SI Text

Preparation of Protein Samples. DNA sequences encoding hMARK3 (residues 48–370) and hMARK3 (residues 320–375) were amplified by PCR from the I.M.A.G.E. clone 5138958 (accession no. BC024773; Open Biosystems, Huntsville, AL); and h14-3-3ε was amplified from I.M.A.G.E. clone 3139004 (accession no. BC001440; Open Biosystems). Site-directed mutagenesis was performed using the QuikChange method (Stratagene, Santa Clara, CA) according to manufacturer's instructions. All sequences were verified and cloned in-frame into the pProEX Htb expression vector (Invitrogen, Carlsbad, CA) to incorporate a tobacco etch virus (TEV) protease-cleavable N-terminal hexa-histidine tag. The construct used to express human ubiquitin (Ub) with a TEV protease-cleavable N-terminal hexa-histidine tag was a kind gift from Silke Wiesner (Hospital for Sick Children, Toronto, ON, Canada). These constructs were expressed in *Escherichia coli* BL21(DE3) cells grown at 37°C to an OD_{600} of 0.6–0.8 before a 16-h induction with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 15°C. Uniform ^{13}C and ^{15}N labeling was achieved by growing bacteria in M9 minimal media containing 2 g/liter D-[^{13}C]glucose and/or 1 g/liter [^{15}N]NH_4Cl as the sole carbon and nitrogen sources, respectively. ^{13}C-methyl/^{15}N labeling was performed by expressing hMARK3 (residues 320–375) in M9 minimal media containing 2 g/liter [1-^{13}C]-D-glucose and 1 g/liter $^{15}NH_4Cl$. Cells were lysed by sonication in 0.2 M NaCl, 5 mM imidazole, 20 mM Hepes (pH 7.5), and 1 mM phenylmethylsulfonyl fluoride, and the cleared supernatant was loaded on to a HiTrap Chelating HP column (GE Healthcare, Bucks, UK) charged with $NiSO_4$ according to instructions manufacturer's. The His-tagged protein was eluted from the Ni column by using a 250–500 mM imidazole (pH 7.5) gradient, the His-tag was cleaved by incubation with TEV protease, and the sample was extensively dialyzed before further Ni chromatography and subsequent application to a Superdex-75 gel-filtration column (GE Healthcare). Samples were eluted in 100 mM NaCl, 20 mM Hepes (pH 7.5), and 5 mM 2-mercaptoethanol for crystal trials or 100 mM NaCl, 20 mM Tris (pH 7.0), and 7.5%(vol/vol) D_2O for NMR studies.

hMARK3 (Residues 48–370) Crystallization and Structure Determination. Crystals were grown at 4°C by the hanging-drop method over ~3 months. Drops were composed of 0.5 μl of a 1:1 mixture of purified hMARK3 (residues 48–370):h14-3-3ε, containing 0.23 mM of each protein, and an equal volume from the 300-μl total reservoir buffer (1.5 M $LiSO_4 \bullet H_2O$ and 0.1 M Hepes, pH 7.5). For data collection, crystals were washed in cryobuffer [1.5 M $LiSO_4 \bullet H_2O$, 0.1 M Hepes (pH 7.5), and 20% (vol/vol) glycerol] and flash-frozen. Analysis of protein crystals on SDS/PAGE gels revealed a single band corresponding to hMARK3 with no evidence for h14-3-3ε. A 2.7-Å data set was collected from a single frozen hMARK3 crystal at the Advanced Photon Source (Argonne, IL) on SBC-CAT beamline BM-19, and data were processed with the HKL2000 suite (1). Phases were obtained by molecular replacement using rMARK2 coordinates (PDB ID code 1ZMU) and the program Phaser1.3 (2). Model building was performed by using Coot (3) with refinement by Refmac (4). Residues 205–208 and 367–370 at the C terminus of hMARK3 were disordered and not included in the final structure. Side chain electron density was not observed for 18 residues (5.7% of total) and are modeled as alanines in the final structure. Structural analysis was performed by using PROCHECK (5). All protein structure figures were generated by using PyMOL (www.pymol.org).

GST Pull-Down Experiments. GST and GST-Ub were expressed from *E. coli* BL21(DE3) and bound to glutathione Sepharose (GE Healthcare) to serve as bait for pull-down experiments with purified UBA domains. Hexa-histidine-tagged hMARK3 UBA domain (residues 320–375), kinase:UBA domain (residues 48–370), and a control UBA domain known to bind monoUb, hHR23A UBA(2) (a kind gift from J. Feigon, University of California, Los Angeles, CA), were expressed via the pPROEX Htb vector from *E. coli* BL21(DE3) and purified by Ni-affinity and size-exclusion chromatography. Then, 20 μg of GST or GST-Ub were incubated with 120 μM (final conc.) of hexa-histidine hMARK3 UBA or kinase:UBA or the positive control hHR23A UBA(2) in binding buffer containing 25 mM Hepes (pH 7.5), 100 mM NaCl, and 2 mM DTT at 4°C for 30 min. The resin was washed four times with 0.5 ml of cold binding buffer and boiled with reducing SDS/PAGE loading buffer, and the resin-associated proteins were resolved by

17% SDS/PAGE. The proteins were visualized by using Colloidal Coomassie staining (Pierce Biochemicals, Rockford, IL).

NMR Spectroscopy. *Sequential amide backbone assignment:* NMR data for resonance assignments were collected for a 1.6 mM hMARK3 (residues 320–375) domain on a Varian INOVA 500-MHz spectrometer equipped with a triple-resonance room-temperature probe and a pulsed-field gradient unit. All data were processed with the NMRPipe/NMRDraw software package (6) with subsequent analysis performed by using XEASY (7). Backbone resonances were assigned for hMARK3 (residues 320–375) by using standard triple-resonance experiments (8, 9). Spectra were collected at 25°C.

Relaxation-dispersion measurements: Conformational exchange processes in the isolated hMARK3 domain were studied via backbone ^{15}N (10, 11) and side-chain methyl ^{13}C (12) single-quantum (SQ) Carr–Purcell–Meiboom–Gill (CPMG) relaxation-dispersion measurements. Data sets were recorded at 500 and 800 MHz (^{1}H frequency) as described previously (13). ^{15}N SQ CPMG experiments were performed on a uniformly ^{15}N-labeled sample at 15°C and 25°C (at 15°C, only 500-MHz data were collected), whereas ^{13}C SQ CPMG experiments were recorded at 5°C by using a sample prepared with [1-^{13}C]-glucose, resulting in close to 50% ^{13}C incorporation in most of the side-chain methyl groups without the generation of ^{13}C-^{13}C spin pairs (unpublished data). The backbone ^{15}N (side-chain methyl ^{13}C) SQ relaxation-dispersion profiles, $R_{2,eff}$ versus ν_{CPMG}, were generated from peak intensities, $I_1(\nu_{CPMG})$ quantified in a series of 2D ^{1}H-^{15}N (^{1}H-^{13}C) correlation maps measured as a function of CPMG frequency $\nu_{CPMG} = 1/(4\delta)$, where 2δ is the interval between consecutive refocusing pulses of the CPMG scheme applied within constant relaxation periods T_{relax} of 30 or 40 ms (20 ms). Peak intensities were converted into effective relaxation rates via $R_{2,eff}(\nu_{CPMG}) = -1/T_{relax} \bullet \ln(I_1(\nu_{CPMG})/I_0)$, where I_0 is the peak intensity in a reference spectrum recorded without the relaxation delay T_{relax}. CPMG frequencies, ν_{CPMG}, used in ^{15}N (^{13}C) SQ CPMG measurements ranged between 50 (100 Hz) and 1,000 Hz. Errors in $R_{2,eff}$ were estimated from repeat spectra measured for at least two CPMG frequencies as described elsewhere (13).

^{15}N CPMG dispersion profiles were obtained for 28 (15°C) or 32 (25°C) amide groups, and all data subsequently were fit together to a model of two-state exchange,

$$A \underset{k_B}{\overset{k_A}{\rightleftharpoons}} B,\ k_{ex} = k_A + k_B\,,$$

by using home-written software (data measured at each temperature were analyzed separately). Dispersion curves for which estimated errors in $R_{2,eff}(\nu_{CPMG}) > 0.2$ $R_{2,eff}(\nu_{CPMG})$ were not included in the fit. At both temperatures (15°C and 25°C), the exchange at most ^{15}N positions is in the fast limit (i.e., $k_{ex} >> \delta\omega$, where $\delta\omega = \varpi_N * \delta\omega$, $\delta\omega$, and $\delta\varpi$ are frequency (rad/s) and chemical shift differences (ppm) between states, respectively and ω_N is Larmor frequency of the ^{15}N nucleus). In this limit, it is only possible to extract the exchange rate constant $k_{ex} = k_A + k_B$ and the product $p_A p_B(\delta\varpi)^2$, where p_A and p_B are the fractional populations of interconverting sites A and B, respectively. ^{13}C SQ CPMG data sets were collected at 5°C (500 and 800 MHz); at this temperature, k_{ex} is reduced so that exchange is no longer fast, and thus it becomes possible to quantify all parameters characterizing a two-state exchange reaction, including p_B, k_{ex}, and $\delta\varpi$. Dispersion profiles from 14 methyl groups could be analyzed.

Amide-solvent exchange: The backbone amide-solvent hydrogen-exchange rates for the isolated hMARK3 UBA domain were measured by using an ^{15}N labeled sample at 25°C, pH = 7.0, with the CLEANEX-PM experiment, as described in Hwang *et al.* (14). Hydrogen-exchange rates, R_{exp}, were obtained from the buildup of peak intensities in a series of 6 2D ^{1}H-^{15}N spectra recorded with mixing times ranging from 9.5 to 63 ms, as described in detail elsewhere (14). Values of R_{exp} ranging from 0.5 to 20 s^{-1} were obtained for 24 amide groups. Protection factors were calculated as R_{int}/R_{exp}, where R_{int} is the residue-specific intrinsic random-coil hydrogen-exchange rate predicted by using the FBMME_HH.xls Microsoft Excel spreadsheet that can be downloaded from http://hx2.med.upenn.edu/download.html (15).

UBA•Ub titrations: ^{1}H-^{15}N HSQC spectra were collected on a Varian INOVA 600-MHz spectrometer with 0.4 mM ^{15}N-labeled hMARK3 (residues 320–375) in the absence or presence of unlabeled human Ub (in the same buffer) to 6.65 molar equivalents. Spectra were analyzed in NMRDraw (6), and average chemical shift perturbations were calculated by using the relationship: $\Delta\delta^{av} = ((\Delta\delta_{1H})^2 + (\Delta\delta_{15N}/5)^2)^{1/2}$, where $\Delta\delta_{1H}$ and $\Delta\delta_{15N}$ are the ^{1}H and ^{15}N shift changes, respectively. The dissociation constant (K_d) was determined for each resonance by plotting $\Delta\delta_{1H}$ versus the molar ratio of Ub/UBA and performing nonlinear curve fitting with the relationship: $y(x) = 0.5*b[(x + 1 + a) - ((x + 1 + a)^2 - 4x)^{0.5}]$, where $y(x)$ is $\Delta\delta_{1H}$ at a given x = Ub/UBA molar ratio and a and b are fitted parameters corresponding to K_d/(total UBA domain concentration) and the maximum $\Delta\delta_{1H}$, respectively (16). ^{1}H-^{15}N HSQC spectra were collected at 25°C on a Varian INOVA 500-MHz spectrometer for 0.4 mM ^{15}N-labeled Ub in the absence or presence of up to 5.3 molar equivalents of unlabeled hMARK3 (residues 320–375) with analysis performed as described above.

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