The fact that translation initiation directed from CrPV IRES, which relies solely on the ribosome itself, is severely impaired in Dicel m' cells strongly implies that the molecular defect intrinsically resides in the inability of Dicel m' ribosomes to efficiently engage the IRES element. Taken together, these findings allow us to propose a model whereby reductions in rRNA modifications due to dyskerin malfunction affect the translation of important cellular IRES mRNAs, which may require more direct interactions with the ribosome for translation initiation, thereby contributing to specific pathological features of X-DC. Although we cannot determine how defects in other cellular functions attributed to dyskerin activity may contribute to X-DC, these findings indicate a previously unknown molecular mechanism by which impairments in rRNA modifications affect translation control and lead to disease pathogenesis.

References and Notes
7. Materials and methods are available as supporting material on Science Online.
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RNA Recognition and Cleavage by a Splicing Endonuclease
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The RNA splicing endonuclease cleaves two phosphodiester bonds within folded precursor RNAs during intron removal, producing the functional RNAs required for protein synthesis. Here we describe at a resolution of 2.85 angstroms the structure of a splicing endonuclease from Archaeoglobus fulgidus bound with a bulge-helix-bulge RNA containing a noncleaved and a cleaved splice site. The endonuclease dimer cooperatively recognized a flipped-out bulge base and stabilizes sharply bent bulge backbones that are poised for an in-line RNA cleavage reaction. Cooperativity arises because an arginine pair from one catalytic domain sandwiches a nucleobase within the bulge cleaved by the other catalytic domain.

The removal of intervening sequences in functional RNAs is required in all domains of life. Introns found in nuclear tRNA and archaeal RNA are removed by protein enzymes—an endonuclease, a ligase, and, in some organisms, a 2'-phosphotransferase (1–5).

Critical to this splicing mechanism are the recognition of the intron-exon junctions and the subsequent breakage of the two phosphodiester bonds. This process is mediated by the endonuclease. The splicing endonucleases characterized so far belong to one of four families: homodimers (α), homotetramers (αβ), homodimers of two heterodimers (αβ), and heterotetramers (αβγ) (6–12). Despite the differences in subunit composition, all splicing endonucleases comprise two conserved catalytic units and two structural units that play roles in correctly orienting the catalytic sites (13). All splicing endonucleases recognize the bulge-helix-bulge (BHB) motif that is composed of two three-nucleotide bulges separated by four base pairs (bp) (6, 8, 9, 14–16). Each bulge contains a cleavage site immediately after the second bulge nucleotide. In addition to the recognition of the canonical BHB RNA motif, splicing endonucleases of different families exhibit distinct substrate recognition properties. The localized structural fold of the BHB motif is sufficient for the removal of archaeal introns from various segments of precursor transfer RNA (tRNA), ribosomal RNA (rRNA), and some mRNA; the secondary structure of the BHB motif is solely responsible for its recognition by the archaeal splicing endonuclease (17–20). Nuclear precursor tRNAs, however, have additional recognition elements in their mature domains that are required for splicing (16, 21–23). The eukaryal ΨΨΨ splicing endonuclease is composed of Sen2, Sen34, Sen54, and Sen15 subunits and locates the splice sites via a “ruler mechanism.” This mechanism requires the specific recognition of two features in precursor tRNAs: the cloverleaf structure of the mature domain (21, 24) and an anticodon-intron base pair (A-I pair) adjacent to the ΨΨΨ bulge (22, 23). It has been proposed that the eukaryal endonuclease, while maintaining the ability to recognize the minimal BHB RNA, has acquired additional RNA recognition properties. Based on the cleavage products and conserved catalytic residues, all splicing endonucleases appear to use a similar mechanism. Cleavage by the splicing endonuclease generates 5'-hydroxyl and 2',3'-cyclic phosphate termini (1, 2, 25). These products suggest an S-type in-line attack by the nearby 2'-hydroxyl on phosphorus and a trinodal bipyrimal translation state, similar to that used by the RNase A family of ribonucleases (26). In previously determined archaeal-splicing endonuclease structures, three invariant residues—a histidine, a lysine, and a tyrosine—form a closely spaced triad. In the Methanococcus jannaschii endonuclease structure, a bound sulfate ion, which was proposed to mimic a phosphate, was found in the center of the triad (27), which further implicates the triad in RNA cleavage. However, there is no structural evidence to confirm the catalytic roles of each of the triad residues, and the catalytic mechanism of the splicing endonuclease remains elusive.

We have determined the cocrystal structure of a dimeric splicing endonuclease from Archaeoglobus fulgidus (AF) bound to a BHB RNA that was formed from two annealed 21-oligomer synthetic oligonucleotide (see Fig. 1A). One strand of the RNA contains a 2'-deoxy-
modified uridine at position 14 (dU14) to prevent its cleavage during crystallization, whereas the other strand was cleaved at a normal 3'-splice site (28). The endonuclease-21-oligomer BHB RNA structure, containing two endonuclease-RNA complexes not related by crystallographic symmetry, was determined by molecular replacement methods and refined at 2.85 Å resolution to an R_free of 29.6% (28). The mean precision of the atomic coordinates is 0.45 Å (table S1).

In the complex, the AF endonuclease is the same symmetric dimer as the free AF endonuclease with the structure deviating only in local regions (29, 30). In contrast, the BHB RNA differs significantly from its unbound solution structure as observed using nuclear magnetic resonance (NMR) methods (Fig. 1B). Curved when in solution (31), the helical portion of the BHB structure displays little bending when bound to the endonuclease (Fig. 1B). In addition, the three bulge nucleotides are completely flipped out of their stacking positions when bound, but not so when in solution. Consequently, the two bulges rise above the helix loop to form two distinct protrusions to interact with the endonuclease (Fig. 1B).

The minor groove of the 4-bp central helix of the BHB RNA docks at the center of a flat surface formed by both subunits of the endonuclease (Fig. 1C). Remarkably, the entire base-paired region of the unbound BHB RNA closely resembles a single A-form helix. The phosphate backbone of the uncleaved 5'-splice-site bulge bends ∼180° and crosses over itself, forming an open knot (Fig. 1C). Similarly, the phosphate backbone of the cleaved 3'-splice-site bulge makes an open knot in preparation for cleavage (Fig. 1C). This tight looping brings the two base pairs flanking the bulge loop, is near the center of the endonuclease and is sandwiched by Arg²⁸⁰ and Arg³⁰² (Fig. 2). Remarkably, the Arg²⁸⁰·Arg³⁰² pair of one subunit stabilizes the bulge that is cleaved by the other subunit (Fig. 2A). Arg²⁸⁰ is strictly conserved, whereas Arg³⁰² may be replaced by an aromatic residue in other archaeal and eukaryal organisms (fig. S2). For each cation–π sandwich, the planes formed by the atoms of the two guanidinium groups are ∼3.8 to 4.1 Å away from and nearly parallel to the adenine ring. This distance corresponds to the most favorable energy of interaction between the guanidinium group and the adenine base according to an ab initio quantum mechanics computation (32). Removal of the nucleobase from nucleotide A13 severely impairs the cleavage activity on the BHB RNA (see fig. S3A). Furthermore, the guanidinium group of Arg²⁸⁰ forms a double hydrogen bond with the phosphate group of the last bulge nucleotide (G16 or g16) and the 2'-hydroxyl oxygen atom of the first bulge nucleotide (A13/a13) (Fig. 2, B and C). This double hydrogen bond constrains the orientation of the guanidinium group and thus maximizes the cation–π interaction while stabilizing the sharp bend bulge backbone. The importance of Arg²⁸⁰ is demonstrated by the fact that it is one of four strictly conserved residues, and its mutation to lysine completely abolishes RNA cleavage activity (fig. S3B).

RNA functional groups of the BHB motif also stabilized the sharply bent bulge backbone. The phosphate group of G16/g16, which closes the bulge loop, is near the center of the negatively charged bulge backbone (Fig. 2D). The nonbridging oxygens of this phosphate form short hydrogen bonds with the 2'-hydroxyl groups of C12 (2.4 Å) and A13 (2.7 Å), and a long hydrogen bond with A15 (3.5 Å) (Fig. 2D). Methylation of the 2'-hydroxyl group of C12 or A13 impairs cleavage activity on the 21-oligomer oligonucleotide BHB RNA substrate (Fig. S3A).

The second bulge nucleotide (dU14/a2314) is also flipped out of helical stacking, pointing in a direction perpendicular to that of the first bulge nucleotide. It is recognized primarily by its ribose ring and its connection to the well-positioned scissile phosphate group (Fig. 2B). Residue Leu²⁵⁵ in loop L7 of the endonuclease forms sequence-independent van der Waals contact with the base (Fig. 2B). This mode of interaction (33) may allow the enzyme to distinguish between families of similar splice sites, as exemplified by the fact that it is one of four strictly conserved residues, and its mutation to lysine completely abolishes RNA cleavage activity (fig. S3B).

Fig. 1. AF endonuclease-BHB RNA complex structure. (A) A precursor tRNA structure with the primary and secondary structure of the bulge-helix-bulge (BHB) RNA used in crystallization represented by letters. (B) Unstacking of three bulge nucleotides and their stabilization in the endonuclease. The bound bulge-helix-bulge (BHB) RNA structure (brown) compared with the ensemble-averaged NMR structure of a BHB RNA (green) (PDB code 2A9L). (C) Overview of the AF endonuclease-BHB RNA complex structure with the intron backbone colored in orange. Nucleotides in helical regions are represented by rods and those in bulges by stick models. The following color scheme for bulge nucleotides is consistently used throughout the article: first bulge nucleotide, bright red; second bulge nucleotide, orange; and the third bulge nucleotide, magenta. The two protein subunits are colored blue and green. (Insert) More detailed RNA-endonuclease interaction.
Report

Fig. 2. Protein-RNA interactions between the bulge-helix-bulge (BHB) RNA and the endonuclease. (A) Cross-subunit stabilization of each splice site. The Arg280/His257 pair from one subunit recognizes the first bulge nucleotide (A13/a13) of the bulge cleaved by the other subunit. The gold spheres indicate scissile phosphates. (B) Key elements of interaction between the endonuclease and bulge nucleotides. Note the stabilization of the sharply bent phosphate backbone mediated by both subunits represented by cyan and green residues. (C) A-minor interaction mediated by the last bulge nucleotide (A15/a15) and the adjacent central helix base pair g9-C12/G9-c12. The g9-C12 base pair corresponds to the A-I pair shown in Fig. 1A.

The unexpected interaction between A15/a15 and the imidazole ring of His257 facilitated the A-minor interaction (Fig. 2E). The A-minor interaction between RNA oxygen atoms at the ends of the bulge. The A-minor interaction mediated by the last bulge nucleotide (A15/a15) and the adjacent central helix base pair g9-C12/G9-c12. Note the stabilization of the A-I base pair (Fig. 2E).

The adenine ring of A15/a15 forms an A-I base pair with the g9-C12 base pair in a eukaryal pre-tRNA, providing the structural basis for their observed interdependence (22, 23). The endonuclease-RNA complex provides structural evidence for cooperativity between the two catalytic subunits. The cross-subunit stabilization of each bulge by cation-π sandwich (Fig. 2A) suggests that cognate interactions between one bulge and both subunits may facilitate the binding of the other bulge. Consequently, disruption of the structural integrity of one bulge would cause impaired cleavage on both bulges. Indeed, in addition to impairing cleavage of the 5′ bulge, 2′-O-methylation of C12 or A13, or removal of the nucleobase from nucleotide A13 at the 5′ bulge, prevented the cleavage of the 3′ bulge (Fig. 2D). These results lend strong support to a model of cooperative binding of the two catalytic endonuclease subunits to the BHB RNA. Remarkably, the observed cooperativity between two catalytic subunits in the AF endonuclease-RNA complex was also demonstrated in the yeast splicing endonuclease by selective mutation of its catalytic subunits (36).

The RNA recognition elements identified here are asymmetrically disposed between the two splice sites in eukaryal pre-tRNAs, although the sites themselves are equally utilized (22, 37). This appears to conflict with cooperativity because the typically unconserved 5′ splice site in nuclear pre-tRNAs would lead to overall inefficient cleavage at both sites. We suggest that the two catalytic subunits are diverged to complement the asymmetrically conserved splice sites. Consistent with this proposal, the Xenopus splicing endonuclease can cleave a miniprecursor tRNA containing only the mature domain and the 3′ splice site (16), and mutation of the Arg280-equivalent residue in yeast Sen2 only mildly impaired cleavage of the 3′ splice site (36).

Interactions between the RNA and the endonuclease at the active sites are consistent with the involvement of catalytic triad residues in cleavage reactions (Fig. 3). At the site of cleavage, the endonuclease facilitates a near in-line geometry of the attacking 2′-oxygen, the scissile phosphate, and the leaving 5′-oxygen that is required for RNA cleavage at this site (Fig. 3, insert). In addition, the scissile phosphates at both 5′ and 3′ splice sites are at the center of three strictly conserved residues, Tyr246, His257, and Lys287, the same as the position occupied by the bound sulfate in the crystal structure of the M. jannaschii endonuclease (27). The pro-Sp nonbridging oxygen of the 5′ splice site is...
Fig. 3. Active site interaction and proposed catalytic mechanisms. (A) Placement of the catalytic triad residues and their distances to functional RNA groups at the 2′-deoxy modified (left) and the cleaved (right) site, respectively. The inset shows the in-line geometry formed by the attacking 2′-nucleophile oxygen, scissile phosphate, and the leaving 5′-oxygen. Gray structural model represents the same nucleotide in 3′-endo sugar-puckering conformation. (B) A proposed catalytic mechanism where His257 acts as the general acid that donates a proton to the 5′ leaving group, Tyr246 facilitates deprotonation of the 2′ nucleophilic oxygen, and Lys287 and the amide group of Ser258 stabilize the developing negative charge at the transition state.

stabilized by a short hydrogen bond formed with Nε of Lys287 (2.7 Å, donor-hydrogen-acceptor angle 114°) and the Nδ atom of His257 is located 3.5 Å away from the leaving 5′-oxygen. The third catalytic triad residue, Tyr246, is 2.2 Å from the modeled 2′-hydroxyl oxygen in the 2′-endo sugar-puckering conformation (3.5 Å for 3′-endo sugar-puckering). The placement of the three residues suggests a catalytic mechanism where Tyr246 deprotonates the nucleophilic 2′-oxygen, His257 protonates the leaving 5′ hydroxyl oxygen [similar to His119 of RNase A (26)], and Lys287 stabilizes the developing negative charge at the transition state (Fig. 3B).

The AF endonuclease-RNA cocrystal structure provides a basis for understanding the mode of RNA recognition by the eukaryal endonuclease. Based on sequence comparisons and pairwise interactions identified by yeast two-hybrid methods, it is believed that the αβγδ subunits of eukaryal endonuclease has the same overall architecture as archael endonucleases, with the structural subunits Sen15 and Sen54 pairing with the 5′-cleaving Sen2 and the 3′-cleaving Sen34, respectively (7, 10, 11, 27). Thus, each AF endonuclease unit can be assigned specifically to one of the αβγδ subunits. We created a structural model of a precursor tRNA substrate by superimposing the anticodon stem of the yeast tRNA^Pre^ structure onto the 5′ stem of the 3′ bulge of the BHB RNA (pre-tRNA^Archeuka^) and analyzed how the pre-tRNA^Archeuka^ substrate interacts with the eukaryal endonuclease subunits (Fig. 4). This showed that the D arm and the acceptor stem of pre-tRNA^Archeuka^ would interact with the Sen54 subunit (Fig. 4). This mode of RNA-protein interaction is consistent with the active but imprecise cleavage of a composite pre-tRNA lacking the acceptor stem by the Xenopus splicing endonuclease (16) and suggests a role of Sen54 in the ruler mechanism of splice-site selection.

The cocrystal structure of the AF endonuclease-RNA complex identifies an evolutionarily conserved RNA recognition and cleavage mechanism for the removal of introns by the splicing endonuclease. Splice-site recognition depends on the chemical features of both protein and RNA residues. The challenge will be to dissect the functional role of each of these components in RNA recognition and in catalysis.

Fig. 4. A structural model of the pre-tRNA^Archeuka^ bound to AF endonuclease (ribbon models) with the corresponding yeast endonuclease subunits represented in colored shapes, and the splice sites indicated by arrows. The model indicates that the structural unit of the AF endonuclease corresponding to Sen54 interacts with the mature domain of the precursor tRNA. The A1 base pair and the third bulge nucleotide involved in the A-minor interaction interact with the subunit corresponding to Sen34.

References and Notes
Molecular Sorting by Electrical Steering of Microtubules in Kinesin-Coated Channels

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Integration of biomolecular motors in nanoscale systems raises the intriguing possibility of manipulating materials on nanometer scales. We have managed to integrate kinesin motor proteins in closed submicron channels and to realize active electrical control of the direction of individual kinesin-propelled microtubule filaments at Y junctions. Using this technique, we demonstrate molecular sorting of differently labeled microtubules. We attribute the steering of microtubules to electric field–induced bending of the leading tip. From measurements of the orientation dependent electrophoretic motion of individual, freely suspended microtubules, we estimate the net applied force on the tip to be in the picoNewton range and we infer an effective charge of \( 12 \, \text{e}^- \) per tubulin dimer under physiological conditions.

Recent years have witnessed a strong interest in the exploration of biomolecular motors in nanotechnology (1–4). A molecular motor such as kinesin, which translocates in 8-nm steps along microtubule filaments through hydrolysis of adenosine triphosphate (ATP) (5), can potentially be used as the workhorse in miniaturized analytical systems or nanoelectromechanical systems (6, 7). In particular, it is envisioned that microtubule shuttles translocating over kinesin-coated tracks can be used to carry a specific cargo to designated places on a chip (8). First steps toward such applications have been realized, such as partial confinement of microtubules to micron-sized kinesin-coated trenches (9–11), docking of shuttles to their tracks (12), rectification of motility (13, 14), and coupling of cargo (15). The much-sought-after goal of dynamic control of the direction of individual microtubules, a key requirement for molecular sorting applications, has not been achieved so far. Attempts to use electric fields to manipulate the negatively charged microtubules have only resulted in large-scale alignment (16) or bulk transport of the filaments (11, 12).

In this report, we reconstituted the kinesin-microtubule transport system in enclosed fluidic channels, which represents two major advances. First, fluidic channels achieved full confinement of the microtubules to their tracks, without the need for any surface modifications or selective patterning of kinesin molecules in open-trench structures. Second, the confined geometry of channels allowed the localized application of strong, directed electric fields, which could be exploited to steer individual microtubules, as well as to perform single-molecule biophysical experiments. By measuring the electrophoretic motion of individual microtubules, we determined the magnitude of the electric field–induced force on the microtubule tip, and we directly confirmed the predicted anisotropy in electrophoretic mobility.

We fabricated fluidic channels 800 nm deep (17) between entrance holes in fused-silica substrates and sealed them (Fig. 1, A and B). Microtubule motility was reconstituted in the channels by a pressure-driven flow to flush the necessary protein constituents (tubulin, kinesin, and fluorescently labeled paclitaxel-stabilized associated protein–free microtubules) from the entrance reservoirs into the channels (Fig. 1C). Using epifluorescence microscopy, we could discern microtubules moving on either the top or bottom surface of a channel by adjusting the focus of the objective (Fig. 1D). The enclosed geometry of the device completely confined the microtubules to their tracks (Fig. 1E). All regions could be coated with kinesin proteins, even less accessible regions in more complicated networks, such as bends (Fig. 1F). The speed of the microtubules in our channels (0.75 ± 0.02 μm/s) was the same as on a glass coverslip in a standard flow cell (0.74 ± 0.04 μm/s). By applying a voltage difference between platinum electrodes inserted in reservoirs at either end of a perpendicular cross-channel, we induced an electric field \( E \) perpendicular to the direction of microtubule motion (Fig. 2A). In this way, the electrical force on the negatively charged microtubules was directed opposite to the electric field.

We demonstrated that microtubules can be directed with an electric field. The trajectory of a microtubule that was subjected to an electric field of strength \( |E| = 35 \, \text{kV/m} \) (70 V over 2 mm) is shown in Fig. 2B. At the beginning of the path, the microtubule was oriented perpendicular to the electric field. As the microtubule progressed, its leading end gradually oriented itself opposite to the applied field, until the microtubule finally changed course by 90° and moved parallel to the electric field and toward the positive electrode. A trace of the leading and trailing-end coordinates of the microtubule showed that they followed exactly the same path (Fig. 2B). This clearly indicates that there was no motion of the microtubule perpendicular to its long axis, which is expected if kinesin molecules hold onto the microtubule. Up to 110 kV/m, we did not observe a measurable increase or decrease of the microtubule velocity due to the electric field (18).

The electric force was used to actively steer individual microtubules into a desired channel of a Y junction, across and through which a perpendicular channel was fabricated in order to confine the electric field. As a microtubule approached the junction, it was steered into the right channel by adjustment of the perpendicular electric field, whose magnitude was between 0