Bimolecular Fluorescence Complementation Analysis of Inducible Protein Interactions: Effects of Factors Affecting Protein Folding on Fluorescent Protein Fragment Association

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Supporting Results and Discussion

Use of the N-terminal fragment of Venus in BiFC analysis

BiFC complexes formed by interaction partners fused to fluorescent protein fragments produce higher fluorescence intensities when one of the proteins is fused to the N-terminal fragment of Venus (VN) compared to fusions to the corresponding YFP fragment (YN) (Fig. 1) [1-3]. In some cases, the enhanced fluorescence intensity can be essential for detection of the protein interaction without overexpression of the interaction partners. In several studies, mutations that disrupt the interaction have been shown to reduce the fluorescence intensities of BiFC complexes formed by the N-terminal fragment of Venus [1; 3-7]. Consistent with these studies, we find that rapamycin treatment enhances BiFC complex formation by the N-terminal fragment of Venus fused to FKBP with FRB fused to YC or CC. Likewise, the N-terminal fragment of Venus fused to several other proteins produces higher fluorescence intensities when the proteins are expressed together with cognate interaction partners fused to YC than when they are expressed with fusions to non-cognate partner fusions. However, when fused to FKBP, the N-terminal fragment of Venus produces higher constitutive BiFC complex formation with FRB-YC relative to the level of inducible BiFC complex formation than the N-terminal fragment of YFP.

Effects of FK506 treatment on transcription activation by FKBP-FRB interaction

FK506 can inhibit rapamycin induction of FKBP and FRB association in cells [8-10]. In some experiments, FK506 can also disrupt pre-formed FKBP-FRB complexes [10]. To investigate the effects of FK506 treatment on FKBP-FRB association independent of BiFC complex formation, we determined the effects of FK506 on rapamycin-inducible transcription activation by FKBP-GAL4 DNA binding domain and FRB-VP16 activation domain fusion proteins using a

reporter gene controlled by a promoter containing GAL4 binding sites [11; 12]. Treatment of cells with FK506 prior to rapamycin induction blocked reporter gene activation (Fig. S5B). However, treatment of cells with FK506 after rapamycin induction of FKBP-FRB association did not attenuate the subsequent increase in reporter mRNA accumulation. Replacement of the medium containing rapamycin with medium lacking rapamycin in combination with or without the addition of FK506 also had little effect on reporter gene transcription. Thus, FK506 treatment subsequent to rapamycin induction of transcription activation does not prevent continued transcription of the reporter at an elevated level. It is therefore possible that FK506 treatment does not disrupt preformed FKBP-FRB complexes in cells regardless of BiFC complex formation. It is also possible that disruption of FKBP-FRB complexes does not restore reporter gene transcription to the level prior to stimulation.

Effects of inhibitors of protein synthesis and folding on transcription activation by FKBP-FRB interaction

The effects of inhibitors or protein synthesis and folding on BiFC complex formation by FKBP and FRB fusions could be due to their effects of FKBP-FRB interaction or on association of the fluorescent protein fragments. To determine the effects of these agents on FKBP-FRB association independent of BiFC complex formation, we measured their effects on rapamycin-inducible transcription activation by FKBP-GAL4(DBD) and FRB-VP16(AD) fusion proteins using a reporter gene controlled by a promoter containing GAL4 binding sites [11; 12]. Anisomycin, tunicamycin or thapsigargin treatment had little effect on rapamycin-inducible transcription (Fig. S5B). Cycloheximide pre-treatment blocked rapamycin induction of reporter gene transcription. Since all of the protein synthesis or protein folding inhibitors did not block rapamycin induction of reporter gene transcription, their effects on BiFC complex formation

were not due to inhibition of FKBP-FRB interaction. The suppression of reporter gene transcription by cycloheximide could be due to inhibition of FKBP-FRB interaction, but could also be due to other effects of cycloheximide on transcriptional activity. These results support the interpretation that inhibitors of protein synthesis and folding attenuate BiFC complex formation by interfering with fluorescent protein fragment association.

Supporting Materials and Methods

Plasmid construction: The sequences encoding amino acids residues 1–154 of the enhanced yellow fluorescent protein (YN), the enhanced cyan fluorescent protein (CN), residues 1–155 of the Venus fluorescent protein (VN), or a chimera containing residues 1–155 of the Venus fluorescent protein with the exception for M153 from YFP (VyN) [2; 13] were fused to the C-terminal end of FKBP12 using a TRGSRSIAT linker [14]. The sequences encoding amino acids 155-238 of the enhanced yellow fluorescent protein (YC) or the enhanced cyan fluorescent protein (CC) were fused to the C-terminal end of FRB using a TSGTAAARPACKIPNDLKQKVMNH linker [14]. The FKBP fusions contained a FLAG epitope and a nuclear localization signal and the FRB fusions contained an N-terminal HA epitope and nuclear localization signal [14]. The coding sequences were cloned downsteam of the constitutive CMV promoter in the pBiFC vector [15]. The sequence encoding the FKBP-YN fusion was cloned downstream of the doxycycline-regulated promoter in plasmid pUHrTG2-1 [16] to control the timing of its expression.

The plasmids encoding Max and the Myc bHLH-ZIP domain as well as the leucine zipper deletion derivative bMycΔZIP fused to the YFP fragments (YC and YN) were described previously [17]. The plasmids encoding the fusions to the Venus fragment (VN) were constructed by replacing the sequences encoding the YN fragment with those encoding the corresponding VN fragment.

Cells, antibodies and chemicals. COS-1 cells were cultured as recommended by the American Type Culture Collection. Fugene 6 was obtained from Roche and used for transfections in accordance with the manufacturer's recommendations. Polyclonal anti-GFP was obtained from Fitzgerald (Cat # 20R-6R011). Rapamycin (R-1018) and FK506 (F-1030) were obtained from

A.G. Scientific, Inc. AP21967 was a generous gift from Ariad. Cycloheximide (C1988), anisomycin (A9789), puromycin (P7255), and doxycycline (D9891), tunicamycin (T7765), thapsigargin (T9033), and geldanamycin (G3381) were obtained from Sigma Aldrich.

Microscopy imaging of bimolecular fluorescence complementation. COS-1 cells were transfected with plasmids encoding the indicated combinations of fusion proteins together with a plasmid encoding a CFP internal control fusion using Fugene-6 and incubated at 37°C for 24 hours. The cells were treated as indicated in each figure legend and either fixed using 3.7% paraformaldehyde in PBS for 10 minutes at 4°C or analyzed live. The fluorescence was imaged using an inverted Nikon TE300 fluorescence microscope as described [14]. Time lapse images were captured and the fluorescence intensities of the cells were measured using SimplePCI software (C-Imaging).

Flow cytometry quantitation of bimolecular fluorescence complementation. COS-1 cells were transfected with plasmids encoding the indicated combination of fusion proteins together with a plasmid encoding CFP using Fugene-6 and incubated at 37°C for 24 hours. The cells were treated as indicated in each figure legend, washed with PBS and incubated in 0.15M KCl and 0.015 M Na-citrate in PBS for 5 minutes at 37 °C, fixed using 3.7% paraformaldehyde in PBS for 10 minutes at 4 °C, collected and re-suspended in 250μL of 0.8% BSA in PBS. The fluorescence intensities were measured using a Beckton Dickinson Biosciences FACS Aria cell sorting system with 488 nm excitation of BiFC or YFP and 407 nm excitation of CFP fluoresence. A minimum of 10,000 cells were analyzed for each combination of proteins analyzed in each experiment. In most experiments cells were analyzed by flow cytometry on the day of collection, and in all cases were analyzed within 24 hours of collection. The spectrum of

BiFC complexes formed by YFP fragments is indistinguishable from that of intact YFP [14; 15], indicating that the BiFC signal can be quantified using instrument parameters optimized for YFP fluorescence.

To analyze the fluorescence intensities of transfected cells, threshold values were set for BiFC and CFP fluorescence that excluded 99% of non-transfected cells. The cross-talk between BiFC and CFP fluorescence signals was corrected for by using compensation factors that were set using cells that expressed YFP and CFP separately. The mean fluorescence intensities for the population of cells that exceeded either the BiFC or CFP threshold were calculated. In some experiments (Fig. 6, Supp. Fig. 4), the mean BiFC fluorescence was shown without normalization to avoid potential effects of the treatments on the normalized ratio.

Effects of small molecule inhibitors on BiFC complex formation. COS-1 cells were cultured with various small molecule inhibitors as indicated in each figure. During pre-treatments, the medium was replaced every 24 hr with fresh medium containing the small molecule inhibitors indicated. To ensure complete removal of small molecule inhibitors after pretreatment, the trypsinized cells were washed twice with PBS and once with growth medium before re-plating in fresh medium. The pre-treated cells were cultured for the times indicated before analysis of the effect of rapamycin on BiFC complex formation.

To examine potential effects of small molecule inhibitors on FKBP-FRB intereactions, COS-1 cells were transfected with the pBJ5-FRB-VP16 [12], pBJ5-Gal4-FKBP [11] expression, and pGal4-LUC reporter (provided by Jason Gestwicki) plasmids. The cells were treated using the same conditions as used for BiFC analysis. RNA was isolated using Qiagen total RNAeasy isolation kits and Qiashredder columns. 2 μg of the isolated RNA was reverse transcribed using

Promega reverse transcriptase (A3500) as recommended by the manufacturer. The resulting cDNA was diluted 1:10, and 3 μl was analyzed by quantitative PCR using primers specific for the luciferase reporter (TTCCACCTGTGCCACGACTACTTC and ACTGCCGCCCACTCAAAATAACTT) and normalized using primers specific for renilla luciferase (GCCTCGTGAAATCCCGTTAG and CCATTTCATCAGGTGCATCTTC).

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Supporting Figure Legends

Figure S1. Levels of fusion protein expression and fluorescence intensities of cells that express intact fluorescent proteins or constitutive BiFC complexes in the absence and presence of rapamycin. (A) COS-1 cells that expressed FKBP-YN and FRB-YC were cultured at 37 ^{o}C or 30 ^{o}C. The cells were harvested at the times indicated and the cell extracts were analyzed by immunoblotting using anti-GFP antibody. **(B)** COS-1 cells were treated and analyzed as described in part (A) with the addition of 100 nM rapamycin at time 0. **(C)** COS-1 cells that expressed the protein(s) indicated were treated with 100 nM rapamycin at time 0 (solid symbols) or left untreated (open symbols). The cells were fixed at the times indicated and analyzed by flow cytometry. The data represent the mean BiFC fluorescence intensities of cells normalized by the fluorescence produced by a co-expressed CFP fusion protein.

Figure S2. Time-course of fluorescence complementation by FKBP and FRB fused to fragments of CFP and effects of the amount of plasmid transfected on inducible and constitutive BiFC complex formation. (A) COS-1 cells that expressed FKBP-CN and FRB-CC were treated with 100 nM rapamycin at time 0. The cells were fixed at the times indicated and were imaged by microscopy. The images show CN-CC fluorescence and were captured using the same acquisition parameters. **(B)** Cells that expressed the indicated combinations of FKBP-YN and FRB-YC or FRB-CC were treated with 50 nM rapamycin at time 0, fixed at the indicated times, and analyzed by flow cytometry. The data represent the mean BiFC fluorescence intensities of cells normalized to the CFP fluorescence produced by a co-expressed internal control protein. **(C)** Cells that expressed FKBP-VN and FRB-YC or FRB-CC were treated and analyzed as described in part B. **(D)** Effects of the amounts of plasmids encoding

FKBP-YN, -VN or -VyN transfected on constitutive and inducible BiFC complex formation with FRB-YC. The indicated amounts of plasmids encoding FKBP-YN, -VN or -VyN were transfected with 500 ng of plasmid encoding FRB-YC and the cells were treated with 100 nM rapamycin for 3 hours. The cells were fixed and analyzed by flow cytometry. The graph shows the mean ratio of BiFC to CFP fluorescence as a function of the amounts of plasmids encoding FKBP-YN, -VN or -VyN transfected into the cells.

Figure S3. Effects of FK506 treatment after BiFC complex formation by the FKBP and FRB fusions on the fluorescence intensities of BiFC complexes formed by different fluorescent protein fragments. (A) Cells that expressed FKBP-YN, FRB-YC and a CFP fusion were cultured 37 ^{o}C and treated with the indicated concentrations of AP21967 or control medium for 3 hours. The cells were washed and transferred into medium containing the same concentration of AP21967, 1 μM FK506 or DMSO vehicle. At the times indicated, the cells were fixed and analyzed by flow cytometry. The ratios of YN-YC to CFP fluorescence were plotted as a function of the time after rapamycin addition. **(B)** Cells were treated and analyzed as described in part (A), except that they were shifted to 30 ^{o}C at the time of AP21967 addition. **(C)** Cells that expressed either FKBP-YN and FRB-YC or FKBP-YN and FRB-CC as indicated were incubated with 25 nM rapamycin or control medium for 4 hours. The cells were washed and transferred into medium containing the same concentration of rapamycin or 1 μM FK506. At the times indicated, the cells were fixed and analyzed by flow cytometry. The ratios of YN-YC or YN-CC to CFP fluorescence were plotted as a function of the time after rapamycin addition. **(D)** Cells that expressed either FKBP-VN and FRB-YC or FKBP-VN and FRB-CC as indicated were treated and analyzed as described in part C.

Figure S4. Effects of inhibitors of protein synthesis on BiFC complex formation by FKBP and FRB fused to different fluorescent protein fragments. (A) Inhibition of FKBP-YN—FRB-CC BiFC complex formation by cycloheximide added prior to rapamycin induction. Cells were incubated in the presence (open symbols) or absence (solid symbols) of 50 μg/ml cycloheximide for the indicated times before the addition of 100 nM rapamycin at time 0. The cells were fixed at the times indicated, analyzed by flow cytometry and the ratio of YN-CC to CFP fluorescence was plotted as a function of time after rapamycin addition. The data represent the mean and standard deviation of two independent experiments. **(B)** Inhibition of FKBP-VN—FRB-YC and FKBP-VN—FRB-CC BiFC complex formation by cycloheximide added before rapamycin induction. Cells were incubated in the presence or absence of 50 μg/ml cycloheximide as indicated before the addition of 100 nM rapamycin at time 0. The cells were fixed at the times indicated, analyzed by flow cytometry and the ratios of VN-YC or VN-CC to CFP fluorescence were plotted as a function of time after rapamycin addition. **(C)** Effects of cycloheximide on the fluorescence intensities of cells that expressed JunD-YFP or Venus-CBX2 fusion proteins [7]. The cells were treated and analyzed as described in part B. **(D)** Inhibition of FKBP-YN—FRB-YC and FKBP-VN—FRB-YC BiFC complex formation by puromycin added before rapamycin induction. Cells were incubated in the presence or absence of 25 μg/ml puromycin as indicated prior to the addition of rapamycin at time 0. The cells were fixed at the times indicated, analyzed by flow cytometry, and the ratio of BiFC to CFP fluorescence was plotted as a function of the time after rapamycin addition. **(E)** Rates of onset of anisomycin inhibition of BiFC complex formation. Cells that expressed FKBP-YN and FRB-YC were incubated in the presence (open symbols) or absence (solid symbols) of 25 μg/ml anisomycin for

the times indicated before addition of rapamycin at time 0. The cells were fixed at the indicated times, analyzed by flow cytometry, and the mean ratios of YN-YC to CFP fluorescence intensities were plotted as a function of time after rapamycin addition. The attenuation of BiFC complex formation by a variety of structurally and mechanistically distinct inhibitors of protein synthesis [18-20] suggests that the attenuation was a consequence of the inhibition of protein synthesis.

Figure S5. Effects of FK506 and inhibitors of protein synthesis and folding on the activation of a reporter gene regulated by FKBP-FRB interaction. (A) Cells that were transfected with the pGal4-LUC reporter and pBJ5-FRB-VP16 [12] and pBJ5-Gal4-FKBP [11] expression plasmids together along with a pCMV-renilla internal control were treated with 100 nM rapamycin for 2 hours followed by addition of 1mM FK506 (FK506) or DMSO vehicle and incubation for an additional 2 or 6 hours. Total RNA was isolated from the cells and the levels of firefly and renilla luciferase mRNA were quantified by qPCR. **(B)** Cells that were transfected with the pGal4-LUC reporter and pBJ5-FRB-VP16 [12] and pBJ5-Gal4-FKBP [11] expression plasmids together with a pCMV-renilla internal control were treated with 50 μg/ml cycloheximide (CHX), 25 ug/ml anisomycin (Aniso), 1 μg/ml tunicamycin (TM), 50 ng/ml thapsigargin (TG), 1mM FK506 (FK506), or DMSO vehicle for one hour prior to the addition of 100 nM rapamycin. After 3 hours, total RNA was isolated and the levels of firefly and renilla luciferase mRNA were quantified by qPCR.

Supporting Movie Legends

Supporting Movie 1. Structure of YFP indicating the positions of amino acid substitutions in the VN and VyN fragments. The segments of YFP corresponding to the YN and YC fragments are shown in green and cyan respectively. F46 and F64, which are substituted by L in VN and VyN are shown in orange. M153, which is substituted by T only in VN is shown in red. The tripeptide fluorophore is shown in yellow. Each frame corresponds to a 6 degree (π/30) rotation about the vertical axis.

Supporting Movie 2. Time-lapse imaging of the induction of BiFC complex fluorescence in cells that expressed FKBP-YN and FRB-YC. The acquisition was started immediately after rapamycin addition and an image was acquired every 10 minutes. The total length of the sequence is 240 minutes.

Supporting Movie 3. Time-lapse imaging of the induction of BiFC complex fluorescence in cells that expressed FKBP-VN and FRB-YC. The acquisition was started immediately after rapamycin addition and an image was acquired every 10 minutes. The total length of the sequence is 240 minutes.

Supporting Movie 4. Time-lapse imaging of the induction of BiFC complex fluorescence in cells that expressed FKBP-VyN and FRB-YC. The acquisition was started immediately after rapamycin addition and an image was acquired every 10 minutes. The total length of the sequence is 240 minutes.

Figure S1

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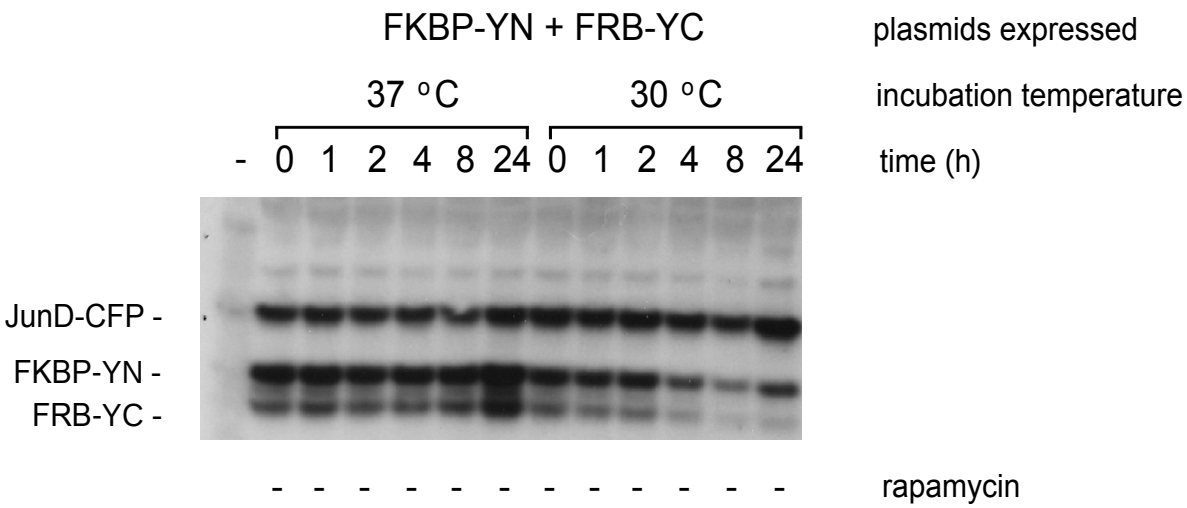

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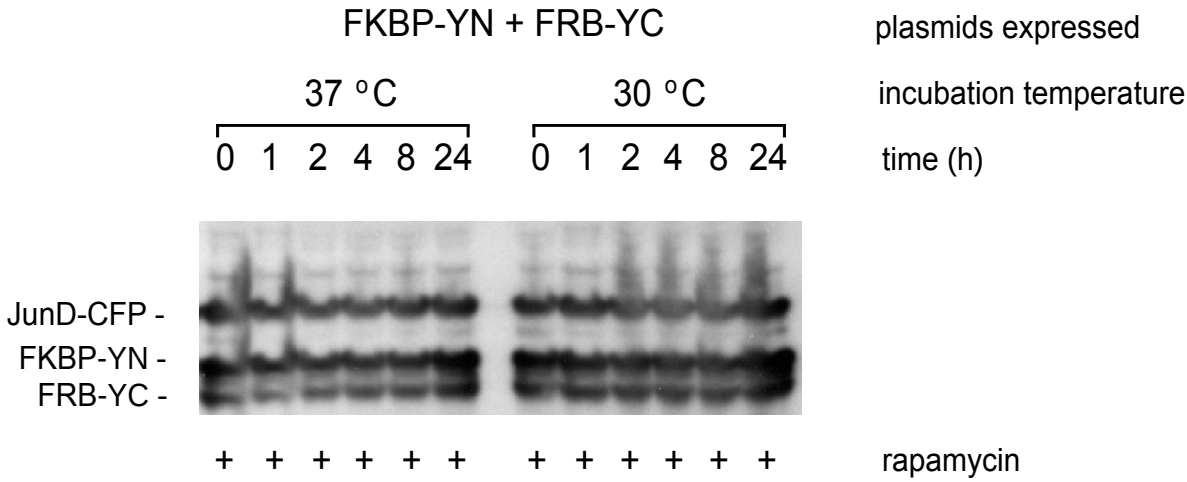

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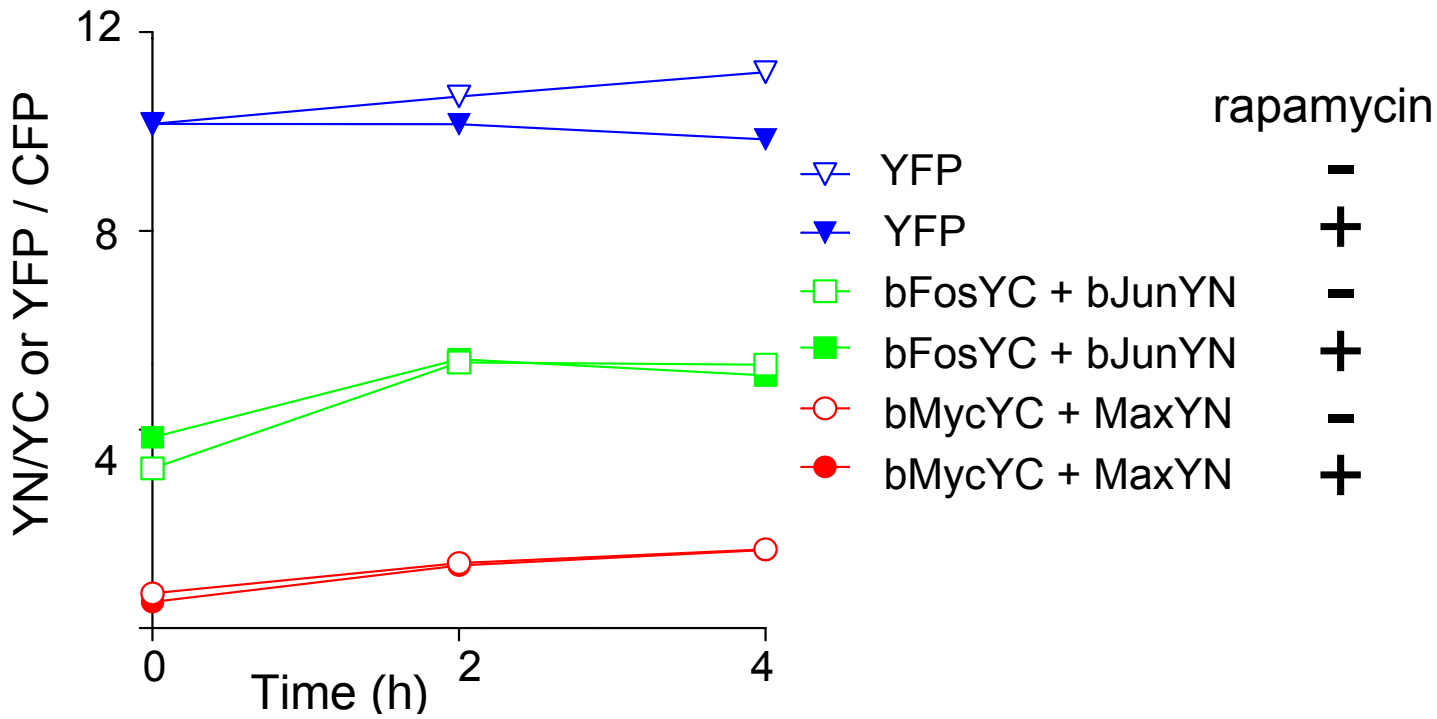

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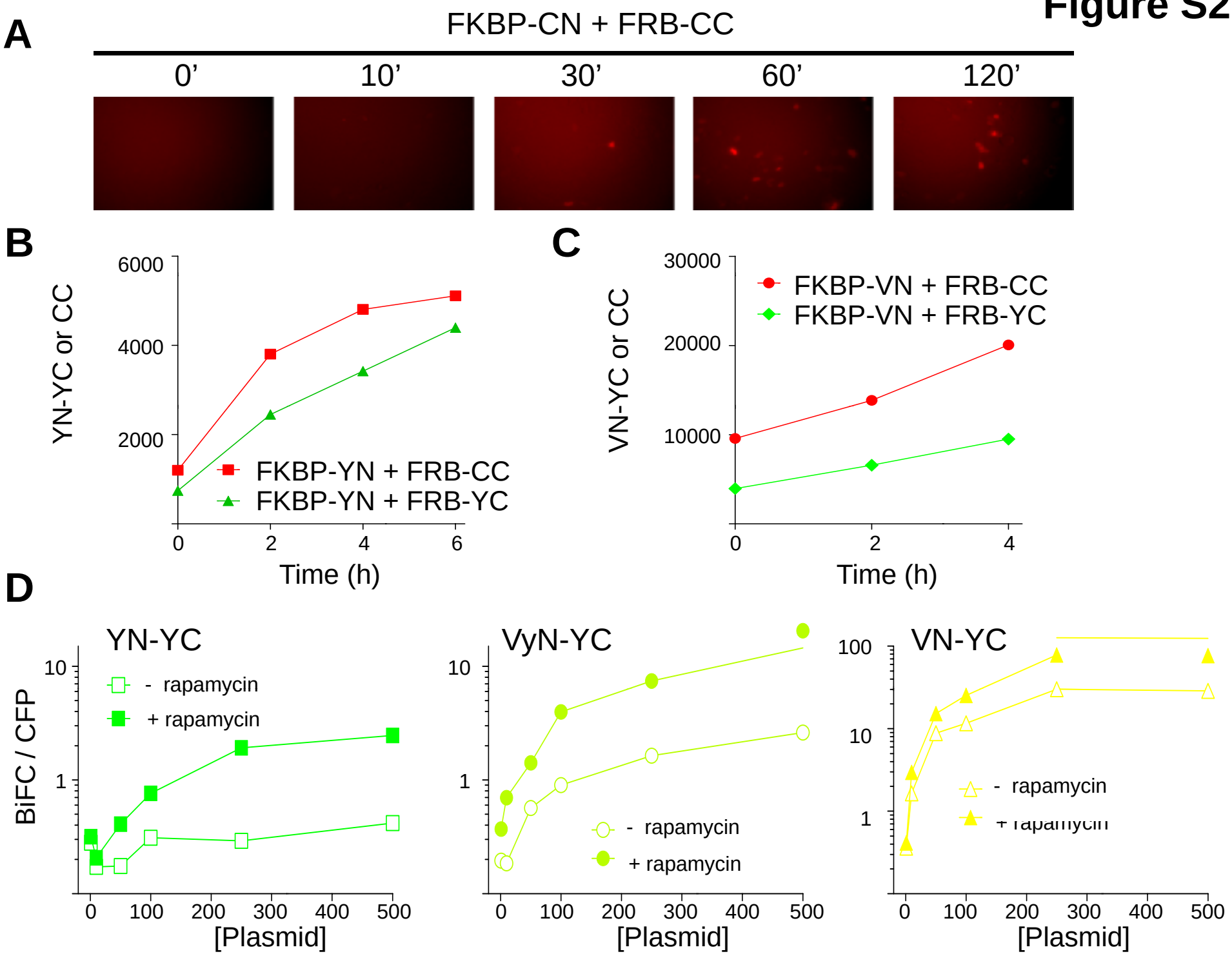
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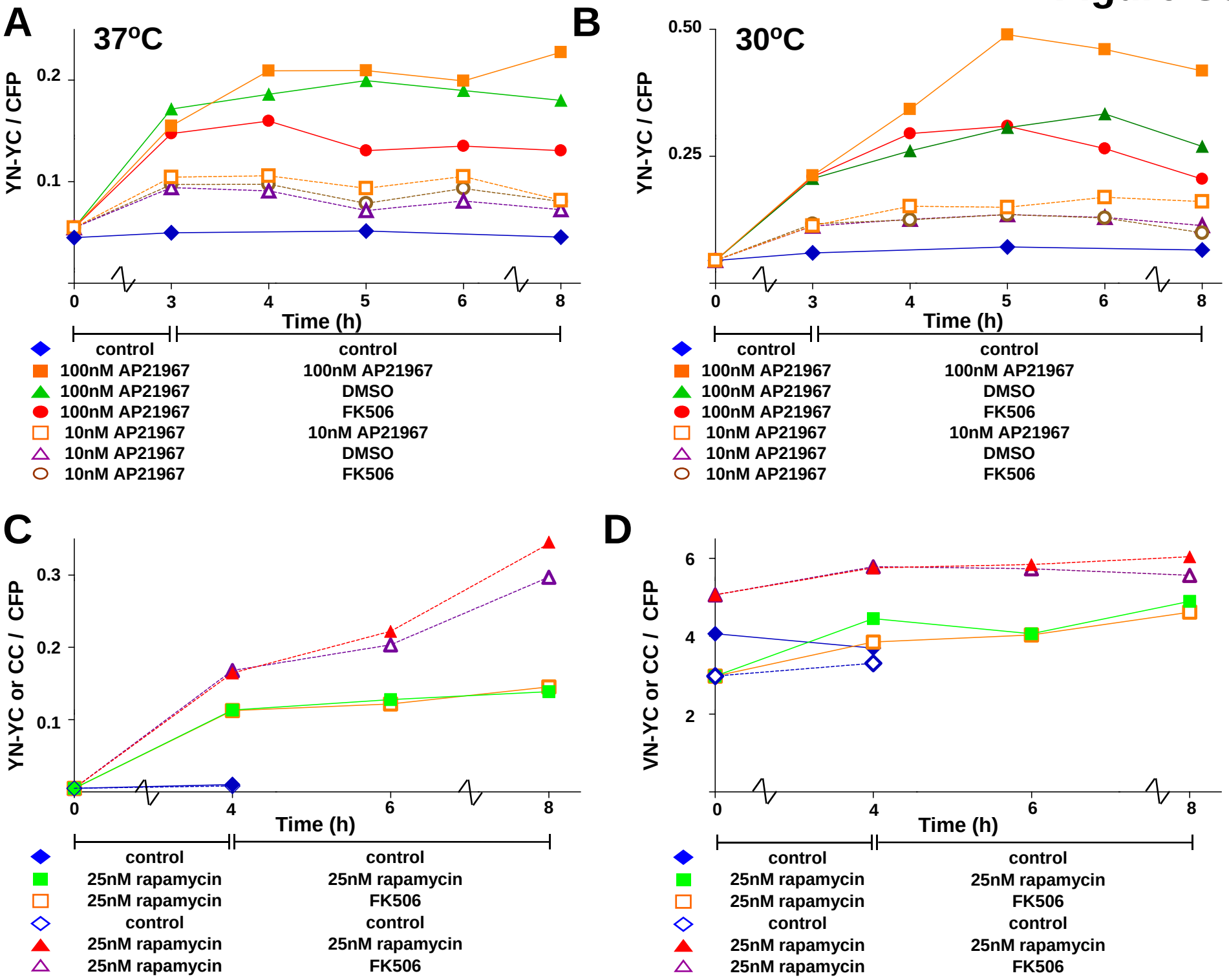
Figure S3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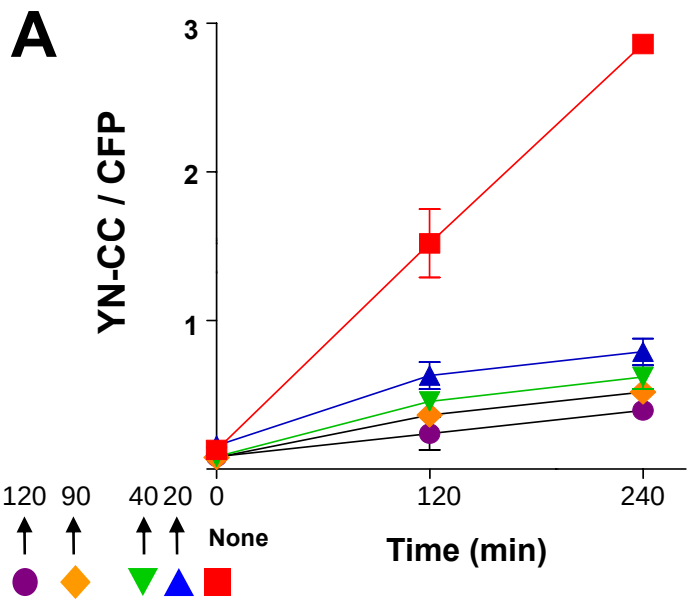
FK506

Figure S4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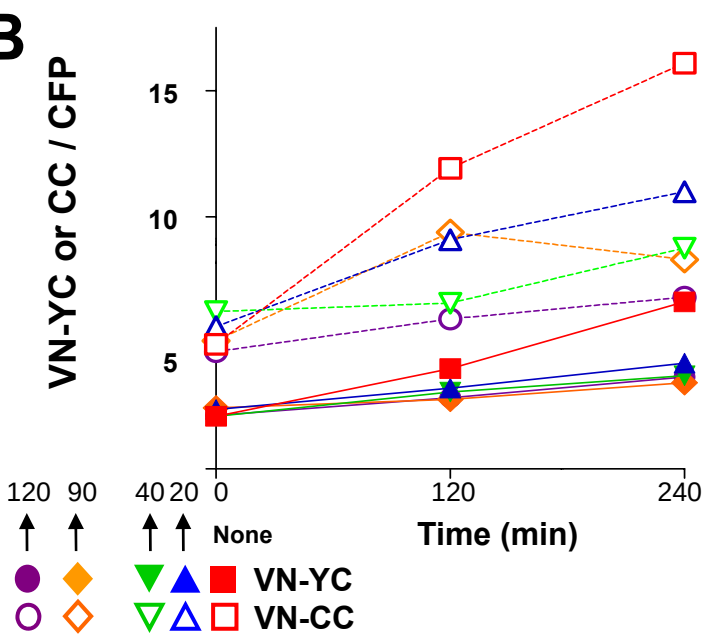
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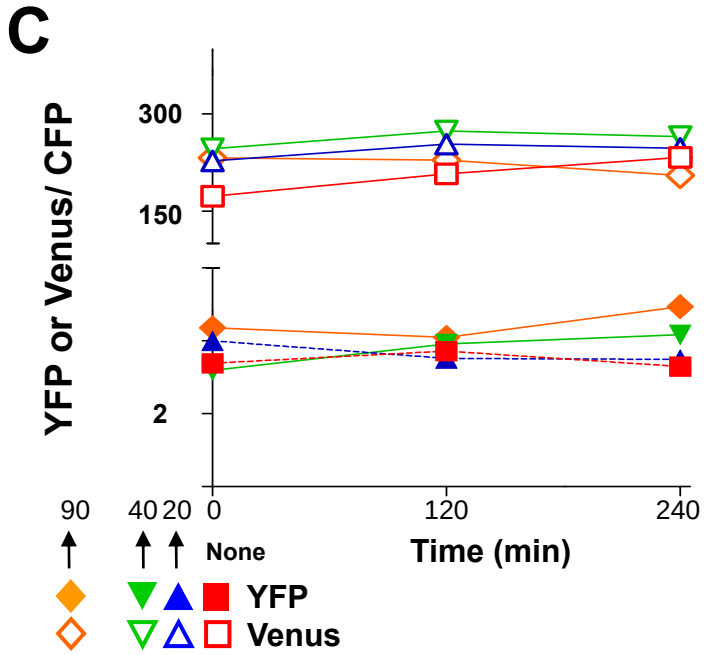
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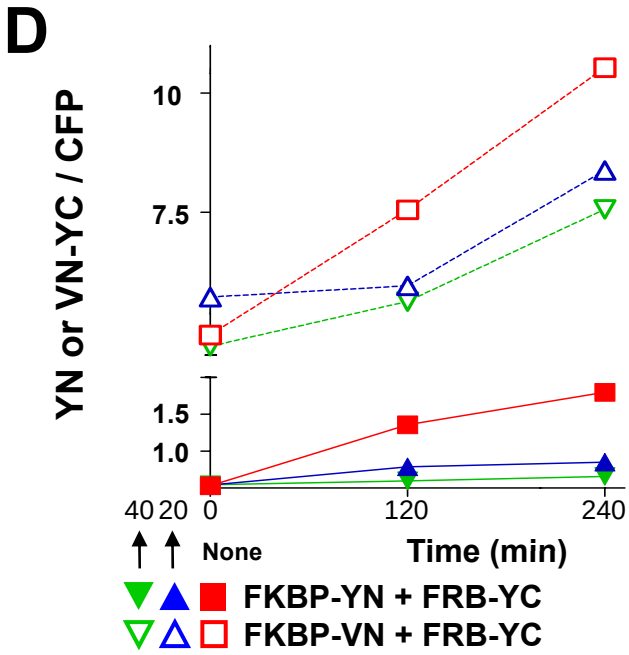
90
40 20
0
120
240
None
Time (min)
YFP
Venus


D
YN or VN-YC / CFP
10
7.5
1.5
1.0
40 20
0
120
240
None
Time (min)
FKBP-YN + FRB-YC
FKBP-VN + FRB-YC

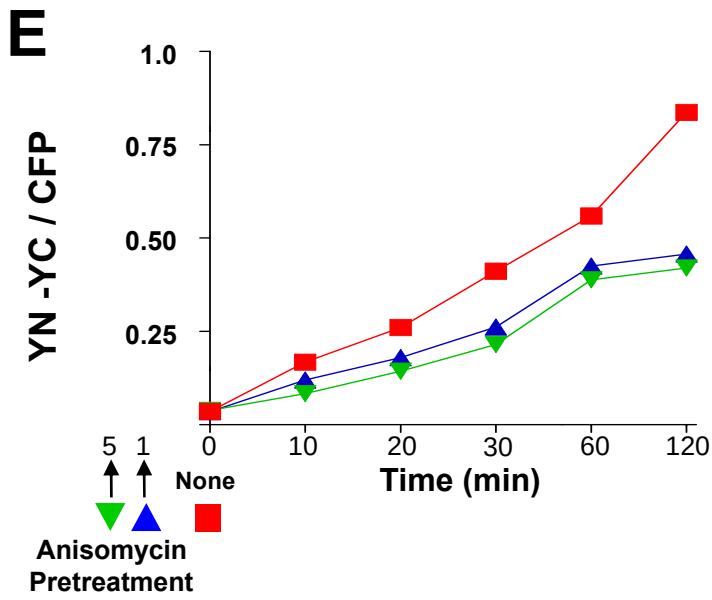
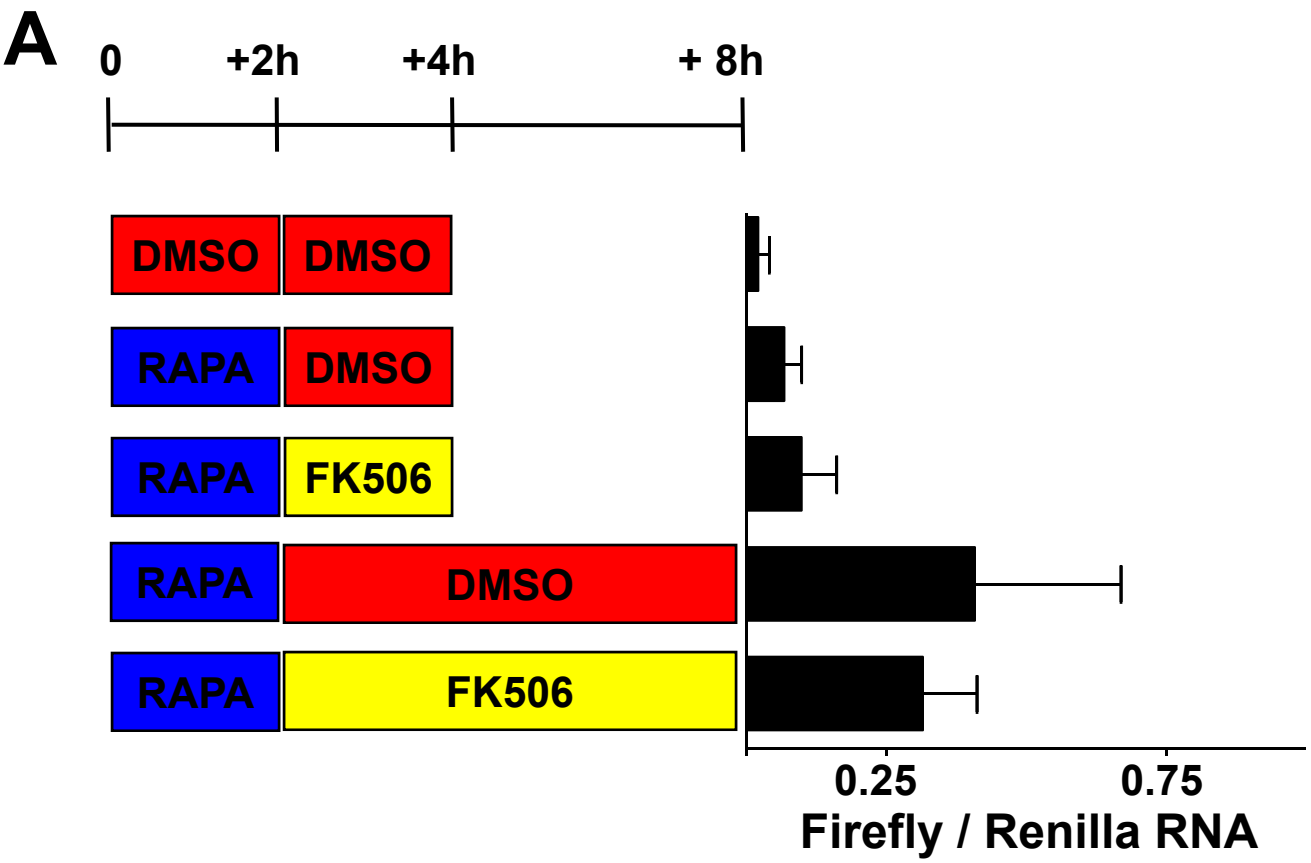
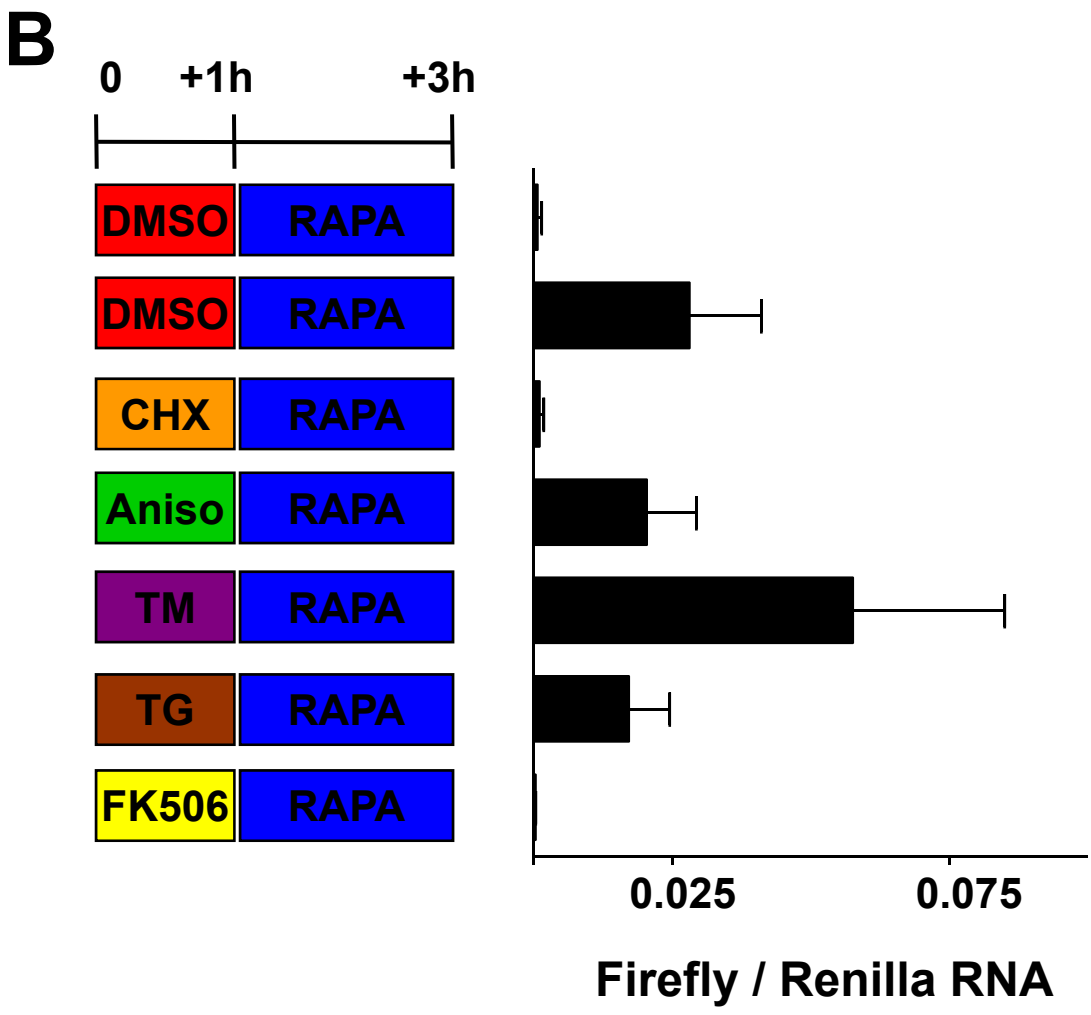

E
YN -YC / CFP
1.0
0.75
0.50
0.25
5 1
0
10
20
30
60
120
None
Time (min)
Anisomycin
Pretreatment

Figure S5


A
0
+2h
+4h
+ 8h
DMSO
DMSO
RAPA
DMSO
RAPA
FK506
RAPA
DMSO
RAPA
FK506
0.25
0.75
Firefly / Renilla RNA


B
0
+1h
+3h
DMSO
RAPA
DMSO
RAPA
CHX
RAPA
Aniso
RAPA
TM
RAPA
TG
RAPA
FK506
RAPA
0.025
0.075
Firefly / Renilla RNA