S4-5). Furthermore, we observed that knockdown of three of the  
229 shortlisted candidates - *NR1D2*, *BHLHE40* and *ARNTL2* had the largest influence on period across  
230 while other genes including those from PC1 had little or no effect on period change (Figure 3a-  
231 c). It is noticeable that individual knockdown of the genes resulted in small magnitude period  
232 changes that cannot entirely account for period differences between the short and long period  
233 clones (Figure 3a). These results suggest that that clonal period-heritability is a multi-gene trait  
234 involving a consortium of multiple medium-effect genes. Notably, all three above-mentioned  
235 genes are transcription factors that are either regulated by and/or act on E-boxes and are part of  
236 both the core and auxiliary molecular clock loops (Ikeda *et al.*, 2000; Okamura *et al.*, 2002;  
237 Kawamoto *et al.*, 2004; Guillaumond *et al.*, 2005; Nakashima *et al.*, 2008; Sasaki *et al.*, 2009;  
238 Takahashi, 2017). This reinforces the idea that while persistence of circadian oscillation requires  
239 a functional core clock loop involving negative feedback by the PER-CRY family, modulation of  
240 clock period might be governed by interaction between multiple loops coupled by E-box  
241 associated transcription factors (Zhang and Kay, 2010; Relógio *et al.*, 2011). Another notable gene  
242 that our analysis revealed happens to be one of the relatively less studied circadian clock genes  
243 *ARNTL2* (*BMAL2*). While *ARNTL2* is a functional paralog of the core clock gene *ARNTL1* (*BMAL1*),  
244 its precise role in the clock loop remains largely elusive (Ikeda *et al.*, 2000; Sasaki *et al.*, 2009; Shi

245 *et al.*, 2010) thus highlighting a potential role of *ARNTL2* in circadian period-modulation, which  
246 awaits further exploration.

247 Intriguingly, in contrast to the above-discussed genes, we find another category among the  
248 assayed genes that exhibit an inverted-U shaped relationship with period. The expression of  
249 these genes (*NR1D1*, *CSNK1D*, *NFIL3*, *CLOCK*, *CIPC*) is high in clones with intermediate periods  
250 (23.8-26.9h) and is drastically reduced in clones with periods deviating from the intermediate  
251 range (Figure 3g). Furthermore, our knockdown studies also confirm that expression patterns of  
252 these genes are not causal but likely to be a response/consequence to period variation (Figure  
253 3b). Such inverted-U shaped responses (Hormesis) is observed in various biological systems and  
254 is regarded as a regulatory/homeostatic mechanism to prevent very large deviations of  
255 cellular/organismal phenotypes from their optimal range (Calabrese and Baldwin, 2001; Baldi  
256 and Bucherelli, 2005; Zhang *et al.*, 2008). As discussed earlier, since a higher degree of period-  
257 heterogeneity can be detrimental to the circadian clock network, we hypothesize that while there  
258 are mechanisms within the clock circuitry that promote period-heterogeneity, the network might  
259 also harbour hormesis-based mechanisms which impose constraints on the range of period that  
260 the circadian clock can exhibit (Baldi and Bucherelli, 2005; Zhang *et al.*, 2008). Such mechanisms  
261 may also explain why we observe a saturation of period divergence after assay generation 2  
262 (Figure 1b).

263 While evidence thus far strongly suggests that clonal period-heterogeneity is driven by  
264 differences in clock gene expressions, we then asked ‘what is the source of these expression  
265 differences?’ One possibility is that the short and long period clones might have accumulated  
266 random mutations resulting in period change and subsequently selected by us. However, we

267 reason that this is highly unlikely because – a) With a mutation rate of  $\sim 2.5 \times 10^{-8}$ /nucleotide in  
268 human cells (Nachman and Crowell, 2000), the probability of occurrence of at least two kinds of  
269 mutations within a small fraction of the genome (comprising clock genes) driving short and long  
270 periods is extremely low. b) We see significant trends in expression of the same subset of genes  
271 across both short and long period clones (Figure 2g; Supplementary Figure S6). This presupposes  
272 that mutations driving short and long periods have occurred within the same genes, which  
273 further drastically reduces the probability that the observed period differences stem from  
274 random mutations. c) Even if the mutation rate is higher than we estimate, the saturation of  
275 divergence in period over the last three assay generations (Figure 1b) cannot be entirely  
276 accounted by mutations since the periods could continue to diverge due to further accumulation  
277 of mutations. Therefore, we argue that the observed period-heterogeneity is unlikely to be due  
278 to random mutations, which leaves us with another alternative – epimutations. Epimutations are  
279 heritable changes in expression of genes and are not associated with DNA mutations.  
280 Epimutations are often associated with changes in methylation states of genes or other heritable  
281 chromatin modifications (Holliday, 2006). The rates of epimutations are observed to be order of  
282 magnitude higher than DNA mutation rates (Van Der Graaf *et al.*, 2015) and successfully explains  
283 phenotypic heterogeneity in many life forms including clonal populations (Kaufmann *et al.*, 2007;  
284 Stockholm *et al.*, 2007; Neildez-Nguyen *et al.*, 2008; Taudt *et al.*, 2016; Springer and Schmitz,  
285 2017). Therefore, we hypothesized that epimutations-driven gene expression differences may  
286 underlie clonal heterogeneity in circadian period. As a preliminary test of this hypothesis, we  
287 studied the effect of a Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid (SAHA)  
288 treatment on the circadian period across our clones. Interestingly, we find that treatment with

289 SAHA significantly shortens (albeit by a small magnitude) the period in long-period clones with  
290 little or no effect on the short and intermediate clones (Figure 3d). The small magnitude effect  
291 of SAHA treatment might be due to one or all of the following reasons. SAHA is broad spectrum  
292 Histone Deacetylase (HDAC) inhibitor and promotes upregulation of genes by acetylation,  
293 whereas other epigenetic mechanisms that might contribute to the gene expression in our clones  
294 are not targeted by this treatment. Alternatively, off-target effects of SAHA might also upregulate  
295 other genes that in turn negatively influence the change in period. In addition, as discussed  
296 previously, if period heterogeneity is indeed a multi-gene trait relying on combined upregulation  
297 and downregulation of two or more genes, mere treatment with epigenetic modifiers that leads  
298 to genome-wide changes in gene expression may not be a good strategy. Nevertheless, the  
299 differential effects of SAHA on short and long period clones is promising and provides preliminary  
300 support to the idea that epigenetic modulation of gene expression might underlie clonal period-  
301 heterogeneity. Future targeted studies along these lines may shed more light on this aspect.

302 In conclusion, our study reports that the heterogeneity in periods observed within circadian clock  
303 networks in mammals is not stochastically driven but has a heritable basis and that this is likely  
304 to be a multi-gene trait. We identified that differential regulation of three E-box associated  
305 transcription factors might govern period-heterogeneity in circadian clock networks and provide  
306 preliminary evidence that epigenetically regulated gene expression differences may underlie  
307 clonal period-heterogeneity. In addition, we also observed a subset of genes that exhibit which  
308 we hypothesize are part of homeostatic mechanisms that may constrain circadian clocks from  
309 deviating largely from their optimal period range. Future studies will help further explore the  
310 phenomenon of period-heterogeneity and its regulation.

## 311 **MATERIALS AND METHODS**

### 312 **Clone selection protocol**

313 All clones used in this study were U-2 OS cells (human, ATCC # HTB-96) stably expressing firefly  
314 luciferase from a 0.9-kb *BMAL1* promoter (Maier *et al.*, 2009), cultured and maintained in DMEM  
315 containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).  
316 See Supplementary Figure S1 for a pictorial description of the selection protocol. Briefly, cells  
317 from ‘founding culture’ expressing a circadian period of  $24.6 \pm 0.16$  h (mean  $\pm$  SD) were plated as  
318 single-cell clones in 96-well ‘parent plates’ and grown to confluency. Upon reaching confluency,  
319 an ‘assay plate’ was established for every ‘parent plate’ by splitting cells. The period of  
320 bioluminescence rhythms from cells in ‘assay plates’ was recorded (see below for recording  
321 protocol) and clones exhibiting short or long periods (tails of the period-distribution) were  
322 selected. Bioluminescence rhythms of every clone was recorded 2-3 times and only clones that  
323 consistently exhibited short/long periods were selected. Following the selection of clones,  
324 corresponding clones from the ‘parent plate’ were single-cell cloned in 96-well plates, and the  
325 procedure was repeated for four assay generations by selecting short and long period clones  
326 every generation.

### 327 **Bioluminescence recording**

328 Cells were plated in white 96-well plate at a density of  $20 \times 10^3$  cells/well and after 72 hours, cells  
329 were synchronized with dexamethasone (1 µM) for 30 minutes, washed twice with PBS and  
330 cultured in Phenol-Red-free DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml  
331 penicillin and 100 µg/ml streptomycin) and 250 µM D-luciferin (Biothema, Darmstadt, Germany).



332 Bioluminescence was recorded at 37°C in a 96-well plate luminescence counter (TopCount,  
333 PerkinElmer, Rodgau, Germany) for up to 7-days. ChronoStar software (Maier *et al.*, *in press*) was  
334 used for data analysis and estimation of rhythms parameters including period, decay constant  
335 (damping), relative amplitude and average bioluminescence (MESOR) of the oscillation as  
336 described previously (Abraham *et al.*, 2010).

### 337 **RNA preparation and NanoString based gene expression analysis**

338 Five days before the RNA extraction, cells were plated at a density of  $\sim 20 \times 10^3$  cells/well in 24-  
339 well plate with DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and  
340 100 µg/ml streptomycin). Since we intended to measure average gene expression levels, the  
341 culture medium was not replaced for five days to prevent accidental synchronization of cells. On  
342 day-5 the medium was removed, 100 µl/well iScript™ RT-qPCR Sample Preparation Reagent  
343 (Biorad) was added on top of the cell-layer and incubated at 37 °C for 5 min. 3µl of the sample  
344 was withdrawn without disturbing the cell-layer and used for further downstream analysis as per  
345 manufacturer's instructions.

346 A previous study of ours combined whole-genome transcriptomics with machine learning and  
347 identified genes that could serve as reliable circadian time-telling markers (Wittenbrink *et al.*,  
348 2018). Based on this, we designed a 24-plex NanoString probe panel comprising 20 circadian clock  
349 and clock associated genes and 4 housekeeping genes (Supplementary Table S1). The custom-  
350 designed probes included a 3'-end biotinylated capture probe and a 5'-fluorescence-barcoded  
351 reporter probe for each gene target. Hybridization of probes and gene expression-count reading  
352 was according to the manufacturer's instructions. Raw expression data was acquired by a

353 NanoString nCounter Digital Analyzer (NanoString Technologies), QC processed and analysed by  
354 nSolver™. QC analysis flagged reads from one (*CIART*) of the 24 genes in the panel as unsuitable  
355 for analysis and was not considered. Data normalization involved three steps: (a) normalization  
356 by the arithmetic mean of the positive spike-in controls, (b) subtraction of the mean of the  
357 negative controls, and (c) normalization by the geometric mean of the four housekeeping genes.

### 358 **Principal Component Analysis and Clustering**

359 Log<sub>2</sub>-transformed gene expression data were first subjected to Bartlett's Test of Sphericity to  
360 validate its adequacy for Principal Component Analysis (PCA) following which correlation-based  
361 PCA was implemented in R (R Core Development Team, 2013) using factoextra and FactoMineR  
362 packages (Kassambara, 2016). Broken-Stick model (Jolliffe, 2011) was used to determine the  
363 number of retainable Principal Components (PCs). Determining the optimal cluster-number is  
364 often a complication in unsupervised exploratory data analysis. Unlike many studies in biology  
365 that employ PCA to identify genes based on expression differences between known cell-types  
366 (which can be used to estimate the optimal number of clusters), our study employs a panel of  
367 clones with a continuous distribution of phenotypes (period) and thus cannot be categorized  
368 trivially. Hence, we adopted two schemes for optimal cluster-number determination. (a) For  
369 agglomerative hierarchical clustering, we assessed the agglomeration schedule to identify the  
370 possible number of clusters (Yim and Ramdeen, 2015). (b) In addition, we also performed k-  
371 means clustering for different values of cluster ( $k = 1-10$ ) and used 5 different indexes –  
372 'silhouette method' (Rousseeuw, 1987), 'elbow method' (Thorndike, 1953), 'gap-statistic'  
373 (Tibshirani *et al.*, 2001), 'Calinski-Harabasz criterion value (variance-ratio method)' (Caliński and  
374 Harabasz, 1974) and Bayesian Information Criterion (BIC; Fraley and Raftery, 2002) to assess the

375 optimal cluster-number. We selected the optimal cluster number based on agreement between  
376 (a) and (b). Heatmapper (Babicki *et al.*, 2016) and 'dendextend' (Galili, 2015) were used for  
377 hierarchical clustering analysis based on 'euclidean-distance' and 'complete-linkage' measures  
378 (D, 2005). 'Nbclust' (Charrad *et al.*, 2014) and 'mclust' (Scrucca *et al.*, 2016) were used for k-  
379 means based clustering analysis while for all other statistical analysis and graphing was  
380 performed using R and Prism version 8.00 for Windows (GraphPad Software, La Jolla California  
381 USA, [www.graphpad.com](http://www.graphpad.com)).

### 382 **RNAi mediated gene knockdown**

383 The GIPZ microRNA-adapted shRNA constructs used for the study were purchased from Open  
384 Biosystems and packaged into lentiviral vectors in HEK293T cells in a 96-well plate format (Maier  
385 *et al.*, 2009). Virus-containing supernatants were then filtered and reporter cells (clonal cell  
386 populations used in the study) were transduced with 150  $\mu$ L of the filtrate containing 8 ng/ $\mu$ L  
387 protamine sulfate. After at least 24h, the filtrate was replaced with fresh medium containing  
388 puromycin (10  $\mu$ g/mL). After 3 days, the transduced reporter cells were synchronized and  
389 bioluminescence rhythms were recorded as described above.

### 390 **SAHA treatment and dose response analysis**

391  $10^3$  cells were plated in a 96-well plate on day-0. After 24h, the culture media was replaced with  
392 media containing 1.6  $\mu$ M Suberanilo Hydroxamic Acid (SAHA) or DMSO vehicle control. The drug  
393 was replaced every day for three consecutive days. On day-4, the cells were rinsed thrice with  
394 PBS and fresh (no drug) culture media was added. The cells were untreated for next 48h and  
395 bioluminescence rhythms were recorded from day-6.

396 The above-described protocol was followed for estimating the IC<sub>50</sub> value as well. Cells were  
397 treated with varying concentrations (0-100 µM) of SAHA from day-1, and cell proliferation was  
398 assayed on day-6 using the Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fischer Scientific,  
399 catalog #V13154) as per manufacturer's protocol. IC<sub>50</sub> was calculated from the resulting dose  
400 response curve using Prism version 8.00 for Windows (GraphPad Software, La Jolla California  
401 USA, [www.graphpad.com](http://www.graphpad.com) ; Supplementary Figure S7).

402

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410

### 411 **AUTHOR CONTRIBUTIONS**

412 Conceptualization: NKL, SK and AK; Experiments and data acquisition: NKL and SK. Data curation  
413 and formal analysis: NKL and SK; Manuscript preparation: NKL and AK.

414

### 415 **COMPETING INTERESTS**

416 The authors declare no conflict of interest.

417

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597

598

599

600 **FIGURE LEGENDS**

601 **Figure 1: Clonal period-heterogeneity is not stochastically driven but largely inherited**

602 **(a)** Divergence of the period-distributions of short (red) and long (blue) period clones from a  
603 common founding culture (green) across multiple assay generations. Dashed black lines depict  
604 the mean of respective period distributions. The grey dashed line extended from assay  
605 generation-1 depicts mean period of the founding culture (assay generation 0) for visual  
606 assessment of the period divergence. Red (short period clone) and blue (long period clone)  
607 arrows indicate the means periods of representative clones selected for the successive  
608 generation **(b)** Divergence of the mean period among three representative clonal lines each for  
609 long-period clonal line (LCL) 1-3 and short-period clonal line (SCL) 1-3. Error bars are SD (n = 3-5  
610 experiments).  $\alpha$  indicates that the period of all three SCLs differs significantly from all three LCLs  
611 for the given assay generation. Asterisks (\*) on top represent LCLs and those at the bottom  
612 represent SCLs. Asterisks of different colours indicate that the period of the three clones in that  
613 generation is significantly different ( $p < 0.001$ ; n = 3-5) from the periods in other generations,  
614 while those with the same colour do not differ significantly. For example, the periods of SCLs 1-3  
615 in assay generation-2 differ significantly from their periods in assay generation-1 and from the  
616 founding culture in assay generation-0, but not from assay generations 3 and 4. **(c)** Regression of  
617 progeny cultures' periods on mean periods of their parental cultures' periods as a proxy-estimate  
618 of heritability. Each data point is an average of 3-5 experiments. Blue solid line is the linear  
619 regression fit with its 95% CI (blue dotted line).

620

621 **Figure 2: Inheritance of clock-gene expression patterns might govern clonal period-**  
622 **heterogeneity**

623 **(a)** Linear regression of progeny mean bioluminescence intensity on parental values suggests a  
624 strong heritability of mean bioluminescence intensity ( $R^2 = 0.76$ ). Each data point is an average  
625 of 3-5 experiments. Blue solid line is the linear regression fit with its 95% CI (blue dotted line). **(b)**  
626 Cross-correlation of average expression values between the 19 analysed genes across all 25  
627 clones indicates a high degree of inter-gene correlation. The colour and size of the circles  
628 represent the strength of correlation (Pearson  $r$ ). **(c)** Scree plot depicting the percentage of  
629 variance explained by the 19 principal components (black bars) and the expected values based  
630 on the Broken-Stick model (red line). **(d)** Factor map of individual clones plotted across the  
631 principal components (PCs) 1 and 2 reveals that the first two PCs clusters the clones in three  
632 clusters of short (red), intermediate (green) and long (blue) period clones. **(e)** Correlation circle  
633 depicting the loading of 19 genes across PCs 1 and 2. **(f)**  $\text{Cos}^2$  values (squared loadings as a  
634 measure of the quality of representation of a gene on a PC) of the 19 genes for PCs 1-5. The  
635 colour and size of circles represent the magnitude of  $\text{Cos}^2$  value. **(g)** Hierarchical clustering based  
636 on the expression of 5 genes selected from PC2. With the exception of one clone, all others  
637 clustered into 3 groups of short, intermediate and long clones (red, green and blue dashed  
638 rectangles respectively). **(h)** Hierarchical clustering based on the expression of 5 genes from PC1  
639 resulted in 2 clusters – i) intermediate period (green dashed rectangle) and ii) short and long  
640 period (blue dashed rectangle). The colour coding of clones in (g) and (h) is the same as in (d).

641



642 **Figure 3: Epigenetically regulated expression of E-Box associated factors may govern clonal**  
643 **period-heterogeneity**

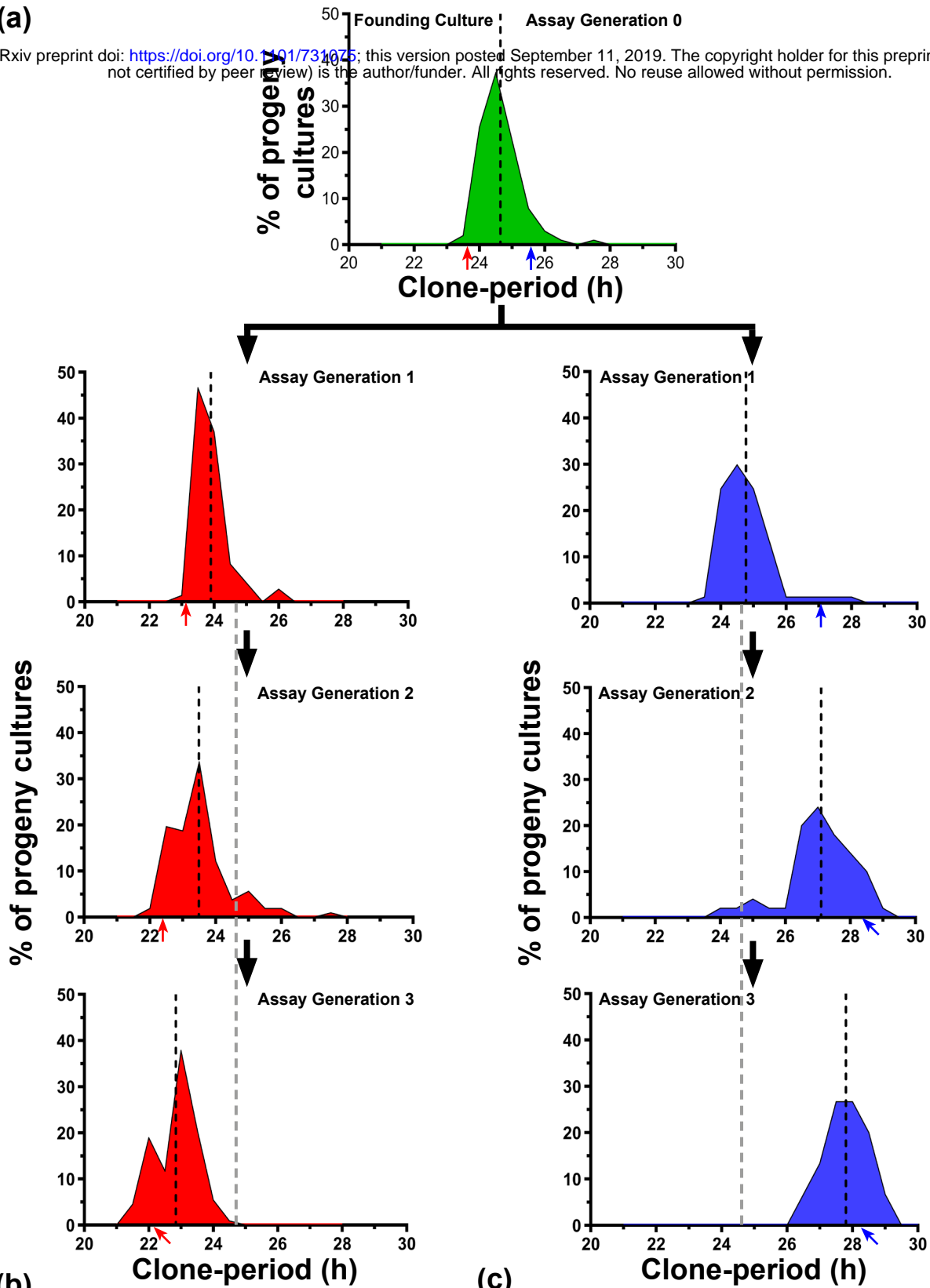
644 Period change (compared to non-silenced control) upon knockdown of the **(a)** five PC2 genes and  
645 **(b)** three PC1 genes for the short (red), intermediate (green) and long (blue) period clones. Bars  
646 with different symbols indicate significant differences ( $p < 0.05$ ) while bars with same symbols  
647 are not significantly different from each other (Mixed model ANOVA followed by Tukey's HSD).  
648 **(c)** Averaged absolute period change across all clones upon knockdown of genes from PC2 (black)  
649 and PC1 (grey). **(d)** Period change (compared to vehicle control) upon treatment of short (red),  
650 intermediate (green) and long (blue) period clones with HDAC (Histone Deacetylase) inhibitor  
651 SAHA (1.6  $\mu$ M). For all panels in this figure,  $n = 3-4$  experiments and error bars are SD. \*:  $p < 0.05$ ;  
652 \*\*\*\*:  $p < 0.0001$ .

653

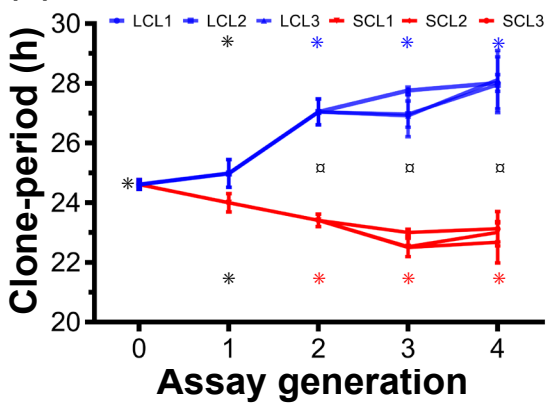
# Figure 1

(a)

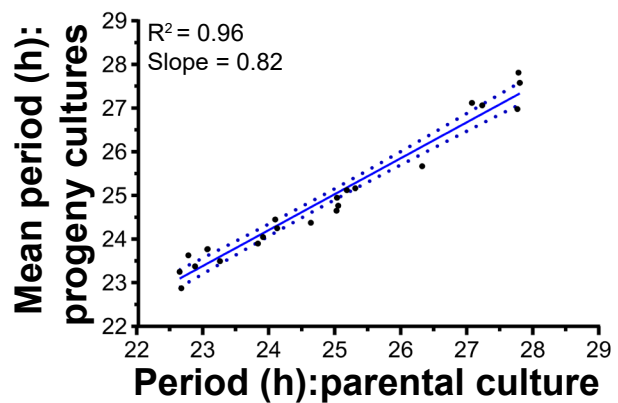
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(b)

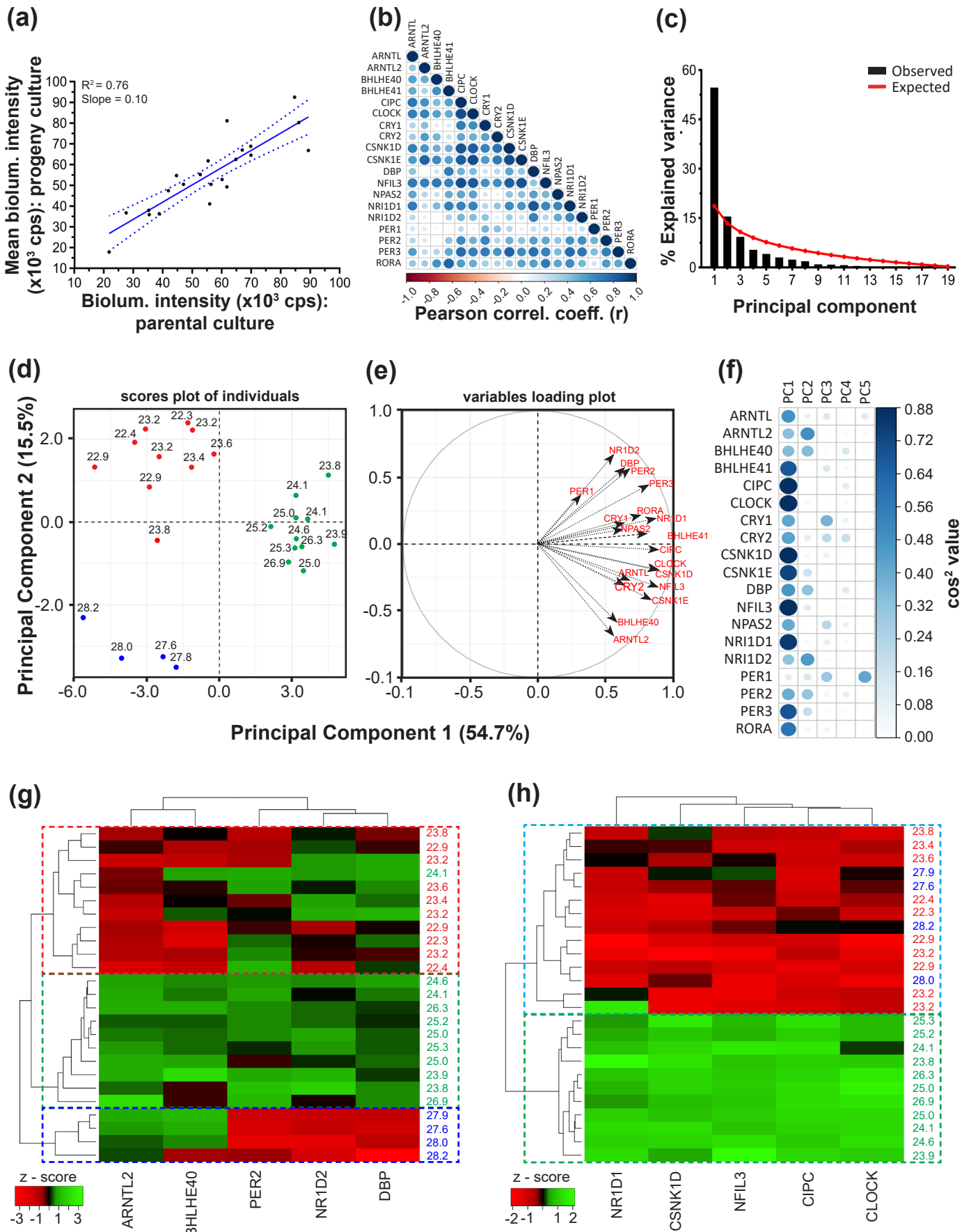


(c)



# Figure 2

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# Figure 3

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