Published in final edited form as:
DNA Repair (Amst). 2007 October 1; 6(10): 1507–1516.

Modes of interaction among yeast Nej1, Lif1 and Dnl4 proteins and comparison to human XLF, XRCC4 and Lig4

Rajashree A. Deshpande[1,2] and **Thomas E. Wilson**[2,*]

2*Department of Pathology, University of Michigan Medical School, Rm. 2065 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200*

Abstract

The nonhomologous end joining (NHEJ) pathway of double-strand break repair depends on DNA ligase IV and its interacting partner protein XRCC4 (Lif1 in yeast). A third yeast protein, Nej1, interacts with Lif1 and supports NHEJ, similar to the distantly related mammalian Nej1 orthologue XLF (also known as Cernunnos). XRCC4/Lif1 and XLF/Nej1 are themselves related and likely fold into similar coiled-coil structures, which suggests many possible modes of interaction between these proteins. Using yeast two-hybrid and co-precipitation methods we examined these interactions and the protein domains required to support them. Results suggest that stable coiled-coil homodimers are a predominant form of XLF/Nej1, just as for XRCC4/Lif1, but that similar heterodimers are not. XLF-XRCC4 and Nej1-Lif1 interactions were instead mediated independently of the coiled coil, and by different regions of XLF and Nej1. Specifically, the globular head of XRCC4/Lif1 interacted with N- and C-terminal domains of XLF and Nej1, respectively. Direct interactions between XLF/Nej1 and DNA ligase IV were also observed, but again appeared qualitatively different than the stable coiled coil-mediated interaction between XRCC4/Lif1 and DNA ligase IV. The implications of these findings for DNA ligase IV function are considered in light of the evolutionary pattern in the XLF/XRCC4 and XLF/Nej1 family.

Keywords

nonhomologous end joining; DNA ligase IV; Nej1; XLF/Cernunnos; yeast; human

1. Introduction

Nonhomologous end joining (NHEJ) is a mode of DNA double-strand break (DSB) repair in which the two DNA ends are directly rejoined [1-3]. The critical step in NHEJ is strand ligation. Accordingly, all organisms use a specialized and dedicated DNA ligase to catalyze NHEJ. In eukaryotes this is DNA ligase IV, whose catalytic subunit is Lig4 (Dnl4 in yeast) [4,5]. Lig4/Dnl4 is comprised of a typical ATP-dependent ligase domain, containing putative DNA binding, adenylation and OB fold subdomains [6], and a tandem pair of C-terminal BRCT domains which mediate interaction with its critical partner protein XRCC4 (Lif1 in yeast) [7-9]. XRCC4/Lif1 is known from crystallographic studies to be homodimeric, with an N-terminal globular head domain followed by a long ~100 amino acid coiled-coil of α-helices that provides extensive and stable interaction between the two monomers [10,11]. The Lig4/

[1]Current Address: Department of Molecular Genetics and Microbiology, University of Texas at Austin, MBB 2.212, 2500 Speedway, Austin, TX 78712
[*]Corresponding author wilsonte@umich.edu, Phone: (734) 764-2212, FAX: (734) 763-2162

Dnl4 BRCT domains bind strongly to XRCC4/Lif1 by encircling this coiled coil at a conserved motif near its C-terminal end [7], supporting robust co-purification of Lig4/Dnl4 with XRCC4/Lif1 [12-14]. Finally, XRCC4/Lif1 contains a C-terminal region that is poorly conserved and structurally uncharacterized, but that is nonetheless phosphorylated and required for NHEJ and interaction with FHA domains of two proteins [15,16] (manuscript in preparation). The precise actions of XRCC4/Lif1 in supporting Lig4/Dnl4 are not clear, but it binds DNA [17] and is required for stability of Lig4/Dnl4 [9,18] and its recruitment to DSBs [19].

The involvement of a third protein in the DNA ligase IV complex was first suggested by discovery of the yeast NHEJ protein Nej1 and the observation that it interacts with Lif1 [20-24]. Nej1 explains the mating type-dependent regulation of yeast NHEJ by virtue of its selective expression in haploid cells [21,22]. This fact, combined with the inability of standard computational approaches to reveal apparent Nej1 homologues outside of budding yeasts, led to the supposition that Nej1 was not a universal NHEJ protein. However, it was recently discovered that people with a form of V(D)J recombination/NHEJ deficiency lack the protein XLF (also called Cernunnos) [25,26]. Like Nej1, XLF interacts directly with XRCC4, is required for NHEJ, and is ~300 residues in length. Indeed, detailed computational analyses aided by this information and new genome sequences verified that Nej1 and XLF are ancestrally related, despite their low primary sequence conservation [27-29]. Even more strikingly, these computational approaches indicated that XLF, and by inference Nej1, is distantly related to XRCC4/Lif1, and that these genes likely arose by an ancestral duplication [25,28]. Although unsubstantiated as yet, the implication is that XLF/Nej1 also has N-terminal globular head, internal coiled coil, and C-terminal domains. The most substantial difference is that XLF/Nej1 likely has a shorter and/or more disrupted coiled-coil due to a higher frequency of prolines [30].

These observations have led to speculative models regarding the types of interactions that might occur within the DNA ligase IV complex [31] (Figure 1). Specifically, one can envision both homodimerization of XLF/Nej1 and XRCC4/Lif1, in addition to heterodimers via mixed coiled coils of XLF-XRCC4 or Nej1-Lif1 pairs. Lig4/Dnl4, in addition to binding XRCC4/Lif1, might bind via a similar BRCT-dependent mode to XLF/Nej1, or to the heterodimer, via a XLF/Nej1 motif that is superficially similar to the Lig4/Dnl4 binding site in XRCC4/Lif1 [27,28]. In addition, XLF/Nej1 and XRCC4/Lif1 might have ligase-independent functions as homo- or hetero-dimers, and indeed XRCC4 supports tetramerization exclusive of Lig4 binding [32]. The importance of this complex set of potential interactions is highlighted by the observation that organisms with Lig4/Dnl4 can have apparent homologues of both or just one of XLF/Nej1 and XRCC4/Lif1, suggesting that functions might be differentially carried by these proteins across species [33]. We have begun to explore these ideas by examining protein-protein interactions between these various proteins, with the specific goals of (i) querying the existence of the interaction modes depicted in Figure 1, and (ii) comparing interactions between yeast and human to test the idea that XLF and Nej1 are functionally homologous. Our results support a model in which both XLF/Nej1 and XRCC4/Lif1 homodimerize but do not constitutively heterodimerize, with the known XLF-XRCC4 and Nej1-Lif1 interactions being less stable and, remarkably, mediated by different regions of XLF/Nej1. Both XLF/Nej1 and XRCC4/Lif1 interact with Lig4/Dnl4, but again in qualitatively different fashion.

2. Materials and Methods

2.1. Yeast strains

Yeast strains PJ69-4a and PJ69-4α [34,35], which harbor Gal4 responsive *HIS3*, *ADE2* and LacZ markers, were used for two-hybrid studies. Yeast strains BY4741 and BY4742 [36] were used for expressing tagged proteins for pull-down studies. Diploids of these a/α strain pairs were made by mating to combine various protein constructs. Alternatively, when indicated,

plasmids were recovered from yeast and co-transformed into haploid yeast. The *lif1*Δ allele was created by PCR-mediated gene replacement with kanMX4 [36] with PCR confirmation of the presence of the kanMX4 insertion as well as loss of the Lif1 coding sequence. All strains were grown at 30°C in a rich medium containing 1% yeast extract, 2% peptone, 2% dextrose, and 40 μg/ml adenine (YPAD) or a synthetic defined (SD) medium with either 2% glucose or galactose as needed [24].

2.2. Plasmid constructs

Expression constructs for two-hybrid and pull-down studies were constructed by gap repair by co-transforming into naive yeast the linearized vector and a tailed PCR product corresponding to the desired coding sequence. Appropriate gap repair constructs were identified by Western blot and sequencing. Yeast two-hybrid Gal4 DNA binding domain (bait, pOBD2) and transcriptional activating domain (prey, pOAD) vectors were as previously described [35]. Vectors for tagged expression for pull-downs were modifications of pTW300 [37] in which the expression cassette was first transferred to 2μ plasmids of the pRS series [38]. PCR with tailed primers was then used to replace the *ADH1* promoter with the *GAL1* promoter and insert various tags in the general configuration: *Xho*I-*GAL1* promoter-*Nco*I-start codon-tag1-*Bam*HI-tag2-stop codon-*Sal*I-*HDF1* terminator-*Not*I. Derivatives included tag pairs FLAG-His6, Myc-His6 and calmodulin binding peptide (CBP)-His6 on *URA3*-, *HIS3*- and *LEU2*-marked backbones, respectively. Appropriate design of gap repair primers and vector digestion allowed selective incorporation of various combination of tags (details are available upon request).

2.3. Yeast two-hybrid assay

Two-hybrid analysis was performed essentially as described [16,35], except that three independent isolates of each clone were initially mated and tested. All clones for which clear and consistent positive results were not obtained were verified by sequencing. Figures show only final experiments using individual confirmed isolates. Empty prey vector is included to reveal the degree of auto-activation for all baits.

2.4. CBP pull-down

In the basic protocol, yeast strains bearing the appropriate combinations of tagged expression constructs were grown in double strength SD media to log phase and harvested by centrifugation. Lysates were prepared from 1 g wet weight cell pellet in lysis buffer (10 mM Tris-HCl buffer pH 7.5, 0.5 M NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM imidazole, 0.1% NP40, 10 % glycerol and 1 mM PMSF) by zirconia bead lysis followed by centrifugation at 15000 g. CBP-tagged proteins were pulled down by incubating lysates with 40 μl of calmodulin affinity resin (Stratagene) at 4°C for 2 h with slow agitation. Beads were washed 5 times with 1.5 ml lysis buffer and boiled in SDS-PAGE sample buffer to elute bound proteins. Variations in this basic protocol are discussed in the text.

2.5. Immunoprecipitation

Proteins were purified from 5 g cell pellet of two separate strains co-expressing either FLAG- and CBP-Nej1 or CBP- and Myc-Lif1 using calmodulin affinity resin essentially as described above for CBP pull-downs, except that here the lysis buffer lacked NP40 and proteins bound to 200 μl calmodulin agarose were eluted into 500 μl buffer C-EGTA (50 mM Tris-HCl buffer pH 7.5, 0.5 M NaCl, 2 mM EGTA, 10 % glycerol and 1 mM PMSF). These purified proteins were mixed in the presence of 0.1% NP40 and 60 μg/ml BSA and allowed to interact at 4°C for 3 h with slow agitation followed by addition of anti-FLAG antibodies (M2, Sigma). Incubation was continued for another hour followed by addition of Protein A/G Plus agarose (Santa Cruz). After overnight incubation, beads were washed 6 times with 0.5 ml buffer C-

EGTA containing 0.1% NP40 and BSA and boiled in SDS-PAGE sample buffer to elute bound proteins.

2.6. Immunoblotting

Proteins from CBP pull-downs or immunoprecipitation were subjected to 8% (Figures 5-7) or 12% (Figure 4) SDS-PAGE followed by electroblotting to nitrocellulose. Blots were probed using primary antibodies to FLAG (M2, from mouse, Sigma), Myc (9E10, from mouse, Santa Cruz) or CBP (C16T, from rabbit, Upstate). Blots were then developed using species-specific secondary antibodies bearing different fluorescent labels (IRDyes 800CW anti-mouse and 680 anti-rabbit, LiCor) and visualized using a LiCor Odyssey scanner to allow simultaneous detection of two proteins in separate channels.

3. Results

3.1. Minimal Nej1-Lif1 and XLF-XRCC4 interacting regions

We first sought to identify more precisely the protein regions responsible for the known Nej1-Lif1 and XLF-XRCC4 interactions, in order to compare yeast and human and explore the validity of different interaction models (Figure 1). To guide these studies, crystallographic analyses of XRCC4 and Lif1 [7,10,11] were used to subdivide the proteins into the regions shown in Figure 2. These include the N-terminal globular head domain, the segment of the coiled coil buried in the interface between the head domains, the segment of the coiled coil exposed to solvent and other proteins, including the C-terminal motif bound by Lig4/Dnl4, and the structurally uncharacterized C-terminus. Nej1 and XLF are predicted to fold similarly to XRCC4 [25,28] so similar regions were defined for Nej1 based on this information, except that there is no clear counterpart to the Lig4/Dnl4 binding motif. Also, the C-terminal regions of XLF and Nej1 are very poorly conserved and may not be homologous to each other or to XRCC4/Lif1. Others have suggested that the XLF/Nej1 C-terminus might be a continuation of the coiled coil [25], and predictions are predominantly helical, but many prolines can be found in this region across many species which are unexpected for a coiled coil [30]. In the absence of structural information it is uncertain where a true XLF/Nej1 coiled coil might end, but the boundaries shown in Figure 2 are useful for interaction mapping in any case.

We first used two-hybrid analysis to detect protein-protein interactions between XLF-XRCC4 and Nej1-Lif1 pairs. Because our prior systematic two-hybrid analysis showed an interaction between residues 150-342 of Nej1 and 1-265 of Lif1 [16], we initially made N-terminal and C-terminal deletion series of XLF/Nej1 and XRCC4/Lif1, respectively, mainly at the boundaries indicated in Figure 2. All fragments were expressed as both Gal4 DNA binding domain (“bait”) and Gal4 activator domain (“prey”) fusions, and all constructs were tested against each other in wild-type diploid yeast. Selected portions of these data are shown in Figure 3, and the combined data summarized in Figure 2. Despite autoactivation by some baits, informative results could be obtained for most pairs. This analysis refined the Nej1-Lif1 interaction to Lif1 residues 1-196, demonstrating that its Dnl4 binding site is not required, and Nej1 residues 173-342, demonstrating that its putative coiled coil is not required (Figure 3A). Loss of the Nej1-Lif1 interaction upon C-terminal deletion in Nej1(1-208) confirmed the importance of the Nej1 C-terminus (Figure 3A) [16,23]. Similar to Lif1, an N-terminal but more focused region of XRCC4, corresponding to its globular head (residues 1-119), supported the XLF interaction (Figure 3B and 3C). However, in contrast to Nej1, not even the smallest N-terminal deletions of XLF (removing residues 1-127) were tolerated (Figure 3B and 3C). We therefore constructed a C-terminal deletion series of XLF and observed that even the smallest XLF bait (residues 1-128), corresponding to its putative globular head, was sufficient for interaction with XRCC4 (Figure 3B). Thus, the globular head of both XLF and XRCC4, devoid of any coiled coil region, appeared to correspond to the minimal interacting region.

To confirm the inferred interacting regions by a different method, we next made N-terminal fusions of relevant protein fragments with either a combined FLAG-His6 epitope or the CBP tag. Protein pairs were co-expressed in diploid yeast and pulled down with calmodulin affinity resin (Figure 4). As expected, CBP-Lif1 was able to specifically pull down FLAG-His6-Nej1 (Figure 4A, lane 2). Consistent with the two-hybrid results, FLAG-His6-Nej1(173-342) was also pulled down effectively (Figure 4A, lane 6). Extending the two-hybrid results, even CBP-Lif1(1-157) proved sufficient to pull down Nej1, demonstrating that, like XRCC4, the Lif1 coiled coil is not required for the interaction (Figure 4A, lane 4). Interaction between the two most truncated Nej1 and Lif1 proteins, CBP-Lif1(1-157) and FLAG-His6-Nej1(173-342), was reduced but nonetheless detectable above background (Figure 4A, lane 8). Overall, confirmation of the two-hybrid results was also seen with the human proteins, although higher non-specific binding of FLAG-His6-XLF to beads weakened the robustness of the conclusion with this construct (Figure 4B). Importantly, CBP-XRCC4(1-119) was readily able to pull down FLAG-His6-XLF(1-128) with no non-specific binding (Figure 4B, lane 8). We thus conclude that the N-terminal globular head of XRCC4/Lif1 is required for interaction with XLF/Nej1, but that different regions of XLF and Nej1 are required (Figure 2).

3.2. Homodimerization of XLF/Nej1 and XRCC4/Lif1

It is clear from prior studies that XRCC4/Lif1 interacts homotypically via a strong coiled coil-mediated association of two molecules of protein, which we will refer to as dimerization [7, 10,11]. The apparent homology of XLF/Nej1 and XRCC4/Lif1 thus predicts a similar strong homodimer interaction for XLF/Nej1. Indeed, we previously observed that human and *Schizosaccharomyces pombe* XLF purified from bacteria eluted from a gel filtration column at a volume consistent with dimer formation [28]. Similarly purified His6-Nej1 eluted at a volume corresponding to a molecular mass of 79 kDa, approximately twice its calculated and electrophoretic molecular weight of 40 kDa (Bianchi J and Doherty AJ, personal communication), again consistent with dimer formation. His6-Nej1 also robustly shifted plasmid DNA in an agarose EMSA (Bianchi J and Doherty AJ, personal communication), very similar to XRCC4 [17] and human and *S. pombe* XLF [28], providing further evidence for their functional correspondence.

Despite the above, homodimerization proved difficult to reproducibly detect by two-hybrid, although importantly this limitation existed for XRCC4/Lif1 as well as XLF/Nej1. XRCC4 homodimerization could be observed that mapped to residues 1-164, consistent with the crystallographically observed dimer interface, but only with the more permissive *HIS3* reporter (Figure 3E). The full length Nej1 (1-342) bait-prey combination showed only very weak growth and again only with the *HIS3* reporter (Figure 3D). An apparent interaction was also seen between Nej1(1-208) and Nej1(173-342), but the latter does not correspond to the putative Nej1 coiled coil, and other constructs that include the coiled coil showed no interaction (Figure 3D). Neither XLF nor Lif1 homodimers were readily apparent, although auto-activiation did make these contructs somewhat less interpretable (data not shown). These findings are generally consistent with previous two-hybrid analyses [9,16,23] and suggest a bias of this method against detecting the stable coiled-coil dimers that clearly exist in the case of XRCC4/Lif1.

Given this uncertainty from the two-hybrid analysis, it was important to validate XLF/Nej1 homodimerization by an independent method. We therefore co-expressed CBP-Nej1 and FLAG-Nej1 in the same wild-type diploid yeast strain and performed pull-down experiments with calmodulin affinity resin. We observed pull-down of FLAG-Nej1 in a manner dependent on co-expression of CBP-Nej1, even at 0.5 M NaCl and 0.1 % NP-40 (Figure 5A, lane 2), consistent with Nej1 dimerization. The same pattern was observed for XLF (Figure 4B, lane2). This pattern was similar to that observed for XRCC4 (Figure 5B, lane 2) and Lif1 (not shown),

and clearly demonstrates formation of stable higher order (dimer or multimer) XLF and Nej1 complexes. For XLF, further support that this represents a coiled-coil dimer interface can be seen in Figure 4B, where full length XLF failed to pull down XLF(1-128) lacking the putative coiled coil region (compare lanes 2 and 6). However, interaction data alone cannot unambiguously reveal the mechanism of the XLF/Nej1 homotypic interaction. One notable feature in Figure 5 and throughout is that we consistently observe two gel bands for XLF, XRCC4, Nej1 and Lif1. The basis of this pattern is unknown, but is unlikely to represent degradation because the upper bands exceed the expected monomer molecular weight. Similar anomalous behavior has been observed for Lif1 [9] and XRCC4 [13,14].

3.3. Modes of heterotypic interaction between XLF/Nej1 and XRCC4/Lif1

The prior observation of XLF-XRCC4 and Nej-Lif1 interactions and the hypothesis that these protein pairs fold similarly have suggested that they might form coiled-coil heterodimers similar to XRCC4/Lif1 and apparent XLF/Nej1 homodimers [31]. Arguing against this possibility, data above demonstrate that the minimal XLF-XRCC4 and Nej-Lif1 interacting regions need not include the coiled coil region of any of these proteins. However, it is possible that more than one mode of interaction exists. In this regard, we might be misled by the two-hybrid analysis, since its apparent bias against detecting coiled-coil homodimerization might extend to coiled-coil heterodimer interactions. To explore the question of heterodimerization using a different method, we next compared the relative ability of CBP-Lif1 and CBP-Nej1 to pull down FLAG-His6-Nej1 under different salt stringency conditions. Our hypothesis was that if dimerization was a strong component of both homotypic and heterotypic interactions then a similar salt sensitivity should be observed. Instead, there was a clear difference, with the heterotypic Nej1-Lif1 interaction being reduced 5-fold in 0.5 M NaCl salt as compared 0.15 M NaCl (Figure 5A, lanes 3 and 6) while the homotypic Nej1-Nej1 interaction was the same in either condition (Figure 5A, lanes 2 and 5). Similar results were observed when tag orientations were reversed (data not shown). The same pattern was seen for the human proteins (Figure 5B and data not shown). Thus, the predominant interaction mode appears to be different for homotypic and heterotypic interactions, consistent with the mapping of the minimal interacting regions.

A corollary of the above interpretation is that homodimeric Nej1 should remain capable of interacting with homodimeric Lif1. To test this we co-expressed Nej1 and Lif1 in separate strains to create CBP-Nej1::FLAG-Nej1 and CBP-Lif1::Myc-Lif1 dimers. Proteins were purified in parallel using the CBP tags and eluted, mixed to allow interaction, and then pulled down using antibodies against the Nej1 FLAG tag. The blot was then developed using antibodies to all tags (Figure 6). As expected based on the purification scheme, both CBP-Nej1 and FLAG-Nej1 were present in large amounts, again indicating that Nej1 was dimeric. Lif1 tagged with both CBP and Myc was also pulled down in a manner dependent on the presence of Nej1 (Figure 6, lanes 1 and 2), indicating interaction between a dimer of Lif1 and a dimer of Nej1. The Lif1 bands were weak because the experiment was performed at high salt to suppress any non-specific binding, although this unfortunately also has disproportionate effects on the Nej1-Lif1 heterotypic interaction (see above). Also, it is possible that Nej1 and Lif1 purified separately form higher order multimers similar to XRCC4 [32] that compete with subsequent heterotypic interactions.

3.4. XLF/Nej1 and XRCC4/Lif1 interactions with Lig4/Dnl4

Lig4/Dnl4 interacts with XRCC4/Lif1 via a well characterized and stable interaction between the tandem BRCT domains of the former and the coiled coil of the latter [7-9]. Might XLF/Nej1 also support this interaction, thereby creating a potential ligase control mechanism? We first tested this possibility by two-hybrid analysis using full length Lig4/Dnl4 constructs in wild-type diploid strains, as well as constructs that split the ligase and BRCT regions (Figure

7A). As expected, the Lig4/Dnl4-XRCC4/Lif1 interactions were readily detected and specific to the BRCT domains. However, no Lig4/Dnl4-XLF/Nej1 interactions were detected. Importantly, all constructs shown in Figure 7A were excellent two-hybrid reagents as they showed strong interactions when paired with the correct proteins (see also Figure 3). Thus, if Lig4/Dnl4-XLF/Nej1 interactions exist, they are likely weaker or of a different type than Lig4/Dnl4-XRCC4/Lif1 interactions.

To test this relationship by a different method we again turned to *in vitro* interactions of tagged proteins. Others have previously observed essentially quantitative co-purification of Lig4-XRCC4 and Dnl4-Lif1 protein pairs, often over several columns [12-14]. We also observed this with our yeast constructs, but did not see similar co-purification of Dnl4-Nej1 or Dnl4-Lif1-Nej1 complexes under high salt washing conditions (data not shown). However, we were able to detect direct Lig4/Dnl4-XLF/Nej1 interactions in experiments in which all three yeast proteins were co-expressed in a haploid strain and pulled down using CBP-Nej1 (Figure 7B). As expected, both Myc-Lif1 and FLAG-Dnl4 were co-precipitated at low but measurable efficiency (Figure 7B, lane 1). This initially suggested a three-way complex supported by Dnl4-Lif1 and Lif1-Nej1 interactions, but surprisingly, FLAG-Dnl4 pull-down could still be specifically observed even when the Lif1 plasmid was omitted and the native *LIF1* gene was deleted from the strain (Figure 7B, lanes 2 and 3). Thus, a direct Dnl4-Nej1 interaction also appears to exist. Similar results were obtained in experiments comparing Lig4-XRCC4 and Lig4-XLF interactions. Both CBP-XRCC4 and CBP-XLF could independently pull down Lig4, but the former was much more efficient under the conditions used (Figure 7C, lanes 2 and 3).

4. Discussion

There is a remarkable pattern of conservation and inferred structural relatedness of XLF/Nej1 and XRCC4/Lif1 that has led to models of how homo- vs. hetero-dimerization of these proteins might regulate Lig4/Dnl4 [31] (Figure 1). Experiments reported here evaluated these many possible modes of interaction to explore these ideas and whether they can be generalized across species.

4.1. Homotypic interactions

The simplest prediction made by computational studies was that XLF/Nej1 should form a strong homodimer, and possibly multimers of dimers, similar to XRCC4/Lif1 [10,11,32] (Figure 1). We addressed this directly by pull-down analysis using two differentially tagged forms of XLF/Nej1 and observed a robust and salt-stable co-purification of one tagged form when pulling down the other. This directly mirrored similar analysis of XRCC4/Lif1 (Figure 5, and data not shown). In the case of XLF, this co-purification was dependent on the presence of the coiled coil in both tagged forms (Figure 4B). Size exclusion chromatography has also revealed that Nej1 in solution has a molecular mass consistent with dimerization (Bianchi J and Doherty AJ, personal communication), similar to human and *S. pombe* XLF [28]. Such observations alone cannot prove that XRCC4/Lif1 and XLF/Nej1 form comparable coiled-coil-dependent homodimers, but strongly support this interpretation. Paradoxically, this interpretation is also supported by the consistently poor ability of the two-hybrid method to detect these dimerization interactions (Figure 3 and data not shown) despite their stability *in vitro*.

Importantly, XRCC4/Lif1 and XLF/Nej1 might also interact homotypically by means other than coiled-coil dimers, perhaps similar to heterotypic interactions (see below). Unfortunately, such interactions would often be masked experimentally by the strong dimer interaction. We were able to detect one apparent homotypic interaction with Nej1 two-hybrid constructs 1-208

and 173-342 which does not appear consistent with coiled-coil formation (Figure 3), although structural information will ultimately be required to state this with certainty.

4.2. Heterotypic interactions

Both XLF and Nej1 were identified by two-hybrid detection of heterotypic interactions with XRCC4/Lif1 [21,25], interactions which we verify and refine here by both two-hybrid and pull-down methods (Figure 2). Prediction of a similar fold of XLF/Nej1 and XRCC4/Lif1 subsequently suggested the possibility of coiled-coil-dependent heterodimerization [25,28, 31] (Figure 1). Several criteria suggest that the earlier described modes of interaction are distinct from heterodimerization, and that true heterodimerization is not readily observable. First, for both human and yeast proteins heterotypic interactions did not require the coiled coil domain (Figures 3 and 4) [21,23]. Also, the heterotypic interactions were more salt-sensitive than the homodimer interactions (Figure 5). Finally, it was possible to detect interactions between proteins in which differential tags provided increased confidence that Nej1 and Lif1 had preformed as homodimers prior to heterotypic interaction (Figure 6). Importantly, no part of our data rules out the possibility of coiled-coil heterodimers, but collectively they suggest that this is not a dominant and/or constitutive mode of interaction. This is consistent with reports that only a fraction of cellular XLF/Nej1 partitions with XRCC4/Lif1 [25,39]. True heterodimers might form only in a context-specific manner, for example only at the site of a DSB or following post-translational modification. However, the high stability of each homodimer would appear to demand an active mechanism for dimer switching under such conditions. Also, it is interesting that a recently observed checkpoint-dependent Nej1 phosphorylation site is in the C-terminal region that interacts with the Lif1 head domain [39], suggesting different possible regulatory roles for such modifications.

4.3. XLF/Nej1 interaction with Lig4/Dnl4

The formation of strong apparent XLF/Nej1 homodimers leads to another predicted possible interaction in which Lig4/Dnl4 binds to the putative XLF/Nej1 coiled coil in a manner analogous to XRCC4/Lif1 (Figure 1). This has also been suggested by partially conserved motifs of the coiled coil within and between XLF/Nej1 and XRCC4/Lif1 families [27,28], although these motifs are in different portions of the coiled coils. We did observe interactions between XLF/Nej1 and Lig4/Dnl4 (Figure 7), as previously suggested for XLF [25,27,28]. However, these appear qualitatively different than the interactions between XRCC4/Lif1 and Lig4/Dnl4. Specifically, XRCC4/Lif1-Lig4/Dnl4 interactions were readily detected by two-hybrid analysis while XLF/Nej1-Lig4/Dnl4 interactions were not (Figures 7). It is possible that Lig4/Dnl4 two-hybrid proteins might bind endogenous Lif1 and thereby affect the readout, but this would simply reinforce the case that the ligase binds with great preference to XRCC4/Lif1. Similarly, we and others have observed that interactions between XRCC4/Lif1 and Lig4/Dnl4 are stable over many column purification steps [12-14]. In contrast, the interactions between XLF/Nej1 and Lig4/Dnl4 were much weaker (Figure 7 and data not shown).

4.4. Correspondence of Nej1 and XLF

Taken together, our data and observations in the literature strongly suggest a functional as well as structural correspondence of XLF and Nej1 in supporting Lig4/Dnl4. One of the more unexpected findings was thus the lack of correspondence of the minimal XRCC4/Lif1-interacting regions in XLF and Nej1. Although both yeast and human heterotypic interactions depended on the globular head domain of XRCC4/Lif1, yeast depended on the ill-defined and less conserved C-terminus of Nej1 while human depended on the putative globular head of XLF. As above, we cannot rule out that some common modes of interaction are below the two-hybrid limit of detection, but it is clear that the major mode differs between species. This may not be surprising given that these interactions depend on the diverged non-coiled-coil portions

of the proteins, and emphasizes that even from a common scaffold there is a potential for drift in the non-coiled-coil interactions.

4.5. Other species

The functional correspondence, or lack thereof, of the interactions discussed above becomes particularly relevant when considering further species. Most strikingly, not all species with Ku and Lig4/Dnl4, and therefore presumably NHEJ, appear to have both XRCC4/Lif1 and XLF/Nej1 homologues [28,33]. In some cases, current algorithms may fail to detect them given the high rate of primary sequence divergence. In other cases the absence of one protein is more certain. For example, syntenic relationships make it apparent that a XRCC4/Lif1 homologue is in fact absent from *Ashbya gossypii* [28,33]. *S. pombe* is another organism in which XRCC4/Lif1 is as yet unidentified despite the presence of a definable XLF/Nej1 homologue [28]. It is thus likely that there will be still further variations of the interaction modes described here as more species are considered.

In summary, the collected results here and from the literature support a model in both budding yeast and humans in which Lig4/Dnl4 is predominantly bound to XRCC4/Lif1 homodimers, with XLF/Nej1 homodimers providing a further supporting role via direct contacts to both Lig4/Dnl4 and XRCC4/Lif1. However, observable differences in these interactions between species, the relatedness of XRCC4/Lif1 and XLF/Nej1, and the weak and non-uniform conservation of these proteins all suggest a substantial flexibility in their use during NHEJ. Greater insight into the specific roles of XRCC4/Lif1 and XLF/Nej1 in supporting DNA ligase IV action will be required to understand the potential importance of these differences in regulating NHEJ outcomes.

Acknowledgements

We thank Drs. Patrick O'Brien and JoAnn Sekiguchi for providing human XLF and XRCC4 coding sequences. We thank Dr. Aidan Doherty for critical reading of the manuscript and sharing information regarding the behavior of Nej1 purified from bacteria. This work was supported by Public Health Service grant CA102563.

References

[1]. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA Repair (Amst) 2006;5:1042–1048. [PubMed: 16822724]

[2]. Daley JM, Palmbos PL, Wu D, Wilson TE. Nonhomologous end joining in yeast. Annu Rev Genet 2005;39:431–451. [PubMed: 16285867]

[3]. Wilson TE, Topper LM, Palmbos PL. Non-homologous end-joining: bacteria join the chromosome breakdance. Trends Biochem Sci 2003;28:62–66. [PubMed: 12575991]

[4]. Wilson TE, Grawunder U, Lieber MR. Yeast DNA ligase IV mediates non-homologous DNA end joining. Nature 1997;388:495–498. [PubMed: 9242411]

[5]. Frank KM, Sekiguchi JM, Seidl KJ, Swat W, Rathbun GA, Cheng HL, Davidson L, Kangaloo L, Alt FW. Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. Nature 1998;396:173–177. [PubMed: 9823897]

[6]. Tomkinson AE, Vijayakumar S, Pascal JM, Ellenberger T. DNA ligases: structure, reaction mechanism, and function. Chem Rev 2006;106:687–699. [PubMed: 16464020]

[7]. Dore AS, Furnham N, Davies OR, Sibanda BL, Chirgadze DY, Jackson SP, Pellegrini L, Blundell TL. Structure of an Xrcc4-DNA ligase IV yeast ortholog complex reveals a novel BRCT interaction mode. DNA Repair (Amst) 2006;5:362–368. [PubMed: 16388993]

[8]. Grawunder U, Zimmer D, Leiber MR. DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. Curr Biol 1998;8:873–876. [PubMed: 9705934]

[9]. Herrmann G, Lindahl T, Schar P. Saccharomyces cerevisiae LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4. EMBO J 1998;17:4188–4198. [PubMed: 9670033]

[10]. Junop MS, Modesti M, Guarne A, Ghirlando R, Gellert M, Yang W. Crystal structure of the Xrcc4 DNA repair protein and implications for end joining. EMBO J 2000;19:5962–5970. [PubMed: 11080143]

[11]. Sibanda BL, Critchlow SE, Begun J, Pei XY, Jackson SP, Blundell TL, Pellegrini L. Crystal structure of an Xrcc4-DNA ligase IV complex. Nat Struct Biol 2001;8:1015–1019. [PubMed: 11702069]

[12]. Chen L, Trujillo K, Ramos W, Sung P, Tomkinson AE. Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. Mol Cell 2001;8:1105–1115. [PubMed: 11741545]

[13]. Grawunder U, Wilm M, Wu X, Kulesza P, Wilson TE, Mann M, Lieber MR. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. Nature 1997;388:492–495. [PubMed: 9242410]

[14]. Critchlow SE, Bowater RP, Jackson SP. Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. Curr Biol 1997;7:588–598. [PubMed: 9259561]

[15]. Koch CA, Agyei R, Galicia S, Metalnikov P, O'donnell P, Starostine A, Weinfeld M, Durocher D. Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV. EMBO J 2004;23:3874–3885. [PubMed: 15385968]

[16]. Palmbos PL, Daley JM, Wilson TE. Mutations of the Yku80 C terminus and Xrs2 FHA domain specifically block yeast nonhomologous end joining. Mol Cell Biol 2005;25:10782–10790. [PubMed: 16314503]

[17]. Modesti M, Hesse JE, Gellert M. DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity. EMBO J 1999;18:2008–2018. [PubMed: 10202163]

[18]. Bryans M, Valenzano MC, Stamato TD. Absence of DNA ligase IV protein in XR-1 cells: evidence for stabilization by XRCC4. Mutat Res 1999;433:53–58. [PubMed: 10047779]

[19]. Teo SH, Jackson SP. Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks. Curr Biol 2000;10:165–168. [PubMed: 10679327]

[20]. Valencia M, Bentele M, Vaze MB, Herrmann G, Kraus E, Lee SE, Schar P, Haber JE. NEJ1 controls non-homologous end joining in Saccharomyces cerevisiae. Nature 2001;414:666–669. [PubMed: 11740566]

[21]. Frank-Vaillant M, Marcand S. NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev 2001;15:3005–3012. [PubMed: 11711435]

[22]. Kegel A, Sjostrand JO, Astrom SU. Nej1p, a cell type-specific regulator of nonhomologous end joining in yeast. Curr Biol 2001;11:1611–1617. [PubMed: 11676923]

[23]. Ooi SL, Shoemaker DD, Boeke JD. A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae. Science 2001;294:2552–2556. [PubMed: 11701889]

[24]. Wilson TE. A genomics-based screen for yeast mutants with an altered recombination/end-joining repair ratio. Genetics 2002;162:677–688. [PubMed: 12399380]

[25]. Ahnesorg P, Smith P, Jackson SP. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. Cell 2006;124:301–313. [PubMed: 16439205]

[26]. Buck D, Malivert L, De Chasseval R, Barraud A, Fondaneche MC, Sanal O, Plebani A, Stephan JL, Hufnagel M, Le Deist F, Fischer A, Durandy A, De Villartay JP, Revy P. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. Cell 2006;124:287–299. [PubMed: 16439204]

[27]. Callebaut I, Malivert L, Fischer A, Mornon JP, Revy P, De Villartay JP. Cernunnos interacts with the XRCC4 x DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1. J Biol Chem 2006;281:13857–13860. [PubMed: 16571728]

[28]. Hentges P, Ahnesorg P, Pitcher RS, Bruce CK, Kysela B, Green AJ, Bianchi J, Wilson TE, Jackson SP, Doherty AJ. Evolutionary and functional conservation of the DNA non-homologous end-joining protein, XLF/Cernunnos. J Biol Chem 2006;281:37517–37526. [PubMed: 17038309]

[29]. Pavlicek A, Jurka J. Positive selection on the nonhomologous end-joining factor Cernunnos-XLF in the human lineage. Biol Direct 2006;1:15. [PubMed: 16749933]

[30]. Gromiha MM, Parry DA. Characteristic features of amino acid residues in coiled-coil protein structures. Biophys Chem 2004;111:95–103. [PubMed: 15381307]

[31]. Sekiguchi JM, Ferguson DO. DNA double-strand break repair: a relentless hunt uncovers new prey. Cell 2006;124:260–262. [PubMed: 16439201]

[32]. Modesti M, Junop MS, Ghirlando R, Van De Rakt M, Gellert M, Yang W, Kanaar R. Tetramerization and DNA ligase IV interaction of the DNA double-strand break repair protein XRCC4 are mutually exclusive. J Mol Biol 2003;334:215–228. [PubMed: 14607114]

[33]. WilsonTENonhomologous end-joining: mechanisms, conservation and relationship to illegitimate recombination. Topics in Current Genetics in press 2006

[34]. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 1996;144:1425–1436. [PubMed: 8978031]

[35]. Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 2000;403:623–627. [PubMed: 10688190]

[36]. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 1998;14:115–132. [PubMed: 9483801]

[37]. Wilson TE, Lieber MR. Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. J Biol Chem 1999;274:23599–23609. [PubMed: 10438542]

[38]. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 1989;122:19–27. [PubMed: 2659436]

[39]. Ahnesorg P, Jackson SP. The non-homologous end-joining protein Nej1p is a target of the DNA damage checkpoint. DNA Repair (Amst). 2006

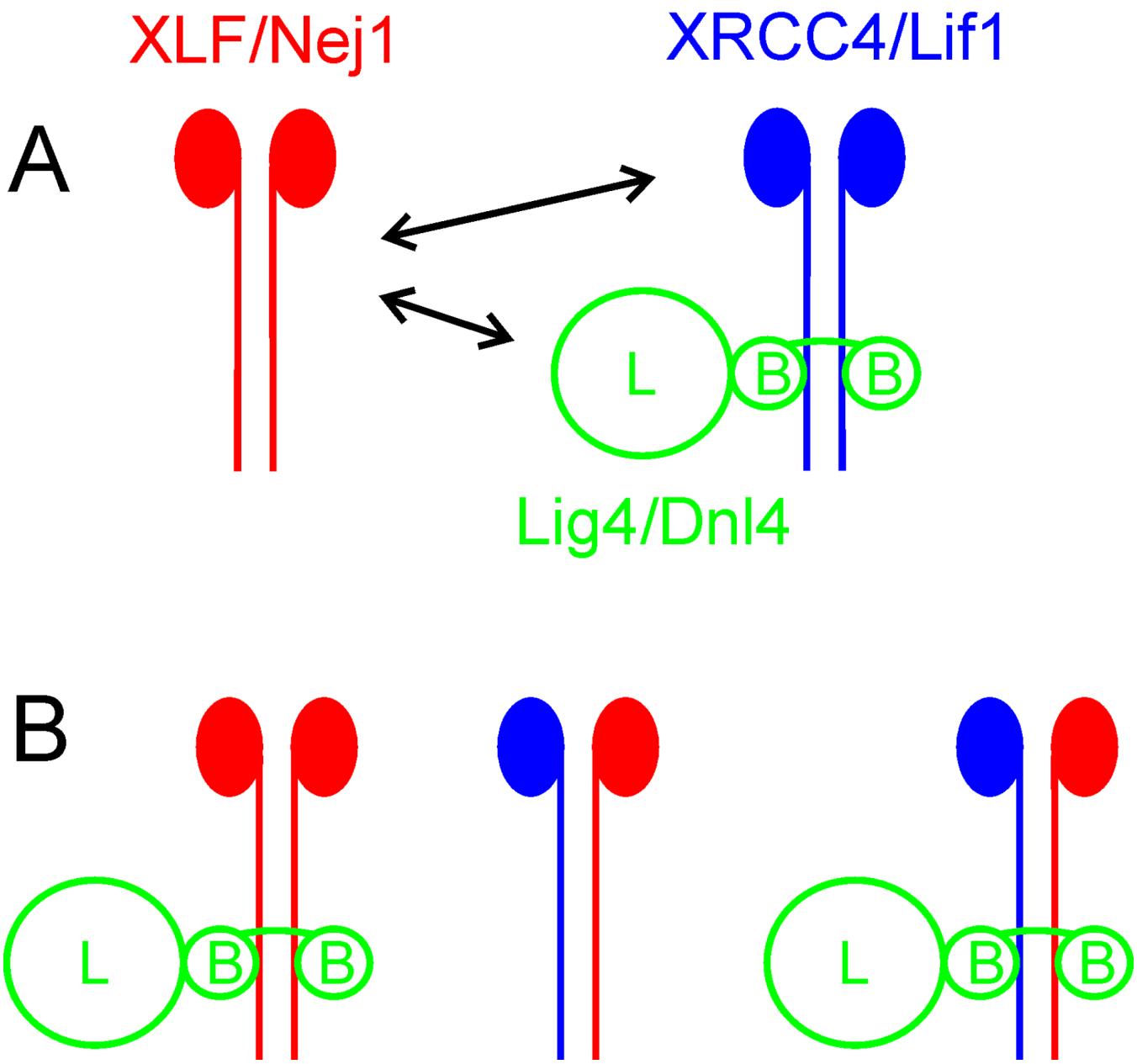

Figure 1. Possible modes of interaction between XLF/Nej1, XRCC4/Lif1 and Lig4/Dnl4

The similar predicted structures of XLF/Nej1 and XRCC4/Lif1 suggest many possible modes of interaction within the overall DNA ligase IV complex. **(A)** Drawings illustrate the modes of interaction strongly supported by work here and elsewhere: strong stable homodimeric interactions between the globular heads and coiled coils of XLF/Nej1 and XRCC4/Lif1; strong stable interaction between the tandem BRCT domains of Lig4/Dnl4 and the coiled coil of XRCC4/Lif1; and interactions between XLF/Nej1 and XRCC4/Lif1 homodimers and between XLF/Nej1 and Lig4/Dnl4. **(B)** Drawings illustrate alternative possible modes of interaction for which clear evidence is currently lacking, including coiled-coil heterodimers of XLF/Nej1 and XRCC4/Lif1 and interaction of the Lig4/Dnl4 BRCT domains with such heterodimers or the XLF/Nej1 homodimer.

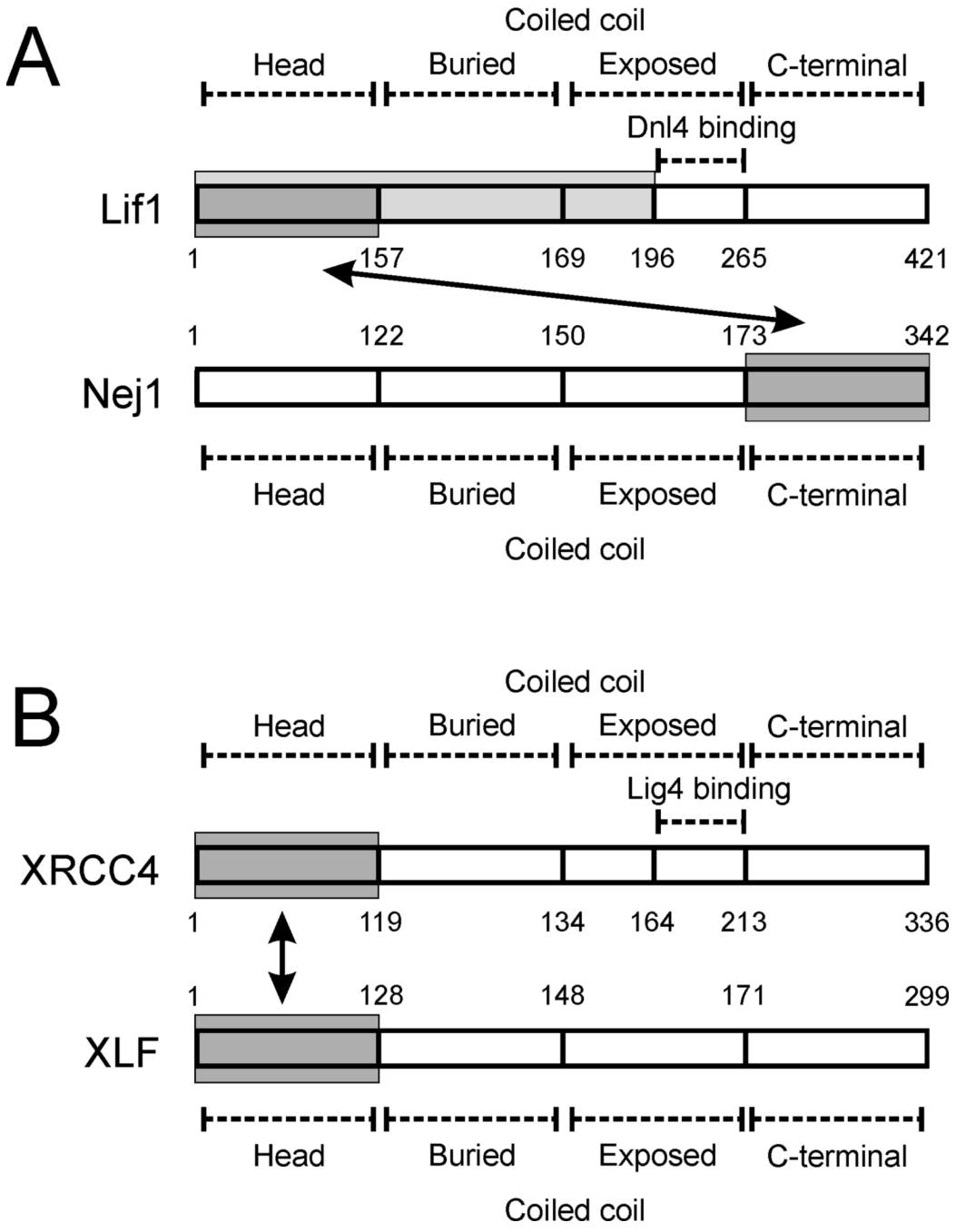

Figure 2. Summary of Nej1-Lif1 and XLF-XRCC4 minimal interacting regions

The relative positions of the inferred globular head, coiled-coil and C-terminal domains are schematically depicted for each protein. Numbers identify residues at the domain boundaries that correspond to most constructs used in Figures 3 and 4. The poorly defined boundaries between the XLF and Nej1 coiled coil and C-terminal domains were set at the end of the most highly conserved stretch and before the increase in proline residues. The drawings are not to scale. Double-headed arrows and shaded boxes indicate the minimal interacting regions between the protein pairs as revealed by two-hybrid (Figure 3) and pull-down (Figure 4) analyses. The top lighter box in Lif1 indicates the minimal interacting region in two-hybrid analysis (1-196), while the bottom darker box indicates this region as refined by pull-down

analysis (1-157). Strikingly, while **(A)** Nej1-Lif1 and **(B)** XLF-XRCC4 pairs each have reproducible interactions, the XLF/Nej1 regions supporting these interactions differ.

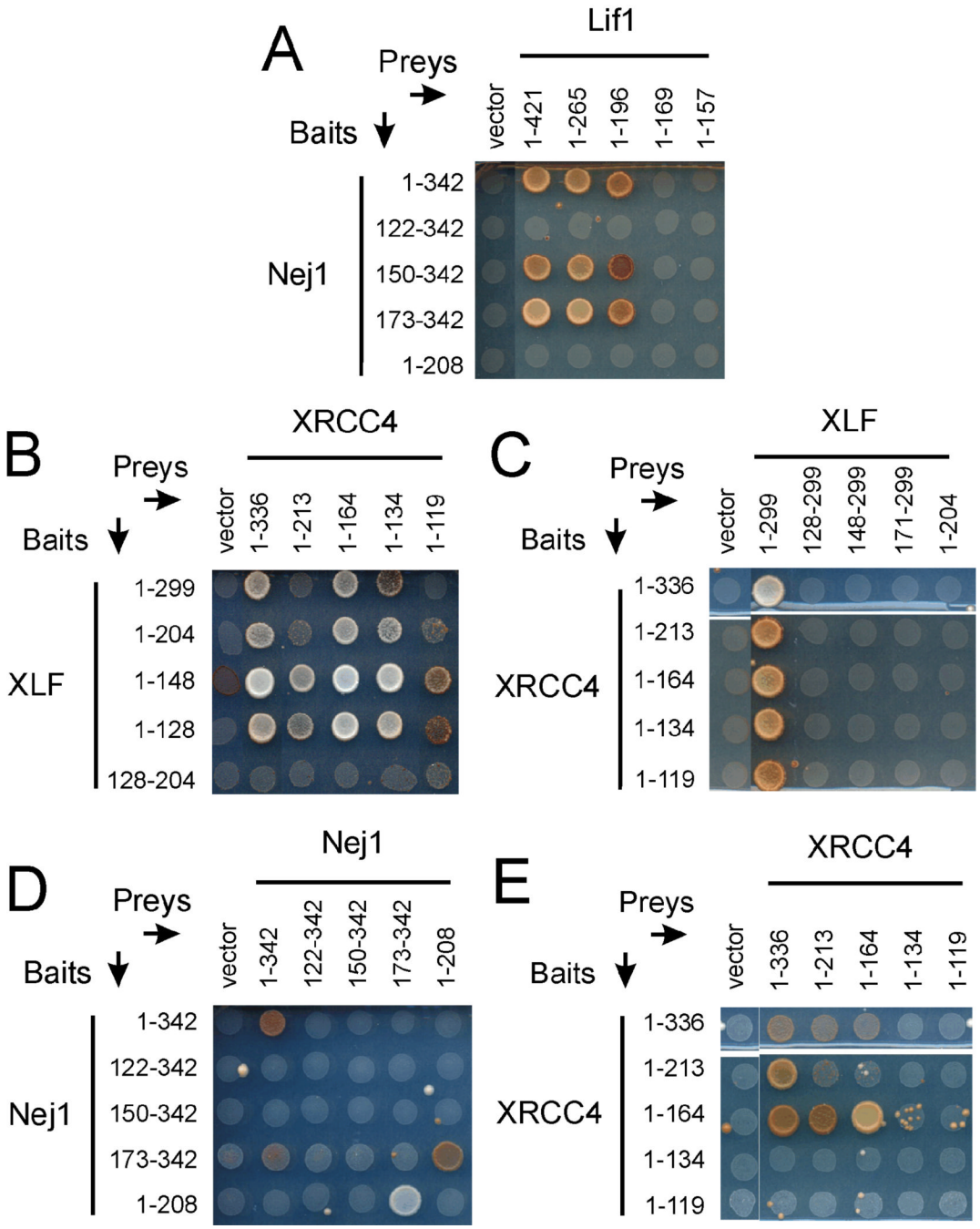

Figure 3. Nej1-Lif1 and XLF-XRCC4 minimal interacting regions, two-hybrid analysis

Wild-type haploid yeasts containing various bait and prey constructs were mated, spotted to two-hybrid indicator medium lacking adenine or histidine, and grown for 5 days at 30° C. Prey columns labeled “vector” contained no insert to show the level of bait auto-activation. Panels show the most important bait and prey combinations from the overall analysis. Results are summarized in Figure 2. Panels show heterotypic interactions between **(A)** Nej1 and Lif1 and **(B)** and **(C)** XLF and XRCC4, spotted to adenine-selective medium, as well as homotypic interactions of **(D)** Nej1 and **(E)** XRCC4, spotted to the more permissive histidine-selective medium.

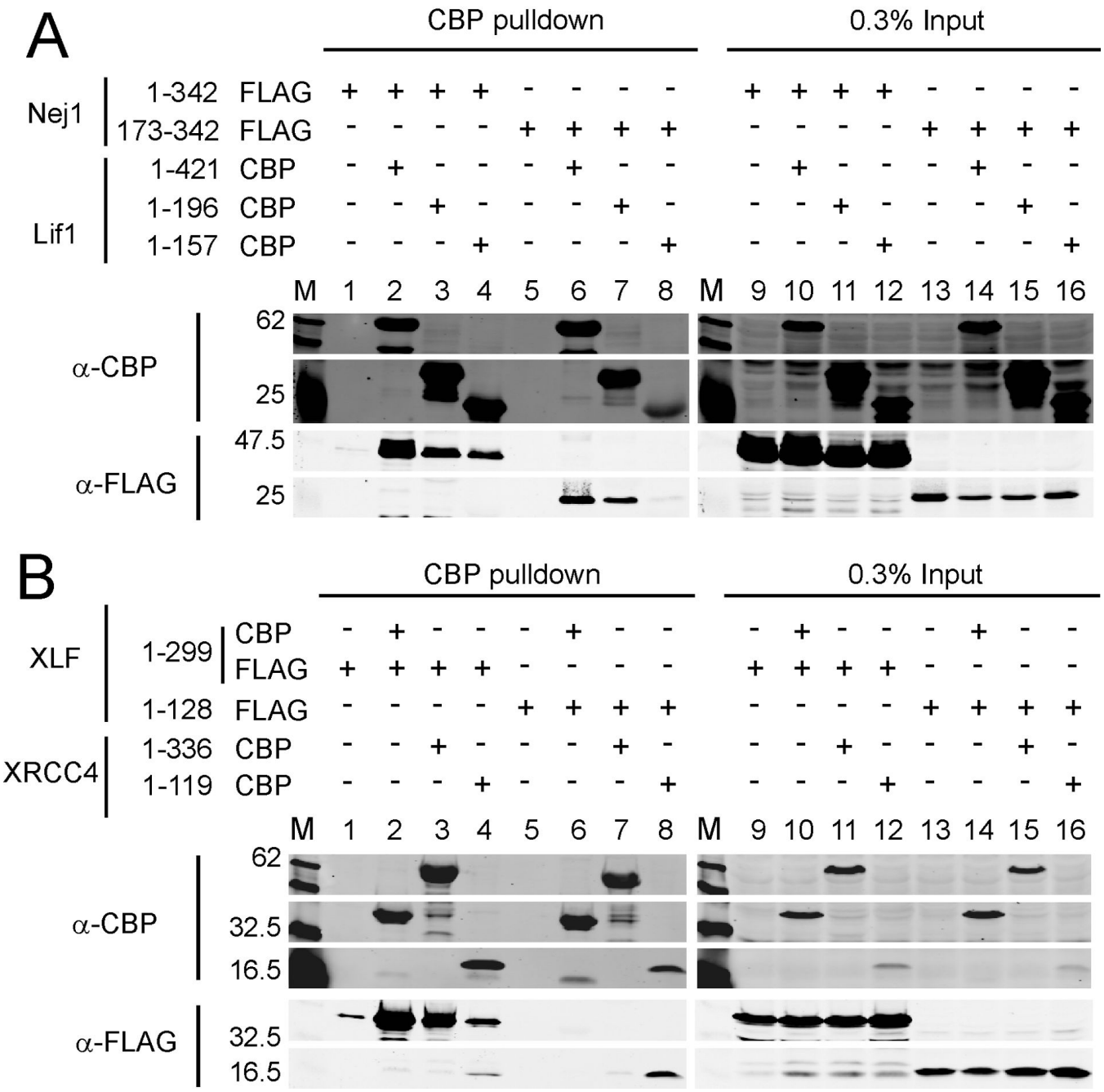

Figure 4. Nej1-Lif1 and XLF-XRCC4 minimal interacting regions, pull-down analysis

Protein fragments corresponding to a subset of the two-hybrid truncation series in Figure 3 were co-expressed in wild-type diploid yeast as pairs of FLAG and CBP fusions. CBP-tagged proteins and any interacting proteins were pulled down from cell-lysates with calmodulin agarose, eluted by boiling in SDS-PAGE sample buffer, electrophoresed and immunoblotted with α-CBP and α-FLAG antibodies (left panels). A portion of the lysates was also immunoblotted to show input proteins (right panels). Pull-downs were performed using the 0.15 M NaCl condition of Figure 5. Lane "M" contains molecular weight markers, indicated in kDa, which are visible in the α-CBP detection channel. **(A)** Nej1-Lif1 interactions. **(B)** XLF-XRCC4 interactions. Results are summarized in Figure 2.

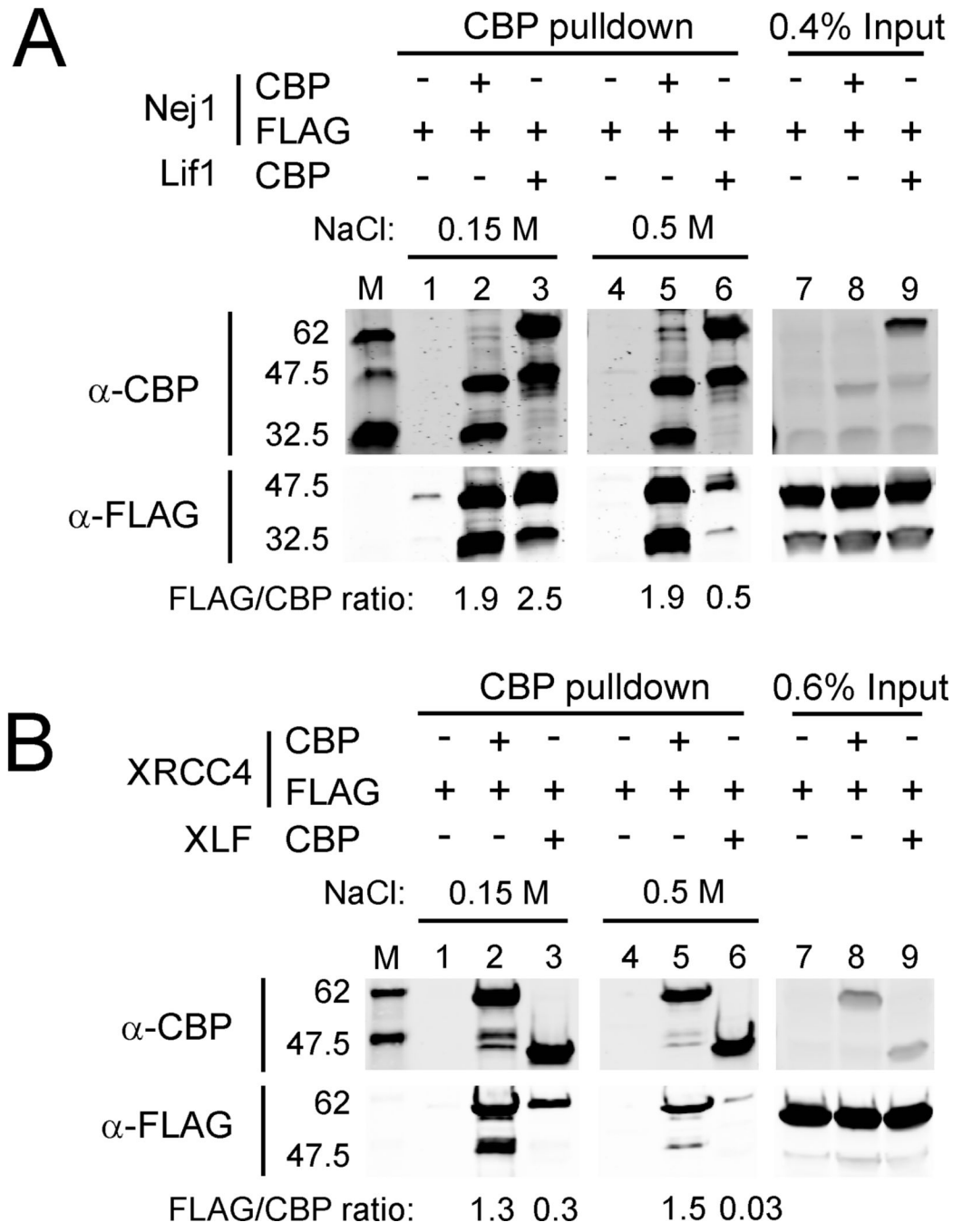

Figure 5. Differential stability of homotypic and heterotypic XLF/Nej1 and XRCC4/Lif1 interactions

XLF/Nej1 and XRCC4/Lif1 were co-expressed in wild-type diploid yeast as combinations of CBP- and FLAG-tagged proteins. Note that the two tags were on separate expression constructs even when the same protein was tagged twice. CBP-tagged proteins and any interacting proteins were pulled-down from cell-lysates with calmodulin agarose at 0.15 M (left panels) or 0.5 M (middle panels) NaCl and immunoblotted as in Figure 4. For relevant lanes, band intensities were determined using the LiCor Odyssey scanner, corrected for any background of non-specifically bound protein, and expressed as a ratio of FLAG to CBP signals. For both **(A)** yeast proteins and **(B)** human proteins, the XLF/Nej1-XRCC4/Lif1 heterotypic interaction

was substantially more sensitive to high salt than the strong and stable Nej1 and XRCC4 homotypic interactions.

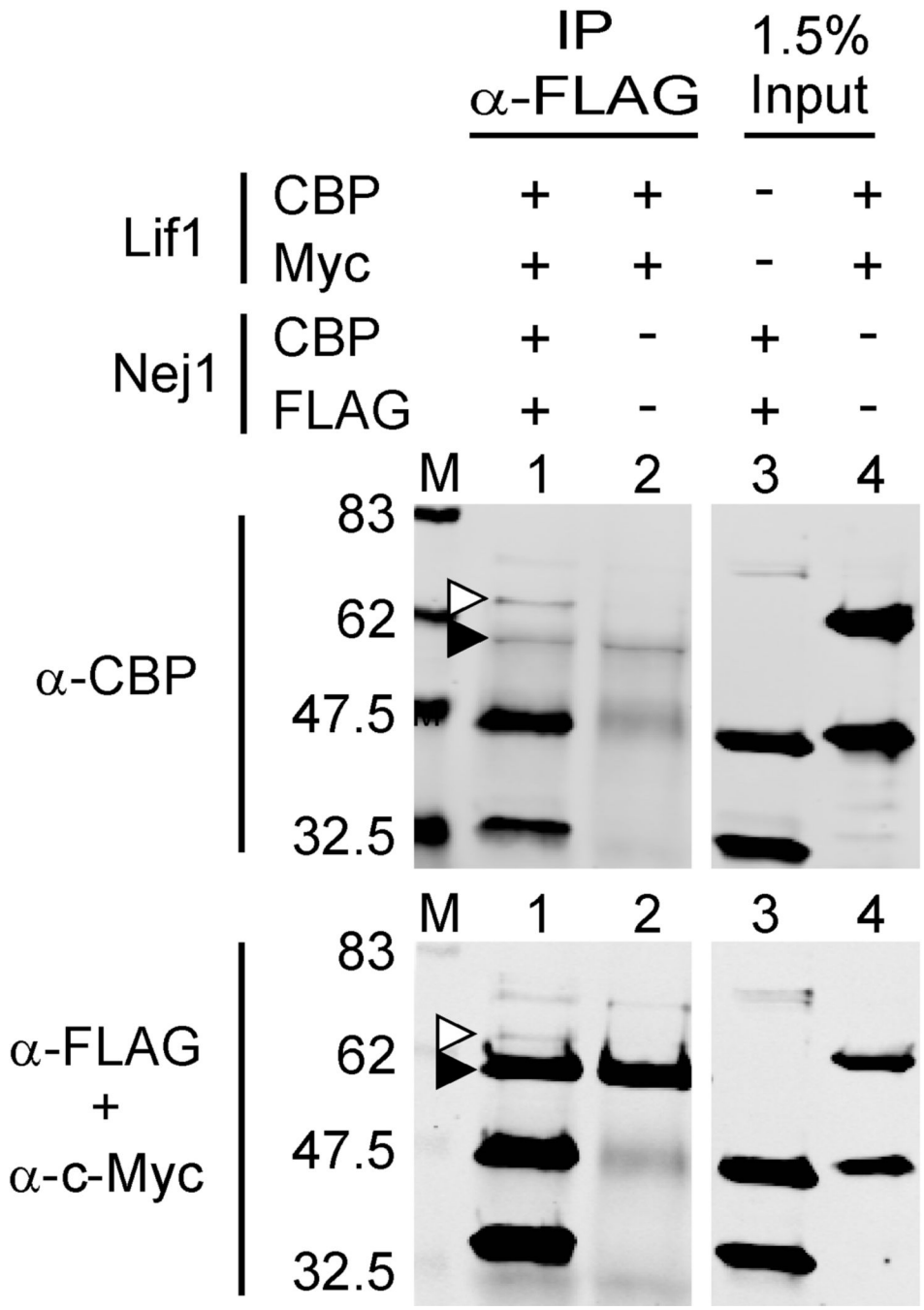

Figure 6. Direct interaction between Nej1 and Lif1 homodimers

Differentially tagged proteins were co-expressed in diploid yeast to allow the formation of dimers containing CBP-Nej1::FLAG-Nej1 and, in a separate strain, CBP-Lif1::Myc-Lif1. Nej1 and Lif1 were purified in parallel from these strains in a first step using calmodulin agarose. The eluted Nej1 and Lif1 fractions were mixed and then immunoprecipitated with α-FLAG antibody to pull down Nej1 dimers and any associated Lif1. Immunoblotting was subsequently performed against all tags. Open triangles indicate the upper Lif1 band, migrating just above the immunoglobulin band marked with filled triangles, which can be visualized separately from Nej1 and that specifically co-precipitated with Nej1. Importantly, any Nej1 or Lif1 molecule tagged with FLAG or Myc is inferred to be in a homodimer with a CBP-tagged Nej1 or Lif1

molecule, respectively, because it bound to calmodulin agarose in the first purification step. Co-immunoprecipitation thus likely represents interaction of Nej1 dimers with Lif1 dimers.

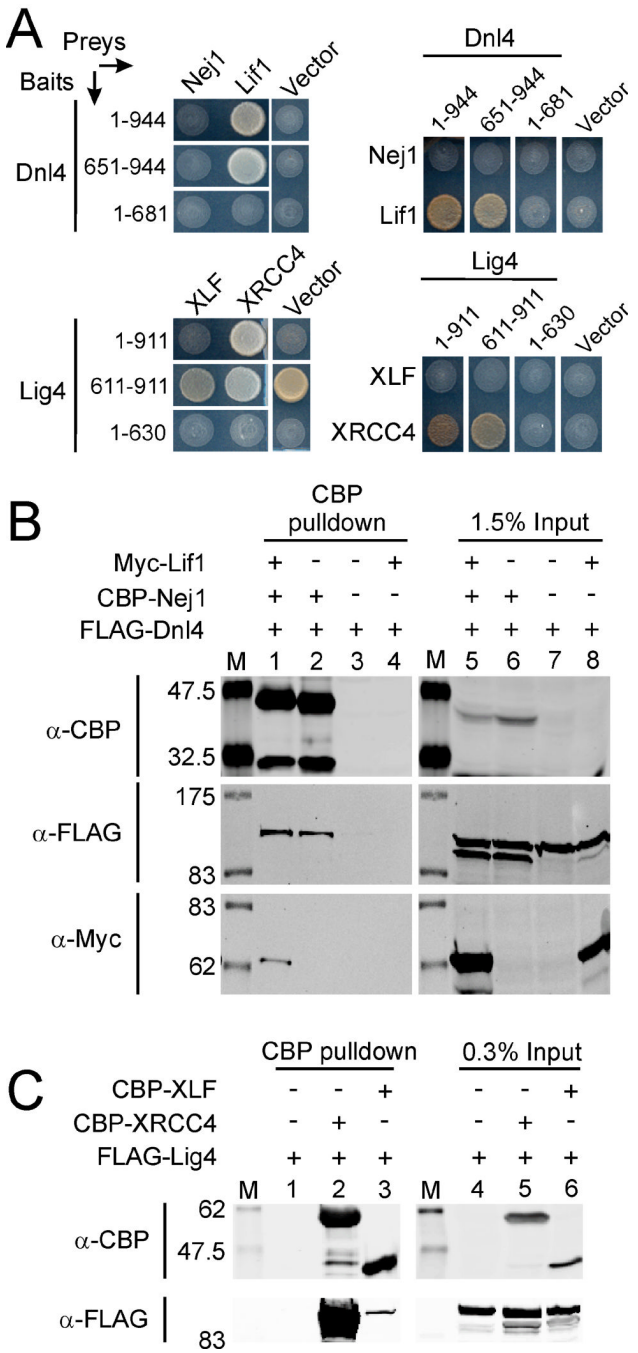

Figure 7. Direct interaction between XLF/Nej1 and Lig4/Dnl4 in pull-down but not two-hybrid analysis

(A) Two-hybrid analysis was performed similar to Figure 3 using XLF/Nej1, XRCC4/Lif1 and various fragments of Lig4/Dnl4. Histidine selective indicator medium is shown. In both orientations, a strong XRCC4/Lif1-Lig4/Dnl4 interaction was detected that depended on the C-terminal BRCT domains of Lig4/Dnl4, but no XLF/Nej1-Lig4/Dnl4 interaction was detected. **(B)** CBP-Nej1, Myc-Lif1 and FLAG-Dnl4 were co-expressed from plasmids as indicated in haploid yeast that carried a chromosomal *lif1*Δ allele. Pull-down of CBP-Nej1 using calmodulin agarose was followed by immunoblotting for all tags. CBP-Nej1 was able to pull down FLAG-Dnl4 regardless of the presence of Lif1, although with low efficiency.

(C) Human proteins were expressed in haploid yeast and pulled down as in (B). Similar to yeast proteins, CBP-XLF was able to pull down FLAG-Lig4 in the absence of XRCC4, although with substantially less efficiency than CBP-XRCC4.