IN VITRO STUDY OF THE EFFECT OF PALB2 PHOSPHORYLATION ON BRCA1-PALB2 DIMERIZATION

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IN VITRO STUDY OF THE EFFECT OF PALB2 PHOSPHORYLATION ON BRCA1-PALB2 DIMERIZATION

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ABSTRACT

BRCA1 and PALB2 are two proteins that bind to efficiently repair DNA damage through homologous recombination. The inability of these proteins to dimerize due to genetic variations can increase an individual's risk of developing breast and ovarian cancer. Currently, most PALB2 genetic variants are classified as variants of unknown significance (VUS) due to insufficient data to predict pathogenicity. *In vivo* methods to predict pathogenicity of these variants are time consuming and costly. As a result, we aimed to create a high-throughput and cell-free assay to test the effect of VUS on the BRCA1-PALB2 binding interaction. Importantly, we wanted to recreate any relevant cellular conditions to obtain the most accurate data, and currently, it is unknown how one such cellular condition, the phosphorylation of PALB2, would affect the BRCA1-PALB2 binding interaction *in vitro*. To determine if phosphorylation affects the binding interaction, we mimic the phosphorylation states of PALB2 using site-directed mutagenesis and test their effect on BRCA1 binding using isothermal titration calorimetry. Our results indicate a surprising finding: PALB2 phosphorylation does not significantly alter the strength of the BRCA1-PALB2 binding interaction with minimized constructs *in vitro*. Thus, we hypothesize it is not critical to recreate the phosphorylation states of PALB2 when testing the effect of VUSs on the BRCA1-PALB2 binding interaction.

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INTRODUCTION

Breast cancer is the most common form of cancer diagnosed in females, and in 2023 alone, it is estimated that there will be an additional 297,790 new cases of breast cancer in the United States.[1] The breast cancer mortality rate, however, has dropped by 42% from 1989 to 2020, and this is in part due to scientific and medical advancements in research and treatment.[1] One of these advancements was due to the pioneering work of Dr. Mary-Claire King. She hypothesized that a genetic link existed for some forms of breast cancer, which led her to discover the breast cancer gene 1 (BRCA1).[2] This discovery then fueled further improvements in our understanding of breast cancer with the identification of other genes involved in hereditary breast cancer, such as breast cancer gene 2 (BRCA2) and partner and localizer of BRCA2 (PALB2).

All three of these genes encode for proteins that are involved in the DNA damage response and cell cycle checkpoints in a cell. Specifically, the binding interaction between BRCA1 and PALB2 is key for activation and maintenance of the G2/M phase checkpoint of cell division, which prevents cell division when DNA damage is present.[5] Inhibition of binding between these proteins causes defects in the activation and maintenance of the checkpoint, which can contribute to genome instability and tumorigenesis.[5] Additionally, when double stranded breaks in DNA occur in cells, homologous recombination (HR) takes place to repair the DNA damage.[4] During this response, BRCA1 binds to PALB2, which then recruits BRCA2 to the site of damage.[4] Together and along with other proteins, this complex performs HR to prevent the accumulation of DNA damage in a cell and subsequent tumor formation.[4]

Some individuals, however, are born with genetic variations in the PALB2 protein that inhibit the binding of BRCA1 and PALB2 and prevent HR from taking place.[6-7] A recent meta-analysis of twelve different studies on breast cancer risk for individuals who carry one of these pathogenic PALB2 mutations were combined, and the estimated risk of developing breast cancer was found to be 48.70% by age 80.[8] Many papers have detailed these variants and their resulting cancer risk, but there still exist many variants of unknown significance (VUS), which are variants not yet researched or studied enough to determine pathogenicity. As a result, it is important to continue classifying VUS to help patients better understand whether their variant is pathogenic and could increase their risk of developing cancer or if it is benign.

Further research on the effect of PALB2 variants is ongoing, but current testing assays are both timely and costly. These assays include *in vivo* experiments such as immunofluorescence and immunoprecipitation. In light of this, we set out to create a high-throughput *in vitro* system to test the effects of PALB2 variants on the BRCA1-PALB2 binding interaction. Importantly, we want to recreate any relevant cellular conditions to ensure we obtain the most accurate data.

For other DNA repair pathways, prior research has shown that one particular protein modification, phosphorylation, plays an important role in signaling DNA damage and helping to recruit various members of the repair pathways, and this idea also appears to be true in the BRCA1-PALB2 pathway.[12] Specifically, in the absence of DNA damage, a serine at position 64 of PALB2 is phosphorylated. However, in the presence of DNA damage, the serine at position 64 becomes hypophosphorylated while a serine at position 59 of PALB2 is phosphorylated (figure 1). Researchers have determined that this event is not only necessary for proper BRCA1-PALB2 binding but also for PALB2 localization to DNA damage sites *in vivo*.[9-11]

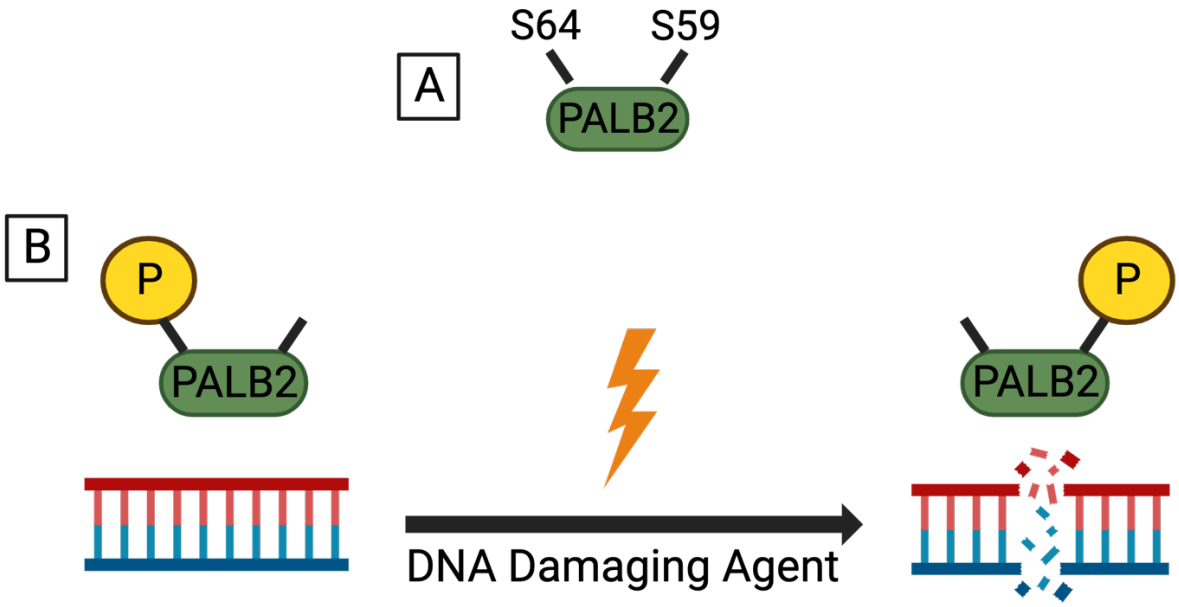

Figure 1. Phosphorylation of PALB2 during different cellular conditions. (A) PALB2 residues of interest (S59, S64) that are phosphorylated during different cellular conditions. (B) In the absence of DNA damage, S64 is phosphorylated. In the presence of DNA damage, S64 is hypophosphorylated, and S59 is phosphorylated.

However, it is unknown how phosphorylation would affect the binding interaction between the coiled-coiled domains of BRCA1 and PALB2 *in vitro*, where these proteins are isolated from other cellular molecules and pathways that could be causing the phosphorylation effects seen *in vivo*. Thus, we set out to mimic the phosphorylation states of PALB2 and assess their effect on the *in vitro* binding interaction using isothermal titration calorimetry (ITC). We found that phosphorylation does not directly affect the binding interaction between the coiled-coil domains of BRCA1 and PALB2 *in vitro*. Consequently, we determined PALB2 phosphorylation is not important to recreate when testing the effects of PALB2 VUSs on the

BRCA1-PALB2 binding interaction *in vitro*. However, additional research is needed to determine the effect of BRCA1 phosphorylation on the binding interaction before we can be confident in the validity of our *in vitro* system.

METHODS

Bacterial Growth and Protein Expression

The BRCA1 WT construct consists of amino acids 1377-1426 of the human BRCA1 protein. The PALB2 WT construct consists of amino acids 1-93 of the human PALB2 protein. PALB2 phosphomimetics were created by performing site directed mutagenesis on the PALB2 WT construct and confirmed via Sanger sequencing. pET-SUMO 6xHis vector plasmids containing each protein construct were then transformed into BL21(DE3) competent *E. coli* cells by heat shocking the cells for 30 seconds at 42ºC. The cells were plated on LB agar plates containing 50 µg/mL kanamycin and incubated for 12 hours at 37ºC. Afterward, the colonies were resuspended from the plates and divided equally between two flasks containing 1 L LB broth and 50 µg/mL kanamycin. These colonies were then incubated at 37ºC while shaking at 250 rpm until the optical density (OD) reached 0.6. The growth of the cells was then slowed by reducing the temperature to 16ºC for one hour. Protein expression was induced with 0.2 mM IPTG for 16 hours at 16ºC, and the cells were then harvested by centrifugation and frozen at -80ºC.

Protein Purification

The harvested cells were removed from the -80ºC freezer and thawed in a beaker of room temperature water. During the process, lysozyme, DNase, and protease inhibitor cocktail were added. The cells were lysed through sonication with 15-second pulses and 30-second rest periods for a total of 10 minutes of pulsing. The cells were then centrifuged at 14,000 rcf and 4ºC for 25

minutes. The resulting supernatant was loaded into the Akta Start GE System for purification by way of cobalt affinity chromatography. Due to the presence of a His tag (six histidine sequence) located upstream of the protein, the protein of interest sticks to the cobalt column while *E. coli* proteins flow through the column. The protein was then eluted from the column using increasing concentrations of imidazole, and the fractions containing the protein of interest were determined using absorbance at 280 nanometers (nm) as measured by the Akta system. These fractions were then combined and dialyzed in buffer containing 50 mM NaCl and 25 mM sodium phosphate buffer system at pH 7.0 for 16 hours at 4ºC to lower the imidazole concentration. The protein was removed from the buffer, and HRV 3C protease was added to cleave the His tag for 1 hour in the presence of dithiothreitol (DTT). The protein was then run through glutathione and nickel affinity chromatography to remove the HRV 3C protease and His tag, respectively. Size exclusion chromatography (SEC) followed using the GE Fast Pressure Liquid Chromatography (FPLC) system to further purify the construct. The fractions containing the protein of interest were determined via absorbances at 215 and 280 nm as measured by the GE FPLC system. The fractions were then concentrated with 3000 Dalton cut-off concentrators until concentrations reached acceptable values for ITC testing. For PALB2 constructs, these values were determined using the absorbance at 280 nm and the extinction coefficient of the single tyrosine residue in the construct. For BRCA1 constructs, a lack of aromatic amino acids present in the protein that absorb at 280 nm necessitated the use of backbone absorbance at 205 nm to estimate concentration.

Isothermal Titration Calorimetry

Before each experiment, excessive cleaning was performed using Sodium Dodecyl Sulfate into water titrations along with water into water titrations. Buffer into buffer runs followed to ensure the instrument was clean. Before loading each protein into the machine, both the syringe and cells were equilibrated with buffer. Between experimental runs, additional cleaning with buffer and water was performed to ensure cleanliness of cell and syringe, and the syringe was dipped into the titration cell filled with water to clean the outside of the syringe. All proteins used in the experimental runs were in a buffer containing 50 mM NaCl and 25 mM sodium phosphate buffer system at pH 6.5. During these runs, BRCA1 WT (0.425 mM) was titrated into each PALB2 construct (0.125 mM) using the parameters listed in table 1. Buffer into buffer runs were also performed to aid in analyzing the data. Analysis involved using Origin software to subtract buffer into buffer runs from the experimental data to account for the heat of dilution. Following, the experimental data was fit to a single binding site model using procedures as described in the Origin instruction manual to determine thermodynamic parameters.

Table 1

Parameter	Value
Syringe Speed (rpm)	300
Cell Temperature (ºC)	25
Initial Delay (sec)	300
Spacing (sec)	200
Injection size (µL)	2

Injection length (sec)	4
Filter Period (sec)	5
# of Injections	16

RESULTS

Many protein kinases are unspecific *in vitro*, phosphorylating many amino acid residues instead of only their intended target(s). Further, it is challenging to separate phosphorylated proteins away from any that remained unphosphorylated in the *in vitro* phosphorylation reaction due to the similar properties of the phosphorylate and unphosphorylated proteins. As a result, the generation of phosphomimetic mutations, a technique that involves mutating a serine residue to a glutamic acid (Glu), was used to mimic phosphorylation for our experiments. This technique attempts to replicate the negative charge a phosphate group possesses by mutating the phosphorylation site (Ser, Thr, Tyr) to a negatively charged amino acid (Asp or Glu). The literature has detailed this method and its validity, including one paper that used it with PALB2.[8,13] After creating these mutants, ITC was performed to elucidate the strength of the BRCA1-PALB2 binding interaction in the presence and absence of "phosphorylation."

When two proteins bind, the binding event is typically exothermic, releasing energy in the form of heat, which can be accurately measured using ITC. Consequently, this technique provides a mechanism by which protein binding can be quantitatively assessed. To do so, one protein construct is loaded into the syringe of the ITC machine and is then titrated into another protein contained within the ITC cell. Throughout the experiment, the ITC machine applies heat to maintain a constant temperature in the ITC cell. Due to exothermic protein binding, however,

the amount of heat the machine needs to apply varies, and this change can be graphed to quantitatively determine protein binding.

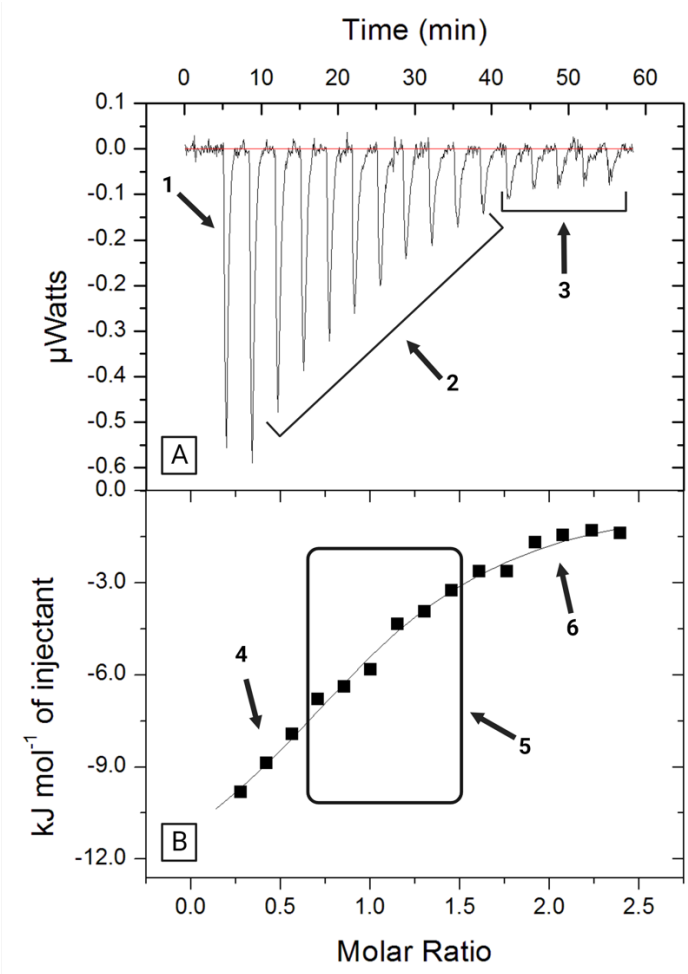

Figure 2. ITC Titration of WT BRCA1 into WT PALB2. Each arrow and number correspond to an important part of the titration referenced in the text. (A) Graph of power applied by the ITC machine over the course of 16 evenly spaced injections over time. (B) Graph of heat per mole of injectant versus the ratio of WT BRCA1 concentration to WT PALB2 concentration.

Figure 2 represents an ITC experiment in which WT BRCA1 was titrated into WT PALB2 over the course of sixteen injections. In the top graph, the change in power (heat/unit time) applied by the system is plotted over the course of the sixteen evenly spaced injections over the course of one hour. During each injection, BRCA1 binds PALB2, releasing heat and

producing a decrease in the amount of power the machine must apply to keep the cell at a constant temperature. This event can be seen as a downward pointing peak **(Figure 2 Arrow 1)**. After each injection of BRCA1, the total amount of unbound PALB2 decreases, so fewer binding events occur. This can be seen by the decreased intensity of peaks as the experiment progresses since the decrease in power the machine must apply to maintain the cell temperature also decreases **(Figure 2 Arrow 2)**. Eventually, all PALB2 inside the cell becomes bound to BRCA1, referred to as the saturation point. Afterward, the only heat exchange seen during injections is due to the heat released as BRCA1 further dilutes the liquid in the cell, referred to as the heat of dilution **(Figure 2 Arrow 3)**.

Peaks in **Figure 2A** can then be integrated and plotted in **2B** as a function of heat per mole of injectant versus the ratio of BRCA1 concentration to PALB2 concentration (molar ratio). At the start of the experiment, with substoichiometric concentrations of BRCA1, all or most of the BRCA1 injected is binding PALB2 **(Figure 2 Arrow 4)**. As the molar ratio approaches 1, less unbound PALB2 is available to interact with BRCA1 **(Figure 2 Arrow 5)**. After PALB2 saturation, the heat per mole of injectant should be zero as no additional BRCA1 binding occurs. However, due to the heat of dilution, the heat per mole of injectant seen is slightly less than zero **(Figure 2 Arrow 6)**.

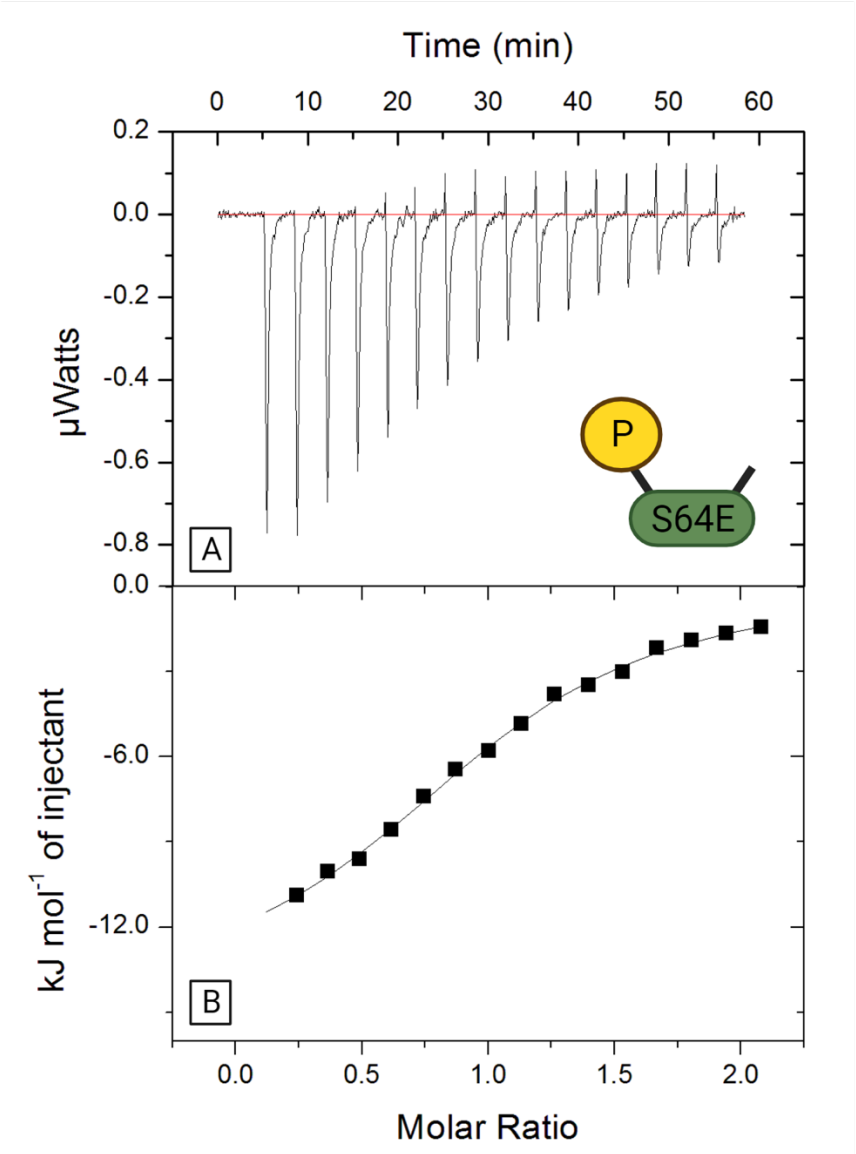

Figure 3. ITC Titration of WT BRCA1 into PALB2 S64E. (A) Graph of power applied by the ITC instrument over the course of 16 evenly spaced injections, which is denoted by time. (B) Graph of heat per mole of injectant versus the ratio of WT BRCA1 concentration to PALB2 S59E concentration.

Figure 3 represents the titration of WT BRCA1 into PALB2 S64E, and binding appears to be similar to the binding between WT BRCA1 and WT PALB2. Towards the middle of the experiment, however, upward peaks are seen **(Figure 3A)**. These peaks represent an endothermic event, in which heat is being absorbed by the system, and thus, the ITC machine must apply more heat to maintain the cell temperature. Causes for these endothermic peaks are unknown but

are unlikely to be derived from the protein-protein interaction as they do not decrease with PALB2 saturation like the peaks resulting from the exothermic event. Additionally, the microwatt values of **Figure 3A** are slightly higher than in the previous figure, but this can be attributed to concentration differences between the titration runs. If the overall concentration of protein is increased in a titration, the microwatt values displayed on the graph also increase due to an increased amount of protein binding and thus heat released. Besides these two small differences, **Figure 3A** is similar to **Figure 2A** (WT BRCA1 titration into WT PALB2).

Figure 3B is also similar to **Figure 2B**. It appears that saturation has just occurred at the end of the experiment or perhaps has not occurred yet as the last few data points still trend upward instead of being equal heats per mole of injectant as seen in **Figure 2B**. This can likely be attributed to minor concentration differences between the titration runs.

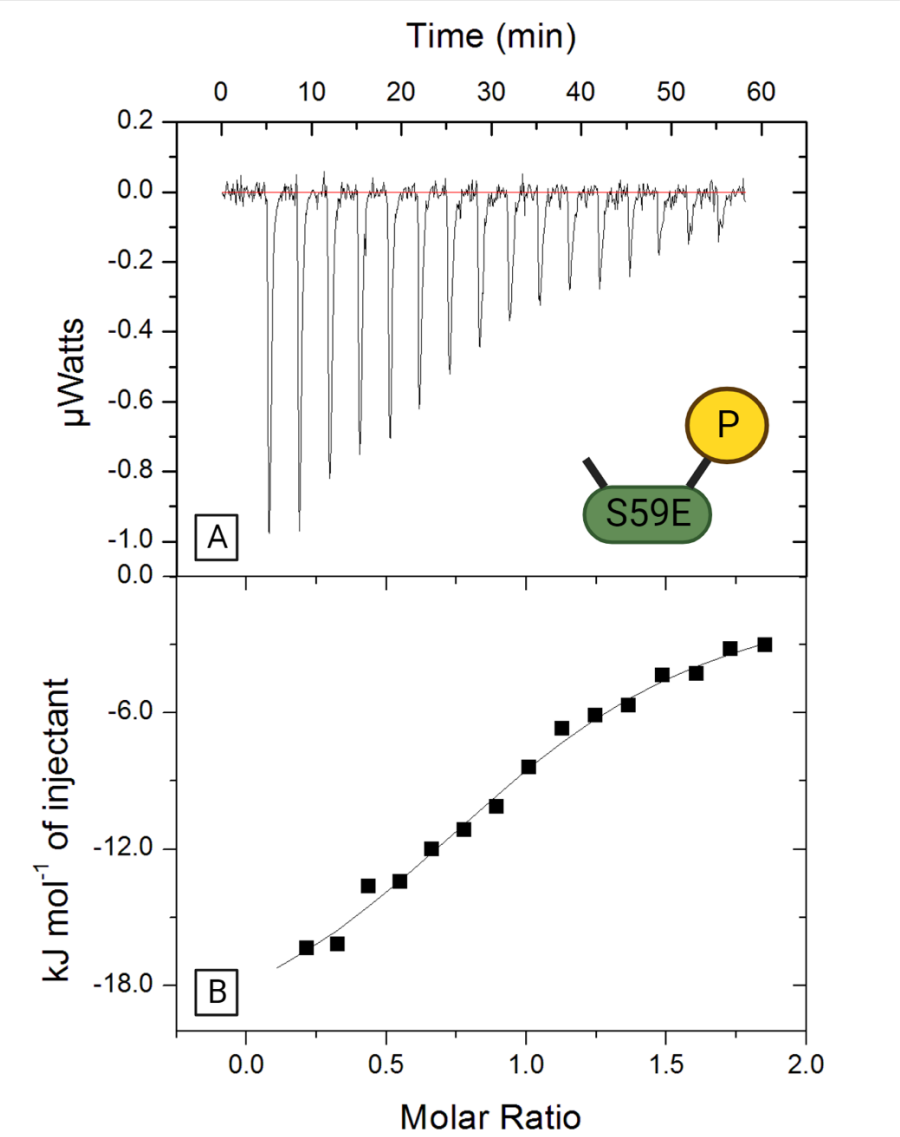

Figure 4. ITC Titration of WT BRCA1 into PALB2 S59E. (A) Graph of power applied by the ITC machine over the course of 16 evenly spaced injections, which is denoted by time. (B) Graph of heat per mole of injectant versus the ratio of WT BRCA1 concentration to PALB2 S59E concentration.

In **Figure 4**, WT BRCA1 was titrated into PALB2 S59E. Graph A is very similar to graph A of the WT BRCA1 titration into WT PALB2. The microwatt values pictured are increased compared to **Figure 2**, but, these differences are due to concentration differences and likely have no effect on the ability to quantify the biological significance of phosphorylation on the BRCA1-PALB2 binding interaction. **Figure 4B** is also similar to **Figure 2B**, however equal

heats per mole of injectant are seen at the beginning of the experiment. Additionally, it appears that saturation just occurred at the last injection or perhaps did not occur as the final points in **Figure 4B** do not have equal heats per mole of injectant. Similarly to **Figure 3B** in which this same scenario was present, these differences can be attributed to concentration differences between the titrations that do not impact the ability to determine the biological relevance of phosphorylation on BRCA1-PALB2 binding.

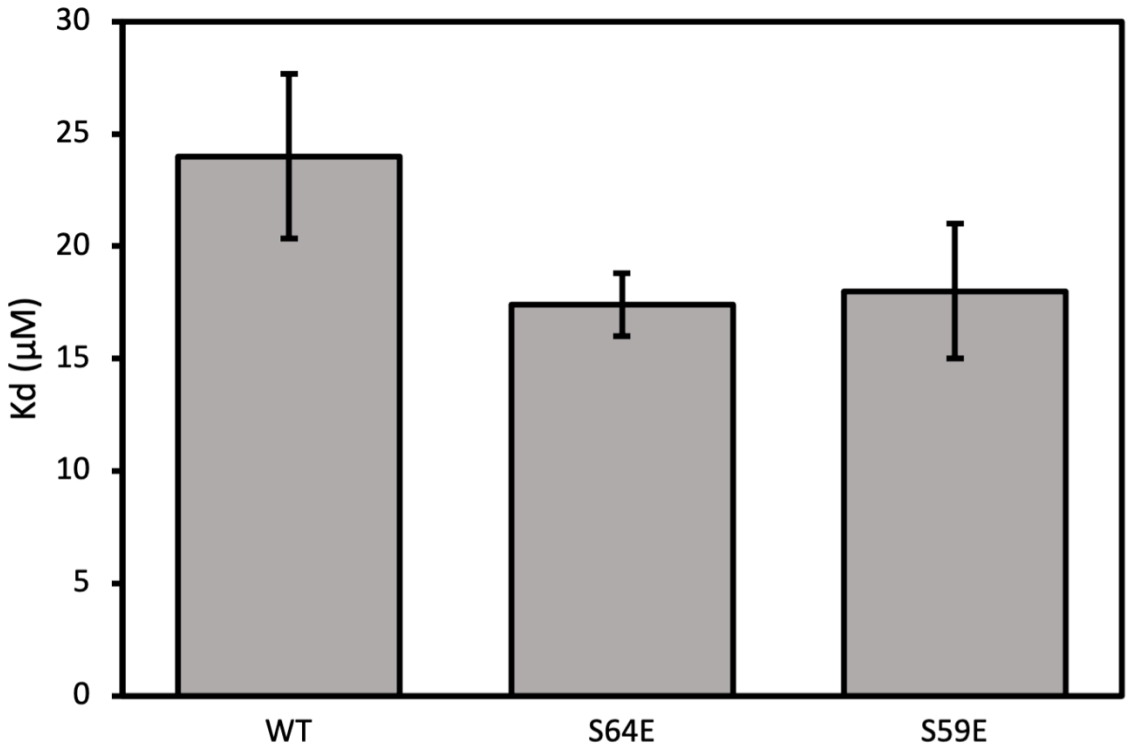

Figure 5. Binding Affinity of Experimental ITC Titrations. Dissociation constant (K_d) and corresponding error of each experimental titration of PALB2 (WT, S64E, S59E) into WT BRCA1 is presented. Dissociation constant and error were determined by fitting each set of experimental data to a single binding site model using Origin software.

Changes in heat plotted in part B can be fit to a single-site binding curve using Origin software to obtain binding parameters of the reaction. One of these binding parameters, K_d, reflects the dissociation constant of a binding interaction. The higher the K_d, the more likely two binding partners are to dissociate and thus if phosphorylation effects binding, we would expect

this value to vary for the PALB2 mutants. For all three titrations, this value along with its corresponding error were plotted in **Figure 5**, and none of the values had a difference large enough to be considered biologically relevant. As a result, we concluded that PALB2 phosphorylation is not biologically relevant to the BRCA1-PALB2 binding interaction, and consequently, we do not need to recreate it when testing the effects of VUSs on the BRCA1-PALB2 binding interaction.

DISCUSSION

We aim to create a high throughput *in vitro* assay to test the effects of VUS on the BRCA1-PALB2 binding interaction. To ensure the accuracy of this method, we want to recreate any necessary cellular conditions. One such condition, phosphorylation, has been shown to affect other DNA repair pathways.[12] Further, the effect of phosphorylation on the *in vivo* interaction between BRCA1 and PALB2 was found to be important.[11] However, the *in vitro* effect of phosphorylation on the binding interaction was unknown. Consequently, we mimicked the phosphorylation states of PALB2 *in vitro* and established that they have no significant effect on the BRCA1-PALB2 binding interaction using ITC.

In our ITC experiments, we encountered difficulty producing characteristic sigmoidal curves that provide the most accurate data. These abnormalities were attributed to a difficulty in determining the concentration of BRCA1. The only method to determine the concentration of BRCA1 was protein backbone absorbance at 205 nm since our BRCA1 construct contained no aromatic amino acids. Therefore, the concentration determined was likely inaccurate because other particles also absorb in this range. The same issue was not encountered for determining PALB2 concentration as this protein construct contains the aromatic amino acid tyrosine; hence,

we were accurately able to measure the concentration using absorbance at 280 nm. To correct for the concentration inaccuracies for the BRCA1 protein, we adjusted the BRCA1 concentrations in ITC software until the stoichiometry calculated by the software was 1:1. Both the protein data bank structure of the BRCA1-PALB2 dimer (PDB ID 7K3S) and a correlation time experiment measured by NMR give evidence to this stoichiometric ratio.[3]

From the ITC experiments, differences in K_d of PALB2 WT and PALB2 S64E extended beyond the differences in error of the fit of the best curve, but they were not larger than the differences in error due to concentration measurements. Taking this into account, we must conclude the K_d differences are not significant. Further, biologically significant changes due to phosphorylation are associated with a large change in K_d like the 10-fold difference reported previously in a different system.[21]

Our findings differ from those concerning the *in vivo* effect of PALB2 phosphorylation on the BRCA1-PALB2 binding interaction. *In vivo,* S59 phosphorylation has been shown to augment binding affinity of the BRCA1-PALB2 binding interaction relative to S64 phosphorylation.[11] A potential cause for this discrepancy could be due to BRCA1 and PALB2 binding to DNA *in vivo*, bringing them in closer proximity to each other and augmenting the binding affinity of the BRCA1-PALB2 heterodimer.[14-18] Additionally, depending on phosphorylation state, other protein players could be present *in vivo* that bind to domains other than the heterodimer binding domains used for our assays.[19-20] These proteins could cause a change in the local concentration of BRCA1 and PALB2, thus affecting the binding affinity. Additionally, the other protein binding partners of BRCA1 and PALB2 could be causing changes in the conformational shape of PALB2 and BRCA1, also affecting binding affinity. Lastly, another possibility arises that the additional domains of PALB2 are autoinhibitory in the presence

of S64 phosphorylation, thus thwarting heterodimer formation and favoring PALB2 homodimer formation. We would not see these differences *in vitro* due to the use of shortened construct lengths that do not include domains outside of the heterodimer binding domains. Further research is needed to determine whether additional factors outside the heterodimer binding domains exist that affect the BRCA1-PALB2 binding interaction.

Furthermore, published findings indicate that PALB2 phosphorylation may affect the binding interaction *in vitro*.[11] This conclusion was drawn from a pulldown assay, which is less quantitative than ITC experiments in regard to binding affinity. Additionally, different construct lengths were used to find these results. The aforementioned study used BRCA1 residues 1385-1429 and PALB2 residues 1-200; whereas, we used BRCA1 residues 1377-1467 and PALB2 residues 1-95. Three potential hypotheses arise to explain this discrepancy. First, the additional PALB2 residues used in the previous study could be autoinhibiting the heterodimer interaction when S64 phosphorylation is present, acting in an intramolecular manner. Second, the additional residues of PALB2 could be interacting intermolecularly with residues of another molecule of PALB2 due to S64 phosphorylation, promoting PALB2 homodimer formation which would inhibit BRCA1 binding. Third, the pulldown assay presented in the report was unquantified, without replicates, and displayed an experimental band with obstructed visibility. Additional testing would be required to understand these differences; however, we do not believe this contradicting data negates our conclusion that it is not critical to recreate PALB2 phosphorylation for the constructs used for our *in vitro* assays.

Although we have determined the importance of PALB2 phosphorylation, the importance of BRCA1 phosphorylation *in vitro* remains unclear. Similarly, we hope to create

phosphomimetics of BRCA1 phosphorylation sites and test their effect on the BRCA1-PALB2 binding interaction using ITC.

Overall, we have determined that PALB2 phosphorylation is not critical to recreate *in vitro* when testing the effects of VUSs on the BRCA1-PALB2 binding interaction. Further research is needed to conclude the effect of BRCA1 phosphorylation on the heterodimer binding affinity before we can be confident in the validity of our high-throughput *in vitro* assay to test the effects of VUS on BRCA1-PALB2 binding affinity. With this new assay, we hope to augment the timeline in which the over 2000 PALB2 and BRCA1 VUS can be classified as pathogenic or benign, aiding patients with these variants in understanding their cancer risk.

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