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# Revisiting the Role of TGF$\beta$ Receptor Internalization for Smad Signaling: It is Not Required in Optogenetic TGF$\beta$ Signaling Systems

*Yuchao Li, Heeyoung Lee, Won Do Heo, and Zhike Zi**

**Endocytosis is an important process by which many signaling receptors reach their intracellular effectors. Accumulating evidence suggests that internalized receptors play critical roles in triggering cellular signaling, including transforming growth factor $\beta$ (TGF$\beta$) signaling. Despite intensive studies on the TGF$\beta$ pathway over the last decades, the necessity of TGF$\beta$ receptor endocytosis for downstream TGF$\beta$ signaling responses is a subject of debate. In this study, mathematical modeling and synthetic biology approaches are combined to re-evaluate whether TGF$\beta$ receptor internalization is indispensable for inducing Smad signaling. It is found that optogenetic systems with plasma membrane-tethered TGF$\beta$ receptors can induce fast and sustained Smad2 activation upon light stimulations. Modeling analysis suggests that endocytosis is precluded for the membrane-anchored optogenetic TGF$\beta$ receptors. Therefore, this study provides new evidence to support that TGF$\beta$ receptor internalization is not required for Smad2 activation.**

## 1. Introduction

Transforming growth factor $\beta$ (TGF$\beta$) is a multifunctional cytokine belonging to a superfamily consisting of 33 structurally related members.[1,2] It regulates a wide range of cellular functions in developmental processes and tissue homeostasis. Malfunctions of TGF$\beta$ signaling have been connected to a variety of diseases such as cancer, developmental defects, and connective tissue diseases.[3,4] Although the functions of TGF$\beta$ signaling are versatile, the core molecular mechanism of the canonical TGF$\beta$ signaling transduction is relatively simple. Briefly, a dimer of active TGF$\beta$ ligands induces the assembly of two type I TGF$\beta$ receptors (T$\beta$RI) and two type II TGF$\beta$ receptors (T$\beta$RII) to form a symmetric 2:2:2 complex (ligand-receptor-complex, LRC).[5] The oligomerization of the receptors promotes activation of T$\beta$RI through transphosphorylation, catalyzed by the constitutively active kinase of T$\beta$RII.[6] In the canonical TGF$\beta$ signaling, the activated kinase domain of T$\beta$RI phosphorylates the receptor-regulated Smad proteins (R-Smads, i.e., Smad2 and Smad3 for TGF$\beta$-like signaling pathway). R-Smads then bind to the common mediator Smad4 (co-Smad) and translocate into the nucleus.[7] The Smad complexes bind to DNA in conjunction with other transcription factors/cofactors, regulating the transcription of various target genes.[8]

Like many signaling receptors, both T$\beta$RI and T$\beta$RII receptors constantly undergo endocytosis independent of ligand stimulation.[9] Endocytosis is an important cellular process that regulates cell signaling, cell proliferation and differentiation, nutrient uptake, and drug delivery.[10] It has become clear that receptor endocytosis plays key roles in both positive and negative regulations of many intracellular signaling cascades. Dynamic trafficking of signaling receptors provides spatial and temporal separation of signaling pathways and determines the specificity and efficiency in cellular responses.[11] Upon internalization, the activated receptors are often sorted through endosome and sent to lysosomes for degradation. The internalized receptors, spatially separated from their cell surface counterparts, have better access to intracellular signaling proteins. Some of them may return to the plasma membrane by vesicular transport and be reused for further signal detection.

Numerous early studies have characterized the endocytosis of TGF$\beta$ receptors and its role in TGF$\beta$ signaling.[9,12] It has been shown that TGF$\beta$ receptors have two major endocytosis routes: clathrin-dependent and caveolin-dependent internalizations.[13–15] However, divergent interpretations were present in different studies regarding the effect of TGF$\beta$ receptor

Y. Li, Z. Zi
Max Planck Institute for Molecular Genetics
Otto Warburg Laboratory
14195 Berlin, Germany
E-mail: zhike.zi@molgen.mpg.de
H. Lee, W. D. Heo
Department of Biological Sciences
Korea Advanced Institute of Science and Technology (KAIST)
Daejeon 305701, Republic of Korea
W. D. Heo
KAIST Institute for the BioCentury
Korea Advanced Institute of Science and Technology (KAIST)
Daejeon 305701, Republic of Korea
Z. Zi
German Federal Institute for Risk Assessment
Department of Experimental Toxicology and ZEBET
10589 Berlin, Germany
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adbi.202101008.



**DOI: 10.1002/adbi.202101008**

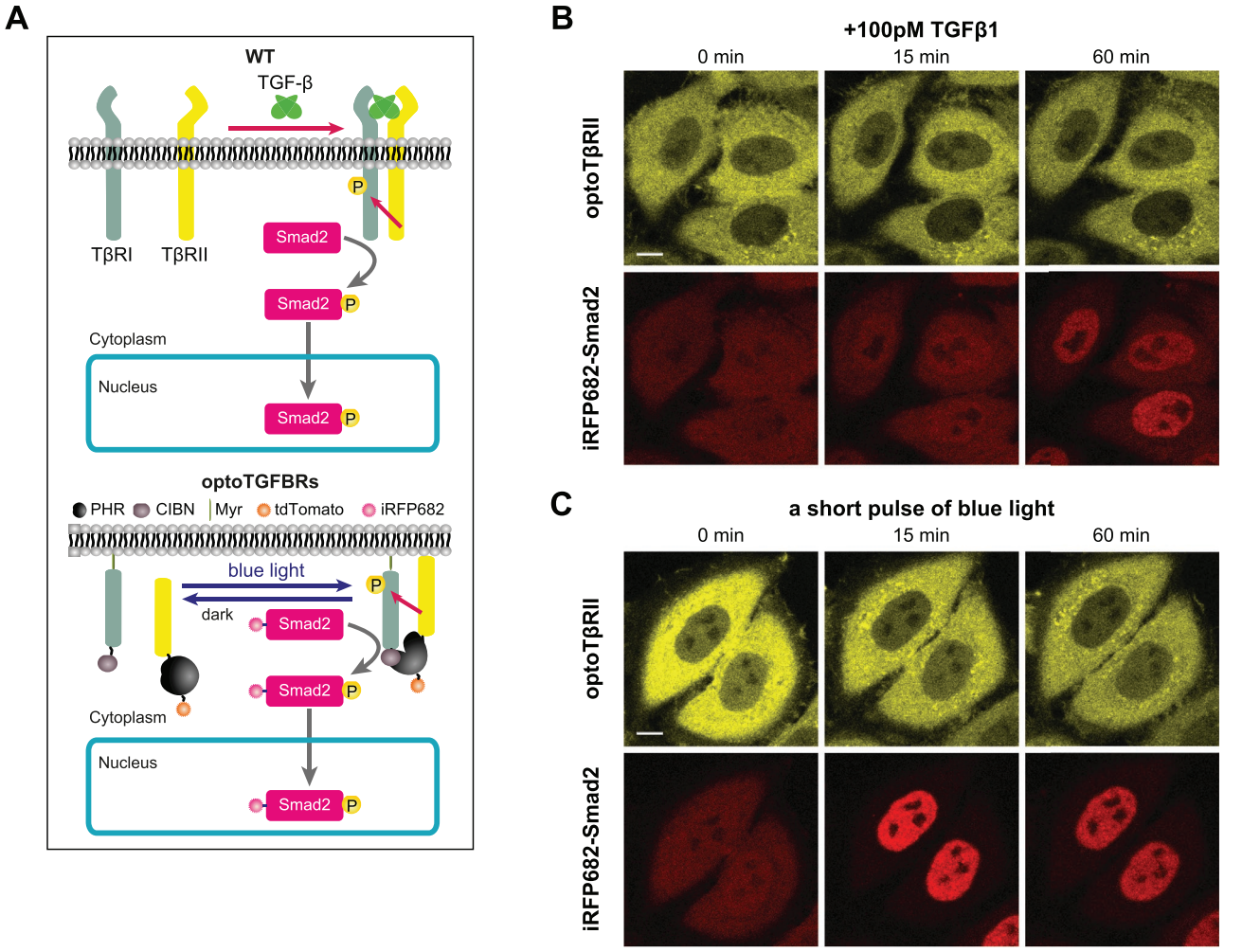


**Figure 1.** Smad signaling can be induced by blue light using an optogenetic TGF$\beta$ system with plasma membrane-tethered T$\beta$RI. A) Overview of the wild-type TGF$\beta$/Smad signaling (WT) and the optoTGFBRs optogenetic system in optoTGFBRs-HeLa cells. B) The responses of optoT$\beta$RII and iRFP-Smad2 proteins to TGF$\beta$ ligand. C) The responses of optoT$\beta$RII and iRFP-Smad2 proteins to blue light (488 nm). Light power: 12.4 µW. Scale bars: 10 µm. Panel A is adapted with permission.[17] Copyright 2018, American Chemical Society.

internalization. For example, Penheiter et al. used various methods (low-temperature treatment, potassium depletion, or the dominant-negative K44A dynamin mutant) to block receptor endocytosis and evaluated the consequent TGF$\beta$ signaling activities.[15] They reported that these clathrin-dependent endocytic blocks do not affect T$\beta$RI activation, but can impair or abolish the phosphorylation and nuclear translocation of Smad2/3. In contrast, Chen's group found that inhibiting the clathrin-mediated endocytosis by potassium depletion or mutant dynamin (K44A) does not affect Smad2 signaling or TGF$\beta$-related transcription.[16] For the requirement of clathrin-dependent endocytosis in Smad signaling, it is still unclear why different groups reported inconsistent results despite using similar experimental systems. One reason could be that the chemical endocytosis inhibitors act in a nonspecific way and their side effects may vary in different cellular or experimental contexts. In addition, genetic approaches of endocytosis inhibition could result in other secondary effects in a cell-type dependent manner, which might affect the crosstalk between TGF$\beta$ and other signaling pathways. Thus, caution is needed in interpreting these findings.

In this study, we combined systems and synthetic biology approaches to address the necessity of TGF$\beta$ receptor internalization for Smad signaling. We have previously developed an optogenetic system (optoTGFBRs) that allows us to induce Smad2 phosphorylation and nuclear translocation with blue light.[17] Here, we developed two mathematical models considering whether the membrane-tethered TGF$\beta$ receptors in the optoTGFBRs system undergo endocytosis or not. The experimental observation of optoTGFBRs signaling dynamics does not support the model with receptor internalization. In addition, we showed that blue light could still induce Smad2 activation when both T$\beta$RI and T$\beta$RII receptors were tethered to the plasma membrane in another similar optogenetic TGF$\beta$ signaling system. Therefore, without using chemical or genetic inhibitors of endocytosis, our work provides new evidence to support that TGF$\beta$ receptor internalization is dispensable for inducing Smad2 signaling.

## 2. Results

### 2.1. Blue Light Can Induce Fast and Strong Smad Signaling Using the optoTGFBRs System with Plasma Membrane-Tethered TβRI

Our previous work has shown that TGFβ signaling could be precisely activated in time and space with blue light by expressing an optoTGFBRs system in HeLa cells (optoTGFBRs-HeLa).[17] In the optoTGFBRs system, the interaction between the chimeric TβRI and TβRII is controlled through the reversible dimerization between the N-terminal end of CIB1 (CIBN) and the PHR domain of Cryptochrome2 (CRY2). The optoTGFBRs-HeLa cell line stably expresses three synthetic TGFβ signaling proteins (**Figure 1**A): the plasma membrane-tethered optoTβRI protein (Myr-cytTβRI-CIBN, in which the cytoplasmic region of TβRI was fused with the CIBN domain and anchored at the plasma membrane by a myristoylation signal peptide), the optoTβRII protein (cytTβRII-PHR-tdTomato, in which the PHR domain of CRY2 was fused to the cytoplasmic region of TβRII tagged with tdTomato), and a Smad2 activation reporter (iRFP-Smad2: human Smad2 protein tagged with iRFP682).

The optoTGFBRs-HeLa cells contain two versions of TGFβ receptor pairs: one is the endogenous TβRI and TβRII pair, which responds to TGFβ ligands in the surrounding medium; the other is the chimeric optoTβRI and optoTβRII pair, which responds exclusively to blue light illumination. As shown in Figure 1B,C, a single pulse of blue light can induce fast and strong Smad2 nuclear accumulation in optoTGFBRs-HeLa cells. Upon blue light stimulation, most

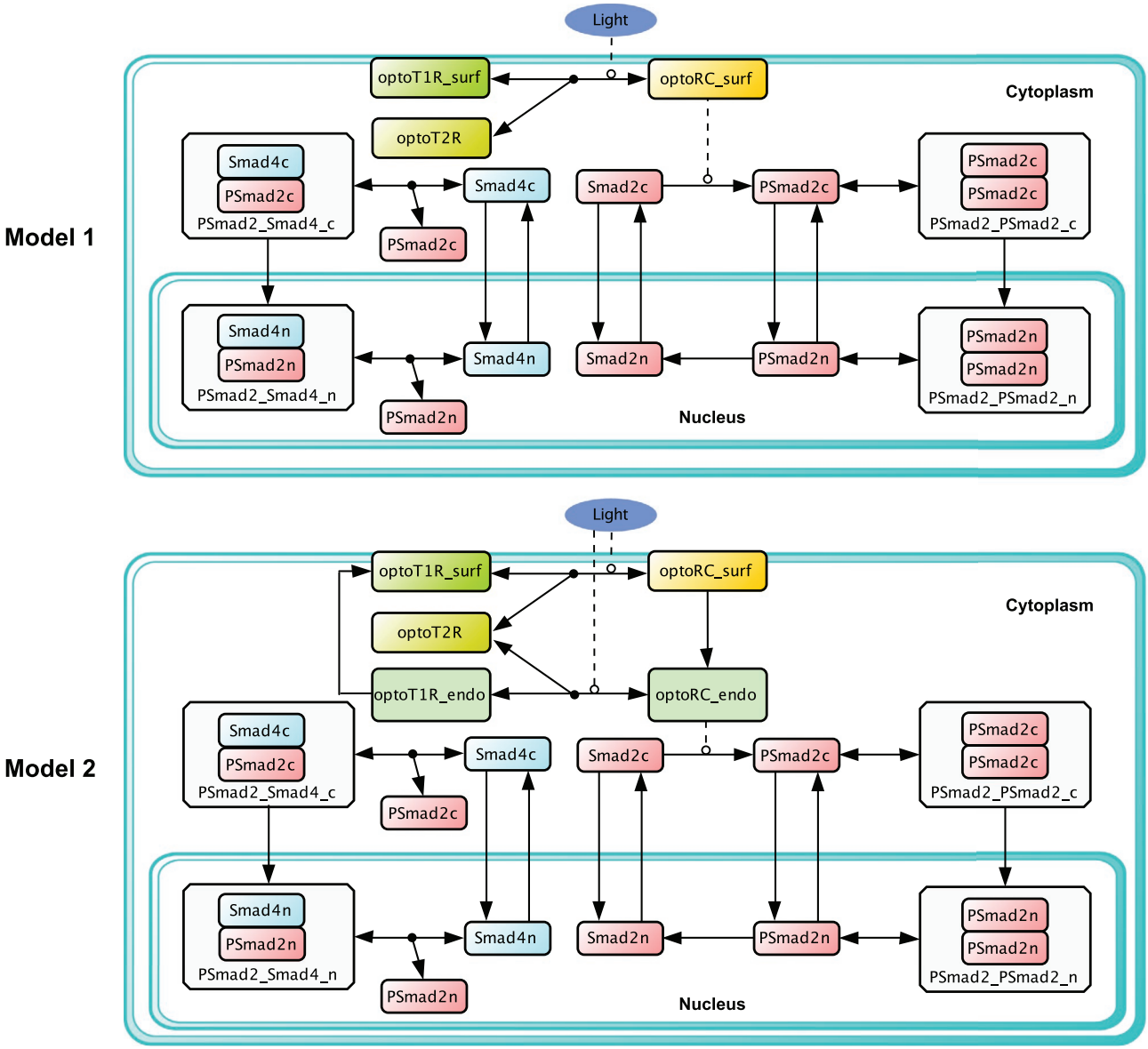


**Figure 2.** Scheme of the mathematical models for the optogenetic TGFβ signaling network (optoTGFBRs). Model 1: no internalization for optogenetic TGFβ receptors. Model 2: with internalization. A detailed description of both models is given in the Supporting Information.

of the iRFP-Smad2 proteins translocate to the nucleus within 15 min. In contrast, there were still observable traces of iRFP-Smad2 remaining in the cytosol at 1 h in cells treated with a saturating dose ($100 \times 10^{-12}$ M) of TGF$\beta$ ligand. This result confirms that the plasma membrane-tethered optoT$\beta$RI is functional and can induce Smad2 signaling completely, indicating that TGF$\beta$ receptor internalization is not required for Smad2 activation.

### 2.2. Mathematical Modeling Analysis Indicate that Endocytosis is Precluded for Plasma Membrane-Tethered optoT$\beta$RI Receptors

In optoTGFBRs-HeLa cells, optoT$\beta$RI with an *N*-terminal myristoylation sequence is anchored to the plasma membrane. Upon blue light stimulation, optoT$\beta$RI associates with optoT$\beta$RII and forms receptor complexes (optoRC). Within optoRC, optoT$\beta$RI is phosphorylated by constitutively active optoT$\beta$RII. However, it is not clear whether the optoRC (including optoT$\beta$RI) undergoes endocytosis. To solve this problem, we employed mathematical modeling. Specifically, two ordinary differential equations (ODE) models were developed based on two different hypotheses concerning the existence of receptor endocytosis in optoTGFBRs-HeLa cells (**Figure 2**). Model 1 assumes that *N*-myristoylated optoT$\beta$RI does not undergo endocytosis independent of light stimulation, while model 2 assumes that optoT$\beta$RI can internalize after forming optoRC with optoT$\beta$RII and they can recycle back to the plasma membrane after disassociation. Rate equations in these models were derived based on the law of mass action kinetics. These two ODE models have the same module to characterize the phosphorylation, dephosphorylation, and nucleocytoplasmic shuttling processes for Smad2 proteins.

To capture the dynamic properties of the optoTGFBRs system, we first determined the association and dissociation kinetics for optoT$\beta$RI and optoT$\beta$RII based on live-cell imaging data (Supporting Information). We also performed fluorescence recovery after photobleaching experiments to determine the import and export kinetics of iRFP-Smad2 (Supporting Information). In addition, we derived the initial conditions and some other model parameters based on previous published work (Supporting Information). Tables S1–S3 in the Supporting Information summarize the initial conditions for the models and the derived model parameters based on experimental data or previous published work. Up to this point, there were four unknown parameters for model 1 and five unknown parameters for model 2. The ordinary differential equations for model 1 and model 2 are listed in Tables S4 and S5 in the Supporting Information.

To estimate the unknown parameters for both model 1 and model 2, we collected three time-course datasets by quanti-

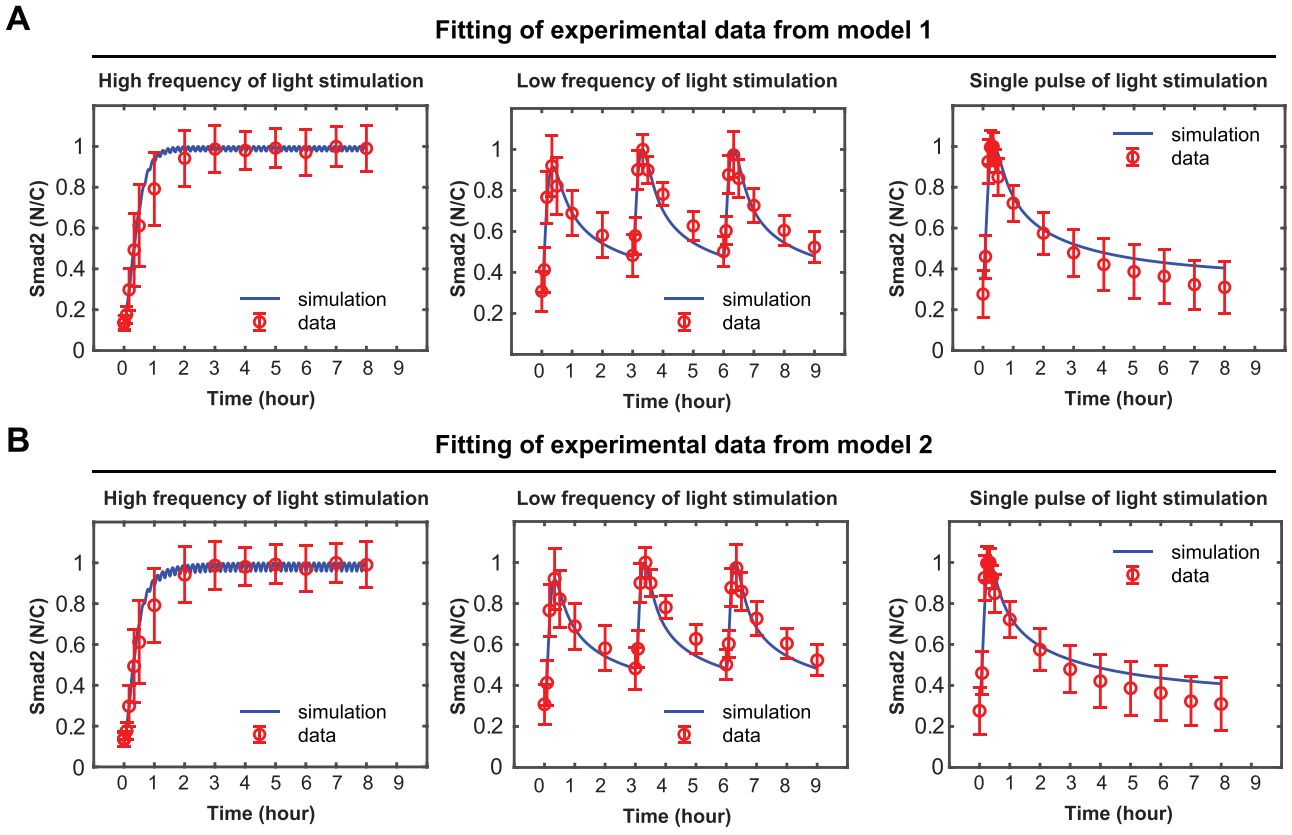


**Figure 3.** Comparison of model simulations and the experimental data for the nuclear-cytoplasmic ratio (N/C) of iRFP-Smad2 proteins. A) Fitting of experimental data for model 1. B) Fitting of experimental data for model 2. High and low frequency of light stimulations were performed by illuminating cells with one short pulse of blue light (pixel dwell time: 3.15 µs) every 10 min and every 3 h, respectively. Experimental data were presented as mean ± SD, $n = 38$ (high frequency), $n = 32$ (low frequency), $n = 42$ (single pulse).

fying the nuclear-to-cytoplasmic (N/C) ratio of iRFP-Smad2 upon three light stimulations patterns. We next used a parallel parameter estimation tool SBML-PET-MPI, which applied the algorithm of stochastic ranking evolution strategy to estimate the unknown model parameters by simultaneously fitting these experimental data sets (51 data points in total).[18,19] Identifiability of the estimated model parameters was evaluated by computing the profile likelihood.[20] All the estimated model parameters were identifiable and their confidence intervals were obtained (Tables S6 and S7, Supporting Information). As shown in **Figure 3**, after parameters optimization, computer simulations based on both model 1 and model 2 can fit these experimental datasets very well. This result suggests that we cannot yet determine whether plasma membrane-tethered optoT$\beta$RI proteins undergo endocytosis based on Smad2 nuclear translocation datasets.

We next asked under which conditions the nonendocytosis model (model 1) and endocytosis model (model 2) would predict different signaling dynamics. When blue light is turned on, optoT$\beta$RII will associate with optoT$\beta$RI. Accordingly, cytoplasmic optoT$\beta$RII will be recruited to the plasma membrane where optoT$\beta$RI locates. This will lead to a temporary depletion of cytoplasmic optoT$\beta$RII signal. If optoTGFBRs-HeLa cells are exposed to frequent blue light pulses, all of optoT$\beta$RI should stay on the plasma membrane if optoRC does not undergo endocytosis (model 1). In this case, cytoplasmic optoT$\beta$RII level will decrease by a similar amount following each light pulse (**Figure 4**A). In contrast, if the endocytosis of optoRC complexes exists (model 2), a certain portion of optoRC complexes will be internalized to the cytoplasm. Therefore, the extent of cytoplasmic optoT$\beta$RII depletion will be reduced after the next blue light pulse before reaching equilibrium, as less optoT$\beta$RI proteins would be present at the plasma membrane after internalization (Figure 4B). To test which model correctly predicts the dynamics of cytoplasmic optoT$\beta$RII under frequent blue light pulse stimulations, we quantified the corresponding cytoplasmic optoT$\beta$RII signal from the live cell imaging experiment with high frequency of light stimulation (Figure 3). As shown in Figure 4, the experimental data are consistent with the prediction of model 1 and it is quite different from the prediction of model 2. These results imply that the *N*-terminal myristoylation prevents the internalization of optoT$\beta$RI receptors, but Smad2 signaling could still be activated in the absence of receptor internalization.

### 2.3. Plasma Membrane-Tethered T$\beta$RI and T$\beta$RII Receptors Can Induce Smad Signaling

To further test whether Smad signaling can be induced without TGF$\beta$ receptor internalization, we constructed a modified version of optoTGFBRs system, in which both cytoplasmic domains of T$\beta$RI and T$\beta$RII receptors (Myr-cytT$\beta$RI-CIBN-mCerulean and Myr-cytT$\beta$RII-PHR-mCitrine) are tethered to the plasma membrane through an *N*-terminal myristoylation sequence (**Figure 5**A). When we transiently expressed Myr-cytT$\beta$RI-CIBN-mCerulean, Myr-cytT$\beta$RII-PHR-mCitrine, and mScarlet-Smad2 in HeLa cells, the images showed that the myristoylation signal peptides successfully anchored the cytT$\beta$RI-CIBN-mCerulean and cytT$\beta$RII-PHR-mCitrine proteins to the plasma membrane (Figure 5B). As shown in Figure 5C, mScarlet-Smad2 proteins can be activated and translocate into the nucleus of these HeLa cells upon blue light illumination. These results indicate that activation of Smad2

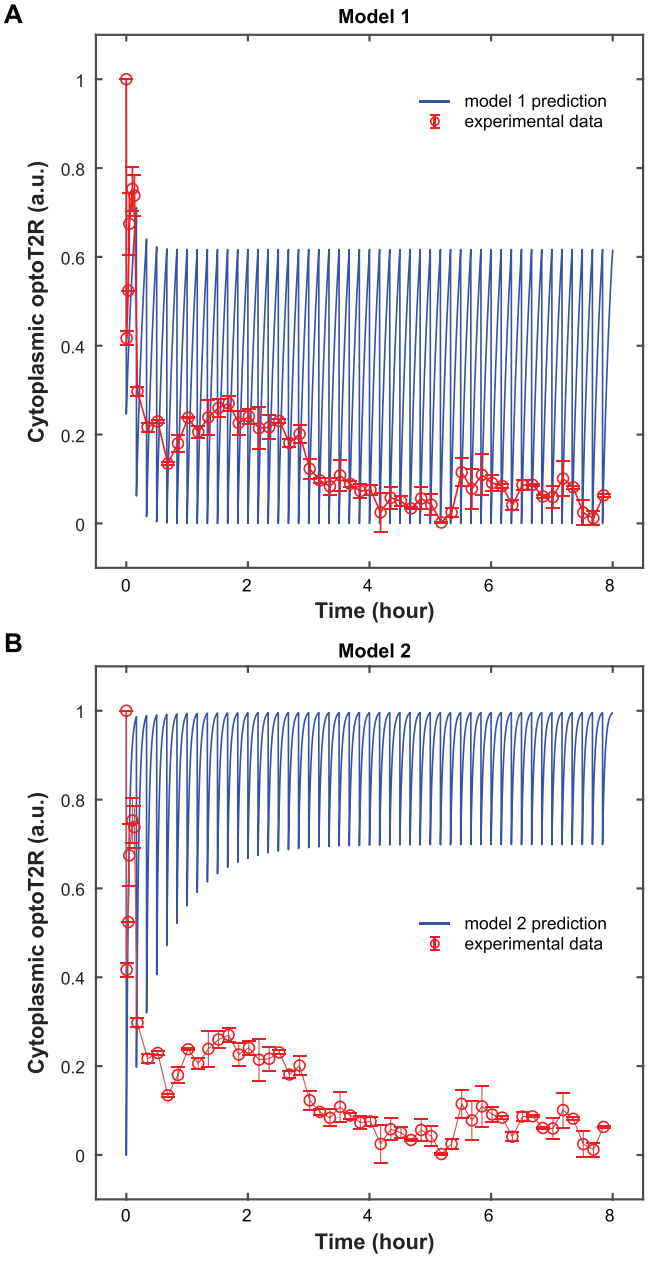


**Figure 4.** The optogenetic TGF$\beta$ receptors do not undergo endocytosis when they are activated. A) A comparison of the model 1 prediction and the experimental data for cytoplasmic optoT$\beta$RII. B) A comparison of the model 2 prediction and the experimental data for cytoplasmic optoT$\beta$RII. optoTGFBRs-HeLa cells were stimulated by one short pulse of blue light (pixel dwell time: 3.15 µs) every 10 min, cytoplasmic optoT$\beta$RII signal (immediately after each pulsed stimulation) was quantified. Experimental data and model predictions were scaled between 0 and 1 for comparison. Experimental data were presented as mean ± SD, $n = 3$.

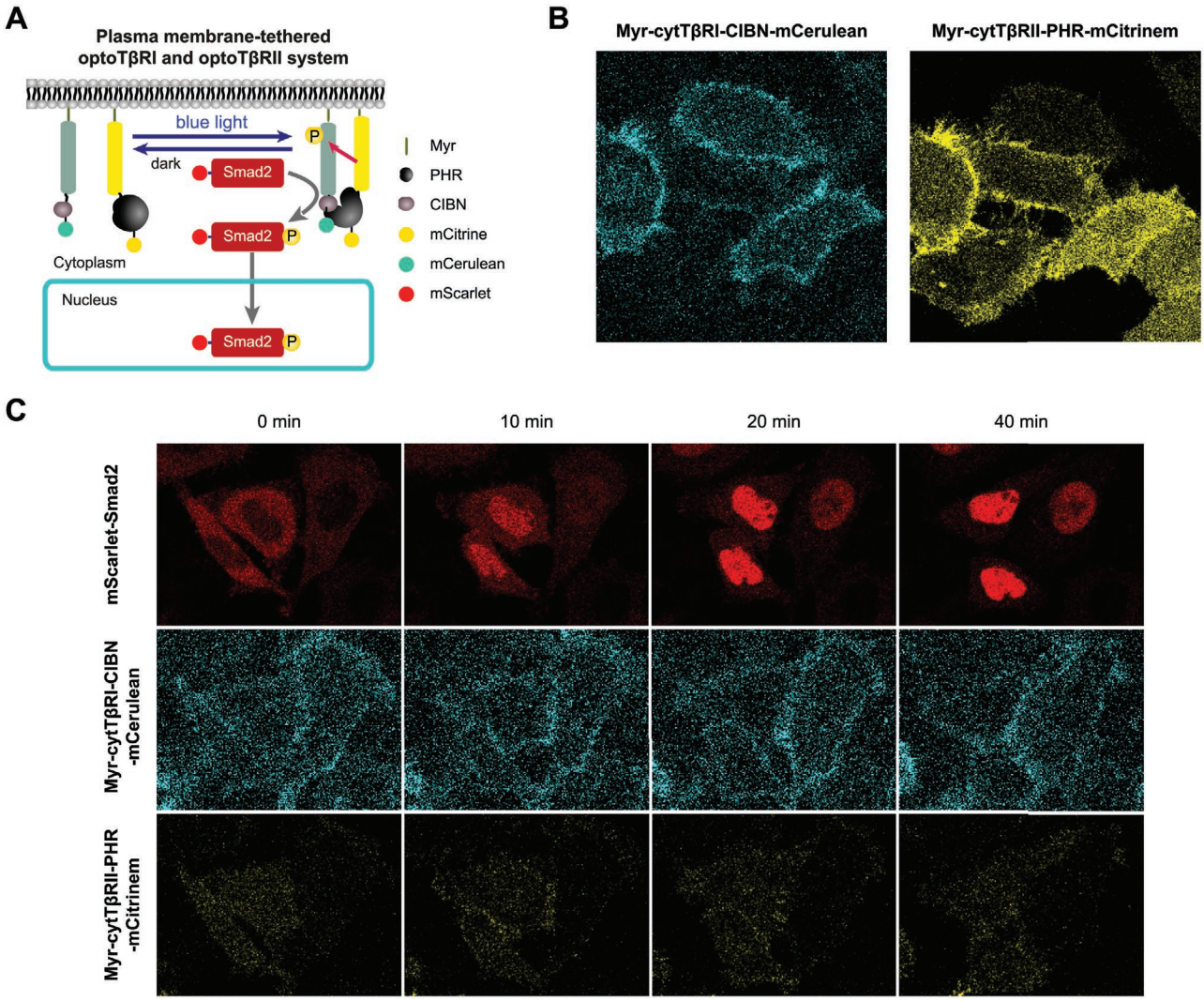


**Figure 5.** Smad signaling is maintained in the optogenetic TGF$\beta$ signaling system with plasma membrane-tethered T$\beta$RI and T$\beta$RII proteins. A) Overview of the plasma membrane-tethered optogenetic TGF$\beta$ signaling system. B) Myr-cytT$\beta$RI-CIBN-mCerulean and Myr-cytT$\beta$RII-PHR-mCitrine proteins show the plasma membrane localization when they are expressed in HeLa cells. C) Smad2 signaling responses to pulses of blue light stimulations (frequency of light stimulation: 1/30 Hz, Light power: 6 μW) in HeLa cells that co-express mScarlet-Smad2, Myr-cytT$\beta$RI-CIBN-mCerulean, and Myr-cytT$\beta$RII-PHR-mCitrine proteins.

signaling can take place when both T$\beta$RI and T$\beta$RII proteins are anchored to the plasma membrane.

## 3. Discussion

Previous studies have clearly shown that TGF$\beta$ receptors are internalized after ligand stimulation in the native TGF$\beta$ signaling pathway.[9,13–15] It was postulated that TGF$\beta$ receptor internalization is necessary for Smad2 activation.[15] Is it really the case? And what kind of role does TGF$\beta$ receptor internalization play for Smad2 activation. In this work, we tested this hypothesis using optogenetic TGF$\beta$ signaling systems (optoTGFBRs), in which TGF$\beta$ receptors are anchored to the plasma membrane and can be activated by blue light illumination. Compared with chemical endocytosis inhibitors, the optogenetic system can avoid nonspecific side effects and largely reduce (if not fully abolish) TGF$\beta$ receptor internalization. Indeed, our modeling analysis results do not support the model with TGF$\beta$ receptor internalization (model 2). Since blue light can activate Smad2 signaling in this optogenetic TGF$\beta$ signaling system, our new data verified that TGF$\beta$ receptor internalization is not essential for Smad2 activation, which agree with some previous studies.[16,21] We further corroborated this conclusion by showing that Smad2 activation could be triggered in another version of optogenetic TGF$\beta$ signaling system, in which both T$\beta$RI and T$\beta$RII receptors are anchored to the plasma membrane. It is worth noting that other groups have recently shown that Smad1/5 activation can be induced in optogenetic BMP and TGFBR/ACVR signaling systems when BMPR1B/BMPR2 or TGFBR1/ ACVR1 proteins are anchored to the plasma membrane through an *N*-terminal myristoylation.[22,23]

If TGF$\beta$ receptor internalization is not necessary for Smad2 activation, to what extent might it contribute to Smad2

signaling? It has been reported that chemical inhibition of endocytosis can impair TGF$\beta$-induced Smad2 nuclear translocation. Hence, TGF$\beta$ receptor endocytosis might enhance the efficiency of Smad2 activation and contribute to maximal Smad2 signaling responses.[12,21] In this work, we have shown that blue light stimulation in optoTGFBRs-HeLa cells induces a faster and stronger Smad2 activation than TGF$\beta$ ligand does in the endogenous TGF$\beta$ signaling. For example, Smad2 activation peaks after about 15 min upon a short pulse of blue light stimulation in the optoTGFBRs-HeLa cells, while Smad2 activation peaks at around 45 min with a short pulse or even a sustained TGF$\beta$ simulation.[17,24] Therefore, our results suggest that the efficiency of Smad2 activation could be also higher even without the contribution from TGF$\beta$ receptor internalization. Previous mathematical modeling studies indicated that the efficiency of Smad2 activation could be regulated at various levels, e.g.,, the endocytosis and degradation of endogenous TGF$\beta$ receptors.[24–26] Here, we postulate that there could be different explanations for the stronger nuclear Smad2 signaling observed in optoTGFBRs-HeLa cells. First, optogenetic TGF$\beta$ receptors are overexpressed compared to endogenous counterparts,[17] higher abundance of TGF$\beta$ receptors might contribute to stronger nuclear Smad2 signaling. Second, the efficacy of Smad2 activation might be increased by the increased local concentration of optogenetic TGF$\beta$ receptors clustering at plasma membrane, which is similar to the effect of signaling regulation by phase separation.[27] Third, negative feedback regulations might have different impacts on the endogenous and light-controllable TGF$\beta$ signaling. In the endogenous system, TGF$\beta$ ligand binds to T$\beta$RII, recruits and phosphorylates T$\beta$RI, which then activates Smads. During this time, the negative feedbacks gradually build up and act on TGF$\beta$ receptors. However, in the optoTGFBRs system, light induces a much faster reaction of receptor binding and activation, which immediately activates Smad2 to a higher extent before the negative regulations build up. In addition, endogenous TGF$\beta$ receptor endocytosis could mediate TGF$\beta$ receptor degradation, which could dampen the Smad signaling and serve as a route to attenuate cellular responses to continuous TGF$\beta$ stimulation.[26,28] It is possible that such endocytosis-mediated negative regulations cannot act on the optogenetic TGF$\beta$ receptors, whereas they can reach the endogenous TGF$\beta$ receptors. Therefore, the plasma membrane-anchored TGF$\beta$ receptors might avoid the attenuation of TGF$\beta$ signaling and thus maintain a maximal Smad2 signaling level.

To conclude, the presented findings with the optogenetic TGF$\beta$ signaling systems confirmed that TGF$\beta$ receptor internalization is not essential for Smad2 activation. However, it remains to be explored how Smad2 activation on the plasma membrane differs from that on the internalized early endosomes.

## 4. Experimental Section

*Cell Culture and Transient Transfection*: HeLa cells were cultured in Dulbecco's modified Eagle medium, which was supplied with 10% fetal bovine serum, 100 units mL$^{-1}$ penicillin and 100 µg mL$^{-1}$ streptomycin, and $2 \times 10^{-3}$ M L-glutamine at 37 °C with 5% or 10% $CO_2$. According to the manufacturer's instructions, cells were transfected using Neon transfection system (MPK5000, Invitrogen) under optimized condition (980 V, 35 ms, 2 pulses). Transfected cells were plated on 96 well-plate coated with 1 mg mL$^{-1}$ of poly-D-lysin (Sigma).

*Plasmid Construction*: The mScarlet-Smad2 was generated by inserting the Smad2 sequence (from the iRFP682-Smad2 plasmid) into the mScarlet-C1 vector using BsrGI and EcoRI restriction enzyme site. The construction of other plasmids was described in previous publication.[17]

*Cell Imaging and Analysis*: Cell imaging experiments were performed using Zeiss LSM 710 NLO 2-photon/confocal laser scanning microscope and Nikon A1R confocal microscope (Nikon Instruments). These microscopes were equipped with an incubator for maintaining environmental conditions for live cell imaging. Cells were stimulated with blue light using a 488 nm laser with a power of 6 or 12.4 µW. The cell imaging experiments and imaging data analysis were done according to the method described in previous study.[17] Briefly, live cell imaging data were manually quantified with ImageJ. The Smad2 signal was quantified as the ratio of nuclear-to-cytoplasmic iRFP-Smad2 signal. As it is difficult to quantify the fluorescence signal at the plasma membrane, the dynamics of optoT$\beta$RII were represented by the depletion of the cytoplasmic optoT$\beta$RII level. For comparison among different images, the mean tdTomato intensity (level of optoT$\beta$RII) in cytoplasmic area was normalized to that in the entire imaging field. Multiple corresponding areas were quantified and the average values were calculated.

*Mathematical Modeling*: Parameter estimation and model simulations were implemented with SBML-PET-MPI.[19] Details of the mathematical modeling, including initial conditions, parameter values, and the system of ordinary differential equations, were described in the Supporting Information.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

## Acknowledgements

This work was supported by a grant to Z.Z. from the Federal Ministry of Education and Research (BMBF)-funded e:Bio SyBioT project (031A309).

Open access funding enabled and organized by Projekt DEAL.

## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

mathematical modeling, receptor endocytosis, synthetic biology, systems biology, TGF$\beta$ signaling

Received: May 23, 2021
Revised: July 21, 2021
Published online: August 31, 2021

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