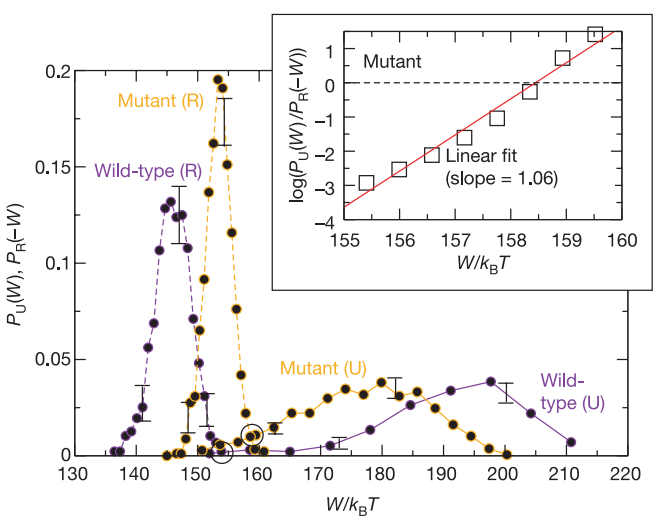

Figure 3 | Free-energy recovery and test of the CFT for non-gaussian work distributions. Experiments were carried out on the wild-type and mutant S15 three-helix junction without Mg^{2+}. Unfolding (continuous lines) and refolding (dashed lines) work distributions. Statistics: 900 pulls and two molecules (wild type, purple); 1,200 pulls and five molecules (mutant type, orange). Crossings between distributions are indicated by black circles. Work histograms were found to be reproducible among different molecules (error bars indicating the range of variability). Inset, test of the CFT for the mutant. Data have been linearly interpolated between contiguous bins of the unfolding and refolding work distributions.

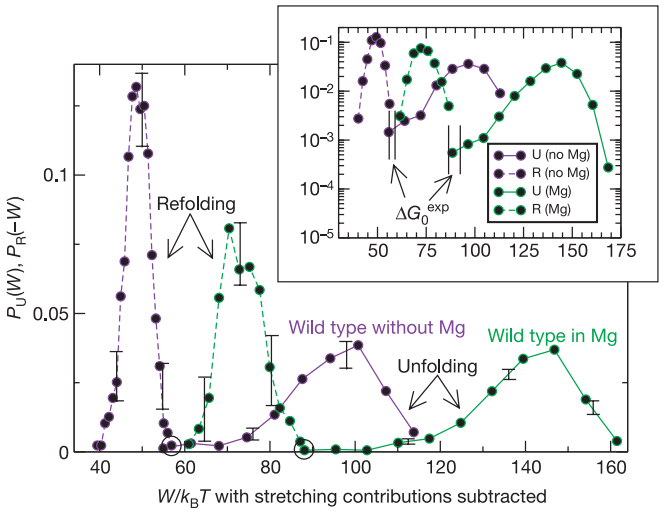

Figure 4 | Use of CFT to extract the stabilizing contribution of Mg^{2+} to the free energy of the S15 three-helix junction (wild type). Unfolding (continuous lines) and refolding (dashed lines) work distributions. Green curves, 450 pulls and two molecules in Mg^{2+}; purple curves, 900 pulls and two molecules without Mg^{2+}. Crossings between distributions are indicated by black circles. Work histograms are reproducible between the molecules (error bars indicating the range of variability). Inset, the same histograms in logarithmic scale (axes labels as for the main panel) showing (vertical black bars) the regions of work values where unfolding and refolding distributions are expected to cross each other by Bennett's acceptance ratio method (Supplementary Information).

and narrow work distributions $P_R(W)$ along the refolding path. These distributions complement each other, one being large where the other is small, thereby providing thermodynamically important information about the free-energy landscape.

Bennett's acceptance ratio method gives $\Delta G^{exp} = 154.1 \pm 0.4\,k_BT$ and $\Delta G^{exp} = 157.9 \pm 0.2\,k_BT$ for unfolding the wild-type and mutant types, respectively, giving a difference between the two forms $\Delta\Delta G_0^{exp} = \Delta\Delta G^{exp} = 3.8 \pm 0.6\,k_BT$. After subtracting the (identical for both molecules) handle and RNA entropy loss contributions ($97 \pm 1\,k_BT$) we get $\Delta G_0^{exp} = 57 \pm 1.5\,k_BT$ (wild type) and $\Delta G_0^{exp} = 60.8 \pm 1.5\,k_BT$ (mutant), the error increasing owing to the uncertainty in the contributions coming from the stretching of ssRNA. Free-energy prediction programs such as Mfold[25] and Visual OMP[21] give a $\Delta\Delta G_0^{mfold} = 2 \pm 2\,k_BT$ between the forms (at 25 °C and 100 mM NaCl). Thus, when combined with acceptance ratio methods, the CFT furnishes a method precise enough to determine the difference in the folding free energies of RNA molecules differing only by one base pair in 34 base pairs.

Finally we apply equation (1) to obtain the free energy of stabilization by Mg^{2+} of the S15 three-helix junction. These values are often difficult to access using bulk methods because melting temperatures of tertiary folded RNAs are frequently higher than the boiling point of water, and Mg^{2+} catalyses the hydrolysis of RNA at increased temperatures[26]. Figure 4 depicts the work histograms in the presence and absence of Mg^{2+} (at constant ionic strength); stretching contributions differ in the presence and absence of magnesium ions ($116.8\,k_BT$ and $97\,k_BT$, respectively). These values have been subtracted from the work data to properly compare the unfolding free energies of both molecules. The strong increase of irreversibility due to Mg^{2+} can be seen in the large value of the average dissipated work (about $50\,k_BT$ along the unfolding reaction and $16\,k_BT$ along the refolding path). Applying Bennett's acceptance ratio method for the molecule in the presence of magnesium yields $\Delta G^{exp} = 205.5 \pm 1.5\,k_BT$ and (after subtracting the stretching contributions) gives $\Delta G_0^{exp} = 88.7 \pm 2.5\,k_BT$ for the unfolding reaction of the wild-type junction in 4 mM $MgCl_2$. The difference in free energies of unfolding in the presence and absence of Mg^{2+}, $\Delta\Delta G_0^{exp} = -31.7 \pm 2\,k_BT$, gives the free energy of stabilization associated with the binding of Mg^{2+}

Table 1 | Summary of results obtained for all molecules

Molecule	W_m^U	W_m^R	σ_U	σ_R	$W_{cum}^{(2)}$	W_J^U	W_J^R	W_J^{est}	ΔG^{exp}	ΔG_0^{exp}	W_{dis}^U	W_{dis}^R	R_U	R_R
Hairpin (1.5 pN s^{-1})*	110.9	108.7	2.35	2.21	109.7 (0.2)	107.4 (0.7)	110.9 (0.2)	109.1 (0.5)	110.0 (0.2)	62.5 (1.2)	0.9	1.3	3.1	1.9
Hairpin (7.5 pN s^{-1})	113.8	106.6	2.63	2.84	110.3 (0.2)	109.7 (0.7)	110.9 (0.5)	110.3 (0.5)	110.3 (0.5)	62.8 (1.5)	3.5	3.7	0.98	1.10
Hairpin (20 pN s^{-1})	115.7	104.1	3.2	3.5	110.1 (0.2)	110.2 (0.7)	108.6 (0.2)	109.4 (0.4)	110.2 (0.6)	62.9 (1.6)	5.4	6.2	0.94	0.98
S15 (wild, no Mg)	191.3	145.9	11.3	2.9	158.7 (0.8)	155.2 (1.4)	149.3 (0.2)	152.2 (0.7)	154.1 (0.4)	57.0 (1.5)	36.3	9.1	1.75	0.46
S15 (mutant, no Mg)	176.5	153.4	10.6	2.1	156.0 (0.4)	152.4 (5.0)	155.7 (0.2)	154.1 (0.3)	157.9 (0.2)	60.8 (1.5)	18.6	4.5	3.02	0.49
S15 (wild, Mg)	256.4	190.3	12.2	5.0	213.0 (1.3)	207.0 (4.0)	199.8 (0.6)	203.6 (2.0)	205.5 (1.5)	88.7 (2.5)	50.9	15.2	1.46	0.82

W_m^U and W_m^R, σ_U and σ_R, W_J^U and W_J^R are the average total work, standard deviations and predictions obtained by using Jarzynski's equality along the unfolding (U) and refolding (R) paths. $W_{cum}^{(2)}$ is the estimate obtained by Hummer[30], $W_{cum}^{(2)} = (W_m^U + W_m^R)/2 - (\sigma_U^2 - \sigma_R^2)/12\,k_BT$, which gives the leading correction to the linear response prediction, W_J^{est} is the average of the estimates obtained by using Jarzynski's equality along the unfolding and refolding paths $W_J^{est} = (W_J^U + W_J^R)/2$, ΔG^{exp} is our best estimate obtained by using the acceptance ratio method; ΔG_0^{exp} is the final estimate for the unfolding free energy at zero force after subtracting the handles contribution, $W_{dis}^{U,R} = |W^{U,R} - \Delta G^{exp}|$ is the average dissipated work (for the analysis of the hairpin data we took $\Delta G^{exp} = 110.3$ for all pulling rates) and $R_{U,R} = \frac{\sigma_{U,R}^2}{2k_BTW_{dis}^{U,R}}$ is a parameter that is equal to 1 for gaussian work distributions[8]. Statistical errors are given for Jarzynski's equality and the crossing estimates. These were obtained using the bootstrap method. All work values (except ΔG_0^{exp}) include the handle and RNA stretching contributions and are given in units of k_BT at $T = 298$ K. In parentheses we indicate the errors in units of k_BT.

*Data for the RNA hairpin at 1.5 pN s^{-1} are also included for completion; however, at such low loading rates drift effects are very large and data are very noisy as revealed by the values of R_U and R_R, which differ too much from 1.