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Using Affinity Pulldown Assays to Study Protein-Protein Interactions of human NEIL1 Glycosylase and the Checkpoint Protein RAD9-RAD1-HUS1 (9-1-1) Complex

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Abstract

Affinity pulldown is a powerful technique to discover novel interaction partners and verify a predicted physical association between two or more proteins. Pulldown assays capture a target protein fused with an affinity tag and analyze the complexed proteins. Here, we detail methods of pulldown assays for two high-affinity peptide fusion tags, Flag tag (DYKDDDDK) and hexahistidine tag (6xHis), to study protein-protein interactions of human NEIL1 glycosylase and the checkpoint protein complex RAD9-RAD1-HUS1 (9-1-1). We uncover unique interactions between 9-1-1 and NEIL1, which suggest a possible inhibitory role of the disordered, phosphorylated C-terminal region of RAD9 in regulating NEIL1 activity in base excision repair through lack of physical association of 9-1-1 and NEIL1.

Keywords

protein-protein interactions; affinity pulldown; Flag pulldown; Ni pulldown; human NEIL1; human RAD9-RAD1-HUS1

1. Introduction

Approximately tens of thousands of oxidative/alkylated base lesions occur daily in the human genome. Most oxidative damage is efficiently repaired by base excision repair (BER) and single-stranded break repair (SSBR) to prevent mutations and maintain the genomic integrity. DNA repair pathways, including BER, is complicated by sub-pathways and alternative pathways, as well as their cross-talks with other DNA processes such as replication and transcription. These DNA transactions involve multiprotein complexes and dynamic macromolecular assemblies of both canonical proteins and non-canonical accessory factors [1]. These megadalton assemblies are highly coordinated and regulated in temporal and spatial manner, often driven by specific protein-protein interactions. To gain insights on how DNA repair machines work, it is necessary to understand the architecture and functional interactions of proteins in these machines.

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Recent development in cutting-edge techniques with increased throughput has enabled qualitative and quantitative analysis of protein-protein interactions and established protein interactome networks. The emerging findings help define the role of the interactions in complex biological processes, shape the molecular basis for human diseases that may arise from dysregulated protein-protein interactions, and facilitate mechanistic understanding and potential therapeutic discovery to human diseases. An array of genetic, cell biological, biochemical, and biophysical methodologies are available to validate, characterize, and confirm protein-protein interactions [2]. Among those methods, affinity pulldown or affinity co-purification **(**Figure 1**)** is a classical, low-to-medium throughput, but powerful technique to identify direct protein-protein interactions and functional associations of proteins by providing qualitative analysis for both stable and transient protein complexes. Specifically, affinity pulldown are : 1) robust to set up *in vitro* by mixing purified proteins of interest or using cell extracts containing co-expressed proteins from recombinant hosts, such as *E. coli* or insect cells; 2) readily adaptable for multi-protein interaction studies, in addition to binary interactions; 3) useful for mapping interaction domains and screening for interaction-defective mutations; 4) easy to adjust assay stringency to increase specificity or detect weak interactions; and 5) affordable by using common reagents and inexpensive laboratory equipment. To increase throughput of affinity pulldown, spin columns, magnetic beads and magnetic microplates are applicable options.

The checkpoint sliding clamp RAD9-RAD1-HUS1 (9-1-1) interacts with and stimulates enzymes involved in base excision repair (BER), such as NEIL1, MYH, TDG, FEN-1, and DNA LIGASE I, thus linking BER activities to checkpoint coordination [3, 4]. Here we describe a general method of pulldown assays to confirm physical associations between NEIL1 glycosylase and the heterotrimeric 9-1-1 complex. We found that NEIL1 interacts with full-length 9-1-1 and a truncated 9-1-1 (9Δ-1-1) that lacks a C-terminal tail of RAD9, when 9-1-1 proteins were expressed and purified from *E. coli* **(**Figure 2A and 2B**)**, consistent with previous reports. When 9-1-1 was expressed and purified from insect cells, surprisingly, NEIL1 does not interact with full-length 9-1-1 (Figure 2C), while retaining interaction with truncated 9Δ-1-1 (Figure 2D**)**. Although the C-terminal region of RAD9 is dispensable for interacting with NEIL1, the absence of the C-terminal RAD9 leads to hyperactivation of NEIL1 *in vivo* [5]. Our finding suggests that the disordered and phosphorylated C-terminal region of RAD9 might regulate NEIL1 activity in human cells by destabilizing or inhibiting interaction of 9-1-1 with NEIL1.

2. Materials

Prepare all reagents using deionized ultrapure water and molecular biology grade reagents. Store all reagents at 4°C, unless indicated otherwise. Filter sterilize buffers and reagents for long-term usage. Follow all waste disposal and safety regulations when disposing waste materials.

2.1. Purified proteins

Avoid repeated freezing and thawing of purified proteins. All purified proteins are stored in the presence of 5-10% glycerol in small aliquots at −80°C. Proteins tagged with an affinity tag are highlighted by bold text.

1. Untagged human NEIL1: purified from *E. coli* as described [6]
2. Flag-tagged full-length human 9-1-1 (**Flag-RAD9**–RAD1–HUS1): expressed and purified from insect cells as described [7, 8]
3. 6xHis-tagged truncated human 9Δ-1-1 lacking the C-terminal region of RAD9 (**RAD9ΔC-6xHis**–RAD1–HUS1): expressed and purified from insect cells as described [7, 8]
4. Human 6xHis-tagged full-length 9-1-1 (RAD9–**RAD1–6xHis**–HUS1) and truncated 9Δ-1-1 complex (RAD9ΔC–**RAD1–6xHis**–HUS1): expressed and purified from *E. coli* as described [9]

2.2. Buffers and affinity chromatography resins

1. Column binding buffer: 50 mM Tris.HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 50 μg/mL BSA, and 1 mM DTT
2. Column washing buffer: 50 mM Tris.HCl, pH 7.5, 300 mM NaCl, 10% glycerol, and 1 mM DTT
3. Ni elution buffer: 300 mM imidazole in the column washing buffer
4. Flag elution buffer: 200 μg/mL 3x Flag peptide in the column washing buffer
5. Glycine buffer: 100 mM Glycine, pH 3.5
6. Anti-Flag M2 resin (Sigma Millipore)
7. Ni-NTA Fast Flow resin (Qiagen)
8. 5x Lane marker reducing sample buffer: 0.3 M Tris.HC1, 5% SDS, 50% glycerol, 100 mM DTT, and proprietary pink tracking dye (Thermo Fisher)
9. 1x Tris-Glycine SDS running buffer for protein gel electrophoresis: 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3
10. 12% Tris-Glycine SDS polyacrylamide gels
11. SimplyBlue SafeStain solution (Thermo Fisher)

2.3. Equipment

1. Rotator
2. High-speed microcentrifuge
3. Heat block
4. Protein electrophoresis apparatus

3. Methods

Carry out all steps of affinity pulldown at 4°C, unless otherwise specified.

3.1 Column Equilibration

3.1.1 Column Equilibration for anti-Flag M2 column

1. Gently mix and fully resuspend the anti-Flag M2 resin. Pipette 25 μL column volume (CV) of anti-Flag M2 resin into a 1.5 mL microtube (*see* Note 1).
2. Add 1 mL of column binding buffer to the microtube to wash the resin and get rid of 50% glycerol in the resin storage medium. Use low speed centrifugation at 500 *x g* for 1 min to collect the resin by packing the resin at the bottom of the tube. Remove and discard the supernatant using a micropipette (*see* Note 2).
3. Wash one more time with column binding buffer as described in **step 2.**
4. Add 1 mL of glycine buffer to the microtube to resuspend and wash the resin. Repeat glycine buffer wash two more times (*see* Note 3).
5. Then wash three times with 1 mL of column binding buffer to equilibrate the resin.

3.1.2 Column Equilibration for Ni-NTA column

1. Gently swirl the Ni-NTA Fast Flow bottle and thoroughly resuspend the resin before use. Pipette 25 μL column volume (CV) of Ni-NTA Fast Flow resin into a 1.5 mL microtube.
2. Wash the Ni-NTA resin two times with 1 mL of distilled water to remove 20% ethanol in the resin storage medium.
3. Wash the resin three times with 1 mL of column binding buffer to equilibrate the resin.

3.2 Column Batch Binding

1. Thaw aliquots of purified proteins on ice. Sample protein load fractions (L) for each protein and save for SDS-PAGE analysis.
2. Mix purified full-length 9-1-1 or 9Δ-1-1 proteins (~10 μg) with purified NEIL1 (~ 12 μg) at 1:3 molar ratio in column binding buffer to final 200 μL. Incubate mixed proteins on ice for 1 h and gently tap the tube every 15 min to mix.

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1. Cutting the end of the micropipette tip is recommended if pipette tip opening is too narrow. Ni-NTA and Anti-Flag M2-Flag resins are stored as 50% slurry in the storage medium. Pipette double the desired column volume of the resin slurry to get the working column volume of resin. For example, pipette 50 μL of resin slurry to have the column volume of 25 μL resin for experiments. Equilibration may be done at room temperature.
2. When removing buffer from the resin after centrifugation, use a micropipette to carefully remove the supernatant and leave a small volume of buffer (15-20 μL) above the resin to minimize loss of the resin. Add buffer with force to the microtube to help dislodge and resuspend the packed resin. Then gently invert the tube several times to wash the resin. Remove the last wash completely from the resin without leaving any residual buffer.
3. Glycine washes remove protein contaminants from antibody-conjugated affinity resins, such as anti-Flag M2 resin. Skip this step if the affinity resin is not antibody-based. Anti-Flag M2 resin can be regenerated and reused by glycine washes as well. Make sure that there is no residual glycine buffer in the final wash, because leftover glycine buffer may denature proteins due to its low pH.

3. Transfer mixed proteins into the microtube containing the equilibrated column resin and tap the tube to resuspend the resin (*see* Notes 4). Incubate for 1 h while mixing gently on a rotator at 4°C (*see* Note 5).

4. Centrifuge at 4°C for 1 min at 500 *x g* to collect the resin. Remove and save the supernatant as column flow though fractions (FT) for SDS-PAGE analysis.

3.3 Column Batch Washing

1. Add 0.5 mL of column wash buffer to the microtube to resuspend and wash the resin. Pack the resin by centrifugation and discard the supernatant (wash buffer).

2. Wash the resin four more times as in **step 1**, and sample the last wash (W5) and save for SDS-PAGE analysis (*see* Note 6).

3.4 Column Batch Elution

1. Add 1 column volume (CV) of elution buffer to the resin, and incubate on ice for 15 min for anti-Flag M2 resin and 5 min for Ni-NTA resin. Pack the resin by centrifugation and collect the supernatant as elution 1, E1.

2. Repeat **Step 1** one more time and collect the supernatant as elution 2, E2 (*see* Note 7).

3.5. SDS-PAGE Analysis

1. Sample 20 μL from the affinity pulldown fractions as described in 3.2, 3.3 and 3.4, including load (L), flow through (FT), last wash (W5), and eluted fractions 1 and 2 (E1 and E2). Add 5 μL of 5x lane marker reducing sample buffer to each sample. Add 1 CV of 5x lane marker reducing sample buffer to the leftover column beads (B) (*see* Note 8). Denature all samples for 5 min at 95°C.

2. Load 20 μL of each denatured sample to 12% Tris-Glycine SDS polyacrylamide gels and electrophorese in 1x Tris-Glycine SDS running buffer at 200 V for 1 h and 15 min.

3. After electrophoresis, rinse polyacrylamide gels three times for 5 min with 100 mL of distilled water with gentle shaking. Add sufficient SimplyBlue SafeStain

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4. Always include a negative control pulldown to ensure that the complexed protein does not bind non-specifically to the targeting affinity resin that may result in false positive interactions. When feasible, perform a reciprocal pulldown using an affinity resin targeting the other protein(s) to confirm interactions.

5. Batch binding is carried out with constant mixing of the resin and proteins in the microtube. Fasten the tube to a rotator and mix gently in a 4°C cold room. Make sure that the resin and proteins are fully mixed on the rotator before beginning the 1-hour binding.

6. Remove the final wash buffer from the resin completely before adding the elution buffer. Leftover wash buffer dilutes the elution buffer and results in inefficient elution. Tap the tube to mix every 5 min during each elution step to resuspend the resin to ensure complete elution.

7. Most published pulldown assays do not elute proteins from the affinity resin, but interpret proteins bound or not bound on the resin as positive or negative interactions. We routinely carry out two runs of elution to ensure complexed proteins, when expressed and purified recombinantly, are properly folded, soluble, non-aggregated proteins to minimize false positive interactions. In addition, the ability of eluting complexed proteins from the affinity resin warrants subsequent biochemical, physical and structural analysis.

8. The 5x lane marker reducing sample buffer contains DTT as a reducing agent and is stored at −20°C for long-term stability, but SDS in the sample buffer tends to precipitate at low temperatures. Warm up the 5x sample buffer at 37°C for 5-10 min to dissolve SDS before use. Do not heat the sample buffer for more than 15 min, because DTT may get oxidized.

solution (~20 mL) to cover the polyacrylamide gel, then incubate at room temperature for 1 h with gentle shaking (*see* Note 9).

4. Discard the staining solution. Wash with 100 mL of distilled water for 1-3 h to destain the polyacrylamide gel. Perform more washes until the background of gels is clear.

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6. References

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9. A water-based, non-hazardous SimplyBlue SafeStain is used to visualize protein bands on polyacrylamide gels. It is important to have thorough water rinse to remove SDS and buffer salt; otherwise, they interfere with binding of the Coomassie dye to proteins and reduce staining sensitivity. Stain polyacrylamide gels in SimplyBlue SafeStain for up to 3 h, but sensitivity decreases after 3 h. Add 2 mL of 20% NaCl (w/v) for every 20 mL of stain, when leaving polyacrylamide gels overnight in the stain.

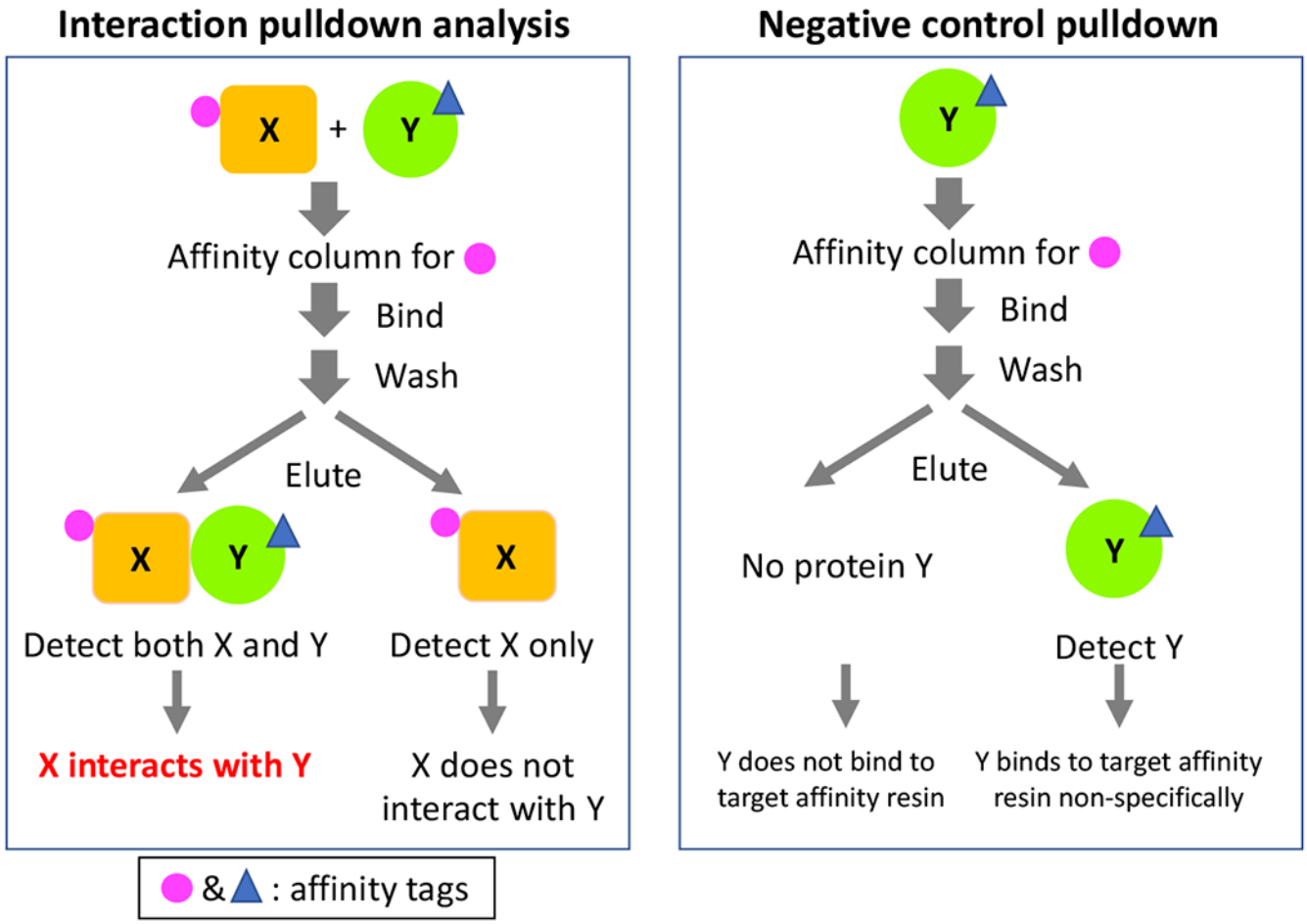

Figure 1.

Schematic of affinity pulldown for direct protein-protein interactions. In the left panel, proteins of interest X and Y are incubated together and bound to an affinity column specific to the affinity tag on protein X (*e.g.,* the bait protein). If both proteins X and Y are detected in the eluted fractions after affinity pulldown, protein Y interacts with protein X. If only protein X is present in the eluted fractions, the two proteins either do not physically interact with each other, or the interaction of the two proteins is not stable to be detected by this method. The right panel depicts a negative control pulldown that should always be performed to ensure the prey protein (*e.g.,* protein Y) does not bind to the affinity column non-specifically to prevent any false positive interaction with the bait protein (*e.g.,* protein X).

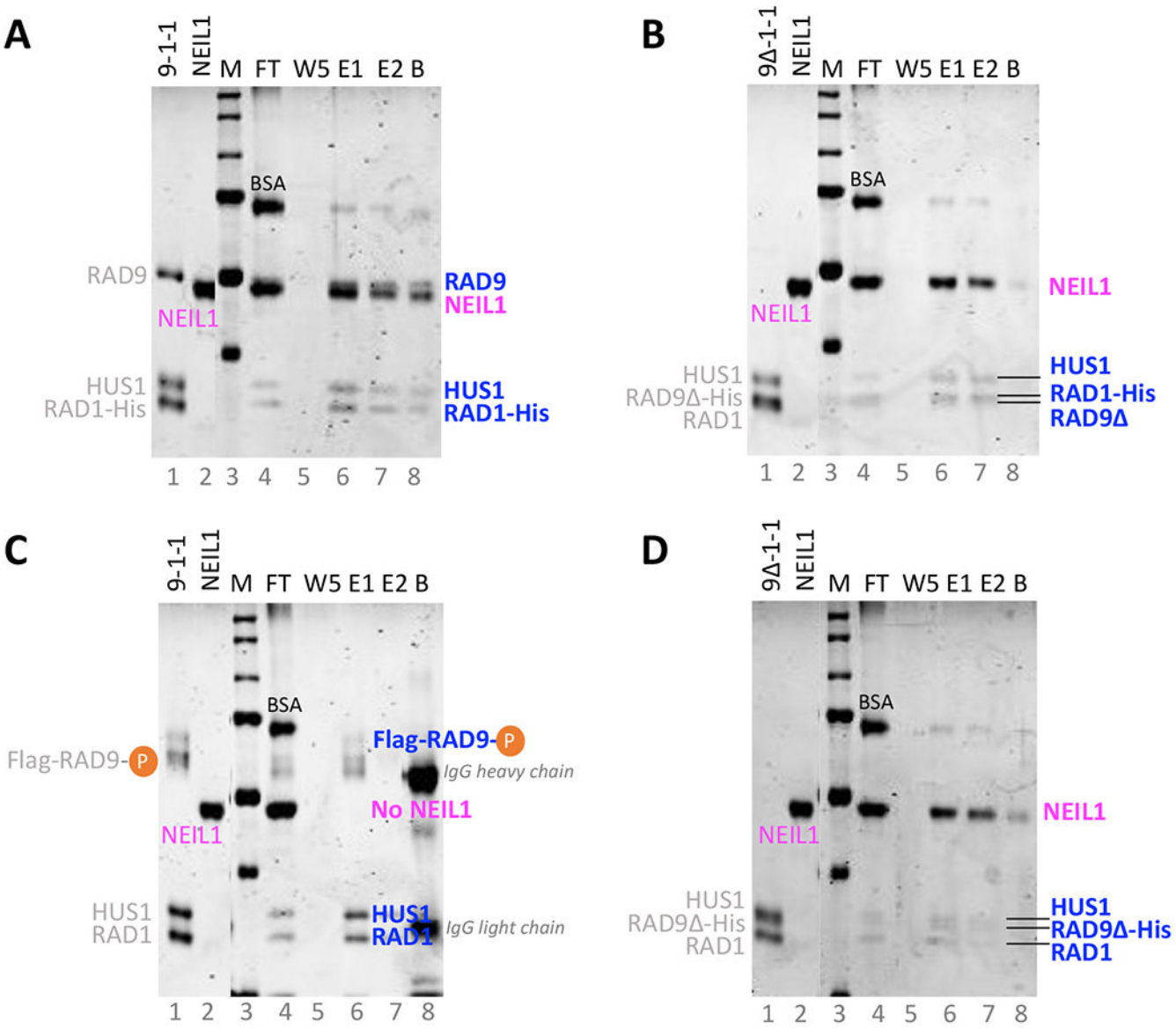

Figure 2.

Physical interaction studies of NEIL1 glycosylase and the 9-1-1 sliding clamp by affinity pulldown. Selected affinity resins, either anti-Flag M2 resin or Ni-NTA resin, were used to pull down Flag- or 6xHis-tagged 9-1-1 proteins and detect if NEIL1 was co-purified with 9-1-1. NEIL1 was pulled down by Ni-NTA resin with 6xHis-tagged **(A)** full-length 9-1-1 and **(B)** truncated 9Δ-1-1, when the 9-1-1 proteins were purified from *E. coli*. NEIL1 was not pulled down with full-length, Flag-tagged 9-1-1 by anti-Flag M2 resin, when the 9-1-1 complex was purified from insect cells **(C),** but was co-purified by Ni-NTA resin with 6xHis-tagged truncated 9Δ-1-1 that was purified from insect cells **(D)**. The affinity pulldown fractions were analyzed by SDS-PAGE with Coomassie staining, as following, in lane 1, 9-1-1 or 9Δ-1-1: protein load for full-length or truncated 9-1-1; lane 2, NEIL1: protein load for NEIL1; lane 3, M: protein molecular weight markers, from top to bottom, including 250, 150, 100, 75, 50, and 37 kDa standard markers; lane 4, FT: flow through fractions; lane 5,

W5: last wash fractions; lane 6, E1: eluted fraction 1; lane 7, E2: eluted fraction 2; and lane 8, B: leftover resin fractions. RAD9, RAD1, HUS1, and NEIL1 with or without an affinity tag are indicated on the Coomassie-stained polyacrylamide gels.