Molecular Basis for Impaired DNA Damage Response Function Associated with the RAP80 ΔE81 Defect*□

Anamika1, Craig J. Markin1, Manoj K. Rout, and Leo Spyropoulos2

From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Background: RAP80 recruits repair protein complexes to DNA damage sites through multivalent polyubiquitin binding.

Results: A single point deletion (ΔE81) in RAP80 abrogates multivalent interactions with polyubiquitin.

Conclusion: Disruption of multivalent binding arises from loss of electrostatic interactions between Glu-81 and ubiquitin.

Significance: A single residue deletion mutation in RAP80 impairs DNA damage repair and is implicated in the pathogenesis of cancer.

Signal transduction within the DNA damage response is driven by the flux of protein-protein interaction cascades that ultimately recruit repair complexes to sites of damage. The protein RAP80 plays a central role in the damage response by targeting BRCA1/BRCA2 tumor suppressors to DNA damage foci through multivalent binding of Lys-63-linked polyubiquitin chains. Mutations within the high penetrance BRCA1/BRCA2 genes account for ~20% of familial breast cancers. The genetic basis for the remaining cancers remains unknown, but may involve defects in binding partners for BRCA1 and BRCA2 that lead to impaired targeting to foci and a concomitant role in the pathogenesis of cancer. Recently, an in-frame deletion mutation (ΔE81) in a conserved region from the first ubiquitin interaction motif of RAP80 has been linked to an increase in chromosomal abnormalities. Using NMR spectroscopy, we demonstrate that the N-cap motif within the α-helix of the first ubiquitin interaction motif from ΔE81 undergoes a structural frameshift that leads to abolishment of multivalent binding of polyubiquitin chains. Loss of this single glutamate residue disrupts favorable electrostatic interactions between RAP80 and ubiquitin, establishing a plausible molecular basis for a potential predisposition to cancer unrelated to mutations within BRCA1/BRCA2 genes.

The recognition of Lys-63-linked polyubiquitin (polyUb)3 chains plays a central role in the DNA damage response by recruiting repair protein complexes to sites of double-stranded DNA breaks, or DNA damage foci (1). The protein RAP80 is involved in multivalent recognition of polyUb chains through N-terminal tandem ubiquitin-interacting motifs (tUIMs) and is fundamental for protein recruitment in the DNA damage response (1, 2). Defects within mechanisms underlying the binding of repair proteins to DNA damage foci may give rise to tumorigenesis and the progression of disease (3, 4). This highlights intense efforts to identify binding partners for BRCA1 and BRCA2, tumor suppressors whose recruitment to sites of DNA damage is essential for repair (5, 6).

RAP80 is a large, 80-kDa protein comprising a number of domains; the polyUb binding function is localized to a pair of closely spaced, or tandem UIMs, short α-helices that bind the canonical hydrophobic interface centered on Ile-44 of Ub (7–9). The individual UIMs of RAP80 have low affinity for Ub monomers (KD ~500 µM). In tandem, the UIMs employ a multivalent binding mechanism to substantially increase the affinity for Lys-63-linked or linear polyUb chains with two to four linked monomers, to KD values ranging from 2 to 75 µM (7, 8).

Approximately 20% of familial breast cancers arise from germ line mutations in the BRCA1 and BRCA2 genes (3). The genetic basis for the remaining cancers is not known, but is suspected to involve mutations in genes with lower penetrance than BRCA1 and BRCA2, potentially in combination with environmental or other factors (3). Given that BRCA1 and BRCA2 proteins function within protein complexes that localize to sites of DNA damage, mutations in genes for proteins that interact with these tumor suppressors may also be involved in cancer susceptibility (3).

Recently, an alteration within the highly conserved N-terminal UIM region from the RAP80 gene was discovered in a group of 112 BRCA1/BRCA2 mutation-negative Finnish breast cancer families (3). The alteration, ΔE81, is an in-frame deletion of the first of three sequential Glu residues that occur at the N terminus of the first UIM α-helix. The RAP80 ΔE81 mutant demonstrated reduced ubiquitin binding in GST pulldown assays and reduced localization to DNA damage foci in confocal microscopy studies with U2OS cells. Furthermore, cells expressing ΔE81 RAP80 show increases in chromosomal abnormalities that are consistent with defective double-stranded DNA break repair (3); these results point to the biological relevance of the ΔE81 mutation and a potential familial link to cancer.

We investigated the molecular basis for impaired recognition of Ub by ΔE81 RAP80 through the use of solution state NMR spectroscopy in conjunction with detailed thermodynamic binding models. In addition, we used the temperature depen-
ENCE OF THE ¹Hα CHEMICAL SHIFTS TO MEASURE THE STABILITY OF THE α-HELICES FOR THE TANDEM UIMs IN ΔE81. THE RESULTS INDICATE THAT THE MULTIVALENT BINDING ADVANTAGE FOR TANDEM UIMs IN RAP80 IS LOST IN THE ΔE81 MUTANT, AND PROVIDE A MOLECULAR DESCRIPTION FOR A PRIMARILY STRUCTURAL DEFECT THAT MAY BE INVOLVED IN THE PATHOGENESIS OF CANCER.

EXPERIMENTAL PROCEDURES

Mutagenesis, Protein Expression, and Purification—The ΔE81-RAP80-tUIM MUTANT WAS MADE USING PCR MUTAGENESIS WITH RECOMBINANT pGEX-6P-1 RAP80-tUIM PLASMID (8) AS A TEMPLATE. INSERTS HARBOURING THE DELETION MUTANT WERE RELIGATED INTO pGEX-6P-1 AND VERIFIED BY SEQUENCING (CLEARVIEW BIOSTRUCTURES, INC., EDMONTON, AB, CANADA). OVEREXPRESSION AND PURIFICATION OF [¹³C]RAP80-tUIM WERE CARRIED OUT AS DESCRIBED PREVIOUSLY (8, 10). OVEREXPRESSION AND PURIFICATION OF [¹³C]RAP80-tUIM WERE ALSO CARRIED OUT AS DESCRIBED PREVIOUSLY FOR WILD TYPE RAP0-tUIM (8, 10), WITH THE EXCEPTION THAT 10 mM UREA WAS ADDED TO THE PURIFIED PROTEIN SOLUTION AND REMOVED BY DIALYSIS BEFORE THE FINAL SIZE-EXCLUSION CHROMATOGRAPHY STEP (SUPERDEX 30). THIS STEP WAS INCLUDED TO DENATURE ANY REMAINING PROTEASE FOLLOWING CLEAVAGE OF THE GST AFFINITY TAG. TANDEM RAP80-tUIM WERE ALSO EXTRACTED AS DESCRIBED PREVIOUSLY FOR WILD TYPE RAP80-tUIM (8, 10), WHEREAS RECOMBINANT UBQ WAS PURCHASED FROM BOSTON BIOCHEM (CAMBRIDGE, MA).

NMR Spectroscopy, Main Chain Relaxation Measurements, and Structure Determination—NMR SPECTRA FOR TITRATIONS OF ΔE81-RAP80-tUIM WITH Ub/Ubα WERE COLLECTED AT 25 °C USING A VARIAN UNITY INOVA 600-MHz SPECTROMETER. SAMPLES (350 µL) WERE PLACED IN 5-MM SHIGEMI MICROCCELL NMR TUBES, WITH BUFFER CONTAINING 50 mM TRIS, 150 mM NaCl, 2 mM DTT, pH 7.3, AND 10% D2O. CHEMICAL SHIFT ASSIGNMENT FOR ΔE81-RAP80-tUIM WAS CONDUCTED AT 5 °C, THE NMR SAMPLE CONTAINED 0.1 mM 4,4-DIMETHYL-4-SILAPENTANE-1-SULFONIC ACID (DSS) AS AN INTERNAL CHEMICAL SHIFT REFERENCE, AND THE PROTEIN CONCENTRATION WAS 0.6 mM, WITH BUFFER CONDITIONS IDENTICAL TO THOSE AT 25 °C. BACKBONE ¹Hα CHEMICAL SHIFTS WERE DERIVED FROM THREE-DIMENSIONAL ¹⁵N NOESY-HSQC SPECTRA (21) WITH THE AID OF ASSIGNMENTS AT 25 °C. INTERPROTON DISTANCE RESTRAINTS WERE DERIVED FROM THREE-DIMENSIONAL ¹⁵N NOESY-HSQC SPECTRA (21) WITH A MIXING TIME OF 200 MS. FOR THESE NMR EXPERIMENTS, SAMPLE CONDITIONS WERE SIMILAR TO THOSE USED FOR CHEMICAL SHIFT ASSIGNMENT AT 5 °C, WITH THE EXCEPTION THAT PROTEIN CONCENTRATIONS WERE 0.3 AND 1.3 mM FOR [¹³C]RAP80-tUIM AND [¹³C]RAP80, RESPECTIVELY. QUANTITATIVE MAIN CHAIN PHI AND PSI DIHEDRAL ANGLE RESTRAINTS WERE ASSESSED AS WELL AS ROTAMER PROBABILITIES FOR THE SIDE CHAIN CHI DIHEDRAL ANGLES WERE DERIVED FROM NMR CHEMICAL SHIFTS USING THE TALOS-N PROGRAM (22). IN ADDITION, HELIX N-CAP BOX MOTIFS WERE IDENTIFIED FROM NMR CHEMICAL SHIFTS USING THE MICS PROGRAM (23).

Structures for wild type and ΔE81-RAP80 were calculated using torsion angle dynamics and the simulated annealing protocol in XPLOR-NIH (24). NOE restraints were sorted into strong (1.8–2.9 Å), medium (1.8–3.5 Å), and weak (1.8–5.0 Å) distance ranges. Backbone torsion angle restraints were obtained from the values calculated by the TALOS-N program. CHI angle restraints were included for residues for which TALOS-N indicated that a single rotamer was favored. Twenty structures were calculated in this manner for wild type RAP80-tUIM and ΔE81-RAP80-tUIM.

ΔE81-RAP80-tUIM α-Helix Stability Measurements—The temperature dependences of ¹Hα chemical shifts for ΔE81-RAP80-tUIM were measured by collecting two-dimensional ¹H-¹³C HSQC spectra from 5 to 50 °C, in steps of 5 °C. The NMR sample was prepared identically to that used for main chain chemical shift assignment at 5 °C, as described above. Due to extensive overlap for ¹Hα resonances, per residue chemical shifts were obtained by deconvolution of the spectra through fitting to a lineshape function defined as the sum of one to four Gaussian curves, depending on the degree of overlap, using Mathematica (25). Spectra were converted to tabular format using the pipe2txt.tcl program implemented in NMRPipe (26), and this text file was subsequently imported into Mathematica for deconvolution. Regions surrounding peaks of interest were defined, and chemical shifts and intensities within these regions were extracted. One-dimensional ¹H projections were obtained by summing ¹³Cα planes together; the projection was subsequently fit to the deconvolution function. Fitted parameters included linewidths, intensity scaling factors, and chemical shifts for the Gaussian peaks. To obtain an estimate of the chemical shift error, the dimensions of the initial selection box were varied within reasonable bounds and the procedure repeated; the chemical shifts thus obtained were averaged, and the S.D. was taken to be the error. The resonances analyzed in this manner were Phe-85, Leu-89, and Ala-113. Chemical shift overlap for most other peaks was too severe for effective decon-
volution. In the case of Phe-85 at 50 °C, an overlapping water artifact precluded chemical shift determination; thus, this point was not included in the subsequent analyses.

To facilitate direct comparison of the helical stabilities of wild type and ΔE81-RAP80-tUIM, we reanalyzed our previously measured $^{1}H_{d}$ data (8) using this deconvolution methodology. The chemical shift changes were fit to a two-state cooperative helix-coil transition, as described previously (8, 27). Errors in the fitted values of $T_{m}$ and $\Delta T$; the midpoint and width of transition, respectively, were obtained from Monte Carlo simulations using the errors determined during the chemical shift deconvolutions.

**Chemical Shift Titrations for ΔE81-RAP80-tUIM with Ub and Tandem Ub2—**Two-dimensional $^{1}H$-15N HSQC NMR spectra (28) for titrations of [U-15N]-ΔE81-RAP80-tUIM with unlabeled human recombinant ubiquitin (Boston Biochem) and of [U-15C,15N]-ΔE81-RAP80-tUIM with tandem Ub$_{2}$ were collected at 25 °C and 600 MHz. Protein concentrations for the stock solutions of ΔE81-RAP80-tUIM, Ub, and Ub$_{2}$ used in NMR-monitored titrations were determined using amino acid analyses. Titrations were conducted by diluting labeled ΔE81-RAP80-tUIM with aliquots from stock solutions of Ub or Ub$_{2}$, and following chemical shift changes, as described previously (8). The concentrations of [U-15N]-ΔE81-RAP80-tUIM were 215, 209, 201, 188, 174, 160, 146, 133, 119, 105, and 53 μM; the concentrations of Ub were 0, 90, 221, 443, 666, 889, 1112, 1334, 1557, 1780, and 890 μM, corresponding to Ub/RAP80 ratios of 0, 0.4, 1.1, 2.4, 3.8, 5.5, 7.6, 10.0, 13.1, 16.9, and 16.9. The decrease in protein concentration during the titration allows for more extensive sampling of the binding isotherm resulting in an increase for the precision of the fitted $K_{D}$ value, as described previously (29). For the Ub$_{2}$ titration, concentrations of [U-15C,15N]-ΔE81-RAP80-tUIM were 206, 203, 196, 190, 182, 160, 136, and 121 μM, and the corresponding concentrations of Ub$_{2}$ were 0, 40, 131, 215, 310, 596, 908, and 1096 μM, corresponding to Ub$_{2}$/RAP80 ratios of 0, 0.2, 0.7, 1.1, 1.7, 3.7, 6.7, and 9.1. For some residues, overlap prevented chemical shift determination for certain titration points; these were not included in the fitting procedure to extract binding constants.

Determination of the dissociation constants for binding of Ub to the N- and C-terminal UIMs, $K_{D,N}$ and $K_{D,C}$, respectively, was achieved using a global fit of the chemical shift changes of all significantly shifting residues to a binding model describing the interaction of Ub with the N-terminal UIM (N-UIM), the C-terminal UIM (C-UIM), or two Ub molecules simultaneously bound to both UIMs (8)

\[
K_{D,N} = \frac{[P][L]}{[PL_{N}]}, \quad K_{D,C} = \frac{[P][L]}{[PL_{C}]},
\]

\[
[PL_{NC}] = \frac{[PL_{N}][L]}{(K_{D,N} + K_{D,C})} + \frac{[PL_{C}][L]}{(K_{D,N} + K_{D,C})}
\]

where [P] is the concentration of free RAP80, [L] is the concentration of free Ub, [PL$_{N}$] is the concentration of RAP80 with Ub bound to the N-UIM, [PL$_{C}$] is the concentration of RAP80 with Ub bound to the C-UIM, [PL$_{NC}$] is the concentration of RAP80 with Ub bound to both the N- and C-UIM, with $K_{D,N}$ and $K_{D,C}$ as described above. The total protein concentration is given by [P] + [PL$_{N}$] + [PL$_{C}$] + [PL$_{NC}$], the total ligand concentration is given by [L] + [PL$_{N}$] + [PL$_{C}$] + 2[PL$_{NC}$]. Chemical shift changes for residues from the N-UIM were taken to reflect either Ub binding to this UIM alone, or with a second Ub bound to the neighboring UIM, but not Ub bound only to the C-UIM; a corollary approach was also used to assess Ub binding for residues belonging to the C-UIM. Unlike binding of Ub to wild type RAP80-tUIM as characterized in our previous work (8), the deletion of Glu-81 abrogates Ub binding to the N-UIM. This is manifested as drastically reduced $\Delta \delta$$_{max}$ values for residues of the N-UIM, the result of greatly reduced levels of saturation. To achieve an estimate of this weakened affinity, it was necessary to restrain $\Delta \delta$$_{max}$ values for some residues of the N-UIM during the global fit as follows: the free chemical shifts and their direction of movement upon titration with Ub matched those observed for wild type RAP80-tUIM; thus, $\Delta \delta$$_{max}$ values for Lys-90 and Ser-92 were used as the corresponding $\Delta \delta$$_{max}$ values for ΔE81-RAP80-tUIM. The $K_{D,N}$ and $K_{D,C}$ values from fits using these constraints individually were then averaged to give the reported values.

The dissociation constants ($K_{D,N}$ and $K_{D,C}$) for binding of Ub$_{2}$ to ΔE81-RAP80-tUIM were obtained in an analogous manner, except with the substitution of a binding model for Ub$_{2}$ rather than Ub, as follows

\[
K_{D,N} = \frac{[P][L]}{[PL_{N}]} \quad K_{D,N} = \frac{[P][L]}{[PL_{C}]},
\]

\[
[PL_{NC}] = \frac{[PL_{N}][L]}{(K_{D,N} + K_{D,C})} + \frac{[PL_{C}][L]}{(K_{D,N} + K_{D,C})}
\]

\[
[P_{N}P_{L_{NC}}] = \frac{[P_{N}L_{N}][P]}{(K_{D,N} + K_{D,C})} + \frac{[P_{L_{C}}][P]}{(K_{D,N} + K_{D,C})}
\]

where [P] is the concentration of free RAP80, [L] is the concentration of free Ub$_{2}$, [PL$_{N}$] is the concentration of RAP80 with the N-Ub from Ub$_{2}$ bound to the N-UIM, [PL$_{C}$] is the concentration of RAP80 with the C-Ub from Ub$_{2}$ bound to the C-UIM, [PL$_{NC}$] is the concentration of RAP80 with the N-Ub of Ub$_{2}$ bound to the C-UIM, [PL$_{NC}$] is the concentration of RAP80 with Ub$_{2}$ bound to the C-UIM, and [PL$_{NC}$] is the concentration of one Ub$_{2}$ molecule with the N-UIM from one RAP80 molecule bound to the N-Ub and the C-UIM from a second RAP80 bound to the C-Ub from another Ub$_{2}$, $K_{D,N}$ and $K_{D,C}$ are as described above. The total protein concentration is given by [P] + [P$_{N}$L$_{N}$] + [P$_{L_{C}}$] + [P$_{N}$L$_{NC}$] + [P$_{C}$L$_{NC}$] + 2[P$_{N}$P$_{L_{NC}}$] the total ligand concentration is given by [L] + [P$_{N}$L$_{N}$] + [P$_{L_{C}}$] + [P$_{N}$L$_{NC}$] + [P$_{C}$L$_{NC}$] + 2[P$_{N}$P$_{L_{NC}}$] + [P$_{N}$P$_{C}$L$_{NC}$]. $\Delta \delta$$_{max}$ values for residues (Lys-90 and Ser-92) of the N-UIM were likewise individually constrained at their wild type RAP80-tUIM values to achieve a reasonable fit, and the fitted $K_{D,N}$ and $K_{D,C}$ values were again
Chemical shift errors were determined using Monte Carlo methods; where possible, isolated peaks used in the $K_D$ determination were extracted from the spectra, noise was added to each point in a random fashion (based on the overall level of spectral noise, obtained from NMRDraw (26)), and the chemical shift was determined using a parabolic fit, as described in Ref. 29. This procedure was repeated 10,000 times, and the S.D. of the chemical shift was determined for the ensemble of values. When it was not possible to extract an isolated peak, the error was estimated as the median of the other chemical shift errors calculated for the spectrum. However, uncertainty in the stock protein concentrations is typically much larger than that for chemical shifts and is the main source of error in the determination of $K_D$ values from NMR titrations. The stock protein concentration errors were taken to be 10% (29, 30). Subsequently, both chemical shift and protein concentration errors were used to estimate errors for the fitted dissociation constants, using 250 Monte Carlo trials.

**Stability Calculations for the Ub-RAP80 N-UIM Interaction**—Molecular dynamics (MD) simulations were conducted to estimate the free energy of interaction for wild type or $\Delta$E81 RAP80 N-UIM with Ub using the AMBER 11/12 suites of biomolecular simulation programs (31). A starting model for the interaction between the N-UIM from RAP80 and Ub was generated from the structure of Lys-63 Ub, bound to wild type RAP80 (Protein Data Bank ID 3A1Q) (9). Given that the crystallographic structure lacks RAP80 residues N-terminal to Glu-81 and contains a GPLGS cloning artifact instead, these five residues were removed and replaced with the residues Met-79 and Thr-80, whose main chain and side chain dihedral angles were set at standard N-cap values. This wild type model was then used to create a starting model for the interaction between $\Delta$E81-RAP80 N-UIM and Ub. However, Met-79 in the $\Delta$E81 mutant cannot be accommodated in the N-cap conformation because of steric clashes with Ub. Thus, the main chain and dihedral angles for Met-79 and Thr-80 were set at standard $\alpha$-helical values, the side chain of Thr-80 was set at $\chi^1 \sim \pm 180^\circ$ to form a favorable electrostatic interaction with Arg-42 from Ub, and the side chain from Met-79 was set at $\chi^1 \sim +60^\circ$ to form favorable van der Waals interactions with Leu-73 from Ub. The starting models were then energy-minimized using a generalized Born implicit solvent model (32). Using these initial models, molecular dynamics simulations were conducted with the ff99SBNMR forcefield (33), the TIP3P water model, and a particle mesh Ewald approach (PMEMD) with default parameters was used for calculating long range electrostatics, as implemented in CUDA (34). SHAKE was used to restrain covalent bonds to hydrogen, temperature was regulated using a Berendsen thermostat, and pairwise nonbonded and electrostatic interactions were cut off at 8 Å. The initial structural models were solvated in a truncated octahedral water box with a distance of 24 Å between protein atoms and their images in adjacent unit cells, and the systems were neutralized with Na$^+$ ions. The systems were heated over 50 ps to 298 K with 2 kcal/mol restraints on solute atoms and equilibrated to 1 atmosphere of pressure for an additional 50 ps. Production dynamics were conducted for $\sim$16 ns. After discarding the first $\sim$4 ns, the free energies of binding were determined from the average over 12 snapshots (1 per ns), using the MMPBSA python scripts in AMBER 11 with the ff99SBNMR forcefield (33), a Poisson-Boltzmann implicit solvent model, and entropy refinement using the normal mode approximation (35).

**RESULTS**

**NMR Spectra for $\Delta$E81-RAP80-tUIM**—The superposition of $^1$H-$^15$N HSQC NMR spectra for wild type and $\Delta$E81-RAP80-tUIM is shown in Fig. 1. The spectra reveal large chemical shift changes near the site of the deletion mutation, with smaller or insignificant chemical shift changes in the remaining N- and C-terminal UIM domains.

**NMR-monitored Titrations for the Interaction of $\Delta$E81-RAP80-tUIM with Ub, and Tandem Ub$_2$**—The interaction between $\Delta$E81-RAP80-tUIM and Ub or tandem Ub$_2$ chains was analyzed using NMR-monitored titrations. $[^1$H-$^15$N]-$\Delta$E81-RAP80-tUIM was titrated with unlabeled Ub (Fig. 2). For a Ub:$\Delta$E81-RAP80-tUIM ratio of 17:1, substantial chemical shift changes are localized to the C-UIM (Fig. 2a). Representative chemical shift changes for residue Glu-82 during titration with monoUb are shown in Fig. 2b. The changes are linear, small in magnitude, and not accompanied by substantial line broadening, indicative of weak 1:1 binding with fast kinetics ($k_{off} > 10,000$ s$^{-1}$). For the C-UIM, representative chemical shift changes for Ala-115 upon titration with Ub are linear and large in magnitude, indicating a 1:1 binding interaction that is substantially stronger than that for the N-UIM (Fig. 2c). As in the case of the N-UIM, lack of substantial line broadening is indicative of fast binding kinetics. The NMR-monitored titration was analyzed using a binding model wherein the N- and C-UIMs bind Ub independently with separate dissociation constants, $K_{D,N}$ and $K_{D,C}$, respectively (Fig. 2d and Equation 1). Representative fits of the chemical shift changes for residue Ser-117 from the C-UIM are shown in Fig. 2e and remain similar to wild type, with $K_{D,C} = 590 \pm 80$ μM. In contrast, Ub
binding to the N-UIM is significantly impaired with \( K_{D,N} \) increasing \( \sim 20 \)-fold to 24 \( \pm \) 8 mM (Fig. 2f).

The binding of \( \Delta E81 \)-RAP80-tUIM to tandem Ub\(_2\) chains was assessed by monitoring chemical shift changes for [\( ^{1-13}C,^{15}N \)]-\( \Delta E81 \)-RAP80-tUIM upon titration with unlabeled Ub\(_2\) (Fig. 3). As in the case of binding to monoUb, large chemical shift changes are confined to the C-UIM for \( \Delta E81 \)-RAP80-tUIM:Ub\(_2\) ratios of 1 to 9 (Fig. 3a). Representative spectral changes for both UIMs (Fig. 3, b and c) are similar to the results for titration with monoUb. The chemical shift changes within the N-UIM are linear, small, and consistent with fast kinetics, whereas those for the C-UIM are linear, but large in magnitude, and indicative of fast binding kinetics. The titration was analyzed using a binding model lacking multivalent effects, wherein the different Ub molecules in tandem Ub\(_2\) bound independently to the individual UIMs in \( \Delta E81 \)-RAP80-tUIM with separate dissociation constants, \( K_{D,N} \) and \( K_{D,C} \) (Equation 2). Representative fits of the chemical shift changes upon addition of Ub\(_2\) to [\( ^{1-13}C,^{15}N \)]-\( \Delta E81 \)-RAP80-tUIM are shown in Fig. 3, e and f. For Ub\(_2\), binding, \( K_{D,N} \) is 8 \( \pm \) 2 mM, modestly stronger than binding of Ub, indicative of severe impairment in Ub recognition compared with wild type. The value for \( K_{D,C} \) remains the same as that observed for binding of Ub, 700 \( \pm \) 122 \( \mu \)M. The slightly enhanced affinity observed for \( K_{D,N} \) for Ub\(_2\) compared with Ub binding likely reflects the presence of some residual multivalency when the C-terminal UIM is bound.

**NMR Chemical Shifts Indicate That the N-UIM Helix N-cap Is Maintained in \( \Delta E81 \)-RAP80-tUIM**—The N-cap is a common structural motif that stabilizes the N termini of \( \alpha \)-helices. The N-cap motif possesses an NMR signature that is defined by an upfield shift for the random coil \( ^{13}C_\alpha \) value of the capping residue and a structural signature that includes main chain \( \phi \) and \( \psi \) dihedral angles of \( \sim -94^\circ \) and +167\(^\circ \), respectively (36, 37). The \( ^{13}C_\alpha \) chemical shift values for the N-terminal residues from the N-UIM show the characteristic signature for a helix N-cap for both wild type and \( \Delta E81 \)-RAP80-tUIM (Fig. 4a). Using \( ^1H^N, ^1H_\alpha, ^{13}C_\alpha, ^{13}C_\beta, ^{13}C_\gamma, ^{13}CO \) (wild type), and \( ^{15}N \) chemical shifts, quantitative values for the main chain \( \phi \) and \( \psi \) angles were derived from the TALOS-N program, and helix capping motifs were predicted from the MICS program. The calculated main chain dihedral angles indicate the presence of an N-UIM helix N-cap for both wild type and \( \Delta E81 \)-RAP80-tUIM (Table 1 and supplemental Tables 1 and 2). In addition, residue Thr-80 is predicted to be in an N-cap conformation with 90 and 80% probability for wild type and \( \Delta E81 \)-RAP80-tUIM, respectively (supplemental Tables 3 and 4).

**NMR Structures for Wild Type and \( \Delta E81 \)-RAP80-tUIM**—In addition to identifying a helix N-cap for the N-UIM, the quantitative values for the main chain \( \phi \) and \( \psi \) angles from the TALOS-N program indicate that the structure of the N- and C-UIMs from wild type and \( \Delta E81 \)-RAP80-tUIM are \( \alpha \)-helical. Additionally, the TALOS-N program predicts that a number of side chains within the helices adopt the expected \( \chi^1 \) angles (typ-
Delta E81 RAP80 Abrogates Multivalent Binding of Polyubiquitin

FIGURE 3. Titration of Delta E81-RAP80-tUIM with tandem Ub2. a, maximum chemical shift changes for the interaction of Ub2 with Delta E81-RAP80-tUIM. b and c, representative regions from two-dimensional 1H-15N HSQC NMR spectra from the N-UIM (b) and C-UIM (c) from Delta E81-RAP80-tUIM titrated with unlabeled Ub2. d, binding model used to analyze the interaction of Delta E81-RAP80 with Ub2 (Equation 2). e, fits of chemical shift perturbation data to binding models for RAP80-tUIM binding to tandem Ub2. Chemical shift changes for Ser-117 within the C-UIM are indicated on the vertical axis, and concentrations of Delta E81-RAP80-tUIM and unlabeled Ub2 titrant are indicated on the horizontal axis. Experimentally determined chemical shift changes are shown as points, and the best fits to the binding isotherms are shown as surfaces. f, chemical shift changes for Lys-90 within the N-UIM. Additional details are as in e.

Structural statistics for the wild type and Delta E81 mutant structures calculated on the basis of NOE and TALOS-derived dihedral angle restraints are given in Table 2. Importantly, the chi angle for Thr-80 for wild type and Delta E81-RAP80-tUIM predominantly adopts the expected +60° rotamer for the N-cap conformation, allowing the side chain hydroxyl to hydrogen bond with the main chain amide of the N+3 residue, or Glu-83 and Gln-84 for wild type and Delta E81, respectively. The TALOS main chain dihedral angles, and distance restraints derived from 15N NOESY HSQC NMR spectra, were used to calculate structures for the N-terminal UIM for wild type and Delta E81-RAP80 (Fig. 4, b–d). The NMR-derived structures indicate that the N terminus from the N-UIM for Delta E81-RAP80 undergoes a structural frameshift, wherein Glu-81 in wild type is replaced by Thr-80 in the Delta E81 mutant.

Main Chain Dynamics for Wild Type and Delta E81-RAP80-tUIM from 15N Relaxation Measurements—The temperature dependence of 1H, chemical shifts indicates that, in general, the N-UIM and C-UIM alpha-helices retain stability for Delta E81 compared with wild type RAP80, with ~70% and >90% helical content at the centers of the alpha-helices at 25 and 5 °C, respectively (residue 89, N-UIM; residue 113, C-UIM) for wild type and Delta E81 (Table 3 and Fig. 5). However, near the site of the deletion mutation (residue 85), there is a decrease in stability, with a Tm -13 ± 6 K, which corresponds to ~10 and 20% less helical content at 5 and 25 °C, respectively, for residue Phe-85 of Delta E81 near the N terminus of the N-UIM compared with wild type (Fig. 5). This modest destabilization at the N terminus is
consistent with results from quantitative chemical shift analysis at 5 °C using the TALOS-N program that indicates Thr-80 has ~10% lower probability of adopting the N-cap conformation for ΔE81 compared with wild type. Assuming that Ub binds

TABLE 1
Main chain dihedral angles for the N-cap motif in wild type and ΔE81-RAP80 N-UIM α-helices from NMR chemical shifts

| Residue | Wild type φ(°) | Wild type ψ(°) | ΔE81 φ(°) | ΔE81 ψ(°) |
|---------|----------------|----------------|------------|------------|
| Met-79  | -77 ± 11       | 141 ± 10       | -71 ± 8    | 141 ± 10   |
| Thr-80  | -72 ± 5        | 162 ± 4        | -74 ± 6    | 161 ± 6    |
| Glu-81  | -58 ± 4        | -40 ± 4        | -59 ± 4    | -37 ± 6    |
| Glu-82  | -67 ± 4        | -40 ± 4        | -59 ± 4    | -37 ± 6    |

TABLE 2
Structural statistics for 20 NMR-derived structures for wild type and ΔE81-RAP80-tUIM

|                  | Wild type RAP80-tUIM | ΔE81 RAP80-tUIM |
|------------------|----------------------|-----------------|
| Distance restraints |                      |                 |
| Total             | 65                   | 120             |
| Intraresidue      | 24                   | 56              |
| Sequential (|i-j| = 1) | 40       | 57              |
| Medium (2 ≤ |i-j| ≤ 4) | 1        | 7               |
| Long (|i-j| ≥ 5) | 0        | 0               |
| Dihedral restraints violation | 57 φ, 57 ψ, 15 χ | 56 φ, 56 ψ, 14 χ |
| Restraints violation |                      |                 |
| Distance of > 0.5 Å | 0                   | 5               |
| Dihedral of >5° | 8                    | 8               |
| φ/ψ in the most favored region (%) | 94.2       | 93.7            |
only the α-helical state of the UIM (conformational selection), the experimentally determined dissociation constants are larger than expected. Using Equations 3 and 4 in reference (8), the dissociation constant for binding to a purely α-helical state can be derived from the experimental values of $K_{DN}$ and $K_{DC}$ using

$$K_D = K_D^{exp} \times f_H,$$

(Eq. 3)

where $K_D^{exp}$ is the experimental, or apparent, $K_D$ value for the N- or C-UIM ($K_{DN}$ or $K_{DC}$), and $f_H$ is the fraction of α-helix.

**Stability Calculations for the Ub-RAP80 N-UIM Interaction**—From the MD simulations for the complex between wild type RAP80 and the N-UIM and Ub (Fig. 6a), MMPBSA calculations using AMBER 11 give a favorable free energy of interaction for wild type RAP80 of $\sim 19 \pm 4$ kcal/mol. The structural frameshift for the ΔE81-RAP80 N-UIM causes residues Met-79 and Thr-80 to rotate 90° about the helix axis. Thus, for the N-UIM of ΔE81 in the N-cap conformation, the side chain from Met-79 will sterically clash with residues 70–73 from Ub at the N-UIM-Ub interface. However, when residues Met-79 and Thr-80 adopt α-helical conformations, the side chain from Met-79 at the N terminus of the N-UIM can be accommodated. Using the MD simulations as reasonable models for the ΔE81 N-UIM-Ub interaction gives a free energy of interaction of $\sim 9 \pm 4$ kcal/mol, leading to an unfavorable change in the free energy of interaction for the mutant with ΔΔG (mutant-wild type) of $+ 10 \pm 5$ kcal/mol. The pairwise energy decomposition with respect to van der Waals, electrostatic, and polar solvation terms is shown in Fig. 6b and demonstrates the unfavorable changes in the energetics of binding between the C terminus of Ub and the N terminus of the N-UIM as a result of the ΔE81 mutation. To facilitate comparison with the experimental values for $K_{DN}$, the experimental free energy of binding was calculated according to

$$\Delta G = RT \ln(K_D),$$

(Eq. 4)

where $R$ is the gas constant (1.987 × 10$^{-3}$ kcal K$^{-1}$ mol$^{-1}$), $T$ is temperature (K), and $K_D$ (molar$^{-1}$) is given by Equation 3, thus including the effects of conformational selection.

**DISCUSSION**

Two-dimensional 1H-15N NMR spectra for wild type and ΔE81-RAP80-tUIM indicate that the overall α-helical structure of the UIM domains remains intact (Fig. 1). As expected, the cross-peak for Glu-81 vanishes and is essentially replaced by Glu-82 in the deletion mutant. Interestingly, the cross-peaks for Glu-83 and Gln-84 for ΔE81-RAP80 shift to the approximate locations for residues Glu-82 and Gln-83, respectively, in the wild type protein. Residue Thr-80, adjacent to the deletion site, also experiences a large chemical shift change. These main chain amide chemical shift changes indicate that the structure of the C-UIM remains unchanged, whereas the N-UIM undergoes a structural frameshift, maintaining helical secondary structure. Consistent with these results, main chain and side chain dihedral angles derived from NMR chemical shifts show that the N-cap structure is maintained in ΔE81-RAP80-tUIM through the structural frameshift, with Glu-82 in ΔE81-RAP80-tUIM taking the role of Glu-81 in wild type RAP80-tUIM. The structural implications for the frameshift are evident in the NMR structures for the N-cap in ΔE81 and wild type RAP80-tUIM (Fig. 4b). For ΔE81-RAP80-tUIM, residues Met-79 and Thr-80 translate $\sim 1.5$ Å along the N-UIM helix axis toward the C terminus and rotate about the helix axis $-90°$. In the context of the interaction between Ub and the N-UIM, the structural frameshift causes Glu-81 in wild type RAP80 to be replaced by Thr-80 in the ΔE81 mutant, and the N-cap can no longer be accommodated at the Ub-UIM interface due to steric clashes with the side chain of Met-79. However, if residues Met-79 and Thr-80 adopt an α-helical conformation, the side chain of Met-79 moves away from the interface, which can facilitate binding, but favorable electrostatic interactions between Glu-81 from RAP80 and residues Arg-42, Arg-72, Leu-73, and Arg-74 from Ub are disrupted and replaced by interactions between the shorter side chain of Thr-80 and Arg-42, Arg-72, Leu-73, and Arg-74 from Ub (Fig. 6a). It is likely that the α-helical conformation at residues Met-79 and Thr-80 can be adopted to some extent, given the flexibility at these residues observed through 15N NMR $S^2$ values (Fig. 4, e–g) as well as

| Residue | $T_m$ (°C) | $\Delta T$ (°C) | $T_m$ (°C) | $\Delta T$ (°C) |
|---------|------------|----------------|------------|----------------|
| 85      | 309 ± 4    | 46 ± 10        | 296 ± 4    | 41 ± 9         |
| 89      | 302 ± 2    | 15 ± 3         | 305 ± 3    | 20 ± 4         |
| 113     | 316 ± 6    | 21 ± 4         | 312 ± 5    | 17 ± 4         |

**FIGURE 5. Temperature dependence for $^1$H, chemical shifts for residues Phe-85, Leu-89, and Ala-113 in wild type (red) and ΔE81-RAP80-tUIM (blue). $f_c$ indicates fraction in the random coil conformation.**
random coil index chemical shift $S^2$ values (38), determined through the TALOS-N program (supplemental Tables 1 and 2).

NMR-monitored $^{1}H$–$^{15}N$ chemical shift titrations of Ub into wild type and $\Delta E81$ RAP80-tUIM reveal that loss of a single N-terminal glutamic acid residue results in near abolishment of Ub binding for the N-UIM. The 20-fold increase in $K_{D,N}$ for binding of Ub to $\Delta E81$ RAP80 N-UIM corresponds to a 2.2 kcal/mol loss in binding affinity (Equations 3 and 4). Similarly, the NMR titration results for binding of Ub$_2$ to $\Delta E81$-RAP80 show a substantial impairment in Ub recognition for the N-UIM, with a 12-fold increase in $K_{D,N}$ or a loss of 1.5 kcal/mol in binding affinity. Taken together, the results for Ub and Ub$_2$ binding to $\Delta E81$-RAP80-tUIM indicate that this single residue mutation leads to abolishment of multivalent binding compared with wild type RAP80-tUIM. Interestingly, stability calculations from MD simulations for the free energy of interaction between wild type or $\Delta E81$ RAP80 N-UIM and Ub are in agreement with the experimental values determined from NMR, with the calculated $\Delta \Delta G$ of $+10 \pm 5$ kcal/mol comparing favorably with the experimental $\Delta \Delta G$ of $+2$ kcal/mol. Importantly, these calculations suggest that disruption of the interaction between RAP80 and polyubiquitin is mainly due to the loss of key electrostatic interactions between Glu-81 within the N-UIM and residues Arg-42, and 72–74 from the C terminus of Ub (Fig. 6).

In further support of a predominantly structural basis for the defective function of $\Delta E81$-RAP80, the stabilities of the $\alpha$-helices for the N- and C-UIMs of the deletion mutant remain similar to wild type, with ~70% $\alpha$-helical content at 25 °C. A modest destabilization near the N terminus of the N-UIM is observed as a 20% loss in helical structure for residue F85 (Table 3). Using Equations 3 and 4, this difference in helical content indicates that the destabilization results in a modest loss of 0.2 kcal/mol in binding energy. In other words, taking into account this loss in inherent helicity, and assuming $K_{D,N} = K_{D,C}$ (8), the $K_{D,N}$ for Ub binding to $\Delta E81$ would be expected to increase from 590 to 767 $\mu M$, rather than to the experimentally measured value of 24 $\mu M$.

In light of intense efforts to investigate the putative association between defects within binding partners for BRCA1/BRCA2 tumor suppressors and a hereditary disposition to breast cancer, the results provide a compelling molecular basis for genomic instability resulting from a single amino acid deletion in RAP80. This defect impedes polyUb recognition, is likely responsible for aberrant targeting of BRCA1 BRCA2 to DNA damage foci, and is potentially involved in the pathogenesis of disease.

Considering the broad, vital role that multivalent interactions play in human biology (39) and the variety of different UIM-Ub pairs (40, 41), it is of interest that near complete abolishment of multivalent binding is implicated in the development of cancer; that is, binding of polyUb by $\Delta E81$-RAP80 is not abolished. The C-UIM binds Ub normally, whereas abrogated binding of Ub by the N-UIM is responsible for the near total loss of multivalent recognition.

Acknowledgments—We thank the Canadian National High Field NMR Centre (NANUC) for assistance and for use of the facilities. Operation of NANUC is funded by the University of Alberta. Computational studies were enabled by the use of computing resources provided by WestGrid and Compute/Calcul Canada.
ΔE81 RAP80 Abrogates Multivalont Binding of Polyubiquitin

Escherichia coli ribonuclease HI: correlations with structure and function in an active enzyme. J. Mol. Biol. 234, 144–163

19. Spyracopoulos, L. (2006) A suite of Mathematica notebooks for the analysis of protein main chain 13C NMR relaxation data. J. Biomol. NMR 36, 215–224

20. Kay, L. E., Xu, G. Y., and Yamazaki, T. (1994) Enhanced sensitivity triple resonance spectroscopy with minimal H2O saturation. J. Magn. Reson. A 109, 129–133

21. Zhang, O., Kay, L. E., Olivier, J. P., and Forman-Kay, J. D. (1994) Backbone 1H and 13C resonance assignments of the N-terminal SH3 domain of drk in folded and unfolded states using enhanced-sensitivity pulsed field gradient NMR techniques. J. Biomol. NMR 4, 845–858

22. Shen, Y., and Bax, A. (2013) Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. J. Biomol. NMR 56, 227–241

23. Shen, Y., and Bax, A. (2012) Identification of helix capping and β-turn motifs from NMR chemical shifts. J. Biomol. NMR 52, 211–232

24. Schwieters, C. D., Kuszewski, J. J., Tjandra, N., and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. J. Magn. Reson. 160, 65–73

25. Wolfram, S. (2003) The Mathematica Book, 5th Ed., Wolfram Media, Champaign, IL

26. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, I., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293

27. Nesmelya, I., Krushelnitsky, A., Iñayutin, D., Blanco, F., Ramirez-Alvarado, M., Daragan, V. A., Serrano, L., and Mayo, K. H. (2001) Conformational exchange on the microsecond time scale in α-helix and β-hairpin peptides measured by 13C NMR transverse relaxation. Biochemistry 40, 2844–2853

28. Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. J. Am. Chem. Soc. 114, 10663–10665

29. Markin, C. J., and Spyracopoulos, L. (2012) Increased precision for analysis of protein-ligand dissociation constants determined from chemical shift titrations. J. Biomol. NMR 53, 125–138

30. Markin, C. J., and Spyracopoulos, L. (2012) Accuracy and precision of protein-ligand interaction kinetics determined from chemical shift titrations. J. Biomol. NMR 54, 355–376

31. Case, D. A., Darden, T. A., Cheatham, T. E., Simmerling, C., Wang, J., Duke, E. R., Luo, R., Walker, R. C., Zhang, W., Merz, K. M., Roberts, B., Wang, B., Hayik, S., Roitberg, A., Seabra, G., Kolossváry, I., Wong, K. F., Paesani, F., Vanicek, I., Liu, J., Xu, W., Brozell, S. R., Steinbrecher, T., Gohlke, H., Cai, Q., Yeh, X., Wang, J., Hsieh, M.-J., Cui, G., Roe, D. R., Mathews, D. H., Seetin, M. G., Sagui, C., Babin, V., Luchko, T., Guerout, S., Kovalenko, A., and Kollman, P. A. (2010) AMBER 11, University of California, San Francisco

32. Morgan, J., Simmerling, C., McCammon, J. A., Case, D. A., and Onufriev, A. (2007) Generalized Born model with a simple, robust molecular volume correction. J. Chem. Theory Comput. 3, 156–169

33. Li, D. W., and Brüschweiler, R. (2010) NMR-based protein potentials. Angew. Chem. Int. Ed. Engl. 49, 6778–6780

34. Götz, A. W., Williamson, M. J., Xu, D., Poole, D., Le Grand, S., and Walker, R. C. (2012) Routine microsecond molecular dynamics simulations with AMBER on GPUs. I. Generalized Born. J. Chem. Theory Comput. 8, 1542–1555

35. Kollman, P. A., Massa, I., Reyes, C., Kuhn, B., Huo, S., Chong, L., Lee, M., Duan, Y., Wang, W., Donini, O., Cieplak, P., Srivinasa, J., Case, D. A., and Cheatham, T. E., 3rd (2000) Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. Acc. Chem. Res. 33, 889–897

36. Gronenborn, A. M., and Clore, G. M. (1994) Identification of N-terminal helix capping boxes by means of 13C chemical shifts. J. Biomol. NMR 4, 455–458

37. Seale, J. W., Srivinasa, R., and Rose, G. D. (1994) Sequence determinants of the capping box, a stabilizing motif at the N termini of α-helices. Protein Sci. 3, 1741–1745

38. Berjanskii, M. V., and Wishart, D. S. (2005) A simple method to predict
protein flexibility using secondary chemical shifts. J. Am. Chem. Soc. 127, 14970–14971

39. Mammen, M., Choi, S. K., and Whitesides, G. M. (1998) Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. Angew. Chem. Int. Ed. Engl. 37, 2755–2794

40. Fisher, R. D., Wang, B., Alam, S. L., Higginson, D. S., Robinson, H., Sundquist, W. I., and Hill, C. P. (2003) Structure and ubiquitin binding of the ubiquitin-interacting motif. J. Biol. Chem. 278, 28976–28984

41. Safadi, S. S., and Shaw, G. S. (2010) Differential interaction of the E3 ligase parkin with the proteasomal subunit S5a and the endocytic protein Eps15. J. Biol. Chem. 285, 1424–1434