Non-equilibrium forces drive the anomalous diffusion of telomeres in the nucleus of mammalian cells

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Abstract
Telomeres are vital nucleotide sequences at both ends of each chromosome, and their motion reports on the local dynamics of decondensed chromatin in the nucleus of interphase cells. Here, we show that the previously reported subdiffusive motion of telomeres is driven by non-equilibrium cytoskeletal forces. In particular, breaking down microtubules leads to a significantly reduced generalized diffusion coefficient of telomeres. This translates into a markedly reduced effective temperature in the stochastic forces that govern the telomeres’ random walk. Moreover, telomere motion in cells that lack microtubules is well described by the monomer dynamics of a Rouse polymer that is embedded in a viscoelastic medium. In contrast, active cytoskeletal forces in untreated cells override the environment’s elastic contributions, resulting in the well-known scaling for conventional Rouse dynamics in viscous media. Our data highlight that even subdiffusive motion in cells in most cases may not be a simple thermal transport process but rather is driven by non-equilibrium events.

1. Introduction
The nucleus is the defining organelle of eukaryotic cells [1]. Between cell divisions, i.e. in interphase, the nucleus harbors the cell’s DNA in a decondensed form that permits transcriptional processes, hence fueling the de novo synthesis of vital proteins. Even in this state, however, the DNA retains its organization into (decondensed) chromosomes whose ends are marked by specific sequences of bases, the so-called telomeres. Telomeres not only act as simple end markers but they also have been shown to stabilize the DNA’s secondary structure and to reflect on the cell’s age and reproduction ability (see, for example, [2] for a recent review). Since decondensed chromosomes occupy distinct nuclear subvolumes [3], telomeres are not randomly dispersed in the nucleus but rather highlight the locations of the associated chromosome territory.

The motion of telomeres has been used as an indicator for the DNA’s dynamics in vivo [4], potentially reflecting how well accessible the DNA is for regulatory proteins or the transcription machinery. Detailed single-particle tracking experiments on telomere-bound TRF-2 proteins have revealed that the motion of telomeres in mammalian cells exhibits a subdiffusive characteristics on short and intermediate time scales [5]: the telomeres’ mean square displacement (MSD) was seen to display a sublinear power-law increase, \( \langle r^2(t) \rangle \sim t^\alpha \) with \( \alpha \approx 1/3 \) and \( \alpha \approx 1/2 \) on short and intermediate time scales, respectively. The observation of a diffusion anomaly \( \alpha < 1 \) has been rationalized partly in terms of the subdiffusive motion of a monomer within the DNA polymer, but also an explanation in terms of a continuous time random walk (CTRW) has been discussed. The most prominent feature of a CTRW is its weak ergodicity breaking [6, 7] which is manifested in different scalings of the time- and ensemble-averaged MSD (see, for example, [8–10] for comprehensive reviews on this aspect). A subsequent analysis suggested that the motion of telomeres rather is consistent with a fractional Brownian/Langevin dynamics [11] rather than to be of a CTRW type. Very recently, a rapid and almost normal diffusive motion of telomeres has been reported when lamin \( A \) was knocked down [12]. This observation strongly suggests that lamin \( A \) proteins not only connect the decondensed chromatin to the nuclear envelope but also act as crosslinkers within the chromatin bundle.
While considerable effort has been invested into elucidating the type of stochastic process that describes the motion of telomeres on a mesoscopic scale, the nature of the involved random forces has been left mostly unexplored. In particular, the tacit assumption that thermal noise is driving the (sub)diffusion of telomeres has not been probed. In fact, the assumption of a mere thermal driving of the (transient) subdiffusion of nanoparticles in the cytoplasm and nucleoplasm has been challenged recently: the observation of an anisotropically varying diffusion anomaly in dividing cells [13], most likely caused by protein traffic along the mitotic spindle, and the emergence of a glass-like diffusion arrest in energy-depleted bacteria [14] highlight the importance of active processes for intracellular diffusion processes.

Here, we show that the subdiffusive motion of telomeres in the nucleus of interphase cells is driven by active stochastic forces of the microtubular cytoskeleton rather than being a mere thermal transport process. Upon disrupting microtubules, the telomeres’ generalized diffusion coefficient was seen to be significantly reduced. As a consequence, also the effective temperature assigned to the stochastic forces of the telomeres’ random walk was markedly lower. Given the strong coupling of nuclear envelope and chromatin, and bearing in mind the lack of long-range transport processes within the nucleus, the stochastic shaking of the entire nucleus by the cytoskeleton arguably is the dominant source of active noise for intra-nuclear diffusion processes. Therefore, telomere motion in cells with disrupted cytoskeleton most likely represents a very good proxy for the thermally driven diffusion process. Consistent with this reasoning we observed that telomere motion in cells that lack microtubules is well described by the monomer dynamics of a Rouse polymer embedded in a viscoelastic medium. In contrast, active cytoskeletal forces in untreated cells seemed to override the environment’s elastic contributions, resulting in the well-known scaling for conventional Rouse dynamics in viscous media.

Altogether, our data suggest that a non-negligible part of the random forces that drive (sub)diffusional processes in the nucleus of mammalian cells are of genuine non-equilibrium nature.

2. Materials and methods

Bone osteosarcoma cells (U2OS, DSMZ- No. ACC 785) were grown at 37 °C and 5% CO₂ in McCoy’s medium with phenol red (Biochrom, Germany) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin. For visualizing telomeres we used a previously characterized GFP-tagged TRF-2 construct [5, 15] that recognizes the human telomeric sequences TTAGGG. Transient transfection with the plasmid, a kind gift of Y. Garini (Bar-Ilan University, Israel), was performed 24 h prior to data acquisition using Peqlab (Peqlab, USA) according to the manufacturer’s protocol (2 µl Peqlab, 1 µg plasmid DNA in 100 µl serum free Opti-MEM from Thermo Fisher Scientific, Germany).

For live-cell microscopy, cells were plated in 4-well µ-slide microscopy chambers (ibidi, Martinsried, Germany) at a density of ~75 000 cells/well. For imaging, the medium was changed to MEM without phenol-red supplemented with 5% FCS and 5% HEPEs (referred to as ‘imaging medium’ in the remainder). For disrupting the microtubular cytoskeleton the imaging medium was supplemented with 20 µM nocodazole (Sigma-Aldrich, Germany) which blocks microtubule growth. Cells were chilled on ice for 10 min to induce microtubule depolymerization. Then cells were allowed to relax at 37 °C for 15–30 min before imaging in the presence of the drug. As control samples, cells were fixed 24 h after transfection by incubating them with 4% paraformaldehyde (PFA) in 1x DPBS (Biochrom, Germany) at room temperature for 15 min. Fixed samples were stored in 1x DPBS containing 1% PFA at 4 °C.

Imaging was performed with a customized spinning-disk confocal microscope based on a DMI 4000 stand (Leica Microsystems, Germany), a CSU-X1 spinning-disk unit (Yokogawa Microsystems, Japan), an HC PL APO 100x/1.40NA oil immersion objective, and an Evolve 512 EMCCD camera (Photometrics, USA). Live-cell imaging was performed with an autofocus feedback at 37 °C using a custom-made incubation chamber. Samples were illuminated at 488 nm and fluorescence light was detected in the range 500–550 nm. Confocal images (single optical slices of the cell) were taken at an interval of 175–200 ms and exposure times of 75–150 ms for a total time of 5–7 min.

3. Results and discussion

In order to study the nature of the stochastic forces that drive the motion of telomeres within the nucleus of mammalian cells, we expressed and monitored TRF-2 (coupled to green fluorescent protein, GFP) that has been shown before to reliably recognize the human telomeric sequences TTAGGG [5, 15] (see Materials and Methods for details). In line with previous reports [5], telomeres were readily visible as punctuate nuclear pattern in confocal fluorescence images, see figure 1(a). Applying to each image a simple sequence of dilation and erosion steps reduced the image contents to a single nucleus while any fluorescence outside the chosen nucleus was masked, see figure 1(b). Time series of masked images were evaluated with the Matlab Particle Tracking Code
(site.physics.georgetown.edu/matlab), and only telomere trajectories that spanned the total time series were retained for further analysis.

The acquired time-lapse movies revealed that telomeres were mobile but also a collective motion of the nucleus and/or the entire cell was occasionally observed. Being interested in the motion of individual telomeres rather than contributions from a putative stage or nucleus drift, we aimed at correcting for this global motion. Since the contrast between cytoplasm and nucleus was very low (see figure 1(b)), identifying the motion of the nucleus via simple masking approaches turned out to be unstable. We therefore used the convex polygonal envelope of the retained telomeres instead, see figure 1(c). The center-of-mass trajectory of this polygonal area turned out to be a stable and faithful approximation of the global nucleus movement, and was hence subtracted from the telomere trajectories of the respective cell. Representative, drift-corrected telomer trajectories are shown in figure 1(d). Please note that the results below are not affected by this correction approach since the global motion disturbs the local motion pattern only on large time scales. In fact, evaluating uncorrected trajectories lead to the same gross results but an earlier crossover of telomeres to a superdiffusive motion was observed.

For each individual telomer trajectory, consisting of \(i = 1, \ldots, N\) two-dimensional coordinates \((x_i, y_i)\) separated by a time lag \(\Delta t\) (about 200 ms, see Materials and Methods), we calculated as a first quantitative observable the time-averaged MSD

\[
\langle r^2(k\Delta t)\rangle_t = \frac{1}{N - k} \sum_{i=1}^{N-k} (x_i - x_{i+k})^2 + (y_i - y_{i+k})^2.
\]  

In addition, we calculated the average of \(\langle r^2 \rangle\) over the telomere ensemble within a single cell, \(\langle \langle r^2 \rangle \rangle_e\). Representative examples of \(\langle r^2 \rangle\) and the corresponding \(\langle \langle r^2 \rangle \rangle_e\) for untreated cells are shown in figure 2(a). A
transient subdiffusive increase of both, \( \langle r^2(t) \rangle \), and \( \langle \langle r^2(t) \rangle \rangle \), is observed for small time scales that eventually crosses over to normal diffusion, consistent with previous observations \([5]\). A similar overall scaling is also seen for cells in which microtubules have been disrupted by nocodazole (see Materials and Methods for details) albeit with markedly lower values for the MSDs, see figure 2(b). In strong contrast to living cells (untreated and with nocodazole treatment), fixed cells only displayed very small and almost constant MSDs that basically reflect the precision limit of the tracking approach, see figure 2(c).

In particular, the MSD offset \( r_0^2 = \langle r^2(\Delta t) \rangle \), of individual trajectories of immobilized telomers in fixed cells followed a lognormal distribution with parameters \( \mu = -8.3 \) and \( \sigma = 0.7 \), yielding an arithmetic mean \( \langle r_0^2 \rangle \approx 3.2 \times 10^{-4} \mu m^2 \). While it is tempting to simply subtract this positive offset from individual MSD curves of mobile telomers to correct for the finite localization precision, another effect challenges this straightforward approach: recording an image takes a finite time \( \Delta t \) during which diffusing objects are constantly moving. As a result, a negative offset emerges in the MSD which depends on the frame time and particle mobility [16]. Using the diffusion constant \( D \approx 7 \times 10^{-4} \mu m^2 s^{-1} \) for freely diffusing telomers in lamin A deficient cells [12] as an estimate for the general short-term motion of telomers, the magnitude of this negative offset \((4D\Delta t/3 \approx 2 \times 10^{-4} \mu m^2)\) approximately equals the (positive) average localization error, \( \langle r_0^2 \rangle \). Hence, the true MSD of mobile telomers is plagued for short times by two opposing additive contributions that approximately cancel out each other in our case. We therefore have chosen to not add or subtract a somewhat arbitrary offset when evaluating the MSDs. In fact, subtracting a residual positive offset \( \langle r_0^2 \rangle = 4D\Delta t/3 \approx 10^{-4} \mu m^2 \) did not alter the conclusions drawn below. The distribution of individual step increments, however, can be corrected easily by defining a cutoff below which step sizes are deemed insignificant. Accounting for the lognormal distribution of localization errors obtained from fixed cells, we have used the geometric mean and standard deviation to define a safe upper bound \( e^{\mu+\sigma} = 5 \times 10^{-4} \mu m^2 \) for this cutoff, below which individual squared displacements were not considered.

Figure 2. Representative time-averaged MSDs, \( \langle r^2(t) \rangle \), as defined in equation (1) (thin gray lines) and the corresponding ensemble averages, \( \langle \langle r^2(t) \rangle \rangle \), of the same cell (red lines) found for telomers in single U2OS cells (a) without treatment, (b) after disrupting the cytoskeleton with nocodazole, and (c) after fixation. In living cells, an initial subdiffusive growth \( r^2(t) \sim t^\alpha \) with \( \alpha \approx 0.5 \) is observed that eventually crosses over to normal diffusion (dashed lines indicate the scaling). As expected, in fixed cells (subfigure c) no significant growth of the MSD is observed (please also note the tenfold lower MSD values as compared to living cells). (d) The distribution \( p(\xi) \), here shown for \( t = 5\Delta t \), that can be used as an indicator for weak ergodicity breaking (see main text and figure 2(c) for definition) is very similar for untreated (gray-shaded) and nocodazole-treated (red) cells. In particular, a vanishing mean \( \langle \xi \rangle \) in both cases indicates the absence of a weak ergodicity breaking in the telomers’ motion.
Figure 3. (a) Distributions of anomaly exponents, $p(\alpha)$, as obtained from the time-averaged MSDs, $\langle r^2(t) \rangle$, of individual telomeres. A highly significant shift to smaller anomaly values (as reported by a Kolmogorov–Smirnov test) is seen for cells in which the cytoskeleton was disrupted by nocodazole (red lines, $n = 762$ telomeres) as compared to unperturbed cells (gray-shaded, $n = 579$). Almost vanishing anomaly exponents, i.e. an almost constant $\langle r^2(t) \rangle$, is found for fixed cells (blue, $n = 268$). Mean values of $p(\alpha)$ are stated explicitly. Anomaly exponents $\alpha$ determined from $\langle r^2(t) \rangle$ (colored dashes above the histograms) are consistent with the distributions $p(\alpha)$ and feature the same mean values. (b) In line with the results for $p(\alpha)$, also the distributions of generalized diffusion coefficients, $p(D_\alpha)$, showed a highly significant shift to smaller values when the cytoskeleton was disrupted (red) as compared to untreated cells (gray-shaded). Mean values changed from $\langle D_\alpha \rangle = 0.0023 \mu m^2/s^\alpha$ to $\langle D_\alpha \rangle = 0.0012 \mu m^2/s^\alpha$ upon treatment with nocodazole. As before, colored dashes above the histograms indicate the consistent results found via $\langle r^2(t) \rangle$. Fixed cells, for which no significant increase of the MSD had been observed $\langle \alpha(t) \rangle \approx 0$ featured even lower transport coefficients (blue). These data provide a method-intrinsic uncertainty level below which individual squared displacements can be rated insignificant (see main text for details).

Complementing an earlier study on the telomeres’ type of random walk [11], we used the relative deviations of $\langle r^2(t) \rangle$ and $\langle r^2(t) \rangle_{\text{fix}}$, defined via

$$\xi_t = \langle r^2(t) \rangle / \langle r^2(t) \rangle_{\text{fix}} - 1,$$

for testing a potential weak ergodicity breaking. In agreement with previous reports that the motion of telomeres is well described by a fully ergodic fractional Langevin equation for testing a potential weak ergodicity breaking. In agreement with previous reports that the motion of telomeres was found to be significantly different (p-values < 0.1%). For fixed cells we observed an almost vanishing mean exponent $\langle \alpha(t) \rangle \approx 0.02$, as expected for an approximately constant MSD.

The generalized diffusion coefficients were affected even stronger by the absence of microtubules, see figure 3(b): the distributions $p(D_\alpha)$ highlight a roughly two-fold reduction of the mean generalized diffusion coefficient upon disrupting the cytoskeleton $\langle D_\alpha^{\text{noc}} \rangle / \langle D_\alpha^{\text{fix}} \rangle \approx 1.9$. Again, a Kolmogorov–Smirnov test as well as a Student’s t-test rated the associated distributions $p(D_\alpha)$ to be significantly different (p-values < 0.1%). Thus, telomeres move significantly less when lacking the microtubule-mediated rocking of the nuclear
envelope, in accordance with the aforementioned lamin-A mediated coupling of chromatin and the nuclear envelope. As expected, fixed cells showed even lower transport coefficients which, together with the previously found $\langle \alpha \rangle \approx 0.02$, indicates a small and approximately constant MSD.

Following an earlier analysis of telomere motion [5] and bearing in mind that TRF-2 only labels a short DNA sequence, an interpretation of the data in terms of the Rouse model for polymers appears adequate. Adopting this view, the reported particle tracks represent the motion of an average monomer within a polymeric coil for which one expects

$$\langle r^2(t) \rangle = D_\beta t^{\beta}, \quad D_\beta = \left( \frac{4 b^2 k_B T}{3 \pi \gamma} \right)$$

with $\beta = 1/2$ on time scales below the Rouse time, $t_R \sim L^2/\langle k_B T \rangle$. Here, $k_B T$ denotes thermal energy, while $b$ and $L$ are the effective monomer size and polymer length, respectively. At thermal equilibrium, the fluctuation-dissipation theorem for a monomer therefore yields the relation $D_\beta \sim \sqrt{k_B T/\gamma}$.

The Rouse exponent $\beta = 1/2$ is in favorable agreement with our experimental data for the mean anomaly exponent $\langle \alpha \rangle$ of telomere motion in (un)treated cells (see figure 3(a)). Neglecting the small, but systematic deviation of $\langle \alpha \rangle$ from $\beta = 1/2$ in case of nocodazole-treated cells (these will be discussed below), the measured generalized diffusion coefficients (see figure 3(b)) can be taken as an experimental proxy for $D_\beta$, as defined in equation (3). Assuming the general form of the fluctuation-dissipation relation to be preserved at non-equilibrium conditions but with an effective temperature, the ratio $\langle \langle D_\beta^{\text{noc}} \rangle \rangle / \langle D_\beta^{\text{eff}} \rangle = T_{\text{eff}}^{\text{noc}} / T_{\text{eff}}^{\text{eff}}$ may be used to estimate the non-equilibrium part of the stochastic forces that govern the telomeres’ random walk: due to a lack of long-range active transport processes within the nucleus, e.g. via molecular motors, the effective temperature found for nocodazole-treated cells presumably is very near to the mere (constant) thermal value, $T_{\text{eff}}^{\text{noc}} \approx T$. Therefore, the roughly twofold difference in $D_\beta^{\text{noc}}$ between untreated and nocodazole-treated cells suggests $T_{\text{eff}}^{\text{noc}} \approx 4T$, i.e. microtubule-based random forces enhance the basic thermal motion of telomeres with an additional active energy of $\sim 3k_B T$. While this additional energy may look quite large at first glance, even higher contributions have been reported for driven colloids [18].

Using an effective temperature in this discussion certainly is somewhat unsatisfactory since for actively driven colloids an additive contribution to thermal noise in the fluctuation-dissipation theorem has been calculated and measured explicitly [18]. Lacking an externally controlled potential, however, a particle’s random walk, e.g. in the formulation of an overdamped Langevin equation, only depends on a single parameter, $T_{\text{eff}} / \gamma$. In other words, without additional knowledge of the precise length and/or time scale that shall be expected, the sole random walk trajectory cannot reveal the magnitude of an active contribution that is hidden in an effective temperature.

Let us now return to the small but significant deviation of the mean anomaly exponent from the Rouse scaling in nocodazole-treated cells. For the common Rouse scenario a Markovian viscous environment is assumed. Yet, intracellular fluids as well as artificial crowded solutions have been shown to exhibit a non-zero viscoelasticity on small length and time scales [19, 20] with a sublinear frequency scaling of the complex shear modulus, $|G(\omega)| \sim \omega^{-\nu} (\nu < 1)$. As a consequence of this viscoelasticity, the diffusion of small particles was shown to be governed by fractional Brownian motion [21] with an MSD $\langle r^2(t) \rangle \sim t^\nu$ as a key feature. Hence, when modeling DNA as a Rouse polymer, a viscoelastic rather than a viscous environment needs to be assumed. Yet, intracellular temperature.

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Considering dense dextran solutions as a reasonable model for the interior of the nucleus suggests $\nu \approx 0.82$ [23] and hence leads to $\beta = 0.41$, in very good agreement with our experimental data for $\langle \alpha \rangle$ in nocodazole-treated cells, c.f. figure 3(a).

The generalized scaling exponent $\beta$ also determines the short-term behavior of the velocity autocorrelation function of monomers within the Rouse chain [22]: the bonding to neighboring monomers leads to a short-term anti-correlation of diffusional steps that decays towards zero as $C(\tau) \sim -C_0 / \tau^{2-\beta}$. To test this prediction, we averaged the negative velocity autocorrelation function over all telomer trajectories in untreated and in nocodazole-treated cells, respectively. Indeed, these data agree well with the anticipated power-law decay when replacing $\beta$ by the respective values found for $\langle \alpha \rangle$, see figure 4(a). Moreover, the prefactor $C_0$ is reduced approximately twofold upon disrupting the cytoskeleton, as expected already from the generalized diffusion coefficients.

So how to rationalize that the nuclear environment appears viscoelastic for cells without cytoskeleton but is more or less viscous for untreated cells? Given that chromosomes occupy distinct territories, the degree of entanglement of the chromatin will be quite low. Therefore, the emergence of an elastic contribution of the medium most likely will be driven by (un)specific interactions in the range of few $k_B T$. Driving the fluid out of equilibrium via microtubules, i.e. adding random forces with an average energy of $\sim 3k_B T$, may be enough to overcome these interactions, i.e. to compensate the environment’s local restoring forces, rendering the
surrounding almost viscous to telomeres. Therefore, also the MSD's scaling exponent $\alpha$ would report on the non-equilibrium driving in the presence of an intact cytoskeleton. Finally, we also inspected the cumulative distribution of normalized step increments, $P(r)$. As discussed above, we have neglected here individual squared increments below a cutoff value $5 \times 10^{-4} \mu m^2$. For two-dimensional Gaussian random walks, the increments' probability distribution function for any observation period $\tau$ is given by $P(r) = 1 - e^{-r^2/\sigma^2}$ [21], with $\sigma^2 = \langle r^2(\tau) \rangle$ being the respective MSD. Plotting $1 - P(r^2)$ versus $r^2$ for the recorded telomere trajectories in a semilogarithmic fashion therefore should reveal a linear decay. Indeed, data for single telomeres agree with the prediction for a Gaussian random walk, $1 - P(r^2) \sim -r^2$ (indicated by gray dashed lines as a guide to the eye). Please see main text for discussion.

In conclusion, we have shown that the subdiffusive motion of telomeres in the nucleus of mammalian cells is strongly dependent on non-equilibrium forces induced by the cytoskeleton. Disrupting microtubules was seen to significantly reduce the effective temperature in the stochastic forces that govern the telomeres’ random walk. Picturing the motion of telomeres as monomer dynamics of a Rouse polymer embedded in a viscoelastic medium, the contributions of the active cytoskeletal forces most likely are responsible for overcoming the environment’s elastic contributions, i.e. the medium appears more viscous than viscoelastic. Our data highlight

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**Figure 4.** (a) The ensemble-averaged velocity autocorrelation function of the telomeres’ motion shows a significant anti-correlation ($\langle C(\tau) \rangle < 0$) for short times in untreated and nocodazole-treated cells (black circles and red squares, respectively). The anticipated power-law decay $-\langle C(\tau) \rangle \sim C_\alpha \tau^{\alpha-1}$ (dashed lines with $\alpha$ as given in figure 3(a)) is in good agreement with the experimental data. In agreement with the ratio of generalized diffusion coefficients also a two-fold reduction in $C_\alpha$ is observed upon depolymerization of microtubules. Please note: the exceptionally high value $C(\tau = \Delta t)$ for the shortest time lag also contains contributions from the finite localization accuracy [17]. (b) The cumulative distribution function of step increments between successive frames, collected from all telomeres in a cell ensemble and displayed here as $1 - P(|r|)$, shows an almost linear decay in a semilogarithmic plot for untreated and treated cells (black and red lines, respectively). This suggests an exponential rather than a Gaussian distribution of increments. Inset: in contrast, data for individual telomeres agree with the prediction for a Gaussian random walk, $\ln(1 - P(r^2)) \sim -r^2$ (indicated by gray dashed lines as a guide to the eye). Please see main text for discussion.

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7
that even subdiffusive motion in cells in most cases may not be a simple thermal transport process but rather should be interpreted as being driven by non-equilibrium events.

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