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A Discrete Loop in SERCA N-Domain Plays a Role in SERCA Headpiece Dynamics and Function

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A DISCRETE LOOP IN SERCA N-DOMAIN
PLAYS A ROLE IN SERCA HEADPIECE
DYNAMICS AND FUNCTION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

OLGA N. RAGUIMOVA

CHICAGO, IL

MAY 2018
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For my daughters, Alina and Alice, two sunshines.
Fools are lucky. We work day and night!

— Vladimir Putin
# TABLE OF CONTENTS

**ACKNOWLEDGMENTS** iii

**TABLE OF CONTENTS** vii

**LIST OF FIGURES** ix

**LIST OF ABBREVIATIONS** xii

**ABSTRACT** xiv

## CHAPTER ONE: INTRODUCTION AND BACKGROUND 1

- The Cardiac Calcium ATPase in the Heart 1
- SERCA in Other Diseases 4
- SERCA Structure and Function 4
- N\(\beta5\)-\(\beta6\) Loop is Predicted to Regulate SERCA Compact Conformation 9
- SERCA Regulation by PLB 10
- SERCA in Regulatory Complexes: HAX-1 and DWORF 13
- 2-color ATPase Transporters: SERCA and MRP-1 15

## CHAPTER TWO: MATERIALS AND METHODS 17

- Molecular Biology and Cell Culture 17
- Fluorescence Microscopy and FRET Quantification 18
- Live Cell Ca\(^{2+}\) Uptake Activity Assay 21
- Measuring Calcium Oscillations by Confocal Microscopy in Live Cells 22
- All-atoms Molecular Dynamics Simulations 23
- Structural Analysis and Visualization 24
- Principal Component Analysis 24
- Domain Orientation Calculation 25
- Root Mean Square Fluctuations Calculations (RMSF) 26
- Root Mean Square Deviation Calculations (RMSD) 26
- AAV-293 Cell Microsomal Membranes Preparations 26
- Ca\(^{2+}\)-ATPase Functional Measurements 27
- Western Blotting 28
- Ligand-induced Stabilization of SERCA Biochemical Intermediates 29
- MRP-1 Functional Measurements 29
- Statistical Analysis 30

## CHAPTER THREE: SERCA STRUCTURAL DYNAMICS DURING CALCIUM TRANSPORT 31

- All-atom Molecular Dynamics Simulations of SERCA Point Mutants 31
- Comparison between Structural Dynamics of WT- and AAA-SERCA 37
- The Role of the N\(\beta5\)-\(\beta6\) Loop in SERCA ATPase Activity 41
- Quantification of FRET of 2-color SERCA Expressed in Microsomal Membranes 42
- SERCA Structural Dynamics in AAV-293 Cells 46
- SERCA Conformational Changes in Response to Intracellular Ca\(^{2+}\) Dynamics 49
<table>
<thead>
<tr>
<th>Chapter Four: Serca Structural Dynamics in Complex with Plb</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-atoms MD Simulations of WT- or AAA-SERCA in the Complex with PLB</td>
<td>56</td>
</tr>
<tr>
<td>Extended MD Simulations of Serca and PLB/PLBpS16 Complexes</td>
<td>58</td>
</tr>
<tr>
<td>Effect of Ser16 Phosphorylation on Serca-PLB Structural Dynamics</td>
<td>64</td>
</tr>
<tr>
<td>Serca-PLB Structural Dynamics Compared to FRET in Live Cells</td>
<td>66</td>
</tr>
<tr>
<td>PLB Structural Dynamics in Complex with Serca</td>
<td>68</td>
</tr>
<tr>
<td>Serca Conformational Changes in Complex with PLB measured by FRET</td>
<td>72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Five: Application of 2-Color FRET Biosensors in Identification of Regulatory Interactions</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serca and Hax-1 Direct Interactions as Assessed by FRET</td>
<td>77</td>
</tr>
<tr>
<td>Analysis of Serca Association with Dworf</td>
<td>79</td>
</tr>
<tr>
<td>Quantification of DOX Uptake by AAV-293 Cells to Assess MRP-1 Transport Activity</td>
<td>82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Six: Discussion</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural Dynamics of WT-Serca During Ca²⁺ Transport</td>
<td>85</td>
</tr>
<tr>
<td>Structural Dynamics of AAA-Serca, the Nβ5-β6 Loop Mutant, During Ca²⁺ Transport</td>
<td>91</td>
</tr>
<tr>
<td>The Nβ5-β6 Loop as a Determinant of Serca Structural Dynamics and Function</td>
<td>92</td>
</tr>
<tr>
<td>Structural Dynamics of Serca-PLB Complex</td>
<td>94</td>
</tr>
<tr>
<td>2-color ATPases as Biosensors for Identification of Regulatory Interactions</td>
<td>96</td>
</tr>
<tr>
<td>Clinical Implications</td>
<td>98</td>
</tr>
<tr>
<td>Limitations of Current Study</td>
<td>100</td>
</tr>
<tr>
<td>Overall Conclusion</td>
<td>104</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>List of References</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>105</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vita</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>118</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic representation of calcium handling in the cardiac myocyte.</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>SERCA structure.</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Simplified schematic overview of SERCA kinetic cycle.</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Acceptor-sensitized FRET.</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Acceptor photobleaching FRET.</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>The diagram of the SERCA domains angular autocorrelation vectors sampling.</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>Schematic representation of the enzyme-linked ATPase assay.</td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>RMSD and RMSF of SERCA structural dynamics calculated from MD simulations.</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>The relative motions of SERCA domains are preserved upon Nβ5-β6 loop mutations.</td>
<td>34</td>
</tr>
<tr>
<td>10</td>
<td>SERCA loop mutations increase A- and N-domains separation <em>in silico.</em></td>
<td>35</td>
</tr>
<tr>
<td>11</td>
<td>First and second principal components of SERCA structural dynamics.</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>Principal component analysis for WT- and AAA-SERCA.</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>SERCA headpiece domains structural dynamics.</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>N-domain β5-β6 loop triple mutation decreases SERCA function.</td>
<td>41</td>
</tr>
<tr>
<td>15</td>
<td>SERCA structural dynamics measured by FRET in microsomes.</td>
<td>43</td>
</tr>
<tr>
<td>16</td>
<td>Modified Post-Albers reaction cycle.</td>
<td>44</td>
</tr>
<tr>
<td>17</td>
<td>SERCA headpiece structural dynamics measured by FRET in AAV-293 cells.</td>
<td>47</td>
</tr>
<tr>
<td>18</td>
<td>SERCA structural dynamics in response to spontaneous Ca²⁺ events measured by FRET in AAV-293 cells.</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 19. SERCA structural dynamics in response to transient and sustained Ca\(^{2+}\) elevation measured by FRET in AAV-293 cells.

Figure 20. SERCA FRET changes in response to intracellular Ca\(^{2+}\) dynamics is not due to intermolecular FRET

Figure 21. SERCA structural dynamics in correlation with ER Ca\(^{2+}\) changes

Figure 22. WT-SERCA and AAA-SERCA complex structural dynamics in complex with non-phosphorylated PLB or PLB phosphorylated on Ser16

Figure 23. RMSD and RMSF of SERCA-PLB complex structural dynamics calculated from MD simulations

Figure 24. SERCA-PLB structural dynamics in response to PLB phosphorylation on Ser16.

Figure 25. PLB cytosolic domain motions are positively correlated with SERCA headpiece structural dynamics.

Figure 26. PLB phosphorylation on Ser16 effect on SERCA TM-domain structural dynamics.

Figure 27. 200 ns MD simulations of SERCA-PLB structural dynamics compared to FRET experiments in cells.

Figure 28. PLB intramolecular interactions upon Ser16 phosphorylation.

Figure 29. PLB conformational changes upon Ser16 phosphorylation.

Figure 30. SERCA structural conformers as determined by FRET in microsomes from AAV-293 cells expressing 2-color SERCA with or without PLB.

Figure 31. FRET values for SERCA E1 and E2 states.

Figure 32. Ca\(^{2+}\) uptake in live cells

Figure 33. 2CS FRET in the presence of Hax-1.

Figure 34. Analysis of DWORF homo-oligomerization and association with SERCA, PLB and itself by acceptor photobleaching FRET.

Figure 35. Analysis of DWORF association with SERCA by acceptor sensitizing FRET.

Figure 36. DOX uptake in the nucleus of AAV-293 cells in the presence of candidate modulators.
Figure 37. WT-SERCA population accumulates in states prior to slow steps during *transient* Ca\(^{2+}\) increase.

Figure 38. WT-SERCA population accumulates in states prior to slow steps during *sustained* Ca\(^{2+}\) increase.

Figure 39. AAA-SERCA population accumulates in a new state prior to slow step during *sustained* Ca\(^{2+}\) increase.

Figure 40. AAA-SERCA structural dynamics measured by FRET in microsomes.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2CS</td>
<td>two-color SERCA</td>
</tr>
<tr>
<td>AAA</td>
<td>triple alanine mutant D426A/E429A/E435A</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus vector producer cell lines</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>A.U.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>Caf</td>
<td>caffeine</td>
</tr>
<tr>
<td>Cer</td>
<td>mCerulean</td>
</tr>
<tr>
<td>Ctrl</td>
<td>control</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>HAX-1</td>
<td>HS-1-Associated-protein-X-1</td>
</tr>
<tr>
<td>Iono</td>
<td>ionomycin</td>
</tr>
<tr>
<td>Kₐ</td>
<td>apparent dissociation constant</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MRP-1</td>
<td>Multidrug resistance protein 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principle component analysis</td>
</tr>
<tr>
<td>pCa</td>
<td>a measure of calcium concentration equal to $-\log_{10}[\text{Ca}^{2+}]$</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>RFP</td>
<td>pTag-red fluorescent protein</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>UT</td>
<td>untransfected</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum rate of reaction</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-Rhod</td>
<td>X-Rhod-1/AM</td>
</tr>
<tr>
<td>YFP</td>
<td>enhanced yellow fluorescent protein</td>
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</tbody>
</table>
ABSTRACT

The sarco/endoplasmic reticulum calcium ATPase (SERCA) and its inhibitor phospholamban (PLB) are major regulators of Ca^{2+} levels in the cardiac cell. Deficient calcium handling in the heart has been linked to heart disease, including heart failure; the leading cause of death in developed countries. As of today, targeting SERCA and SERCA-PLB complex to enhance cardiac function has not been successful due to lack of details on the SERCA regulatory mechanisms. Previous molecular dynamics (MD) simulations by my lab predicted that a small loop in SERCA N-domain (Nβ5-β6 loop) regulates SERCA transition from open-to-closed conformation and may interact with PLB cytosolic domain. Thus, I hypothesized that the Nβ5-β6 loop plays a role in (1) SERCA structural dynamics during Ca^{2+} transport and (2) SERCA regulation via interaction with PLB cytosolic domain.

Two-color SERCA (2CS) labeled with fluorescent proteins on two of the headpiece domains was used in FRET measurements, and open SERCA conformers were detected during transient Ca^{2+} elevations. I believe these open conformers represent a steady-state population of SERCA in the sloe step of the Ca^{2+} cycle. In the presence of sustained Ca^{2+} elevation the wild-type SERCA, but not the mutated transporter, acquired a new slow step and accumulated in non-physiological high-Ca^{2+} affinity closed conformation. Measurement of SERCA function in vitro showed that the mutated transporter still hydrolyzed ATP and have normal Ca^{2+} affinity, but maximal ATPase activity was reduced by
63%. I attribute this functional deficit to the decrease in SERCA headpiece compact assembly as was detected in silico and in live cells.

2CS FRET quantifications in microsomes detected ligand-stabilized compact conformers for high-affinity (E1) transporter, while low-affinity (E2) states were characterized by more open conformations. Additional MD simulations have shown steady interactions of N\(\beta\)5-\(\beta\)6 loop with PLB cytoplasmic domain irrespective of loop mutations or PLB phosphorylation status. SERCA ligand-stabilized conformers detected with FRET obtained more closed structure in the presence of PLB, and extra compact assembly upon PLB Ser16 phosphorylation. Overall, these data reveal a discrete structural element that facilitates a compact conformation during Ca\(^{2+}\) transport and enhanced scientific understanding of ATPase transporters function and regulation.

Additionally, I utilized 2CS FRET biosensor to confirm long-time predicted direct interaction with the indirect SERCA regulator HAX-1 and to study the association with the recently identified regulatory micropeptide DWORF. Even more, my lab applied 2CS labeling technique to generate another 2-color ATPase transporter biosensor, 2-color MRP-1, which was used in FRET high-throughput screening to identify novel MRP-1 substrates. I further determined functional effect of identified ligands on MRP-1 function in live cells and confirmed the 2-color labeling technique to be useful for generation of FRET biosensors in drug discovery.
CHAPTER ONE

INTRODUCTION AND BACKGROUND

The Cardiac Calcium ATPase in the Heart

Calcium (Ca\(^{2+}\)) serves as a signaling molecule in the heart to drive muscle contraction, thus removal of Ca\(^{2+}\) from the sarcoplasm is essential for myocardium relaxation (Bers, 2002). In particular, Ca\(^{2+}\) binding to a Ca\(^{2+}\) sensor Troponin C induces conformational changes in myofilament arrangement, which results in contraction. Sarco-endoplasmic reticulum calcium ATPase (SERCA) transports Ca\(^{2+}\) from the cytosol into the endoplasmic/ sarcoplasmic reticulum (ER/SR) lumen and in this way regulates relaxation of the heart. Sequestering Ca\(^{2+}\) from the cytosol by SERCA also affects contractile force, as restored SR Ca\(^{2+}\) load determines the force of the myofilament contractions. Figure 1 shows the Ca\(^{2+}\)-induced myofilament contraction/relaxation cycle in cardiac myocyte (Fig. 1A) and changes in cytosolic Ca\(^{2+}\) during this cycle (Fig. 1B). Depolarization across the transverse tubule membrane opens L-type calcium channels, causing an influx of Ca\(^{2+}\) into sarcoplasm (1). The influx of Ca\(^{2+}\) induces Ca\(^{2+}\) release from the SR lumen through ryanodine receptors (RyR) into sarcoplasm, where Ca\(^{2+}\) binds to myofilament force-generating structures and induces contraction (2). Relaxation (3) is produced when Ca\(^{2+}\) is removed from the sarcoplasm primarily through SERCA (approximately 70% of Ca\(^{2+}\) removal (Bassani et al., 1994)) and calcium transporters on the plasma membrane (not shown). During influx of
sarcoplasmic Ca\textsuperscript{2+} (Fig. 1, (2)), SERCA is active and continuously undergoes structural changes in order to progress throughout the enzymatic cycle presented in Fig. 3.

Previous studies have shown that SERCA function is reduced in heart failure (HF) (Gwathmey et al., 1987; Roe et al., 2015). HF is defined as the inability of the heart to keep up with its workload. Normally, the heart provides adequate blood flow to supply oxygen and nutrients to the cells and remove carbon dioxide and metabolites. In HF, the body may not get the oxygen it needs. HF is a chronic and progressive condition and a major cause of morbidity and mortality worldwide (Mosterd and Hoes, 2007; Mozaffarian et al., 2016).
While patients are treated with medications, lifestyle changes, surgical procedures and device implantation (Yancy et al., 2017), there is no cure for HF available.

HF has been linked to deficient Ca^{2+} handling in failing cardiac cells (Bers, 2006). More specifically, in humans failing myocardium SR Ca^{2+} load is decreased (Lindner et al., 1998; Piacentino et al., 2003; Pieske et al., 1999), while cytosolic calcium levels remain elevated during the relaxation period (Beuckelmann et al., 1992; Gwathmey et al., 1991). As a result, the failing heart has weak muscle contraction and reduced cardiac output. Since SERCA is a primary regulator of calcium handling in myocardium, it is an appealing target to treat cardiac diseases. In particular, increasing SERCA activity in failing cardiac cells is predicted to improve heart function (Hayward et al., 2015; Inesi et al., 2008). Recently, phase 2 of gene therapy clinical trials aimed at upregulation of SERCA, the most promising heart failure treatment option (Horowitz et al., 2011), had failed to significantly improve patient’s cardiac function (Zsebo et al., 2014). This study was aimed at increasing SERCA levels in heart failure patients by the adeno-associated viral expression. At the dose tested, increased SERCA levels did not improve the clinical outcome of heart failure patients (Greenberg et al., 2016). One possible explanation is that simply increasing SERCA levels is not sufficient to overcome heart failure symptoms. Instead, enhancing SERCA function is an alternative approach for the discovery of novel therapeutic options. The present study was aimed to add to understanding of SERCA function by investigation of SERCA structural dynamics during Ca^{2+} transport.
SERCA in Other Diseases

Besides being a key component in heart failure manifestation, various SERCA isoforms have been implicated in other pathologies. Recently SERCA has been implicated in cancer stem-like cell survival by preventing Ca\(^{2+}\)-induced apoptosis in glucose-deprived conditions due to sequestering Ca\(^{2+}\) from cytosol (Park et al., 2017). Other reports emphasize SERCA importance in skin health, where somatic mutations in SERCA2b lead to Darier’s disease, characterized by epidermal lesions (Savignac et al., 2011; Takagi et al., 2016). This disorder results from a tissue-specific missense, non-sense, frameshift, and splicing mutations, which affect both SERCA2b and SERCA2a. Being a major isoform expressed in the epidermis, loss-of-function SERCA2b mutation has a detrimental effect on the health of the epidermis with variety of clinical manifestations.

Germline mutations of skeletal SERCA1a isoform result in pathological skeletal muscle stiffness, called Brody myopathy (Odermatt et al., 1996), while overexpression of SERCA2a rescues dystrophic phenotype in mice (Goonasekera et al., 2011). Based on the variety of SERCA function in all of these disorders, SERCA is a high value therapeutic target. Nevertheless, the details of SERCA structural dynamics during Ca\(^{2+}\) cycle are still not resolved to the degree that would allow targeted transporter regulation. This study was aimed at discovering the detail of SERCA structural dynamics during Ca\(^{2+}\) cycle.

SERCA Structure and Function

SERCA is the ion transporter that is responsible for sequestration of calcium Ca\(^{2+}\) in the ER/SR. There are three isoforms of SERCA: SERCA1-3 encoded by ATP2A1-3 respectively.
Sarcoplasmic reticulum ATPase SERCA2 belongs to a Ca^{2+} P-type ATPase2 family which has two isoforms: SERCA2a and SERCA2b resulting from alternative splicing of the exon 20 and only differ in their C-terminus (SERCA2b has an additional transmembrane helix 11).

SERCA2a is expressed in cardiac myocytes and slow-twitch skeletal muscles. SERCA2b is expressed in almost all tissues. SERCA2 isoforms are found in ER/SR and involved in the Ca^{2+} transport from the cytoplasm/sarcoplasm to the lumen of ER/SR (Periasamy and Kalyanasundaram, 2007). Orthologues of SERCA2 pumps are common in bacteria and most likely human SERCA2 transporters evolved as a mechanism to transport Ca^{2+} against a
steep gradient (Deves and Brodie, 1981). The main focus of the current study is on the cardiac isoform SERCA2a.

The first SERCA to have the crystal structure determined was SERCA1a of rabbit muscle (Toyoshima et al., 2000). Today, there is an abundant collection of SERCA structures in the PDB database, including conformers in various ligand-stabilized states. Based on these data, a clear picture of SERCA structure emerged. As illustrated in Fig. 2, SERCA2a contains four distinct domains: three cytoplasmic domains (A, actuator; N, nucleotide binding; P, phosphorylation) comprise a cytoplasmic headpiece, while 10-helical transmembrane (TM) domain transports Ca\(^{2+}\) (Toyoshima, 2008). Each of the domains has its unique function. The nucleotide binding N-domain (Fig. 2, yellow) has two main functions: binding of ATP and phosphorylation of P-domain. The N-domain core structure is conserved among SERCA isoforms and contains crucial residues (e.g. Phe487) for ATP binding (Sorensen et al., 2004). In ATP bound state, the N-domain undergoes structural rearrangements and becomes linked to P-domain via nucleotide-mediated interactions which allows phosphorylation of P-domain to take place (Toyoshima, 2008).

The phosphorylation P-domain (Fig. 2, black) contains the phosphorylated residue Asp351 placed in conserved Asp-Lys-Thr-Gly (DKTG) motif, and Mg\(^{2+}\)-coordinating residue Asp703 positioned in conserved Thr-Gly-Asp-Asn (TGDN) sequence (Aravind et al., 1998; Picard et al., 2007). Besides being linked to the N-domain, P-domain’s flat surface allows docking and rotation for A-domain (Toyoshima, 2009), thus potentially mediating A–N-domains interaction.
The actuator A-domain (Fig. 2, blue) has a conserved Thr-Gly-Glu-Ser (TGES) motif (Moller et al., 1996) which plays an important role in dephosphorylation of P-domain residue Asp351 (Clausen et al., 2004), thus serving a protein phosphatase function. A-domain connects to TM-domain via a long linker that allows A-domain to be flexible and serve as a gating mechanism (actuator) for Ca\textsuperscript{2+} binding and release (Toyoshima et al., 2007).

SERCA transports two Ca\textsuperscript{2+} ions from cytosol to the ER/SR lumen for every ATP hydrolyzed (Inesi et al., 1980). In addition, SERCA counter-transport 2 or 3 H\textsuperscript{+} ions (Stokes and Green, 2003; Yu et al., 1993). According to classical theory for ion transport, when P-domain gets phosphorylated via ATP hydrolysis, the ATP-mediated interaction between N- and P-domains breaks, and P-domain shifts and pulls along the A-domain, which in turn tugs the A-domain linker connected to TM-domain. These complex structural rearrangements of cytoplasmic headpiece result in TM reorganization suitable for Ca\textsuperscript{2+} transport (Toyoshima, 2008).

There are two Ca\textsuperscript{2+} transport sites in the center of the TM-domain, which can be in high or low affinity states, and sequential Ca\textsuperscript{2+} binding is cooperative (Inesi et al., 1980). Ca\textsuperscript{2+} binding to the site I is maintained by oxygen side chains and one water molecule on cytosolic side of helixes M5, M6, and M8. Only after the first Ca\textsuperscript{2+} binds, can the second Ca\textsuperscript{2+} bind to site II on nearby helix M4 which provides 3 main chain carbonyl groups for ion coordination (Toyoshima et al., 2000). If Ca\textsuperscript{2+} binding sites are exposed to the cytoplasmic site, then the ATPase is in a high-affinity (E1) conformation. On the other hand, if Ca\textsuperscript{2+} binding sites are exposed to the luminal side of ER membrane, the transporter is in a low-
affinity state (E2) (Moller et al., 2010). Transition of the transporter from E1 to E2 conformation has been proposed to be induced by phosphorylation event. Specifically, P-domain phosphorylation results in distortion of the high-affinity binding sites and opens an ion exiting channel on the other side of the membrane, while repositioning the dephosphorylation TGES motif of A-domain closer to the phosphorylation site on P-domain (Moller et al., 2010; Toyoshima and Mizutani, 2004). The consequent repositioning of A-domain during hydrolysis of aspartylphosphate of P-domain (dephosphorylation event) results in transition of the pump from the E1 to E2 conformation, which is predicted to be the slowest step of the Ca\(^{2+}\) catalytic cycle (Fig. 2, (c)) (Champeil et al., 1986; Hanel and Jencks, 1990; Petithory and Jencks, 1986). Fig. 3 presents a simplified schematic overview of SERCA Ca\(^{2+}\) transport cycle steps, including Ca\(^{2+}\) binding (a), phosphorylation (b), Ca\(^{2+}\) release (c), dephosphorylation (d), and reset of cycle by ATP binding (e). All of these steps of the enzymatic cycle are intrinsically regulated by means of the transporter structural determinants’ responses to the ligand (Ca\(^{2+}\), ATP, Mg\(^{2+}\)) binding and their or their metabolites release events. For example, SERCA structural rearrangement during transition from open to close conformation upon Ca\(^{2+}\) binding (Toyoshima and Mizutani, 2004) was proposed to result in the restriction of ATP delivery to phosphorylation site, and thus prevent the sequence of events that would lead to TM-domain rearrangement and backflow of Ca\(^{2+}\) ions from the SR luminal side (Toyoshima, 2008; Toyoshima, 2009). This idea of internal structural determinants regulating the pump catalytic cycle is a powerful approach to understanding SERCA functional mechanisms. Identification of the structural determinants that regulate SERCA catalytic cycle would be beneficial in rational design of
Figure 3. Simplified schematic overview of SERCA kinetic cycle.
E1 state are yellow and E2 states are green. (a) ATP binding induces conformational changes that facilitate Ca\(^{2+}\) binding at the cytosolic membrane side. (b) P-domain phosphorylation and ATP hydrolysis. (c) Ca\(^{2+}\) release at the other side of membrane. (d) Dephosphorylation. (e) ATP binding. Steps (c-d) are predicted to be the slowest.

SERCA targeted modulators. Up to this point, only few SERCA specific activators have been identified, and their potency is still under investigation (Cornea et al., 2013; Dahl, 2017; Kang et al., 2016). One of the goals of this work was to contribute to understanding of SERCA function via thorough investigation of SERCA structural dynamics during Ca\(^{2+}\) transport.

**Nβ5-β6 Loop is Predicted to Regulate SERCA Compact Conformation**

The advances in crystallography and electron microscopy techniques allowed science to capture SERCA short-lived transitional structures in biochemically-stabilized
environments. Nevertheless, the structural dynamics of SERCA during Ca\textsuperscript{2+} cycling have not been fully discovered and alternative approaches are needed to investigate transitional conformations. A recent computational study by Smolin et al. (Smolin and Robia, 2015a) has identified a β-loop in SERCA N-domain as a potential regulator of SERCA transition from open-to-closed conformation. This short (10 amino acids) N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop has three negatively charged residues (Asp426, Glu429, and Glu435) which are predicted to interact with positive cluster on the A-domain surface. Formation of H-bonds/salt bridges between the N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop and polar/basic residues of A-domain is predicted to penetrate the poorly diffusible water cushion separating A- and N-domains and facilitate the closure of the SERCA cytoplasmic headpiece during Ca\textsuperscript{2+} transport cycle. The N-domain N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop is highly conserved among SERCA isoforms and multiple species (Smolin and Robia, 2015a). Analysis of two major human genome variance databases (OMIM and COSMIC) revealed that N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop carries no reported mutations (Amberger et al., 2015; Forbes et al., 2015). High preservation and absence of variance in this discrete structure suggest that SERCA N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop residues may play an important role in transporter assembly/function. One of the goals of the current study is to investigate in detail the predicted role of N\textbeta\textsubscript{5}-\textbeta\textsubscript{6} loop in SERCA structural regulation and function.

**SERCA Regulation by PLB**

The SERCA’s main inhibitor is phospholamban (PLB), a 52-amino-acid residue polypeptide (Fig. 1, PLB in red) (Kirchberber et al., 1975; Tada and Inui, 1983; Toyoshima et al., 2003). PLB is composed of amino-terminal cytosolic α-helix, connected via an unstructured hinge
to a carboxyl hydrophobic transmembrane single-spin α-helix (Simmerman et al., 1986). PLB binds to SERCA at the TM groove composed of helixes M2 (residues 89-119), M4 (residues 313-330), M6 (residues 789-809), and M9 (residues 932-950) and stabilizes SERCA conformation in reduced affinity for Ca\(^{2+}\), and thus reduces maximal Ca\(^{2+}\) transport rate (\(V_{\text{max}}\)) (Chen et al., 2006; Morita et al., 2008). SERCA inhibition by PLB is relieved when PLB cytosolic domain is phosphorylated by protein kinase A (PKA) on Ser16 or calcium/calmodium-dependent protein kinase II (CaMKII) on Thr17 (Simmerman et al., 1986; Tada and Inui, 1983). Several studies suggest that PLB phosphorylation increases SERCA maximal rate of Ca\(^{2+}\) transport beyond of SERCA \(V_{\text{max}}\) alone (Antipenko et al., 1997; Reddy et al., 2003), suggesting that PLB binding even ‘primes’ SERCA for more efficient enzymatic cycling.

In the SR membrane PLB forms homo-pentamers, and upon de-oligomerization into active monomers is able to bind and inhibit SERCA (Simmerman and Jones, 1998). PLB mutations that prevent homo-pentamer formation and thus enhance a monomeric form of PLB have been shown to improve PLB ability to inhibit SERCA (Kimura et al., 1997). It is assumed that PLB monomers bind to SERCA, while PLB homo-pentamers serve as a non-inhibitory reserve pool (Simmerman and Jones, 1998). PLB monomeric to pentameric equilibrium is highly dynamic (Robia et al., 2007) with different affinities of PLB monomers to different SERCA conformational states (Bidwell et al., 2011). The interaction between PLB subunits and SERCA conformers are still under investigation.

Currently, there are two models how PLB phosphorylation causes relief of SERCA inhibition. The dissociation model suggests that PLB phosphorylation results in PLB
dissociation from SERCA, which allows SERCA to return into a high Ca$^{2+}$ affinity conformational state (Chen et al., 2006; James et al., 1989; Kimura et al., 1997). Nevertheless, this model does not explain why phosphorylation of PLB results in SERCA maximal rate of Ca$^{2+}$ transport increase beyond $V_{\text{max}}$ of SERCA alone (Antipenko et al., 1997; Reddy et al., 2003). The alternative model, the so called ‘subunit’ model, predicts that PLB remains bound to SERCA after phosphorylation (Bidwell et al., 2011; Negash et al., 2000). This model predicts that PLB binding serves as a SERCA “shortcut” to efficient Ca$^{2+}$ transport by shifting ATPase conformation to an ordered, compact and high Ca$^{2+}$ affinity (high-efficiency) structural state (Pallikkuth et al., 2013). Additionally, the significant structural changes in SERCA-PLB complex toward more compact conformation upon PLB phosphorylation have been supported by the decrease in SERCA-to PLB FRET (Hou et al., 2008). Thus, this model of PLB shift becomes more and more predominant over dissociation model.

Unfortunately, the crystal structure of SERCA-PLB regulatory complex has been determined without the PLB cytosolic domain (residues 1-23 not resolved) due to its highly dynamic structure (Akin et al., 2013). To date, it remains unclear how PLB cytosolic domain phosphorylation can induce SERCA-PLB compact conformation and increased Ca$^{2+}$ transport rate. My lab’s recent MD simulations of SERCA spontaneous motions have shown that SERCA N$\beta$5-$\beta$6 loop was in position to interact with PLB cytosolic domain (Smolin and Robia, 2015a); thus, it is reasonable to hypothesize that N$\beta$5-$\beta$6 loop could be an intrinsic regulator of SERCA function via interaction with PLB. Specifically, N$\beta$5-$\beta$6 loop has three negatively charged residues, Asp426, Glu429, and Glu435, which are potential candidates
for interaction with multiple positive residues on PLB cytosolic domain. Negative charges introduced to Ser16 and Thr17 with addition of phosphoryl groups upon phosphorylation may cause repulsion of PLB cytosolic domain from negative loop residues and in this way become that force that shifts SERCA-PLB complex and relieves it from inhibition. One of the goals of current study is to examine the regulatory complex in more detail and investigate the possible role of Nβ5-β6 loop in structural regulations in SERCA-PLB regulatory complex.

**SERCA in Regulatory Complexes: HAX-1 and DWORF**

SERCA regulation is a plausible target to improve cardiac function in heart related disorders. Even though PLB remains a major SERCA regulator, recently other molecules were predicted to regulate pump activity. Two of them, HAX-1 and DWORF, were included in the scope of current study and are described below.

HS-1-Associated-protein-X-1 (HAX-1) is a ubiquitously expressed protein that has been shown to play an anti-apoptotic role in striated muscles via interaction with the mitochondrial enzyme caspase-9 (Han et al., 2006). In failing heart, Hax-1 has been shown to interact with SERCA inhibitor phospholamban residues 16-22, which overlaps with phosphorylation sites (Vafiadaki et al., 2007). The HAX-1 pull-down experiments detected SERCA in complex with HAX-1 and PLB, which suggests that PLB can interact with both HAX-1 and SERCA simultaneously, or alternatively, HAX-1 can interact with both SERCA and PLB at the same time (Kimura and Inui, 2002). HAX-1 binding to SERCA has been confined to residues 575-594 on N-domain by additional in vitro pull-down experiment
(Vafiadaki et al., 2009). Recently, Hax-1 has been reported to promote cell survival via positive regulation of SERCA levels (Bidwell et al., 2017; Vafiadaki et al., 2009) and mediate PLB inhibitory activity on SERCA (Bidwell et al., 2018), which suggests that SERCA is under HAX-1 regulation. Nevertheless, direct binding of HAX-1 to SERCA in live cells that is not mediated by simultaneous association with PLB is still under investigation (Vandecaetsbeek et al., 2011). I examined the direct effect of Hax-1 on SERCA headpiece closure.

Long noncoding RNAs (lncRNA) were long viewed as junk nucleic acids that are not translated into proteins. Nevertheless, over the last decade, lncRNAs have been demonstrated to carry out transcriptional regulation, serve as scaffold, and to functionally regulate proteins and RNA molecules (Ulitsky and Bartel, 2013). Even more strikingly, some lncRNAs were identified as transcripts that encode short peptides (Anderson et al., 2015; Matsumoto et al., 2017; Nelson et al., 2016). The Dworf RNA transcript encodes the muscle-specific short peptide named DWORF (Nelson et al., 2016). DWORF has been shown to localize in SR with SERCA and has been predicted to physically interact with SERCA at the same groove and with similar affinity as PLB. Moreover, DWORF enhances SR Ca\(^{2+}\) uptake by SERCA through displacement of the inhibitory PLB, and is proposed to be a promising therapeutic target to enhance cardiac contractility. I examined the detail of DWORF interaction with and regulation of SERCA in this study, which is discussed further.
2-color ATPase Transporters: SERCA and MRP-1

My laboratory developed a 2-color SERCA FRET biosensor for a high-throughput screening strategy that allowed identification of molecule’s effectors with high precision and speed in live cells. This technique is used for drug discovery and has a potential for rapid and precise identification of small-molecule regulators of transporter activity (Cornea et al., 2013). The transport ATPases include not only Ca^{2+} pumps like SERCA, but a wide variety of proteins that move different types of ions or molecules across the biological membranes (Pedersen, 2005). All ATPase transporters hydrolyze ATP in order to move substrates across membranes and are involved in variety of physiological processes. Mutations observed in ATPases result in a large number of diseases. While SERCA belongs to P-type ATPase class, there are three more major classes: V, F, and ABC types.

Multidrug resistance protein 1 (MRP-1) is an ABC (ATP-binding cassette) family plasma membrane transporter that exports a wide variety of drugs from the cell (Deeley et al., 2006). As a result, MRP-1 is a major contributor to the failure of chemotherapy in cancer patients. It is only recently a high resolution MRP-1 structure was resolved with electron cryomicroscopy (Johnson and Chen, 2017), and before that, homologous ABC-family transporters were used to predict MRP-1 structure. MRP-1 has been expected to be composed of two membrane spanning domains and two cytoplasmic nucleotide-binding domains (Dean and Allikmets, 2001). Two structural conformers of MRP-1 were predicted to exist. The first one is a low substrate-affinity conformation with nucleotide-binding domains locked together and ligand-binding site exposed to the extracellular environment (Fig. 36A, bottom panel), while a second one is a high substrate-affinity conformation with
binding site exposed to cytoplasm and nucleotide-binding domains separated (Fig. 36A, top panel) (Aller et al., 2009). Without the MRP-1 structure resolved, the real conformational dynamics of this important transporter were not defined, and an alternative approach was needed to investigate the MRP-1 structural determinants. My lab used similar to 2CS labeling strategy (Hou et al., 2012; Pallikkuth et al., 2013) in generating 2-color MRP-1 biosensor. Fluorescent MRP-1 sensor was used for identification of transporter’s structural dynamics and to monitor the conformational changes of MRP-1 nucleotide binding domains in the presence of small molecules. As a result, several substrates that induced MRP-1 conformational changes were identified as candidates into MRP-1 allosteric modulators (Iram et al., 2015). This study describes advanced investigation of functional significance of identified compounds in MRP-1 activity as a part of present project.

Overall, this dissertation work concentrates on structural dynamics and regulation of several ATPases, predominantly SERCA. Specifically, I investigate SERCA headpiece structural transitions during the enzymatic cycle and a role of Nβ5-β6 loop during these transitions. I examine SERCA’s regulatory interactions, mostly with PLB and in a smaller scale with DWORF and HAX-1. In my investigations, I utilize FRET biosensor labeling strategy to carry out SERCA structural measurements. I successfully apply this strategy to generating MRP-1 FRET sensor and showed that this approach can be used as a wide-ranging tool in structural biology and drug discovery.
CHAPTER TWO
MATERIALS AND METHODS

Molecular Biology and Cell Culture

The engineering and functional characterization of 2CS has been previously described in detail by Hou et al (Hou et al., 2012). I used a canine SERCA2a construct labeled with Cer on the N-terminus and a YFP intra-sequence tag inserted before residue 509 in the N-domain. In some experiments, like ATPase assay, I used an analogous construct, with Tag-RFP on the N-terminus and an EGFP tag inserted before residue 509 in the N-domain. The Cer-YFP pair has a Förster distance ($R_0$) of 49.8 Å and the EGFP-TagRFP pair has an $R_0$ of 58.3 Å (Gadella, 2011). Ala mutations in Nβ5-β6 loop residues Asp426, Glu429, Glu435 were introduced with QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Stratagine, La Jolla, CA) according to the manufacture's protocol. Single mutations and a triple mutation were made: Asp426Ala, Glu429Ala, Glu435Ala, and Asp426Ala/Glu429Ala/Glu435Ala (AAA). Adenoviral vectors of TagRFP-EGFP-SERCA (WT and AAA) were obtained from the Loyola Cardiovascular Research Institute virus production facility. All constructs were validated with nucleotide sequencing and in fluorescent signal in cell expression.

AAV-293 cells were cultured and transiently transfected using MBS mammalian transfection kit (Agilent Technologies, Stratagine, La Jolla, CA) as described previously (Hou et al., 2012). Briefly, the transfected cells were trypsinized for 1 min and plated onto
poly-D- and allowed to adhere for 1–2 hr prior to imaging.

**Fluorescence Microscopy and FRET Quantification**

Acceptor-sensitized emission FRET was used to measure intermolecular protein-protein or intramolecular protein interactions. This technique allows excited-state energy to be transferred directly from excited fluorophore (donor) to a proximate molecule (acceptor) and thus provides evidence of molecule interactions (Day and Davidson, 2012; Gordon et al., 1998). FRET was calculated from fluorescence intensities of donor (Cer) and acceptor
(YFP) images, as well as an image that captures the acceptor emission with donor excitation.

Wide-field fluorescent microscopy was done as described previously (Hou et al., 2008). Briefly, cells expressing 2-color SERCA (Fig. 4A) were imaged with an inverted microscope (Nikon Eclipse TE2000-U) equipped with a metal halide lamp and a back-thinned CCD camera (iXon 887: Andor Technology, Belfast, Northern Ireland). For each sample, acquisition of the field was performed with a 60×1.49 N.A. objective with 100 or 150–ms exposure for each channel: Cer, YFP, and FRET. Fluorescence intensity was automatically quantified with a multiwavelength cell scoring application in MetaMorph software (Molecular Devices, Sunnyvale, CA). The background threshold was set to 100 counts. Cell size criteria were limited to 30-80 μm diameter or manually defined by drawing a cell contour. FRET efficiency $E$ was calculated according to $E=F_{em}/(F_{em}+G\times F_{Cer})$ where $F_{em}=F_{FRET}-a\times F_{YFP}-d\times F_{Cer}$ (Bidwell et al., 2011; Hou et al., 2008), $F_{FRET}$, $F_{YFP}$, and $F_{Cer}$ are the matching fluorescence intensity from FRET, YFP, and Cer images, respectively, and $G$ represents FRET intensity corrected for the bleed-through of the channels calculated as in photobleaching-correction method described by Zal and Gascoigne (Zal and Gascoigne, 2004). $G$ was calculated as $G = \frac{(F_{em}) - (F_{em}^{post})}{F_{FRET} - F_{Cer}}$ where $F_{em}$ calculated before and $F_{em}^{post}$ calculated after photobleaching, and $G$ determined to be 3.20 for experimental set up. The acceptor photobleaching technique details are described below. The parameters $a$ and $d$ are bleed-through constants calculated as $a= F_{FRET}/F_{Cer}$ for a control sample transfected with only YFP-SERCA, and $d= F_{FRET}/F_{Cer}$ for a
control sample transfected with only Cer-SERCA. These calibrations indicated $a=0.074$ and $d=0.70$ in my experimental setup.

Each cell that expressed CFP and YFP above background threshold was plotted as percent FRET over a range of expressed protein as determined by YFP intensity (in arbitrary units, A.U.). The plotted data were fit by equation: $FRET = \frac{FRET_{max} \times I_{YFP}}{K_d + I_{YFP}}$, where $I_{YFP}$ is YFP intensity, $FRET_{max}$ is maximum FRET, and $K_d$ is the apparent dissociation constant of the donor- and acceptor-labeled proteins for each other.

Another FRET technique used in investigation of protein-protein interactions is progressive acceptor photobleaching. This method utilizes irreversible selective destruction of the acceptor fluorophore through prolonged excitation. Once destroyed, the acceptor fluorophore no longer can quench the donor emission (assuming FRET pair was in
distance suitable for FRET), and donor fluorescence intensity increases (Patel et al., 2002). Acceptor photobleaching experiments were performed on a similar set up as described for acceptor-sensitized emission FRET, except that YFP was selectively photobleached by exposure to YFP excitation (504/12nm bandpass filter) for 10 s, and then YFP and CFP were imaged. The first 10 images were acquired in 10 s intervals without photobleaching to obtain fluorescence intensities baseline values. Images were analyzed with ImageJ software (Schindelin et al., 2012) by manually selecting cells. The fluorescence intensity values were plotted over time either as arbitrary units (A.U.) or average intensity was normalized to baseline intensity using the equation: \( \frac{F}{F_0} = \frac{F - F_{bg}}{F_0 - F_{0bg}} \), where \( F \) is the fluorescence intensity after photobleaching, \( F_0 \) is last baseline fluorescence intensity recorded, and \( F_{bg} \) and \( F_{0bg} \) are background fluorescence signals. Figure 5 shows a representative field of cells expressing Cer-PLB and YFP-PLB and changes in their corresponding fluorescence intensities before and after YFP photobleaching.

**Live Cell Ca\(^{2+}\) Uptake Activity Assay**

To evaluate Ca\(^{2+}\) transport activity of SERCA, I transfected AAV-293 cells with Cer-SERCA and YFP-PLB DNA constructs and performed Ca\(^{2+}\) uptake activity assay as described previously (Bidwell et al., 2011). 24 hrs post-transfection, cells were labeled with the cell-permeant Ca\(^{2+}\) indicator dye X-Rhod-1 (AM) (ThermoScientific, Waltham, MA). Transfected and untransfected cells were distinguished based on the intensity of Cer and YFP fluorescence emissions. Release of Ca\(^{2+}\) from ER stores was accomplished by stimulating purinergic receptors with extracellular application of 200 \( \mu \)M ATP, which activated inositol 1,4,5-trisphosphate receptors on ER membrane (Dubyak and el-Moatassim, 1993). Accumulation of Ca\(^{2+}\) in the cytosol was quantified as an increase in X-Rhod-1 fluorescence.
SERCA Ca\textsuperscript{2+} transport activity was detected as decrease in ATP-stimulated cytosolic Ca\textsuperscript{2+} accumulation relative to untransfected cells. 4 min after application of extracellular ATP, cells were treated with 50 μM TG to determine the size of the Ca\textsuperscript{2+} store remaining in the ER.

**Measuring Calcium Oscillations by Confocal Microscopy in Live Cells**

AAV-293 cells were transiently co-transfected with three plasmids containing genes of human GFP-Ryanodine receptor-2 (RyR), low affinity ER calcium sensor R-CEPIA1er, and 2-color canine SERCA2a wild type (WT) or triple loop mutant AAA (ratio of DNA constructs was 3 unites of RyR to 1 unit of R-Cepia1er, and 10 unites of SERCA). Transfected cells were cultured for 24 hrs and seeded into poly-D-lysine coated glass bottom chamber slides in MDM. 24 hrs after seeding, media was changed with PBS with Ca\textsuperscript{2+}/Mg\textsuperscript{2+} and experiments were conducted with a Leica SP5 laser scanning confocal microscope equipped with a 63× water objective. R-CEPIA1er was excited with the 543 nm line of a He-Ne laser, and emitted fluorescence was measured at wavelength ≥580 nm. 2CS fluorophores Cer and YFP were excited with the 430 and 514 nm lines of an Argon laser, respectively, and emitted fluorescence was measured at wavelength ≥485±15 nm and ≥537±15 nm, respectively. Images were acquired in line-scan mode for up to 8–12 min with addition of 10 mM caffeine or 100 μM ionomycin (Iono) at 2 min. Fluorescence image analysis was performed with Fiji (ImageJ) software (Schindelin et al., 2012).

To load cells with the low-affinity Ca indicator X-Rhod-1/AM (X-Rhod) (Lock et al., 2015) cells were incubated with 10 μM X-Rhod (ThermoScientific, Waltham, MA) for 15 min in PBS with Ca\textsuperscript{2+}/Mg\textsuperscript{2+}. Before conducting experiments, cells were washed twice with PBS with Ca\textsuperscript{2+}/Mg\textsuperscript{2+}. 
MRP-1 functional measurements were accomplished with Argon laser illumination at 488 nm for GFP and DOX, and detection of emission bands of 496-522 and 565-650 nm respectively.

**All-atoms Molecular Dynamics Simulations**

All-atoms MD simulations were performed with the GROMACS software package (Hess et al., 2008; Pronk et al., 2013) with CHARMM 27 force field (MacKerell et al., 2000). TIP3P water model (Jorgensen W. L. and L., 1983) was used to carry out MD simulations with an integration time of 2 fs. The reference 1SU4 crystal structure of SERCA (Toyoshima et al., 2000) was used to introduce Nβ5-β6 loop mutations and perform energy minimization with the steepest descent method for 1000 steps. Then the structures were embedded into a POPC lipid bilayer and solvated into triangular water box with dimension sizes 130 × 130 × 160 Å. Na⁺ and Cl⁻ ions were added to the solution to concentration of 150 mM. The Berendesen method (Berendsen H. J. C., 1984) with relaxation time of 0.1 ps was used to increase the temperature of the system to 300 K and to increase the pressure to 1 bar. After 1 ns equilibration, the production run was performed in the NPT assemble using the Nose-Hoover thermostat (Hoover, 1985; Nose, 1984) and the Parrinello-Rahman barostat (Parrinello, 1981) with relaxation time of 1 ps. Six independent production runs for each wild-type or mutant models were started with different sets of assigned velocities at 300 K. The atoms coordinates were saved every 1 ps. The production runs were carried out for 100 ns.
Structural Analysis and Visualization

The VMD program (Humphrey et al., 1996) was used for visualization and rendering structures. The GROMACS (Hess et al., 2008; Pronk et al., 2013) program was used for the computational analysis of secondary structures of AAMD run productions. The A-N domains distance was computed as minimum distance between atoms of domain A-(residues number 1–40 and 128–241) and atoms of N-domain (residues 360–603). The number of contacts was calculated within 4 Å between atoms of A- and N-domains. A contact of an N-domain atom with multiple atoms of A-domain was counted as one contact (i.e., instead of multiple contacts). The first 10 ns of MD runs were considered as equilibration time and not included into calculations (except for SERCA-PLB complex, where whole trajectories were included into quantification).

Principal Component Analysis

To identify the major motions of the SERCA headpiece during MD trajectories, I aligned SERCA structures using the 10-helix TM-domain as a reference and used principle component analysis (PCA) (Amadei et al., 1993; Kitao et al., 1998; Tournier and Smith, 2003). In order to compare structural ensembles with respect to the same eigenvectors, I combined three SERCA reference X-ray crystal structures (1SU4 (Toyoshima et al., 2000), 3W5B (Toyoshima et al., 2013) and 1VFP (Toyoshima and Mizutani, 2004)), six MD trajectories of WT-SERCA, and six MD trajectories of AAA-SERCA into a single trajectory. To obtain sets of eigenvectors and eigenvalues corresponding to principle components I built covariance matrixes of the atomic fluctuations in GROMACS (Hess et al., 2008; Pronk et al., 2013). The diagonalization of matrixes yielded the eigenvectors (which are principle components) and their associated eigenvalues.
The GROMACS program was used for the computational analysis of SERCA structures from AAMD productions. To perform correlation analysis of domains orientation, I quantified the orientation of the vector which connects the domain hinge and domain center of mass. I computed the hinge disorder function defined as

$$\theta_x(t) = \cos^{-1}\left(\frac{q_x(t) \cdot \bar{q}_x(t)}{|q_x(t)| \cdot |\bar{q}_x(t)|}\right)$$

where $x$ is N, A, or P domain. Thus, $\theta_x(t)$ defines the time-dependent orientation of each domain relative to the starting orientation (Fig. 6).
**Root Mean Square Fluctuations Calculations (RMSF)**

RMSF was calculated in GROMACS (Hess et al., 2008; Pronk et al., 2013) as the root mean square fluctuations (i.e. standard deviation) of atomic positions in the trajectory after fitting to TM-domain with a reference to structure at 10 ns.

**Root Mean Square Deviation Calculations (RMSD)**

RMSD was calculated in GROMACS (Hess et al., 2008; Pronk et al., 2013). SERCA structures of production runs were aligned using 10-helix TM-domain as a reference. RMSD was calculated for Cα positions with reference to starting structure at 10 ns.

**AAV-293 Cell Microsomal Membranes Preparations**

Microsomal membrane containing SERCA were isolated from AAV-293 cells infected (for ATPase assay) or transfected (FRET measurement) with two-color SERCA constructs as described (Clifford and Kaplan, 2009). Cells were grown to confluency on 7–10 x 150 mm² dishes for 2 days, washed twice with PBS, harvested by scraping, and pelleted at 4°C for 10 min at 1000 × g. To prepare cell homogenate, the cell pellets were (1) resuspended in cold homogenizing solution (0.5 mM MgCl₂, 10 mM Tris-HCl pH 7.5, EDTA-free complete protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Dallas, TX)), (2) disrupted in Potter-Elvehjem homogenizer, (3) supplemented with equal volume of sucrose solution (100 mM MOPS pH 7.0, 500 mM sucrose, EDTA-free complete protease inhibitor cocktail), and (4) passed through a 27-gauge needle. To prepare microsomal membranes, (1) cell homogenates were centrifuged for 10 min at 1000 × g at 4°C, (2) the low-speed supernatants were centrifuged at 30 min at 55000 rpm (Beckman Coulter Optima Ultracentrifuge, rotor TLA-110, ~ average 130000 × g), (3) the high-speed membrane
pellets were resuspended in a 1:1 mix of homogenizing and sucrose solutions, and (4) the membrane suspensions were passed through a 27-gauge needle. The protein concentration of microsomal membranes was determined with Pierce BCA assay kit (ThermoScientific, Waltham, MA). ATPase assay was performed the same day; otherwise, microsomal membranes were aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. Light SR (LSR) microsomal samples enriched in SERCA (approximately 90% of proteins in the LSR is SERCA) were a gift from Dr. David D. Thomas at the University of Minnesota.

**Ca^{2+}^-ATPase Functional Measurements**

Two-color SERCA enzymatic activity was measured and quantified in microsomes by spectrophotometric measurement of the rate of NADH consumption as a function of Ca concentration in an enzyme-coupled activity assay in a 96-well plate (Lockamy et al., 2011; Reddy et al., 2003). Figure 7 shows the schematic of the enzyme-linked ATPase assay. The
time-dependent change of NADH absorbance was measured at 25°C in a PHERAnstar FSX microplate reader (BMG Labtech, Cary, NC) at 340 nm. Each well contained 3–4 μg of microsomal membranes, 50 mM MOPS at pH 7.0, 0.1 M KCl, 5.0 mM MgCl2, 1.0 mM EGTA, 2.5 mM ATP, 0.2 mM NADH, 5 IU of pyruvate kinase, 5 IU of lactate dehydrogenase, and 0.7 μg of Ca2+ ionophore (A23187) (chemicals were obtained from SigmaAldrich, St. Louis, MO). Free Ca2+ concentrations were calculated using Ca2+/Mg2+/ATP/EGTA Calculator from Theo Schoenmakers’ Chelator (Schoenmakers et al., 1992). Data were fitted using a Hill function: 

\[ V = V_{\text{max}} / [1 + 10^{−n(pK_{\text{Ca}}−pCa)}], \]

where \( V \) is the initial ATPase rate, \( n \) is the Hill coefficient, and \( K_{\text{Ca}} \) is the apparent Ca2+ dissociation constant and \( V_{\text{max}} \) was obtained from the fit of the Hill equation at saturated Ca2+ concentrations.

**Western Blotting**

The protein concentrations were determined with a bicinchoninic acid protein assay (BCA) (ThermoScientific, Waltham, MA) according to the manufacture's instructions. Microsomal membrane preparations were diluted in 2-fold SDS buffer (BioRad, Hercules, CA), heated at 55°C for 10 min and loaded on a 4–15% polyacrylamide precast gel (BioRad, Hercules, CA). The proteins were separated at 110V for 1 hr and transferred to polyvinylidene difluoride membrane at 4°C for one hr at 100 V. The membrane was blocked in Odyssey PBS blocking buffer (LI-COR, Lincoln, NE) for one hr at room temperature followed by incubation with primary antibody: mouse anti-SERCA2 (IID8, 1:1,000; Abcam, Cambridge, UK) or mouse anti-PLB (2D12, 1:1,000; Abcam, Cambridge, UK) overnight at 4°C. Blots were incubated with anti-mouse secondary antibodies IRDye680RD or IRDye800CW (1:10,000, LI-COR, Lincoln, NE) for 1hr at room temperature. The blots were imaged using LI-COR Odyssey
biomolecular imager and intensities were analyzed with Image Studio Lite Version 5.2 software (LI-COR, Lincoln, NE).

**Ligand-induced Stabilization of SERCA Biochemical Intermediates**

To fix SERCA in substrate-stabilized biochemical intermediates, various substrate solutions were prepared by addition of corresponding substrates to the calcium-free base solution which included KCl 100 mM, Imidazole 10 mM, MgCl$_2$ 5 mM, EGTA 2 mM, pH 7.0 (KOH). The following ligands were used to prepare specific solutions corresponding to SERCA biochemical state (in parenthesis): 100μM thapsigargin (E2-TG), 3mM ATP (E1-ATP), 2.1 mM CaCl$_2$ (E1-2Ca) with free [Ca$^{2+}$]=100 μM; 2.1 mM CaCl$_2$ and 3 mM ATP (E1-2Ca+ATP); 2.1 mM CaCl$_2$ and 3 mM ATP non-hydrolysable analog AMP-PCP (E1-2Ca+AMMPPCP); 2.1 mM CaCl$_2$, 3 mM ADP, 3mM KF and 50 μM AlCl$_3$ (E1P-2Ca+AlF$^-$+ADP); 0.1 mM orthovanadate (E2-Vi); 50 μM AlCl$_3$ and 3 mM KF (E2-AlF$_4^-$). Chemicals were obtained from SigmaAldrich, St. Louis, MO.

To measure SERCA intramolecular FRET in ligand-stabilized biochemical intermediates, 1μl of membrane preparations (7–10 μg total protein concentration) was thawed on ice and mixed with 9 μl of substrate solutions and immediately imaged using fluorescent microscopy, as described above.

**MRP-1 Functional Measurements**

I measured doxorubicin (DOX) extrusion from GFP-MRP-1 expressing cells to determine function of MRP-1 in the presence of candidate modulators. Transiently transfected cells plated on glass-bottom coverslip were pre-incubated with 10 μM each candidate compound for 15 min, and then 2 μM DOX was added for 1 hour. Cells were imaged by
confocal microscopy, and the intensity of the red fluorescence was used as an index of DOX accumulated in the cell nucleus. Cells expressing MRP-1 were identified by the presence of GFP fluorescence and compared to cells in the same field.

**Statistical Analysis**

Data are presented as the mean ± S.E.M. of ≥ 3 experiments unless specified in figure legend. All statistical tests were done using OriginPro 9.1 (OriginLab Corporation, Northampton, MA). Student’s t test (unpaired two-tailed) was used to compare the difference between two groups, one-way analysis of variance (ANOVA) was used to compare the difference between three or more groups, and two-way ANOVA was used to compare the difference between different groups under different treatment conditions. ANOVA was followed by Tukey's post hoc test. A probability (p) value of <0.05 was considered significant.
CHAPTER THREE

SERCA STRUCTURAL DYNAMICS DURING CALCIUM TRANSPORT

All-atom Molecular Dynamics Simulations of SERCA Point Mutants

Previous MD simulations of SERCA spontaneous movements indicated that a SERCA headpiece open-to-closed transition was initiated by an interaction between the A-domain and the Nβ5-β6 loop of the N-domain via electrostatic/H-bond interactions (Smolin and Robia, 2015a). The Nβ5-β6 loop (426-DYNEAKGYEK-436) was predicted to help in piercing the poorly-diffusible water layer and to support a stable, ordered arrangement of the N- and A-domains during Ca\textsuperscript{2+} cycling. When this loop was mutated in silico to remove the three negative charges (D426A/E429A/E435A, or AAA), SERCA was not able to obtain closed conformations.

To further investigate the role of Nβ5-β6 loop in SERCA headpiece dynamics, I extended these simulations for WT- and AAA-SERCA from 40 ns up to 100 ns as a more physiologically relevant time point, and performed additional simulations of SERCA Nβ5-β6 loop single-point mutants. Fig. 2 shows the starting structure of SERCA used for MD simulations (PDB accession code 1SU4 (Toyoshima et al., 2000)), highlighting the A-, N-, P-, and TM-domains, plus the Nβ5-β6 loop.

First, I examined SERCA global structural dynamics to confirm that the gross structure and motions of SERCA were not disrupted by the introduction of loop mutations.
I observed comparable domain RMSD for WT-, AAA- and SERCA point mutants, indicative of similar domain dynamics (Fig. 8A). Although the N-domain was the most dynamic, no substantial differences in its motions were observed between SERCA starting structures (Fig. 8B-C). SERCA Cα atoms RMSF, that present residue-by-residue motions, showed higher RMSF values for N-domain (Fig 8D). This observation also confirms that N-domain is the most dynamic for all SERCA structures. Nevertheless, all of the SERCA
structures had dynamics comparable to WT as shown in Fig. 8D by overlapping plots. Small RMSF increases (like for the A-domain’s and TM-helix’s residues for point mutants in Fig. 8D) was regarded as non-significant changes compared to RMSF changes reported for SERCA different structures 1SU4, 1WPG, and 2ZBD (Smolin and Robia, 2015a).

To inspect SERCA dynamics in more detail, I calculated angular autocorrelation of cytoplasmic domains for all SERCA structures, and compared the relative motions of the cytoplasmic domains to the starting orientation. This analysis allowed sampling of domains’ redistribution in the space and detected larger dynamics for N-domain as represented by larger angle values (Fig. 9A top panel). A-domain had the intermediate rotational dynamics (Fig. 9A center panel), while P-domain presented with smallest angular correlation values (Fig. 9A bottom panel). Similar angular autocorrelation values were observed for the WT and mutant SERCA structures for all of the domains (Fig. 9A), indicating the preservation of the cytosolic domains’ dynamics upon mutations.

Fig. 9B shows representative covariance matrices for WT-SERCA (upper left) and AAA-SERCA (lower right) as a residue-by-residue correlation of transporter motions. If residues move in the same direction (follow each other), they are considered to be positively correlated and presented in red color, while negative correlations represent motions in the opposing directions (toward or away from each other) and colored in blue. I observed a positive self-correlation within N-domain (residues 369-603) for all structures (Fig. 9B, red). This observation was expected, as generally the domain residues move in the same direction as a part of the whole domain. On the contrary, the correlation between the N- and A-domains was negative for WT-SERCA due to independent motions of these domains in opposing directions. Interestingly, motions of AAA-SERCA A- and N-domains
had null correlation (Fig. 9B, white), indicative of reduction of dependent motions of these domains. This observation that AAA-SERCA has reduced correlation between A- and N-domains motions signify that Nβ5-β6 loop mutations decrease domain-to-domain dependent dynamics. Besides that element, the rest of the WT- and AAA-SERCA structural motions were similar.

Even though the primary SERCA structural dynamics were conserved, the quantitative analysis of MD trajectories proved a very specific structural effect of Nβ5-β6
SERCA loop mutations increase A- and N-domains separation in silico.

WT-SERCA (black), AAA (red), D426A (blue), E429A (green), E435A (pink). Data represent average of 6 MD run productions. (A) Number of contacts between the N- and A-domains during MD trajectories. (B) Distribution of data in (A). (C) AAA-SERCA shows an increase in N–A-domains separation distance compared to WT-SERCA. (D) Distribution of data in (C). (E) Negative correlation of separation distance on domain–domain contacts, from results in (A) and (C). Pearson correlation $r = -0.99$, $p=0.002$, * $p=0.037$ for number of contacts.

loop mutations on the separation of the SERCA headpiece domains. In particular, substitution of three negative Nβ5-β6 loop residues to alanine decreased the N- and A-domains contacts (Fig. 10A) and increased minimal distance (Fig 10C). Figures 10B and 10D show distribution of sampled trajectories. When means of six trajectories for each of the number of contacts and distance between A- and N-domains were plotted against each other, the domains minimal distance and the number of contacts showed a negative correlation ($r = -1$) for WT-SERCA structure (Fig. 10E, black). This observation means that a short distance between domains was maintained by a large number of contacts. On the contrary, AAA-SERCA structure showed an opposing negative correlation presented by a
large domain separation and significantly fewer contacts (Fig. 10E, red). The single-point mutants were well-tolerated and resulted in intermediate phenotypes between AAA and WT values for domain-domain separations (Fig. 10E, blue, green, and magenta). This result supports the importance of Asp426, Glu429 and Glu435 residues in SERCA structural assembly.

Interestingly, single point mutant Glu429A had shown number of contacts between A- and N-domains comparable to WT, but increased domains separation (Fig. 10E, green). This observation suggests that one or both of the remaining residues, Asp426 and/or Glu435, are redistributed in space to form a large number of contacts, while keeping domains apart. Considering the fact that Glu429 is at the very tip of the SERCA Nβ5-β6 loop (Fig. 1), it is likely that residue Glu429 is the first residue of the N-domain to interact with the A-domain, and this residue may be defining for the decrease in the distance between cytosolic domains.

In conclusion, my in silico analysis of SERCA spontaneous motions detected preserved gross dynamics of SERCA mutants compared to WT (Fig. 8-9), suggesting maintenance of structural assembly upon introduction of mutations. On the other hand, SERCA A- and N-domains separation was significantly increased by removal of the Nβ5-β6 loop negative charges (mutation of Asp426, Glu429 and Glu435 to Alanine), which was aimed to prevention of the salt bridges/H-bonds between the Nβ5-β6 loop and the A-domain basic/positive residues. Thus, my all-atoms MD simulations implicated the Nβ5-β6 loop as a discrete structure that regulates SERCA headpiece closure (Fig. 10). Next, I set up to determine what effect this structural deficit in the cytosolic headpiece closure has on the transporter structural dynamics.
Comparison between Structural Dynamics of WT- and AAA-SERCA

To examine in detail SERCA motions and the effect of Nβ5-β6 loop mutations on SERCA structural dynamics, I performed a principal component analysis (PCA) of the ensembles of WT-and AAA-SERCA trajectories. PCA analysis determines how each trajectory sampled the major motions compared to the starting structure. Here I present two most dominant motions, PC1 (48% of all motions) and PC2 (15% of all motions). The principal motion PC1 was SERCA headpiece opening/closing, while the second major motion PC2 was twisting of the cytoplasmic domains. Fig. 11A summarizes positive and negative motions of the A- and N-domains along each component. To visualize the distribution of PC1 and 2, I plotted them against each other for each structure sampled every 10 ns in all of the trajectories (Fig. 11B). As a result, 6 different WT-SERCA trajectories sampled values ranging from -36 Å (closed) to +25 Å (open) (Fig. 11B9G, black), while 6 different AAA-SERCA trajectories (Fig. 11B, red) showed more population of open structures at the expense of closed
conformations (minimum of -30 Å, maximum of +31 Å). This result is consistent with the observed increased separation between N- and A-domains upon Nβ5-β6 loop mutations (Fig. 10). Along the second dominant motion PC2 axis (twisting of the cytoplasmic domains), WT showed several trajectories ranging from -27 to +23 Å, which was 56% greater than the range of AAA-SERCA along PC2 (from -14 to +18 Å) (Fig. 11B). Individual trajectories for WT- and AAA-SERCA are highlighted in different colors in Fig. 12B and C respectively and show a wider distribution of individual WT trajectories. The representative structures were extracted at the representative point of trajectory in each of

**Figure 12. Principal component analysis for WT- and AAA-SERCA.**

(A) Sampling of the first and second principal components for six WT-SERCA (black) trajectories and six AAA-SERCA (red) trajectories. Each graphical point (black and red) represents a structure extracted from trajectories at a time interval of 0.1 ns over the time course of 100 ns simulations. Turquoise dots indicate selected structures (shown in inset) from each structural quadrant. The arrows represent specific motions of the N-domain (yellow) and A-domain (blue). (B, C) Individual PC analysis for WT-SERCA (B) and AAA-SERCA (C), where the six different colors represent each of six individual MD simulations.
the quadrants as in figure 12A (green dots) and represent the conformational extremes. Overall, PCA indicates that the AAA-loop mutations decrease the range of motions of SERCA cytosolic domains along the first two principal components and shifts the population of structures toward a more open ensemble.

Additionally, I looked into SERCA headpiece ensemble in more detail by examining separation of cytosolic domains (Fig. 13). Minimal distance between A–N-domains was increasing over time, while distances between P- and A-domains or P-and N-domains were not changed (Fig. 13A). This result suggests that while A- and N-domains are separate from each other, it is the P-domain that retains contacts with both A- and N-domains constantly. Separation of A–N domains was expected due to high RMSD of these domains, especially N-domain (Fig. 8A). Consistent with the preceding observation (Fig 13A), numbers of contacts between P–N or P–A-domains were the highest, and I observed their matching changes along the trajectories (Fig. 13B). This result indicates that the P-domain may serve as an anchoring structure for cytosolic domains, while A- and N-domains separate from each other in distance (Fig. 13A) but still retain a minimal number of contacts (Fig. 13B). This deduction may be an alternative hypothesis to my earlier observation that residue Glu429 is critical for A- and N-domains minimal distance decrease (Fig. 10E).

Analysis of distance between each domain’s center of mass shows redistribution of domains in space. Specifically, I observed the same pattern for A–N and P–N-domains separation, while the P–A-domains position remained unchanged, which suggests that the P-domain is moving together with the A-domain, and they both follow more dynamic N-
domain motions. Overall, this analysis gives more insight on SERCA cytoplasmic dynamics: the P-domain serves as an anchoring structure for A- and N-domains, and dynamic motions of N-domain are principal for structural changes in remaining cytosolic domains.

Overall, my MD simulations detected a decrease in SERCA structural dynamics for the Nβ5-β6 loop triple mutation. Specifically, PC1 analysis sampled more open conformations in the expense of the closed ensembles, while PC2 had overall decreased distribution (Fig. 11-12). I believe that the direct consequence of the AAA-SERCA reduced dynamics is the observed deficit in AAA-SERCA headpiece closure (Fig. 10) and the Nβ5-β6 loop serves as SERCA structural determinant. Additionally, analysis of SERCA cytosolic domains’ spatial distribution suggested that P-domain serves as a coordinating domain between highly dynamic A–N-domains (Fig. 13). These insights into SERCA structural dynamics and identification of the Nβ5-β6 loop as SERCA structural determinant are important for understanding transporter function, which will be examined next.
The Role of the Nβ5-β6 Loop in SERCA ATPase Activity

To determine the possible functional significance of the Nβ5-β6 loop, I prepared microsomal vesicles from AAV-293 cells expressing 2-color WT-SERCA or AAA-SERCA and measured Ca²⁺-dependent ATPase activity. Fig. 14A shows a representative example of n = 4 independent experiments and demonstrates that the maximal activity of AAA-SERCA was decreased. Quantification of all 4 experiments yielded a 63±5% difference in ATPase maximal rate between WT and AAA transporter (p = 0.015) (Fig. 14C). This result is consistent with the previous finding by Inesi lab that mutation of Asp426 to Ala decreased SERCA ATPase activity by 61% (Inesi et al., 1988). Based on K_{Ca} values of 7.2±0.2 (WT) and 7.1±0.3 (AAA), the apparent Ca²⁺ affinity of AAA-SERCA was not significantly different from
that of WT-SERCA (p = 0.61) (Fig. 14B). I detected 2-color SERCA viral expression in microsomal preparations by the Western blotting analysis (Fig 14D). Since I observed a band for endogenous SERCA, I confirmed that endogenous SERCA activity was not significant and thus negligible by measuring Ca^{2+}-dependent ATPase activity in microsomes from AAV-293 cells transduced with control GFP vector (Fig. 14E, pink).

Overall, the data indicate that SERCA ATPase activity is decreased by mutations of negatively charged loop residues; nevertheless, the mutated transporter is still able to perform a complete enzymatic cycle. This functional ATPase assay confirmed the significance of the Nβ5-β6 loop in SERCA ATPase activity in vitro. To further investigate SERCA structural intermediates during the ATPase enzymatic cycle, I performed in vitro structural measurements of SERCA transitional states in the substrate-stabilized environment.

Quantification of FRET of 2-color SERCA Expressed in Microsomal Membranes

It has been previously demonstrated that 2-color SERCA FRET is increased with increasing Ca^{2+} (Hou et al., 2012). I performed 2-color FRET measurements in microsomal membranes prepared from AAV-293 cells expressing WT-SERCA with controlled Ca^{2+} concentrations. Fig. 15A shows that increasing Ca^{2+} resulted in a progressive shift toward a higher FRET values. The WT 2-color SERCA FRET response to Ca^{2+} had a K_{Ca} of 1.25±0.22 μM, which was different from 0.1 μM value obtained for the ATPase assay. I attribute this nearly 12-fold difference in K_{Ca} to the different composition of buffers for different assays. Even though I calculated free Ca^{2+} concentration using the Ca^{2+}/Mg^{2+}/ATP/EGTA Calculator from Theo Schoenmakers’ Chelator (Schoenmakers et al., 1992), it is not uncommon to observe a 10-fold discrepancy in K_{Ca} between published reports (Hou et al., 2012; Hughes
The observed Hill coefficient of 0.76 suggests the apparent lack of cooperativity, which is consistent with the proposition that the structural transition from low- to high-affinity states is complete after binding of the first Ca\(^{2+}\) to the transport site I (Cantilina et al., 1993; Chen et al., 2006; Henderson et al., 1994a; Henderson et al., 1994b; Inesi et al., 1988; and Middleton, 2014; Sahoo et al., 2015; Sahoo et al., 2013; Shaikh et al., 2016). The

**Figure 15. SERCA structural dynamics measured by FRET in microsomes.**

(A) WT 2-color SERCA showed an increase in FRET with Ca\(^{2+}\) increase. (B) FRET of 2-color SERCA stabilized in key enzymatic states. (C) FRET values for SERCA E1 and E2 conformers. Yellow highlights E1 states, while green highlights E2 states. Data represent at least 5 independent experiments. (D) Real-time measurements of SERCA FRET upon addition of Ca\(^{2+}\) with ATP solution shows SERCA FRET decrease and eventually gradual recovery. Red line represents exponential FRET recovery by Hill1 fit. Data represent 12 cells from 2 independent experiments.
Trieber et al., 2009). Overall, the data are compatible with my previous observation that upon increase in Ca\textsuperscript{2+} concentration with ionophore treatment, 2-color SERCA exhibit a high FRET compact state (Hou et al., 2012).

To investigate SERCA intermediates in the ATPase enzymatic cycle, I stabilized Ca\textsuperscript{2+} transporter conformers with biochemical substrates (MacLennan et al., 1997). I observed low FRET in the presence of ligands for the E2 (low Ca\textsuperscript{2+} affinity) intermediates and high FRET in the presence of ligands for E1 (high Ca\textsuperscript{2+} affinity) states. Specifically, E2-H\textsuperscript{+} (protonated SERCA) showed 9.9±0.6% FRET, and E2-TG, which locks SERCA in the Ca\textsuperscript{2+}-free E2 state (Sagara et al., 1992a; Sagara et al., 1992b) resulted in 10.3±0.5% FRET. E2P,

**Figure 16. Modified Post-Albers reaction cycle.** Blue boxes enclose states with similar intramolecular FRET efficiencies. E1 state are yellow and E2 states are green. Major transitional states are shown in black, while grey represent non-physiological state (TG-bound. E2-TG) and alternative pathway (Ca\textsuperscript{2+} binding prior to ATP binding, E1-2Ca). (a) ATP binding induces conformational changes that facilitate Ca\textsuperscript{2+} binding at the cytosolic membrane side. (b) P-domain phosphorylation and ATP hydrolysis. (c) Ca\textsuperscript{2+} release at the other side of membrane. (d) Dephosphorylation. (e) ATP binding. Steps (c-d) are predicted to be the slowest steps.
phosphoenzyme intermediates, (E2-AlF$_4^-$ and E2-V$_{ij}$) showed even lower ~8% FRET. In contrast, I observed higher FRET (above 15%) for microsomes in E1 states: E1-ATP, E1-2Ca, E1-2Ca+ATP, 2Ca+AMP-PCP, and E1-2Ca+ADP+AlF$_4^-$. The FRET values for each state are summarized in Table 1 (Fig. 15C). Overall, these data indicate that SERCA E1 conformers are more compact, while E2 conformers are more open headpiece conformations.

SERCA progression through the Ca$^{2+}$ transport cycle is powered by ATP hydrolysis. In my FRET detection of biochemically-stabilized conformers in SERCA-rich microsomal membranes I used 3 mM ATP, which is on the higher side of the physiological ATP concentration. Nevertheless, I wanted to confirm that ATP remains non-depleted and I detect true FRET values for the E1-ATP and E1-2Ca+ATP states. As an alternative to ATP, I used non-hydrolysable ATP analog- AMP-PCP, which showed FRET similar to E1-ATP state (~15%) (Fig. 15B). Additionally, I monitored FRET changes after ATP addition in real-time and observed an initial decrease with following recovery in 2-color SERCA FRET (Fig. 15D). I interpreted this result as a demonstration of several cycling SERCA phases: entering in active cycling (decrease), build-up of luminal Ca$^{2+}$ (increase), and steady-state cycling with inhibition by high luminal Ca$^{2+}$ or build-up of ADP (plateau) (Inesi and de Meis, 1989).

Based on these findings, it is most likely that in Fig. 15B for E1-2Ca+ATP state I was measuring a plateau steady-state cycling phase when SERCA is continuously cycling following addition of activated solution (2Ca+ATP) as in Fig. 15, and while ATP is not depleted, but rather significant ADP build-up results in SERCA accumulation in this compact conformation.
SERCA FRET measurements in a substrates-stabilized environment allowed identification of SERCA headpiece structural conformers for several intermediate states during Ca\(^{2+}\) transport (Fig. 15). As a result, in Fig. 16 I present a Post-Alberts reaction cycle supplemented with my data. I highlighted states with similar intramolecular FRET efficiencies in blue boxes: E1 states (yellow) have high FRET (~15%), while E2 states (green) have predominantly low FRET (~8-10%). My data demonstrates compact SERCA E1 conformers, while E2 conformers have more open headpiece conformation and indicate a shift in SERCA conformers depending on ligand presence during specific cycle steps. To provide complementary details for SERCA structural dynamics in physiological conditions, I next detected FRET responses to regulated Ca\(^{2+}\) levels in live cells.

**SERCA Structural Dynamics in AAV-293 Cells**

To determine if SERCA headpiece structural differences observed in MD simulations and *in vitro* were present in the cells, I used widefield microscopy and acceptor-sensitized FRET to measure SERCA headpiece dynamics in AAV-293 cells in response to Ca\(^{2+}\) elevations. The average basal FRET for 2-color SERCA was significantly lower for AAA- and all point mutant SERCA structures compared to WT-SERCA (Fig. 17A), which is indicative of a more open headpiece conformation upon N5\(\beta\)-6\(\beta\) loop mutations and supports my MD calculations (Fig. 10). It is remarkable that single point mutants D426A, E429A, and E435A showed decreased basal FRET intermediate between WT- and AAA-SERCA, which is consistent with intermediary opened headpiece phenotype I observed for these mutants in MD simulations (Fig10E). Thus, 2-color SERCA intramolecular FRET measurements in cells show the same specific structural effect of N\(\beta\)5-\(\beta\)6 loop mutations on separation of the SERCA headpiece domains as in my MD data.
Our lab had previously shown that 2-color SERCA FRET is markedly decreased with binding to TG (Hou et al., 2012). In accordance with this result, the average FRET observed for WT-, AAA-SERCA and all point mutants decreased momentarily with addition of 50 μM of TG (Fig. 17B). This decrease in average FRET indicates that TG binding at TM-domain induced structural changes in SERCA cytosolic headpiece. Thus, I believe the protein structure is intact and preserved after loop mutation. Notably, addition of DMSO vehicle did not result in FRET change (Fig 17B, navy).
Application of the Ca\textsuperscript{2+} ionophore ionomycin (Iono) allowed influx of Ca\textsuperscript{2+} into the cells expressing 2-color WT-SERCA and resulted at first in the immediate FRET decrease (phase 1 in Fig. 17C, black) and secondly, in the slow FRET recovery to around the starting FRET value (phase 2 in Fig. 17C, black). This observation indicates that rapid Ca\textsuperscript{2+} increase allows reorganization of SERCA population into a very open conformation, followed by emerging of closed SERCA conformers until reaching an equilibrium of mixed SERCA ensembles within a few minutes. All point mutants behaved similar to the WT-SERCA population in response to Iono (Fig17C, blue, green and pink) with similar kinetics of phase 2 FRET recovery (Fig.17D). In contrast, AAA-SERCA only exhibited phase 1 FRET change and was lacking recovery phase 2 (Fig17C, red). This result indicates that the mutation of just one of the negative residues of N5\beta-6\beta loop is not enough to abolish SERCA headpiece closure, while removal of all three negative H-bonding residues from the N5\beta-6\beta loop has a detrimental effect on SERCA obtaining compact state. Since AAA-SERCA responded normally to TG, I believe that the deficit in phase 2 of FRET change in response to Ca\textsuperscript{2+} influx was not due to a global structural defect, but instead due to lack of H-bonding N5\beta-6\beta loop residues that are regulate SERCA headpiece closure.

One of the future directions for this study is the detection of FRET during real-time structural changes that SERCA undergoes with addition of Ca\textsuperscript{2+} (or other substrates) in a fast-flow set up (Schaaf et al., 2017). These measurements will allow the detection of SERCA intermediate states in real-time with the variety of substrates and will significantly enhance the scientific understanding of SERCA structural dynamics during Ca\textsuperscript{2+} transport.

Overall, the in-cells FRET data (Fig. 17) are compatible with the MD simulation analysis that detected a significant defect in SERCA headpiece closure for AAA-SERCA,
while the point mutants exhibited intermediate between WT and AAA, or similar to WT-SERCA phenotype (Fig. 10). In discussion chapter I will further elaborate on the observed SERCA phase 1 and 2 FRET changes in response to sustained Ca$^{2+}$ influx. To advance scientific understanding of structural rearrangement that SERCA has to undergo during Ca$^{2+}$ cycling, I simultaneously monitored real-time changes in SERCA intramolecular FRET and spontaneous Ca$^{2+}$ oscillations in the cytosol or ER in live cells.

**SERCA Conformational Changes in Response to Intracellular Ca$^{2+}$ Dynamics**

To monitor simultaneous real-time changes in SERCA intramolecular FRET and spontaneous Ca$^{2+}$ fluctuations, I utilized the muscle-like system as described previously (Bovo et al., 2016). Briefly, I reconstituted elements of muscle cell Ca$^{2+}$ handling in AAV-293 cells by co-expression of GFP-RyR and 2CS. Ca$^{2+}$ was monitored in the cytosol with X-rhod-1 Ca$^{2+}$ indicator or in the ER with low-affinity Ca$^{2+}$ sensor R-CEPIA1er by confocal microscopy. SERCA conformational changes were detected as anti-correlated changes in Cer and YFP fluorescence (Fig. 18A) and intramolecular FRET was quantified as the YFP/Cer fluorescence ratio (Fig. 18B). In preceding experiments I observed high intramolecular FRET measured in microsomal membranes in the presence of high Ca$^{2+}$ and for all E2-2Ca states (Fig. 15A-B), as well as accumulation of SERCA in high FRET state after Iono treatment (Fig. 17C). Thus, I expected to detect high intramolecular FRET correlated to elevation of cytosolic Ca$^{2+}$ during spontaneous Ca$^{2+}$ release events. Surprisingly, Ca$^{2+}$ elevations in the cytosol corresponded to *decreases* in FRET (Fig. 18B), suggesting that SERCA population shifted to an open conformation. Similarly, SERCA intramolecular FRET dropped at the same time when ER Ca$^{2+}$ store was at the lowest (release event) (Fig. 18C), indicating that SERCA cytosolic headpiece shifted towards open conformation during Ca$^{2+}$
I noticed that while SERCA intramolecular FRET decrease was instantaneous at the moment of spontaneous Ca\(^{2+}\) release events (Fig. 18B-C), FRET increase happened in the middle of the gradual increase in ER Ca\(^{2+}\) before the full restoration of ER Ca\(^{2+}\) stores (Fig. 18C). This observation indicates that the ER continues to fill even after SERCA FRET is reverted to baseline and cytosolic Ca\(^{2+}\) is returned to its lowest (Fig. 18B). I believe that this phenomenon is due to the activity of store-operated Ca\(^{2+}\) entry mechanisms that function irrespective of cytosolic Ca\(^{2+}\) levels to fill ER Ca\(^{2+}\) store (Hogan and Rao, 2015). Nevertheless, the biggest paradox was the observation of SERCA

Figure 18. SERCA structural dynamics in response to spontaneous Ca\(^{2+}\) events measured by FRET in AAV-293 cells.

(A) Anti-correlated changes in Cer and YFP fluorescence intensity indicate rhythmic FRET fluctuations in intact cells. (B) The ratio of YFP/Cer, as in (A) was used as an index of FRET (top panel, grey). FRET was inversely correlated to cytosolic Ca\(^{2+}\), as measured by X-Rhod-1 fluorescence (black, bottom panel). (C) A decrease in 2-color SERCA intramolecular FRET (top trace) occurs simultaneously with depletion of ER Ca\(^{2+}\) stores (bottom trace). (D) AAA-SERCA FRET decreased during spontaneous cytosolic Ca\(^{2+}\) elevations.
intramolecular FRET decrease upon cytosolic Ca\textsuperscript{2+} increase, as I expected the complete opposite.

In addition to observed anti-correlated FRET/Ca\textsuperscript{2+} oscillations paradox during transient Ca\textsuperscript{2+} elevations, AAA-SERCA showed similar to WT anti-correlated intramolecular FRET/ intracellular Ca\textsuperscript{2+} oscillations (Fig. 18D). This observation was the opposite from what I expected based on my previous results when I observed that AAA-SERCA has a deficit in obtaining high FRET compact conformation in response to persistent Ca\textsuperscript{2+} influx upon Iono treatment (Fig. 17C, red).
I detected the same unexpected anti-correlated FRET and Ca\textsuperscript{2+} fluctuations during a different experimental set which was aimed to induce a transient cytosolic Ca\textsuperscript{2+} increase. The transient cytosolic Ca\textsuperscript{2+} elevation was triggered by treating cells expressing GFP-RyR and 2-color SERCA with caffeine (Caf), a RyR activator, which resulted in an immediate increase in cytosolic Ca\textsuperscript{2+} and its rapid decrease to a low level of cytosolic Ca\textsuperscript{2+} (Fig. 19B) due to activation of plasma membrane Ca\textsuperscript{2+} extruders. As expected for transient Ca\textsuperscript{2+} elevation, this event was mirrored by anti-correlated FRET changes (rapid decrease, then sustained increase). Again, opposing my previous observations that N\textbeta5-\textbeta6 loop triple mutant was not able to obtain high FRET compact conformation (Fig. 17C), AAA-SERCA reacted similarly to WT-SERCA to Caf-induced transient increase in cytosolic Ca\textsuperscript{2+}, presenting an initial FRET decrease which was followed for FRET increase (Fig. 19D).

To further investigate these unexpected observations, I measured SERCA response to Iono immediately after detecting unexpected anti-correlated FRET and cytosolic Ca\textsuperscript{2+} fluctuations. First, I monitored the puzzling negative correlation in SERCA intramolecular FRET and cytosolic Ca\textsuperscript{2+} levels for both WT- and AAA-SERCA before the addition of Iono (Fig. 19A, C). Then, immediately after Iono addition, WT-SERCA intramolecular FRET exhibited a biphasic response (rapid decrease and slow increase) (Fig. 19A), and AAA-SERCA intramolecular FRET only presented phase 1 (rapid decrease) and was lacking phase 2 of FRET recovery (Fig. 19C), as observed earlier (Fig. 17C). Combining FRET measurements during transient and then sustained Ca\textsuperscript{2+} elevations in one experiment showed that both of the opposing FRET changes indeed take place. Since the major difference that was determining the FRET change directions in response to Ca\textsuperscript{2+} elevations was the level of Ca\textsuperscript{2+} influx, I concluded that the anti-correlated FRET and cytosolic Ca\textsuperscript{2+}
Oscillations are characteristic for transient Ca\(^{2+}\) elevations, while correlation between FRET and cytosolic Ca\(^{2+}\) levels is a distinctive response to sustained cytosolic Ca\(^{2+}\) increase. Thus, the anti-correlated simultaneous FRET and cytosolic Ca\(^{2+}\) fluctuations are observed during transient intramolecular Ca\(^{2+}\) dynamics.

It is noteworthy, that intracellular spontaneous Ca\(^{2+}\) oscillations were hard to detect in cells expressing AAA-SERCA compared to cells transfected with WT-SERCA. Additionally, in those AAA-SERCA cells that displayed intracellular spontaneous Ca\(^{2+}\) fluctuations, the frequency of Ca\(^{2+}\) release events was lower compared to cells transfected with WT-SERCA (compare Fig. 18B vs. 18D). These observations demonstrate that in AAA-SERCA cells, ER Ca\(^{2+}\) stores are not sufficient to activate RyR for spontaneous Ca\(^{2+}\) release, and/or AAA-SERCA has slower Ca\(^{2+}\) transport kinetics and requires longer time to fill in the ER lumen than WT-SERCA. I propose that these functional deficiencies arise from the AAA-SERCA defective headpiece closure mechanism.

It has been reported that SERCA forms dimers which enhances SERCA activity (Blackwell et al., 2016) and potentially SERCA headpiece dynamics. I tested if observed
FRET fluctuations were due to changes in intermolecular FRET between two SERCA molecules rather than representing SERCA headpiece dynamics. As expected, I detected intermolecular FRET between Cer-SERCA and YFP-SERCA in muscle reconstituted experimental conditions (Fig. 20A). Nevertheless, intermolecular FRET did not change in response to spontaneous Ca\(^{2+}\) releases (Fig. 20B) or Iono addition (Fig. 20C). Thus, I concluded that observed FRET changes in response to cytosolic Ca\(^{2+}\) elevation were a result of genuine intramolecular structural rearrangements and not induced by SERCA oligomerization events. More elaborate explanation on the cause of the FRET differences in response to Ca\(^{2+}\) levels are provided in the discussion section.

It has been previously proposed that SERCA saturation in E2-2Ca state can occur as a result of the inhibition by increased luminal Ca\(^{2+}\) (Inesi and de Meis, 1989). To test if observed slow FRET recovery phase 2 (Fig. 16C) was due to saturation of SERCA in the E2-2Ca state, I monitored SERCA intramolecular FRET simultaneously with ER Ca\(^{2+}\) buildup (Fig. 21A). After I detected correlated FRET decrease upon ER Ca\(^{2+}\) store release events, I saturated cytosol with Ca\(^{2+}\) by means of Iono treatment, and observed expected biphasic fluctuations.

**Figure 21. SERCA structural dynamics in correlation with ER Ca\(^{2+}\) changes**

(A) Addition of Iono causes an increase in ER Ca\(^{2+}\) content, but this increase occurs more slowly than the second phase of the observed FRET response of 2-color SERCA. (B) 2CS FRET does not change with addition of PBS. Data represent representative traces.
FRET response along with a slow accumulation of Ca\(^{2+}\) in the ER. Ca\(^{2+}\) build-up in ER occurred much more slowly that the phase 2 FRET change, which suggests that the phase 2 FRET change in Fig. 16C is not due to saturation of SERCA by luminal Ca\(^{2+}\) binding.

I also confirmed that the observed change in the FRET ratio or ER Ca\(^{2+}\) fluctuations was not triggered by the addition of control PBS solution, which indicate that my technique does not introduce motion artifacts that could interfere with fluorescence quantification (Fig. 21B).

Overall, monitoring of simultaneous real-time changes in SERCA intramolecular FRET and spontaneous Ca\(^{2+}\) fluctuations detected SERCA population accumulated in low FRET state (open headpiece architecture) with increased cytosolic Ca\(^{2+}\) levels, while a decrease in the cytosolic Ca\(^{2+}\) resulted in the redistribution of SERCA population into high FRET state (closed headpiece conformation) (Fig. 18B). This observation was the opposite from what I expected based on my in cells Iono experiments, where I observed a FRET increase in response to Ca\(^{2+}\) elevations (Fig. 17C). Another unexpected observation was that the defective in headpiece closure AAA-SERCA population had displayed similar to WT anti-correlated FRET response to cytosolic Ca\(^{2+}\) oscillations. I propose that both of these paradoxical to my previous observations are due SERCA accumulation in the specific slow steps of Ca\(^{2+}\) transport cycle. I will elaborate in the substantial detail on both the anti-correlated FRET and Ca\(^{2+}\) changes and the normal AAA-SERCA FRET response to transient Ca\(^{2+}\) elevation in discussion chapter.
CHAPTER FOUR
SERCA STRUCTURAL DYNAMICS IN COMPLEX WITH PLB

All-atoms MD Simulations of WT- or AAA-SERCA in the Complex with PLB

Previous MD simulations of SERCA structural dynamics predicted that the Nβ5-β6 loop was pointing toward PLB binding cleft and in acidic loop residues Asp426, Glu429, and Glu435 were in the position to interact with various positive residues of PLB cytosolic domain (Smolin and Robia, 2015a). Here, I investigated this possibility by performing runs and analysis of 100 ns MD simulations of structural dynamics of WT- or AAA-SERCA complexes with non-phosphorylated PLB or with PLB phosphorylated on Ser16 (PLBpS16). Fig. 22A shows the starting x-ray crystal structure of SERCA-PLB complex that was used for MD simulations: PDB accession code 4KYT (Akin et al., 2013). Since PLB cytosolic domain was not resolved due to its dynamic structure, the cytoplasmic part of PLB from crystal structure PDB access code 1ZLL was added (by former research assistant Dr. Nikolai Smolin). The A-domain is highlighted in blue, N-domain is in yellow, P-domain is in black, TM-domain is in grey, Nβ5-β6 loop is in orange, and PLB is in red. For each complex, three independent production runs were executed.

Previously, my lab’s MD simulations of SERCA spontaneous motions detected an increase in SERCA A-N domains separation when the triple Nβ5-β6 loop mutation was introduced (Fig. 10). PLB association with SERCA was not able to interfere with this specific effect of
loop mutations, and I observed a 2 Å increase in distance between centers of A- and N-domains’ masses for AAA-SERCA compared to WT-SERCA complexes (Fig. 22C-D).

Additionally, I observed a greater deviation in distances between A- and N-domains of individual trajectories upon triple loop mutation as detected by greater distribution of trajectories from the starting separation distance of approximately 40 Å (Fig. 22B, red line).

This observation indicates that triple Nβ5-β6 loop mutation interferes with SERCA cytosolic headpiece closure, and also destabilizes A- and N-domain interactions.
Nevertheless, phosphorylation of Ser16 did not facilitate detectable differences in distance between A- and N-domain for either WT- or AAA-SERCA structures (Fig. 22B-D).

Considering that phosphorylation introduces negative charges to the residue with phosphoryl group addition, and according to my structural analysis A-domain has a cluster of positive residues (133-139) accessible for PLB binding, I hypothesized that Ser16 interaction with A-domain would be enhanced by PLB phosphorylation. As predicted, phosphorylation increased Ser16 interaction with A-domain from 4.7±3 to 7.6±1.6 for WT-SERCA and from 1.2±1.5 to 6.9±1.3 for AAA-SERCA (Fig. 22E). These results indicate that PLB interaction with A-domain is enhanced by Ser16 phosphorylation. Additionally, I observed a similar number of contacts between the Nβ5-β6 loop and PLB independent of PLB phosphorylation on Ser16 or Nβ5-β6 loop mutations which indicated a stable interaction between the Nβ5-β6 loop and PLB (Fig. 22F).

Based on the observations that PLB stably interacts with SERCA Nβ5-β6 loop and that phosphorylated Ser16 has an increased interaction with A-domain, I further hypothesized that PLB serves as a connecting structure between N- and A-domains via interaction with Nβ5-β6 loop, while Ser16 phosphorylation allows more effective 'bridging' of A–N-domains. To further investigate this hypothesis, I extended MD simulations for WT-SERCA in complex with non-phosphorylated PLB or phosphorylated on Ser16 PLBpS16 from 100 ns up to 200 ns.

**Extended MD Simulations of SERCA and PLB/PLBpS16 Complexes**

I started with analysis of the effect of Ser16 phosphorylation on the global structure and dynamics of SERCA-PLB complex.
observed comparable global structural dynamics for SERCA in complex with PLB and PLBpS16 by detecting similar RMSD trajectories in SERCA-domains motions (Fig. 23A) and relatively comparable residue-specific RMSF for both complex structures, SERCA (Fig. 23B) and PLB (Fig. 23C). It may be worth noticing that PLB Ser16 phosphorylation induced a slight increase in motion (~1 Å) for unstructured linker between cytosolic and TM-domains (Fig. 23C, residues 20-26). This result suggests that Ser16 phosphorylation changes PLB structural dynamics, which will be investigated in more detail below (Fig. 27, 28).

Interestingly, the amplitude of SERCA N-domain motions was reduced from the maximal of ~11 Å for SERCA alone (Fig. 8D) to a maximal of ~5 Å in complex with PLB (Fig. 23B). This result indicates that PLB binding results in more compact conformation of SERCA headpiece.

Figure 23. RMSD and RMSF of SERCA-PLB complex structural dynamics calculated from MD simulations

(A) Individual SERCA domains (orange N-domain, blue A-domain, grey P-domain, and black TM-domain) show similar global Cα RMSD fluctuations by WT-SERCA in complex with PLB and Ser16 phosphorylated PLB. (B) RMSF analysis of SERCA Cα atoms revealed that the A- and N-domains are the most mobile, while with similar relative structural dynamics. (C) RMSF analysis of PLB Cα atoms shows similar residue-specific fluctuations for non-phosphorylated (black) and phosphorylated on Ser16 PLB (red). Data represent mean ± S.D. of n=3.
Next, I confirmed that SERCA Nβ5-β6 loop interaction with PLB was still preserved on the extended time-scale. The number of contacts between PLB and Nβ5-β6 loop was similar with a slight increase from 17.7±6.4 to 21.3±14.3 upon Ser16 phosphorylation (Fig. 24 A-B), suggesting that PLB phosphorylation could increase interaction with Nβ5-β6 loop on extended time-scale. I also tested to determine if my previous observation that PLB Ser16 phosphorylation increases the number of contacts with the A-domain for the extended time-scale. Fig. 24C shows a red trace that presents an average of three independent trajectories for SERCA-PLBpS16 complexes above the black trace (SERCA-PLB) for most of the interval of 0-100 ns. Nevertheless, at the interval of 100-200 ns
SERCA-PLBpS16 is characterized by the decreased number of contacts compared to SERCA-PLB (Fig. 24C), indicating an alteration of the PLB cytosolic domain position in regard to the A-domain. This result is unexpected but not surprising, as PLB cytosolic domain is known to be very dynamic, and extending my MD simulations is necessary to provide more details on the PLB cytosolic domain dynamics.

Since my preceding hypothesis implied that negative charges introduced to Ser16 by phosphorylation strengthen the interaction with SERCA A-domain positively charged cluster (residue 133-139), I tested if the number of contacts between Ser16 and two positive residues on A-domain surface, Arg139 and Arg134, was affected by phosphorylated Ser16 separation from A-domain. I observed a reduction in the number of contacts between SERCA Arg139 and PLB Ser16 from 5.8±7.6 to 0.9±1.5 upon phosphorylation (Fig. 24E), while for Arg134 the number of contacts was below 0.2 (Fig. 24F) independent of Ser16 phosphorylation, meaning that this interaction is not likely to occur. In Figure 24E-F I observed a large deviation in the SERCA-PLB number of contacts measured between PLB Ser16 and Arg134 and 139. This result was most likely due to the fact that PLB cytoplasmic domain (residues 1-23, including Ser16), is extremely dynamic and briefly samples variety of sites on A-domain, including analyzed residues Arg134 and Arg139, and in this way displays a great variation in the numbers of contacts it forms. These data indicate that phosphorylation of PLB on Ser16 does not just reduce interaction between PLB Ser16 and SERCA A-domain, but specifically with the positive cluster on the A-domain surface.

I tested to determine if an extension of SERCA-PLB complex MD simulations resolved the effect of PLB Ser16 phosphorylation on SERCA headpiece dynamics and
calculated the number of contacts between N-A-domains for the 200 ns production runs. I detected no difference between SERCA-PLB and SERCA-PLBpS16 complexes in A- and N-domains separation (Fig. 24G-H). Interestingly, SERCA headpiece closure was observed as the distance between centers of masses of A- and N-domains decreased by over 5 Å during MD trajectories (Fig. 24G). This observation indicates SERCA spontaneous headpiece closure in the presence of PLB, and is complementary to induction of compact headpiece conformation which I observed as a decrease in RMSF of the most dynamic N-domain in the presence of PLB (Fig. 8D and 23B).

Since I was not able to resolve specific effects of PLB Ser16 phosphorylation on SERCA structural dynamics during the simulated time, I decided to test if I could identify differences in the relative motions within the SERCA-PLB complexes by covariance correlation.

**Figure 25.** PLB cytosolic domain motions are positively correlated with SERCA headpiece structural dynamics.
Representative of n=3 covariance matrices Cα atoms for SERCA-PLB (A) and SERCA-PLBpS16 (B). Covariance analysis of residue dynamics as measured from Cα revealed positively (red) and negatively (blue) correlated motions. PLB residues are plotted after SERCA as residues 995-1046.
Fig. 25 shows representative covariance matrices for the SERCA-PLB (A) and the SERCA-PLBpS16 (B) complexes as a residue-by-residue correlation of motions. If residues move in the same directions, they are considered to be positively correlated (red), while negative correlations represent motions in the opposing directions (blue). As for SERCA alone (Fig 9D), I detected a positive self-correlation within the N-domain (residues 369-603). In contrast to SERCA structure alone, the correlations within the A- and N-domains and between A–N-domains were positive for both SERCA-PLB complexes, suggesting that PLB association with SERCA increases dependent motions of these domains. Overall, the covariance matrixes for SERCA in complex with PLB were presented by more red colors (positively correlated motions) (Fig. 25) compared to SERCA alone matrix (Fig. 9D), which indicates that PLB binding induces coordinated motions within SERCA intramolecular interactions. Another interesting observation was the occurrence of mostly positive correlation between the motions of PLB cytoplasmic domain (residues 1-23) and SERCA A- and N-domains for both non-phosphorylated and phosphorylated Ser16 complexes (Fig. 25), which indicates that dynamics of PLB cytoplasmic helix are coordinated with SERCA headpiece dynamics. This conclusion is consistent with the preceding MD observation that PLB cytosolic domain interaction with SERCA headpiece is persistent (Fig. 24B).

To summarize, my all-atom MD simulations of SERCA-PLB/PLBpS16 complexes identified more ordered SERCA conformation induced by PLB binding as detected by the reduction in the N-domain dynamics (Fig. 8D and 23B) and more coordinated motions of SERCA cytoplasmic domains (Fig. 9 and 25). Additionally, I observed a stable interaction of PLB cytosolic domain with the SERCA N5β-6β loop irrespective of Ser16 phosphorylation (Fig. 24A-B). These data suggest that the induction of SERCA ordered conformation is due
to the PLB coordination via SERCA N5β-6β loop. Thus, it is the PLB binding itself, not the Ser16 phosphorylation, which regulates SERCA compact conformation and ordered structural dynamics. I further performed MD simulations analysis targeted to resolve effects of Ser16 on SERCA-PLB structural dynamics.

**Effect of Ser16 Phosphorylation on SERCA-PLB Structural Dynamics**

According to the subunit model, when SERCA inhibition by PLB is relieved by PLB phosphorylation, PLB shifts to a new position on SERCA TM-domain (Bidwell et al., 2011; Negash et al., 2000). Nevertheless, the precise position of phosphorylated PLB on SERCA TM-domain after the shift is not yet determined. Therefore, I examined the change in interaction between TM-domain of PLB and SERCA TM-domain. I calculated the number of contacts between PLB TM-helix (Fig. 26A, red licorice structure, residues 23-52) and SERCA M-helices that comprise the PLB docking groove (Fig. 26A, M2-helix, residues 89-119, pink; M4-helix, residues 313-330, blue; M6-helix, residues 789-809, violet; M9-helix, residues 932-950, green). I detected a slight reduction in the number of contacts between PLB TM-helix and SERCA M2-helix from 99.2±10.8 to 88.9±8.4 upon PLB Ser16 phosphorylation. At the same time I detected an increase in the number of contacts from 52.4±5.0 to 56.8±6.6 between PLB TM-helix and SERCA M9-helix, and no change for M4- and M6-helices. These data correspond to a displacement of PLB TM-domain from SERCA M2-helix onto M9-helix. Nevertheless, the detected changes in the number of contacts are relatively low (~10% for M2-helix and ~3% for M9-helix), and further investigations are required to determine if the PLB TM-domain shift from the SERCA M2- to the M9-helix is a considerable event.
The SERCA acidic residues Glu309, Glu770, Asp779, and Glu908 have been identified as the Ca\textsuperscript{2+} transport site II with Glu309 being the ‘gating’ residue (Clarke et al., 1989). A recent publication by Autry et al (Autry et al., 2016) described structural
rearrangements in the Ca\(^{2+}\) transport site II induced by sarcolipin, an inhibitory peptide, specifically emphasizing displacement of the gating residue Glu309. Since sarcolipin binds to the same SERCA helixes as PLB, I tested to determine if PLB phosphorylation induced a change in the transport site II gating mechanism by comparing minimal distances between SERCA Glu309 and remaining residues of Ca\(^{2+}\) transport site II (residues 799, 770, and 908). I detected an increase in distance between Glu309 and transport site II from 12.6±1.0 Å to 13.3±0.2 Å upon PLB S16 phosphorylation (Fig. 26C-D), confirming distortion of the Glu309 gating mechanism for Ca\(^{2+}\) transport site II upon PLB Ser16 phosphorylation. Since the size of a Ca\(^{2+}\) ion is only 1.14 Å, the 0.7 Å increase in transport site may translate into considerable structural rearrangements for Ca\(^{2+}\) accommodation or displacement.

I detected displacement of PLB TM-domain from SERCA M2-helix onto M9-helix and distortion of the Glu309 gating mechanism for Ca\(^{2+}\) transport site II upon PLB Ser16 phosphorylation. These specific changes induced by PLB Ser16 phosphorylation indicate that phosphorylation of PLB cytosolic domain on Ser16 has an effect on SERCA-PLB transmembrane interactions which allow transport of Ca\(^{2+}\) ions. This counter-intuitive conclusion has been suggested by several reports (Autry et al., 2016; Espinoza-Fonseca et al., 2015a; Espinoza-Fonseca et al., 2015b; Espinoza-Fonseca and Ramirez-Salinas, 2015) and required further investigation.

**SERCA-PLB Structural Dynamics Compared to FRET in Live Cells**

Previously, my lab had reported a decrease in FRET efficiency between the SERCA N-terminus and the N-terminus of PLB from 25±1.2% for non-phosphorylatable PLB-Ser16Ala to 14±1.3% for phosphomimetic PLB-Ser16Glu (Hou et al., 2008), which indicated a decrease in distance between the SERCA N-terminus and the N-terminus of PLB upon PLB
phosphorylation. I calculated the minimal distance between the SERCA N-terminus and the PLB N-terminus (Fig. 27A) from MD simulations and were able to detect an average of a 4.5 Å decrease in minimal distances upon PLB phosphorylation (Fig. 27B-C). This result suggests that while MD simulations and FRET measurements are different, these techniques can be used to complement each other.

**Figure 27.** 200 ns MD simulations of SERCA-PLB structural dynamics compared to FRET experiments in cells.
For B-C and E-F data represent mean ± S.D. of 3 trajectories. (A) SERCA-PLB complex structure with SERCA N-terminus and residue 509 highlighted in red. (B) Minimal distance between SERCA N-terminus and residue 509. (C) Quantification of C. (D) SERCA-PLB complex structure with SERCA N-terminus and PLB N-terminus highlighted in red. (E) Minimal distance calculated for SERCA-PLB complexes. (F) Quantification of (E) shows a decrease between SERCA and PLB upon PLB phosphorylation on Ser16.
I used fluorescently labeled 2-color SERCA in acceptor sensitization FRET experiments in live cells to investigate SERCA cytosolic headpiece structural dynamics (Gruber et al., 2014; Hou et al., 2012; Pallikkuth et al., 2013). The Cer fluorophore is attached to N-terminus (before residue 1) while YFP is inserted before residue 509 within A-domain (Fig. 27D). I hypothesized that I can predict in silico the structural dynamics of SERCA headpiece upon PLB Ser16 phosphorylation, as would be detected by FRET in live cells. I used MD generated trajectories to calculate the minimal distance between SERCA N-terminus and residue 509. Analysis of MD production runs showed no significant separation of SERCA N-terminus and residue 509 upon PLB phosphorylation on Ser16 (Fig. 27E-F). This observation suggests that the PLBpS16 effect on SERCA structure in live cells could be hard to resolve. On the other hand, the structural changes observed in real-time in live cells are happening on the scale of μs and higher, and most likely further extension of my MD runs is necessary to observe substantial SERCA structural changes in complex with PLB that will correspond to in-cells observations.

**PLB Structural Dynamics in Complex with SERCA**

It has been shown that PLB phosphorylation affects PLB structure and intramolecular interactions (Li et al., 2003; Petithory and Jencks, 1986; Sayadi and Feig, 2013; Sugita et al., 2006). It is plausible to propose that PLB intramolecular changes induced by Ser16 phosphorylation could regulate SERCA structural dynamics to increase SERCA activity, especially with my preceding observations that PLB phosphorylation increased SERCA N-domain order (Fig. 9B vs. 23B) and that relative motions of A–N-domains and PLB are correlated (Fig. 25). Thus, I set to examine some potential changes in PLB structure and intramolecular interactions that has been proposed by numerous studies.
PLB Ser16 phosphorylation has been reported to decrease the distance between TM-domain residue Cys24 and cytosolic domain residue Tyr6 by 3 Å, as well as stabilize the hinge region between PLB TM- and cytosolic domains (Li et al., 2003). Calculated minimal distance for these residues (Fig. 28A) from my MD production runs showed 5.9 Å increase in distance between Cys24 and Tyr6 upon Ser16 phosphorylation (Fig. 28B-C). This contradiction may arise from the differences in physiological solutions used for FRET experiments and current MD simulation system set-up, and stresses an importance of complementary techniques. Nevertheless, these results implicated a separation of PLB cytosolic domain from TM-domain upon Ser16 phosphorylation.

Figure 28. PLB intramolecular interactions upon Ser16 phosphorylation. For B-C and E-G data represent the mean ± S.D. of 3 trajectories. (A) SERCA-PLB complex structure with PLB Cys24 and Tyr6 residues highlighted in violet licorice representation. (B) Minimal distances calculated between PLB Cys24 and Tyr6 show an increase in separation between these residues upon Ser16 phosphorylation (red). (C) Quantification of B. (D) SERCA-PLB complex structure with PLB Ser16, Arg9, Arg13 and Arg14 highlighted in violet licorice representation. (E-F) Minimal distance between PLB Ser16 and Arg14 (E) and Ser16 and Arg13 (F) is reduced upon Ser16 phosphorylation (red). (G) Minimal distance between PLB Ser16 and Arg14 is not affected by Ser16 phosphorylation (red).
The phosphoryl group on Ser16 has been shown to interact with PLB cytosolic residues Arg9, Arg13 and Arg14 (Fig. 28D) to unwind the cytosolic helix and affect PLB’s inhibitory interactions with SERCA (Sugita et al., 2006). I tested to determine if Ser16 interaction with the positive arginine cluster on PLB cytosolic domain was increased with phosphorylation by calculating minimal distances between Ser16 and each of Arg9, Arg13, and Arg14. I observed a decrease in separation between Ser16 and neighboring Arg14 by 2.2 Å, between Ser16 and Arg13 by 0.8 Å, and no detectable changes between Ser16 and furthermost Arg9 (Fig. 28E-G). These data confirm that the Ser16 interacts with the cytosolic arginine cluster upon Ser16 phosphorylation, supposedly via H-bonding formed
by the negative phosphoryl group. Importantly, such interaction of neighboring residues will most likely introduce a natural kink in PLB structure and result in more compact and stabilized structural composition of PLB. I next examined PLB structural composition.

In order to investigate changes in PLB structure upon Ser16 phosphorylation, I calculated the percentage of structured and unstructured elements. I detected an increase from 60.0±4.5% to 65.0±4.6% in PLB overall structure as a result of Ser16 phosphorylation (Fig. 29A). Further investigation revealed that α-helical organization was increased by from 52.0±4.0% to 56.0±3.6% (Fig. 29B), while more unstructured configurations, such as coil and bend, were decreased by from 26.3±5.0% to 23.3±4.9% and from 10.7±1.5% to 9.3±1.5% respectively (Fig. 29C-D). Specifically, α-helicity of the cytosolic domain was increased by from 60.9±13.1% to 77.3±6.0% upon PLB phosphorylation (Fig. 29E). Thus, my data suggest that PLB phosphorylation on Ser16 induces more structure and order in PLB conformation.

With PLB Ser16 phosphorylation I detected an increased separation of PLB cytosolic and TM domains while predating a kink in PLB domains’ linker due to phosphorylated Ser16 increased interaction with Arg13/Arg14 cluster (Fig. 28). Such interesting structural PLB arrangement would be possible if the kink results in twisting of PLB cytosolic domain upward. To investigate PLB structural arrangement in more detail, I calculated the angle between PLB cytoplasmic and TM-domains by assigning vector generated between Cα of residues 12 and Cα of residues 4 as the cytoplasmic domain vector, while the vector of Cα of residues 23 and Cα of residues 52 as TM-domain vector (Fig. 29F) (Sayadi and Feig, 2013). I detected a 31.6° increase in PLB intra-domain angle (Fig. 28G-H), suggesting that PLB cytosolic domain unfolds/twists from TM-domain and obtains more upward
conformation upon Ser16 phosphorylation. This result complements a detected increase in minimal distance between TM residue Cys24 and cytoplasmic domain Tyr6 (Fig. 28B-C) and confirms the presents of the kink in the Ser16 region introduced by increased interaction between Ser16 and cytosolic Arginine cluster (Fig. 87E-F). One possible explanation for such strange structural arrangement when the PLB cytosolic domain is directed upwards with a kink in Ser16 region could be the preceding observation that the PLB cytoplasmic domain strongly interacts with the SERCA Nβ5-β6 loop (Fig. 2A-B) and thus oriented upward, toward SERCA headpiece domain.

Overall, PLB Ser16 phosphorylation has a pronounced effect on PLB structure. I detected a separation of PLB cytosolic domain from the TM-domain by an increase in distance (Fig. 28) and an angle (Fig. 29G-H) between these two domains. Additionally, I observed an increase in PLB structural organization with detecting more structured and less unstructured PLB intramolecular assemblies with Ser16 phosphorylation (Fig. 29A-E). These results provide an alternative explanation to the question how the phosphorylation of PLB cytosolic domain results in SERCA TM domain rearrangement to accommodate Ca\(^{2+}\) ions transport: detected reorganizations of PLB structural composition result in adjusted PLB-SERCA TM interactions, which may lead to PLB shift to a distinct binding groove and rearrange Ca\(^{2+}\) transport sites. I extended my studies of PLB-SERCA structural dynamics upon PLB binding and PLB Ser16 phosphorylation in vitro.

**SERCA Conformational Changes in Complex with PLB measured by FRET**

To further investigate the SERCA-PLB regulatory complex, I performed an analysis of SERCA structural conformers in vitro. Acceptor-sensitized FRET was used to examine conformation of the SERCA cytosolic headpiece in the presence of non-phosphorylatable or
phosphomimetic PLB. I prepared microsomal membranes from cells co-expressing WT 2-color SERCA alone, SERCA with phosphomimetic PLB-Ser16Glu, and SERCA with non-phosphorylatable PLB-Ser16Ala. Fig. 30A shows FRET increase with Ca\(^{2+}\) concentration for SERCA alone or in complex with PLB. The apparent Ca\(^{2+}\) affinity was not changed by PLB binding as \(K_{Ca}\) was not significantly changed in PLB presence (Fig. 30B). Overall, I detected an increase in 2-color SERCA FRET with Ca\(^{2+}\) concentration, and SERCA intramolecular FRET increase was observed in complex with PLB, especially in complex with phosphomimetic PLB-Ser16Glu. This observation indicates that phosphorylated PLB stabilizes SERCA in the compact conformation.

SERCA conformational states were stabilized with substrates to characterize the intermediates of the Ca\(^{2+}\) transport cycle (Fig. 30C-D) (MacLennan et al., 1997). As for SERCA alone (Fig. 15B), I observed low FRET for SERCA conditions under which E2 conformers are expected to be predominant: protonated SERCA (H\(^+\)), TG-bound, and E2P phosphoenzyme intermediates (AlF\(_4^-\) and V\(_i\)). Higher FRET was observed for microsomes in E1 states: ATP-bound, Ca\(^{2+}\)-bound (2Ca), and both ATP and Ca\(^{2+}\)-bound (2Ca+ATP) intermediates. The FRET values for each intermediate are presented in Fig. 31. In agreement with my previous observations (Fig. 30A), I detected higher FRET for SERCA in complex with PLB, with the highest values attained in the presence of phosphomimetic PLB-Ser16Glu. These data confirm that SERCA headpiece obtains very compact conformations during high-affinity E1 states of enzymatic cycle, and, overall, the SERCA headpiece structure obtains even more compact conformations upon PLB phosphorylation.

It has been shown that overexpression of SERCA in AAV-293 cells dramatically enhances SERCA ability to transport Ca\(^{2+}\) from the cytosol, while PLB decreases SERCA
mediated Ca\(^{2+}\) sequestering into ER (Bidwell et al., 2011). To determine the differences in SERCA Ca\(^{2+}\) transport in complex with phosphomimetic or wild-type PLB, I performed Ca\(^{2+}\) uptake in live cells which enabled the monitoring of SERCA function as Ca\(^{2+}\) transient in the presence of PLB (1 nM SERCA to 5 nM PLB transfection ratio of DNA constructs). Since PLB phosphorylation has been shown to relieve SERCA inhibition (Simmerman et al., 1986; Tada and Inui, 1983), I predicted that phosphomimetic PLB should have similar to or more of overexpressed WT-SERCA phenotype in sequestering Ca\(^{2+}\) from cytosol. Fig. 32A shows AAV-293 cells expressing Cer-SERCA (blue) and YFP-PLB (yellow), while the cytosolic Ca\(^{2+}\) is labeled with Ca\(^{2+}\) indicator X-Rhod-1 (red), and white arrows are indicating untransfected (UT) cells in the same field. I triggered inositol 1,4,5-trisphosphate receptors-mediated Ca\(^{2+}\) release from ER by activation of inositol 1,4,5-trisphosphate receptors on ER membrane via stimulation of purinergic receptor with extracellular ATP (Dubyak and el-Moatassim, 1993). Ca\(^{2+}\) accumulation in the cytosol was detected by an increase in X-Rhod-1 fluorescence (Fig. 32B). I detected endogenous SERCA activity (Fig. 32B, black) as a decrease in cytosolic Ca\(^{2+}\) fluorescence. Subsequent application of SERCA
inhibitor TG was designed to determine Ca\(^{2+}\) store remaining in ER and resulted in a rise in Ca\(^{2+}\) signaling as I detected an increase in X-Rhod-1 fluorescence. Cells co-transfected with SERCA and WT-PLB (Fig. 32B, red) displayed similar to exogenous SERCA response to ATP and Ca\(^{2+}\) transport, as was expected, considering that PLB inhibits SERCA function.

Nevertheless, ER Ca\(^{2+}\) storage was larger than for UT cells, which suggests that SERCA inhibited by PLB has greater than endogenous SERCA capabilities to sequester Ca\(^{2+}\). This observation is contradictory to published reports, where SERCA in complex with PLB has been shown to have similar to exogenous SERCA Ca\(^{2+}\) transport activity (Bidwell et al., 2011). Even more surprising was Ca\(^{2+}\) transient observed for SERCA in complex with phosphomimetic PLB-Ser16Glu/Thr17Glu (Fig. 32B, blue). Instead of expected smaller than UT response to ATP and larger Ca\(^{2+}\) release from ER stores upon TG treatment, I observed responses similar to SERCA in complex with WT-PLB, which suggests that phosphorylation of PLB has no effect on SERCA activity in this experimental set-up. Since I performed Ca\(^{2+}\) measurements in live cells, it is possible that overexpressed WT-PLB gets phosphorylated in response to increased cytosolic Ca\(^{2+}\) by the cells’ endogenous PKA. To

<table>
<thead>
<tr>
<th>E1/E2 states</th>
<th>no PLB Mean FRET (%)</th>
<th>S.E.</th>
<th>PLB-Ser16 Ala Mean FRET (%)</th>
<th>S.E.</th>
<th>PLB-Ser16Glu Mean FRET (%)</th>
<th>S.E.</th>
</tr>
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<tbody>
<tr>
<td>TG</td>
<td>10.8 1.1</td>
<td></td>
<td>15.4 1.3</td>
<td></td>
<td>18.2 1.4</td>
<td></td>
</tr>
<tr>
<td>H(^{+})</td>
<td>12.1 1.3</td>
<td></td>
<td>16.3 2.3</td>
<td></td>
<td>18.6 2.4</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>17.7 2.6</td>
<td></td>
<td>20.8 3.2</td>
<td></td>
<td>23.9 3.4</td>
<td></td>
</tr>
<tr>
<td>2Ca</td>
<td>16.6 1.4</td>
<td></td>
<td>20.0 1.3</td>
<td></td>
<td>23.2 2.3</td>
<td></td>
</tr>
<tr>
<td>2Ca+ATP</td>
<td>16.9 0.8</td>
<td></td>
<td>19.7 1.0</td>
<td></td>
<td>23.0 2.1</td>
<td></td>
</tr>
<tr>
<td>V(_{i})</td>
<td>9.9 0.8</td>
<td></td>
<td>13.5 2.4</td>
<td></td>
<td>16.5 3</td>
<td></td>
</tr>
<tr>
<td>AlF(_{4})</td>
<td>9.2 3.4</td>
<td></td>
<td>13.1 3.1</td>
<td></td>
<td>18.24 1.4</td>
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</tr>
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**Figure 31. FRET values for SERCA E1 and E2 states.**

Intramolecular FRET was measured in ER microsomes from AAV-293 cells expressing 2-color SERCA without PLB or with non-phosphorylatable PLB-Ser16Ala or phosphomimetic PLB-Ser16Glu. Yellow highlights E1 states, while green highlights E3 states. Data represent the mean ± S.E. of at least 3 independent experiments.
optimize this experiment, I would need to repeat measurements with a non-
phosphorylated version of PLB-Ser16Ala, as well as determine the true ratio of PLB.
Another aspect for optimization would be determination of the in-cell SERCA: PLB ratio to
determine that enough PLB is expressed to overcome PLB pentamers formation and allow
association with SERCA (I have used up to 1 nM SERCA to 20 nM PLB ratio of constructs’
expression for FRET measurements). Overall, PLB seems to inhibit overexpressed SERCA
activity, and ER Ca\textsuperscript{2+} stores are increased for overexpressed SERCA in complex with PLB.

Our FRET structural data indicate that PLB binding to SERCA leads to SERCA
cytosolic domain compact conformation, which is further enhanced by PLB Ser16
phosphorylation (Fig. 30A). Based on the substrate-stabilized SERCA intermediates of
enzymatic cycle experiment, PLB binding and phosphorylation induce compact
conformation in all transitional states of Ca\textsuperscript{2+} transport (Fig. 30C), which suggests that
SERCA compact conformation may be of a regulatory significance. To elucidate the details
of structural aspects of SERCA regulation by PLB, further investigations are necessary. In
particular Ca\textsuperscript{2+} handling in live cells in the presence of PLB variants are of great interest, as
well as extended MD simulation of SERCA-PLB complex spontaneous dynamics.
CHAPTER FIVE
APPLICATION OF 2-COLOR FRET BIOSENSORS IN IDENTIFICATION OF REGULATORY INTERACTIONS

Our lab has generated the 2-color SERCA FRET sensor (Hou et al., 2012) which was used in this work, as well as in multiple assays aimed to determine SERCA structural dynamics (Blackwell et al., 2016; Hou et al., 2012; Pallikkuth et al., 2013) and potential substrates (Cornea et al., 2013; Gruber et al., 2014). Additionally, my lab has demonstrated that the technique to generate 2-color FRET biosensor can be applied to other molecules. For example, 2-color MRP-1, another transporter ATPase, was generated and used in FRET high-throughput screening for identification of novel substrates (Iram et al., 2015). For both SERCA and MRP-1 transporters, the most mobile domains were labeled with donor and acceptor fluorescent proteins to monitor structural changes upon interaction with potential modulators. The identified substrates were further tested to determine their functional effect. Thus, my lab has developed a general technique of 2-color FRET biosensor generation which can be applied to a wide variety of molecules. In support to this statement, I will next describe several examples of the 2-color FRET biosensors use for detections and investigations of regulatory interactions.

SERCA and Hax-1 Direct Interactions as Assessed by FRET

Hax-1 has been shown to interact with SERCA in vitro (Vafiadaki et al., 2009). Nevertheless, the direct interaction of HAX-1 and SERCA in vivo has not been observed and is highly
To observe the potential interaction in cells, I tested to determine if Hax-1 has an effect on SERCA intramolecular FRET in AAV-293 cells expressing unlabeled Hax-1 and 2-color SERCA (Cer fluorophore on N-terminus and YFP before residue 509 within N-domain). I detected an apparent decrease in intramolecular FRET for 2-color SERCA in the presence of Hax-1 using the acceptor sensitization technique, but not the acceptor photobleaching method (Fig. 33A-B). Even though the 2-color SERCA by itself showed FRET values similar to those reported previously (Hou et al., 2012), the main concern about 2-color SERCA FRET in the presence of Hax-1 is the possibility that the YFP fusion site on the SERCA N-domain before residue 509 can be too close to the proposed Hax-1 binding site (Fig. 32C, loop consisting of residues E575-V594, red) (Vafiadaki et al., 2009; Vandecaetsbeek et al., 2011). Binding of Hax-1 to this highly accessible N-domain loop would result in Hax-1 being physically in the way of measuring
Nevertheless, the detection of a FRET decrease in the presence of HAX-1 (Fig. 33A-B) indicates direct binding of HAX-1 to SERCA, and that this interaction indeed occurs in live cells. Following investigations are needed to determine how stable this interaction is and if there is a functional aspect to this direct association of HAX-1 with SERCA.

**Analysis of SERCA Association with DWORF**

Recently identified by Dr. Eric Olson group micropeptide encoded by IncRNA DWORF has been proposed to bind to SERCA in the same binding site as SERCA inhibitor PLB as presented in Fig. 34A-B, thus relieving SERCA inhibition by displacement of PLB (Nelson et al., 2016). Nevertheless, the details of SERCA-DWORF association have not been investigated due to the fact that discovery of DWORF micropeptide is relatively recent. I used progressive acceptor photobleaching FRET measurements to investigate DWORF interaction with SERCA and/or PLB, as well as DWORF ability to form homo-dimers. Fig. 34C shows that DWORF-SERCA complex has lower FRET compared to PLB-SERCA complex (Fig. 34D). The lower FRET can be explained by shorter topology of DWORF (Fig. 34A) compared to PLB (Fig. 34B). PLB truncated all the way to TM-domain has been shown to have decreased FRET compared to full length PLB (data by previous lab member Phil Bidwell, not published). Plotting Cer vs. YFP resulted in a linear relationship of donor and acceptor fluorophores (Fig. 34E) consistent with 1:1 stoichiometry. This result means that DWORF binds to SERCA as a monomer to form a heterodimer (one Cer for one YFP detected), the same way as PLB (Blackwell et al., 2016; Kelly et al., 2008). I detected no FRET between DWORF-DWORF (Fig. 34F), consistent with the suggestion that DWORF is monomeric (Nelson et al., 2016). Additionally, I did not detect FRET between DWORF-PLB (Fig. 34G), indicating that DWORF does not form hetero-oligomers with PLB. Overall, these
data confirmed DWORF association with SERCA in 1:1 stoichiometry and showed that DWORF most likely exists as a monomer and does not form homo- or heterodimers.

When using the acceptor sensitization FRET method, DWORF-SERCA FRET was also decreased compared to PLB-SERCA FRET (Fig 35A-B). I observed smaller $K_d$ of DWORF compared to PLB (Fig. 35C), which indicates that DWORF has higher apparent affinity for SERCA than PLB. This observation indicates that targeting PLB displacement with DWORF overexpression can become a novel approach to enhance SERCA activity in cardiac dysfunctions.
To investigate if DWORF competes with PLB for association with SERCA, I performed competition experiments. Specifically, I measured FRET between Cer-SERCA and YFP-DWORF or YFP-PLB in the presence of increasing DWORF expression. The maximal SERCA-to-PLB FRET was progressively decreased by increased co-expression of DWORF (Fig. 35D-E), which indicated that DWORF indeed competes with PLB for the same binding site. Since I have shown that DWORF does not oligomerize with PLB (Fig. 34G), this decrease is not due to PLB being sequestered away from SERCA into DWORF-PLB hetero-oligomeric complexes. Additionally, I saw a progressive decrease in the apparent PLB-to-SERCA $K_d$.
parameter (Fig. 35F). This result indicates that PLB’s apparent affinity to SERCA increases in the presence of DWORF. This is exactly what I would expect if PLB is competed away- a positive correlation between an increased number of free PLB molecules and their affinity for SERCA.

With the present FRET measurements, I still cannot exclude the possibility that DWORF binds to SERCA at different from the PLB binding site(s) and alters SERCA-PLB complex structure which results in a FRET decrease. Further investigations are needed to determine the details of structural regulations of SERCA activity by DWORF.

Overall, I confirmed that DWORF binds to SERCA with higher affinity than PLB (Fig. 35C) most likely at the same binding site (Fig. 35D-E) in one to one ratio (one DWORF molecule per one SERCA) (Fig. 34E). I showed that primarily DWORF exists as a monomer and does not form oligomers with itself or PLB. Since DWORF positively regulates SERCA activity (Nelson et al., 2016) and has a higher affinity to SERCA than PLB (Fig. 35C), DWORF is very likely to become a high significance novel therapeutic target to enhance cardiac function.

**Quantification of DOX Uptake by AAV-293 Cells to Assess MRP-1 Transport Activity**

2-color SERCA pump has been successfully used to identify modulators in a high-throughput screen of a library of biologically active compounds (Gruber et al., 2014). My lab had applied the same labeling technique to generate 2-color multi-drug resistance protein-1 (MRP-1) biosensor with GFP and TagRFP fluorophores attached to the two most dynamic cytosolic nucleotide-binding domains, which were predicted to undergo transition from open-to-closed conformation during extrusion of substrates from the cytosol (Aller et al., 2009). 2-color MRP-1 FRET measurements were performed to screen for and identify
prospective transporter modulators (Iram et al., 2015). As a result, several MRP-1 substrates were detected: MK571 (known MRP1 inhibitor), epigallocatechin gallate (antioxidant), mesalamine (anti-inflammatory), calcipotriol (analog of vitamin D), and meropenem (antibiotic). To define these substrates' functions, I tested to determine if these compounds are able to alter MRP-1 activity.

I used doxorubicin (DOX), one of the MRP-1 substrates, to perform assessment of MRP-1 function in the presence of the identified substrates. When DOX is not transported out of the cell, it accumulates in the nuclei as detected by the presence of red florescence (Fig. 36B-C, UT). Overexpressed MRP-1 prevents DOX accumulation in the nuclei and I saw great reduction of red signal in cells nuclei (Fig. 36B-C, MRP-1). Treatment of cells with
known MRP-1 inhibitor MK571, one of the high-throughput screening hits, resulted in DOX accumulation in cells’ nuclei as would be expected for a transporter inhibitor. Calcipotriol inhibited MRP-1 transport comparable to MK571 with similar DOX levels detected in cells’ nuclei (Fig. 36B-C, calcipotriol). Since calcipotriol is a vitamin D analog, this result explains the fact that vitamin D has been demonstrated to potentiate the effect of chemotherapeutic agents (Ma et al., 2010). Epigallocatechin gallate and meropenem exhibited no effect on MRP-1 transport activity, even though they induced structural changes to MRP-1 organization in preceding high-throughput screening. Mesalamine interfered with DOX redistribution in UT cells, and was not used to evaluate MRP-1 function (Fig. 36B-C, mesalamine).

Overall, I was able to confirm two hit compounds as MRP-1 inhibitors, MK571 and calcipotriol, which demonstrated the potential of utilizing 2-color FRET sensors in combination of high-throughput screening analysis with functional assay in order to identify novel modulators of protein of interest. This technique has a potential to become a powerful tool in clinical drug discovery.
CHAPTER SIX
DISCUSSION

While SERCA is one of the major regulators of Ca\(^{2+}\) handling in the heart, and thus the ideal target to improve cardiac function (Hayward et al., 2015; Inesi et al., 2008), at the moment, no successful therapeutic approaches have been developed to increase SERCA activity. The promising gene therapy clinical trials designed to increase SERCA expression in patients’ failing hearts have not yet achieved success in improving patients’ cardiac function (Zsebo et al., 2014) and highlighted the apparent lack of understanding of SERCA structure and function. One of the goals of this work was to contribute to understanding of SERCA function via thorough investigation of SERCA structural dynamics during Ca\(^{2+}\) transport. The strength of the current study was in a combination of in silico, in vitro, and ex vivo assays that resulted in a detailed evaluation of SERCA structural and regulatory dynamics.

**Structural Dynamics of WT-SERCA During Ca\(^{2+}\) Transport**

SERCA FRET measurements in a substrates-stabilized environment allowed identification of SERCA headpiece structural conformers for several intermediate states during Ca\(^{2+}\) transport (Fig. 15). I modified a Post-Alberts reaction cycle by adding my FRET data and emphasizing states with similar intramolecular FRET efficiencies in blue boxes: SERCA high-calcium affinity E1 states (yellow) are presented by compact conformers (~15% FRET), while SERCA low-calcium affinity E2 states (green) have more open headpiece conformation (~8% and ~10% FRET) during specific cycle steps (Fig. 16).
Figure 16 presents SERCA ground protonated state E2 and biochemically equivalent TG-bound state (E2-TG) (Satoh et al., 2011; Toyoshima et al., 2000), both of which displayed low FRET states (~10%) suggesting SERCA open conformers. These in vitro results were confirmed by ex vivo measurements of SERCA structural conformation in cells, where TG also sequestered SERCA population into low FRET state (Fig. 17B). Thus, both measurements consistently resulted in detection of low FRET.

On the other hand, SERCA biochemically-stabilized intermediate states of enzymatic cycle representing physiological Ca\(^{2+}\) bound states E1-2Ca-ATP and E1P-2Ca, as well as non-physiological state E1-2Ca, all displayed high FRET states (~15%) characterizing SERCA closed conformers. I also detected high 2-color SERCA FRET in AAV-293 cells treated with Iono (Fig. 17C, black). However, I observed low FRET during spontaneous Ca\(^{2+}\) release events (Fig. 18B). Here, I will elaborate on these apparently inconsistent observations, which are actually the major findings of this work.

To resolve the deceptive discrepancies of the detected FRET changes in response to intracellular Ca\(^{2+}\) oscillations in cells, I treated cells with Iono after detection of the spontaneous anti-correlated FRET and cytosolic Ca\(^{2+}\) fluctuations (Fig. 19A). Figure 37, left panels, shows a schematic representation of one spontaneous anti-correlated FRET/Ca\(^{2+}\) change and FRET biphasic response to Iono treatment. High-starting SERCA FRET (Fig. 37A, blue arrows) was observed in live cells, which are characterized by low basal Ca\(^{2+}\) (up to 200 nM) and high ATP (up to 5mM). Thus, SERCA is saturated with ATP but not Ca\(^{2+}\) (Fig. 37A, E1-ATP), the same conditions as in non-muscle cells and in relaxed muscle cells.

I observed high SERCA FRET in microsomal preparations when both ATP and Ca\(^{2+}\) were high (Fig. 15B and Fig. 16, E1-2CA-ATP). However, in the cells during intracellular
Ca$^{2+}$ oscillations, I observed low FRET upon cytosolic Ca$^{2+}$ increase (Fig 18B). Numerous reports suggested that exposure to low Ca$^{2+}$ shifts ATP-bound SERCA (E1-ATP) into high-affinity conformer primed for efficient Ca$^{2+}$ binding and transport upon major Ca$^{2+}$ influx (Inesi et al., 2006; Jensen et al., 2006; Mintz et al., 1995). Thus, SERCA in resting E1-ATP state is readily activated upon influx of cytosolic Ca$^{2+}$. In support to this statement and in contrast to biochemically ‘fixed’ SERCA intermediates, in live cells, where ATP levels are high, SERCA starts cycling immediately upon Ca$^{2+}$ release event. However, because of the

Figure 37. WT-SERCA population accumulates in states prior to slow steps during transient Ca$^{2+}$ increase.

Schematic representation of simultaneous FRET and cytosolic Ca$^{2+}$ fluctuations as detected in Fig. 19A is on the left, while corresponding transitional steps are highlighted by blue arrows on the Post-Albers cycle (left panels). (A) In basal Ca$^{2+}$, SERCA population is at resting E1-ATP state (high FRET- open conformers) (B) During transient Ca$^{2+}$ increase, SERCA population accumulates in E2P prior to slow P$_i$ release step (low FRET- open conformers). I propose the existence of SERCA Ca$^{2+}$-bound low FRET state (open conformers) just before Ca$^{2+}$ release event, another slow step of enzymatic cycle.
slow steps in SERCA enzymatic cycle, like during release of P_i group (Champeil et al., 1986; Hanel and Jencks, 1990; Petithory and Jencks, 1986), SERCA population accumulates in E2 low FRET state (open conformers) (Fig. 37B, E2P). These conditions occur during contractions of cardiac or skeletal muscle cells and in non-muscle cells during intracellular Ca^{2+} waves.

Ca^{2+} release was also identified as one of the slow steps of SERCA enzymatic cycle (Fernandez-Belda et al., 1984). Based on this statement and the fact that I detected accumulation of majority of pumps in low FRET state, I propose the existence of Ca^{2+}-bound state, which is most likely short-lived and exists right before Ca^{2+} ions are released into ER lumen (Fig. 37B, '?'). After each spontaneous Ca^{2+} release event, probably due to SERCA function and temporary inactivation of RyR, cytosolic Ca^{2+} concentration drops and SERCA population returns to low Ca^{2+}/high ATP conformation of high FRET state (Fig. 37A, E1-ATP).

In contrast to transient intracellular Ca^{2+} increases, during sustained Ca^{2+} elevation upon Iono treatment, even though SERCA population originally accumulates in high Ca^{2+}/high ATP low FRET state (open conformers), the majority of SERCA pumps gradually shifts into high FRET state (compact conformers) (Fig. 17B and 19A). The immediate decrease in phase 1 FRET upon the addition of Iono matches the prediction that the transition of pumps to E2 conformation (Fig. 38A) is the slowest step of the Ca^{2+} catalytic cycle, and the majority of SERCA pumps accumulates in E2P (or anticipated low FRET Ca^{2+}-bound) state(s).

Less clear was an increase in phase 2 FRET change observed in Iono-treated cells (Fig. 17C). In this experiment the cytosolic Ca^{2+} remained high while FRET gradually
increased over a period of several minutes. This observation was surprising as I only expected to detect phase 1 low FRET of SERCA population accumulated in the slow step of Ca\textsuperscript{2+} release (Fig. 38A, E2P). I confirmed my observation in a muscle reconstituted setup when Iono was applied to cells after detection of anti-correlated FRET/Ca\textsuperscript{2+} changes and caused both the initial low FRET phase 1 and the following high FRET phase 2 (Fig. 19A). I propose several possible explanations for the phase 2 FRET change in Iono-treated cells.

Figure 38. WT-SERCA population accumulates in states prior to slow steps during sustained Ca\textsuperscript{2+} increase.
Schematic representation of simultaneous FRET and cytosolic Ca\textsuperscript{2+} fluctuations as detected in Fig. 19A is on the left, while corresponding transitional steps are highlighted by blue arrows on the Post-Albers cycle (left panels). (A) Upon Iono treatment, phase 1 FRET decrease (open conformers) are observed due to accumulation of WT-SERCA in E2P step of enzymatic cycle. (B) Phase 2 FRET increase (closed conformers) of WT-SERCA during sustained Ca\textsuperscript{2+} increase are observed due to accumulation of WT-SERCA in E1-2Ca-ADP bound step of enzymatic cycle.
First, Iono application as low as 2 μM has been shown to activate Ca²⁺/H⁺ exchangers and increase intracellular pH due to Ca²⁺ influx (Yamada et al., 1996). Fluorescent proteins are known to be highly sensitive to pH outside of cellular levels range (Schmitt et al., 2014). Therefore, an increase in intracellular pH (alkalinization) could affect fluorescent proteins and result in non-relevant FRET change. Nevertheless, the experiment with Cer-SERCA and YFP-SERCA (Fig. 20C) did not detect intermolecular FRET change upon addition of Iono. This, it is unlikely that cell alkanization was the direct cause of observed phase 2 FRET change.

Second, luminal Ca²⁺ accumulation have been shown to inhibit SERCA in a non-active state (Inesi and de Meis, 1989). Nevertheless, I detected slow accumulation of Ca²⁺ in ER lumen after Iono treatment, much slower than phase 2 FRET change (Fig. 21A). Therefore, I do not believe that the luminal Ca²⁺ may inhibit SERCA in non-active state.

Third, I cannot exclude inhibition of SERCA cycling by the ADP build-up (Inesi and de Meis, 1989) as ADP to ATP ratio was shown to be dramatically increased upon cells perturbation (up to 100 times) due to distortion of the mitochondrial membrane (Tantama et al., 2013). Therefore, SERCA activation may gradually deplete ATP and increase ADP levels in Iono-treated cells, which would shift SERCA population into non-physiological high Ca²⁺/ADP bound state (Fig. 38B, E1-2Ca-ADP) which is consistent with observed high FRET (~15%) in ligand-stabilized conformations E1-2Ca-ADP-AlF₄⁻ (Fig. 15B and 16).

To summarize, in the cells with low basal Ca²⁺ and high ATP levels, SERCA accumulates in E1-ATP high FRET state (compact conformation). During temporary cytosolic Ca²⁺ elevation, SERCA population progresses through E1-2Ca-ATP and phosphoenzyme E1P-2Ca states and accumulates in the slow E2P low FRET states (open
conformers), which is the main and novel funding of current project. Thus, in physiological conditions during contractions of cardiac or skeletal muscle cells, majority of the actively cycling SERCA population has an open headpiece conformation.

**Structural Dynamics of AAA-SERCA, the Nβ5-β6 Loop Mutant, During Ca²⁺ Transport**

The experiments with AAA-SERCA, which has key residues of the Nβ5-β6 loop mutated, allowed the understanding of the SERCA conformers’ redistribution during Ca²⁺ cycling in the presence of a defective headpiece closure mechanism. Even though, I observed AAA-SERCA phase 1 FRET decrease after Iono application similar to WT, the subsequent recovery and accumulation of SERCA in high FRET state was not achieved (Fig 17C and 19C). These data indicate that AAA-SERCA population was accumulating in a specific for AAA-SERCA slow step, E2 low FRET states (open conformers), and was not able to progress to E1 high FRET states (compact conformers) due to decreased kinetics of structural transition from open to closed headpiece conformations (Fig. 39).

Based on these results, I was surprised to observe AAA-SERCA FRET change kinetics similar to WT during spontaneous Ca²⁺ release events (Fig. 18D). I speculate that while overexpressed AAA-SERCA has a deficit in obtaining closed conformation, endogenous SERCA sequesters Ca²⁺ back into ER lumen while Na²⁺/Ca²⁺ exchangers and plasma membrane Ca²⁺ ATPases remove Ca²⁺ from the cell, which results in cytosolic Ca²⁺ levels decrease. Thus, when intracellular Ca²⁺ levels return to basal level, AAA-SERCA population redistributes back to the resting E1-ATP high FRET state (Fig. 16, E1-ATP). This statement is supported by the observed AAA-SERCA FRET decrease in response to transient elevations of Ca²⁺ upon RyR inhibitor Caf treatment, and following the increase in FRET as Ca²⁺ gets
exported from the cell, supposedly via \( \text{Na}^{2+}/\text{Ca}^{2+} \) exchangers and plasma membrane \( \text{Ca}^{2+} \) ATPases (Fig. 19D).

To summarize, when AAA-SERCA is exposed to persistent elevation of \( \text{Ca}^{2+} \), like upon Iono treatment, the defective in opened-to-closed transition pumps accumulate in a new and AAA-SERCA specific slow step, E2 low FRET states (open conformers). As a consequence, phase 2 FRET recovery is not observed (Fig. 17C and 17C).

**The N\( \beta \)5-\( \beta \)6 Loop as a Determinant of SERCA Structural Dynamics and Function**

Previously, Dr. Nikolai Smolin had predicted that the SERCA N\( \beta \)5-\( \beta \)6 loop plays an important role in SERCA headpiece transition from open-to-closed conformation (Smolin and Robia, 2015a). Specifically, salt bridges/H-bonds between the N\( \beta \)5-\( \beta \)6 loop acidic/negative residues (Asp426, Glu429, and Glu435) and basic/positive residues of the A-domain (residues133-139) were predicted to initiate the contact between N- and A-
domains and subsequent closure of the headpiece. The current study was designed to investigate a role of the Nβ5-β6 loop in the SERCA dynamics during Ca^{2+} transport and transporter function. To prevent the formation of the salt bridges/H-bonds between Nβ5-β6 loop and the A-domain, I designed triple Alanine mutant of these three negatively charged residues (AAA) or point mutants (Asp426Ala, Glu429Ala, and Glu435Ala). Generated SERCA constructs were used to analyzed SERCA structure and function during Ca^{2+} transport.

The all-atoms MD simulations showed that even though AAA-SERCA had conserved overall structural integrity (Fig. 8-9), it was deficient in headpiece closure (Fig. 10) and had reduced structural dynamics (Fig. 11). Interestingly, mutation of just one of the Nβ5-β6 loop residues was not sufficient to induce these detrimental changes; although, Glu429 may have a more prominent role in A- and N-domains closure than other two residues (Fig. 10D). Overall, my MD simulations indicated that the Nβ5-β6 loop facilitate SERCA structural transition to compact headpiece conformation and regulate transporter structural dynamics.

The in silico results were confirmed by ex vivo measurements of SERCA structural dynamics by FRET, which detected a deficit in SERCA triple loop mutant transition from open-to-closed conformation (Fig. 17C). Again, the point loop mutants showed similar to WT-SERCA kinetics during transition from open-to-closed conformation (Fig. 17D). Since I detected AAA-SERCA population in a low FRET state with application of TG similar to WT(Fig. 17B), I excluded global defects in transporter structure upon Nβ5-β6 loop mutations and attributed AAA-SERCA deficiency in headpiece closure to a specific role of the Nβ5-β6 loop negative residues.
I revealed functional significance of the SERCA Nβ5-β6 loop in ATPase assay by detecting a 63% reduction in SERCA maximal ATPase activity for AAA-SERCA compared to WT transporter (Fig. 15A, C). Detected decline of SERCA function was not attributed to the reduction in Ca²⁺ affinity as mutated transporter binds Ca²⁺ with similar to WT apparent affinity (Fig. 15B). I propose that detected functional deficit in SERCA ATPase activity is a direct consequences of AAA mutant deficit in structural transition from opened to closed headpiece conformation.

Overall, I identified the SERCA Nβ5-β6 loop as a determinant of SERCA structural dynamics and function by presenting evidence that the Nβ5-β6 loop facilitates closure of SERCA headpiece and regulates ATPase activity.

**Structural Dynamics of SERCA-PLB Complex**

Previous MD simulation by my lab predicted that the SERCA Nβ5-β6 loop can interact with PLB cytoplasmic domain. (Smolin and Robia, 2015a). Here I performed additional MD simulations to confirm that interaction between the SERCA Nβ5-β6 loop and PLB was stabilized throughout the MD trajectories and independent of loop mutations (Fig. 22F) or PLB phosphorylation (Fig. 24A-B). Additionally, the SERCA structure was stabilized by association with PLB as detected by reduction in RMSF N-domain (Fig. 8D vs. 23B) and *in vitro* (Fig. 30), which indicates that PLB induces more compact SERCA conformation as proposed by my lab previously (Bidwell et al., 2011). I speculate that the induction of more ordered SERCA headpiece conformation may be a result of overall structural stabilization by PLB. In support to this statement, I observed positive correlation between motions of the PLB cytosolic domain and the SERCA headpiece domains (Fig. 25), as well as increase in SERCA intramolecular motions as represented by predominantly red color (positive
correlation) of SERCA-PLB covariance matrixes (Fig. 25) versus white color (no correlation) of SERCA alone (Fig. 9).

Even though I did not detect a particular effect of Ser16 phosphorylation on SERCA headpiece structural dynamics (Fig. 23 and 24G-H), I resolved structural rearrangements within the SERCA TM-domain upon phosphorylation. Ser16 phosphorylation induced a slight shift of PLB from the SERCA TM-helix M2 toward helix M9 (Fig. 26A-B) at the same time as the Ca$^{2+}$ transport site II was increased by displacement of gating residue Glu309 by 0.7 Å, which may be a substantial structural rearrangements to accommodate passage of 1.14 Å Ca$^{2+}$ ion. This observation supports SERCA relief-of-inhibition subunit model, which states that upon phosphorylation PLB shifts to a new position on the SERCA TM-domain (Bidwell et al., 2011; Negash et al., 2000). Additionally, I demonstrated that like sarcoplin (Autry et al., 2016), PLB can induce structural rearrangements in the Ca$^{2+}$ transport site II by displacement of gating residue Glu309, possibly for accommodation of Ca$^{2+}$ ion passage.

Overall, these data demonstrate that phosphorylation of the cytosolic PLB domain induces SERCA compact conformation and structural rearrangements within the SERCA TM-domain, which most likely has functional significance for efficient Ca$^{2+}$ transport.

When I examined changes within PLB itself, I detected that PLB phosphorylation introduced a kink in the area of the phosphorylation site Ser16 (Fig. 27), increased separation of the PLB TM- and cytosolic domains (Fig. 27 D-G), and oriented PLB more upward (Fig. 28F-H). At the same time Ser16 phosphorylation resulted in an increase of PLB overall structural organization (Fig. 28). I propose that these specific changes in PLB structure are the preceding cause of changes in SERCA TM-domain. This statement is
supported by the report that PLB phosphorylation alters structure of the SERCA-PLB regulatory complex (Hou et al., 2008).

In summary, I propose that SERCA overall structure is stabilized by association with PLB, and structural reorganization of PLB upon Ser16 phosphorylation results in PLB displacement from SERCA M2- toward M9-helix. As a consequence, SERCA TM-domain Ca\(^{2+}\) transport site II is able to accommodate Ca\(^{2+}\) passage, leading to SERCA activation.

2-color ATPases as Biosensors for Identification of Regulatory Interactions

SERCA targeting in cardiac dysfunctions is aimed at increasing SERCA activity which is predicted to improve cardiac function (Hayward et al., 2015; Inesi et al., 2008). Thus, it is important to identify and characterize SERCA direct regulators. My lab has successfully used the 2-color SERCA pump to investigate transporter structural dynamics, function, and regulatory interactions (Blackwell et al., 2016; Gruber et al., 2014; Hou et al., 2012). I utilized 2-color SERCA as a tool to investigate SERCA interactions with proposed direct regulators of SERCA activity: HAX-1 and DWORF.

HAX-1 has been shown to directly interact with SERCA in vitro (Vafiadaki et al., 2009), but no evidence has been reported for this interaction in vivo. My measurements with acceptor-sensitized and acceptor photobleaching FRET in live cells detected a decrease in FRET values in the presence of HAX-1 (Fig. 33), confirming HAX-1 direct binding to SERCA. It is worthwhile to indicate that for the 2-color SERCA, the YFP fusion site on the SERCA N-domain before residue 509 is too close to the proposed Hax-1 binding site (residues E575-V594) (Vafiadaki et al., 2009; Vandecaetsbeek et al., 2011). Thus, binding of Hax-1 to this site on the N-domain could result in Hax-1 being physically in the
way of measuring true FRET values. Nevertheless, this detail does not disprove the demonstrated direct interaction of HAX-1 and SERCA.

Another SERCA proposed regulator is DWORF, a recently identified micropeptide encoded by IncRNA. It has been proposed that DWORF binds to SERCA in the same binding groove as SERCA inhibitor PLB and relieves SERCA inhibition by displacement of PLB (Nelson et al., 2016). I investigated the details of SERCA-DWORF regulatory interaction by utilizing 2-color SERCA in live cell FRET measurements. I confirmed direct DWORF interaction with SERCA (Fig. 34D) and detected a higher DWORF apparent affinity to SERCA compared to PLB (Fig. 35C). The competition FRET experiments indicated that DWORF competes with PLB for SERCA binding (Fig. 35A). Since I did not detect DWORF-PLB oligomerization (Fig. 34G), I can exclude the possibility that PLB is sequestered into heterodimers and the observed decrease in FRET between SERCA-PLB is due to true competition with DWORF for a binding site. These results make DWORF a promising target for gene therapy to enhance SERCA activity and thus improve cardiac function. I noted that it was hard to compete PLB off with increasing DWORF levels (the decrease in maximal FRET was not proportional to an increase in DWORF construct expressed) (Fig. 35D-E). This observation suggests that when PLB gets competed away from SERCA and its free concentration increases, it attains higher affinity for SERCA (Fig. 35F), which should be taken into consideration if DWORF is to be used in gene therapy.

Utilization of 2-color SERCA FRET sensor in this work and multiple other studies is aimed to determine SERCA structural dynamics (Blackwell et al., 2016; Hou et al., 2012; Pallikkuth et al., 2013) and potential substrates (Cornea et al., 2013; Gruber et al., 2014). It has been shown that the technique to generate 2-color FRET biosensor can be applied to
other molecules. For example, 2-color MRP-1, another transporter ATPase, was generated and used in high-throughput screening to identify potential substrates (Irám et al., 2015). Here, I confirmed that several of the compounds identified as hits were MRP-1 modulators, among which I recognized a novel MRP-1 inhibitor, the vitamin D analog- calcipotriol (Fig. 36). This discovery can potentially explain the mechanism of action by which vitamin D has been demonstrated to potentiate the effect of chemotherapeutic agents (Ma et al., 2010).

In summary, 2-color FRET sensors have proved to be an effective tool in investigation of various transporters structural dynamics, regulatory interaction, identification of novel substrates, and analysis of ATPase function. Utilization of 2-color FRET biosensors is a promising tool for the high-throughput screening assays that are important in the drug discovery field (Cornea et al., 2013; Gruber et al., 2014; Irám et al., 2015).

Clinical Implications

SERCA regulates Ca\textsuperscript{2+} handling in cardiac cells by sequestering Ca\textsuperscript{2+} from cytosol into SR lumen, which allows heart relaxation and also restores SR Ca\textsuperscript{2+} load and determines heart contractile force (Fig. 1) (Bers, 2006; Gwathmey et al., 1987). SERCA is an attractive target for improving cardiac function in heart failure patients (Roe et al., 2015).

In heart failure a deficiency in SERCA levels and function has been reported (Hasenfuss and Pieske, 2002; Hasenfuss et al., 1994). Restoration of SERCA levels in various models of heart failure has been shown to improve cardiac performance of failing cardiomyocytes and hearts (Kho et al., 2012). Nevertheless, latest human clinical studies failed to significantly improve clinical outcome of heart failure patients, presumably due to the viral delivery failure (Greenberg et al., 2016; Hayward et al., 2015). The alternative
reason for gene therapy failure could be that solely an increase in SERCA levels is not enough to override the regulatory processes of failing heart. Instead, enhancing SERCA function and positive regulation are alternative strategies for improving cardiac output in heart failure patients. Thus, it is extremely important to understand details of SERCA function and regulation.

SERCA function and regulation have been under investigation for decades. Nevertheless, only few SERCA specific activators have been identified so far (Cornea et al., 2013; Dahl, 2017; Kang et al., 2016). Small molecular allosteric activators of SERCA have been shown to increase SERCA Ca\(^{2+}\) transport to relive ER stress and attenuate apoptosis and mitochondrial dysfunction (Dahl, 2017; Kang et al., 2016). However, the mechanisms of action for SERCA pharmacological activators are still not described. Based on the FRET method used for initial high-throughput screening in identification of these compounds (Cornea et al., 2013; Gruber et al., 2014), I reason that binding of small-molecule activators results in SERCA cytosolic headpiece structural rearrangement. Thus, understanding of the SERCA structural dynamics upon binding of pharmacological activators is important for the rational design of SERCA novel activators.

This study provided ample details of SERCA structural dynamics during Ca\(^{2+}\) transport cycle to enhance scientific understanding of SERCA function. Specifically, I described SERCA structural dynamics in regard to slow steps of Ca\(^{2+}\) transport. Ultimately, designing small-molecules which facilitate SERCA ‘fast’ transition through these relatively slow steps that I described can enhance SERCA function and become beneficial for improving cardiac function.
Additionally, I have identified and investigated one of SERCA functional determinant, the Nβ5-β6 loop. The Nβ5-β6 loop facilitates SERCA structural transition from opened to closed conformations, which is one of the fundamental steps of SERCA enzymatic cycle. I predict that enhancing SERCA cycling ‘speed’ would allow boosted Ca\(^{2+}\) transport and result in improved cardiac function. Since I showed that the Nβ5-β6 loop facilitate A- and N-domains closure, I propose that a small pharmacological molecule that is designed to interact with this loop and simultaneously serve as an extension of this structure even further toward A-domain, could allow more efficient cytosolic headpiece closure and enhanced cycling.

Overall, this project provided insights into SERCA structural transitions through catalytic cycle, including identification of the SERCA novel structural and functional determinant, the Nβ5-β6 loop. These insights contribute to understanding of SERCA function and provide novel justifications for design of SERCA small molecular activators.

**Limitations of Current Study**

In present work I utilized a variety of techniques, including *in silico, in vitro, and ex vivo* measurements. There are several limitations in each of these techniques that I had encountered. For example, I performed MD simulations for SERCA spontaneous motions for WT and mutants up to 100 ns due to limited resources of computer time available to perform MD runs. This short timescale excludes a variety of structural changes that are happening on a much longer timeline. Recently, MD studies of SERCA structural dynamics as long as 1 µs were conducted (Espinoza-Fonseca et al., 2015b; Espinoza-Fonseca and Ramirez-Salinas, 2015; Fernandez-de Gortari and Espinoza-Fonseca, 2017) and MD
simulations at 1 µs timescale are much anticipated in scientific publications. Nevertheless, I was able to resolve specific differences induced by loop mutations for all SERCA structures in a short 100 ns timeline. However, I had a setback in investigation of SERCA-PLB complex structural dynamics upon PLB Ser16 phosphorylation due to limitation of computer time resources. Specifically, the differences detected between Ser16 and the A-domain number of contacts for SERCA-PLB and SERCApS16 complexes at 100 ns MD simulation runs (Fig. 22E) became opposing at 200 ns (Fig. 24C). This is not surprising, considering that in physiological conditions structural changes such as SERCA headpiece closure or Ca\textsuperscript{2+} ion transport are predicted to take place on a µs-scale. Thus, my studies are limiting to the detection of short-lived events and do not include the full spectrum of spontaneous structural changes. Due to this limitation, I decided to supplement, and in this way strengthen, my MD simulations data with physical experiments.

The basis for FRET technique have been established 70 years ago ( Förster, 1948) and up-to-date this assay plays a major role in detection and characterization of proteins’ inter- and intramolecular structural changes. The structural dynamics of 2-color SERCA captured by acceptor photobleaching and acceptor-sensitized emission FRET measurements have been well characterized (Bidwell et al., 2011; Hou et al., 2012; Smolin and Robia, 2015b). Nevertheless, such measurements only capture average FRET, which represents average conformation of SERCA population. Most likely, SERCA conformers exist in a significant structural heterogeneity which is not detected with these methods. For example, x-ray crystallography and electron microscopy identified only one structural state of SERCA stabilized by TG (Takahashi et al., 2007; Toyoshima et al., 2000; Young et al., 2001), while time-resolved fluorescence measurements showed that SERCA bound to TG
exists in at least two conformations (Pallikkuth et al., 2013). Thus, my FRET results do not report all the variety of SERCA conformers which presumably exists.

In this study I used muscle-like HEK-293 cell model which was initially developed for monitoring intracellular Ca\(^{2+}\) oscillations (Bovo et al., 2016). This model was supplemented with 2-color SERCA and adapted for simultaneous detection of SERCA FRET and spontaneous Ca\(^{2+}\) release events. I obtained clear traces of the FRET ratio and intracellular Ca\(^{2+}\) oscillations for cells expressing WT-SERCA (Fig. 18B-C, 19A-B). Nevertheless, cells transfected with AAA-SERCA and exhibiting simultaneous FRET change/Ca\(^{2+}\) release events were harder to find. Additionally, the Ca\(^{2+}\) fluctuations were much rare, while traces were noisier compared to those of the cells expressing WT-SERCA (Fig. 18D, 19C-D). This difficulty of finding ‘firing’ cells limited my AAA-SERCA analysis by the number of cells evaluated compared to WT-SERCA. On the other hand, the inability to detect number of cells with simultaneous FRET and Ca\(^{2+}\) fluctuations similar to WT indicates the deficiency of AAA-SERCA to store Ca\(^{2+}\) in the ER for the release event, which I believe to be the direct consequence of AAA-SERCA deficiency in structural transitions from open-to-closed conformation.

In my DWORF acceptor-sensitized FRET detection experiment I observed a higher DWORF’s apparent affinity to SERCA than PLB (Fig. 35C). Nevertheless, in my competition experiments, when I increased DWORF levels to outcompete PLB from SERCA, non-linear SERCA-PLB FRET and \(K_d\) decreases were observed, suggesting that PLB has higher affinity to SERCA in the presence of DWORF (Fig. 35E-F). This observation may seem as contradictory to my previous observation that DWORF binds to SERCA better than PLB (Fig. 35C). I believe that the increased apparent affinity of PLB to SERCA was due to the
increase of PLB monomers as a result of competition with DWORF. Availability of PLB monomers enhanced PLB ability to bind to SERCA and in this way increased PLB apparent affinity. Ideally, I need to perform the competition experiments when instead of outcompeting PLB with DWORF from SERCA binding site, I compete DWORF from SERCA with gradually increasing co-expression of PLB. In reality, this experiments required a large amount of PLB DNA construct transfection, and cells, overwhelmed with DNA load, became unhealthy, which resulted in inconstant measurements between experiments. Therefore, optimization of the expression system is required to overcome this limitation for successful execution of the PLB-DWORF competition experiments. One of possible strategy could be the use of smaller DNA vectors.

Another difficulty that I encountered was the struggle to produce functional microsomes. As presented in Figure 15, I measured WT-SERCA FRET in microsomal membrane preparations from AAV-293 cells transiently transfected with SERCA DNA constructs. The FRET increase curve over increasing Ca\(^{2+}\) levels (Fig. 15A) was regarded as a control experiment in determination if the microsomal preparations are suitable for
substrate-stabilized structural FRET measurements (Fig. 15B). However, for AAA-SERCA microsomal preparations I constantly observed no significant FRET response to increasing Ca\(^{2+}\) levels (Fig. 39A) or in substrate-stabilized structural measurements (Fig. 39B). Since I prepared several batches all of which showed this result, I am unsure if AAA-SERCA headpiece was not responsive to substrates tested, or microsomal samples were compromised. Based on the ATPase curves, AAA-SERCA has similar to WT pump Ca\(^{2+}\) affinity (Fi. 14B). Thus, I believe that Ca\(^{2+}\) binds to mutated transporter and should induce a structural response similar to WT. However, since AAA-SERCA has a deficit in progression from open to closed conformer, I may only detect this specific to AAA-SERCA slow step of enzymatic cycle. Thus, the interpretation of this experiment is ambiguous and for that reason it was not included in the main chapter.

Overall, I believe that I was able to provide a complex review of the SERCA structural dynamics during Ca\(^{2+}\) transport and identified the N\(\beta\)5-\(\beta\)6 loop as SERCA structural and functional determinant by combining in silico, in vitro, and ex vivo experiments, which were aimed at complementing and strengthening each other.

**Overall Conclusion**

The novel insights and substantial details of SERCA transient conformations during Ca\(^{2+}\) transport revealed in this study extend the scientific understanding of SERCA structural dynamics and redistribution of SERCA conformers in response to intracellular Ca\(^{2+}\) fluctuations. The SERCA N\(\beta\)5-\(\beta\)6 loop, identified as a structural determinant of transporter headpiece closure with a functional implication in SERCA ATPase activity and Ca\(^{2+}\) transport, can become a promising target of a novel small-molecule therapeutics aimed to enhance cardiac output in heart failure patients.
LIST OF REFERENCES


Olga N. Raguimova was born on November 26, 1982 to Valentina and Nikolai Shmantsar. She was raised and lived in Russia until moving to the United States in 2003. Olga had attended Truman College in Chicago, IL, where she joined Biochemistry program and discovered her passion for science. She transitioned to the University of Illinois in Chicago in 2009, where she earned a Bachelor of Arts in Biology, with Honorable Distinction, in 2011. In 2012 she joined Dr. Toru M. Nakamura's laboratory at the University of Illinois in Chicago as a research assistant.

In 2012, Olga matriculated in the Loyola University Stritch School of Medicine Integrated Program in Biomedical Sciences to pursue doctoral degree. In 2014 she joined the laboratory of Dr. Seth L. Robia in the Physiology Department and continued her graduate education under his mentorship. Olga’s doctoral work focused on the role of the Nβ5-β6 loop, a distinct structure of sarco/endoplasmic reticulum calcium ATPase, in transporter structural dynamics and function. Her work was supported by Loyola University Chicago and by the National Institute of Health through a grant to Seth L. Robia (HL092321).

Following graduation, Olga will begin her post-doctoral research position in the laboratory of her Ph.D. mentor Dr. Seth L. Robia, with a future transition into industry. Currently Olga lives in the Chicago area of Illinois, with her two school age daughters, Alina and Alice, and her partner, Matthew E. Klich.