Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Modification of transmembrane and GPI-anchored proteins on living cells by efficient protein *trans*-splicing using the *Npu* DnaE intein

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SUPPORTING FIGURES

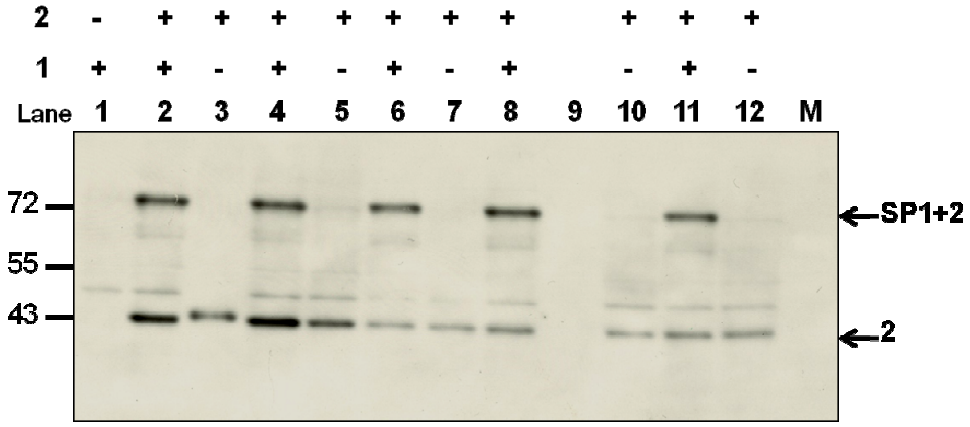

Figure S1: Control experiments for removal of excess protein 2 after protein trans-splicing. These experiments were performed to address the cause for the residual protein **2** that can be seen in the western blot analyses after protein *trans*-splicing and after washing of the cells (e.g. in Figures 2C, 2D, and 3C). For heparin wash conditions, heparin (50U/ml) was added to the wash media. For harder wash conditions, cells were washed during the second wash until they detached from the plate; cells were then subsequently pelleted by centrifugation and the supernatant removed. Lanes 1 & 2: protein **2** at 5 μM with normal wash conditions; lane 3: no cells with protein **2** at 5 μM with normal wash conditions; lanes 4 & 5: protein **2** at 5 μM with heparin wash conditions; lanes 6 & 7: protein **2** at 5 μM with harder wash conditions; lanes 8 & 10: protein **2** at 2 μM with normal wash conditions; lane 9: blank; lanes 11 & 12: protein **2** at 2 μM with heparin wash conditions. To summarize these control experiments, lower protein concentrations of protein **2** and harder washing decreased the residual band of protein **2**. Thus, at least a fraction of the residual protein **2** was unspecifically attached to the cells or to the plates. Binding of protein 2 to the plates was observed. It is thus possible that all of the background observed was a consequence of this unspecific binding, although we cannot rule out that some protein **2** was taken up by the cells. However, this latter possibility seems unlikely to occur by a specific mechanism. Importantly, the reaction can also be performed with protein **2** at only 2 μM concentration.

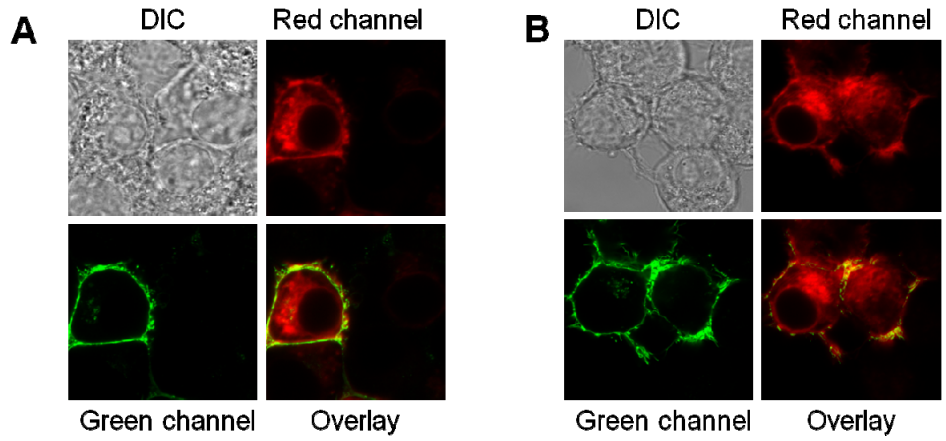

Figure S2: Non-covalent split intein fragment association using an intein mutant inactive for splicing.

(A) In construct **2a** the catalytically important cysteine residue at position 1 of the Int^N fragment was replaced by a serine residue (C1S). Adding of purified **2a** (5 µM final concentration) for 1 h to the growth medium of N2a cells expressing **1** showed staining of the plasma membrane of transfected cells. See Figure S3 for the confirmation that no covalent splice product was formed in this case. **(B)** N2a cells were transfected with construct **1a** where the cysteine residue at position +1 of the Int^C fragment was replaced by a serine residue (C+1S). Addition of protein **2** for 1 h to the growth media of these cells again showed staining of the plasma membrane. However, in this case splice product formation took place at a reduced level as seen in Figure S3.

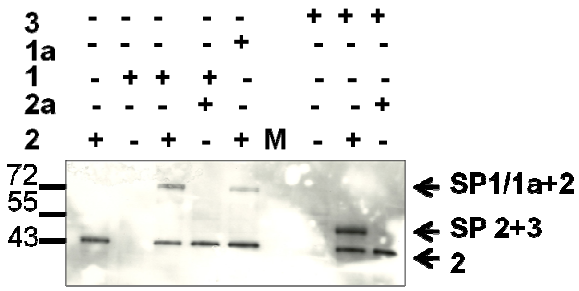

Figure S3: Intein mutants show no or reduced splice product formation.

N2a cells transfected with expression plasmids for Int^C fusion constructs **1**, **1a**, and **3**, respectively, were incubated with purified Int^N proteins **2** or **2a**. Following washing of the cells, cell lysates were prepared. Shown is an immuno blot analysis of these lysates with an anti-GFP antibody. **1a** contains a mutation of the catalytically important cystein residue of the Int^C fragment (C+1S), which reduces, however, not completely abolished protein *trans*-

splicing activity of the *Npu* DnaE intein. **2a** contains a C1S mutation in the Int^N fragment and abolishes protein *trans*-splicing.

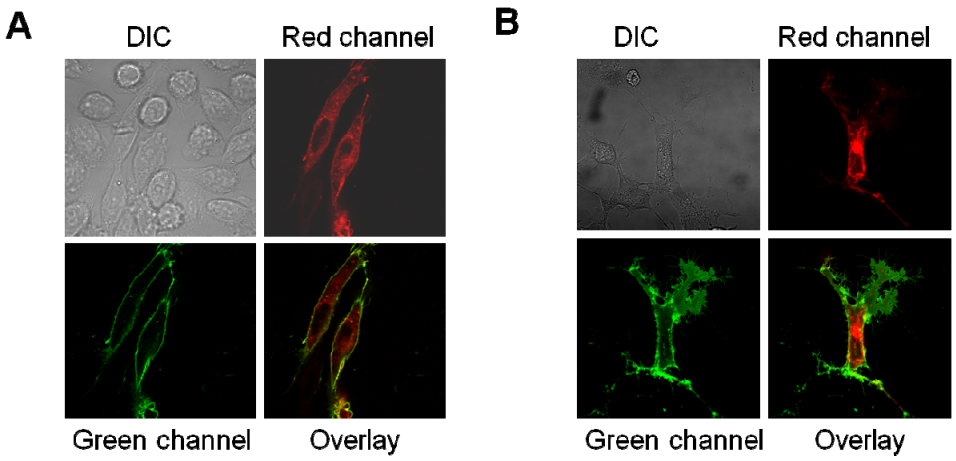

Figure S4: Protein *trans*-splicing on different mammalian cells.

Cells were transiently transfected with a plasmid encoding **1**. 24 h after transfection protein **2** was added to the growth media and cells were subsequently washed. **(A)** Confocal microscopy of fixed CHO cells that were incubated with **2** for 1 h. **(B)** Confocal image of fixed COS-7 cells incubated with **2** for 1h.

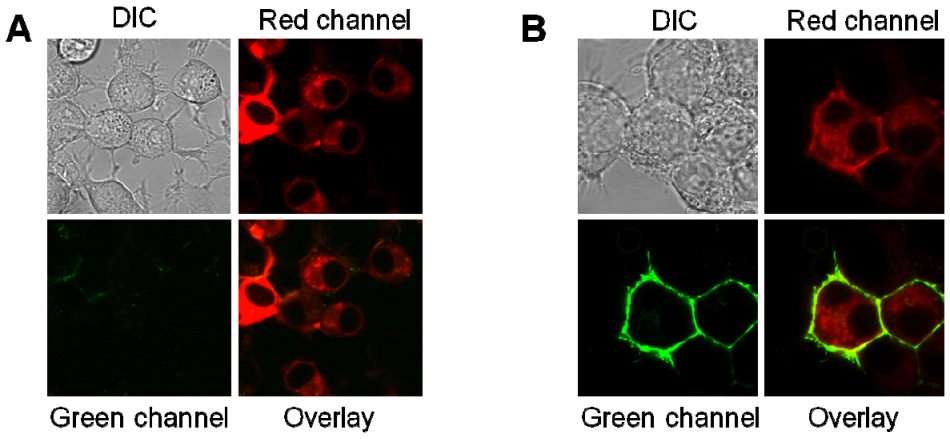

Figure S5: Effect of reducing agent on the splicing reaction.

N2a cells were transfected with plasmid expressing Int^C construct **1**, incubated with Int^N construct **2**, washed, and fixed. Shown are confocal microscopy images of the cells. **(A)** Protein **2** was non-reduced and no DTT was added to the growth medium. **(B)** Protein **2** was reduced with DTT prior to addition to the cells but no extra DTT was added to the growth medium.

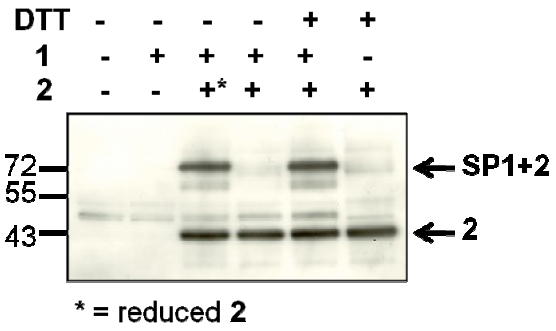

Figure S6: Effect of reducing agent on the splicing reaction.

Shown is the western blot analysis of the cell lysates obtained from experiments as described in Figure S5 using an anti-GFP antibody.

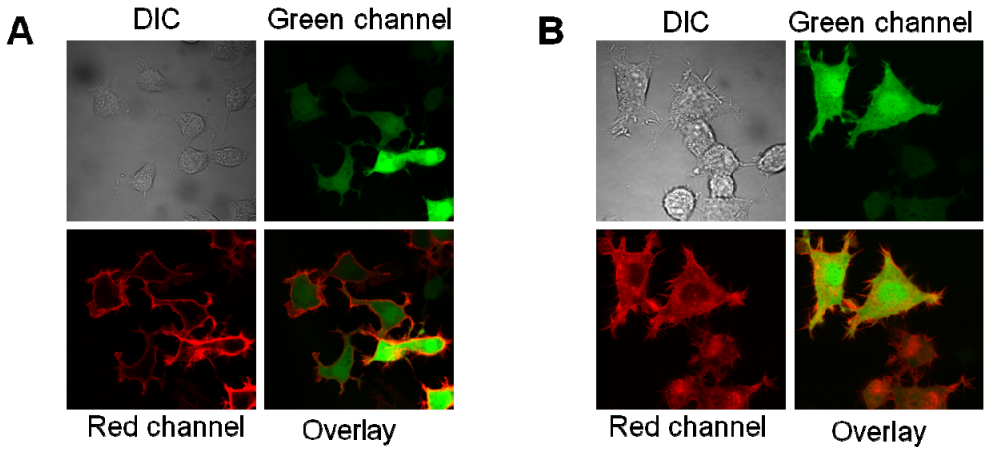

Figure S7: Correct processing and localization of the Int^C fragment targeted for C-terminal modification with a GPI anchor.

Shown are N2a cells immunostained against an HA-antibody tagged with Alexa fluor 568 (Invitrogen). Cells were transfected with a plasmid encoding cytoplasmic eGFP and a plasmid expressing Int^C construct **3**. **(A)** Cells were not permeablized prior to staining. **(B)** Cells were permeablized with Triton X-100 prior to immunostaining. This data demonstrates that the Int^C fusion protein with the N-terminal HA-tag was correctly targeted to the cell surface on the plasma membrane. In case of the permeablized cells, the anti-HA antibody also recognized protein still in the secretory pathway.

SUPPLEMENTARY METHODS

General procedures

General molecular biology techniques were used for cloning and protein expression and purification. All cell culture media were purchased from PAN-Biotech. DPBS was purchased from Invitrogen. HCHO used was of histological grade and purchased from Sigma. BSA and Triton X-100 were purchased from Roth. Pfusion polymerase used for PCR was from Finnzymes. Primers were purchased from Operon Biotechnologies. The sequences of all plasmids constructed were confirmed by sequencing from GATC-biotech. The ECL advanced kit from GE healthcare was used for western blotting. Antibiotics were purchased from Roth and stocks prepared were filter sterilized. All experiments were performed at least in triplicate.

Construction of expression plasmids

*Construction of plasmid pTD038 encoding **1***: The mammalian expression vector pDisplay (Invitrogen) contains a murine Ig κ-chain leader sequence and the hemagglutinin A at the 5'-end of the multiple cloning site (MCS) to direct the fused encoding protein sequence to the secretory pathway. At the 3'-end the transmembrane domain of the platelet derived growth factor receptor (PDGFR) is encoded, which anchors the protein product to the plasma membrane, displaying it on the extracellular side. The mCherry encoding gene was PCR amplified from plasmid mCherry-N1(Clontech) using the following primers: forward primer 5'- ATA GCT AGC ATG GTG AGC AAG GGC GAG GAG G -3'; reverse primer 5'- TAT GCG GCC GCT ACT TGT ACA GCT CGT CC -3'. The product obtained was double digested with NheI/NotI (Fermentas) and cloned downstream of the PDGFR domain encoding sequence of the p-Display vector, resulting in plasmid pTD037. In a second step, the gene fragment encoding *Npu*DnaE$_C$-Trx-His$_6$ was amplified using PCR from bacterial plasmid pBAD- *Npu*DnaE$_C$-Trx-His$_6$ (pVS07)[1] using the following primers: forward primer 5'- ATA GGG CCC AGA TGA TCA AAA TAG CCA CAC GTA AAT -3'; reverse primer 5'- TAT GTC GAC GTA GTG ATG GTG ATG GTG ATG AGA. The PCR product obtained and plasmid pTD037 were digested with ApaI/SalI and ligated to yield the

mammalian expression plasmid pTD038 for expression of Ig κ-HA- *Npu*DnaE$_C$-Trx-His$_6$-TMD-mcherry (**1**).

*Construction of plasmid pTD056 encoding **1a***: The C+1S mutant gene fragment was PCR amplified from bacterial plasmid pBAD-*Npu*DnaE$_{C+1S}$-Trx-His$_6$ (pVS08)[1] using the following primers: forward primer 5'- ATA GGG CCC AGA TGA TCA AAA TAG CCA CAC GTA AAT -3'; reverse primer 5'- TAT GTC GAC GTA GTG ATG GTG ATG GTG ATG AGA. The resulting sequence was double digested with Apa1/Sal1 and ligated into plasmid pTD038 (encoding **1**) to obtain the construct pTD056 encoding Ig κ-HA-*Npu*DnaE$_{C+1S}$-Trx-His6-TMD-mCherry (**1a**).

*Construction of plasmid pTD057 encoding **3***: The gene fragment coding for the Gas1p sequence was amplified using PCR from mammalian expression plasmid pCDNA3.1/Zeo Kre-Prp-Gas1p (kindly provided by Martin Engelhard and Miria Schumacher),[2] using the following primers: forward primer 5'- ATA GTC GAC TCT TCT TCT TCT TCT TCA GCT TCA TC -3'; reverse primer 5'- TAT GCG GCC GCT TAA ACC AAA GCA AAA CCG AC -3'. The resulting PCR product and plasmid pTD038 (encoding **1**) were digested with SalI/NotI and ligated to yield the plasmid pTD057 encoding Ig κ-HA- *Npu*DnaEc-Trx-His$_6$-Gas1p (**3**).

Bacterial expression plasmids pVS07 for expression of **2** (StreptagII-eGFP-*Npu*DnaE$_N$) and pVS18 for expression of **2a** (StreptagII-eGFP-$_{C1S}$*Npu*DnaE$_N$) were previously reported.[1]

Protein expression and purification

Bacterial expression plasmids pVS07 and pVS18 were used for transformation of *E.coli* BL21 (DE3 Gold) cells. The cells were grown in 600 mL of LB medium containing 50 μg/mL kanamycin at 37ºC to an O.D.$_{600}$ of 0.6. At this point the temperature was lowered to 28ºC and protein expression was induced by adding 0.4 mM isopropyl thiogalactoside (IPTG) and the cells were grown for an additional 4-5 hours. The cells were harvested by centrifugation and re-suspended in Strep-Tactin® Buffer W: 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0 and further lysed by using a high pressure homogenizer (Emulsiflex-C5, Avestin). The proteins were purified from the soluble fraction by Strep-tactin affinity chromatography using manufacturer's instructions (iBA technologies) and dialyzed against PBS buffer, pH 7.4. The protein concentrations were determined using the calculated molar extinction coefficient at λ= 280nm.

Cell culture and plasmid transfection

Mouse neuro-2 cells were cultured in Modified Eagle's medium (MEM; PAN Biotech) containing 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin,1 % sodium pyruvate and 1 % L-Glutamine at 37 ºC under 5 % CO_2 . CHO cells were cultured in HAM's F-12 media (PAN Biotech) containing 10 % FBS, 1% penicillin/streptomycin and 1 % L-Glutamine at 37 ºC under 5 % CO_2. COS-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; PAN Biotech) containing 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin and 1 % L-Glutamine at 37 ºC under 5 % CO_2 .Transfection was carried out using Gene Juice (Novagen) transfection reagent according to the manufacturer's protocol.

Protein *trans*-splicing on living cells

5 to 10 x 10^4 N2a cells were plated on glass bottom dishes (35mm; Mat-tek corporation) and incubated at 37 ºC and 5 % CO_2. After 24 hours, when a confluency of 50-70 % was reached, the cells were transfected with 1 μg of plasmid DNA and incubated for another 24 h. In case of plasmid pTD057 encoding protein **3**, 0.1 μg of plasmid mCherry was co-transfected as a transfection control. 24 h post-transfection cells were washed once with 1 mL of serum-free DMEM. The splicing reaction was carried out by incubating the cells in serum-free DMEM along with 5 μM protein **2** or **2a** and 2 mM DTT in a total volume of 300 μL, at 37 ºC for 1 h unless otherwise stated. After the incubation the cells were washed twice with serum-free DMEM, fixed by adding 1 mL of fixing solution (1 mL 37% HCHO + 7 mL DPBS) and incubated for 20 min at 37 ºC. The cells were washed 3 times with DPBS and finally 1 mL of DPBS was added to the cells. The cells were observed using an Olympus fluoview confocal (Olympus) laser microscope. The 488 nm line of argon laser and the 568 nm line of krypton laser were used for exciting the samples. The cells were viewed using the 60x oil immersion objective lens. For *trans*-splicing on the surface of COS-7 cells DMEM was used but for CHO cells the DMEM media was replaced by the HAM's-F12 media

Immunostaining

The immunostaining reactions were carried out on cells plated on Mat-tek dishes. Post transfection, cells were fixed with fixing solution as mentioned above. Washing was carried out 3 times with DPBS and the cells were permeablized with 1 mL permeablization solution (0,25% Triton X-100 in DPBS) for 15 min at room temperature. Again washing was carried

out 3 times with DPBS. In the next step, blocking was carried out using 150 µL of blocking solution (10% BSA in DPBS)/ dish. Blocking solution was replaced with 250 µL of anti-HA monoclonal primary antibody used at a dilution of 1:500. Cells were incubated at 37 ºC for 1 h and then washed 3 times with DPBS. 250 µL of goat anti-mouse secondary antibody tagged with Alexa-fluor 568 (Invitrogen) at a dilution of 1:1000 was then added to the cells and again an incubation of 1h at 37 ºC was carried out. The cell layer was again washed with DPBS 3 times and finally 1 mL of DPBS was added to prevent the cell layer from drying. The cells were either immediately observed or stored at 4 ºC for later analysis.

Western blot analysis

5 to 10 x 10^4 Neuro-2a cells were plated on 6 well-plates (Sarstedt), transfected and treated according the protein *trans*-splicing protocol as mentioned above. Following washing, 100 µL of 2x SDS loading buffer was added to the cells and mixed. The cells were thoroughly scraped off from the plates and boiled for 10 mins. The protein samples were separated on a 15% SDS-PAGE gel and transferred onto a PVDF membrane for western blotting. The splice products **SP1+2** and **SP2+3** were detected using a primary monoclonal antibody against GFP (1 mg/mL; mouse IgG1, Covance, used at 1:10,000 dilution). Construct **1** was detected using a primary monoclonal anti-HA antibody (1 mg/mL; mouse IgG1, Covance, used at 1:100,000 dilution).

SUPPORTING REFERENCES

1. J. Zettler, V. Schutz and H. D. Mootz, *FEBS Lett*, 2009, **583**, 909-914.
2. M. C. Schumacher, U. Resenberger, R. P. Seidel, C. F. Becker, K. F. Winklhofer, D. Oesterhelt, J. Tatzelt and M. Engelhard, *Biopolymers*, 2010, **94**, 457-464.