Nano-mechanics of HaloTag Tethers

Ionel Popa[§], Ronen Berkovich[§], Jorge Alegre-Cebollada[§], Carmen L. Badilla[§], Jaime Andres Rivas-Pardo[§], Yukinori Taniguchi[¶], Masaru Kawakami[¶], and Julio M. Fernandez[§]

[§]Dept. of Biological Sciences, Columbia University, 1212 Amsterdam Ave., New York, USA.; [¶]School of Materials Science, Japan Advanced Institute of Science and Technology (JAIST), 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan.

Supplementary Information

Table of contents

S1. Surface and cantilever functionalization

Glass coverslips were cleaned overnight at room temperature using Helmanex II (Helma) cleaning detergent or for 30 min using Piranha cleaning (H_2SO_4:H_2O_2 30% = 3:1), washed thoroughly with double distilled water, dried and plasma-cleaned for 30 min (Harrick Palsma). The surfaces were functionalized to have amine surface groups with (3-aminopropyl)triethoxysilane (Sigma-Aldrich) using standard vapor silanization procedure for >24 h under vacuum. We also used an alternative liquid silanization procedure. In this case, we incubated the glass surfaces in (3-aminopropyl)triethoxysilane at 90 °C for 15 min, followed by rinsing with ethanol and water and curing at 150 °C for 30 min. The silane layer was visually verified from the contact angle of a water drop. While the pick-up rate was slightly higher for the vapor silanization procedure, the measured detachment forces were identical.

The chloroalkane ligand was attached to the surface using HaloTag Thiol O4 bifunctional ligand (Promega), following a thiol-based covalent attachment procedure, as described elsewhere [1]. Briefly, the amine functionalized glass was treated with 10 mM succinimidyl-[(N-maleimidopropionamido) tetracosaethyleneglycol] ester ($SMPEG_{24}$ – Thermo) diluted in 100 mM borax buffer (pH 8.5) for 1 h, washed and reacted overnight in a humid chamber with 7.5 mM HaloTag Thiol O4 ligand, which was previously dissolved in the same borax buffer. The reaction was quenched with 50 mM 2-mercaptoethanol (Sigma-Aldrich) for 5 min.

In the case of the maleimide functionalized surfaces, the protein was added right after the 1 h $SMPEG_{24}$ reaction step. Gold surfaces were prepared by evaporating a layer of 5-10 nm of Cr/Ni

and 15-30 nm of Au (Edwards Auto 306 Vacuum Coater). Immediately before usage, the gold surfaces were cleaned with ethanol 100%, rinsed extensively with water, dried with nitrogen and the protein was left to adsorb for 30 min at 4 °C.

Gold cantilevers were prepared from MLCT silicon nitride cantilevers (Bruker) by coating them with a 2 nm Cr/Ni layer and 13-15 nm of Au. Chloroalkane functionalized cantilevers were exposed to oxygen plasma for 10 min and silanized in a similar way as the surfaces. The cantilevers were then reacted for 1 h with the $SMPEG_{24}$ and overnight with HaloTag Thiol O4 ligand. Finally the reaction was quenched with 2-mercaptoethanol.

S2. Assessment of the chloroalkane chemistry

Two methods were used to analyze the chloroalkane chemistry. In the first method a HaloTag-GFP protein was adsorbed to the surface in the presence and absence of TEV protease (0.5-1 mg/mL). The experiments were done in 10 mM HEPES buffer pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT. TEV was added immediately after the adsorption to the surface. Images of the two drops were taken before and after washing with the same HEPES buffer, using G-Box Gel Imaging system (Syngene). We illuminated the surface with blue light at 455-487 nm, and measured the florescence at 516-600 nm wavelength. Covalently attached polyproteins render the glass fluorescent. This fluorescence disappears in the presence of TEV protease, which cleaves away the GFP fluorescent protein.

In a second method four microscope slides (VWR, 22x40 mm^2) were functionalized with chloroalkane ligand and sandwiched together a humid chamber for 1 h with HaloTag-$(I27)_8$ polyprotein. Slides were then washed three times for a total time of 1-2 h with 10 mM HEPES pH 7.2, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT buffer and sandwiched again with 2 mg/mL TEV for 1 h in a humid chamber. The sandwiches were then opened, a small volume of water was added to increase the washing efficiency and the wash was collected and concentrated using a vacuum concentrator (DNA SpeedVac). 15-well 4-20% Precise Tris-Glycine gels (Thermo Scientific) were loaded with the collected sample, together with a HaloTag-$(I27)_8$ and TEV controls. Electrophoresis was ran for ~ 60 min at 0.05 A constant current and at voltages below 200 V. Gels were then stained with Coomassie Blue (BioRad), followed by silver staining. When digested with TEV protease, covalently attached polyproteins (original mass of HaloTag-$(I27)_8$ - 117kDa) give rise to a protein fragment of 82 kDa (molecular mass of $(I27)_8$). The missing 35 kDa correspond to the HaloTag, which remains covalently attached to the surface. Therefore, the absence of a band at ~35 kDa shows that the contribution of non-specific adsorption is negligible.

S3. Single molecule AFM experiments

In a typical **force-extension** experiment the cantilever was pushed against the protein-covered surface for ~1 s at a force of 800 pN and then retracted with constant velocity of 400 nm/s. Traces showing saw-tooth like patterns in the measured force were saved and further used for the data analysis. The importance of a unique mechanical fingerprint is highlighted in Figure S1, which displays the outcome of a typical experiment with Halo- L_8-Cys construct adsorbed on a chloroalkane functionalized surface and pulled using a gold-coated cantilever. In this experiment, out of 736 consecutive traces 61 % show attachment of a molecule between the cantilever and the surface. This pick-up rate is significantly higher than the standard case of gold surface – silicon nitride cantilevers, which is ~ 10 %. These traces can be grouped in seven categories: tethering of the construct to both termini (~12%), tethering only to the HaloTag terminus or the breaking of the C-terminus anchoring before the unfolding of all component domains (~3 %), non-specific tethering in random places along the polyprotein chain (~6 %), tethering of more than one protein (~22 %), hard to resolve traces due to non-specific pick-up of molecules in parallel with the protein construct (~3%), and tethering of something else than the polyprotein (~1%). From the traces showing the specific attachment of the HaloTag (~16 %) we could evaluate the covalent thiol-gold bond formation. While pick-up rate is an elusive parameter that can vary from experiment to experiment (errors in the preparation of proteins or surfaces may even lead to complete failure of the experiment), a highly reproducible parameter that reliably quantifies the success of the attachment is the end-to-end specificity. Traces that show the HaloTag fingerprint were considered to have specific attachment if all the other eight domains in the polyprotein also unfolded. From the approaches used, chloroalkane surface – gold cantilever showed the highest proportion of traces with HaloTag fingerprint (~16%), followed by gold surface – chloroalkane cantilever (~9%) and maleimide surface – chloroalkane cantilever (~5%). The pick-up rate in the latest configuration decreased abruptly after ~ 1h.

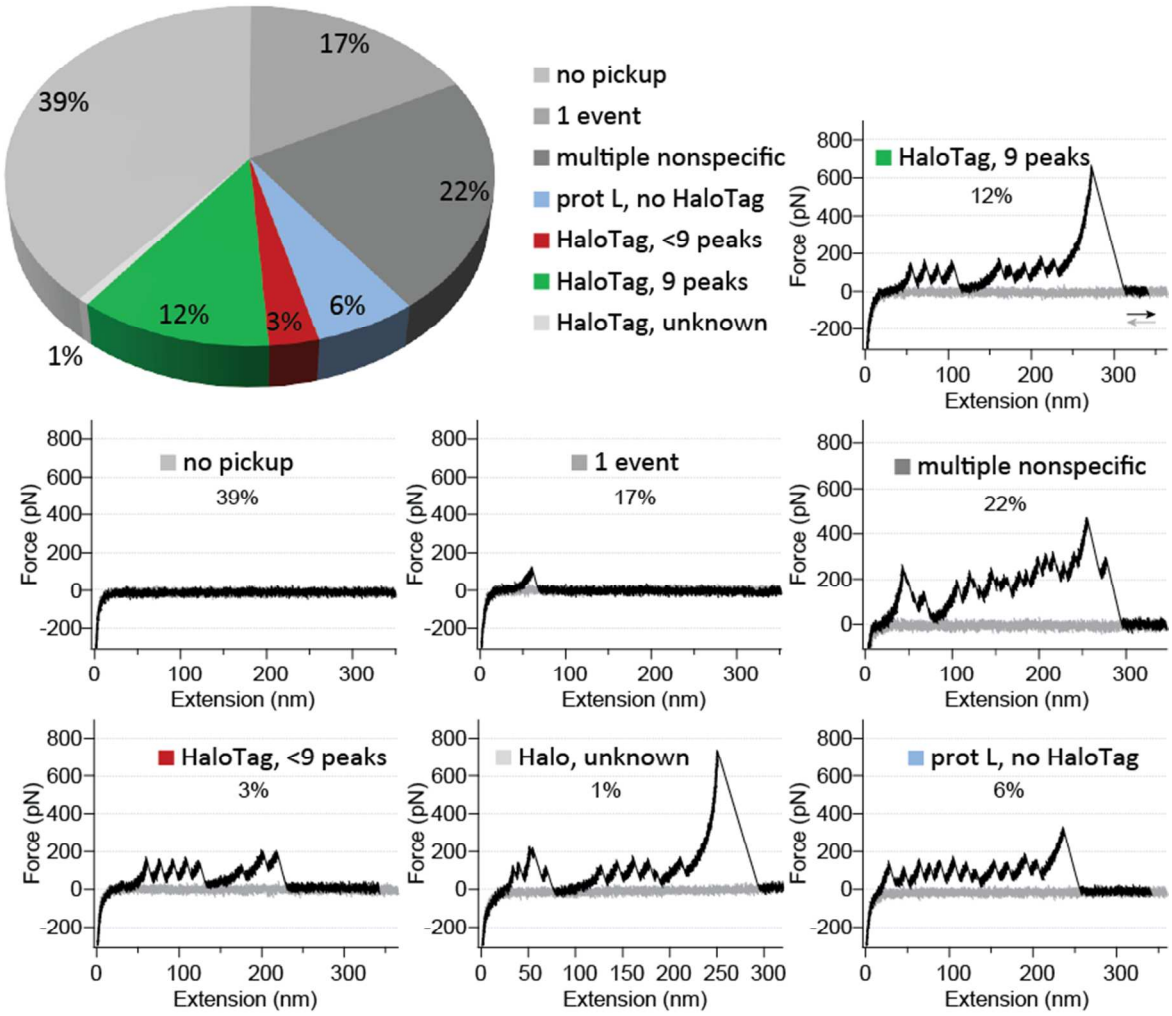

Figure S1. Success rate of the attachment chemistry. Pie chart of 736 consecutively measured traces of a typical experiment. The Halo-L_8-Cys construct (with C-terminus HaloTag) was adsorbed on a chloroalkane surface and pulled with a gold coated cantilever. All the attempts were recorded and analyzed, without selection. The grey traces represent the approach of the cantilever to the surface, while the black traces represent the retraction of the cantilever from the surface. The pie chart shows the percentage traces designated to one of the seven categories.

Halo-$I27_8$-cys construct with the HaloTag at the C-end of the polyprotein, showed the unfolding of the N-terminus HaloTag as the last most stable event, competing with the detachment from the gold-coated cantilever (Figure S2). From the traces showing eight I27 unfolding events, ~28 % (n = 227) detached before the unfolding of the N-terminus HaloTag.

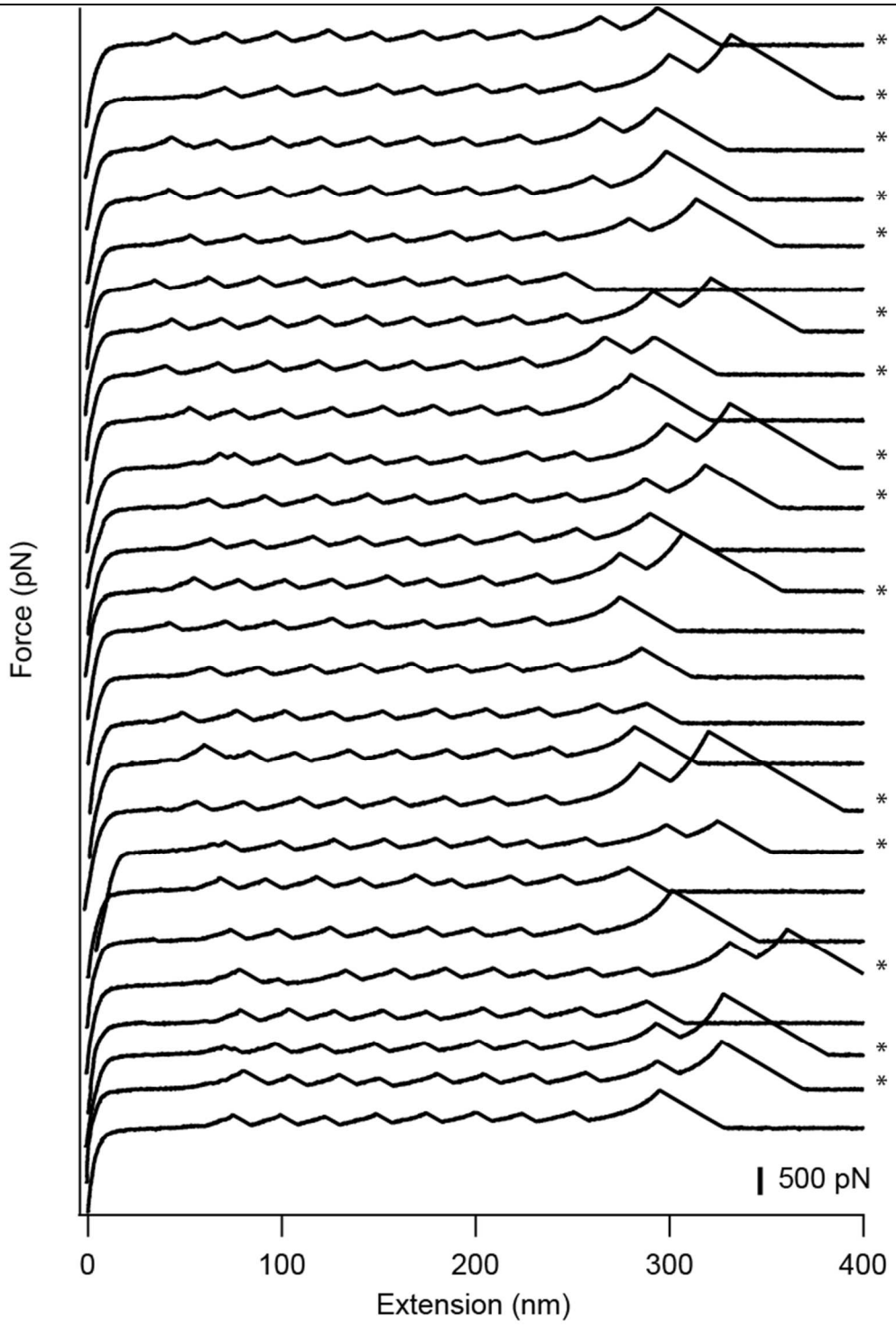

Figure S2. Force extension curves of the Halo-$(I27)_8$-cys (with N-terminus HaloTag) showing the unfolding of eight I27 domain, followed by the unfolding of HaloTag. When adsorbed on a chloroalkane surface and pulled with a gold cantilever, detachment competes with the unfolding of HaloTag. Hence, some traces only show the unfolding of eight I27 domains. The curves showing unfolding of HaloTag before detachment are marked with asterisk.

In **force-clamp** mode, an active feedback system maintained the force experienced by the polyprotein at a given setpoint value (time response < 3 ms). In these experiments, the cantilever was pressed into the protein layer with a force of -800 pN for 1 s, and then the surface was retracted under feed-back control to -100 pN for 0.2 s to determine the position of the surface, followed by a constant pulling force (for the kinetic measurements) or a linear increase in force (for the force-ramp experiments).

In order to quantify the unfolding kinetics of the component proteins from the data obtained for Halo-L_8-Cys in force-clamp mode, each measured curve was decomposed into two end-to-end traces: a first trace corresponding to the unfolding of protein L and a second trace corresponding to the denaturation of HaloTag (Figure 5A, marked with blue for protein L and magenta for HaloTag). Each trace was then populated with the value of the closest plateau to reflect the measured dwell times before and after unfolding. For a given force, each population of traces was then averaged (Figure 5C). A boot-strap procedure was then used to calculate the average unfolding rate, which was assumed to follow an exponential behavior, and the error of the measurement [2].

Folding kinetics of protein L was measured by exposing Halo-L_8-Cys construct to 80 pN of pulling force for 5 s, followed by a quench in force to 0 pN for varying periods of time and a force-ramp where folded protein L domains could be clearly see. Computing the number of folded domains for different folding times yielded the folding ratio. The folding rate was then calculated by assuming protein folding as having an exponential behavior [3].

When Halo-L_8-Cys construct was exposed to repeating unfolding-refolding cycles, the force protocol was designed as follows: the cantilever was pushed against the protein layer for 1 s at a force of -800 pN, then repeatedly pushed with -50 pN of force and pulled with 100 pN of force. During each push phase the position of the surface was compared with the position from the previous cycle and possible cantilever drift was then corrected from this difference.

S4. Mechanical properties of the studied proteins

The three proteins used in the AFM single molecule experiments (HaloTag, protein L and I27) show specific mechanical fingerprint, given by both their contour length increment and their unfolding force. While I27 is significantly more mechanically stable, protein L shows only slightly higher unfolding force when compared to C-terminus HaloTag. This organization is confirmed by the unfolding sequence (Figure S3). Force-extension traces of the Halo–L_8–Cys have the HaloTag mechanical denaturation as the first peak in 34.1 % of the cases. On the other hand, Halo–$I27_8$–Cys construct shows the denaturation of the HaloTag as the first occurring event in 95.3 % of the cases.

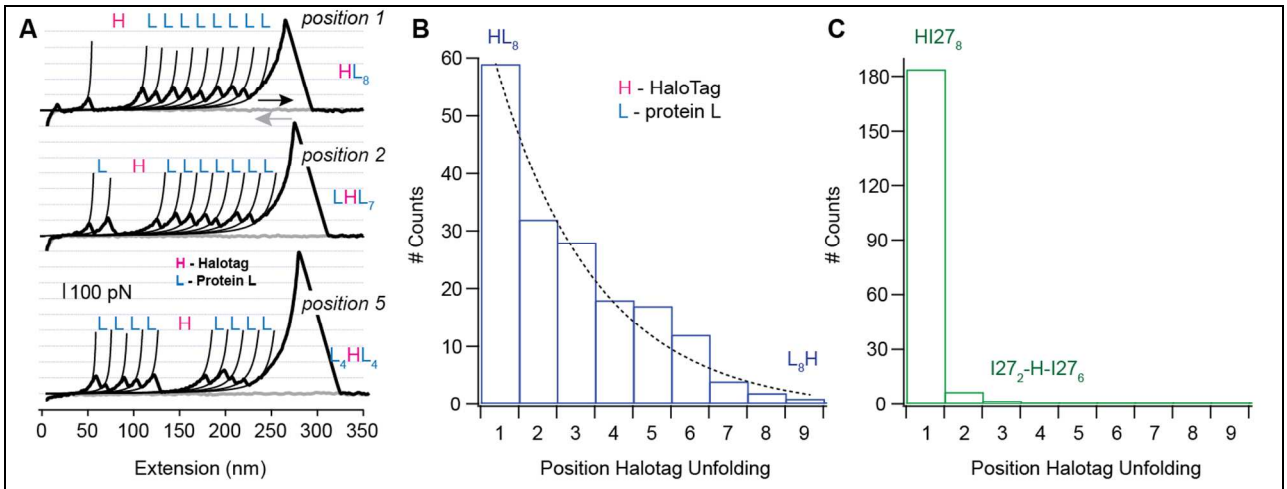

Figure S3. **Unfolding order measured from force-extension traces showing unfolding of all the nine protein domains, at a pulling rate of 400 nm/s.** A) Traces of Halo–L_8–Cys with the HaloTag protein unfolding in different positions; B) Unfolding position of the HaloTag in the Halo–L_8–Cys construct ; C) Unfolding position of the HaloTag in the Halo–$I27_8$–Cys.

Unfolding of mechanically stable proteins shows in force-extension mode saw-tooth pattern in the measured force as a function of extension. As shown in Fig. 2, we estimated the contour length increment by fitting the data using a standard worm-like chain model for polymer elasticity:

$$F = \frac{k_B T}{p}\left[\frac{1}{4}\left(1-\frac{x}{L_c}\right)^{-2}+\frac{x}{L_c}-\frac{1}{4}\right]$$

where F is the force, p the persistence length, L_C the contour length, x the extension and k_BT the thermal energy.

HaloTag is an alpha/beta protein with six consecutive parallel beta strands at its core (A-F, Figure S4). The attachment point of the enzyme to its chloroalkane ligand is at the end of the C beta-strand (amino acid 106; Figure S4). The measured unfolding contour length increments are due to the extension of the amino acid chain placed behind the mechanical transition state structure (clamp) in the protein. Under force, each amino acid contributes with 0.4 nm to the measured contour length increase (ref. [4]). We estimated the mechanical clamp of N and C-terminus HaloTag from the measured contour length increases of 26.5 and 66 nm respectively. Our data suggest that N-terminus HaloTag has the mechanical clamp between strands B and C, thus protecting amino acids 35 through 106 (Figure S4A). Since residues 35 and 106 are located 1.7 nm away (PDB code: 3g9x), the expected contour length increment is 27.1 nm (72 aa x 0.4 - 1.7 nm). The C-terminus HaloTag has its transition state somewhere in strands C, D, E and F, protecting amino acids 106-270 (Figure S4B). Since the distance between these residues is 1.4 nm in the crystal structure, the predicted contour length increment induced by C-terminus HaloTag unfolding is 64.2 nm (164 aa x 0.4 – 1.4 nm). In both cases, the transition state structures involve parallel beta strands in the core of the protein, which are known to withstand high mechanical forces [5,6].

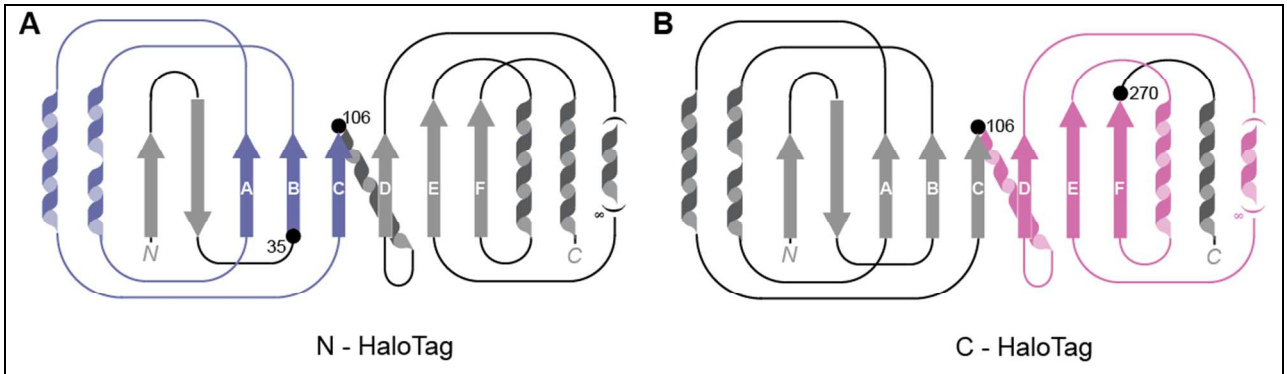

Figure S4. Topology of the HaloTag protein. A) Topology of the N-terminus HaloTag with the residues marked in blue representing those released by unfolding. B) Topology of the C-terminus HaloTag with the residues marked in magenta representing the polypeptide released by unfolding. The chloroalkane ligand is covalently anchored to residue 106 in both cases. Residues outside the core parallel beta strands (A-F) unravel at low force prior to unfolding (1-35 for N-terminus HaloTag; 270-299 for the C-terminus HaloTag), and do not contribute to the measured unfolding contour length increase.

S5. Effect of the homo-polyprotein on the attachment chemistry

In the first approach (chloroalkane surface – gold coated cantilever) we measured the detachment forces and the selectivity for two polyprotein constructs, Halo–L_8–Cys (Figure 4B) and Halo–$I27_8$–Cys (Figure S5). Both proteins show similar detachment forces and selectivity of attachment toward the two termini.

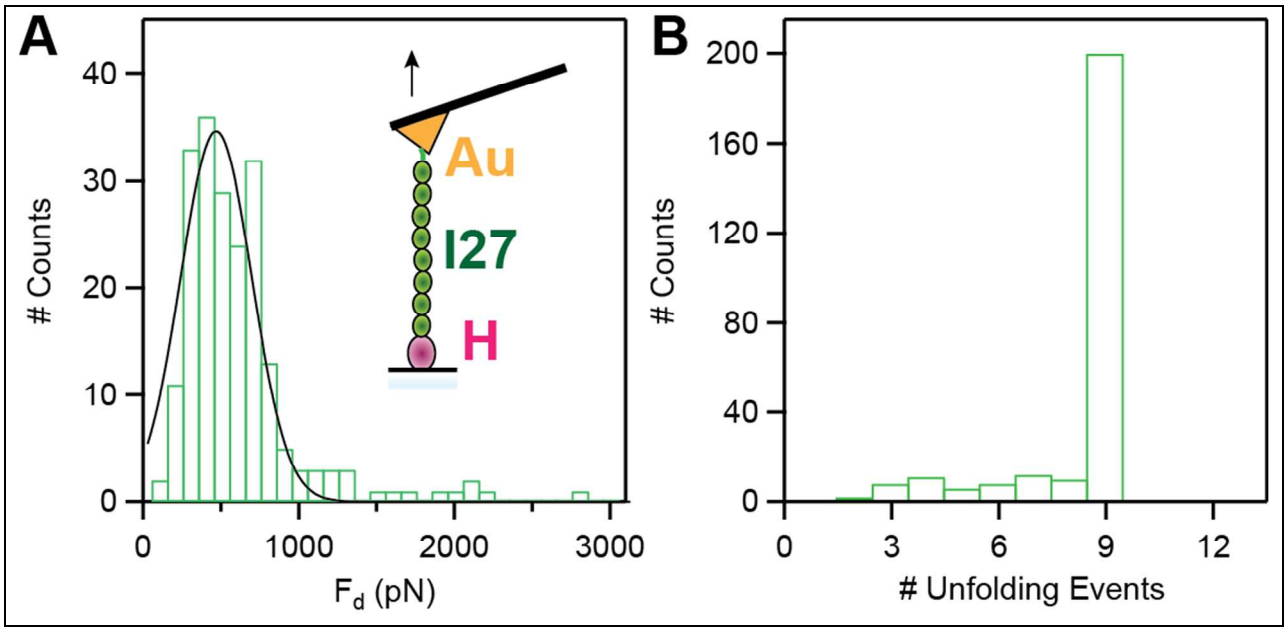

Figure S5. AFM experiments of the Halo-I27$_8$-Cys construct adsorbed on a chloroalkane surface and pulled using a gold coated cantilever. A) The measured detachment force is 533 ± 375 pN (s.d., n = 260). The black line represents a Gaussian fit to the data. Insert – Schematics of the experiment, where the polyprotein construct is tethered between the cantilever and the surface. B) Histogram of the number of unfolding events from force-extension traces of the *Halo- I27$_8$-Cys* construct, obtained from curves showing the HaloTag unfolding fingerprint. The measured specificity of the attachment to the termini of the polyprotein construct is 78%.

S6. Comparison of thiol-gold and thiol-maleimide surface

Polyproteins were covalently attached to gold coated and maleimide functionalized surfaces and pulled using chloroalkane functionalized cantilevers. A change in the measured detachment force from ~1013 pN to ~2001 pN is an indirect indication that indeed we are probing these two covalent attachments (Figure S5 and S6).

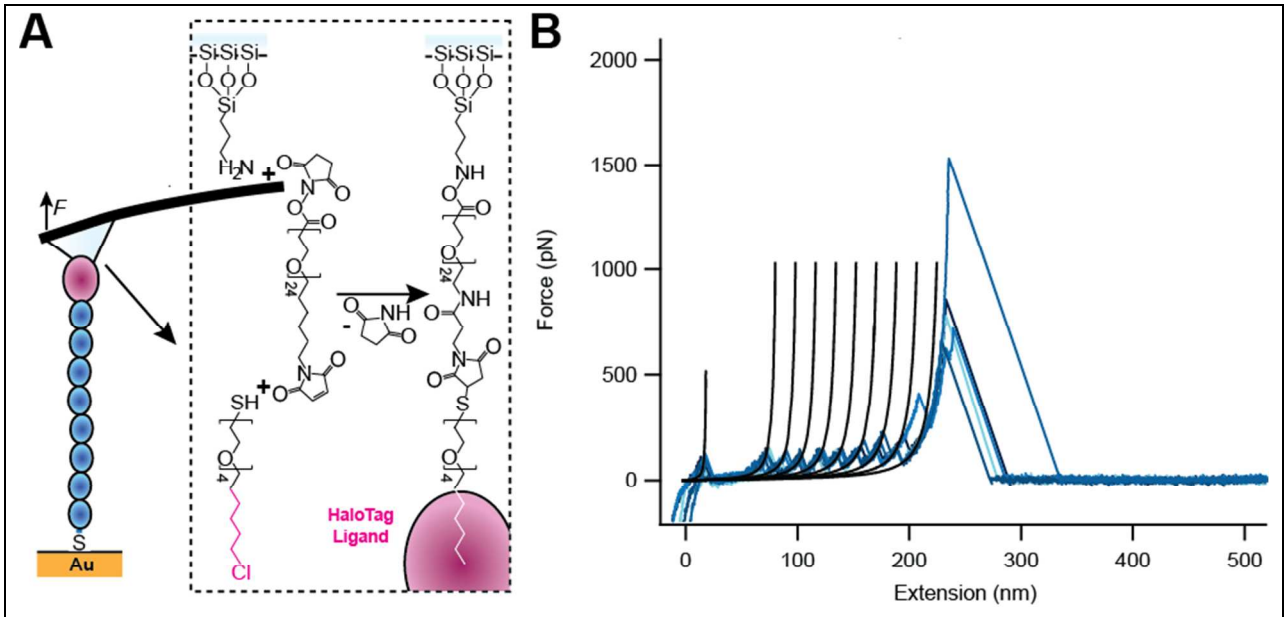

Figure S6. AFM experiments using Halo-L$_8$-Cys polyproteins attached to the surface through gold–thiol reaction and pulled through the HaloTag – chloroalkane chemistry. A) Schematics detailing the tethering configuration. B) Examples of curves measured in this configuration. The black lines represent fits to the worm-like chain model. Note that for Halo-L8-Cys only curves having the HaloTag as the first unfolding event are displayed for clarity purposes. This arrangement has a detachment force of 1013 ± 347 pN (n = 173) and a selectivity of 66% (Figure 4C).

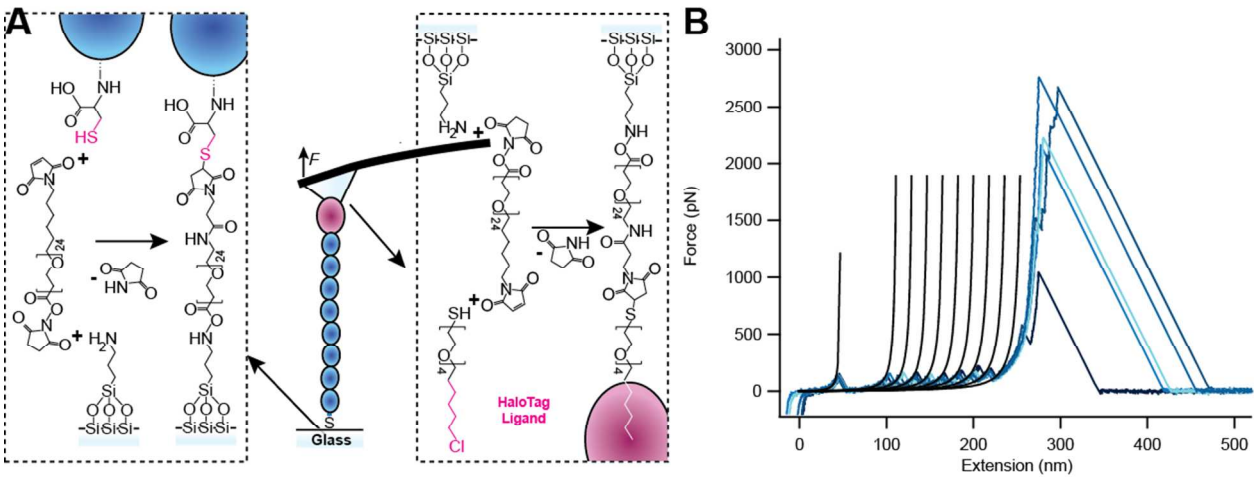

Figure S7. AFM experiments using Halo-L$_8$-Cys polyproteins attached to the surface through maleimide–thiol reaction and pulled through the HaloTag – chloroalkane chemistry. A) Schematics detailing the tethering configuration. B) Examples of curves measured in this configuration. The black lines represent fits to the worm-like chain model. Note that for Halo-L8-Cys only curves having the HaloTag as the first unfolding event are displayed for clarity purposes. This arrangement has a detachment force of 2001 ± 585 pN (n = 108) and a high selectivity of 86% (Figure 4D).

References

(1) Zimmermann, J. L.; Nicolaus, T.; Neuert, G.; Blank, K. *Nat Protoc* 2010, *5*, 975.

(2) Wiita, A. P.; Perez-Jimenez, R.; Walther, K. A.; Grater, F.; Berne, B. J.; Holmgren, A.; Sanchez-Ruiz, J. M.; Fernandez, J. M. *Nature* 2007, *450*, 124.

(3) Kosuri, P.; Alegre-Cebollada, J.; Feng, J.; Kaplan, A.; Inglés-Prieto, A.; Badilla, Carmen L.; Stockwell, Brent R.; Sanchez-Ruiz, Jose M.; Holmgren, A.; Fernández, Julio M. *Cell* 2012, *151*, 794.

(4) Ainavarapu, R. K.; Brujic, J.; Huang, H. H.; Wiita, A. P.; Lu, H.; Li, L. W.; Walther, K. A.; Carrion-Vazquez, M.; Li, H. B.; Fernandez, J. M. *Biophys J* 2007, *92*, 225.

(5) Sikora, M.; Sulkowska, J. I.; Cieplak, M. *Plos Comput Biol* 2009, *5*.

(6) Lu, H.; Schulten, K. *Biophys J* 2000, *79*, 51.