

# Bimolecular fluorescence complementation (BiFC): Investigation of composition and cellular localization of homo- and heterodimeric transmembrane receptors and intracellular signalling pathways

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Bimolecular fluorescence complementation (BiFC) is an imaging technology in order to study protein-protein interactions, which allows for the visualization of the intracellular location of protein interactions in fixed or living cells. Here we will present two examples for two different protein classes both involved in the same signalling pathway. Many functional components in the plasma membrane of mammalian cells that take part in regulatory processes are composed of more than one subunit. In example, transmembrane receptors that resemble the first step in a signal transduction pathway are often composed of homo- and or heterodimers. To investigate the formation of such homo- and heterodimers for BiFC analysis we used split EYFP-tagged wild-type and mutant constructs of the auxiliary TGF-beta receptor endoglin. We show that homodimeric WT receptors localize normally in the plasma membrane, while homo- and heterodimeric receptors with mutant compositions cannot be transported to the membrane but remain within the endoplasmic reticulum. In addition, further downstream components of the TGF-beta pathway like Smad4 and hDcp1a/SMIF, an RNA processing protein in P-bodies, were also analysed by BiFC.

Based on the presented examples, we will discuss the benefit of BiFC analysis and provide information on how to perform such experiments and point out the limitations of this method.

**Keywords:** BiFC (Bimolecular Fluorescence Complementation), transmembrane receptor dimerization, protein interactions, endoglin, Smad4, hDcp1a, SMIF

## 1. Introduction

Many cellular functions are realized through a complex network of protein interactions. Especially processing of external stimuli involves many different proteins that mediate the propagation of the signal within the cell. Extracellular ligands bind to integral membrane receptors which relay the signal inward and trigger protein phosphorylation, the release of ions, membrane potential changes and translocation of transcription factors from the cytoplasm into the nucleus. Changes in gene regulation, protein expression levels, release of cytokines, or other cellular behaviour like proliferation and cell migration are influenced by extracellular signals. In all of these processes protein interactions perform different molecular duties.

To study the individual interactions of the involved proteins many techniques have become available. Yeast and mammalian two-hybrid assays allow the screening for putative interaction partners or the observation of individual protein interactions. Such assays are conducted in living cells but still demand additional downstream analysis for verification and further characterization, e.g. by biochemical *in vitro* methods like co-immuno precipitation. Next to this fluorescence microscopy has become a useful tool for establishing and confirming experimental data by visualizing the cellular localization and relative intensity of an interaction event *in vivo* and *in situ*. The most prominent microscopic technique for the visualization of protein interactions in living cells is Förster-Resonance Energy Transfer (FRET). Alternatively, the relatively new and well-characterized technique of bimolecular fluorescence complementation (BiFC) is available for the study of protein interactions in living cells and has already been applied for the visualization of most different interactions of proteins residing in virtually every cellular compartment (reviewed in [1])

Here, we present the use of BiFC to visualize two structurally different protein interactions that are both involved in the same signalling pathway. In one case, BiFC was used to visualize different assemblies of the homodimeric transmembrane receptor endoglin. In the other case, we applied the BiFC method to visualize the interaction between the two cytoplasmic proteins Smad4 and hDcp1a/Smif.

### 1.1. Biological background: endoglin, Smad4 and SMIF

Our research interest is in signalling by the TGF- $\beta$  family and here particularly in endothelial cells in the context of the dominantly inherited vascular disorder Hereditary Hemorrhagic Telangiectasia (HHT), also known as Morbus Osler. A disease caused by mutations in endoglin or ALK1 [2]. Further members of the TGF- $\beta$  family are e.g. the Bone Morphogenetic Proteins (BMPs) and activins. Signalling by members of the TGF- $\beta$  family is a relatively straightforward series of events. TGF- $\beta$  for example, binds to two pairs of the TGF- $\beta$  type II and type I receptors, T $\beta$ RII and T $\beta$ RI [3]. These receptors belong to the family of serine/threonine kinase receptors with a single transmembrane

domain. Upon TGF- $\beta$  binding, the type I receptors become phosphorylated by the type II receptors and, in turn, phosphorylate members of the receptor-regulated Smads (R-Smads, Smad-1 -2 -3 -5 -8). Subsequently, phosphorylated R-Smads bind to the mediator protein Smad4 and translocate into the nucleus, where the R-Smad/Smad4 complex coregulates gene expression. Along the way there are several modifier proteins involved for example, at the receptor level, receptors like the TGF- $\beta$  type III receptor endoglin, which can enhance or inhibit signalling depending on the ligand and the receptor composition. There are seven type I receptors, ALK1-7, specific for one or more TGF- $\beta$ /BMP members. These receptors usually act as homodimers, but functional heterodimers like ALK1/ALK5 have also been reported. A further modifier protein is the Smad4 interacting factor Smif, reported to enhance TGF- $\beta$  signalling by binding to Smad4 upon which the Smif/Smad4 complex is shuttled into the nucleus [4]. Interestingly, SMIF is also known as the human decapping protein 1a, hDcp1a, and predominantly located in P-bodies [5, 6]. Translationally repressed mRNAs, in conjunction with mRNA decay related proteins and other translation repressors, accumulate in cytoplasmic foci referred to as P-bodies [7]. Thus, P-bodies are aggregates of translationally repressed mRNPs associated with the translation repression and mRNA decay machinery.

### 1.2. Aim of the protein interaction analyses

In HHT1 endoglin, as a homodimer, is exposed to a heterozygous genetic background that leads to different receptor compositions. A direct interaction of wild-type and endoglin missense mutants had previously been shown by biochemical methods [8], but this finding is controversially discussed in the literature [9]. In this context we were interested in confirming this interaction and in investigating in detail the cellular localisation of the different receptor compositions, using the BiFC method as it had been used before for homo- and hetero-dimerization analysis [10]. Moreover, the method seemed to be suitable as a tool to investigate the different protein interactions of the signalling pathway for live cell imaging. As a first approach to establish the method for this analysis, we chose SMIF and Smad4, as the interaction between the two had previously been shown and characterized [4].

The BiFC method proved to be suitable for the visualization of both protein interactions. It was possible to investigate the different receptor compositions of endoglin and their localisation, and, in addition, we were able not only to confirm the interaction between SMIF and Smad4, but also to gain some simplified insight into the relative orientation of the proteins through the application of BiFC.

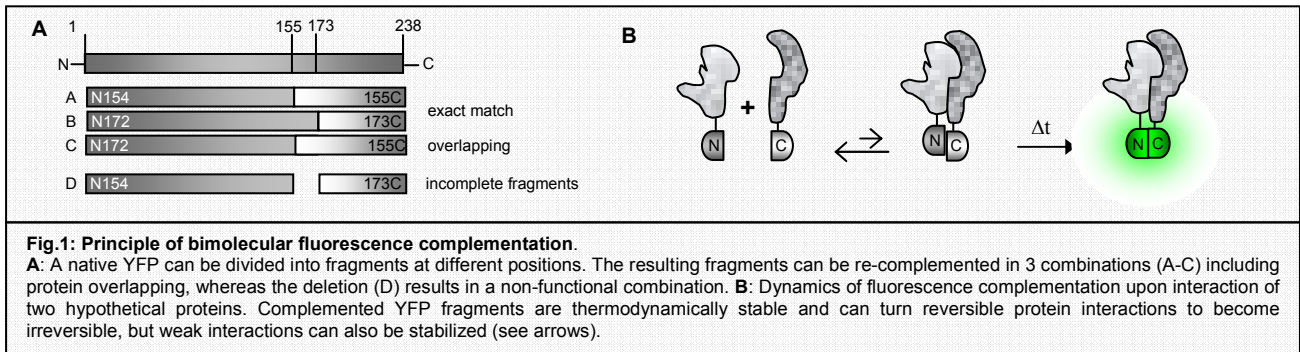
## 2. Bimolecular fluorescence complementation: principle and characteristics

A variety of proteins are known to reconstitute functionality upon complementation of their fragments [11]. The principle of bimolecular fluorescence complementation is thus based on the fact that a fluorescent protein can be split into two portions, which can reconstitute the fluorescent property of the original protein, when the two counterparts are brought back into direct proximity to each other, re-complementing to the functional protein. The functional complementation for a fluorescent protein was shown for GFP and subsequently for EYFP (rev.in [1]), in which two amino acid positions for the division into complementary fragments were identified [12]. The resulting portions can be used to achieve a fluorescence complementation by combining them in 3 different ways (Fig.1A), two of which exactly match the primary structure (A+B), and one of which partially overlaps it (C). Other available GFP variants were also tested for complementation, such as CFP, Venus, and other engineered fluorophores. This led to the experimental approach of combining fragments of different fluorescent proteins. Such complemented hybrid proteins have altered spectral characteristics, which can be used for simultaneous visualization of multiple protein interactions in a multicolour-based assay [13].

### 2.1. Dynamics of fragment association and influence on complex formation of interacting proteins

The dynamics of BiFC fragment association have been characterized in *in-vitro* and *in-vivo* experiments [12, 14]. Fluorescence intensities produced by free BiFC fragments are relatively weak, compared to the intact proteins. This can be explained through incomplete protein folding and varying conformational changes of the fragments minimizing the statistical chances of coming into contact in the right orientation and conformation, so that only a small amount of free fragments is able to mutually complement. This is advantageous to study protein-protein interactions.

The affinity for complementation of fragments fused to non-interacting proteins is low and reduces the detection of false positive interactions caused by random establishment of fragment complementation. But, in the case of an interaction, the fragment complementation is facilitated, as the interacting proteins bring the corresponding counterparts into proximity to each other and keep them there. The fragments fused to the interactors have a reduced degree of free sterical orientations and conformational changes, and the interaction of the two proteins prolongs the temporal encounter of the fragments to perform and finalize the complementation.



After complementation the fragment association is thermodynamically stable, and is then also able to stabilize the protein complex in such a way that the interaction can become irreversible (Fig.1B) [12]. This can be an obstacle to the observation of association-dissociation dynamics of certain protein complexes in live cell imaging, but it also renders it possible to visualize very weak protein interactions not observable under other conditions. This does not seem to be mandatory for all protein interactions, however, as dissociation of BiFC-stabilized protein complexes has also been observed [15].

Besides the given specificity of BiFC signals resulting from protein interactions, this does not overcome the need of controls for a clear interpretation of the result and to exclude false positive interactions. Such controls are recommended by testing mutants of the interactors with deleted binding domains, or by experiments using a known competitor for one of the two interactors that can be added as a third element. Moreover a protein known not to interact with the one or other interactor can be used for complementation. If this is not available, a simple control can always be conducted by combining one fusion interactor with the corresponding free BiFC fragment, or by additionally adding one interactor not fused to a fluorescent fragment to the BiFC interaction as an internal competitor. Attention also has to be paid to the relative ectopic expression levels, as overly high protein concentrations can favour false positive fragment association.

## 2.2. Fluorescence intensities of BiFC complexes

Apart from the positive characteristics of the complementation dynamics for the study of protein interactions, the method has the technical disadvantage of drastically lower detectable signal levels of complemented fluorophores. The intensities resulting from protein-interaction complementations typically reach only about 1-10% when compared to the intact fluorophores. This, however, is not the result of a lower intrinsic fluorescence characteristic of a BiFC complex. BiFC complexes have been shown to possess the same fluorescence property as the native fluorophore of EYFP [12]. The explanation for this is that only a small percentage of the protein interactors perform the interaction, due to partial separation of the interactors in different compartments or through competitive interactions with other proteins. Moreover, the fused fragments can negatively influence the effectiveness of the protein interaction. And, as mentioned above, the encounter time of the interactors must also be sufficient for the fragments to perform the complementation. This is problematic when studying interactions of proteins with relatively low physiological expression levels, and/or when the interactors are involved in multiple protein associations and the interaction of interest takes place at only a small proportion. It is thus crucial to test the cell system used for minimal autofluorescence background under the given experimental condition. A way to overcome weak signals is stronger expression (overexpression) for the visualization of real protein interactions, but this is risky when the interaction is not known to occur. Strong expression levels can lead to false positive BiFC associations owing to the great abundance of molecules, raising the probability of fragment complementation demanding more controls (e.g. DNA titration) for verification. To resemble the native conditions for a protein interaction, strong expression should be avoided, but adjusted for proper signal detection. Another method is the choice of brighter fluorophores than EYFP, that are applicable for BiFC, and which have recently been tested, but exhibit different association dynamics that might not be useful for every interaction experiment [16].

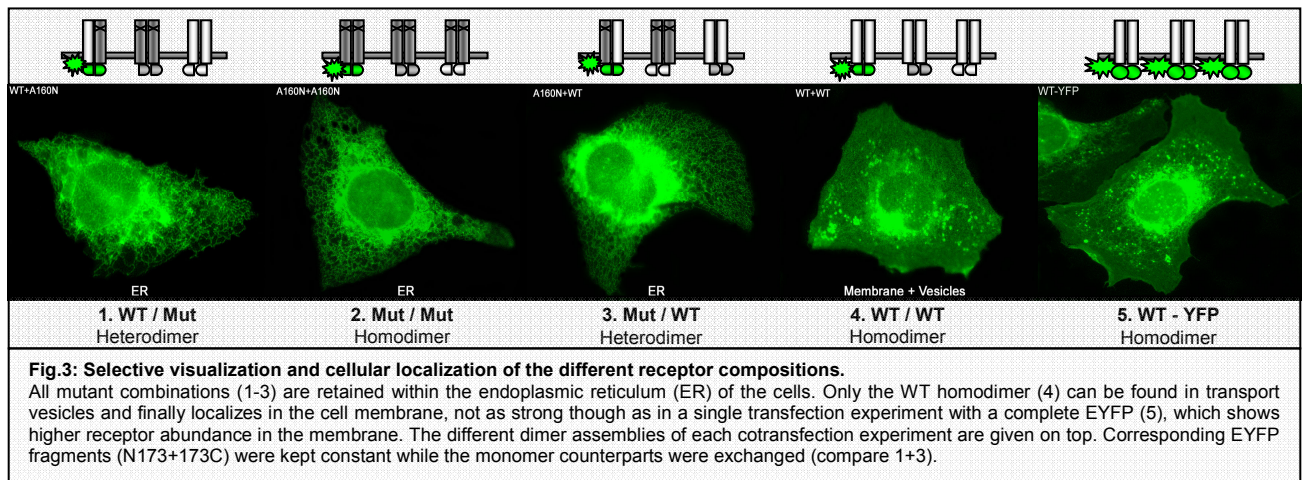
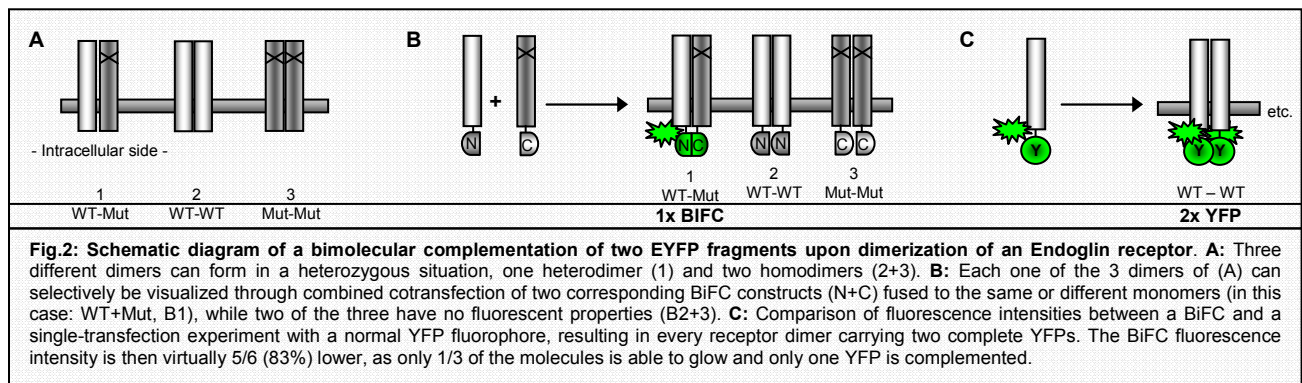
## 2.3. Sterical considerations of BiFC fusion protein interactors

The sterical orientation of an unknown protein interaction is not predictable. To study protein interactions using BiFC requires testing multiple combinations of the various fusion proteins. Even the outcome of well-characterized protein interactions cannot be predicted using BiFC, as it is not certain whether, and to what extent, the fluorophore portions interfere with the interaction. It is thus advantageous to create any possible N- and C-terminal fusion with the respective fragments, and to test the fusion proteins in any combination excluding incompatible fragment combinations. Alternatively, to support sufficient free motility of the fragments, polypeptide linkers can also be inserted between the fragments and the proteins.

### 3. Visualization of transmembrane receptors: homo- and heterodimers of endoglin

HHT is a dominantly inherited vascular disease that is caused by mutations in the endoglin or ALK1 genes. In the case of an endoglin mutation, three different molecule assemblies of the affected receptors are theoretically possible (Fig.2A), with different or same wild-type or mutant subunits.

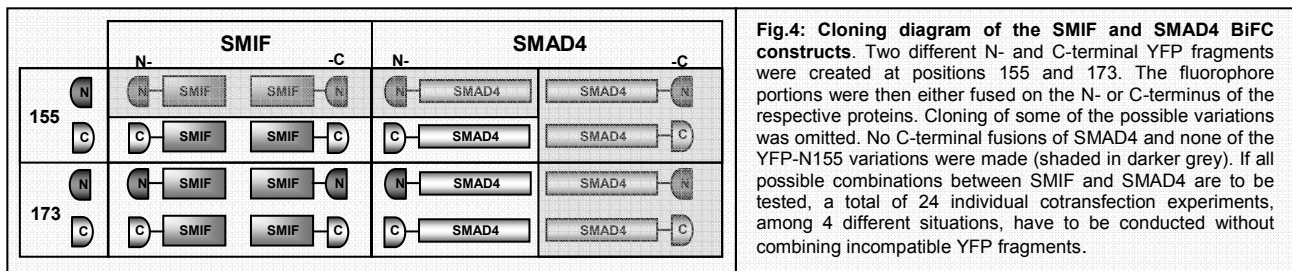
In order to investigate the fate of the different homo- and heterodimers resulting from such a genetic background, constructs were created for WT-endoglin and the endoglin missense mutant A160N. The two complementary EYFP fragments (N173 and 173C) were fused to the intracellular ends of the respective WT- and mutant- endoglin monomers, resulting in four different constructs, carrying either the one or the other BiFC fragment (not shown). The dimers of interest can then be visualized through cotransfection of the respective two complementary endoglin-BiFC constructs, which results in the complementation of the two EYFP fragments upon dimerization of the monomers (Fig.2B). For the visualization of WT or mutant homodimers, the corresponding BiFC constructs carrying the same respective WT or mutant inserts were used (Fig.3.2+4). For the visualization of the WT/mutant heterodimers, the two EYFP fragments fused to the individual monomers can be interchanged, which results in two combinations for complementation and two different BiFC molecules (Fig.3.1+3).



#### 3.1. Mutant compositions of endoglin dimers are retained within the endoplasmic reticulum (ER)

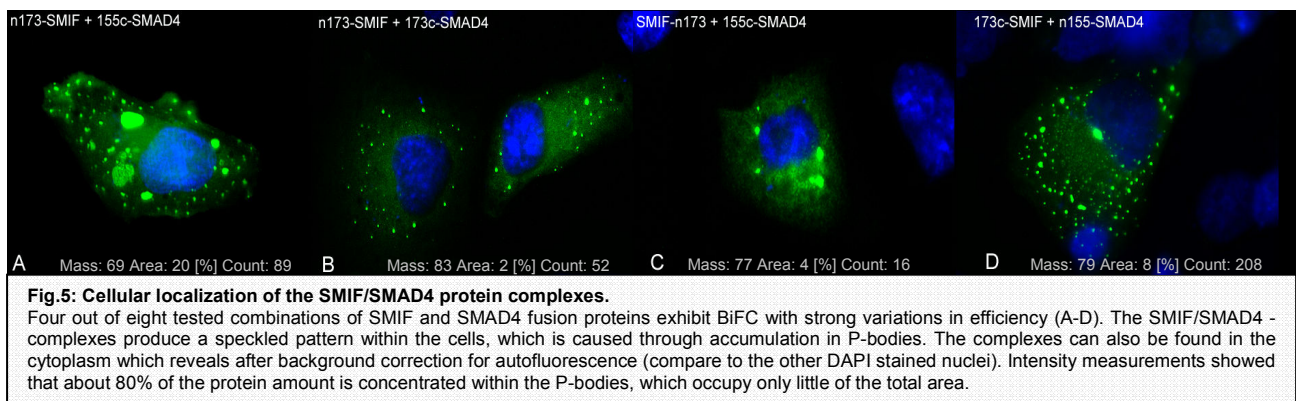
As a result of the transfection experiments it was clear that all of the three possible dimer compositions are truly formed but localize differently depending on their individual composition (Fig.3). To exclude any influence of the BiFC-fluorophore on the localization pattern of the receptors, we also expressed an endoglin-EYFP fusion protein for comparison. As expected, in both cases the WT homodimers localized normally in the membrane and transport vesicles. However, due to the visibility of every formed endoglin-EYFP receptor (see Fig.2C) the cells showed a much stronger fluorescence in the membrane than for the BiFC receptors (Fig.3.5). This demonstrates that the BiFC fluorophore has no influence on the localization of the proteins. To the contrary all of the mutant compositions of the receptor were retained within the ER. This confirmed a previous finding for the mutant-homodimer, in which only a non-membrane but intracellular localization was shown [8]. BiFC revealed its subcellular localization in the ER and even more interesting showed the same for the WT/mutant heterodimer, thus confirming a dominant negative effect of the mutant as previously suggested [8].

It is important to note that the interchange of BiFC fragments within the WT/mut heterodimers led in both situations to a functional complementation, proving that both variations are able to complement. This observation is impossible to differentiate within homodimers, and delivers some information about the protein assembly of the monomers. It appears that there is no preferential orientation between the one ("left") and the other ("right") monomer for dimerization.



#### 4. Visualization of cytoplasmic proteins: interaction of SMIF and Smad4

For the visualization of the interaction between SMIF and Smad4, a variety of constructs were cloned in order to test different combinations of the fusion proteins for fragment complementation (Fig.4). To raise the number of possibilities for combination, both of the two sites for EYFP fragmentation were used (positions 155 and 173) and the different fragments were fused to the N- and C-terminal sites of the two interactors. To obtain a minimal set of testable constructs, N155 fusions and C-terminal fusions of Smad4 were omitted. The first are less versatile and can only be used together with fusions of 155C (Fig.1), the latter allow to test all of the possible 4 situations as outlined in Fig.6.



##### 4.1. Four out of eight tested SMIF/Smad4 combinations result in BiFC and localize in P-bodies

As in the experiments with endoglin, cells were co-transfected in any possible complementary combination of the available constructs (Fig.6.). As a result of this, fluorescence was only observed in 4 out of 8 tested combinations that are in the following referred to as A-D (Fig.5). In those cells exhibiting BiFC signals the protein complexes had a high abundance in tiny to big vesicle-like structures, which most likely represent P-bodies, since this is the predominant localization of hDcp1a/SMIF [17, 18]. A small portion of SMIF/Smad4 complexes seemed not to be located in P-bodies as diffuse fluorescence was also observed in the cytoplasm. Image analysis revealed a proportional distribution of the complexes of around 80% in P-bodies and 20% in the cytoplasm. The occurrence of the speckled localization pattern was comparable within the different combinations except for the combination with opposing BiFC fragments (Fig.6.1), which showed a somewhat diffuse distribution (Fig.5.C).

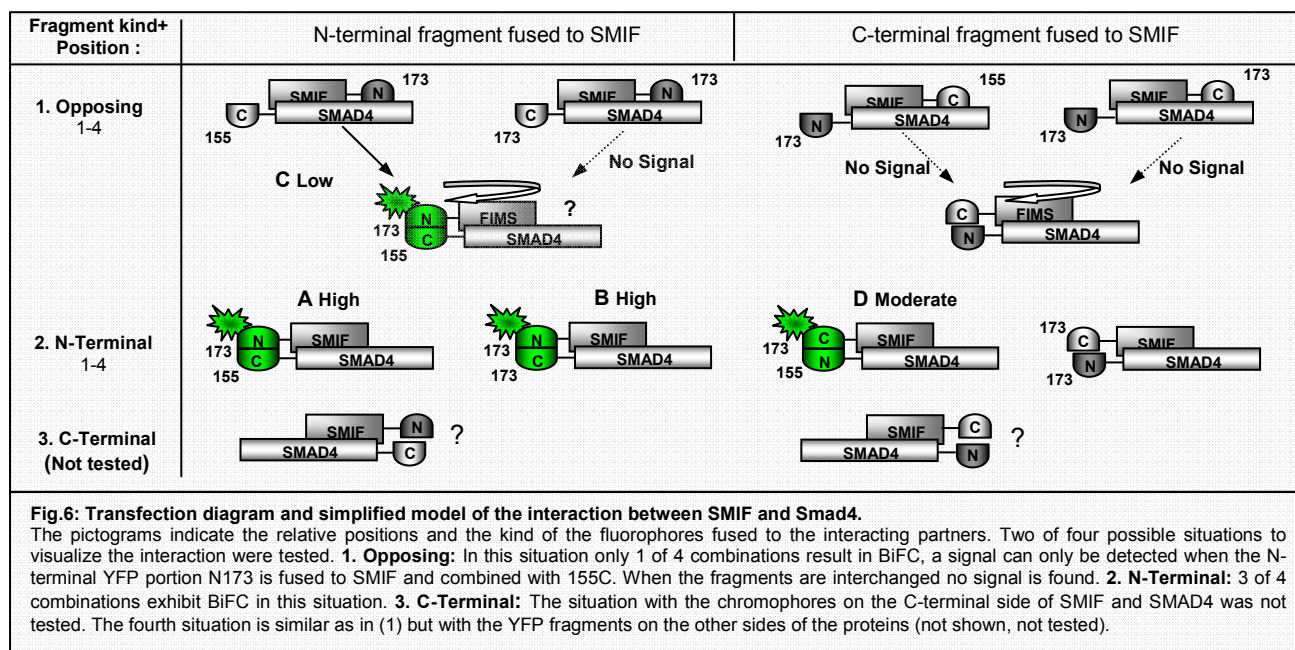
##### 4.2. The SMIF/Smad4 complexes show strong variations in BiFC efficiency

The complementation efficiencies of the BiFC positive complexes differed strongly in intensity and frequency. From the number of BiFC positive complexes among the different combinations (Fig.6), we observed that the efficiency for fragment complementation was subjected to 3 influences, depending on the kind of the fragments and their relative position within the complex:

1. Combinations with fragments fused to the same, N-terminal, side (Fig.6.2) were much more efficient (3 of 4 Combinations) than combinations with the fragments fused to the opposing ends of the proteins (1 of 4 combinations, Fig.6.1). The latter showed the lowest efficiency of all of those tested.
2. SMIF fusions with the N-terminal EYFP fragment were more efficient (3 of 4 combinations) than fusions with the C-terminal EYFP fragment (1 of 4 combinations).

3. Fragment combinations of 'N173+155C' were more efficient (3 of 4), than combinations of 'N173+173C' (1 of 4).

The first effect is caused by the relative (sterical) orientation of the interaction partners within the complex reducing the chances for the fragments to come into contact when they are fused to the opposing ends. The second effect is caused by the relative directional orientation of the fragments themselves when fused differently to the interactors, probably reducing their mobility and number of conformational states. The third effect resembles an intrinsic affinity of the fragments to complement with each other, that depends on the position of fragmentation (refer to figure 1A), which has also been reported elsewhere [16].



The combination of these three oppositional effects led either to no detectable signals, or to BiFC signals of very low (C), a moderate (D), and a higher efficiency (A+B) (Fig.6 /Fig.7). This might become clearer when an individual combination is examined.

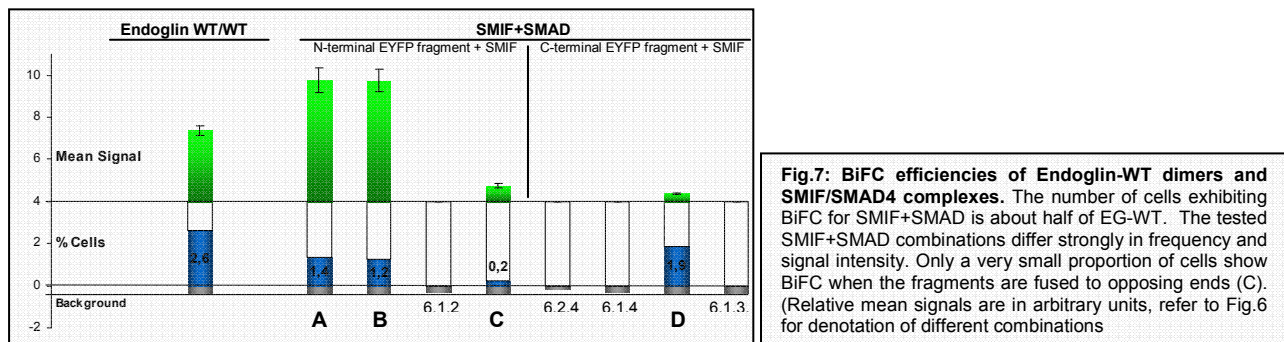
For example: When viewing the last combination in the first row in Fig.6.1 (opposing), this combination unites all of the three, non-favourable, effects for a complementation to take place: 1. the fragments are on the opposing ends, 2. the C-terminal EYFP portion is fused to SMIF, and 3. the fragments 173+173 are used. In contrast to this, the first combination of the second row in Fig.6.2 (A) unites all of the favourable influences: 1.the fragments are on the same side, 2. the N-terminal EYFP fragment is fused to SMIF, and 3. the fragments 173+155 are used. This leads to the greatest possible efficiency for the second combination, and to the least (resp. no) efficiency for the first.

For the BiFC negative combinations it cannot be proven whether the lack of a BiFC signal is caused by an impairment of the interaction through the fragments, or whether the interaction does take place, but the ability of the fragments to complement is insufficient for detection. However, when compared to the positive combinations, the attachment of the BiFC fragments apparently does not interfere with the protein interaction. At least one combination each proves the possibility for protein interaction throughout the different situations: one interaction occurs for the opposing situation (C), and one interaction occurs for the situation, when the C-terminal fragment is fused to SMIF (D). Thus, it can be assumed that protein interaction also applies for the respective negative combinations in the given situations. Therefore, if the protein interaction takes place within the BiFC negative combinations, the lack of a BiFC signal is caused by an unfavourable orientation of the fragments within the complex that somehow minimizes the chances for a successful complementation. This also explains the differences in signal intensity within the different combinations, which resembles the amount of fragment complementation after protein interaction, and not the amount of the protein interactions that have occurred in totality. Depending on the different effects that influence the complementation efficiency, only a certain proportion of all of the interactions performed will exhibit BiFC.

Moreover, the persistence of the BiFC positive complexes is promoted through the stabilizing effect of the complementation that may lead to an accumulation of steadily formed complexes in a given time. This might not occur for the negative combinations, or at a rate that does not reach the detection limit.

The microscopic investigation (Fig.5) of the four BiFC positive combinations delivered additional information about the differing efficiencies. As already mentioned, the lowest total efficiency was detected within the opposing combination of the fragments, and it was revealed that the localization of these complexes was rather diffuse and with the lowest count of P-bodies (Fig.5.C). In this case, the relative position of the fragments within the complex results in such an adverse sterical orientation of the fragments within the complex, that complementation occurs only at a very

low rate (0,2%, Fig.7C). According to this and the localization pattern, this might suggest that, if complementation takes place, the complex becomes slightly deformed and is forced to stay in this shape through the stabilizing effect of the complemented fragments. This results in loss or strong reduction of its biological function, which leads to some extent to the intracellular dislocation of the complexes apart from the P-bodies, as observed by their low number.



**Fig.7: BiFC efficiencies of Endoglin-WT dimers and SMIF/SMAD4 complexes.** The number of cells exhibiting BiFC for SMIF+SMAD is about half of EG-WT. The tested SMIF+SMAD combinations differ strongly in frequency and signal intensity. Only a very small proportion of cells show BiFC when the fragments are fused to opposing ends (C). (Relative mean signals are in arbitrary units, refer to Fig.6 for denotation of different combinations)

On the other hand, the N-terminal combinations, A and B, had high fluorescence intensities and a moderate frequency, when compared to combination D (Fig.7). These intensities are caused by a high number of complexes concentrated within the P-bodies, which became huge in some cases, as a result of the accumulation (Fig.5.A). This also resembles the above-mentioned influences on fragment complementation, which strongly promote the complementation for these two combinations. All of these three combinations of SMIF and Smad4 (A,B and C), however, do not seem to be physiological and do not resemble any of the biological functions of the complex. For the first two combinations (A+B), the promoting effects on fragment complementation are too strong, leading to an exaggerated accumulation of the complexes. In the third combination (C), the effects are so unfavourable for complementation that, when performed, the shape of the complex is altered in such a way that it loses its biological function.

Surprisingly, this extra strong effect of the combinations A and B cannot be observed when the N-terminal BiFC fragment fused to SMIF is exchanged with a C-terminal fragment, as is the case in combination D. The C-terminal fragment has a less favourable orientation, but complementation is promoted through the higher affinity of the fragment combination 173+155 instead of 173+173. In this last combination (D), the influences on the complementation seem best to meet the physiological conditions. As a result, the cells have the highest number of P-bodies of a tiny to small size and exhibit moderate fluorescence intensity, and the frequency of the occurrence for BiFC within the cells is highest.

## 5. Conclusions

### 5.1. BiFC proved to be a reliable method for the visualization of specific protein interactions

The known homodimeric character of the endoglin receptor was clearly confirmed through the application of BiFC. It was possible to show that all of the endoglin receptor compositions are truly formed and that the endoglin mutant tested here restrains the wild-type endoglin within the ER. Thus, BiFC allowed us to solve the longstanding debate about whether endoglin missense mutants dimerize with wild-type and what the fate of these heterodimers is.

As well as for endoglin, we also could confirm the previously described interaction between SMIF and Smad4. The interaction of the two proteins had initially been found in a yeast two-hybrid screen, and was further characterized by co-immunoprecipitation. Owing to the BiFC method, we gained additional information about the subcellular localization of the complex to be most likely located in P-bodies. This had not been described before and was only known for hDcp1a/SMIF

### 5.2. Advantages and disadvantages of BiFC: High specificity versus low fluorescence intensity/frequency

In our findings the method combines the advantages of a high specificity for protein interaction analysis together with a low instrumental effort. For the instrumental setup standard laboratory cloning techniques for construct generation, cell culture and a conventional fluorescence microscope are sufficient. For evaluation of BiFC efficiencies image analysis can be conducted instead of flow cytometry.

The specificity of fluorescence signals that resulted from the protein interactions was displayed through at least three different observations. 1. The examined protein complexes of endoglin and SMIF/Smad4 showed in all cases but one distinct localization patterns instead of diffuse fluorescence without a clear localization. The localization patterns also met the expectation, as they confirmed the localization of wt-endoglin and the mutant, which had previously been examined using constructs with native EYFP fusions. The visualization of SMIF/Smad4 produced the same pattern like known for hDcp1a/SMIF alone, which is abundantly used as a P-body marker. The only exception of the latter interaction was found within the combination with the opposing fragments. But even the diffuse localization of the

complexes in this combination showed to be a specific result of the different influences on BiFC formation (as explained in section 4.2). 2. The negative controls that were conducted by cotransfecting unfused EYFP fragments together with a single complementary BiFC interactor-protein resulted in no detectable signals (data not shown). 3. We found that it was rather more difficult to obtain any detectable BiFC signals than to be confronted with a high number of BiFC events, additionally illustrating the specificity of the method.

In contrast to the high specificity, low fluorescence intensities and a low frequency of BiFC events proved to be the two main technical difficulties to be overcome. This is displayed by the results of the image analysis that was conducted using a 20x magnification lens for global signal detection and measurement, to replace flow cytometry analysis (Fig.7). The maximal frequency of BiFC events throughout all of the experiments reached not even 4% of the total number of cells. And the mean intensities, shown as arbitrary units in Fig.7, revealed to be so low, that it became problematic to distinguish BiFC positive cells from the overall autofluorescence, during the microscopic investigation under a higher magnification. In most cases BiFC positive cells could rather be identified through their distinct localization pattern, than through an outstanding brightness.

### 5.3. Reasons for low BiFC efficiencies depend on the nature of the protein interactions

The low intensities of the investigated protein interactions are not due to a lower intrinsic fluorescence property of a complemented EYFP fluorophore [12], but the result of the relative amount of BiFC exhibiting protein complexes. In the case of endoglin, the fluorescence intensities are influenced by the ratio of dimer formation, which leads to 2/3 of “blind” dimer molecules (see Fig.2B). This ratio, however, is found only under the hypothetical assumption of an equilibrated transfection event of the two constructs. As soon as one of the two constructs is in quantitative excess in a transfected cell, the affinity for self-assembly of the respective monomer is strongly favoured and the possibility for BiFC becomes lower than 1/3. At the same time, however, the quantitative assembly of dimers with compatible fragments is also raised to the disadvantage of the third (incompatible) dimer. Due to this fact, it is actually possible that BiFC can finally take place when different constructs amounts are present. Detection of BiFC fluorescence is then only limited by the total DNA amount of the minimal transfected counterpart. With respect to all possible construct ratios and the respective individually necessary amount of the minimal counterpart, the threshold for the occurrence of BiFC becomes very small. Due to this circumstance, the fluorescence of a relative high proportion of all the transfected cells is below the detection level, which leads to the very low number of BiFC-positive cells. Thus, even a relative high transfection efficiency can nevertheless lead to a low number of BiFC positive cells. This was seen when we compared endoglin-EYFP with endoglin-BiFC transfections. Typical transfection efficiencies for endoglin-EYFP reach about 40-60%. However, when cells were transfected with the same amount of BiFC constructs under the same conditions only about 3% of the cells exhibited BiFC signals.

For the interaction between SMIF and Smad4 the frequencies were even lower than for endoglin, but could reach substantially higher intensities. When viewing the different BiFC efficiencies throughout the experiments with endoglin and with SMIF and Smad4, the structural differences of the two protein interactions accounting for the individual outcomes have to be taken into consideration.

The dimerization of the endoglin receptor is, in fact, a protein interaction that is not comparable to the one occurring between SMIF and Smad4. As shown in Fig.7, the BiFC frequency of the endoglin dimers was higher than for the SMIF/Smad4 interactions. This is due to the fact, that endoglin exists exclusively as a dimer, which is already assembled in the ER, as it was shown for the WT/mut dimers. And the dimerization of the endoglin receptors occurs at a rate of virtually 100%, but their visibility is limited by the proportional assembly of monomers with incompatible EYFP fragments (which theoretically leads to the 2/3 reduction).

In contrast to this “forced” dimerization, the protein interaction between SMIF and Smad4 can be influenced through competitive interaction with other proteins, and it also underlies the biological regulation of the cell. Moreover, the chance for BiFC to occur is strongly influenced by the kind of the attached fragments and their relative position within the complex, as it is displayed through the high variability of complementation efficiencies observed within the BiFC positive combinations. The sum of these influences leads to a lower probability for the protein interaction to take place than it is the case for endoglin.

The differences of the signal intensities among endoglin-WT and the different SMIF/Smad4 complexes are the result of the respective cellular localization of the molecules. The main amount of the endoglin-WT homodimers is equally distributed throughout the cell membrane which spans the total available cell area, which leads to a lower mean fluorescence intensity than for the SMIF/Smad4 complexes. The main amount of the latter is concentrated in P-bodies that occupy only a very small proportion of the total cell area (Fig.5). Due to this concentration of the complexes in the P-bodies the fluorescence intensities can become higher than for the endoglin-WT homodimer.

### 5.4. Summary

Besides a low instrumental effort, the BiFC method combines the advantage of a high expectable specificity for the visualization of protein interactions of highly different nature. Moreover, BiFC offers the possibility of gaining insight into the relative orientation of the interacting partners [19]. Opposed to this is the difficulty entailed by low intensities and maybe low occurrence of events, which however, might reflect the physiological relevance of these events. As

outlined, these difficulties can be caused by absolutely different reasons depending on the nature of the protein interaction. For the identification of a BiFC positive protein interaction multiple combinations of the interacting proteins have to be tested, and BiFC positive combinations have to be further characterized. The need for further functional characterization of a given BiFC interaction is clearly displayed through the high variability of BiFC events and their respective cellular localization pattern as exemplary shown for the interaction between SMIF and Smad4, and in particular for combination C. This at last also illustrates the strong influence of the fragment complementation dynamics that a protein interaction can be exerted to.

## 6. Material and Methods

The native EYFP in the original vectors EYFP-N1 and -C1 (Clontech) was replaced by PCR generated BiFC fragments. The obtained vectors were used for cloning of the mentioned constructs. Constant seeds of CHO cells were transfected with constant DNA amounts (250ng/100.000cells/construct). Cells were fixed and DAPI stained 20h after transfection and mounted on slides for microscopy. Cell counts and intensity measurements of the different SMIF/Smad4 combinations were conducted by image processing (ImageJ) of 50 images each taken with a 20x lens under constant exposure. Cell counts were expressed as % of cells exhibiting BiFC to the DAPI nucleus total number. Fluorescence intensities were corrected for autofluorescence of untransfected samples. Negative controls for BiFC of SMIF+Smad4 were conducted by equal amount co-transfection of free complementary EYFP fragments that resulted in no detectable signals.

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