Induced heterodimerization and purification of two target proteins by a synthetic coiled-coil tag

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Abstract: A synthetic *de novo* designed heterodimeric coiled-coil was used to copurify two target fluorescent proteins, Venus and enhanced cyan fluorescent protein (ECFP). The coiled-coil consists of two 21-amino acid repetitive sequences, (EIAALEK)₃ and (KIAALKE)₃, named E3 and K3, respectively. These sequences were fused to the C-termini of ECFP or Venus followed by either a strep- or a his-tag, respectively, for affinity purification. Mixed lysates of Venus-K3 and ECFP-E3 were subjected to consecutive affinity purification and showed highly specific association between the coiled-coil pair by SDS-PAGE, gel filtration, isothermal titration calorimetry (ITC), and fluorescence resonance energy transfer (FRET). The tagged proteins eluted as heterodimers at the concentrations tested. FRET analysis further showed that the coiled-coil pair was stable in buffers commonly used for protein purification, including those containing high salt concentration and detergent. This study shows that the E3/K3 pair is very well suited for the copurification of two target proteins expressed *in vivo* because of its high specificity: it forms exclusively heterodimers in solution, it does not interact with any cellular proteins and it is stable under different buffer conditions.

Keywords: synthetic coiled-coil; heterodimer; copurification; ECFP; Venus; FRET

Introduction

Proteins can associate using a wide range of mechanisms. One of the simplest motifs in tertiary or quaternary protein–protein interactions is the coiled-coil, which can be found in all kingdoms of life. Some analyses suggest that up to 10% of eukaryotic proteins contain coiled-coil motifs.¹

A coiled-coil consists of two or more alpha-helices that wind around each other, generating a higher-order supercoil structure. Coiled-coils have a seven amino acid long basic unit (heptad: *abcdefg*) that is repeated several times.^{2,3} The individual heptad residues occupying the positions *a* and *d* are hydrophobic and permit the association of different coiled-coil strands following the knobs-into-holes model proposed by Crick in 1953.⁴ The association of residues in positions *a* and *d* create a tight hydrophobic core that is stabilized by ionic interactions of polar and charged amino acids in positions *e* and *g*.^{2,5–7} The residues in the remaining positions are normally polar and are exposed to the solvent (Fig. 1).

Coiled-coils can homo- or hetero-associate, forming dimers, trimers, or higher-order oligomers in parallel or antiparallel configurations.⁸ The regularity and simplicity of the motif has proved to be a very good candidate for *de novo* protein design in

Abbreviations: ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; TEV, tobacco etch virus.

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the past;^{2,9–15} for example, properties like the association constant, specificity, and oligomerization state of interacting peptides in solution could be controlled to some extent.^{12,16} A synthetic, *de novo* designed coiled-coil offers the possibility of specifically copurifying weakly interacting proteins that otherwise would not withstand conventional purification procedures and to analyze their interaction *in vitro*, even at low concentration. From the small size of a coiled-coil tag, we hypothesize that steric hindrance effects within the heterodimer would also be minimized when fused to a weakly interacting protein pair. In this work, we have used a coiled-coil pair (E3/K3)^{11,12,17} as a tag to heterodimerize the two GFP variants ECFP and Venus, purified the heterodimer by a two-step affinity chromatography to homogeneity, and analyzed the E3/K3 interaction. The E3/K3 pair has the advantages of being short (21 amino acids per tag) and behaving as a heterodimer when the free peptides are analyzed in solution. The E3/K3 pair consists of two separate three-heptad repeats: E3 contains the sequence (EIAALEK)₃ and K3 is composed of three repeats of KIAALKE [Fig. 1(A,B)]. Using gel filtration, microcalorimetry, and FRET experiments, we found that the addition of the E3/K3 pair allows ECFP and Venus to be specifically and tightly heterodimerized with a 1:1 stoichiometry at nanomolar affinity, and that the heterodimer is stable under different conditions. Our results indicate that the E3/K3 coiled-coil pair is a useful tag for inducing dimerization and enabling copurification of weakly or noninteracting proteins.

Results

Influence of peptide linkers and concentration on the in-solution behavior of the K3 and E3 chimeric proteins

When free K3 and E3 peptides are mixed, they form a stable heterodimeric coiled-coil interaction.^{12,17} However, free K3 peptides alone can also form homodimers in solution.¹⁸ To test whether Venus-K3 chimeras form homodimers and/or higher-ordered oligomers, three Venus-K3 constructs containing different linkers (construct-1: Venus-K3-KLAATLE-His containing the sequence KLAATLE as linker; construct-2: Venus-TEV-K3-pG-His containing a polyglycine linker; construct-3: Venus-TEV-K3-PPG-His containing a di-proline motif as linker) were generated [Fig. 1(C)], affinity-purified and subjected to gel filtration. We found that the linker construct-1, which contains the endogenous linker encoded on the pET-21a expression plasmid preceding the hexahistidine tag, elutes as a protein species of 65 kDa [Fig. 2(A)]. This is double the size of a monomer (33.1 kDa). As the linker in construct-1 (KLAATLE) strongly resembles a heptad repeat and only a single protein band can be observed by SDS-PAGE, it is

Figure 1. Depiction of the E3/K3 interaction. (A) Helical wheel representation of the E3/K3 coiled-coil. (B) NMR solution structure of the E3/K3 coiled-coil (PDB code 1U0I). The K3 peptide is shown on the left, the E3 on the right. Leucines and isoleucines are depicted by spheres, glutamic acids and lysines by sticks. (C) Depiction of the four constructs used in this study, showing both the encoded sequences and the restriction cleavage sites, as well as the calculated molecular weights. The asterisk denotes a stop codon. “pG,” polyglycine linker (GGSG); “PPG” diproline motif linker; “TEV” TEV protease cleavage site; “His” hexahistidine tag; “Strep” Strep-TagII (WSHPQFEK).

likely that the 65 kDa protein corresponds to a homodimeric species that is generated by introducing an additional “repeat”, similar to what has been observed for synthetic peptides in solution previously.^{11,12} The exchange of the linker to either a polyglycine- or a diproline-motif caused the proteins to elute as monomers [Fig. 2(B,C)].

Moreover, all chimeric constructs exist as higher oligomeric species when analyzed at a higher protein concentration. The maximum molecular weight species observed with the pG and diproline-linker is a dimer. In contrast, the KLAATLE construct can form a 112-kD protein consistent with a tetrameric species, indicating an intrinsic propensity of homooligomerization at elevated concentrations in accordance to what has been shown previously for free peptides.¹⁸ Thus, both the concentration and the type of linker affect the oligomerization state of the K3 peptide when fused to a protein.

In the case of ECFP-TEV-E3-Strep, the concentration-dependent homodimerization effect is very low [Fig. 2(D)]. The fact that only a slight increase in molecular weight is observed at higher protein concentration indicates that any homotypic interactions are very weak and most probably transient. It

Figure 2. Effect of concentration on the association state of K3- and E3-tagged variants. All samples were first affinity purified and analyzed by SDS-PAGE (inset) prior to gel filtration. The samples were then brought to the desired concentration (4 and 0.2 mg mL⁻¹) in a final volume of 250 μ L and loaded onto a Superdex200 column. Theoretical molecular weights: Venus-K3-KLAATLE-His 33.1 kDa (K3-KLAATLE variant); Venus-TEV-K3-pG-His 33.7 kDa (K3-pG variant); Venus-TEV-K3-PPG-His 33.8 kDa (K3-PPG variant); ECFP-TEV-E3-Strp 32 kDa. The molecular weights of the eluted peaks have been calculated by extrapolating from a gel filtration standard curve and are noted above each elution peak in kDa. (A) K3-KLAATLE variant gel filtration profiles of 4 mg mL⁻¹ (dashed line) and 0.2 mg mL⁻¹ (continuous line) loaded sample. (B) K3-pG variant gel filtration profiles of 4 mg mL⁻¹ (dashed line) and 0.2 mg mL⁻¹ (continuous line) loaded sample. (C) K3-PPG variant gel filtration profiles of 4 mg mL⁻¹ (dashed line) and 0.2 mg mL⁻¹ (continuous line) loaded sample. (D) ECFP-TEV-E3-Strp gel filtration profiles of 4 mg mL⁻¹ (dashed line) and 0.2 mg mL⁻¹ (continuous line) loaded sample.

is, however, also possible that the observed behavior for ECFP-TEV-E3-Strp is due to a weak interaction between ECFP monomers¹⁹ and thus does not originate from the E3 peptide itself. Taken together, the E3 and two of the K3 constructs behave as monomers at concentrations of 0.2 mg mL⁻¹ or below and are suitable to test for their heterodimerization properties.

E3/K3-driven heterodimerization of the fusion proteins and distance estimation

We next wanted to determine if the E3- and K3-tagged proteins can form stable heterodimers that can be purified. In the first set of experiments, we mixed equimolar amounts of previously purified, monomeric chimeric proteins (Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp) and analyzed the oligomeric state by gel filtration and SDS-PAGE. We found that the chimeras associate to generate a species with a molecular weight equivalent to the heterodimer [Fig. 3(A)]. Then we tested whether heterodimerized proteins can be specifically obtained when bacterial lysates containing either overexpressed Venus-TEV-K3-pG-His or ECFP-TEV-E3-Strp are incubated and subsequently purified by a two-step

affinity chromatography (NiNTA- followed by Strep-Tactin-affinity chromatography). The eluate was subjected to gel filtration and SDS-PAGE. The purified species behave as a heterodimer of $\sim$ 1:1 stoichiometry and is clearly distinguishable from the monomeric E3- and K3-tagged proteins alone [Fig. 3(B)]. Additionally, the concentration of the E3/K3 heterodimer does not affect its association state, indicating that the E3/K3 interaction is stable and highly specific.

In the case of the K3/E3 heterodimer formed by mixing *E. coli* lysates of overexpressed proteins and isolated by a two-step affinity purification, the sample is >95% pure as judged by Coomassie staining [Fig. 3(B)]. Even after a single affinity purification, the eluted K3- and E3-tagged samples are highly pure (Fig. 3), independent of the construct used. The fact that the eluted samples are free of any contaminants suggests that the K3 and E3 peptides only associate with their designated partner and are inert toward binding to other cellular proteins.

We further subjected the purified Venus-K3/ECFP-E3 heterodimer to ITC measurements to determine the stoichiometry as well as the affinity of the E3/K3 interaction. ITC measurements of the

Figure 3. Formation of heterodimers. (A) Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp were purified from cell lysates and mixed at equimolar amounts. Middle panel, SDS-PAGE analysis of the eluted fraction from the affinity column (Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp) and gel filtration peak of the mixed monomers. Right panel, Superdex200 gel filtration profile of the purified monomers (“K3” = Venus-TEV-K3-pG-His; “E3” = ECFP-TEV-E3-Strp) versus mixed monomers at equimolar amounts (“E3/K3,” continuous line). (B) Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp cell lysates were mixed and subjected to a two-step affinity purification (StrepTactin after NiNTA). Right panel, Superdex200 gel filtration profile of the two-step affinity purified heterodimer (dashed line, 4 mg mL⁻¹ loaded sample; continuous line, 0.2 mg mL⁻¹ loaded sample) and SDS-PAGE analysis of the peak fraction as an inset within the graph, “K3” = Venus-TEV-K3-pG-His; “E3” = ECFP-TEV-E3-Strp).

combined K3- and E3-tagged fusion proteins (Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp) revealed a 1:1 molar ratio and a high affinity interaction [K_d of 89.3 ± 5.2 nM] [Fig. 4(A)]. This is in good agreement with the dissociation constant determined for the free E3/K3 coiled-coil (K_d in the range of 70 nM).^{12,17,18} To test the heterodimerization ability of the purified monomers, Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp were mixed at equimolar concentrations (2 μ M each) in 200 μ L lysis buffer. The fluorescence levels in the different channels (Venus, FRET, and ECFP) were measured and compared to those of a 2 μ M solution of the two-step affinity purified heterodimer [Fig. 4(B)]. The FRET efficiency and distance estimation were calculated [Fig. 4(C)] by the fluorescence reduction of the donor (ECFP) in the presence of the acceptor (Venus). The fluorescence levels of the mixed monomers and the purified heterodimer are the same, confirming that virtually all the monomeric molecules are able to heterodimerize. The FRET efficiency (E) and distance estimation (d) are also equal ($E = 0.46 \pm 0.03$ for the mixed samples and 0.45 ± 0.03 for the two-step affinity purified heterodimer; $d = 51.3 \pm 0.5$ Å for the mixed samples and 51.6 ± 0.5 Å for the two-step affinity purified heterodimer). The distance that a given linker provides between the E3/K3-tag and

the proteins of interest can be crucial for their successful copurification. If the proteins are bulky and the linker too short, the K3 and E3 peptides might fail in forming a stable coiled-coil interaction due to steric hindrance.

The E3/K3 interaction is stable under different buffer conditions

If the E3/K3 system is to be introduced as a general tool for protein purification, it has to be resistant to commonly used reagents and treatments, such as detergent solubilization and high salt washes. Previous studies have shown that coiled-coils with similar sequences to the E3/K3 pair are stable at high salt concentration.¹⁵ To test the stability of the E3/K3 interaction when fused to a protein, FRET efficiency and distance was measured in buffers containing high salt, detergent, glycerol, and ethanol. Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp monomers were dialyzed against the chosen buffer (except for Triton X-100, where the detergent was added to the desired final concentration) and mixed to a final concentration of 2 μ M. We found that the E3/K3 coiled-coil is stable under a variety of buffer conditions [Fig. 5(A,B)]. The small differences seen in the FRET efficiency could be due to the conformation of the C-terminus, comprising the TEV cleavage site,

Figure 4. Properties of heterodimerized Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp. **(A)** Isothermal titration calorimetry of ECFP-TEV-E3-Strp binding to Venus-TEV-K3-pG-His. Top panel, data from 30 injections of 100 μ M ECFP-TEV-E3-Strp into the cell containing 10 μ M of Venus-TEV-K3-pG-His in lysis buffer at 25°C. Bottom panel, best Chi-square fit (continuous line) to the calorimetry data (squares), analyzed with MicroCal Origin software. The calculated dissociation constant is $K_d = 89.3 \pm 5.2$ nM. **(B)** Equimolar amounts of the affinity purified Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp monomers were mixed (gray bars) and its fluorescence compared to the affinity purified heterodimer (white bars) in triplicate. Final concentration 2 μ M of each species in 200 μ L lysis buffer. Error bars show the standard error (σ/n). **(C)** Comparison of efficiency and distance estimation for the “mixed” and “two-step affinity purified” samples (Fig. 3).

the coiled-coil region and the affinity tag. These could have a tighter or more extended conformation depending on the buffer used, which would translate into a modest distance change between the monomers.

A moderate change in the FRET efficiency produces almost no effect in the distance estimation, since the latter is proportional to the sixth root of the inversed efficiency.²⁰ If the E3/K3 tags are cleaved with TEV, the FRET signal is lost [Fig. 5(A,B)]. A recent study has shown that the stability of free peptides of the E3/K3 coiled-coil is pH-dependent and disrupts at pH 5: E3 forms homotrimers and K3 remains largely as monomers with a small fraction of homodimers.¹⁸ However, when the two-step affinity purified Venus-E3/ECFP-K3 heterodimer is subjected to size exclusion chromatography in acetate buffer at pH 5, a single-peak elution profile is seen [Fig. 5(C)]. The elution peak has a larger molecular weight than a monomer but smaller than a dimer. The peak is not symmetric, and has a “tail” that extends up to the expected elution volume of the monomers. This indicates that the E3/K3 interaction in chimeric proteins is disrupted to some extent at pH 5. The fact that the sample does not elute at the expected volume for the monomers suggests that the interaction at pH 5 is weak but

not entirely lost, with monomers and dimers in equilibrium. The absence of the E3-homotrimers observed in previous studies is probably due to steric hindrance and charge effects of the linker, coiled-coil sequence and affinity tag at lower pH, which would prevent homomeric interactions. However, FRET analysis of this sample was not possible due to the pH sensitivity of the fluorescent proteins.^{21,22} Taken together the E3/K3 system is a highly specific heterodimerization tool, displaying a strong affinity suitable for purification of two weakly interacting proteins that would not withstand conventional copurification and resistant to many commonly used reagents.

Discussion

In this study we have shown how a small *de novo* designed heterodimeric coiled-coil can be used as a dimerization tool. The addition of an affinity tag at the C-terminus allows efficient affinity purification of heterodimers. The E3/K3 interaction is highly specific and very stable. The properties of the system make it a very powerful tool for experiments that need a strong association of two target proteins, while avoiding the use of a bulky dimerization tag. Although other coiled-coils have been used in protein

Figure 5. Stability of the E3/K3-tagged heterodimer under different buffer conditions. Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp monomers were mixed at a final concentration of $2 \mu\text{M}$ each in different buffer conditions. The FRET efficiency, measured as the decrease in donor fluorescence in the presence of acceptor, was calculated. This value was used to estimate the distance between monomers. **(A)** FRET efficiency of the mixed sample in different buffer conditions. If the tags are cleaved with TEV protease, no FRET occurs. **(B)** Distance calculations of the mixed samples in different buffer conditions. Note that the estimated distance of the TEV cleaved samples ($89 \pm 0.6 \text{ \AA}$) is very close to the effective limits for FRET (around $100 \text{ \AA}$). **(C)** pH dependency of the E3/K3 interaction. “E3/K3 pH 5” = 0.2 mg mL^{-1} of the “two-step affinity purified” (Fig. 3) heterodimer at pH 5, as compared to Venus/ECFP heterodimer (“E3/K3”) and Venus-TEV-K3-pG-His monomer (“K3”).

purification,^{9,10,15,22,23} we have chosen the E3/K3 pair because of its small size and its optimal association properties.¹² E3/K3 has also recently been used as a fluorescent marker to study surface membrane receptors and their internalization,²⁴ which suggests that it can also be used in eukaryotic cells. The K3 peptide has a tendency to homodimerize at higher concentrations, as observed in Ref. 18, although this effect seems to be more pronounced with protein fusions than in the free peptide. The E3 fusion constructs behave as monomers in solution, up to the concentrations tested. However, the results at pH 5 considerably differ from those obtained in earlier studies.¹⁸ E3 fusion variants do not form homotrimers, and the E3/K3 interaction seems to be disrupted only to some extent. These differences, as well as the ones observed at pH 7, could be explained by the presence of different neighboring residues, which would contribute additional electric charge to the construct or even change the conformation of the tag. Further investigation on these mechanisms could allow the generation of heterodimerization tags with tuned association properties.

The induced dimerization of proteins has previously been examined in different experimental setups. Bimolecular complementation, such as split luciferase,²⁵ bimolecular fluorescence complementation (BiFC),^{26,27} and the TEV protease split system,²⁸ as well as the dimerization domains FRB and FKBP²⁹ have been used to study conformational changes and the association states of proteins within cells. However, these systems are not compatible with affinity purification because they were designed to observe natural interactions between protein subunits, but not to maintain them. Another coiled-coil pair, GCN4, has been used as a tool for the purification of VEGF receptor ectodomains³⁰ and in EGFR kinase domain studies,³¹ but it is based on the natural activator GCN4 and thus may interact with endogenous proteins. We wanted to generate a completely synthetic pair to avoid any potential interactions with cellular proteins. Additionally, the GCN4 interaction is homomeric, meaning that if two different proteins were tagged with dimeric GCN4, two thirds would form homodimers. In light of the results, the E3/K3 pair allows to specifically study

protein homo- and heterodimers that are not stable enough to survive purification and it could be easily transplanted to other protein systems.

Materials and Methods

Cloning of Venus/ECFP E3/K3 constructs

Four chimeric proteins were generated: (1) Venus-K3-KLAATLE-His, containing the endogenous pET21 linker sequence (KLAATLE) before the his-tag. (2) Venus-TEV-K3-pG-His, containing a TEV cleavage site before and a polyglycine linker after the K3 sequence followed by a his-affinity tag; (3) Venus-TEV-K3-PPG-His, containing a TEV cleavage site before and a diproline motif after the K3 sequence followed by a his-affinity tag; and (4) ECFP-TEV-E3-Strp, containing a TEV cleavage site before and a Strp-affinity tag after the E3 sequence.

The open reading frames for Venus and ECFP were amplified from the plasmids pPWV and pPWC, a kind gift from Leonie Ringrose (IMBA, Vienna), and cloned between the NdeI and HindIII sites of a pET-21a vector (Novagen). An S-tag (KETAAK-FERQHMDs) coding sequence was added at the 5' end of Venus, to increase its molecular weight and differentiate it from ECFP in subsequent analyses. Both Venus and ECFP sequences contain an internal Bsp1407I site at the C-terminus. For construct-1 (KLAATLE variant), two complementary oligos coding for a polyglycine linker just after the Venus sequence and followed by the K3 coding sequence were annealed. They included the overhangs for Bsp1407I and HindIII, and were cloned into these sites of the pET-21-S-Tag-Venus-His. The oligo sequences are [K3+: 5'-GTACAGCGGAGGCTCTGG CAAGATTGCCGCTCTGAAAGAGAAAATCGCCG CTCTGAAGGAGAAAATCGCTGCCCTGAAGGA GA-3']; [K3-: 5'-AGCTTCTCCTTCAGGGCAGCG ATTTTCTCCTTCAGAGCGGCGATTTTCTCTTC CAGAGCGGCAATCTTGCCAGAGCCTCCGCT-3'] where the bold sequence corresponds to the K3 peptide. For construct-2 (polyglycine variant), the same strategy was followed, but this time the annealed sequences contained a BamHI site at the 5' end (underlined), followed by the same K3 sequence as above and a polyglycine linker just before a XhoI overhang at the 3' end [K3pG+: 5'-GTACAGCGGC GGATCCGGC-(K3)-GGTGGCAGTGGCC-3'; K3pG-: 5'-TCGAGGCCACTGCCACC-(K3)-GCCGGATCCGC CGCT-3'].

The linker between the K3 peptide and the hexahistidine tag in this case is GGSGLLE.

A TEV cleavage site was then introduced by annealing two complementary oligos coding for the TEV cleavage site (ENLYFQG) and cloning them into the Bsp1407I and BamHI sites resulting to Venus-TEV-K3-pG-His [TEV+: 5'-GTACAGCGGC-

GAGAACCTGTACTTCCAGGGCG-3'; TEV-: 5'-GATC CGCCCTGGAAGTACAGTTCTCGCCGCT-3']

Cloning of construct-3 (di-proline motif, PP) was done similarly as for construct-2 but using coding sequences for a diproline motif [PPG+: 5'-GTAC AGCGGCGGATCCGGC-(K3)-CCTCCTAGTGGCC-3'; PPG-: 5'-GGCCACTAGGAGG-(K3)-GCCGGATCCGC CGCT-3'].

For the E3 construct, two oligos coding for a BamHI site (underlined) within a polyglycine linker followed by the E3, polyglycine and Strep-TagII sequences were annealed and cloned into the Bsp1407I and HindIII sites of the pET21-ECFP vector. A stop codon was included after the Strep-TagII sequence:

- [E3+: 5'-GTACAGCGGAGGATCCGGCGAGATCG CTGCCCTGGAGAAGGAAATTGCTGCTCTGG AAAAGGAGATTGCTGCCCTGGAGAAGGGCG GCTCCGGCTGGTCCCCACCCCAAGTTCGAGAAG TGAA-3';
- E3-:5'-AGCTTTCACCTTCTCGAACTGGGGGTGGG ACCAGCCGGAGCCGCCCTTCTCCAGGGCAGC AATCTCCTTTTCCAGAGCAGCAATTTCTTC TCCAGGGCAGCGATCTCGCCGATCCTCCG CT-3']

A TEV cleavage site was introduced by annealing two complementary oligos coding for the TEV cleavage site (ENLYFQG) and cloning them into the Bsp1407I and BamHI sites of pET-21-ECFP-E3-Strp.

Expression, purification, and TEV cleavage of E3/K3-tagged ECFP/Venus

The different vectors were transformed into BL21 Rosetta cells and grown on LB plates supplemented with ampicillin. Single colonies were picked and transferred to 1 L LB medium in the presence of ampicillin and chloramphenicol (100 and 25 $\mu\text{g mL}^{-1}$, respectively). Protein expression was induced overnight at $\text{OD}_{600} = 0.7$ at 25°C with a final concentration of 1 mM IPTG. Cells were then harvested at 6000g, 15 min and the pellet resuspended in 70 mL lysis buffer (Tris 20 mM, NaCl 150 mM, imidazole 10 mM, pH 7.5). The suspension was sonicated three times for 1 min and centrifuged at 20,000g, 25 min, 4°C. The supernatant was filtered with a 0.25- μm pore size membrane and applied to either a HisTrap 1 mL or a StrepTrap 1 mL (GE Healthcare) using an ÄKTA HPLC chromatography system (GE Healthcare). For HisTrap purification, the column was washed with lysis buffer plus 20 mM imidazole and eluted in lysis buffer plus 300 mM imidazole (Sigma-Aldrich Handels GmbH, Cat. No. 56749). For StrepTrap purification, the column was washed with lysis buffer and eluted with lysis buffer plus 2.5 mM D-desthiobiotin (Sigma-Aldrich Handels

GmbH, Cat. No. D1411). Eluted fractions were pooled and the protein concentration measured with the Bradford method (20 μ L sample in 1-mL Bradford reagent, Bio-Rad, Cat. No. 500-0006) in triplicate. For the two-step affinity purification, the ECFP-TEV-E3-Strp lysate was incubated with 500 μ L NiNTA beads (Qiagen NiNTA Superflow, Cat. No. 30430) to clear the sample of any proteins whose synthesis may have not terminated at the stop codon and continued through the pET21 endogenous hexahistidine tag. Both Venus-TEV-K3-pG-His and ECFP-TEV-E3-Strp lysates were mixed and a sequential affinity purification (StrepTrap after HisTrap) was carried out. The purity of the samples was assayed by SDS-PAGE and Coomassie staining. For the gel filtration analysis, purified samples were brought to the desired concentration in 250 μ L lysis buffer and loaded onto a Superdex 200 10/300 GL column (GE Healthcare, bed volume 24 mL) at a flow rate of 0.6 mL min⁻¹, previously equilibrated with 35 mL lysis buffer. Eluted fractions were collected in 250 μ L aliquots and the concentration of the highest peak was measured by Bradford assay (20 μ L sample in 1 mL Bradford reagent). Samples were analyzed by SDS-PAGE and Coomassie staining. For the TEV cleavage, purified, his-tagged TEV (a kind gift from Stefan Westermann, IMP Vienna) was added to the purified Venus and ECFP samples and incubated overnight at 4°C in lysis buffer plus 1 mM DTT. Samples were then loaded onto a HisTrap column and the flow-through collected.

Molecular weight determination of gel filtration samples

All molecular weights were calculated based on a standard curve using a mixture of gel filtration molecular weight markers (BioRad, Cat. No. 151-1901). About 100 μ L of this mixture were brought to a volume of 250 μ L in PBS buffer and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) connected to an ÄKTA HPLC system (GE Healthcare). The markers used and the observed elution volumes are:

Thyroglobulin: 670 kDa, 9.30 mL; gamma-globulin: 158 kDa, 12.81 mL; Ovalbumin: 44 kDa, 15.37 mL; myoglobin: 17 kDa, 17.33 mL.

The molecular weight is given by the equation $\text{Log}(\text{MW, kDa}) = -0.2014x + 4.7379$, where x is the observed elution volume of a sample. $R^2 = 0.998$.

Fluorescence measurements, FRET efficiency, and distance estimation

Samples (Venus-TEV-K3-pG-His; ECFP-TEV-E3-Strp; equimolar amounts of individually purified species; and affinity purified heterodimer) were diluted to 2 μ M in a final volume of 200 μ L lysis buffer and the fluorescence measured in a Tecan GeniosPro fluorescence reader (Tecan Group), manual gain 25, four measurements per well, per tripli-

cate. For buffer screening, the samples were dialyzed against the desired buffer before mixing (except for the Triton X-100 buffer, in which the samples were brought to a final detergent concentration of 1% by mixing them with a 2 $\times$ concentrated buffer). Venus channel excitation 505 nm, emission 535 nm; FRET channel excitation 420 nm, emission 535 nm; and ECFP channel excitation 420 nm, emission 465 nm. The error bars show the standard error (σ/n) of three measurements. The FRET efficiency (E) was calculated with the fluorescence reduction of the donor (ECFP) in the presence of the acceptor (Venus), according to the following relationship²⁰:

$$E = 1 - \frac{R_{\text{mix}}}{R}$$

where R is the fluorescence ratio of an ECFP-only sample over a Venus-only sample; and R_{mix} is the fluorescence ratio of ECFP over Venus when both proteins are mixed, subtracting the expected spill over of each protein in other fluorescence channels when measured alone, as follows:

$$R_{\text{mix}} = \frac{\text{ECFPChannel}_{\text{mixed}} - \text{ECFPChannel}_{\text{Venus}}}{\text{VenusChannel}_{\text{mixed}} - \text{VenusChannel}_{\text{ECFP}}}$$

Isothermal titration calorimetry

ECFP-TEV-E3-Strp was adjusted to a final concentration of 100 μ M in the syringe and Venus-TEV-K3-pG-His to 10 μ M in the chamber, both in lysis buffer. Thirty injections of 10- μ L each, with a delay of 1 min between injections, were carried out at 25°C using a VP-ITC Microcalorimeter (MicroCal, LLC). The results were analyzed and plotted using MicroCal Origin software.

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References

1. Liu J, Rost B (2001) Comparing function and structure between entire proteomes. *Protein Sci* 10:1970–1979.
2. Kohn WD, Kay CM, Hodges RS (1998) Orientation, positional, additivity, and oligomerization-state effects of interhelical ion pairs in alpha-helical coiled-coils. *J Mol Biol* 283:993–1012.
3. Lupas A (1996) Coiled coils: new structures and new functions. *Trends Biochem Sci* 21:375–382.
4. Crick FHC (1953) The packing of alpha-helices: simple coiled-coils. *Acta Cryst* 6:689–697.
5. Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol* 11:82–88.
6. Kohn WD, Monera OD, Kay CM, Hodges RS (1995) The effects of interhelical electrostatic repulsions between glutamic acid residues in controlling the

- dimerization and stability of two-stranded alpha-helical coiled-coils. *J Biol Chem* 270:25495–25506.
7. Krylov D, Mikhailenko I, Vinson C (1994) A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions. *EMBO J* 13: 2849–2861.
 8. Parry DA, Fraser RD, Squire JM (2008) Fifty years of coiled-coils and alpha-helical bundles: a close relationship between sequence and structure. *J Struct Biol* 163:258–269.
 9. Catimel B, Faux MC, Nerrie M, Rothacker J, Otvos LJ, Wade JD, Nice EC, Burgess AW (2001) The use of coiled-coil interactions for the analysis and micropreparative isolation of adenomatous polyposis coli protein complexes. *J Pept Res* 58:493–503.
 10. Chao H, Bautista DL, Litowski J, Irvin RT, Hodges RS (1998) Use of a heterodimeric coiled-coil system for biosensor application and affinity purification. *J Chromatogr B Biomed Sci Appl* 715:307–329.
 11. Litowski JR, Hodges RS (2001) Designing heterodimeric two-stranded alpha-helical coiled-coils: the effect of chain length on protein folding, stability and specificity. *J Pept Res* 58:477–492.
 12. Litowski JR, Hodges RS (2002) Designing heterodimeric two-stranded alpha-helical coiled-coils. Effects of hydrophobicity and alpha-helical propensity on protein folding, stability, and specificity. *J Biol Chem* 277: 37272–37279.
 13. Liu J, Deng Y, Zheng Q, Cheng CS, Kallenbach NR, Lu M (2006) A parallel coiled-coil tetramer with offset helices. *Biochemistry* 45:15224–15231.
 14. Liu J, Zheng Q, Deng Y, Cheng CS, Kallenbach NR, Lu M (2006) A seven-helix coiled coil. *Proc Natl Acad Sci USA* 103:15457–15462.
 15. Tripet B, Yu L, Bautista DL, Wong WY, Irvin RT, Hodges RS (1997) Engineering a de novo designed coiled-coil heterodimerization domain for the rapid detection, purification and characterization of recombinantly expressed peptides and proteins. *Protein Eng* 10:299.
 16. Grigoryan G, Keating AE (2008) Structural specificity in coiled-coil interactions. *Curr Opin Struct Biol* 18: 477–483.
 17. Lindhout DA, Litowski JR, Mercier P, Hodges RS, Sykes BD (2004) NMR solution structure of a highly stable de novo heterodimeric coiled-coil. *Biopolymers* 75:367–375.
 18. Apostolovic B, Klok HA (2008) pH-sensitivity of the E3/K3 heterodimeric coiled coil. *Biomacromolecules* 9: 3173–3180.
 19. Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. *Nat Methods* 2:905–909.
 20. Jares-Erijman EA, Jovin TM (2003) FRET imaging. *Nat Biotechnol* 21:1387–1395.
 21. Felber LM, Cloutier SM, Kundig C, Kishi T, Brossard V, Jichlinski P, Leisinger HJ, Deperthes D (2004) Evaluation of the CFP-substrate-YFP system for protease studies: advantages and limitations. *Biotechniques* 36: 878–885.
 22. Wolber V, Maeda K, Schumann R, Brandmeier B, Wiesmuller L, Wittinghofer A (1992) A universal expression-purification system based on the coiled-coil interaction of myosin heavy chain. *Biotechnology (N Y)* 10:900–904.
 23. Riley LG, Ralston GB, Weiss AS (1996) Multimer formation as a consequence of separate homodimerization domains: the human c-Jun leucine zipper is a transplantable dimerization module. *Protein Eng* 9:223–230.
 24. Yano Y, Yano A, Oishi S, Sugimoto Y, Tsujimoto G, Fujii N, Matsuzaki K (2008) Coiled-coil tag—probe system for quick labeling of membrane receptors in living cell. *ACS Chem Biol* 3:341–345.
 25. Ozawa T, Kaihara A, Sato M, Tachihara K, Umezawa Y (2001) Split luciferase as an optical probe for detecting protein–protein interactions in mammalian cells based on protein splicing. *Anal Chem* 73:2516–2521.
 26. Hu CD, Chinenov Y, Kerppola TK (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell* 9:789–798.
 27. Kerppola TK (2006) Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat Protoc* 1:1278–1286.
 28. Wehr MC, Laage R, Bolz U, Fischer TM, Grunewald S, Scheek S, Bach A, Nave KA, Rossner MJ (2006) Monitoring regulated protein–protein interactions using split TEV. *Nat Methods* 3:985–993.
 29. Pollock R, Clackson T (2002) Dimerizer-regulated gene expression. *Curr Opin Biotechnol* 13:459–467.
 30. Ruch C, Skiniotis G, Steinmetz MO, Walz T, Ballmer-Hofer K (2007) Structure of a VEGF-VEGF receptor complex determined by electron microscopy. *Nat Struct Mol Biol* 14:249–250.
 31. Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, Wemmer DE, Zhang X, Kuriyan J (2009) Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. *Cell* 137: 1293–1307.