USING AUREOCHROME TO CONTROL PROTEIN-PROTEIN INTERACTIONS WITH LIGHT

Xin Zou

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Approved by :

Klaus Hahn, Ph. D.

Gary Johnson, Ph. D.

Brian Kuhlman, Ph. D.

ABSTRACT

XIN ZOU: Using Aureochrome to Control Protein-protein Interactions with Light

(Under the direction of Klaus Hahn)

Protein-protein interactions occurring with precise timing and subcellular localization are critical for regulating various cellular behaviors, yet it is difficult to study these behaviors because there are no practical means to generate protein-protein interactions at precise times and places in live cells. Photoactivatable proteins provide a way to manipulate protein-protein interactions with light *in vivo*. Recently, a blue light receptor Aureochrome was discovered in stramenopile algae *Vaucheria frigida*. It has a basic region domain, leucine zipper domain and a LOV (Light, Oxygen, Voltage) domain. Blue light treatment strongly enhances Aureochrome binding to target DNA, implying that Aureochrome is a blue light-regulated transcription factor. To control protein-protein interactions by taking advantage of Aureochrome, we characterized the light-regulated dimerization of Aureochrome. With co-immunoprecipitation assays, we showed that a leucine zipper coupled with a LOV domain (F144-K348) is sufficient for light-dependent dimerization. Critical mutations or deletion of the leucine zipper destroy the dimerization, indicating that the leucine zipper domain is critical for dimerization. Mutation of the LOV domain also disrupts the response to light. Deletion of 25 amino acids at the C-terminus leads to light-independent dimerization, implying that an autoinhibition mechanism is involved. By introducing a salt bridge mutation in the leucine zipper domain, we are able to re-engineer Aureochrome to generate homodimerization rather than heterodimerization, which could potentially be valuable in many applications. Further to verification of the dimerization of AUREO1 in living cells, we expect AUREO1 to be useful for precisely controlling protein-protein interactions temporally and spatially with light.

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LIST OF ABBREVIATIONS

AUREO1: Aureochrome1

AUREO2: Aureochrome2

BRET: bioluminescence resonance energy transfer

bZIP: basic region/leucine zipper

co-IP: co-immunoprecipitation

DNA: Deoxyribonucleic Acid

FMN: flavin mononucleotide

FV: Flag-mVenus

HM: His-Myc

LOV: light, oxygen and voltage

LZ: leucine zipper

PCR: polymerase chain reaction

CHAPTER 1

BACKGROUND AND INTRODUCTION

Proteins activities and protein-protein interactions are highly dynamics in living cells[1,2]. Precise control of proteins activities and their interactions are essential for regulating various cellular behaviors. Through perturbing cells and monitoring the response, we are able to study the role of proteins and their interaction. However, it remains difficult to control proteins activities or protein-protein interactions at precise times and places in live cells. Traditional methods, such as knockdown, knockout and exogenous expression, perturb protein activities in hours or even days. The application of these methods are greatly limited when they are used to study cell behaviors happening within minutes or even seconds, such as membrane ruffling, cell protrusion and migration[3]. Chemical induced dimerization has been widely used to regulate protein-protein interactions in living cells, especially control of signal transduction and transcription processes.[4] For example, a Rapamycin-triggered heterodimerization strategy has been used to control protein dimerization in living cells to activate and inhibit small GTPase signaling pathways[5,6]. Rho GTPases were directly activated or inhibited within seconds, followed by cell morphological changes. However, it remains challenging to control proteins dynamics spatially within subcellular dimensions. Cell behaviors are not only controlled by proteins activities but also regulated by localization. Therefore, although chemical-induced protein dimerization methods have provided new ways to perturb biological systems, lack of tissue specificity and temporal resolution restricted by

cell permeation and diffusion limit applications. Besides, it could be difficult to deliver molecules for these methods to tissues for *in vivo* studies. A system that is able to control protein activities or protein-protein interactions with high temporal and spatial resolution will benefit cell behavior research tremendously.

Recently, genetically encoded light regulated systems have been developed based on plant photoreceptors. One such system[7] is based on the LOV (light, oxygen and voltage) domain from phototropin[8,9]. Rac1 mutants were fused to the LOV domain and were sterically blocked by the LOV domain until blue light irradiation. Upon blue light activation, photoactivatable Rac1 (PA-Rac1) could generate precisely localized cell protrusions and membrane ruffling. Localized Rac1 activation by laser was sufficient to drive directed cell migration. This system could be applied to Cdc42, but not to RhoA, which is also in the Rho family. Proper three dimensional orientations between the LOV domain and the fused protein are required and limit the extension of this photoactivation approach to other proteins with very different three dimensional structures.

Light regulated protein-protein interactions via genetically encoded dimerization based on plant domains are also being developed in different labs[10-13]. One of them is based on the *Arabidopsis thaliana* photoreceptor phytochrome B (PhyB) and phytochrome interaction factor 3 (PIF3), which dimerize upon red light illumination and dissociate upon far-red light illumination. Despite the rapid reversibility of dimerization with far-red light illumination, this system requires a bilin cofactor found only in plants and some light-sensing lower organisms, limiting the application to other organisms as the cofactor needs be added exogenously, especially for *in vivo* studies. Another system took advantage of the light-dependent dimerization of FKF1 and GI, two proteins that control flowering in *Arabidopsis*

thaliana[11]. FKF1 contains a LOV domain which responds to light via a flavin mononucleotide (FMN). Blue light illumination induces formation of a covalent bond between FMN and cysteine 91 of FKF1, leading FKF1 to bind to protein GI[9]. Although this system does not need exogenous chromophore, the kinetics of the dimerization are slow, requiring tens of minutes[11]. In 2010, Kennedy et al developed a rapid light-regulated dimerization system based on a basic helix-loop-helix protein *Arabidopsis* CIB1 and cryptochrome 2 (CRY2)[14]. No exogenous cofactors are required for the system. The response upon light activation happens within seconds and the dimerization is reversible. Although the CIB1 and CRY2 are a little oversized, this system provides a very powerful potential platform for controlling a wide range of protein-protein interactions.

Recently, a blue light receptor Aureochrome was discovered in stramenopile algae *Vaucheria frigida*[15]. Two homologs, AUREO1 and AUREO2, were identified. They were composed of 348 and 343 amino acids, respectively. Each has one putative basic region/leucine zipper (bZIP) transcription-regulation domain and a single LOV domain near the C-terminus (Figure 1A). The LOV domains of AUREO1 and AUREO2 are similar to the LOV1 and LOV2 domains from plant phototropins. 11 and 9 conserved amino acids residues necessary for the FMN binding and cysteinyl adduct formation[16] were found in AUREO1 and AUREO2 respectively, indicating that these proteins may functions as photoreceptors (Figure 1B[15]).

AUREO1 binds flavin mononucleotide (FMN) via its LOV domain, possibly forming a cysteinyl adduct to the C94a carbon of the FMN upon light activation. This process is reversible, with a half-life of around 5 minutes. Gel shift assays showed that AUREO1 binds to DNA upon light activation, indicating that AUREO1 functions as a transcription factor

which is regulated by light. However, AUREO2 does not rely on light activation. Besides, transcription factors with bZIP domains usually bind to DNA by forming

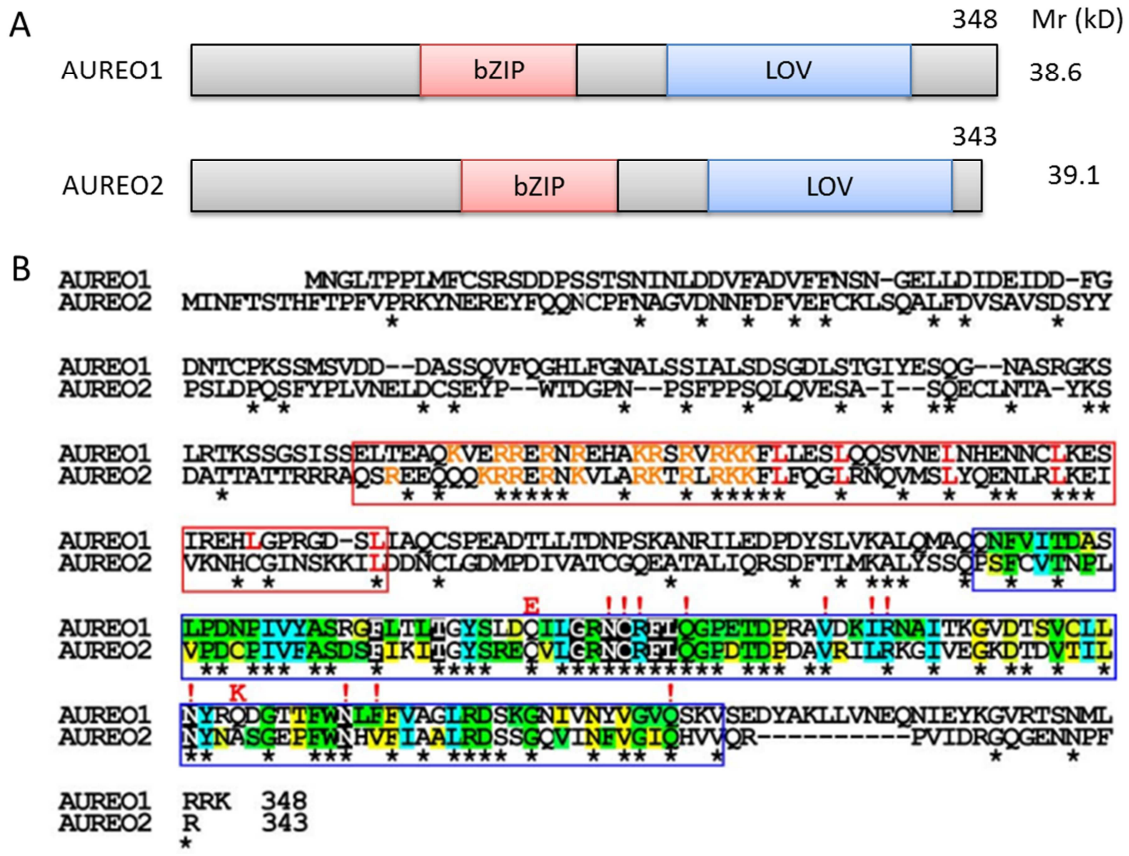

Figure 1 Structure and aligned sequences of Aureochromes from *V. frigida*. A, basic region/leucine zipper (bZIP) and LOV domains are colored red and blue, respectively. B, sequence alignment of AUREO1 and AUREO2. bZIP and LOV domains are indicated with red and blue frames, respectively. Basic amino acids are colored in orange and heptad leucine residues of bZIP domains are colored in red. The asterisks indicate identical amino acids between AUREO1 and AUREO2[14].

dimers via a leucine zipper, which is typically a 7 residue repeat α-helix (heptad). These results indicated that AUREO1 possibly dimerizes upon light activation. By fusing AUREO1 with target proteins, we could potentially take advantage of the light regulated dimerization to control protein-protein interactions with light. It could provide us with a powerful

genetically encoded tool to control protein-protein interactions or protein activities in living cells with high spatial and temporal resolution.

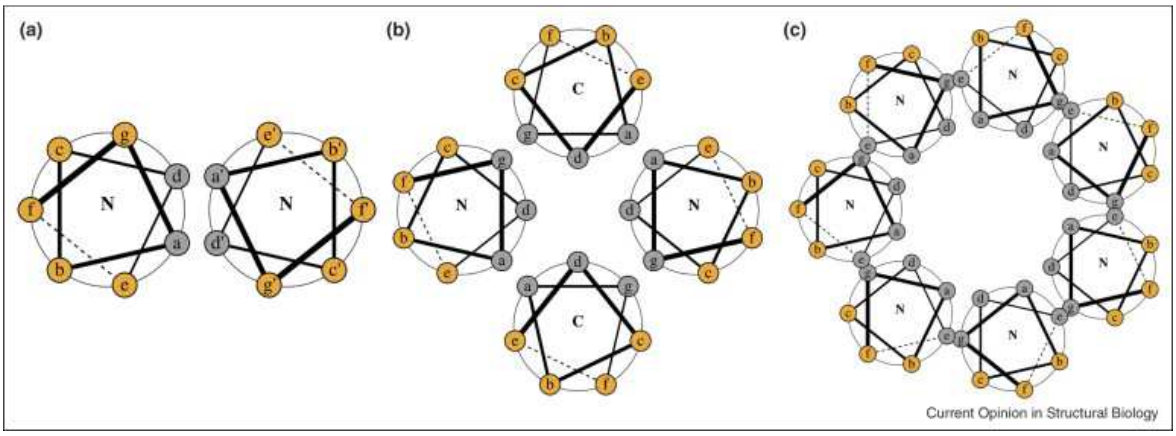

Figure 2 **Varying topology structures of coiled coils.** Heptad positions (denoted abcdefg) are shown in small letters. Predominantly hydrophobic and predominantly polar/charged residues are colored with gray and orange, respectively. A, the canonical 3-4 heptad repeat, in which hydrophobic residues are located at a and g positions, is found for many coiled coils including dimers, trimers, and tetramers. B, An antiparallel tetramer with a 3-3-1 repeat. C. A parallel seven-helix coiled coil with a 3-1-2-1- hydrophobic pattern[16].

A leucine zipper, which is a very common structure found in transcription factors, is typically a 7 residue repeat α-helix (heptad). The heptad repeat typically has hydrophobic residues at a and d position for interactions, and polar/charged residues at e and g position (Figure 2). A large number of structural variations are found among coiled coils, such as dimers, trimers, tetramers and pentamers. Their helix orientations and alignments may vary as well as they can form homocomplexes or heterocomplexes. Protein designers have shown great interest in the coiled coil structures, which were among the first rationally designed structures[16]. Hydrophobic-polar patterning imposes association of helices, and charge patterning and other features can be used to confer specificity. For example,

homodimerization could be engineered to heterodimerization by making one helix basic and another acidic[17,18] Besides this, they could be engineered for various purposes[18,19], such as increasing binding affinity.

In this study, we propose to develop blue light regulated dimerization based on AUREO1. This system will provide a platform to control protein-protein interactions by light within seconds and subcellular dimension. It has several advantages, such as fast response, reversibility, and no requirement for exogenous cofactors. The leucine zipper domain, with potential in various engineering possibilities, such as homodimerization or heterodimerization, different kinetics, also could provide a wide application to control protein-protein interactions in living cells.

CHAPTER 2

RESULTS AND DISCUSSION

2.1 Characterization of light regulated dimerization of AUREO1

To develop a light regulated protein-protein interaction based on AUREO1, we first investigated whether the dimerization of AUREO1 is light regulated. It was previously reported[15] that AUREO1 contains a bZIP (basic region + leucine zipper) domain and LOV domain. Typically the basic region binds to the DNA major groove while the leucine zipper dimerizes. Therefore we propose that portions of AUREO1 containing a leucine zipper and LOV domain (F144-K348) are sufficient for dimerization.

As showed in Figure 3A, we labeled the AUREO1 (F144-K348) containing leucine zipper and LOV domains with His-Myc (HM) tag or Flag-mVenus (FV) tags and studied their dimerization by co-immunoprecipitation (co-IP). Dimerization of AUREO1was examined in HEK 293 cells coexpressing HM and FV tagged AUREO1 (F144-K348). Two groups were examined at the same time with ambient light activation or without light activation (performed under red light). Cell lysates were co-immunoprecipitated with anti-Flag beads and detected with anti-Myc or anti-mVenus in the following western blotting. As showed in Figure 3B, HM-AUREO1 was detected in the co-IP with ambient light activation, which means HM-AUREO1 co-immunoprecipitated with FV-AUREO1. However, there was no co-IP of HM-AUREO1 detected by anti-Myc in the dark. This indicated that AUREO1 (F144-K348) was sufficient for light regulated dimerization. The ambient light was sufficient

to activate the dimerization of AUREO1 in the *in vitro* biochemical studies. When the AUREO1 is in the dark without light activation, it does not dimerize.

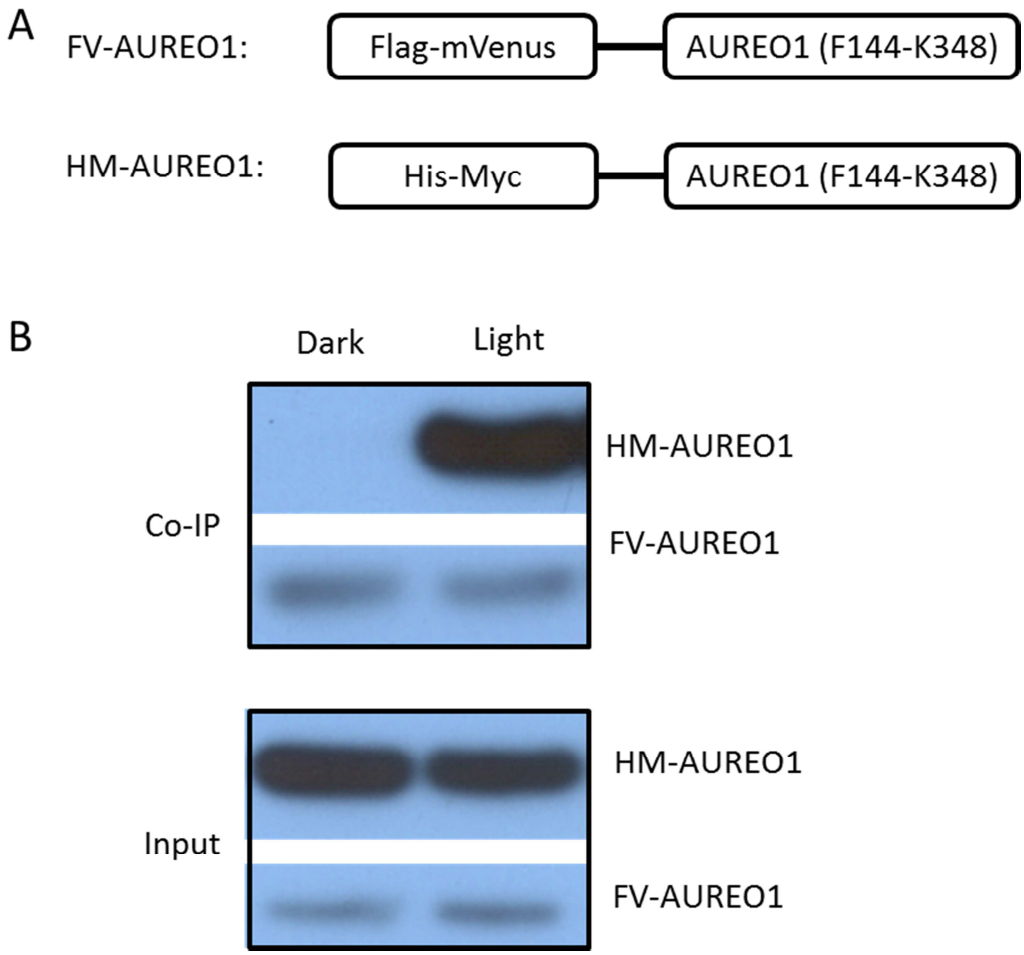

Figure 3 AUREO1 (F144-K348) is sufficient for light regulated dimerization. A, AUREO1 (F144-K348) was tagged with Flag-mVenus (FV) or His-Myc (HM) respectively. B, AUREO1 (F144-K348) dimerizes upon light activation, but not in the dark. HM-AUREO1 was detected by anti-Myc and FV-AUREO1 was detected by anti-mVenus.

Furthermore, we investigated which regions are critical for the light regulated dimerization of AUREO1. It has been known that the leucine zipper domain forms a parallel or anti-parallel coiled-coil motif that functions as a flexible DNA binding arms for transcription factors[20]. To study if the leucine zipper is involved in the dimerization of

AUREO1, we deleted the leucine zipper but only kept the LOV domain (O217-K348) and tagged this with Flag-mVenus (Figure 4A). Using a similar strategy, as discussed in Figure 3, we studied the role of the leucine zipper with co-IP assay. As showed in Figure 4B, LOV domain alone (O217-K348) does not dimerize with AUREO1 (F144-K348), indicating that the leucine zipper is required for the dimerization of AUREO1. In addition, as a 7 residue repeat α-helix, the leucine zipper normally dimerizes via hydrophobic interactions in a and d positions[19].

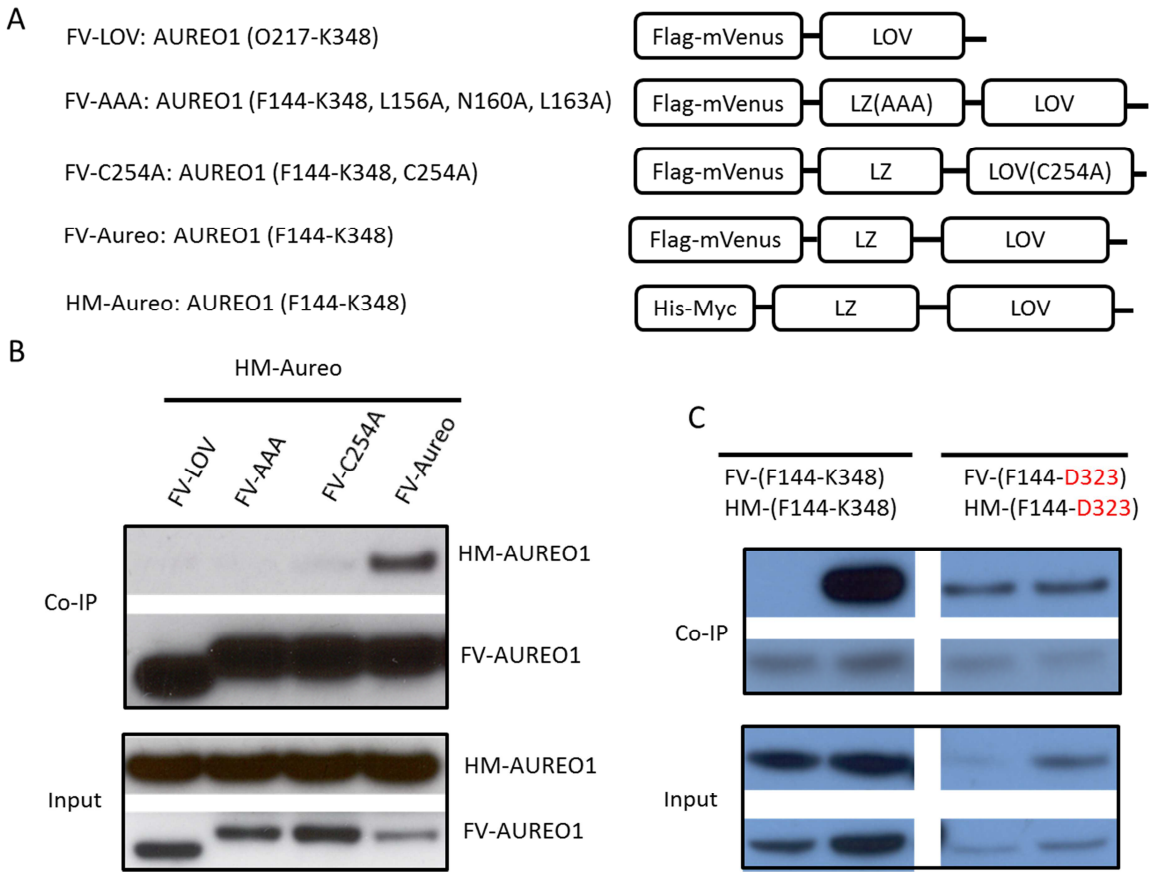

Figure 4 Both the leucine zipper and the LOV domain are critical for light regulated dimerization of AUREO1. A, the constructs used in the co-IP experiments to characterize the light regulated dimerization of AUREO1. B, deletion or mutations of the critical residues on the leucine zipper destroys the dimerization. Cysteine 254 to Alanine mutation destroys the dimerization too. C, deletion of the C-terminus (D323-K348) leads to dimerization independent of light regulation.

To test if the AUREO1 dimerizes via the hydrophobic interactions between the leucine zipper motifs, we introduced site mutations in the a and d positions. The residues leucine 156, asparagine 160 and leucine 163 of AUREO1 (F144-K348) were mutated to alanine and tagged with Flag-mVenus (FV-AAA). As showed in the co-IP in Figure 4B, the dimerization of AUREO1 was destroyed by the site mutations. It suggests that the AUREO1 dimerizes by the hydrophobic interactions of its leucine zipper motif.

Although there is no crystal structure of AUREO1, sequence blast shows that it may have a structure similar to the LOV2 domain from *Arabidopsis.* AUREO1 has a cysteine at residue 254, which aligns with cysteine 450 of LOV2. This led to our hypothesis that AUREO1 responds to light activation by binding to flavin mononucleotide (FMN) via residue cysteine 254. To test this hypothesis, we mutated residue cysteine 254 to alanine and tagged with Flag-mVenus for a co-IP test. As showed in Figure 4b, FV-C254A binds to HM-AUREO1 very weakly. The binding affinity is much lower than for wild type protein. Therefore, we believe that the LOV domain responds to light activation by binding to FMN via its cysteine 254.

We wonder how the LOV domain binding to FMN leads to the dimerization of the leucine zipper domain. Interestingly, Aureochrome has two homologs AUREO1 and AUREO2. Although they have similar motifs and sequences, only AUREO1, but not AUREO2, binds to FMN, indicating that only AUREO1 is regulated by light[15]. Comparing these two homologs, AUREO2 lack ten amino acids (D323-Q332) in the c-terminus, which is likely a α-helix predicted by second structure prediction. We propose the hypothesis that the leucine zipper is blocked by the C-terminus of AUREO1 in the dark. When it is exposed to light activation, LOV domain binding to FMN leads to conformational change and further

releases the block of the leucine zipper. To test this hypothesis, we compared the light regulation of AUREO1 with or without the C-terminus (D323-K348). As showed in Figure 4C, AUREO1 (F144-K348) only dimerize when it is activated by light. Meanwhile, AUREO1 (F144-D323) always dimerizes, even without light activation. It indicates there is an interaction between the leucine zipper and C-terminus and the dimerization of leucine zipper is blocked. Conformational change of the LOV domain caused by binding to FMN diminishes this interaction and releases the block.

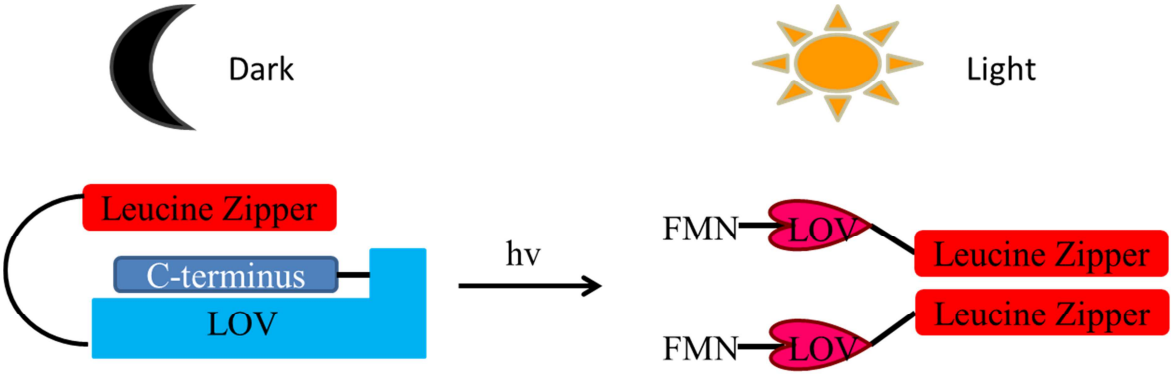

Figure 5 The autoinhibition model of the light regulated dimerization of AUREO1. To sum up, AUREO1 (F144-K348), which mainly contains a leucine zipper and LOV domain, is sufficient for light regulated dimerization. Both the leucine zipper and LOV domain are critical for the dimerization. Deletion of the leucine zipper or site mutations at critical positions destroys the dimerization. It is highly possible that the C-terminus blocks the dimerization of the leucine zipper domain. Activated by light, the LOV domain binds to the FMN and goes through a conformational change, which releases the blockage of the C-terminus leucine zipper and further leads to the dimerization. We propose the auto-inhibition model to explain the light regulated dimerization of AUREO1 as showed in figure 5. A crystal structure of the AUREO1 in the dark will provide more accurate explanations and insight about the model.

2.2 Engineering AUREO1 to control heterodimerization rather than homodimerization

One advantage of developing light regulated protein-protein interactions with Aureochrome relies on its leucine zipper, which is critical for the dimerization. The leucine zipper widely exists in transcription factors for dimerization interaction. Previous studies have shown that engineering could be used to alter the dynamics, affinities, or switch from homodimerization to heterodimerization. If we could engineer Aureochrome to produce herterodimerization, this light regulated protein-protein interactions system would provide far wider applications.

As shown in Figure 6A, the leucine zipper is a 7 repeat α-helix (view from top to bottom). Residues at positions a and d are responsible for hydrophobic interactions. Besides this, positively or negatively charged residues at positions e and g could interact with each other. It has been shown that homodimerization of leucine zippers can be converted to heterodimerization by introducing salt bridges at positions e and g[17]. To engineer Aureochrome to produce heterodimerization, we designed several pairs of salt bridge (R-E or K-E) mutations at positions e and g in different repeats. Then we tested if they can form heterodimers but not homodimer, using co-IP experiments similar to what we did previously. With extensive trials, we finally found that introduction of an R-E salt bridge at residues E159 and K164 generated constructs that worked as a pair of heterodimers.

We mutated E159 and K164 to R or E, and tagged them with Flag-mVenus or His-Myc respectively. The denotation is shown in Figure 6B. With strategy similar to that applied previously, we investigated the dimerization of the EE and RR mutants. We found that HM-EE and FV-RR dimerize upon light activation but not in the dark, which is very similar to the dimerization of HM-AUREO1 and FV-AUREO1. Moreover, the

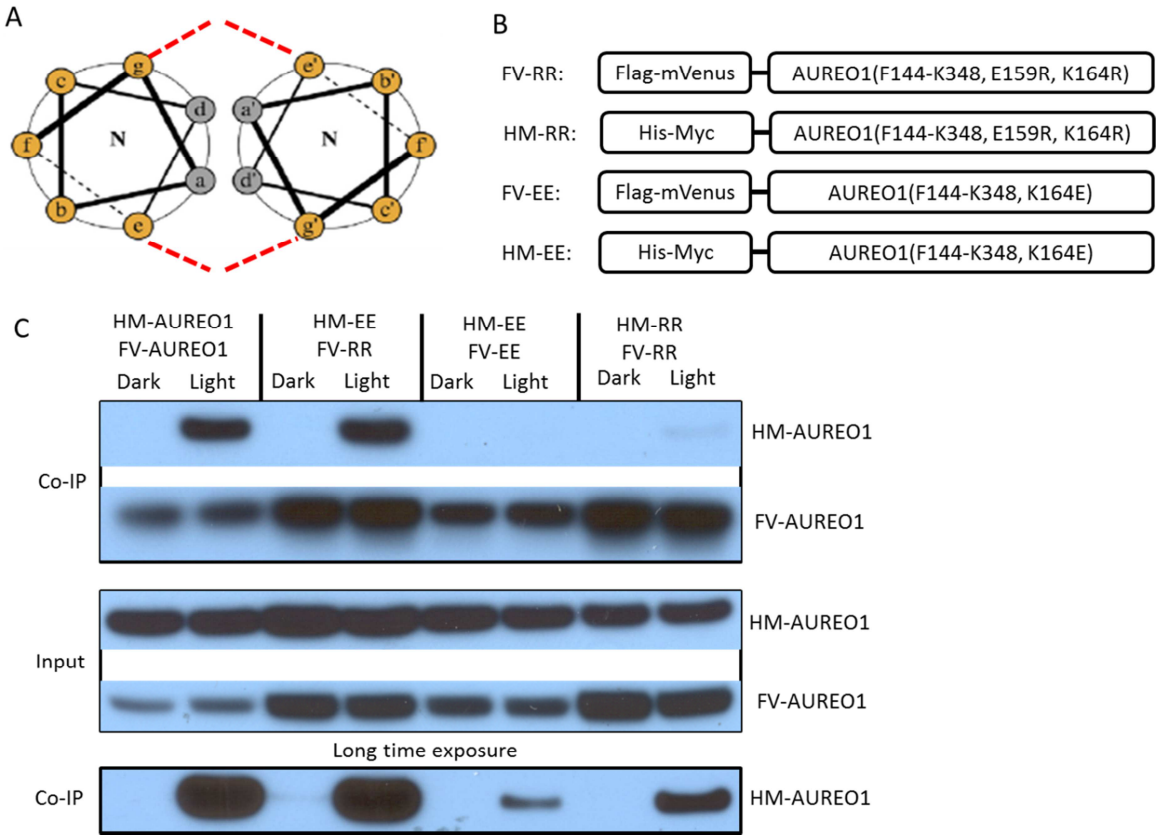

Figure 6 AUREO1 is engineered to light regulated heterodimerization by introducing salt bridges at positions a and g. A, the leucine zipper is typically a 7 repeat α-helix (from top to bottom view). Heptad positions (denoted abcdefg) are shown in small letters. Predominantly hydrophobic and predominantly polar/charged residues are colored in gray and orange, respectively. B, the constructs of the heterodimerization pairs. C, the co-IP results of homo- and hetero-dimerization. HM: His-Myc; FV: Flag-mVenus; EE: AUREO1 (F144-K348, K164R); RR: AUREO1 (F144-K348, E159R, K164R).

heterodimerization affinity of HM-EE and FV-RR is comparable to that of HM-AUREO1 and FV-AUREO1. On the other hand, neither the dimerization of HM-EE and FV-EE or HM-RR and FV-RR is comparable to that of HM-AUREO1 and FV-AUREO1. We did see some low levels of homodimerization for the EE and RR pairs with long exposure. However, the dimerization affinities were much lower than wild type AUREO1. This suggests that introduction of EE and RR mutations converted the homodimerization into heterodimerization. Moreover, it reduced the homodimerization affinity significantly,

possibly by the repulsion of the charged residues. The heterodimer pair provides us a great module to use light to control two different target proteins interacting with each other, but not with themselves.

To sum up, we successfully engineered AUREO1 from a homodimerization to a heterodimerization domain by introducing the salt bridge R-E at E159 and K164. Both mutants AUREO1 (F144-K348, E159R, K164R) and AUREO1 (F144-K348, K164E) have no or much lower dimerization affinities.

2.3 Test the heterodimerization of AUREO1 in living cells

To regulate protein-protein interactions with AUREO1 in living cells, we needed to verify the interactions of AUREO1 *in vivo* as a proof of principle. We proposed a membrane translocation system to investigate the dimerization of AUREO1 in mammalian cells. As showed in Figure 7, we tagged one of the heterodimerizing pair with a fluorescent protein (such as GFP) and anchored it on the plasma membrane with a membrane localization tag. We then tagged the other member of the heterodimer pair with another fluorescent protein with a different spectrum, for example mCherry. Without light activation in the dark, the AUREO1 with membrane localization tag should localize on the membrane while the other AUREO1 without membrane localization tag should localize in the cytoplasm. After light activation, if the AUREO1 dimerizes in living cells, the AUREO1 without membrane localization tag will be translocated to the membrane.

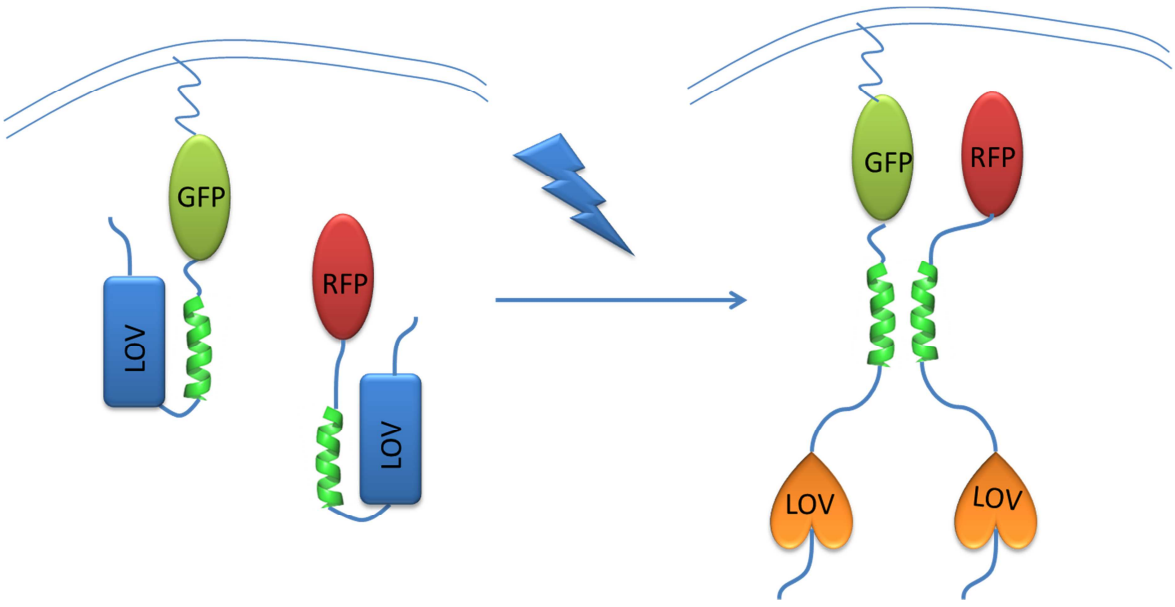

Figure 7 Testing light regulated dimerization of AUREO1 in vivo with a membrane translocation system. Leucine zipper is shown as a green α-helix.

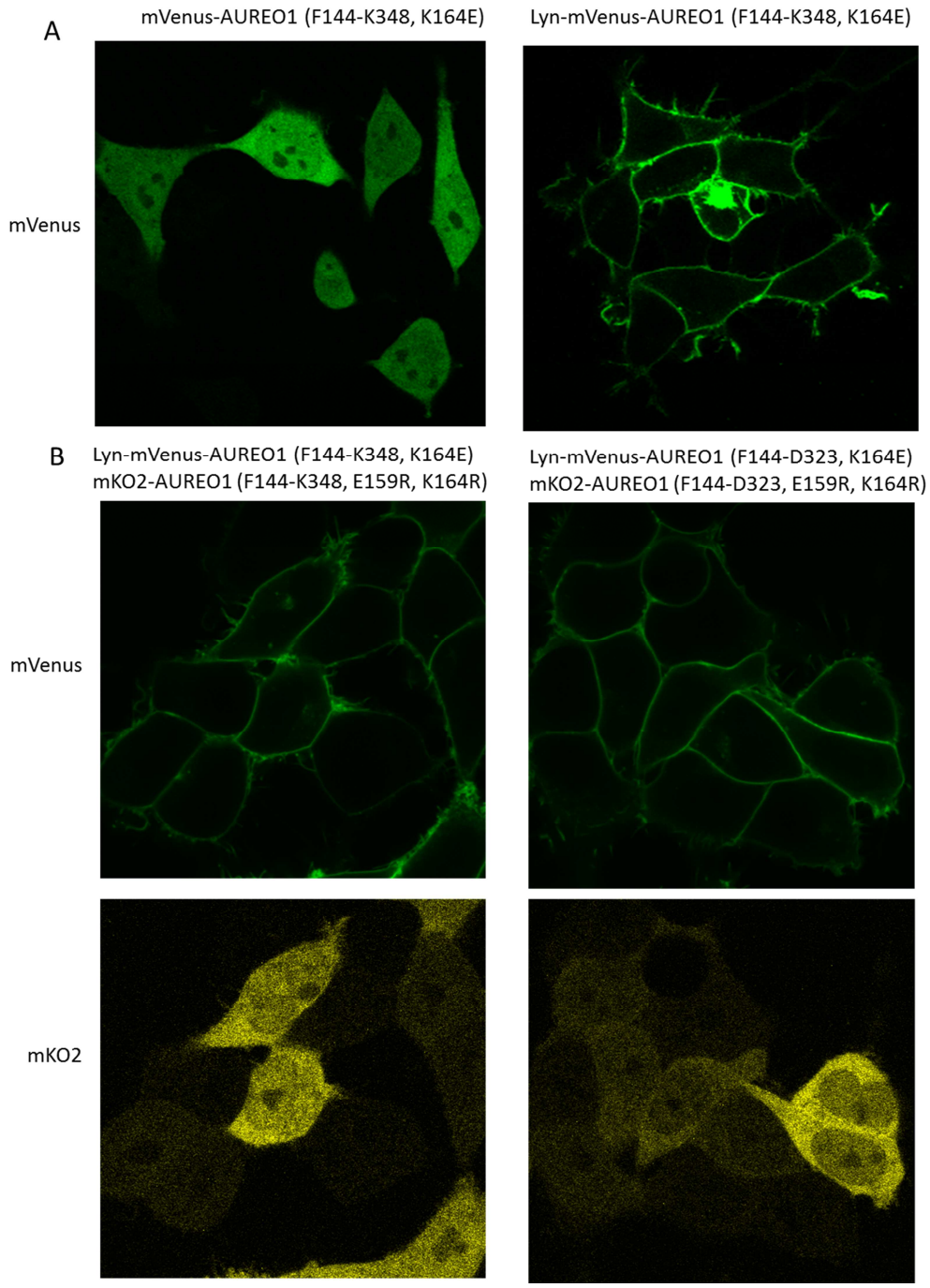

Figure 8 Testing AUREO1 dimerization in HEK283 cells with membrane translocation system. A, without membrane tag, AUREO1 localizes in the cytosol evenly, while it localizes on the membrane when it is fused with a Lyn membrane tag. B, left panel, co-expression of Lyn-mVenus-AUREO1 (F144-K348, K164E) and mKO2-AUREO1 (F144-K348, E159R, K164R); right panel, co-expression of Lyn-mVenus-AUREO1 (F144-D323, K164E) and mKO2-AUREO1 (F144-D323, E159R, K164R).

Considering that the C-terminus of AUREO1 is involved in the light regulation, we chose the N-terminus membrane localization signal from Lyn kinase. As showed in Figure 8A, mVenus-AUREO1 (F144-K348, K164E) localizes in HEK 293 cells uniformly, while Lyn-mVenus-AUREO1 (F144-K348, K164E), which has the membrane localization signal, mostly localizes on the plasma membrane, as showed by confocal imaging. We co-expressed Lyn-mVenus-AUREO1 (F144-K348, K164E) and mKO2-AUREO1 (F144-K348, E159R, K164R) in HEK293 cells and they localized on the membrane or in the cytosol respectively (as showed in Figure 8B, left panel). We activated the cells with ambient light for up to 10 minutes but saw no membrane translocation. We also tried other light sources such as 473 nm or 488 nm lasers, again with no membrane translocation after light activation. To rule out the possibility that the activation of AUREO1 dimerization needed a long time, we in investigated the dimerization *in vivo* by co-expression of lit state mutants. As showed in Figure 4C and 5, it is known that deletion of D323-K348 leads to the constitutive dimerization of AUREO1 with or without light activation, which suggests AUREO1 (F144-D323) mutants could be used as lit state mutants. We co-expressed Lyn-mVenus-AUREO1 (F144-D323, K164E) and mKO2-AUREO1 (F144-D323, E159R, K164R) in HEK293 cells. As shown in Figure 8B (right panel), although Lyn-mVenus-AUREO1 (F144-D323, K164E) localizes to the membrane clearly, mKO2-AUREO1 (F144-D323, E159R, K164R) is still uniformly distributed in the cytosol, indicating there is no dimerization in the living cells.

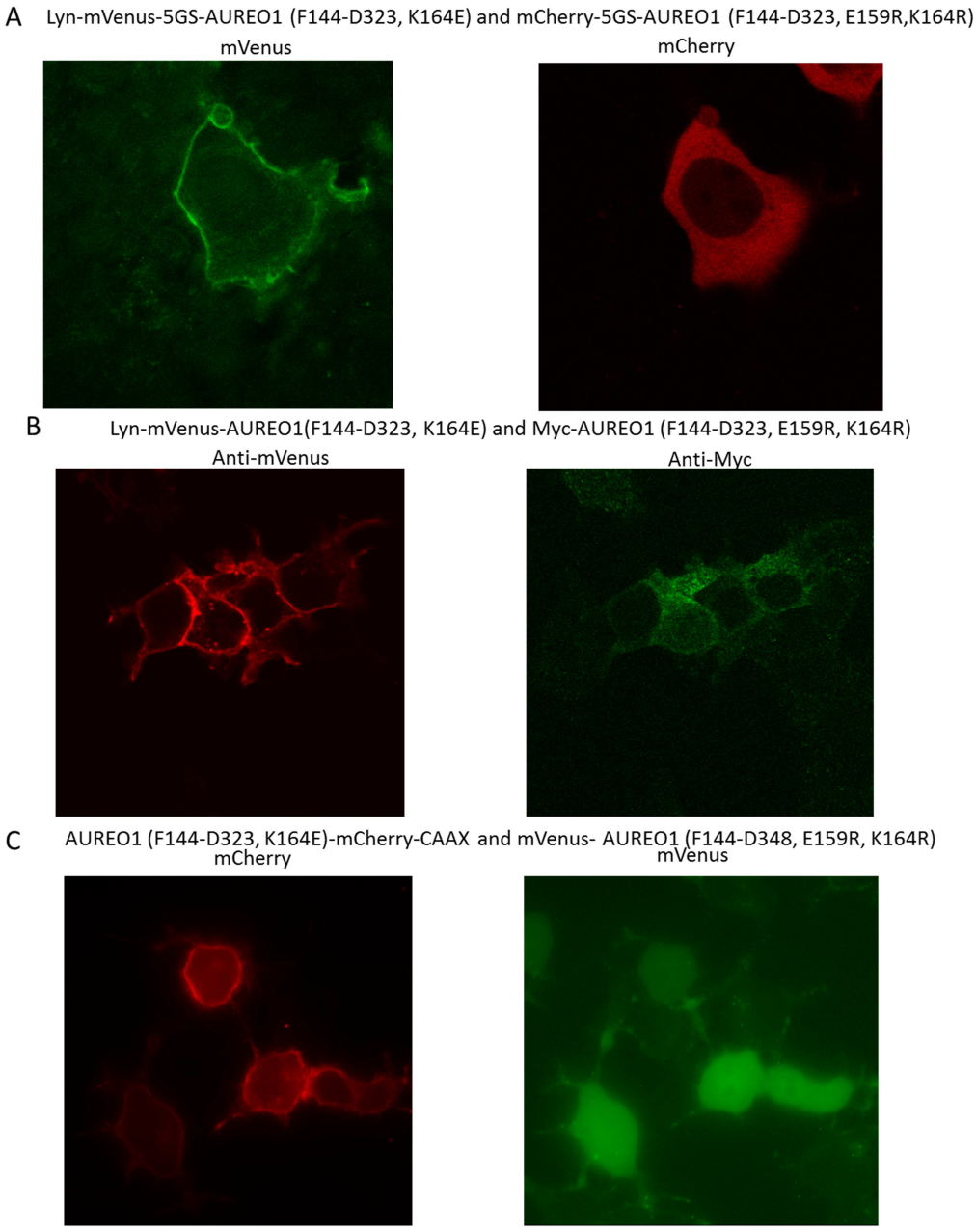

Figure 9 Optimization of the membrane translocation system.
A, co-expression of Lyn-mVenus-5GS-AUREO1 (F144-D323, K164E) and mCherry-5GS-AUREO1 (F144-D323, E159R,K164R). Left panel: mVenus channel; right panel: mCherry channel. B, co-expression of Lyn-mVenus-AUREO1(F144-D323, K164E) and Myc-AUREO1 (F144-D323, E159R, K164R). Left panel: immunoblotting by anti-mVenus; right panel: immunoblotting by anti-Myc. C. co-expression of AUREO1 (F144-D323, K164E)-mCherry-CAAX and mVenus- AUREO1 (F144-D348, E159R, K164R). Left panel: mCherry channel; right panel: mVenus channel. All the images were performed with confocal microscopy.

There are several possibile explanations for the contradictory results in the co-IP experiments and live cell imaging. Firstly, the bulky fluorescent proteins may inhibit or weaken the dimerization of the AUREO1. We introduced a series of GS linkers between the fluorescent proteins and AUREO1 to increase the flexibility between the fluorescent protein and the leucine zipper domain. However, even with co-expression of lit state mutants, there was no membrane translocation in living cells. Figure 9A shows the co-expression of Lyn-mVenus-5GS-AUREO1 (F144-D323, K164E) and mCherry-5GS-AUREO1 (F144-D323, E159R, K164R) which have a GSGSGSGSGS (5GS) linker between the fluorescent proteins and AUREO1. Other pairs with 1GS, 2GS, 3GS or 4GS linker did not show membrane translocation either (data not shown).

In addition, we deleted the fluorescent proteins and studied the membrane translocation with immunostaining. We co-expressed membrane tagged Lyn-mVenus-AUREO1 (F144-D323, K164E) and Myc-AUREO1 (F144-D323, E159R, K164R), which were the same constructs used in the co-IP experiments except that they had the additional membrane localization tag. We fixed the cells and immunostained with anti-mVenus and anti-Myc and imaged with confocal microscopy. As showed in Figure 9B, the construct Lyn-mVenus-AUREO1 (F144-D323, K164E) was localized on the membrane clearly. However, the co-expressed Myc-AUREO1 (F144-D323, E159R, K164R) was not recruited to the membrane but remained in the cytosol homogenously, indicating that these two constructs did not dimerize in cells.

Thirdly, the N-terminus membrane localization signal might influence the dimerization of AUREO1 in live cells. We replaced it with a CAAX box which is a C-terminus membrane localization sequence and tested with a similar membrane translocation

system as shown in Figure 2.5. We co-expressed AUREO1 (F144-D323, K164E)-mCherry-CAAX and mVenus-AUREO1 (F144-D348, E159R, K164R) in HEK293 cells and investigated with confocal microscopy. As showed in Figure 9C, the protein with CAAX (membrane localization signal) localized on the membrane clearly. However, mVenus-AUREO1 (F144-D323, E159R, K164R) was not recruited to the membrane and stayed in the cytosol evenly, suggesting that there was no dimerization between these two co-expressed constructs.

Although we tried different ways to optimize the translocation system, including different activation methods, various linkers, different membrane tags and orientations etc, we did not detect membrane translocation in living cells. It could be possible that the dimerization of AUREO1 is different when it is anchored to the membrane. Our results could also be due to the sensitivity of the microscopy detection. For example, if there is only a small portion of the AUREO1 dimerizing, we may not detect the accumulation of AUREO1 on the membrane. Therefore, we propose several strategies to further investigate the dimerization of AUREO1 in living cells as we will discuss in Chapter 2.4.

2.4 Future directions

In this study, we investigated the light regulated dimerization of AUREO1 with co-IP experiments and successfully engineered AUREO1 to heterodimerize by introducing salt bridges. We further tested the dimerization of AUREO1 in live cells with membrane translocation systems. Although we tried multiple ways to activate the dimerization and different combinations of construct, linker or membrane location tag, we detected no light regulated membrane translocation in living cells. There are several possible reasons. For example, it is possible that the dimerization of AUREO1 varies when it is distributed in the cytosol or anchored on the membrane. Besides, the current method we use may not be sensitive enough to detect the change of protein localization. For example, if only a very small portion of the AUREO1 is recruited to the membrane by dimerization, the scope may not be sensitive to detect the subtle change.

In future study, we propose strategies to study the AUREO1 dimerization in living cells. One strategy is the luciferase-based protein complementation assay, which is thought to have the most sensitive and highest dynamic range among protein-protein interaction detection methods in living cells, better than fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET)[21]. As shown in Figure 10A, AUREO1 will be fused with the N-terminal domain of luciferase (NLuc) or the C-terminal domain of luciferase (CLuc) respectively and co-expressed in cells. Before light activation, NLuc and CLuc are separated and do not interact with each other, therefore the luminescence is low and close to background levels. Upon light activation the AUREO1 dimerizes and NLuc and CLuc will be brought close, which leads to the increase of the luminescence

produced by luciferase. Vice versa, the luciferase activity decreases when the AUREO1 dissociates.

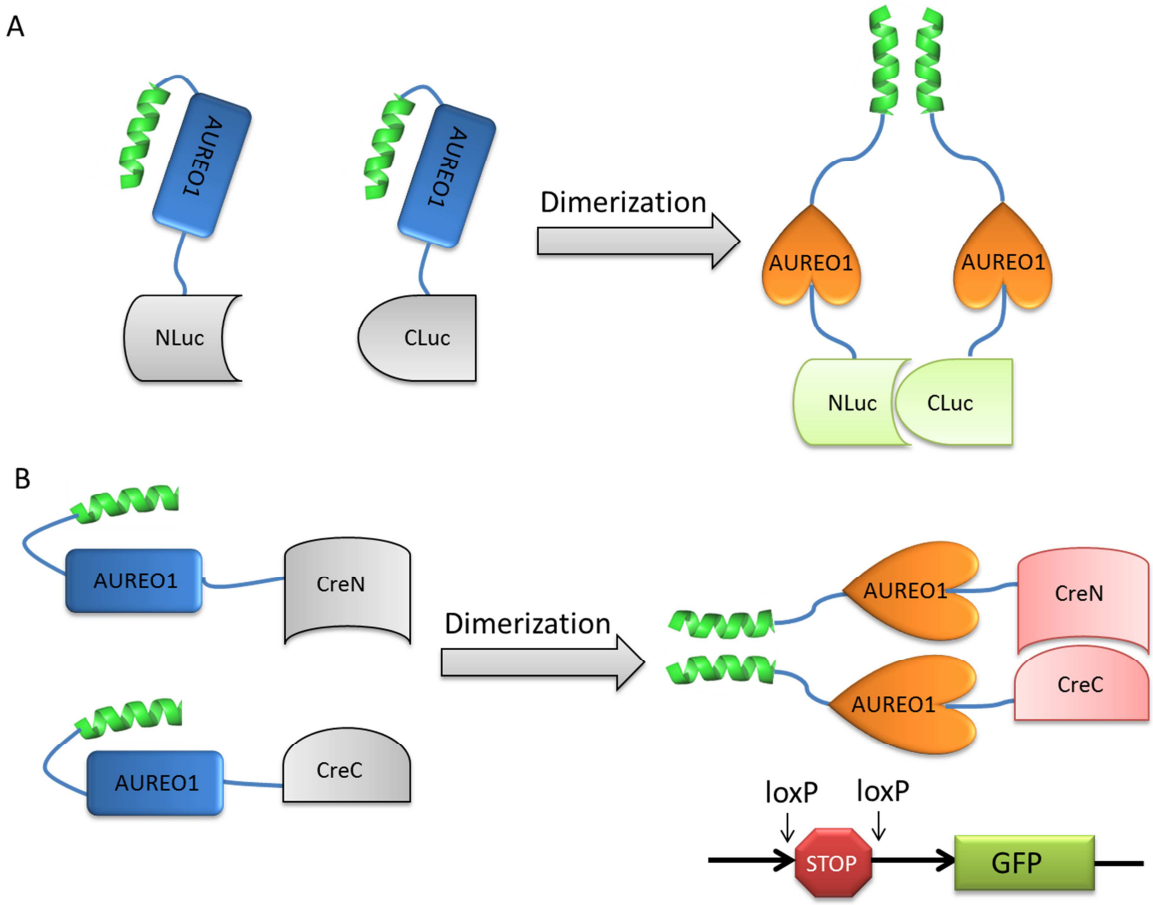

Figure 10 Other systems to investigate the dimerization of AUREO1 in living cells. The leucine zipper domain is shown as green α-helix. A, split luciferase complementation assay. Luciferase is split into N-terminal domain (NLuc) and C-terminal domain (CLuc). B, split Cre-loxP system. The arrow shows the loxP recombination site. The red STOP sign shows the stop codon. Cre protein is split into N-terminal domain (CreN) and C-terminal domain (CreC).

Similarly, we could also use split Cre-loxP system which was initially used in activating gene expression *in vivo* and *in vitro*[22,23]. It could also be used to delete DNA sequences in selected cell types of transgenic animals at high efficiency[24]. It has been shown that the Cre recombinase can be split into two polypeptides (N-Cre and C-Cre, as showed in Figure 10B). Both N-Cre and C-Cre do not have detectable recombination activity until they interact with each other[25,26]. Before light activation, the STOP codon prevents the

downstream GFP from transcription. Upon light activation, AUREO1 dimerizes to recruit CreN and CreC together, which will function normally. The STOP codon will be removed by the loxP site recombination and GFP will start to express, which could be detected by fluorescence microscopy.

With these systems, we could not only test the dimerization of AUREO1 in living cells, but also provides examples of controlling protein-protein interactions and protein activities *in vivo*. This could be further applied to controlling other protein-protein interactions or protein activities with optimization.

CHAPTER 3

CONCLUSIONS

In this study, we successfully characterized the light regulated dimerization of AUREO1. With co-IP experiments, we found that the AUREO1 (F144-K348) containing the leucine zipper and LOV domain is sufficient for the light regulated dimerization of AUREO1. Destroying the leucine zipper interactions with deletions or mutations abolishes the dimerization. Critical site mutation at cysteine decreases the dimerization dramatically. These results suggest that both the leucine zipper and the LOV domain are critical for light regulated dimerization. In addition, deletion of the C-terminus (from D323 to K348) leads to dimerization even in the dark, indicating that an auto-inhibition between the leucine zipper and C-terminus might be involved in the light regulation.

Furthermore, we successfully engineered AUREO1 to convert it from a homodimerizer to heterodimerizer by introducing salt bridges. Based on previous works on leucine zipper engineering and some trials, we found that residues E159 and K164 are critical for dimerization. Introducing R-E salt bridges at these sites leads to heterodimerization but no or very weak homodimerization. These mutants potentially provide a platform for wider applications.

In addition, we investigated the dimerization of AUREO1 in living cells with a membrane translocation system. With AUREO1 anchored on the membrane or even distributed in the cytosol, we expected to see the AUREO1 was recruited to the membrane

upon dimerization. We tagged the constructs with different fluorescent proteins and detected them by confocal microscopy or immunostaining. However, we did not see a membrane translocation with extensive trials, including different ways to active the dimerization, various linkers between AUREO1 and fluorescent proteins or membrane tags, and different membrane tags. In future study, we proposed two ways to investigate the dimerization of AUREO1 in living cells, including split luciferase complementation assay and split Cre-loxP system.

Characterization of the light regulated dimerization of AUREO1 established a possible platform for controlling protein-protein interactions. Engineering heterodimerization greatly widened the potential applications. Further verification of dimerization of AUREO1 in living cells will provide a very important genetically encoded platform that can be activated easily with common light sources. With future improvement to the dynamic range, efficiency and binding affinity by engineering the leucine zipper and LOV domains, we expect these modules to be useful for understanding sophisticated biological questions by precisely controlling protein-protein interactions temporally and spatially with light.

CHAPTER 4

METHODS AND MATERIALS

DNA cloning. The cDNA encoding the AUREO1 of *Vaucheria frigida* was a gift from Dr. Hironao Kataoka[15]. AUREO1 was cloned into pTriEX vectors with His-Myc or Flag-mVenus. Lyn membrane tag was fused with AUREO1 by extension PCR. Site specific mutations were introduced by overlap extension PCR[27]. Fluorescent proteins mVenus, mCherry and mKO2 were inserted with a short GS linker to monitor expression and subcellular localization. Phusion High-fidelity DNA polymerase (NEB) was used in PCR reactions and all plasmids were verified by DNA sequencing.

Cell culture. HEK293 cells were maintained in DMEM containing 10% FBS following the supplier's culturing instructions. Cells were transfected transiently for 16-20 hours using FuGENE 6 (Roche) for co-IP assay or imaging.

Co-IP assay. The co-IP assay were adopted from a previous work form our lab[7]. Flag-mVenus tagged AUREO1 and His-Myc tagged AUREO1 constructs were co-expressed in HEK293 cells by transient transfection using FuGENE 6 (Roche). The cells were lysed in 50mM Tris pH 7.5, 150 nM NaCl and 1% Trition X-100 (lysis buffer) with addition of EDTA-free protease inhibitor cocktail (Roche) for 10 minutes at 4 ℃. After centrifugation for 2 minutes at 4 ℃, the supernatants were incubated with Flag/M2-agarose (Sigma) at 4 ℃ for 1 hour. The supernatants was washed with lysis buffer for 3 times and eluted with lysis

buffer containing 200μg/ml 3X Flag peptide (Sigma). All procedures were done at 4 °C under red light (in the dark) or ambient light, facilitated by Handee spin columns (Pierce). The purified protein complexes as well as cell lysates were fractionated on 4-12% NuPAGE pre-cast gels (Invitrogen) followed by western blot analysis using antibodies against mVenus (JL-8, Clontech, 1:5000 dilution) and Myc (9E10, Sigma, 1:1000 dilution).

Live cell imaging. Cells for live cell imaging were seeded on coverslips coated with 5 μg/ml fibronectin in Ham's F-12K medium free of Phenol red and containing 2% FBS. Coverslips were mounted in an Attofluor live cell chamber (Invitrogen) placed on a microscope stage with a heated stage adaptor (Warner).

Immunostaining. Cells were washed with media without serum and 2X PBS and then blocked with PBS containing 1% BSA for 15 minutes and followed by 2X washes with PBS. Cells were fixed in PBS containing 4% paraformaldehyde (PFA) for 20 minutes and washed for 3 times with PBS. Then the cells were permeabilized with 0.2% Trition X-100 in PBS for 10 minutes and washed for 3 times with PBS. Fixed and permeabilized cells were incubated with primary antibody dilution in PBS-BSA for 1 hour at room temperature or 4 °C overnight and then washed for 2 times with PBS. Then the cells were incubated with secondary antibody dilution containing a fluorescent label for 30 minutes in the dark and washed with PBS for 3 times.

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