Phosphorylation on residue serine-88 of DYNLL1 plays central role in functional regulation

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Abstract:
The Dynein Light Chain LC8-Type 1(DYNLL1 or LC8) protein in its homodimeric form is a major dimerization hub for many cellular processes, including transcription factor regulation and internal cellular transport via dynein and myosin motor protein complexes. Variations in homodimeric to monomeric LC8 ratios have also been linked to diseased states such as some forms of breast cancer. Previous studies have suggested that the major regulatory pathway for LC8 is by phosphorylation of residue serine-88, which leads to homodimeric dissociation. However, these studies were based on inferences using incorporated phosphomimetic residues. Here we directly test the impact of phosphorylation on residue serine-88 by means of direct insertion of phosphoserine into residue-88(non-canonical amino acid insertion). Successful incorporation of phosphoserine into residue-88 of LC8 was achieved with protein yields of about 10 mg(protein)/L(culture), approximately 20% average yield of wild type LC8. Analysis of $LC8_{pSer\text{-}88}$ using Native-PAGE and circular dichroism spectroscopy suggests phosphorylation of serine-88 affects binding partner affinities but not through loss of homodimeric structure. It is postulated here that phosphorylation of serine-88 does not cause the dissociation of the homodimer form into monomeric forms. Rather, binding affinity is regulated by phosphorylation of serine-88 through conformational changes to the homodimeric form.

Introduction:

Dynein Light Chain LC8-Type 1, often abbreviated DYNLL1 or LC8, is a small protein homodimer found in all animal species. First discovered for its role in helping assemble the cytoplasmic dynein complex[1-3], DYNLL1 has been found to interact with a wide variety of other proteins including myosin V, neuronal nitric acid synthetase, p53, transcription factors Swallow and Trpsl, and others[4-7]. It is believed that LC8 acts as a major dimerization hub for many protein networks, making it a crucial protein in a vast array of cellular processes[8]. This theory is supported by its ubiquity and conserved sequence across many animal species, as well as by experiments involving knockout mutations that resulted in organismal lethality[9].

Monomeric DYNLL1 has a primary sequence around 89 aminos acids in length and approximately 11,000 Da in weight. In most cells DYNLL1 is found mainly in its dimeric form, with exceptions being in some lymphoma and leukemia cells[10]. Crystallographic and NMR studies have revealed the dimeric DYNLL1 to be comprised of two central β domains each containing 4 beta folding sheets, and flanking α helices (see figure 1).

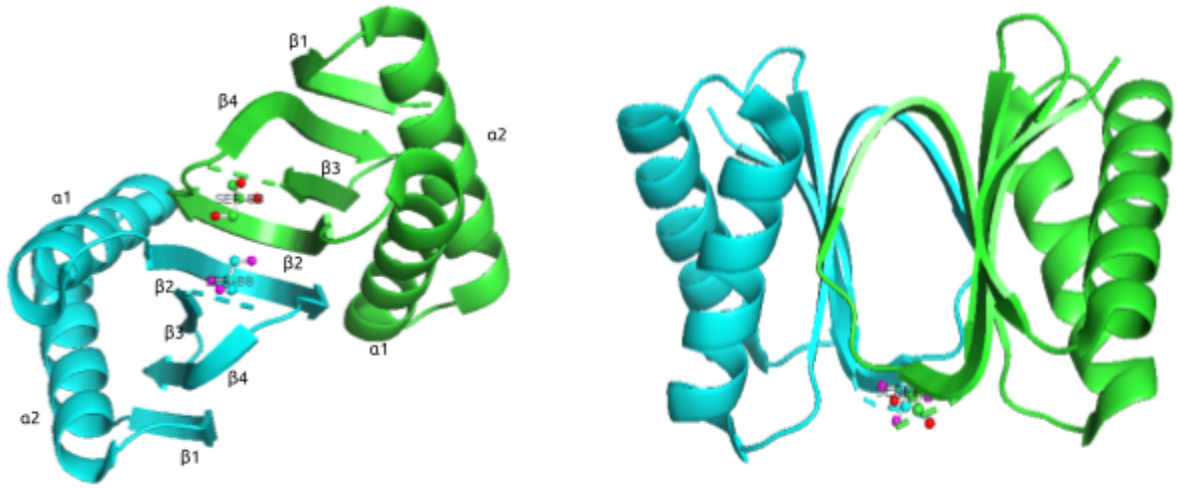

Figure 1: Ribbon diagrams of homodimeric LC8(PDB-3DVH) with monomeric subunits highlighted in green and turquoise.
(a) Serine-88 residue represented in ball and stick form, showing it location at the dimer interface between the 2 monomeric subunits. Aplha helcies and beta sheets labeled α and β respectively
(b) Same as (a) but rotated horizontally 90°

Protein binding assays along with sequence motif analysis has suggested that the KXTQTX motif is the primary binding site for LC8[11]. This sequence recognizes Thr-Gln-Thr (TQT) triplet motifs, along with other non-TQT motifs, in the intrinsically disordered domains of its binding partners[12], inducing the folding of disordered monomeric binding partners and ultimately leading to their dimerization[13].
Cells employ a wide array of strategies to regulate proteins and their expression, from methylation on DNA before transcription to post-translational covalent modifications of the proteins themselves[14]. Protein phosphorylation is one of the most common forms of regulation the cell implements and is performed by protein kinases that transfer the phosphate group of an ATP molecule to, most commonly, a serine, threonine, or tyrosine residue on the protein. It has been suggested that phosphorylation of the residue serine-88 is the key mechanism in DYNLL1 regulation because of its ability to disrupt binding with protein partners such as Bim [15], however structural analysis of the DYNLL1-Bim complex shows serine-88 tucked away and hidden from the binding site[16]. Monomeric LC8 does not have the binding potential that the dimerized form exhibits and dimer-monomer equilibria disruptions have been linked to diseased states[17-18]. These studies have used phosphomimetics, such as glutamic acid, to mimic the chemical characteristics of phosphorylated amino acids (like phosphoserine), allowing them to infer the characteristics of phosphorylated proteins.
It is hypothesized here that serine-88 plays a role in DYNLL1 functional regulation not through interactions with binding partners but through its interactions in forming the LC8 homodimer and will be tested via direct insertion of phosphoserine into LC8 at

residue 88. Incorporation of phosphoserine in de novo LC8 will be attempted using genetic code expansion tools in a process involving selectively mutated seryl tRNA synthetase/$tRNA_{CUA}$ pair[19]. Selective mutagenesis of amino acetyl tRNA synthetase has been shown to produce charged non canonical amino acid-$tRNAs$[20]. The biochemical synthesis of serine in most cells involves a step where the phosphate group of phosphoserine is hydrolysed by phosphoserine phosphatase, resulting in L-serine (see figure 2).

By combining selectively mutated seryl tRNA synthetase/$tRNA_{CUA}$ pair and dynll1 gene sequence in plasmid vectors, with *Escherichia coli* cells lacking phosphoserine phosphatase, de novo production of LC8 with a phosphoserine on residue 88 will be tested; as well as its impacts on homodimeric to monomeric ratios in vitro.

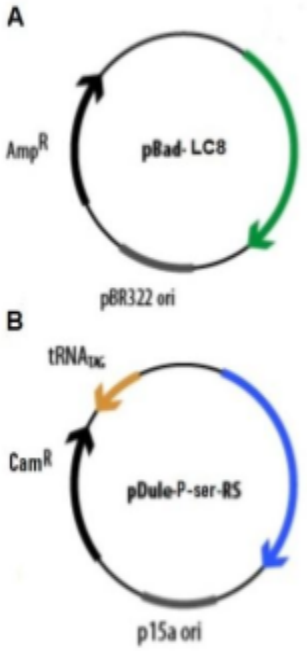

Figure 2. Expression and machinery plasmids (A and B respectively) transformed into TOP10ΔSerB E. coli for production of the $LC8_{pSer-88}$.

(a) The pBad plasmid contains the gene sequence of interest, along with a gene sequence (penicillin beta-lactamase) for ampicillin resistance. Expression is controlled by arabinose operon.

(b) The pDule plasmid encodes for translational machinery (seryl tRNA synthetase/tRNA pair) which incorporates phosphoserine when the ribosome encounters an amber stop codon. Also incorporated is a gene sequence for chloramphenicol resistance. Expression is controlled by lactose operon.

Materials and Methods:

Buffers and Stock Solution. LC8 is a very stable protein with an isoelectric point of 6.8. All buffers prepared for LC8 collection and storage were within a pH range of 6.78-7.38, in order to carry proper charge for SDS-PAGE analysis. Immobilised metal affinity equilibrium and elution buffers were prepared to 300 mM sodium chloride and 50 mM sodium phosphate concentrations. Immobilised metal affinity elution buffer contained 100 mM imidazole for freeing covalently bonded His-LC8 from Talon® metal affinity resin. Both equilibrium and elution buffers for immobilised metal affinity chromatography were brought to pH 7.0 PBS buffers were used for long term LC8 storage. Sodium chloride, potassium dihydrogen phosphate, and disodium phosphate concentrations were 140 mM, 1.8 mM, and 10 mM respectively. Sodium fluoride was added to a concentration of 40 mM to stabilize the phosphoserine in $LC8_{pser-88}$. PBS buffers were adjusted to a

final pH 7.38. 1.5 M stock Tris-HCl at pH 8.8, 0.5 stock Tris-HCl at pH 6.78, 2x Bromophenol Blue loading die, and 10x running buffer for SDS-PAGE analysis were prepared as described elsewhere[21]. Sodium phosphate buffer at pH 7.00 and sodium citrate buffer at pH 2.99 were made for CD analysis. Sodium phosphate contained x,y,z at concentrations x1,y1,z1 respectively. Sodium citrate buffer contained x,y,z at concentration x1,y1,z1 respectively. Both CD buffers contained 40 mM sodium fluoride to stabilize the $LC8_{pSer\text{-}88}$.

Protein Expression. The DYNLL1 gene sequence encoding *Homo sapien* LC8 was cloned into *Escherichia coli* expression plasmid vector pBad with His tagged amine Auto induction medium were produced following protocol described elsewhere[22]. Non terminal and ampicillin resistance coding region. A machinery plasmid pDule was produced with a $TCT\text{-}TAG_{264}$ codon switch in the DYNLL1 gene sequence encoding *Homo sapien* LC8. The pDule plasmid contained a chloramphenicol resistant and an aminoacyl tRNA synthetase coding regions. pBad was transformed into *Escherichia coli* TOP10ΔserB cell lines for expression of wild-type LC8. pBad and pDule were transformed into TOP10ΔserB cell lines from expression of mutant $LC8_{pser\text{-}88}$. TOP10ΔserB cell strains were chosen for their lack of phosphoserine phosphatase encoding gene, to prevent native enzymatic dephosphorylation activity. $pBad_{sfGFP\ WT}$ and $pBad_{sfGFP\text{-}TAG150}$ plasmids containing GFP protein encoding sequence in place of LC8 were cloned and transformed into TOP10ΔserB cell lines. pBad vectors were regulated by arabinose operons and pDule vectors were regulated by lactose operons. inducing medium followed the same recipes without the addition of lactose or arabinose. Cells were grown for 48 hours in 37℃ incubator while shaking at 250 rpm. 100 mL cultures of wild type LC8 and 750 mL cultures of $LC8_{pser\text{-}88}$ were grown alongside 100 mL cultures of wild type sfGFP and $sfGFP\text{-}TAG_{150}$. 5 mL cultures of all 4 cell types were grown in non-inducing media. The induction media's induction ability was tested by the visual expression of GFP in the TOP10ΔserB $pBad_{sfGFP\ WT}$ and $pBad_{sfGFP\text{-}TAG150}$ growth cultures. SDS-PAGE analysis was performed on crude cell extracts to determine relative size and expression of denatured monomeric LC8 and GFP. Wild type LC8 and $LC8_{pser\text{-}88}$ cell samples were pelleted by centrifugation, weighed, and stored at -80℃

Protein Purification. The cell pellets were resuspended in 20 mL of immobilised metal affinity equilibrium buffer per 1g weight of cell pellet. Microfluidization at 18,000 psi and centrifugation for 20 minutes at 11,000g (4℃) was performed on resuspend cells and the crude lysate was applied to equilibrated Talon® metal affinity columns. The small scale expression of LC8 wild type used a resin bed volume of 350 µL and the larger scale expression of $LC8_{pSer\text{-}88}$ used a resin bed volume of 1.0 mL. Elution buffer containing 100 mM imidazole was used to elute His tagged wild type LC8 and $LC8_{pser\text{-}88}$. Purified protein runoff was collected in 500 uL aliquots as described in

protocol elsewhere[23] SDS-PAGE analysis was performed on collected aliquotes to determine relative purity and concentrations. Aliquots with visible and pure banding on SDS-PAGE were transferred to PBS buffer (with NaF) using PD-10 Desalting Columns containing *Sephadex G-25 resin*. The 500 uL aliquots that qualified for buffer exchange were pipetted together if they had visually similar concentrations based on reactions with 1x Bradford reagent. Total volume of applied protein samples to PD-10 Desalting Columns was 2.5 mL and eluted proteins were collected in 600 mL aliquots. Samples were stored in 4℃ for up to 3 weeks. For longer protein storage it is recommended to follow a freezing with glycerol protocol.

Bradford Assay and NanoDrop: Protein concentrations of PBS protein aliquots were tested using standard Bradford protein assay[24] and verified by using nanodrop. Bovine serum albumin was used as the protein standard. The spectrophotometer was set to 595 nm wavelength and blanked with PBS buffer (with NaF)/1x Bradford reagent.

SDS-PAGE Analysis: There were 2 variants of SDS-PAGE gels used. First were SDS-PAGE gels where both the resolving and the stacking gels were kept at 18% acrylamide/bisacrylamide. Second were the SDS-PAGE gels where the resolving gel was kept at 18% acrylamide/bisacrylamide but the stacking gel was changed to 4%. The 18% resolving/ 4% stacking gels were used when running gels containing wild type LC8 because they had better segregation and resolution. However, the pure 18% SDS-PAGE gels seemed to resolve the $LC8_{pser\text{-}88}$ better. Regardless of which variant was used, wells containing protein samples were loaded at 1:1 sample to loading die ratios and sample volumes never exceeded 40 µL. All electrophoresis chambers were run at 200V with x1 running buffer for between 45-60 minutes.

Discontinuous Native PAGE: DYNLL1, being a smaller protein, is best resolved on 10% percent gels. The recombinant protein, sfGFP-Swa, was used for a simple binding Native-PAGE assay with wild type LC8 and $LC8_{pSer\text{-}88}$. sfGFP-Swa contained the Swallow protein sequence (which is known to bind wild type LC8) attached to a Green Fluorescent Protein in order to determine LC8 samples' binding affinities. sfGFP-Swa with LC8 samples were incubated on ice for 20 minutes to allow adequate time for binding. More complex Native-PAGE binding assays were performed with Rabies Virus Phosphoprotein [RavP] (also known to bind wild type LC8) at physiological (pH 7) and denaturing (pH 3) conditions. Both wild type LC8 and $LC8_{pSer\text{-}88}$ samples were transferred to sodium phosphate (pH 7.00) and sodium citrate (pH 2.99) buffers using dialysis buffer exchange before analysis. LC8 samples were combined with varying concentrations of RavP (from low to high concentrations) and incubated on ice for 20 minutes to allow for proper binding. All electrophoresis chambers containing Native-PAGE gels were run at 70V for between 1.5-4 hours.

Phos-Tag™ Acrylamide Gel: Successful incorporation of phosphoserine in mutant $LC8_{pSer\text{-}88}$ was tested using a Phos-Tag™ Acrylamide gel. In theory phosphorylated

proteins migrate more slowly on this type of gel because of interactions with the special Phos-tag™ Acrylamide AAL-107 medium (available for purchase from Wako Pure Chemical Corporation). Gel and samples were prepared using standard SDS-PAGE procedure, with phosphorylated proteins run side by side to their wild type counterparts (no ladder is needed). Gel was run at 150V for approximately 90 minutes.
Circular Dichroism (CD) Spectroscopy: CD analysis was performed on LC8 samples at physiological and denaturing conditions. Both wild type LC8 and $LC8_{pSer\text{-}88}$ samples were transferred to sodium phosphate pH 7.00 and sodium citrate pH 2.99 buffers using dialysis buffer exchange before analysis. Cuvettes used were 1 cm in path length and data was recorded in millidegrees(mdeg), then converted into molar ellipticity(θ) for presentation. Only near-UV (250-300 nm) data was collected as secondary structure data was not under consideration. Concentrations of samples were not considered when performing CD analysis and concentrations of wild type LC8 and $LC8_{pSer\text{-}88}$ used were simply their respective concentrations in buffer.

Results and Discussion:

Protein Expression and Purification: After 48 hours of incubation all 4 auto induction media cultures showed robust growth. Both wild type TOP10ΔserB-GFP and TOP10ΔserB-$GFP_{TAG\text{-}150}$ showed expected visual production of GFP whereas the 5 mL non-inducing cultures did not; suggesting both expression and machinery plasmids were expressing. LC8 production of wild type LC8 and $LC8_{pSer\text{-}88}$ cultures was visually confirmed on SDS-PAGE of crude cell extract. Expected large bands in the 11 kDa region of the gel indicated successful production of LC8 in cultures. Cultures were pelleted for storage and had pellet masses within the range of 0.48-1.09g. Eluted proteins, after purification with immobilised metal affinity chromatography, were collected in two (approximately 1.5 mL) aliquots for the wild type LC8 and one (large 5 mL) sample for the $LC8_{pSer\text{-}88}$. Protein purity was tested by SDS-PAGE analysis on 4% stacking and 15% resolving acrylamide gel. Both aliquots of wild type LC8 showed relatively high purity of about 95% and the $LC8_{pSer\text{-}88}$ showed moderate purity of about 85%.(Figure 3)

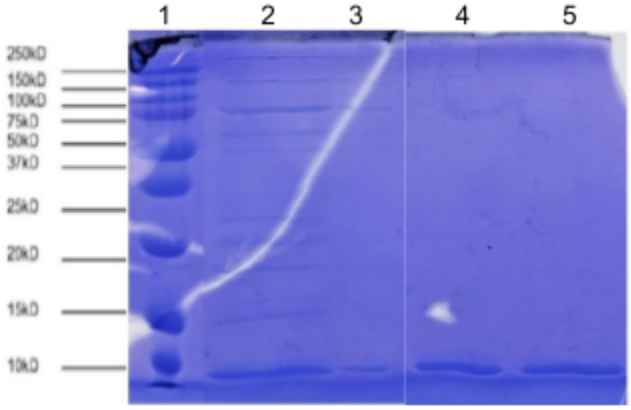

Figure 3: Image of 15% resolving and 4% stacking acrylamide SDS-PAGE gel. The sizes of the molecular weight standards(Precision Plus Protein™ Dual Color Ladder) are shown is well 1. Well 2-3 contains $LC8_{pSer\text{-}88}$ and wells 4-5 contain wild type LC8. Protein purity is estimated to be 85% and 95% for $LC8_{pSer\text{-}88}$ and wild type LC8 respectively.

The purity $LC8_{pSer-88}$ is thought to be the result of the larger expression and bed volume used, with more opportunities for background proteins to bind the TALON™ resin. Protein samples after buffer exchange had an average concentration (between Bradford assay and nanodrop testing) of 1.5 mg/mL for the $LC8_{pSer-88}$ and 0.5 mg/mL for the wild type LC8. From these concentrations an approximate protein yield of 50 mg/L culture for wild type LC8 and 10 mg/L culture for $LC8_{pSer-88}$ was calculated. Although the $LC8_{pSer-88}$ is expressing about 20% the rate of the wild type LC8, it is expressing higher than had been anticipated. Wild type LC8 has been known to express between 60-80 mg/L culture depending on the methods used. Protein yields for non canonical amino acid incorporations are often much smaller. Of course the $LC8_{pSer-88}$ has some impurities that are contributing to this high calculated protein yield, but this method of expression and purification seems to be effective.

Validation of Phosphoserine Incorporation: The first indication of successful incorporation of phosphoserine was observed not from a test but during the preparation for CD analysis. During the dialysis buffer exchange into the sodium phosphate buffer and sodium citrate buffer it was observed that the $LC8_{pSer-88}$ sample precipitated out of the buffer solutions but wild type LC8 did not. There was more precipitation observed in the sodium phosphate buffer but both had significant precipitation. This suggested that the wild type LC8 and $LC8_{pSer-88}$ were different in some capacity. Perhaps the $LC8_{pSer-88}$ was exposing some hydrophobic residues normally buried in the wild type LC8. To confirm $LC8_{pSer-88}$ had successfully incorporated phosphoserine a Phos-Tag™ acrylamide gel analysis was performed. As seen on Figure 4 the $LC8_{pSer-88}$ sample migrated more slowly compared to the wild type LC8 sample, which is what is expected if the $LC8_{pSer-88}$ had successfully incorporated the phosphoserine. Additionally, it was seen that $LC8_{pSer-88}$ samples and wild type LC8 samples migrated at different rates on Native-PAGE gels. This is consistent with what is expected if there is a

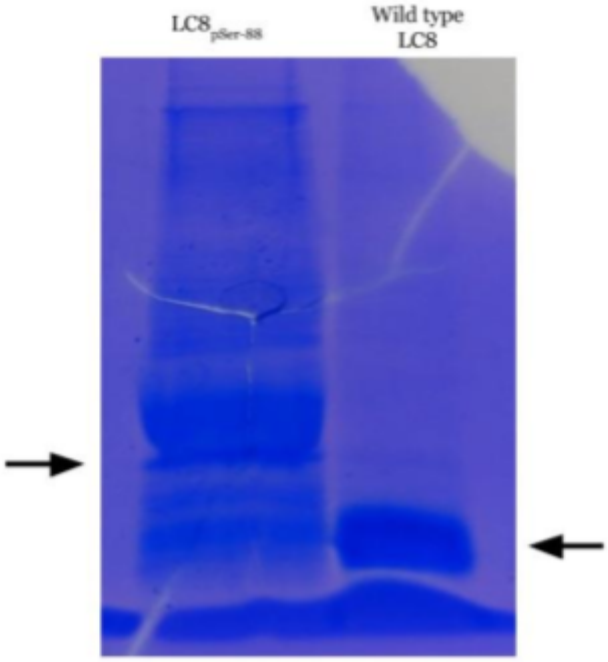

Figure 4. Phos-Tag™ Acrylamide AAL-107 Gel showing $LC8_{pSer-88}$ to the left and wild type LC8 to the right. $LC8_{pSer-88}$ lane is slower than the wild type, indicating phosphorylation of mutant LC8

phosphoserine carrying an additional negative charge compared to the wild type LC8 and that it is changing the 3D structure.

Discontinuous Native-PAGE Analysis: The simple binding assay using sfGFP-Swa on a 10% Native-PAGE gel suggests that $LC8_{pSer-88}$ is binding the Swallow region of the sfGFP-Swa. The second band is thought to be the result of a difference in concentrations between sfGFP-Swa and $LC8_{pSer-88}$, with the $LC8_{pSer-88}$ being more concentrated. This would result in all the sfGFP-Swa being bound and the rest of the $LC8_{pSer-88}$ migrating to its normally observed region. It should be noted that there is not 100% consensus about these results between other colleagues. It has been suggested that the gel picture was taken upside-down and the well order is reversed. There is evidence of this both in the expected migration of the $LC8_{pSer-88}$ and the bands observed. If this is the cause then Figure 6 would suggest $LC8_{pSer-88}$ is not binding the GFP-Swa. Repeated experiments are needed for more confidence in these results and their interpretations.

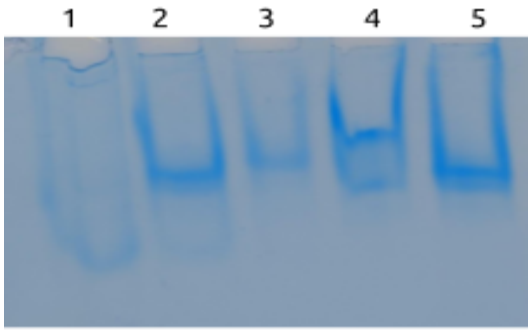

Figure 6: Photo of Native-PAGE 10% acrylamide gel.

1. GFP-Swa
2. Wild type LC8 + GFP-Swa
3. Wild type LC8
4. $LC8_{pSer-88}$ + GFP-Swa
5. $LC8_{pSer-88}$

Native-PAGE analysis of RavP binding in physiological and denaturing conditions can be seen in Figure 5. These gels suggest that wild-type LC8 is binding RavP as expected but interestingly enough $LC8_{pSer-88}$ is not binging RavP. If this is combined with sfGFP-Swa binding assay (under the interpretation of $LC8_{pSer-88}$ binding sfGFP-Swa) it would suggest that $LC8_{pSer-88}$ is selectively binding some of its partners but not others and that its binding affinities are different than wild type LC8.

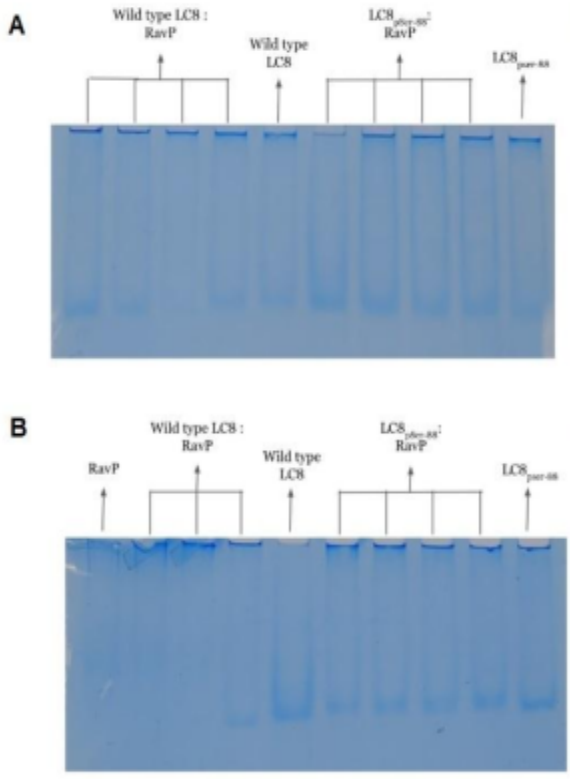

Figure 5 Photos of 10% acrylamide native gel RavP binding assay run at pH 3 and pH 7 (A and B respectively). Ratio of RavP to LC8 increases by well left to right. Bound and unbound RavP can be seen with a dark band pooled at the top of some wells and unbound LC8 is seen migrating down the length of the gel. Wells containing a mixture of RavP with bound LC8 will pool at the top of the gel. At pH 3 neither the WT LC8 or LC8 P-ser is seen to bind RavP while at pH 7 the WT LC8 binds RavP but LC8 P-ser does not.

Circular Dichroism (CD) Spectroscopy: CD analysis of LC8 samples at physiological and denaturing conditions can be seen in Figure 7.

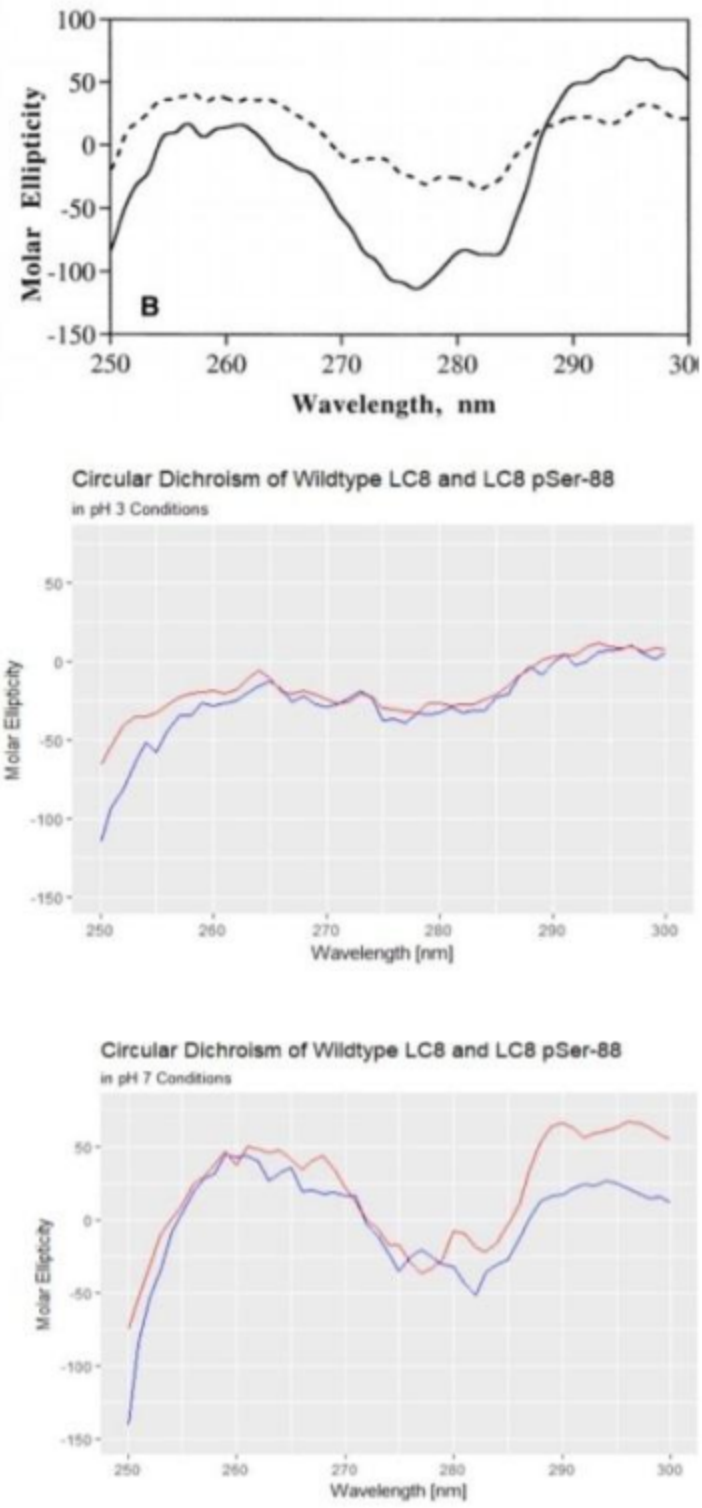

Figure 7: **A**. Graphs from Barbar et. al. paper depicting CD spectra of monomeric LC8 (dashed line) and dimeric LC8 (solid line) $(1)_{[8]}$. **B.** Spectra of wild type LC8 and $LC8_{pSer-88}$ at pH 3 conditions both produce signals characteristic of monomeric tertiary structure (see part A for reference). **C.** Spectra of wild type LC8 and $LC8_{pSer-88}$ have significant overlap with highest level of identity in the phenylalanine region (~250-260 nm) and some degree of variation in the tyrosine region (~280-300 nm). Even with the degree of uncertainty in the tyrosine region, this data suggests a high likelihood that $LC8_{pSer-88}$ exists as a homodimer at physiological pH.

The difference between the $LC8_{pSer-88}$ in sodium citrate buffer (pH 2.99) and sodium

phosphate buffer (pH 7.00) suggests that $LC8_{pSer-88}$ is in its homodimeric form. This is supported by the difference in spectra at different conditions and its similarity to known homodimeric structure in physiological conditions. The 280-300 nm region is slightly different than might be expected if the $LC8_{pSer-88}$ was fully homodimeric. This could mean that the $LC8_{pSer-88}$ is existing in some ratio of homodimeric to monomeric forms at physiological conditions. We suggest that the slight difference in this region is not due to monomeric/dimeric ratios but due to the impurity of the sample, with the background proteins causing this slight difference in the 280-300 nm region.

Conclusions:

Other studies that have examined LC8 and Pak1 (the suggested kinase of LC8) interactions have suggested that LC8 phosphorylation is responsible for the dissociation of the homodimeric form into its monomeric forms[25-26]. Based on our circular dichroism results we suggest that the $LC8_{pSer-88}$ samples are in their homodimeric form at physiological conditions and thus phosphorylation is not the (atleast main) avenue for dissociation. Perhaps Pak1 phosphorylates LC8 in monomeric form, which is supported by the fact that serine-88 is buried in the homodimeric form and is less accessible. Then the phosphorylated monomeric LC8 dimerizes to form a homodimer that is phosphorylated. This new homodimeric form could have a new 3D structure in which the binding groves have different binding affinities to protein partners. This is supported by our Native-PAGE binding assays in which the $LC8_{pSer-88}$ has different binding abilities compared to the wild type LC8 samples. It is proposed here that phosphorylation of LC8 is a major regulatory pathway for the protein but not because of its ability to cause dissociation into monomeric form. Instead we postulate that the phosphorylated LC8 regulates its function by changing the binding affinities to its different partners in its homodimeric form.

Successful incorporation of phosphoserine into LC8 is possible through methods described in this paper. Further studies using $LC8_{pSer-88}$ are needed for validation of results found in this paper. Protein purity of $LC8_{pSer-88}$ needs to be improved, potentially via the incorporation of a cleavable site before the polyhistidine tail of $LC8_{pSer-88}$ or through size-exclusion chromatography. Size-exclusion chromatography could also be used to examine whether or not $LC8_{pSer-88}$ is existing purly in its homodimeric form. More binding assays are needed as well, to test the $LC8_{pSer-88}$ binding affinity to different partners. Finally, phosphorylated $LC8_{pSer-88}$ structure should be examined in greater detail through Crystallography and NMR studies.

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