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The properties of the RNA-binding protein NF90 are considerably modulated by complex formation with NF45

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ABSTRACT

Nuclear factor 90 (NF90) is an RNA-binding protein (RBP) that regulates post-transcriptionally the expression of various mRNAs. NF90 was recently shown to be capable of discriminating between different RNA substrates. This is mediated by an adaptive and cooperative interplay between three RNA-binding motifs (RBMs) in the protein’s C-terminus. In many cell types, NF90 exists predominantly in a complex with nuclear factor 45 (NF45). Here, we compared the RNA-binding properties of the purified NF90 monomer and the NF90-NF45 heterodimer by biophysical and biochemical means and demonstrate that the interaction with NF45 considerably affects the characteristics of NF90. Along with a thermodynamic stabilization, complex formation substantially improves the RNA-binding capacity of NF90 by modulating its binding mode and by enhancing its affinity to single- and double-stranded RNA substrates. Our data suggest that features of both the N- and C-termini of NF90 participate in the heterodimerization with NF45 and that the formation of NF90-NF45 changes the conformation of NF90’s RBMs to a status in which the cooperative interplay of the RBMs is optimal. NF45 is considered acting as a conformational scaffold for NF90’s RBMs, which alters the RNA-binding specificity of NF90. Accordingly, the monomeric NF90 and the NF90-NF45 heterodimer may exert different functions in the cell.

Summary statement
Interaction of NF45 with NF90 leads to conformational rearrangements of the RNA-binding motifs of NF90, which changes the affinities and binding modes of NF90 to different RNA substrates. Our data suggest the monomeric NF90 and the NF90-NF45 complex functioning differently.

Short title
NF45 modulates NF90 activity

Keywords
NFAR-1, ILF-3, RBP, RNA-protein interactions, dsRBM, RGG, post-transcriptional regulation
Abbreviations

INTRODUCTION

Dynamic interactions of RNA-binding proteins (RBPs) with coding and non-coding RNAs contribute significantly to the regulation of mammalian gene expression. For example, the binding of RBPs to mRNAs may decisively affect their processing, subcellular distribution, half-life or translation rate.[1] A characteristic feature of RBPs involves the ability to associate with a large repertoire of RNA molecules. Yet, with certain RNA ligands, the proteins display a marked binding specificity. Crucial determinants of the conformational flexibility of RBPs are a small number of RNA-binding modules. These may be present in several copies and/or different arrangements within the proteins.[2] While there is a paucity in our knowledge regarding how RBPs discriminate between different RNA molecules, there is increasing evidence that binding-specificity and binding-affinity are determined by a close functional interplay of the RBMs and by multiple and flexible interactions of these motifs with the substrate.[3, 4]

Prominent RBMs are double-stranded (ds) RNA-binding motifs (RBM), dsRBMs, and arginine-glycine-rich RGG/RG motifs. dsRBMs are conserved domains with a typical size of 65-70 amino acids (aa) that fold into a $\alpha\beta\beta\alpha$ conformation and exhibit a particularly high affinity for dsRNA.[5-9] The fold and RNA-binding properties of dsRBMs are decisively determined by a set of highly conserved residues, e.g., a conserved phenylalanine located in the N-terminal part of the central $\beta$-sheet.[6, 10-12] With RBPs containing several dsRBM copies, these may act jointly during RNA binding, though different motifs may display different RNA-binding activities. This was first observed during studies on isolated tandem dsRBMs of the RBPs ADAR2 (adenosine deaminase acting on RNA) and PKR (protein kinase RNA-activated).[8, 13-15] RGG/RG motifs, which commonly consist of several copies of arginine and glycine repeats [16], interact with different types of nucleic acids, preferentially with single-stranded (ss) RNA.[16-19] The binding activity of RGG/RG motifs may be regulated by post-translational modifications like methylation or phosphorylation, but also by interacting proteins.[20, 21]

Nuclear factor 90 (NF90), also termed NFAR-1, DRBP76, MPP4, or TCP80, is a multifunctional protein [22], which regulates gene expression at several levels. NF90 was reported to be involved in transcription, [23, 24] to interact with the nuclear export machinery [25], and to affect translation [26, 27] [28] and miRNA biogenesis.[29] Along with these and other functions, NF90 undergoes multiple interactions with
other proteins.[30] For example, by binding to PKR, NF90 was shown to modulate the antiviral interferon response.[31-33]

The best established role of NF90 acting as an RBP is its interaction with the 3' untranslated regions (UTRs) of various mRNAs, whereby it modulates their turnover and expression levels.[34-37] Additionally, NF90 was found to bind to the genomes of positive-strand RNA viruses such as bovine viral diarrhea virus, hepatitis C virus and Dengue virus. These viruses recruit NF90 as a host factor that supports their replication by yet undefined mechanisms.[38-42]

NF90 is encoded by the human ILF3 gene locus. Alternative splicing in the pre-mRNA’s exon 14 generates two NF90 isoforms, NF90a (702 aa) and NF90b (706 aa), which differ by a NVKQ insert in the C-terminus (Fig. 1A).[43] NF90a, b contain a bipartite nuclear localization signal (NLS) and an N-terminal DZF (domain associated with zinc fingers) that functions as an interaction site for other DZF-containing proteins (see below).[44] Moreover, NF90a, b comprise three RBMs, a tandem pair of dsRBMs (dsRBM-1 and dsRBM-2) and an RGG/RG motif, all which are essential for RNA binding and are localized in the otherwise unstructured C-terminus of the protein (Fig. 1A).[45-47]

Recently, we systematically examined the RNA-binding mechanisms of purified NF90, analyzing specifically the activities of the dsRBM and RGG/RG motifs.[45] The two dsRBMs participate very differently in RNA binding. While dsRBM2 is a major determinant in the association of NF90 with dsRNA, the impact of dsRBM1 on RNA binding is minimal. Nevertheless, both dsRBMs contribute to the binding of NF90 to the same RNA molecule, which occurs cooperatively and simultaneously. These findings fit with crystal structure data of the tandem dsRBMs of NF90 (aa. 393 – 592) in complex with an 18 bp dsRNA, which suggests similar dsRBM-RNA interaction interfaces of the NF90 and ADAR2 dsRBMs.[48] The NF90 RGG/RG motif was indicated to crucially determine the overall RNA binding properties of NF90. That is, the immediate C-terminus of the protein including the RGG/RG motif is indispensable for interactions of the protein with ssRNA. Moreover, the NF90 C-terminus is involved in conformational changes of the protein during RNA binding, with the RGG/RG motif acting as a central regulatory element.

In sum, these studies revealed a dynamic, cooperative and coordinated action of the three motifs in the RNA-binding process of NF90, which enables the protein to distinguish different features of target RNA ligands.[45]
In most investigated cell types, NF90 was not detected as an individual protein but was tightly complexed with the nuclear factor 45 (NF45; also known as ILF2). NF45, which comprises 390 aa, contains an incompletely characterized RGG/RG motif in the N-terminus and a DZF domain. The DZF domain was shown to be a major molecular determinant of complex formation by interacting with the structurally similar domain of NF90 (Fig. 1A).[44, 47, 49] This was confirmed by a crystal structure of the N-terminal part of NF90 (aa. 1-380) with NF45 (aa. 29–390) lacking its N-terminus.[44] In the majority of cell types, NF90 and NF45 are localized predominantly in the nucleus. However, the proteins shuttle between nucleus and cytoplasm, and the transport of NF90 was indicated to be regulated by RNA binding and phosphorylation.[50-53] While it is currently uncertain whether NF90 and NF45 function as monomers, as a complex, or in both forms, the available data support the idea that many of the functions of NF90 mentioned above may be mediated by the NF90-NF45 complex.

In this study, we compared the properties of the monomeric NF90 and the NF90-NF45 complex. The results suggest that NF90, while forming a heterodimer with NF45, undergoes conformational alterations in its C-terminus that improve the structural orientation and functional interplay of its RBMs. In agreement with this notion, complex formation with NF45 significantly affects the RNA-binding properties of NF90.
MATERIAL AND METHODS

Plasmid constructs. The cDNA sequence encoding NF90b (*H. sapiens*) was cloned into a pET-21(a)+ vector, the cDNA sequence encoding NF45 was cloned into a pETSUMO vector. Both vectors were used for heterologous synthesis of the proteins in *Escherichia coli*. The NF90b variants were generated by site-directed mutagenesis and PCR (Table S1) as described earlier.[45]

Expression and purification of proteins. The cDNA sequence encoding NF90b and NF45 were expressed in *E. coli* BL21-CodonPlus® (DE3)-RP. Biomass production was carried out using fermentation. Protein synthesis was induced by adding 1 mM isopropyl-β-1-thio-D-galactopyranoside. NF90b, its variants and NF45 were isolated and purified from inclusion bodies according to Schmidt *et al.*[45] Briefly, the cells were harvested, centrifuged, re-suspended in 0.1 M Tris/HCl pH 7.0, 1 mM EDTA and lysed using a French Press homogenizer. Following DNase I treatment and incubation with Triton X-100, the inclusion bodies (IB) were collected by centrifugation and washed. The isolated IB fractions of NF90b and NF45 were re-solubilized using 8 M or 6 M urea as denaturant, respectively, and clarified by ultracentrifugation.

The denatured NF90 solution was fractionated by cation exchange chromatography (CEC, GE Healthcare), and the bound protein eluted by applying an ionic strength gradient.[45]

Re-solubilized SUMO-NF45 was applied to a cation-exchange chromatography column (MonoS, GE Healthcare) at pH 3.0, followed by an anion exchange chromatography step (HiLoad Q-Sepharose, GE Healthcare). Pooled fractions of SUMO-NF45 were refolded using a high ionic strength buffer (50 mM sodium phosphate, pH 7.2, 0.5 M NaCl) prior to cleavage of the SUMO-fusion by SUMO-protease. Since the authentic NF45 tended to precipitate under these conditions, the protein solution was adjusted to 6 M GuHCl and applied to an affinity chromatography (HisTrap, GE Healthcare) in order to remove un-cleaved material. The NF45 solution was then used for the following refolding procedure to yield the native protein.

For the final refolding of NF45 and NF90b as well as its variants, purified fractions of denatured protein were slowly dropped into, and rapidly diluted in, refolding buffer [50 mM Na-phosphate, 100 mM NaCl, 5 % (v/v) glycerol, 500 mM arginine, 10 mM DTT, pH 7.2]. The final dilution factor was at least 10-fold and the final protein concentration was less than 0.1 mg/mL. After refolding, the proteins were further purified.
by affinity- and size-exclusion chromatographies. Final protein fractions were concentrated and aliquots stored in assay buffer [50 mM Na-phosphate, 100 mM NaCl, 5 % (v/v) glycerol, 1 mM TCEP, pH 7.2] at -80 °C. The oligomerization state was addressed by analytical ultracentrifugation. The protein concentration of NF90b was determined by measuring the absorbance at 280 nm using $\varepsilon_{280} = 44350$ M$^{-1}$ cm$^{-1}$. For NF45 $\varepsilon_{280} = 26930$ M$^{-1}$ cm$^{-1}$ was used. The extinction coefficients were calculated using the ExPASy server.[54] To estimate the theoretical 280 nm / 260 nm ratio of the recombinant proteins, we calculated their $\varepsilon_{260}$ using extinction coefficients at 260 nm for Trp, Tyr and Phe from the PhotoChemCAD database.[55, 56] NF45 contains 3 Trp, 15 Phe and 7 Tyr giving $\varepsilon_{260} = 15583$ M$^{-1}$ cm$^{-1}$. NF90 contains 4 Trp, 15 Tyr and 15 Phe giving $\varepsilon_{260} = 26584$ M$^{-1}$ cm$^{-1}$. The final preparations used for the functional assays were stable during the period of the assays (data not shown).

**Reconstitution of NF90b-NF45.**

For the formation of the NF90b-NF45 complex and variants thereof, equimolar amounts of CEC-purified NF90b and NF45 (after cleavage and HisTrap) were mixed and subsequently refolded together following the approach for the refolding of the monomeric NF90.[45] Fractions containing the complex were concentrated and aliquots stored in assay buffer at -80 °C. The oligomerization state was determined by analytical ultracentrifugation. The protein concentration was determined by measuring the absorbance at 280 nm using $\varepsilon_{280} = 71280$ M$^{-1}$ cm$^{-1}$. The extinction coefficients were calculated using the ExPASy server.[54] To estimate the theoretical 280 nm / 260 nm ratio of NF90b-NF45, we calculated its $\varepsilon_{260}$ using extinction coefficients at 260 nm for Trp, Tyr and Phe from the PhotoChemCAD database.[55, 56] Heterodimeric NF90b-NF45 contains 7 Trp, 30 Phe and 22 Tyr; this resulted in $\varepsilon_{260} = 42167$ M$^{-1}$ cm$^{-1}$.

**Analytical ultracentrifugation.** Sedimentation velocity and equilibrium measurements were performed at 128794 x g (40000 rpm) and 5152 x g (8000 rpm) in an analytical ultracentrifuge XL-A (Beckman Instruments, Inc., Fullerton, CA) using double-sector cells and an An50Ti rotor. All centrifugation experiments were performed at 20 °C. Analyses were carried out at a protein concentration of 4.2 - 11 µM in assay buffer. The data obtained were analyzed using the Sedfit program.[57]
**Circular dichroism.** Experimental details are essentially described by Schmidt et al.[45] Briefly, measurements were performed with a Jasco J-810 spectropolarimeter using the following instrumental setup: 0.5 nm pitch, 40 accumulations, 50 nm/min scanning speed, 1 nm slit widths, response time 1 s. All experiments were carried out in assay buffer at 20 °C. Far-UV circular dichroism spectra were recorded at a protein concentration of 10 µM using cuvettes with optical path lengths of 0.1 mm. Acquired protein spectra were corrected for buffer contribution using the Spectra Manager I software (Jasco). The data were converted to mean residue ellipticity Θ_{MRW}.

**Intrinsic fluorescence.** Fluorescence spectra were recorded on a Fluoromax-4 Spectrofluorometer (Jobin Yvon, France) using 3 µM of protein in assay buffer. The excitation monochromator was set to 280 nm or 295 nm, respectively. The excitation and emission slit widths were set to 1 nm and 5 nm, respectively, and the scanning speed was 100 nm/min with a response time of 1 s. At least five spectra were measured, averaged and normalized to the fluorescence intensity of NF90b at 343 nm.[45]

**Chemical unfolding / refolding**

Chemical denaturation of the proteins was induced by incubation of 3 µM protein in assay buffer supplemented with indicated concentrations of a denaturant composition (GuHCl and urea in equal ratio) for 24 h at 8 °C. Each sample buffer condition was prepared using a Hamilton Diluter. After incubation, the circular dichroism at 225 nm of each sample was monitored using a Jasco J-810 spectropolarimeter with the following instrumental setup: time course of 60 s, λ = 225 nm, 4 s response, 1 s data pitch, 2 accumulations, 1 nm slit widths and standard sensitivity using 1 mm cell path. A monitoring wavelength of 225 nm was chosen because in pilot experiments this wavelength displayed the best signal quality at high ionic strength that was supplemented with the highest denaturant concentration. All transitions were carried out at 20 °C. The data were analyzed and quantified according to a three-state model (**Equation 1**) regarding an equilibrium, in which an accumulation of an intermediate is considered (see below).[58] For refolding transitions, native protein fractions were first completely unfolded by incubation in 10 M of denaturant (see above) composition for 24 h, and then used for sample preparation as described above.

\[
U \leftrightarrow I \leftrightarrow N
\]
F_{obs} = F_N + F_I \cdot e^{\frac{\Delta G_{N-I}^0 \cdot c_{N-I}[D]}{RT}} + F_U \cdot e^{\frac{\Delta G_{I-U}^0 \cdot c_{I-U}[D]}{RT}} \cdot e^{\frac{\Delta G_{N-I}^0 \cdot c_{N-I}[D]}{RT}} + e^{\frac{\Delta G_{I-U}^0 \cdot c_{I-U}[D]}{RT}} \cdot e^{\frac{\Delta G_{N-I}^0 \cdot c_{N-I}[D]}{RT}}

F_{obs}, F_N, F_I and F_U are the observed signal amplitudes - (N) of the native, (I) intermediate and (U) unfolded state, respectively. $\Delta G_{x-y}^0$ represents the change in free energy of the indicated transition x-y. [D] is the denaturant concentration, $R$ is the gas constant, $T$ is the absolute temperature and $c_{x-y}$ is the slope at the transition midpoint and a measure for the cooperativity of the respective transition.

**Substrates used in the nucleic acid-binding studies.** Fluorescently labeled oligonucleotides were purchased from IBA GmbH (Göttingen, Germany). The RNA molecules were labeled with 6-carboxyfluorescein at the very 5'-nucleotide (Table 1). The concentrations were determined by absorbance at 260 nm using the respective extinction coefficients. Double-stranded nucleic acids were prepared by mixing the corresponding single-strands in equimolar ratio, heating to 95 °C, subsequent cooling and final purification by non-denaturing PAGE before storage at -80 °C. The RNA molecules that were used in this study are summarized in Table 1.

**Measurement of RNA-binding constants and binding mode analysis (LEM).** Experimental details are essentially as described by Schmidt et al. [45] The protein of interest was added to 5′FAM-EX-labeled RNA (25 – 300 nM) in assay buffer supplemented with the indicated concentrations of NaCl. Fluorescence changes were monitored on a Fluoromax-4 Spectrofluorometer (Jobin Yvon, France) at 20 °C. After attaining equilibrium, the signal amplitudes of the 5′FAM-EX-labeled RNAs were measured (excitation at 491 nm, emission at 515 nm) and corrected for the volume change. Fluorescence intensities relative to the starting fluorescence were plotted against the protein concentration. Fitting the binding isotherms according to Equation 2 [59-61] with the program KaleidaGraph™ (Synergy software) yielded the $K_D$-values of the interaction of the protein and the labeled RNA (see supplemental material for the

$$
\Delta F = 1 + \gamma \cdot \left( [\text{RNA}]_0 + [\text{protein}]_0 + K_D \right) - \sqrt{\left( [\text{RNA}]_0 + [\text{protein}]_0 + K_D \right)^2 - 4 \cdot [\text{RNA}]_0 \cdot [\text{protein}]_0} 
$$

(2)
mathematical derivation). $\Delta F$ is the relative change in fluorescence intensity, $\gamma$ is the signal amplitude. $[\text{RNA}]_0$ and $[\text{protein}]_0$ are the total RNA and protein concentrations, respectively; $K_0$ is the dissociation constant.

The equilibrium constants of the RNA-protein complexes in dependence on the salt or urea concentration, respectively, were determined and analyzed according to a linear free energy relationship (LFER).[62-64] The logarithm (ln) of the association constants $K_A$ at the different NaCl or urea concentrations were plotted versus the concentration of sodium chloride or urea to avoid data clustering (Equation 3 and Equation 4). In the applied narrow concentration rage of NaCl this simplified linear free energy extrapolation (LEM) [65] approach turned out to be valid.

$$\Delta G_{\text{bs}}^0 = -RT \cdot \ln(K_A \cdot 1 \text{ M})$$

$$\Delta G_{\text{bs}}^0 = \Delta G_0^0 - m \cdot [\text{BS}]$$

$$\ln(K_A \cdot 1 \text{ M}) = \frac{m}{RT} \cdot [\text{NaCl}] - \frac{\Delta G_0^0}{RT}$$

$$\ln(K_A \cdot 1 \text{ M}) = \frac{m'}{RT} \cdot [\text{urea}] - \frac{\Delta G_0^0}{RT}$$

$\Delta G_0^0$ and $\Delta G_{\text{bs}}^0$ represent the changes in free energy at the ionic strength of the assay buffer at 0 M NaCl or 0 M urea, respectively, and at the indicated concentration of the buffer supplement (BS) NaCl or urea, respectively. $R$ is the gas constant, $T$ is the absolute temperature, $m$ and $m'$ reflect the dependencies on the ionic strength or the urea concentration, respectively. 1 M is the standard molar concentration to eliminate units. All shown measurements were carried out at least in triplicates. Errors of the $m$ or $m'$ values obtained from the fitting routine were in the range of 15%.
Results

Preparation of NF45, NF90b and of the NF45-NF90b heterodimer. To reconstitute the functional NF90b-NF45 complex, it was most important to generate homogeneous preparations of the authentic, full-length NF90b and NF45 proteins. Likewise, to enable unequivocal interpretation of mechanistic RNA-protein interaction data, the proteins had to be free of nucleic acids (NA). During the subsequent experiments, we used the largest NF90 isoform, NF90b, and to prepare it we took advantage of a previously established purification strategy in which NF90b and variants thereof were generated from inclusion bodies (IB), depleted of NA and refolded.[45] A comparable strategy was used for NF45, which was produced as a SUMO (small ubiquitin-like modifier)-fusion protein and purified from IB after heterologous synthesis in E. coli. The contaminating NA were removed by cation exchange chromatography under denaturing conditions and low pH. Subsequently, the fusion-protein was refolded and the SUMO-portion removed, which yielded NF45 with an authentic N-terminus. The protein was further purified by affinity- and size-exclusion chromatographies (see Material and Methods and Fig. 1A, B). The near-UV absorbance spectra of the proteins displayed 280 nm / 260 nm ratios of about 1.8 (NF90b) and 1.7 (NF45), respectively (Fig. S1A). These corresponded closely to the theoretical value of 1.7 indicating negligible NA contamination. Analytical ultracentrifugation demonstrated the homogeneity of the purified monomeric NF90b [45] and NF45 proteins in assay buffer (Fig. S1B).

The NF90b-NF45 complex was generated by a ‘rapid-dilution-pulse-refolding’ approach, whereby the purified and denatured NF90b and NF45 proteins were refolded in an equimolar mixture of both protein preparations using the same protocol as with the individual components (see Materials and Methods for details). The purity of the complex was initially estimated by SDS-PAGE (Fig. 1B); its near-UV absorbance spectrum displayed a 280 nm / 260 nm ratio of about 1.8 indicating the same level of purity as was achieved for the individual proteins (see Fig. 1C and above). The homogeneity of the reconstituted NF90b-NF45 complex was confirmed by analytical ultracentrifugation (Fig. 1D). Moreover, these data demonstrated that under the applied assay buffer conditions NF90b-NF45 exists exclusively as a heterodimer. Reconstitution of the authentic complex with both full-length proteins (NF90b - 706 aa; NF45 - 390 aa) accordingly verified the heterodimeric status of the NF90b-NF45 complex, which was earlier suggested by immunoprecipitation studies [49] and by the crystal structure of a truncated complex, NF90b1.
Formation of the NF90b-NF45 heterodimer is associated with structural changes of NF45 and NF90b. The formation of a protein complex often correlates with considerable changes in the biological function(s) of its individual components. Assuming such a close structure/activity-relationship also for NF90b-NF45, we examined the heterodimer for conformational changes in comparison to the individual NF90b and NF45 proteins. For this, we first measured the far-UV circular dichroism (CD) spectra of the purified NF45, NF90b and of the reconstituted NF90b-NF45 (Fig. 2A; red, blue, black). To assess the situation of the NF90b-NF45 complex also in the absence of conformational changes, we calculated a reference spectrum that considered the contributions of the spectra of the individual NF90b and NF45 and an equimolar ratio of both proteins (Fig. 2A; purple). Interestingly, the profiles of the far-UV CD spectra of all protein species generally resembled the characteristics of the spectrum of the individual NF90b protein (see Fig. 2A and [45]). That is, the spectra of NF45 and NF90b as well as that of NF90b-NF45 showed two negative maxima at 208 and 225 nm and a positive maximum at about 190 nm indicating considerable helical content and other pronounced secondary structural elements (Fig. 2A; see also Discussion). However, in comparison to the reference that was calculated from the individual components, the measured spectrum of the NF90b-NF45 heterodimer revealed a pronounced decrease in signal intensity at \( \lambda < 200 \text{ nm} \) (Fig. 2A; compare black and purple traces). This was a first indication that the formation of the NF90b-NF45 heterodimeric complex is associated with changes in the protein’s secondary structures.

As a second means to detect potential protein structure changes during NF90b-NF45 complex formation, we measured the intrinsic fluorescence of identical concentrations of NF45, NF90b and NF90b-NF45 applying excitations at 280 nm (Fig. 2B) and 295 nm (Fig. 2C). As above, the obtained data were compared with calculated reference spectra (Fig. 2B and C; purple). The shape of a protein’s fluorescence spectrum essentially depends on the intramolecular arrangement of the fluorophores tyrosine (Tyr) and tryptophan (Trp) and on the fluorescence resonance energy transfer (FRET) between them. Considering that the distance between the participating intramolecular amino acids (aa) residues
represents a major determinant of the intrinsic FRET, variations in the microenvironment of the aromatic aa residues and of the ‘solvent-accessible surface area’ (SASA) should lead to changes in the fluorescence signal. As shown in Fig. 2B and C, at both excitation wavelengths, the reconstituted NF90<sub>b</sub>-NF45 complex showed an evident quenching of the combined Tyr / Trp or Trp-only intrinsic fluorescence in comparison to the reference spectra (compare black and purple traces). Furthermore, the maxima of the measured fluorescence spectra of the heterodimer were blue-shifted, which pointed to a more polar environment of fluorophores of the heterodimer in comparison to the reference, and, accordingly, to a decrease in the SASA. Both types of spectroscopy data hence suggested conformational changes of the tertiary structure of NF45 and/or of NF90<sub>b</sub> during formation of the heterodimer.

Heterodimer formation increases the thermodynamic stability of NF45 and NF90<sub>b</sub>. Owing to the release of binding energy, the formation of a protein complex may result in a higher thermodynamic stability of its components. To investigate NF90<sub>b</sub> and NF45 for changes in the thermodynamic stability during heterodimerization, we conducted a chemical equilibrium unfolding of the individual, purified proteins and of the reconstituted NF90<sub>b</sub>-NF45 heterodimer (see Material and Methods). Following an incubation period of 24 h at different concentrations of denaturant, the signal amplitudes of the protein’s CD were monitored at 225 nm (Fig. 2D; Table 2). As shown above, we calculated a reference unfolding transition considering the simultaneous unfolding of both individual monomers in the absence of NF90 / NF45 interactions (Fig. 2D; purple). The folding of the proteins was reversible; this was indicated by the purification strategy and by a refolding experiment that was carried out with the monomeric NF90<sub>b</sub> (Fig. S2A). Most interestingly, the transition curves proceeded in a biphasic manner displaying two transition midpoints (Fig. 2D; Table 2) at concentrations between 1.5 and 3 M of the denaturant. The folding pathway of the monomeric NF90<sub>b</sub> and NF45, as well as that of the NF90<sub>b</sub>-NF45 heterodimer, comprised the population of at least one stable intermediate. NF90<sub>b</sub> and NF45 shared the first transition ([D<sub>50%</sub>] = 0.46 M, Table 2), while, in comparison with both the individual proteins and the reference, the heterodimer’s first transition was shifted to higher concentrations of denaturant ([D<sub>50%</sub>] = 1.19 M; Table 2). In the second transition, NF90<sub>b</sub> and NF45 proceeded with different midpoints (Table 2) while the measured folding transition of the heterodimer corresponded exactly to the optical contributions of the individual components that were considered in the reference (Fig. 2D; compare black and purple traces). To determine the thermodynamic stabilities of
NF90_b and of NF45, data investigations were performed applying a three-state folding model of a monomer (see Equation 1 in Material and Methods) that considers the population of a stable intermediate (Fig. 2D).[58] This revealed overall thermodynamic stabilities of NF90_b and of NF45 that were in the range of $\Delta G_{N-U}^{0} \approx 4.1$ kcal mol$^{-1}$ (N, native state; U, unfolded state; see Table 2).

To evaluate the thermodynamic stability of a complex, an association / dissociation step and the concentrations of the monomeric components need to be considered.[66] Thus, to calculate the thermodynamic stability of the heterodimeric NF90_b-NF45 complex, it was crucial to determine the association / dissociation step in the complete transition. Performing analytical ultracentrifugation and size-exclusion chromatography, we observed that the heterodimer does not dissociate during the first transition (Fig. S2B). The association / dissociation step of the heterodimer was accordingly assumed to occur during the second transition in the fully denatured state. However, as outlined, the signal amplitude of the second transition was composed essentially of the optical contributions of the individual components where the folding was determined to proceed according to a three-state model without an association / dissociation step (see above). In conclusion, the oligomerization during the heterodimer’s folding process was not directly discernable; for that reason, we focused the quantitative analysis on the first transition only. In comparison to the individual NF90_b and NF45, this revealed an increase in the thermodynamic value of the heterodimer NF90_b-NF45 by about 1.0 kcal mol$^{-1}$ ($\Delta\Delta G_{N-I}^{0}$; Table 2; N-native state; I-intermediate state) demonstrating that the formation of the complex leads to a stabilization of structural elements of the NF90_b and NF45 components.

The RNA binding properties of the NF90_b-NF45 complex are considerably improved. As explained, there is significant evidence that NF90_b and / or the NF90_b-NF45 complex are important players in post-transcriptional gene regulation that affect the expression levels of several cellular mRNAs. Accordingly, it was important to figure out, if NF90_b-NF45 complex formation had an impact on RNA binding. NF45 does not bind RNA effectively ([44, 53]; see Fig. S3A). In contrast, NF90_b binds RNA with high affinity ($K_d$ in the nM range), particularly dsRNA ([45]; see also Table 3).

To examine the RNA-binding properties of NF90_b-NF45, we took advantage of a quantitative fluorometric binding assay that was previously established to characterize the RNA-binding properties of the monomeric NF90_b.[45] Three 5'-FAM-EX-labeled model RNA substrates were used: a randomly
composed 16-nucleotide single-stranded oligonucleotide (ssRNA), a partly double-stranded version of ssRNA containing 13 bp (dsRNA-1), and an elongated version of dsRNA-1 containing 33 bp (dsRNA-2) (Table 1). These RNA molecules exemplify several basic features of RNA substrates, namely short, longer, single- and double-stranded RNA structures. Importantly, the sequence composition of the ssRNA does not support the formation of base-pairs, and the sequence composition of the dsRNAs does not enable the formation of base-pairs other than the intended ones. In fact, all three RNA substrates were certain not to undergo conformational transitions under the chosen experimental conditions (see [45] and below).

Active-site titrations with purified NF90\textsubscript{b} and dsRNA-1 or dsRNA-2 had earlier confirmed that the binding of both RNAs occurs in an equimolar manner; one molecule of NF90\textsubscript{b} binds to one RNA molecule, independently of its length.[45] In pilot experiments, we confirmed this to be the case also with the NF90\textsubscript{b}-NF45 complex (Fig. S3B). This permitted a direct comparison of the binding activities of NF90\textsubscript{b} and of the complex, and it enabled the use of Equation 2 (see Material and Methods) for the interpretation of the binding studies with the different 5'-FAM-EX-RNAs.

Binding experiments of NF90\textsubscript{b}-NF45 with each of the RNA substrates revealed that under standard conditions (assay buffer, 20° C; see Material and Methods and [45]) the heterodimer displayed the highest affinity for the dsRNA-2 ($K_D = 2$ nM), followed by dsRNA-1 ($K_D = 20$ nM) and ssRNA ($K_D = 110$ nM, see Table 3 and below). Thus, compared to NF90\textsubscript{b}, the NF90\textsubscript{b}-NF45 heterodimer turned out to show a similar affinity for dsRNA-2, while the affinities to ssRNA-1 and dsRNA-1 were about 10-fold higher.

Next, we investigated the specific RNA binding modes, i.e. the different sets of ionic and non-ionic contacts (see below and [45]), of NF90\textsubscript{b} and NF90\textsubscript{b}-NF45 to the different RNA substrates. For this, we analyzed the RNA-protein interactions systematically at increasing ionic strengths, i.e., at increasing concentrations of sodium chloride (example given in Fig. S3C; Fig. 3). Competing with the biopolymers’ hydration shell, the additional ions enhance hydrophobic interactions and perturb ionic contacts that support the formation of the protein-RNA complexes. The correlation of the association constant of the protein-RNA interaction and the applied concentration of sodium chloride is given by Equation 3, which describes a linear free energy extrapolation method (LEM; see Material and Methods). The $m$-value is a component of the slope of Equation 3 and, in this case, reflects the dependency of protein-RNA complex formation on the concentration of sodium chloride. The $m$-value was accordingly taken as a reference for
the “mode of binding” of the proteins to the respective RNAs. That is, a decrease in \( m \) (more negative slope) is indicative of an increase in the sensitivity of the protein–RNA interactions to the ionic strength and, accordingly, a more important role of ionic interactions in the formation of the respective protein-RNA complex. An increase in \( m \) (less negative slope) is indicative of a lower sensitivity to the ionic strength and suggests an increased relevance of hydrophobic interactions during formation of the protein-RNA complex. Note that a conformational impact of the salt conditions on the individual protein and RNA components could be excluded (see [45] and Fig. S3D).

The measurements of the \( m \)-values of the association of NF90\( \text{b} \)-NF45 to the three RNA ligands revealed that the heterodimer, like the NF90\( \text{b} \) monomer, showed different modes of binding to the different RNA substrates. The \( m \)-value of the binding of NF90\( \text{b} \)-NF45 to ssRNA was -14.4 kcal mol\(^{-2}\) L (vs. -24.8 kcal mol\(^{-2}\) L of NF90\( \text{b} \)), with dsRNA-1 -23.1 kcal mol\(^{-2}\) L (vs. -12.8 kcal mol\(^{-2}\) L of NF90\( \text{b} \)), and with dsRNA-2 -9.4 kcal mol\(^{-2}\) L (vs. -8.1 kcal mol\(^{-2}\) L of NF90\( \text{b} \); Table 3).

These data demonstrated that the NF90\( \text{b} \)-NF45 complex, like the NF90\( \text{b} \) monomer, is capable of specifically discriminating between different RNA substrates by forming different protein–RNA contacts. However, compared to NF90\( \text{b} \), the complex displayed a significantly higher level of ionic interactions with dsRNA-1 and a higher level of hydrophobic interactions with ssRNA. Conversely, with the longer dsRNA-2, NF90\( \text{b} \) and NF90\( \text{b} \)-NF45 showed comparable association constants and binding modes; actually, with dsRNA-2, the binding modes of NF90\( \text{b} \) and NF90\( \text{b} \)-NF45 displayed the highest fraction of hydrophobic interactions as compared to the other RNA substrates (Table 3). In conclusion, it can be stated that formation of the heterodimer considerably improves the capability of NF90\( \text{b} \) to interact with the ssRNA and the dsRNA-1 substrates. This is associated with changes in the binding modes of NF90\( \text{b} \) to these RNAs.

*Structural elements of NF90\( \text{b} \) that become stabilized during heterodimer formation are not involved in RNA binding.* We could show above that the formation of the NF90\( \text{b} \)-NF45 heterodimeric complex leads to a stabilization of structural elements within NF90\( \text{b} \) and/or NF45. Consequently, it was interesting to understand whether this stabilization correlated with the improved binding affinity of NF90\( \text{b} \)-NF45 to the ssRNA and dsRNA-1 substrates (Table 3). In view of the scenario that the complex does not dissociate during the first transition of chemical unfolding (Fig. 2D), we decided to perform a similar set of binding experiments with ssRNA-1 and dsRNA-1 as those shown above but using increasing concentrations of
urea. The concentration range of urea was chosen such that it covered the first transition during the denaturation process (see also Fig. S2B); the dsRNA-1 was assured not to denature under the conditions of the experiment (not shown). The data were investigated according to a modified LEM (see above; Equation 4). Since urea is a denaturant, the \( m \)-value (termed here \( m' \)) was in this case taken as a combined measure of the stability of the intrinsic secondary structural elements contributing to the binding event(s) and of the influence of the denaturant on the solvent shell of the interacting species.[64, 67] As shown in Fig. 4, the \( m' \)-values that were measured for the binding of NF90\(_b\) or NF90\(_b\)-NF45 to the ssRNA were nearly congruent, while in the case of the dsRNA-1, the heterodimeric complex showed a slightly increased sensitivity towards the treatment with urea. However, the overall binding to RNA was considerably less affected by the urea treatment than it was during treatment with sodium chloride (compare Fig. 3 and Fig. 4). These results revealed that the intrinsic structural elements that are involved in RNA binding apparently do not unfold during the first transition of chemical denaturation. The deficiency in the RNA interaction of NF90\(_b\)-NF45 that was detected at increasing urea concentrations was accordingly attributed to marginal reorientations and / or changes of the solvent shell. In contrast, we reasoned that the structural elements that are stabilized upon heterodimerization of NF90\(_b\)-NF45 are not involved in RNA binding and do not directly account for the improved RNA-binding properties of the complex.

Within NF90\(_b\)-NF45, the NF90 C-terminus is involved in interactions with NF45. Throughout our earlier studies with the monomeric NF90\(_b\), we generated and tested a series of protein variants. The variants were purified by the same protocol as used for the NF90\(_b\)wt protein, and all showed similar far-UV CD spectra indicating that the overall secondary structures remained unchanged with respect to that of NF90\(_b\)wt.[45] With one of these variants, NF90\(_b^{S651E}\), the Ser651 residue, which is part of the RGG/RG region, was modified by pseudo-phosphorylation. This modification causes a more than 50-fold reduction in the binding affinity of the protein to dsRNA-2, and we obtained initial experimental indications that it leads to local changes in the conformational properties of the RGG/RG region.[45]

To gain insights into the molecular features that caused the modified RNA binding properties of the heterodimeric complex, we generated the complex variant NF90\(_b^{S651E}\)-NF45 using the earlier established pulse-refolding approach. Complex formation was confirmed by analytical ultracentrifugation (not shown).
Interestingly, when we compared the far-UV CD spectra of the wt and pseudo-phosphorylated complexes, the spectrum of NF90_b^{S651E}–NF45 showed a significantly larger signal amplitude at λ < 200 nm (Fig. 5A; red). The trace of the spectrum of NF90_b^{S651E}–NF45 thus closely corresponded to that of the reference, the spectral contributions of the individual NF90_b^{wt} and NF45 components (Fig. 5A; compare red and purple traces). These data indicated that the formation of the NF90_b^{wt}-NF45 complex involves conformational changes, which apparently do not occur during the formation of the NF90_b^{S651E}–NF45 complex. Moreover, the observations suggested that within the heterodimer, the C-terminus of NF90_b is involved in interactions with NF45.

To address this further, we next compared the thermodynamic stabilities of the monomeric NF90_b^{wt} and NF90_b^{S651E} and of the corresponding wt and mutant heterodimer complexes applying the denaturation approach described. As shown in Fig. 5B and 5C, the chemical unfolding processes of NF90_b^{S651E} and of NF90_b^{S651E}–NF45 generally resembled those of the NF90_b^{wt} and NF90_b^{wt}-NF45. In all cases, we obtained the biphasic transition profile with two transition midpoints. However, with the monomeric NF90_b^{S651E}, the cooperativity (termed c-value; see Material and Methods) was less pronounced in the first transition and, in comparison to NF90_b^{wt}, the calculated thermodynamic stability, ΔG_{N-I}^{0}, was decreased by 0.64 kcal mol\(^{-1}\) (see Table 2). The second transition of NF90_b^{S651E} displayed a larger cooperativity (as indicated by the c-value), with a ΔG_{I-U}^{0} value that was increased by 1.41 kcal mol\(^{-1}\). In total, the pseudo-phosphorylated NF90_b variant shows a thermodynamic stability that was elevated by 0.77 kcal mol\(^{-1}\) vs. the wt protein.

With the NF90_b^{S651E}–NF45 heterodimer, the thermodynamic stability of the first unfolding transition closely resembled the value of the wt complex, though with a less pronounced signal amplitude (20% relative amplitude vs. 30% of the wt complex; Fig. 5C; red). The second unfolding transition corresponded to the individual contributions of NF90_b^{S651E} and NF45, which served here as a reference (Fig. 5C, purple).

Thus, in agreement with our earlier observations, [45] these findings pointed to local structural and / or stability alterations that are caused by the pseudo-phosphorylation of Ser651. Moreover, they suggested that the decrease in the thermodynamic stability of the first intermediate, which was monitored with the monomeric NF90_b^{S651E}, was compensated by the interaction with NF45 in the complex. The data further support the idea that within the heterodimer, the RGG/RG motif-containing C-terminus of NF90_b is involved in interactions with NF45 and that it also participates in the conformational changes that are
associated with complex formation. However, we obtained no indications that this interaction of the NF90\(_b\) C-terminus with NF45 contributes to the increase in the thermodynamic stability of the complex in comparison to the monomers (see Discussion).

*Formation of the NF90\(_b\)-NF45 heterodimer modulates the activity of the NF90\(_b\) RGG/RG motif.* As explained, we could previously demonstrate that the RGG/RG motif of monomeric NF90\(_b\) decisively determines the protein’s overall RNA-binding properties. The subsequent experiments should disclose whether and how a modified RGG/RG motif affected the interaction of the NF90\(_b\)-NF45 complex to ssRNA and dsRNA-1. ssRNA and dsRNA-1 are the substrates that are bound by the complex in a different binding mode and at significantly higher affinity, as it is the case with the monomeric NF90\(_b\) (Fig. 3; Table 3). Performing essentially the same analysis as above, we compared the affinities and binding modes of NF90\(_b\)\(^{S651E}\) and NF90\(_b\)\(^{S651E}\)-NF45 as well as of NF90\(_b\)\(^{wt}\) and the NF90\(_b\)\(^{wt}\)-NF45 complex to ssRNA and dsRNA-1 (see Fig. 6 and Table 3). As already described,[45] the monomeric NF90\(_b\) and NF90\(_b\)\(^{S651E}\) display a similar, low affinity for ssRNA (K\(_D\) = ca. 1200 nM) and a moderate affinity for dsRNA-1, which, with the NF90\(_b\)\(^{S651E}\) variant, is reduced by a factor of ca. 4 (K\(_D\) NF90\(_b\)\(^{wt}\) = ca. 160 nM; K\(_D\) NF90\(_b\)\(^{S651E}\) = 500 nM; Table 3). In comparison to the NF90\(_b\)\(^{S651E}\) monomer, the binding affinity of the NF90\(_b\)\(^{S651E}\)-NF45 complex to the ssRNA was also improved; however, it was only 1.7-fold vs. 11-fold with the NF90\(_b\)\(^{wt}\)-NF45 (when compared to NF90\(_b\)\(^{wt}\); see above). With the dsRNA-1, and again in comparison with the respective monomers, the binding affinity of NF90\(_b\)\(^{S651E}\)-NF45 was improved by ca. 6-fold (vs. 8-fold with NF90\(_b\)-NF45\(^{wt}\); Table 3). When compared directly with the NF90\(_b\)\(^{wt}\)-NF45 complex, the affinity of the NF90\(_b\)\(^{S651E}\)-NF45 complex for ssRNA was lowered by a factor of 6; with dsRNA-1, it was reduced by ca. 4-fold (Table 3).

The measured m-values (binding modes) revealed that with the ssRNA, the variant NF90\(_b\)\(^{S651E}\)-NF45 complex showed a generally similar behavior as the NF90\(_b\)\(^{wt}\)-NF45 complex by binding this type of RNA through an enlarged fraction of hydrophobic interactions in comparison to the monomer (indicated by an increase of \(m\); Table 3). However, with NF90\(_b\)\(^{S651E}\)-NF45, this increase in the relevance of hydrophobic interactions was not as pronounced as with the NF90\(_b\)\(^{wt}\)-NF45 complex; this is best exemplified by the \(\Delta m\)-value (difference of m-values of heterodimer and monomer; see Table 3). Alterations were also
observed with dsRNA-1. As outlined earlier, with the NF90b-wt-NF45 complex and dsRNA-1, we observed a significant increase in the level of ionic interactions (indicated by a decrease of $m$) with respect to NF90b. The monomeric NF90b$^{S651E}$ already showed a high level of ionic interactions with dsRNA-1. However, in further contrast to the situation with the wt protein, formation of the heterodimer had no additional consequences because the $m$-values of the monomer and of the heterodimer were very similar (Table 3). When directly compared with the NF90b-wt-NF45 complex, the $m$-values of the variant NF90b$^{S651E}$-NF45 complex were slightly increased (indicating a slightly increased fraction of hydrophobic interactions) when interacting with ssRNA and dsRNA-1.

Pseudo-phosphorylation of the NF90 RGG/RG motif thus has a drastic, negative effect on ssRNA binding and a moderately negative effect on dsRNA-1 binding by NF90b-NF45. Accordingly, these results yielded additional hints that the interaction of NF45 and NF90b in the heterodimeric complex considerably improves the binding of NF90b’s C-terminus and the included RGG/RG motif to ssRNA. The fact that dsRNA-binding was also affected by the pseudo-phosphorylation of the RGG/RG motif suggests that formation of the NF90b-NF45 complex increases the cooperativity between the C-terminus of NF90 and the dsRBMs (see below and Discussion).

Heterodimer formation with NF45 improves the activity of NF90b’s dsRBMs, in particular its capability to bind ssRNA. The last experiments of this series aimed to understand further the impact of complex formation on the activities of the tandem dsRBMs of NF90b. For this, we generated the heterodimeric complex with an NF90b variant in which the two conserved phenylalanines (F432 and F559) in dsRBM1 and dsRBM2 were exchanged by alanines. Complex formation again was confirmed by analytical ultracentrifugation (not shown). The aa substitutions F432A and F559A considerably impaired the RNA-binding properties of the mutated dsRBM [10-12]; in fact, the monomeric NF90b$^{F432,559A}$ showed a drastically (ca. 10-fold) reduced affinity for dsRNA ([45]; see also Table 3). Like with NF90b$^{S651E}$, the overall secondary structure of the monomeric NF90b$^{F432,559A}$ was shown to remain unchanged with respect to that of NF90b-wt.[45] When we measured the far-UV CD of the reconstituted NF90b$^{F432,559A}$-NF45, it showed essentially the same spectral profile as the NF90b-wt-NF45 complex. This indicated that complex formation of NF90b$^{F432,559A}$-NF45 was accompanied by comparable conformational changes as it was
observed with the wt complex. This was in evident contrast to the situation with NF90b\(^{S651E}\)-NF45 (Fig. 5A).

Using again the earlier explained experimental scheme, we next measured the affinities and binding modes of the NF90b\(^{F432,559A}\)-NF45 complex for the ssRNA and dsRNA-1 substrates and compared the data obtained with those of the monomeric NF90b\(^{F432,559A}\) as well as with those of NF90b\(^{wt}\) and NF90b\(^{wt-NF45}\). As shown in Table 3, heterodimerization with NF45 also enhanced the affinity of the variant NF90b\(^{F432,559A}\) to ssRNA and dsRNA-1. Compared with the respective NF90b monomers, the affinity of NF90b\(^{F432,559A-NF45}\) was observed to be increased 7-fold with the ssRNA (vs. 11-fold with NF90b\(^{wt-NF45}\)); with the dsRNA-1, it was increased by a factor of 8 (vs. 8-fold with NF90b\(^{wt-NF45}\)). However, it is important to note that the affinities of the variant complexes were considerably lower than with the wt complex, namely by a factor of 2.5 (ssRNA) and 10 (dsRNA-1), respectively (Table 3).

Finally, we compared the binding modes (\(m\)-values) of the monomeric NF90b\(^{F432,559A}\) and the NF90b\(^{F432,559A-NF45}\) complex. These data revealed that the changes of the RNA-binding mode during NF90b\(^{F432,559A-NF45}\) complex formation generally resembled the situation that was observed with NF90b\(^{wt-NF45}\). However, the fraction of hydrophobic interactions with the ssRNA that becomes newly formed during complex formation turned out to be detectably lower with NF90b\(^{F432,559A-NF45}\) as with NF90b\(^{wt-NF45}\) (compare \(\Delta m\) values in Table 3) indicating that complex formation slightly improves the capability of the dsRBMs to interact with ssRNA (see Discussion). With the dsRNA-1 substrate, we observed an increase of the ionic interactions during complex formation, which is in the same range as with the wt complex.

In summary, the data that were obtained with the variant NF90b\(^{S651E-NF45}\) and NF90b\(^{F432,559A-NF45}\) complexes support the following assumptions. As already outlined, formation of the NF90-NF45 heterodimer significantly impacts the conformation and activity of NF90's immediate C-terminus, and it markedly improves the capability of this part of the protein to interact with ssRNA. In contrast, complex formation does not affect the general RNA binding properties of the dsRBMs but improves the capability of these motifs to bind the shorter dsRNA-1 substrate. These data suggest that when NF90 is associated with NF45 the cooperative actions of the two dsRBMs and of the dsRBMs with the RGG/RG element are
considerably enhanced (see Fig. 7 and Discussion). Correspondingly, the NF90 C-terminus shows an increased binding to dsRNA-1, while the dsRBMs associate with ssRNA more efficiently (Table 3).
Discussion

The RNA-binding protein NF90, an important regulator of post-transcriptional gene regulation, exists in multiple cell types predominantly as a complex together with its most prominent interaction partner NF45. A clear-cut differentiation of the properties of the monomeric NF90 and the NF90-NF45 complex had not been possible, and one important reason for this was that functional studies could not be performed because the full-length proteins were not available in purified form. Recently, we established protocols that enabled the purification of the monomeric NF90, and a detailed characterization of its RNA-binding properties.[45] Consequently, in the current study our aim was to provide information on the constitution of the NF90-NF45 complex and to understand whether and how the NF45 interaction partner modulates the properties of NF90. We successfully reconstituted NF90-NF45 from the heterologously expressed and purified individual NF90 and NF45. Analytical ultracentrifugation of the homogeneous complex demonstrated that the two proteins form a stable heterodimer under physiological conditions, which is congruent with the findings of earlier reports.[32, 49] Higher oligomeric states of the heterodimer were not detected. Furthermore, we were able to ascertain that the interaction of NF90 and NF45 occurs independently of the presence of RNA (Fig. 1), and that complex formation could be achieved by using either a pulse-refolding approach or by co-incubating fractions of refolded monomeric NF90 and NF45, with both preparations showing identical properties (not shown). Taken together, these observations demonstrate that we reconstituted native, functional preparations of NF90, NF45 and NF90-NF45.

The general relevance of the NF90-NF45 complex was first documented by Guan et al.[49] These authors performed complementary RNAi knock-down experiments of NF90 and NF45 in cells and observed that depletion of one of the proteins also reduced the level of the other. Based on further data in their study, Guan et al. came to the conclusion that this co-regulation of NF90 and NF45 is a post-translational phenomenon that may result from protein destabilization in the absence of binding partners. The authors further suggested that NF45 is a short-lived protein, which is stabilized by binding to its partners, and that one of its functions is to stabilize and/or to regulate the activity of NF90.[32, 49] In support of this hypothesis, we observed in this study that the interaction of NF90 and NF45 indeed induces a mutual thermodynamic stabilization of the proteins (Fig. 2; Table 2; \( \Delta \Delta G^0_{N-I} = 1 \text{ kcal mol}^{-1} \)). As indicated by the chemical denaturation experiments, folding of NF90, NF45 and the NF90-NF45 heterodimer involves the
formation of at least one stable intermediate, with the monomeric NF90 and NF45 sharing a homologous first transition of unfolding. NF90 and NF45 show about 30% primary structure identity in their DZF-domain. Therefore, it can be reasonably assumed that some structural elements of these domains unfold in the course of the first transition. Due to the fact that the first transition of the NF90-NF45 heterodimer was shifted to a higher concentration of denaturant, this supports the further assumption that formation of the heterodimeric complex is accompanied by a stabilization of structural elements of the DZF domains. Such a scenario again is in agreement with earlier findings [47, 49], and data obtained from the crystal structure of the truncated NF90^{1-380}-NF45^{29-390} complex [44] indicate that structural elements of the DZF-domains form an interaction interface for both proteins.

Surprisingly, the first transition of denaturation of the NF90-NF45 complex does not comprise a dissociation / association step, and the second transition of the heterodimer resembles the contributions of the individual monomers. This situation prohibited the calculation of the overall thermodynamic stability of NF90-NF45; nevertheless, it suggests that the association of NF90 and NF45 is a diffusion-controlled step during the second transition of chemical unfolding. Moreover, it pointed to at least one additional interaction site between NF90 and NF45. Our experimental data support this idea and propose that, besides the DZF domains, also the C-terminus of NF90 is involved in the heterodimerization with NF45 (see Fig. 7). First indications supporting this notion were gathered from the spectra of the protein's intrinsic fluorescence. Here, we observed that heterodimerization is accompanied by a quenching of tryptophan fluorescence (Fig. 2), although no Trp is present in the interaction interface apparent in the NF90^{1-380}-NF45^{29-390} crystal structure (see Fig. S4). The second hint derived from the CD spectra of the NF90_{wt}-NF45 and NF90_{S651E}-NF45 complexes. These measurements showed that formation of the wt complex is accompanied by conformational changes of NF90 and / or NF45, and that these are inhibited by pseudo-phosphorylation of the RGG/RG motif in NF90's C-terminus (Fig. 2). Third, heterodimerization was able to compensate for the destabilization of structural elements during the first transition of chemical unfolding that were measured with the pseudo-phosphorylated NF90_{S651E} monomer. The idea of an interaction of the C-terminus of NF90 with NF45 is closely compatible with earlier observations of Guan et al.,[49] which were obtained during immunoprecipitation studies. The data revealed that NF90's dsRBMs do not participate in interactions with NF45. Conversely, an NF90 variant with a shorter C-terminus, NF90_{c}, was found to be less capable of heterodimerizing with NF45 than the NF90_{a, b} isoforms having the
entire C-terminus. We also performed immunoprecipitation experiments at low protein concentrations (< 60 nM) to evaluate the efficacy of complex formation of the purified NF90wt or NF90\textsuperscript{S651E} with NF45. In agreement with Guan et al., we observed that co-precipitation of NF45 was less efficient with the pseudophosphorylated NF90 (Fig. S5). This further supports the idea of a contact of NF90's C-terminus with NF45; however, the interaction does not significantly contributes to the heterodimer's stability, as the wildtype and pseudophosphorylated complexes show comparable thermodynamic stabilities (Fig. 5).

Interactions of NF90 and NF45 involving the part of the protein that is crucial for RNA binding provides a plausible explanation for the modulation of NF90’s RNA-binding activity during complex formation. This is further supported by the observation that complex formation is accompanied by substantial conformational changes of the proteins (see Fig. 2 and 5 and below).

As outlined, RBPs are assumed to achieve a specific and high-affinity nucleic acid binding based on three characteristics. These involve a modular architecture comprising a certain number of RBMs, a combination of multiple low-affinity / low-specificity RNA-interactions ('combinatorial binding'), and dynamic, intra- and / or inter-molecular protein-protein interactions modifying the binding interface(s).[3, 4] Along these lines, the monomeric NF90\textsubscript{b} was shown to discriminate the properties of different substrate RNAs by a close functional interplay of its RGG/RG and dsRBM motifs and by multiple, flexible interactions of these RBMs with the individual RNA ligand.[45] Here, we assessed the RNA-binding properties of the NF90\textsubscript{b}-NF45 heterodimer by applying the same strategies as in the preceding study, namely by measuring the affinities and binding modes to three differently organized RNA ligands. The following technical prerequisites enabled unbiased RNA-protein interaction studies and direct comparisons of the characteristics of the NF90\textsubscript{b} monomer and the NF90\textsubscript{b}-NF45 heterodimer. First, purification and processing of the wt and NF90\textsubscript{b} variants and of the wt and NF90\textsubscript{b}-NF45 complex variants could be performed by using essentially the same protocols. As a result, all protein preparations showed the same, negligible levels of contaminating nucleic acids; differences in the stability of the wt and variant complexes were not apparent (Fig. 5 and Table 2). Second, NF90\textsubscript{b} as well as NF90\textsubscript{b}-NF45 showed the same 1:1 binding stoichiometry of the applied RNA substrates. Finally, we applied three different types of RNA substrates, which showed a minimum of flexibility. Melting-curve analyses and CD spectroscopy ensured that the ssRNA, dsRNA-1 and dsRNA-2 substrates did not undergo conformational transitions under the applied experimental conditions and that our measurements addressed solely the behavior of the
interaction site of the investigated RNA-protein complexes.[45] Considering that yet defined natural RNA-binding sites of NF90 could only be narrowed to segments of approximately 100 nucleotides in size, which do not display obvious, common features [37-39], the here-applied RNA substrates were not designed to closely resemble such sites but to define the basic features of RNA-protein interactions. Nevertheless, the information obtained by these data is expected to facilitate the future characterization of natural NF90 binding sites.

Thus, the heterodimeric complex was shown to display an approximately 10-fold higher affinity to ssRNA and dsRNA-1. In contrast, its binding parameters for dsRNA-2, the substrate that is bound with the highest affinity (K<sub>D</sub> = 2 nM), were essentially the same as for monomeric NF90<sub>b</sub> (Fig. 2). The 33 bp-long dsRNA-2 was previously shown to be bound effectively by both the dsRBMs of NF90, while this was not the case with the 13 bp dsRNA-1.[45] The fact that complex formation with NF45 did not change NF90<sub>b</sub>’s effective binding of dsRNA-2 was accordingly explained by the fact that heterodimerization does not alter the intrinsic properties of the dsRBMs. This fits well with the observation that double-mutation of the dsRBMs strongly inhibits the binding of dsRNA-2 to NF90<sub>b</sub> ([45]; see also Table 3) but has no negative impact on the NF45-mediated improvement of the binding of NF90 to ssRNA and dsRNA-1 in the heterodimer (see Table 3 and for further Discussion below).

In agreement with their functions as RBPs, NF90 and the NF90-NF45 heterodimer show generally high affinities (nM-µM) to all tested types of RNA substrates. Moreover, the protein(s) bind the RNAs at high reaction rate constants. This was shown when we studied the kinetics of the different protein-RNA binding reactions (Fig. S6). While on-rates (association) could not be determined for technical reasons, because they were too fast to be monitored (data not shown), we defined two monomolecular steps (a fast step k<sub>1</sub> > 1 s<sup>-1</sup> and a slower step k<sub>2</sub> < 1 s<sup>-1</sup>) in the dissociation process (off-rates, Fig. S6). Assuming diffusion-controlled initial binding reactions, this suggested that the observed differences in the affinities of the monomer and heterodimer were caused by differences in the off-rates.

As explained, the binding of dsRNA-2 by NF90 involves a close cooperativity of both dsRBMs and, with the monomer, we obtained strong evidence that a cooperative action of all the RBMs is induced by the association of RNA ([45]; Fig. 7). The evident improvement of the binding activity of NF90<sub>b</sub> to ssRNA and dsRNA in the heterodimer hence suggests that NF90, when it is assembled in the NF90-NF45 complex, exists in a conformation where all RBMs are in a state of maximal cooperativity, even in the absence of
RNA. The mutual stabilization of the proteins during heterodimerization could not explain the improved RNA-binding affinities (Fig. 4). Actually, the denaturation experiments revealed that within the first transition of denaturation, the structural integrity of the RBMs of NF90 remains unchanged, which is best indicated by the fact that an enhanced binding of ssRNA and dsRNA-1 by NF90-NF45 is detectable even throughout this transition (Fig. 4). The data therefore exclude the possibility that the improved binding properties of the NF90-NF45 heterodimer can be ascribed to a stabilization of the domain structure of the RBMs. Rather, several indications support the idea that a major function of NF45 within the heterodimer, besides generally stabilizing NF90, is to induce conformational changes in the RNA-binding site that increase the cooperation between NF90’s RBMs (Fig. 7). The first evidence supporting this was provided by the denaturation experiments, which showed that the binding properties of the complex are noticeably more sensitive to the treatment with denaturant (Fig. 4). Further support was obtained when we compared the binding modes of NF90b and NF90b-NF45 to the different RNAs. Thus, with the NF90 monomer we determined that RNA-binding via the dsRBMs predominantly involves hydrophobic (non-ionic) interactions, while RNA-interactions via the RGG/RG motif show a prevalent fraction of ionic contributions. The enhanced binding activity of the NF90-NF45 complex correlated with increasing hydrophobic interactions with the ssRNA and increasing ionic interactions with the dsRNA-1. Considering that the dsRBMs represent the major determinants of the binding of dsRNA by NF90 while the RGG/RG motif was demonstrated to be a critical component for the binding of ssRNA, [45] it can be accordingly concluded that NF90-NF45 heterodimerization leads to improved contributions of the NF90 dsRBMs to the binding of ssRNA and of the C-terminal RGG region to the binding of dsRNA (see also Discussion above). This idea was further supported by a van’t Hoff analysis, which was performed for the binding of ssRNA to NF90 and the NF90-NF45 heterodimer. It revealed a considerably higher binding enthalpy with the heterodimer and accordingly suggested a significantly greater number of protein-RNA interactions with the heterodimer NF90-NF45 compared with the monomeric NF90 (Fig. S7). Moreover, further support for this conclusion was provided when we investigated variants of the heterodimeric complex in which either the dsRBMs or the RGG/RG motif were mutated. As outlined, these data revealed that the NF45-mediated improvement of NF90’s RNA-binding capacity was unaffected by the inactivation of both the dsRBMs. In contrast, however, this function of NF45 was clearly restricted by pseudo-phosphorylation of NF90’s C-terminal RGG region (see Table 3). These results strengthen the earlier proposal that heterodimerization does not
alter the intrinsic properties of the dsRBMs, for example, by stabilizing these motifs. Instead, formation of
the NF90-NF45 heterodimer was confirmed to have a positive impact on the RNA-binding activity of the C-
terminus of NF90. Phosphorylation of Ser-651 within the RGG/RG region, which, interestingly, fosters
nuclear export of the human NF90, [52] was defined here as a clear-cut negative regulator of RNA binding
by NF90 and NF90-NF45. However, the RNA-binding process of the protein may be also regulated by
other means, as in the murine NF90 residue 651 is Asn.
In sum, we propose a model suggesting that NF45’s main function in heterodimerization is to increase
the cooperative interplay of all the RBMs of NF90 (Fig. 7). This is suggested to occur by tertiary structural
changes that result in an improved RNA-binding affinity of NF90 to short ssRNA and dsRNA. Although
NF45 also contains an RGG motif, the protein appears to play no important role in RNA binding. This
notion is supported by the observation that the monomeric NF45 does not bind RNA effectively (Fig.
S3A). Moreover, in comparison to the monomeric NF90, the NF90-NF45 complex does not generally
show increased ionic interactions with each of the RNA substrates tested, which is what would be
expected if the NF45 RGG motif does participate directly in RNA binding (see Table 3). Our model of
NF45-induced changes in the tertiary structure is congruent with the idea that in the NF90-NF45 complex,
NF45 should be in direct contact with the C-terminal region of NF90. Along this line, it is important to note
that besides NF90a and NF90b, the ILF3 locus also encodes a C-terminally extended isoform [68], NF110
(ILF3), which interacts with NF45 as well.[49] It will be interesting to perform a similar study with the
monomeric NF110 and the NF110-NF45 heterodimer to gain further insights into the role of the C-
terminus in the RNA-binding activity of this class of RBPs.
At present, one can only speculate about the structural changes of NF90 and NF45 that occur in the
heterodimeric NF90-NF45 complex. An intriguing idea is that the effective inter-domain linker lengths
between the NF90 RBMs might be shortened by NF45, due to its interaction with both termini of NF90.
This would be in line with previously performed systematic correlations of linker lengths and apparent
binding affinities of tandem-RNA recognition motifs, which demonstrated that shorter inter-domain
distances may increase the RNA-binding affinities by several orders of magnitudes.[69] For example, with
the KH3 domain of the RBP NOVA-1, such a rearrangement was shown to increase the protein’s rigidity,
which, in turn, displayed a platform for an improved cooperativity of multiple binding sites.[70] In fact,
support for an extended RNA-binding interface in the ssRNA binding process of NF90-NF45 compared
with NF90 was provided by our studies. A similar situation was found with the protein Rna14, which serves as a scaffold for the interaction of the RBPs Rna15 with Hrp1. As a result of this interaction, three RBMs (two of Hrp1, one of Rna15) jointly contribute to RNA binding in the ternary complex, which significantly increases both affinity and specificity.[71, 72].

Interestingly, our findings about NF90-NF45 are in close congruence with earlier observations on the human splicing factor U2AF, which consists of a large, U2AF65, and a small subunit, U2AF35. Heterodimerization of both subunits was shown leading to a 15-fold increase in the RNA-binding affinity of U2AF. Besides providing an additional RNA-binding interface [73], U2AF35 was discussed to affect the conformational states of the RNA-recognition motifs (RRM) of U2AF65 towards open conformations showing increased RNA-binding affinities [74]. Moreover, it was demonstrated that on binding to U2AF65, the isolated, degenerated RRM of U2AF35 adopts a stable conformation [75]. Heterodimerization of U2AF is of crucial biological importance as it considerably increases the specificity of interaction of the factor to precise 3’ splice-sites.

There are also other studies, which describe a clear correlation between cooperativity and affinity of RBMs with a specific function of an RBP [76, 77]. Altogether, this supports the idea that the association of NF45 with NF90 increases the RNA-binding specificity of NF90 and, in this way, modulates its function in the post-transcriptional regulation of gene expression. Taking this idea further, the monomeric NF90 thus may represent a less specific and less active RBP, which nevertheless is capable of flexibly binding very different RNA substrates.[45] The heterodimeric form would thus represent a more active form that supports specific functions, for example, by regulating the expression of defined mRNAs.[26, 37] However, such a functional link between heterodimerization and biological function of NF90 remains to be demonstrated.
Acknowledgements

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Declaration of interests

The authors have no competing interests to declare.

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Supplementary data statement

Supporting information available:
Supplementary Methods (active-site titration, van’t Hoff parameters, fast kinetics of RNA binding, derivation of Equation 2).
Supplementary Figures S1 Quality of the recombinant, purified NF45), S2 Chemical unfolding / refolding of NF90; Integrity of NF90\text{b wt-NF45} in urea, S3 Stoichiometry of RNA binding of NF90\text{b wt-NF45}; structural integrity of NF90\text{b wt-NF45} at different salt concentrations, S4 Location of Trp and Tyr residues along the structure of NF90 and NF45, S5 Fast kinetics of the dissociation of protein-RNA complexes and S6 van’t Hoff-analysis of the binding of ssRNA by NF90\text{b wt} and NF90\text{b wt-NF45}.
Supplementary Table S1 Oligonucleotides used for mutagenesis of NF90\text{b}.
Supplementary References.

Author contributions

TS, PK, RPG performed experiments. HL performed and analyzed analytical ultracentrifugation experiments. TS, PK, SF, RPG and SEB were involved in designing the study and interpreting results. TS, RPG and SEB wrote the manuscript.
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### TABLES

#### Table 1. Synthetic 6-carboxyfluorescein (FAM-EX)-5’-labeled RNA molecules that were applied for binding studies

<table>
<thead>
<tr>
<th>substrate</th>
<th>sequence</th>
<th>$\epsilon_{260}$ (M$^{-1}$cm$^{-1}$)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssRNA</td>
<td>FAM-EX-5’-CUAAGAUGCUCCGCUCG-3’</td>
<td>148600</td>
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<tr>
<td>dsRNA-1</td>
<td>FAM-EX-5’-CUAAGAUGCUCCGCUCG-3’ 3’-UCUACGAGCGACG-5’</td>
<td>273800</td>
</tr>
<tr>
<td>dsRNA-2</td>
<td>FAM-EX-5’-CUAAGAUGCUCCGCUCG-3’ 3’-UCUACGAGCGACGCUUCUUCUCCUAA-5’</td>
<td>669200</td>
</tr>
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</table>

$^a$ Extinction coefficients were provided by manufacturer IBA

#### Table 2. Thermodynamic parameters of the chemical unfolding of NF90b, NF45 and NF90b-NF45 transition

<table>
<thead>
<tr>
<th>transition</th>
<th>[D]50% (M)</th>
<th>c (kcal L mol$^{-2}$)</th>
<th>$\Delta G^0$ (kcal mol$^{-1}$)</th>
<th>$\Delta G^0_{N-U}$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF90b</td>
<td>N-I</td>
<td>0.48 ± 0.07</td>
<td>3.06 ± 0.26</td>
<td>1.46 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>I-U</td>
<td>2.85 ± 0.12</td>
<td>0.87 ± 0.03</td>
<td>2.48 ± 0.06</td>
</tr>
<tr>
<td>NF45</td>
<td>N-I</td>
<td>0.46 ± 0.21</td>
<td>3.42 ± 0.4</td>
<td>1.59 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>I-U</td>
<td>5.73 ± 0.04</td>
<td>0.51 ± 0.02</td>
<td>2.92 ± 0.06</td>
</tr>
<tr>
<td>NF90b-NF45</td>
<td>N-I</td>
<td>1.19 ± 0.07</td>
<td>2.05 ± 0.09</td>
<td>2.44 ± 0.12</td>
</tr>
<tr>
<td>NF90b$^{S651E}$</td>
<td>N-I</td>
<td>0.82</td>
<td>1</td>
<td>0.82 ± 0.10</td>
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<tr>
<td></td>
<td>I-U</td>
<td>3.19 ± 0.12</td>
<td>1.22 ± 0.10</td>
<td>3.89 ± 0.35</td>
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<tr>
<td>NF90b$^{S651E}$-NF45</td>
<td>N-I</td>
<td>0.94 ± 0.13</td>
<td>2.35 ± 0.20</td>
<td>2.21 ± 0.21</td>
</tr>
</tbody>
</table>

N, I, U – native, intermediate and unfolded state
Table 3. RNA binding parameters of NF90b, NF90b-NF45, and variants to different RNA substrates

<table>
<thead>
<tr>
<th></th>
<th>ssRNA</th>
<th>dsRNA-1</th>
<th>dsRNA-2</th>
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<tr>
<td></td>
<td>$K_D$</td>
<td>$m$</td>
<td>$K_D$</td>
</tr>
<tr>
<td></td>
<td>(nM)</td>
<td>(kcal L mol$^{-2}$)</td>
<td>(nM)</td>
</tr>
<tr>
<td>NF90b*</td>
<td>1200 ± 49</td>
<td>-24.8</td>
<td>160 ± 19</td>
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<tr>
<td>NF90b-NF45</td>
<td>110 ± 9</td>
<td>-14.4</td>
<td>20 ± 2</td>
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<tr>
<td>NF90b$^{F432, S559A*}$</td>
<td>1800 ± 130</td>
<td>-22.1</td>
<td>1800 ± 170</td>
</tr>
<tr>
<td>NF90b$^{F432, S559A-NF45}$</td>
<td>250 ± 10</td>
<td>-18.6</td>
<td>222 ± 20</td>
</tr>
<tr>
<td>NF90b$^{S651E*}$</td>
<td>1100 ± 78</td>
<td>-16.5</td>
<td>500 ± 22</td>
</tr>
<tr>
<td>NF90b$^{S651E-NF45}$</td>
<td>660 ± 30</td>
<td>-10.8</td>
<td>84 ± 7</td>
</tr>
</tbody>
</table>

* data taken from Schmidt et al. [45]

** $K_D$ is obtained from extrapolation of the experimental data to 100 mM NaCl

$^c$ Binding mode observed at < 200 mM NaCl

$^d$ Binding mode observed at > 200 mM NaCl
FIGURE LEGENDS

Figure 1. Features of NF90, the NF90 isoforms, the applied NF90 variants and NF45. Quality of the purified proteins and of the reconstituted NF90-NF45 complex. (A) Organization of the NF90 isoforms NF90a and NF90b, and of NF45. The double-stranded RNA-binding motifs (dsRBM), the RGG motifs, the ‘domain associated with zinc fingers’ (DZF) motifs and isoform-specific elements are indicated. Arrows mark the amino acid positions that were mutated in the variants used in this study. The panels below the schemes show a prediction of NF90b’s and of NF45’s secondary structural elements using the PredictProtein-Server[78] (full boxes: α-helix, open boxes: β-sheet). (B) Coomassie Blue-stained SDS gel of purified and refolded NF45 (lane 1), NF90bwt (lane 2) and the NF90bwt-NF45wt heterodimer (lane 3). Each lane was loaded with 30 pmol of the individual proteins or of the NF90bwt-NF45wt complex, respectively. (C) UV absorption spectrum (see text) and (D) sedimentation equilibrium analysis (analytical ultracentrifugation) of purified and reconstituted NF90bwt-NF45 in assay buffer. The molecular mass was determined with an apparent Svedberg constant of 4.38, corresponding to 105 kDa. This revealed the heterodimeric state of the complex (theoretical mass 119 kDa).

Figure 2. Heterodimerization of NF90 and NF45 induces conformational changes in the proteins as well as a mutual thermodynamic stabilization. (A) Far-UV circular dichroism (CD) spectrum of purified NF90bwt (blue), NF45 (red) and NF90bwt-NF45 complex (black). The acquired data were normalized to mean residue weight (MRW) ellipticities. The raw data of NF90bwt and NF45 were used to calculate a reference spectrum for the NF90bwt-NF45 complex (purple). The molecular weight of the heterodimer was considered for its normalization to MRW ellipticity. Accordingly, the reference reflects the spectral progressions if the protein interactions would not induce conformational changes. (B) Fluorescence spectra of equal concentrations of NF90bwt (blue), NF45 (red) and NF90bwt-NF45 (black) excited at 280 nm and (C) at 295 nm in assay buffer. At both excitation wavelengths, the raw data of NF90bwt and NF45 were used to calculate the sum as the reference spectrum of the NF90bwt-NF45 heterodimer (purple). As indicated above, the reference reflects the fluorescence of NF90bwt-NF45 if the protein interactions would not induce conformational changes. The individual spectra were normalized to the signal intensity of NF90bwt at 343 nm. (D) Chemical unfolding of NF90bwt (blue), NF45 (red) and NF90bwt-NF45 (black) using a denaturant composition of GuHCl and urea (see Material and Methods). Signal amplitudes were monitored using the protein’s CD at 225 nm. Obtained ellipticities were plotted as the fraction unfolded as a function of the denaturant concentration and fitted according to Equation 1. Thermodynamic parameters are summarized in Table 2. The raw data for NF90bwt and NF45 were used to calculate a reference transition of chemical unfolding for the NF90bwt-NF45 heterodimer (purple). Accordingly, this reference reflects the unfolding transition of NF90bwt-NF45 if the protein interactions would not induce changes in thermodynamic stability.
Figure 3. RNA binding of NF90<sub>b</sub>wt and NF90<sub>b</sub>wt-NF45. Binding of NF90<sub>b</sub>wt (open symbols) and NF90<sub>b</sub>wt-NF45 (full symbols) to dsRNA-2 (circle), dsRNA-1 (square) or ssRNA (triangle) was determined as a function of the concentration of sodium chloride. Fluorescence data were analyzed according to Equation 2. The binding parameters were further analyzed according to a linear free energy extrapolation method (LEM; Equation 3), the parameters of which are summarized in Table 3.

Figure 4. Increased thermodynamic stability of NF90<sub>b</sub>wt-NF45 does not explain its improved RNA-binding capacity. Binding of NF90<sub>b</sub>wt (open symbols) and NF90<sub>b</sub>wt-NF45 (full symbols) to dsRNA-1 (circle) or ssRNA (triangle) was determined as a function of the urea concentration. Fluorescence data were analyzed according to Equation 2. The binding parameters were further analyzed according to a linear free energy extrapolation method (LEM, Equation 4); the m'-values are given in the Table.

Figure 5. The NF90<sub>b</sub> C-terminus is involved in interactions with NF45. (A) Far-UV circular dichroism (CD) spectra of NF90<sub>b</sub>wt-NF45 (black), NF90<sub>b</sub>S651E-NF45 (red) and NF90<sub>b</sub>F432559A-NF45 (blue). The acquired data were normalized to mean residue weight (MRW) ellipticities. As in Fig. 2, the raw data for NF90<sub>b</sub>wt and NF45 were used to calculate a reference spectrum for heterodimeric NF90<sub>b</sub>wt-NF45 (purple). Accordingly, the reference reflects the spectral progression if the protein interactions would not induce conformational changes. (B) Chemical unfolding of NF90<sub>b</sub>wt (black) and NF90<sub>b</sub>S651E (red) and in (C) of NF90<sub>b</sub>wt-NF45 (black) and NF90<sub>b</sub>S651E-NF45 (red) using a denaturant composition of GuHCl and urea (see Material and Methods). Signal amplitudes were monitored using the protein’s CD at 225 nm. The ellipticities obtained were plotted as the fraction unfolded as a function of the denaturant concentration and fitted according to Equation 1. Thermodynamic parameters are summarized in Table 2. In (C) the raw data of NF90<sub>b</sub>wt and NF45 were used to calculate a reference transition of chemical unfolding of NF90<sub>b</sub>wt-NF45 (purple). Accordingly, this reference reflects the unfolding transition of NF90<sub>b</sub>wt-NF45 if the heterodimerization of the proteins would not induce changes in the thermodynamic stability.

Figure 6. RNA binding of variants of NF90<sub>b</sub>-NF45. Binding of NF90<sub>b</sub>wt-NF45 (diamond), NF90<sub>b</sub>S651E-NF45 (circle) and NF90<sub>b</sub>F432559A-NF45 (square) to (A) ssRNA and (B) dsRNA-1 was determined as a function of the sodium chloride concentration. The fluorescence data were analyzed according to Equation 2. The binding parameters were further analyzed according to a linear free energy extrapolation method (LEM; Equation 3), the parameters of which are summarized in Table 2.

Figure 7. Heterodimerization of NF90<sub>b</sub> and NF45 results in a reorientation of NF90’s RNA-binding motifs. Schematic model of the proposed interaction mechanism of NF90<sub>b</sub> and NF45. (A) Within the monomeric NF90<sub>b</sub>, the RBMs within the protein’s C-terminus, the RGG motif (red) and both dsRBMs (blue), show a flexible and loose spatial arrangement. (B, C) RNA binding of the monomeric NF90<sub>b</sub> is realized by an RNA-dependent coordinated interplay of the RGG motif and both dsRBMs. Each RBM
contributes differently to the binding process of different RNA substrates. ssRNA (B) is bound primarily via NF90’s RGG motif, while dsRNA (C) is bound primarily via the dsRBMs. The flexibility of RNA-protein interactions is shown schematically as dashed lines. (DC) Interaction of NF45 with NF90\textsubscript{b} involves the protein’s N-terminal DZF domains but also the C-terminus of NF90\textsubscript{b}. As a result, the heterodimerization induces conformational changes in NF90\textsubscript{b} such that both dsRBMs and the RGG/RG region of NF90 rearrange to close spatial proximity and adopt a state of maximal cooperativity even in the absence of RNA. This changes the overall RNA-binding modes such that the binding affinity to short RNA molecules is significantly increased. Thus, in comparison to the monomeric NF90, the RNA-binding process of the NF90-NF45 heterodimer involves higher contributions of the dsRBMs to ssRNA binding (E) and of the RGG motif to dsRNA binding (F).
FIGURES

Figure 1
Figure 3

\[ \ln K^x \]

vs

\[ \text{NaCl (M)} \]
Figure 4

<table>
<thead>
<tr>
<th>NF90&lt;sub&gt;α&lt;/sub&gt; and NF90&lt;sub&gt;α&lt;/sub&gt;-NF45 binding to dsRNA-1 and ssRNA in the presence of urea</th>
<th>$m'(\text{ssRNA})$ (kcal L mol$^{-1}$)</th>
<th>$m'(\text{dsRNA-1})$ (kcal L mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF90&lt;sub&gt;α&lt;/sub&gt;</td>
<td>-0.64</td>
<td>-0.62</td>
</tr>
<tr>
<td>NF90&lt;sub&gt;α&lt;/sub&gt;-NF45</td>
<td>-0.77</td>
<td>-0.96</td>
</tr>
</tbody>
</table>
Figure 6

A

B

\[ \ln K_n \]

\[ \text{NaCl (M)} \]

\[ \begin{array}{cccccc}
0.08 & 0.1 & 0.12 & 0.14 & 0.16 & 0.18 \\
\end{array} \]

\[ \begin{array}{cccccc}
13.0 & 13.5 & 14.0 & 14.5 & 15.0 & 15.5 \\
16.0 & 16.5 & 17.0 & 17.5 & 18.0 \\
\end{array} \]
Figure 7