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Studying Cell Signal Transduction with Biomimetic Point Mutations

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1. Introduction

Post-translational modification (PTM), the chemical modification of a protein after its translation, represents an evolutionarily conserved mechanism of regulation of protein function. (Deribe et al., 2010) Through modulation of conformational change, enzymatic activity or interaction with other proteins, it often provides a transient switch between various intracellular signals to orchestrate an integrated response of the cell to environmental cues.

Site-directed mutagenesis has proven to be an essential tool in studying the molecular and signaling mechanisms of complex cellular functions through the ability to mutate a specific residue in the sequence of native protein in order to change its function. In combination with proteomics and database analysis of primary sequences, it offers a tool for exploring the transient nature of protein-protein interactions along with activity of enzymes, the primary assemblies of signaling nodes.

This chapter will highlight the use of site-directed mutagenesis for studying signal transduction networks. It will first describe how the diversity of the natural 22 amino acid 'toolbelt' results in fortuitous isostructural and isoelectronic similarity to several key post-translationally modifications (e.g. phosphorylation, sulfoxidation and nitrosylation). This similarity has allowed researchers to introduce gain- or loss-of-function substitutions into primary sequences and thus produce a form of the protein that mimicks its active or inactive state. The substitution of serine or threonine for phosphomimetic aspartic or glutamic acid or non-phosphorylatable residues, such as alanine, will be discussed, along with other permutations, including natural analogs of oxidized methionine and cysteine.

While discussing how site-directed mutagenesis offers to researchers a tool to engineer a constitutively active OFF and ON forms of a protein, we will also provide examples of how PTM translates into a chemical phenotype through the regulation of enzyme activity or modulation of binding affinity to partners. Also discussed in this chapter are limitations and considerations to be taken when interpreting results obtained with biomimetic tools.

With advanced computing analysis and algorithms operating on primary sequences, the predictability of PTM sites is rapidly becoming an automated process. Complex networks can be accessed and verified. Even in the age of high-resolution mass spectrometry, site-directed mutagenesis will continue to be a powerful tool for studying and validation of signaling networks.

2. Harnessing nature's toolbelt with biomimetic point mutations

Site-directed mutagenesis, a molecular biology technique used to introduce a point mutation into primary DNA sequence at a defined site, was first described by Michael Smith in 1978. (Hutchison et al, 1978) His group induced specific mutations into the viral DNA strand of the bacteriophage phiX174 *am3* using complementary oligodeoxyribonucleotides and specific mutagens. "It appears to us that general methods for the construction of any desired mutant sequence would greatly increase the ability of sequencing methods to yield biologically relevant information. Such methods should also make it possible to construct DNAs with specific modified biological functions" M. Smith and his colleagues proposed in the paper. This was proven to be correct by vast number of studies, which exploited site-directed mutagenesis as a tool for testing structure-function relationships over the course of three decades. Providing a constitutively activated or deactivated form of the protein or removing entirely the potential for protein modification, it has also been instrumental in dissecting signal transduction networks. It is instructive to understand the precise strengths and limitations of biomimetic approaches in order to extract the most meaningful data from experiments.

2.1 Layers of diversity

The quest to understand and control structure-function relationship has revealed that Nature is an exquisite chemist. The already significant chemical diversity of the amino acid pool is amplified profoundly when individual amino acids are condensed into peptide chains. Individual residues can act in a seemingly modular fashion, or residues can coordinate to bestow more complex and macroscopic functional and catalytic characteristics to the proteins and peptides on which they reside. This diversity, however, is even more dramatic than the genetic message may initially predict.

As summarized in an excellent review by Wash and co-authors, (Walsh et al., 2005), Nature has devised two mechanisms of 'proteome diversification' over the course of evolution. The amount of unique proteins per unit RNA transcript has been dramatically amplified to be far more diverse than Central Dogma of protein expression would have suggested. The first method lies at the level of the transcript. Alternate splicing of the same gene product can result in a variety of unique proteins with unique functions. The second is through targeted post-translational modification of existing proteins and peptides to chemically 'tag' them. These "tags" can directly influence the structure and function of the protein, or it may be interpreted by another docking small molecule, protein, or assembly of proteins as a biological ON/OFF switch. This largely reversible labelling offers the ability to tune and coordinate signalling networks on a timescale faster than is required for protein synthesis and proteolysis. A necessity for life, it is not surprising that many aspects of signalling cascades predate species divergence from unicellular organisms. (Tan, 2011)

While Nature has found a way to amplify the diversity of the pool of amino acid building blocks (in direct analogy to the diversification of DNA messages through arrangement of the nucleotide code), the selection pressures of evolution have still adhered to chemical intuition and energy conservation. In fact, evolution exerts less pressure on similar chemically structured residues. It seems the idea of a structural mimic is, ironically, bio-inspired.

2.2 Post-translational modification offers fast and dynamic response to stimulus

Post-translational modification of proteins offers another layer of complexity in the regulation of cellular processes. It provides a powerful tool for fast and reversible modification of protein structure, which effects dynamic changes in protein function, and ultimately translates into a cellular phenotype. The most easily deciphered systems are those in which there is a large chemical difference between the modified and non-modified group and systems that are controlled by a unimodal 'adding' or 'removing' of an effector moiety, as in the prototypical case of phosphorylation (*i.e.*, addition or removal of a phosphate group with a kinase or phosphatase, respectively). Chemical modification of an amino acid residue on an existing polypeptide is an energy-efficient means of reversibly and quickly altering the charge or steric properties of a peptide to result in a new molecular phenotype.

For example, a mere phosphorylation event induces a large structural change in the bacterial receiver domain of the nitrogen regulatory protein C (NtrC) (Kern et al., 1999) (Fig. 1). Phosphorylation of NtrC at this position Asp_{54} causes the movement of three alpha-helices, resulting in the formation of a hydrophobic patch thought to be necessary for downstream signal transduction.

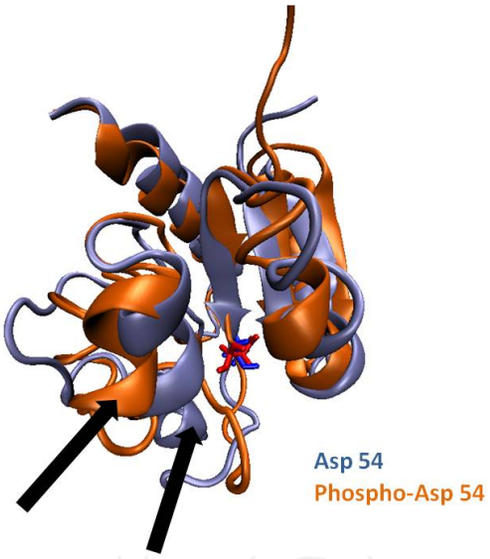

Fig. 1. Phosphorylation induces conformational changes**.** Crystal structure of NtrC before (blue) and after (orange) phosphorylation of Asp_{54}. A single residue modification from Asp_{54} (blue sticks) to the phosphorylated form (red sticks) results in major protein restructuring, including movement of α-helix 4 (black arrows) away from the active site to generate a hydrophobic patch. Structural coordinates were obtained from the Protein Databank PDB code: (1DC7, 1DC8).

To date, over 200 post-translation modifications have been identified; and they have been explored to varying extents, based on a mixture of propensity and technical accessibility. Phosphorylation, although overshadowed in propensity by glycosylation, is by far the most largely studied and characterized modification in the context of a signal relay in cellular systems. This is owed in large part to the availability of tools to efficiently mimic the isostructural property of phosphorylated residues, not the least of which is site-directed mutagenesis and the use of natural amino acids as constitutive substitutes.

2.3 Natural amino acids as substitutes for modified amino acids

Invention of site-directed mutagenesis offered scientists, for the first time, access to the entire amino acid regimen for a given residue location. Chemical similarity between certain post-translationally modified residues and some natural amino acids became central to the field of genetic-based manipulation of protein properties. Shown in Fig. 2 are examples of natural mimics of some well-studied post-translational modifications, highlighting structural similarities.

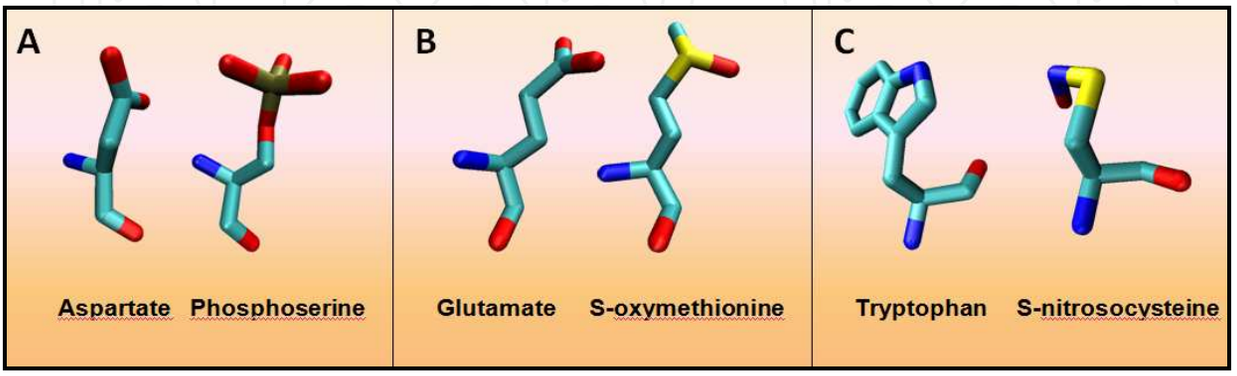

Fig. 2. Isostructural similarities between natural amino acids and post-translational modifications. Structural coordinates for natural amino acids and products of **(A)** phosphorylation **(B)**, sulfoxidation, and **(C)** nitrosylation. Carbon atoms are shown in cyan; oxygen, in red; nitrogen, in blue; and phosphorous in gold. Hydrogen atoms have been omitted for clarity.

While site-directed mutagenesis was first described in 1978 (Hutchison et al., 1978), the first examples of a PTM mimicking appeared in 1988 and 1989; the latter was described four years before the Nobel Prize in Chemistry was awarded to Michael Smith and Kary B. Mullis, for the development of site-directed mutagenesis and the polymerase chain reaction, respectively. When investigating the effect of phosphorylation of the large T antigen portion of Simian Virus 40 (SV40) on DNA-binding and replication, it was found that substitution of a known phosphorylatable Thr_{124} residue by glutamic acid, as opposed to alanine or cysteine, resulted in partial retention of native DNA replication activity. (Schneider et al., 1988) It was suggested that a negative charge, and not a phospho-group *per-se*, was required to promote DNA replication. Shortly thereafter, two papers revealed a remarkable structural and functional similarity between the phosphorylated form of serine and glutamic acid. In agreement with similar structural deviations of phosphorylated serine and glutamic acid, the substitution of the regulatory Ser_{46} for glutamic acid in HPr (Wittekind et al., 1989), a protein component of the bacterial PEP-dependent sugar-transport system, resulted in enzyme inactivation indistinguishable from the native phosphorylation. (Reizer et al., 1989) The crystal structure of unmodified and phospho-Ser_{46} would later be determined (Jia et al, 1994) (Audette et al., 2000), and an image of the electrostatic potential shows the large surface change that accompanies phosphorylation (Figure 3).

This provided the first direct evidence for the structural mimicking of a phosphate group using site-directed mutagenesis. It showed that an easily genetically encoded glutamic acid residue was a strong phosphoserine mimic. The result was an explosion in the usage of point mutations as surrogates for phosphorylated or phospho-defective forms of potential regulatory sites.

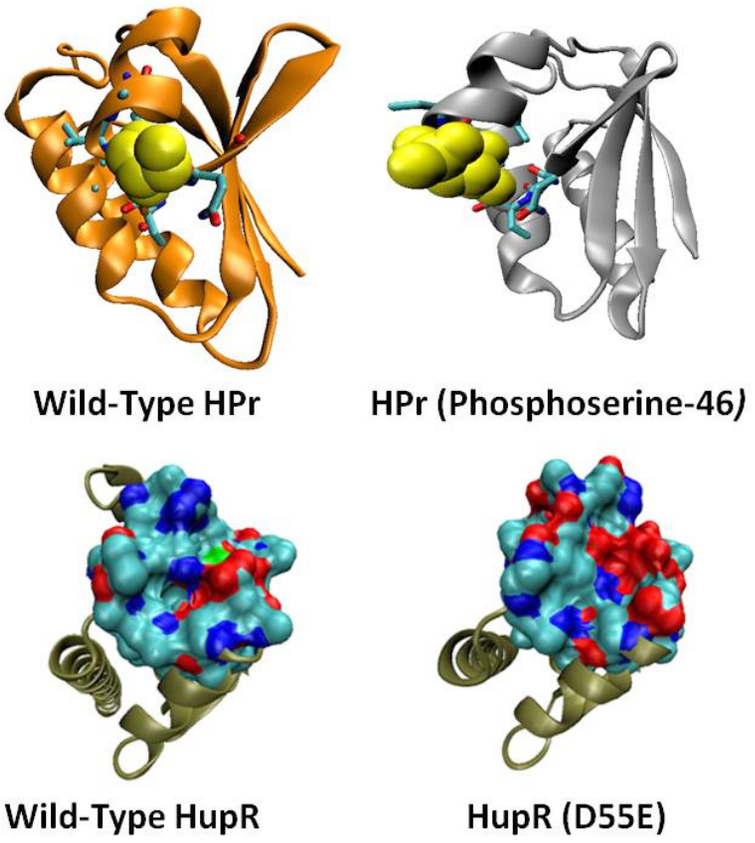

Fig. 3. Phosphorylation induces marked structural and surface-contour changes. (Top) Ribbon diagrams of crystal structures of native and phosophorylated forms of HPr; Ser_{46} is highlighted in yellow, and nearby residues are shown. (Bottom) Surface representation of the wild-type and the phosphomimetic $Asp_{55}E$ variants of Hydrogen Uptake Protein Regulator (*HupR*). A phosphomimetic mutation induced marked surface-contour changes that affected protein dimerization and function. Structural coordinates were obtained from the Protein Databank (PDB code: 1FU0, 1PTF, 2JK1, 2VUH). Highlighted residues and the surface layer proximal to residue 55 in HupR were color-coded by atom type: carbon atoms are cyan, nitrogen atoms are blue, oxygen atoms are red, and phosphorous atoms are green (inhibitor compound).

Another example of how substitution with phospho-mimetic glutamic acid can lead to a conformational change is shown in Fig. 3 (bottom). A surface-contour map of the receiver domain of nitrogen regulatory protein HupR (in which phosphorylation is actually inhibitory to transcription) before and after mutation of Asp_{55} to glutamic acid (Davies et al., 2009) illustrates a marked surface change due to mutation of a single residue, which leads to deactivation of the receiver domain in a manner similar to that observed for authentic phosphorylation.

Phosphorylation is not the only form of post-translation regulation that has been investigated with natural amino acid mimics. Biomimetic point mutations have been fundamental for studying some other means of protein modification including sulfoxidation, S-nitrosylation, tyrosine nitration, acetylation, methylation, etc. Several examples are illustrated in Table 1.

Modification	Protein	Substitution	Readout	Ref
Phosphorylation	CLIP-170	Ser→Ala Ser→Glu	Co-immunoprecipitation, protein kinetics at MT tip	(Lee et al., 2010)
	Inositol 1,4,5-Trisphosphate Receptor (InsP3R)	Ser→Ala Ser→Glu	Ca^{+2} release from ER	(Wagner et al., 2004)
	AMPA (GluA2 subunit)	Ser→Ala Ser→Glu	Conductance	(Kristensen et al., 2011)
	Itch (E3-Ubiquitin Ligase)	Ser→Ala Ser→Glu Thr→Ala Thr→Glu	Co-immunoprecipitation /Proteolysis/ Ubiquitinylation activity	(Gallagher et al., 2006)
Sulfinic acid	Peroxiredoxin	Cys→Glu	Crystallography	(Jonsson et al., 2009)
Sulfoxidation	Slo-1	Met→Leu Met→Glu	Ionic current (Inside-out patch clamp)	(Santarelli et al., 2006)
Acetylation	Heat shock protein-(HSP-90)	Lys→Arg Lys→Gln Cys→Ala	Chaperon activity	(Scroggins et al., 2007)
S-nitrosylation	Syntaxin-1	Cys→Ser Cys→Trp Cys→Met	Exocytosis (amperometry)	(Palmer et al., 2008)
	Heat shock protein-(HSP-90)	Cys→Ala Cys→Asn Cys→Asp	Protein dimerization (FRET) ATP-ase activity Reverse Transcriptase activity	(Retzlaff et al., 2009)
Methylation	Flap endonuclease 1 (FEN1)	Arg→Ala Arg→Phe Arg→Lys	Endonuclease activity	(Guo et al., 2010)

Table 1. Representative post-translational modifications mimicked by natural point mutations using site-directed mutagenesis.

Amino acid oxidation is a common mechanism for diversification of certain residues; and, like phosphorylation, there are both addition and removal effector proteins associated with the modification. The reactive sulfur group of the methionine residue can be oxidatively modified with a number of reagents to methionine sulfoxide or methionine sulfone, dramatically increasing the polarity and hydrophobicity of the residue. (Black and Mould, 1991) (Hoshi and Heinemann, 2001) A family of enzymes known as methionine sulfoxide reductases have been found competent to reduce the methionine sulfoxide back to a thioether, a necessary requirement for a signalling relay. Substitution of a leucine residue for methionine offers a suitably isostructural null mimic, removing the oxidizeable sulphur atom while maintaining similar geometric and hydrophobic characteristics. (Ciorba et al., 1997) (Chen et al., 2000) In one example, this modification, which adds electron density onto the long methionine residue, has been mimicked *in vitro* by glutamic acid substitution. Interestingly, it was recently found that calcium and voltage gated BK (Big Potassium) ion channels, responsible for regulating K^+ ion flux, are sensitive to oxidation. This has been accomplished through lining of ion-selective pore with the hydrophobic Met_{536}, Met_{712}, and Met_{739} residues, which can be oxidatively modified to the more polar methionine sulfoxide, offering tuning of the hydrophobicity of channel. Oxidation of these methionine residues results in a decrease in half-activation voltage, increasing channel activity. Interestingly, one must mutate all three methionine residues

within the channel to leucine to render the channel insensitive to oxidant treatment. If, however, one replaces any one of these three residues with glutamic acid, the channel behaves just like it does in the wild-type protein after treatment with the oxidant. (Santarelli et al., 2006) Thus, the technique of site-directed mutagenesis revealed that any of the three separate methionine residues can confer the oxidant-sensitivity phenotype to the protein, highlighting a robust selection pressure for that function.

Oxidized forms of cysteine, including nitrosyl, sulfenic acid, and sulfinic acid variants, have been implicated in reversible signalling arrays (Reddie and Carroll, 2008), and some have been mimicked with the use of natural amino acids. For example, cysteine sulfinic acid differs from aspartic acid by a single-atom replacement of sulphur atom with a carbon atom. This phosphomimicking strategy has been used in the investigation of the mechanism of peroxiredoxin (Pxr) (Jonsson et al., 2009), an enzyme involved in the ATP-dependent reduction of cysteine sulfinic acid back to a thiol residue. Reduction of an active site cysteine-sulfinic acid is first accomplished through ATP-dependent phospho-deficient of the sulfinic acid residue, followed by attack of a neighboring cysteine from sulfiredoxin (Srx) enzyme on the sulfinic phosphory ester intermediate. Remarkably, replacement of the catalytic cysteine in Pxr with an aspartic acid residue results in phosphorylation of the aspartic acid, but does not allow subsequent reactivity with the docked Srx. Thus, the single-atom replacement offered by native site-directed mutagenesis allowed for detailed mechanistic study of a trapped intermediate complex, inaccessible with other techniques. Substitution of a cysteine residue with a serine residue offers an effective single-atom replacement of a sulphur atom with an oxygen atom and eliminates sulphur-based chemistry from the site, while minimizing steric differences.

In several cases, tryptophan was reported to be an effective mimic of nitroso-cysteine (Palmer et al., 2008) (Wang et al., 2009) as computer simulations of the cysteine to tryptophan mutant suggest it contains steric bulk not offered by the next closest steric match, methionine, despite the absence of a sulphur atom in the former. In another case, nitroso-cysteine modification was mimicked by substitution with asparagine in studies investigating the effect of nitrosylation on ATPase activity of the 90 kDa heat shock protein (γHSP90). (Retzlaff et al., 2009) (Scroggins and Neckers, 2009) In this study an NO-insensitive γHSP90 was rendered sensitive to inactivation by NO treatment due to replacement of an alanine residue in the C-terminal domain with a cysteine, as this corresponding site is known to be a cysteine in all other HSP variants. Mutation of the engineered site to asparagine (γHSP90 A577N) recapitulates NO treatment of the engineered cysteine mutant, in contrast to a control isoleucine mutant (γHSP90 A577I), which serves as an unmodifiable mimick (shown in Fig 4). Although no citation or rationale was used for the mutant choices, these mimicking residues indeed recapitulated ATPase activity and chaperone activity of the NO-free and NO-bound forms of engineered γHSP90 protein.

As virtually every intracellular process is under dynamic regulation, it is no surprise that PTM-mimicking mutations have been used in conjunction with a myriad of analytical techniques. Shown in Table 1 are some of examples relevant to native site-directed mutagenesis - giving a flavour of the breadth of the strategy. In a manuscript that would bring the strategies of the last nearly 35 years full circle, it has recently been suggested that throughout evolution, phosphoserine residues on proteins have been mutated, through genetic drift. In one study they were more frequently converted into glutamic acid and

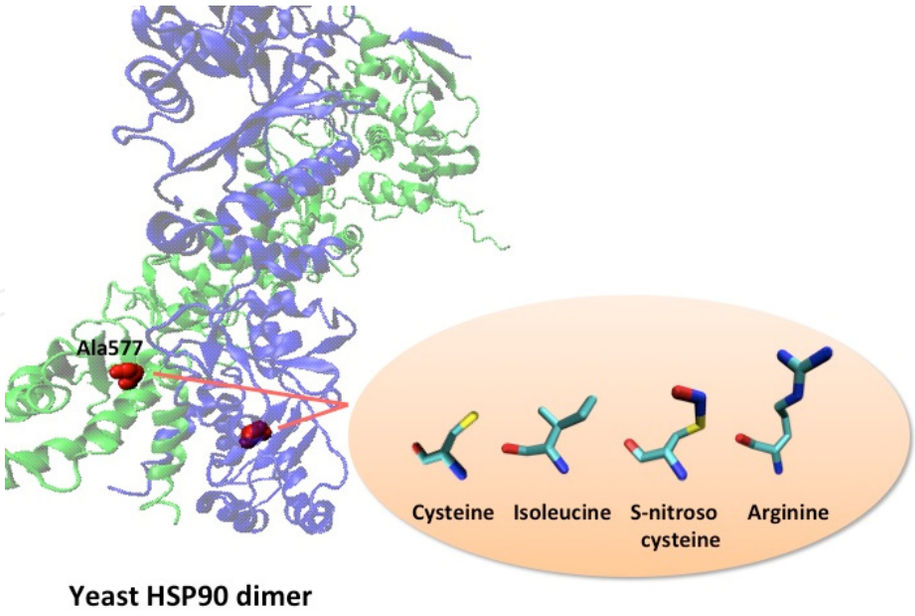

Fig. 4. Mimicking S-nitrosylation in an engineered regulatory site. Ribbon representation of X-ray crystal structure of the Yeast HSP90 dimer; native Ala 577 (left) and engineered variants at position 577 (right) are highlighted in red. Note that there are structural similarities between cysteine and the non-modifiable isoleucine and between S-nitrosocysteine and arginine. Structural coordinates were obtained from the Protein Databank (PDB code: 2CG9) Stick-models of residues were color-coded by atom type: carbon atoms are cyan, nitrogen atoms are blue, and oxygen atoms are red.

aspartic acid than into any other residue, demonstrating evolution away from the switchable into permanent ON or OFF functions. (Kurmangaliyev et al., 2011) Although this work focused on non-structured regions and solely on phosphoserine evolution (the only post-translational modification for which there were sufficient data sets for statistical interpretation), these results might be viewed as an acknowledgement from biology of a mere requirement for a well-placed charge to induce the biological phenotype for which the switch evolved.

2.4 Site-directed mutagenesis in animal models

The ultimate verification of the experimental models obtained in *in vitro* assays or even in cultured cells comes with experiments in living organisms. Site-directed mutagenesis, in combination with other technologies for delivering a tailored plasmid to more complex genomes, has allowed the knock-in of mutations for individual engineered proteins in entire organisms, including mammals. (Yang et al., 1997) This technology has been used to investigate the role of individual amino acids in many biological processes and disease states, such as ASD (Autism Spectrum Disorder) (Bader et al., 2011), malignant hyperthermia (associated with heat stroke susceptibility) (Durham et al., 2008), and depression (Talbot et al., 2010), in the context of a living mammals. Phosphomimetic and phospho-deficient mutations have been knocked-in as well, leading to new understanding of downstream events to protein modification in the context of a living organism. In one prominent example, phosphorylation of ribosomal protein S6 was linked to control of cell size and glucose homeostasis in certain cell types through the use of a phospho-deficient

knock-in mouse model. (Ruvinsky et al., 2005) In this model, all five potentially phosphorylatable serine residues in the putative region of rS6 were mutated to alanine ($rpS6^{P-/-}$) to abolish the potential for their phosphorylation. Smaller cell size, increased protein synthesis, and a severe decrease of circulating insulin were among the phenotypic signatures of the phospho-deficient variant. The phenotype was later shown to include decreased muscle mass and energy availability.

In another salient example, both phosphomimetic and phospho-deficient mutations were generated in a transgenic mouse model of Huntington's disease - a neurodegenerative disorder characterized by motor impairments and various psychiatric symptoms. (Gu et al., 2009) In this model, two phosphorylatable serine residues (13 and 18) in the N-terminus of a disease-promoting mutant of the huntingtin protein (*mhtt*) were mutated to either glutamate (phosphomimetic) or alanine (phospho-deficient) residues to investigate effect of phosphorylation on modification of the *htt* protein with a polyglutamine (PolyQ) extension, and on the onset of Huntington's Disease. Both mutants were viable and successfully rescued the embryonic lethality of an *htt* knockout mutant. A remarkable difference was observed between the Ala and Glu variants. The mice that were phosphodeficient (Ala) in the two serine residues showed onset of disease pathogenesis from the mutant *mhtt* - as measured by neuropathological examination of brain tissue sections, as well as by various motor and psychological tests. Remarkably, the phosphomimetic mutant mice (Glu) showed resistance to *mhtt*-induced disease pathogenesis. This technique clearly demonstrated that loss–of-phosphorylation of these two serine sites is a key signature of the pathogenesis *in vivo*. These results further suggested that the N-terminus of *htt* may be a target for future drug design to treat disease, even after the initializing mutation is present.

Indeed these examples show the power of site-directed mutagenesis in studying the signaling events associated with the disease state. However, caution must always be taken in the interpretation of data obtained from studies using biomimetic constructs.

2.5 Limitations of site-directed mutagenesis for targeting signaling nodes

Despite vast precedent for the utilization of amino acid surrogates and mimics of post-translational modifications in the literature, it must be remembered that the data and interpretation are only as robust as the chemical match of the surrogate moiety to the target. For example, the carboxylic acid functionality of glutamic acid has differing fundamental chemical properties from a serine-phosphate group, including, but not limited to, access to a (-2) charge state (in the case of phosphate) and different acid dissociation constants. Despite this, the single mutation has proven a valuable tool in the mimicking of phosphorylated residues. In addition it has become a common and quite inexpensive laboratory procedure, which is qualified as a 'quick-check' protocol to produce constitutively active, inactive or null mutants of potential phosphorylation sites.

As mentioned, PTMs that result in a significant size or charge difference from the unmodified states have been among the earliest developed. For example, while phosphorylation is the most thoroughly studied form of modification, it has been predicted that protein methylation will be among the slowest developed signalling pathways. This is due to the subtle spatial and charge density differences between methylated and non-methylated forms of an amino acid residue. With mass spectrometry virtually alone in the struggle to address such modifications, the decoding of such signalling networks awaits the

development of sufficient chemical tools to recognize such subtle nodes and expedite interpretation and clarification of mass spectral data. (Huttlin et al., 2010)

It is probable that certain interactions, or classes of interactions, may be more stringent in which the electronic features of post-translationally modified residues are recognized and interpreted as signals. The importance of a cautious eye when utilizing signalling mimics was recently made salient when a phosphometic serine to glutamic acid mutant recapitulated one aspect of a signalling cascade, but not another. (Paleologou et al., 2010) While investigating potential phosphorylation sites in α-synuclein - a major component of the Alzheimer's disease amyloid - the authors generated serine to glutamate and serine to alanine mutants and compared them to wild-type and authentic phosphorylated material in ability to localize at the membrane and participate in fibril formation. In a model wherein phosphorylation of Ser_{87} blocks membrane recruitment and fibril formation, it was shown that substitution of native Ser_{87} with alanine resulted in wild-type-like membrane association and fibril formation, while authentic phospho-Ser_{87}, along with the Ser_{87}E mutant, showed blunted formation of fibrils and poor membrane recruitment. Circular dichroism studies showed that, in a micellar environment, the authentic phospho-Ser_{87} modification resulted in an unstructuring of the random coiled protein, whereas the phosphomimic mutant could not recapitulate the membrane-associate phenotype. This unstructuring has been shown to be important in the interaction with membrane contents, and may further alter protein specificity. Thus, the phosphomimetic mutation introduced with site-directed mutagenesis could only reproduce one aspect of the biological phenotype. In this case, the authors were able to view the limits of the phosphomimic approach because they had access to constructs containing the authentic phosphoprotein for comparison. Nevertheless, it serves as an instructive case in which it is acknowledged that a given PTM can have multiple roles or be required in multiple steps of a signalling cascade or pathway.

3. Conclusion

We have demonstrated that site-directed mutagenesis has played an indispensable role in the unravelling of signal transduction pathways by offering access to constitutively active or inactive forms of several common PTMs. A critical eye must be used when utilizing surrogate residues that lack the capacity for a switchable context, and it is important to understand the limits of any biomimetic approach before results can be properly interpreted. Even with these limitations, it is no wonder the technique of site-directed mutagenesis has penetrated the field of signal transduction since the very conception of the technology. Indeed the utility of site-directed mutagenesis for the development and verification of models for biological processes and disease states will remain beneficial for years to come.

4. Acknowledgment

This work is supported by NIH Training Grant T32 HL007829 to Sieracki N. and RO1 HL103922 to Komarova Y.

5. References

Audette G.F., Engelmann R., Hengstenberg W., Deutscher J., Hayakawa K., Quail J.W., Delbaere L.T.J., 2000. The 1.9 Å Resolution Structure of Phospho-Serine 46 HPr from Enterococcus Faecalis. J. Mol. Biol. 303, 545-553.

Bader P.L., Faizi M., Kim L.H., Owen S.F., Tadross M.R., Alfa R.W., Bett G.C.L., Tsien R.W., Rasmusson R.L., Shamloo M. 2011. Mouse model of Timothy syndrome recapitulates triad of autistic traits. Proc. Natl Acad. Sci. USA 108, 15432-15437

Black S.D., Mould D.R., 1991. Development of Hydrophobicity Parameters to Analyze Proteins Which Bear Post- or Cotranslational Modifications. Anal. Biochem. 193, 72-82.

Chen J., Avdonin V., Ciorba M.A., Heinemann S.H., Hoshi T., 2000. Acceleration of P/C-Type Inactivation in Voltage-Gated K^+ Channels by Methionine Oxidation. Biophys. J. 78, 174-187.

Ciorba M.A., Heinemann S.H., Weissbach H., Brot N., Hoshi T., 1997. Modulation of Potassium Channel Function by Methionine Oxidation and Reduction. Proc. Natl Acad. Sci. USA 94, 9932-9937.

Davies K.M., Lowe E.D., Vénien-Bryan C., Johnson L.N., 2009. The HupR Receiver Domain Crystal Structure in its Nonphospho and Inhibitory Phospho States. J. Mol. Biol. 385, 51-64.

Deribe Y.L., Pawson T., Dikic I., 2010. Post-Translational Modifications in Signal Integration. Nat. Struct. Mol. Biol. 17, 666-672.

Durham W.J., Aracena-Parks P., Long C., Rossi A.E., Goonasekera S.A., Boncompagni S., Galvan D.L., Gilman C.P., Baker M.R., Shirokova N., Protasi F., Dirksen R., Hamilton S.L. 2008. RyR1 S-Nitrosylation Underlies Environmental Heat Stroke and Sudden Death in Y522S RyR1 Knockin Mice. Cell 133, 53-65.

Gallagher E., Gao M., Liu Y.-C., Karin M., 2006. Activation of the E3 Ubiquitin Ligase Itch Through a Phosphorylation-Induced Conformational Change. Proc. Natl Acad. Sci. USA 103, 1717-1722.

Guo Z., Zheng L., Xu H., Dai H., Zhou M., Pascua M.R., Chen Q.M., Shen B., 2010. Methylation of FEN1 Suppresses Nearby Phosphorylation and Facilitates PCNA Binding. Nat. Chem. Biol. 6, 766-773.

Hoshi T., Heinemann S.H., 2001. Regulation of Cell Function by Methionine Oxidation and Reduction. J. Physiol. 531, 1-11.

Hutchison C.A., Phillips S., Edgell M.H., Gillam S., Jahnke P., Smith M., 1978. Mutagenesis at a Specific Position in a DNA Sequence. J. Biol. Chem. 253, 6551-6560.

Huttlin E.L., Jedrychowski M.P., Elias J.E., Goswami T., Rad R., Beausoleil S.A., Villén J., Haas W., Sowa M.E., Gygi S.P., 2010. A Tissue-Specific Atlas of Mouse Protein Phosphorylation and Expression. Cell 143, 1174-1189.

Jia Z., Vandonselaar M., Hengstenberg W., Wilson Quail J., Delbaere L.T.J., 1994. The 1.6 Å Structure of histidine-Containing Phosphotransfer Protein HPr from Streptococcus Faecalis. J. Mol. Biol. 236, 1341-1355.

Jonsson T.J., Johnson L.C., Lowther W.T., 2009. Protein Engineering of the Quaternary Sulfiredoxin-Peroxiredoxin Enzyme-Substrate Complex Reveals the Molecular Basis for Cysteine Sulfinic Acid Phosphorylation. J. Biol. Chem. 284, 33305-33310.

Kern D., Volkman B.F., Luginbuhl P., Nohaile M.J., Kustu S., Wemmer D.E., 1999. Structure of a Transiently Phosphorylated Switch in Bacterial Signal Transduction. Nature 402, 894-898.

Kristensen A.S., Jenkins M.A., Banke T.G., Schousboe A., Makino Y., Johnson R.C., Huganir R., Traynelis S.F., 2011. Mechanism of Ca^{2+}/Calmodulin-Dependent Kinase II Regulation of AMPA Receptor Gating. Nature 14, 727-735.

Kurmangaliyev Y., Goland A., Gelfand M., 2011. Evolutionary Patterns of Phosphorylated Serines. Biol. Direct 6, 8.

Lee H-S., Komarova Y.A., Nadezhdina E.S., Anjum R., Peloquin J.G., Schober J.M., Danciu O., van Haren J., Galjart N., Gygi S.P., Akhmanova A, Borisy G.G., 2010.

Phosphorylation Controls Autoinhibition of Cytoplasmic Linker Protein-170. Mol. Biol. Cell 21, 2661-2673.

Paleologou K.E., Oueslati A., Shakked G., Rospigliosi C.C., Kim H.-Y., Lamberto G.R., Fernandez C.O., Schmid A., Chegini F., Gai W.P., Chiappe D., Moniatte M., Schneider B.L., Aebischer P., Eliezer D., Zweckstetter M., Masliah E., Lashuel H.A., 2010. Phosphorylation at S87 Is Enhanced in Synucleinopathies, Inhibits a-Synuclein Oligomerization, and Influences Synuclein-Membrane Interactions. J. Neurosci. 30, 3184-3198.

Palmer Z.J., Duncan R.R., Johnson J.R., Lian L.-Y., Mello L.V., Booth D., Barclay J.W., Graham M.E., Burgoyne R.D., Prior I.A., Morgan A., 2008. S-Nitrosylation of Syntaxin 1 at Cys145 is a Regulatory Switch Controlling Munc18-1 Binding. Biochem. J. 413, 479-491.

Reddie K.G., Carroll K.S., 2008. Expanding the Functional Diversity of Proteins Through Cysteine Oxidation. Curr. Opin. Chem. Biol. 12, 746-754.

Reizer J., Sutrina S.L., Saier M.H., Stewart G.C., Peterkofsky A., Reddy P., 1989. Mechanistic and Physiological Consequences of HPr(ser) Phosphorylation on the Activities of the Phosphoenolpyruvate:Sugar Phosphotransferase System in Gram-Positive Bacteria: Studies with Site-Specific Mutants of HPr. EMBO J. 8, 2111-2120.

Retzlaff M., Stahl M., Eberl H.C., Lagleder S., Beck J., Kessler H., Buchner J., 2009. Hsp90 is Regulated by a Switch Point in the C-terminal Domain. EMBO Rep. 10, 1147-1153.

Santarelli L.C., Wassef R., Heinemann S.H., Hoshi T., 2006. Three Methionine Residues Located Within the Regulator of Conductance for K+ (RCK) Domains Confer Oxidative Sensitivity to Large-Conductance Ca2+-Activated K+ Channels. J. Physiol. 571, 329-348.

Schneider J., Fanning E., 1988. Mutations in the Phosphorylation Sites of Simian Virus 40 (SV40) T Antigen Alter its Origin DNA-Binding Specificity for Sites I or II and Affect SV40 DNA Replication Activity. J. Virol. 62, 1598-1605.

Scroggins B.T., Neckers L., 2009. Just Say NO: Nitric Oxide Regulation of Hsp90. EMBO Rep. 10, 1093-1094.

Scroggins B.T., Robzyk K., Wang D., Marcu M.G., Tsutsumi S., Beebe K., Cotter R.J., Felts S., Toft D., Karnitz L., Rosen N., Neckers L., 2007. An Acetylation Site in the Middle Domain of Hsp90 Regulates Chaperone Function. Mol. Cell 25, 151-159.

Tan C.S.H., 2011. Sequence, Structure, and Network Evolution of Protein Phosphorylation. Sci. Signal. 4, 6.

Wagner L.E., Li W.-H., Joseph S.K., Yule D.I., 2004. Functional Consequences of Phosphomimetic Mutations at Key cAMP-Dependent Protein Kinase Phosphorylation Sites in the Type 1 Inositol 1,4,5-Trisphosphate Receptor. J. Biol. Chem. 279, 46242-46252.

Walsh C.T., Garneau-Tsodikova S., Gatto G.J., 2005. Protein Posttranslational Modifications: The Chemistry of Proteome Diversifications. Angew. Chem. Int. Edit. 44, 7342-7372.

Wang P., Liu G.-H., Wu K., Qu J., Huang B., Zhang X., Zhou X., Gerace L., Chen C., 2009. Repression of Classical Nuclear Export by S-nitrosylation of CRM1. J. Cell Sci. 122, 3772-3779.

Wittekind M., Reizer J., Deutscher J., Saier M.H., Klevit R.E., 1989. Common Structural Changes Accompany the Functional Inactivation of HPr by Seryl Phosphorylation or by Serine to Aspartate Substitution. Biochemistry 28, 9908-9912.

Yang X.W., Model P., Heintz N., 1997. Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. Nat Biotechnol. 15(9):859-65.