In vitro actin polymerization assay

The *in vitro* actin polymerization assay was done as described[12] using rhodamine-labelled rabbit skeletal muscle actin (Cytoskeleton). For *in vivo* shift experiments with the temperature-sensitive strains, cells were shifted to 36 °C for 180 min before and during collection. In *in vitro* shift assays, cells were grown at 25 °C (permissive temperature), permeabilized, shifted to 36 °C (restrictive temperature) for 30 min *in vitro*, and then kept at this temperature during incubation with rhodamine–actin. Cold-sensitive *arp3-c1* cells were shifted to 19 °C for 30 min before collection and kept at this temperature during collection. Cells were treated with 50 μM Lat A or 1% DMSO during rhodamine–actin incorporation. Cells were treated with 200 nM cytochalasin D after permeabilization and then with 20 nM cytochalasin D during rhodamine–actin incorporation[16]. To test whether F-actin incorporates into permeabilized cells, we added pre-assembled rhodamine–actin filaments[14] to permeabilized cells with and without 10 μM phalloidin (Molecular Probes).

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Competing interests statement

The authors declare that they have no competing financial interests.

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The U1 snRNP protein U1C recognizes the 5′ splice site in the absence of base pairing

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Splicing of precursor messenger RNA takes place in the spliceosome, a large RNA/protein macromolecular machine[1]. Spliceosome assembly occurs in an ordered pathway *in vitro* and is conserved between yeast and mammalian systems. The earliest step is commitment complex formation in yeast or E complex formation in mammals, which engages the pre-mRNA in the splicing pathway and involves interactions between U1 small nuclear ribonucleoprotein (snRNP) and the pre-mRNA 5′ splice site[2,3]. Complex formation depends on highly conserved base pairing between the 5′ splice site and the 5′ end of U1 snRNA, both *in vivo* and *in vitro*[4–7]. U1 snRNP proteins also contribute to U1 snRNP activity[8–10]. Here we show that U1 snRNP lacking the 5′ end of its snRNA retains 5′-splice-site sequence specificity. We also show that recombinant yeast U1C protein, a U1 snRNP protein, selects a 5′-splice-site-like sequence in which the first four nucleotides, GUAU, are identical to the first four nucleotides of the yeast 5′-splice-site consensus sequence. We propose that a U1C 5′-splice-site interaction precedes pre-mRNA/U1 snRNA base pairing and is the earliest step in the splicing pathway.

We previously showed that a truncated U1 snRNP, lacking the 5′-splice-site complementary 5′ end of U1 snRNA, can form a pre-mRNA/U1 snRNP complex and still has some sequence specificity for a normal 5′ splice site[11]. To pursue this observation, we carried out *in vitro* selection (SELEX) experiments with U1 snRNP (Fig. 1, WT). We used a standard yeast commitment complex substrate in which the six-nucleotide 5′ splice site (normally GUAUGU) had been randomized. The pool was incubated with a yeast extract under standard commitment complex conditions and then immunoprecipitated with an antibody against Prp40 (a U1 snRNP protein)[8]. After six rounds of selection, only the sequence GUAAGU was obtained. The selected sequence is predicted to form a more stable interaction with U1 snRNP than with a canonical GUAUGU 5′ sequence, because the uracil to adenine change and the extra guanine of the selected sequence allows the formation of three additional base pairs.

To test whether the selection of the GUAAGU sequence was exclusively due to the 5′ end of U1 snRNA, we repeated the selection

experiment in an extract treated with a complementary oligonucleotide and RNase H to digest that portion of the U1 molecule. Digestion was virtually complete and the U1 snRNA was 10 nucleotides shorter (data not shown)[11]. With this truncated U1 snRNP, we were unable to identify a discrete, selected sequence under these same conditions (Fig. 1b, 25 °C). As the complexes that are formed with truncated U1 snRNP are less stable than wild-type complexes[11], this result was not unexpected. With incubation at 4 °C, however, the six-round selection protocol and RNA sequencing identified the same unique sequence, GUAAGU (Fig. 1); 9 out of 10 clones had this same sequence (data not shown). If this had been caused by small amounts of undigested U1 snRNP, we would not expect a temperature effect on the selection. The result suggests that one or more commitment complex proteins and/or another portion of U1 snRNA selects the same 5′-splice-site sequence. Similar results have been reported for mammalian U1 snRNP[12].

We focused on the U1 snRNP U1C protein because it is important in both mammalian and yeast splicing[13–16] and crosslinking data indicate that U1C associates directly with the 5′ splice site[8]. To test whether U1C also contacts the 5′-splice-site sequence in the absence of the canonical 5′-splice-site/U1 snRNA base pairing, we labelled unique 5′-splice-site phosphates adjacent to single 4-thiouracils in the pre-mRNA substrates. Standard crosslinking experiments were carried out on the truncated U1 snRNA extract (Fig. 2). The pattern was very similar to that observed with intact U1 snRNP[8]. U1C was the most intensely labelled protein, especially when crosslinked with RNAs that were labelled at phosphates between the 5′-splice-site A3 and U4 or between G5 and U6 (Fig. 2).

As the truncated U1 snRNP could successfully select a 5′-splice-site sequence at low temperature, we compared complex formation efficiency at 25 °C and at 0 °C. In addition to the truncated U1 snRNP extract, we also tested a U1C-depleted extract and a wild-type extract (Fig. 3). With the modified extracts there was only a small, reproducible increase in efficiency at low temperature as compared with at 25 °C (Fig. 3a and data not shown). With a wild-type extract, however, there was a marked reduction in the amount of wild-type complex at low temperature (Fig. 3a). This effect could not be due to a decrease in complex stability, as this should be enhanced at low temperature. A more likely explanation, based on the lack of a comparable temperature effect in the truncated extract, is that complex formation is qualitatively different at low temperature and predominantly based on RNA/protein interactions rather than on the canonical RNA/RNA pairing interaction.

To test this possibility, we assayed 5′-splice-site/U1 snRNA base pairing by psoralen crosslinking (Fig. 3b). As previously shown, there were no bands in the truncated extract at either temperature[11]. There were also no detectable bands in the wild-type extract at 0 °C, which is consistent with our hypothesis and indicates that there is little or no RNA/RNA base pairing (Fig. 3b, lanes 1 and 2). The same result was observed at 5 °C, and the normal crosslinked products were observed at 10 °C and above (Fig. 3c). In the U1C-depleted extract, the crosslinked bands were present at 0 °C and at 25 °C (Fig. 3b, lanes 4 and 5). The results suggest that wild-type U1 snRNP does not efficiently undergo 5′-splice-site/U1 snRNA duplex formation at low temperature, but base pairing occurs under these conditions in the absence of U1C.

To address directly RNA/protein interactions, we assayed the crosslinking of U1 snRNP proteins to a 4-thiouracil-labelled substrate (Fig. 3d). The standard crosslinking pattern was obtained with the wild-type extract at low temperature, although the U1C and Snup56p bands were more intense, and the SmD1 and SmD3 bands were less intense, than at 25 °C. Increases in U1C and Snup56 intensity were also apparent in the truncated extract, which suggests that there is enhanced interaction or stability of these RNA/protein interactions at low temperature even without the 5′ end of U1 snRNA. In the U1C-depleted extract, the U1C band was noticeably less intense and the Snup56 band was absent, which is consistent

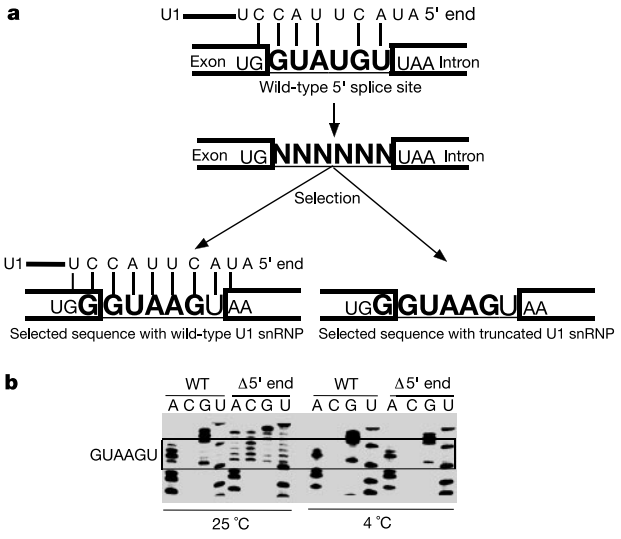

Figure 1 *In vitro* selection with U1 snRNP. **a**, Summary of selected sequences. The wild-type 5′ splice site was randomized and used to select the preferred sequence for U1 snRNP binding, either with wild-type or truncated U1 snRNA. Bold letters indicate the selected sequences; large letters between brackets (GUAAGU) indicate the putative 5′-splice-site sequences. **b**, Winning sequences were determined by sequencing the double-stranded DNA pool after six rounds of selection. RNA pools were incubated in standard splicing extracts containing wild-type (WT) or truncated U1 snRNP, at either 25 °C or 4 °C. These two selections were done side by side simultaneously. Immunoprecipitated RNA was amplified by PCR with reverse transcription to generate an enriched double-stranded DNA pool, which was then sequenced. Ten individual clones from each pool were sequenced to confirm the winning sequence (data not shown).

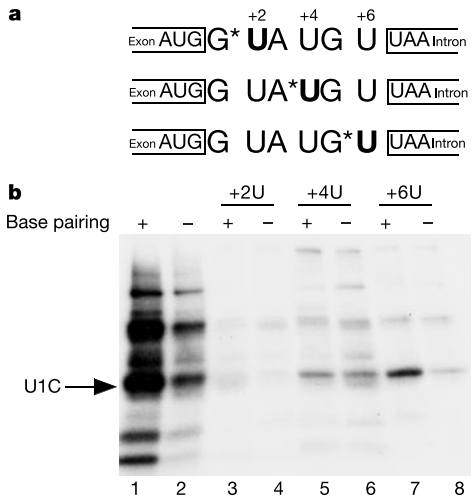

Figure 2 Site-specific crosslinking of commitment complex proteins to the 5′-splice-site region in the presence and absence of pre-mRNA/U1 snRNA base pairing. **a**, Site-specifically labelled RNA substrates. Asterisks indicate ^{32}P-labelled positions. The 4-thiouracil is indicated in bold. **b**, Protein profiles that crosslink to the 5′-splice-site region. In lanes 1 and 2, body labelled RNA substrate was used with either wild-type or truncated U1 snRNP splicing extract[11], respectively. In lanes 3–8, site-specifically labelled substrates were used as indicated. The position of U1C is shown on the left.

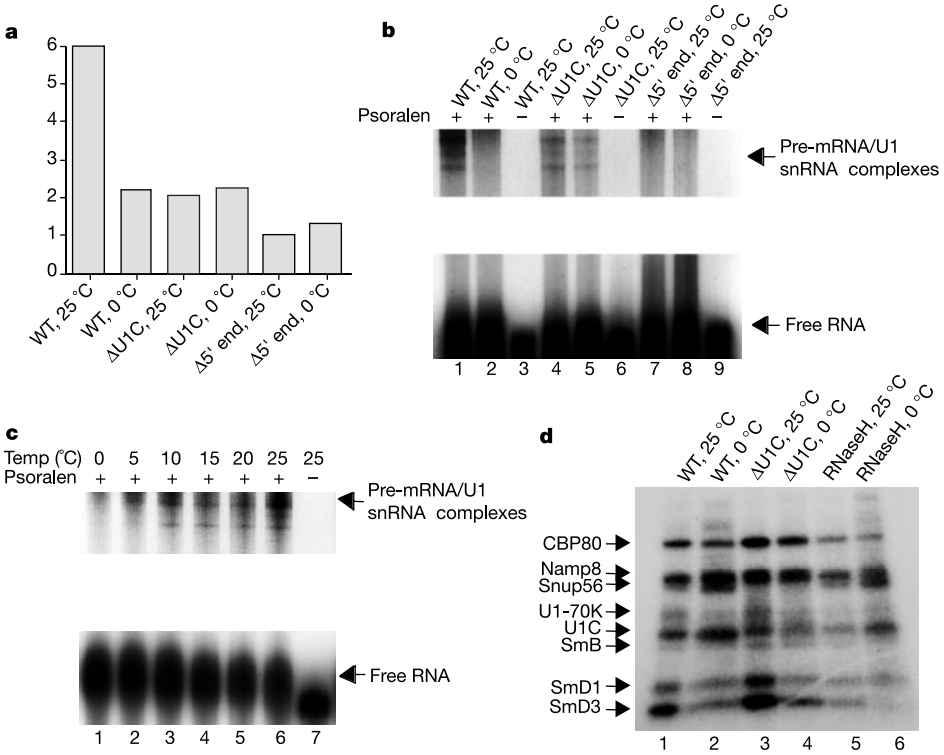

Figure 3 Characterization of complex formation in different extracts and at different temperatures. **a**, Quantitative analysis of complex formation. Three different splicing extracts were used to form RNA/protein complexes for 30 min with a standard body-labelled ^{32}P substrate of 72 nucleotides (WT-72)[8]. The reactions were at equilibrium as longer incubations had no effect, and identical results were obtained at 4 °C and at 0 ° (not shown). Complex formation was measured by immunoprecipitation with an antibody against Prpr40 (ref. 11). **b**, U1 sRNA/5′-splice-site base pairing assayed by psoralen crosslinking. Crosslinking was assayed in the three extracts after 30 min of incubation at the two temperatures. Results and procedures for the wild-type and RNase-H-treated extracts at 25 °C were as described[11]. **c**, Psoralen crosslinking as a function of incubation temperature. The wild-type extract was incubated for 30 min at different temperatures, and base pairing was assayed by psoralen. **d**, Comparison of the protein crosslinking patterns of the three extracts at two different incubation temperatures. 4-Thiouracil-labelled ^{32}P WT-72 RNA[8] was used as a substrate for protein ultraviolet crosslinking, and samples were normalized for counts before being loaded on the SDS gels so that similar amounts of complex were compared. Results for the wild-type and RNase-H-treated extract at 25 °C were as described[8,11]. Previously identified proteins[8] are indicated on the left.

with the idea that the U1C protein is missing from most of this U1 snRNP population and that the Snup56 interaction with RNA is dependent on U1C. The weak signal at the U1C position is due to residual U1C protein and/or to a co-migrating protein, which is presumably detectable when most of U1C is depleted.

Together, the results indicate that the predominant interaction at low temperature is between U1 snRNP proteins and the single-stranded 5′-splice-site region. This suggests that selection with wild-type U1 snRNP at 4 °C is based predominantly on RNA/protein interactions. Even at 25 °C, wild-type U1 snRNP selection might still be substantially due to RNA/protein interactions. This is because it is difficult to estimate the efficiency of canonical 5′-splice-site/U1 snRNA complex formation at 25 °C; in other words, there might still be a substantial fraction of RNA/protein complexes that have not converted to base pairing.

These results (Figs 2 and 3) suggest that U1C contributes to 5′-splice-site sequence specificity. We found that in electrophoretic mobility shift assays (EMSAs) recombinant yeast U1C could shift a substrate containing a normal 5′ splice site but not one with several 5′-splice-site mutations (AUUUGU versus GUAUGU; Fig. 4a). To identify a preferred sequence, we carried out *in vitro* selection experiments using the RNA pool described above. In this case, however, filter binding was used to isolate RNAs that bound to recombinant U1C. After five rounds, the affinity was about 20-fold higher than with the initial pool (Fig. 4b). Only one winner sequence, GUAUAA, was apparent in the RNA sequence; it was also the only sequence in clones of this RNA (Fig. 4c). The first four nucleotides are identical to a correctly positioned 5′-splice-site sequence, and the fifth nucleotide is a purine. (The yeast consensus sequence is a guanosine at this position.) This result does not exclude additional contacts between recombinant U1C and other regions of the 72-nucleotide RNA, which might contribute to the selection of the GUAU motif.

We presume that U1C interacts with the GUAUAA sequence as predominantly single-stranded RNA, because there are no more than three adjacent complementary bases in the substrate RNA (data not shown). To verify this notion, we carried out EMSA experiments with a partially double-stranded RNA. Before the addition of recombinant U1C protein, the winner substrate was preincubated with an antisense RNA roughly centred on the 5′ splice site. There was no evidence for an interaction of U1C with the duplex RNA (Fig. 5). In addition, the results indicated that a double-stranded 5′-splice-site region competes with RNA/U1C complex formation.

Mutations of L13 in the conserved zinc-finger domain of U1C bypass the function of an essential helicase, Prp28 (ref. 10). It was proposed that the mutations decrease the stability of the U1 snRNA/5′-splice-site interaction, which then no longer requires the helicase for duplex melting and progression along the splicing pathway[10]. Previous results are also consistent with a duplex stabilization role for U1C[13,14]. Although our data do not exclude this interaction, such an interaction does not account for the selection experiment with recombinant U1C protein. Taking into account our biochemical data (Fig. 3), another possibility is that the L13 mutation allows

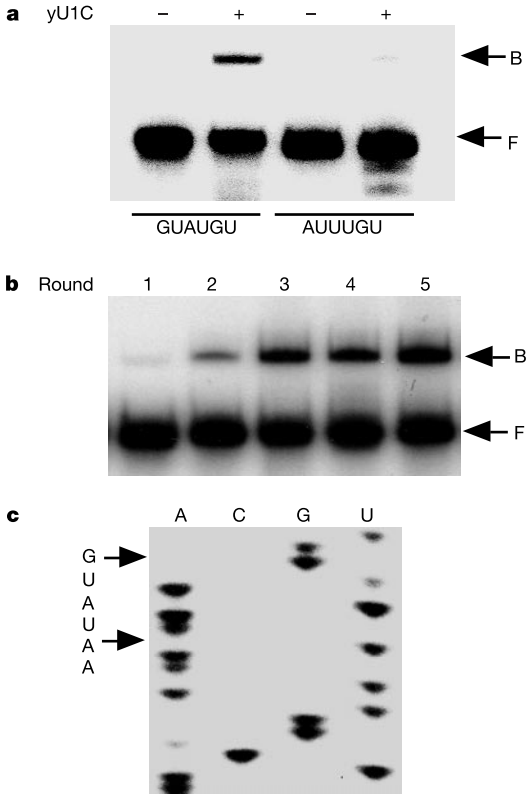

Figure 4 Characterization of sequence specificity of recombinant yU1C. **a**, EMSA for recombinant yU1C with either wild-type or 5′-splice-site mutant RNA. Bands corresponding to free (F) and bound (B) RNA are indicated on the right. **b**, EMSA of yU1C and RNA pools after successive rounds of selection, as indicated. **c**, A single winning sequence selected by yU1C was identified by DNA sequencing. Identical results were obtained with two independent preparations of U1C and starting RNA pools. The initial pools were verified as random.

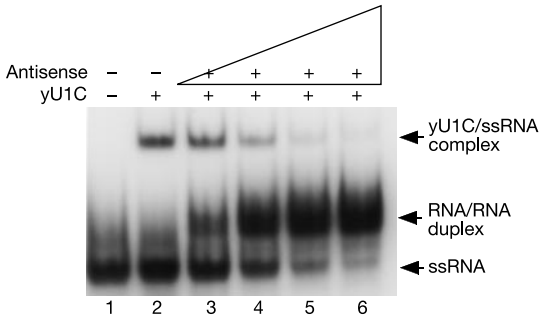

Figure 5 yU1C binding to single-stranded (ss) RNA and duplex substrates. A 24-nucleotide antisense RNA (GGTCCATATTATTATACCATTTTG), complementary to the GUAUAA-containing winner 72-nucleotide sequence, was used. ^{32}P-labelled winner 72-nucleotide was pre-incubated for 10 min with an increasing amount of cold antisense RNA under EMSA conditions, before the addition of yU1C protein (lanes 3–6). There was no antisense RNA in lane 2 and no protein in lane 1. Bands corresponding to ss RNA, duplex RNA and the yU1C/RNA complex are indicated on the right.

spliceosome assembly to proceed without base pairing. This interpretation is based on the apparent protein-only recognition of the 5′-splice-site region by U1 snRNP at low temperature (Fig. 3). Base pairing requires more elevated temperatures, which indicates that there is a temperature-dependent step required for the U1 snRNA/5′-splice-site region interaction. In the U1C-depleted extract, however, base pairing occurs at low temperature, which suggests that the U1C protein suppresses base pairing at low temperature. This fits well with data that indicate that U1C limits pre-mRNA access to the 5′ end of U1 snRNA[15].

Together, these results suggest a model in which the initial interaction between U1 snRNP and the pre-mRNA 5′-splice-site region is mediated by protein. U1C contributes to this 5′-splice-site selection, which is then followed by the canonical U1 snRNA/5′-splice-site base pairing. The temperature-dependent step might reflect a rate-limiting conformational change required for base pairing. This might even be a unimolecular structural rearrangement, by analogy to ribosome assembly[16]. On the basis of *in vivo* evidence, we had previously considered that U1C might function as a local, dedicated RNA chaperone to accelerate duplex formation—in addition to its putative contribution to duplex stabilization[15]. In contrast to the mammalian system[17–21], there is no evidence in the yeast system for U1 snRNP-independent splicing, and there is no *in vivo* evidence for yeast 5′-splice-site recognition in the absence of U1 snRNP function.

RNA/protein interactions before RNA/RNA base pairing also form the basis of the current model for the U2 snRNP/branchpoint interaction, where proteins identify and bind to the branchpoint region before U2 snRNA/branchpoint base pairing[22–24]. The stability of the base-paired complexes may make the snRNAs ill-suited to function as the initial selectors for pre-mRNA substrates[25]. Although we have no data on the mammalian system, the notion that U1C acts as an initial 5′-splice-site selector fits with a study indicating that the absence of human U1C from U1 snRNP decreases the on-rate but not the off-rate of U1 snRNA/5′-splice-site base pairing[26]. There are other proteins that probably recognize the 5′ splice site in a sequence-specific manner, that is, there are direct contacts between the U5 snRNP protein Prp8 and the 5′-splice-site GU[27,28]. There is also evidence from an *in vitro* yeast *trans*-splicing system for early, ATP-dependent contacts between U4/U6 snRNP and the 5′ splice site[29]. If U1C can identify and recruit potential 5′-splice-site sequences, then the canonical base pairing might have a proofreading function in addition to contributing to initial 5′-splice-site choice. This view suggests that 5′-splice-site selection has at least three steps comprising sequential interactions with U1C, U1 snRNA and U6 snRNA. □

Methods

yU1C overexpression and purification

We cloned the yU1C open reading frame into pETBlue-2, which incorporates a polyhistidine tag at the carboxy terminus. The recombinant protein was overexpressed in either *Escherichia coli* strain Origami (DE3)pLacI or Tuner (DE3)pLacI and purified according to the manufacturer's protocol (Novagen). We dialysed the eluate twice for 1.5 h against 1 l of buffer D (20 mM HEPES-KOH (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol and 20% glycerol).

DNA pool construction

The double-stranded DNA pool was synthesized by polymerase chain reaction (PCR). The upstream primer R1 (5′-GCGGAATTCTAATACGACTCACTATAGGTCGAGACTAGC AATAAC-3′) contains an *Eco*RI site and a T7 promoter; the downstream primer R3 (5′-GCGGATCCTCAATATTACGTGTCCT-3′) contains a *Bam*HI site. Oligonucleotide R2 (5′-CTCAATATTACGTGTCCTTAAAAGCCTCCTTTAGTCCATATTANNNNNNCA TTTTGTTATTGCTAGTCTCGACC-3′), where N represents a degenerate nucleotide, was used as a template for PCR with primers R1 and R3. The generated RNA sequence is identical to the 72-nucleotide described previously[8], except for the six random nucleotides at the 5′-splice-site region.

EMSA

Recombinant yU1C was incubated with either radiolabelelled individual RNA substrate or RNA pool in 12.5 μl of binding buffer (25 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA and 0.2 μg of transfer RNA) for 30 min at 25 °C. We separated the reaction mixtures on 6% native polyacrylamide gels at 4 °C.

Filter binding assay

Radiolabelled RNA was incubated with recombinant yU1C in 20 μl of binding buffer (see

above) for 30 min at 25 °C. Mixtures were passed over nitrocellulose filters under vacuum. Filters were washed with 3 ml of binding buffer before RNA recovery.

Other methods

We carried out ultraviolet crosslinking, immunoprecipitation, RNase H treatment and U1C-depletion as described[11,15].

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The authors declare that they have no competing financial interests.

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RNA aptamers as reversible antagonists of coagulation factor IXa

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Many therapeutic agents are associated with adverse effects in patients. Anticoagulants can engender acute complications such as significant bleeding that increases patient morbidity and mortality[1]. Antidote control provides the safest means to regulate drug action. For this reason, despite its known limitations and toxicities, heparin use remains high because it is the only anticoagulant that can be controlled by an antidote, the polypeptide protamine[2–4]. To date, no generalizable strategy for developing drug–antidote pairs has been described. We investigated whether drug–antidote pairs could be rationally designed by taking advantage of properties inherent to nucleic acids to make antidote-controlled anticoagulant agents. Here we show that protein-binding oligonucleotides (aptamers) against coagulation factor IXa are potent anticoagulants. We also show that oligonucleotides complementary to these aptamers can act as antidotes capable of efficiently reversing the activity of these new anticoagulants in plasma from healthy volunteers and from patients who cannot tolerate heparin[5]. This generalizable strategy for rationally designing a drug–antidote pair thus opens up the way for developing safer regulatable therapeutics.

To determine if properties inherent to nucleic acids can be used to develop an antidote-controlled anticoagulant, we first sought to generate an aptamer with anticoagulant activity. Procoagulant proteins that promote fibrin clot formation have been targeted in the development of many anticoagulant agents[6], and anticoagulant aptamers have been isolated against coagulation factors VIIa[7] and thrombin[8–10]. Here we describe the isolation of aptamers specific for coagulation factor IXa (FIXa).

We employed iterative *in vitro* selection techniques[11,12] to screen a nucleic-acid-based combinatorial library containing about 10^{14} species for those members capable of binding FIXa with high affinity. To ensure that the resultant aptamers would be stable in human plasma, the starting library contained 2′-fluoropyrimidines[13]. *In vitro* selection was performed for eight rounds against FIXa. The RNAs present in the round-eight library were converted to complementary DNAs and sequenced. Sixteen of the RNAs obtained bind FIXa, and all share a conserved primary sequence and secondary structure (Fig. 1a, b). Of these RNAs, aptamer 9.3 bound FIXa with the highest affinity (dissociation constant $K_d = 0.65 \pm 0.2$ nM). Covariation analysis of this sequence family[14] aided in the generation of a truncated version of this aptamer, termed 9.3t ($K_d = 0.58 \pm 0.1$ nM), and an inactive mutant version, 9.3tM (K_d for FIXa $> 10\,\mu$M) (Fig. 1b). Aptamer 9.3t exhibits greater than 5,000-fold specificity for FIXa versus the structurally similar coagulation factors VIIa, Xa, XIa and activated protein C (K_d values $> 5\,\mu$M). Attachment of a polyethlyene glycol of relative molecular mass $M_r = 40{,}000$ to the 5′ end of an aptamer has been shown to enhance the bioavailability of aptamers *in vivo*[15,16], and its attachment to aptamer 9.3t had a nominal impact on the affinity of this aptamer for FIXa, (K_d of Peg-9.3t, 2.83 ± 0.4 nM).

We next determined if aptamer 9.3t inhibits FIXa activity. *In vivo*, FIXa forms a complex with coagulation factor VIIIa on a cell surface

