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# Protein Scaffold-Activated Protein Trans-Splicing in Mammalian Cells

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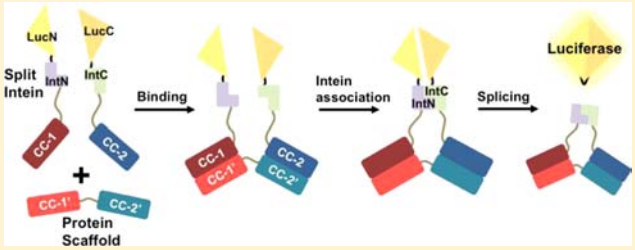
Supporting Information

**ABSTRACT:** Conditional protein splicing is a powerful biotechnological tool that can be used to rapidly and post-translationally control the activity of a given protein. Here we demonstrate a novel conditional splicing system in which a genetically encoded protein scaffold induces the splicing and activation of an enzyme in mammalian cells. In this system the protein scaffold binds to two inactive split intein/enzyme extein protein fragments leading to intein fragment complementation, splicing, and activation of the firefly luciferase enzyme. We first demonstrate the ability of antiparallel coiled-coils (CCs) to mediate splicing between two intein fragments, effectively creating two new split inteins. We then generate and test two versions of the scaffold-induced splicing system using two pairs of CCs. Finally, we optimize the linker lengths of the proteins in the system and demonstrate 13-fold activation of luciferase by the scaffold compared to the activity of negative controls. Our protein scaffold-triggered conditional splicing system is an effective strategy to control enzyme activity using a protein input, enabling enhanced genetic control over protein splicing and the potential creation of splicing-based protein sensors and autoregulatory systems.




## INTRODUCTION

Protein splicing is a post-translational modification that can control the activity of a protein by assembling it from inactive fragments. Analogous to RNA splicing, protein splicing is the process by which an intervening protein domain, or intein, self-excises out of a larger polypeptide, ligating the two flanking polypeptides—termed exteins—into a single protein.[1] Protein splicing can occur in *cis*- or in *trans*-.[2−4] For trans-splicing the intein sequence is split into two fragments, and the splicing reaction occurs between two distinct polypeptides. Intein domains are highly modular and have been used by researchers to assemble several different proteins in various experimental contexts.[5−8]

The development of conditional protein splicing has enabled researchers to post-translationally control protein activity in response to specific molecular inputs and has already become a useful research tool. By engineering split inteins that contained the rapamycin ligand-binding domains, Mootz et al. first demonstrated that trans-splicing could be induced by the small molecule rapamycin.[6] Other conditional splicing systems have been generated to induce splicing in response to temperature, light, and chemical ligands such as 4-HT.[9−13]

Here we propose a novel conditional splicing system in which splicing is induced by the presence of a genetically encoded protein scaffold. In contrast to previously developed conditional splicing systems activated by exogenously administered inducers (small molecules and light), a protein inducer can be directly linked to endogenous biological pathways. This characteristic enables the potential to monitor or rewire biological pathways at the protein level. The engineering of synthetic post-translational signaling pathways reflects a common strategy used by natural systems and is a major research focus. However, only a few general approaches exist.[14−18] The protein-induced protein splicing system offers a general mechanism to post-translationally convert a protein input into a fully formed output protein through engineered binding domains. The general reaction mechanism demonstrated by our work also suggests methods to create splicing-based protein sensors and autoregulatory systems.

Biomolecular scaffolds have previously been used to increase the yield of biochemical synthesis pathways by bringing together enzymes operating in a pathway.[19−22] We hypothesized that protein scaffolds could also be used to bring together split inteins and trigger protein splicing. Our system consists of two fusion proteins—each containing split intein/extein domains fused to a scaffold binding domain—and the input scaffold protein. In the presence of the scaffold the two fusion proteins bind to the scaffold leading to association of the intein fragments, splicing, and activation of the output protein.

We constructed, tested, and optimized a scaffold-induced splicing system comprising well-characterized protein components. We used two pairs of previously characterized antiparallel coiled-coils termed LZA/LZB and EE/RR.[23−25] LZA is known to bind strongly to LZB and EE to RR, but no binding is expected to occur between proteins in the different pairs. These coiled-coils drive the specific association of the intein/extein fusion proteins and the synthetic scaffold. We

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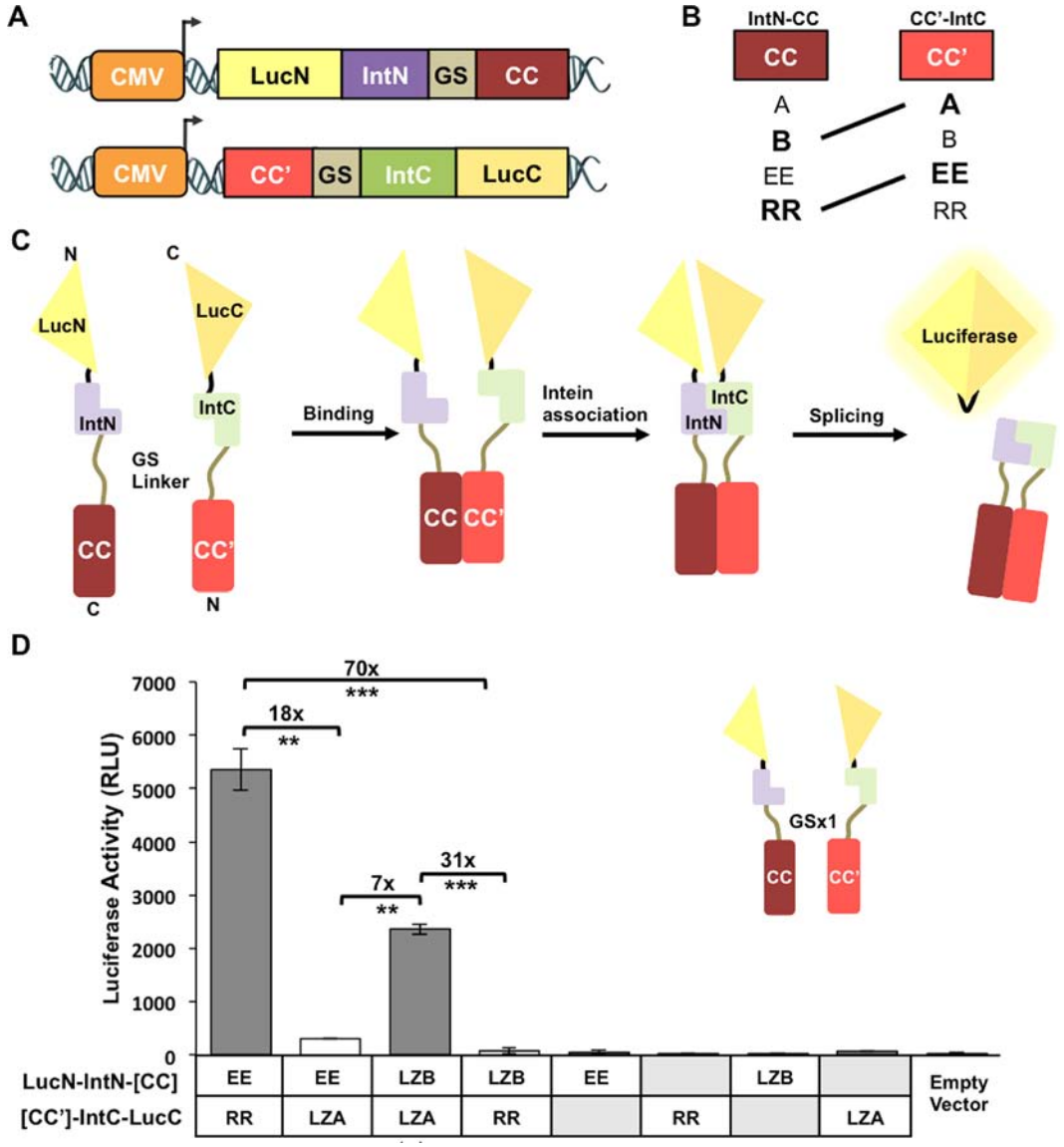


**Figure 1.** Coiled-coil-mediated protein trans-splicing. (A) Schematic representation of the expression system used to test trans-splicing of CC-intein/extein fusion proteins. Each CC-intein/extein fusion protein is expressed from the CMV promoter. Coiled-coil domains CC and CC′ are fused to split inteins and luciferase exteins, IntN/LucN and LucC/IntC, via flexible glycine-serine linkers (GS). (B) Schematic representation of the complementary CC binding assay design. CC-intein/extein pairs tested are in boldface, and those connected by lines are expected to interact. (C) Binding of complementary coiled-coil domains leads to intein fragment complementation and splicing and activation of firefly luciferase. (D) Activity of recombinant intein pairs as measured by luciferase output. Shaded boxes represent transfection with an empty expression vector. Data are presented as mean ± s.d., $n = 3$. Two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

chose to use the *Saccharomyces cerevisiae* vacuolar ATPase (VMA) split intein because these split fragments display very weak splicing activity in the absence of outside protein-binding domains.[8,26] For the output protein we used firefly luciferase, as it has a sensitive biochemical readout and previously determined extein split sites.[8,27] After demonstrating the ability of the CCs to mediate splicing, we optimized the linker lengths of the individual component proteins of the system. Finally, we showed that our protein scaffold system had comparable efficacy to that of the established rapamycin-induced splicing system.[8]

## ■ RESULTS AND DISCUSSION

We first sought to determine whether coiled-coil binding could mediate trans-splicing between proteins containing split VMA intein fragments in mammalian cells. We generated *Cytomegalovirus* (CMV) promoter driven expression plasmids encoding fusion proteins comprising a CC domain fused by a flexible glycine-serine (GS) linker to an N- or C-terminal split VMA intein domain and an N- or C-terminal firefly luciferase extein fragment, respectively (Figure 1A). Amino acid sequences of the coiled-coil domains are listed in Supporting Information, Table S1. We expected that complementary CCs (LZA with LZB and EE with RR) would bind, leading to split intein complementation, protein splicing, and luciferase activity. Conversely, we anticipated that proteins with noncomplementary CCs would not interact, resulting in inefficient splicing and low luciferase activity (Figure 1B,C).

To test for CC-mediated splicing we transiently cotransfected different combinations of N-intein and C-intein expression plasmids into U2OS osteosarcoma cells and assayed


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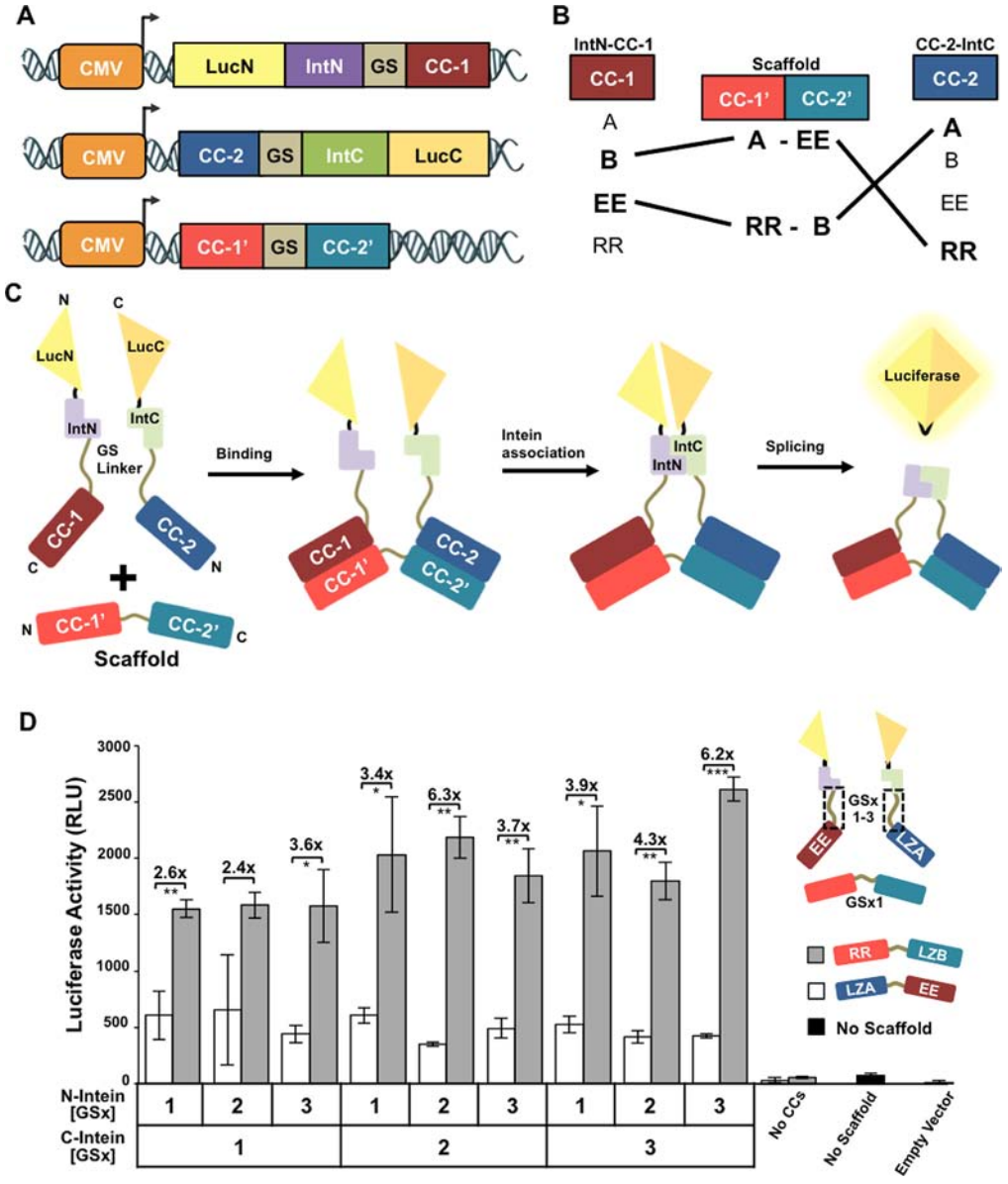


**Figure 2.** Protein scaffold-activated protein trans-splicing. (A) Schematic representation of the expression system used to test conditional splicing of CC-intein/extein fusions in response to CC scaffolds. (B) Schematic representation of the expected complementary CC binding for each synthetic scaffold and CC-intein/extein pair. Proteins tested are in boldface, and those connected by lines are expected to interact. (C) Recombinant inteins containing noncomplementary coiled-coil domains CC-1 and CC-2 associate only in complex with a protein scaffold containing complementary CCs, CC-1′ and CC2′. Presence of the scaffold results in the splicing and activation of firefly luciferase. (D) Induction of splicing between EE and LZA split inteins of varying GS linker lengths by a protein scaffold. Constructs encoding each intein pair were cotransfected with a construct encoding an ON-target scaffold RR-GS1-LZB (gray) or an OFF-target scaffold LZA-GS1-EE (white). The 'No Scaffold' control consists of the 3 GS linker EE and LZA split inteins transfected with an empty vector instead of a scaffold. The 'No CCs' control represents cotransfections of inteins not fused to coiled-coils. Data presented as mean ± s.d., $n = 3$. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

for luciferase activity at 48 h. The specific CC combinations that we built and transfected are shown in boldface, and connecting lines indicate expected interactions in Figure 1B.

We found that luciferase activity was significantly higher when the coexpressed proteins contained complementary CCs, indicating that CCs can coordinate specific protein trans-splicing. Proteins containing the EE/RR pair showed higher overall luciferase activity than the LZA/LZB pair (5360 RLU and 2360 RLU, respectively) (Figure 1D). This result agrees with higher activities reported for other protein systems using the EE/RR CC pair compared to those using the LZA/LZB pair.[23,26]

Importantly, Schwartz et al. previously demonstrated that any luciferase activity observed from the intein/extein pairs used in the scaffold system is due to protein splicing and not protein fragment complementation.[8] Expression constructs encoding intein/extein protein fragments lacking CCs and single CC-intein/extein fragment transfections showed no significant luciferase activity above the vector-only control, further supporting the role of coiled-coil binding in mediating the splicing reaction (Figure 1D). Given the large number of CC pairs present in the literature, these results suggest that combining CCs with the VMA split intein fragments could be a general strategy to produce a large number of new functionally


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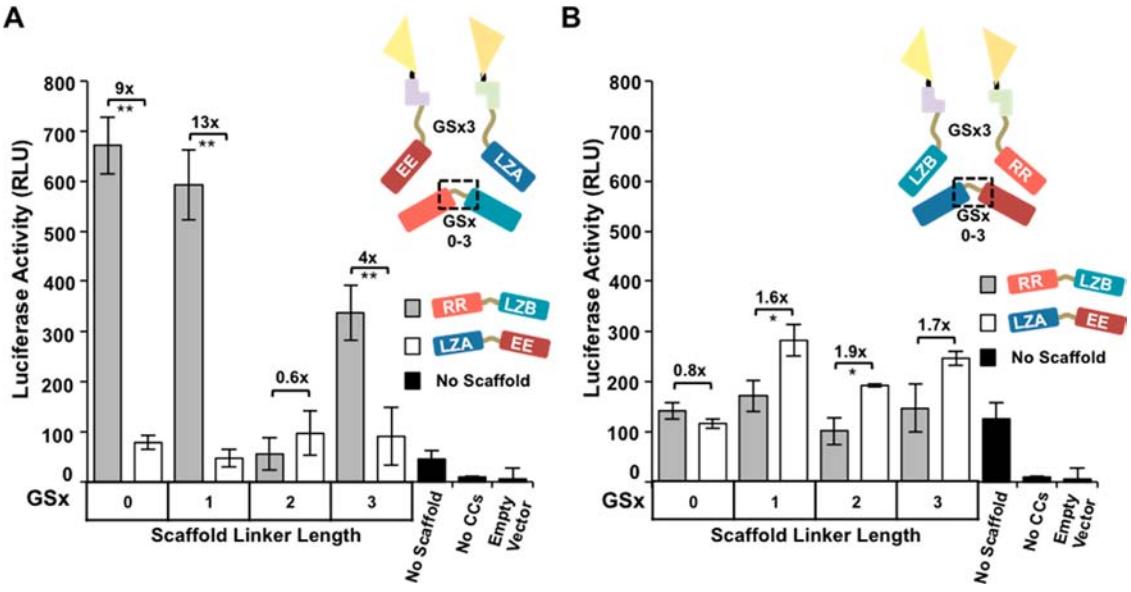


**Figure 3.** Effects of scaffold linker length on scaffold-induced protein splicing. (A) Luciferase activity of the LZA-GS3-intC/LucC and LucN/IntN-GS3-EE proteins induced by CC scaffolds with GS linkers of varying lengths. CCs for both ON-target (RR-LZB) and OFF-target (EE-LZA) scaffolds were fused together by 0−3 GS linkers. (B) Luciferase activity of the RR-GS3-LucC/LucC and LucN/IntN-GS3-LZB proteins induced by CC scaffolds with GS linkers of varying lengths. CCs for both ON-target (EE-LZA) and OFF-target (RR-LZB) scaffolds were fused together by 0−3 GS linkers. The 'No Scaffold' control consists of the intein pair transfected with an empty vector instead of a scaffold. The 'No CCs' control represents cotransfections of inteins not fused to coiled-coils. Data presented as mean ± s.d., $n = 3$. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

orthogonal intein pairs with minimal cross-reactivity.[28] Additionally, the low levels of luciferase activity for protein pairs with noncomplementary CCs indicate that these proteins could potentially serve as substrates in a scaffold-induced conditional splicing system.

Next, we investigated whether we could induce trans-splicing of two noninteracting CC-intein/extein proteins using a protein scaffold. In this system the scaffold protein is comprised of two CCs that are complementary to the CCs of the CC-intein/extein fragments. We expect both CC-intein/extein fragments to bind to the scaffold leading to split intein complementation, splicing of the luciferase exteins, and luciferase activity (Figure 2A,B,C). We first focused on scaffolding the LZA/EE pair of CC-intein/extein fragments. As previous results indicated the importance of spacing between scaffolded molecules, we created scaffolds and CC-intein/extein proteins with flexible linkers of varying lengths.[19,20,29] We generated CMV expression constructs encoding fusion proteins with 1-, 2-, or 3-copies of the glycine-serine linker $(GGGS)_3$ between CCs and the split intein/extein domains (Figure 2A). To test for scaffold-mediated splicing and the effect of CC-intein/extein linker length we cotransfected different combinations of expression plasmids encoding the N-intein and C-intein proteins with different linker lengths along with an 'ON-target' RR-LZB scaffold or an 'OFF-target' LZA-EE scaffold. We assayed for luciferase activity at 48 h. The expected CC interactions for the scaffolds and intein fragments that we tested (in boldface) are shown with connecting lines in Figure 2B.

The results of the luciferase assays demonstrated that the protein scaffold could induce specific trans-splicing of two noninteracting intein/extein proteins. We found that the ON-target scaffold led to significantly higher levels of luciferase activity than the OFF-target scaffold for all linker lengths of the LZA/EE proteins tested (2.4−6.3 fold). The CC-intein/extein proteins with 3-GS linkers exhibited the highest luciferase levels (~2600 RLU) (Figure 2D). These 3-GS linker proteins also exhibited significantly higher levels of luciferase in the presence of the ON-target scaffold compared to the 'No Scaffold' control. Neither scaffold affected the splicing and luciferase activity of the control intein/extein proteins containing no CCs. We also found that the behavior of this system was robust to changes in amounts of DNA transfected (Supporting Information [SI], Figure 1).

Next, we investigated the effect of the scaffold linker length on scaffold-induced splicing. We created DNA constructs encoding scaffolds with 0−3 × GS linkers. We also generated expression constructs encoding CC-GS3-intein/extein proteins with the LZB/RR CC pair. The full list of constructs generated and their subparts are listed in SI, Table S2. We transfected these CC-intein/extein constructs and scaffolds with different linker lengths and assayed for luciferase activity.

We found that most of the scaffolds of different GS-linker lengths were capable of inducing splicing and that the linker length had variable effects on luciferase activity (Figure 3A). For the LZA/EE-intein/extein system the luciferase activity was highest for the shortest, 0-GS linker, scaffold and correlated negatively with the length of the scaffold GS linker. The 0-, 1-, and 3-GS scaffolds all induced higher splicing levels than both the OFF-target scaffold and 'No Scaffold' controls. The 2-GS scaffold showed no significant induction of splicing activity, possibly due to steric constraints (Figure 3A).[29] While the induction levels for the LZB/RR-intein/extein system were lower than those of the LZA/EE system, the 1-and 2-GS LZB/RR systems showed significantly higher levels of luciferase with ON-target scaffolds compared to 'OFF-target' and 'No Scaffold' controls (Figure 3B). Of note, observed differences in the activity of the LZA/EE-intein/extein system in Figures 2D, 3A, 4B are most likely due differences in the luciferase assay kits used. While experiments within a single figure used the same


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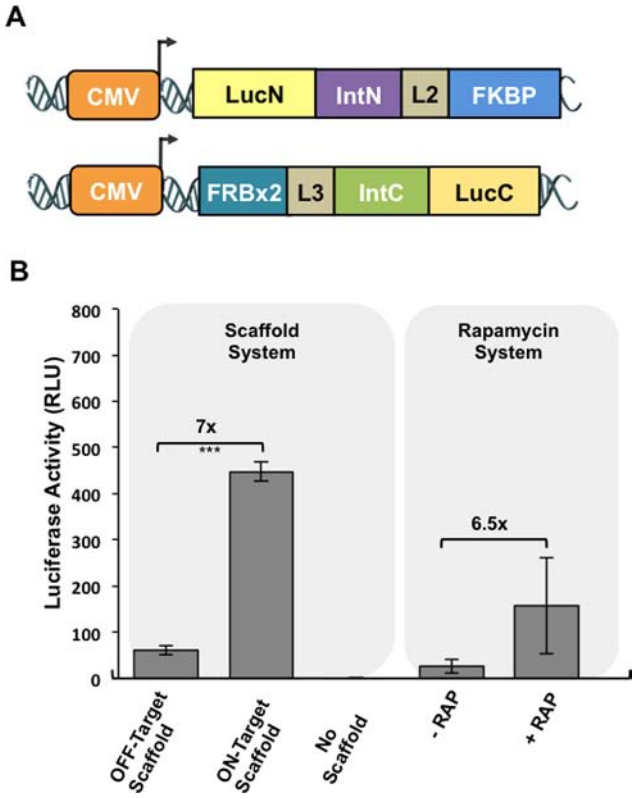


**Figure 4.** Comparison of the protein scaffold-induced splicing system to the rapamycin-induced splicing system. (A) Schematic representation of the expression system used to test rapamycin-induced conditional splicing. Rapamycin-binding domains FRB (two copies) and FKBP were fused via flexible linkers L2 and L3 to split intein-firefly luciferase extein fusions.[8] (B) Comparison of luciferase activity of the rapamycin-induced splicing system and the scaffold-induced system (LZA-GS3-LucC and LucN-GS3-EE and GS1 scaffolds). The 'No Scaffold' control consists of the intein pair transfected with an empty vector instead of a scaffold. Data presented as mean ± s.d., $n$ = 3. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

assay kit and can be quantitatively compared, results between figures should not be directly compared.

Finally, we compared the efficacy of the scaffold-induced splicing system to that of the established rapamycin-induced splicing system reported in Schwartz et al. In this system the rapamycin-binding domains FRB and FKBP bind simultaneously to rapamycin, leading to split intein complementation, protein splicing, and luciferase activity. We cloned CMV expression plasmids encoding the rapamycin inducible system, transfected it into U2OS cells and assayed for activity following 48 h. For the rapamycin system transfected cells were incubated with rapamycin or DMSO-only (Figure 4A). We found that rapamycin successfully induced luciferase activity 6.5-fold compared to the DMSO-only vehicle control. In comparison, under the same experimental conditions our scaffold-induced system exhibited similar behavior with a 7-fold induction in the presence of scaffold (Figure 4B). It should be noted that the rapamycin-inducible system has been previously shown to have optimal activity at 25 °C in *Drosophila* S2 cells; however, whenever implemented in mammalian cells, reactions are performed at 37 °C.[8] These results demonstrate that the efficacy of our scaffold-induced system is similar to that of an established conditional splicing system.

The successful implementation of our protein scaffold-induced splicing system provides the mechanistic foundation for further adaptations and applications. As the VMA split inteins have been used in various organisms and contexts, it is likely that the scaffold-induced system could also be adapted to control protein activity in a number of instances.[5−8] Additionally, the modularity of the VMA split intein with respect to binding domains and extein proteins suggests that other CCs or protein binding domains such as the PDZ, SH3, or zinc finger domains could be used and that the system could be adapted to splice together other output proteins.[30−33] That the scaffold is a protein allows for the potential to link its presence—and thus also the activation of the splicing reaction—to other biological processes *in vivo*. This goal could be accomplished by expressing the scaffold from a promoter specific to the desired process, or by post-translationally controlling its activity or localization.[14] Converting the presence of one protein into the production of a second protein also implies a design for a general protein sensor in which the protein being sensed serves as the scaffold protein for the splicing reaction (Figure 5A). Finally, the efficacy of this protein-induced system using two separate pairs of inteins with little cross-reactivity provides the basis for higher-order functioning systems. These systems could include autoregulatory networks such as protein splicing cascades or amplifiers analogous to established DNA-based systems (Figure 5B).[34]

## ■ CONCLUSIONS

We have developed a novel conditional splicing system in which a genetically encoded protein scaffold induces the trans-splicing and activation of an enzyme. We first demonstrated the ability of complementary CCs to mediate protein trans-splicing between two proteins, effectively generating two new sets of orthogonal inteins. We then demonstrated the efficacy of the scaffold-induced splicing system with two different sets of complementary CC proteins. We optimized the system based on component linker lengths, yielding a system capable of 13-fold induction over the 'No scaffold' and 'OFF-target' controls. Finally, we showed that the system had comparable efficacy to that of an established conditional protein splicing system. In sum, the protein scaffold-induced splicing system adds to the repertoire of modular approaches that researchers can employ to precisely control protein activity and biological functions in living cells.

## ■ EXPERIMENTAL SECTION

**Recombinant DNA constructs.** Recombinant plasmids were created using the Biobrick Cloning method. DNA sequences encoding the VMA intein fragments, LZA, LZB, EE, RR, FRB, FKBP were flanked with Biobrick ends and synthesized for order by Integrated DNA Technologies (IDT). The intein−luciferase fusion parts were constructed via PCR and BspQI restriction enzyme methods. The complete list of constructs and their constituent BioBrick parts can be found in Table S2 of SI. Sequences of all BioBrick subparts are listed in Table S3 of SI. For CMV expression constructs coding regions were cut with Xba1 and Not1 and cloned into the NheI and NotI sites of the CMV expression plasmid "pCDNA5ins."[35]

**Mammalian Cell Culture and Transfection.** Human osteosarcoma-derived U2OS cells (ATCC no. HTB-96) were cultured at 37 °C, 5% $CO_2$ in growth medium (McCoy's 5A medium, 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin). For transfections, cells were plated in 12-well plates at ∼150,000 cells per well in 1 mL growth medium. Transient transfections were performed 24 h after plating at 80% confluency using Lipofectamine LTX with Plus


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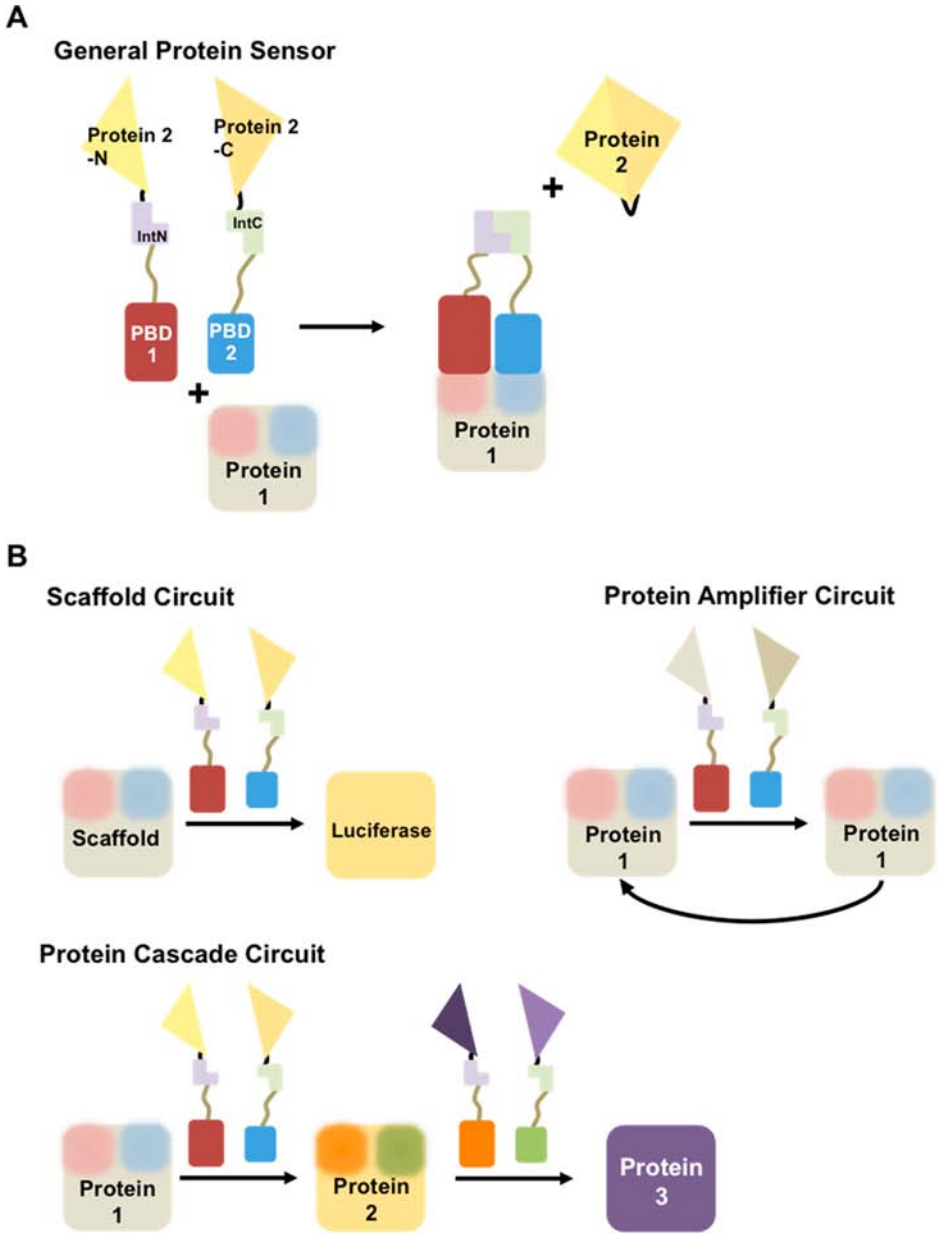


**Figure 5.** Potential applications of the protein-induced protein splicing system. (A) Schematic representation of a general protein sensor. The sensor comprises two fusion proteins consisting of protein binding domains (PBDs) for an arbitrary protein - Protein 1, split intein fragments IntN and IntC, and exteins of the output protein - Protein 2. Upon binding to Protein 1, the intein fragments complement and Protein 2 is spliced and activated. (B) Potential protein-based autoregulatory networks. The scaffold circuit described in this manuscript is represented in abbreviated diagrammatic form. In a hypothetical protein amplification circuit, Protein 1 induces the splicing of additional Protein 1, creating positive feedback and amplification of Protein 1. For a protein cascade circuit, Protein 1 induces the splicing of Protein 2 which in turn catalyzes the splicing of Protein 3.

(Invitrogen) and a total of 1 $\mu$g DNA per reaction according to the manufacturer's protocol. All transfections were performed in triplicate with the precise DNAs and amounts as specified in Table S4 of SI. Transfected cells were incubated at 37 °C for 48 h prior to analysis by luciferase assay.

**Luciferase Assay.** Luciferase activity of transfected cells was measured using Dual Luciferase Reporter Assay (Promega) according to manufacturer's instructions. Briefly, a luciferase lysis buffer (1 × passive lysis buffer supplemented with 1 $\mu$M ZnCl) was used to lyse the cells and inhibit background splicing. To each transfection well we added 250 $\mu$L of the modified buffer, and the plates sat on the shaker for 15 min prior to aliquotting in a 96-well plate. For each transfection well, 100 $\mu$L of Luciferase Assay Buffer (LARII) was pipetted over 20 $\mu$L of cell lysate and photometer readings were taken for each well. Luciferase activity is reported in relative light units (RLU) calculated by subtracting the raw output of each transfection well by an initial blank value taken on a well containing only cell lysate. All charts contain data collected in a single assay run using pooled LARII buffer.

## ■ ASSOCIATED CONTENT

### Supporting Information

DNA titration of LZA-EE scaffold system, DNA constructs and subparts, DNA transfection amounts, DNA subpart sequences, amino acid sequences of the coiled-coil domains. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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