Developing Mutant FKBP•Rapamycin•FRB Ternary Complexes

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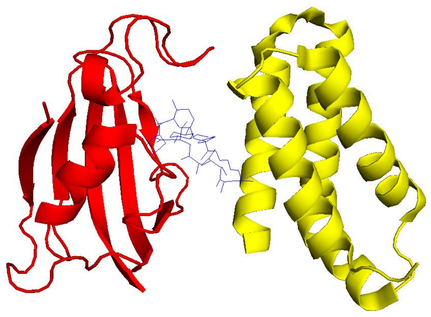
Abstract

Transient protein-protein interactions (PPIs) occur in signaling and metabolic pathways. Studying PPIs will provide insight to these pathways, which is important for developing drugs that target PPIs and disrupt pathways related to disease. The binding affinities of transient PPIs will be quantified by utilizing two new techniques: nano-reaction chambers and super-spatiotemporal resolution microscopy. To validate these new techniques, a series of complexes with various binding affinities and interaction times will be developed.

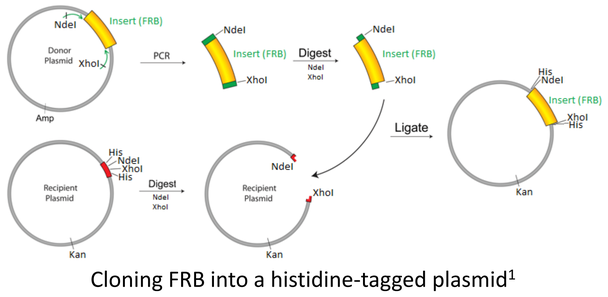
Rapamycin binds to the FK506 binding protein (FKBP) and the mammalian target of rapamycin (mTOR) to form a ternary complex. The part of mTOR that participates in binding to rapamycin is called the FKBP-rapamycin binding domain (FRB). Binding affinities have previously been quantified for all components of the FKBP•rapamycin•FRB ternary complex, with the exception of the protein-protein interaction between FKBP and FRB, which may be too fast for previous techniques to characterize. The rapamycin binding pocket of FRB was modified by site-directed mutagenesis to form six FRB mutants that bind more weakly to and dissociate more quickly from the FKBP•rapamycin complex. The transient interactions between the FRB mutants and the FKBP•rapamycin complex will be characterized at the single-molecule level with new nano-reaction chambers. A consecutive fusion mechanism enables a single mutated ternary complex to form inside a single nano-reaction chamber. Interaction traces will be collected over time to detect the association and dissociation of this complex. The mutated ternary complexes will also be characterized by a new super-spatiotemporal resolution microscope that will provide sub-millisecond temporal resolution of this in vitro system. The characterized mutant complexes will be used as binding affinity markers to validate the kinetic properties of transient PPIs in various biological pathways.

FKBP•Rapamycin•FRB Ternary Complex

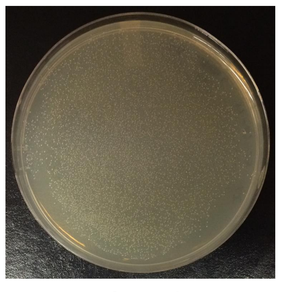


Wild-type FKBP•rapamycin• FRB ternary complex

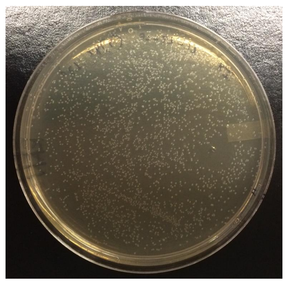
Plasmid Cloning by PCR



Cloning FRB into a histidine-tagged plasmid[1]



Transformed FRB S1081C (donor plasmid)



Transformed pET28b (recipient plasmid)

Site-Directed Mutagenesis

Table 1. EC_{50} of Rapamycin and Four Rapalogs for Frb Variants Annotated According to the Amino Acids at Positions 2095, 2098, and 2101

Mutant Frb	Rapamycin	C20-Marap	C16-BSrap	C16-iRap	AP21967 (C16-AiRap)
KTW	0.45	—[a]	2.7	2.3	—[a]
PLF	0.80	4.5	—[a]	6.1	—[a]
KLW	3.2	52	16	1.2	37
PLW	5.0	30	26	13	26
TLW	2.1	34	7.4	4.6	19
ALW	1.7	34	7.5	4.3	19
PTF	3.3	4.5	—[a]	—[a]	ND
ATF	2.7	15	—[a]	—[a]	ND
TTF	3.0	13	—[a]	—[a]	ND
KLF	0.93	4.5	—[a]	6.1	—[a]
PLF	0.8	4.5	—[a]	6.4	—[a]
TLF	1.6	8.5	—[a]	4.3	—[a]
ALF	1.6	1.0	—[a]	12	—[a]
KTF	1.8	9.3	—[a]	—[a]	—[a]
KHF	1.5	6.5	—[a]	14	ND
KFF	2.2	15	—[a]	—[a]	ND
KLF	0.93	4.5	—[a]	12	—[a]

Values are measured in the rapalog-dependent transcriptional switch and restandardized for transfection efficiency between experiments according to the EC_{50} of rapamycin against KTW or PLF. ND, not determined.
[a]A half-maximal concentration greater than 150 nM that cannot be determined accurately.

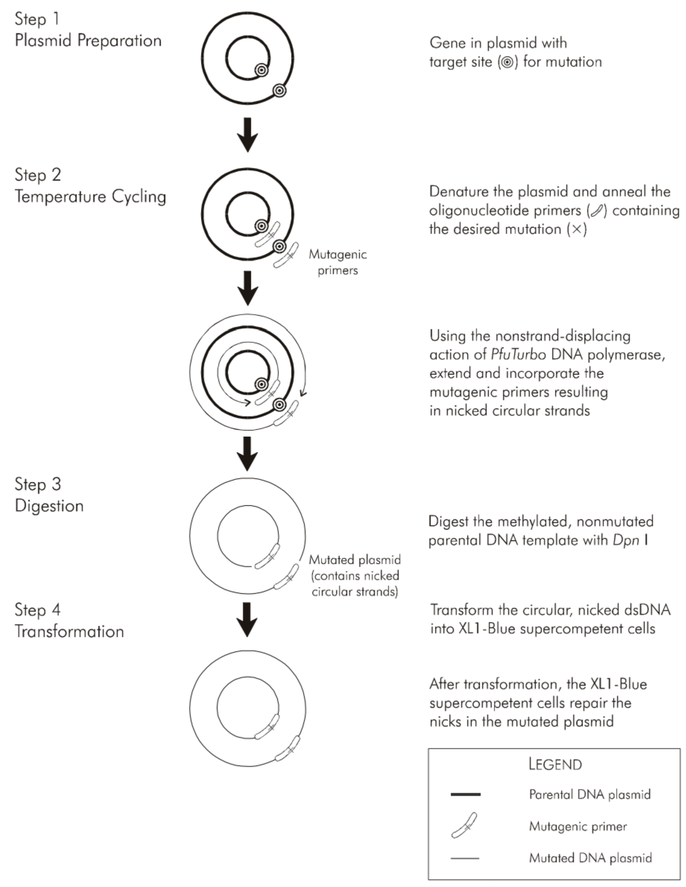
Table used to select the FRB K2095P and T2098L mutations[2]

TABLE I
Statistical data of cell cycle progression upon microinjection of wild-type and mutant FRB proteins

Microinjections of synchronized MG63 cells were carried out as described under "Experimental Procedures" and in legends to Figs. 2 and 4. Three to eight independent experiments were carried out for each injection condition, and 50–200 cells were successfully injected in each experiment. All injected cells were scored for S phase entry by BrdUrd incorporation, and data are presented as the percentage of injected cells in S phase. On average, about 97% cells without injection are in S phase under the same growth conditions.

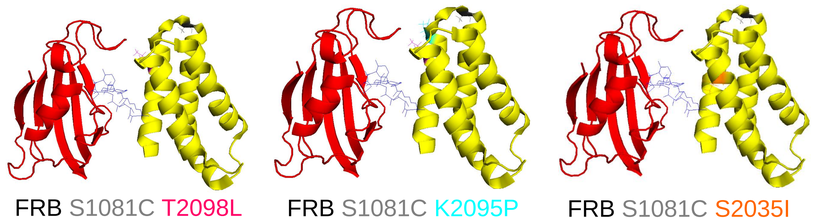
Protein injected	Percentage of cells in S phase
	%
No injection	~97
Dextran	91.0 ± 3.6
IgG	88.7 ± 6.1
Wild-type FRB	16.2 ± 7.2
Ser^{2035} → Ile FRB	22.4 ± 5.0
Tyr^{2105} → Ala FRB	18.8 ± 6.0
Trp^{2027} → Phe FRB	86.1 ± 9.7

Table used to select the FRB S2035I mutation[3]

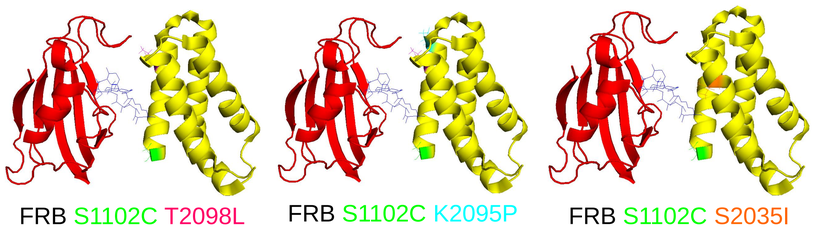


Site-directed mutagenesis protocol[4]

FRB Mutations:

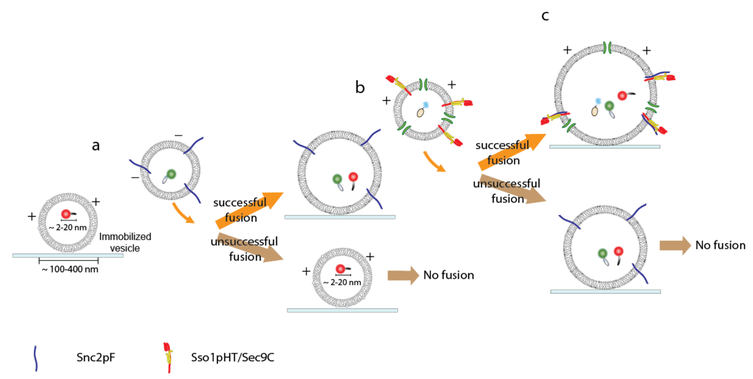


FRB S1081C T2098L | FRB S1081C K2095P T2098L | FRB S1081C S2035I



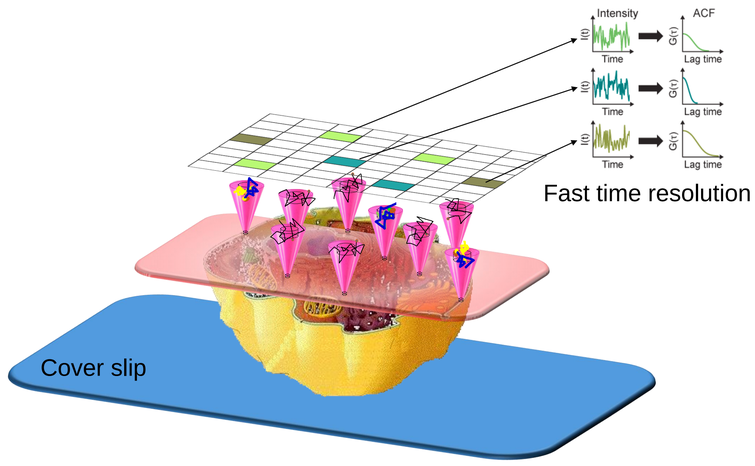
FRB S1102C T2098L | FRB S1102C K2095P T2098L | FRB S1102C S2035I

Smart Nano-Reaction Chambers



Charge-to-charge and SNARE vesicle fusion mechanisms

Interactive Multifocal Correlation Microscope



Fast time resolution

Tracking multi-spatial signalings with super-spatiotemporal resolution in live cells

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- Dr. Marcin Ptaszek

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2. Bayle, J. H.; Grimley, J. S.; Stankunas, K.; Gestwicki, J. E.; Wandless, T. J.; Crabtree, G. R. Rapamycin Analogs with Differential Binding Specificity Permit Orthogonal Control of Protein Activity. *Chemistry & Biology* **2006**, *13*, 99-107.
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4. QuikChange® Site-Directed Mutagenesis Kit Instruction Manual. Stratagene. http://kirschner.med.harvard.edu/files/protocols/Stratagene_quickchangepdf.pdf (accessed Aug 3, 2017).