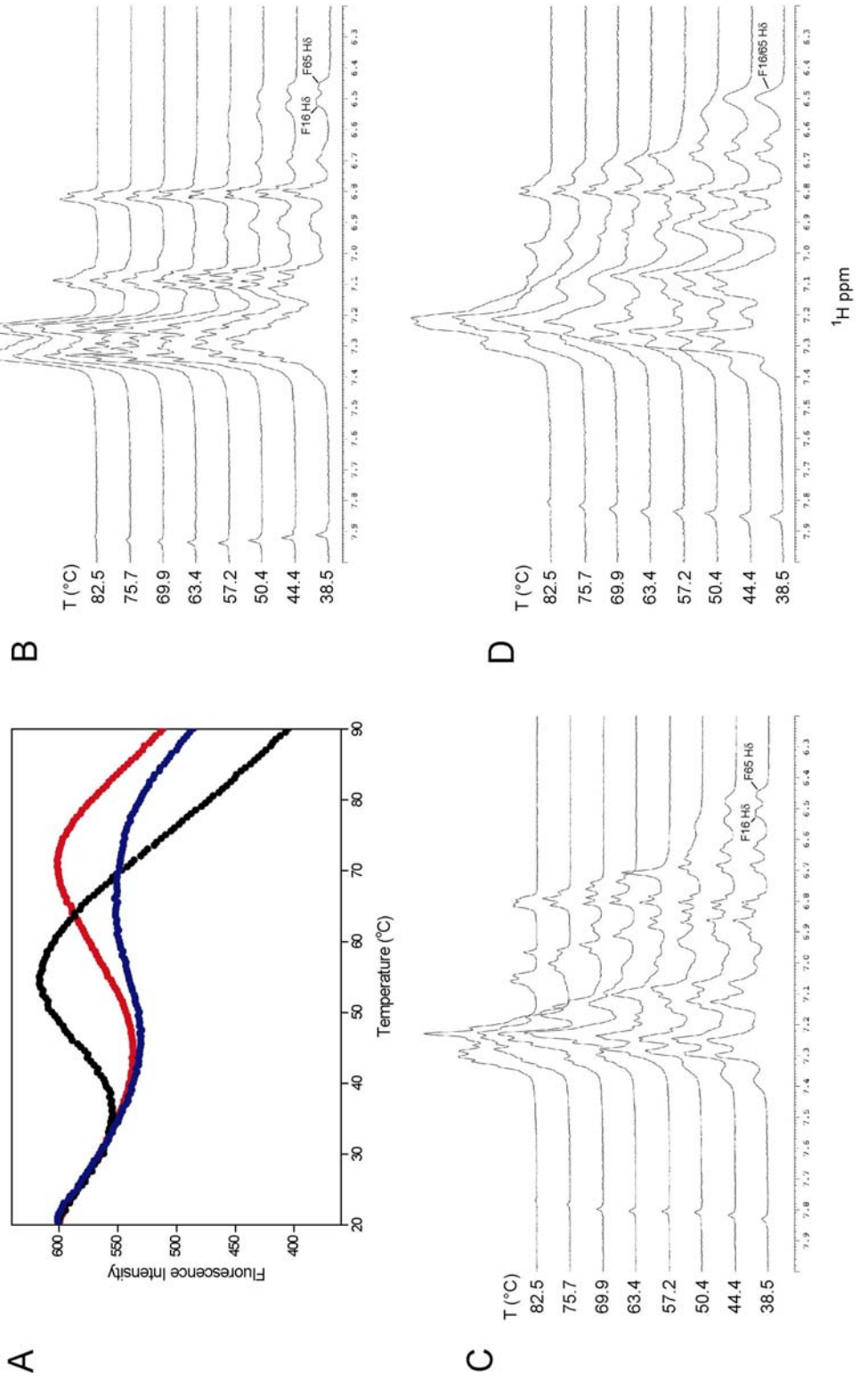


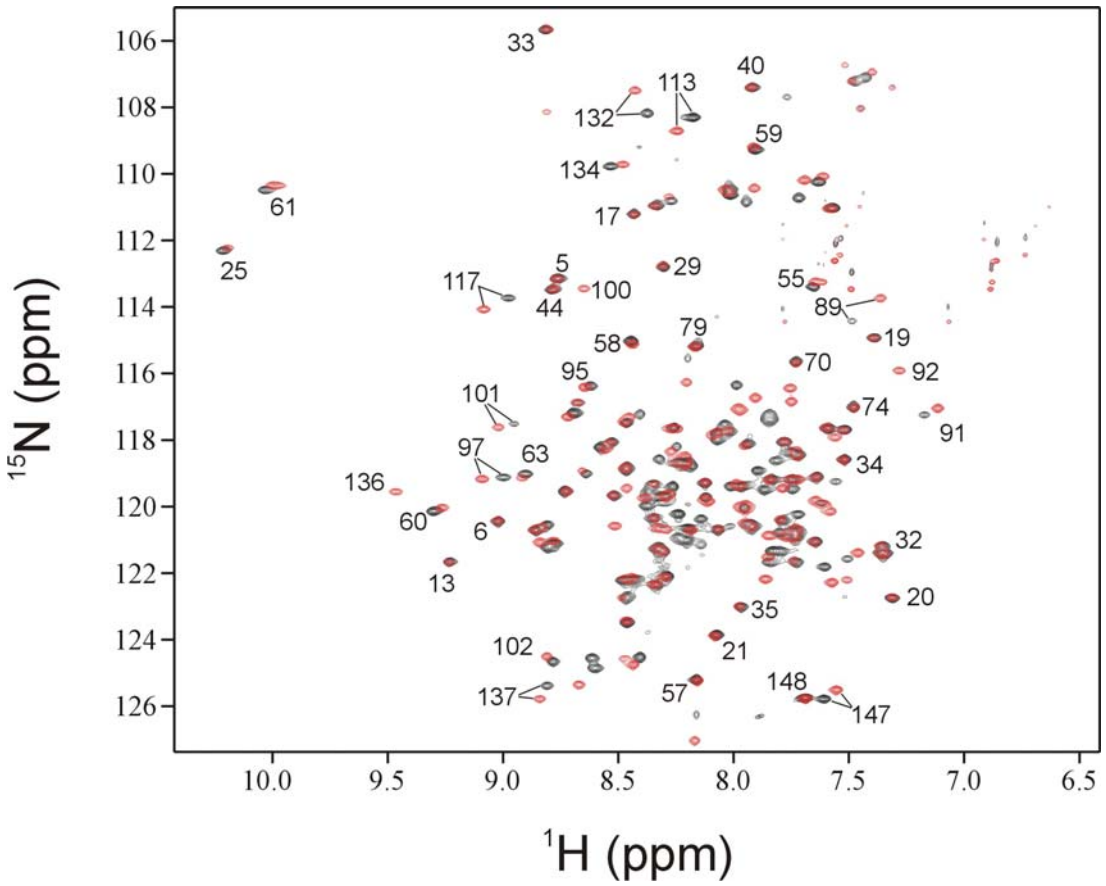
Supplementary Fig. 1

Supplementary Fig. 1: Fluorescence and NMR spectroscopy data confirm that the C-domains of apo-CT-CaM and apo-Nle-CaM unfold at higher temperatures than the N-domains of these proteins, which is the reversed order of stability in comparison to apo-wt-CaM.

A) Fluorescence monitored temperature melts show a large shift to higher temperatures for the thermal transition of the C-domains of apo-CT-CaM (red) and apo-Nle-CaM (blue) in comparison to apo-wt-CaM (black). Each sample was heated from 20–90^{o}C at scan rate of 1^{o}/min in a Varian Cary Eclipse spectrofluorimeter using excitation and emission wavelengths of 280 nm and 305 nm respectively. Since CaM contains no Trp residues in its amino acid sequence, these conditions can be used to specifically study the melting behavior of the 2 Tyr residues in CaM (Y99 and Y138) both of which are found in the C-domain of the protein (Masino et al, *Protein Sci.*, 2000, 9:1519–29). For clarity of presentation, the data are normalized to a common fluorescence intensity of 600 arbitrary units at 20^{o}C.

Changes in the aromatic region of the one-dimensional ^{1}H NMR spectra for B) apo-wt-CaM, C) apo-CT-CaM and D) apo-Nle-CaM show that the Leu and Nle substitutions do not significantly influence the melting temperature of the apo-N-domain. The well-resolved reporter signals for the Phe16 and Phe65 Hδ resonances from the N-domain of each protein shift downfield with increasing temperature and disappear near 57.2^{o}C, consistent with the T_m values of 55-60^{o}C that were observed for the N-domain of each protein using DSC (see Table 1 in the main text). The Phe16 and Phe65 Hδ resonances were assigned by (Torok et al, *Biochemistry*, 1992, 31:3452–62). NMR spectra were recorded on a Bruker Avance 400 MHz NMR spectrometer using samples of 0.8 mM CaM protein in 100 mM KCl, 0.5 mM DSS, 0.03 % NaN_3, 2 mM d_{16}-EDTA, 99.99% D_2O, pH 7.2.

In agreement with the DSC data (Table 1 and Figure 3 of the main text), these fluorescence data (panel A) and NMR data (panels B–D) demonstrate that the C-domains of apo-CT-CaM and apo-Nle-CaM unfold at higher temperatures than the N-domains of these mutant proteins, which is opposite to apo-wt-CaM.



Supplementary Fig. 2: ^{1}H, ^{15}N HSQC NMR spectral overlay of apo-^{15}N-wt-CaM (black) and apo-^{15}N-CT-CaM (red). Spectra were each recorded using samples of 0.8 mM protein in 100 mM KCl, 2 mM EDTA, 10% D_2O, 0.5 mM DSS and 0.03% NaN_3 at a field strength of 500 MHz. Resonance assignments for apo-^{15}N-wt-CaM were obtained using standard triple resonance NMR experiments (see Materials and Methods in the main text), and resonance assignments for apo-^{15}N-CT-CaM were obtained from the resonance overlap with the apo-^{15}N-wt-CaM spectrum. For clarity, spectral assignments are only provided for the most dispersed backbone amide resonances. Note that some of the peaks for apo-^{15}N-wt-CaM (such as those corresponding to F92 and I100) are not clearly visible at the contour level used to generate this Figure, but these peaks can be detected as weak resonance signals at lower contour levels as shown for example, in Figure 4 A and C in the main text.