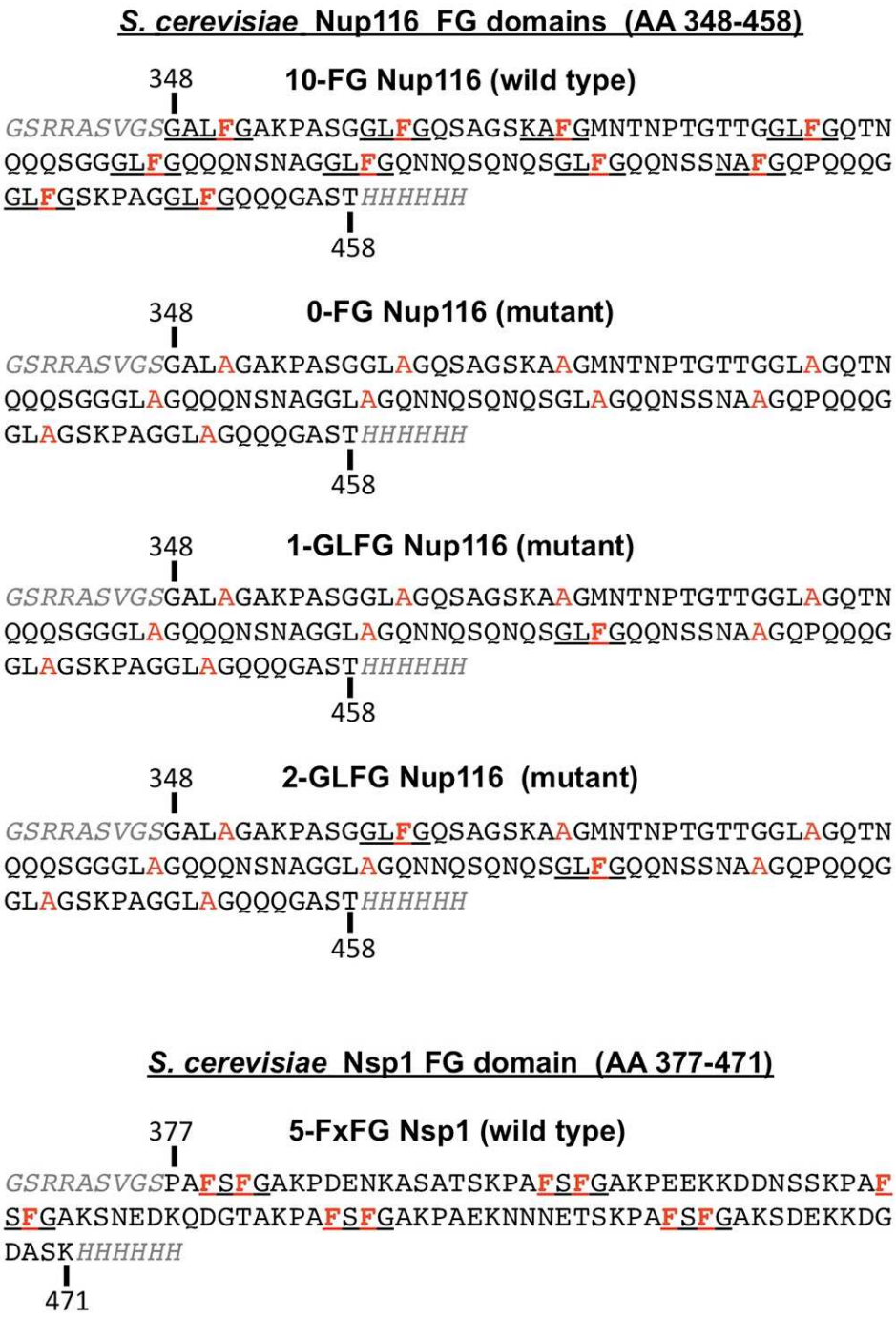


**Figure S1:** The amino acid (AA) sequence of nup FG domains used in this study. Phenylalanine residues, and the alanine residues used to substitute them, are highlighted in red font. FG motifs are underlined. AAs in gray are not part of the nup, but were used during its purification. Numbers in black bold font mark the corresponding AA position in the wild type protein.

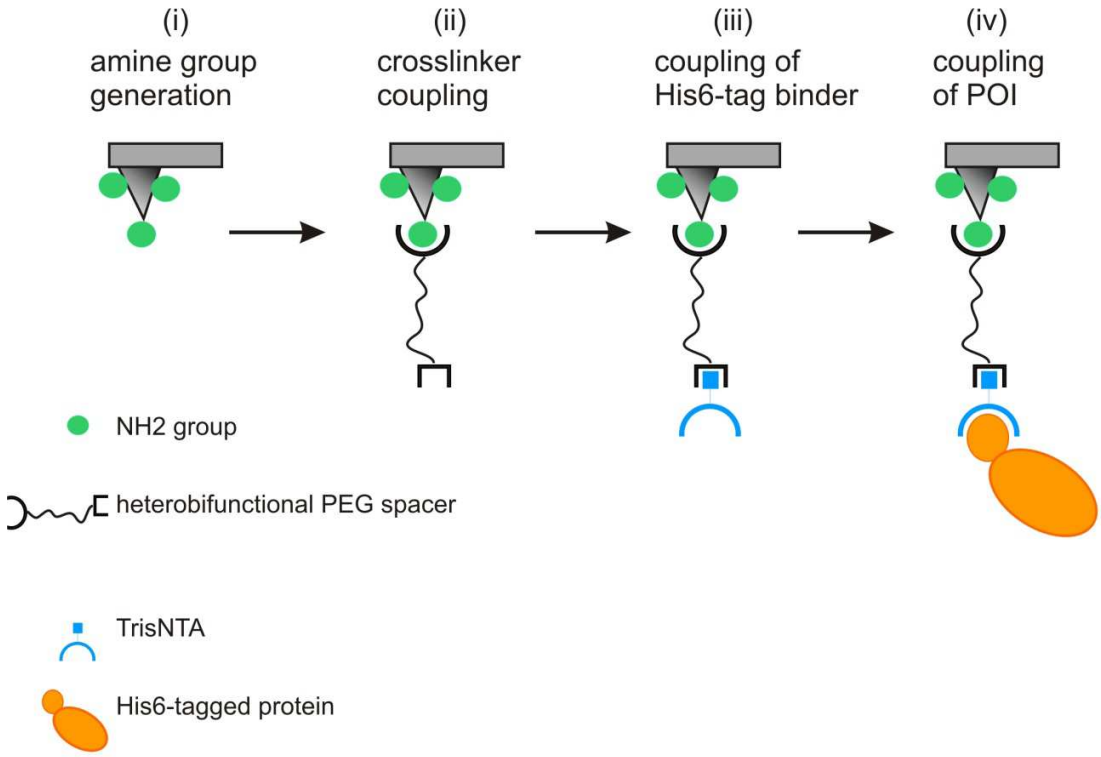


**Figure S2:** MRFS tip and surface chemistry. Purified nups and importin were immobilized onto AFM tips and/or flat mica surfaces via C-terminal $His_6$ tags, as depicted. Coupling was performed in four steps: (*i*) the AFM-tip and mica surfaces were coated with APTES (3-aminoprobyl triethoxysilane) to introduce amine groups; (*ii*) the heterobifunctionalcrosslinker NHS-PEG-malemeide was coupled to the APTES-modified surfaces by a covalent bond between the amine-functionalized surface and the NHS ester group of the PEG linker; (*iii*) dithiodipropionyl-tris-NTA was attached to the linker by breaking the sulfide bonds in the "thiol-tris-NTA" group using TCEP; Dithiopropionyl-tris-NTA was prepared by reacting COOH-protected tris-NTA (compound 13 published in (35)) with dithiobis(succinimidyl propionate) (DSP, Pierce) and subsequent removal of the tert-butyl ester functions, in close analogy to the synthesis of aminohexanoyl-tris-NTA (compound 19 in (35)); (*iv*) finally, the $His_6$-tagged protein(s) was coupled to the PEG-functionalized surface via the formation of non-covalent Tris-NTA-Ni-$His_6$-complex.

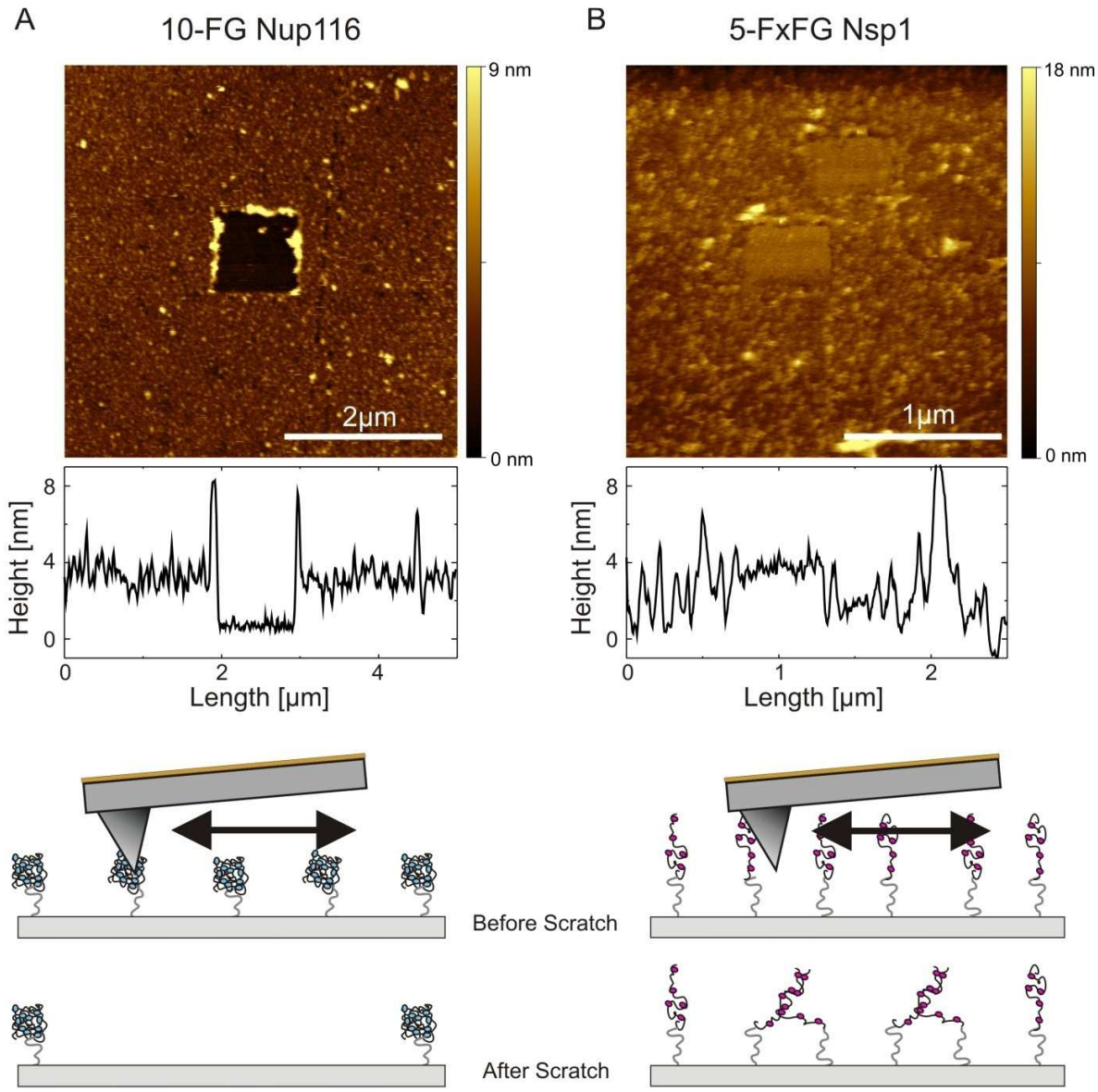


**Figure S3:** AFM images of nups immobilized on mica. A molecular layer of nup on the flat mica was verified by AFM imaging in MAC-mode. By increasing the amplitude of the cantilever, a high force was applied to scratch-away the molecules in a 1 µm x 1 µm area, as depicted. AFM height measurements are highly accurate, but lateral measurements are confounded by tip geometry. (**A)** In the case of 10-FG Nup116 (left), the bare mica was exposed revealing the height of the molecular layer as ~3 nm, consistent with Nup116 adopting a 'mushroom' configuration. (**B)** In the case of 5-FxFG Nsp1, a similar attempt to remove it from the mica resulted in areas with decreased roughness.This suggested that the Nsp1 molecules became tangled during the scratching process (as depicted) or were "forced-flattened' unto the mica aided by non-specific charge effects.

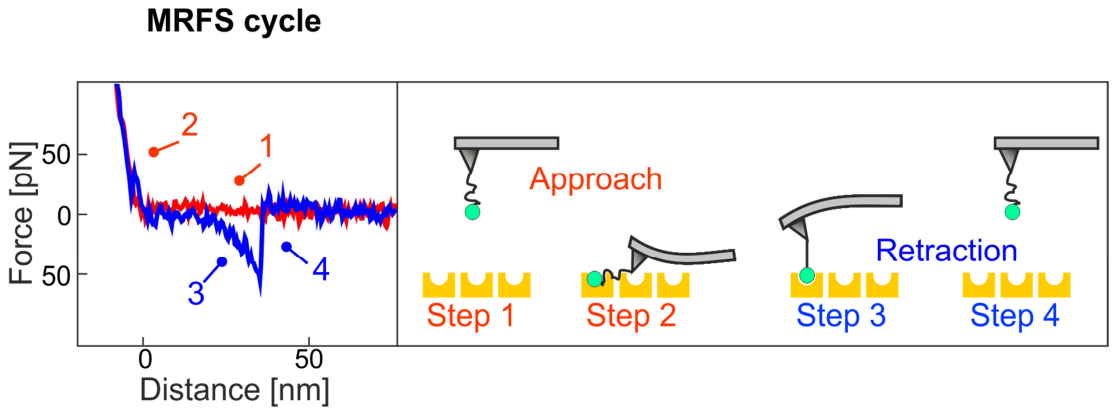


**Figure S4:** MRFS force-distance cycle. Interaction forces were recorded through deflections of the AFM cantilever. A horizontally-fixed cantilever is lowered (approach phase, red line) until the tip touches a molecule on the surface, or the surface (step 1). Upon further approach, the cantilever bends upwards (step 2). Upon withdrawal (retraction phase, blue line) the cantilever relaxes as it bends back to the zero-force line. When a ligand-receptor complex forms (between the tip and surface), the tip remains connected to the ligand during further retraction, resulting in downward-bending of the cantilever (step 3). When the bond breaks at a measurable force, the cantilever returns to the zero-bend reference line (step 4).

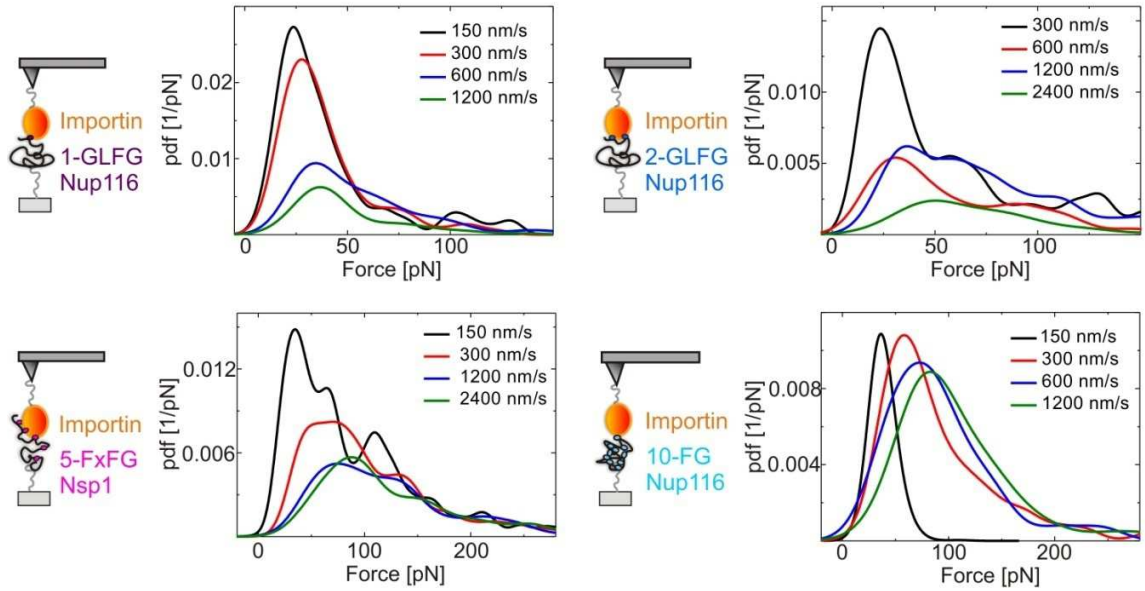


**Figure S5:** Pdf analysis of importin-nup interactions at various pulling velocities. The unbinding forces increased in relation to pulling velocity, consistent with Evan's theory. Importin captured only one FG repeat when interacting with 1-GLFG Nup116 (*upper left*), but captured one and sometimes two FG repeats simultaneously when interacting with 2-GLFG Nup116 (*upper right*). Importin also interacted with one, two, and three FxFG repeats simultaneously when binding 5-FxFG Nsp1 (*lower left*). In contrast, importin/10-FG Nup116 interactions only exhibited single unbinding forces (*i.e.* one FG repeat captured) despite the nup having ten FG repeats (*lower right*).

## *Experimental Section*

Tip and Surface Chemistry: Commercially-available AFM cantilevers (Bruker) were amine-functionalized via APTES silanisation as described.[35] A heterobifunctional PEG linker (malemeide-PEG-NHS, Polypure, Norway) was coupled to the tip via an amide bond by incubating the cantilevers for 2 h in 1mL chloroform containing 1mg malemeide-PEG-NHS and 0.5% v/v triethylamine.[36] After rinsing with chloroform and drying with a stream of nitrogen, the tips were incubated in a mixture of 50 µL of 4mM dithiodipropionyl-tris-NTA,[37] 3µl of 100 mM TCEP and 3 µL of 1M HEPES, pH 10 resulting in a free Tris-NTA coupling group at the distal end of the PEG linker. The tips were then pre-loaded with Ni by a 10 min incubation in Ni-buffer (50mM Tris, 5mM NiCl2, pH 7.5). Finally the $His_6$-tagged protein was coupled to the tip in the presence of 200 µM Ni by immersing the AFM tip in 0.1 mg/mL protein overnight at 4°C. Force Measurements: All measurements were carried out in NIM buffer (10mM NaCl, 90mM KCl, 2.2 mM MgCl2, 1.1 mM EGTA and 10mM HEPES, pH 7.4) by using a PicoPlus AFM (Agilent Technologies, Chandler, AZ). Force-distance cycles were performed at room temperature using mica-immobilized nups, and importin- or nup-coated tips with 0.01–0.03 N/m nominal spring constants. Spring constants were determined using the thermal noise method.[38] Empirical force distributions of rupture forces were calculated as described.[39] A sweep range of 150–300 nm and sweep rate of 0.5–4 Hz was used, resulting in loading rates from 200 to 24,000 pN/s. The loading rates were determined by multiplying the pulling velocity with the effective spring constant (i.e. the mean slope at rupture). For single molecular interaction forces, a linear rise of the unbinding force with respect to a logarithmically-increasing loading rate is expected: $F^* = f_\beta \cdot \ln(r/k_{off} \cdot f_\beta)$, where $f_\beta$ is the ratio of the thermal energy kBT and of $x_\beta$.[29] From such dependency, $x_\beta$ and $k_{off}$ were deduced.[30]

## Experimental Section References


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