Dynamic clustering regulates activity of mechanosensitive membrane channels

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Mechanosensitive channels regulate the flux of solutes between the cell and its environment in response to mechanical stimuli. Recent *in vitro* experiments have suggested that bacterial mechanosensitive channels separate into 2D liquid-like clusters, exhibiting cooperative activity. To understand the functional role of the process and its physical mechanism, we develop a coarse-grained model of mechanosensitive channels embedded in a membrane, exposed to an osmotic shock. We find that the channel aggregation into liquid-like clusters promotes the closure of individual channels. The extent of clustering is highly dependent on the channel concentration and membrane stress. We show that this behaviour yields a tightly regulated gating system, whereby at high tensions channels gate individually, and at lower tensions the channels spontaneously aggregate and inactivate. We suggest that this positive feedback protects the cell against excessive loss of cytoplasmic content. Our results point to a novel role of functional micro-phase separation in the cell membrane.

Both eukaryotic and prokaryotic cells harbour a phospholipid membrane packed with proteins, which enables separation of cellular content from the external environment. This physical barrier facilitates transport of signals and materials between the cell and its environment, thus sustaining life [1]. In addition, membranes of unicellular organisms separate the cell from the outside world, and as such need to be able to respond quickly and efficiently to sudden changes in the cell's surroundings. One of the ways the membranes respond to external stimuli is by reorganising associated macromolecules [2]. A characteristic example of such a behaviour are membrane mechanosensitive channels (MSCs), which respond to mechanical cues from the cell's surrounding, and are central to senses of hearing, balance, and touch, as well as for ensuring cell osmotic homeostasis [3–5].

Arguably, the best studied MSCs are those of bacterium *Escherichia coli*, whose role is to protect the cell against sudden drops in the environmental solute concentration, so called hypoosmotic shock [6, 7]. *E. coli's* cell envelope is composed of two membranes and a stiffer cell-wall between them, which allows it to sustain internal pressures in the range of 0.3 to 3 atm [8, 9]. Upon hypoosmotic shock water rushes into the cell, resulting in the cell swelling and increased tension in the bacterial envelope [6, 7]. If left unchecked, this pressure can lead to cell death by rupturing the envelope [10, 11]. To prevent it, a portfolio of MSC in *E. coli's* inner membrane act as "pressure release valves" that open and create a nano-sized pore at the centre of the protein. This in turn enables solute and water efflux, reestablishing desired osmotic pressure inside the cell [12–14]. This response is fast and solely regulated by the membrane tension and chemical potential of water and solutes [14].

Bacterial MSCs consist of closely-packed transmembrane helices connected by loops [15, 16]. Driven by membrane tension, the helices are thought to tilt with respect to one another, creating a space between them (up to 3 nm in diameter) for small solutes to non-selectively pass through [17]. Recent studies debate the existence and the role of spontaneous clustering of one of the MSCs found in *Escherichia coli*, MSC of large conductance (MscL) [18, 19]. Indeed, membrane clustering appears to be a common mechanism in cellular signaling, and has been observed for many transmembrane proteins and signaling receptors [20]. Clustering of MSC *in vitro* has been shown to result in collective, non-linear gating behavior [18], suggesting that it could tamper with cell's passive response during a hypoosmotic shock recovery, for better or worse. However, assessing the extent of MscL aggregation via imaging techniques *in vivo* has proven to be difficult due to the potential artifacts of the MscL tags on the process [19], and hence opens a need to explore orthogonal ways to assess the aggregation phe-

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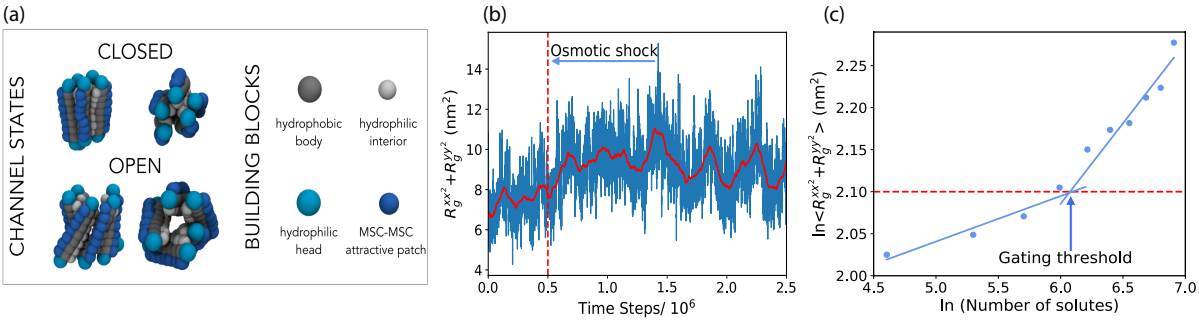

FIG. 1: **Coarse-grained model and single channel properties.** (a) MSC is presented as a collection of rods connected by weak springs. Each rod consists of overlapping hydrophobic beads (depicted in gray) and hydrophilic heads (in cyan), and is $\sim$ 10 nm long. Channel inside is lined with hydrophilic stripes (silver). Explicit inter-channel attractions can be turned-on via an external hydrophobic patch (dark blue). (b) Pore size oscillations of a single MSC. The dashed vertical red line marks the occurrence of an instantaneous osmotic shock corresponding to the membrane tension of $\gamma = 1.2$ mN/m, which leads to an increase in the average pore size. The solid red line represents the moving time average (window size 10^5 time steps). (c) The variation of the pore size with the shock magnitude for a single channel. The sudden expansion of the pore size can be seen around $\gamma = 0.50$ mN/m, corresponding to ${R_G^{xx}}^2 + {R_G^{yy}}^2 = 8.2$ nm^2, which we choose as the threshold for channel opening.

nomenon.

By developing a near-minimal computer model of MSCs embedded in a fluid membrane, we investigate the physical mechanisms behind the MSC cluster formation and cooperative gating, and their implications on cell-volume regulation. We characterise the gating properties of a single channel and subsequently consider minimal requirements for the formation of stable MSCs clusters. We find that channels form clusters of liquid-like properties that promote the closure of individual channels, and that the gating activity is strongly dependent on the cluster size. Furthermore, the cluster size itself is strongly dependent on the magnitude of the osmotic shock, establishing a positive feedback between the channel aggregation and gating properties. We then expand our previously reported model of *E. coli's* volume recovery to hypoosmotic shocks [14] to include the preceding positive feedback, and find that the MSC clustering protects the cell from losing excess solutes without compromising cell volume and pressure recovery upon a hypoosmotic shock.

Near-minimal model of MSCs. The scale at which the key physics of MSC function takes place is between the single-channel and continuous approaches, and suited for minimal coarse-grained models that capture multiple channels' interaction on a membrane patch. Guided by the known structures of single isolated MSCs [15, 16, 21], we built a generic MSC model out of rod-shaped subunits connected by weak springs (Fig.1a). While bacterial MSCs possess varying number of repetitive helical subunits, from $\sim$ 4 to several tens [7], without loss of generality, we choose to include five rod-shaped subunits. Each rod is made of seven core hydrophobic, and two hydrophilic head beads (Fig. 1 a). The rods are longer than the membrane thickness to reproduce the hydrophobic mismatch between the protein and the lipid layer found in structural studies [15]. To ensure the α-helical transmembrane core is straight, the beads belonging to a single rod are connected by strong elastic bonds and angular potential. We describe the lipid bilayer with a previously published three-beads-per-lipid model [22] (Fig. S1 and S2). The single rod diameter is twice the radius of a lipid bead, and the inner part of the channel is lined with hydrophilic beads to prevent lipids from overflowing inside the channel. Finally, to model explicit non-specific inter-protein attractions, an attractive patch of beads is added on the external side of each rod. To generate a hypoosmotic shock, we place a gas of inert "solute" beads on one side of the membrane, allowing it to interact with the channels and the membrane via volume-excluded interactions only. The collisions of solute beads with the membrane create membrane tension, which is linearly proportional to the solute concentration difference across the membrane (Fig. S4). For further details see Supplementary Information.

Coarse-grained model of single channels captures gating under tension. We first focus on the behaviour of a single MSC. The application of the hypoosmotic shock causes an increase in the membrane tension and area. Since the transmembrane components of the MSC interact attractively with the hydrophobic layer of the membrane, they maintain contact with the expanding lipid bilayer. Consequently, channel rods tilt with respect to one another, resulting in the overall lateral expansion of the channel, Fig. 1 and Video1. To quantify the channel pore size we measure the in-plane compo-

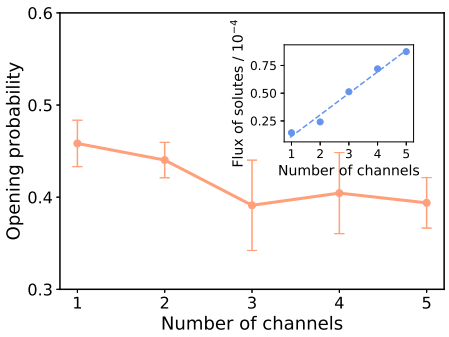

FIG. 2: **Multiple channels interacting via membrane-mediated interactions gate as single channels.** The probability of channel pore opening versus the number of channels present in the system. The error bars correspond to the standard deviation of five runs with different initial seeds. Inset: total flux of solutes through the channels versus the number of channels in the system. Channel area fraction ranges from 0.03 ($N = 1$) to 0.15 ($N = 5$).

nents of the MSC radius of gyration tensor (see Supplementary Information). We find that the pore size oscillates stochastically, and that the application of hypoosmotic shock leads to an immediate increase in the pore size (Fig. 1b), allowing for the passage of the solutes and channel gating. To determine the threshold of opening of the channel we measure the average pore size at different values of hypoosmotic shocks. As shown in Fig. 1(c), the pore size increases sharply around the surface tension of $\gamma = 0.50$ mN/m, which we take as the threshold tension for the pore opening. Above this threshold value we consider the MSCs "open"; for values below it, we deem the channel "closed".

Multiple channels interacting via membrane-mediated interactions gate as single channels. We now look at multiple MSCs interacting only via volume exclusion and effective membrane-mediated interactions. Fig. 2 shows the gating properties of such MSCs as a function of the number of channels in the system. The flux of solutes passing through the portion of open MSCs is also tracked (inset in Fig. 2). It is evident that each channel behaves independently. Indeed, we find that the membrane-mediated interactions in our system are minimal (Fig. S7), despite imposing a significant hydrophobic mismatch of $\approx$ 5nm between the proteins and the membrane. Based on these results, we chose a top-down strategy instead. We know that: (i) non-specific inter-protein interactions can drive protein aggregation in trans-membrane protein systems [20] (ii) MSC aggregation has been reported *in vitro* [18], and *in vivo* while working with MSCs labelled with a small covalent dye [19]. Therefore, to drive MSC aggregation we incorporated weak inter-protein attractions, modelled via a hydrophobic stripe on the outer side of the channel (Fig. 1a).

Multiple channels interacting via non-specific inter-protein attractions exhibit cooperative gating Incorporating weak non-specific inter-protein attraction leads to the assembly of MSCs into small clusters of sizes between 2 and $\sim$ 15 MSCs. The stronger the inter-protein attraction, the larger and more stable the clusters are. We find that MSCs exhibit strong cooperative gating within the clusters. Fig. 3(a) shows the pore sizes of two attractive channels as a function of the separation between them. Sharp decrease in the pore sizes at $\sim$9.5 nm channel separation corresponds to the cooperative closure of individual MSC (Video 2). The reason for this is purely geometrical and is encoded in the general channel shape - two closed channels can achieve larger contact area between them, maximising their attraction. For multiple channels diffusing in the bilayer we observe dynamic rearrangement and aggregation into larger clusters that leads to decreased gating activity per channel, which scales with the cluster size (Fig. 3b). Simply, the more neighbours a channel has, the more favourable the closed conformation that maximises the inter-channel attraction. We also find that the clusters are dynamic in nature, whereby individual channels within the clusters oscillate between the closed and open states, (Fig. 4b) can move within, leave the cluster, or join another.

Since the channel activity depends on the number of neighbours, individual channel activity within a single cluster is consequentially inhomogeneous. The channels on the cluster interior will on average gate less than the channels sitting at the aggregate rim (inset in Fig. 3b). The average channel activity will hence depend not only on the cluster size, but also on its shape, e.g. elongated clusters exhibit higher gating activity compared to the compact ones.

Osmotic shock magnitude controls protein aggregation We now perform a computational experiment to mimic the situation in which a bacterial cell, living under quiescent conditions, encounters a sudden hypoosmotic shock. We start our simulation with a membrane that contains channels all aggregated into a single cluster. We then applied a sudden tension of 1.5 mN/m, and monitored the system in time. As a control, we repeated the same simulation at zero tension (Fig. 3c). We find that the high magnitude shock breaks up the MSC cluster into individual channels, switching the system from the clustered to the mixed state (Video 3 and 4). Such isolated channels open with higher probability (inset in

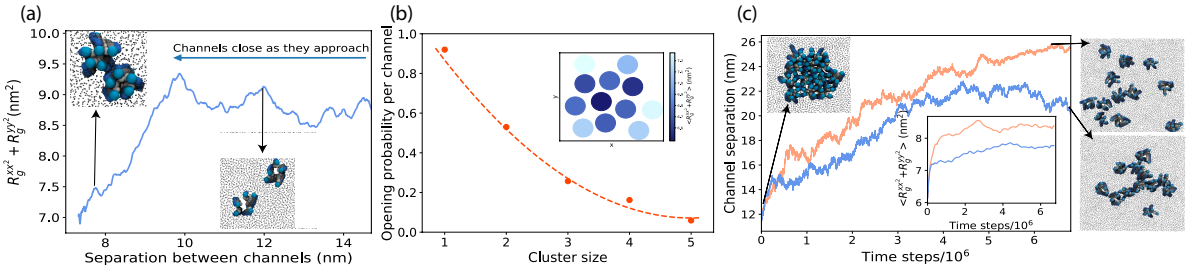

FIG. 3: **Channels interacting via explicit attractive interactions exhibit cooperative gating.** (a) Average pore size of two MSCs as a function of distance between them ($\gamma = 1.30$ mN/m). (b) Single channel opening probability versus aggregate size ($\gamma = 1.70$ mN/m and the channel area fraction is 0.16). Inset: The average pore size of channels within a single cluster formed of twelve MSCs. (c) The average distance between the channels increases in time and is larger for higher membrane tension ($\gamma = 1.70$ mN/m, shown in orange versus $\gamma = 0.70$ mN/m, shown in blue) Far right: The snapshot of the cluster configuration in the last time-frame. Inset: The probability of channel opening increases as the simulation progresses. The interchannel attraction in all the subfigures is $\varepsilon_{protein-protein} = 0.9$kT.

Fig. 3c), enabling efficient gating when it is needed most. On the other hand, at low applied tensions the channels remain in a cluster, albeit the cluster shape dynamically elongates (Fig. 3c).

These findings suggest that the spontaneous formation of liquid-like MSC clusters enables an additional level of control over their gating and signal transduction. This control is implemented in the system in a passive way, hard-wired in the system's physical properties. On average, single channels are more closed at low membrane tensions, making them more aggregation-prone, which in turn further deactivates their gating. When the cell encounters a hypoosmotic shock, the membrane tension increases and channels open, making them less aggregation prone, which results in spontaneous dispersion of clusters and further opening of individual channels. The positive feedback between the membrane tension and the cluster formation hence dynamically adjusts the extent of channel clustering, as well as their gating properties.

Channel clustering fine-tunes cell volume. To make experimentally testable predictions, we build on our previously developed continuum model of *E. coli* cell volume recovery upon hypoosmotic shock [14] and include the observed effect of channel clustering. Experimentally, we observed total cell volume expansion within seconds after the hypoosmotic shock, followed by a period of slower volume recovery that exhibits a characteristic "overshoot" below the value of initial volume (Fig. S10). To explain the response, we consider the change of the cellular volume (V) and solute concentration (n_i) in time. In our continuum model, cell volume changes, and consequentially the cell membrane tension, are governed by the flux of water, proportional to the difference between osmotic pressure and Laplace pressure on the cell wall. Solute concentration changes are governed by the diffusive fluxes through the MSCs, enhanced by the surface tension build up (see Supplementary Information). Our coarse-grained model of MSCs aggregation predicts that the channel clustering *(i)* decreases at higher membrane tensions, *(ii)* decreases opening probability per channel and *(iii)* increases for higher MSCs surface fractions. Combining the above, we define a parameter β, which gives the effective channel gating due to the channel clustering: $\beta = \sum_{N=1}^{30} P_{opening}(N,\gamma) \cdot P_{formation}(N,\rho,\gamma)$, where $P_{opening}$ is the probability of channel opening for a cluster consisting of N channels and at a given bilayer tension (γ), and $P_{formation}$ is the probability of forming a cluster of size N at a given tension and at a certain channel surface density (ρ). We introduce β in the Master equations that describe the changes of cellular V and n_i in time (Eq.S23 and S24), and fit them to the experimental volume trace (Fig. S10).

Fig. 4a illustrates the predicted dynamics of cluster aggregation and disaggregation as the volume expands and recovers during hypoosmotic shock. Probability of observing channels as monomers ($N = 1$) and as aggregates (shown for $N = 5$) is given as a color scale for each time point post-hypoosmotic shock, showing that larger clusters are less likely to form at the point of maximum volume expansion (largest tension), and more likely to form as the volume recovers and the membrane tension decreases.

To demonstrate the consequence of MSCs clustering on the cell volume recovery, we compare volume dynamics when we allow the channels to form clusters and when force them to exist solely as isolated channels (inset in Fig. 4a). Channel clustering can reduce the volume "overshoot" commonly found upon recovery, without jeopardizing

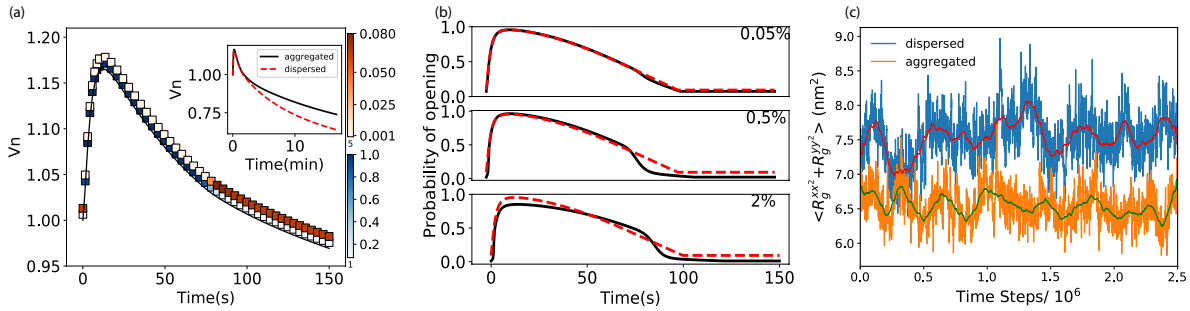

FIG. 4: **Clustering tightly regulates channel closing to overcome leaky cell membrane.**(a) Dynamics of channel aggregation-disaggregation as a result of varying normalized cell volume (gray faded line), V_n, upon 0.96 Osmol hypoosmotic shock and 0.5% channel packing fraction. Colorbars: Probability of finding a single isolated channel (orange) and a cluster of 5 channels (blue). Inset: Comparison of predicted volume response due to channel clustering (black line) against isolated channels (red dashed line) for the same shock and packing fraction as in the main figure. (b) Comparison of channel opening probabilities for the case of clustered (black line), and isolated channels (red dashed line) for the same conditions as in (a). (c) Oscillations in the average MSC pore size at zero membrane tension for a system of 12 MSCs in a dispersed (blue line) and aggregated (orange line) states. Red lines show the moving time average in each case (10^5 time steps window).

channel opening at the point of maximum tension (and volume). This is further depicted in Fig. 4b that shows MSCs' probability of opening during a hypoosmotic shock and subsequent recovery, for three different channel surface densities. For the physiological value of the channel surface density (middle panel, 0.5%), the membrane tension upon a hypoosmotic shock is high, and channels are mostly monomers. As the membrane tension relaxes during the volume recovery the channels cluster, resulting in a tighter regulation of channel closing and reduction of cytoplasmic solute leakage. If the surface density of channels is lower (upper panel), the cell loses the ability to tightly shut the MSCs at post-recovery membrane tensions, leading to larger "overshoot". On the other hand, if the channels' surface density is high due to increased MSCs expression (bottom panel), the high channel aggregation will delay the channel opening at the point of maximum volume, to the detriment of the cell's ability to recover from the shock (Fig. S11).

Discussion. Our results show that spontaneous aggregation of mechanosensitive membrane channels, driven by non-specific inter-protein attractions, results in liquid-like clusters that exhibit lower gating activity than dispersed clusters. Our findings align well with the study by [18] showing that the aggregation of *E. coli* MscLs into clusters in reconstituted in lipid vesicles leads to a significant decrease in the total gating activity. Their patch-clamp experiments show that a number of active channels in a patch was consistently lower than the total number of channels in the patch, for example, a membrane patch containing 11 channels was reported to gate as if it contained only 5 isolated channels. The results were further reinforced by small angle neutron scattering measurements of the total membrane area increase when the channels were open, which was not as large as expected if the channels were behaving independently.

The results from our model are in contrast with previous theoretical studies that, based on continuum modeling, predict that membrane-mediated interactions lead to collective channel opening [23–27]. The coarse-grained model used here does not reproduce the theoretically predicted protein aggregation due the hydrophobic mismatch, possibly due to the high stiffness of the coarse-grained lipids. However, since the channels constantly fluctuate between the open and the closed state, the sign of the corresponding bending-mediated interactions is not obvious either. Membrane mediated interactions might still be present, but are too weak to be measurable in our system. Nevertheless, these interactions have been notoriously difficult to experimentally quantify, making it hard to assess their importance in driving MSC aggregation observed in experiments [18, 19].

We demonstrated that coupling between the membrane tension, channels' conformational change and clustering produces a tightly controlled gating system, exhibiting positive feedback encoded in the system's physical properties. Based on the results, we predict the effects of the feedback on the cell volume regulation. We suggest that MSC aggregation serves to protect the cells from excessive gating during the cell volume recovery post-shock and in steady-state.

Our simulations also show that isolated channels

have a non-zero probability of gating even at zero tension (Fig. 4c). This agrees with patch-clamp characterisation of single-channel gating, where it is evident that the channel opening does not follow a sharp step function [28]. Hence, if MSCs are over-expressed, e.g. when bacteria grow under hyperosmotic conditions or when they enter stationary phase [29], the probability of single channel gating even under quiescent conditions would be sufficiently high to cause a potentially significant loss of cytoplasmic content. For example, our model predicts the probability of opening of 2% for the case of aggregated channels and 10% for the case of single channels in a cell at steady-state, which, without any further action from the cell, could lead to up to 20% volume loss in a period of 10 min. This can be significant, especially for a dormant cell in stationary phase; thus channel aggregation, which is more pronounced at higher channel numbers, could be a natural self-defence mechanism of bacteria against unnecessary gating, contributing to bacterial survival. Along the same lines, our results indicate that the MSC expression in the healthy cell is possibly finely tuned (Fig. 4b), such that it enables efficient cell volume recovery, but prevents unnecessary volume loss.

The generic MSC model developed identifies the basic physical mechanisms behind mechanosensing of membrane channels. Due to their generality, our results can also be helpful in guiding the design of synthetic nanomechanosening systems [30] and artificial membrane channels [31, 32].

Methods

The details of the potentials used in our computer model and the associated parameters are given in the Supplementary Information. The simulation box is cuboidal in shape with the initial dimensions $L_x = L_y = \frac{1}{3}L_z$. The side length of the box is allowed to fluctuate in the x and y directions with periodic boundary conditions applied. The simulation box was kept fixed in size in the z direction with weak Lennard-Jones walls at both ends, and with the wall depth $\varepsilon = 1.0kT$. The membrane was tethered to the centre of the simulation box with a weak spring of spring constant $k = 1.0kT/\sigma^2$ to prevent its drift under the collisions with the solutes. The simulations were run in the isoenthalpic-isobaric (NPH) ensemble with zero lateral pressure $P_x = P_y = 0$, coupled to a Langevin thermostat with the friction coefficient η set to unity: $\eta = m/\tau_0$, where m is the particle mass (set to unity for all particles) and τ_0 the simulation unit of time. The simulation time step was taken to be $\tau = 0.008\tau_0$. We used the LAMMPS molecular dynamics package to run the simulations [33] and the VMD package to visualise the trajectory files [34]. The continuum model for describing cell volume recovery [14], extended to incorporate the channel clustering, and its coupling to the results of computer simulations, are detailed in Supplementary Information.

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