**Supplemental Information**

1 **Table S1. Plasmids used in this study**

| Plasmid | Description |
|---|---|
| pHIV-1_356_WT | pUC19, contains T7 recognition sequence followed HIV-1 NL4-3 5′UTR 356 (nt 1-356) |
| pHIV-1_356_ΔDIS | pUC19, contains T7 recognition sequence followed HIV-1 NL4-3 5′UTR 356 ΔDIS (nt 1-356; $^{255}$GAAGCGCGCA → $^{254}$GAGA) |
| pHIV-1 356 3M-1 | pUC19, contains T7 recognition sequence followed HIV-1 NL4-3 5′UTR 356 M2 (nt 1-356; $^{34}$A→T, $^{108}$G→C, $^{258}$G→C) |
| p6G-Mini-MSL | pKD-HIV(GFP-I-Hy), contains ~3000 nt of NL4-3 viral genome ($^{259}$GCGCGC → $^{259}$GGGGGG) |
| p6C-Mini-BSL | pKD-HIV(GFP-I-Hy), contains ~3000 nt of NL4-3 viral genome ($^{259}$GCGCGC → $^{259}$CCCCCC) |
| pLYSF119 | pFVAL119, T7 RNA promoter followed by human tRNA$^{Lys3}$ sequence. Insert sequence flanked by Fok1 recognition sites. |

2 **Table S2. Primers used in this study**

| Primer | Description | 5′-3′ sequence |
|---|---|---|
| FRET FH238 Fwd | 5′UTR forward primer containing T7 promoter and handle annealing sequence | GAATTTAATACGACTCACTATAGGTTGGAGGTTATGGAGCAGTGCCCGTCTGTTGTGTGA |
| 238 Fwd | 5′UTR forward primer containing T7 promoter | GAATTTAATACGACTCACTATAGGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGA |
| FRET FH238 2M Fwd | 5′UTR forward primer containing T7 promoter, handle annealing sequence, and 2M mutation | GAATTTAATACGACTCACTATAGGTTGGAGGTTATGGAGCAGTCCCCGTCTGTTGTGTGA |
| FRET FH238 Rev | 5′UTR Reverse primer | GCACCCATCTCTCTCCTTCTAGCCTCCGCTAGTCAAAATTTTTGG |



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**Table S3. RNA and DNA sequences used in this study**

| Name | Nucleic acid type | Source | 5′-3′ Sequence |
|---|---|---|---|
| FH238.WT 5′UTR *FRET Handle (FH) is in italics* | RNA | *In vitro* Transcription | *GGUUGGAGGUUAUGGAGCA*GUGCCCGUCUG UUGUGUGACUCUGGUAACUAGAGAUCCCUCA GACCCUUUUAGUCAGUGUGGAAAAUCUCUAG CAGUGGCGCCCGAACAGGGACUUGAAAGCGA AAGUAAAGCCAGAGGAGAUCUCUCGACGCAG GACUCGGCUUGCUGAAGCGCGCACGGCAAGA GGCGAGGGGCGGCGACUGGUGAGUACGCCA AAAAUUUUGACUAGCGGAGGCUAGAAGGAGA GAGAUGGGUGC |
| 238.WT 5′UTR | RNA | *In vitro* Transcription | GUGCCCGUCUGUUGUGUGACUCUGGUAACUA GAGAUCCCUCAGACCCUUUUAGUCAGUGUGG AAAAUCUCUAGCAGUGGCGCCCGAACAGGGA CUUGAAAGCGAAAGUAAAGCCAGAGGAGAUC UCUCGACGCAGGACUCGGCUUGCUGAAGCGC GCACGGCAAGAGGCGAGGGGCGGCGACUGG UGAGUACGCCAAAAAUUUUGACUAGCGGAGG CUAGAAGGAGAGAGAUGGGUGC |
| FH238.2M 5′UTR *FH is in italics and mutations are underlined* | RNA | *In vitro* Transcription | *GGUUGGAGGUUAUGGAGCA*GUCCCCGUCUG UUGUGUGACUCUGGUAACUAGAGAUCCCUCA GACCCUUUUAGUCAGUGUGGAAAAUCUCUAG CAGUGGCGCCCGAACAGGGACUUGAAAGCGA AAGUAAAGCCAGAGGAGAUCUCUCGACGCAG GACUCGGCUUGCUGAAGGGCGCACGGCAAGA GGCGAGGGGCGGCGACUGGUGAGUACGCCA AAAAUUUUGACUAGCGGAGGCUAGAAGGAGA GAGAUGGGUGC |
| FH238.ΔDIS 5′UTR *FH is in italics and mutations are underlined* | RNA | *In vitro* Transcription | *GGUUGGAGGUUAUGGAGCA*GUGCCCGUCUG UUGUGUGACUCUGGUAACUAGAGAUCCCUCA GACCCUUUUAGUCAGUGUGGAAAAUCUCUAG CAGUGGCGCCCGAACAGGGACUUGAAAGCGA AAGUAAAGCCAGAGGAGAUCUCUCGACGCAG GACUCGGCUUGCUGGAGACGGCAAGAGGCGA GGGGCGGCGACUGGUGAGUACGCCAAAAAUU UUGACUAGCGGAGGCUAGAAGGAGAGAGAUG GGUGC |
| FH238.6G 5′UTR *FH is in italics and mutations are underlined* | RNA | *In vitro* Transcription | *GGUUGGAGGUUAUGGAGCA*GUGCCCGUCUG UUGUGUGACUCUGGUAACUAGAGAUCCCUCA GACCCUUUUAGUCAGUGUGGAAAAUCUCUAG CAGUGGCGCCCGAACAGGGACUUGAAAGCGA AAGUAAAGCCAGAGGAGAUCUCUCGACGCAG GACUCGGCUUGCUGAAGGGGGGGACGGCAAGA GGCGAGGGGCGGCGACUGGUGAGUACGCCA AAAAUUUUGACUAGCGGAGGCUAGAAGGAGA GAGAUGGGUGC |

| | | | |
|---|---|---|---|
| 238.6C 5′UTR *Mutations are underlined* | RNA | *In vitro* Transcription | GUGCCCGUCUGUUGUGUGACUCUGGUAACUA GAGAUCCCUCAGACCCUUUUAGUCAGUGUGG AAAAUCUCUAGCAGUGGCGCCCGAACAGGGA CUUGAAAGCGAAAGUAAAGCCAGAGGAGAUC UCUCGACGCAGGACUCGGCUUGCUGAACCCCC CCACGGCAAGAGGCGAGGGGCGGCGACUGG UGAGUACGCCAAAAAUUUUGACUAGCGGAGG CUAGAAGGAGAGAGAUGGGUGC |
| FH.Oligo | DNA | Purchased from Integrated DNA Technologies | TTTTGCTCCATAACCTCCAACC |
| FH.Oligo-5′AmMC6-3′Bioteg | DNA | Purchased from Integrated DNA Technologies | AmMC6-TTTTGCTCCATAACCTCCAACC-BioTEG |
| FH.Oligo-5′Alexa488 | DNA | Purchased from Integrated DNA Technologies | Alexa488-TTTTGCTCCATAACCTCCAACC |
| FH.Oligo-5′647 | DNA | Purchased from Integrated DNA Technologies | Alexa647-TTTTGCTCCATAACCTCCAACC |
| anti-PBS cDNA | DNA | Purchased from Integrated DNA Technologies | 5’-GTCCCTGTTCGGGCGCCA-3’ |
| $tRNA^{Lys3}$ | RNA | *In vitro* Transcription | 5’-GCCCGGAUAGCUCAGUCGGUAGAGCAUCAGA CUUUUAAUCUGAGGGUCCAGGGUUCAAGUCC CUGUUCGGGCGCCA-3’ |
| Minihelix | RNA | Purchased from Integrated DNA Technologies | 5’-GCCCGGACAGGGUUCAAGUCCCUGUUCGGGC GCCA-3’ |

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**Table S4. Steady-state fluorescence anisotropy.**

| Sample | Anisotropy |
|---|---|
| LD650 | 0.11 ± 0.01 |
| WT-LD650 | 0.15 ± 0.01 |
| ΔDIS-LD650 | 0.14 ± 0.01 |
| 2M-LD650 | 0.14 ± 0.01 |
| WT-LD650 + $tRNA^{Lys}$ | 0.14 ± 0.01 |
| WT-LD650 + NC | 0.13 ± 0.01 |
| WT-LD650 + NC + $tRNA^{Lys}$ | 0.14 ± 0.01 |
| LD550 | 0.15 ± 0.01 |
| WT-LD550 | 0.17 ± 0.01 |
| ΔDIS -LD550 | 0.18 ± 0.01 |
| 2M-LD550 | 0.18 ± 0.01 |
| WT-LD550 + $tRNA^{Lys}$ | 0.18 ± 0.01 |
| WT-LD550 + cDNA | 0.17 ± 0.01 |
| WT-LD550 + NC | 0.22 ± 0.01 |
| WT-LD550 + NC + $tRNA^{Lys}$ | 0.22 ± 0.01 |

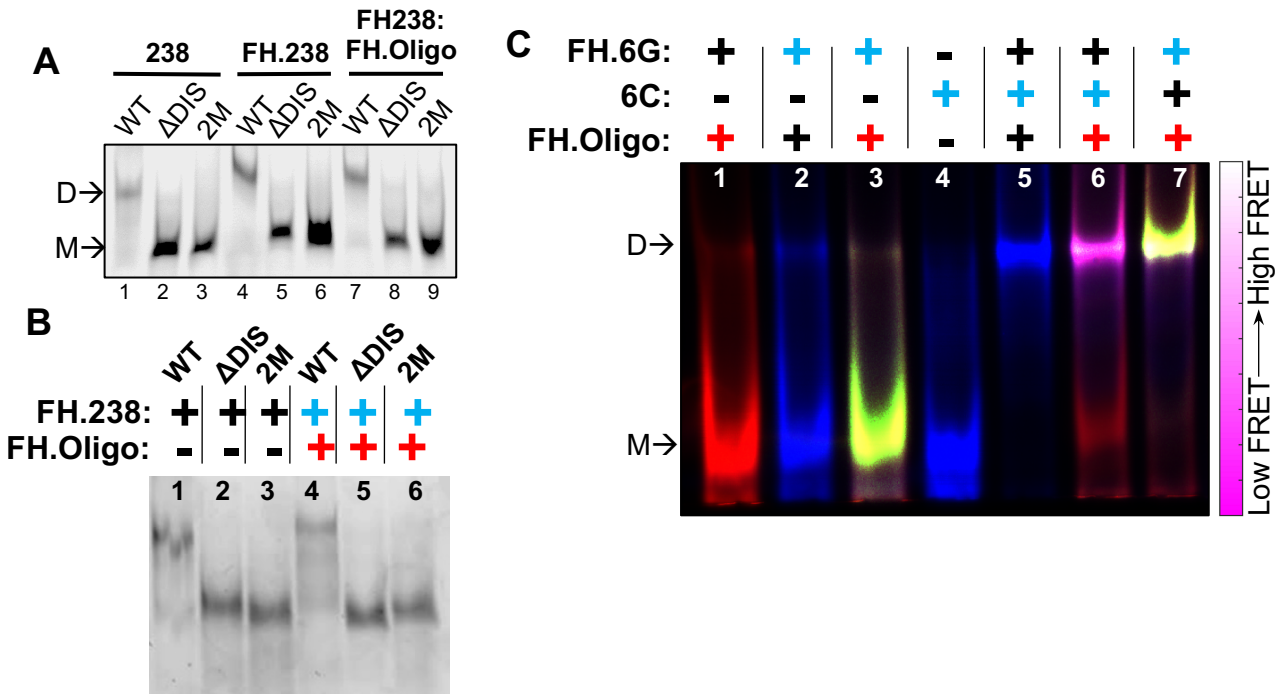


**Figure S1. Native gel and in-Gel FRET of FH238 constructs.** (**A**) Fluorescence image of a native 4.5% polyacrylamide gel showing 3′-fluorophore labeled 5′UTR constructs (indicated above each lane). The gel was run in the presence of 10 mM $Mg^{2+}$ in the running buffer and gel matrix. The 238-nt 5′UTR constructs without the 5′ FH extension (lanes 1-3), with the 5′ FH extension (lanes 4-6) and annealed to FH.Oligo (lanes 7-8) are shown. The locations of the monomer (M) and dimer (D) bands are indicated. (**B**) Native 4.5% polyacrylamide gel electrophoresis of HIV-1 5′UTR constructs in the presence (Lane 1-3) and absence (4-6) of 3′ FRET probe and FH.Oligo. Gel imaged by ethidium bromide staining. (**C**) Native 4.5% polyacrylamide gel electrophoresis of FH238.6G (FH.6G) and 238.6C (6C) 5′UTR constructs. Lanes 1-3 are the FH.6G 5′UTR, lane 4 is the 6C 5′UTR, and lanes 5-7 are the FH.6G 5′UTRs bound to the 6C 5′UTR.

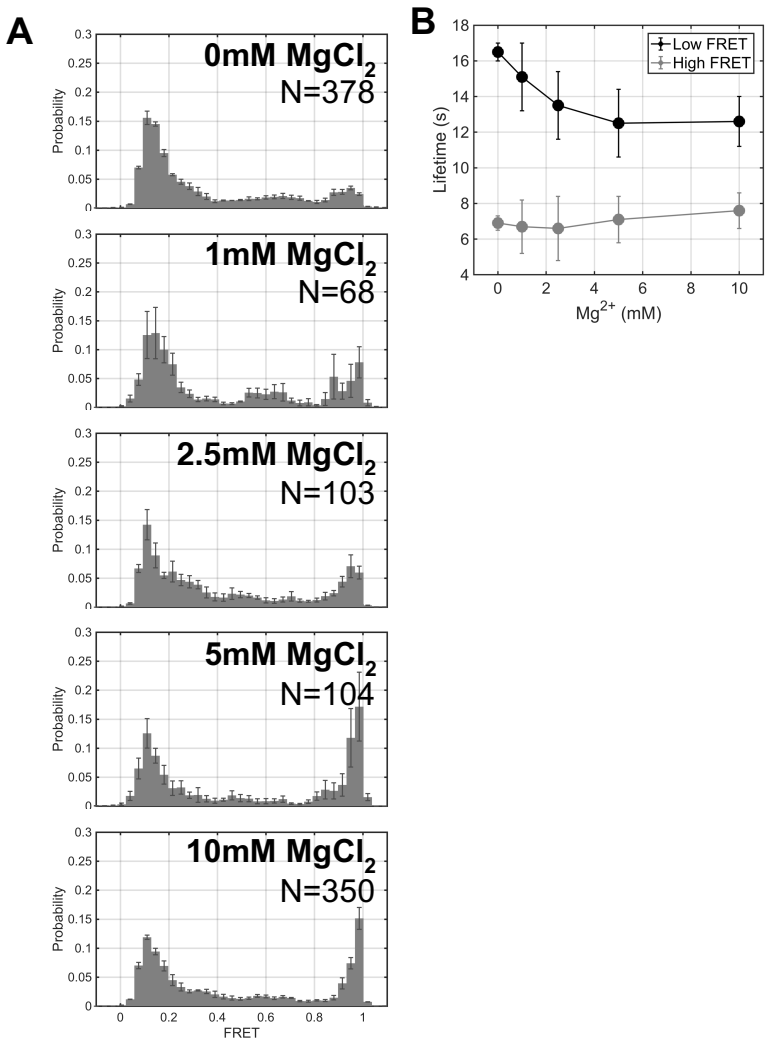


**Figure S2. $Mg^{2+}$ promotes transition of the monomeric 5′UTR to the dimerization-competent conformation.** (**A**) Population FRET histograms from FH238.WT molecules imaged with the Intra-UTR FRET assay in the indicated $MgCl_2$ concentration. The number of FRET trajectories compiled into the histogram is indicated (N). The quantification of the low- and high-FRET state occupancies is shown in Figure 2F. (**B**) The lifetime of the low-FRET state decreases with increasing $Mg^{2+}$, while the lifetime of the high-FRET state is comparatively insensitive to $Mg^{2+}$.

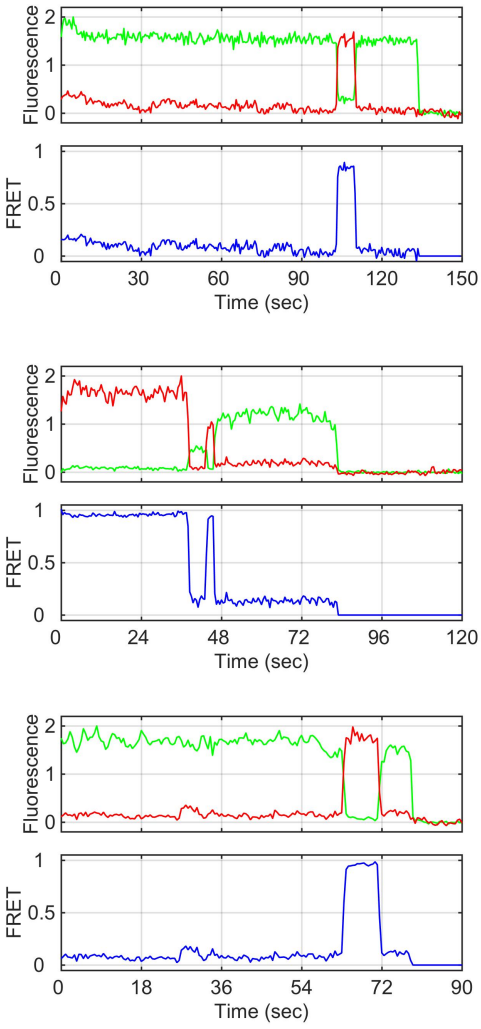


**Figure S3. Transitions out of extended dimer are observed in the Inter-UTR smFRET assay.** Example fluorescence (donor, green; acceptor, red) and FRET (blue) traces from the Inter-UTR smFRET assay showing transitions into and out of the high-FRET extended dimer conformation.