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Structural interconversions modulate activity of *Escherichia coli* ribonucleotide reductase

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Edited by Rowena G. Matthews, University of Michigan, Ann Arbor, MI, and approved October 31, 2011 (received for review August 3, 2011)

Essential for DNA biosynthesis and repair, ribonucleotide reductases (RNRs) convert ribonucleotides to deoxyribonucleotides via radical-based chemistry. Although long known that allosteric regulation of RNR activity is vital for cell health, the molecular basis of this regulation has been enigmatic, largely due to a lack of structural information about how the catalytic subunit ($\alpha_2$) and the radical-generation subunit ($\beta_2$) interact. Here we present the first structure of a complex between $\alpha_2$ and $\beta_2$ subunits for the prototypic RNR from *Escherichia coli*. Using four techniques (small-angle X-ray scattering, X-ray crystallography, electron microscopy, and analytical ultracentrifugation), we describe an unprecedented $\alpha_2\beta_2$ ring-like structure in the presence of the negative effector dATP and provide structural support for an active $\alpha_2\beta_2$ configuration. We demonstrate that, under physiological conditions, *E. coli* RNR exists as a mixture of transient $\alpha_2\beta_2$ and $\alpha_4\beta_4$ species whose distributions are modulated by allosteric effectors. We further show that this interconversion between $\alpha_2\beta_2$ and $\alpha_4\beta_4$ entails dramatic subunit rearrangements, providing a stunning molecular explanation for the allosteric regulation of RNR activity in *E. coli*.

Author contributions: N.A., E.J.B., C.M.Z., F.J.A., J.S., and C.L.D. designed research; N.A., E.J.B., C.M.Z., F.J.A., J.S., and C.L.D. performed research; M.A.F. and K.Y. prepared samples and performed AUC and SAXS experiments and image analysis; C.M.Z. performed crystallization and crystal structure determination; M.A.F. and K.Y. prepared samples and performed AUC and SAXS experiments; N.A., E.J.B., J.S., and C.L.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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2This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112715108/DCSupplemental.

Important targets of anticancer and antiviral drugs, ribonucleotide reductases (RNRs) are classified by the metallocofactor used to generate a thyl radical (1) that initiates reduction of ribonucleotides to deoxyribonucleotides (2, 3). Class Ia RNRs are found in all eukaryotes and many aerobic bacteria, with the *Escherichia coli* enzyme serving as the prototype. These RNRs reduce ribonucleotide 5′-diphosphates and are composed of two homodimeric subunits: $\alpha_2$ and $\beta_2$ (Fig. 1A). The $\alpha_2$ subunit contains the active site, where ribonucleotide reduction occurs, and two types of allosteric effector binding sites (4, 5). One effector site tunes the specificity for all four ribonucleotides in substrates in response to intracellular levels of deoxyribonucleoside 5′-triphosphates (dATP, dTTP, dGTP) and ATP (6, 7) such that balanced pools of deoxyribonucleotides are maintained (8). The second effector site controls the rate of reduction, binding ATP to turn the enzyme on or dATP to turn it off (4, 6). This activity site is housed in an N-terminal domain (5, 9) and provides a means for negative feedback regulation to safeguard against cytotoxic elevation of deoxyribonucleotide levels (2, 3, 10). The $\beta_2$ subunit harbors the essential diferric-tyrosyl radical (Y122· in *E. coli*) cofactor (11) that initiates radical chemistry.

Active RNR has long been proposed to be a transient $\alpha_2\beta_2$ complex (Fig. 1B) with enhanced subunit affinity upon binding of substrates and effectors (12–15). For each turnover, $\alpha_2$, $\beta_2$, substrate and effector must interact, triggering long-range proton-coupled electron transfer (PCET) reducing Y122· in $\beta_2$ and oxidizing C439 to a thyl radical in the active site of $\alpha_2$, over an unprecedented distance of >35 Å (13, 15, 16) (Fig. 1C). Once the thyl radical is generated in the active site of $\alpha_2$, ribonucleotide reduction proceeds through a conserved mechanism (17). Allosteric regulation of this activity is key to cell survival and involves conformational changes as well as oligomeric state changes in both prokaryotic (12, 14, 18) and eukaryotic systems (19–23). For *E. coli* (12, 14, 18), mouse (20–22), yeast (19, 23), the negative effector dATP has been linked to increases in oligomeric state with a recent gas-phase electrophoretic molecular mobility analysis (GEMMA) study estimating a molecular mass of 510 kDa for the dATP-inhibited *E. coli* RNR (most consistent with an $\alpha_2\beta_2$ state) (18), whereas for human and yeast RNR, dATP has been linked to $\alpha$-hexamerization (19, 22, 23).

To understand the role of oligomeric state in the activity regulation of this prototypic class Ia RNR from *E. coli*, we have combined data from four complementary structural techniques. Using small-angle X-ray scattering (SAXS) and analytical ultracentrifugation (AUC), we provide evidence that supports a compact $\alpha_2\beta_2$ structure for the active complex that can be reversibly converted via a dynamic conformational rearrangement to an inactive $\alpha_4\beta_4$ state in the presence of elevated dATP or protein concentrations. Additionally, using SAXS, single-particle EM, and X-ray crystallography, we demonstrate that the $\alpha_2\beta_2$ complex induced by the negative effector, dATP, is an unexpected ring-like structure composed of alternating $\alpha_2$ and $\beta_2$ subunits. Together, these results explain how activity can be modulated by oligomerization, which is induced by effector binding at an allosteric site that is approximately 42 Å from the active site.

Results

**dATP Shifts Oligomeric Equilibrium.** Sedimentation velocity AUC was employed to investigate the oligomeric distributions of *E. coli* RNR at multiple protein concentrations and in the presence of effectors at physiologically relevant concentrations (approximately 175 μM for dATP, 3 mM for ATP, and 0.1 mM for dTTP) (24–27). Using $\beta_2$ that was pretreated with hydroxyurea to prevent turnover and thus oxidation of the active site during measurements, three different modes of RNR regulation were investigated: (i) under negative feedback regulation with dATP in both specificity and activity sites and CDP as the substrate; (ii) under enhanced CDP reduction with ATP in both specificity and activity sites and CDP as the substrate; and (iii) under GDP reduction with dTTP in the specificity site and no effector in the activity site.
Fig. 1. Previously determined structures and proposed models for E. coli class Ia RNR. (A) Homodimeric α2 with nucleotides bound (spheres) and the core domain (residues 1–100) colored in green. Homodimeric β2 with diiron centers (green) and disordered C termini (gray lines). Protein Data Bank ID codes 4R1R, 3R1R, 1RIB (5, 15, 28). (B) Proposed α2β2 model in which the subunits are docked along their symmetry axis (15). (C) Docking model rendered as a surface. Radical pathway involving Y122 → W48 → Y356 in β2 (Y122 and W48 shown as orange spheres) and residues Y731 → Y730 → C439 in α2 (dark-blue spheres). Y356 in β2 lies in the disordered C termini (15).

In the presence of saturating dATP and CDP, α2 alone and β2 alone sediment at 5.2 and 8.4 S, respectively (Fig. 2A). These sedimentation coefficients are in good agreement with previously reported experimental values (12, 14) as well as theoretical values calculated from their crystal structures (5, 28) of 5.8 and 8.4 S. Similar results are obtained for the individual subunits with ATP/CDP and dTTP/GDP (SI Appendix, Fig. S1). When α2 and β2 are combined in the presence of saturating dATP/CDP, a distinct peak is observed at 15.6 S at a physiological RNR concentration of 1 μM (26, 27) (Fig. 2A). Increasing the protein concentration does not result in peak shifts, indicating that the 15.6-S peak can be attributed to a single slowly dissociating species (29). Gaussian fitting to the peak yields a molecular mass of 533 kDa by the Svedberg relation (30) (SI Appendix, Fig. S2), similar to the 510-kDa estimate for the dATP-inhibited E. coli enzyme reported by GEMMA (18), and consistent with an α2β2 complex. A typical globular protein of this molecular mass has a frictional ratio of 1.2, giving an expected sedimentation coefficient of 19 S (31). This value is much larger than the observed value for the dATP-inhibited complex, suggesting that this complex is highly nonglobular.

In contrast, broad peaks with maxima near 10–11.5 S were observed in the presence of saturating dTTP/GDP or ATP/CDP at 1 μM protein concentration (Fig. 2B). Gaussian fitting of these peaks did not yield molecular mass estimates that were consistent with any one single species of RNR, including the expected α2β2 active complex. Furthermore, increasing the protein concentration by 10-fold shifted the dominant peak toward 15.6 S (dashed line in Fig. 2B), approaching the sedimentation coefficient of the dATP-inhibited complex. The observation of broad peaks that shift with increasing protein concentration is the hallmark of a mixture of rapidly exchanging species rather than a single species (29). Contrary to our previous understanding of E. coli class Ia RNR, the peak shifts observed here indicate that a large complex dominates at high protein concentration in the absence of dATP, with no effector in the activity site (Fig. 2B, dTTP/GDP) or even with a positive activity regulator occupying the activity site (Fig. 2B, ATP/CDP). Significantly, the peak shifts suggest that, under these conditions, this large complex is able to rapidly exchange with smaller species and is therefore in the equilibrium mixture even at physiological protein concentrations.

dATP-Inhibited Complex Contains Both α2 and β2. To determine the subunit composition and stoichiometry of the dATP-inhibited complex, we used SAXS, a structural technique that provides protein size and shape information in solution. α2 was titrated into β2 under identical dATP/CDP conditions investigated by AUC, leading to a rapid and dramatic increase in radius of gyration (Rg) up to the equimolar point, consistent with the formation of a large complex with a 1:1 subunit stoichiometry (SI Appendix, Fig. S3). Given the 533-kDa molecular mass determined by AUC, a 1:1 complex is most consistent with a 517 kDa α2β2 oligomerization state.

As a control, α2 was examined under identical conditions without β2. The Rg of α2 alone showed minimal concentration dependence with a value of 39.7 ± 0.3 Å when extrapolated to zero protein concentration to eliminate the effects of interparticle interactions (SI Appendix, Fig. S3B), in excellent agreement with the theoretical value of 39.3 Å calculated from the α2 crystal structure (5). The reverse titration and corresponding control with β2 alone yielded similar results (SI Appendix, Fig. S4), showing that both α2 and β2 must be present for the formation of higher order oligomers.

Structure of the dATP-Inhibited Complex Is an α4β4 Ring. EM and X-ray crystallography were used to investigate the architecture of the dATP-induced α4β4 complex. EM images were acquired of 0.15 μM α2 and β2 in the presence of 1 mM CDP and 0, 1, or 50 μM dATP (Fig. 3A and SI Appendix, Fig. S5). In the absence of dATP, the subunits are largely dissociated, as expected from the measured micromolar subunit affinity (32). The addition of dATP drives the formation of distinct ring-like complexes approximately 200 Å in outer diameter. Images of these ring-shaped particles were aligned and classified (SI Appendix, Fig. S6) and the resulting class averages are consistent with α2 and β2 subunits arranged in an alternating pattern, forming an α4β4 complex (Fig. 3B, Average). Because the EM images were collected as tilted—untilted pairs, we were able to generate a 3D

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map of the αβ1 ring at approximately 23-Å resolution (Fig. 3B and SI Appendix, Fig. S7). Crystal structures of the individual dimeric subunits (5, 28) could be unambiguously fit to the 3D map of the αβ1 ring, confirming the alternating subunit arrangement suggested by the 2D class averages. To obtain a more detailed picture of the αβ1 complex, a 5.65-Å resolution crystal structure was obtained by cocrystallizing α2 and β2 with 10 mM dATP (Fig. 3C and SI Appendix, Table S1). The structure, which was solved by molecular replacement, displays electron density for dATP bound at both activity and specificity sites (SI Appendix, Fig. S8 D and E) and reveals the same αβ1 ring-shaped species in the asymmetric unit of the crystal as that observed by EM (Fig. 3 B and C). The individual α2 and β2 subunits of the αβ1 structure align well with structures of uncomplexed α2 and β2 subunits, showing only small variations in the α2 cone domain, and sharing overall average root-mean-square derivations of 1.11 and 1.01 Å for α2 and β2 subunits, respectively (SI Appendix, Fig. S8 B and C). In the αβ1 structure, α2 and β2 contact each other at two points. In addition to the previously identified (15) binding pocket on α2 for the C terminus of β2, we find a second interaction between the activity site, housed in the N-terminal cone domain of α2 (Fig. 3 C and D, green), and each lobe of β2 (Fig. 3 C and D, red/orange). This direct contact between the dATP-bound α2 activity site and the β2 subunits provides a molecular explanation for how the presence of activity effectors can be sensed, leading to the regulation of activity. From these structural data, we can also rationalize why a dATP-induced αβ1 complex would be inactive. We find that residues on the radical propagation pathway (Fig. 1C) are not aligned appropriately for electron transfer, instead facing a large central opening approximately 100 Å in diameter (Fig. 3C) such that W48 of β2 and Y731 of α2 are solvent exposed, and Y356 on the flexible C-terminal tail of β2 remains disordered. Also, the distance between W48 and Y731 is too long (ca. 55–58 Å) for PCET via only a single residue in the middle (Y356). In contrast, for active E. coli RNR, expected W48-Y731 distances are approximately 23–25 Å based on the proposed docking model (15) (Fig. 1B), which has been supported experimentally using distance restraints from pulsed electron–electron double resonance (PEL-DOR) spectroscopy (13).

The unanticipated αβ1 structure observed by EM and crystallography is supported by SAXS and AUC. Importantly, the use of these four methods allows for the interrogation of this RNR structure at a wide range of protein and dATP concentrations. Protein concentrations of 0.15 μM (EM) to 25 μM (X-ray), and dATP concentrations of 50 μM (EM) to 10 mM (X-ray) were examined. In addition, physiological concentrations of protein (1–2 μM) and dATP (175 μM) were investigated by AUC and SAXS in solution. Over this wide range of conditions, we find a consistent view of the αβ1 structure. The theoretical sedimentation coefficient calculated from the αβ1 structure is 15.6 S, in excellent agreement with that observed experimentally by AUC (Fig. 2A). The extrapolated Rs of 71.1 ± 1.1 Å for αβ1 determined by SAXS (SI Appendix, Fig. S9) is a larger value than expected for a globular protein of this molecular mass, but consistent with an open ring structure. Moreover, theoretical scattering curves calculated from the αβ1 structure are well described with the experimental scattering obtained by SAXS (Fig. 3E), demonstrating that the αβ1 ring is not only stable in solution, but also the dominant form of the complex under negative feedback regulation by dATP near physiological protein and effector concentrations.

**dATP Promotes Subunit Rearrangement.** With the structures of free α2 and β2 determined previously (5, 28) and the structure of the dATP-inhibited αβ1 complex now determined by both EM and crystallography, SAXS was used to study the interconversion between different oligomeric states in solution. SAXS is ideal...
for probing structural interconversions as the relative fractions of individual states can be deconvoluted from mixtures (33). Titration of dATP into RNR in the presence of CDP leads to a dramatic change in the shape of the scattering curves and their corresponding Kratky curves, indicative of a transition from a predominantly compact state to a nonglobular state (Fig. 4 A and B). Above 24 μM dATP, or 4 molar equivalents of dATP per α2, the scattering curves are nearly superimposable and the Rs approaches that of the αβ2 ring (SI Appendix, Fig. S10). This result is consistent with full dATP occupancy in the effector sites of αδβ4, as expected from the reported dATP binding affinities for the specificity and activity sites of 0.5 and 5 μM, respectively (4, 34).

Isointensity points in the scattering curves are observed, indicating that this structural change can be explained by a two-state process (35) (Fig. 4A). Consistent with this interpretation, singular value decomposition shows that the scattering curves can be fit to linear combinations of two independent states with low residual values from the individual subunits as calibrants, the average molecular mass in the dATP-driven transition was calculated to increase from 223 kDa in the absence of dATP to 512 kDa at saturating dATP (SI Appendix, Fig. S12). These values are in close agreement with the 259 kDa αβ2 expected in the absence of dATP and the 517 kDa αβ4 expected under saturating dATP. The excellent molecular mass estimation and compact shape, this result is consistent with full dATP occupancy in the effector sites (SI Appendix, Tables S2 and S3, and Fig. S10). The small additional density observed in the molecular envelope of αβ2 can be explained by the presence of approximately 3% αδβ4 (Fig. 4C).

Discussion

Allosteric regulation of activity in class Ia RNR provides a mechanism to prevent the accumulation of cytotoxic levels of deoxyribonucleotides. dATP is unique among allosteric effectors in its ability to down-regulate RNR activity (4, 6). Here, using SAXS, EM, and X-ray crystallography, we present a structural model to explain dATP inhibition of activity for the prototypic class Ia RNR. We find that the dATP-inhibited complex has an αβ2 stoichiometry as predicted by GEMMA (18), but with an unprecedented arrangement of alternating subunits in an open ring structure. Importantly, we have confirmed this surprising structure using four different techniques and have shown that this state is the predominant species stabilized by dATP under physiological solution conditions. Using the same concentration of protein in the absence of dATP the SAXS data agree well with the predicted αβ2 docking model of the active RNR (15), providing a structural depiction of the elusive αβ2 oligomerization state.

In addition to high dATP concentrations driving the formation of the αβ2 state of E. coli RNR, AUC results show that increasing protein concentrations also shift the conformational equilibrium toward the higher molecular mass αβ4 (Fig. 2B). At lower protein concentrations and in the absence of dATP, αβ2 is still present but to a smaller degree, in rapid equilibrium with the active αβ2 state. Taken together, the SAXS and AUC results suggest that the active RNR complex is an intermediate between two inactive states, the dissociated α2 and β2 and the αδβ4 ring (Fig. 4D),

[Image: SAXS investigation of the transition between lower and higher order structures of E. coli class Ia RNR. (A) Scattering curves measured as 0–175 μM dATP was titrated into a 6 μM solution of α2 and β2 in the presence of 1 mM CDP (red to violet) display iso- intensity points, suggesting a two-state transition. (B) Kratky representations of the scattering curves (Iq2 vs. q) at 0 μM (red), 12 μM (green), and 40 μM (blue) dATP show a transition from a compact globular state, as indicated by the monomodal peak, to a large nonglobular state, indicated by a bimodal curve (36). (C) Fitting linear combinations of the αβ2 docking model (15) and the αδβ4 ring to the titration data provided relative fractions of the two states. (D) Ab initio SAXS reconstructions of free subunits aligned with deposited crystal structures (5, 39), a compact αβ2 state aligned with the proposed docking model (15), and the αδβ4 ring aligned with the crystal structure (SI Appendix, Tables S2 and S3, and Fig. S10).]
and that the equilibrium between these states is sensitive to both protein and effector concentrations.

Although it has long been known that the α₂ cone domain binds the activity effectors ATP and dATP (5, 19), this α₂β₂ structure provides a molecular explanation for how the cone domain regulates the activity of *E. coli* RNR. We find that each α₂ cone domain contacts a lobe of β₂, burying a surface area (556 Å² at each of four interfaces; Fig. 3D) that is large enough to stabilize the ring structure as the dominant species under physiologically relevant protein (1–2 μM) and dATP (175 μM) concentrations in the absence of ATP, although not so large as to inhibit interconversion back to the active α₂β₂ state in the presence of ATP and other effectors. In the α₂β₂ state, the radical transfer pathway from β₁ to α₁ is disrupted and solvent exposed, unable to propagate radicals. In contrast, in a compact α₂β₂ state, these residues are expected to be closer together, buried, and shielded from bulk water (15). Thus the cone domain of α₂ “communicates” the nucleotide levels of the cell to β₂, and the equilibrium of conformers can be shifted toward the α₂β₂ state, disrupting the radical pathway and inhibiting activity, or toward α₂β₁, restoring the radical pathway and enzyme activity, as nucleotide levels dictate.

Interestingly, a recently published 6.6-Å structure of yeast RNR also shows the α₂ cone domain to be present at a protein–protein interface. In this case, however, the interface connects two α₂-subunits in a dATP-induced α₂ hexamer (19). Although it is still early in terms of understanding structure/function for eukaryotic RNRs, this recent structural work along with the results presented here provide compelling support for the involvement of the cone domain in the formation of high-order RNR oligomers and pave the way for future studies.

Twenty-one years after the structure of αβ was published (39), a structure of an αβ-complex for this prototypic class Ia RNR is now available. Through the power of multiple structural techniques and comparisons of multiple laboratories, the structures of all dominant forms of this enzyme can now be described. From crystallography and SAXS, views of the dissociated subunits are available (this work and refs. 5 and 28), from SAXS and PELDOR, low-resolution structural data support an active α₂β₂ model (this work and ref. 13), and from crystallography, EM, and SAXS, the structure of an allosterically inhibited α₂β₁ ring has been determined (this work). These structures have broad applications, providing a molecular framework for considering the relationship between in vivo *E. coli* nucleotide concentrations and RNR activity, as well as offering a basis for the rational design of a class of inhibitors that could act by stabilizing inactive oligomeric states of the enzyme. This open ring structure of alternating subunits is the latest surprise as this prototypic RNR enzyme, in all of its states, comes into focus.

### Materials and Methods

*E. coli* α₂ and β₂ were isolated as previously described (40, 41). All of the β₂ used in these structural studies was treated with hydroxyurea (HU) to reduce the essential tyrosyl radical to prevent substrate turnover. All experiments were performed in the standard assay buffer (50 mM HEPES, pH 7.6, 15 mM MgCl₂, 1 mM EDTA) with 5 mM DTT and the nucleotide concentrations adjusted to the indicated levels.

Crystallization of α₂ and β₂ with each dATP was performed by hanging drop vapor diffusion at pH 7.5. Data were collected at the Advanced Light Source beamline 8.2.2, and the structure was solved by molecular replacement using the coordinates 2R1R (5) and 1MXR (42) to 5.65-Å resolution and R factors of 27.5 (work) and 30.3 (free) (*SI Appendix, Table S1*).

SAXS images were collected at the Cornell High Energy Synchrotron Source G1 station and processed following previously described protocols (43). Data analyses were performed using the ATSAS package (44) and MATLAB (MathWorks). The momentum transfer variable, q, is defined as q = 4π/λ sin(θ/2), where θ/2 is the scattering angle, and λ is the X-ray wavelength.

Sedimentation velocity AUC was performed using a Beckman XL-1 analytically ultracentrifuge equipped with interference optics. Sedimentation coefficient distributions g(s) and-lg(s) were generated in DCDT+ (30) and Sedfit (45), respectively. The values were corrected to standard values g(20,w) using Sednterp (46). Theoretical g(20,w) values were calculated with MATD (47).

EM specimens were prepared by staining with uranyl acetate. Eighty-nine pairs of CCD images of untitled and –5° tilted specimens were collected on a Tecnai F20 electron microscope (FEI). Particles were selected in untitled images using e2boxer.py (48) and were matched with particles from tilted images using TiltPicker (49). A final dataset of 13,895 untitled particles were iteratively aligned and classified using SPIDER as described previously (50) and the corresponding set of tilted images used to generate a 3D reconstruction for each class.

### Acknowledgments

We thank Deborah Pheasant [Massachusetts Institute of Technology (MIT) Biophysical Instrumentation Facility] and Dr. Walter Stafford (Boston Biomedical Research Institute) for helpful discussions on AUC. For assistance with SAXS data collection, we thank Mackenzie Frer- ing and Tamar N. Kung (MIT). Funding for this work was provided by the Synchrotron Source (CHESS) scientists, Drs. Arthur Woll and Richard Gillilan. We thank Prof. Sol Gruner (Cornell) for access to SAXS equipment and wet lab space. CHESS is supported by the National Science Foundation (NSF) and National Institutes of Health/National Institute of General Medical Sciences (NIH/NIGMS) via NSF award DMR-0963868, and the Macromolecular Diffraction Facility at CHESS resource is supported by NIH/National Center for Research Resources (NCRR) award RR-01646. Electron microscopy was performed at the National Resource for Automated Molecular Microscopy, which is supported by the NIH through the NCRR P41 program (RR017573). Crystallographic data collection was conducted at Advanced Light Source, a Department of Energy (DOE) national user facility (Contract DE-AC02-05CH11231), at beamline 8.2.2 operated by the Berkeley Center for Structural Biology, which is supported in part by the DOE and NIH/NIGMS. This work was supported by NIH grants F32GM094862 (to N.A.), F32DK080622 (to E.J.B.), T32GM08334 (to C.M.Z.), GM07167 (to F.J.A.), and GM25959 (to J.S.), and the NSF Graduate Research Fellowship under Grant 0645960 (to M.A.F.). C.L.D. is a Howard Hughes Medical Institute Investigator.


