



## 32 Introduction

33 Cell division requires the entire genome to be duplicated once and only once during the S-  
34 phase of the cell cycle, followed by segregation of the sister chromatids into two daughter cells.  
35 To ensure the complete and correct duplication of genomes, the initiation of DNA replication is  
36 highly regulated and begins with the assembly of a pre-Replication Complex (pre-RC) at origins  
37 of DNA replication throughout the genome (Bell and Labib, 2016). Among eukaryotes, studies in  
38 *Saccharomyces cerevisiae* have proved to be the best characterized system, from which  
39 individual proteins involved in DNA replication have been identified and studied extensively,  
40 including reconstitution with purified proteins of pre-RC assembly and the regulated initiation of  
41 DNA replication from pre-RCs (Evrin et al., 2009; Remus et al., 2009; Yeeles et al., 2015). In *S.*  
42 *cerevisiae*, pre-RC assembly begins with the hetero-hexameric Origin Recognition Complex  
43 (ORC), comprising Orc1-6 subunits, binding to each potential DNA replication origin (Bell et al.,  
44 1993; Bell and Labib, 2016; Bell and Stillman, 1992; Gibson et al., 2006). Chromatin-bound  
45 ORC then provides a platform for the assembly and recruitment for other pre-RC proteins. Cdc6  
46 binds to ORC, followed by the binding of the Cdt1-Mcm2-7 complex to form head-to-head  
47 Mcm2-7 double hexamers to complete the formation of the pre-RC (Araki, 2011; Bell and Labib,  
48 2016; Bleichert et al., 2017; Evrin et al., 2009; Heller et al., 2011; Remus et al., 2009). The  
49 Mcm2-7 double hexamer helicase precursor complex remains bound to DNA in an inactive state  
50 until it is activated by additional proteins and protein kinases (Bell and Labib, 2016). During S  
51 phase, Cyclin-dependent protein kinase (CDK) and the Cdc7-Dbf4-dependent protein kinase  
52 (DDK), Sld2, Mcm10, Dpb11, Sld3/7, DNA polymerase  $\epsilon$ , Cdc45 and the GINS complex are  
53 recruited to activate MCM2-7 helicase (Araki, 2016; Araki et al., 1995; Bell and Labib, 2016;  
54 Kamimura, 2001; Kamimura et al., 1998; Takayama et al., 2003; Yeeles et al., 2015). The  
55 functional helicase consists of Cdc45-Mcm2-7-GINS (CMG) and when activated it unwinds the  
56 DNA in a bidirectional and temporally regulated manner from each origin (Bleichert et al., 2017).

57 In all eukaryote cells, including those from both yeast *S. cerevisiae* and human, each ORC1-5  
58 subunit consists of a AAA+ or a AAA+-like domain and a winged helix domain (Bleichert et al.,  
59 2017; Chen et al., 2008; Li et al., 2018; Ocaña-Pallarès et al., 2020; Tocilj et al., 2017). In yeast,  
60 Orc1-6 remains as a stable complex bound to the chromatin throughout the cell division cycle  
61 (DePamphilis, 2003; Weinreich et al., 1999). ORC binds to A and B1 DNA sequence elements  
62 within the Autonomously Replicating Sequence (ARS), which contains a conserved ARS  
63 Consensus Sequence (ACS) (Bell and Labib, 2016; Bell and Stillman, 1992; Celniker et al.,  
64 1984; Deshpande and Newlon, 1992; Marahrens and Stillman, 1992; Rao and Stillman, 1995).

65 On the other hand, in human cells, there is no sequence-specific binding of ORC to the DNA,  
66 and the binding of ORC to chromosomes is dynamic (Vashee et al., 2001). ORC subunits do  
67 localize to specific sites within the chromosome, most likely via interactions with modified  
68 histones (Hossain and Stillman, 2016; Long et al., 2019; Miotto et al., 2016). One or more of the  
69 human ORC subunits dissociate from the complex soon after the pre-RC is formed. For  
70 example, ORC1 is ubiquitinated by the SCF<sup>skp2</sup> ubiquitin ligase during the G1-S transition and  
71 then re-appears as cells enter mitosis (Kara et al., 2015; Kreitz et al., 2001; Méndez et al., 2002;  
72 Ohta et al., 2003). Human cell ORC1 is the first ORC subunit to bind to mitotic chromosomes  
73 and is inherited into the daughter cells where it recruits other ORC subunits and CDC6 to form  
74 the new pre-RCs (Kara et al., 2015; Okuno et al., 2001).

75 ORC is a conserved complex in eukaryotes, and it is essential for DNA replication in *S.*  
76 *cerevisiae*, *S. pombe*, *Xenopus* and *Drosophila*, since mutation or depletion of ORC prevents  
77 CDC6 binding and MCM loading onto DNA (Chuang et al., 2002; Pflumm and Botchan, 2001;  
78 Romanowski et al., 1996; Speck et al., 2005). Besides its function in the initiation of DNA  
79 replication, ORC protein subunits also play other important roles. In yeast, Orc1 directly  
80 interacts with silencing regulator Sir1 at the silent mating type loci to mediate transcriptional  
81 gene silencing and maintain heterochromatin (Bell et al., 1993; Fox et al., 1995; Hou et al.,  
82 2005; Triolo and Sternglanz, 1996). ORC1 also plays a role in transcriptional gene silencing in  
83 human cells (Hossain and Stillman, 2016). ORC2 depletion after pre-RC assembly resulted in  
84 spindle and DNA damage checkpoint activation, and impaired sister-chromatid cohesion  
85 (Shimada and Gasser, 2007). In *Drosophila*, *Orc2* mutants showed reduced S phase cells,  
86 increased number of mitotic cells with abnormally condensed chromosomes and chromosome  
87 alignment defects, and more importantly, those mutants could not survive at late larval stage  
88 (Loupart et al., 2000; Pflumm and Botchan, 2001). In human, mutations in ORC1, ORC4,  
89 ORC6, CDT1, and CDC6 are detected in Meier-Gorlin syndrome patients (Bicknell et al., 2011b,  
90 2011a; Guernsey et al., 2011; Hossain and Stillman, 2012; Munnik et al., 2015). ORC1 and  
91 ORC2 localize to centrosomes and ORC1 regulates the re-duplication of the centriole (Hemerly  
92 et al., 2009; Prasanth et al., 2004). ORC also localizes to telomeres via the TRF2 shelterin  
93 protein (Deng et al., 2009; Tatsumi et al., 2008). It was also shown that siRNA knockdown or  
94 CRISPR/Cas9 knockout of ORC1 resulted in loss of MCM2-7 from chromatin, abnormal  
95 duplication of centrioles, and a change in cell cycle stage distribution (Hemerly et al., 2009; Kara  
96 et al., 2015; McKinley and Cheeseman, 2017). ORC1, ORC2, ORC3, and ORC5 associate with  
97 heterochromatin, and depletion of any ORC subunits disrupt localization of heterochromatin and  
98 also causes abnormal heterochromatin decondensation in cells (Giri et al., 2016; Prasanth et

99 al., 2010, 2004). ORC2 and ORC3 also specifically localize to centromeric heterochromatin  
100 during late S phase, G2 and mitosis and removal of these proteins causes decondensation of  
101 centromeric  $\alpha$ -satellite (Craig et al., 2003; Prasanth et al., 2010, 2004).

102 There is an emerging debate, however, about the essential nature of ORC in human cells (Bell,  
103 2017). ORC is overexpressed in numerous cancerous cell lines (McNairn and Gilbert, 2005) and  
104 HCT116 colorectal cancer cells can survive with only 10% of the ORC2 protein level (Dhar et  
105 al., 2001). More importantly, it was reported that HCT116  $p53^{-/-}$  ( $TP53^{-/-}$ , but we henceforth use  
106  $p53^{-/-}$ ) cells in which expression of either ORC1 or ORC2 subunit was eliminated using  
107 CRISPR-Cas9 mediated gene ablation could still proliferate (Shibata et al., 2016). Here we  
108 developed a genetic method to address the function of the pre-RC proteins ORC and CDC6,  
109 particularly focusing on the ORC1 and ORC2 subunits. We demonstrate that ORC proteins are  
110 essential for normal cell proliferation and survival in human cells. Moreover, ORC1 or ORC2  
111 depleted cells showed multiple defects in progression through cell division cycle, including DNA  
112 replication and mitosis, as well as defects in nuclear structure.

## 113 Results

### 114 ORC1-6 and CDC6 are essential for cell survival

115 Despite the implication that ORC in performs a critical role in DNA replication as well as cellular  
116 proliferation, there are conflicting observations about the essential role for the ORC1 and ORC2  
117 subunits in human cells (Shibata et al., 2016). To further confound the issue, examination of the  
118 genes essential for cell survival in the DepMap database that summarize results from whole-  
119 genome CRISPR screens (GeCKO 19Q1 and Avana 20Q2 libraries) (Meyers et al., 2017;  
120 Tsherniak et al., 2017), show that ORC1 is found to be a common essential gene while ORC2 is  
121 listed as non-essential in tested cell types (Figure 1—Figure supplement 2a). Other members of  
122 the pre-RC proteins - ORC3-6, CDC6, MCM2-7 (data not shown) and CDT1 (data not shown)  
123 are all classified as common essential by at least one of the two whole genome CRISPR  
124 screens (GeCKO and Avana) but not necessarily both in DepMap.

125 To address the question of essentiality and to map functional domains within the ORC and  
126 CDC6 proteins, we used unbiased tiling-sgRNA CRISPR depletion screens which have been  
127 shown to be informative of not only the overall essentiality of the protein to cell fitness but also  
128 directly help nominate previously unknown, functionally essential regions in these in proteins,  
129 DNA and RNA (He et al., 2019; Hsu et al., 2018; Montalbano et al., 2017; Shi et al., 2015; Wang

130 et al., 2019). We employed a tiling sgRNA CRISPR scheme using designed libraries of guide  
131 RNA targeting every possible PAM sequence 5'-NGG-3' (*Streptococcus pyogenes* Cas9) across  
132 each exon of *ORC1-6* and *CDC6*. These CRISPR libraries also included control guide RNAs  
133 targeting either known core essential genes or those targeting non-essential gene loci or no loci  
134 at all (Miles et al., 2016). The total library comprised 882 guides targeting *ORC1-6* and *CDC6*,  
135 1602 negative controls (Used in GeCKO V2 library - "NeGeCKO" (Sanjana et al., 2014),  
136 negative controls used in The Sabatini/Lander CRISPR pooled library (Park et al., 2016),  
137 Rosa26, CSHL in-house negatives (Lu et al., 2018; Tarumoto et al., 2018) and 43 positive  
138 controls (median of 3 guides targeting essential genes such as those encoding CDK1, CDK9,  
139 RPL9, PCNA etc.) (Supplement Table1\_guides). Parallel screens were done in HCT116-Cas9  
140 and RPE-1-Cas9 cell lines and the relative depletions of guide RNA in the cell populations  
141 between day 3 and day 21 were compared using the guide read counts generated by illumina  
142 based next-generation sequencing (n = 2 for HCT116, n = 1 for RPE-1) and the data was  
143 analyzed with MAGeCK (Li et al., 2014). The screens performed well as shown by the  
144 consistent log fold change (LFC) pattern of depletion or enrichment of positive and negative  
145 controls respectively - although the absolute values and range of LFCs were cell-line specific.  
146 As expected, overall the CRISPR-Cas9 screen had a more profound effect on the diploid RPE-1  
147 cells as compared to a transformed cell line like HCT116. The LFC threshold of 'essentiality'  
148 was set at the value at which a guide RNA was depleted more than every negative control as  
149 well as  $\geq$  to the median depletion of guides targeting positive controls (Figure 1—Figure  
150 supplement 1b-d). In two replicate screens of HCT116, LFC  $\geq -1$  and LFC  $\geq -5$  in RPE-1, were  
151 found to be the cut-off for log fold depletion, above which the regions of the protein can be  
152 called essential. The fact that many such regions exist across the protein of course also imply  
153 the protein itself is essential to the cell line. Importantly, screens showed significant drop out of  
154 guide RNAs that target regions that have been previously annotated by structure and function  
155 (Figure 1a-b, 1f, Figure 2a-c, Figure 2—Figure supplement 1a, 3a-c, 3g, 4a-c, 4g, 4h-j, 4n, 5a-c,  
156 5g, 5h-j, 5n). To visualize the tiling sgRNA data mapped to translated protein sequence in  
157 relation to its amino acid conservation and disorder we used NCBI RefSeq coding sequences  
158 (NP\_004144.2, NP\_006181.1, NP\_862820.1, NP\_859525.1, NP\_002544.1, NP\_055136.1,  
159 NP\_001245.1) for three analyses - (1) FrPred (Adamczak et al., 2004; Fischer et al., 2008)  
160 (<https://toolkit.tuebingen.mpg.de/frpred>) server that calculates a conservation score based on  
161 amino acid variability as well as the probability of it being a functional ligand binding or catalytic  
162 site at each amino acid position of the input sequence (Figure 1c, Figure 2—Figure supplement  
163 1b, 3d, 4d, 4k, 5d, 5k); (2) ConSurf (Ashkenazy et al., 2016) (<https://consurf.tau.ac.il/>) server  
164 which analyses the probability of structural and functional conservation despite amino acid

165 variability for any given position of input sequence (Figure 1d, Figure 2—Figure supplement 1c,  
166 3e, 4e, 4l, 5e, 5l). We ran this server overall with default parameters except for the number of  
167 species to include. In one analysis we chose 50 representative species homologues with  
168 maximum and minimum percent identity set to 95 and 50 respectively, and in the other we  
169 increased the species to 150 and set max. and min. percent identity to 95 and 35 to compare a  
170 larger evolutionary subset. In both analyses the UNIREF90 database was used, which consists  
171 of cluster sequences that have at least 90% sequence identity with each other into a single  
172 UniRef entry, thus increasing the representative diversity of species considered in the output.  
173 And lastly, 3) Disopred tool (Buchan and Jones, 2019; Jones and Cozzetto, 2015)  
174 (<http://bioinf.cs.ucl.ac.uk/psipred/>) that scores for intrinsically disordered regions (IDRs) that are  
175 usually not well conserved yet found to be functionally essential in many proteins (Figure 1e,  
176 Figure 2—Figure supplement 1d, 3f, 4fm 4m, 5f, 5m). In this similar way, we also performed  
177 tiling sgRNA screens for MCM2-7 and CDT1 and found them to be essential, but including that  
178 data was currently beyond the scope of this work.

179 For the purpose of this paper, we focus on discussing the tiling-sgRNA screens for *ORC1* and  
180 *ORC2* and compare the phenotypic differences with DepMap datasets as well as the previous  
181 study that classified these two subunits as non-essential. The pattern of depletion for *ORC1*  
182 showed that the strongest (higher in the plot) depletions map within the *Bromo-Adjacent*  
183 *Homology* (BAH) domain, ATPase Associated with diverse cellular Activities domain (AAA+)  
184 and the *Winged-Helix Domain* (WHD), all of which are crucial for its function (Figure 1a, 1b, 1f).  
185 The same is evident in both cell lines for *ORC2* where the highly depleted guides cluster to the  
186 ATPase-like C terminal end of *ORC2* (Figure 2a-c). Interestingly, we found there were  
187 functionally essential regions in the lesser conserved IDR regions of both *ORC1* and *ORC2*. Our  
188 lab has recently reported that a Cyclin-motif bearing region of *ORC1* (180 - 240 aa) is essential  
189 for binding to CDC6 at the right time during the cell cycle to enable pre-RC formation (Hossain  
190 et al., 2019). And similarly, based on homology, a previously identified putative NLS motif  
191 (Lidonnici et al., 2004) also had hits nominating that region as essential in our *ORC1* screen  
192 (Figure 1f). There are other essential regions that correspond to novel protein binding sites that  
193 are separately being investigated.

194 When we compared DepMap CRISPR Achilles (Avena 20Q2 library) dataset and a combined  
195 RNAi dataset of cell lines, it indicated that using the CRISPR method, with a gene effect score  
196 of less than -0.5, *ORC1* classified as common essential in > 90 percent of the cell lines, while  
197 with RNAi datasets with that same cut-off, it classified as essential in only about 45 percent of

198 the same cell lines (Figure 1—Figure supplement 2b-c). It is evident that the method of choice  
199 did have a bearing on the phenotypic outcome of the knock-down. The study by Shibata et al.  
200 (2016) that found *ORC1* and *ORC2* to be non-essential also used CRISPR editing as the  
201 method of knock-down, but also performed long term selection for cell proliferation to obtain  
202 *ORC1*<sup>-/-</sup> or *ORC2*<sup>-/-</sup> cells. We therefore determined our screen had guide RNAs that were used in  
203 either of the DepMap dataset or used in the directed study (Shibata et al., 2016). For *ORC1* and  
204 *ORC2* sgRNAs that were used in DepMap datasets, there was a variation in their phenotype as  
205 measured by LFC values, with some guides classifying *ORC1* and *ORC2* as essential and  
206 others not (Figure 2—Figure supplement 2a-c). Of note is the fact that the guide used to target  
207 *ORC2* in the Shibata et al. (2016) study showed activity very close to the cut-off in HCT116 cells  
208 and scored as non-essential in RPE-1. It is important to note that when a guide targeting a  
209 relatively non-essential region allows for the cells to proliferate, no conclusion can be made  
210 about the protein being essential. The Shibata et al. (2016) study used that single guide to insert  
211 a blasticidin and a poly A cassette into the locus, presumably disrupting its transcription  
212 significantly, while our single-guide-per-locus type of screen did not introduce such large  
213 insertions. We find that *ORC1-6*, *CDC6* are essential in both cell lines tested, and that the  
214 depletion of these proteins negatively affected the diploid RPE-1 cells more. The results of  
215 these screens and the published DepMap conclusions, especially about *ORC2*, suggest that  
216 using too few guides to target proteins can lead to artifactual observations both in terms of  
217 essentiality or non-essentiality, and that overall gene-effect is affected by the combination of the  
218 choice of guide RNA and the cell line studied. At this point we selected guide RNAs that target  
219 *ORC2* from our tiling sgRNA screen to study them individually (Figure 2—Figure supplement  
220 2d). We also received *ORC1* and *ORC2* deficient stable cell lines from the authors of the  
221 previous study (Shibata et al., 2016) for further analysis.

## 222 **Rapid *ORC2* removal in cancer cells impedes cell growth and causes DNA damage**

223 Depleting *ORC2* with an siRNA approach was a slow process that took at least 24-48 hours,  
224 however, using this approach various phenotypes have been observed, including G1 arrest, an  
225 S-phase defect, and abnormally condensed chromosomes as well as defects in mitosis  
226 (Prasanth et al., 2010, 2004). These phenotypes can be an outcome of accumulated errors that  
227 happen during any phase of cell cycle and thus it is hard to distinguish between primary and  
228 secondary phenotypes associated with the loss of *ORC2*. Therefore, we used CRISPR/Cas9 in  
229 combination with tagging *ORC2* with an auxin inducible degron (mAID) to construct cell lines in  
230 which the endogenous *ORC2* was knocked out by CRISPR, and the complementing CRISPR-

231 resistant mAID-ORC2 could be then rapidly removed from cells, allowing exploration of the  
232 importance of ORC2 at different stages of cell division cycle (Natsume et al., 2016; Nishimura et  
233 al., 2009). To mediate the endogenous ORC2 knockout, four sgRNAs were selected from the  
234 CRISPR screen to validate the screening result. For complementation, mAID-tagged sgRNA  
235 resistant ORC2 (mAID-ORC2<sup>9f</sup>) was constructed with the mAID degron fused to the ORC2  
236 amino-terminus, and the *ORC2* cDNA was edited to harbor multiple mismatches based on two  
237 of the sgRNAs, ORC2-1 and ORC2-2 (Figure 2d). The mAID-ORC2<sup>9f</sup> was transduced into the  
238 TO-HCT116 cell line, which expresses a doxycycline-inducible *Oryza sativa* (Asian rice) *TIR1*  
239 (*OsTIR1*) gene that encodes a plant auxin-binding receptor that interacts with the conserved E3  
240 ubiquitin ligase SCF complex to degrade mAID-tagged proteins (Natsume et al., 2016;  
241 Nishimura et al., 2009), or the U2OS cell line to perform ORC2 depletion or genetic  
242 complementation, respectively. The effects of four sgRNAs were tested. In the negative-  
243 selection CRISPR/Cas9 experiment, cells expressing a positive control RPA3 sgRNA and all  
244 four ORC2 sgRNAs, but not the negative control Neg15 sgRNA, were outcompeted over 3  
245 weeks in culture by non-transduced cells that lacked any sgRNA (Figure 2e-f). The CRISPR  
246 competition effects by ORC2-1 and ORC2-2 sgRNAs were rescued by mAID-ORC2<sup>9f</sup> in both  
247 TO-HCT116\_mAID-ORC2<sup>9f</sup> and U2OS\_mAID-ORC2<sup>9f</sup> cell lines (Figure 2f-h).

248 To acquire cloned cells to study ORC2 depletion phenotypes, TO-HCT116\_mAID-ORC2<sup>9f</sup> cells  
249 were depleted of the *ORC2* gene with sgRNA ORC2\_1 by the CRISPR/Cas9 editing technique  
250 then individual clones isolated after single cell sorting. Five cell lines, ORC2\_H-1, ORC2\_H-2,  
251 ORC2\_H-3, ORC2\_H-4, and ORC2\_H-5, were obtained from two independent CRISPR/Cas9  
252 knockout experiments done about 6 months apart. Sequencing of the target sites showed that  
253 the ORC2\_H-1 and H-3 cell lines had heterozygous mutations at the sgRNA targeting site which  
254 create premature stop codons downstream of the target site (Figure 3—Figure supplement 1a).  
255 On the other hand, H-2, H-4, and H-5 are homozygous with an identical two-nucleotide-deletion,  
256 creating a nonsense mutation at the sgRNA targeting site. Although ORC2\_1 sgRNA targets the  
257 C-terminus of ORC2, no truncated form of protein was detected by western blot. Our ORC2  
258 rabbit polyclonal antibody was produced against amino-terminus half of the ORC2 protein. The  
259 LTR-driven mAID-ORC2<sup>9f</sup> was low in expression compared to expression in the TO-HCT116,  
260 RPE-1 and IMR90 cells, but was sufficient to complement the loss of endogenous ORC2 done  
261 by CRISPR knockout (Figure 3a).

262 We focused on analysis of the effects of auxin-induced ORC2 depletion in the H-2, H-4, and H-5  
263 cell lines because they have rapid depletion of mAID-ORC2<sup>9f</sup> after auxin treatment. We



264 excluded off target effects by confirming the ORC2-H-2 cloned cell line was resistant to both  
265 ORC2-1 and ORC2-2 sgRNAs, but not to the ORC2-3 and ORC2-4 sgRNAs (Figure 3—Figure  
266 supplement 1b). Compared with the parental TO-HCT116 cell line, the human diploid cell RPE-1  
267 had about 50% of the amount of ORC2, while IMR-90 cells had about 15% (Figure 3a). The  
268 relative levels of ORC3 reflect the levels of ORC2 since they are known to form an intertwined  
269 complex throughout the cell cycle (Dhar et al., 2001; Jaremko et al., 2020; Vashee et al., 2001).  
270 ORC2\_H-2, H-4, and H-5 had no endogenous ORC2 detected (note, a nonspecific smaller band  
271 was detected in between the two endogenous ORC2 proteins) (Figure 3a). In addition,  
272 ORC2\_H-2 cells expressed mAID-ORC2<sup>gr</sup> at about 5% compared to endogenous ORC2 level in  
273 TO-HCT116, and H-4 and H-5 cells expressed about 10% of the endogenous ORC2 level. It is  
274 known that cancer cells can proliferate normally with 10% of the levels of ORC2 (Dhar et al.,  
275 2001).

276 Next, we compared the proliferation rates in these cell lines. In normal media conditions the  
277 ORC2\_H-4, H-4, and H-5 cells grew only slightly slower than the parental TO-HCT116 cells  
278 (Figure 3b). When doxycycline only was added to induce the OsTIR protein expression, the  
279 proliferation rate of all cell lines decreased, possibly due to some toxicity of doxycycline or the  
280 expression of the OsTIR1 protein itself (Figure 3c). Importantly, the ORC2\_H-4, H-4, and H-5  
281 cells proliferated like the parental TO-HCT116 cells. Moreover, auxin alone did not affect the  
282 proliferation rate of wild type TO-HCT116, H-4, and H-5 cells, but it reduced the proliferation  
283 rate of H-2 cells substantially (Figure 3d). This phenotype was probably due to the leaky  
284 expression of Tet-OsTIR1 in the ORC2\_H-2 cells. Importantly, when both doxycycline and auxin  
285 were added, all three ORC2 KO cell lines stopped proliferating, whereas the parental TO-  
286 HCT116 cells continued to proliferate (Figure 3e).

287 Concomitant with the lack of cell proliferation, the cell cycle profile changed after mAID-ORC2<sup>gr</sup>  
288 was depleted in these cells. Cells were treated with doxycycline and auxin to deplete mAID-  
289 ORC2<sup>gr</sup> for 0 hr., 4 hr., 24 hr., and 50 hr. at most. At the 50 hr. time point, all three ORC2 KO  
290 cell lines had less cells progressing from G1 into S phase, and more cells accumulated at the  
291 end of S phase or at the G2/M phase (Figure 3f, Figure 3—Figure supplement 2). Cells with a  
292 4C DNA content (late S/G2/M phase) continued to incorporate EdU suggesting that DNA  
293 replication was not complete, even though the bulk of the genome was duplicated. This  
294 phenotype was consistent with previous observations that cells treated with ORC2 siRNA  
295 arrested in interphase (70%) or as rounded, mitotic-like cells (30%) (Prasanth et al., 2004).

296 To analyze whether the cell cycle arrest was due to checkpoint activation in response to DNA

297 damage, cell extracts were prepared from doxycycline and auxin treated cells and proteins  
298 detected by immunoblotting with various DNA damage markers. CHK1 is essential for the DNA  
299 damage response and the G2/M checkpoint arrest and is primarily phosphorylated by ATR,  
300 while phosphorylation by ATM has also been reported (Gatei et al., 2003; Goto et al., 2019;  
301 Jackson et al., 2000; Liu et al., 2000; Wilsker et al., 2008). ORC3 and mAID-ORC2<sup>gr</sup> proteins in  
302 ORC2\_H-2, H-4, and H-5 cell lines were depleted after 4 hours of auxin treatment, while ORC1  
303 and CDC6 levels remained unchanged (Figure 3g). Phosphorylation of ATM(S1981),  
304 ATR(T1989), and CHK1(S345) was detected in H-2, H-4, and H-5 after 50 hr. of auxin  
305 treatment, but not in the parental TO-HCT116 cells (Figure 3g, Figure 3—Figure supplement  
306 3a). Higher levels of P- $\gamma$ H2AX(S139) in H-2, H-4, and H-5 cells were detected even when no  
307 auxin was added (Figure 3g). This showed that although cells can divide with only 5-10 % of  
308 ORC2, a certain degree of DNA damage existed. In this experiment, the level of  
309 phosphorylated-CHK2(T68) showed no difference between control and mutant cells, but did  
310 increase along with mAID-ORC2<sup>gr</sup> depletion. Supporting results were also found using  
311 immunofluorescent staining of individual cells. When doxycycline and auxin were added for 48  
312 hours, substantially more ATM(S1981) and CHK1(S345) phosphorylation were detected in all  
313 three ORC2 KO cell lines (Figure 3h-i). In the absence of doxycycline and auxin, the P-  
314  $\gamma$ H2AX(S139) signal was more abundant in ORC2\_H-2, H-4, and H-5 cells than in wild type  
315 (Figure 3—Figure supplement 3b). To conclude, insufficient ORC2 protein in cells resulted in  
316 abnormal DNA replication and DNA damage, and in response to DNA damage, CHK1 was  
317 activated and cells arrested in G2 phase.

### 318 **Loss of ORC2 results in heterochromatin decompaction and abnormal nuclear** 319 **morphology**

320 The ORC2 depleted ORC2\_H-2 and ORC2\_H-5 cells had twice the nuclear volume following  
321 treatment with doxycycline and auxin for 48 hr. (Figure 4a-b) compared to cells without  
322 treatment. The volume of the nucleus was greater than the volume of the largest parental cells,  
323 and thus was not due to their arrest with a 4C DNA content. This phenotype could be due to  
324 cells arrest in G2 phase with heterochromatin decompaction, since ORC2 depletion using  
325 siRNA decondenses centromere associated  $\alpha$ -satellite DNA (Prasanth et al., 2010). During  
326 interphase, ORC2 and ORC3 localize to the heterochromatin foci and interact with  
327 heterochromatin protein 1 $\alpha$  (HP1 $\alpha$ ) through ORC3 (Prasanth et al., 2004). To detect  
328 heterochromatin decompaction, immunofluorescent staining of the centromeric protein C  
329 (CENP-C) was performed. In TO-HCT116 cells, CENP-C staining showed multiple, compact

330 foci, but in the doxycycline and auxin treated cells that were dependent on mAID-ORC2<sup>gr</sup>,  
331 CENP-C foci were larger and more prominent (Figure 4c). These observations were consistent  
332 with heterochromatin decompaction in ORC2 depleted cells.

### 333 **ORC2 is essential for initiation of DNA replication**

334 When cells were treated with siRNA against *ORC2* for 72 hours, 30% of the cells arrested in a  
335 mitosis-like state (Prasanth et al., 2004). This observation led to the conclusion that ORC2 is not  
336 only required for the initiation of DNA replication, but also during mitosis. To examine the role of  
337 ORC2 in G1 and mitosis following acute depletion, TO-HCT116 and the ORC2\_H-2 cells were  
338 synchronized at the G1/S phase boundary with a 2C DNA content with a double thymidine  
339 block, with doxycycline added during the second thymidine block. When indicated, auxin was  
340 added 4.5 hours before the release from the second thymidine block and the cells followed for  
341 progression through two cell division cycles (Figure 5a). Cells were harvested at several  
342 timepoints after release and pulse-labeled with EdU for two hours. During the first cell cycle  
343 following release into S phase, no obvious change in DNA content and EdU labeling was  
344 observed, whether doxycycline and auxin was added or not (Figure 5b-c; Figure 5—Figure  
345 supplement 1). During the second cell cycle, however, doxycycline and auxin-treated TO-  
346 HCT116 cells progressed through S phase only slightly slower than the untreated cells. In  
347 contrast, serious cell cycle defects were observed between the ORC2\_H-2 auxin-treated or  
348 untreated cells, starting with the second cell cycle (Figure 5b-c). First, auxin treated ORC2\_H-2  
349 cells exhibited a very slow S phase, indicating that cells were struggling to correctly replicate the  
350 DNA. Second, cells arrested with a 4C DNA content, which could be in the stage of late S or  
351 G2/M phase. Thirdly, after 48 hours of releasing from the double thymidine block, some cells  
352 were arrested in G1 phase and couldn't enter S phase. Since auxin was added 4.5 hours before  
353 second thymidine release and it required about 4 hours to knockdown mAID-ORC2<sup>gr</sup>, those  
354 phenotypes were observed only during the second cell cycle, suggesting that, for the first cell  
355 cycle, the pre-RC was already formed and the cells were primed to replicate the complete  
356 genome. ORC2 depleted cells then either arrested during G1 or went through an incomplete S  
357 phase and arrested at the G2/M phase and did not progress further. This experiment indicated  
358 ORC2 primarily functions in establishing DNA replication initiation, as expected, but based on  
359 the results so far, we could not conclude a role during mitosis because most ORC2 depleted  
360 cells with a 4C DNA content continued to replicate DNA.

### 361 **An MCM complex loading and pre-RC assembly defect in ORC2 depleted cells**

362 The auxin-treated, mAID-ORC2<sup>gr</sup> depleted cells could not replicate normally, possibly due to  
363 insufficient ORC to form the pre-RC. To test this hypothesis, the DNA-loaded MCM2-7 was  
364 measured by extracting the asynchronous cells with detergent and examining the chromatin  
365 bound MCM2 in relation to cell cycle stage using flow cytometry, as described previously  
366 (Matson et al., 2017). Asynchronous TO-HCT116, ORC2\_H-2, and H-5 cells with or without  
367 doxycycline and auxin treatment were pulse-labeled with EdU, harvested and stained with anti-  
368 MCM2 antibody and DNA dye. The results showed that in normal media without detergent  
369 extraction, nearly 100% of the cells were positive for MCM2 in all three cell lines (Figure 6a;  
370 Figure 6 –Supplement 1). When extracted, about 78 % of TO-HCT116, 65.2 % of ORC2\_H-2,  
371 and 76.9 % of ORC2\_H-5 cells were positive for MCM2. When cells were treated with both  
372 doxycycline and auxin for 20 hours, 84.5 % of TO-HCT116 cells had DNA-loaded MCM2, while  
373 only 4.42% of H-2 and 26.2% of H-5 cells did (Figure 6b; Figure 6—Figure supplement 1). The  
374 different degrees of reduced MCM2 between H-2 and H-5 was expected because the level of  
375 mAID-ORC2<sup>gr</sup> in H-2 was only half the amount compared to H-5 cells, and auxin knockdown  
376 was not 100% efficient. Of particular importance was the lack of MCM2 on chromatin in the G1  
377 phase cells when mAID-ORC2<sup>gr</sup> was depleted. In conclusion, low number of pre-RC formation  
378 on DNA origins results in cells arresting in G1. Those cells that still initiated DNA replication  
379 have a very slow S phase and arrested at G2/M with DNA damage and checkpoint activation.

### 380 **ORC2 depletion in cells leads to aberrant mitosis**

381 In order to know if the mAID-ORC2<sup>gr</sup> depleted cells entered mitosis, we evaluated the mitotic  
382 index by staining cells with anti-pH3S10 and followed by flow cytometry analysis. In a normal  
383 asynchronous cell population, about  $4.53 \pm 0.59$  % and  $1.57 \pm 0.33$  % were pH3S10-positive in  
384 TO-HCT116 and ORC2\_H-2 cells respectively, while  $31.4 \pm 2.88$  % of TO-HCT116 cells and  
385  $15.6 \pm 1.25$  % of ORC2\_H-2 cells were at G2/M (Figure 7a; Figure 7—Figure supplement 1).  
386 When only doxycycline was added, there was no significant change. When treated with  
387 doxycycline and auxin for 28 hours, the pH3S10-positive cell population percentage was about  
388  $2.39 \pm 0.26$  in TO-HCT116 and only  $0.79 \pm 0.09$  in ORC2\_H-2, while  $17.23 \pm 0.78$  % of TO-  
389 HCT116 cells and  $36.7 \pm 1.61$  % of ORC2\_H-2 cells were at G2/M. After 50 hr. dox and auxin  
390 treatment, the pH3S10-positive cell population percentage was  $3.95 \pm 0.16$  in TO-HCT116 and  
391 only  $0.96 \pm 0.15$  in ORC2\_H-2, while  $20.77 \pm 1.76$  % of TO-HCT116 cells and  $79.57 \pm 1.2$  % of  
392 ORC2\_H-2 cells were at G2/M phase. In normal media conditions, TO-HCT116 already had 2.9  
393 times as many mitotic cells as ORC2\_H-2. When treated with doxycycline and auxin, although  
394 the G2/M population increased 2.3- fold and 5- fold at 28 hr. and 50 hr., respectively, the

395 number of mitotic cells in ORC2\_H-2 was reduced 50 - 80 % compared to the non-treated H-2  
396 cells. This showed that most ORC2\_H-2 cells accumulated at the 4C DNA peak after ORC2  
397 depletion were in the G2 stage and most cells did not enter into mitosis.

398 Nevertheless, mitosis still occurred at a very low frequency. To observe how mitosis progression  
399 was affected after ORC2 depletion, we constitutively-expressed H2B-mCherry in TO-HCT116  
400 and ORC2\_H-2 cells via lentiviral transduction and performed time lapse fluorescent imaging of  
401 the mitotic chromosomes. Cells were synchronized using a single thymidine block and auxin  
402 was added or omitted 2 hours before releasing into fresh media with or without doxycycline and  
403 auxin. As expected, the first cell cycle after releasing from the thymidine block in both cell lines  
404 with or without doxycycline and auxin was normal and cells that progressed into mitosis and into  
405 the second cell cycle. During the second cell cycle, in the absence or presence of doxycycline  
406 and auxin, it took TO-HCT116 cells about 35 min. to progress from prophase to chromosome  
407 segregation (Figure 7b and c). The ORC2\_H-2 cells in the absence of doxycycline and auxin  
408 also progressed through mitosis like the parental cells (Figure 7d). In stark contrast, the  
409 ORC2\_H-2 cells in the presence of doxycycline and auxin condensed chromatin and attempted  
410 to congress chromosomes at the metaphase plate but never achieved a correct metaphase  
411 alignment of chromosomes even after seven hours (Figure 7e). Those few cells that did attempt  
412 anaphase had abnormal chromosome segregation, producing lagging chromosomes,  
413 micronuclei and eventually apoptosis.

#### 414 **Characterization of previously published *ORC1*<sup>-/-</sup> and *ORC2*<sup>-/-</sup> cell lines**

415 The results so far confirm previous observations that ORC is essential for cell proliferation in  
416 human cells, but there remains the curious case of the viable knockout of *ORC1* and *ORC2*  
417 genes in p53<sup>-/-</sup> HCT116 cells which needs to be explained (Shibata et al., 2016). We obtained  
418 two of the *ORC1*<sup>-/-</sup> and *ORC2*<sup>-/-</sup> cell lines used in that study and performed several experiments.  
419 We first tested whether using CRISPR/Cas9 to target *ORC2* with sgRNAs in the *ORC2*<sup>-/-</sup> cell  
420 line, whether negative selection occurred like that in the wild type HCT116 cells as shown in  
421 (Figure 2e). The sgRNA target GFP competition assay showed that in both parental HCT116  
422 p53<sup>-/-</sup> and *ORC2*<sup>-/-</sup> cell lines, cells targeted with *ORC2* sgRNA were outgrown by sgRNA-  
423 negative cells (Figure 8a-b). More importantly, both cell lines expressing mAID-ORC2<sup>9r</sup> rescued  
424 or partially rescued the sgRNA targeting effect with a mAID-ORC2<sup>9r</sup> that was resistant to the  
425 ORC2-1 and ORC2-2 sgRNAs, showing that the depletion was not due to an off-target effect of  
426 the *ORC2* sgRNAs (Figure 8c-d). This assay suggested that there is some form of functional  
427 ORC2 in the *ORC2*<sup>-/-</sup> cells that could be targeted by the tested sgRNAs. In addition, an

428 immunoblot of the cell lysates showed a reduced level of ORC3 in the *ORC2*<sup>-/-</sup> cells, and since  
429 ORC2 and ORC3 form stable heterodimers in cells, this result again indicated that some form of  
430 ORC2 was expressed in cells, albeit at a lower level (Figure 8—Figure supplement 1a). When  
431 immunoprecipitated with an antibody against ORC3, we detected ORC3 and a putative  
432 truncated form of ORC2 which was seen only in *ORC2*<sup>-/-</sup> cells (Figure 8—Figure supplement  
433 1b). Next, we designed primer pairs that span the exon junction for every exon in *ORC2* and  
434 performed quantitative RT-PCR (qPCR) to determine the nature of the *ORC2* transcripts in the  
435 *ORC2*<sup>-/-</sup> cells (Figure 8e). The fold change (FC) indicated that in *ORC2*<sup>-/-</sup> cells, about 60% of the  
436 mRNAs had exon 7 skipped, whereas other exons remained the same (Figure 8—Figure  
437 supplement 2).

438 With the idea that genomic instability caused by the absence of or mutation within either *ORC1*  
439 or *ORC2* in these cell lines might give rise to copy number variations, we performed SMASH  
440 (Wang et al., 2016) analysis on the two parental HCT116 with the p53 WT and p53 null  
441 background as well as the Shibata et al. (2016) *ORC1* and *ORC2* deficient lines. Both the  
442 parental cell HCT116 cell lines showed very similar chromosome copy number, while both  
443 *ORC1* deficient and *ORC2* deficient cell lines had additional CNVs in chromosomes unrelated to  
444 those harboring either *ORC1* or *ORC2* (Figure 8—Figure supplement 3). The significance of  
445 these specific loci which showed alterations in copy number when either *ORC1* or *ORC2* was  
446 deleted remains to be seen. However, it was in this analysis that we also noticed that part of the  
447 *ORC2* gene locus was hugely amplified (Figure 8—Figure supplement 3 solid arrow). To study  
448 in detail the *ORC2* gene region on chromosome 2 in the putative *ORC2*<sup>-/-</sup> cells, we performed  
449 long-read Nanopore sequencing analysis that showed the *ORC2* gene in *ORC2*<sup>-/-</sup> cells was  
450 highly rearranged and heterogenous (Figure 8f). Aside from the aforementioned heterozygous  
451 deletion of the 7<sup>th</sup> exon, the region near the 3<sup>rd</sup> and 4<sup>th</sup> exons also exhibited overwhelming  
452 amplification and structural rearrangement signatures (Figure 8f). Thus, the *ORC2* gene is  
453 present but has heterogenous expression of exon 7 and is sensitive to sgRNAs that target  
454 *ORC2*. We conclude that the cell line is not a true *ORC2* knockout.

455 With regard to the HCT116 *ORC1*<sup>-/-</sup> (B14) cell line, we confirmed that they lacked ORC1 protein  
456 using multiple antibodies and confirmed that they duplicated at a much slower rate than the  
457 parental line, as previously reported (Shibata et al., 2016). Furthermore, we were unable to  
458 passage these cells for many generations (by 30 generations they stopped proliferating). We  
459 also compared HCT116 *ORC1*<sup>-/-</sup> (B14) and HCT116 *ORC2*<sup>-/-</sup> (P44) cell lines with the parental  
460 lines, both either p53 WT or null background by confocal microscopy [Figure 8g (1-4), 8h; Figure

461 8—Figure supplement 4a-e, Figure supplement 5a-c]. There was a myriad of nuclear  
462 morphology defects in the *ORC1* deficient cell line. When the nuclei were stained with Hoechst  
463 dye, up to 10 percent contained abnormally large nuclei or sometimes what seemed to be  
464 multiple nuclei aggregated together in single cell, while many other of the cells appeared  
465 normal. When probed further by staining for F-actin and Lamin B1 for overall cellular and  
466 morphology and nuclear membrane integrity respectively, we saw that despite the staining for  
467 DNA content looking normal, up to 50 percent of the *ORC1*<sup>-/-</sup> cells showed highly abnormal,  
468 involuted nuclear membranes (Figure 8i). In addition, most of the gigantic nuclei seemed to  
469 have lost the nuclear membrane, while those cells that had Lamin B1 staining displayed  
470 abnormal nuclear membrane integrity.

471 The chromatin organization in the *ORC1* deficient cells was observed by transmission electron  
472 microscopy (TEM) and revealed a huge difference in cell size and nuclear structure between the  
473 wild type HCT116 *p53*<sup>+/+</sup> and *ORC1*<sup>-/-</sup> (Figure 8—Figure supplement 5). About 35 % of *ORC1*<sup>-/-</sup>  
474 cells were grossly larger than wild type cells. Those multinucleate/polyploid giant cells were full  
475 of membrane invagination, vacuoles and apoptosis. Most likely they were formed due to  
476 extensive DNA damage and nuclear structural defects and underwent a different type of cell  
477 division called neosis, in which intracellular cytokinesis occurs and some mononuclear cells are  
478 produced from nuclear budding or asymmetric cell division (Sundaram et al., 2004). All those  
479 phenotypes pointed to the fact that although *ORC1*<sup>-/-</sup> cells do not survive in culture in the long  
480 term and even when they were slowly proliferating, they were grossly abnormal. It may well be  
481 the case that the *p53*<sup>-/-</sup> status was required for these cells to be produced in the first place.

## 482 Discussion

483 The *ORC2*<sup>-/-</sup> cell line believed to be a complete knockout via the use of 3 sgRNAs, one targeting  
484 the exon 4, and the others targeting the 6<sup>th</sup> and 7<sup>th</sup> introns retained a truncated form of ORC2 that  
485 could interact with ORC3 and was expressed from a rearranged gene. These cells were still  
486 susceptible to ORC2 knockdown using four sgRNAs selected from our CRISPR screens and also  
487 partially rescued the phenotype with of two sgRNAs using a CRISPR-sgRNA resistant mAID-  
488 ORC2<sup>gr</sup>. Similar to what we found for *ORC2*<sup>-/-</sup> cells, CRISPR-induced frameshifts in cells often  
489 generate truncated proteins that, although they may not be recognized by western blot, still  
490 preserve whole or partial protein function (Smits et al., 2019). Based on these observations with  
491 ORC2 and the results with *ORC1* deficient cells, that despite the over-production of CDC6, were  
492 unable to proliferate for many generations and produced abnormally structured cells, as well as  
493 data analyzed by tiling-sgRNA CRISPR screens, we conclude that ORC is essential in human

494 cells. This conclusion is consistent with existing literature (Hemerly et al., 2009; McKinley and  
495 Cheeseman, 2017; Ohta et al., 2003; Prasanth et al., 2010, 2004, 2002) and is not surprising  
496 since ORC has multiple functions in human cells and ORC is essential in all other eukaryotic cells  
497 examined.

498 The pooled CRISPR/Cas9 domain-focused screen has become a common and powerful tool for  
499 uncovering genes that are essential for cell proliferation, cell survival, and for identification of  
500 essential functional domains in proteins (Adelmann et al., 2018; Park et al., 2016; Shi et al., 2015;  
501 So et al., 2019). However, if the screens use only a handful of guides targeting annotated essential  
502 regions, it may still result in data which may or may not score a gene as essential. Tiling-sgRNA  
503 CRISPR-Cas9 screens on the other hand test 'functional' or 'essential' domains in a more  
504 unbiased way. Using this approach targeting sgRNAs across entire open reading frames of  
505 ORC1-6 and CDC6 enabled us to classify them as essential, and correlate functional domains  
506 within these proteins. The combined results also confirmed that all ORC1-6 and CDC6 proteins  
507 were essential in cancer cells as well as a human diploid cell line, including ORC2 that was  
508 characterized in the DepMap portal (<https://depmap.org/portal/>) as non-essential based on  
509 multiple shRNA and whole genome CRISPR-Cas-9 screens in multiple cells. We were able to  
510 identify many sgRNAs that targeted *ORC2* in the tiling-sgRNA CRISPR screen and the two  
511 chosen cloned sgRNAs that killed cells were successfully complemented using a *mAID-ORC2<sup>gr</sup>*  
512 transgene, demonstrating specificity of the knockdowns. Thus, large scale experiments,  
513 especially negative results, should be interpreted with caution, such that the essential nature of a  
514 gene should be examined in depth as we have done here.

515 The known functional domains in ORC1, including the BAH, AAA+ and WHD were identified using  
516 the open reading frame tiling CRISPR-Cas9 sgRNA screen, as well as other regions of ORC1,  
517 including the intrinsically disordered region (IDR; amino acids 180-480, Figure 1e) which we know  
518 binds Cyclins E and A-CDK2 and CDC6 (Hossain et al., 2019) as well as many other proteins we  
519 have identified and are characterizing in detail. The screen also identified an essential region of  
520 ORC1 in and around amino acid 750-790 (Figure 1a-b) which may represent the pericentrin-  
521 AKAP450 centrosomal targeting (PACT) domain that localizes ORC1 to centrosomes to regulate  
522 correctly centrosome and centriole copy number (Hemerly et al., 2009).

523 In ORC2, multiple, essential domains were identified, including the AAA+-like domain and the  
524 WHD. The WHD of human ORC2 controls access of human ORC to DNA by inserting itself into  
525 the DNA binding channel prior to activation of the protein by binding of ORC1 and subsequent  
526 binding of CDC6 (Hossain et al., 2019; Jaremko et al., 2020). The ORC2-carboxy terminus binds



527 to ORC3 and ORC2 is also known to bind to PLK1, the mitotic protein kinase (Song et al., 2011).  
528 Interestingly, ORC2 also has an IDR (Figure 2—Figure supplement 1d; amino acids 30-230) and  
529 a gRNA tiling screen of this region shows limited essential amino acids, but a conserved region  
530 surrounding amino acid 190 is reproducibly essential in both HCT116 cancer cells and diploid  
531 (Figure 2a-b and Figure 2—Figure supplement 1a). The entire IDR, however, may contribute to  
532 DNA mediated ORC liquid phase transition (Parker et al., 2019).

533 The use of a mAID-ORC2<sup>gr</sup> enabled rapid removal of ORC2 from cells and analysis of the resulting  
534 phenotypes. It was not surprising that ORC2 is essential for loading MCM2, and hence MCM2-7,  
535 to establish pre-RCs and origins of DNA replication across the genome. In the absence of ORC2,  
536 cells loaded little MCM2, most likely resulting in too few origins of replication and a consequent  
537 slow S phase and arrest with a near 4C DNA content and ongoing DNA synthesis. ORC2  
538 depletion yielded other phenotypes, including large nuclei and a failure to execute mitosis. The  
539 large nuclei, also observed in the *ORC1*<sup>-/-</sup> cells, have large CENP-C foci, probably due to  
540 decompaction of the centromeric associated  $\alpha$ -satellite DNA, as observed previously (Prasanth  
541 et al., 2010). We suggest a general role for ORC in nuclear organization and organizing chromatin  
542 domains in the nucleus, including heterochromatin. In yeast, ORC is essential for transcriptional  
543 silencing at the silent mating type heterochromatic loci *HMRa* and *HML $\alpha$*  loci and its function in  
544 replication are separable from that in silencing (Bell et al., 1993; DeBeer et al., 2003; Ehrenhofer-  
545 Murray et al., 1995). In *Drosophila*, ORC localizes and associates with heterochromatin protein  
546 HP1 during interphase and mitosis and heterozygous, recessive lethal mutations in *DmORC2*  
547 suppress position effect variegation (Huang et al., 1998; Pak et al., 1997). In human, ORC1  
548 interacts with RB and SUV39H1, a histone methyltransferase that tri-methylates histone H3K9  
549 which HP1 binds to repress E2F1-dependent CCNE1 transcription (Hossain and Stillman, 2016).  
550 ORC1 and ORC3 (a tight ORC2 binding partner) directly interact with HP1, and depletion of ORC  
551 subunits disrupt localization of HP1 and the compaction of chromosome 9  $\alpha$ -satellite repeats DNA  
552 (Prasanth et al., 2010). The mechanism by which the nuclei become large as a result of ORC  
553 depletion is under further investigation.

554 A final phenotype we observed in the acute removal of ORC2 is that the cells that replicate DNA  
555 and enter into mitosis attempt chromosome congression at the metaphase plate, but never make  
556 it, even after 7 hours. Eventually the cells die of apoptosis. We had observed abnormal mitotic  
557 cells following long term (72 hr.) treatment of cells with shRNAs that targeted *ORC2* but it was  
558 not clear if this phenotype was due to incomplete DNA replication (Prasanth et al., 2004). However,  
559 in the current study, acute removal of ORC2 captured some cells with a clear defect in

560 chromosome congression during mitosis. Moreover, both ORC2 and ORC3 localize to  
561 centromeres (Craig et al., 2003; Prasanth et al., 2004), suggesting that they play a role in spindle  
562 attachment or centromeric DNA organization, particularly the centromere associated satellite  
563 repeat sequences. We speculate that in ancestral species, ORC localized at origins of DNA  
564 replication and this ORC also functioned in organization of chromosomes and in chromosome  
565 segregation, but upon separation of DNA replication and chromosome segregation with the  
566 advent of mitosis, separate functions of ORC in DNA replication, chromatin or nuclear  
567 organization and chromosome segregation were retained, but executed at different times during  
568 the cell division cycle.

## 569 **Materials and methods**

### 570 **Cell Culture**

571 HCT116 (WT cell lines is  $p53^{+/+}$ ), U2OS, and RPE1 cell lines were obtained from the Cold  
572 Spring Harbor Laboratory and cultured in DMEM (Gibco) and supplemented with 10% Fetal  
573 bovine serum and 1 % Penicillin/Streptomycin. IMR-90 cell line is culture in EMEM with 10 %  
574 Fetal bovine serum and 1 % Penicillin/Streptomycin. Plat-E cells and HEK293T cells were  
575 cultured in DMEM supplemented with 10 % FBS and penicillin/streptomycin. Plat-E cells were  
576 used for retroviral production and HEK293T cells were used for lentiviral production. HCT116  
577 ( $p53^{-/-}$ ), HCT116  $ORC1^{-/-}$  ( $p53^{-/-}$  background, clone B14), HCT116  $ORC2^{-/-}$  ( $p53^{-/-}$  background,  
578 clone P44) were a kind gift from Dr. Anindya Dutta (University of Virginia, Charlottesville, VA,  
579 USA). Tet-OsTIR1 HCT116 (TO-HCT116) cell line was a kind gift from Dr. Masato Kanemaki  
580 (National Institute of Genetics, Mishima, Japan). All gifted cell lines were cultured in McCoys 5A  
581 (Gibco) supplemented with 10 % fetal bovine serum and 1% Penicillin/Streptomycin. All cell  
582 lines were cultured at 37 °C with 5 % CO<sub>2</sub>. All of the cell lines used in this study were tested for  
583 mycoplasma and were negative.

584

### 585 **Tiling-sgRNA guide design**

586 Every possible guide directly upstream of a sp-Cas9 canonical PAM (NGG) sequence in the 5'-  
587 >3' direction is extracted from the target exon sequences. Guides with the canonical PAM  
588 (NGG) are aligned to the hg38 genome using the BatMis exact k-mismatch aligner (Tennakoon  
589 et al., 2012). A maximum of three mismatches are considered for off-target evaluation. The  
590 resulting alignment file is parsed, and each off-target location is assessed a penalty according to  
591 the number of mismatches to the target sequence, the exact position of each mismatch in the  
592 guide, where the further the mismatch is from the PAM the higher the penalty, the proximity of  
593 the mismatches to each other; assigning higher penalties to mismatches that are further apart.

594

595 The resulting penalties from each assessed off-target site are then combined in to a single off-  
596 target score for each guide similar to (Hsu et al., 2013), with 1.00 as the maximum possible  
597 score for guides not having any off-target site with up to three mismatches. The final results  
598 include the guide sequence, the PAM, the number of off-target sites in the genome with 0, 1, 2  
599 and 3 mismatches, the cut site location, the calculated off-target score, and any yes  
600 (Supplement Table 1\_guides).

601

## 602 **Plasmid construction and sgRNA cloning**

603 Clonal HCT116-Cas9 expressing cell lines were a gift from Dr. Chris Vakoc and RPE1-Cas9  
604 expressing cell lines were derived from Dr. Jason Sheltzer (Cold Spring Harbor Laboratory, NY,  
605 USA). In this study, all the sgRNAs targeting genes of interest as well as controls were cloned  
606 into LRG2.1 (derived from U6-sgRNA-GFP, Addgene: 108098 - as described in ref (Tarumoto et  
607 al., 2018)). Single sgRNAs were cloned by annealing sense and anti-sense DNA oligos followed  
608 by T4 DNA ligation into a BsmB1 digested LRG2.1 vector. To improve U6 promoter transcription  
609 efficiency, an additional 5' G nucleotide was added to all sgRNA oligo designs that did not  
610 already start with a 5' G. Sequences of all arrayed sgRNA libraries used in this study are  
611 provided in (Supplement Table1\_guides)

612

613 For an unbiased tiling CRISPR domain screen, pooled sgRNA libraries were constructed. All  
614 possible sgRNAs (PAM - NGG) were designed across each exon of the 7 target genes.  
615 Targeting or positive/negative control sgRNAs were synthesized in duplicate or triplicate in a  
616 pooled format on an array platform (Twist Bioscience) and then PCR cloned into the BsmB1-  
617 digested LRG2.1 vector using Gibson Assembly. To ensure the representation and identity of  
618 sgRNA in the pooled lentiviral libraries, a deep-sequencing analysis was performed on a MiSeq  
619 instrument (Illumina) and verified that 100 % of the designed sgRNAs were cloned in the  
620 LRG2.1 vector and that the abundance of >95 % of the sgRNA constructs was within 5-fold of  
621 the mean. While this was as a means to QC for ORC1-6 and CDC6 libraries, for time  
622 considerations, in case of MCM2-7, CDT1 and Control libraries, presence of all guides in the  
623 T=0 sampling during the pooled CRISPR screening (described later) served as a similar QC.

624

625 For CRISPR complementation assays, *ORC2* sgRNA resistant synonymous mutations were  
626 introduced to *ORC2* by PCR mutagenesis using Phusion high fidelity DNA polymerase (NEB).  
627 Guide RNA resistant *ORC2* (*ORC2<sup>gr</sup>*) was amplified by PCR and cloned into *NheI*-digested  
628 *mAID-mCherry2-NeoR* plasmid (*mAID-mCherry2-NeoR*, Addgene 72830) in order to add *mAID*

629 degron sequence to the N-terminus. The mAID-ORC2<sup>gr</sup> was then PCR and assembled into  
630 BglII/XhoI digested pMSCV-hygro retroviral vector (TaKaRa #634401). All cloning was done  
631 using In-Fusion cloning system (TaKaRa). In this experiment, sgRNAs targeting ORC2 and  
632 control sgRNAs were cloned into BsmB1 digested LgCG\_cc88 lentiviral vector by the same  
633 sgRNA cloning strategy described above.

634

635 For knocking out endogenous *ORC2* in TO-HCT116 cells, we used sgRNA\_ORC2-1-epCas9-  
636 1.1-mCherry plasmid to perform CRISPR/Cas9 in the cells. Sequence of sgRNA\_ORC2-1 was  
637 cloned into epCas9-1.1-mCherry plasmid which was a kind gift from Dr. David Spector (Cold  
638 Spring Harbor Laboratory, NY, USA). Single sgRNA were cloned by annealing sense and anti-  
639 sense DNA oligos followed by T4 DNA ligation into a BbsI -digested sgRNA\_ sgRNA\_ORC2-1-  
640 epCas9-1.1-mCherry vector.

641

642 To construct lentiviral vector that constitutively express H2B-mCherry in TO-HCT116 and  
643 ORC2\_H-2 cells, H2B-mCherry sequence were PCR and cloned into BamHI/BspDI -digested  
644 pHAGE-CMV-MCS-IZsGreen vector which was a kind gift from Dr. Alea Mills (Cold Spring  
645 Harbor Laboratory, NY, USA).

646

#### 647 **Viral Transductions**

648 Lentivirus was produced in HEK293T cells by co-transfecting target plasmid and helper  
649 packaging plasmids psPAX2 and pVSVG with polyethylenimine (PEI 25000, cat#) transfection  
650 reagent. HEK293T cells were plated a day before to achieve 80 % - 90 % confluency on the day  
651 of transfection. Plasmids were mixed in the ratio of 1:1.5:2 of psPAX2, pVSVG and target  
652 plasmid DNA in OptiMEM (Cat#). 32  $\mu$ l of 1 mg/mL PEI (calculated based on the final volume of  
653 transfection) was added, mixed and incubated, before addition to the cells. Cell culture medium  
654 was changed 7 h after transfection, then supernatant collected at 36 and 72 h following  
655 transfection was pooled. For the high throughput lentiviral screening, virus supernatant was  
656 concentrated with Lenti-X™ Concentrator (Takara, #631231) as per the manufacturer's  
657 protocol.

658 Retrovirus was produced in Plat-E cells by co-transfecting target plasmid and packaging  
659 plasmids pCL-Eco and pVSVG in the ratio of 1.25:1:9 with PEI. Cell culture medium was  
660 changed 7 h after transfection, and the supernatant was collected at 36 hr. post-transfection.

661

662 For either lenti- or retroviral transductions, target cells were mixed with viral supernatant,  
663 supplemented with 8  $\mu$ g/mL polybrene and centrifuged at 1700 rpm for 30 min. at room

664 temperature. Fresh medium was replaced 24 h after transduction. Antibiotics (1 µg/mL  
665 puromycin; 10 µg/mL of blasticidin; 200 µg/ml of hygromycin) were added 72 h after infection  
666 when selection was required.

667

### 668 **Pooled sgRNA screening**

669 CRISPR-based negative selection screenings using sgRNA libraries targeting proteins ORC1-6,  
670 CDC6 as well as positive and negative controls, were performed in stable Cas9 expressing  
671 HCT116 and RPE1 cell lines. The screens were performed as previously described (Lu et al.,  
672 2018; Miles et al., 2016; Shi et al., 2015) with a few optimizations for scale. Briefly, to ensure a  
673 single copy sgRNA transduction per cell, multiplicity of infection (MOI) was set to 0.3-0.35. To  
674 achieve the desired representation of each sgRNAs during the screen, the total number of cells  
675 infected was determined such that while maintaining the MOI at ~0.3, the sgRNA positive cells  
676 were at least 2000 times the sgRNA number in the library. Cells were harvested at day 3 post-  
677 infection and served as the initial time-point (T=0) of the pooled sgRNA library, representing all  
678 guides transduced to begin with. Cells were cultured for 10 population doublings (T=10) and  
679 harvested as the final time point. All experiments were performed in triplicates. Genomic DNA  
680 was extracted using QIAamp DNA midi kit (QIAGEN) according to the manufacturer's protocol.

681

682 Next Generation Sequencing library was constructed based on a newly developed protocol. To  
683 quantify the sgRNA abundance of initial and final time-points, the sgRNA cassette was PCR  
684 amplified from genomic DNA using Amplitaq Gold DNA Polymerase (Invitrogen, 4311820) and  
685 primers (F2: TCTTGTGGAAAGGACGAAACACCG; R2: TCTACTATTCTTTCCCCTGCACTGT).  
686 The resulting DNA fragment (~ 242 bp) was gel purified. In a 2<sup>nd</sup> PCR reaction illumina-  
687 compatible P7 and custom stacked barcodes (Supplement Table 2\_BClist) including the  
688 standard illumina P5 forward primer were introduced into samples by PCR amplification and gel  
689 purified for the final product (~180-200 bp). The final product was quantified by Agilent  
690 Bioanalyzer DNA High-sensitivity Assay (Agilent 5067-4626) and pooled together in equal molar  
691 ratio and analyzed by NGS. A 5% PhiX spike in used was for quality purposes. Illumina libraries  
692 were either sequenced with a 300cycle MiSeqv2 kit or a 76 cycle NextSeq 500/550 kit by single-  
693 end sequencing using NextSeq mid-output. (DRYAD doi to be generated)

694

### 695 **Quantification and analysis of screen data:**

696 The quantification of guides was done using a strict exact match to the forward primer, sample  
697 barcode, and guide sequence. MAGeCK was used for the identification of essential sgRNAs by  
698 running the "mageck test" command on the raw sgRNA counts. MAGeCK employs median

699 normalization followed by a Negative Binomial modeling of the counts, and provides the log fold  
700 change (lfc) and p-values at both the guide and gene levels (Li et al., 2014).

701

### 702 **GFP competition and sgRNA complementation assay**

703 TO-HCT116, TO-HCT116\_mAID-ORC2<sup>9f</sup>, U2OS, U2OS\_mAID-ORC2<sup>9f</sup>, HCT116 *p53*<sup>-/-</sup>,  
704 HCT116 *p53*<sup>-/-</sup>\_mAID-ORC2<sup>9f</sup>, *ORC2*<sup>-/-</sup> p44, and *ORC2* p44<sup>-/-</sup>\_mAID-ORC2<sup>9f</sup> cells were  
705 transduced with different sgRNA-Cas9-GFP lentivirus respectively with the MOI at 0.3-0.4 to  
706 ensure one copy of sgRNA transduction per cell. Cells were passaged every 3 days from day 3  
707 (P1) post-transduction to day 21(P7), and at the same time, GFP percentage were evaluated by  
708 guava easyCyte™ flow cytometer. Three technical repeats were done for each datapoint. GFP  
709 percentage of day 3 (P1) for each sgRNA was normalized to 100 %, and then result of each  
710 passage was normalized to day 3 correspondingly. All experiments were repeated twice at  
711 least.

712

### 713 **Generating endogenous ORC2 KO mAID-ORC2<sup>9f</sup> cell lines**

714 TO-HCT116 cells were transduced with mAID-ORC2<sup>9f</sup> via retroviral transduction and selected  
715 with 200 µg/ml of hygromycin to grow TO-HCT116-mAID-ORC2<sup>9f</sup> cells. sgRNA\_ORC2-1-  
716 epCas9-1.1-mCherry plasmid was transiently transfected into TO-HCT116-mAID-ORC2<sup>9f</sup> cells  
717 using Lipofectamine 2000 Transfection Reagent (ThermoFisher #11668019) following  
718 manufacturer's protocol. Fresh medium was replaced 6 h after transfection. Cells were harvest  
719 by 0.25% trypsin-EDTA after 24 hr., washed once with PBS, and then resuspended in sorting  
720 buffer containing 2% FBS, 2 mM EDTA, and 25mM HEPES pH7.0. Single Cell was FACS  
721 sorted by gating on mCherry fluorescence intensity into 96-well plates with 200 µl fresh medium  
722 containing 200 µg/ml of hygromycin. Single cell clone was expanded by transferring to 24-well  
723 plate, 6-well plate, and 10 cm culture dish once they reached 90% confluency.

724

### 725 **Cell Proliferation assays**

726 TO-HCT116, *ORC2*\_H-2, *ORC2*\_H-4, and *ORC2*\_H-5 were treated with or without 0.75µg/ml  
727 doxycycline for 24 hours before seeding. For each cell line, 150,000 cells were seeded in each  
728 well at day 1, and medium was changed every day. Every day we harvested 3 wells for each  
729 cell lines for counting. Cells were stained with 0.4 % trypan blue solution and live cells were  
730 counted by automated cell counter.

731

### 732 **Immunoprecipitation, Immunoblotting and quantitation**

733 Cells were incubated with RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % Sodium deoxycholate,  
734 0.1 % SDS, 25 mM Tris-HCl PH 7.4) on ice for 15 minutes. Cell lysates were added with  
735 Laemmli buffer and ran on SDS-PAGE followed by western blotting to detect proteins by  
736 indicated antibodies: Primary antibodies used include anti-ORC2 (rabbit polyclonal #CS205, in-  
737 house), anti-ORC3 (rabbit polyclonal #CS1980, in-house), anti-ORC1 (mouse monoclonal  
738 #pKS1-40, in-house), anti-CDC6 (mouse monoclonal #DCS-180, EMD Millipore), anti-ATM  
739 (rabbit monoclonal #ab32420, abcam), anti-pATM(S1981) (rabbit monoclonal #ab81292,  
740 abcam), anti-CHK1 (rabbit monoclonal #ab40866, abcam), anti-pCHK1(S345) (rabbit polyclonal  
741 #2348, Cell Signaling), anti-pCHK2(T68) (rabbit polyclonal #2197, Cell Signaling), anti-ATR  
742 (rabbit polyclonal #ab2905, abcam), anti-pATR(T1989) (rabbit polyclonal #ab227851, abcam),  
743 anti-pATR(S428) (rabbit polyclonal #2853, Cell Signaling), anti-p- $\gamma$ H2AX(S139) (rabbit  
744 monoclonal #9718, Cell Signaling), anti- $\beta$ -Actin (mouse monoclonal #3700, Cell Signaling).  
745 Secondary antibodies include ECL<sup>TM</sup> anti-Rabbit IgG Horseradish Peroxidase linked whole  
746 antibody (#NA934V, GE Healthcare) and ECL<sup>TM</sup> anti-mouse IgG Horseradish Peroxidase linked  
747 whole antibody (#NA931V, GE Healthcare).  
748 Relative ORC2 (or mAID-ORC2<sup>9r</sup>) and ORC3 Protein level in each cell line was quantitated by  
749 normalizing band intensity to  $\beta$ -Actin of each cell line and then eventually normalized to HCT116  
750 cells using ImageJ software.

751

### 752 **Cell cycle analysis and pulse EdU label**

753 In double-thymidine block and release experiment, cells were first incubated with 2 mM  
754 thymidine for 18 hours. After 3 times PBS wash, cells were released into fresh media with or  
755 without (0.75  $\mu$ g/ml) doxycycline for 9 hours. Next, 2 mM thymidine were added into the media  
756 for 16 hours. 500 nM of auxin was added into the medium if needed 4.5 hr. before released.  
757 When released from thymidine block, 0 hr. time point cells were harvest, and the rest were  
758 washed with PBS for 3 times and released into fresh medium  $\pm$ dox and auxin. Cells were  
759 collected at indicated time point and 10  $\mu$ M EdU were added into the medium 2 hours before  
760 each harvest (Including time 0). Cells were fixed and processed following Click-iT<sup>TM</sup> EdU Alexa  
761 Fluor<sup>TM</sup> 488 Flow Cytometry Assay Kit manufacturer's manual (ThermoFisher #C10420) and  
762 DNA was stained with FxCycle<sup>TM</sup> Violet Stain (ThermoFisher #F10347).

763

### 764 **Mitotic index flow cytometry**

765 TO-HCT116 and ORC2\_H-2 cells were pre-treated with doxycycline for 24 hr. when needed in  
766 this experiment. Cells were trypsinized and harvest at different time points after auxin treatment,

767 and immediately fixed with 4 % Paraformaldehyde in PBS for 15 min, centrifuged at 1000 xg for  
768 7 min. to remove fixation and washed with 1 % BSA-PBS and centrifuged. Next, cells were  
769 permeabilized with 0.5 % triton x-100 in 1 % BSA-PBS for 15 min. at room temperature,  
770 centrifuged, and washed with 1% BSA-PBS and centrifuged. Next, primary antibody anti-  
771 pH3S10 antibody (mouse monoclonal #9706, Cell Signaling) were incubated at 37 °C for 45 min.  
772 Cells were then washed 3 times in 1 % BSA-PBS +0.1 % NP-40, and incubated with secondary  
773 antibody (Donkey anti-Mouse Alexa Fluor 647 #715-605-151 Jackson ImmunoResearch) at  
774 37 °C for 50 min. in the dark. Lastly, after 3 washes cells were incubated with FxCycle™ Violet  
775 Stain (ThermoFisher). The positive/negative gates for pH3S10 were gated on a negative  
776 control, which is unstained cells.

777

### 778 **Cell extraction and MCM2 flow cytometry**

779 EdU-pulse-labeled asynchronous TO-HCT116, ORC2\_H-2, ORC2\_H-5 cells with or without  
780 doxycycline and auxin treatment were harvest, washed with PBS, and processed based on the  
781 protocol from (Matson et al., 2017) with slight optimization. In brief, for non-extracted cells, cells  
782 were fixed with 4 % Paraformaldehyde in PBS for 15 min, and then centrifuged at 1000 xg for 7  
783 min. to remove fixation and washed with 1 % BSA-PBS and centrifuged. Next, cells were  
784 permeabilized with 0.5 % triton x-100 in 1 % BSA-PBS for 15 min, centrifuged, and washed with  
785 1% BSA-PBS and centrifuged. For chromatin extracted cells, cells were lysed on ice for 5 min.  
786 in CSK buffer (10mM PIPES/KOH pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 1 mM  
787 MgCl<sub>2</sub>, 1 mM DTT) with 0.5 % triton x-100 with protease and phosphatase inhibitors. Cells were  
788 centrifuged and washed with 1 % BSA-PBS twice and then fixed in 4% paraformaldehyde in  
789 PBS for 15 min. After one wash with PBS, cells were processed following Click-iT™ EdU Alexa  
790 Fluor™ 488 Flow Cytometry Assay Kit manufacturer's manual (ThermoFisher #C10420), but  
791 instead of saponin-based permeabilization and wash reagent, we used 1 % BSA-PBS +0.1 %  
792 NP-40 for all washing steps. Next, cells were incubated with Anti-MCM2 antibody (mouse  
793 monoclonal #610700, BD Biosciences) at 37 °C for 40 min. in the dark. Cells were then washed  
794 3 times in 1 % BSA-PBS +0.1 % NP-40, and incubated with secondary antibody (Donkey anti-  
795 Mouse Alexa Fluor 647 #715-605-151 Jackson ImmunoResearch) at 37 °C for 50 min. in the  
796 dark. Finally, cells were washed for 3 times and incubated with FxCycle™ Violet Stain  
797 (ThermoFisher). The positive/negative gates for MCM were gated on a negative control, which  
798 is unstained cells.

799

### 800 **Immunofluorescence Staining**



801 TO-HCT116, ORC2\_H-2, ORC2\_H-4, and ORC2\_H-5 cells were grown on coverslips for 2 days  
802 with or without doxycycline and auxin treatment. When harvest, coverslips were transferred to 6-  
803 well plate and rinse with PBS. Cells were fixed in 4 % Paraformaldehyde for 10 min. at room  
804 temperature. Next, cells were washed three times for 5 min. in cold PBS. Cells were then  
805 permeabilized in 0.5 % Triton x-100 -PBS for 9 min. After three PBS wash, cells were blocked  
806 with 5 % normal goat serum (NGS) -PBS +0.1 % Tween for 1 hr. For primary antibody  
807 incubation, antibodies were diluted in 1 % NGS-PBS +0.1 % Tween and incubated for 17 hr. in  
808 the cold room. Primary antibodies used include anti-CENP-C antibody (Mouse monoclonal  
809 #ab50974, Abcam), anti-pCHK1(S345) (rabbit polyclonal #2348, Cell Signaling), anti-p-  
810  $\gamma$ H2AX(S139) (rabbit monoclonal #9718, Cell Signaling), and anti-pATM(S1981) (Mouse  
811 monoclonal #ab36180, Abcam). Cells were washed three times for 5 min. in 1 % NGS-PBS  
812 +0.1 % Tween before 1 hr. secondary antibody incubation at room temperature. Secondary  
813 antibodies used are Goat Anti-Mouse IgG H&L Alexa Fluor® 647 (#ab150115, Abcam) and  
814 Goat Anti-Rabbit IgG H&L Alexa Fluor® 488 (#ab150077, Abcam). Next, cells were stained with  
815 1  $\mu$ g/ml DAPI and mounted with VECTASHIELD® Antifade Mounting Medium (#H-1000-10,  
816 Vector Laboratories). Images were taken using a Perkin Elmer spinning disc confocal equipped  
817 with a Nikon-TiE inverted microscope using 60X objective oil lens with an Orca ER CCD  
818 camera. Images presented are maximum intensity projections of a z-stack (z=0.3 $\mu$ M).

819  
820 To study the nuclear and cellular morphology HCT116 p53 WT and p53 null cells and *ORC1*<sup>-/-</sup>  
821 (B14) and *ORC2*<sup>-/-</sup> (P44) cells were plated on coverslips. On day 2 cell were fixed with 4% PFA  
822 and the method described above was followed. Primary antibody against Lamin B1 (Abcam  
823 ab16048) was used as a marker for nuclear envelope. Secondary antibody used is Goat Anti-  
824 Rabbit IgG Alexa Fluor® 594 (Abcam ab150084). In addition, Phalloidin iFluor® 488 (Abcam  
825 ab176753) was used to stain for cytoskeleton and DNA was detected with 1 $\mu$ g/ml Hoechst dye  
826 (ThermoFisher #62249). Mounted coverslips were imaged with Perkin Elmer spinning disc  
827 confocal equipped with a Nikon-TiE inverted microscope using 40X objective lens with an Orca  
828 ER CCD camera. Images presented are single channel average intensity projections or merged  
829 multi-channel maximum intensity projections of z-stacks.

830

### 831 **ORC2 Nuclear volume quantitation**

832 Cells nuclear were fixed and stained with DRAQ5™ Fluorescent Probe Solution as per the  
833 manufacturer's guidelines (ThermoFisher #62251). Images were taken using a Perkin Elmer  
834 spinning disc confocal equipped with a Nikon-TiE inverted microscope using 60X objective lens

835 with an Orca ER CCD camera. Images presented are maximum intensity projections of a z-  
836 stack ( $z=0.3\mu\text{M}$ ). Nuclear size was analyzed with volocity software (version 6.3.1).

837

### 838 **Live cell microscopy**

839 TO-HCT116 and *ORC2\_H-2* cells were seeded in ibidi  $\mu$ -Slide 8 Well Glass Bottom and in the  
840 presence or absence of 0.75  $\mu\text{g/ml}$  doxycycline for 24 hours. Next, 2 mM thymidine were added  
841 and incubated  $\pm$ dox for 24 hours. Two hours prior to washing out thymidine, 500 nM auxin were  
842 added to the dox treated wells. Samples were then imaged starting at 4 hours after thymidine  
843 release and the timepoints reconstructed as from a movie using volocity software. Images were  
844 taken using a Perkin Elmer spinning disc confocal equipped with a Nikon-TiE inverted  
845 microscope using 40X objective lens with an Orca ER CCD camera. Images presented are  
846 maximum intensity projections of a z-stack ( $z=3\mu\text{M}$ ). Frames were taken approximately every 5  
847 minutes.

848

### 849 **Quantitative PCR**

850 Total RNA of HCT116 *p53<sup>-/-</sup>* and *ORC2<sup>-/-</sup>* cells were extracted using Rneasy Mini Kit (Qiagen  
851 #74104) following manufacturer's handbook and quantified by Nanodrop (ThermoFisher). RNA  
852 was then converted to cDNA by doing reverse transcription using oligo(dT) or random hexamer  
853 primers provided by TaqMan Reverse Transcription Reagents (#N8080234, Applied  
854 Biosystems). Primer pairs for quantitative PCR are designed to PCR exon-exon junction  
855 (Supplement Table 3\_exon primers) and each PCR was done in triplicates. The delta-Ct ( $\Delta\text{Ct}$ )  
856 value was obtained from subtracting Actin mean Ct from each primer pair mean Ct in each cell  
857 line. The delta-delta-Ct ( $\Delta\Delta\text{Ct}$ ) value was calculated by subtracting HCT116 *p53<sup>-/-</sup>*  $\Delta\text{Ct}$  from  
858 *ORC2<sup>-/-</sup>*  $\Delta\text{Ct}$  for each primer pair individually. Fold change (FC) for each primer pair in *ORC2<sup>-/-</sup>*  
859 cells compared to HCT116 *p53<sup>-/-</sup>* cells was calculated as  $\text{FC} = 2^{(\text{to the power of } \Delta\Delta\text{Ct})}$ .

860

### 861 **Transmission electron microscopy**

862 HCT116 and *ORC1<sup>-/-</sup>* cells were harvest and washed twice with PBS. Cells were pelleted and  
863 resuspended in 1 mL of 2.5 % glutaraldehyde in 0.1 M sodium cacodylate solution (pH 7.4)  
864 overnight at 4 °C. Fixative was removed, and in each step, 200  $\mu\text{l}$  of the solution was left in the  
865 tubes. Pellet was washed with 0.1 M sodium cacodylate buffer. Next, 4 % Melt agarose solution  
866 was added to the tube and centrifuged immediately at 1,000 x g for 10 min. at 30 °C, and then  
867 directly transferred the tube to 4 °C or ice for 20 min. to solidify the agarose. Agarose was  
868 washed twice with 0.1 M cacodylate buffer. Next, 1 % osmium tetroxide ( $\text{OsO}_4$ ) solution was  
869 added and let stand for 1 hr. followed by three 0.1 M cacodylate buffer washes. Samples were

870 then dehydrated by a graded ethanol series (50 %, 60 %, 70 %, 80 %, 90 %, 95 %, 100 %,  
871 respectively). Finally, samples were embedded in 812 Embed resin and sectioned in 60-90  $\mu$ M  
872 using Ultramicrotome. Hitachi H-7000 Transmission Electron Microscopy was used to image the  
873 sample.

874

### 875 **Nanopore Long read sequencing and analysis**

876 High molecular weight DNA was isolated using the MagAttract kit (Qiagen # 67563). The quality  
877 of the DNA from the was assessed on femtopulse (Agilent) to ensure DNA fragments were  
878 >40kb on average DNA was sheared to 50kb via Megarupter (diagenode). After shearing, the  
879 DNA was size selected with a SRE kit (Circulomics) to reduce the fragments <20kb. After size  
880 selection, the DNA under when a-tailing and damage repair followed by ligation to sequencing  
881 specific adapters. The  $\frac{1}{2}$  prepared library was mixed with library loading beads and loaded on to  
882 a PROM-0002 flow-cell and was allowed to sequence for 24 hours. After 24 hours the flow-  
883 cell was treated with DNase to remove stalled DNA followed by a buffer flush. The second  $\frac{1}{2}$  of  
884 the library was then loaded and allowed to sequencing for 36 hours. The DNA was base called  
885 via Guppy 3.2 in High accuracy mode. Long reads were aligned to the reference human  
886 genome using NGMLR (<https://github.com/philres/ngmlr>) and structural variants were  
887 identified using Sniffles (<https://github.com/fritzsedlazeck/Sniffles>) (Sedlazeck et al., 2018).  
888 The alignments and structural variants were then visualized using IGV (<https://igv.org/>).

889

890 **Acknowledgements:** We thank Dr. Leemor Joshua-Tor for comments on the manuscript and  
891 Dr. Anindya Dutta for providing cell lines. We also thank Jennifer Shapp for technical  
892 assistance. This work was supported by grants from the National Cancer Institute (P01-  
893 CA13106 and a Cancer Center Support Grant P50-CA045508.

894

895 **Author Contributions:** All the experiments were performed by H-C.C. and K.B., microscopy  
896 was performed in collaboration with H.A., the computational analysis of genomic data was done  
897 in collaboration with O.E.D., the CRISPR-Cas9 genome tiling methods and library preparation  
898 were done in collaboration with O.K., K.H. K.C. and C.V. and the genomic DNA sequencing was  
899 done in collaboration with R.W.M. and data analyzed by S.A. and M.S. Genomic copy number  
900 determination was done with P.A.. Experiments were designed and analyzed by H-C.C, K.B,  
901 O.K, C.V. and B.S. H-C.C, K.B. and B.S. wrote the paper.

902

903 **References**

904  
905

906 Adamczak R, Porollo A, Meller J. 2004. Accurate prediction of solvent accessibility using neural  
907 networks-based regression. *Proteins Struct Funct Bioinform* **56**:753–767.  
908 doi:10.1002/prot.20176

909 Adelman CH, Wang T, Sabatini DM, Lander ES. 2018. Methods in Molecular Biology. *Methods*  
910 *Mol Biology Clifton N J* **1907**:125–136. doi:10.1007/978-1-4939-8967-6\_10

911 Araki H. 2016. Elucidating the DDK-dependent step in replication initiation. *Embo J* **35**:907–8.  
912 doi:10.15252/embj.201694227

913 Araki H. 2011. Initiation of chromosomal DNA replication in eukaryotic cells; contribution of  
914 yeast genetics to the elucidation. *Genes & Genetic Systems* **86**:141–149.  
915 doi:10.1266/ggs.86.141

916 Araki H, Leem SH, Phongdara A, Sugino A. 1995. Dpb11, which interacts with DNA polymerase  
917 II(epsilon) in *Saccharomyces cerevisiae*, has a dual role in S-phase progression and at a cell  
918 cycle checkpoint. *Proc Natl Acad Sci USA* **92**:11791–11795. doi:10.1073/pnas.92.25.11791

919 Ashkenazy H, Abadi S, Martz E, Chay O, Mayrose I, Pupko T, Ben-Tal N. 2016. ConSurf 2016:  
920 an improved methodology to estimate and visualize evolutionary conservation in  
921 macromolecules. *Nucleic Acids Res* **44**:W344–W350. doi:10.1093/nar/gkw408

922 Bell SP. 2017. Rethinking origin licensing. *eLife* **6**:1027. doi:10.7554/elife.24052

923 Bell SP, Kobayashi R, Stillman B. 1993. Yeast origin recognition complex functions in  
924 transcription silencing and DNA replication. *Science* **262**:1844–1849.  
925 doi:10.1126/science.8266072

926 Bell SP, Labib D. 2016. Chromosome Duplication in *Saccharomyces cerevisiae*. *Genetics*  
927 **203**:1027–1067. doi:10.1534/genetics.115.186452

928 Bell SP, Stillman B. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication  
929 by a multiprotein complex. *Nature* **357**:128–134. doi:10.1038/357128a0

930 Bicknell LS, Bongers EMHF, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY,  
931 Bober M, Brown PAJ, Bokhoven H van, Dean J, Edrees AY, Feingold M, Fryer A, Hoefsloot  
932 LH, Kau N, Knoers NVAM, MacKenzie J, Opitz JM, Sarda P, Ross A, Temple IK, Toutain A,  
933 Wise CA, Wright M, Jackson AP. 2011a. Mutations in the pre-replication complex cause  
934 Meier-Gorlin syndrome. *Nat Genet* **43**:356–359. doi:10.1038/ng.775

935 Bicknell LS, Walker S, Klingseisen A, Stiff T, Leitch A, Kerzendorfer C, Martin C-A, Yeyati P,  
936 Sanna NA, Bober M, Johnson D, Wise C, Jackson AP, O'Driscoll M, Jeggo PA. 2011b.  
937 Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause  
938 microcephalic primordial dwarfism resembling Meier-Gorlin syndrome. *Nat Genet* **43**:350–  
939 355. doi:10.1038/ng.776

- 940 Bleichert F, Botchan MR, Berger JM. 2017. Mechanisms for initiating cellular DNA replication.  
941 *Science* **355**:eaah6317. doi:10.1126/science.aah6317
- 942 Buchan DWA, Jones DT. 2019. The PSIPRED Protein Analysis Workbench: 20 years on.  
943 *Nucleic Acids Res* **47**:W402–W407. doi:10.1093/nar/gkz297
- 944 Celniker SE, Sweder K, Srienc F, Bailey JE, Campbell JL. 1984. Deletion mutations affecting  
945 autonomously replicating sequence ARS1 of *Saccharomyces cerevisiae*. *Molecular and*  
946 *Cellular Biology* **4**:2455–2466. doi:10.1128/mcb.4.11.2455
- 947 Chen Z, Speck C, Wendel P, Tang C, Stillman B, Li H. 2008. The architecture of the DNA  
948 replication origin recognition complex in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA*  
949 **105**:10326–10331. doi:10.1073/pnas.0803829105
- 950 Chuang R-Y, Chretien L, Dai J, Kelly TJ. 2002. Purification and Characterization of the  
951 *Schizosaccharomyces pombe* Origin Recognition Complex: Interaction With Origin DNA and  
952 Cdc18 Protein. *J Biol Chem* **277**:16920–16927. doi:10.1074/jbc.m107710200
- 953 Craig JM, Earle E, Canham P, Wong LH, Anderson M, Choo KHA. 2003. Analysis of  
954 mammalian proteins involved in chromatin modification reveals new metaphase centromeric  
955 proteins and distinct chromosomal distribution patterns. *Hum Mol Genet* **12**:3109–3121.  
956 doi:10.1093/hmg/ddg330
- 957 DeBeer MAP, Müller U, Fox CA. 2003. Differential DNA affinity specifies roles for the origin  
958 recognition complex in budding yeast heterochromatin. *Gene Dev* **17**:1817–1822.  
959 doi:10.1101/gad.1096703
- 960 Deng Z, Norseen J, Wiedmer A, Riethman H, Lieberman PM. 2009. TERRA RNA Binding to  
961 TRF2 Facilitates Heterochromatin Formation and ORC Recruitment at Telomeres. *Mol Cell*  
962 **35**:403–413. doi:10.1016/j.molcel.2009.06.025
- 963 DePamphilis ML. 2003. The ‘ORC cycle’: a novel pathway for regulating eukaryotic DNA  
964 replication. *Gene* **310**:1–15. doi:10.1016/s0378-1119(03)00546-8
- 965 DepMap B. 2019. DEMETER 2 Combined RNAi. doi:10.6084/m9.figshare.9170975.v1
- 966 Deshpande AM, Newlon CS. 1992. The ARS consensus sequence is required for chromosomal  
967 origin function in *Saccharomyces cerevisiae*. *Mol Cell Biol* **12**:4305–4313.  
968 doi:10.1128/mcb.12.10.4305
- 969 Dhar SK, Yoshida K, Machida Y, Khaira P, Chaudhuri B, Wohlschlegel JA, Leffak M, Yates J,  
970 Dutta A. 2001. Replication from oriP of Epstein-Barr Virus Requires Human ORC and Is  
971 Inhibited by Geminin. *Cell* **106**:287–296. doi:10.1016/s0092-8674(01)00458-5
- 972 Ehrenhofer-Murray AE, Gossen M, Pak DTS, Botchan MR, Rine J. 1995. Separation of Origin  
973 Recognition Complex Functions by Cross-Species Complementation. *Science* **270**:1671–  
974 1674. doi:10.1126/science.270.5242.1671

- 975 Evrin C, Clarke P, Zech J, Lurz R, Sun J, Uhle S, Li H, Stillman B, Speck C. 2009. A double-  
976 hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA  
977 replication. *Proc Natl Acad Sci USA* **106**:20240–20245. doi:10.1073/pnas.0911500106
- 978 Fischer JD, Mayer CE, Söding J. 2008. Prediction of protein functional residues from sequence  
979 by probability density estimation. *Bioinformatics* **24**:613–620.  
980 doi:10.1093/bioinformatics/btm626
- 981 Fox CA, Loo S, Dillin A, Rine J. 1995. The origin recognition complex has essential functions in  
982 transcriptional silencing and chromosomal replication. *Gene Dev* **9**:911–924.  
983 doi:10.1101/gad.9.8.911
- 984 Gatei M, Sloper K, Sörensen C, Syljuäsen R, Falck J, Hobson K, Savage K, Lukas J, Zhou B-B,  
985 Bartek J, Khanna KK. 2003. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent  
986 Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation. *J Biol Chem*  
987 **278**:14806–14811. doi:10.1074/jbc.m210862200
- 988 Gibson DG, Bell SP, Aparicio OM. 2006. Cell cycle execution point analysis of ORC function  
989 and characterization of the checkpoint response to ORC inactivation in *Saccharomyces*  
990 *cerevisiae*. *Genes Cells* **11**:557–573. doi:10.1111/j.1365-2443.2006.00967.x
- 991 Giri S, Chakraborty A, Sathyan KM, Prasanth KV, Prasanth SG. 2016. Orc5 induces large-scale  
992 chromatin decondensation in a GCN5-dependent manner. *J Cell Sci* **129**:417–429.  
993 doi:10.1242/jcs.178889
- 994 Goto H, Natsume T, Kanemaki MT, Kaito A, Wang S, Gabazza EC, Inagaki M, Mizoguchi A.  
995 2019. Chk1-mediated Cdc25A degradation as a critical mechanism for normal cell-cycle  
996 progression. *J Cell Sci* **132**:jcs.223123. doi:10.1242/jcs.223123
- 997 Guernsey DL, Matsuoka M, Jiang H, Evans S, Macgillivray C, Nightingale M, Perry S, Ferguson  
998 M, LeBlanc M, Paquette J, Patry L, Rideout AL, Thomas A, Orr A, McMaster CR, Michaud  
999 JL, Deal C, Langlois S, Superneau DW, Parkash S, Ludman M, Skidmore DL, Samuels ME.  
1000 2011. Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. *Nat*  
1001 *Genet* **43**:360–364. doi:10.1038/ng.777
- 1002 He W, Zhang L, Villarreal OD, Fu R, Bedford E, Dou J, Patel AY, Bedford MT, Shi X, Chen T,  
1003 Bartholomew B, Xu H. 2019. De novo identification of essential protein domains from  
1004 CRISPR-Cas9 tiling-sgRNA knockout screens. *Nat Commun* **10**:4541. doi:10.1038/s41467-  
1005 019-12489-8
- 1006 Heller RC, Kang S, Lam WM, Chen S, Chan CS, Bell SP. 2011. Eukaryotic Origin-Dependent  
1007 DNA Replication In Vitro Reveals Sequential Action of DDK and S-CDK Kinases. *Cell*  
1008 **146**:80–91. doi:10.1016/j.cell.2011.06.012
- 1009 Hemerly AS, Prasanth SG, Siddiqui K, Stillman B. 2009. Orc1 controls centriole and centrosome  
1010 copy number in human cells. *Sci New York N Y* **323**:789–93. doi:10.1126/science.1166745
- 1011 Hossain M, Bhalla K, Stillman B. 2019. Cyclin binding Cy motifs have multiple activities in the  
1012 initiation of DNA replication. *Biorxiv* 681668. doi:10.1101/681668

- 1013 Hossain M, Stillman B. 2016. Opposing roles for DNA replication initiator proteins ORC1 and  
1014 CDC6 in control of Cyclin E gene transcription. *Elife* **5**:e12785. doi:10.7554/elife.12785
- 1015 Hossain M, Stillman B. 2012. Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory  
1016 domain and cause centrosome reduplication. *Gene Dev* **26**:1797–1810.  
1017 doi:10.1101/gad.197178.112
- 1018 Hou Z, Bernstein DA, Fox CA, Keck JL. 2005. Structural basis of the Sir1-origin recognition  
1019 complex interaction in transcriptional silencing. *Proc Natl Acad Sci USA* **102**:8489–8494.  
1020 doi:10.1073/pnas.0503525102
- 1021 Hsu JY, Fulco CP, Cole MA, Canver MC, Pellin D, Sher F, Farouni R, Clement K, Guo JA,  
1022 Biasco L, Orkin SH, Engreitz JM, Lander ES, Joung JK, Bauer DE, Pinello L. 2018. CRISPR-  
1023 SURF: discovering regulatory elements by deconvolution of CRISPR tiling screen data. *Nat*  
1024 *Methods* **15**:992–993. doi:10.1038/s41592-018-0225-6
- 1025 Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X,  
1026 Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. 2013. DNA targeting specificity of  
1027 RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**:827–32. doi:10.1038/nbt.2647
- 1028 Huang DW, Fanti L, Pak DTS, Botchan MR, Pimpinelli S, Kellum R. 1998. Distinct Cytoplasmic  
1029 and Nuclear Fractions of Drosophila Heterochromatin Protein 1: Their Phosphorylation  
1030 Levels and Associations with Origin Recognition Complex Proteins. *J Cell Biol* **142**:307–318.  
1031 doi:10.1083/jcb.142.2.307
- 1032 Jackson JR, Gilmartin A, Imburgia C, Winkler JD, Marshall LA, Roshak A. 2000. An  
1033 indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest  
1034 caused by DNA damage. *Cancer Res* **60**:566–72.
- 1035 Jaremko MJ, On KF, Thomas DR, Stillman B, Joshua-Tor L. 2020. The dynamic nature of the  
1036 human Origin Recognition Complex: Conformational states revealed through five cryoEM  
1037 structures. *eLife (under revision)*.
- 1038 Jones DT, Cozzetto D. 2015. DISOPRED3: precise disordered region predictions with  
1039 annotated protein-binding activity. *Bioinformatics* **31**:857–863.  
1040 doi:10.1093/bioinformatics/btu744
- 1041 Kamimura Y. 2001. Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA  
1042 replication in *Saccharomyces cerevisiae*. *The EMBO Journal* **20**:2097–2107.  
1043 doi:10.1093/emboj/20.8.2097
- 1044 Kamimura Y, Masumoto H, Sugino A, Araki H. 1998. Sld2, Which Interacts with Dpb11 in  
1045 *Saccharomyces cerevisiae*, Is Required for Chromosomal DNA Replication. *Molecular and*  
1046 *Cellular Biology* **18**:6102–6109. doi:10.1128/mcb.18.10.6102
- 1047 Kara N, Hossain M, Prasanth SG, Stillman B. 2015. Orc1 Binding to Mitotic Chromosomes  
1048 Precedes Spatial Patterning during G1 Phase and Assembly of the Origin Recognition  
1049 Complex in Human Cells. *J Biol Chem* **290**:12355–12369. doi:10.1074/jbc.m114.625012

- 1050 Kreitz S, Ritzi M, Baack M, Knippers R. 2001. The Human Origin Recognition Complex Protein  
1051 1 Dissociates from Chromatin during S Phase in HeLa Cells. *J Biol Chem* **276**:6337–6342.  
1052 doi:10.1074/jbc.m009473200
- 1053 Li N, Lam WH, Zhai Y, Cheng J, Cheng E, Zhao Y, Gao N, Tye B-K. 2018. Structure of the  
1054 origin recognition complex bound to DNA replication origin. *Nature* **559**:217–222.  
1055 doi:10.1038/s41586-018-0293-x
- 1056 Li W, Xu H, Xiao T, Cong L, Love MI, Zhang F, Irizarry RA, Liu JS, Brown M, Liu SX. 2014.  
1057 MAGECK enables robust identification of essential genes from genome-scale CRISPR/Cas9  
1058 knockout screens. *Genome Biol* **15**:554. doi:10.1186/s13059-014-0554-4
- 1059 Lidonnici MR, Rossi R, Paixão S, Mendoza-Maldonado R, Paolinelli R, Arcangeli C, Giacca M,  
1060 Biamonti G, Montecucco A. 2004. Subnuclear distribution of the largest subunit of the human  
1061 origin recognition complex during the cell cycle. *J Cell Sci* **117**:5221–5231.  
1062 doi:10.1242/jcs.01405
- 1063 Liu Q, Guntuku S, Cui X-S, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo  
1064 F, Bradley A, Donehower LA, Elledge SJ. 2000. Chk1 is an essential kinase that is regulated  
1065 by Atr and required for the G2/M DNA damage checkpoint. *Gene Dev* **14**:1448–1459.  
1066 doi:10.1101/gad.14.12.1448
- 1067 Long H, Zhang L, Lv M, Wen Z, Zhang W, Chen X, Zhang P, Li T, Chang L, Jin C, Wu G, Wang  
1068 X, Yang F, Pei J, Chen P, Margueron R, Deng H, Zhu M, Li G. 2019. H2A.Z facilitates  
1069 licensing and activation of early replication origins. *Nature* **16**:360–6. doi:10.1038/s41586-  
1070 019-1877-9
- 1071 Loupart M-L, Krause S, Heck MS. 2000. Aberrant replication timing induces defective  
1072 chromosome condensation in Drosophila ORC2 mutants. *Curr Biol* **10**:1547–1556.  
1073 doi:10.1016/s0960-9822(00)00844-7
- 1074 Lu B, Klingbeil O, Tarumoto Y, Somerville TDD, Huang Y-H, Wei Y, Wai DC, Low JKK, Milazzo  
1075 JP, Wu XS, Cao Z, Yan X, Demerdash OE, Huang G, Mackay JP, Kinney JB, Shi J, Vakoc  
1076 CR. 2018. A Transcription Factor Addiction in Leukemia Imposed by the MLL Promoter  
1077 Sequence. *Cancer Cell* **34**:970-981.e8. doi:10.1016/j.ccell.2018.10.015
- 1078 Marahrens Y, Stillman B. 1992. A yeast chromosomal origin of DNA replication defined by  
1079 multiple functional elements. *Science* **255**:817–823. doi:10.1126/science.1536007
- 1080 Matson JP, Dumitru R, Coryell P, Baxley RM, Chen W, Twaroski K, Webber BR, Tolar J,  
1081 Bielinsky A-K, Purvis JE, Cook JG. 2017. Rapid DNA replication origin licensing protects  
1082 stem cell pluripotency. *eLife* **6**:114. doi:10.7554/elife.30473
- 1083 McFarland JM, Ho ZV, Kugener G, Dempster JM, Montgomery PG, Bryan JG, Krill-Burger JM,  
1084 Green TM, Vazquez F, Boehm JS, Golub TR, Hahn WC, Root DE, Tsherniak A. 2018.  
1085 Improved estimation of cancer dependencies from large-scale RNAi screens using model-  
1086 based normalization and data integration. *Nat Commun* **9**:4610. doi:10.1038/s41467-018-  
1087 06916-5



- 1088 McKinley KL, Cheeseman IM. 2017. Large-Scale Analysis of CRISPR/Cas9 Cell-Cycle  
1089 Knockouts Reveals the Diversity of p53-Dependent Responses to Cell-Cycle Defects. *Dev*  
1090 *Cell* **40**:405-420.e2. doi:10.1016/j.devcel.2017.01.012
- 1091 McNairn AJ, Gilbert DM. 2005. Overexpression of ORC subunits and increased ORC-chromatin  
1092 association in transformed mammalian cells. *J Cell Biochem* **96**:879–887.  
1093 doi:10.1002/jcb.20609
- 1094 Méndez J, Zou-Yang XH, Kim S-Y, Hidaka M, Tansey WP, Stillman B. 2002. Human Origin  
1095 Recognition Complex Large Subunit Is Degraded by Ubiquitin-Mediated Proteolysis after  
1096 Initiation of DNA Replication. *Mol Cell* **9**:481–491. doi:10.1016/s1097-2765(02)00467-7
- 1097 Meyers RM, Bryan JG, McFarland JM, Weir BA, Sizemore AE, Xu H, Dharia NV, Montgomery  
1098 PG, Cowley GS, Pantel S, Goodale A, Lee Y, Ali LD, Jiang G, Lubonja R, Harrington WF,  
1099 Strickland M, Wu T, Hawes DC, Zhivich VA, Wyatt MR, Kalani Z, Chang JJ, Okamoto M,  
1100 Stegmaier K, Golub TR, Boehm JS, Vazquez F, Root DE, Hahn WC, Tsherniak A. 2017a.  
1101 Computational correction of copy number effect improves specificity of CRISPR-Cas9  
1102 essentiality screens in cancer cells. *Nat Genet* **49**:1779–1784. doi:10.1038/ng.3984
- 1103 Meyers RM, Bryan JG, McFarland JM, Weir BA, Sizemore AE, Xu H, Dharia NV, Montgomery  
1104 PG, Cowley GS, Pantel S, Goodale A, Lee Y, Ali LD, Jiang G, Lubonja R, Harrington WF,  
1105 Strickland M, Wu T, Hawes DC, Zhivich VA, Wyatt MR, Kalani Z, Chang JJ, Okamoto M,  
1106 Stegmaier K, Golub TR, Boehm JS, Vazquez F, Root DE, Hahn WC, Tsherniak A. 2017b.  
1107 Computational correction of copy number effect improves specificity of CRISPR-Cas9  
1108 essentiality screens in cancer cells. *Nat Genet* **49**:1779–1784. doi:10.1038/ng.3984
- 1109 Miles LA, Garippa RJ, Poirier JT. 2016. Design, execution, and analysis of  
1110 pooled in vitro CRISPR/Cas9 screens. *Febs J* **283**:3170–3180. doi:10.1111/febs.13770
- 1111 Miotto B, Ji Z, Struhl K. 2016. Selectivity of ORC binding sites and the relation to replication  
1112 timing, fragile sites, and deletions in cancers. *Proc Natl Acad Sci USA* **113**:E4810–E4819.  
1113 doi:10.1073/pnas.1609060113
- 1114 Montalbano A, Canver MC, Sanjana NE. 2017. High-Throughput Approaches to Pinpoint  
1115 Function within the Noncoding Genome. *Mol Cell* **68**:44–59.  
1116 doi:10.1016/j.molcel.2017.09.017
- 1117 Munnik SA de, Hoefsloot EH, Roukema J, Schoots J, Knoers NV, Brunner HG, Jackson AP,  
1118 Bongers EM. 2015. Meier-Gorlin syndrome. *Orphanet J Rare Dis* **10**:114.  
1119 doi:10.1186/s13023-015-0322-x
- 1120 Natsume T, Kiyomitsu T, Saga Y, Kanemaki MT. 2016. Rapid Protein Depletion in Human Cells  
1121 by Auxin-Inducible Degron Tagging with Short Homology Donors. *Cell reports* **15**:210–218.  
1122 doi:10.1016/j.celrep.2016.03.001
- 1123 Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M. 2009. An auxin-based  
1124 degron system for the rapid depletion of proteins in nonplant cells. *Nature methods* **6**:917–  
1125 922. doi:10.1038/nmeth.1401
- 1126 Ocaña-Pallarès E, Vergara Z, Desvoves B, Tejada-Jimenez M, Romero-Jurado A, Galván A,  
1127 Fernández E, Ruiz-Trillo I, Gutierrez C. 2020. Origin Recognition Complex (ORC) Evolution

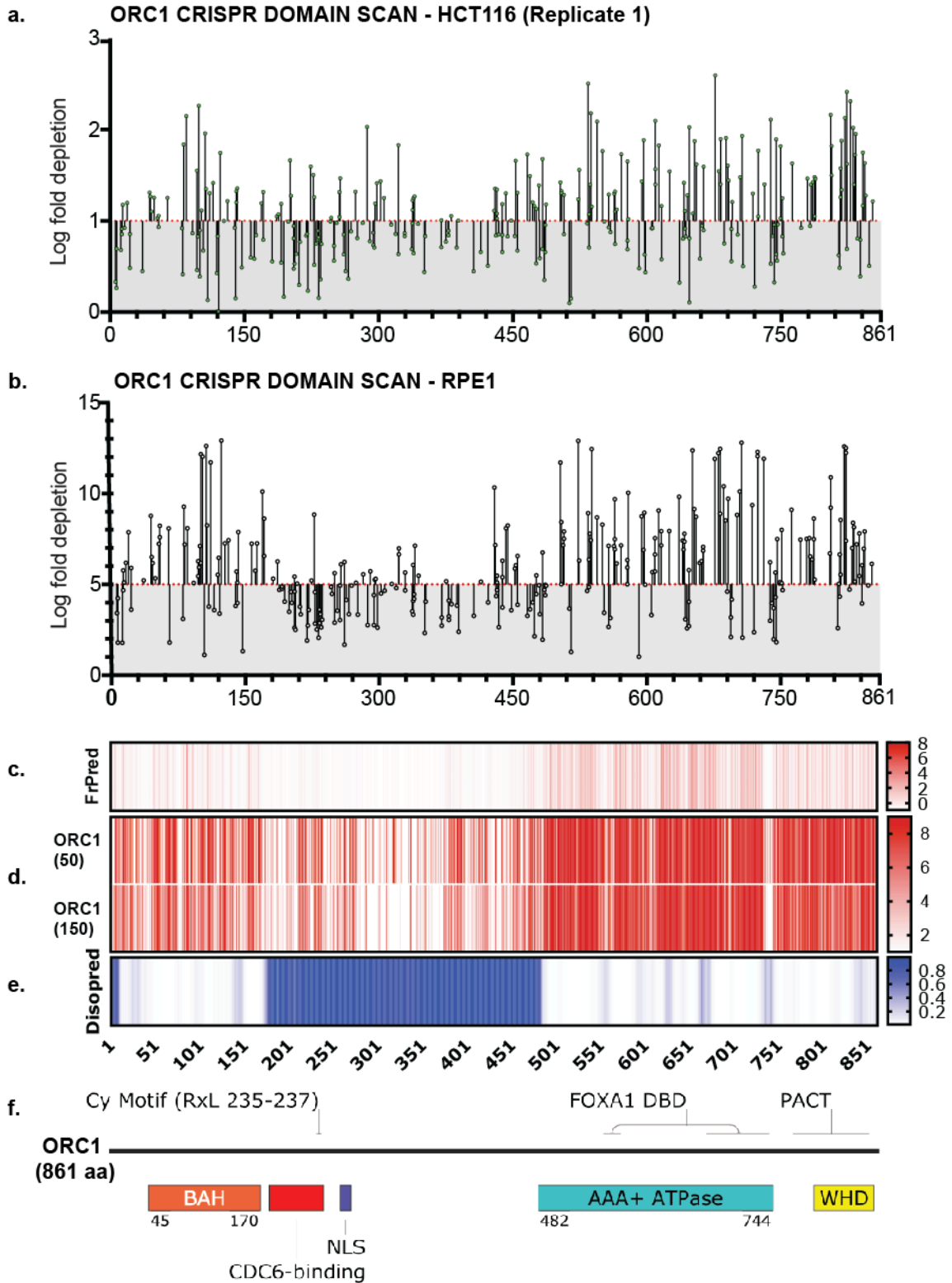
- 1128 Is Influenced by Global Gene Duplication/Loss Patterns in Eukaryotic Genomes. *Genome*  
1129 *Biol Evol* **12**:3878–3889. doi:10.1093/gbe/evaa011
- 1130 Ohta S, Tatsumi Y, Fujita M, Tsurimoto T, Obuse C. 2003. The ORC1 Cycle in Human Cells: II.  
1131 Dynamic Changes in the Human ORC Complex During the Cell Cycle. *J Biol Chem*  
1132 **278**:41535–41540. doi:10.1074/jbc.m307535200
- 1133 Okuno Y, McNairn AJ, Elzen N den, Pines J, Gilbert DM. 2001. Stability, chromatin association  
1134 and functional activity of mammalian pre-replication complex proteins during the cell cycle.  
1135 *Embo J* **20**:4263–4277. doi:10.1093/emboj/20.15.4263
- 1136 Pak DTS, Pflumm M, Chesnokov I, Huang DW, Kellum R, Marr J, Romanowski P, Botchan MR.  
1137 1997. Association of the Origin Recognition Complex with Heterochromatin and HP1 in  
1138 Higher Eukaryotes. *Cell* **91**:311–323. doi:10.1016/s0092-8674(00)80415-8
- 1139 Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu  
1140 H, Krupczak KM, Garcia-Beltran W, Piechocka-Trocha A, Krogan NJ, Marson A, Sabatini  
1141 DM, Lander ES, Hacohen N, Walker BD. 2016. A genome-wide CRISPR screen identifies a  
1142 restricted set of HIV host dependency factors. *Nat Genet* **49**:193–203. doi:10.1038/ng.3741
- 1143 Parker MW, Bell M, Mir M, Kao JA, Darzacq X, Botchan MR, Berger JM. 2019. A new class of  
1144 disordered elements controls DNA replication through initiator self-assembly. *Elife* **8**:e48562.  
1145 doi:10.7554/elife.48562
- 1146 Pflumm MF, Botchan MR. 2001. Orc mutants arrest in metaphase with abnormally condensed  
1147 chromosomes. *Dev Camb Engl* **128**:1697–707.
- 1148 Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B. 2004. Human Orc2 localizes to  
1149 centrosomes, centromeres and heterochromatin during chromosome inheritance. *The EMBO*  
1150 *Journal* **23**:2651–2663. doi:10.1038/sj.emboj.7600255
- 1151 Prasanth SG, Prasanth KV, Stillman B. 2002. Orc6 Involved in DNA Replication, Chromosome  
1152 Segregation, and Cytokinesis. *Science* **297**:1026–1031. doi:10.1126/science.1072802
- 1153 Prasanth SG, Shen Z, Prasanth KV, Stillman B. 2010. Human origin recognition complex is  
1154 essential for HP1 binding to chromatin and heterochromatin organization. *Proc Natl Acad Sci*  
1155 *USA* **107**:15093–15098. doi:10.1073/pnas.1009945107
- 1156 Rao H, Stillman B. 1995. The origin recognition complex interacts with a bipartite DNA binding  
1157 site within yeast replicators. *Proc Natl Acad Sci USA* **92**:2224–2228.  
1158 doi:10.1073/pnas.92.6.2224
- 1159 Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, Diffley JFX. 2009. Concerted loading of  
1160 Mcm2-7 double hexamers around DNA during DNA replication origin licensing. *Cell*  
1161 **139**:719–30. doi:10.1016/j.cell.2009.10.015
- 1162 Romanowski P, Madine MA, Rowles A, Blow JJ, Laskey RA. 1996. The Xenopus origin  
1163 recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr Biol*  
1164 **6**:1416–1425. doi:10.1016/s0960-9822(96)00746-4

- 1165 Sanjana NE, Shalem O, Zhang F. 2014. Improved vectors and genome-wide libraries for  
1166 CRISPR screening. *Nat Methods* **11**:783–784. doi:10.1038/nmeth.3047
- 1167 Sedlazeck FJ, Rescheneder P, Smolka M, Fang H, Nattestad M, Haeseler A von, Schatz MC.  
1168 2018. Accurate detection of complex structural variations using single-molecule sequencing.  
1169 *Nat Methods* **15**:461–468. doi:10.1038/s41592-018-0001-7
- 1170 Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. 2015. Discovery of cancer drug  
1171 targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol* **33**:661–7.  
1172 doi:10.1038/nbt.3235
- 1173 Shibata E, Kiran M, Shibata Y, Singh S, Kiran S, Dutta A. 2016. Two subunits of human ORC  
1174 are dispensable for DNA replication and proliferation. *eLife* **5**:1844. doi:10.7554/elife.19084
- 1175 Shimada K, Gasser SM. 2007. The origin recognition complex functions in sister-chromatid  
1176 cohesion in *Saccharomyces cerevisiae*. *Cell* **128**:85–99. doi:10.1016/j.cell.2006.11.045
- 1177 Smits AH, Ziebell F, Joberty G, Zinn N, Mueller WF, Clauder-Münster S, Eberhard D, Savitski  
1178 MF, Grandi P, Jakob P, Michon A-M, Sun H, Tessmer K, Bürckstümmer T, Bantscheff M,  
1179 Steinmetz LM, Drewes G, Huber W. 2019. Biological plasticity rescues target activity in  
1180 CRISPR knock outs. *Nat Methods* **16**:1087–1093. doi:10.1038/s41592-019-0614-5
- 1181 So RWL, Chung SW, Lau HHC, Watts JJ, Gaudette E, Al-Azzawi ZAM, Bishay J, Lin LT-W,  
1182 Joung J, Wang X, Schmitt-Ulms G. 2019. Application of CRISPR genetic screens to  
1183 investigate neurological diseases. *Mol Neurodegener* **14**:41. doi:10.1186/s13024-019-0343-3
- 1184 Song B, Liu XS, Davis K, Liu X. 2011. Plk1 Phosphorylation of Orc2 Promotes DNA Replication  
1185 under Conditions of Stress. *Mol Cell Biol* **31**:4844–4856. doi:10.1128/mcb.06110-11
- 1186 Speck C, Chen Z, Li H, Stillman B. 2005. ATPase-dependent cooperative binding of ORC and  
1187 Cdc6 to origin DNA. *Nat Struct Mol Biol* **12**:965–971. doi:10.1038/nsmb1002
- 1188 Sundaram M, Guernsey DL, Rajaraman MM, Rajaraman R. 2004. Neosis: A Novel Type of Cell  
1189 Division in Cancer. *Cancer Biol Ther* **3**:207–218. doi:10.4161/cbt.3.2.663
- 1190 Takayama Y, Kamimura Y, Okawa M, Muramatsu S, Sugino A, Araki H. 2003. GINS, a novel  
1191 multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes &*  
1192 *Development* **17**:1153–1165. doi:10.1101/gad.1065903
- 1193 Tarumoto Y, Lu B, Somerville TDD, Huang Y-H, Milazzo JP, Wu XS, Klingbeil O, Demerdash  
1194 OE, Shi J, Vakoc CR. 2018. LKB1, Salt-Inducible Kinases, and MEF2C Are Linked  
1195 Dependencies in Acute Myeloid Leukemia. *Mol Cell* **69**:1017-1027.e6.  
1196 doi:10.1016/j.molcel.2018.02.011
- 1197 Tatsumi Y, Ezura K, Yoshida K, Yugawa T, Narisawa-Saito M, Kiyono T, Ohta S, Obuse C,  
1198 Fujita M. 2008. Involvement of human ORC and TRF2 in pre-replication complex assembly  
1199 at telomeres. *Genes Cells Devoted Mol Cell Mech* **13**:1045–59. doi:10.1111/j.1365-  
1200 2443.2008.01224.x

- 1201 Tennakoon C, Purbojati RW, Sung W-K. 2012. BatMis: a fast algorithm for k-mismatch  
1202 mapping. *Bioinform Oxf Engl* **28**:2122–8. doi:10.1093/bioinformatics/bts339
- 1203 Tocilj A, On KF, Yuan Z, Sun J, Elkayam E, Li H, Stillman B, Joshua-Tor L. 2017. Structure of  
1204 the active form of human origin recognition complex and its ATPase motor module. *Elife*  
1205 **6**:1822. doi:10.7554/elife.20818
- 1206 Triolo T, Sternglanz R. 1996. Role of interactions between the origin recognition complex and  
1207 SIR1 in transcriptional silencing. *Nature* **381**:251–253. doi:10.1038/381251a0
- 1208 Tsherniak A, Vazquez F, Montgomery PG, Weir BA, Kryukov G, Cowley GS, Gill S, Harrington  
1209 WF, Pantel S, Krill-Burger JM, Meyers RM, Ali L, Goodale A, Lee Y, Jiang G, Hsiao J, Gerath  
1210 WFJ, Howell S, Merkel E, Ghandi M, Garraway LA, Root DE, Golub TR, Boehm JS, Hahn  
1211 WC. 2017. Defining a Cancer Dependency Map. *Cell* **170**:564-576.e16.  
1212 doi:10.1016/j.cell.2017.06.010
- 1213 Vashee S, Simancek P, Challberg MD, Kelly TJ. 2001. Assembly of the Human Origin  
1214 Recognition Complex. *J Biol Chem* **276**:26666–26673. doi:10.1074/jbc.m102493200
- 1215 Wang Y, Zhong Y, Zhou Y, Tanaseichuk O, Li Z, Zhao JC. 2019. Identification of a Xist silencing  
1216 domain by Tiling CRISPR. *Scientific reports* **9**:2408. doi:10.1038/s41598-018-36750-0
- 1217 Wang Z, Andrews P, Kendall J, Ma B, Hakker I, Rodgers L, Ronemus M, Wigler M, Levy D.  
1218 2016. SMASH, a fragmentation and sequencing method for genomic copy number analysis.  
1219 *Genome Res* **26**:844–851. doi:10.1101/gr.201491.115
- 1220 Weinreich M, Liang C, Stillman B. 1999. The Cdc6p nucleotide-binding motif is required for  
1221 loading mcm proteins onto chromatin. *Proc Natl Acad Sci USA* **96**:441–446.  
1222 doi:10.1073/pnas.96.2.441.
- 1223 Wilsker D, Petermann E, Helleday T, Bunz F. 2008. Essential function of Chk1 can be  
1224 uncoupled from DNA damage checkpoint and replication control. *Proc Natl Acad Sci USA*  
1225 **105**:20752–20757. doi:10.1073/pnas.0806917106
- 1226 Yeeles JTP, Deegan TD, Janska A, Early A, Diffley JFX. 2015. Regulated eukaryotic DNA  
1227 replication origin firing with purified proteins. *Nature* **519**:431–435. doi:10.1038/nature14285
- 1228
- 1229

1230 FIGURES AND FIGURE LEGENDS

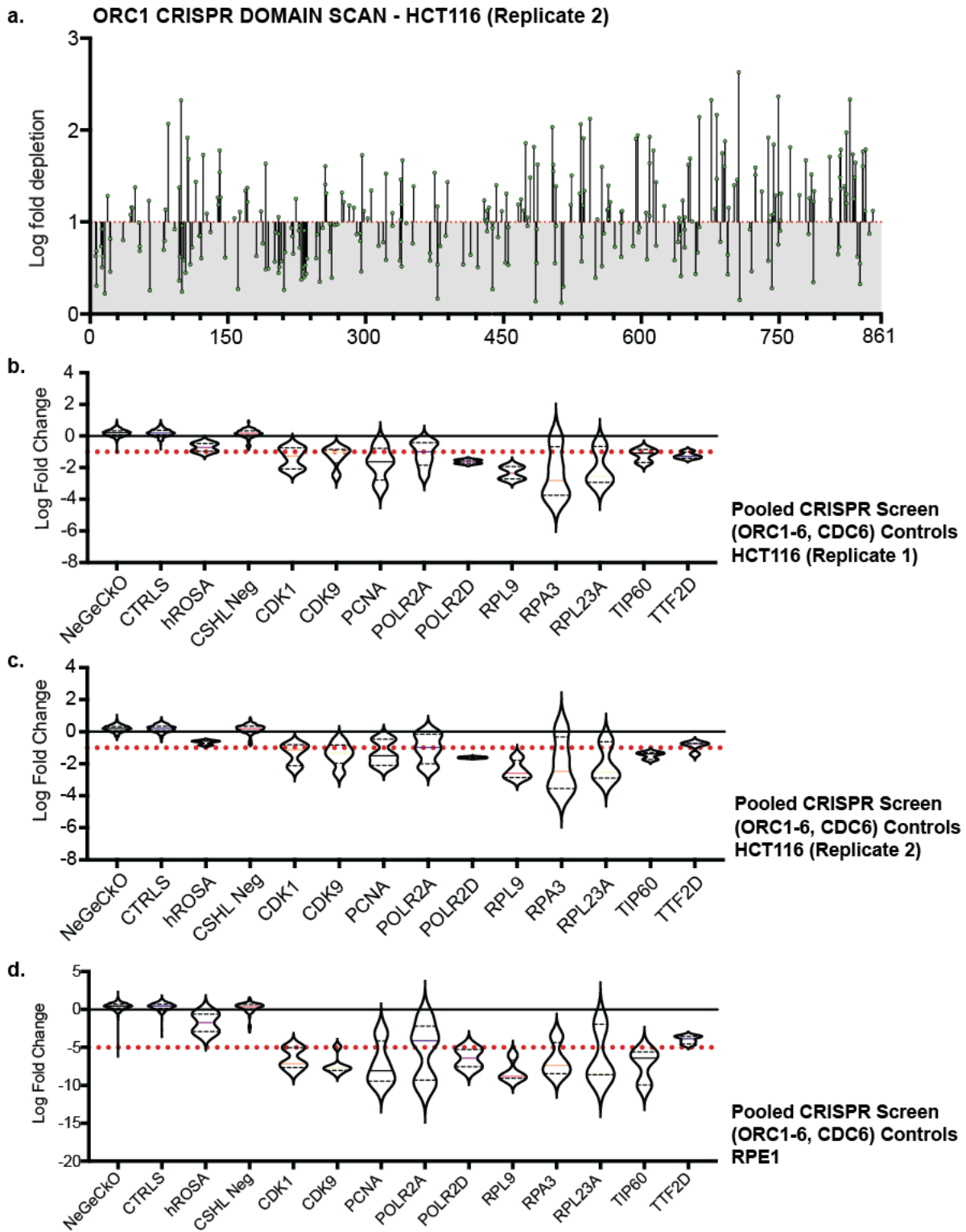
1231 Figure 1.



1232

1233 Figure 1. ORC1 is essential to HCT116 and RPE-1 cell lines. (a) tiling sgRNA map ORC1  
1234 (replicate 1) in HCT116. Mapped as Log fold depletion (inverted LFC scale) as calculated by  
1235 MaGeCK (Li et al., 2014) on y axis vs the codon that is disrupted by the guide RNA on the x  
1236 axis. Effect of guide RNA is interpreted as essential if its depletion is more than 1 log fold (red  
1237 dotted line). (b) tiling sgRNA map of ORC1 in RPE-1. Effect of guide RNA is interpreted as  
1238 essential if its depletion is more than 5 log fold (red dotted line). (c) FrPred  
1239 (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC1 (NP\_004144.2) shown as gradient heat map  
1240 of conservation score vs amino acid position. (d) Consurf (<https://consurf.tau.ac.il/>) of hORC1 –  
1241 (upper) ORC1 (50) subset (50 HMMER Homologues collected from UNIREF90 database, Max  
1242 sequence identity = 95%, Min sequence identity 50, Other parameters = default), and (lower)  
1243 ORC1 (150) subset (150 HMMER Homologues collected from UNIREF90 database, Max  
1244 sequence identity = 95%, Min sequence identity 35, Other parameters = default). Data  
1245 represented as heat map of Conservation scores of each amino acid position. (e) Disopred  
1246 (<http://bioinf.cs.ucl.ac.uk/psipred/>) plot of hORC1 – heat map representing amino acids within  
1247 intrinsically disordered regions of the protein. (f) Schematic of domain architecture of ORC1.  
1248

1249 Figure 1—Figure supplement 1.

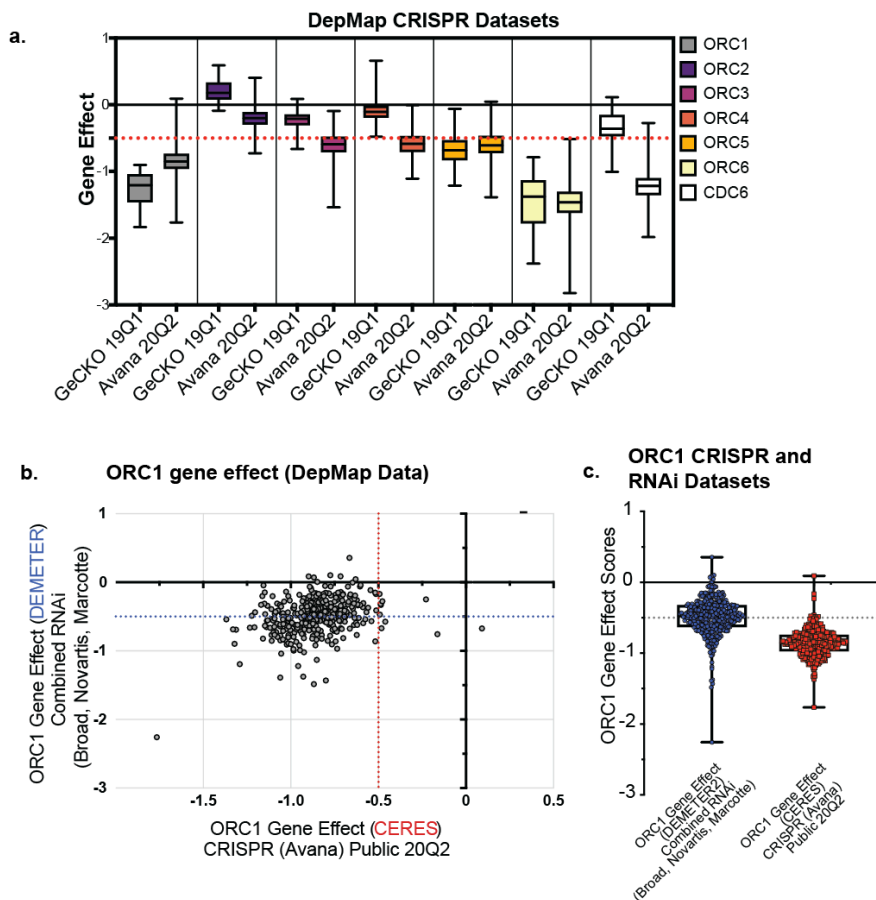


1250

1251 Figure 1—Figure supplement 1. Tiling sgRNA CRISPR screen data and controls. (a) tiling  
1252 sgRNA map of ORC1 (replicate 2) in HCT116. Mapped as Log fold depletion (inverted LFC  
1253 scale) as calculated by MaGeCK (Li et al., 2014) on y axis vs the codon that is disrupted by the  
1254 guide RNA on the x axis. Effect of guide RNA is interpreted as essential if its depletion is more  
1255 than 1 log fold (red dotted line). (b) tiling sgRNA controls for HCT116 (replicate 1). Violin plots  
1256 mapped as distribution of Log fold depletion (MaGeCK) for each guide RNA from for negative  
1257 (NeGeCKO, CTRLS, hROSA, and CSHL-neg library) or positive control (CDK1, CDK9, PCNA,  
1258 POLR2A, POLR2D, RPL9, RPA3, RPL23A, TIP60, TTF2D) subset. The median and quartiles of  
1259 LFC for each subset are indicated within each violin plot. Cut-off of essentiality is  $LFC \geq -1$ ,  
1260 indicated by red dotted line (Highest log fold depletion value of all negative controls and more  
1261 than the median of positive controls. (c) tiling sgRNA controls for HCT116 (replicate 2). (d) tiling  
1262 sgRNA controls for RPE-1. Cut-off of essentiality is  $LFC \geq -5$ , indicated by red dotted line.  
1263



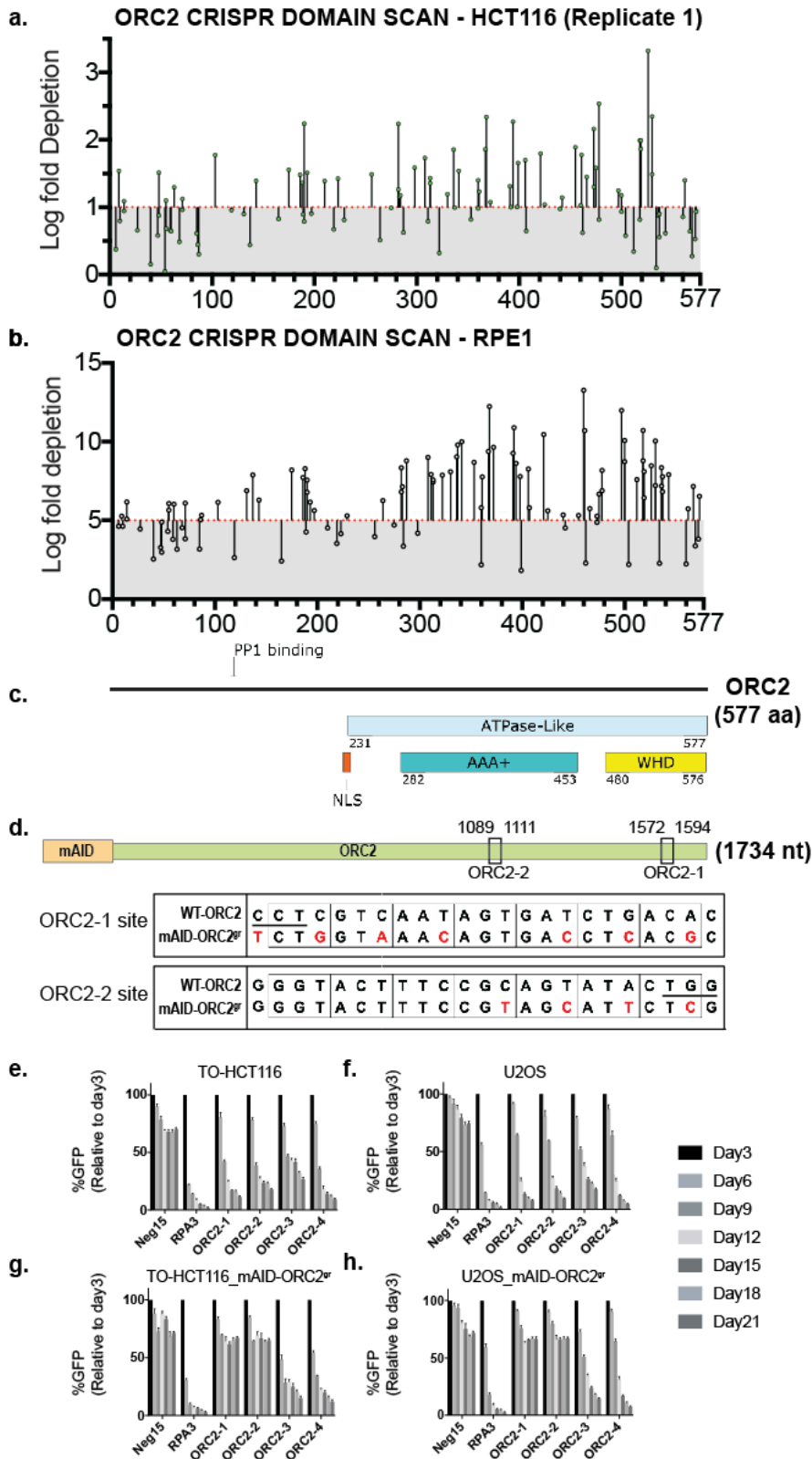
1264 Figure 1—Figure supplement 2.



1265

1266 Figure 1—Figure supplement 2. DepMap analyses of *ORC1* data. (a) Distribution of Gene Effect  
 1267 scores of *ORC1-6* and *CDC6* across all the cell lines used in either the GeCKO 19Q1 or Avana  
 1268 20Q2 CRISPR screens reported on DepMap [<https://doi.org/10.6084/m9.figshare.7668407>,  
 1269 <https://doi.org/10.6084/m9.figshare.12280541.v4>; (Meyers et al., 2017b)]. Each box plot  
 1270 represents gene effect range displayed in the tested cell lines. The red dotted line represents  
 1271 the gene effect score below which genes are scores as essential. (b) *ORC1* gene effect values  
 1272 for CRISPR (CERES; (Meyers et al., 2017b) vs RNAi (DepMap, 2019; McFarland et al.,  
 1273 2018) mapped as xy scatter for ~390 common cell lines used in the screens. Red dotted line  
 1274 bifurcates the plot at CRISPR based gene effect score of less than -0.5 is considered essential  
 1275 to cell line. Blue dotted line bifurcates the plot at RNAi based gene effect score of less than -0.5  
 1276 is considered essential to cell line. (c) Distribution of *ORC1* gene effect scores across all the cell  
 1277 lines used in CRISPR Avana 20Q2 and RNAi datasets respectively ((McFarland et al., 2018;  
 1278 Meyers et al., 2017b).

1279 Figure 2.



1280

1281 Figure 2. *ORC2* is essential in HCT116 and RPE-1 and both by tiling sgRNA and single guide  
1282 CRISPR knock-down in presence of sgRNA-resistant mAID-*ORC2*<sup>gr</sup>. (a) tiling sgRNA map of  
1283 *ORC2* (replicate 1) in HCT116. Mapped as Log fold depletion (inverted LFC scale) as calculated  
1284 by MaGeCK (Li et al., 2014) on y axis vs the codon that is disrupted by the guide RNA on the x  
1285 axis. Effect of guide RNA is interpreted as essential if its depletion is more than 1 log fold (red  
1286 dotted line). (b) tiling sgRNA map of *ORC2* for RPE-1. (c) schematic of *ORC2* protein showing  
1287 annotated structural or functional domains. (d) The top panel shows the mAID degron was fused  
1288 to *ORC2* transgene at the N-terminus, and the two black boxes indicate *ORC2*-1 and *ORC2*-2  
1289 sgRNAs targeting regions. The numbers represent nucleotide positions in the *ORC2* cDNA. The  
1290 lower two panels show the comparison of wild type and mAID-*ORC2*<sup>gr</sup> with silent mutations at  
1291 the indicated sgRNA targeting sites. The red color marks the mismatches. Protospacer-adjacent  
1292 motif (PAM) site is underlined in the wild type sequence. (e-h) Negative-selection time course  
1293 assay that plots the percentage of GFP positive cells over time following transduction with the  
1294 indicated sgRNAs with Cas9. Experiments were performed in (e) TO-HCT116, (f) U2OS, (g)  
1295 TO-HCT116\_mAID-*ORC2*<sup>gr</sup>, and (h) U2OS\_mAID-*ORC2*<sup>gr</sup> cell lines. The GFP positive  
1296 percentage was normalized to the Day3 measurement. N = 3. Error bars, mean ± SD.

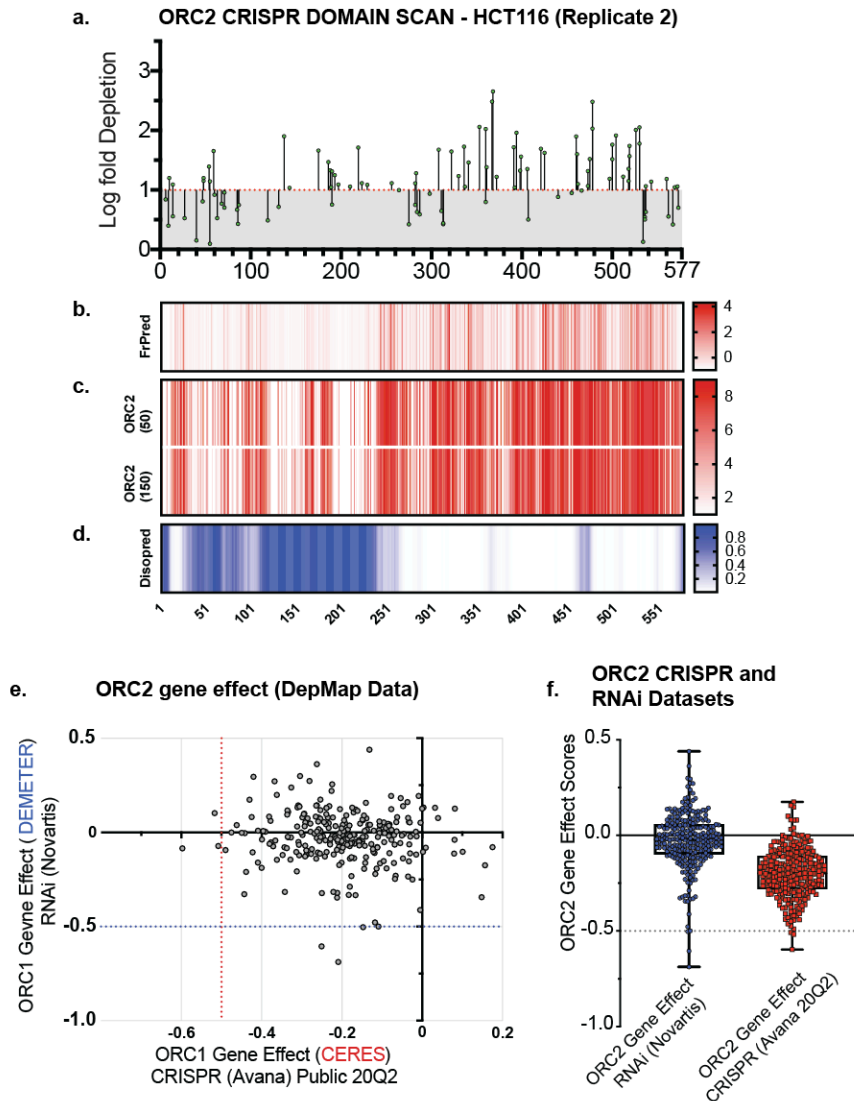
1297

1298

1299

1300

1301 Figure 2—Figure supplement 1.



1302

1303 Figure 2—Figure supplement 1. (a) Tiling sgRNA map of ORC2 (replicate 2) in HCT116. (b)  
1304 FrPred (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC2 (NP\_006181.1) shown as gradient  
1305 heat map of conservation score vs amino acid position. (d) Consurf (<https://consurf.tau.ac.il/>) of  
1306 hORC2 – (upper) ORC2 (50) subset (50 HMMER Homologues collected from UNIREF90  
1307 database, Max sequence identity = 95%, Min sequence identity 50, Other parameters = default),  
1308 and (lower) ORC2 (150) subset (150 HMMER Homologues collected from UNIREF90 database,  
1309 Max sequence identity = 95%, Min sequence identity 35, Other parameters = default). Data  
1310 represented as heatmap of Conservation scores of each amino acid position. (e) Disopred  
1311 (<http://bioinf.cs.ucl.ac.uk/psipred/>) plot of hORC2 – heatmap representing amino acids within  
1312 intrinsically disordered regions of the protein.

1313 Figure 2—Figure supplement 2.

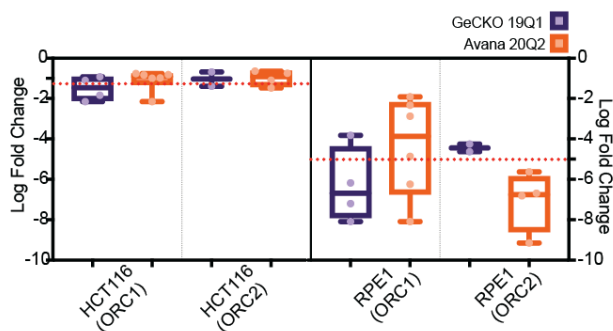
a. **GeCKO library: ORC1 and ORC2 sgRNA**

No.	Gene	sgRNA	HCT116 (LFC; $\leq -1$ )	RPE1 (LFC; $\leq -5$ )
1	ORC1	TGTACTCGAGCACGTTTCTT	-1.837	-7.1963
3	ORC1	AGAAACGTGCTCGAGTACAG	-2.149	-8.0847
5	ORC1	ACCGAGATTACATCCAGAT	-1.1019	-6.1747
6	ORC1	ACCACATCCTTTGGGGCTAA	-0.92417	-3.82
7	ORC2	GAACCTTGTCTGAAGCAGAA	-1.3909	-4.6301
9	ORC2	TCTTCCTCAAATCATATTC	-0.68492	-4.2515

b. **Avana 20Q2 library: ORC1 and ORC2 sgRNA**

No.	Gene	sgRNA	HCT116 (LFC; $\leq -1$ )	RPE1 (LFC; $\leq -5$ )
1	ORC1	TTACCCCAAGAGCCAGAAAG	-0.81863	-2.8709
2	ORC1	AGAAACGTGCTCGAGTACAG	-2.149	-8.0847
3	ORC1	AGTTTGCGGAGATTTGGAGG	-0.83692	-1.9165
4	ORC1	TCTGGTTTCAGAATCACGGA	-0.76629	-2.3272
5	ORC1	ATTCTCCAGGAAGAATAAAA	-0.98108	-6.2369
6	ORC1	AAGTCTGCTGGGACATCTGA	-0.99435	-4.8626
8	ORC2	AAGTTCGGCAGAAAAGGAAG	-0.75093	-6.8116
9	ORC2	GGTTGCACAGGAACATGAAG	-1.094	-5.625
10	ORC2	TTTGGAGGAAGATGACCAGG	-0.6455	-6.6874
12	ORC2	ATATTCTGCTTCCAACCTCAG	-1.4795	-9.1468

c.



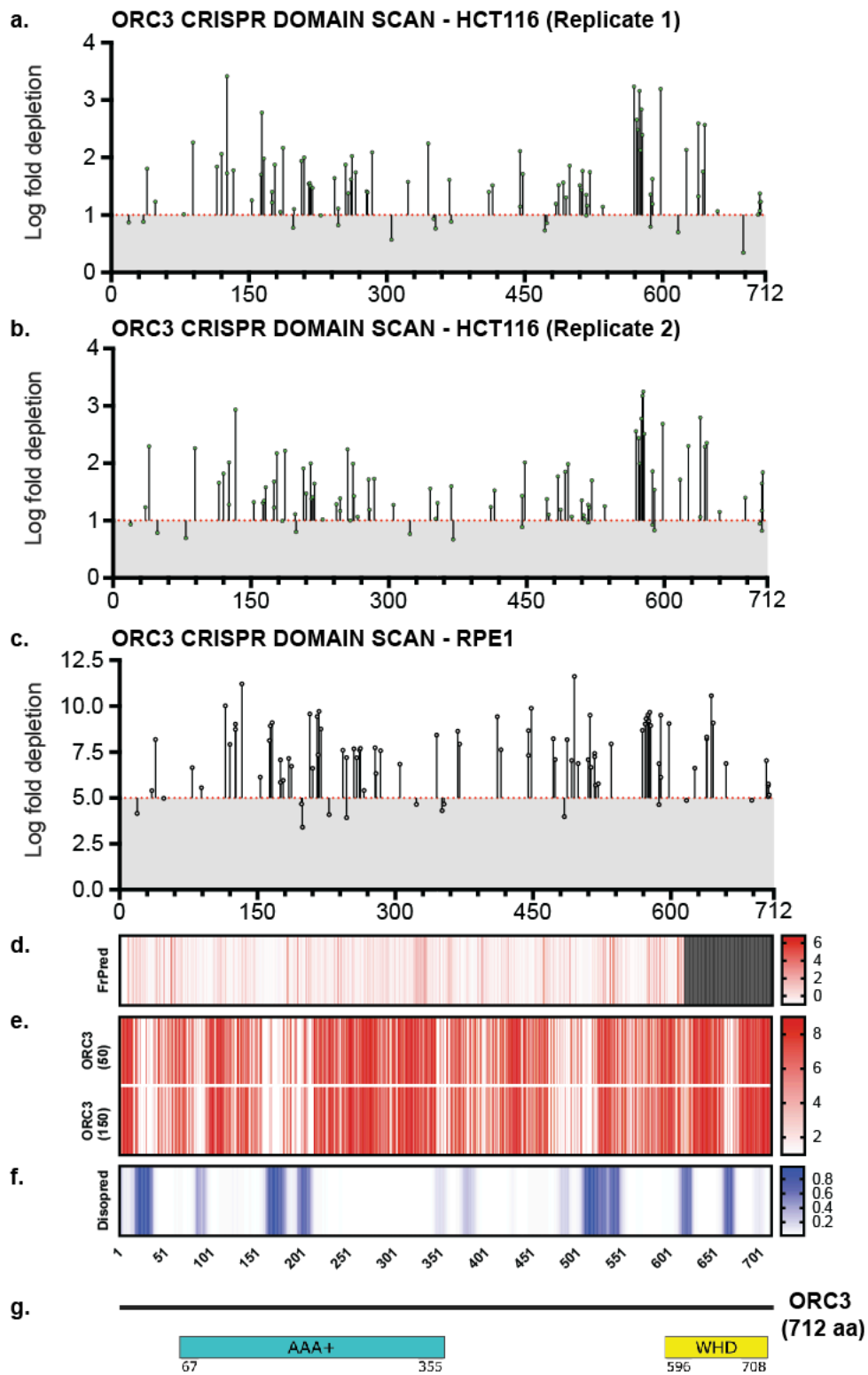
d. **ORC2 guides used in this study**

Gene	sgRNA	aa	HCT116-I (LFC; $\leq -1$ )	HCT116-II (LFC; $\leq -1$ )	RPE1 (LFC $\leq -5$ )
ORC2-1	GTGTCAGATCACTATTGACG	526	-3.3226	-2.0084	-8.468
ORC2-2	GGTACTTTCCGCAGTATAC	367	-1.8567	-2.4848	-9.3786
ORC2-3	GGATGATGAAGGGTTGCAC	193	-1.5095	-1.2495	-6.1627
ORC2-4	AAAGCACAATGTTGAACCCA	308	-1.7298	-1.6785	-8.9999
Shibata 2016	GAAGGAGCGAGCGAGCCTT T	40	-1.5134	-1.1548	-4.4581

1314

1315 Figure 2—Figure supplement 2. (a) Table listing guide RNA sequences used in GeCKO 19Q1  
 1316 library against *ORC1* and *ORC2* that were also present in our tiling sgRNA screen; columns  
 1317 HCT116(LFC  $\leq -1$ ) and RPE1(LFC  $\leq -5$ ) show the LFC of the GeCKO guides in our screen. (b)  
 1318 Table listing guide RNA sequences used in Avana 20Q2 library against *ORC1* and *ORC2* that  
 1319 were also present in our tiling sgRNA screen; columns HCT116(LFC  $\leq -1$ ) and RPE1(LFC  $\leq -5$ )  
 1320 show the LFC of the GeCKO guides in our screen. Rows highlighted blue represent the only  
 1321 common guide against *ORC1* used between GeCKO and Avana screens (c) Distribution of the  
 1322 LFC values for the GeCKO and Avana guides in HCT116 and RPE-1 (graphical representation  
 1323 of the tables (a) & (b)). (d) *ORC2* guides selected for single guide studies. Row highlighted  
 1324 yellow – guide RNA sequence used in the Shibata et. Al 2016 study.

1325 Figure 2—Figure supplement 3.

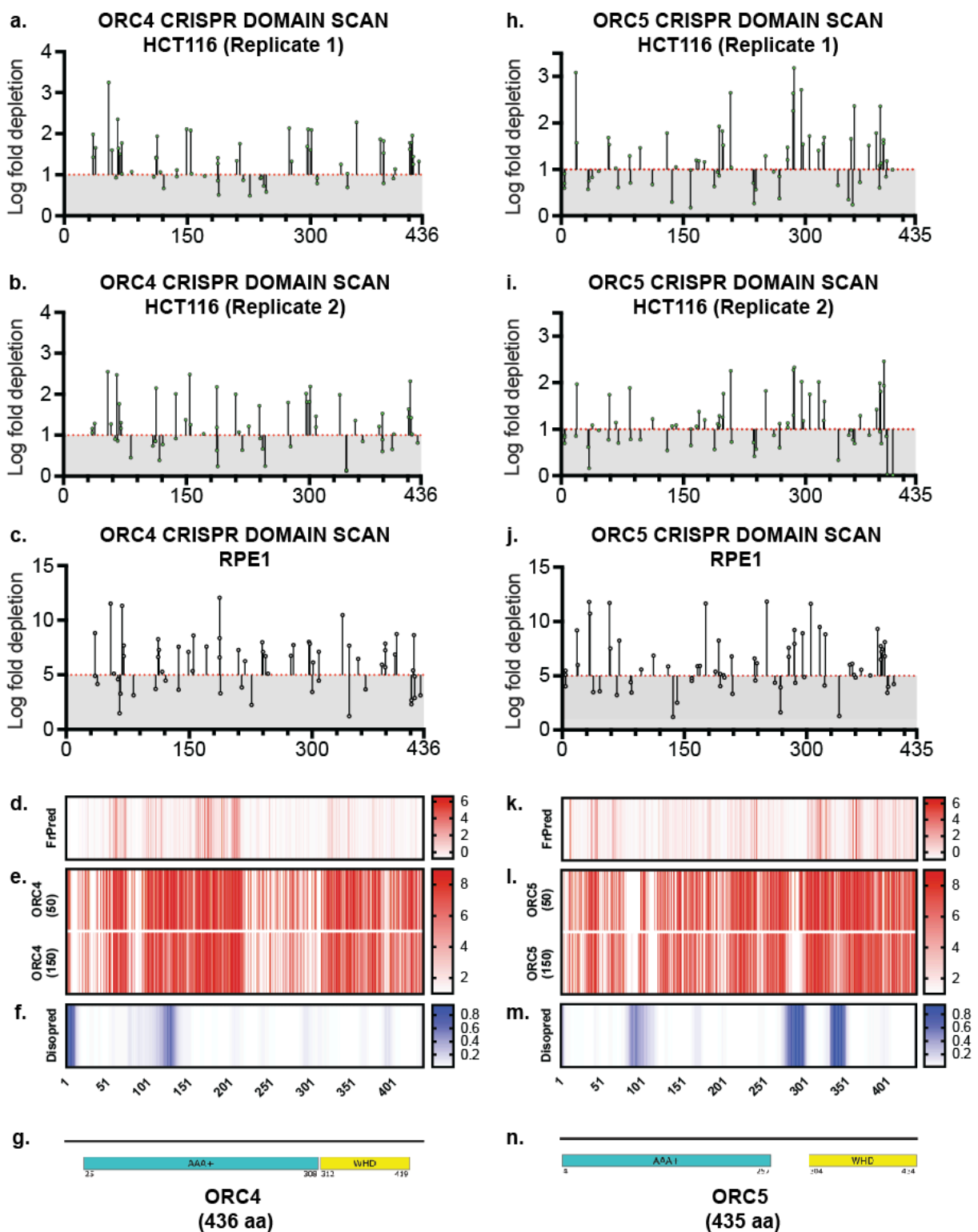


1326

1327

1328 Figure 2—Figure supplement 3. Tiling sgRNA CRISPR screen data contd. (a) tiling sgRNA map  
1329 of ORC3 (replicate 1) in HCT116. Mapped as Log fold depletion as calculated by MaGeCK on y  
1330 axis vs the codon that is disrupted by the guide RNA on the x axis. Effect of guide RNA is  
1331 interpreted as essential if its depletion is more than 1 log fold (red dotted line). (b) tiling sgRNA  
1332 map of ORC3 for HCT116 (replicate 2). (c) tiling sgRNA map of ORC3 for RPE-1 cell line. Cutt-  
1333 off of essentiality is  $LFC \geq -5$ , indicated by red dotted line. (d) FrPred  
1334 (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC3 (NP\_862820.1) shown as gradient heat map  
1335 of conservation score vs amino acid position. (e) Consurf (<https://consurf.tau.ac.il/>) of hORC3 –  
1336 (upper) ORC3 (50) subset (50 HMMER Homologues collected from UNIREF90 database, Max  
1337 sequence identity = 95%, Min sequence identity 50, Other parameters = default), and (lower)  
1338 ORC3 (150) subset (150 HMMER Homologues collected from UNIREF90 database, Max  
1339 sequence identity = 95%, Min sequence identity 35, Other parameters = default). Data  
1340 represented as heatmap of Conservation scores of each amino acid position. (f) Disopred  
1341 (<http://bioinf.cs.ucl.ac.uk/psipred/>) plot of hORC3 – heatmap representing amino acids within  
1342 intrinsically disordered regions of the protein. (g) Schematic of domain architecture of ORC3.

1343 Figure 2—Figure supplement 4.

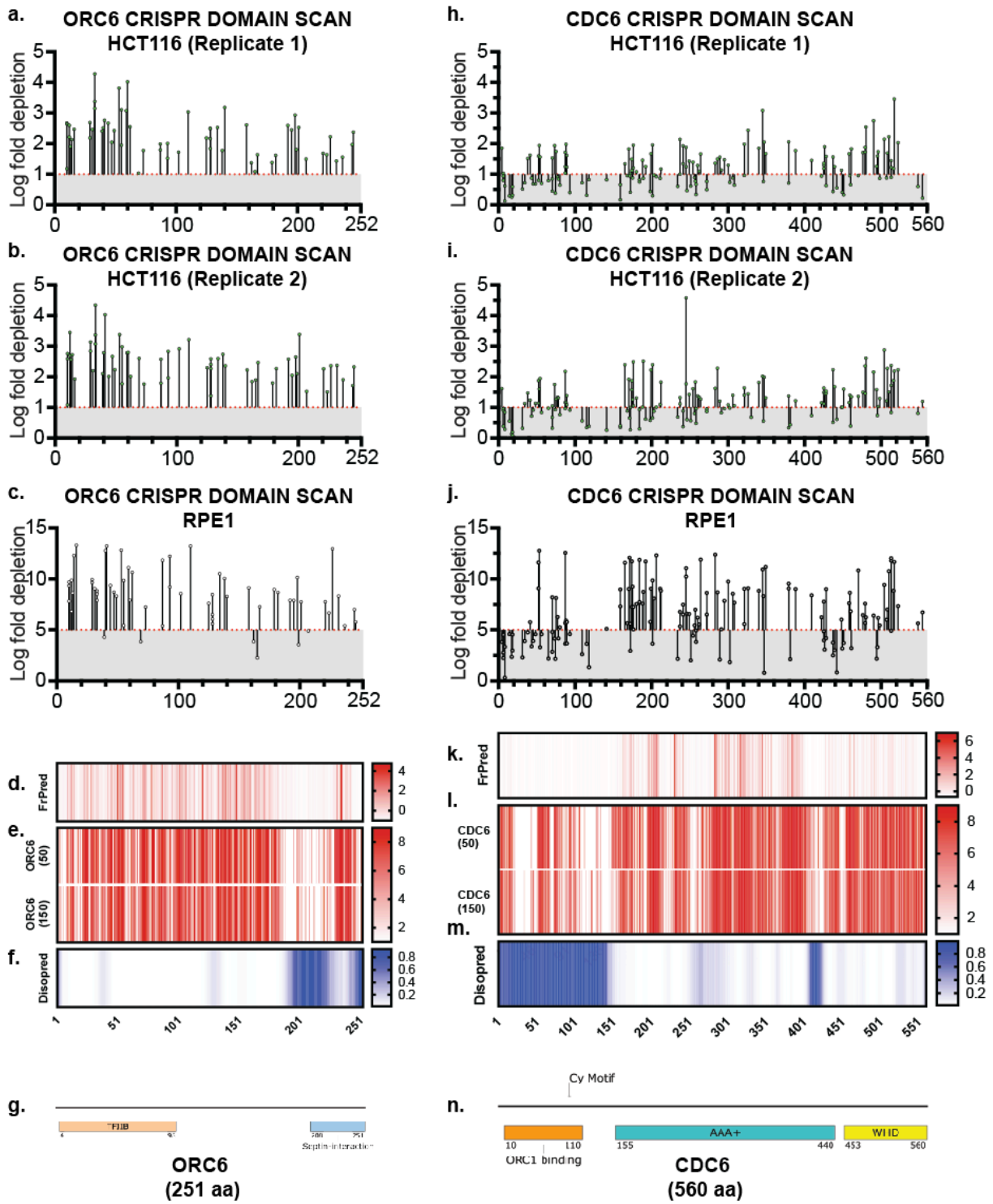


1344



1345 Figure 2—Figure supplement 4. Tiling sgRNA CRISPR screen data contd. (a) tiling sgRNA map  
1346 of ORC4 (replicate 1) in HCT116. Mapped as Log fold depletion as calculated by MaGeCK on y  
1347 axis vs the codon that is disrupted by the guide RNA on the x axis. Effect of guide RNA is  
1348 interpreted as essential if its depletion is more than 1 log fold (red dotted line). (b) tiling sgRNA  
1349 map of ORC4 for HCT116 (replicate 2). (c) tiling sgRNA map of ORC4 for RPE-1 cell line. Cutt-  
1350 off of essentiality is LFC  $\geq$  -5, indicated by red dotted line. (d) FrPred  
1351 (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC4 (NP\_859525.1) shown as gradient heat map  
1352 of conservation score vs amino acid position. (e) Consurf (<https://consurf.tau.ac.il/>) of hORC4 –  
1353 (upper) ORC4 (50) subset (50 HMMER Homologues collected from UNIREF90 database, Max  
1354 sequence identity = 95%, Min sequence identity 50, Other parameters = default), and (lower)  
1355 ORC4 (150) subset (150 HMMER Homologues collected from UNIREF90 database, Max  
1356 sequence identity = 95%, Min sequence identity 35, Other parameters = default). Data  
1357 represented as heatmap of Conservation scores of each amino acid position. (f) Disopred  
1358 (<http://bioinf.cs.ucl.ac.uk/psipred/>) plot of hORC4 – heatmap representing amino acids within  
1359 intrinsically disordered regions of the protein. (g) Schematic of domain architecture of ORC4. (h)  
1360 tiling sgRNA map of ORC5 (replicate 1) in HCT116. (i) tiling sgRNA map of ORC5 for HCT116  
1361 (replicate 2). (j) tiling sgRNA map of ORC5 for RPE-1 cell line. (k) FrPred  
1362 (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC5 (NP\_002544.1) shown as gradient heat map  
1363 of conservation score vs amino acid position. (e) Consurf (<https://consurf.tau.ac.il/>) of hORC5 –  
1364 (upper) ORC5 (50) subset and (lower) ORC5 (150) subset. Data represented as heatmap of  
1365 Conservation scores of each amino acid position. (f) Disopred (<http://bioinf.cs.ucl.ac.uk/psipred/>)  
1366 plot of hORC5 – heatmap representing amino acids within intrinsically disordered regions of the  
1367 protein. (g) Schematic of domain architecture of ORC5.  
1368

1369 Figure 2—Figure supplement 5.

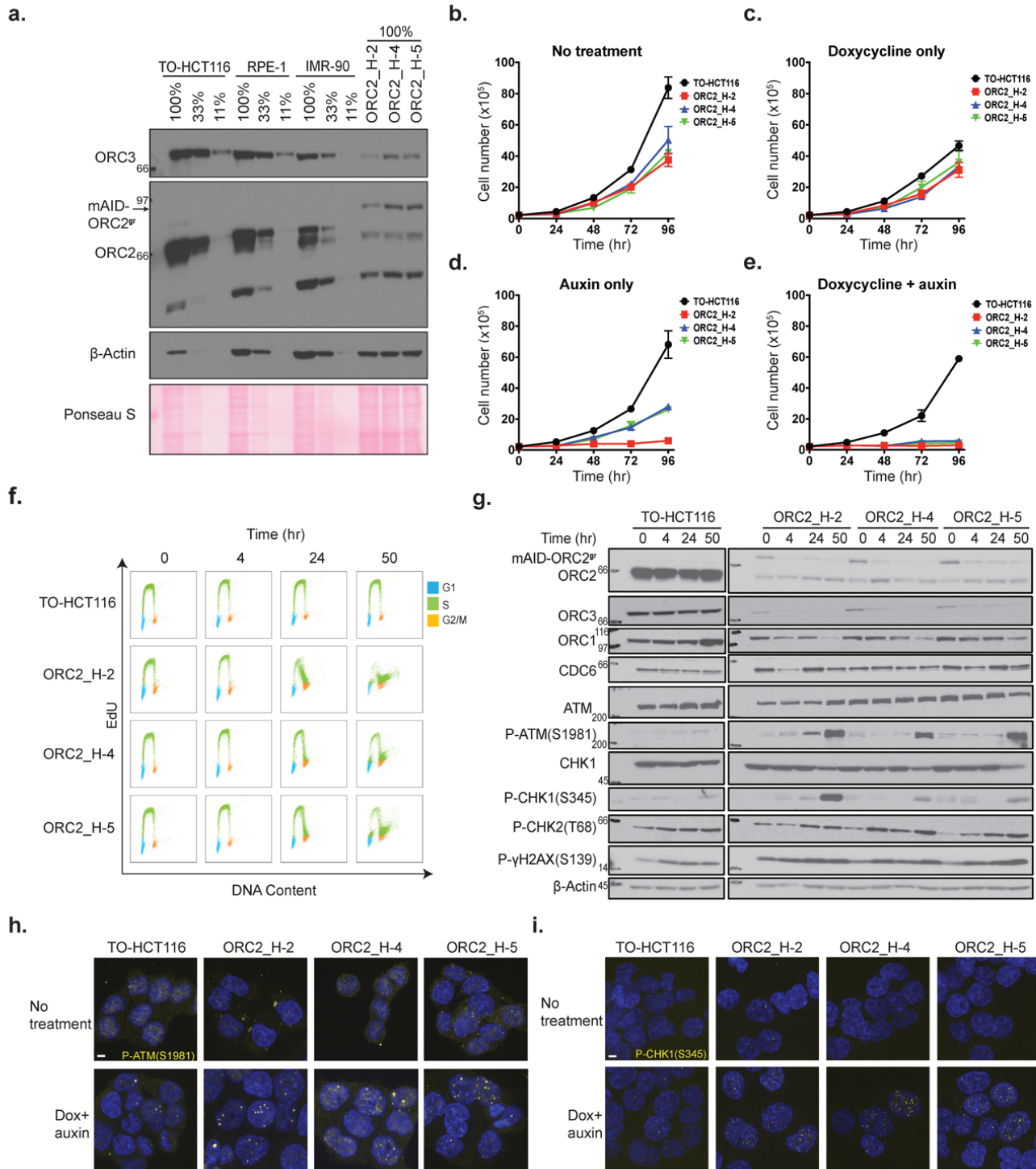


1370

1371

1372 Figure 2—Figure supplement 5. Tiling sgRNA CRISPR screen data contd. (a) Tiling sgRNA  
1373 map of ORC6 (replicate 1) in HCT116. Mapped as Log fold depletion as calculated by MaGeCK  
1374 on y axis vs the codon that is disrupted by the guide RNA on the x axis. Effect of guide RNA is  
1375 interpreted as essential if its depletion is more than 1 log fold (red dotted line). (b) Tiling sgRNA  
1376 map of ORC6 for HCT116 (replicate 2). (c) Tiling sgRNA map of ORC6 for RPE-1 cell line. Cutt-  
1377 off of essentiality is LFC  $\geq$  -5, indicated by red dotted line. (d) FrPred  
1378 (<https://toolkit.tuebingen.mpg.de/frpred>) of hORC6 (NP\_055136.1) shown as gradient heat map  
1379 of conservation score vs amino acid position. (e) Consurf (<https://consurf.tau.ac.il/>) of hORC6 –  
1380 (upper) ORC6 (50) subset and (lower) ORC6 (150) (f) Disopred  
1381 (<http://bioinf.cs.ucl.ac.uk/psipred/>) plot of hORC6-heatmap representing amino acids within  
1382 intrinsically disordered regions of the protein. (g) Schematic of domain architecture of ORC6. (h)  
1383 tiling sgRNA map of CDC6 (replicate 1) in HCT116. (i) Tiling sgRNA map of CDC6 for HCT116  
1384 (replicate 2). (j) Tiling sgRNA map of CDC6 for RPE-1 cell line. (k) FrPred  
1385 (<https://toolkit.tuebingen.mpg.de/frpred>) of hCDC6 (NP\_001245.1) shown as gradient heat map  
1386 of conservation score vs amino acid position. (e) Consurf (<https://consurf.tau.ac.il/>) of hCDC6 –  
1387 (upper) CDC6 (50) subset and (lower) ORC5 (150) subset. Data represented as heatmap of  
1388 Conservation scores of each amino acid position. (f) Disopred (<http://bioinf.cs.ucl.ac.uk/psipred/>)  
1389 plot of hCDC6 – heatmap representing amino acids within intrinsically disordered regions of the  
1390 protein. (g) Schematic of domain architecture of hCDC6.

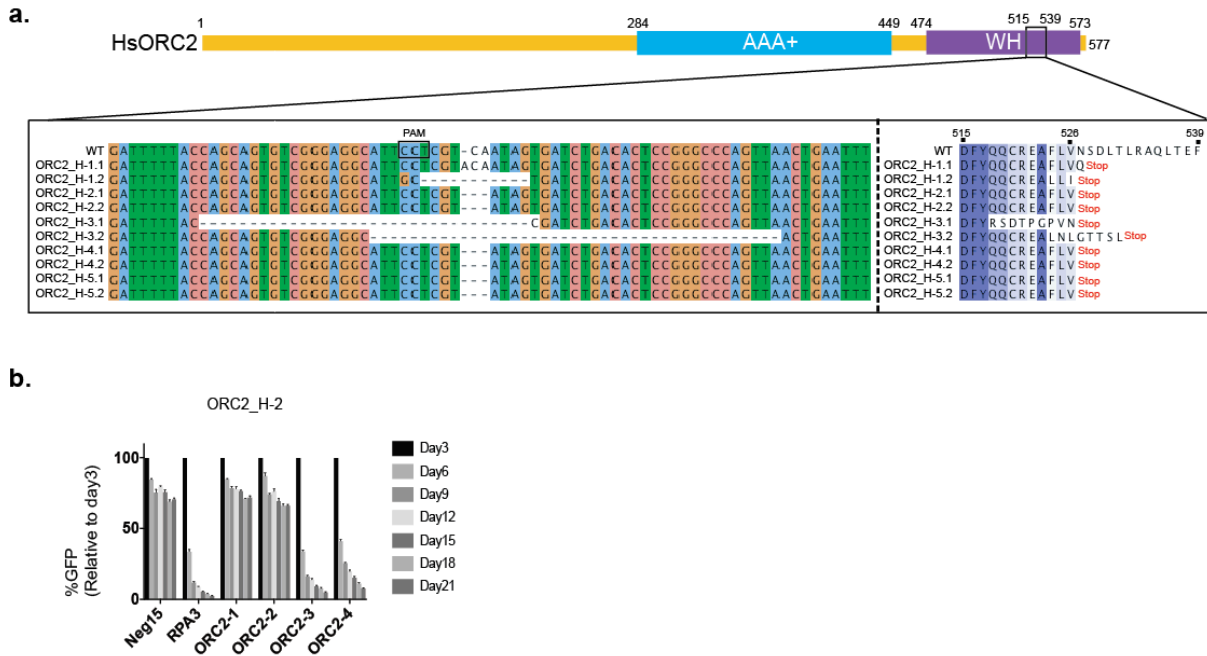
1391 Figure 3.



1392

1393 Figure 3. Characterization of CRISPR/Cas9 ORC2 knockout complemented with sgRNA  
1394 resistant ORC2<sup>gr</sup> cell lines. (a) Protein level of ORC2, mAID-ORC2<sup>gr</sup>, and ORC3 in human TO-  
1395 HCT116, hTERT-RPE1, human diploid IMR-90 cell line, and three ORC2 KO cell lines. (b-e)  
1396 Cell lines growth rates under (b) normal condition, (c) doxycycline only, (d) auxin only, and (e)  
1397 Dox+auxin containing medium, respectively. The x axis indicates hours after adding doxycycline  
1398 or auxin if any. The y axis reflects the cell number ( $\times 10^5$ ). N = 3 (biological repeats). Error bars,  
1399 mean  $\pm$  SD. (f) Cell cycle analysis of TO-HCT116, ORC2\_H-2, ORC2\_H-4, and ORC2\_H-5 cell  
1400 lines after mAID-ORC2<sup>gr</sup> depletion. Cells were treated with 0.75  $\mu$ g/ml of doxycycline for 24  
1401 hours before auxin treatment. Cells were pulse labeled with 10  $\mu$ M EdU for 2 hours before  
1402 harvesting at 0, 4, 24, and 50 hr. time points. The x axis indicates DNA content, and the y axis  
1403 refers to EdU incorporation. Overlay plots show colors in different cell cycle phases. (G1-blue;  
1404 S-green; G2/M-orange) (g) Protein expression profile of mAID-ORC2<sup>gr</sup>, ORC2, ORC1, ORC3,  
1405 CDC6, ATM, p-ATM(S1981), CHK1, p-CHK1(S345), p-CHK2(T68), and p- $\gamma$ H2AX(S139) in 4 cell  
1406 lines after dox and auxin treatment for 0, 4, 24, and 50 hr. Cells were pretreated with  
1407 doxycycline for 24 hr. before auxin treatment. Immunoblots for each protein were developed on  
1408 the same film at the same time for comparison between four cell lines. (h) Immunofluorescent  
1409 staining of p-ATM(S1981) in four cell lines with or without dox+auxin treatment. (i)  
1410 Immunofluorescent staining of p-ATM(S1981) in four cell lines with or without dox+auxin  
1411 treatment. For (h) and (i), dox+auxin treated cells were pre-treated with doxycycline for 24 hr.  
1412 before adding auxin for 48 hours. Scale bar indicated 4  $\mu$ M.  
1413

1414 Figure 3—Figure supplement 1.

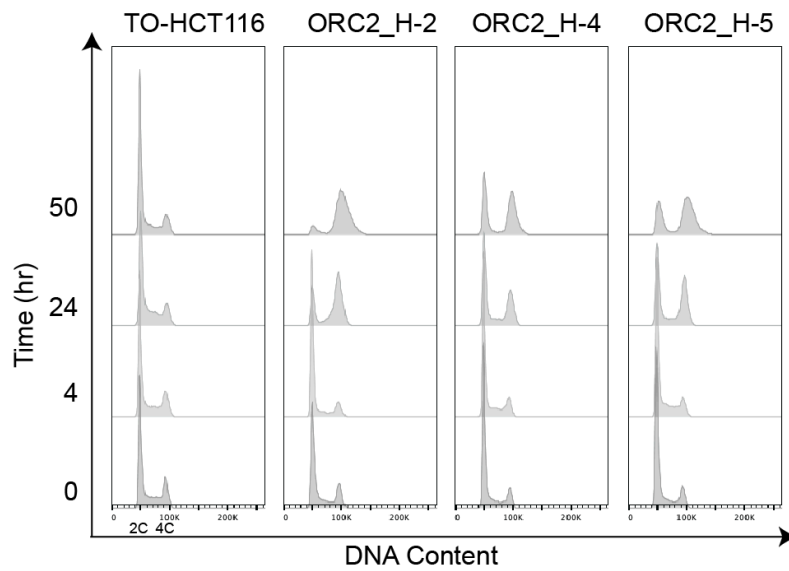


1415

1416 Figure 3—Figure supplement 1. CRISPR/Cas9 ORC2-1 sgRNA mutagenesis in five cell lines. (a)  
 1417 Nucleotide and amino acid alignments near the sgRNA targeting site in parental TO-HCT116 and  
 1418 five cloned ORC2 KO cell lines. (b) ORC2\_H-2 is resistant to ORC2\_1 and ORC2\_2 sgRNAs.  
 1419 Negative-selection time course assay that plots the percentage of GFP<sup>+</sup> cells over time  
 1420 following transduction with the indicated sgRNAs with Cas9. The GFP positive percentage was  
 1421 normalized to the Day3 measurement. N = 3. Error bars, mean ± SD.

1422

1423 Figure 3—Figure supplement 2.



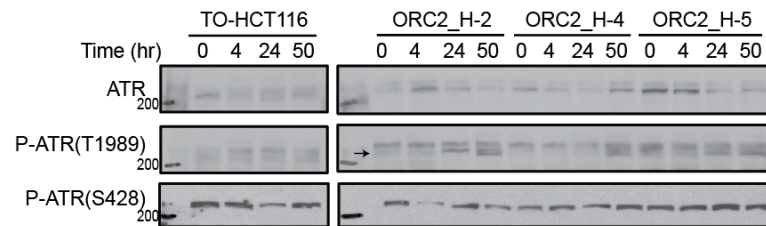
1424

1425 Figure 3—Figure supplement 2. Flow cytometry analysis of cell cycle after dox and auxin  
1426 treatment in TO-HCT116, ORC2\_H-2, ORC2\_H-4, and ORC2\_H-5 cell lines. X axis refers to DNA  
1427 content. Y axis represents the period of time (hr) for auxin treatment.

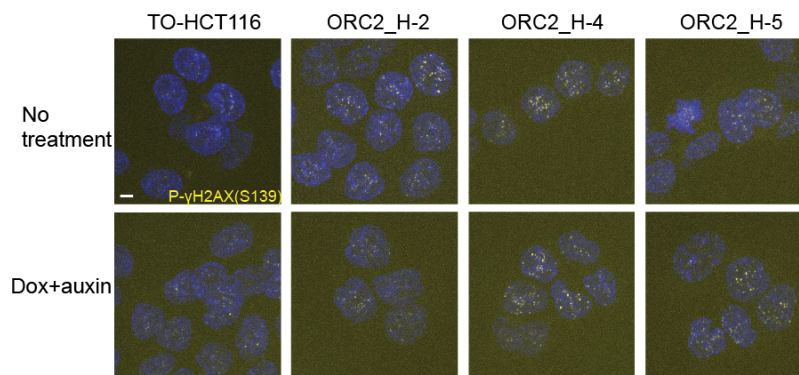
1428

1429 Figure 3—Figure supplement 3.

a.



b.



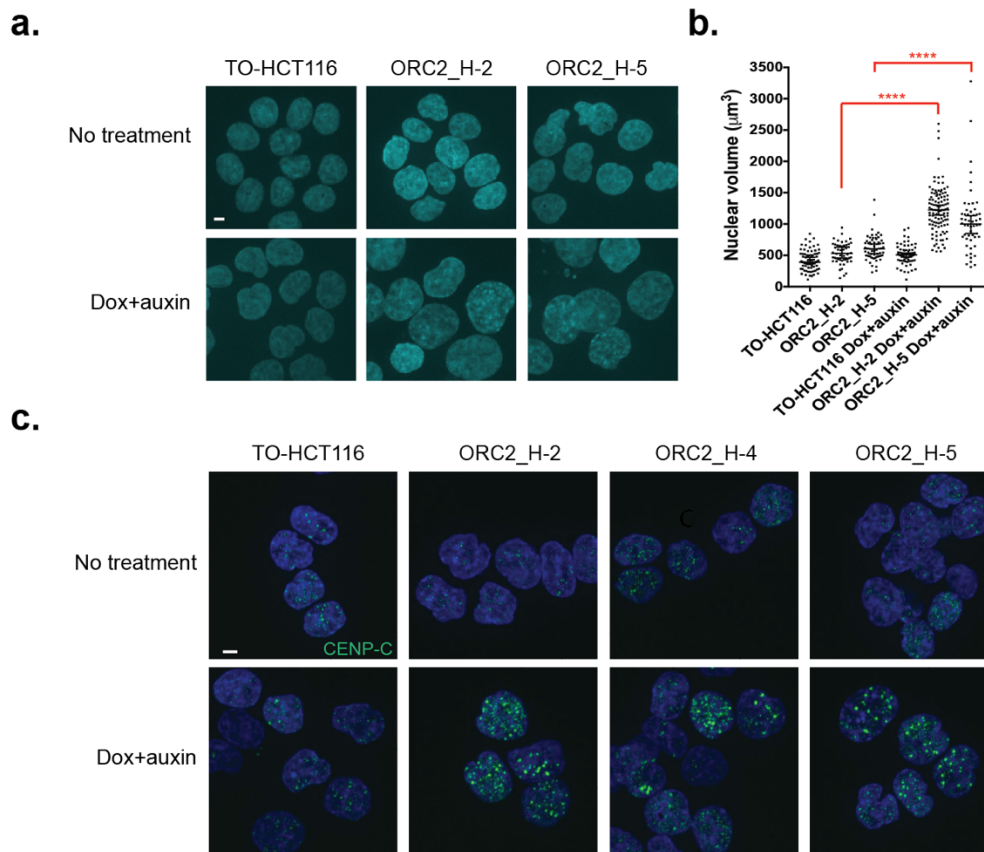
1430

1431 Figure 3—Figure supplement 3. DNA damage checkpoint was activated in ORC2\_H-2, ORC2\_H-  
1432 4, and ORC2\_H-5 cell lines. (a) ATR(T1989) was phosphorylated in ORC2\_H-2, -4, -5 cell lines  
1433 after dox and auxin treatment for 50 hours. There was no change in p-ATR(S428) level. (b)  
1434 Phosphorylation of  $\gamma$ H2AX(S139) was seen in ORC2\_H-2, -4, -5 cell lines either in the absence  
1435 or presence of dox and auxin.

1436



1437 Figure 4.

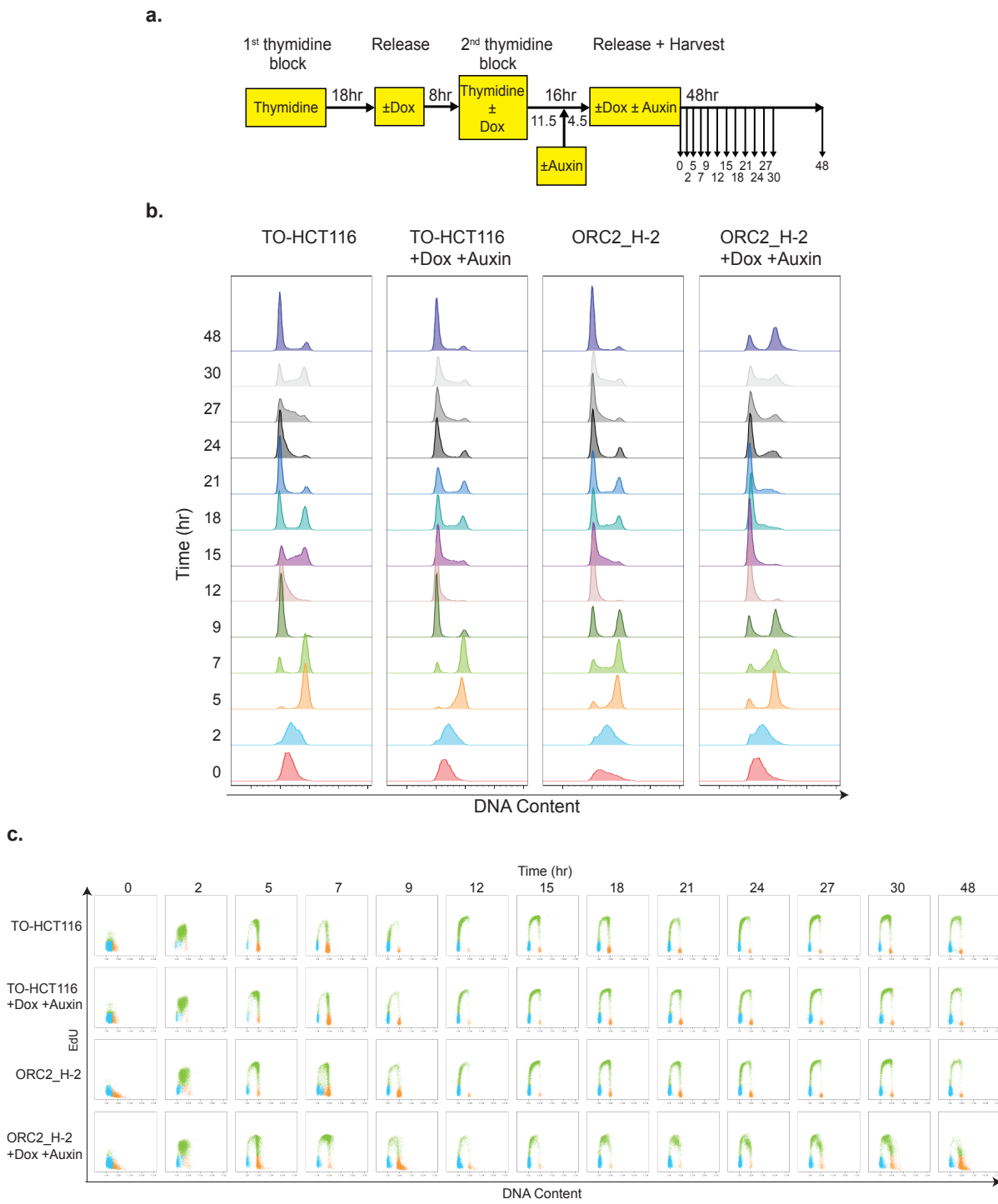


1438

1439 Figure 4. Auxin-treated ORC2\_H2, H-4, and H-5 cells had abnormal nuclear phenotypes. (a)  
1440 Nuclear morphology of TO-HCT116, ORC2\_H-2 and H-5 cells after 48 hr. auxin treatment. Scale  
1441 bar indicated 4  $\mu\text{M}$ . (b) Scatter plot illustrating the nuclear volume after 48 hr. auxin treatment.  
1442 Untreated: TO-HCT116, n=77; ORC2\_H-2, n=52; ORC2\_H-5, n=63. Dox and auxin treated: TO-  
1443 HCT116, n=66; ORC2\_H-2, n=110; ORC2\_H-5, n=54. Error bars, medium  $\pm$  95% CI. Nuclear  
1444 volume decreased significantly in both dox and auxin treated ORC2\_H-2 and H-5 cells. (Student  
1445 t-test, \*\*\*\* p<0.0001) (c) Immunofluorescent staining of CENP-C after mAID-ORC2<sup>9r</sup> depletion for  
1446 50 hrs. Scale bar indicated 4  $\mu\text{M}$ .

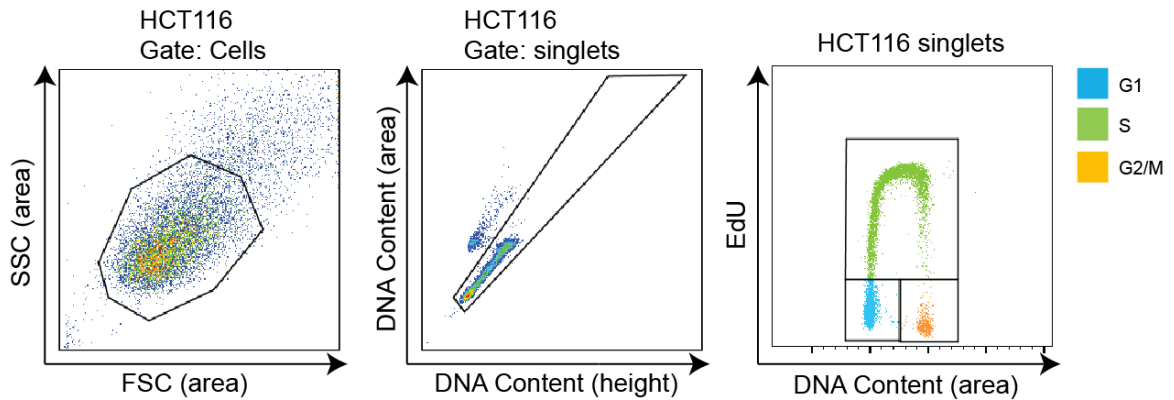
1447

1448 Figure 5.



1449 Figure 5. ORC2\_H-2 cells show abnormal cell cycle progression after mAID-ORC2<sup>gr</sup> depletion.  
1450 (a) Experimental scheme of TO-HCT116 and ORC2\_H-2 cells synchronization by a double  
1451 thymidine block. (b) Flow cytometry analysis of FxCycle™ Violet stained cells (singlets)  
1452 released from double thymidine block in indicated treatment. (c) Cell cycle analysis of TO-  
1453 HCT116 and ORC2\_H-2 cell lines released from double thymidine block in indicated treatment.  
1454 Cells were pulse labeled with 10μM EdU for 2 hours before harvesting at reported time points.  
1455 The x axis indicates DNA content, and the y axis refers to EdU incorporation. Overlay plots  
1456 show colors in different cell cycle phases. (G1-blue; S-green; G2/M-orange)  
1457

1458 Figure 5—Figure supplement 1.

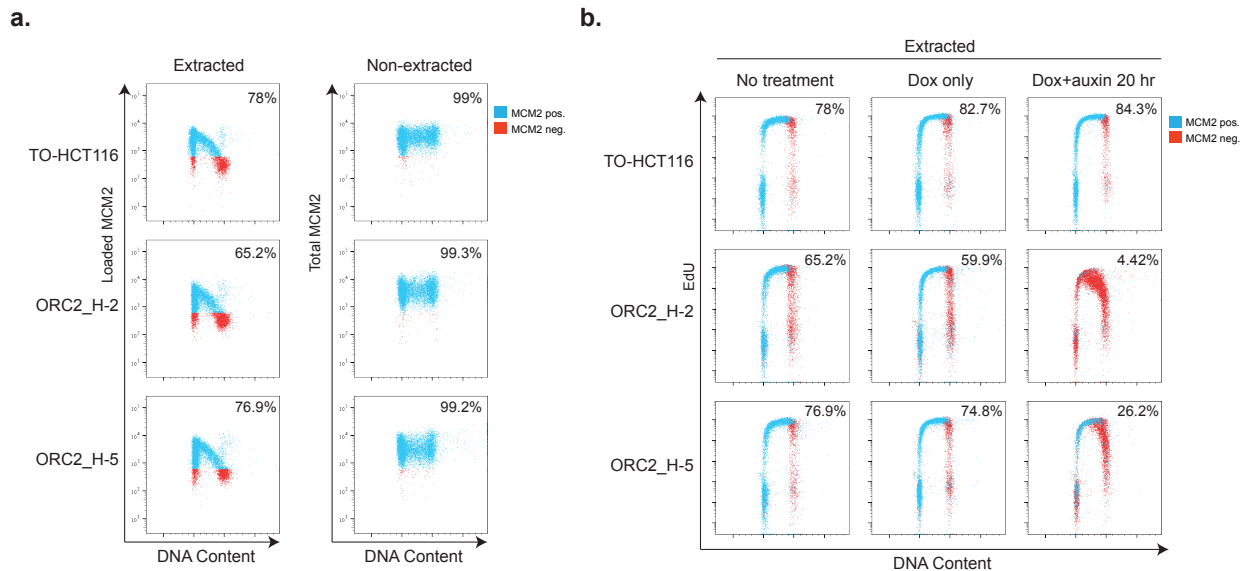


1459

1460 Figure 5—Figure supplement 1. Flow cytometry gating. Example of flow cytometry gating for  
1461 Figure 5b and c. First of all, FSC (area) vs SSC (area) gating was used to exclude the cell  
1462 debris. Next, singlets were gated on FxCycle™ Violet (DNA content) height vs area. Cell  
1463 population in G1, S, and G2/M phase was gated on the FxCycle™ Violet (DNA content) area vs  
1464 EdU plot.

1465

1466 Figure 6.

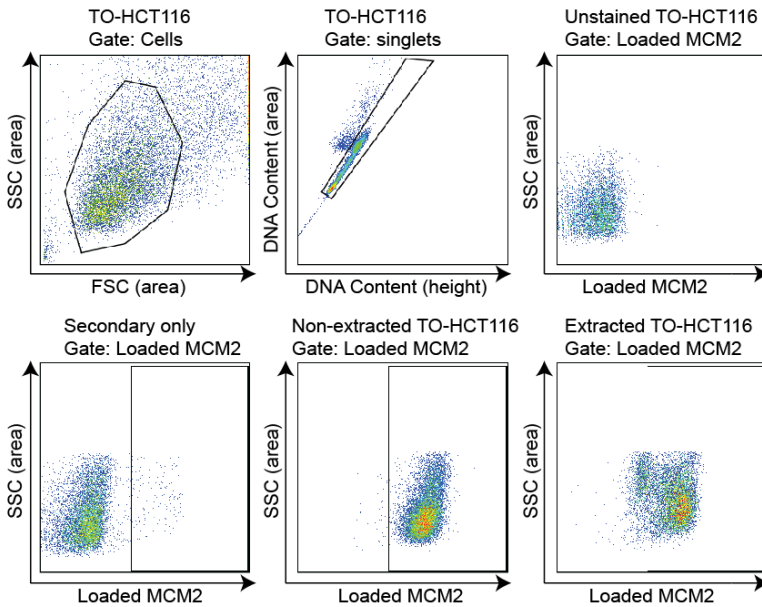


1467 Figure 6. Depletion of mAID-ORC2<sup>gr</sup> in ORC2\_H-2 and H-5 cells result in decreased DNA-loaded  
1468 MCM. (a) Flow cytometry analysis of DNA-bound MCM2 and total MCM2 in asynchronous cells.  
1469 Extracted: Cells were treated with nonionic detergent to wash off unbound MCM2 before fixation,  
1470 and then stained with anti-MCM2 antibody and Alexa Fluor 647- conjugated secondary antibody.  
1471 Non-extracted: Cells were fixed right after harvest and detected for MCM2 with the same  
1472 procedure. Blue cells are MCM2 positive, and red cells are MCM2 negative. The x axis indicates  
1473 DNA content, and the y axis refers to MCM2 level. (b) Flow cytometry measuring DNA content,  
1474 EdU incorporation, and DNA-bound MCM2 in asynchronous cells in different condition. Cells were  
1475 pulse labeled with 10  $\mu$ M EdU for 2 hours before harvesting. The x axis indicates DNA content,  
1476 and the y axis refers to EdU incorporation. MCM2 positive and negative cells are shown as blue  
1477 and red cells. Numbers at the upper right corner indicates percentage of MCM2 positive  
1478 population.

1479

1480

1481 Figure 6—Figure supplement 1.

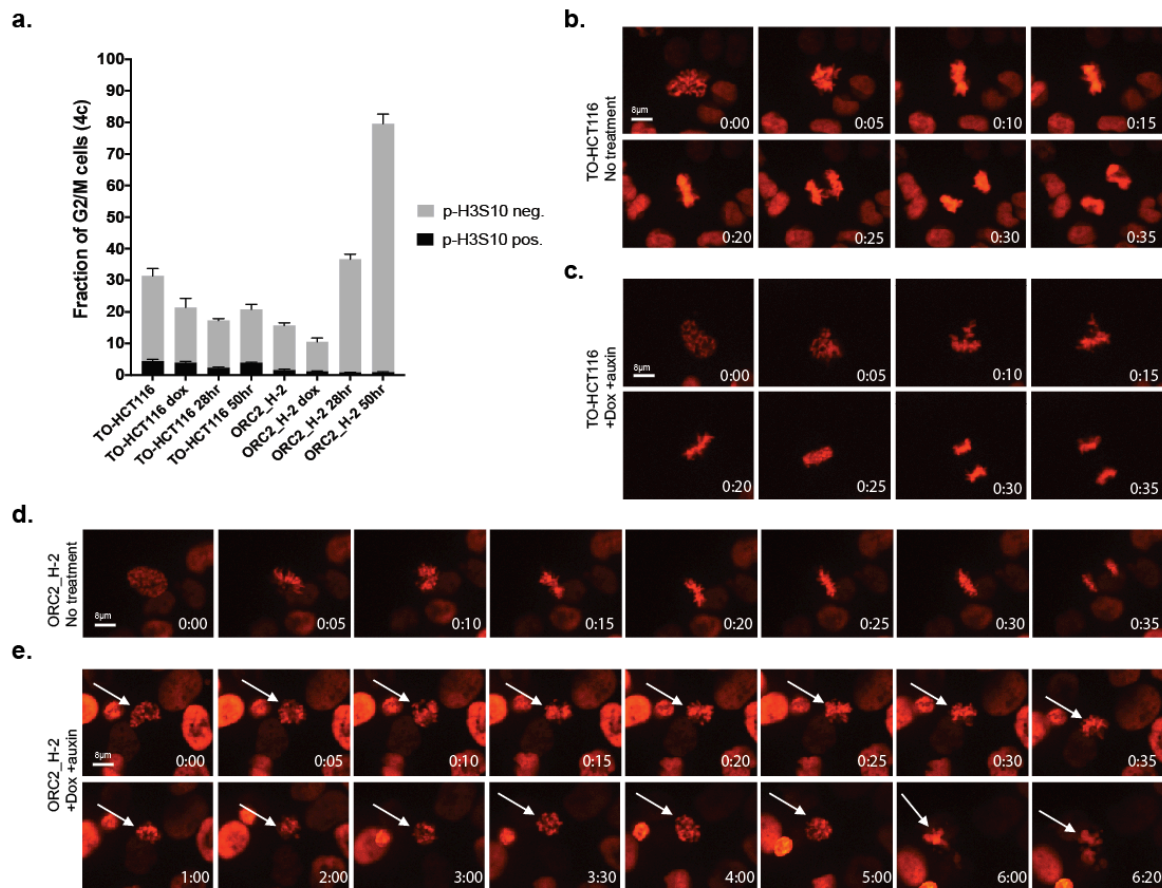


1482

1483 Figure 6—Figure supplement 1. Flow cytometry gating. Example of flow cytometry gating for  
1484 Figure 6. FSC (area) vs SSC (area) gating was used to exclude the cell debris. Next, singlets  
1485 were gated on FxCycle™ Violet (DNA content) height vs area. MCM2 positive population was  
1486 gated on the loaded MCM2 (area) vs SSC (area) of the unstained negative control. Cells stained  
1487 for secondary Donkey anti-Mouse Alexa Fluor 647 antibody only showed minimum background  
1488 for loaded MCM2.

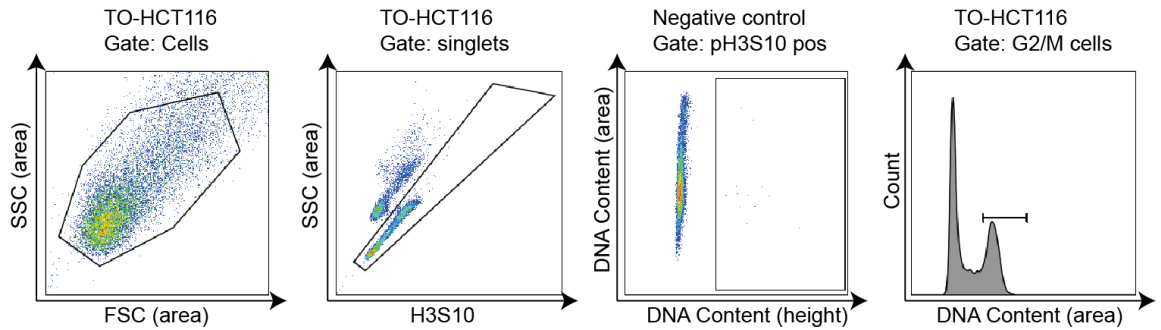
1489

1490 Figure 7.



1491  
 1492 Figure 7. ORC2\_H-2 cells have aberrant mitosis after auxin treatment. (a) Mitotic index of TO-  
 1493 HCT116 and ORC2\_H-2 G2/M cells with or without auxin. 0.75 μg/ml Doxycycline were added  
 1494 for 24 hr. before auxin treatment. Cells were harvest after 0, 28, or 50 hr. of auxin treatment  
 1495 followed by staining with anti-pH3S10 antibody for mitotic cells and FxCycle™ Violet for DNA  
 1496 content. The x axis refers to cell line and different conditions, including no treatment,  
 1497 doxycycline only, dox+auxin for 28 hr, and dox+auxin for 50 hr. The y axis is the fraction of 4c  
 1498 G2/M cells. Cell population positive or negative for p-H3S10 were shown as black or grey color.  
 1499 N=3 (biological repeats). (b-e) Time lapse imaging of TO-HCT116 and ORC2\_H-2 cell lines.  
 1500 Cells were first arrested with single thymidine block (± dox) for 24 hr. and then released into  
 1501 desired medium. Time shown in lower left corner indicates time (hour : minute) since early  
 1502 prophase. (b) Images of TO-HCT116 cells without auxin were taken at 36 hr. 33 min. after  
 1503 released from thymidine block. (c) Auxin treated TO-HCT116 cells were taken at 32 hr. 58 min.  
 1504 after released from thymidine block. (d) ORC2\_H-2 cells were taken at 41 hr. 38 min. after  
 1505 released from thymidine block. (e) Dox and auxin treated ORC2\_H-2 cell were taken at 33 hr.  
 1506 44 min. after released from thymidine block. White arrows in (e) pointed to the same cell. Scale  
 1507 bar indicated 8 μm.

1508 Figure 7—Figure supplement 1.



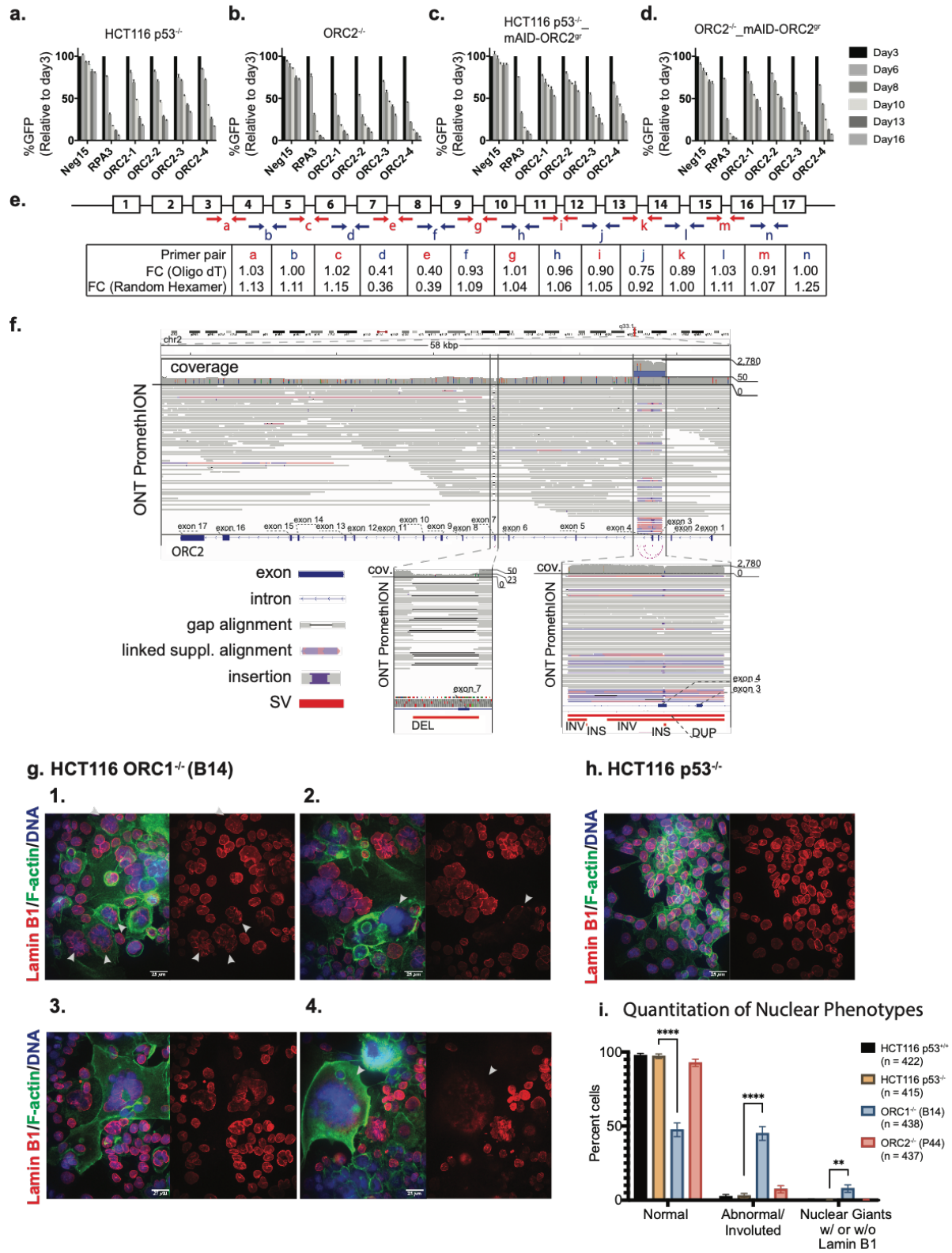
1509

1510 Figure 7—Figure supplement 1. Flow cytometry gating. Example of flow cytometry gating for  
1511 Figure 7a. FSC (area) vs SSC (area) gating was used to exclude the cell debris. Next, singlets  
1512 were gated on FxCycle™ Violet (DNA content) height vs area. Phospho-H3S10 positive  
1513 population was gated on DNA content (height) vs DNA content (area) of the unstained negative  
1514 control cells. G2/M cell population was gated on the DNA content (area) histogram.

1515



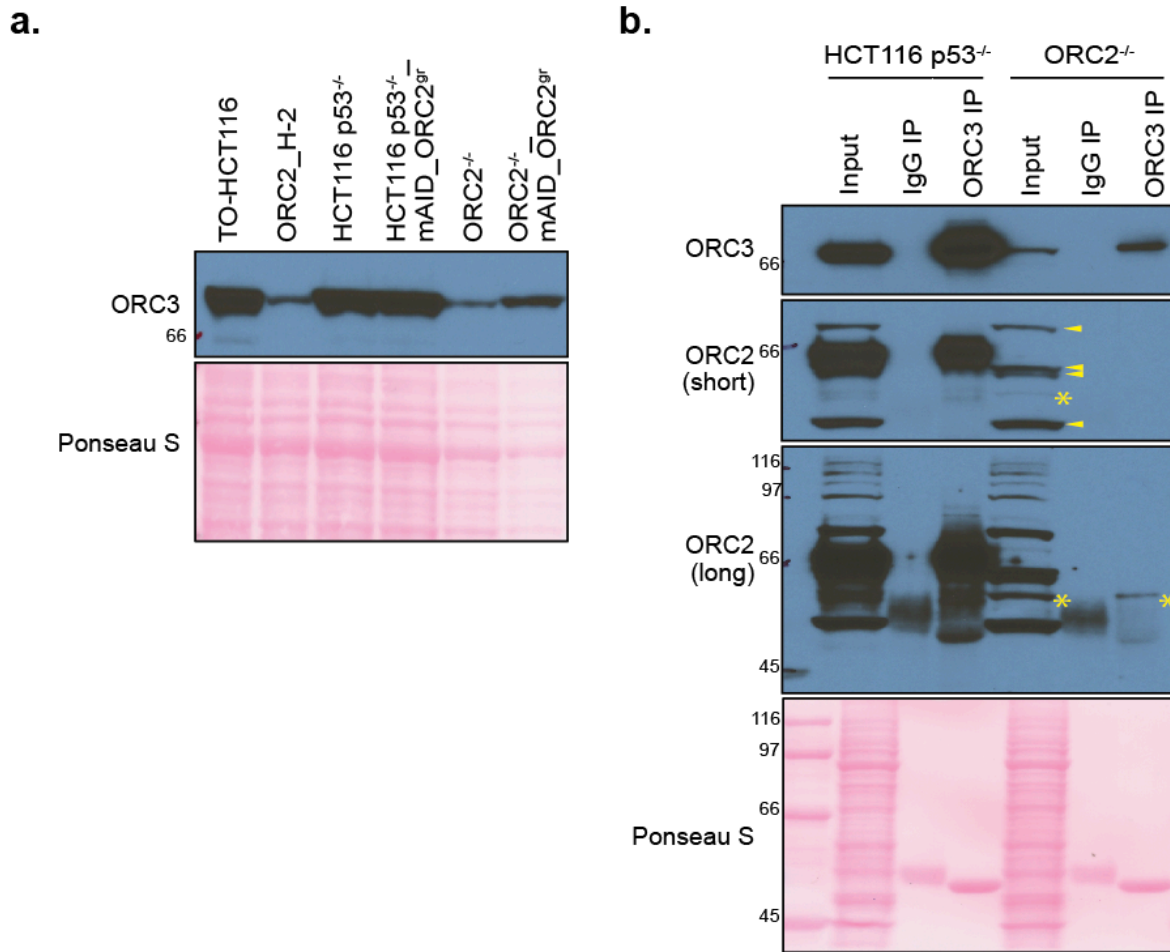
1516 Figure 8.



1517

1518 Figure 8. Characterization of previously published *ORC1*<sup>-/-</sup> and *ORC2*<sup>-/-</sup> cell lines. (a-d)  
1519 Negative-selection time course assay that plots the percentage of GFP<sup>+</sup> cells over time  
1520 following transduction with the indicated sgRNAs with Cas9. Experiments were performed in  
1521 HCT116 *p53*<sup>-/-</sup>, *ORC2*<sup>-/-</sup>, HCT116 *p53*<sup>-/-</sup>\_mAID-*ORC2*<sup>9f</sup>, and *ORC2*<sup>-/-</sup>\_mAID-*ORC2*<sup>9f</sup> cell lines.  
1522 The GFP positive percentage was normalized to the Day3 measurement. *n* = 3. Error bars,  
1523 mean ± SD. (e) Calculated fold change (FC) for each primer pairs in *ORC2*<sup>-/-</sup> cells compared  
1524 to HCT116 *p53*<sup>-/-</sup> cells. The red and blue arrows indicate each the primer pair. Two kinds of  
1525 primers, Oligo dT and Random Hexamer, were used in the reverse transcription step. Bar  
1526 diagram view is shown in Figure 8—Figure supplement 1. (f) Structural Variations (SVs) in  
1527 *ORC2* gene. Top panel shows the alignment overview of the ONT Promethion long reads over  
1528 the *ORC2* gene region with coverage track showing an average coverage of ~50x for the  
1529 majority of the gene sequence, with exceptions in regions around exon 3, 4, and 7. Zoomed-in  
1530 region with heterozygous deletion spanning the exon 7 of the *ORC2* gene, with supporting gap  
1531 aligned ONT reads and coverage track with a characteristic “dip” over the deleted segment.  
1532 Zoomed-in rearranged region around exons 3 and 4 of the *ORC2* gene, with linked supplement  
1533 read alignments pileup over the affected area supporting the spanning duplication SV, with 2  
1534 more nested inversions and 2 more nested insertion highlighted in the SV track. (g) 1-4:  
1535 Immunofluorescence of HCT116 *ORC1*<sup>-/-</sup> (B14) cell line stained with Anti-Lamin B1 antibody  
1536 (Red), Phalloidin (F-actin) (Green), Hoechst Dye (Blue). Images show either merge of all three  
1537 channels or Lamin-B1 staining of the nuclei. White arrows indicate abnormal and involuted  
1538 nuclei in image 1. White arrows also show extremely large (nuclear giants) that have lost  
1539 nuclear membrane integrity (2,4). (h) parental cell line for the *ORC1*<sup>-/-</sup> line presented as control  
1540 for quantitative and qualitative comparison. More fields of control cells HCT116 *p53* WT and *p53*  
1541 null background, HCT116 *ORC1*<sup>-/-</sup> and HCT116 *ORC2*<sup>-/-</sup> cell lines are shown in Figure 8—Figure  
1542 supplement 3 and 4. Scale bar is 25 μm (i) Quantitation of abnormal nuclei between cell lines.  
1543 Significance calculated using two-way ANOVA for multiple comparisons keeping HCT116 *p53*<sup>-/-</sup>  
1544 as control. \*\*\*\* *p* < 0.0001, \*\* *p* = 0.0028.  
1545

1546 Figure 8—Figure supplement 1.

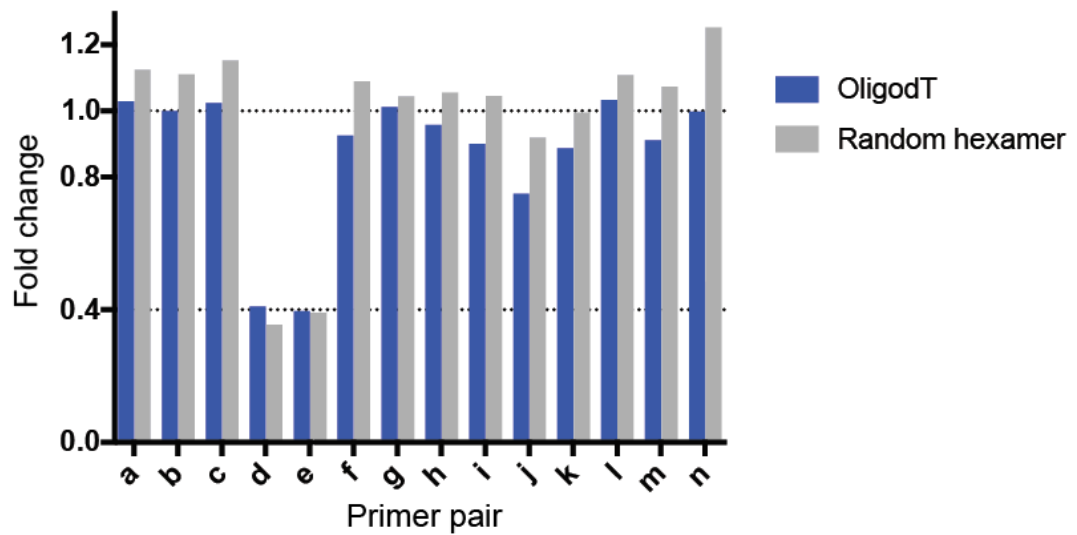


1547

1548 Figure 8—Figure supplement 1. ORC3 exists in *ORC2*<sup>-/-</sup> cell line. (a) ORC3 expression in TO-  
 1549 HCT116, *ORC2\_H-2*, HCT116 *p53*<sup>-/-</sup>, HCT116 *p53*<sup>-/-</sup>\_mAID-ORC2<sup>gr</sup>, *ORC2*<sup>-/-</sup>, and *ORC2*<sup>-/-</sup>  
 1550 \_mAID-ORC2<sup>gr</sup> cell lines. Whole cells were boiled in Laemmli buffer and followed by western  
 1551 blotting and detected with anti-ORC3 antibody. (b) ORC3 immunoprecipitation (IP) in HCT116  
 1552 *p53*<sup>-/-</sup> and *ORC2*<sup>-/-</sup> cell lines. Cells were lysed in lysis buffer and incubated with mouse IgG or  
 1553 ORC3 mouse monoclonal antibody for immunoprecipitation, followed by western blotting and  
 1554 detected with antibodies against ORC2 and ORC3. The loaded input was 2.5% and IP was  
 1555 30%. Both short and long exposure of ORC2 detection were shown here. Asterisks (\*) indicated  
 1556 the putative truncated ORC2 which was only found in *ORC2*<sup>-/-</sup> cell line. In the short exposure  
 1557 only, arrows pointed to nonspecific bands detected by the anti-ORC2 antibody.

1558

1559 Figure 8—Figure supplement 2.



1560

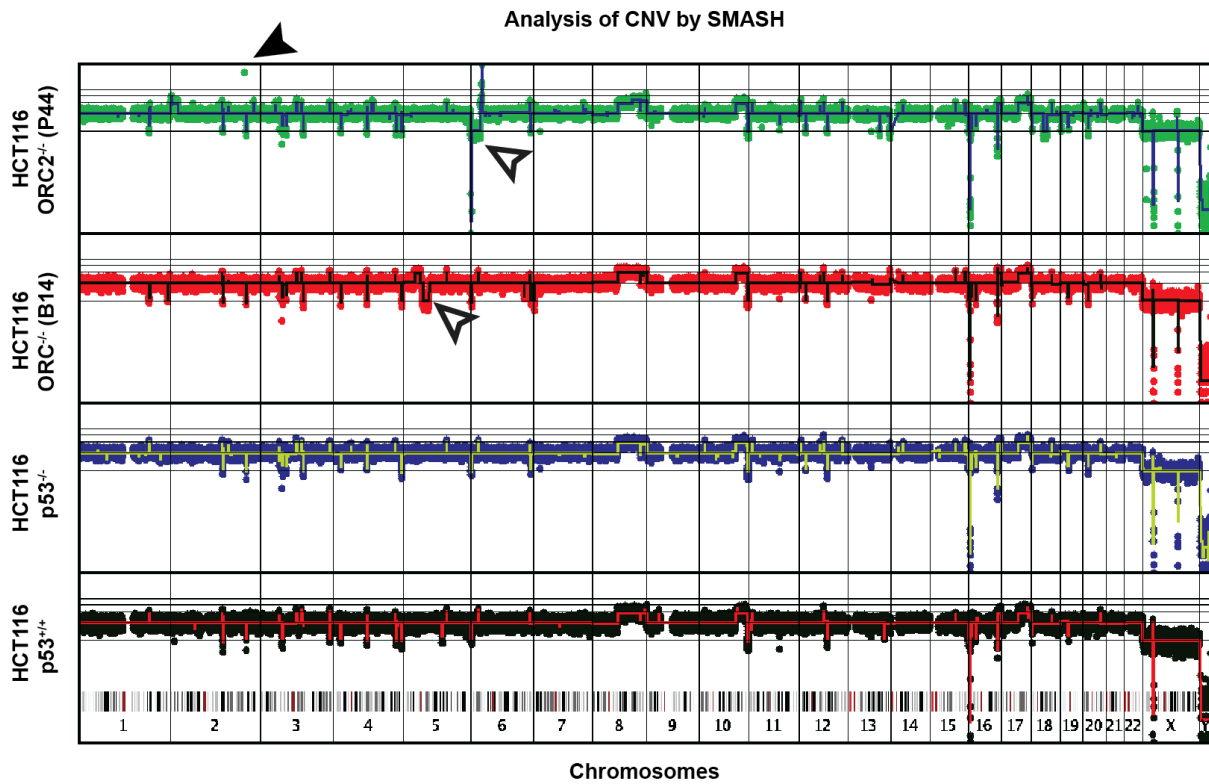
1561

1562 Figure 8—Figure supplement 2. Real time quantitative PCR fold change in a bar diagram view.  
1563 Blue, fold change of OligodT primer sample. Grey, fold change of random hexamer sample. The  
1564 exon junctions in the *ORC2* cDNA are shown as indicated in Figure 8e and the Fold change  
1565 (FC) for each primer pair in *ORC2*<sup>-/-</sup> cells compared to HCT116 *p53*<sup>-/-</sup> cells was calculated as FC  
1566 = 2<sup>(to the power of ΔΔCt)</sup>.

1567

1568

1569 Figure 8—Figure supplement 3.



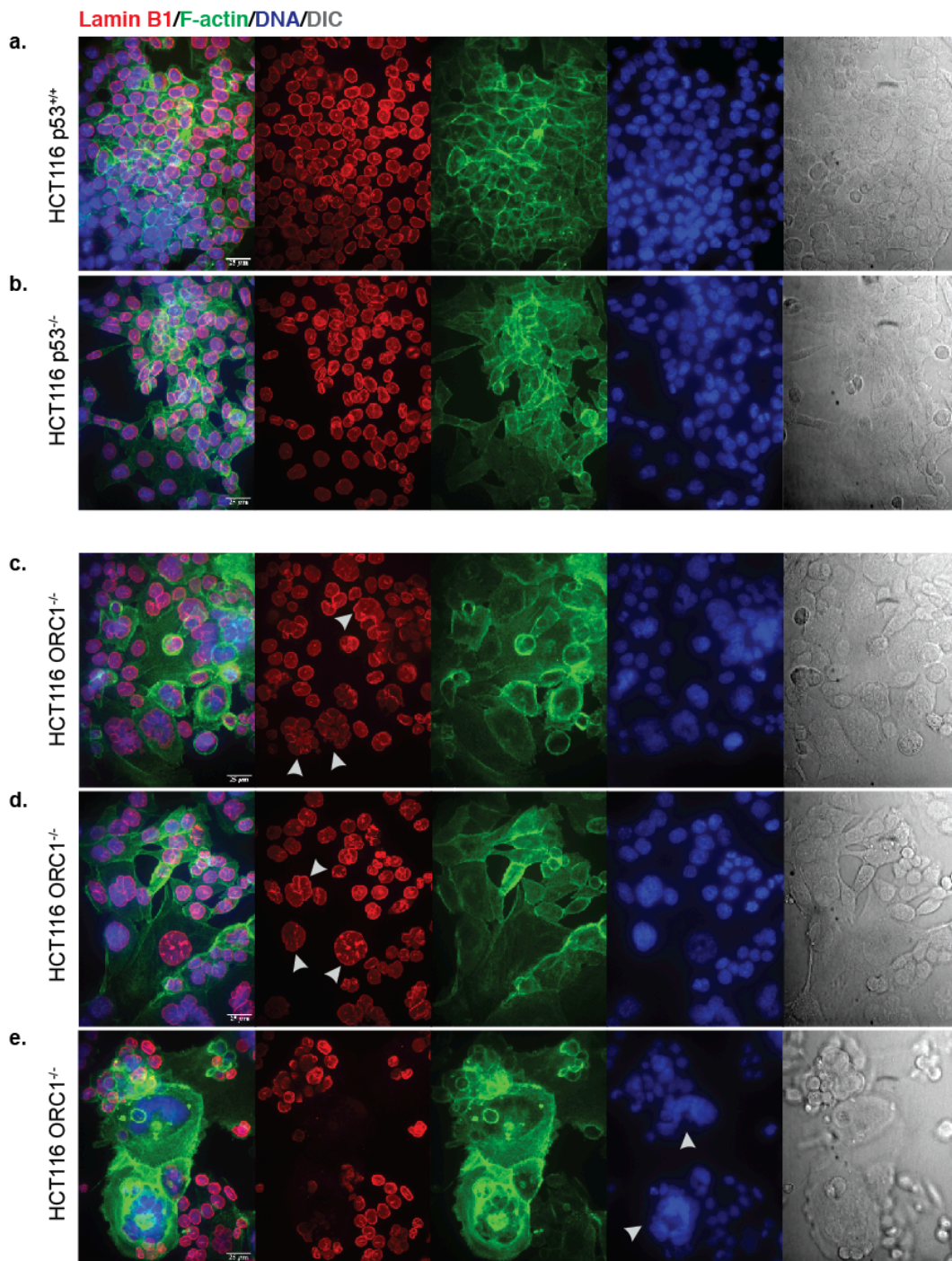
1570

1571 Figure 8—Figure supplement 3. Copy number analysis of the genomes of four cell lines using  
1572 the SMASH method. The amplification of the ORC2 gene sequences in HCT116 ORC2<sup>-/-</sup> (P44)  
1573 cells is shown by the green dot and filled arrow. The open arrows show acquired CNVs in the  
1574 ORC1<sup>-/-</sup> and ORC2<sup>-/-</sup> cells compared to the parent cells.

1575

1576

1577 Figure 8—Figure supplement 4.



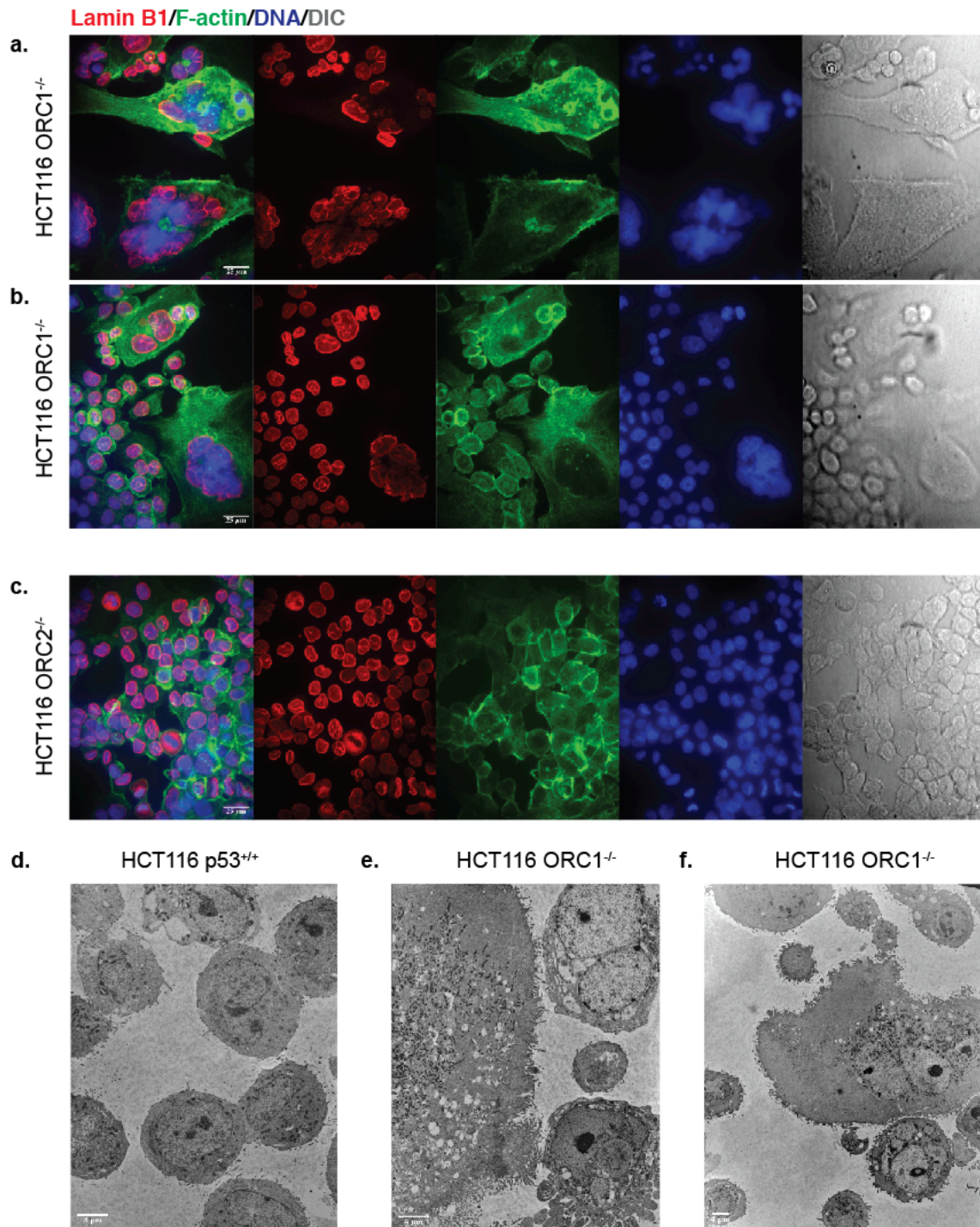
1578

1579 Figure 8—Figure supplement 4. Confocal Microscopy images of HCT116 cell lines. Imaged  
1580 acquired as z-stack of 25  $\mu\text{m}$  ( $z = 1\mu\text{m}$  each). Images presented maximum intensity projections  
1581 in the merge and average intensity projections in single channel images. Channel reference:

1582 Lamin B1 (Red), F-actin (Green), DNA (blue), DIC (grey) (a) HCT116 p53<sup>+/+</sup>. (b) HCT116 p53<sup>-/-</sup>.

1583 (c-e) HCT116 ORC1<sup>-/-</sup> (B14). Scale bar is 25  $\mu\text{m}$ .

1584 Figure 8—Figure supplement 5.



1585

1586 Figure 8—Figure supplement 5. Confocal (a-c) and Transmission electron microscopy (d-f)

1587 (TEM) images of HCT116 cell lines. Confocal: (a-b) HCT116 *ORC1*<sup>-/-</sup> (B14), (c) HCT116 *ORC2*<sup>-/-</sup>

1588 <sup>-/-</sup>. Scale bar is 25 μm. TEM: (d) HCT116 *p53*<sup>+/+</sup> cells with 2000x magnification. (e) *ORC1*<sup>-/-</sup> cells

1589 with 2000x magnification. (f) *ORC1*<sup>-/-</sup> cells with 1000x magnification. Scale bar in (d-e) is 4 μm.

1590 Supplement Table 1. The sequences of all guide RNAs used for gene editing, including those  
1591 directed to ORC1-6 and CDC6 as well as positive and negative guides for the tiling CRISPR  
1592 screens.

1593

1594 Supplement Table 2. Sequence of Barcode primers used for Next Gene Sequencing analysis in  
1595 tiling CRISPR screens.

1596

1597 Supplement Table 3. Primers used for exon analysis qPCR of the *ORC2* gene cDNAs from  
1598 various cell lines.