Supporting Information

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SI Materials and Methods

Materials. Deuterium oxide was obtained from Apollo Scientific. Sodium trimethylsilyl [2,2,3,3-2H4] propionate (TSP), imidazole, Trizma base, nickel resin, and thermolysin from Bachem Biosciences (Spectrum Laboratories). Amicon centrifugal devices with a cutoff molecular weight of 3,000 Da were from Millipore.

Protein Expression and Purification. Wild-type NUPR1 and C-RING1B were produced and purified in LB media as described (17, 30) in C41 cells. For 15N-labeled NUPR1 and C-RING1B, the same protocol as in LB was used, containing 15NH4Cl as the sole source of nitrogen. RYBP was expressed in LB as described (19).

We chose to mutate the positions, namely Ala33 and Thr68, based on our previous MD results of the isolated wild-type protein (38), where those residues are involved in hydrophobic clusters, acting as small “tongs” ready to interact with other regions (and/or molecules). We decided to mutate both residues to Gln to disrupt any possible contact with the rest of the polypeptide chain. The three mutants (a single at each position and a double one) were obtained by using the Quick Site Directed Mutagenesis kit (Stratagene), with the following pair of primers for Ala33Gln mutation: forward: tgcacctctagctgcattcctcgtgcc and reverse: ctcctccgaggtaggaatgctccccgaggctatagaggtcag. The following primers were used for the Thr68Gln mutation: forward: ggcaagaggaagactggtgcagaagctgcagaattcaga and reverse: cctccgaggtaggaatgctccccgaggctatagaggtcag. Mutations were double-checked by DNA sequencing at GATC Biotech.

Expression of the three mutants was carried out initially under the same conditions as for the wild type. However, we observed that their expression was basically different from that of the wild type. The Thr68Gln was expressed nearly at the same amount as the wild-type NUPR1 (8 mg/L of culture versus 12–12 mg/L culture); however, the expression of Ala33Gln/Thr68Gln was substantially decreased (2–3 mg/mL of culture) and we did not obtain any expression of Ala33Gln. In the latter mutant, we tried several strains (C41, C43, and BL21) with different temperatures (25, 30, and 37 °C) after induction with several final amounts of isopropyl β-D-1-thiogalactopyranoside (0.5 and 1 mM); in all cases, we were unsuccessful. A possible explanation is that Ala33 maintains a local compactness of the protein that favors its expression; loss of this compactness of the protein that favors its expression; loss of this compactness in the Ala33Gln mutant is partially (but not fully) prevented by the concomitant Thr68Gln mutation. Although indirectly, this result further supports the presence of long-range interactions between the two regions, which has been suggested by in silico results (38) and biophysical characterization of the electrophoresis of the wild-type NUPR1 (41). Given the poor expression of the mutants in rich media we did not attempt to express them in minimal one. The mutants were purified in rich media following the same protocol as in the wild type (30).

CRISPR/Cas9n Clone Development. Cells were seeded in 12-well plates and transfected with 1 μg of NUPR1 double nickase plasmid or double control nickase plasmid (Santa Cruz Biotechnology), delivered with 2 μL of Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) per well, following the manufacturer’s protocol. Transfected cell selection was performed with puromycin and after 72 h and isolation of single-cell colonies was done. Complete allelic knockouts were confirmed by Sanger sequencing.

CRISPR/Cas9n clones were developed to test whether the interaction between the two proteins is specific, by using a PLA assay. Control clones, with wild-type NUPR1 expression, and knockout clones, with a complete lack of expression of the protein NUPR1, have been used for this purpose. In these experiments, red fluorescence corresponds to the PLA-positive signal, and it indicates that the two molecules belong to the same protein complex. The objective of the assay was to demonstrate that the positive interaction that we showed in the PLA experiments in wild-type cells (red fluorescence) corresponds exclusively to the interaction between NUPR1 and RING1B, and it is not due to any possible interaction with other unstructured proteins with similar amino acid composition or to an uncommon unspecific binding of the primary antibodies. For this reason, we used wild-type and knockout clones, whose sole difference is the presence or the absence of one of the proteins of the complex under study (in this case, NUPR1). If the interaction between the proteins is specific, no positive signal should be observed in the knockout clones. However, if the interaction observed in the wild-type cells corresponded to an unspecific binding with other proteins, red fluorescence would be detected.

Basis of the PLA Approach. Proximity ligation assay is a technology that allows us to visualize protein interactions with an easy-to-execute immunodetection protocol that is similar to traditional immunofluorescence. Briefly, the fixed samples are incubated with two primary antibodies from two different species and each one will bind one of the proteins of the complex under study. Then, secondary antibodies conjugated with oligonucleotides (named PLA probe MINUS and PLA probe PLUS) are added to the reaction and incubated. After that, the ligation solution, consisting of two oligonucleotides and ligation enzyme, is added to the samples. The oligonucleotides will hybridize to the two PLA probes and join to form a closed circle if they are in close proximity. Finally, the amplification solution, consisting of nucleotides and fluorescently labeled oligonucleotides, is added together with polymerase enzyme. The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a repeated sequence product. At the microscope, we are able to visualize red fluorescent spots because fluorescently labeled oligonucleotides will hybridize to the RCA product.

We have chosen this technique because it combines the specificity of two primary antibodies (already validated in one of our laboratories by immunofluorescence) and two secondary antibodies, with the sensitivity afforded by RCA to detect protein interaction in cells. The pair of oligonucleotide-labeled secondary antibodies generates an amplified signal only when the probes are in close proximity (<40 Å).

Moreover, the PLA approach allows us to test in cellulo the results obtained in vitro, providing valuable information about the interaction of wild-type or mutant proteins on a more realistic model. Therefore, it is possible to demonstrate whether these proteins interact within a physiological context, in the presence of many more factors compared with the in vitro assays (where only the two proteins are present in solution).

Immunofluorescence. HeLa cells were seeded in six-well plates on coverslips and transfected as previously described with NUPR1-FLAG and the different mutants. After fixation, cells were incubated with a mouse anti-FLAG (Sigma-Aldrich) primary antibody. After washing out the first antibody, cells were incubated with Alexa Fluor 488-labeled secondary antibody. Image acquisition of
Alexa Fluor 488-derived fluorescence and DAPI staining was performed using a Nikon Eclipse 90i fluorescence microscope.

**Proteolysis by Thermolysin.** Thermolysin was dissolved in Tris (50 mM, pH 7) containing NaCl (150 mM) and CaCl \(_2\) (100 mM), and stored at −80 °C; its concentration was measured by using \(e_{280\text{nm}} = 66,086 \text{ M}^{-1}\cdot\text{cm}^{-1}\). Proteolysis was performed at 25 °C in Tris 50 mM, NaCl 150 mM, pH 7, using 10 μM of wild-type NUPR1 and 0–90 μM of C-RING1B (protomer units); the reaction was initiated by the addition of thermolysin (at a final concentration of 10 nM and 10 mM CaCl \(_2\)) in a final volume of 200 μL. At different times, 20 μL of the reaction mixture were withdrawn and proteolysis was quenched by addition of 5 μL of EDTA, 100 mM, pH 8, mixed with Laemmli’s buffer and denatured at 95 °C for 5 min. Samples were resolved in 16% acrylamide Tris-Tricine PAGE gels, stained with Coomassie Blue, and densitometered using ImageJ (https://imagej.nih.gov/ij/). After normalization of the intensities by using that of the control sample in the absence of thermolysin, the intensity (I) versus time (t) curves were analyzed using a single exponential function:

\[
I = I_0 \exp^{-kt},
\]

where \(k\) is the proteolysis rate constant and \(I_0\) the initial intensity.

To estimate the \(K_d\) for the interaction between the wild-type NUPR1 and C-RING1B we considered first the initial cleavage of NUPR1 (where \(N\) is the intact NUPR1 and \(F\) is the proteolyzed products) by thermolysin to follow a one-step model with first-order kinetics (\(k\) is the rate constant):\[N\xrightarrow{k} F,\]

for which the rate law is

\[
\frac{d[N]}{dt} = -k[N].
\]

In the presence of C-RING1B, the cleavage of wild-type NUPR1 is considered to follow first-order kinetics, with a rate constant \(k_{\text{app}}\) (whose value depends on [C-RING1B]): \[NL\xrightarrow{k_{\text{app}}} F,\]

where \(NL\) is the 1:1 C-RING1B/NUPR1 complex. In this case, the rate law is

\[
\frac{d[NL]}{dt} = -k_{\text{app}}[NL].
\]

Assuming that wild-type NUPR1 in complex with C-RING1B is largely protected toward proteolysis, we can use the following model: \[NL \xrightarrow{k_{\text{app}}} \text{F} + \text{L},\]

where the rate law is

\[
\frac{d[NL]}{dt} = -k[N].
\]

Because \(K_d = \frac{[N][L]}{[NL]}\), we can substitute \([N]\) into Eq. S4, and combine it with Eq. S3 to yield

\[
\frac{d[NL]}{dt} = k_{\text{app}}[N]/[L] = k_{\text{app}}.
\]

This leads to

\[
\ln(k_{\text{app}}) - \ln(k) = \ln(K_d) - \ln([L]).
\]

Therefore, a plot of \(\ln(k_{\text{app}}) - \ln(k)\) versus \(\ln([\text{C-RING1B}])\) yields a slope of \(-1\) (if the stoichiometry of the interaction is 1:1) and a y axis intercept equals to \(\ln(K_d)\).
Fig. S1. Fluorescence characterization of the binding between C-RING1B and NUPR1 mutants. (A) Changes in the intensity at 330 nm with increasing amounts of Thr68Gln after excitation at 295 nm. The line through the data is the fitting to Eq. 1, yielding a $K_d$ of $9 \pm 2 \mu M$. (B) Changes in the intensity at 330 nm with increasing amounts of Ala33Gln/Thr68Gln after excitation at 295 nm. The line through the data is the fitting to Eq. 1, yielding a $K_d$ of $14 \pm 5 \mu M$. a.u., arbitrary units.
Fig. S2. Far-UV CD characterization of the binding between C-RING1B and NUPR1 mutants by using steady-state spectra. (A) Far-UV CD spectra of the complex (black line) and that obtained by the addition of the spectra of each protein (red line) in the mutant Thr68Gln. (B) Far-UV CD spectra of the complex (black line) and that obtained by the addition of the spectra of each protein (red line) in the mutant Ala33Gln/Thr68Gln. mdeg, millidegrees.
Fig. S3. Far-UV CD characterization of the binding between C-RING1B and NUPR1 mutants by using thermal denaturations. Thermal denaturations followed by the changes in ellipticity at 222 nm of the complex with C-RING1B of the two NUPR1 mutants and that of isolated C-RING1B. To allow for a comparison the traces of the thermal denaturations have been scaled up, and then the y axis does not represent the measured ellipticity. mdeg, millidegrees.

Fig. S4. Interaction of RYBP and C-RING1B mapped by HSQC spectra of C-RING1B. Overlay of spectra of C-RING1B in the presence of different amounts of RYBP at 0 (black), 20 (red), 40 (blue), and 60 (green) μM (all in protomer units).
Fig. S5. Fitting of the absolute intensity (corrected by the value of the receiver gain in each titrating point) of the cross-peak of C-RING1B, appearing at 9.40 ppm to Eq. 1 for the Thr68Gln (A) and Ala33Gln/Thr68Gln (B) mutants. The $K_d$ for Thr68Q was $65 \pm 15 \mu M$ and $115 \pm 85 \mu M$ for the double mutant. a.u., arbitrary units.

Fig. S6. Hydropathy plot of C-RING1B. Selected protein residues are labeled, and the most hydrophobic segments are highlighted in red. The hydrophobicity scale of Kyte-Doolittle was used, by considering a window size of nine consecutive residues.
Fig. S7. Docking poses of selected portions of mutated NUPR1 sequence to C-RING1B. Fragments of NUPR1 mutants Ala33Gln and Thr68Gln centered on (A) Gln33 and (B) Gln68. Fragment length for NUPR1 is either seven (cyan), five (orange), or three (purple) residues, with Cα atoms of the central amino acid represented as spheres; clusters Cl-1, Cl-2, and Cl-4 are also labeled. Regions with high hydrophobicity in C-RING1B are represented as transparent gray surfaces. Figures were produced with VMD (66).

Fig. S8. Specific interaction between C-RING1B and wild-type NUPR1. In situ PLA in CRISPR/Cas9n control cells (with wild-type NUPR1) and CRISPR/Cas9n NUPR1 knockout cells. Mouse anti-HA and rabbit anti-NUPR1 were used to detect the protein interaction by PLA. DAPI staining was used to detect nuclei and combined with the PLA fluorescence in the merged panel. (Magnification: 40×.)
Fig. S9. Immunofluorescence in HeLa cells transfected with wild-type and NUPR1 mutants. Mouse anti-FLAG primary antibody and Alexa 488-labeled goat anti-mouse secondary antibody were used to reveal the localization of the protein. DAPI staining was used to detect nuclei and it was combined with the Alexa 488 fluorescence in the merged panel. (Magnification: 20×.)

Fig. S10. In situ PLA between C-RING1B and NUPR1 mutants. Mouse anti-FLAG and rabbit anti-HA were used to reveal the interaction between the proteins. Nuclei staining was performed with DAPI and combined with the PLA fluorescence in the merged images. (Magnification: 10×.)