

1 **Transcription-dependent regulation of replication dynamics modulates genome stability**

2 **(85/90)**

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23 Replication stress is a primary threat to genome stability and has been implicated in
24 tumorigenesis^{1,2}. Common fragile sites (CFSs) are loci hypersensitive to replication stress³
25 and are hotspots for chromosomal rearrangements in cancers⁴. CFSs replicate late in S-phase³,
26 are cell-type dependent⁴⁻⁶ and nest within very large genes^{4,7-9}. The mechanisms responsible
27 for CFS instability are still discussed, notably the relative impact of transcription-replication
28 conflicts^{7,8,10} *versus* their low density in replication initiation events^{5,6}. Here we address the
29 relationships between transcription, replication, gene size and instability by manipulating the
30 transcription of three endogenous large genes, two in chicken and one in human cells.
31 Remarkably, moderate transcription destabilises large genes whereas high transcription levels
32 alleviate their instability. Replication dynamics analyses showed that transcription
33 quantitatively shapes the replication program of large genes, setting both their initiation
34 profile and their replication timing as well as regulating internal fork velocity. Noticeably,
35 high transcription levels advance the replication time of large genes from late to mid S-phase,
36 which most likely gives cells more time to complete replication before mitotic entry.
37 Transcription can therefore contribute to maintaining the integrity of some difficult-to-
38 replicate loci, challenging the dominant view that it is exclusively a threat to genome stability.
39
40 It is largely agreed that CFSs tend to remain incompletely replicated until mitosis upon
41 replication stress. Incompletely replicated regions are processed by specific endonucleases
42 promoting mitotic DNA synthesis and sister chromatid separation, eventually at the cost of
43 chromosomal rearrangements¹¹⁻¹⁵. Two main mechanisms have been suggested to explain this
44 delayed replication completion. One postulates that secondary DNA structures¹⁰ or
45 transcription-dependent replication barriers, notably R-loops^{7,8,10}, lead to fork stalling and
46 collapse. The other proposes that replication of the core of the CFSs by long-travelling forks
47 due to their paucity in initiation events is specifically delayed upon fork slowing^{5,6}. Here we

48 directly addressed the impact of transcription on the replication program and fragility of very
49 large genes. For this purpose, we manipulated the transcription of such genes in chicken
50 DT40 cells, where targeted DNA modification by homologous recombination is very
51 efficient, and in human colon carcinoma HCT116 cells using the CRISPR-Cas9 technique.
52 We first focused on *DMD*, the largest annotated avian gene (Fig. 1). *DMD* extends over 996
53 kb and is neither transcribed (Fig. 1a) nor fragile (Fig. 1b) in DT40 cells. Determination of the
54 replication timing showed that *DMD* replicates late (S4 fraction) in S-phase (Fig. 1e). We
55 activated its transcription by inserting a tetracyclin-inducible promoter (Tet-promoter) on both
56 alleles (Supplementary Fig. 1). A low level of *DMD* transcription from the Tet-promoter was
57 detected in $DMD^{\text{Tet/Tet}}$ cells grown without tetracycline (Fig. 1a). Importantly, fluorescent *in*
58 *situ* hybridization (FISH) analysis of breaks induced in *DMD* by the DNA polymerase
59 inhibitor aphidicolin revealed that the gene became fragile in these cells (Fig. 1b). Addition of
60 tetracycline increased both *DMD* mRNA levels (Fig. 1a) and *DMD* instability (Fig. 1b). Our
61 results therefore strongly support a major role of transcription in fragility, further illustrating
62 that transcription constitutes an intrinsic source of genomic instability¹⁶⁻¹⁸.
63 We then examined the replication program of *DMD* in wild-type (WT) cells and in $DMD^{\text{Tet/Tet}}$
64 cells grown in the presence of tetracycline. We used DNA-combing to visualize neo-
65 synthesized DNA along single molecules spanning *DMD*, which permitted us to measure the
66 speed of individual forks and to map initiation and termination events along the locus⁵
67 (Supplementary Figs 2, 3a and Supplementary Table 1). Initiation and termination events
68 appeared randomly distributed along *DMD* in WT cells (Fig. 1c). In striking contrast,
69 initiation events accumulated in the Tet-promoter region when *DMD* was transcribed (Fig.
70 1d). This initiation zone was followed by a large region depleted of initiation events
71 extending over the 5' part of the gene. Initiation events also clustered in a second, ≈ 300 kb-
72 large zone located in the 3' half of active *DMD*. As expected, modifications of the initiation

73 profile resulted in a redistribution of termination events. Active *DMD* remained late-
74 replicating (Fig. 1f), confirming that transcription *per se* is not sufficient to impose early
75 replication^{19,20}. Thus, our results show that transcription modulates origin distribution, in line
76 with recent analyses²¹⁻²³, and account for the frequent localization of origins near transcription
77 start sites^{24,25}. In addition, activation of *DMD* transcription specifically increased fork speed
78 along the *DMD* locus (Fig. 1g). One hypothesis is that transcription could regulate chromatin
79 permissiveness to fork progression. Consistently, chromatin factors like the histone chaperone
80 FACT (Facilitates Chromatin Transcription), which participates in the disassembly of the
81 nucleosomes upstream of the RNA polymerase, influence the rate of fork progression on *in*
82 *vitro* reconstituted chromatin templates²⁶.

83 We next modulated the transcription of the 616 kb-long *CCSER1* gene in DT40 cells (Fig. 2).
84 *CCSER1* is transcribed (Fig. 2a) and is one of the most fragile regions of the DT40 genome
85 upon aphidicolin treatment (Fig. 2b and ⁴). We enhanced *CCSER1* transcription by placing
86 both alleles of the gene under the control of the strong chicken β -actin promoter
87 (Supplementary Fig. 4). Unexpectedly, the ensuing ≈ 20 fold increase in *CCSER1* transcription
88 (Fig. 2a and Supplementary Fig. 4f) was accompanied by a dramatic reduction of aphidicolin-
89 induced *CCSER1* instability (Fig. 2b). To understand why, we compared *CCSER1* replication
90 in WT and *CCSER1* ^{β a/ β a} cells (Fig. 2c-g, Supplementary Fig. 3b and Supplementary Table 1).
91 A strong initiation zone overlapped *CCSER1* promoter region in WT cells, with some
92 additional initiation events apparently randomly distributed along the gene (Fig. 2c).
93 Importantly, *CCSER1* overexpression further favoured initiation in the promoter region at the
94 expense of the rest of the gene (Fig. 2d). In addition, termination events clustered in the third
95 quarter of *CCSER1* in *CCSER1* ^{β a/ β a} cells (Fig. 2d), suggesting that *CCSER1* ^{β a} allele was
96 mainly replicated by forks proceeding inward from the initiation zone located upstream of the
97 β -actin promoter and from a second initiation region located 3' of *CCSER1*. Together with

98 *DMD*, our results therefore demonstrate that transcription quantitatively dictates the
99 replication initiation program of active large genes, as well as the ensuing termination profile.
100 In agreement with the results obtained for *DMD*, enhancing *CCSER1* transcription also
101 significantly increased fork velocity inside the locus (Fig. 2g).
102 Remarkably, *CCSER1* overexpression advanced the replication time of the 5' part of the gene,
103 which shifted from mid (S2/S3 fractions) to early S-phase (S1/S2 fractions) (Fig. 2f). It also
104 markedly advanced the replication time of the 3' end of *CCSER1* and its flanking region (Fig.
105 2f). These data are consistent with those obtained by combing (Fig. 2d), reciprocally
106 validating each other and further supporting that transcription, at least at a sufficient level,
107 stimulates initiation both upstream and downstream of large genes. One intriguing possibility
108 is that the coordinated timing shift observed in 5' and 3' of *CCSER1* upon overexpression
109 might be mediated by long-range chromatin interactions. Advanced initiation at both ends of
110 *CCSER1* collectively led to a replication timing shift of the whole *CCSER1* locus in
111 *CCSER1*^{βa/βa} cells, mainly from late (S4 fraction) to mid-late (S3/S4 fractions) S-phase (Fig.
112 2e, f). These results suggest that the stability of *CCSER1*^{βa} alleles stems from the
113 advancement of replication timing induced by the strong increase in transcription, which
114 gives cells more time to complete replication before entering mitosis. Together with the data
115 obtained for *DMD*, they also suggest that only high transcription levels may be capable of
116 advancing the replication timing of large genes.
117 To test this hypothesis, we used the β-actin promoter in place of the Tet-promoter to increase
118 *DMD* transcription (Supplementary Fig. 5a-f). We failed to recover homozygous cells but
119 heterozygous *DMD*^{+βa} cells were alive and exhibited no growth defect (Supplementary Fig.
120 5g). We therefore compared these cells to heterozygous *DMD*^{+Tet} cells treated with
121 tetracycline (Fig. 3). *DMD* transcription was ≈10 times higher in *DMD*^{+βa} than in *DMD*^{+Tet}
122 cells (Fig. 3a and Supplementary Fig. 5h) yet, in line with *CCSER1* results, *DMD* was two-

123 fold less fragile upon aphidicolin exposure (Fig. 3b). As anticipated, we detected a strong
124 shift to earlier replication timing at the β -actin promoter insertion site, from S4 to S2/S3
125 fractions (Fig. 3c, d).

126 Finally, we sought to extend our conclusions to FRA3B, the archetypal human CFS nested in
127 the 1.5-Mb-long *FHIT* tumour suppressor gene²⁷ (Fig. 4). We used the CRISPR-Cas9 system
128 to up-regulate *FHIT* transcription in HCT116 epithelial cells, where FRA3B is the most active
129 CFS⁴ (Supplementary Fig. 6). *FHIT* promoter region was substituted on both alleles with a
130 cassette containing the strong human EF1 α promoter (Supplementary Fig. 6), which resulted
131 in a \approx 20 fold increase in *FHIT* mRNA levels (Fig. 4a). This increase elicited a massive
132 reduction of FRA3B instability upon aphidicolin treatment (Fig. 4b) accompanied by a shift to
133 earlier replication of the *FHIT* locus, from late (S3/S4 fractions) to mid S-phase (S2/S3
134 fractions) (Fig. 4c, d). Thus, data from human cells are perfectly in agreement with those from
135 DT40 cells, further substantiating that transcription directly and quantitatively influences the
136 replication time of large genes and that advancing replication timing protects active large
137 genes from fragility. Interestingly, the 5' and 3' ends of *FHIT* replicated earlier than its
138 central part in WT cells (Fig. 4c). This profile is accentuated in *FHIT*^{EF1 α /EF1 α} cells (Fig. 4d),
139 reminiscent of the replication timing profile of *CCSER1* ^{β a} alleles (Fig. 2f). These results
140 confirm that high levels of transcription favour advanced initiation of replication both 5' and
141 3' of active large genes, contributing to an earlier replication of the entire locus. Noticeably, it
142 has been reported that the insertion in the genome of DT40 cells of the strong β -actin
143 promoter upstream of the 423 bp-long blasticidin resistance gene has little impact on the
144 replication timing of the targeted chromosomal region¹⁹, suggesting that transcription-
145 dependent shift in timing may be limited to genes over a certain size.

146 We show here that the degree of instability of large genes upon replication stress relies on
147 their transcription level in an atypical way, with low transcription being more deleterious than

148 high transcription levels. Strikingly, while cause-and-effect relationships between
149 transcriptional regulation and replication timing have fuelled an intense debate²⁸, we
150 demonstrate that transcription regulates replication timing in a quantitative rather than
151 qualitative manner. Noticeably, high levels of transcription advance the replication time from
152 late to mid S-phase of all the three large genes that we studied and protect them from
153 replication stress-induced breaks, which establishes a causal role for late replication timing in
154 CFS instability. The high transcriptional-level dependent shift in replication timing most
155 likely gives cells more time to complete replication prior to the onset of mitosis. Importantly,
156 it was observed in multiple cell types that active large genes are generally transcribed at low
157 levels^{8,9} and, accordingly, tend to replicate late during S-phase⁸. Our results are therefore in
158 line with recent genome-wide analyses concluding that CFSs specifically correspond to large,
159 transcribed, late-replicating genes and also explain why not all transcribed large genes are
160 fragile^{4,29}.

161 The role of transcription in origin distribution observed for both *DMD* and *CCSER1* prompted
162 us to propose that initiation-poor regions and associated long-travelling forks observed in
163 CFSs^{3,5,6} actually originate from and reflect the transcription of the cognate large genes.
164 Whether CFS instability results from transcription-driven paucity in initiation events or
165 transcription-dependent formation of barriers impeding fork progression or both phenomena
166 remains to be determined.

167 In conclusion, while transcription is considered today as a primary threat to genome stability,
168 our study reveals complex relationships between transcription, replication and chromosome
169 fragility. The multifaceted impact of transcription on replication calls for a careful assessment
170 of the mutational consequences of transcriptional changes, especially in cancer cells where
171 up-regulation of transcription is suspected to be a major contributor of oncogene-induced
172 replication stress³⁰.

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274

Methods

275

276 **Cell culture.** DT40 cells were grown in RPMI 1640 GlutaMAX medium (Gibco) with 10%
277 fetal bovine serum (Biowest), 2% chicken serum (Gibco) and 0.1 mM β -mercaptoethanol
278 (Millipore). A tetracycline-free fetal bovine serum (Biowest) was used when appropriate.
279 HCT116 human colon carcinoma cells were cultured in McCOY'S 5A medium (Gibco) with
280 10% fetal calf serum and 20 mM L-glutamine (Gibco) except for replication timing
281 experiments for which they were cultured in DMEM (4.5 g/L D-Glucose, L-glutamine)
282 (Gibco) with 10% fetal calf serum and 1 mM sodium pyruvate. All media were supplemented
283 with 100 U.mL⁻¹ penicillin and 100 μ g.mL⁻¹ streptomycin (Gibco). Cells were grown at 37°C,
284 20% O₂, 5% CO₂. DT40 and HCT116 cells and derivative clones were routinely confirmed to
285 be negative for mycoplasma contamination.

286

287 **Tet-ON inducible system.** A Tet-ON system based on the T-REx System (Invitrogen) was
288 used to induce the transcription of *DMD* upon addition of the tetracycline antibiotic. Briefly,
289 the tetracycline-inducible promoter (Tet-promoter) used consists of a CMV promoter into
290 which 2 copies of the Tet-operator 2 (TetO) sequence have been inserted in tandem. In the
291 absence of tetracycline, transcription is repressed by the high affinity binding of the Tet-
292 repressor (TetR) to the TetO. Addition of tetracycline results in the release of TetO by the
293 TetR and derepression of the promoter. The Tet-promoter was amplified from a pcDNA4/TO
294 vector (Invitrogen). The TetR was expressed from a pcDNA6/TR vector (Invitrogen).

295

296 **Constructs.** Targeting constructs for homologous recombination in DT40 cells were created
297 from LoxP vectors¹ using standard molecular biology and cloning protocols. \approx 2 kb-long 5'
298 and 3' homology regions were amplified from DT40 genomic DNA with primers listed in

299 Supplementary Table 2. The chicken β -actin promoter was synthesized by GeneScript. All
300 constructs were checked for mutations by sequencing. Guide RNAs targeting *FHIT* promoter
301 (gRNA1: GCCAAATGCCATGTGGGTGC; gRNA2: TCAATTTAGATTTCGGCTTC) were
302 designed using the gRNA design tool from DNA2.0. gRNA/Cas9 plasmid containing
303 sequences for the two gRNAs and wtCas9 was synthesized by DNA2.0. \approx 1 kb-long 5' and 3'
304 homology regions flanking the CRISPR/Cas9 cutting sites were amplified from RP11-
305 137N22 BAC with primers listed in Supplementary Table 2 and cloned into the HR710PA-1
306 plasmid (System Biosciences) on each side of the EF1 α promoter-hygromycin selection
307 cassette. Detailed cloning procedures are available upon request.

308

309 **Transfection and genotyping.** 10^7 exponentially growing DT40 cells were electroporated
310 with 35 μ g of the linearized construct using a Biorad electroporator set at 25 μ F and 550 V for
311 targeted integrations, 960 μ F and 250 V for the random insertion of the pcDNA6/TR vector.
312 Recombinant clones were selected with either 21.75 μ g.mL⁻¹ blasticidin, 0.5 μ g.mL⁻¹
313 puromycin or 450 μ g.mL⁻¹ zeocin, identified by PCR using LA Taq DNA Polymerase
314 (Takara) and confirmed by Southern blot. At least two positive clones were randomly selected
315 and amplified for further studies. Primers used for genotyping are listed in Supplementary
316 Table 2. Probes used for Southern blot were amplified from the targeting constructs using
317 primers listed in Supplementary Table 2. The 2-log DNA ladder (NEB) was used as
318 molecular-weight size marker for agarose gel electrophoresis except in Supplementary Fig. 1f
319 where the GeneRuler 1 kb Plus DNA ladder (ThermoFisher Scientific) was used.

320 For transient transfection, 3×10^6 exponentially growing DT40 cells were transfected with 5 μ g
321 of a reporter plasmid expressing the luciferase gene under the control of the Tet-promoter
322 using the Amaxa Nucleofector system (T solution, program B-023).

323 HCT116 cells were co-transfected with 1 μg gRNA/Cas9 plasmid and 1 μg HR710PA-1-*FHIT*
324 plasmid using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's
325 instructions. Recombinant cells were selected with 200 $\mu\text{g}\cdot\text{mL}^{-1}$ hygromycin B (Invitrogen)
326 48 h post-transfection. 2 μM ganciclovir was added to the culture medium 7 days post-
327 transfection to counter-select cells containing randomly integrated HR710PA-1-*FHIT*
328 plasmid. After a further 12 days, single clones were selected and amplified. Positive clones
329 were identified by PCR and confirmed by sequencing.

330

331 **Luciferase reporter assay.** DT40 clonal cell lines expressing the Tet-repressor were tested
332 for their ability to induce transcription of the luciferase reporter gene upon addition of
333 tetracycline, which was quantified by the light emitted during an enzymatic reaction of
334 bioluminescence using the Dual-Glo Luciferase Assay System (Promega) according to the
335 manufacturer's instructions. Luminescence was measured using a luminometer 24 h after
336 addition of 1 $\mu\text{g}\cdot\text{mL}^{-1}$ tetracycline (SIGMA) to the culture medium.

337

338 **Tetracycline-induced transcriptional activation of *DMD*.** Transcriptional activation of
339 *DMD* was achieved with a 24 h treatment of $\text{DMD}^{\text{Tet/Tet}}$ and $\text{DMD}^{+/Tet}$ cells with 1 $\mu\text{g}\cdot\text{mL}^{-1}$
340 tetracycline. A low transcriptional background from the Tet-promoter is detected even
341 without tetracycline (Figs 1a and 3a), as already observed².

342

343 **Floxed cassette excision.** The DT40 cell line used in this study contains a stably integrated
344 MerCreMer plasmid¹. The protein is inactive in the absence of 4-hydroxytamoxifen (4-OHT)
345 due to the hormone binding domains of the Mutated estrogen receptor (Mer) fused either side
346 of the Cre recombinase. For excision of floxed cassettes, cells were cultured for 24 h with 0.5
347 μM 4-OHT then distributed into 96-well flat-bottom microtiter plates at a concentration of 1,

348 3 and 30 viable cells per well. Isolated colonies were tested for cassette excision by loss of
349 antibiotic resistance and PCR.

350

351 **mRNA quantification.** DT40 cells were collected during their exponential growth phase.
352 Total RNA was extracted using miRNeasy Mini Kit (Qiagen) according to the manufacturer's
353 instructions and treated with DNase (Roche). 1 µg of total RNA was reverse-transcribed using
354 the iScript cDNA Synthesis Kit (Biorad). Real-time quantitative PCR reactions were set up
355 using GoTaq qPCR Master Mix (Promega) and run on a LightCycler 480 II (Roche). Each
356 reaction was performed in triplicate. DNA contamination was quantified in reverse
357 transcriptase free reactions. Primer sequences are listed in Supplementary Table 3. Each
358 primer pair spanned one intron to avoid unwanted amplification of genomic DNA. The
359 efficiency of each primer pair was tested by performing qPCR with the same protocol on
360 increasing dilutions of cDNA (from 1/10 to 1/10,000 dilutions) and calculated using the
361 coefficient of amplification. The efficiency of each pair being similar, it is possible to directly
362 compare the amount of each amplification product. mRNA levels were quantified relative to
363 β -actin (*ACTB*) mRNA. For DMD^{Tet/Tet}, DMD^{+Tet} and DMD^{+/ β a} cells, mRNA levels were
364 quantified before the excision of the floxed selection cassettes. For each construct, mRNA
365 quantification experiments were performed using at least two different clonal cell lines.

366 Total RNA from HCT116 cells was isolated using RNeasy Mini Kit (Qiagen) according to the
367 manufacturer's instructions. cDNA was synthesized with Maxima First Strand cDNA
368 Synthesis Kit for RT-qPCR (ThermoFisher Scientific). Real-time quantitative PCR was
369 performed using Luminaris Color HiGreen qPCR Master Mix, low ROX (ThermoFisher
370 Scientific) on a 7500 Real-Time PCR System (Applied Biosystems). Primer sequences are
371 listed in Supplementary Table 3. *FHIT* mRNA levels were quantified relative to RNA
372 polymerase II subunit F (*POLR2F*), ribosomal protein L11 (*RPL11*) and cyclophilin B

373 (*PPIB*) mRNA. mRNA quantification experiments were performed using one FHIT^{EF1 α /EF1 α}
374 clonal cell line.

375

376 **Quantification of 5-Ethynyl uridine (EU) incorporation into nascent RNAs.** 10⁷
377 exponentially growing DT40 cells were treated for 1 h with 0.5 mM EU. EU-labelled RNA
378 was captured using the Click-iT Nascent RNA Capture kit (Invitrogen) according to the
379 manufacturer's instructions. Briefly, 1 μ g of total RNA was biotinylated thanks to the click
380 reaction between EU and azide-modified biotin, then purified using streptavidin magnetic
381 beads. Nascent RNAs were reverse-transcribed on the beads using Superscript kit
382 (Invitrogen). Real-time quantitative PCR reactions were performed as described above for
383 DT40 samples using intra-intronic primers listed in Supplementary Table 3. All primers had
384 similar efficiencies. RNA levels were calculated relative to β -actin mRNA quantities.

385

386 **Fluorescent *in situ* hybridization (FISH) on metaphase chromosomes.** DT40 and HCT116
387 cells were grown for 16 h with 0.6 and 0.15 μ M aphidicolin, respectively. Metaphase spreads
388 were prepared according to standard cytogenetic procedures after a 3 h treatment with 0.1
389 μ g.mL⁻¹ colcemid for DT40 cells and a 2 h treatment with 100 nM nocodazol for HCT116
390 cells. For untreated DT40 cells, metaphase spreads were prepared after a 1.5 h treatment with
391 0.1 μ g.mL⁻¹ colcemid.

392 For DT40 FISH analyses, probes correspond to adjacent 5 kb-long PCR products spread over
393 \approx 50 kb delimiting *DMD*, *CCSER1* or *PARK2* genes (see probe coordinates in Supplementary
394 Table 4); probes were amplified from DT40 genomic DNA with primers listed in
395 Supplementary Table 4. Probes were labelled either with biotin using the BioPrime DNA
396 labelling system (Invitrogen) or with digoxigenin using the DIG DNA labelling mix (Roche)
397 and subsequently purified on Illustra ProbeQuant G-50 Micro Columns (GE Healthcare).

398 FISH was performed essentially as described³ without the proteinase K step and using 75 ng
399 of each probe. Chicken Hybloc DNA (Amplitech) was used as a repetitive sequence
400 competitor DNA. Immunodetection was performed by successive incubations in the following
401 reagents: (i) for biotinylated probes, (1) Alexa Fluor 555-conjugated streptavidin (Invitrogen),
402 (2) biotin-conjugated rabbit anti-streptavidin (Rockland Immunochemicals), (3) Alexa Fluor
403 555-conjugated streptavidin (Invitrogen) (ii) for digoxigenin-labelled probes, (1) FITC-
404 conjugated mouse anti-digoxin (Jackson ImmunoResearch), (2) Alexa Fluor 488-conjugated
405 goat anti-mouse (Invitrogen). Chromosomes were counterstained with 49,6-diamidino-2-
406 phenylindole (DAPI) (Vectashield mounting medium for fluorescence with DAPI; Vector
407 Laboratories) and metaphases were observed by fluorescence microscopy. Aphidicolin-
408 induced breaks at the 679.5 kb-long *PARK2* gene were used as a control to demonstrate that
409 modulation of *DMD* or *CCSER1* transcription specifically impacts the fragility of those genes
410 and that the limited perturbations of cell physiology observed in *CCSER1*^{βa/βa} cells
411 (Supplementary Fig. 4d, e) do not perturb break induction by aphidicolin. For each construct,
412 FISH experiments were performed using at least two different clonal cell lines.

413 FISH analysis of FRA3B fragility in HCT116 cells was performed using 50 ng of biotinylated
414 RP11-641C1, RP11-32J15 and RP11-147N17 BACs selected from the human genome project
415 RP11 library. Immunodetection was performed by successive incubations in Alexa Fluor 488-
416 conjugated streptavidin (Invitrogen) and biotin-conjugated rabbit anti-streptavidin (Rockland
417 Immunochemicals). 15 μg Cot-1 DNA (Invitrogen) were used as a repetitive sequence
418 competitor DNA. FISH experiments were performed using one FHIT^{EF1α/EF1α} clonal cell line.

419
420 **Bivariate Fluorescence-Activated Cell Sorting (FACS) analysis.** Cell cycle analyses were
421 performed as follows. Exponentially growing DT40 cells were pulse-labelled for 15 min with
422 30 μM bromodeoxyuridine (BrdU) and fixed in ethanol. After partial denaturation of DNA

423 following HCl/pepsin treatment, immunodetection was performed by incubations with rat
424 anti-BrdU (Bio-Rad, formerly AbD Serotec) then Alexa Fluor 488-conjugated chicken anti-
425 rat (Invitrogen). DNA was counterstained with propidium iodide in the presence of 25 $\mu\text{g.mL}^{-1}$
426 RNase. Samples were analyzed using a BD Biosciences LSRII flow cytometer with BD
427 FACSDiva software. Data were processed using FlowJo v8.7.3. Gating strategy is illustrated
428 in Supplementary Fig. 7.

429

430 **Cell growth and doubling time.** The cumulative growth curves of the DT40 cell lines used
431 in this study were determined after normalization for dilution at each subculture. Doubling
432 times were estimated based on the growth curves. Indeed, for exponentially growing cells, the
433 final number of cells (N) is given by the formula $N = \text{initial number of cells} \times 2^{\text{number of doublings}}$,
434 where the number of doublings corresponds to the duration of culture divided by the doubling
435 time (T); it follows that $T = [\text{duration of culture} \times \log(2)] / [\log(\text{final number of cells}) - \log(\text{initial}$
436 $\text{number of cells})]$.

437

438 **Replication timing analyses.** Timing analyses in DT40 cells were made as previously
439 described⁴. Briefly, exponentially growing DT40 cells were pulse-labelled for 1 h with 3 mM
440 BrdU, fixed in ethanol and stored at -20°C overnight. Fixed cells were re-suspended in 1X
441 PBS with 50 $\mu\text{g.mL}^{-1}$ propidium iodide and 1 mg.mL^{-1} RNase, and incubated for 30 min at
442 room temperature. Cells were sorted by flow cytometry based on their nuclear content using a
443 BD Biosciences INFLUX cell sorter with the BD FACSTTM Software software (v 1.0.0.650).
444 S-phase was divided into four fractions from early to late S-phase named S1 to S4. 50,000
445 cells were sorted in each fraction. DNA was extracted by phenol/chloroform, sonicated to
446 obtain fragments between 500-1000 bp in size, and BrdU-labelled DNA was
447 immunoprecipitated using mouse anti-BrdU antibody (BD Biosciences). Real-time

448 quantitative PCR was performed using the Roche LightCycler 2.0 detection system with the
449 Absolute QPCR-SYBR Green mix (ThermoFisher Scientific). For each reaction,
450 amplification of the purified BrdU-labelled DNA was performed in duplicate. As
451 mitochondrial DNA replicates throughout the cell cycle and should be equally represented in
452 every fraction, the amount of immunoprecipitated DNA in each S-phase fraction was
453 normalized by the abundance of mitochondrial DNA measured using a specific primer pair
454 (MIT). Quality control experiments were performed to confirm enrichment of known early-,
455 mid- and late-replicated loci in the expected fractions (Supplementary Table 5). In
456 heterozygous cells, primer pairs overlapping the site of insertion and next to the site of
457 insertion of the Tet- or β -actin promoters were used to detect the timing of the wild type allele
458 ("without" primers) and both alleles ("both" primers), respectively. A primer pair specific to
459 the transgene ("with" primers) was used to analyze the timing of the modified allele. For each
460 construct, replication timing analyses were performed on two different clonal cell lines.
461 For replication timing analyses of *FHIT*, exponentially growing HCT116 cells were pulse-
462 labelled for 1 h with 50 μ M BrdU, fixed in ethanol and incubated overnight at -20°C in the
463 presence of 15 μ g.mL⁻¹ Hoescht 33342 (ThermoFisher Scientific). Fixed cells were re-
464 suspended in 1X PBS and cells were sorted by flow cytometry based on their nuclear content
465 using a BD Biosciences INFLUX cell sorter. S-phase was divided into four fractions, from
466 early to late S-phase (S1 to S4). To check the quality of sorted HCT116 fractions, the post-
467 sort cells, already stained with Hoescht 33342, were directly re-analyzed by flow cytometry.
468 DNA was isolated using Maxwell RSC Blood DNA kit (Promega) according to the
469 manufacturer's instructions, and sonicated. For each fraction, immunoprecipitation of BrdU-
470 labelled DNA was performed on 5 μ g of DNA using mouse anti-BrdU antibody (BD
471 Biosciences). Immunoprecipitated DNA was further extracted by phenol/chloroform, and
472 real-time quantitative PCR was performed using QuantiNova SYBR Green PCR kit (Qiagen)

473 on a 7300 Real-Time PCR System (Applied Biosystems). Replication timing analyses were
474 performed using one FHIT^{EF1 α /EF1 α} clonal cell line.

475 All primer pairs used for replication timing analyses are listed in Supplementary Table 6.

476

477 **DNA-combing.** Neo-synthesized DNA was labelled as described⁵ with the following
478 changes: exponentially growing DT40 cells were pulse-labelled for 20 min with 20 μ M
479 iododeoxyuridine (IdU) followed by a 20 min pulse with 100 μ M chlorodeoxyuridine (CldU),
480 then by a 5 min chase with 1 mM thymidine. Genomic DNA was extracted and combing was
481 performed on silanized coverslips prepared by plasma cleaning and liquid-phase silanization
482 as described^{6,7} using a Genomic Vision apparatus.

483

484 **FISH on combed DNA and immunofluorescence detection of neo-synthesized DNA.**

485 Morse-codes were designed as described⁸ to specifically identify DNA molecules spanning
486 the *DMD* or *CCSER1* loci. Morse-code probes correspond to a collection of \approx 5 kb-long PCR
487 products spread all over the locus of interest, separated by precise distances and divided into
488 voluntarily different and asymmetrical patterns to correctly orient the DNA fibre. Morse-
489 codes for *DMD* and *CCSER1* detection are made of 32 probes and 27 probes, respectively.
490 PCR products were prepared and labelled with biotin as described above for FISH on
491 metaphases. Primer pairs used are listed in Supplementary Table 7. Hybridization of the
492 probes was carried out as described previously⁵. Immunodetection was performed by
493 successive incubations in the following reagents: (1) Alexa Fluor 488-conjugated streptavidin
494 (Invitrogen), (2) biotin-conjugated rabbit anti-streptavidin (Rockland Immunochemicals), (3)
495 Alexa Fluor 488-conjugated streptavidin (Invitrogen), mouse anti-BrdU (BD Biosciences) and
496 rat anti-BrdU (Bio-Rad, formerly AbD Serotec), (4) biotin-conjugated rabbit anti-streptavidin
497 (Rockland Immunochemicals), Alexa Fluor 350-conjugated goat anti-mouse (Invitrogen) and

498 Alexa Fluor 594-conjugated donkey anti-rat (Invitrogen), (5) Alexa Fluor 488-conjugated
499 streptavidin (Invitrogen), Alexa Fluor 350-conjugated donkey anti-goat (Invitrogen) and
500 mouse anti-single stranded DNA (Millipore), (6) Cy5.5-conjugated goat anti-mouse (Abcam)
501 and (7) Cy5.5-conjugated donkey anti-goat (Abcam). For bulk genome analyses,
502 immunodetection was performed as follows: (1) FITC-conjugated mouse anti-BrdU (BD
503 Biosciences) and rat anti-BrdU (Bio-Rad, formerly AbD Serotec), (2) Alexa Fluor 488-
504 conjugated goat anti-mouse (Invitrogen) and Alexa Fluor 555-conjugated goat anti-rat
505 (Invitrogen), (3) mouse anti-single stranded DNA (Millipore), (4) Cy5.5-conjugated goat anti-
506 mouse (Abcam) and (5) Cy5.5-conjugated donkey anti-goat (Abcam). Antibody incubations,
507 washes and slide mounting were performed as reported previously⁵.

508

509 **Image acquisition.** Images were acquired on a motorized XY stage of an Axio Imager Z2
510 (Carl Zeiss) or a DM6000 B (Leica) epifluorescence microscope connected to a CoolSNAP
511 HQ2 charge-coupled device camera (Roper Scientific) and run by Metamorph software
512 (Molecular Devices). A X100 objective was used for imaging metaphase chromosomes and
513 a X63 objective was used for imaging combed DNA fibres. For images of the *DMD* and
514 *CCSER1* loci, two overlays of images were set up for each microscope field. The first one
515 combined the IdU/CldU and FISH signals to identify the fibres of interest, replicating or not.
516 The second overlay combined the IdU, FISH and DNA signals to determine the length of the
517 DNA fibre bearing the Morse-code. DNA counterstaining was systematically used to ensure
518 (i) that several fibres are not overlapping (ii) that replication signals belong to the same fibre
519 and (iii) that replication signals are intact.

520

521 **Signal treatment and statistical analyses.** Measurement of DNA fibre and replication tract
522 length was performed on imaged DNA molecules using Adobe Photoshop CS5.1. DNA fibres

523 bearing Morse-code signals were modelled and aligned on a schematic representation of the
524 *DMD* or *CCSER1* loci using Adobe Illustrator CS5.1. Graphical representations of *DMD* and
525 *CCSER1* were drawn based on the locus length, the theoretical ≈ 2 kb/ μ m stretching factor of
526 the combing apparatus indicated by the manufacturer, and the resolution of the camera at a
527 magnification of X63 (0.1024 μ m/pixel). For instance, the 996,168 bp *DMD* gene
528 corresponded to $996.168/2/0.1024=4864.1$ pixels. Morse-code probes were used to calculate
529 the actual stretching factor of each DNA molecule by comparing the length of the fibre
530 imaged with the microscope with the theoretical length defined according to the position of
531 the probes. Overall, we found a mean stretching factor of 1.93 kb/ μ m, which is extremely
532 close to the expected value of 2 kb/ μ m. Still, the stretching factor of individual fibres
533 fluctuated from 1.7 to 2.4 kb/ μ m. Therefore, to prevent that variations in the stretching of the
534 DNA molecules during the combing step introduce a bias in tract measurement, the lengths of
535 IdU and CldU tracts were normalized by the stretching factor of the DNA fibre on which they
536 were located. For bulk genome analyses, the lengths of IdU and CldU tracts were normalized
537 by the mean stretching factor calculated when analyzing *DMD* or *CCSER1* loci using the
538 exact same sample and coverslip batch. Replication fork speed was then estimated on
539 individual forks as (i) the ratio $(l_{IdU}+l_{CldU})/(t_{IdU}+t_{CldU})$ for forks displaying an intact IdU tract
540 flanked on one side by an intact CldU tract, (ii) the ratio l_{IdU}/t_{IdU} for forks with an intact IdU
541 signal flanked on one side by a broken CldU signal and (iii) the ratio l_{CldU}/t_{CldU} for forks with
542 an intact CldU signal flanked on one side by a broken IdU signal, with l_{IdU} and t_{IdU} being the
543 measured length (in kb) and labelling time (in min) for IdU incorporation, respectively, and
544 l_{CldU} and t_{CldU} the corresponding parameters for CldU incorporation. For all experiments,
545 $t_{IdU}=t_{CldU}=20$ min. Replication signal integrity was ascertained by DNA counterstaining. For
546 fork progression analyses in *DMD* and *CCSER1*, only forks overlapping at least partially
547 these loci were taken into account. Coordinates and length of DNA molecules and replication

548 signals were compiled using Microsoft Excel for Mac 2011. Statistical comparisons of fork
549 speed distributions were assessed with the nonparametric Mann-Whitney-Wilcoxon test (two-
550 tailed) using GraphPad Prism 6 (GraphPad Software). No assumptions or corrections were
551 made. Statistical significance was set to $p \leq 0.05$. All DNA fibres for *CCSER1*^{ba} allele
552 originate from one biological sample, DNA combing results for WT *CCSER1* and *DMD* loci
553 compile DNA fibres from two distinct biological WT samples and DNA combing results for
554 *DMD*^{Tet/Tet} cells compile DNA fibres from two clonal cell lines. Replication analysis of WT
555 and modified *CCSER1* and *DMD* loci by DNA combing was performed once.

556

557 **Coverage profile.** Graphical representations of "Total DNA" and "DNA with replication"
558 coverages of *DMD* and *CCSER1* were made with R⁹ using custom scripts adapted from¹⁰.

559

560 **Genomic coordinates and genome annotations.** Coordinates are given according to the
561 ICGSC/galGal4 chicken genome or the GRCh37/hg19 human genome assemblies. *DMD* is
562 the largest annotated gene of the chicken genome both in the latest RefSeq and Ensembl gene
563 annotations available when this manuscript was written.

564

565 **Code availability.** Custom R scripts are available upon request.

566

567 **Data availability.** The data that support the findings of this study are available upon request.

568

569

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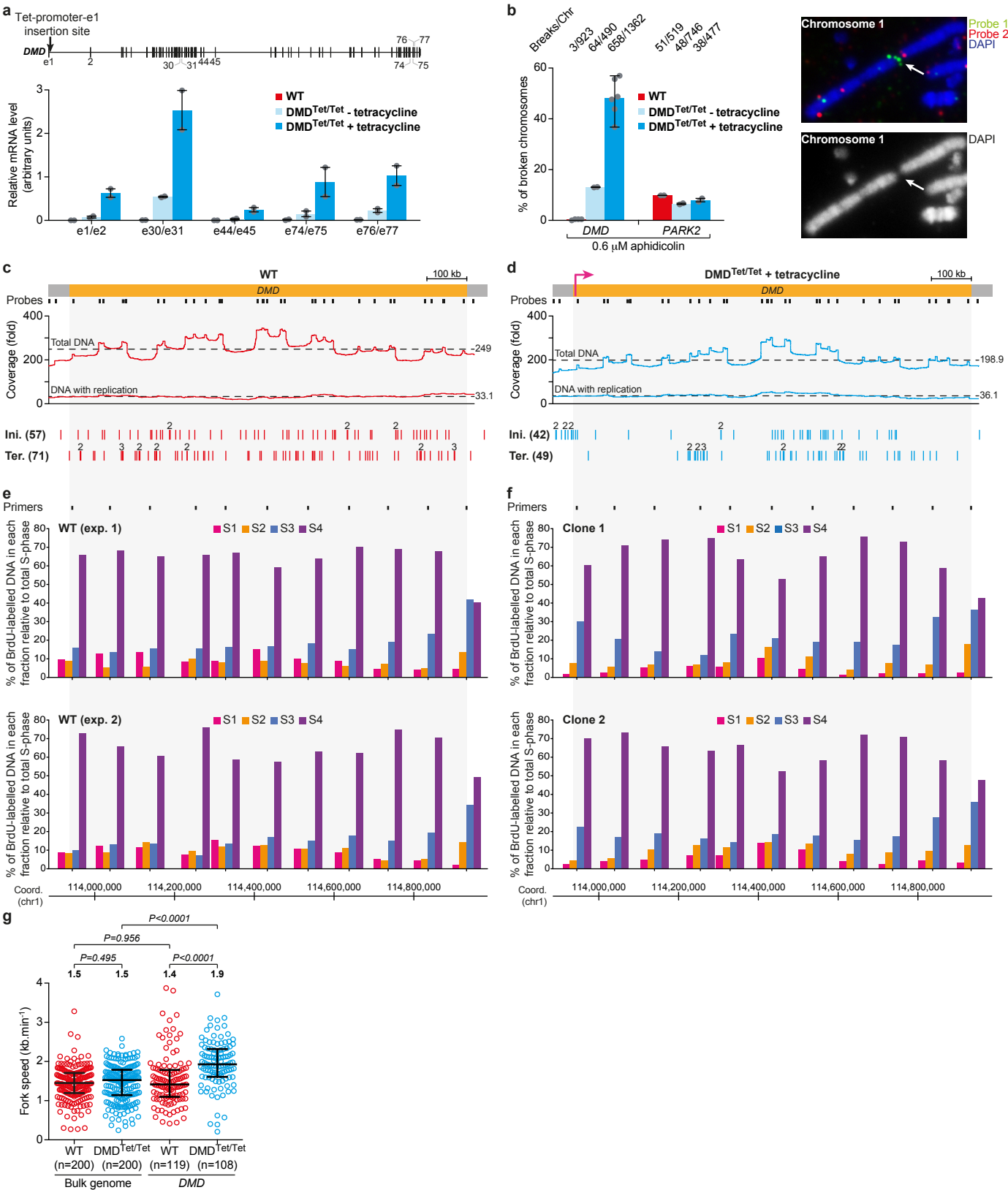
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Figure 1



600 **Figure 1. Impact of *DMD* transcription activation on its replication and fragility.**

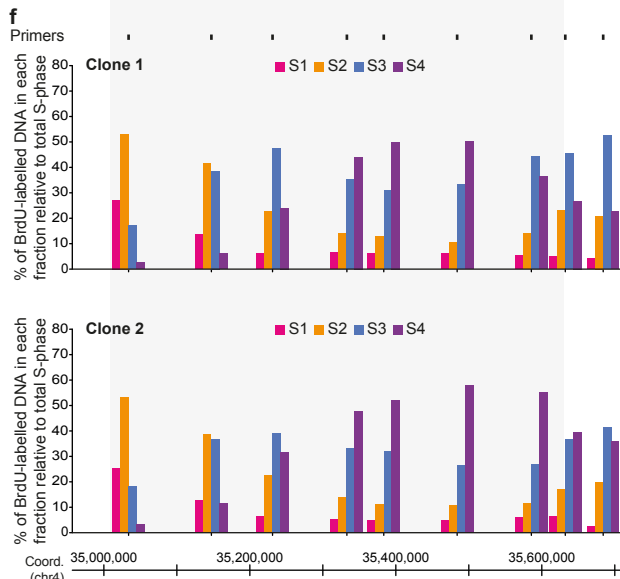
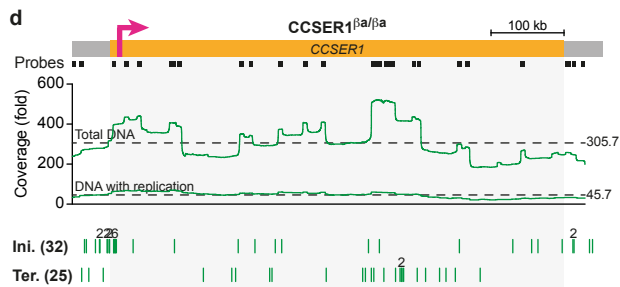
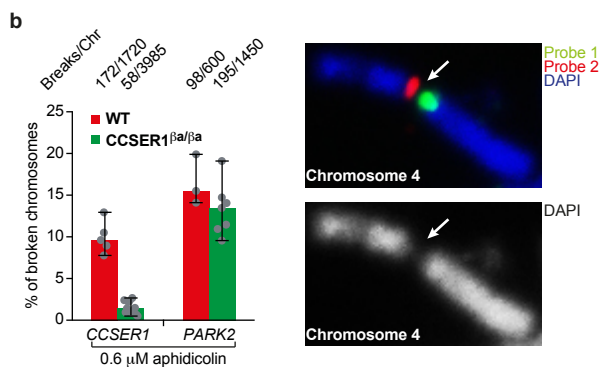
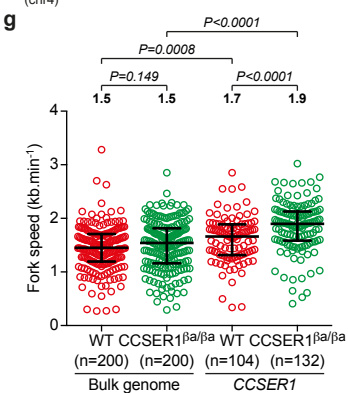
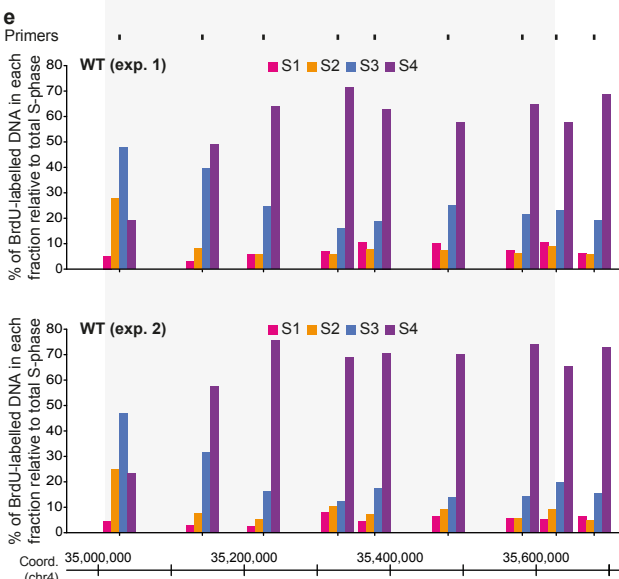
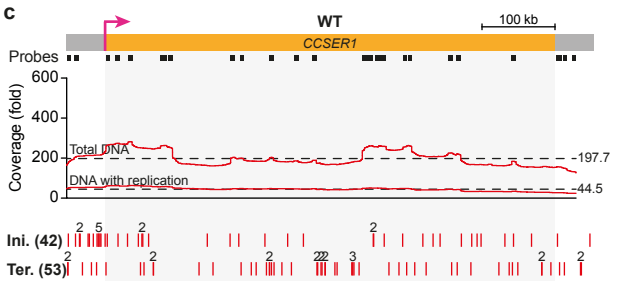
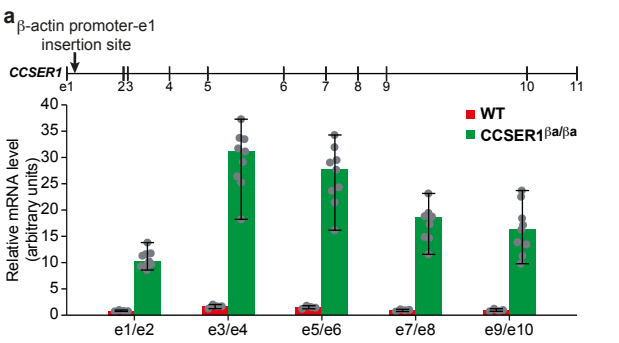
601 **a**, *DMD* mRNA levels relative to β -actin mRNA in WT and $DMD^{Tet/Tet}$ cells (median,
602 extreme values and individual data points). Tested exonic junctions are indicated. Top: Map
603 of *DMD* with its exons and the position of the Tet-promoter-*DMD* exon 1 insertion site.

604 **b**, Left panel: Aphidicolin-induced breaks at *DMD* and *PARK2* in WT and $DMD^{Tet/Tet}$ cells
605 (median, extreme values and individual data points; aggregate numbers are presented on top).
606 Breaks at the 679.5 kb-long *PARK2* gene were used as a control. Right panel: Example of
607 two-color FISH with probes flanking *DMD*. Reverse-DAPI staining is also shown. The arrow
608 indicates the position of a break. We also verified that *DMD* is not fragile in the absence of
609 aphidicolin in WT and $DMD^{Tet/Tet}$ cells (see Supplementary Fig. 1i).

610 **c, d**, Mapping of initiation and termination events along the *DMD* locus in WT cells (**c**) and
611 $DMD^{Tet/Tet}$ cells with tetracycline (**d**). From top to bottom: *DMD* locus (yellow box. A pink
612 arrow represents the active Tet-promoter in $DMD^{Tet/Tet}$ cells) with the name of the cell line
613 indicated above; Morse-code probes (black boxes) used in DNA-combing experiments to
614 identify *DMD*; Total DNA and DNA with replication coverages of the locus (local peaks in
615 total DNA coverage map to Morse-code probes; see Supplementary Figs 2, 3. Dotted lines
616 represent mean coverages, indicated on the right); Map of initiation and termination events
617 (the total number of events is indicated on the left. Figures above some lines indicate
618 colocalized events).

619 **e, f**, Replication timing profile of *DMD* in WT cells (**e**) and $DMD^{Tet/Tet}$ cells with tetracycline
620 (**f**). BrdU pulse-labelled cells were sorted into four S-phase fractions and neo-synthesized
621 DNA was quantified by real-time PCR using the indicated primers (black boxes). Two
622 experiments are shown; for $DMD^{Tet/Tet}$ cells, replication timing analyses performed on two
623 different clonal cell lines are presented.

624 **g**, Replication fork progression in WT and DMD^{Tet/Tet} cells with tetracycline in the bulk
625 genome or in *DMD*. Median with interquartile range (horizontal black lines), P-value and
626 number of forks measured (n) are indicated.

Figure 2

627 **Figure 2. Impact of *CCSER1* transcription upregulation on its replication and fragility.**

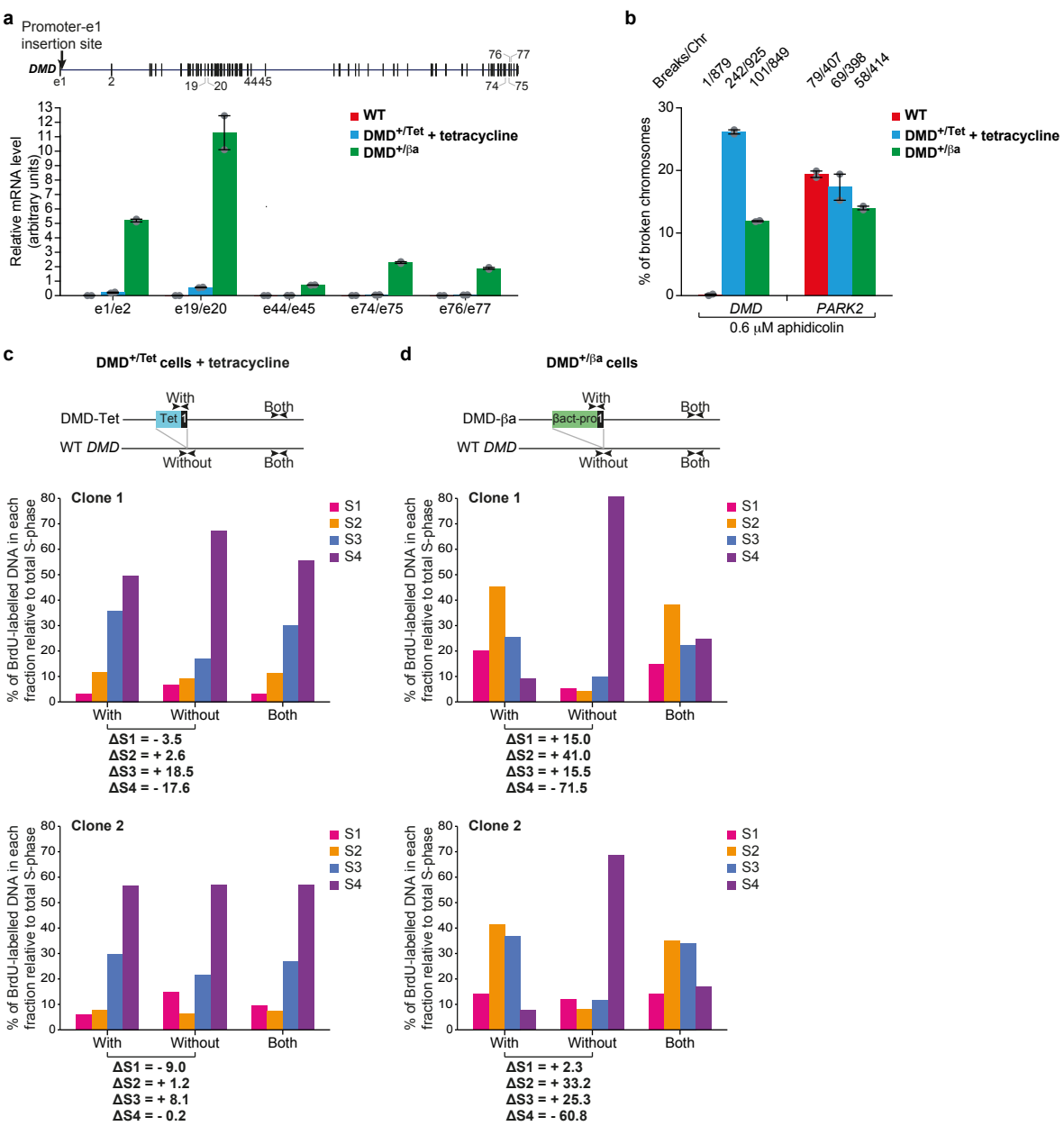
628 **a**, *CCSER1* mRNA levels relative to β -actin mRNA in WT and *CCSER1* ^{β a/ β a} cells (median,
629 extreme values and individual data points). Tested exonic junctions are indicated. Top: Map
630 of *CCSER1* with its exons and the position of the chicken β -actin promoter-*CCSER1* exon 1
631 insertion site.

632 **b**, Left panel: Aphidicolin-induced breaks at *CCSER1* and *PARK2* in WT and *CCSER1* ^{β a/ β a}
633 cells (median, extreme values and individual data points; aggregate numbers are presented on
634 top). Breaks at *PARK2* were used as a control. Right panel: Example of two-color FISH with
635 probes flanking *CCSER1*. See Fig. 1b for details. We also verified that *CCSER1* is not fragile
636 in the absence of aphidicolin in WT and *CCSER1* ^{β a/ β a} cells (see Supplementary Fig. 4g).

637 **c, d**, Mapping of initiation and termination events along the *CCSER1* locus in WT (**c**) and
638 *CCSER1* ^{β a/ β a} (**d**) cells. From top to bottom: *CCSER1* locus (yellow box; a thin and a thick
639 pink arrow represents active *CCSER1* promoter in WT cells and active β -actin promoter in
640 *CCSER1* ^{β a/ β a} cells, respectively) with the name of the cell line indicated above; Morse-code
641 probes (black boxes) used in DNA-combing experiments to identify *CCSER1*; Coverage of
642 the locus; Map of initiation and termination events. See Fig. 1c for details.

643 **e, f**, Replication timing profile of *CCSER1* in WT (**e**) and *CCSER1* ^{β a/ β a} (**f**) cells. Two
644 experiments are shown; for *CCSER1* ^{β a/ β a} cells, replication timing analyses performed on two
645 different clonal cell lines are presented. See Fig. 1e for details.

646 **g**, Replication fork progression in WT and *CCSER1* ^{β a/ β a} cells in the bulk genome or in
647 *CCSER1*. See Fig. 1g for details.

Figure 3

648 **Figure 3. Impact of *DMD* transcription modulation on its replication timing and**
649 **fragility.**

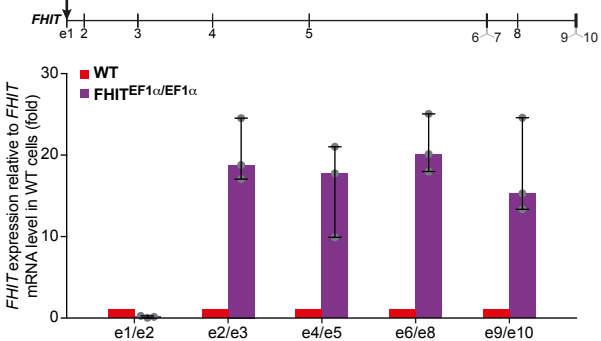
650 **a**, *DMD* mRNA levels relative to β -actin mRNA in WT cells, $DMD^{+/Tet}$ cells with tetracycline
651 and $DMD^{+/\beta a}$ cells (median, extreme values and individual data points). Tested exonic
652 junctions are indicated. Top: Map of *DMD* with its exons and the position of the Tet- or β -
653 actin promoter-*DMD* exon 1 insertion site.

654 **b**, Aphidicolin-induced breaks at *DMD* and *PARK2* in WT cells, $DMD^{+/Tet}$ cells with
655 tetracycline and $DMD^{+/\beta a}$ cells (median, extreme values and individual data points; aggregate
656 numbers are presented on top). Breaks at *PARK2* were used as a control.

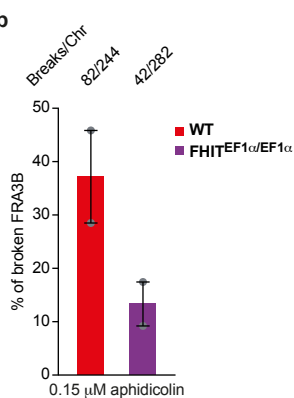
657 **c, d**, Replication timing at the Tet- (**c**) and β -actin (**d**) promoter insertion site. BrdU pulse-
658 labelled $DMD^{+/Tet}$ cells with tetracycline and $DMD^{+/\beta a}$ cells were sorted into four S-phase
659 fractions, and neo-synthesized DNA was quantified by real-time PCR using primer pairs
660 specific of the *DMD*-Tet or *DMD*- βa allele (with), of the WT *DMD* allele (without) or
661 hybridizing on the two alleles (both). The differences in replication timing at the target site
662 following promoter integration ($\Delta S = S_{with} - S_{without}$) were calculated for each sub-fraction.
663 Results for two different clonal cell lines are presented for each construct.

Figure 4

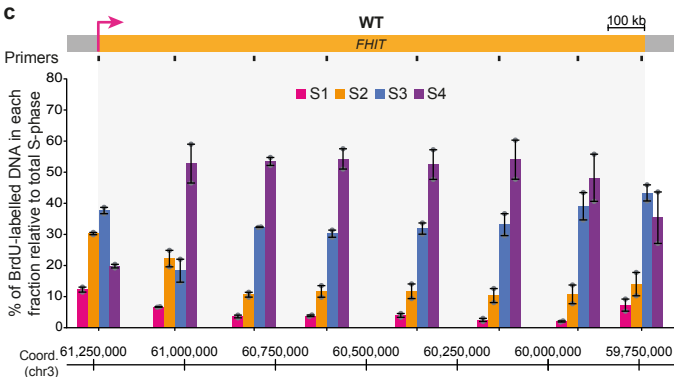
a EF1 α promoter-hygromycin selection cassette insertion site



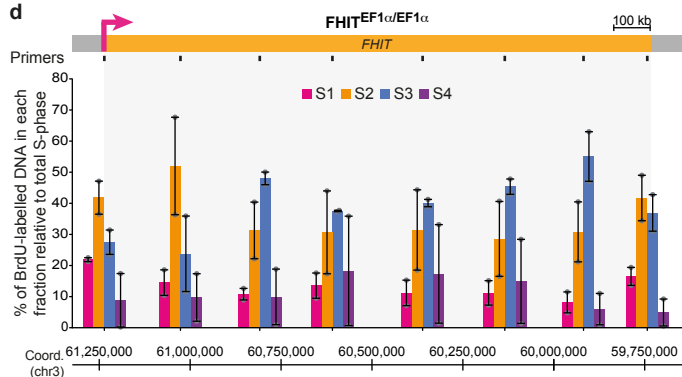
b



c



d



664 **Figure 4. Impact of *FHIT* transcription upregulation on its replication timing and**
665 **fragility in human HCT116 cells.**

666 **a**, *FHIT* expression in WT and $FHIT^{EF1\alpha/EF1\alpha}$ cells relative to *FHIT* mRNA in WT cells
667 (median, extreme values and individual data points). *FHIT* mRNA level was normalized to 3
668 housekeeping genes (*POLR2F*, *RPL11* and *PPIB*). Tested exonic junctions are indicated. Top:
669 Map of *FHIT* with its exons and the position of the EF1 α promoter-hygromycin selection
670 cassette insertion site.

671 **b**, Aphidicolin-induced breaks at *FHIT* in WT and $FHIT^{EF1\alpha/EF1\alpha}$ cells (median, extreme
672 values and individual data points; aggregate numbers are presented on top).

673 **c, d**, Replication timing profile of *FHIT* in WT (**c**) and $FHIT^{EF1\alpha/EF1\alpha}$ (**d**) cells (median and
674 extreme values of 2 experiments). See Fig. 1e for details.