Online methods

Protein expression and purification. NTF2 was purchased from Sigma-Aldrich. All other proteins were expressed and purified as described below. *E. coli* (BL21 DE3) were transformed with the appropriate plasmid and a small number of colonies were picked from the plate into flasks containing 1 L of LB medium plus the appropriate antibiotic. Biotinylated proteins were expressed in the presence of biotin (stock: 100 mM biotin, pH 11; 1 ml per 1L culture) and a biotin ligase. Cells were grown at 37 °C to A600 of 0.6 and then induced with 0.5 mM IPTG overnight. Cells were harvested by centrifugation at 5000 g at 4 °C for 15 min and the pellet was resuspended in phosphate buffer (pH 7.4) containing 0.5 M NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol and protease inhibitors. Proteins were initially purified by Ni-NTA affinity chromatography, followed by size-exclusion chromatography (Superdex 75, Amersham Pharmacia). Proteins were typically dialysed into XB buffer (10 mM HEPES pH 7.7, 1 mM $MgCl_2$, 100 mM KCl, 50 mM sucrose), flash frozen in liquid nitrogen and stored at -80 °C. Protein purity was judged by SDS-PAGE, and concentrations determined by UV absorbance (using calculated extinction coefficients) or Bradford assays. Nucleotide loading of Ran: the protein was incubated for 40 min on ice with 6 mM EDTA and a 50 fold excess of nucleotide. The reaction was stopped with a final concentration of 25 mM $MgCl_2$, added slowly. The protein was then dialysed against 30 mM potassium phosphate pH7.6, including 2 mM Mg-acetate, 2 mM GDP, 7% glycerol and 2 mM β-mercaptoethanol, at 4 °C overnight.

Quantum dot functionalisation and characterisation. Amino PEG functionalised QDs, with an emission peak of 605 nm, were acquired from Invitrogen. QD functionalisation efficiency varied from batch to batch. Upon receipt from the manufacturer, QDs were analyzed for size, coupling efficiency and traces of aggregation; about ½ of the batches were unsuitable and discarded. Good batches were used within 4 weeks of purchase. A two-step conjugation reaction was used. QDs were first coupled to Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SulfoSMCC) in standard PBS. Excess SulfoSMCC was removed via spin filtration. Activated QDs were coupled to cysteine-terminated recombinant protein in PBS supplemented with 350mM NaCl and 2mM EDTA. Uncoupled protein was removed by a second round of spin-filtration. Protein functionalized quantum dots were used within 8 hours of coupling. The mean hydrodynamic diameter of QD cargos was determined by dynamic light scattering (DLS). DLS measurements were performed on a Malvern Zetasizer Nano to measure the hydrodynamic radius. QD cargos were diluted to a final concentration of 8-10 nM in Transport buffer (20 mM HEPES, 110 mM KOAc, 5 mM NaOAc, 2mM MgOAc, 2 mM DTT, pH 7.3) and filtered (0.1 μm) to remove any aggregates. This concentration range yielded the best quality of data as judged by the polydispersity index (PDI). Measurements of the cargo in complex were performed in the presence of 3.8 or 1.0 μM importin-β. Count rates were typically above 200 kcps and multiple measurements were taken. Data were analysed using the Malvern software, using the Multiple Narrow Bands fitting algorithm and Refractive Index and Absorption settings for proteins (RI = 1.45, A = 0.001).

Import assays. HeLa cells were cultured in DMEM media (Gibco) and plated on glass bottomed (size 0 thickness) poly-lysine coated chambers (Mattek Corp.). Cells were permeabilised as follows. The chambers were washed for 3 x 5 min with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.4), followed by a 2 min wash with permeabilisation buffer (50 mM HEPES, 50 mM KOAc, 8 mM MgCl2, pH 7.3), followed by a 10 min permeabilisation with digitonin (50 mg) in permeabilisation buffer. Digitonin was subsequently removed by washing for 3 x 5 min with transport buffer (20 mM HEPES, 110 mM KOAc, 5 mM NaOAc, 2mM MgOAc, 2 mM DTT, pH 7.3). After the final wash, excess liquid was wicked off and a transport mix containing the QD probe and the recombinant import system was quickly added. Control experiments with fluorescently (FITC) labelled dextrans (70 kDa) were used to confirm that the nuclear envelope remained intact following digitonin permeabilisation. The final transport mix (100 μl) contained 50 pM QD, 0.5 μM importin β, 4 μM RanGDP, 1 μM NTF2, and 1 mM GTP in transport buffer. Once the transport mix had been added, the chamber was allowed to settle before starting single-molecule data collection.

Single-molecule microscopy. The microscope was based on a Nikon TE-2000 total internal reflection fluorescence (TIRF) microscope. A beam from a solid-state continuous-wave laser (532 nm, 100 mW, Coherent Compass 315M-100) was passed through a λ/4 plate to circularly polarize the light, a beam expander to enlarge the illumination area, and steered through free space to the microscope using a gimbal mirror mounted on a translation stage. The beam was focussed on the back focal plane of a high-NA objective (Apo 100x NA1.65, Olympus). The illumination beam was angled at just above the critical angle for TIR, passing at a shallow angle through the cells. This configuration allows high signal to noise movies to be recorded at a greater depth into the sample than conventional TIRF. Typical power at the objective was 10mW. A micrometer stage (Semprex) with piezo controlled nanopositioner (Mad City Labs) was used to position the cells. Movies were captured using a cooled electron multiplying CCD camera (Andor iXon+) running at 40 Hz at -78 °C. All instrumentation was controlled using custom C++ code. Image scaling was determined using a stage micrometer (SPI; 10 µm divisions), yielding a single pixel of dimensions 144.5 x 144.5 nm. We experimentally determined the localisation precision by immobilising QDs on a glass coverslip and imaging the particles over time. The mean localisation precision for our experiment is 6 nm, with a practical range for the dataset of 5-8 nm. We quantified drift of the sample/stage by tracking 40 nm and 100 nm gold nanoparticles immobilised onto the same poly-lysine coated glass Mattek chambers used for import experiments. The mean drift rate in our temperature-stabilized room was 11 nm/minute (1.8 Angstrom per second).

Image and data analysis. All data were analysed using custom written code in MATLAB/C++ as described in the supplementary materials.