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1 **Real-time imaging of a single gene reveals transcription-initiated local**
2 **confinement**

3 Running title: Real-time single gene tracking

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30 **Abstract**

31 Genome dynamics are intimately linked to the regulation of gene expression, the most fundamental
32 mechanism in biology. Yet we still do not know whether the very process of transcription drives spatial
33 organization at specific gene loci. Here, we have optimized the ANCHOR/ParB DNA-labeling system for
34 real-time imaging of a single-copy estrogen-inducible transgene in human cells. Motion of an ANCHOR3-
35 tagged DNA locus was recorded in the same cell prior to and during appearance of nascent MS2-labelled
36 mRNA. We found that transcription initiation by RNA polymerase 2 resulted in confinement of the
37 mRNA-producing gene domain within minutes. Transcription-induced confinement occurred in each
38 single cell independently of initial, highly heterogeneous mobility. Constrained mobility was maintained
39 even when inhibiting polymerase elongation. Chromatin motion at constant step-size within a largely
40 confined area hence leads to increased collisions that are compatible with the formation of gene specific
41 chromatin domains, reflect assembly of functional protein hubs and DNA processing during the rate-
42 limiting steps of transcription.

43

44 **Introduction**

45 3D organization of the genome contributes significantly to regulation of all major nuclear processes.
46 Changes in average position of chromosome loci in a population of cells correlate with local or global
47 changes in DNA metabolism (1–9). This is notably the case for gene transcription, where active genes
48 tend to associate with clusters of RNA polymerase II (pol2) (10). By imaging pol2, its cofactors and mRNA,
49 these transcription hubs have been shown to be relatively immobile (11–14), but the motion of the
50 associated DNA has not been reported. Consequently, we do not know if the observed reduced protein
51 mobility is an intrinsic property of the transcription machinery or an indirect effect of changes in
52 chromatin conformation. Whichever the cause, precise kinetics of this reorganization at timescales short
53 enough to determine chromatin physical properties have not been analyzed in mammalian cells. Indeed,

54 real time analysis of chromatin at short time scales relevant for the analysis of transcription activation
55 (minutes) has been hampered by methodological limitations. Existing technologies to visualize DNA loci
56 usually rely on highly repetitive sequences, based on insertion of hundreds of repeats of bacterial
57 operator sequences to which fluorescent repressor fusion proteins bind with high affinity (called FROS
58 for fluorescent repressor operator system (8)), or using multiplexed short guide RNAs that stably recruit
59 catalytically inactive dCas9-GFP fusion proteins to a large, repetitive genomic region and partially unwind
60 the target DNA sequence (15, 16). These technologies confirmed that transcription impacts nuclear
61 localization of gene domains. However, they do not allow tagging of genes within immediate vicinity of
62 regulatory elements by fear to disturb their very function. Nevertheless, it was shown that, in yeast, the
63 mobility of a gene was increased by permanently recruiting the potent activator VP16 or chromatin
64 remodeling factors (17). This effect could stem from constitutive local decondensation of chromatin near
65 the labelled gene. In mouse ES cells, in contrast, using dCas9-GFP targeted to MS2 sequence repeats
66 inserted near the Nanog gene, it was reported that motion of the transcribed gene was reduced (18). In
67 both studies gene motion was compared in different cells. To truly assess immediate changes in
68 chromatin motion during transcription activation, DNA dynamics of a single-copy gene have to be
69 analyzed in real-time while simultaneously monitoring steps of mRNA synthesis in the same cell.

70 To achieve this, we developed a novel ANCHOR DNA labeling system (ANCHOR3) for use in human cells.
71 ANCHOR is based on insertion of a non-repetitive, short (<1kb) DNA sequence (ANCH) to which a limited
72 number of OR (bacterial partition protein or ParB) bind site-specifically. Oligomerization via N-terminal
73 protein-protein interaction leads to accumulation of OR proteins which non-specifically and dynamically
74 associate with adjacent DNA and form a fluorescent focus (19–21). In yeast, OR/ParB was shown to
75 spread over DNA within chromatin up to 2 kb from the ANCH sequence (22). The ANCH site can be
76 placed immediately next to a genomic locus of interest without disturbing DNA function in eukaryotic
77 cells. For example, DNA resection from a HO cleavage site in yeast was normal despite the presence of

78 an OR-bound ANCH site only 76bp next to it, viral gene expression and subsequent infectious behavior in
79 mammalian cells was preserved when inserting an ANCH and OR-GFP expressing cassette into the dense
80 herpes virus genome (Mariame et al, under revision; www. Neovirtech.com), and transcription activation
81 was fully functional from transgenes placed on a plasmid or within the mammalian genome when ANCH
82 was inserted immediately next to promoters (this study and unpublished observations). Hence, stable
83 insertion of the ANCHOR system into cells in which transcription of target genes can be activated under
84 physiological conditions enables fluorescence imaging of a single locus in the same cell over time,
85 without interfering with gene expression.

86 In this study we demonstrate that the optimized ANCHOR3 system in combination with the MS2 system
87 (23) is ideally suited for simultaneous visualization of DNA and mRNA at a single gene level in living
88 human cells at high spatio-temporal resolution. We show that transcription initiation, not elongation,
89 constrains local displacement of the hormone-induced Cyclin D1 gene as an immediate response to the
90 transcription process in human cells.

91

92 **Materials and Methods**

93 **Cell line**

94 The human breast cancer cell line MCF-7 (purchased from ATCC) was used to generate stable FRT/LacZeo
95 clones. Cells were grown in Dulbecco's modified Eagle medium F-12 (red DMEM/F12) completed with
96 10% FBS (Gibco), 1% Sodium Pyruvate (Gibco) and 0.5% Gentamycine (Gibco) or in phenol red free
97 DMEM/F12 completed with 10% charcoal stripped serum, 1% Sodium Pyruvate (Gibco) and 0.5%
98 Gentamycine (Gibco), in a water-saturated atmosphere containing 5% CO₂ at 37°C. Transfections were
99 carried out using FuGENE HD Transfection Reagent (Promega), according to the supplier's
100 recommendation.

101

102 **ANCHOR3 labeling system**

103 ANCH3 corresponds to a specific chromosome partition sequence and was amplified directly by PCR
104 from the genome of an undisclosed exotic bacteria (the ANCHOR system is the property of NeoVirTech
105 SAS; requests of use: contact@neovirtech.com). The PCR product was then cloned using BamH1/HindIII
106 into pCDNA FRT vector digested by BgIII/HindIII (Invitrogen). Insertion was verified by ApaI digestion and
107 sequencing. OR3 corresponding to the cognate ParB protein was amplified by PCR and cloned via
108 BgIII/KpnI directly into pGFP-c1 digested by the same enzyme. Insertion was verified by digestion and
109 sequencing. Functionality of the construct was verified by co-transfection of both vectors in HeLa cells,
110 and produced clearly identifiable fluorescent spots. To construct the ANCH3-CCND1-MS2 transgene (Fig.
111 1a) the ANCH3 sequence was amplified by PCR with primers containing EcoRV restriction sites and
112 ligated into the EcoRV digested pCDNA5-FRT/CCND1pr-HA-CCND1/24MS2/3'UTR plasmid (kindly
113 provided by Yaron Shav-Tal (23)).

114 **Engineering of stable FRT/LacZeo clones expressing the CCND1 transgene**

115 MCF-7 cells were transfected using pFRT//lacZeo kit from Invitrogen according to the supplier's protocol.
116 Selection of FRT clones was performed using 75µg/ml Zeocin (InvivoGen) in red DMEM/F12 medium.
117 Selection medium was renewed every 3 days. Three clones, showing the same growing behavior and
118 ER α -target gene expression profiles as MCF-7 cells were kept for use: G7, A11 and D11. Clones G7, A11,
119 D11 were transfected with 1µg of ANCH3-CCND1-MS2 or ANCH3 plasmids and 6µg of plasmid pOG44
120 encoding Flipase (Invitrogen). Selection of positive clones was performed with 75µg/ml Hygromycin
121 (Invitrogen) and presence of a single fluorescent focus was verified by fluorescence microscopy 24h after
122 cell transfection with 500ng OR3-Santaka. Santaka is ATUM's synthetic non-aequorea fluorescent protein
123 that can be amplified by PCR and cloned into any daughter expression vector of choice
124 (<https://www.atum.bio/products/expression-vectors/mammalian?hl=santaka>).

125 ANCH3-CCND1-MS2 cells as well as control cells with only an ANCH3 insertion were maintained in red
126 DMEM/F12 completed with 75µg/ml Hygromycin.

127 **Fluorescence correlation spectroscopy and fluorescence recovery after photobleaching experiments**

128 For fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP)
129 experiments, 2×10^4 cells were seeded on 8 well Lab-Tek chambered cover glass (Nunc). To visualize
130 spots, cells were transfected 1h after cell seeding with a plasmid encoding OR3-EGFP using FuGENE HD
131 transfection reagent (Promega) according to the manufacturer's instructions for a 3:1 transfection
132 reagent:DNA ratio. To express free mEGFP as a control for FCS, cells were transfected 1h after cell
133 seeding with a plasmid encoding mEGFP (kindly provided by J. Lippincott-Schwartz) using FuGene6
134 transfection reagent (Promega) according to the manufacturer's instructions for a 3:1 transfection
135 reagent:DNA ratio. FCS and FRAP experiments were performed 24 to 48h after cell seeding/transfection
136 in CO₂-independent imaging medium (Gibco, custom-made) supplemented with 20% (v/v) FBS, 1% (v/v)
137 sodium pyruvate and 1% (v/v) L-glutamine.

138 **Fluorescence recovery after photobleaching**

139 Imaging was performed on a Zeiss LSM780 ConfoCor3 confocal microscope using a 40x, 1.2 NA, water
140 Korr FCS objective. Prior to each FRAP time-lapse, an overview image of the measured interphase cell
141 with a nuclear spot was taken. GFP was excited with a 488 nm laser (Ar, 25 mW, 0.4% AOTF transmission,
142 2.3 µW at probe) and detected with a GaAsP detector using a 492-552 nm detection window ($\Delta x, \Delta y = 83$
143 nm, $\Delta z = 0.4$, 60x60 pixels, 9 z-slices). 40 images were acquired at a 5 s time interval. After three pre-
144 bleach images a circular ROI (12 pixels wide) containing the ANCHOR spot was bleached with five
145 iterations and maximal laser power. For bleaching controls, cells with spots were acquired with the same
146 conditions as in the FRAP experiments but without bleaching the fluorescent spot. The z-position was
147 stabilized using Zeiss Definite Focus. Due to hardware limitations the first four images have a time
148 interval between 3.4 s and 6.6 s. These reproducible differences were taken into account in the analysis.

149 The image analysis was performed using Fiji (<http://fiji.sc/Fiji>). For each FRAP time-course the position of
150 the OR3-EGFP ANCHOR spot was tracked in 3D using the Fiji MOSAIC plugin for 3D single-particle
151 tracking ([http://mosaic.mpi-cbg.de/?q=downloads/imageJ,\(24\)](http://mosaic.mpi-cbg.de/?q=downloads/imageJ,(24))). If a spot was not detectable after
152 photobleaching, its position was interpolated. The mean fluorescence intensity F_s was measured in a 5x5
153 rectangular region around the tracked spot. The background fluorescence intensity F_{bg} was measured
154 from a ROI in the unbleached part of the nucleus. Relative fluorescence intensity RI at time point t_i (Fig.
155 S1d) is given by

$$RI(t_i) = \frac{(F_s(t_i) - F_{bg}(t_i))}{\sum_{j=1}^3 [F_s(t_j) - F_{bg}(t_j)]/3} e^{-k_{bl}t_i}. \quad (\text{Eq. S1})$$

156 whereby the data was normalized to the average of the first three pre-bleach images and bleach
157 corrected using an exponential factor. The bleaching rate k_{bl} was estimated from the FRAP time-courses
158 without bleaching the ANCHOR3 spots. Overall bleaching was below 10% within the acquired 40 frames.
159 The post-bleach kinetics were fitted to a single exponential function as described in the legend to Fig.
160 S1d.

161 **Fluorescence correlation spectroscopy and protein number estimation**

162 Imaging and photon counts were acquired on a Zeiss LSM780 ConfoCor3 confocal microscope using a
163 40x, 1.2 NA, water Korr FCS objective. Cells were imaged ($\Delta x, \Delta y = 83 \text{ nm}$, $\Delta z = 0.4$, 164x164 pixels, 9 z-
164 slices) and subsequently two positions in the nucleus outside of ANCHOR3 spots were selected for FCS at
165 the z-position of the central slice. For imaging, GFP was excited with a 488 nm laser (Ar, 25 mW, 0.4%
166 AOTF transmission, 2.3 μW at probe) and detected with a GaAsP detector using a 492-552 nm detection
167 window. For pre-FCS imaging the same detector settings, pixel size, pixel dwell time, and laser settings
168 were used as for FRAP, allowing the conversion of fluorescence intensities in the first pre-bleach FRAP
169 images to concentrations and protein numbers based on an FCS calibration (Eq. S3). For FCS, GFP was

170 excited with a 488 nm laser (Ar, 25 mW, 0.05% AOTF transmission, 0.28μW at probe) and the photon
 171 counts were recorded for 30 s using an avalanche photodiode detector (APD; 505-590 nm detection). To
 172 estimate the effective volume, a water solution of Alexa488 (Lifetech) with a known diffusion coefficient
 173 ($D_{\text{alexa}} = 441 \mu\text{m}^2\text{sec}^{-1}$, M. Wachsmuth, EMBL, personal communication) was measured before each
 174 experiment.

175 The raw photon counts were processed using FluctuationAnalyzer 4G
 176 (<http://www.embl.de/~wachsmut/downloads.html>). This program computes the autocorrelation
 177 function (ACF), correction factors, e.g. due to background, and fits the ACF to physical models of
 178 diffusion (see Wachsmuth et al., 2015, for further details). The ACF $G(\tau)$ was fit to

$$G(\tau) = \frac{1}{N} [1 - \theta + \theta e^{-\tau/\tau_{\text{trip}}}] \left[\left(1 + \left(\frac{\tau}{\tau_D} \right)^\alpha \right)^{-1} \left(1 + \frac{1}{\kappa^2} \left(\frac{\tau}{\tau_D} \right)^\alpha \right)^{-\frac{1}{2}} \right]. \quad (\text{Eq. S2})$$

179 Equation S2 describes anomalous diffusion: N denotes the number of particles in the effective volume,
 180 $\kappa = 5.5$ the structure factor, i.e. the ratio of axial to lateral radius of the effective volume, τ_D the
 181 characteristic diffusion correlation time, α the anomaly parameter. The parameter θ is the fraction of
 182 molecules in a non-fluorescent state and τ_{trip} the apparent life-time in this state. The value of τ_{trip} was
 183 set to 100 μs and the other parameters were fit to the data.

184 The concentration of molecules in the effective focal volume V_{eff} is given by $C = N/V_{\text{eff}}N_A$, where
 185 $V_{\text{eff}} = \pi^{3/2} \kappa w_0^3$ and N_A is the Avogadro's constant. The lateral focus radius w_0 is given by
 186 $w_0 = 2\sqrt{\tau_{\text{alexa}} D_{\text{alexa}}}$ where τ_{alexa} is the characteristic diffusion correlation time measured for Alexa488.
 187 The counts per molecule are given by $\text{CPM} = N / \langle I \rangle$, where $\langle I \rangle$ is the average photon counts.

188 For each experiment, a calibration curve (Fig. S1c) was calculated from the mean fluorescence intensity
189 (FI) and the concentration obtained from FCS. The mean FI was measured in a 5x5 px large square at the
190 location of the FCS measurement. The data points were fit to a line that describes the relationship
191 between fluorescence intensity FI and concentration C :

$$C = a + b FI. \quad (\text{Eq. S3})$$

192 Based on Eq. S3, each pixel of images acquired with the same settings as the pre-FCS images can be
193 converted to a concentration. The protein number in each voxel was obtained by multiplying the
194 concentration C with the voxel volume V_{vox} and N_A (see Fig. S1e).

195 To estimate the protein number at the ANCHOR site, the first pre-bleach image of the FRAP time-course
196 was used. The FI was converted to protein number per pixel using the calibration curve in Eq. S3 and V_{vox} .

$$N(x, y) = \frac{A}{2 \pi \sigma^2} \exp\left(-\frac{(x - x_s)^2}{2 \sigma^2} - \frac{(y - y_s)^2}{2 \sigma^2}\right) + bg \quad (\text{Eq. S4})$$

197 In a 14x14 rectangular region at the location of the ANCHOR3 site a 2D Gaussian was fit (Eq. S4), where x_s
198 and y_s are the coordinates of the spot, bg the background nuclear signal and A the total protein number
199 at the Anchor site. For the fit the Gauss Fit on Spot ImageJ plugin ([http://imagej.nih.gov/ij/plugins/gauss-
200 fit-spot/index.html](http://imagej.nih.gov/ij/plugins/gauss-fit-spot/index.html)) was used.

201 **Fluorescence live cell imaging under stimulation**

202 For live cell tracking during transcription activation, 100 000 cells were plated in 35mm glass-bottom
203 culture plates (Ibidi, Biovalley) in red DMEM/F12 medium and allowed to attach for 24h. Red DMEM/F12
204 medium was changed for phenol red free DMEM/F12 medium supplemented with 10% charcoal stripped
205 serum, without hygromycin, and cells were kept in the latter medium for 72h. Cells were co-transfected
206 with 500ng OR3-Santaka and 1 μ g MCP-GFP DNA vectors 24h before observation.

207 To activate transcription of the ANCH3-CCND1-MS2 transgene, cells were maintained in L-15 medium
208 (Liebovitz's, Gibco) supplemented with 10% charcoal stripped serum, a buffer appropriate for live cell
209 imaging.

210 Observations were made using a Nipkow-disk confocal system (Revolution; Andor) installed on a
211 microscope (IX-81), featuring a confocal spinning disk unit (CSU22;Yokogawa) and a cooled electron
212 multiplying charge-coupled device camera (DU 888; Andor). The system was controlled using the
213 Revolution IQ software (Andor). Images were acquired using a 60× Plan Apo 1.42 oil immersion objective
214 and a two-fold lens in the optical path. Single laser lines used for excitation were diode-pumped
215 solid-state lasers exciting GFP fluorescence at 488 nm (50 mW; Coherent) and Santaka fluorescence at
216 561 nm, and a Quad band pass emission filter (Di01-T405/488/568/647-13x15x0,5; Semrock) allowed
217 collection of the green and red fluorescence. Pixel size was 110 nm. Movies containing 200 image frames
218 acquired with an exposure time of 250 ms were recorded. Images were processed using ICY and FIJI
219 software.

220 After imaging of the cells without transcription stimulation, 100nM 17β-estradiol (E2; Sigma) was added
221 directly under the microscope and dynamics of the OR3-Santaka spot in the same cells were recorded
222 45min later. Before acquisition under E2 stimulation, presence of the MCP-GFP foci, indicating active
223 transcription elongation, was verified in each analyzed cell (Fig. 1c). To block transcription elongation, we
224 added fresh L-15 medium containing 50μM DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Sigma).
225 The cells were imaged 30min after DRB addition and disappearance of the MCP-GFP foci was verified
226 (Fig. 3b). To examine the impact of transcription initiation on chromatin motion in living cells, we added
227 fresh L-15 medium containing 500nM Triptolide (TPL, Sigma) before or after stimulation by E2. The cells
228 were imaged under the same conditions as for DRB treatment. To study the effect of OH-Tamoxifen (OH-
229 Tam; Sigma), cells were maintained in L-15 medium and imaged before and 45min after 1μM OH-Tam
230 treatment.

231 **Lateral drift test**

232 Lateral drift was analyzed by cross correlation. Cross correlation assumes that shape of the structures
233 imaged in the sample is not expected to change significantly during the acquisition; the structure itself
234 can be used to determine whether spatial shift between subsequent images exists. For single cell
235 analysis, movies were cropped and registered by the ImageJ plug-in StackReg using translation and rigid
236 body functions for drift correction, before tracking of the DNA locus using ICY tracker (2). The translation
237 function calculates the amount of translation (Δr) by a vectoring analysis from $\mathbf{x} = \mathbf{r} + \Delta \mathbf{r}$. Where, \mathbf{x} and \mathbf{r}
238 are the output and input coordinates. Rigid body transformation is appropriate because coordinates
239 are $\mathbf{x} = \{\{\cos \vartheta, -\sin \vartheta\}, \{\sin \vartheta, \cos \vartheta\}\} \cdot \mathbf{r} + \Delta \mathbf{r}$, considering both the amount of translation (Δr) and the
240 rotation by an angle ϑ (2).

241 **Mean square displacement**

242 Particle tracking experiments and MSD calculations were carried out using ICY and MatLab software.
243 Tracks of 200 frames were scored. OR3-Santaka spots were detected and tracked using the Spot detector
244 and spot tracking plug-in from ICY in single cells. Mean square displacements (MSD) were calculated in
245 Matlab using the following equation:

246
$$dI2(j,i)=(x(j+i)-x(i))^2+(y(j+i)-y(i))^2 \quad (\text{Eq. S5})$$

247 Averaged MSD resulted from averaging the MSD at each time interval (Fig. 2b, n=14). Mean MSD were
248 extracted from the average squared displacement from 2s to 40 s of the ANCH3 locus during the time of
249 acquisition in one cell (Fig. 2c, 3c, 3d, 3f and 3g, n=15,n=21, n=9, n=7, n=3 and n=10 respectively). Areas
250 of confinement were obtained using the raw trajectories over 50 s and fitted based on an ellipse using
251 the freely available software package GNU Octave to draw a confidence ellipse that contains 95% of all
252 recorded positions ([www.visiondummy.com/2014/04/draw-error-ellipse-representing-covariance-
253 matrix/](http://www.visiondummy.com/2014/04/draw-error-ellipse-representing-covariance-matrix/)).

254

255 **Statistics**

256 Results were analyzed using two different tests. A Student's t-test with a confidence interval of 95% was
257 used for data presented in Fig. 2c, 2d and 2e. A Wilcoxon signed-rank test was used for data presented in
258 Fig. 3c, 3d, 3f and 3g

259 **Results**

260 To simultaneously visualize DNA and mRNA of a single gene, we labeled a Cyclin D1 (CCND1) transgene
261 with a new, improved ANCHOR3 system (see materials and methods). The ANCHOR system was derived
262 from prokaryotic chromosome partitioning components and originally implemented in yeast (22).
263 Specific association of a few ParB/OR protein dimers to a limited number of parS binding sites within the
264 bacterial chromosome's partitioning site initiates formation of a large nucleoprotein complex dependent
265 on non-specific, dynamic ParB/OR binding and ensuing oligomerization (19–21). The ANCHOR system
266 thus relies simply on a short ANCH/INT sequence (<1kb) that can be inserted immediately adjacent,
267 within a few base-pairs, to regulatory elements.

268 The transgene is further composed of the endogenous CCND1 promoter, a CCND1 cDNA cassette
269 including its 3'enhancer region and 24 repeats of the MS2 MCP protein-binding sequence within the
270 CCND1 3'-UTR (23) (Fig. 1a). The construct was inserted into an FRT site within the genome of estrogen
271 receptor *alpha* (ER α)-positive MCF-7 human mammary tumor cells (Fig. 1a). In the engineered,
272 monoclonal cells, called ANCH3-CCND1-MS2, fluorescent OR3-fusion proteins form a single focus at the
273 ANCH3 site of the transgene that can be readily tracked in real time (Fig. 1b; Videos S1, S2). To
274 characterize the binding kinetics of OR3 proteins at and around the ANCH3 site, we used fluorescence
275 recovery after photo-bleaching (FRAP) of OR3-EGFP labeled spots (Fig. 1b). Association and dissociation
276 of OR3-EGFP at the ANCH3-tagged site was in a dynamic steady state with a measured half-life of $57\pm 2s$
277 (Fig. 1b). To estimate the copy number of OR3 proteins at an ANCH3 site in steady state, we performed
278 confocal imaging calibrated by fluorescence correlation spectroscopy (FCS), which allowed us to convert

279 pixel fluorescence intensity to protein concentration or number (Fig. S1a-e). The fluorescence intensity
280 of the ANCHOR3 spot increased with OR3-EGFP abundance (Fig. S1f). Under our OR3 expression
281 conditions, we calculated an average of 481 ± 274 fluorescent molecules per site, corresponding to a
282 significant amplification of the nine OR3 dimers bound specifically to the parS sites of the ANCH3
283 sequence (Fig. S1f).

284 The MCP-EGFP signal corresponding to accumulated CCND1 transcripts is detectable near the ANCHOR3-
285 labeled DNA site 45 minutes after 17β -estradiol (E2) addition to G1-synchronized cells grown in steroid-
286 stripped media (Fig. 1c; video S2), consistent with the fact that ER α target gene expression is triggered 10
287 minutes after E2 addition (28, 29). Fluorescence intensity of the OR3 spot did not vary upon transcription
288 activation, confirming that events leading to mRNA production of the tagged locus do not interfere with
289 OR3 accumulation. If any, displaced OR3 molecules re-associate faster than variations in intensity can be
290 detected using our acquisition settings. Thus, the engineered cell line enabled real-time imaging of a
291 single copy gene during transcription activation by the endogenous ER α , under physiological conditions.

292 To directly test whether changes in gene expression impact local chromatin dynamics, we recorded the
293 motion of the fluorescent ANCHOR3-Santaka-tagged gene in the same cell prior to and 45 min after
294 adding 100 nM E2, while monitoring the appearance of MCP-EGFP-labelled mRNA signals (Fig. 1c). Live
295 cell tracking revealed that movement of the CCND1 gene is locally constrained upon induction of its
296 transcription (Fig. 1d, table 1). We quantified the average displacement of the tagged transgene by
297 plotting the mean square displacement (MSD) to each time interval Δt (see materials and methods).
298 MSD curves calculated from time-lapse image series acquired with an inter-frame interval of 250 ms for
299 a total of 50 s are shown in Fig. 2a. We found that MSD plots of E2-activated CCND1 differed significantly
300 from those of non-activated cells (Fig.2a left panels, Fig. S2, S3). In contrast, E2 had no effect on the
301 behavior of a non-genic ANCH3-only construct integrated at the same genomic location (Fig. 2a right
302 panels, Fig. S2, S3), confirming that the measured decline in mobility was due to transcription of the

303 transgene rather than to unspecific, genome-wide effects of hormone addition. We also examined the
304 motion of the ANCH3-CCND1-MS2 or ANCH3-only constructs inserted into distinct chromosomes (G7,
305 A11 and D11 clones; Fig. 2a, Fig. S2). At the single cell level, we recorded large variations in MSD of the
306 transgene in all clones, but these variations did not correlate with any specific insertion site (Fig. S2).
307 Coherent with the observation that mobility of genetic loci are highly heterogeneous, modelling of
308 nucleosome dynamics in HeLa cells suggests that subdiffusive behavior of chromatin depends on
309 compaction levels and chromatin domain structure (30).

310 Intriguingly, motion of the ANCH3-tagged single gene locus followed two distinct regimes with an
311 increase in the slope of the recorded MSD at time intervals >5 s in these human mammary tumor cells
312 synchronized in G1 (Fig. 2b left panel, Fig. S2). The average MSD curves over 21 trajectories followed a
313 non-linear, anomalous diffusive behavior characteristic of objects moving in complex environments such
314 as the nucleoplasm (31)(Fig. S2). Hence, we analyzed these MSDs on the basis of a generalized diffusion
315 model obeying a power law $MSD \sim k\tau^\alpha$ with k as prefactor, τ the time interval, and α the anomalous
316 diffusion exponent, a model shown to reflect chromatin motion in several cell types and under various
317 conditions (32). Applying this model to our data, we demonstrate that at short <5 s time intervals
318 diffusion of the chromatin fiber of a single gene domain is highly anomalous ($\alpha < 0.4$) and subjected to
319 local constraints. At greater time intervals, the slope of the population averaged MSD curve increases
320 ($\alpha \sim 1$) suggesting that the fiber of the non-transcribed gene locus (ANCH3-CCND1-MS2 or ANCH3 only) is
321 rather mobile, almost freely diffusing. In contrast, the dynamic behavior of the mRNA producing CCND1
322 loci differs significantly: the MSD slope remains low ($\alpha \sim 0.5$) consistent with significant confinement of
323 the actively transcribed locus (Fig. 2b).

324 To fully exploit our ability to track a single gene, we computed an average squared displacement from 2 s
325 to 40 s (mean MSD) before and after induction of transcription in the same cell. For both constructs, in
326 the absence of E2, the mean MSD ranged from 0.009 to 0.290 μm^2 (Fig. 2c). This heterogeneity in the

327 amplitude of motion is coherent with variations in the nuclear environment owing to crowding in
328 mammalian nuclei (18, 33, 34). Independently of the initial mobility, mean MSDs recorded for each cell
329 producing MCP-labeled mRNA were consistently reduced after addition of E2 to ANCH3-CCND1-MS2
330 cells (Fig.2c left panel; between 0.006 and $0.090\mu\text{m}^2$; $n=15$; $p\text{-value}=0.006$). In contrast, variations in
331 mean MSD of the non-genic ANCH3 locus were not significantly affected by E2 addition (Fig. 2c right
332 panel; $n=21$; $p\text{-value}=0.3$).

333 To more accurately describe the behavior of the tracked chromatin locus, we determined its area of
334 confinement and speed in addition to time averaged MSDs which suffer from approximating
335 experimental errors (35). We found that spatial confinement of the transcribed locus reflected
336 obstructed diffusion. The nuclear area explored by the tagged locus over a 50 s time interval in the
337 absence of hormone was reduced by 27% upon addition of E2, from $0.156 \pm 0.08 \mu\text{m}^2$ (no E2, no
338 detectable MCP labeled mRNA) to $0.114 \pm 0.06 \mu\text{m}^2$ (visible mRNA accumulation at the tagged locus after
339 45 min E2, $n=26$) (Fig. 2d, table 1).

340 Confinement of the chromatin fiber could stem from steric hindrance due to protein loading or a change
341 in the physical parameters of the fiber, or both (36). We first tested whether recruitment of large
342 transcriptional co-factor complexes by hormone-bound $\text{ER}\alpha$ to the CCND1 promoter could influence
343 chromatin mobility. Similarly to E2, Tamoxifen (OH-Tam) triggers $\text{ER}\alpha$ binding to responsive promoters,
344 which leads to recruitment of numerous proteins and chromatin remodeling complexes; in contrast to
345 E2, OH-Tam-bound $\text{ER}\alpha$ attracts transcriptional co-repressors (37, 38). Following addition of $1 \mu\text{M}$ OH-
346 Tam motion of the ANCH3-tagged CCND1 locus was not confined. Motion was similar to the dynamic
347 behavior of the constructs in the absence of hormone although more homogeneous likely due to the
348 presence of co-repressor complexes (Fig. S3). We conclude that association of a multitude of
349 transcriptional co-factors recruited by OH-Tam-bound $\text{ER}\alpha$ alone, in the absence of transcription, cannot
350 explain the observed local confinement of the chromatin fiber.

351 We next assessed the role of pol2 activity on motion of the tagged CCND1 locus using two distinct pol2
352 inhibitors (Fig. 3). E2-stimulated cells were treated with an elongation-inhibitor, the adenosine analogue
353 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). In contrast to flavopiridol, DRB action is reversible,
354 rapid and homogenous after 30 min (39). CCND1 mRNA signals disappeared 30 min after addition of 50
355 μ M DRB to E2-treated ANCH3-CCND1-MS2 cells indicating that completion of elongation, i.e.
356 transcription of the 3'UTR including MS2 repeats, was efficiently inhibited (Fig. 3b). The appearance of
357 the MSD plot of the CCND1 locus tracking after DRB addition to cells maintained in E2-containing
358 medium was similar to the one in cells which had not been treated with DRB (Fig. 3b, single cell MSD
359 curves), suggesting that confined motion was sustained despite blocking elongation. Mean MSD values
360 and area of confinement for several cells analyzed 45 min after E2 stimulation and 30 min after adding
361 DRB did also not change significantly (Fig. 3c, table 1). When pre-treating cells for 30 min by DRB prior to
362 adding hormone, we again observed a rapid decline in CCND1 motion upon mRNA production in every
363 single cell analyzed (Fig. 3d). It is further known that in DRB-treated cells, transcription initiation by pol2
364 is preserved but elongation aborts rapidly within the first transcribed exon (40). Our observations let us
365 speculate that initiating but not elongating pol2 confines chromatin dynamics locally. To confirm this
366 hypothesis, we analyzed the tagged gene's motion in cells treated with 500 nM Triptolide (TPL), an
367 inhibitor of TFIIH that blocks pol2 at the promoter after transcription pre-initiation complex (PIC)
368 assembly (41, 42) (Fig. 3a). An increase in mean MSD values and in area of confinement of the ANCHOR3
369 spot's track 30 min following addition of TPL to E2-stimulated mRNA-producing cells suggests that
370 constraint of the ANCH3-tagged transgene was released by blocking initiation (Fig. 3e) (Fig. 3f, table 1).
371 Furthermore, addition of E2 to TPL pre-treated cells did not alter the recorded motion of the transgene
372 ($p=0.6$; Fig. 3g, table 1) compared to DRB pre-treated cells (Fig. 3d). Hence, our data suggest that events
373 linked to PIC formation induce changes in chromatin mobility, leading to increased local mobility at time
374 scales > 5 s within a largely confined area.

375 **Discussion**

376 Real-time tracking of a single-copy Cyclin D1 gene in the same cell prior to and during hormone-induced
377 mRNA synthesis revealed that transcription initiation rapidly confines the mRNA-producing gene and
378 alters its diffusive behavior. Confinement was maintained even upon inhibition of pol2 elongation, but
379 did not occur when recruitment of pol2 or transcription initiation was blocked by anti-estrogens or
380 Triptolide. Our results suggest that PIC formation and concomitant reorganization of the chromatin
381 domain constrain freedom of movement of an induced gene's promoter within minutes, compatible with
382 the establishment of a transcriptional hub. Indeed, pol2 aggregates in numerous rather immobile foci
383 (13, 43). Several models concur in saying that a small fraction of active pol2 forms clusters with reduced
384 mobility as transcription initiates (11, 13, 44). This clustering is dependent on the presence of initiating
385 pol2 complexes (45). But what causes pol2 to stop moving? In principle, our observation that
386 transcription initiation locally confines chromatin dynamics within minutes is compatible with the idea
387 that pol2 foci assemble at active genes, and that, as the transcription initiation bubble forms, a decline in
388 DNA freedom of movement leads to reduced pol2 mobility.

389 Regulation of the CCND1 locus has been shown to involve intragenic looping (46). Such conformational
390 changes in gene domain organization, similar to those observed during glucocorticoid stimulated
391 transcription of MMTV tandem array gene loci in mouse adenocarcinoma cells (47), are likely to have
392 direct consequences for chromatin dynamics. For instance, anchoring of several chromosome fibers
393 within pol2 foci may increase the drag coefficient and hence reduce chromatin displacements. The
394 changes in local dynamics we describe are thus compatible with reorganization of pre-existing chromatin
395 folding within the gene domain at the 100 kb range via long-range looping (1, 48, 49). Reduced roaming
396 area of the transcribed locus at constant step size increases the frequency of interaction with
397 transcriptional cofactors and polymerases of the gene within its regulatory compartment similar to what

398 was recently modelled as a ‘nanoreactor’(50). Increased collisions are compatible with the formation of
399 gene domain specific chromatin clustering (also called ‘topologically associated domains’ or TADs) readily
400 detected by crosslinking methods in mammary tumor cells (unpublished;(51, 52)). In turn, confined
401 dynamics may prevent formation of unwanted long range contacts as transcription proceeds.

402 At the sub-megabase level, TADs comprise one or a few open reading frames and their regulatory
403 elements (gene domains) (53), particularly in human mammary tumor cells (48, 51, 54, 55)(Kocanova et
404 al. in preparation). If the existence of TADs is elusive in yeast, increased ligation frequencies also occur
405 around gene bodies at the 2 kb range (56). Most of our knowledge of chromatin dynamics stems from
406 work in budding yeast (57–60). In particular, live cell chromatin motion of a series of tagged genomic
407 yeast loci fits a Rouse model of polymer dynamics, in which the MSD increases with time with a power-
408 law scaling and an anomaly exponent $\alpha \sim 0.5$ (61). Assuming that within a ~ 100 kb chromatin domain
409 around any of the tagged sites, at least one gene is actively transcribed in a population of yeast nuclei,
410 the reported dynamic behavior characterizes active chromatin. In agreement, obstructed diffusion
411 characterizes the active CyclinD1 transgene locus in human cells here. In the absence of transcription the
412 tagged single human transgene domain was highly dynamic, nearly freely diffusing. Similarly, our
413 unpublished observations in yeast evidence increased motion when mutating RNA pol2 (Socol et al,
414 submitted). Transcription-induced, pol2 dependent, intra-domain contacts therefore likely result from
415 the apparent diffusive behavior of chromatin in living cells. In mammalian cells, ATP-driven processes by
416 chromatin remodeling factors (62) or other chromatin-associated proteins such as SAF-A (63) may
417 contribute to the observed local motion and variation in anomaly. Anomalous diffusion was also
418 reported for telomeres (64) and for gene arrays (65) but changes were derived from comparing motion
419 in cells monitored under different conditions. The computed MSD curves of these loci characterized by

420 specific structures differ from the ones we compute for a single gene locus which emphasizes the need
421 for future modeling to better define physical parameters of the chromatin fiber in human cells.

422 The powerful real-time imaging approach of a single DNA locus undergoing functional changes presented
423 here using the ANCHOR system is widely applicable to other loci and genomes for studying rapid
424 biological processes with single cell resolution, and completing the picture emerging from imaging RNA
425 pol2 and mRNA, but also from chromosome conformation capture data.

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433

434 **Author contributions**

435
436 Performed experiments: TG, SK, FM
437 Analysed data: TG, SK, AB, HSh, KB
438 NW performed and analysed, AZP analysed and JE supervised the FRAP and FCS analysis.
439 Generated material: TG (cell lines), HSe (cell lines, constructs), FG (ANCHOR3 system)
440 Wrote paper: KB, TG, SK;

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446 **References**

447

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Table 1: Area of confinement listed by treatment. Indicated drugs were added sequentially (at 45 min and 75 min, or 0 min and 30 min; see also Fig. 3b, Fig. 3d, Fig. 3e and Fig. 3g). Data are shown as average +/- standard deviation.

Cell lines	Treatment	Time (min)	Area of confinement (μm^2)
ANCH3-CCND1-MS2 cells	E2 N=26	0	0.156 ±0.08
		45	0.114 ±0.06
		75	0.129 ±0.06
		105	0.101 ±0.05
	E2 + DRB N=8	0	0.143 ±0.054
		45	0.121±0.041
		75	0.172±0.150
		105	0.173±0.106
	DRB + E2 N=7	0	0.163±0.075
		30	0.254±0.114
		75	0.205±0.24
	DRB N=5	0	0.148 ±0.040
		45	0.190 ±0.064
		75	0.160 ±0.058
		105	0.109 ±0.061
	E2 + TPL N=3	0	0.154±0.057
		45	0.099±0.045
		75	0.213±0.055
		105	0.168±0.016
	TPL + E2 N=10	0	0.219±0.189
		30	0.143±0.083
		75	0.253±0.179
	No treatment N=6	0	0.118 ±0.035
		45	0.112±0.066
75		0.126±0.068	
105		0.085±0.053	
ANCH3 cells	E2 N=20	0	0.132±0.090
		45	0.137±0.096

Figures legends

Fig. 1: Real-time visualization of a single Cyclin D1 (CCND1) gene locus in human cells

(a) Schematic representation of a stably inserted construct (ANCH3-CCND1-MS2) comprising the CCND1 gene under its endogenous promoter, adjacent to a unique ANCH3 sequence, 24xMS2 repeats within the 3'UTR and a hygromycin selection gene (HYG). The construct is flanked by FRT sites for integration into MCF-7 FRT cells. Transient transfection with OR3 and MCP-tagged fluorescent proteins results in their accumulation at the ANCH3 and MS2 sequences (after estradiol (E2) stimulation), respectively (raw 3D images in supplemental video 1). (b) Fluorescent spots are easily detectable in transfected cells. A representative cell with an OR3-EGFP spot is shown. Region imaged during fluorescence recovery after photobleaching (FRAP) is indicated in orange. At time $t=0$ s a circular region enclosing the ANCHOR spot was bleached and fluorescence recovery of the spot was followed over time. Relative fluorescence intensity (RFI) was calculated according to (see Materials and methods online and Fig. S1; right panel; solid line: mean, shadowed region: lower and upper quartile; $n = 44$ cells, 4 experiments with $n \geq 6$ cells per experiment). Data were fitted to a single exponential. The 95% confidence interval is indicated in brackets. Scale bar: 2 μm . (c) Representative images of transiently transfected ANCH3-CCND1-MS2 cells expressing OR3-Santaka and MCP-EGFP (raw images in videos S1, S2). CCND1 DNA (red spot) colocalizes with transcribed mRNA (green spot) as MCP-EGFP associates with MS2 stem loops 45min after adding 100nM estradiol (E2). The same cell is shown before and after addition of E2. Scale bar = 5 μm and 2 μm (for cropped images). (d) Example of 2D trajectories and area explored over 50 s (250 ms acquisition, 200 steps) of the OR3-Santaka labeled CCND1 locus recorded before (-E2) and after (+E2) transcription activation.

Fig. 2: Cyclin D1 motion is confined during estradiol induced transcription activation.

(a) Representative single cell MSD curves of OR3-Santaka loci in ANCH3-CCND1-MS2 and ANCH3 cells at two different chromosomal insertion sites (clones G7 and A11; distinct stable FRT insertions in MCF-7 cells) before and 45 minutes after E2 addition. (b) Averaged MSD curves of OR3-Santaka loci tracking in ANCH3-CCND1-MS2 ($n=14$) and ANCH3 ($n=14$) cells before and after E2 addition. The arrow indicates the 5 s time point separating two diffusive regimes (Fig. S4 b). (c) Average squared displacement between 2 s and 40 s (mean MSD) of tracking of the OR3-Santaka spot in ANCH3-CCND1-MS2 cells (left panel, $n=15$, $p\text{-value}=0.006$) and ANCH3 cells (right panel, $n=21$, $p\text{-value}=0.3$) before and 45 min after E2 addition. (d) Area of constraint of the OR3-Santaka locus in ANCH3-CCND1-MS2 cells (left panel, $n=24$, $p\text{-value}=0.006$)

and ANCH3 cells (right panel, n=20, p-value=0.860) before and after E2 addition. The boxes show the median and 25–75 percentiles of the data. Asterisks indicate data points beyond the 95th percentile. Student's t-test: *P-values: >0.05 (ns), <0.05 (*), <0.01 (**), <0.001 (***), <0.0001 (****).

Fig.3: Transcription initiation but not elongation confines chromatin motion.

(a) Schematic representation of the steps of the E2-induced ER α -bound CCND1 gene inhibition by Triptolide and DRB during transcription initiation or elongation, respectively. (b, e) 100 nM of E2 was used to activate transcription and 50 μ M DRB to block elongation (b) or 500 nM of Triptolide (TPL) to block initiation of transcription (e). The CCND1 locus was tracked at time points before and after treatment as specified. Representative images from live cell imaging of ANCH3-CCND1-MS2 cells (clone G7) treated first with E2 for 45 min and then with DRB or TPL for 30 min, respectively (b, e). mRNA production is activated by E2 and then blocked by inhibiting transcription elongation as evidenced by the appearance and disappearance of the MCP-EGFP signal. Scale bar = 5 μ m. Successive positions of the OR3-Santaka spot are displayed in the bottom squares (122 steps, 250 ms interval). Representative single cell MSD curves of OR3 loci detected in a cell treated with E2 and then DRB. (c) Average squared displacement between 2 s and 40 s (mean MSD) of single cells (n=9) treated successively with E2 and then DRB. (d) Mean MSD (250 ms interval, 200 steps, n=7) of single cells pre-treated with 50 μ M DRB for 30 min (p-value=0.578) and then treated with 100 nM E2 for 45 min (p-value=0.031). (e, f) as (b, c), but after timed treatment with TPL. (g) Mean MSD (250 ms interval, 200 steps, n=10) of single cells pre-treated with 500 nM TPL for 30 min (p-value=0.117) and then treated with 100 nM E2 for 45min (p-value=0.065).





