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Mitosis, Diffusible Crosslinkers, and the Ideal Gas Law

David J. Odde[1,*]
[1]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN 55455, USA
*Correspondence: oddex002@umn.edu
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During mitosis, molecular motors hydrolyze ATP to generate sliding forces between adjacent microtubules and form the bipolar mitotic spindle. Lansky et al. now show that the diffusible microtubule crosslinker Ase1p can generate sliding forces between adjacent microtubules, and it does so without ATP hydrolysis.

The mitotic spindle is organized by an ensemble of molecular motors that hydrolyze ATP to actively transport microtubules. For example, the kinesin-5 family molecular motors (Cin8/Eg5/Kif11) generate sliding forces between antiparallel microtubules to push spindle poles apart, establish the metaphase bipolar spindle, and ultimately physically separate replicated genomes (Subramanian and Kapoor, 2012). These motors are resisted by passive diffusible crosslinkers, such as Ase1/PRC1/Map65, that have previously been viewed as mere frictional elements (Braun et al., 2011; Pringle et al., 2013). Since friction always acts against the direction of relative movement, the Ase1p-mediated frictional force in this overdamped system would then be predicted to drop to zero once an applied force was removed. In this issue, Lansky et al. show that this prediction is not observed, but rather that Ase1p drives microtubule sliding to maximize overlap in the absence of any applied force or ATP (Lansky et al., 2015).

To investigate force generation mediated by Ase1p crosslinkers, Lansky et al. used an in vitro experiment with purified Ase1p-GFP and red fluorescent microtubules. One "template" microtubule was firmly attached to a coverslip, and then a second microtubule was crosslinked to the template via Ase1p and the ensemble imaged via total internal reflection fluorescence microscopy. The ensemble was then subjected to a variety of forces, including hydrodynamic flow, optical tweezers, and molecular motors, that displaced the microtubules relative to each other, thus reducing the overlap region, as depicted in Figure 1A. As shown previously, continued force application will eventually slide the two apart completely (Braun et al., 2011). However, when the applied force was suddenly removed before all overlap was lost, a strange thing occurred: the second microtubule slowly slid backward to regain the lost overlap between the two microtubules. On the nanometer scale of the molecules, the observed displacements were large covering micrometers. The equivalent macroscopic experiment might be dragging a pencil across a table until it hangs over the edge of the desk, then letting go and seeing the pencil creep back onto the desk. Where does the force come from when there is no ATP or micrometers-long spring to drive the recovery of the overlap? Surprisingly, the familiar ideal gas law, PV = nRT, governs the system.

Unlike the pencil experiment, the microtubule experiment is strongly influenced by thermal forces. As a result, Ase1p can explore a variety of positions within the overlap. As the overlap increases, more positions become available to the Ase1p, as shown in Figure 1B. Thus, the greatest number of positions is accessed when overlap is maximal. Since these positions are energetically equivalent, the most probable state of the system is maximal overlap. If one were to apply a force, this would limit the number of accessible states and compress Ase1p into a smaller overlap region. This is the same physics of an ideal gas, as expressed in the ideal gas law. In this linear system, the ideal gas law can be written $FL = nk_BT$, where F is the force, L is the overlap length, n is the number of crosslinkers, k_B is Boltzmann's constant, and T is the absolute temperature. As the overlap decreases, the force builds as $F \sim 1/L$, which is observed experimentally.

This is a beautiful experimental demonstration of entropy maximization at work. The entropy, S, for any state of the system is given by

$$S = k_B \ln W$$

where W is the multiplicity of the state given by

$$W = \frac{M!}{N!(M-N)!}$$

where M is the number of configurations and N is the number of molecules. When S is maximal, the Gibbs free energy, G, is minimal (assuming no net change in the number of crosslinking bonds). The more probable a state is, the greater the entropy of that state. In the case of microtubule sliding, the more overlap between the microtubules, the more possible configurations there are that achieve that state, as illustrated in Figure 1B. For a single diffusing molecule, N = 1, and

$$W = \frac{M!}{1!(M-1)!} = M$$

For example, for overlap = 1, there is only one possible configuration of the single crosslinker (W = 1). Thus, for overlap = 1, the entropy is

$$S = k_B \ln(1) = 0$$

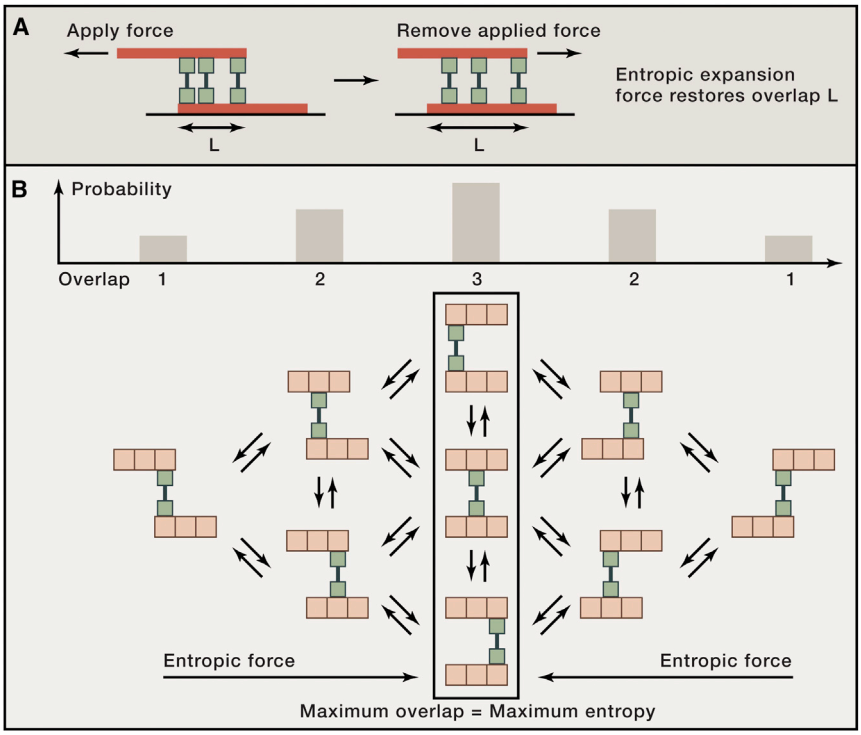

Figure 1. Diffusible Crosslinkers Drive an Entropic Expansion Force to Maximize Overlap between Adjacent Microtubules

(A) Microtubules (red) are crosslinked with Ase1p (green), which can diffuse along the microtubule surfaces. Ase1p exerts passive frictional resistance to applied forces that displace one microtubule relative to the other. Lansky et al. show that when the force is relieved, the microtubule slides back to re-establish maximal overlap, L, between the microtubules. Like a compressed ideal gas, the expansion of Ase1p along the lattice creates the restoring force.

(B) Origin of the entropic expansion force. In this example, two microtubules of length 3 are crosslinked by one Ase1p. Since there is only 1 way to achieve the left-most configuration, it is less probable than the overlap = 2 (2 possible configurations) and overlap = 3 (3 possible configurations) cases, and equally probable to the rightmost overlap = 1 case. Therefore, the most overlapped (overlap = 3) state is the most probable, and so the entropy is maximal. This creates a driving force toward maximal overlap, as observed by Lanksy et al.

For overlap = 2, there are two possible configurations (W = 2), and so

$$S = k_B \ln(2)$$

and for the most overlapped state (overlap = 3), there are 3 possible configurations (W = 3), and so the entropy is

$$S = k_B \ln(3)$$

So we see that the entropy is maximal for the most overlapped state, and driving the system away from this state requires an applied force.

In terms of free energy, $\Delta G = -T\Delta S$, the biggest change in Figure 1B occurs when overlap increases from 1 to 2, which is $\Delta G = -T\Delta S = -\ln(2)k_BT = -0.69k_BT$. Since the force, $F = -\Delta G/\delta$, where δ is the distance over which the energy change occurs, we can then estimate the entropic expansion force. Assuming a step size of δ = 4 nm, which is the size of a tubulin monomer, and an energy unit conversion of 1 k_BT = 4.28 pN-nm, then the entropic force is F = (0.69 k_BT) (4.28 pN-nm/k_BT)/(4 nm) = 0.7 pN, comparable to the force exerted by a molecular motor. Adding more crosslinkers would cause the force to increase proportionately, which Lansky et al. also demonstrate experimentally. Thus, the authors view the crosslinkers as exerting an "entropic expansion force" that acts to maximize the overlap between the two microtubules.

The entropic force is distinct from molecular motor forces in that it does not require ATP hydrolysis. It is also distinct from the microtubule depolymerization force, which drives kinetochore poleward movements in mitosis, a.k.a. the Hill sleeve mechanism (Hill, 1985; Powers et al., 2009). More generally, the importance of entropic forces is already appreciated in determining disordered protein acid structure, and in the packaging of viral genomes (Bustamante et al., 1994). Lansky et al. now reveal another entropy-driven force generating mechanism based on diffusible crosslinkers driving increased overlap between two adjacent self-assembled linear polymers.

So what do these findings mean for cells? It seems strange that Ase1p has the ability in vivo to *enhance* pole separation (Syrovatkina et al., 2013), but this counterintuitive effect is perhaps explained by Ase1p's bundling activity. This activity makes kinesin-5 more efficient as recently reported for the minus end-directed motor Kar3-Cik1 (Hepperla et al., 2014). What it does mean is that the pole-separating kinesin-5 motors may be working harder than we previously thought because they must overcome the extra entropic force that acts in the background to collapse the spindle. In this light, the entropic force may therefore help stabilize the spindle midzone in late mitosis. Beyond microtubules, Lansky et al. speculate that the same principles might drive sliding of actin filaments in cytokinesis due to diffusible crosslinking by myosin II, for example, rather than by its motor activity. At the cellular scale, it seems possible that diffusible crosslinkers that bridge between adjacent cells, such as cadherins, could also exert an entropic force that by itself would act to maximize contact area between adherent cells. In general, the ideal gas law is likely embedded in the background of a multitude of thermally driven cellular processes, exerting forces in the constant search for maximal entropy.

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Transcription Gets to the Checkpoint

John D. Laver[1] and Howard D. Lipshitz[1,*]
[1]Department of Molecular Genetics, University of Toronto, 1 King's College Circle, Toronto, ON M5S1A8, Canada
*Correspondence: howard.lipshitz@utoronto.ca
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The rapid cell proliferation characteristic of early animal embryos is accomplished with an abbreviated cell cycle and no DNA replication checkpoint. Blythe and Wieschaus provide evidence that nascent zygotic transcription precedes—and may trigger—this checkpoint at the midblastula transition.

During the cell cycle, the DNA replication checkpoint pauses entry into M phase until replication is complete. Activation of this checkpoint is essential in early embryos of many animals. In *Drosophila*, for example, a deficient checkpoint results in severe mitotic defects and death (Sibon et al., 1997). Although the importance of the checkpoint is clear, how and why it is activated in early embryos is less so. In this issue of *Cell*, Blythe and Wieschaus (2015) present evidence that checkpoint activation in *Drosophila* is triggered by the onset of zygotic transcription (Figure 1).

The earliest phase of development in *Drosophila* consists of 13 rapid, synchronous nuclear cycles (NCs)—composed only of S and M phases—directed by maternally supplied mRNAs and proteins. As development proceeds, maternal products are degraded and the zygotic genome is activated, a process known as the maternal-to-zygotic transition (MZT). Concurrently, gradual lengthening of the NCs culminates in the introduction of gap phases and cellularization of the blastoderm during NC14, an event known as the midblastula transition (MBT). These processes depend on a functional replication checkpoint.

A long-standing model posits that, with increasing nucleocytoplasmic ratio, essential maternal replication factors are titrated, resulting in replication stress and checkpoint activation (Sibon et al., 1997). In a series of ingenious experiments, Blythe and Wieschaus (2015) use compound chromosomes to alter the total DNA content of the embryo or to modulate the amount of transcriptionally active DNA in embryos with the same total DNA content. By precisely measuring the length of NC13 as a proxy for the extent of checkpoint activation, they demonstrate that this activation correlates best not with total embryonic DNA content but with the amount of transcriptionally engaged DNA, leading to the hypothesis that checkpoint activation is a consequence of the onset of zygotic transcription.

To test this model, Blythe and Wieschaus (2015) perform RNA polymerase II (Pol II) chromatin immunoprecipitation sequencing (ChIP-seq) on carefully staged embryos to accurately define changes in transcriptional activity in NC12, NC13, and NC14. While hundreds of genes are already occupied and undergoing transcription at NC12, NC13 marks the large-scale recruitment of Pol II, largely in a "poised" state, to the transcriptional start sites of thousands of additional genes, which is consistent with the results of an earlier study (Chen et al., 2013). Importantly, these early phases of global zygotic genome activation are largely unaffected in checkpoint mutants, implying that transcription precedes and occurs independently of checkpoint-mediated NC lengthening.

To monitor replication stress at the molecular level, Blythe and Wieschaus (2015) next use fluorescently labeled RPA70, which binds to sites of single-stranded DNA generated upon replication stalling, leading to checkpoint activation. They demonstrate a striking correlation between RPA70-bound and Pol II-occupied DNA, which is consistent with the hypothesis that sites of transcriptionally engaged DNA are sources of replication stress. This interpretation is complicated by the fact that, in budding yeast, RPA70 is generally associated with sites of active transcription independent of replication (Sikorski et al., 2011), so it remains possible that the correlation reflects not sites of replication stalling but a role for the RPA complex in transcription. Indeed, Blythe and Wieschaus (2015) speculate that RPA may directly link transcription to the checkpoint independent of replication stress. Assessing additional and highly specific markers of replication stress, such as phosphorylated RPA30, may be illuminating.

The most compelling evidence for a transcription-induced checkpoint model