SUPPLEMENTARY METHODS

DNA constructs

PCA protein-fragment fusions were created from cDNAs for the interacting proteins of interest fused to either 5’ or 3’ of the hGLuc fragments (e.g., protein A-hGLuc[1] or hGLuc[1]-protein A, protein B-hGLuc[2] or hGLuc[2]-protein B). As a simple positive control, we used GCN4 leucine zipper-forming sequences (Zip) that homodimerize. PCA fragments were amplified by PCR from hGLuc (pCMV-GLUC, Nanolight Technology). hGLuc[1] (N-terminal) corresponds to amino acids 1-93, and hGLuc[2] (C-terminal) corresponds to amino acids 94-169 of hGLuc (excluding the 16 amino acid N-terminal secretory signal sequence) (See Supplementary Figure 1 online). The fusions were subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). We inserted a sequence coding for a 10 amino acid flexible polypeptide linker consisting of (Gly.Gly.Gly.Gly.Ser)2 between the protein of interest and the hGLuc fragments (for both fusions). This linker was chosen because it is the most flexible possible and we have empirically observed that linkers of this length are sufficiently long to allow for fragments to find each other and fold, regardless of the size of the interacting proteins to which the fragments are fused.

Bioluminescence assays in live cells

Plasmids harboring the PCA fusions (e.g., Zip-hGLuc(1) and Zip-hGLuc(2)) were cotransfected in a 1:1 ratio (100 ng DNA total/well) into HEK293 or Hep3B cells plated on tissue culture treated 96-well clear-bottomed white microtiter plates (Corning), using Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). The assays were performed 48 hours after the beginning of transfection (there were approximately 20,000 cells per well on the day of the assay). Medium was exchanged for 100 µl/well of DMEM without phenol red (Invitrogen) and drugs were added to the cell medium for the desired incubation time prior to reading. Cells expressing the FRB-hGLuc(1) and FKBP-hGLuc(2) fusions were treated with a final concentration of 20 nM rapamycin, 1 µM FK506 or 1 µM Cyclosporin A for 3 hours, or left untreated, prior to luminometric analysis, unless otherwise indicated. Cells expressing the Fm-hGLuc(1) and Fm-hGLuc(2) fusions were treated with a final concentration of 1 µM AP21998, 1 µM FK506 or 1 µM Cyclosporin A for various incubation times (see the corresponding Figure legends) prior to luminometric analysis. Cells expressing the hGLuc(1)-Smad3 and PKB-
hGLuc(2) fusions were serum-starved overnight (0.2% fetal bovine serum) and treated with TGFβ (200 pM or 1nM) and/or insulin (1 or 5 µM) for 15 minutes, or left untreated, prior to luminometric analysis. In all experiments, a white backing tape was applied to the bottom of the plate prior to reading. Native coelenterazine (Nanolight Technology) was reconstituted as a stock solution of 1 mg/ml in methanol and diluted in DMEM without phenol red (Invitrogen) at room temperature for injection (injection volume: 100 µl). Unless otherwise indicated, native coelenterazine was used at a final concentration of 20 µM. Signal intensities (integrated over 10 seconds, with an injection delay of 2 seconds) were read on a LMax II 384 plate-reader (Molecular Devices).

**Bioluminescence assays in cell lysates**

Plasmids harboring the PCA fusions were cotransfected in a 1:1 ratio (1 µg DNA total/well) in HEK293 cells plated on tissue culture treated 12-well plates (Corning), using Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). 48 hours after the beginning of transfection, cells were trypsinized and resuspended in 500 µl of cold PBS 1X (there were approximately 2.5 x 10^5 cells per well). Cells were pelleted by centrifugation at 4°C for 1 minute and each pellet was resuspended in 400 µl DMEM without phenol red (Invitrogen) containing protease inhibitors (20 µg/ml aprotinin and 5 mM AEBSF). Cell samples were flash-frozen in dry ice/ethanol for 10 minutes and thawed in a water bath at 37°C for 10 minutes. This freeze-thaw cycle was repeated twice. Cell membrane and other debris were removed by centrifugation at 4°C for 5 minutes (10,000 g). The supernatants (cell lysates) were collected and total protein concentration in each sample was determined with a protein assay (Bio-Rad). Samples were aliquoted (100 µl per well) into a 96-well white microtiter plate (Corning) and drugs were added directly to the cell lysates for the desired incubation time prior to reading. For the F_M-hGLuc(1) and F_M-hGLuc(2) fusions, a final concentration of 1 µM AP21998, 1 µM FK506 or 1 µM Cyclosporin A was used for various incubation times (see the corresponding Figure legends) prior to luminometric analysis. For the FRB-hGLuc(1) and FKBP-hGLuc(2) fusions, a final concentration of 50 nM rapamycin was used (see Supplementary Figure 4 online). Native coelenterazine (Nanolight Technology) was diluted in DMEM without phenol red for injection (injection volume: 100 µl) and, unless otherwise indicated, was used at a final concentration of
20 µM. Signal intensities (integrated over 10 seconds, with an injection delay of 2 seconds) were read on a LMax II 384 plate-reader (Molecular Devices).

**In vitro luciferase assay with purified proteins**

For affinity purifications, we added a polyhistidine (Hisx6) coding sequence to the original constructs in pcDNA3.1 (Zip-hGLuc(1), Zip-hGLuc(2), Zip-hRLuc(1) and Zip-hRLuc(2)). These fusions were transfected and expressed individually in COS-1 cells, and the fusion proteins were affinity purified on Ni-NTA columns (Ni-NTA Spin Kit, Qiagen) following the manufacturer’s instructions for native protein purification by elution with 50 mM NaH$_2$PO$_4$, 300 mM NaCl and 250 mM imidizole, pH 8.0. Purified proteins were quantified with a protein assay (Bio-Rad) and an *in vitro* luciferase assay was performed at final concentrations of 50 nM of purified protein per sample in a final volume of 200 µl of DMEM without phenol red (hGLuc PCA: 50nM Zip-hGLuc(1) + 50nM Zip-hGLuc(2); hRLuc PCA: 50nM Zip-hRLuc(1) + 50nM Zip-hRLuc(2)). Eluted proteins were first diluted into 100 µl of DMEM without phenol red per well in a 96-well white microtiter plate (Corning). Native coelenterazine (Nanolight Technology) was diluted in DMEM without phenol red for injection (injection volume: 100 µl) and assays performed at varying concentrations of coelenterazine (from 1 to 60 µM). Signal intensities (integrated over 10 seconds, with an injection delay of 2 seconds) were read on a LMax II 384 plate-reader (Molecular Devices).

**Immunoblot analysis**

Cell samples were lysed (lysis buffer: 1% Nonidet P-40, 0.1 % SDS, 150 mM NaCl, 20 mM Tris pH 8.0, 20 µg/ml aprotinin and 5 mM AEBSF) and expression levels of Smad3 and PKB (in their fusion form or endogenous) in total lysates were determined by immunoblotting with anti-Smad3 (Zymed Laboratories) or anti-PKB (Cell Signaling) antibodies. Anti-α-tubulin (Sigma) was used as an internal control for loading (Sigma).