along the unfolding path while (absolute) values smaller than ΔG occur more often along the refolding path. As can be seen from equation (1), the CFT states that although $P_U(W)$, $P_R(-W)$ depend on the pulling protocol, their ratio depends only on the value of ΔG. Thus the value of ΔG can be determined once the two distributions are known. In particular, the two distributions cross at $W = \Delta G$:

$$P_U(W) = P_R(-W) \Rightarrow W = \Delta G \quad (3)$$

regardless of the pulling speed. Although the simple identity (3) already gives an estimate of ΔG, it is not necessarily very precise because it uses only the local behaviour of the distribution around $W = \Delta G$. Using the whole work distribution increases the precision of the free-energy estimate[19]. In particular, as we show below, when the overlapping region of work values between the unfolding and refolding work distributions is too narrow (as may happen for large values of the average dissipated work, defined as $\langle W_{dis} \rangle = \langle W \rangle - \Delta G$), the use of Bennett's acceptance ratio method[20] makes it possible to extract accurate estimates of ΔG using the CFT (see the Supplementary Information).

We first experimentally test the validity of the CFT for a molecular transition occurring near equilibrium. For this, we use a short interfering (si)RNA hairpin that targets the messenger RNA of the CD4 receptor of the human immunodeficiency virus (HIV)[11] and that unfolds irreversibly but not too far from equilibrium at accessible experimental pulling speeds (dissipated work values less than $6k_BT$). Under these conditions, the unfolding and refolding work distributions overlap over a sufficiently large range of work values to justify the use of the direct method to experimentally test equation (1). The work done on the molecules during either pulling or relaxation is given by the areas below the corresponding force–extension curves (Fig. 1).

Unfolding and refolding work distributions at three different pulling speeds are shown in Fig. 2. Irreversibility increases with the pulling speed and unfolding–refolding work distributions become progressively more separated. Note, however, that the unfolding and the refolding distributions cross at a value of the work $\Delta G = 110.3 \pm 0.5\, k_BT$ that does not depend on the pulling speed, as predicted by equation (3). Moreover, the work distributions also satisfy the CFT, that is, equation (1) (see the Supplementary Information). We also notice that work distributions are compatible with, and can be fitted to, gaussian distributions (data not shown). After subtracting the contribution arising from the entropy loss due to the stretching of the molecular handles attached on both sides of the hairpin ($\Delta G^{handles} = 23.8\, k_BT$) and of the extended single-stranded (ss)RNA ($\Delta G^{ssRNA} = 23.7 \pm 1\, k_BT$) from the total work, $\Delta G^{exp} = 110.3 \pm 0.5\, k_BT$, we obtain for the free energy of unfolding at zero force $\Delta G_0^{exp} = 62.8 \pm 1.5\, k_BT = 37.2 \pm 1$ kcal mol^{-1} (at 25° C, in 100 mM Tris-HCl, pH 8.1, 1 mM EDTA), in excellent agreement with the result obtained using the Visual OMP from DNA software[21] $\Delta G_0^{mfold} = 38$ kcal mol^{-1} (at 25° C, in 100 mM NaCl).

To extend the experimental test of the validity of the CFT to the very-far-from-equilibrium regime where the work distributions are no longer gaussian, we apply the CFT to determine: (1) the difference in folding free energy between an RNA molecule and a mutant that differs only by one base-pair, and (2) the thermodynamic stabilizing effect of Mg^{2+} ions on the RNA structure. The RNA we consider is a three-helix junction of the 16S ribosomal RNA of *Escherichia coli*[12] that binds the S15 protein. The secondary structure of this RNA is a common feature in RNA structures[22–24] that plays, in this case, a crucial role in the folding of the central domain of the 30S ribosomal subunit. For comparison, and to verify the accuracy of the method, we have pulled the wild type and a C·G to G·C mutant (C754G to G587C) of the three-helix junction.

Figure 3 depicts the unfolding and refolding work distributions for the wild-type and mutant molecules (work values were binned into about 10–20 equally spaced intervals). For both molecules, the distributions display a very narrow overlapping region. In contrast with the hairpin distribution, the average dissipated work for the unfolding pathway is now much larger—in the range 20–40 k_BT — and the unfolding work distribution shows a large tail and strong deviations from gaussian behaviour. Thus, these molecules are ideal to test the validity of equation (1) in the far-from-equilibrium regime. As shown in the inset of Fig. 3, the plot of the log ratio of the unfolding to the refolding probabilities versus total work done on the molecule can be fitted to a straight line with a slope of 1.06, thus establishing the validity of the CFT (see equation (1)) under far-from-equilibrium conditions. Our measurements reveal the presence of long tails in the work distribution $P_U(W)$ along the unfolding path

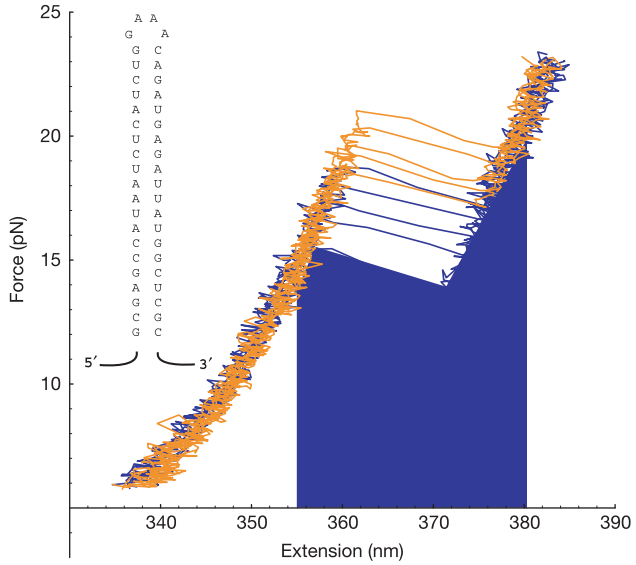

Figure 1 | Force–extension curves. The stochasticity of the unfolding and refolding process is characterized by a distribution of unfolding or refolding work trajectories. Five unfolding (orange) and refolding (blue) force–extension curves for the RNA hairpin are shown (loading rate of 7.5 pN s^{-1}). The blue area under the curve represents the work returned to the machine as the molecule switches from the unfolded to the folded state. The RNA sequence is shown as an inset.

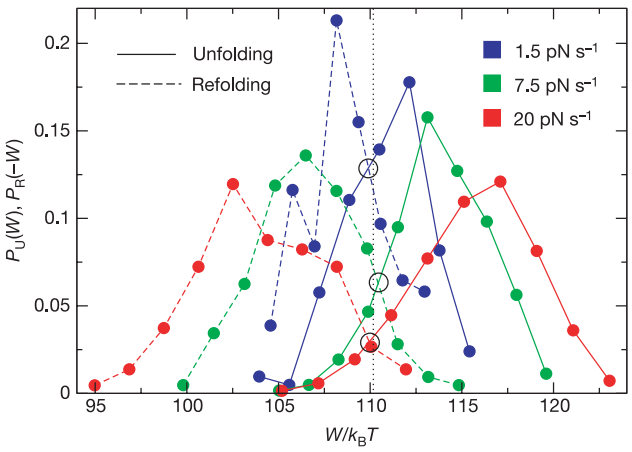

Figure 2 | Test of the CFT using an RNA hairpin. Work distributions for RNA unfolding (continuous lines) and refolding (dashed lines). We plot negative work, $P_R(-W)$, for refolding. Statistics: 130 pulls and three molecules ($r = 1.5$ pN s^{-1}), 380 pulls and four molecules ($r = 7.5$ pN s^{-1}), 700 pulls and three molecules ($r = 20.0$ pN s^{-1}), for a total of ten separate experiments. Good reproducibility was obtained among molecules (see Supplementary Fig. S2). Work values were binned into about ten equally spaced intervals. Unfolding and refolding distributions at different speeds show a common crossing around $\Delta G = 110.3\, k_BT$.