Ligand-induced and small-molecule control of substrate loading in a hexameric helicase

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Processive, ring-shaped protein and nucleic acid protein translocases control essential biochemical processes throughout biology and are considered high-prospect therapeutic targets. The *Escherichia coli* Rho factor is an exemplar hexameric RNA translocase that terminates transcription in bacteria. As with many ring-shaped motor proteins, Rho activity is modulated by a variety of poorly understood mechanisms, including small-molecule therapeutics, protein–protein interactions, and the sequence of its translocation substrate. Here, we establish the mechanism of action of two Rho effectors, the antibiotic bicyclomycin and nucleic acids that bind to Rho’s primary RNA recruitment site. Using small-angle X-ray scattering and a fluorescence-based assay to monitor the ability of Rho to switch between open-ring (RNA-loading) and closed-ring (RNA-translocation) states, we found bicyclomycin to be a direct antagonist of ring closure. Reciprocally, the binding of nucleic acids to its N-terminal RNA recruitment domains is shown to promote the formation of a closed-ring Rho state, with increasing primary-site occupancy providing additive stimulatory effects. This study establishes bicyclomycin as a conformational inhibitor of Rho ring dynamics, highlighting the utility of developing assays that read out protein conformation as a prospective screening tool for ring-ATPase inhibitors. Our findings further show that the RNA sequence specificity used for guiding Rho-dependent termination derives in part from an intrinsic ability of the motor to couple the recognition of pyrimidine patterns in nascent transcripts to RNA loading and activity.

Rho is a hexameric helicase responsible for controlling ∼20% of all transcription termination events in *Escherichia coli* (9). Rho is initially recruited to nascent transcripts in an open, lock-washer-shaped configuration (Fig. 1A) (10, 11), where it binds preferentially to pyrimidine-rich sequences (termed “Rho utilization of termination” sequences, or “rut” sites) using a primary RNA-binding site located in the N-terminal OB folds of the hexamer (12–14). Following *rut* recognition, Rho converts into a closed-ring form (Fig. 1B), locking the RNA strand into a secondary RNA-binding site formed by two conserved sequence elements known as the “Q” and “R” loops (15) located within the central pore of the hexamer. This conformational change, which we show in an accompanying paper to be both RNA- and ATP-dependent (16), rearranges residues in the Rho ATP-binding pockets into a hydrolysis-competent state (17). Once engaged, Rho maintains primary-site contacts with the *rut* sequence as it translocates 5’ to 3’ along the RNA strand in an ATP-dependent manner, a process known as “tethered tracking” (18–21). Rho elicits termination by applying direct or indirect forces to RNA polymerase (22, 23), dislodging it from DNA and the newly made RNA in the transcription bubble.

The basic steps of Rho-dependent transcription termination have been largely elucidated, and it has become increasingly clear that Rho’s activity, like that of many helicases and translocases, can be controlled by a variety of intrinsic and extrinsic factors. The small molecule bicyclomycin (Fig. 1C), a highly specific chemical inhibitor of Rho that has been shown to wedge itself in between subunits of an open Rho ring (Fig. 1D and Fig. S1) (24), is one such example. Previous studies have shown that bicyclomycin is a noncompetitive inhibitor of Rho ATPase activity and a mixed inhibitor of RNA binding to Rho’s secondary site (25, 26). Although consequent structural studies suggested that bicyclomycin antagonizes Rho by sterically preventing the binding of the nucleophilic water molecule that initiates ATP hydrolysis (24), how bicyclomycin might interact with catalytically competent Rho states, such as those thought to accompany ATPase activity and translocation (17), has not been defined.

It is similarly unclear how other dissociable factors, e.g., regulatory proteins and/or nucleic acids, influence Rho activity. It is well established that the sequence of the RNA itself has a pronounced impact on whether a transcript will be acted upon by Rho (12, 27–29). Binding of pyrimidine-rich sequences to the N-terminal RNA-binding domains of Rho is a particularly well-known accelerator of Rho’s ATPase activity (30), with pre-steady-state ATPase assays showing that the formation of a catalytically competent Rho ring is governed by a rate-limiting RNA- and ATP-dependent conformational change (8). The ligand-dependence of this isomerization event correlates with the requirements for ring closure identified in an accompanying study (16). These findings raise the intriguing possibility that the sequence specificity of Rho-dependent termination is caused in part by an increased efficiency of ring closure when the N-terminal RNA-binding domains are occupied by pyrimidine-rich sequences.

Here we show that bicyclomycin and primary-site occupancy affect the structural state of Rho in an opposing manner. Using antibiotic | ATPase | helicase | motor protein | transcription

RING-SHAPED HEXAMERIC HELICASES AND TRANSLASCES ARE MOTOR PROTEINS THAT CONTROL MYRIAD ESSENTIAL AND CELLULAR PROCESSES. MANY HEXAMERIC MOTORS UNDERGO SUBSTRATE-DEPENDENT CONFORMATIONAL CHANGES THAT COUPLE ACTIVITY TO THE PRODUCTIVE BINDING OF CLIENT SUBSTRATES (1-4). INTERNAL REGULATORY DOMAINS AND EXOGENOUS PROTEINS OR SMALL MOLECULES FREQUENTLY IMPACT CLIENT SUBSTRATE RECRUITMENT AND ENGAGEMENT BY THESE ENZYMES (5-8); HOWEVER, IT IS GENERALLY UNCLEAR HOW SUCH FACTORS CONTROL HELICASE OR TRANSLASCE DYNAMICS. RHO IS A HEXAMERIC HELICASE RESPONSIBLE FOR CONTROLLING ~20% OF ALL TRANSCRIPTION TERMINATION EVENTS IN *ESCHERICHIA COLI* (9). RHO IS INITIALLY RECRUITED TO NASCENT TRANSCRIPTS IN AN OPEN, LOCK-WASHER-SHAPED CONFIGURATION (FIG. 1A) (10, 11), WHERE IT BINDS PREFERENTIALLY TO PYRIMIDINE-RICH SEQUENCES (TERMED “RHO UTILIZATION OF TERMINATION” SEQUENCES, OR “RUT” SITES) USING A PRIMARY RNA-BINDING SITE LOCATED IN THE N-TERMINAL OB FOLDS OF THE HEXAMER (12-14). FOLLOWING RUT RECOGNITION, RHO CONVERTS INTO A CLOSED-RING FORM (FIG. 1B), LOCKING THE RNA STRAND INTO A SECONDARY RNA-BINDING SITE FORMED BY TWO CONSERVED SEQUENCE ELEMENTS KNOWN AS THE “Q” AND “R” LOOPS (15) LOCATED WITHIN THE CENTRAL PORE OF THE HEXAMER. THIS CONFORMATIONAL CHANGE, WHICH WE SHOW IN AN ACCOMPANYING PAPER TO BE BOTH RNA- AND ATP-DEPENDENT (16), RERANGERS RESIDUES IN THE RHO ATP-BINDING POCKETS INTO A HYDROLYSIS-COMPETENT STATE (17). ONCE ENGAGED, RHO MAINTAINS PRIMARY-SITE CONTACTS WITH THE RUT SEQUENCE AS IT TRANSLATES 5’ TO 3’ ALONG THE RNA STRAND IN AN ATP-DEPENDENT MANNER, A PROCESS KNOWN AS “TETHERED TRACKING” (18-21). RHO ELICITS TERMINATION BY APPLYING DIRECT OR INDIRECT FORCES TO RNA POLYMERASE (22, 23), DISLODGING IT FROM DNA AND THE NEWLY MADE RNA IN THE TRANSCRIPTION BUBBLE.
Fig. 1. Bicyclomycin is an inhibitor of the Rho helicase. (A) Crystal structure of open-ring and bicyclomycin-bound Rho [PDB ID code 1XPO (24)]. Rho subunits are alternatingly colored yellow and gold for distinction, bicyclomycin is black, and primary-site RNAs are magenta. (B) Crystal structure of closed-ring and translocation-competent Rho [PDB ID code 3ICE (17)]. Rho subunits are alternatingly colored cyan and dark blue, and RNA bound in the secondary site is red. Magenta-colored ovals denote the location of the primary sites that are not occupied in this crystal form. (C) Chemical structure of bicyclomycin. (D) A close-up view of the bicyclomycin-binding pocket shows that bicyclomycin nestles into a small pocket between Rho subunit interfaces. (E) Modeling of bicyclomycin into the closed-ring Rho structure shows clear steric clashes between the drug and Rho.

small-angle X-ray scattering (SAXS) and a fluorescence-based assay to track ring status in vitro, we demonstrate that bicyclomycin inhibits RNA binding to the central pore of Rho by sterically impeding ring closure. In contrast, the binding of pyrimidine-rich nucleic acids to the Rho N-terminal RNA-binding domains promotes Rho ring closure, aiding the capture of nonideal, purine-rich RNA sequences within Rho’s translocation pore. Collectively, these findings help highlight diverse mechanisms by which ligand binding to discrete sites on ring-type ATPases can activate or repress conformational changes and govern the function of the motor.

Results

The Bicyclomycin-Binding Pocket Collapses upon Rho Ring Closure. During the course of examining the bicyclomycin-binding pocket across different Rho structural states (17, 16), we noticed that the site appeared substantially smaller in closed-ring states than in the open-ring states. Computational analysis confirmed that the pocket volume is much larger in the open-ring conformation (272 Å³) (Fig. S2A) than in the closed-ring state (56 Å³) (Fig. S2C), a trend that held true with all open- and closed-ring Rho structures that have been observed to date (Fig. S2 and Table S1). Correspondingly, alignment of a bicyclomycin-bound open ring structure [Protein Data Bank (PDB) ID code 1XPO (24)] with a closed-ring Rho model [PDB ID code 3ICE (17)] revealed extensive steric clashes with bicyclomycin and residues P180, K184, and E211 in the closed-ring state that would appear to preclude binding (compare Fig. 1 D and E). From these observations, we postulated that bicyclomycin might not sterically block the attacking water needed for ATP hydrolysis, as previously proposed, but instead antagonize the RNA- and ATP-dependent conformational switching of Rho from an open- to a closed-ring state.

Bicyclomycin Inhibits Rho Ring Closure over a Range of Nucleotide Concentrations. To determine the impact of bicyclomycin on the Rho conformational state, we first used SAXS to monitor structural dynamics in solution directly. An accompanying study demonstrates that open-ring Rho molecules close in the presence of RNA and the nonhydrolyzable ATP analog ADP·BeF₃ but not in the presence of either ligand individually (16). Because bicyclomycin is relatively modest inhibitor of Rho ATPase activity (IC₅₀ = 20 μM) (25), we reasoned that its effects on ring closure might be most evident when examined over a range of nucleotide concentrations. As a control, we carried out SAXS studies using a series of ADP·BeF₃ concentrations in the presence of RNA and the absence of bicyclomycin. When the average intensity of the observed scattering (I) as a function of scattering vector (q) was plotted for a variety of ADP·BeF₃ concentrations (Fig. 2A), nucleotide-dependent differences in curve shapes were clearly evident, similar to those reported in the accompanying study (Fig. S3) (16). Visual inspection of the curves over a q range from 0.07-0.13 Å⁻¹, the region of the curve in which nucleotide-dependent differences are most pronounced (Fig. 2A, Insert), revealed that the profiles converged between 1.5 and 4 mM ADP·BeF₃, suggesting that under these conditions Rho exists predominantly as a closed ring. Indeed, quantification of the percentage of open vs. closed states using a Minimal Ensemble Search (MES) (31) suggested that 1.5 mM ADP·BeF₃ was sufficient to close the majority of Rho rings in solution, with an increase in the percentage of closed rings observed at 2, 3, and 4 mM ADP·BeF₃ (Fig. 2C and Table S2). Similar trends also were observed in the reduction of the radius of gyration (Rg) at increasing ADP·BeF₃ concentrations, consistent with the expected nucleotide-dependent compaction of the Rho ring upon transition to a predominantly closed-ring state (Figs. S4A and S5 and Table S3).

After establishing a SAXS regime for looking at changes in ring state, we next set out to determine whether bicyclomycin would antagonize the ability of the Rho ring to close in a nucleotide-dependent fashion. We first preincubated Rho with a high concentration of bicyclomycin (400 μM final concentration, an eightfold molar excess over Rho monomer) and then added RNA and a variety of ADP·BeF₃ concentrations. The resulting SAXS curves (Fig. 2B) also displayed differences that were clearly dependent upon nucleotide concentration; however, at intermediate concentrations of ADP·BeF₃ (0.5, 1, and 1.5 mM), curves were skewed toward the open-ring state relative to the drug-free curves (compare Insets in Fig. 2 A and B). Quantification of ring state by MES showed a substantial shift toward the open form when bicyclomycin was present; this shift was particularly notable at 1.5 and 2 mM ADP·BeF₃ (Fig. 2C and Table S2). This bicyclomycin-dependent inhibition of Rho ring closure also was consistent with observed increases in Rg upon addition of the drug (Figs. S4A and S6 and Table S3).

The Rho Ring State Can Be Controlled by Varying the Bicyclomycin Concentration. After observing that bicyclomycin inhibits ring closure, we wondered whether it would be possible to control the Rho ring state by varying the concentration of bicyclomycin with Rho before adding RNA and ADP·BeF₃. To address this question, we conducted a SAXS experiment in the presence of 1.5 mM ADP·BeF₃, a nucleotide concentration that, based on our previous titration, is sufficient to close a substantial fraction (but not all) of the Rho rings present in solution. Preincubating Rho with varying concentrations of bicyclomycin yielded significant differences in the observed SAXS curves (Fig. 3A). When we focused on the mid-q region (Fig. 3A, Insert), it was immediately evident that the ring state can be affected by bicyclomycin. Quantification of the percentage of the open vs. closed ring populations by MES (Fig. 3B and Table S4) shows that the ring state changed from predominantly closed in the absence of bicyclomycin to mostly open at high bicyclomycin concentrations. Observed Rg values ranged from 46.3 Å in the absence of bicyclomycin to 48.2 Å in the presence of 3.200 μM bicyclomycin, a finding that is consistent with bicyclomycin concentration-dependent inhibition of Rho ring closure (Figs. S4B and S7 and Table S3).

A Fluorescence-Based Assay Tracks Ligand-Dependent Effects on Rho Ring Status in Vitro. Because SAXS studies constitute only one assessment of Rho state, we next set out to develop an in vitro assay to further characterize the ligand-dependent effects on the conformation of the hexamer. We reasoned that a short, fluorescein-derivatized RNA would have a significantly faster tumbling rate when free in solution than when stably bound to the secondary (translocation pore) site inside a closed Rho ring (see the diagram in Fig. 4A), thus enabling us to track ring closure by monitoring changes in fluorescence anisotropy (FA). To rule out the possibility that the binding of labeled RNA to Rho’s primary (RNA-recruitment) site
might convolute the signal, we first preincubated the protein with a short pyrimidine-rich DNA (dC5) at a concentration equimolar to that of Rho monomer [polypyrimidine DNAs bind both tightly and selectively to the primary sites of Rho (12, 30)]. No anisotropy changes were observed with a fluorescein-labeled poly-U RNA (rU12*), indicating that rU12* alone does not have high affinity for the secondary site in open-ring Rho. By contrast, robust rU12* binding was observed as nucleotide was added (Fig. 4B), showing that the secondary site becomes capable of stably binding RNA as the Rho ring closes. These findings are consistent with the SAXS studies carried out in the accompanying study (16), showing that both RNA and nucleotide are needed to promote ring closure cooperatively in Rho.

To characterize further the mechanism by which bicyclomycin inhibits Rho, we next conducted an order-of-addition experiment examining the impact of bicyclomycin on rU12* binding either before or after Rho ring closure. As expected, preincubation of bicyclomycin with Rho before the addition of RNA and nucleotide inhibited rU12* binding in a bicyclomycin concentration-dependent manner (Fig. 4C). Conversely, the impact of bicyclomycin on rU12* binding was far less evident when closed rings were formed before the addition of the drug (Fig. 4D). These data indicate that, although bicyclomycin is able to bind to an open Rho ring and prevent ring closure, the molecule is unable to force open a preclosed Rho ring.
Rho Ring Closure Is Promoted by the Binding of Pyrimidine-Containing Nucleic Acids to the N-Terminal Primary-Site Domains. Although RNA binding to Rho’s secondary site (the helicase translocation pore) is a major effector of ring closure, RNA binding to Rho’s primary site (its OB folds) also has been implicated in controlling Rho function (27). To probe the role of primary-site ligands in modulating ring closure, we tested whether the presence or absence of dC₅ could impact the binding of rU₁₂⁺ to the secondary site. We also conducted the same experiments with fluorescein-labeled poly-A RNA (rA₁₂⁺), which is a weaker secondary-site ligand than poly-U and is incompatible with primary-site binding (14, 32). A clear increase for rA₁₂⁺ affinity was evident in the presence of dC₅ (Fig. S8), especially at low ADP·BeF₃ concentrations. Under all nucleotide concentrations tested, rU₁₂⁺ bound more tightly to Rho than did rA₁₂⁺ (Fig. S8), with dC₅ subtly promoting ring closure at moderate (1 mM ADP·BeF₃) nucleotide concentrations. Primary-site occupancy appeared to have either no effect (1.5 mM ADP·BeF₃) or a slight inhibitory effect (2–3 mM ADP·BeF₃) on rU₁₂⁺ binding at higher nucleotide concentrations. Collectively, these results signify that nucleic acids bound to the primary site promote ring closure under conditions that are suboptimal for RNA binding to the secondary site.

Based on the spacing of the OB folds in the Rho structure and the observation that the isolated N-terminal domain engages only a pyrimidine dinucleotide (Fig. 1A and B) (10, 14), each dC₅ substate would be expected to bind one primary site at a time. Having observed that this short oligonucleotide nonetheless can promote Rho ring closure, we wondered whether longer DNAs capable of bridging multiple primary sites in a single Rho hexamer would have a more pronounced impact on ring state (see the diagram in Fig. 5A). We repeated the rU₁₂⁺- and rA₁₂⁺-binding assays with either a 15-mer DNA capable of bridging two primary sites (dC₁₅) or a 75-mer DNA potentially sufficiently long to bridge all six primary sites (dC₇₅), using an ADP·BeF₃ concentration (1 mM) at which primary-site binding of DNA has the most significant effect on RNA binding to the secondary site. The number of putative sites that could be occupied by the DNAs was held constant relative to the dC₅ experiments by adjusting the relative molar ratios between Rho and the DNA (Methods). For both rU₁₂⁺ and rA₁₂⁺, inclusion of dC₁₅ or dC₇₅ promoted RNA binding more robustly than the shorter dC₅ oligo (Fig. 5 B and C). Using fluorescein-labeled derivatives of the various primary-site ligands (denoted as dC₅⁺, dC₇₅⁺, and dC₇₅⁺), titration experiments revealed that DNAs long enough to bridge multiple primary sites have a much higher affinity for Rho than does dC₅ (Fig. 5D): by fixing Rho at a concentration insufficient to drive ring closure with rU₁₂⁺ or rA₁₂⁺ and varying the concentration of primary-site ligands, we found that ring closure can be driven by sufficiently high concentrations of dC₅, dC₁₅, or dC₇₅ (Fig. 5 E and F). These data indicate that primary-site occupancy favors the binding of both optimal (polypyrimidine) and nonoptimal (polypurine) RNAs to Rho’s secondary site.

Discussion

Ring-type helicases and translocases control numerous essential biological processes (33, 34). Although these enzymes generally are capable of stand-alone motor activity, multiple lines of evidence increasingly highlight complex mechanisms by which these enzymes are regulated. Protein–protein and protein–ligand interactions, small molecule effectors, and posttranslational modifications have all been implicated in controlling disparate types of ring-ATPases; how a majority of these factors exert their various stimulatory or antagonistic effects is generally not well understood.

In the present study, we discovered that bicyclomycin, one of the few small-molecule agents known to inhibit a ring-type motor, acts on its target, the Rho transcription termination factor, by sterically blocking a conformational change from an open- to a closed-ring state. Rho ring closure, which an accompanying study shows is dependent upon both ATP and secondary-site–bound RNA (16), triggers RNA strand engagement and rearranges residues in the ATP-binding pocket into a hydrolysis-competent state. By comparing a bicyclomycin-bound, open-ring conformation of Rho with other structures of Rho substates, we found that the drug-binding pocket collapses upon ring closure (Fig. 1). Follow-up SAXS studies show that bicyclomycin does not simply

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**Fig. 4.** FA-based RNA-binding assay to track Rho ring closure in vitro. (A) Schematic illustrating how the tumbling rate of a short fluorescein-tagged RNA is slowed by capture within the Rho ring. The Rho primary sites were blocked with short DNAs, represented in magenta. (B) Binding of rU₁₂⁺ to Rho is dependent upon the ADP·BeF₃ concentration. (C) Preincubation of Rho with bicyclomycin before the addition of RNA and ADP·BeF₃ demonstrates bicyclomycin concentration-dependent inhibition of rU₁₂⁺ binding. (D) Incubation of bicyclomycin with a preclosed Rho ring has little effect on rU₁₂⁺ binding.
occlude the binding of a catalytic water, as previously proposed (24), but instead directly impedes nucleotide-dependent closure of the Rho ring (Figs. 2 and 3). Using an FA-based assay to track ring closure in vitro, we further observed that bicyclomycin acts on preopened Rho rings and has little effect on ring topology after ring closure (Fig. 4). Because inhibition of secondary-site RNA binding is indicative of bicyclomycin binding to Rho (26), these results strongly indicate that bicyclomycin is unable to bind to preclosed Rho rings. Thus, bicyclomycin appears to inhibit Rho by stabilizing a conformation that is incapable of ATP hydrolysis and is incompatible with stable RNA binding to its motor regions.

In the course of investigating bicyclomycin’s effects on Rho, we also found that binding of nucleic acids to Rho’s primary RNA recruitment site promotes the switching of Rho from an open- to a closed-ring state. This finding is in accord with prior studies showing that Rho’s ATPase activity can be stimulated by primary-site occupancy (30) and that Rho’s ATPase rate is regulated in part by an RNA- and ATP-dependent conformational change (8), presumably into a closed-ring state (16, 17). The inclusion of polypuridine DNAs, which bind selectively to the primary site and also promote ATPase activity (30), increased Rho’s affinity for both rU12 and rA12 substrates at nucleotide concentrations that otherwise are too low to drive ring closure stably (Fig. S8). This result suggests that the binding of a ntr sequence to Rho’s primary site, which is formed by an N-terminal OB-fold in the protein (12, 13), allosterically promotes ring closure.

We further found that DNAs capable of bridging two (dC15) or six (dC30) primary sites within the Rho hexamer promoted ring closure more robustly than short (dC5) DNAs capable of occupying only one primary site (Fig. 5 A–C). The more robust impact of these longer DNAs is likely caused by their higher affinity for Rho (Fig. 5 D) rather than by a particular signal that might be propagated by a nucleic acid segment bridging multiple primary sites, because at sufficiently high concentrations all three ligands drive complete ring closure (Fig. 5 E and F). We also found that ring closure was more sensitive to primary-site occupancy by rA12 than with rU12: this finding is of interest because polypurines bind to the secondary site more weakly than polypyrimidines (18). Collectively, these results suggest that the RNA recruitment sites on Rho work together to count the number and spacing of pyrimidine recruitment motifs in an RNA transcript and promote ring closure around nonoptimal secondary-site binding sequences such as purine-rich regions (Fig. 6). Future studies looking at different types of di-pyrimidine patterns on both natural and synthetic substrates will be needed to probe how this interrogation occurs at a molecular level.

In using an auxiliary ligand-binding element to aid activity, Rho joins a growing list of processive ring helicases and translocases that are subject to both intrinsic and extrinsic regulation. These factors include the bacterial DnaB helicase (6), the MCM2-7 helicase (7, 35), and the protoenosomal Rpt1-6 ATPases (5). In each of these motors, a built-in accessory domain or a dissociable set of
Methods

Calculation of Bicyclomycin-Binding Pocket Volumes. Pocket volumes were calculated using POVMC (see SI Methods for program parameters) (37).

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SAXS Sample Preparation. Rho was purified as described previously (17). SAXS samples were assembled as described in SI Methods, flash-frozen in liquid nitrogen, and stored at −80 °C.

SAXS Data Collection and Analysis. SAXS data were collected using an automated, high-throughput system at the Advanced Light Source Beamline 12.3.1 (ALS BL12.3.1), Lawrence Berkeley National Laboratory (38). SAXS curves were generated, analyzed, and plotted as described in SI Methods.

FA-Based RNA-Binding Data Collection and Analysis. DNA oligonucleotides (dC8, dC10, and dC12 (dC815TC1515C1515TC1515C1515) were purchased from IDT. FA samples were prepared, measured, and analyzed as described in SI Methods.