Figures and figure supplements

Evolution of cation binding in the active sites of P-loop nucleoside triphosphatases in relation to the basic catalytic mechanism

Daria N Shalaeva et al
Figure 1. Mg-NTP complexes and their binding in the active sites of P-loop NTPases. Phosphate chains of NTP molecules and their analogs are colored by atoms: oxygen atoms in red, phosphorus in orange. The K⁺ ion is shown as a purple sphere, Na⁺ ion is shown as a blue sphere, Mg²⁺ ions are shown as green spheres. Phosphate chain is shown in stick representation with oxygens in red and phosphorus atoms in orange; γ-phosphate mimicking groups (AlF₄⁻ and MgF₃⁻) are shown in black, coordination and hydrogen bonds are shown as black dashed lines. (A) Active site of the small Ras-like GTPase RhoA in complex with the activating protein RhoGAP [PDB entry 1OW3]; the bound GDP-MgF₃⁻ mimics the transition state. The P-loop with the preceding α-helix is shown as green cartoon; Switch I motif with the conserved Mg²⁺-binding Thr residue is shown in magenta; Switch II motif (DxxG motif, which starts from the conserved Asp of the Walker B motif) is colored turquoise. (B) Active site of the K⁺-dependent GTPase MnmE with bound GDP-AlF₄⁻ [PDB: 2GJ8]. Switch I region and the K-loop are shown in magenta. (C) The active site of dynamin, a Na⁺-adapted GTPase with bound GDP-AlF₄⁻ [PDB: 2X2E]. The P-loop and K-loop (Switch I region) are colored as in panels A and B. (D) Structure of the NTP triphosphate chain with Mg²⁺ ion in a bidentate coordination, referred to as the βγ conformation. The pink dotted arch indicates the Pβ≡O3B≡Pγ angle; the blue dashed line indicates the Pα−Pγ distance. The atom names are in accordance with the CHARMM naming scheme (Vanommeslaeghe et al., 2010) and the recent IUPAC recommendations (Blackburn et al., 2017).

DOI: https://doi.org/10.7554/eLife.37373.002
Figure 2. Binding of monovalent cations to the Mg-ATP in water. The color scheme is as in Figure 1. (A) Superposition of the ATP phosphate chain conformations observed in the MD simulations in the presence of K⁺ ions (shown in purple), Na⁺ ions (shown in blue) and NH₄⁺ ions (nitrogen atoms of NH₄⁺ ions are shown in yellow/green). The ribose and adenine moieties are not shown, the phosphate chain is shown with Pᴬ on top and Pᴳ at the bottom. All cations within 5 Å from the phosphate chain are shown and colored in different shades depending on the nearby oxygen atoms to illustrate the distinction between binding in the AG and BG sites (see text for details). Transparent spheres signify the ions outside the AG and BG sites. The constellation of ions in the vicinity of γ-phosphate is referred to as the site G. For the visualization, we have selected every 100th simulation frame to Figure 2 continued on next page.
sample the conformational states of the Mg-ATP complex with 5-ns intervals. The conformations were superposed to achieve the best possible match between coordinates of the phosphorus and ester oxygen atoms of the ATP phosphate chain. (B) Geometry of the Mg-ATP complex with two monovalent cations bound, one in the AG site and one in the BG site. Distances to the AG and BG binding sites ($R_{AG}$ and $R_{BG}$) were calculated as averages of the distances to the two corresponding oxygen atoms. The distances to the oxygen atoms (e.g. $r^A$) were defined as the shortest distances between a particular M$^+$ ion and any oxygen atom of the respective phosphate group (including the bridging oxygen atoms). (C-E) distance distributions for K$^+$, NH$_4^+$, and Na$^+$ ions in the AG and BG sites.

DOI: https://doi.org/10.7554/eLife.37373.003
Figure 2—figure supplement 1. Radial distribution of cations in the proximity of each oxygen atom. Radial distributions are shown for all atoms of the ATP phosphate chain. (A) Atom names are in accordance with the CHARMM naming scheme (Vanommeslaeghe et al., 2010) and the recent IUPAC recommendations (Blackburn et al., 2017). (B) Radial distributions of cations around individual oxygen atoms. The distributions of cations around ester bond oxygen atoms O\textsuperscript{3A} and O\textsuperscript{3B} are shown by dashed lines. The peak distances from the cation to the oxygen atoms were the same 2.7 Å for K\textsuperscript{+} and NH\textsubscript{4}\textsuperscript{+} ions, while for Na\textsuperscript{+} this distance was 2.2 Å. For the NH\textsubscript{4}\textsuperscript{+} ion, the distance was measured from each oxygen atom to the nitrogen atom of NH\textsubscript{4}\textsuperscript{+}. There are two ester bond oxygens in the phosphate chain, but only the oxygen (O\textsuperscript{3B}) that connects \(\beta\)- and \(\gamma\)-phosphates was seen involved in the cation binding, it interacted more often with K\textsuperscript{+} and Na\textsuperscript{+} than with NH\textsubscript{4}\textsuperscript{+}. Monovalent cations were found near oxygen atoms of \(\gamma\)-phosphate more often than near oxygens of \(\beta\)- and \(\alpha\)-phosphates.

DOI: https://doi.org/10.7554/eLife.37373.004
Figure 2—figure supplement 2. Properties of cation binding to the ATP as derived from MD simulations. (A) Probability distribution functions for cations around the phosphate chain. We have plotted the number of atoms inside the area centered on phosphorus atoms of the ATP phosphate chain as a function of the radius of the selected area. This number was estimated by measuring the distance between each cation in the system and the nearest phosphorus atom of ATP during MD simulations. The plot indicates the presence of 1.5 cations on average in the 4 Å radius around the phosphate chain in the case of Na\(^+\) and NH\(_4\)\(^+\), and 0.75 ions on average in the case of K\(^+\). For all three ions, the first inflection occurs at the distances shorter than 4 Å and a less prominent second inflection can be seen at around 6 Å. (B) Free energy of the cation binding as a function of the distance from the phosphate chain, as estimated from the probability data in panel A. In addition to the two binding sites at the distances of approx. 4 Å and 6 Å, the free energy plot revealed a less pronounced third binding site at a distance of approx. 8–9 Å from the phosphorus atoms. The most prominent is the first peak, corresponding to cation binding around the phosphate chain, within the 4 Å distance of at least one of the phosphorus atoms, so further focus was specifically on cation binding around the phosphate chain.

DOI: https://doi.org/10.7554/eLife.37373.005
Figure 2—figure supplement 3. Binding of monovalent cations to the Mg-GTP in water. Distances to the AG and BG binding sites (R^AG and R^BG) were calculated as averages of the distances to the two corresponding oxygen atoms (see Figure 2 in the main text). The distances to the oxygen atoms (e.g. r) were defined as the shortest distances between a particular M^+ ion and any oxygen atom of the respective phosphate group (including ester oxygen atoms). (A-C) distance distributions for K^+, NH_4^+, and Na^+ ions in the AG and BG sites.

DOI: https://doi.org/10.7554/eLife.37373.006
Figure 3. Cation binding induces eclipsed conformation of the phosphate chain. (A) Conformations of Mg-ATP complexes with one and two $K^+$ ions bound as inferred from MD simulations; left structure, no $K^+$ ion bound in the AG site; right structure, a $K^+$ ion is bound in the AG site. The a-

---

Shalaeva et al. eLife 2018;7:e37373. DOI: https://doi.org/10.7554/eLife.37373
phosphate is in on the top, β- and γ-phosphates are below; the α-phosphate is shown in green, β-phosphate in blue, γ-phosphate in red. (B) Distribution histograms for dihedral angles between phosphate groups in ATP, calculated from MD simulations of Mg-ATP with one K⁺ cation bound in the BG site (green) and with two cations bound in the AG and BG sites (red). Normalized histograms of dihedral angle distribution (thin lines) were calculated from MD trajectories and fitted with normal distribution function (thick lines). Dashed lines indicate the centroid values of the fits by Gaussian function. All distributions were fitted with one-term Gaussian models, except for the $\Psi_{\alpha\gamma}$ angle in case of Mg-ATP with two cations bound, this distribution was fitted with a two-term Gaussian, parameters for the highest peak are shown. (C) The phosphate chain of GTP, illustrating the dihedral angle $\Psi_{\alpha\gamma}$. Dihedral angle is an angle between two planes and is defined by four atoms. In this case, the angle $\Psi_{\alpha\gamma}$ is an angle between the plane that contains atoms $P^G$, $P^A$ and $O^{1A}$ (green), and the plane that contains atoms $P^A$, $P^G$ and $O^{3G}$ (red). In the fully eclipsed conformation, both P-O bonds are coplanar, so that the two planes overlap and the dihedral angle between them is 0°.

DOI: https://doi.org/10.7554/eLife.37373.007
Coupling between cation binding in the AG site and rotation of γ-phosphate relative to α- and β-phosphates. Data from MD simulations with restraints on the positions of K⁺ ions (see the text and Supplementary file 1C). The top graph shows free energy calculated from normalized probabilities of ATP conformations and plotted as function of the dihedral angle between γ- and β-phosphates. The bottom plot displays free energy of coupling the binding of the second K⁺ ion with the γ-phosphate rotation, calculated as the difference between the free energy plots shown on the top graph. The lowest energy value was set to zero. These plots show that the presence of second K⁺ ion in the AG site induces a...
near-eclipsed state of the phosphate chain, by bringing both $\Psi^{\alpha\beta}$ and $\Psi^{\alpha\gamma}$ angles close to 0°, at the expense of $\Psi^{\beta\gamma}$, which increases slightly (see Supplementary file 1D). Binding of the second K+ ion in the AG site stabilizes this almost eclipsed state by ~27 meV.

DOI: https://doi.org/10.7554/eLife.37373.008
Figure 4. Dynamics of the phosphate chain of the Mg-ATP complex with and without monovalent cations. Each left panel shows the $P^A-P^G$ distance (upper trace) and the $P^A-O^B-P^G$ angle (bottom trace) in the course of MD simulations. Thin gray lines show actual values measured from each frame of the MD simulation, the bold black lines show moving average with a 2-ps window. Black boxes indicate fragments of simulations chosen for the analyses of particular types of interaction between the Mg$^{2+}$ ion and the triphosphate chain; the respective conformations of Mg-ATP are shown on the right. The analysis was performed as shown in Figure 2B. The color scheme is as in Figure 1. (A) no added ions; (B–D) MD simulations in the presence of K$^+$, Na$^+$, and NH$_4^+$, respectively.

DOI: https://doi.org/10.7554/eLife.37373.009
Coordination of the Mg$^{2+}$ ion by the oxygen atoms of the ATP phosphate chain during MD simulations. Black vertical lines indicate borders between independent simulations, thick colored lines show moving averages of distances measured during MD simulations. Oxygen atoms are labeled as in Figure 1D. The most populated conformation in each of the four systems is characterized by the Mg$^{2+}$ ion coordinated by three oxygen atoms: one of the free oxygens of the $\alpha$-phosphate (O$^{1A}$ or O$^{2A}$), O$^{3B}$ atom, and an oxygen atom from the $\gamma$-phosphate (O$^{1G}$, O$^{2G}$, or O$^{3G}$). This conformation resembles the $\alpha\beta\gamma$ conformation of the Mg-ATP complex seen in other studies but differs in the inclusion of an ester oxygen atom in the Mg$^{2+}$ coordination sphere.

DOI: https://doi.org/10.7554/eLife.37373.010
Figure 4—figure supplement 2. Estimation of correlation times for the $P^A\cdot P^G$ distances. A, B, Changes of the distance value upon MD simulations of $\beta\gamma$-coordinated Mg-ATP complexes with no additional monovalent cations (A) and with $K^+$ ions (B) provided as examples. (C) Autocorrelation values
plotted as functions of the time lag. Based on this plot, the correlation time of 1 ns of simulation time was anticipated for the all types of interactions between the Mg$^{2+}$ ion and the triphosphate chain and in the presence of all tested M$^+$ ions.

DOI: https://doi.org/10.7554/eLife.37373.011
Estimation of correlation times for the Pβ-O-Pγ angles. A, B, Changes of the angle value upon MD simulations of βγ-coordinated Mg-ATP complexes with no additional monovalent cations (A) and with K⁺ ions (B) provided as examples. (C) Autocorrelation values.

Figure 4—figure supplement 3 continued on next page
plotted as functions of the time lag. Compared to the distance measurements, the angle values oscillated on a much shorter timescale and accordingly had shorter correlation times. From this plot, the correlation time of 5 frames or 250 ps of simulation time was estimated. The general shape of the autocorrelation function was the same for all types of interactions between the Mg$^{2+}$ ion and the triphosphate chain and in the presence of all tested M$^+$ ions.

DOI: https://doi.org/10.7554/eLife.37373.012
Figure 5. Heat maps of the Mg-ATP phosphate chain conformations distribution characterized by the P$^A$-P$^G$ distances (X-axis) and P$^B$-O$_3$-P$^G$ angles (Y-axis). Heat maps for systems with monovalent cations include only conformations of Mg-ATP complexes with at least one cation present within 4 Å radius. The color intensity is proportional to the probability (normalized frequency) of the respective conformation. Magenta dashed lines outline the areas corresponding to the conformations of transition state analogs; blue dashed lines outline the areas corresponding to the conformations of the non-hydrolyzable analogs, calculated from crystal structures of P-loop NTPases (Figure 5—figure supplement 2). (A) Data from the 3 × 170 ns simulations (no. 1–4 in Supplementary file 1C). (B) Data from 4 × 20 ns simulations of Mg-ATP in βγ conformations (no. 5–8 in Supplementary file 1C). DOI: https://doi.org/10.7554/eLife.37373.014
Figure 5—figure supplement 1. Heat maps of the Mg-GTP phosphate chain conformations distribution characterized by the $P^A-P^G$ distances (X-axis) and $P^B-O^3P^D$ angles (Y-axis). Heat maps for systems with monovalent cations include only conformations of Mg-GTP complexes with at least one cation present within 4 Å area, and with Mg$^{2+}$ in βγ coordination. The color intensity is proportional to the probability (normalized frequency) of the respective conformation. Magenta dashed lines outline the areas corresponding to the conformations of transition state analogs; blue dashed lines outline the areas corresponding to the conformations of the non-hydrolyzable analogs, calculated from crystal structures of P-loop NTPases, see text.

DOI: https://doi.org/10.7554/eLife.37373.015
Figure 5—figure supplement 2. Phosphate chain shape of ATP and GTP analogs in the X-ray structures of P-loop NTPases. PDB entries for structures of P-loop NTPases were extracted from InterPro database entry IPR027417 ‘P-loop containing nucleoside triphosphate hydrolase’ and filtered to contain only those X-ray structures that contain Mg$^{2+}$ ions, resulting in a list of 1,333 PDB IDs. Selected structures were analyzed with custom MATLAB scripts to select only those structures which contain either an NTP molecule, or its non-hydrolyzable analog, or a transition state analog. Additionally, we only considered NTP-like molecules bound in the proximity of at least one Lys residue (with less than 4.5 Å distance from NZ atom of Lys to any of the phosphate chain P atoms or the corresponding atoms in mimicking groups). In total, 1,357 NTP-like molecules from 670 PDB entries were used in the measurements. Isotherms for the heat map of the structure shape distribution are shown to indicate the most and least populated areas. Bold lines indicate isotherms chosen to represent crystallographic data in comparison with the MD results. (A) Shapes of ATP and GTP molecules. Native ATP and GTP molecules are most likely to be crystallized with inactive proteins, so the majority of them represent non-productive conformations of the phosphate chain. (B) Shapes of non-hydrolyzable analogs (PDB IDs: ANP, GNP, ACP, GCP, AGS, GSP). Non-hydrolyzable analogs cover lower values of the angle that is analogous to the P$_B$-O$^{3B}$-P$_G$ angle, since in such molecules, the ester oxygen between P$_B$ and P$_G$ is replaced with another atom (N in ANP, GNP; C in ACP, GCP); or one of free oxygens of γ-phosphate is replaced with S (GSP, AGS).

DOI: https://doi.org/10.7554/eLife.37373.016
Figure 6. Location of positive charges around the phosphate chain of Mg-NTP complexes in solution and in protein structures. The color scheme is as in Figure 1; dark blue spheres indicate positions of positively charged side-chain nitrogen atoms of Lys and Arg residues, P-loop regions are shown as cartoons in grey. (A) Superposition of phosphate chain conformations observed in MD simulations with K⁺ ions. Only conformations with βγ coordination of Mg²⁺ are shown. (B) Superposition of P-loop regions of crystal structures of cation-dependent P-loop NTPases: GTPase MnmE [PDB: 2GJ8], Fe transporter FeoB [PDB: 3S58], dynamin-like protein [PDB: 2X2E], and translation factor eIF-B5 [PDB: 4TM2], see Table 3 for details. (C) Superposition of P-loop regions of crystal structures of cation-independent P-loop NTPases: Ras/RasGAP complex [PDB: 1WQ1], septin [PDB: 3FTQ], atlastin [PDB: 4IDQ], Gα₁₂ protein [PDB: 12CA], DNA polymerase III subunit τ [PDB: 3GLF], F₁-ATPase [PDB: 2JDI].

DOI: https://doi.org/10.7554/eLife.37373.017
Figure 6—figure supplement 1. Active sites of P-loop NTPases with established K⁺-dependent activity (see Supplementary file 1A for the full list and references). Each of the proteins shown has both Asn residues that were shown to be associated with binding of monovalent cations in related proteins (Ash et al., 2012). Switch I, including the K-loop, and its flanking regions are shown in magenta, switch II motif DxxG is shown in orange. NTP-like molecules are shown as sticks, Mg²⁺ ions are shown as green spheres, water molecules in the area of supposed cation binding are shown as red spheres.

DOI: https://doi.org/10.7554/eLife.37373.018
Figure 6—figure supplement 2. Activation of the MnmE GTPase upon dimerization. (A) Inactive dimer of the full-length MnmE in the GTP-bound form (the structure (PDB: 3GEI) was resolved with non-hydrolyzable GTP analogs). The P-loop domain is shown in grey, the K-loop is not resolved (its position is indicated by red asterisks), the N-terminal and helical domains are shown in blue and green for different monomers. (B) An active dimer of isolated G-domains of MnmE, as resolved in complex with a transition state analog and K⁺ ion (PDB: 2GJ8). The K-loops are shown in red, K⁺ ions are shown as purple spheres. (C) Schematic representation of the conformational changes in MnmE dimers, reproduced after (Klare, 2013), domains are colored the same way as on panel A.

DOI: https://doi.org/10.7554/eLife.37373.019
Figure 6—figure supplement 3. Activation of the GTPase Era upon RNA binding. (A) Inactive Era in the GDP-bound form [PDB: 3IEU] (Tu et al., 2009) in two projections. (B) Active Era in complex with nucleotides 1506–1542 of 16S rRNA and a non-hydrolyzable analog of GTP [PDB: 3R9W] (Tu et al., 2011) in two projections. (C) Cation-binding site of active Era, occupied by a water molecule (shown as a red sphere) [PDB: 3R9W] (Tu et al., 2011). The black line indicates, for comparison, the position of the K-loop in the inactive structure [PDB: 3IEU] (Tu et al., 2009). The P-loop domain is shown in grey, the P-loop region shown in green, the K-loop region shown in magenta, nucleotide analogs are shown as sticks, Mg$^{2+}$ ions are shown as green spheres.

DOI: https://doi.org/10.7554/eLife.37373.020
Figure 6—figure supplement 4. Positively charged moieties in the active site of RecA-like recombinases. (A) Cation-dependent RadA recombinase from *Methanococcus voltae* [PDB: 2F1H] (Qian et al., 2006). (B) Cation-independent RecA recombinase from *E. coli* [PDB: 3CMX] (Chen et al., 2008). The protein structure is shown as grey cartoon, the adjacent monomer is shown in blue, the P-loop region is shown in green; catalytic Glu residues are shown as orange sticks, conserved Asp residues of the Walker B motif are shown as red sticks. Functionally relevant residues from adjacent monomers are shown as blue sticks. Mg$^{2+}$ ions are shown as green spheres, K$^+$ ions as purple spheres.

DOI: https://doi.org/10.7554/eLife.37373.021
Figure 7. Molecular dynamics of MnmE GTPase. (A) Superposition of the GTP-binding sites of the inactive, monomeric G-domain of MnmE (the 2GJ8 system, blue) and the active K⁺-bound dimer of G-domains (the 2GJ8 system, red); representative structures were sampled from the last 10 ns of 100 ns simulations. The protein backbones are shown as cartoons; GTP and surrounding amino acid residues are shown as sticks; Mg²⁺ and K⁺ ions are shown as spheres. Black dashed lines indicate hydrogen bonds and coordination bonds for cations that are present in both structures; the red dashed line indicates the H-bond that is present only in the K⁺-containing 2GJ8 system. (B) Conformational space of GTP in different states of MnmE GTPase. Scatter plot of the ψ²⁻⁹ dihedral angle (Y-axis) against the length of the hydrogen bond between the O²⁺ atom and the backbone nitrogen of Asn226 (X-axis) as sampled from the MD simulations of three systems: (1) red/orange, active dimer of G-domains with K⁺ ions bound (the 2GJ8 system, red and orange for individual monomers); (2) blue, monomeric G-domain of MnmE with the K⁺ ion replaced by a water molecule (the 2GJ8 system); and (3) black, inactive monomer G-domain of MnmE without a full-fledged K-loop (the 3GEI system). (C) Distribution histograms for dihedral angles between the phosphate groups in GTP, calculated from MD simulations of the dimeric G-domain of MnmE with bound K⁺ ions (the 2GJ8 system, red and magenta colors represent individual monomers in the dimer) and the monomeric G-domain with the K⁺ ion replaced by a water molecule (the 2GJ8 system, blue). Normalized histograms of dihedral angle distribution (solid lines) were calculated from MD trajectories and fitted with normal distribution function (dotted lines). Vertical lines indicate the centroid values of the fits by Gaussian function. Black vertical lines indicate ψ = 0°, which corresponds to the fully eclipsed conformation, while ψ = ±60° corresponds to the fully staggered conformation.

DOI: https://doi.org/10.7554/eLife.37373.024
Figure 7—figure supplement 1. Hydrogen bonds lengths during MD simulations of the G-domain of MnmE. Distances between phosphate chain oxygen atoms and surrounding amino acid residues were measured in the course of 100-ns MD simulations. (A) inactive monomer without a full-fledged K-loop (the 3GEI system), (B) inactive, monomeric G-domain of MnmE with the K$^+$ ion replaced by a water molecule (the 2GJ8$_W$ system), (C) active dimer of G-domains with K$^+$ ions bound (the 2GJ8$_K$ system, the blue and black traces correspond to individual monomers in the dimer.

DOI: https://doi.org/10.7554/eLife.37373.025
Figure 7—figure supplement 2. The distance between O^{2A} and O^{3G} in GTP bound to MnmE as inferred from MD simulations. Plotted are data for the active dimer of G-domains with K^+ ions bound (the 2GJ8 system, red and magenta for individual monomers) and inactive, monomeric G-domain of MnmE with the K^+ ion replaced by a water molecule (the 2GJ8 system, blue). (A) Distribution histograms of the O^{2A}-O^{3G} distances. The distribution histograms were fitted with Gaussians by using MATLAB software, the fitted curves and the corresponding average values are shown as dashed lines. (B) Correlation between the O^{2A}-O^{3G} distance (X axis) and the value of dihedral angle \( \Psi^{\phi} \) (Y axis). Individual conformations observed in MD simulations are plotted as dots.

DOI: https://doi.org/10.7554/eLife.37373.026
Figure 8. Effects of Na⁺ binding on the shape of phosphate chain in solution and in Na⁺-adapted P-loop NTPases. The color scheme is as in Figure 1, except that Al and F atoms in the GDP-AlF₄⁻ complexes are colored grey and cyan, respectively. (A) Superposition of the K⁺-bound (solid structure) and Na⁺-bound (transparent structure) conformations of the triphosphate chain as obtained from MD simulations of an ATP molecule in water. Data from MD simulations 4–8 in Supplementary file 1C. (B) Superposition of the P-loop NTPase structures with a bound K⁺ ion (MnmE GTPase, PDB: 2GJ8 (Scrima and Wittinghofer, 2006), purple) and Na⁺ ion (dynamin, PDB: 2X2E (Chappie et al., 2010), blue). Proteins are shown as a cartoon. Dashed lines indicate hydrogen bonds and coordination bonds. Bonds that occur in all P-loop NTPases are shown in green, those that occur in K⁺-binding proteins are in purple, those bonds that occur in Na⁺-binding dynamin-like proteins are in blue. The thick dashed purple line indicates the bond between the K⁺ ion and the oxygen atom of α-phosphate, which is absent in dynamins. The thick dashed blue line indicates the dynamin-specific H-bond between O₂⁻³ atom and the backbone amide group of the shortened K-loop.

DOI: https://doi.org/10.7554/eLife.37373.027