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Specific amino acids in the BAR domain allow homodimerization and prevent heterodimerization of sorting nexin 33

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Short title: BAR domain-mediated homodimerization of SNX33

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SYNOPSIS

Sorting nexin 33 (SNX33) is a homolog of the endocytic protein SNX9 and has been implicated in actin polymerization and the endocytosis of the amyloid precursor protein. SNX33 belongs to the large family of BAR domain-containing proteins, which alter cellular protein trafficking by modulating cellular membranes and the cytoskeleton. Some BAR domains engage in homodimerization, whereas other BAR domains also mediate heterodimerization between different BAR domain-containing proteins. The molecular basis for this difference is not yet understood. Using coimmunoprecipitations we report that SNX33 forms homodimers, but not heterodimers with other BAR domain containing proteins, such as SNX9. Domain deletion analysis revealed that the BAR domain, but not the SH3 domain, was required for homodimerization of SNX33. Additionally, the BAR domain prevented the heterodimerization between SNX9 and SNX33, as determined by domain swap experiments. Molecular modelling of the SNX33 BAR domain structure revealed that key amino acids located at the BAR domain dimer interface of the SNX9 homodimer are not conserved in SNX33. Replacing these amino acids in SNX9 with the corresponding amino acids of SNX33 allowed the mutant SNX9 to heterodimerize with SNX33. Taken together, this study identifies critical amino acids within the BAR domains of SNX9 and SNX33 as determinants for the specificity of BAR domain-mediated interactions and suggests that SNX9 and SNX33 have distinct molecular functions.

Keywords: BAR domain / endocytosis / sorting nexins / protein dimerization / molecular modelling / Alzheimer's disease

INTRODUCTION

BAR domain containing proteins have been implicated in a variety of cellular functions, such as endocytosis, protein trafficking, cell polarity, regulation of the actin cytoskeleton, signal transduction, tumor suppression, learning and memory [1-3]. The BAR domain is 250 to 280 amino acids long and was named after the founding members of this family, Bin1, amphiphysin 1 and Rvs167 (BAR). Most BAR domains consist of a three-helix bundle, which can dimerize and form a crescent-shaped structure. The positively charged, concave surface of this dimeric structure senses and induces membrane curvature by binding to curved, negatively charged membranes [3, 4].

The BAR domain protein family includes several members of the sorting nexin (SNX) family, SNX1 and 2, SNX4 – 9, SNX18, SNX30, SNX32 and SNX33 [5]. Sorting nexins (SNXs) are a family of 33 cytosolic and membrane-associated proteins characterized by the presence of a SNX-type phox homology (PX) domain, which is a subgroup of the phosphoinositide-binding PX domain superfamily [5-7]. In addition to the PX domain, SNXs may contain additional lipid or protein interaction domains. Few SNXs have been functionally studied, but are generally assumed to be involved in endosomal trafficking [7]. SNX9 (also known as SH3PX1), SNX18 and SNX33 form the SNX9-subfamily of SNXs and share the same domain structure. An N-terminal src homology 3 (SH3) domain is followed by a variable linker region, the PX domain and the C-terminal BAR domain (Fig. 1a). Among the three proteins, SNX9 has been best studied. It is involved in endocytosis and actin assembly and appears to couple actin dynamics to membrane remodelling during the endocytic process [8, 9]. Through its N-terminal SH3 domain it binds different cellular proteins, such as the endocytic GTPase dynamin and the Wiskott-Aldrich syndrome protein [8-11].

The function of SNX18 and SNX33 is less well understood than that of SNX9. SNX18 appears to have a similar trafficking function as SNX9 and also binds dynamin [12]. SNX33 has been implicated in the endocytosis and processing of the amyloid precursor protein and the prion protein [13, 14] as well as in actin polymerization [15]. In agreement with these findings SNX33 binds through its SH3 domain to dynamin and WASP [12, 14, 15]. Additionally, SNX33 binds the metalloprotease ADAM15, but the physiological function of this interaction remains to be established [16, 17]. SNX33 also appears to form homodimers [15], but it remains unknown, whether this occurs through the SH3 or the BAR domain or through both. Both domains can mediate protein homodimerization. Although the SH3 domain is well known to bind to proline-rich regions in target proteins and thereby link distinct proteins, it can also form homodimers, for example of the proteins IB1 and IB2 (also known as JIP1 and JIP2) and the tyrosine kinase Csk (C-terminal Src kinase) [18, 19]. Additionally, BAR domains form dimers [4, 20] and can mediate the homodimerization of the corresponding full-length proteins, such as amphiphysin 1 and SNX9 [21, 22].

BAR domains are not only able to form homodimers, but in some cases also heterodimers between distinct BAR domain containing proteins. This has been described for the close homologs SNX1 and SNX2 or amphiphysin1 and amphiphysin 2 [23, 24]. Heterodimerization has also been observed between more distant BAR domain family members, such as SNX4 and amphiphysin 2 [25]. Heterodimerization may increase the functional versatility of the corresponding proteins, as the heterodimers may have different functions or subcellular localizations than the homodimers. However, the molecular mechanisms, which determine whether a BAR domain is able to form heterodimers, remain unknown. In order to address this question we studied SNX33. Previously, conflicting results have been reported for a potential heterodimerization of SNX33 with its homolog SNX9. One study did not find evidence for SNX9-SNX33 heterodimers in HeLa cells expressing the endogenous proteins [12]. This contrasts another study reporting that transiently overexpressed SNX9 and SNX33 do form heterodimers in HEK293 cells [15]. Here, using molecular modelling and mutational

analysis we show that critical amino acids within the BAR domain of SNX33 determine the specificity of the interaction with other BAR domains. We find that the BAR domain allows SNX33 homodimer formation, but prevents heterodimerization with the BAR domains of the SNX33 homologs SNX9 and SNX18 and the more distant homolog SNX1.

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EXPERIMENTAL

Reagents and Antibodies

The following antibodies were used: anti-HA HA.11 (Covance), anti-Flag FlagM2 (Sigma), HRP-coupled goat anti-mouse and anti-rabbit (Promega), Alexa555-coupled anti-mouse (Molecular Probes).

Plasmid construction

SNX33 and SNX9 cDNAs were described [14]. SNX1 and SNX18 cDNAs were obtained from ATCC. cDNAs without UTRs and with C- or N-terminal fusions to HA- or FLAG-Tag and/or lacking the SH3 and LC domains (PXBAR) or part of the BAR domain (Δ BARH3) were generated by PCR and cloned into vector peak12 (SH3 region corresponding to amino acids 1-60 in SNX33, PX-BAR region corresponding to amino acids 159-574 in SNX33 and 185-595 in SNX9, the last helix of the BAR domain corresponding to amino acids 510-574 in SNX33 and 531-595 in SNX9). In order to generate SNX33BAR9HA and SNX9BAR33HA an EcoRV site was introduced before the start of the BAR domain in SNX33 (amino acid 371, L to I) and SNX9 (amino acid 392, L to I) by PCR. The obtained constructs (SNX33L371IHA and SNX9L392IHA) were subsequently used to swap the BAR domains by EcoRV digestion and cloning into vector peak12. 9mod-HA was generated by PCR in multiple rounds and the resulting fragments were cloned into vector peak12 via triple ligation (see table 1 for individual mutations). The identity of all constructs obtained by PCR was confirmed by DNA sequencing.

Cell culture, Western blot, immunoprecipitation

Human embryonic kidney 293-EBNA (HEK293) and HeLa cells were cultured as described [26]. HEK293 cells stably expressing Flag-SNX33 were generated using plasmids peak12-FlagSNX33 using 0.5 μ g/ml puromycin (Sigma). Transfections were done using Lipofectamine 2000 (Invitrogen). One day after transfection cell lysates (in 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40) were collected and analyzed as described [27-29]. Phosphatase inhibitors (50mM NaF, 1mM NaVO₄, phosphatase inhibitor (1:100, Sigma)) were added to cell lysates. Protein concentration in the cell lysate was measured and corresponding aliquots of lysate was separated by SDS-PAGE. For SNX33/SNX9 co-immunoprecipitation, lysates were incubated with 5 μ g antibody (HA.11, 1:100) for 2 hours (4°C) using protein-G dynabeads (Dyna). After washing with STEN-NaCl (STEN buffer + 0.35 M NaCl) and twice with STEN (0.05 M Tris/HCl pH7.6, 0.15 M NaCl, 2 mM EDTA and 0.2% NP-40), bound proteins were resolved by SDS-PAGE. Western blots were quantified using the luminescent image analyzer LAS-4000 (Fujifilm).

Immunofluorescence

HeLa cells were plated on poly-L-Lysin coated glass coverslips and transfected 24h later with SNX33 and SNX9 deletion constructs. Medium was changed 5h after transfection. 16h after the medium change, cells were washed in PBS, fixed for 20 min in 4% paraformaldehyde/sucrose, quenched for 2 min with 50mM NH₄Cl and washed with PBS. Cells were permeabilized with 0.1% saponin in 8 mM PIPES, 0.5mM EGTA, 0.1mM MgCl₂, 2% BSA. Then cells were stained with HA.11 (1:1000), Alexa Fluor 488 Phalloidin (6.6 μ M, Invitrogen), washed with 0.05% saponin in PBS and incubated with Alexa555-conjugated secondary antibody (1:500). Cells were then washed in 0.05% saponin in PBS and water and fixed with Moviol. Fluorescence was imaged using a Zeiss LSM 510 Meta inverted confocal microscope, equipped with Zeiss LSM software and a Plan Apochromat 100x lens. Expression levels of individual cells were monitored using the imaging processing software

Fiji (<http://pacific.mpi-cbg.de>).

Molecular Modelling

The atomic structure of SNX9 dimers was analyzed with the PROTORP server [30] and molecular graphics [31] using its published crystal structure (PDB-accession code 2RAI)[20]. Amino acid differences to SNX33 and SNX18 and their effect on the formation of potential heterodimers were evaluated manually, thereby identifying key-residues of the interface for mutational analysis. Figures were created using Pymol (<http://www.pymol.org>). The analysis of the potential SNX18-SNX33 heterodimer relies solely on the assumption that amino acid residues pairing in a sequence alignment between SNX18 or SNX33 with interface residues of SNX9 do also form the interface in SNX18 and SNX33.

RESULTS

Homodimerization and membrane tubulation requires the SNX33 BAR domain

First, we tested whether SNX33 is able to form homodimers. To this aim, human embryonic kidney 293 cells stably expressing FLAG-tagged SNX33 (HEK293/FLAG-SNX33) were used. They were transiently transfected with HA-epitope tagged SNX33-HA. SNX33 was present as a doublet band in the immunoblot at around 75 kDa (Fig. 1b), which represents the phosphorylated (upper band) and non-phosphorylated (lower band) form of SNX33, as we demonstrated previously [14]. Immunoprecipitation of SNX33-HA from the cell lysate co-precipitated FLAG-SNX33 (Fig. 1b). The immunoprecipitation was also possible in the opposite way. Immunoprecipitation of FLAG-SNX33 coprecipitated SNX33-HA (Fig. 1c). This demonstrates that SNX33 is able to homodimerize. Next, we analyzed whether the homodimerization of SNX33 is mediated by the SH3 domain, by the BAR domain or by both. Some cytosolic proteins, such as IB1 and Csk dimerize through their SH3 domains [18, 19]. Other proteins, such as several BAR domain containing proteins, homodimerize through their BAR domains [21, 22]. To test the involvement of the SH3 and the BAR domains, two SNX33 mutants were used and tagged with an HA epitope tag. One mutant lacked the N-terminal SH3 domain (Δ SH3). A second mutant lacked the third helix at the C-terminus of the BAR domain (Δ BARH3). This truncation may induce a misfolding and consequently a loss of function of the BAR domain, as shown previously for SNX9 [22]. A loss of function was indeed found in a membrane tubulation assay for the Δ BARH3 mutant (see below in Fig. 2). Similar to full-length SNX33 the mutant lacking the SH3 domain (Δ SH3) coimmunoprecipitated FLAG-SNX33, whereas Δ BARH3 did not, although it was expressed at similar levels as SNX33 and Δ SH3 (Fig. 1b). This experiment demonstrates that the homodimerization of SNX33 requires the intact BAR domain but not the SH3 domain of SNX33. This was confirmed in a second experimental setting. HEK293 cells were used, which stably express SNX33 lacking the SH3 domain (Δ SH3-FLAG). As in the experiment with the full-length FLAG-SNX33 (Fig. 1b), SNX33 or the two deletion mutants Δ SH3 and Δ BARH3 were transiently transfected and tested for coimmunoprecipitation with the stably expressed Δ SH3-FLAG. Again, coimmunoprecipitation was only observed for the full-length SNX33 and Δ SH3, but not for the mutant with the truncated BAR domain (Fig. 1d). Taken together, these experiments demonstrate that the homodimerization of SNX33 requires the intact BAR domain, but not the SH3 domain.

Dimerization of BAR domains generates the crescent-shaped structure, which is required for the membrane-binding and -tubulating activity of BAR domains [4]. Having found that the Δ BARH3 deletion mutant of SNX33 was not able to dimerize, we next tested, whether it had also lost its membrane-tubulating activity. The intact BAR domains of SNX33 and SNX9 induced membrane tubulation in about 15% of HeLa cells when they were expressed together with their PX domains (SNX33 PXBAR and SNX9 PXBAR) (Fig. 2a-c), in agreement with previous reports [12, 32]. The observed membrane-tubulating activity correlated with the expression level of the PXBAR domain (Fig. 2d). However, as expected, the deletion of the C-terminal helix (Δ BARH3) of SNX33 or SNX9 completely abolished the membrane-tubulating activity (Fig. 2a-c), demonstrating that the truncation of the BAR domain results in a loss of its membrane-tubulating activity in addition to the loss of its homodimerization capability (Fig. 1b).

SNX33 does not form heterodimers

Next, we analyzed whether SNX33 is able to form heterodimers with other BAR domain-containing proteins, in particular with its homologs SNX9 and SNX18, but also with the more distantly related SNX1. As a positive control, SNX33 was used. All four proteins were

transiently expressed as HA-tagged proteins in HEK293/FLAG-SNX33 cells and probed for coimmunoprecipitation with FLAG-SNX33. In contrast to SNX33, neither SNX1, nor SNX9 or SNX18 coimmunoprecipitated significant amounts of FLAG-SNX33, although all four proteins were expressed at similar levels (Fig. 3a). This clearly demonstrates that SNX33 does not form heterodimers with its homologs SNX9 and SNX18 or with SNX1. The only condition, where we observed heterodimer formation between SNX33 and SNX9 was upon strong transient overexpression of both proteins (Fig. 3b). However, this interaction was much less than the coimmunoprecipitation observed for SNX33 (Fig. 3b). We conclude that under conditions, where SNX9 and SNX33 are only mildly overexpressed, there is no heterodimerization of both proteins, similar to what has been reported for both proteins expressed at endogenous levels [12]. What prevents the heterodimerization between SNX33 and the other proteins? In view of the finding that the BAR domain is required for the homodimerization of SNX33, we speculated that the BAR domain of SNX33 may also determine the specificity of interactions with other BAR domains. To test this possibility, we made domain swap experiments in which the BAR domains of SNX9 and SNX33 were exchanged (Fig. 3c and d). Both proteins were expressed with an HA epitope tag. In contrast to wild-type SNX33 a SNX33 mutant carrying the SNX9 BAR domain (33BAR9) had lost the ability to coimmunoprecipitate wild-type FLAG-SNX33, similar to the SNX33 mutant with a truncated BAR domain (Δ BARH3)(Fig. 3d). Conversely, the SNX9 mutant carrying the SNX33 BAR domain (9BAR33) was able to interact with FLAG-SNX33. This demonstrates that the BAR domain determines the specificity of the interaction with other BAR domains. The mutant 9BAR33 coimmunoprecipitated less FLAG-SNX33 than the wild-type SNX33, but was also expressed at lower levels than wild-type SNX33 (Fig. 3d lower panel). This indicates that 9BAR33 is likely to be as efficient as wild-type SNX33-HA in forming dimers with FLAG-SNX33. As a control experiment one more mutant of SNX9 and SNX33 was tested. For the exchange of the BAR domains, an EcoRV restriction site had been introduced into the cDNAs at the position where the BAR domain codons started. This resulted in a single amino acid change for SNX33 (L371I) and for SNX9 (L392I). Both mutants SNX33L371I and SNX9L392I were tested for coimmunoprecipitation with FLAG-SNX33 and showed the same result as the corresponding wild-type proteins Fig. 3d). This demonstrates that the single point mutations did not affect the binding behaviour to SNX33. Taken together, the BAR domain swap experiments for SNX9 and SNX33 demonstrate that the BAR domains control the specificity of the BAR domain dimerizations and prevent the heterodimerization between SNX9 and SNX33.

Mechanism of prevention of heterodimer formation

In order to determine why the BAR domains of SNX9 and SNX33 are not able to heterodimerize, molecular graphics and modelling were used. The known crystal structure of the dimerized SNX9 BAR domain [20] was compared to SNX33, for which a crystal structure is not available. Within their BAR domains SNX9 and SNX33 are 36.3 % identical (74 out of 204 amino acids) and share an even larger number of similar amino acid residues, strongly suggesting that the overall fold of the BAR-domain of SNX9 is conserved in SNX33. A detailed analysis of the large dimer interface of SNX9 ($\sim 3000 \text{ \AA}^2$ buried accessible surface area per protomer) using the PROTORG-server was carried out. Mapping of the amino acid conservation between SNX9 and SNX33 to the molecular interface (Fig. 4a, b) revealed that the dimer interface region shows a degree of conservation ((23 out of 64 residues, 35.9 %) similar to that observed for the whole BAR domain. This lack of a higher degree of conservation at the dimer interface is typical for molecules that evolved independently from one another. Correspondingly, no evolutionary pressure forced the dimer interface to retain the ability for heterodimerization between SNX9 and SNX33. These findings are in excellent

agreement with the lack of coimmunoprecipitation between SNX9 and SNX33 (Fig. 3a) and indicates that both proteins may have different biological functions.

A deeper analysis of the interface showed that many strong intermolecular salt bridges, hydrophobic interactions and hydrogen bonding networks are responsible for the formation of tight SNX9 homodimers (Table 1). Out of the 64 residues that line the symmetrical interface in each protomer, 24 contribute each to more than 2 % of the total molecular interface area, making a total of more than 50% of the interface area. 24 hydrogen bonds are formed across the interface. A total of eight tight salt bridges link the two protomers further together, including the bifurcated E579-R586-E583 charged hydrogen bonding network (Fig. 4d). Many of the amino acids at the dimer interface of SNX9 are not conserved in the SNX33 sequence (Table 1). As a consequence, many of the interactions between the two BAR domains would be disrupted in case of a heterodimerization between SNX9 and SNX33, providing a molecular explanation for the observed lack of heterodimerization between both proteins. The *in silico* analysis was also carried out for the potential interaction between SNX9 and SNX18 as well as between SNX33 and SNX18 (suppl. table 1 and 2). In agreement with a previous study [12] and our own data (Fig. 3a), a heterodimerization of SNX9/18 and SNX33/SNX18 does not appear possible due to the lack of conserved amino acids at the dimer interface. To further test the validity of the *in silico* analysis, the interaction between rat amphiphysin 1 and amphiphysin 2 was analyzed (suppl. table 3). In contrast to the SNXs, both amphiphysins have been shown to heterodimerize through their BAR domains [24]. In agreement with the experimental data, the *in silico* analysis revealed that the residues at the dimer interface are either conserved between both proteins or replaced by structurally tolerated amino acids.

The *in silico* analysis of the BAR domains of SNX9 and SNX33 suggests that it should be possible to induce heterodimerization between a modified SNX9 and SNX33, if relevant amino acids in the BAR domain of SNX9 are replaced by the corresponding amino acids of SNX33. To test this hypothesis, 19 amino acid residues - each making a major contribution to the dimer interface in SNX9, but not being conserved in SNX33 - were mutated to their counterparts in SNX33 (see table 1). Indeed, this SNX9-variant with the modified BAR domain (9mod) was able to coimmunoprecipitate SNX33 (Fig. 4e), showing that the chosen amino acids were critical for dimer formation. The observed reduction in interaction strength between FLAG-SNX33 and SNX9mod as compared to either wild-type SNX33 or SNX9 homodimers shows that for an optimal BAR domain dimerization also the many smaller alterations across the BAR domain interface are necessary. The introduced mutations significantly increase the number of compatible surface patches within the larger interface, but a significant number of “non-conserved” residues still exists for the SNX33-SNX9mod dimer (Fig 4c).

To further clarify whether a heterodimer between SNX33 and SNX9mod can exist, we roughly estimated the dissociation constant (K_d) for the SNX33-SNX9mod heterodimer in comparison to the SNX33 homodimer using a mild overexpression of the interaction partners in HEK293 cells. First, the K_d of the SNX33 homodimer was estimated in relation to the SNX9 homodimer, which has a reported K_d of 7.9 μ M [33]. The amount of precipitated SNX33-FLAG after immunoprecipitation of SNX33-HA was in the same range, but slightly higher than the amount of SNX9-FLAG precipitated by SNX9-HA (Fig. 5a,b). Densitometric quantification revealed ~ 2.5 fold higher levels of SNX33 dimer compared to SNX9 dimer, leading to an estimated K_d value of ~ 3 μ M for the SNX33 homodimer. In contrast, the estimated K_d value of the SNX33-SNX9 heterodimer is ~30 fold higher (Fig. 5c), based on the ~30 fold lower coprecipitation between SNX9 and SNX33 (Fig. 3a). The mutations in SNX9mod increase the coprecipitation efficiency ~ 6-fold, leading to an estimated K_d of ~ 13 μ M, which is ~ 1.5 fold higher than the K_d reported for the SNX9 homodimer [33]. This further supports our experimental finding of SNX33-SNX9mod dimers.

DISCUSSION

This study shows that SNX33 forms homodimers but not heterodimers with its closest homologs SNX9 and SNX18. Using mutational analysis as well as molecular modelling we present a molecular mechanism, by which the BAR domain allows homodimerization and prevents heterodimerization of SNX33. We expect that similar modelling approaches should allow to predict the potential of other BAR domains for heterodimerization.

Dimerization of BAR domains generates the crescent-shaped structure, which is required for the membrane-binding and -tubulating activity of BAR domains [4]. BAR domain containing proteins form homodimers [34], which we also find here for SNX33, in agreement with a previous study [15]. Mutational analysis revealed that the BAR domain, but not the SH3 domain is required for SNX33 homodimerization. This is in line with the BAR domain being the dimerization domain in other proteins [4, 20, 34]. A similar result was also reported for the SNX33 homolog SNX9, in which a truncation of the 13 C-terminal amino acids of the BAR domain resulted in a loss of homodimer formation [22].

Although some BAR domains are able to form heterodimers with the BAR domain of other proteins, we find that SNX33 does not form heterodimers with its homologs SNX9 and SNX18 or with the more distant homolog SNX1. This lack of heterodimer formation is in agreement with a previous study, in which endogenous SNX9 or SNX33 were immunoprecipitated from HeLa cells, but failed to coprecipitate the other protein [12]. However, that study and our own data are in contrast to another study reporting heterodimer formation between SNX33 and SNX9 in HEK293 cells [15]. In this study, both SNX33 and SNX9 were transiently overexpressed. We also observed coimmunoprecipitation between SNX33 and SNX9 when both proteins were strongly overexpressed. However, the use of lower plasmid concentrations for the transfections as well as the generation of stable cell lines, expressing the target protein at lower levels, abolished the coimmunoprecipitation between both proteins. Thus, we conclude that under physiological conditions there is no heterodimer formation between SNX33 and SNX9. This conclusion is further supported by mechanistic studies, including mutational analysis and molecular modelling. Swapping of the BAR domains of SNX9 and SNX33 revealed that the SNX33 BAR domain does only form dimers with the SNX33 BAR domain, regardless of whether the remaining part of the protein is of SNX9 or SNX33 origin. The molecular basis for this exclusive homodimer formation of the SNX33 BAR domain was revealed upon modelling of the SNX33 BAR domain structure in analogy to the SNX9 BAR domain structure, which was determined by X-ray crystallography [20]. In the SNX9 structure we analyzed the amino acids, which form the dimer interaction interface. However, in the SNX33 sequence, several amino acids at the interface are not conserved and are exchanged to amino acids, which are not compatible with an energetically favourable interaction to the BAR domain of SNX9. Indeed, mutation of several of these amino acids in the SNX9 sequence to the corresponding amino acids of SNX33 allowed the mutant SNX9 protein to heterodimerize with wild-type SNX33. This clearly shows that the wild-type BAR domains of SNX33 and SNX9 are incompatible for heterodimer formation, indicating that both proteins may have distinct cellular functions, for example by acting at different cellular membranes. This possibility is in agreement with the lack of cellular colocalization of both proteins [12]. However, SNX9 and SNX33 may act at their respective membranes by similar molecular mechanisms. SNX9 couples actin dynamics to membrane remodelling during the endocytic process [8, 9]. Likewise, SNX33 has been implicated in endocytosis and actin remodelling and binds to proteins, such as dynamin, WASP and ADAM15, which are also binding partners of SNX9 [10-12, 14-17, 35].

At present only few BAR domain containing proteins have been shown to form heterodimers through their BAR domains, such as the close homologs SNX1 and SNX2 and amphiphysin 1

and 2 [23, 24]. But even for the two more distantly related BAR domains of SNX4 and amphiphysin 2 heterodimerization has been reported [25]. For many other BAR domain containing proteins it is not yet known whether they are able to form heterodimers, either with close homologs or with more distant homologs. Based on our modelling of the potential interaction between the BAR domains of SNX9 and SNX33 we expect that a similar modelling of the potential dimer interface should allow to predict if a heterodimerization between other BAR domains is possible or not. If the relevant amino acids at the dimer interface are not conserved between two distinct BAR domains, it is likely that they are not able to heterodimerize. In contrast, if the essential amino acids are conserved, a heterodimerization should be possible. This assumption was further validated by analyzing the interaction among the other SNX9 family members. The *in silico* analysis predicted that there is no heterodimerization between SNX18 and either SNX9 or SNX33. This is in agreement with our data and a previous study analyzing the endogenous proteins [12]. In contrast, another recent study reported a coimmunoprecipitation between SNX9 and SNX18 [36]. However, that study used a transient transfection assay, such that the interaction may be due to the strong overexpression, as discussed above. The *in silico* analysis also provided the correct prediction for the experimentally shown interaction between the two BAR domain containing proteins amphiphysin 1 and 2 [24], which are unrelated to the SNX9 family. In this case the amino acids at the dimer interface are either conserved or show conservative mutations, providing a molecular explanation for the heterodimerization between two distinct BAR domain containing proteins.

The different examples show that the *in silico* analysis provides a valuable approach for the prediction of BAR domain dimerization. One limitation is that the structure of one of the two BAR domains should be known. Although distinct BAR domains may have a similar overall fold [34], the presence of non-conserved amino acids may subtly alter the structure, such that different amino acids constitute the dimer interface in different BAR domains. Amino acid conservation may not be the only factor determining successful interaction between two distinct BAR domains. Additional factors may be the length of the BAR domain monomers, the curvature of the crescent-shaped dimer structure, as well as kinks in the individual helices of the BAR domain monomers [34]. Although the extent and functional consequence of heterodimer formation between BAR domain containing proteins remains to be determined, heterodimerization could be a means to increase the functional versatility of the corresponding proteins, as the heterodimers may have different functions or subcellular localizations than the homodimers. Additionally, heterodimerization may allow the formation of large molecular complexes consisting of the distinct binding partners of the two different BAR-domain containing proteins.

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TABLE

Table 1: Stabilization and conservation of the SNX9/SNX33 dimer interface

Amino acid (SNX9)	Partial inter-face contact area > 2%	Number of hydrogen bonds	Conservation (+) or amino acid in SNX33	Mutated in 9mod	Comment
F 405	+		+	-	
A408			K	-	Surface/edge of interface - no major energetic contribution
M409			+	-	
G412			S	-	Surface/edge of interface - no major energetic contribution
V413			+	-	
E415			Q	-	Surface/edge of interface - no major energetic contribution
L416	+		+	-	
V419	+		+	-	
G420			A	-	A spatially well accommodated
E422		1	+	-	Salt bridge to K437
H423	+	1	L	yes	Not compatible with heterodimer
R426	+	1	K	yes	Salt bridge to E434 is still possible
P430			G	-	Surface/edge of interface - no major energetic contribution
L431			F	yes	Not compatible with heterodimer, no space for F431
E434	+	2	+	-	Salt bridge to R426
Y435		2	F	yes	Strong H-bond to Y578 is lost
K437		1	+	-	Salt bridge to E422
I438	+		L	-	L spatially well accommodated
K440			S	-	Surface/edge of interface - no major contribution to dimer
A441	+		+	-	
L442			F	yes	Not compatible with heterodimer
S444			A	-	A spatially well accommodated
L445	+		I	yes	Not compatible with heterodimer
V448	+		S	yes	Not compatible with heterodimers, hydrophobic contact lost
F449	+		+	-	
S451			Q	-	Surface/edge of interface - no major contribution to dimer
S452			M	yes	Not compatible with heterodimer - no space for M452
Y454	+	1	P	yes	Not compatible with heterodimers - P is too small
Q455			F	-	Surface/edge of interface - no major contribution to dimer
G456			C	-	Surface/edge of interface - no major contribution to dimer
E457		1	S	yes	Salt bridge to R559 is lost
L460	+		+	-	
I464			+	-	
A467			T	yes	Not compatible with heterodimer - no space for T467
Y471		1	+	-	
L486			+	-	
L489			M	yes	Not compatible with heterodimer - no space for M489
N553		1	+	-	
H556			+	-	
S557			+	-	
R559		1	+	-	Salt bridge to E547
I560	+		E	yes	Not compatible with heterodimer - opposite Charge
Y561			L	-	Surface/edge of interface - no major contribution to dimer
Y563			F	-	F spatially well accommodated
N564	+	1	K	yes	Not compatible with heterodimer - no space for K564
I567	+		M	yes	Not compatible with heterodimer - no space for M567
R568	+	1	Q	yes	Strong H-bond to P593 is lost
Y570			+	-	
L571	+		+	-	
E572			R	-	Surface/edge of interface - no major contribution to dimer
Q574		1	+	-	
V575	+		I	-	I spatially well accommodated
Y578	+	2	+	-	
E579		2	Q	yes	Salt bridge to R568 is lost
I581			V	-	V spatially well accommodated
A582			G	-	G spatially well accommodated
E583			+	-	*
L585	+		+	-	
R586		2	E	yes	Salt bridge to E579 is lost
A588			T	yes	Not compatible with heterodimer - no space for T588
L589	+		+	-	
F592	+	1	Y	-	Y spatially well accommodated
P593		1	D	-	Surface/edge of interface - no major contribution to dimer
V594			N	-	Surface/edge of interface - no major contribution to dimer
M595	+		L	-	Surface/edge of interface - no major contribution to dimer

Table footnote:

* E583 is not part of the interface but it forms the E579-R586-E583 H-bonding network.

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FIGURE LEGENDS

Fig. 1: Homodimerization of SNX33 requires its BAR domain. (a) Schematic domain structure of SNX33 and deletion mutants. (b) HEK293 cells stably expressing FLAG-SNX33 (FLAG33) were transiently transfected with HA-tagged full-length SNX33 (33HA), or truncated SNX33 variants lacking the N-terminal SH3 domain (Δ SH3HA) or the C-terminal helix of the BAR domain (Δ BARH3HA). Aliquots of the cell lysates (lower two panels) show that Δ SH3HA is expressed at slightly lower levels than full-length SNX33 and Δ BARH3HA. HA-tagged proteins were immunoprecipitated (IP) and detected by anti-HA antibody (second top panel) or stained for coimmunoprecipitated FLAG-SNX33 (uppermost panel). 33HA and Δ SH3HA coimmunoprecipitate FLAG-SNX33. (c) HEK293-FLAG SNX33 cells were transiently transfected with control vector or SNX33-HA. FLAG-SNX33 was immunoprecipitated and tested for coimmunoprecipitated SNX33-HA (uppermost panel). (d) HEK293 cells stably expressing SNX33 lacking its SH3 domain (Δ SH3FLAG) were transiently transfected with constructs as in C. Coimmunoprecipitation with Δ SH3FLAG was observed for full-length SNX33 (33HA) and Δ SH3HA, but not for Δ BARH3HA. Shown are representative western blots of three independent experiments (b-d).

Fig. 2: Membrane-tubulating activity of SNX33 requires the intact BAR domain.

(a) Constructs encoding the HA-tagged PX and BAR domain of SNX33 and SNX9 were expressed in HeLa cells (33PXBAR, 9PXBAR). As a control, an unrelated cytosolic protein (HA-tagged Protein Kinase C alpha; PKCalphaHA) as well as the empty vector were expressed. Cells were co-stained with phalloidin in order to visualize the actin cytoskeleton. Immunofluorescence analysis revealed membrane-tubule formation, which was abolished when the BAR domains lacked the C-terminal third helix of SNX33 and SNX9 (PX Δ BARH3). The control protein PKCalphaHA showed diffuse cytosolic staining and no tubulation, similar to the PX Δ BARH3 constructs. (b) Expression levels of individual cells were monitored using the imaging processing software Fiji. Only cells exceeding a predetermined signal intensity threshold (i.e. a mean grey value above background) were counted. The numbers above the lines indicate whether the construct derives from SNX9 or SNX33. PXB: PXBAR construct; PX Δ BH3: PX Δ BARH3 construct. (c) Cells were scored for the presence or absence of membrane-tubule formation. (d) Based on the expression level of HA-tagged constructs the cells were divided into high-, medium- and low expressing cells (based on the signal intensity measured in (b)). The percentage of cells with observed membrane-tubule formation within each category is shown. Shown are mean and standard deviation of three independent experiments (b-d). (e) Western blot analysis of transfected HeLa cells showing similar expression levels of all four SNX9 and SNX33 constructs (with HA antibody) as well as of actin.

Fig. 3: SNX33 forms homodimers, but not heterodimers. (a) HEK293 cells stably expressing FLAG-SNX33 were transiently transfected with HA-tagged constructs encoding SNX1, SNX9, SNX18 or SNX33. Coimmunoprecipitation was analyzed as in Fig. 1b. FLAG-SNX33 coimmunoprecipitated with SNX33, but not with SNX1, SNX9 or SNX18. (b) HEK293 cells were transiently co-transfected with FLAG-SNX33 and either the empty vector, HA-tagged SNX33 or HA-tagged SNX9. Transient overexpression of both interaction partners leads to artificial coimmunoprecipitation of FLAG-SNX33 with SNX9HA, which is not observed if only one of the interaction partners is stably expressed at lower levels (compare to Fig. 3a). (c) Domain structure of BAR domain swap constructs. SNX33 domains are shown in grey, SNX9 domains are indicated in black. (d) FLAG-SNX33 (FLAG33) expressing HEK293 cells were transiently transfected with HA-tagged constructs encoding full-length SNX33 (33HA), SNX33 lacking the C-terminal helix of its BAR domain

(ΔBARH3), SNX33 carrying the BAR domain of SNX9 (33BAR9), SNX9 carrying the BAR domain of SNX33 (9BAR33) or SNX33 and SNX9 carrying the indicated L->I point mutation (33L371I, 9L392I). SNX33 carrying the SNX9 BAR domain (33BAR9) did not coimmunoprecipitate with FLAG-SNX33, whereas 9BAR33 was able to coimmunoprecipitate. Shown are representative western blots of three independent experiments (a-b, d).

Fig. 4: Molecular analysis and modelling of the SNX9-, SNX33-, and SNX9mod-SNX33 BAR domain dimers

(a), (b), (c) Conservation of amino acid residues within the BAR-domain dimer of SNX9, SNX33 and the mutant SNX9mod carrying 19 SNX33-like mutations. The atomic coordinates were always taken from PDB-entry 2RAI [20]. Molecule B (MolB) is shown as cartoon (red) in front of the solid surface representation of molecule A (MolA, yellow and blue). The surface patch of molecule A that is buried upon dimer formation is shown in blue. Panel (b) is rotated with respect to (a) by 180° around a horizontal axis. In Panels A and B, light colours indicate residues that are conserved between SNX9 and SNX33 while dark colours indicate exchanges across this comparison. Panel (c) shows the comparison between the mutated SNX9-variant SNX9mod and SNX33, exhibiting a significantly larger surface area that is amenable to productive interactions (light blue / light red). (d) Close-up of the SNX9-dimer, showing the amino acid residues contributing to the strong, bifurcated E579-R586-E583 charged hydrogen bonding network with all atoms in stick representation. Oxygens and nitrogens are shown in red and blue respectively, while the carbons and the cartoon representation of the helices are shown in orange and green for molecules A and B (MolA and B), respectively. Hydrogen bonding distances are given in Å. E583 does not directly participate in the dimer interface but is essential for this H-bond network. This figure was rotated about a horizontal axis by ~90 °C with respect to Panel (a), thus looking from the bottom at Panel (a). (e) HEK293 cells stably expressing FLAG-SNX33 (FLAG33) were transiently transfected with HA-tagged constructs encoding SNX33 (33HA), SNX9 (9HA) or SNX9 with a modified BAR domain (9modHA). Coimmunoprecipitation was analyzed as in Fig. 1c. FLAG-SNX33 coimmunoprecipitated with SNX33 and to a lower degree with 9modHA, but not with SNX9. IP: immunoprecipitation.

Fig. 5: Estimation of SNX33-SNX33 and SNX33-SNX9 dissociation constants. (a) HEK293 cells were cotransfected with FLAG- and HA-tagged SNX33 or SNX9 (33HA, 33FLAG, 9HA, 9FLAG). As a control empty vector was cotransfected with the HA-tagged SNX9/33. Coimmunoprecipitation was analyzed as in Fig. 1b. IP: immunoprecipitation. (b) The relative amount of FLAG-SNX33 that coimmunoprecipitated with SNX33-HA was calculated (Enrichment of FLAG-tagged constructs in HA IP divided by the IP efficiency = [(IP HA and Blot Flag : loading control FLAG):(IP HA and Blot HA : loading control HA)]) and compared to the relative amount of SNX9-FLAG precipitated by SNX9-HA. (c) The relative amount of FLAG-SNX33 that coimmunoprecipitated with HA-tagged SNX9/SNX9mod was calculated (as in Fig. 5b) and compared to the relative amount of SNX33-FLAG precipitated by SNX33-HA (compare to Fig. 4e). Shown are mean and standard deviation of three independent experiments (b-c).

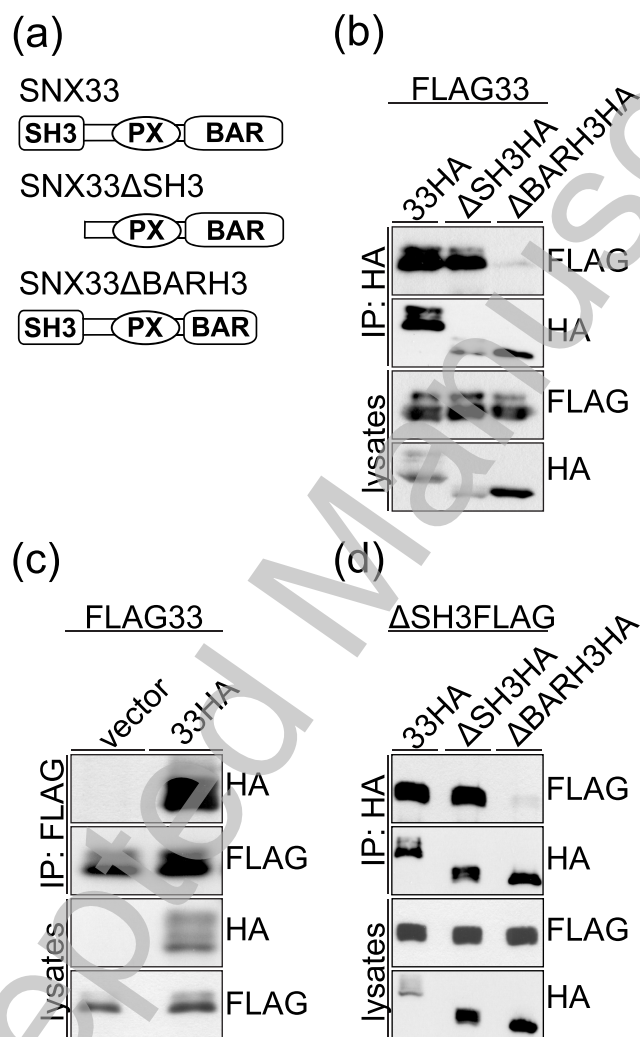


Figure 1

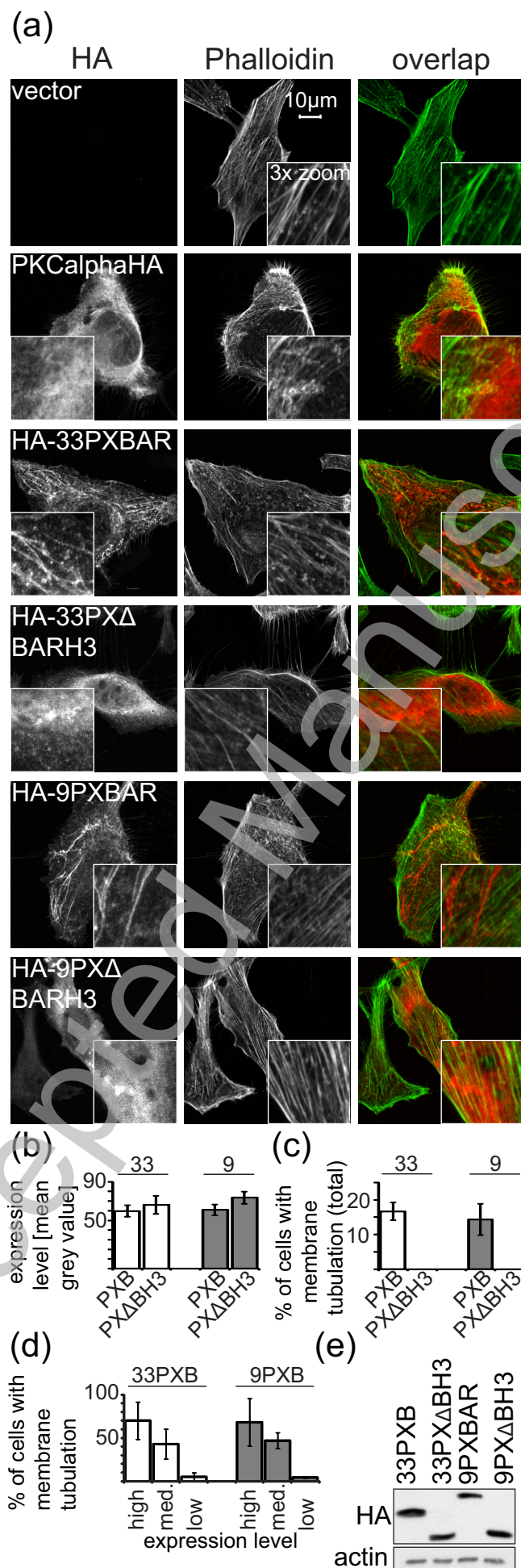


Figure 2

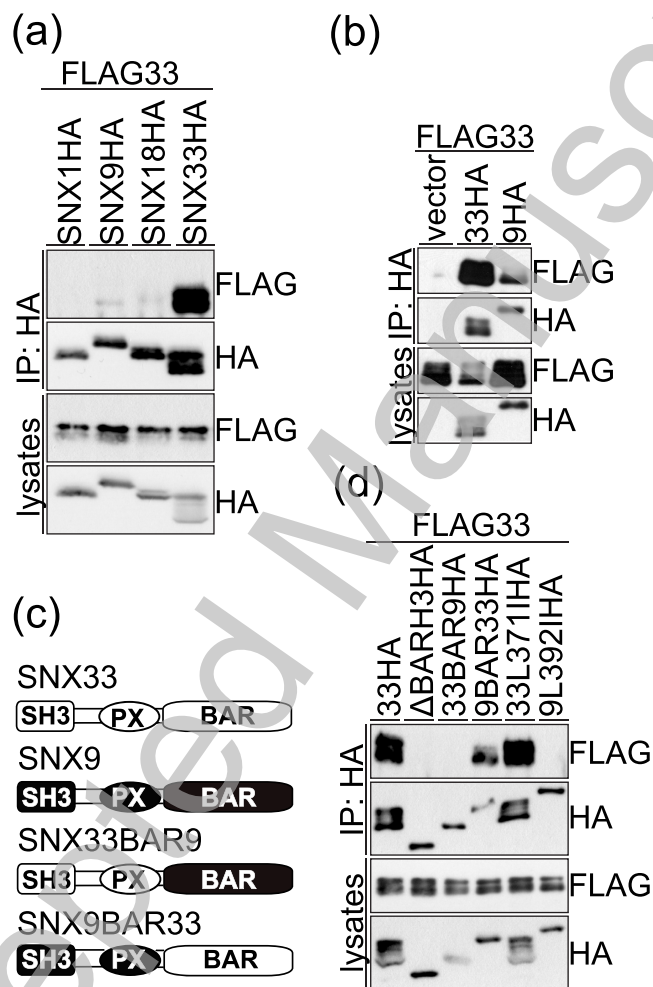


Figure 3

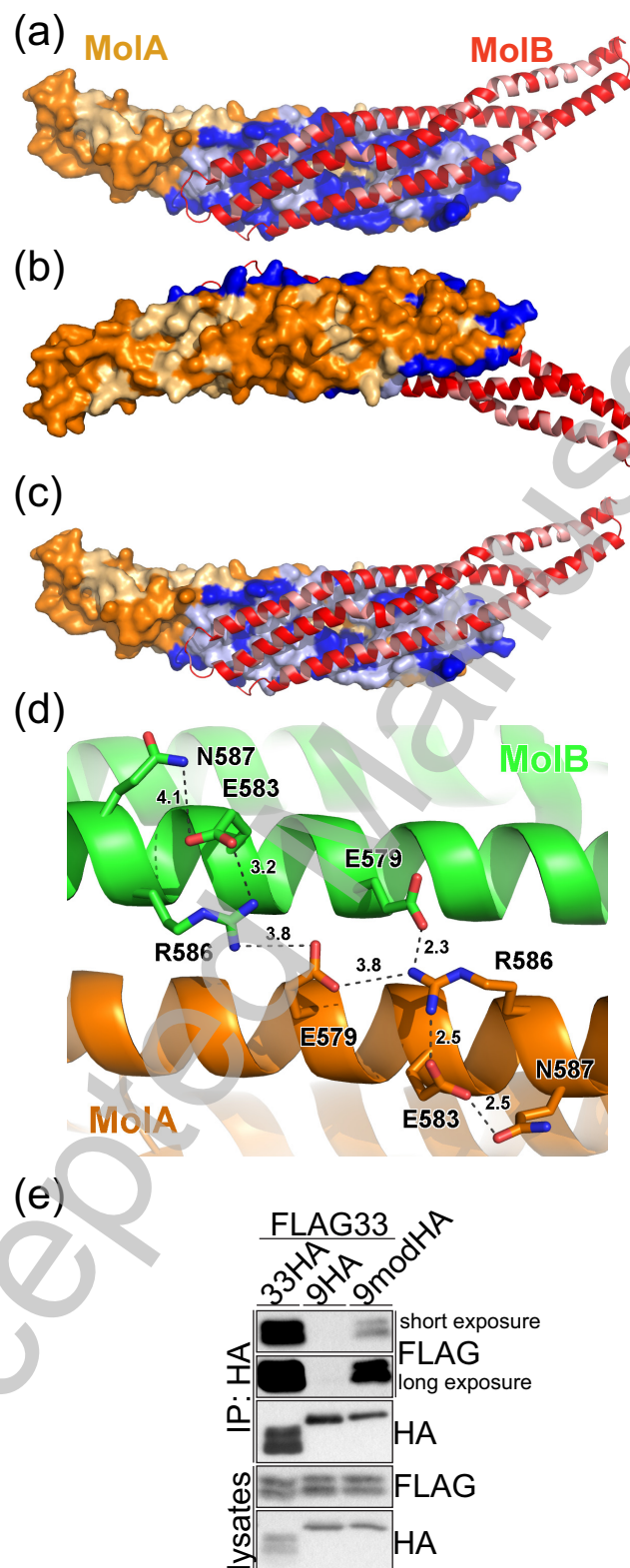


Figure 4

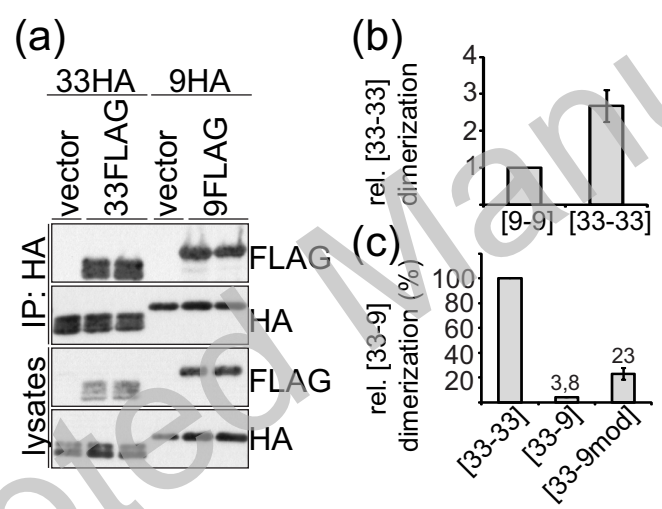


Figure 5