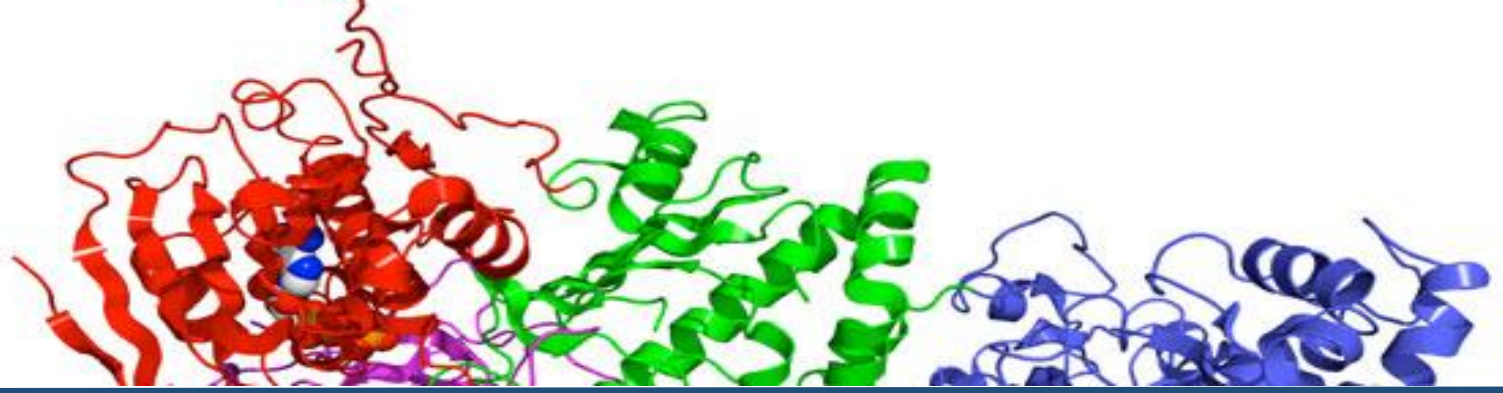


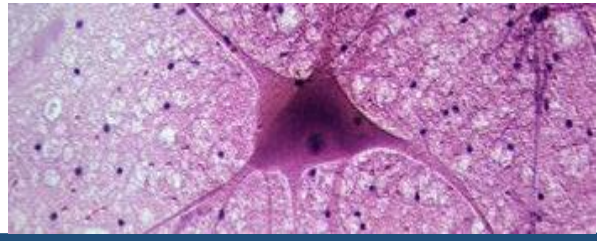
Determining the structural effects of Hsp90 nitration on protein interactions that lead to cell death

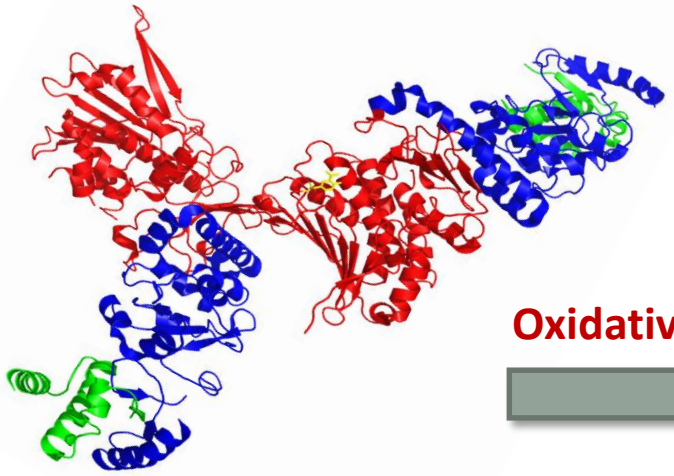
Taylor Bundy

Advisor: Dr. Ryan Mehl, Associate Professor of Biochemistry

Overview: Hsp90 nitration and cell death

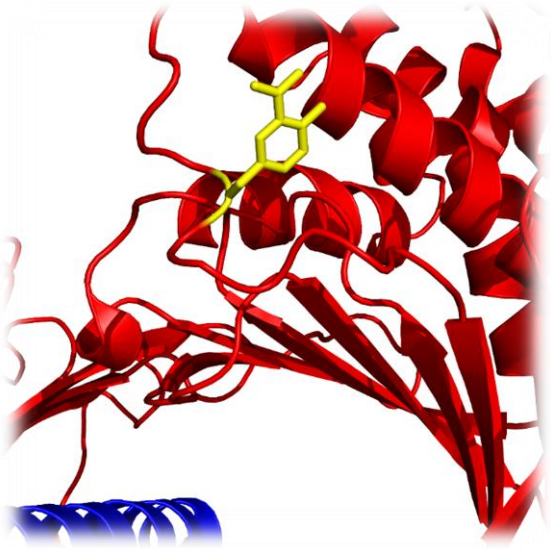
A role in oxidative stress-induced neurodegeneration





Heat Shock Protein 90

Oxidative stress



Nitration of tyrosine 33 or 56

Cell death

Neurodegeneration

Nitration of tyrosine residues 33 or 56 within Hsp90's active site leads to cell death through a toxic gain of function (Franco, Ye, et al. 2012)

Neurodegeneration

A prominent issue in medicine and society

An incurable and debilitating condition resulting in progressive degeneration through the malfunction and death of nerve cells

Common Diseases:

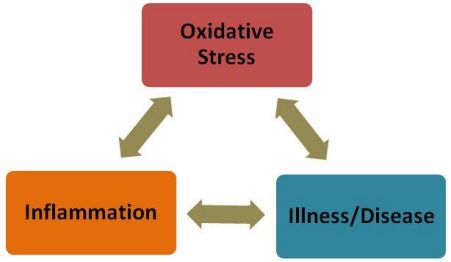
- Amyotropic Lateral Sclerosis (ALS)
- Parkinson's
- Huntington's
- Alzheimer's and other dementias

Subcellular Factors:

- Genetic mutations
- Protein misfolding and aggregation
- Intracellular mechanisms
- Oxidative stress

Oxidative stress

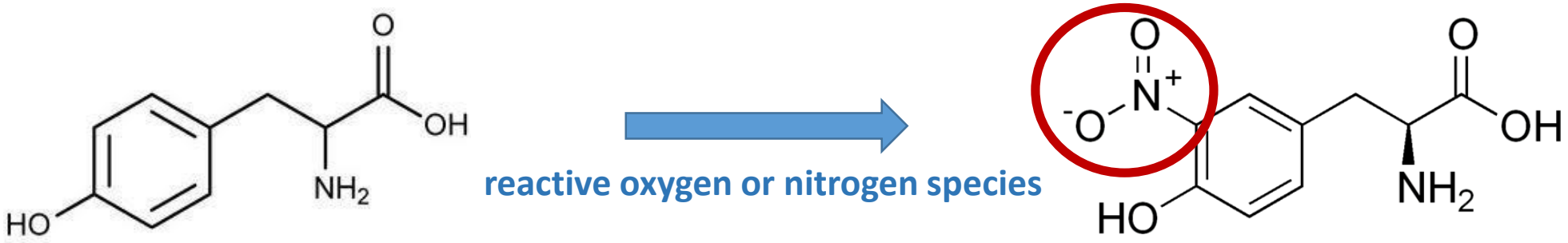
Reactive species altering biochemistry



An imbalance in free radical formation within a cell or organism, most commonly in the form of ***reactive oxygen or nitrogen species***

Has been linked with many different pathologies, originating as an intracellular issue

Leads to the formation of oxidized and nitrated molecules—including tyrosine residues in proteins such as **Hsp90**

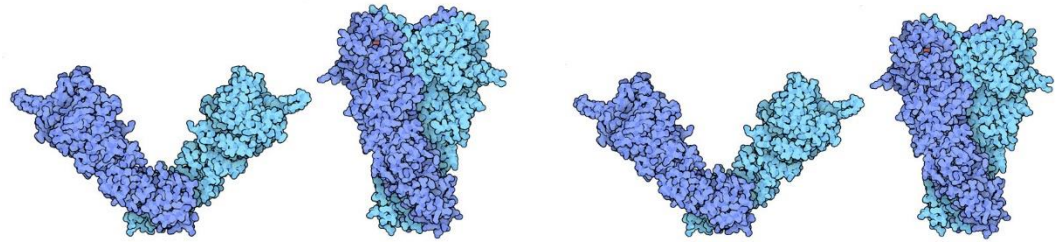


Tyrosine residue in a protein

Nitrated tyrosine residue in a protein

Hsp90

Function and cellular role



A 90kd, ubiquitous heat-shock protein with many diverse functions sorted into two categories:

1. **Chaperone activity**
   - Assists in the folding and stabilization of cellular proteins
   - Activates and inactivates proteins depending on the cell's needs

2. **Cellular signaling role**
   - Hsp90 is known to be involved in several signaling pathways, including the regulation of receptors in cell death pathways

Hsp90's function is generally dependent on its ability to

hydrolyze ATP within its active site

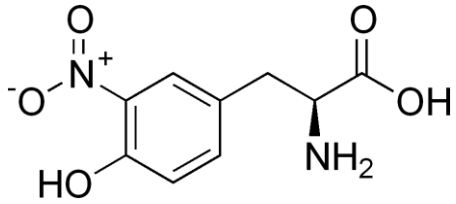
Non-canonical amino acids

Expanding the genetic code

- **Beyond the 20 natural amino acids**
  - More building blocks to create new proteins with potential new functions

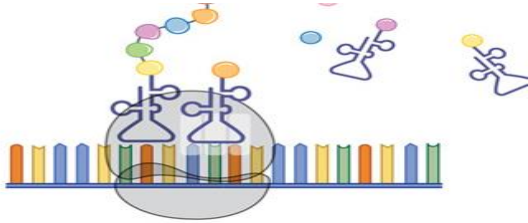
- **Manipulation of protein form and function**
  - Allows for the direction and observation of intracellular reactions and activities
  - Advances the capabilities of biochemical studies and technological development with bio-molecules

One very important ncAA is **nitrotyrosine**



Incorporating nitrotyrosine

Mimicking conditions of cellular stress



64 different codons—sets of three nucleotides—coding for natural amino acids or the cessation of translation. The latter are referred to as ***nonsense*** *or* ***stop codons.***

Nonsense Suppression

- E. coli can be made to have mutations that replace normal codons with nonsense codons, called a **TAG** site
- Rather than truncating the protein, these nonsense codons can be suppressed
- This allows for the ***site-directed incorporation*** of a non-canonical amino acid via a corresponding tRNA

Site-directed incorporation of ncAA's

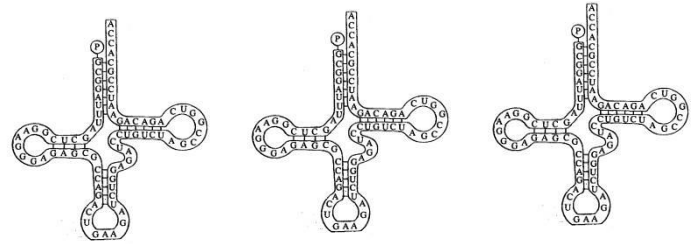
- Site specific
- Can select any residue(s) within a protein to become an ncAA
- Allows for the observance of functional changes in a specific manner
- **What is needed to accomplish this?**

Post-translational Chemical modification

- Not site specific
- Little control over how and where the protein is modified
- Alteration of residues by disease states or the addition of a chemical

Aminoacyl tRNA Synthetases

Enzymes essential to translation

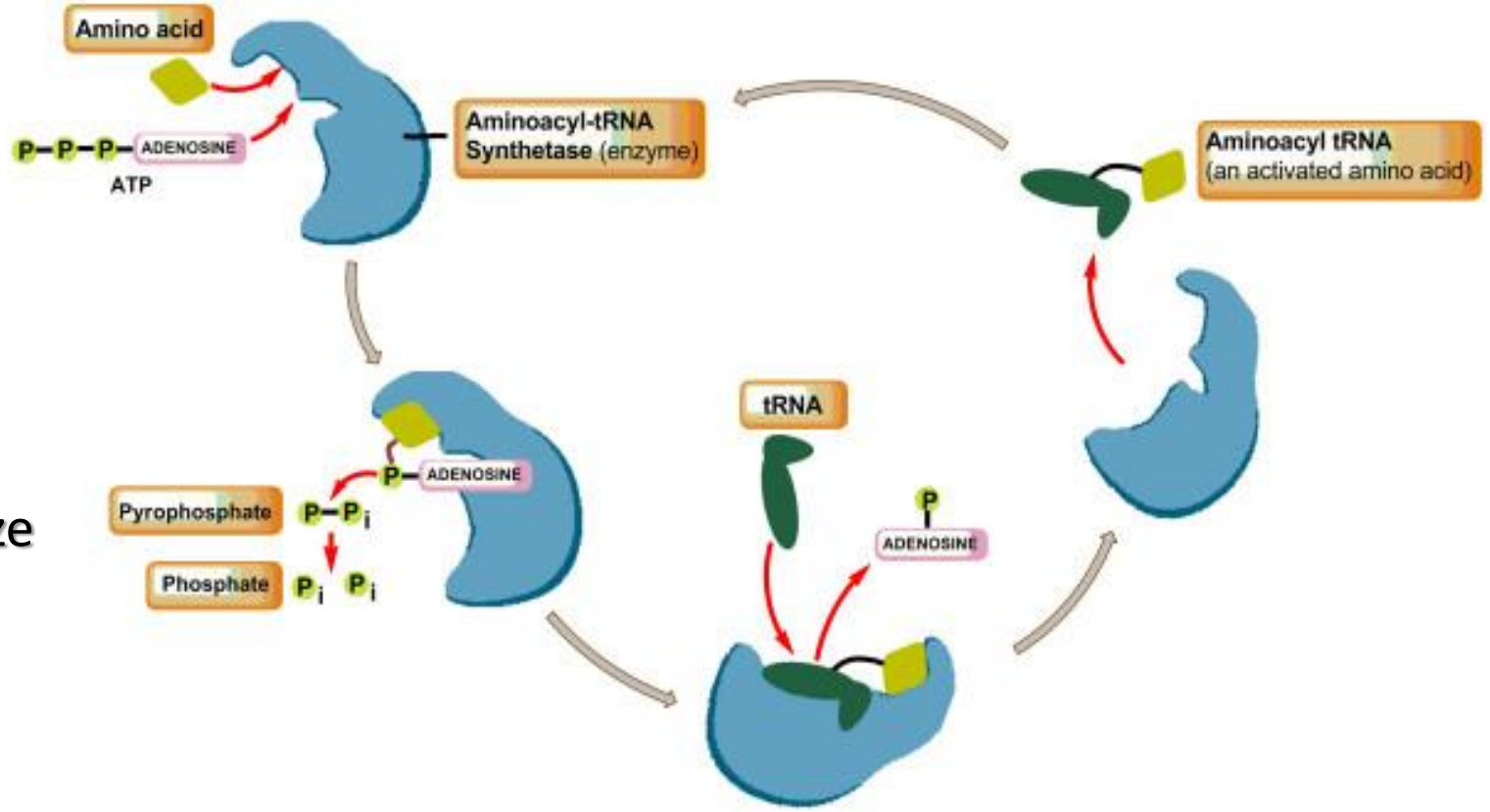


- During the process of translation, a polypeptide chain is formed

- The addition of amino acids is facilitated by tRNAs

- Synthetase enzymes are able to recognize a specific tRNA and catalyze its binding to a particular amino acid

- Synthetases can be selected to recognize and incorporate non canonical amino acids



Synthetase characterization in GFP

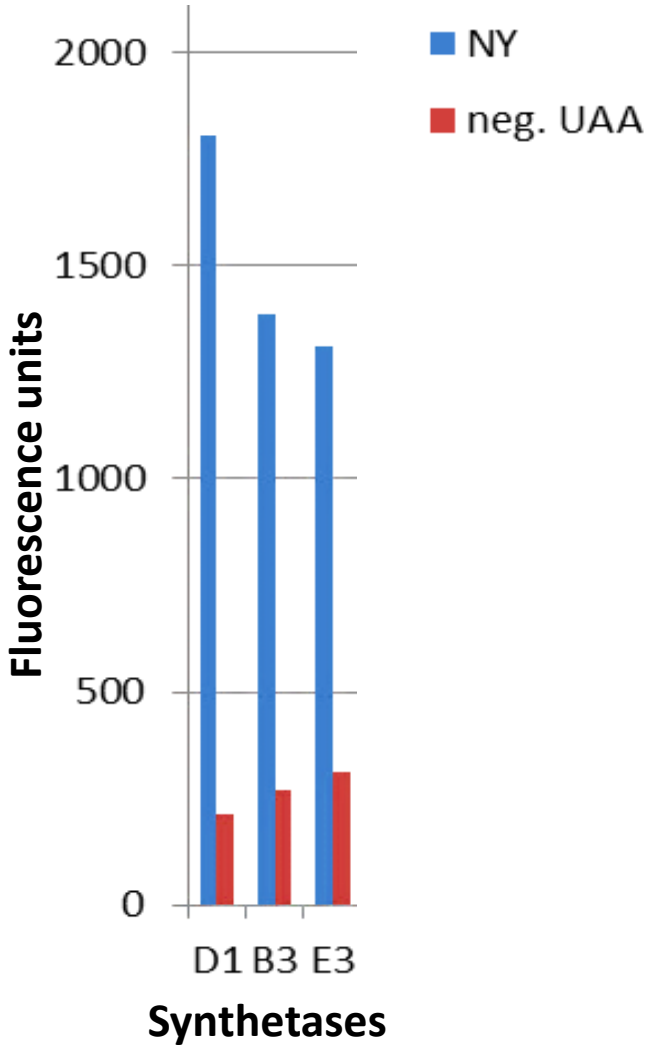
Measuring efficiency and fidelity

Green Fluorescence Protein (GFP)

- Synthetases are used to incorporate nitrotyrosine into GFP with a **TAG** site
- If successfully incorporated, the protein will fold properly and fluoresce, a phenomenon that can be measured and graphed

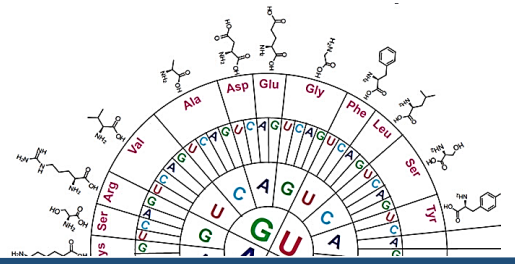
Efficiency — **How efficiently an ncAA such as nitrotyrosine is incorporated into a protein, assessed in GFP**

Fidelity — **In the absence of a ncAA, to what extent the synthetase incorporates other things into the protein**



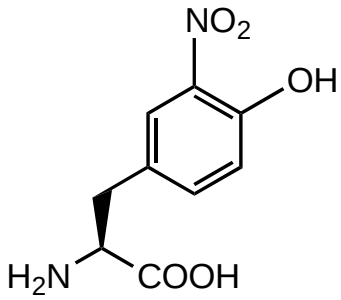
ncAAs other than NY (mimics)

Assessing the role of structure

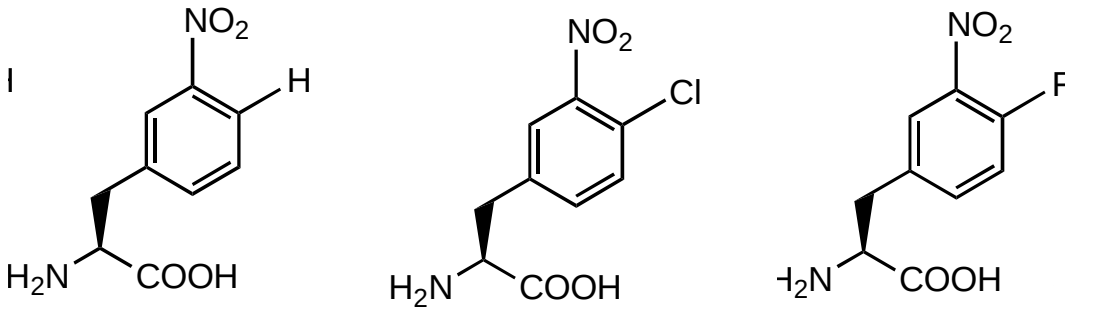


- What is it about the structure of nitrotyrosine that causes death in motor neuron cells?
- Will ncAA's with similar structure incorporated into Hsp90 yield similar results?

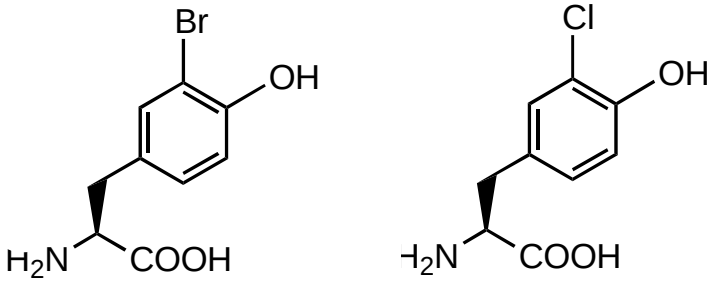
Nitrotyrosine



Retain nitro group, replaced hydroxyl group



Retain hydroxyl group, replaced nitro group with other electron withdrawing groups



GFP can again be used to determine which synthetases best incorporate these ncAA's

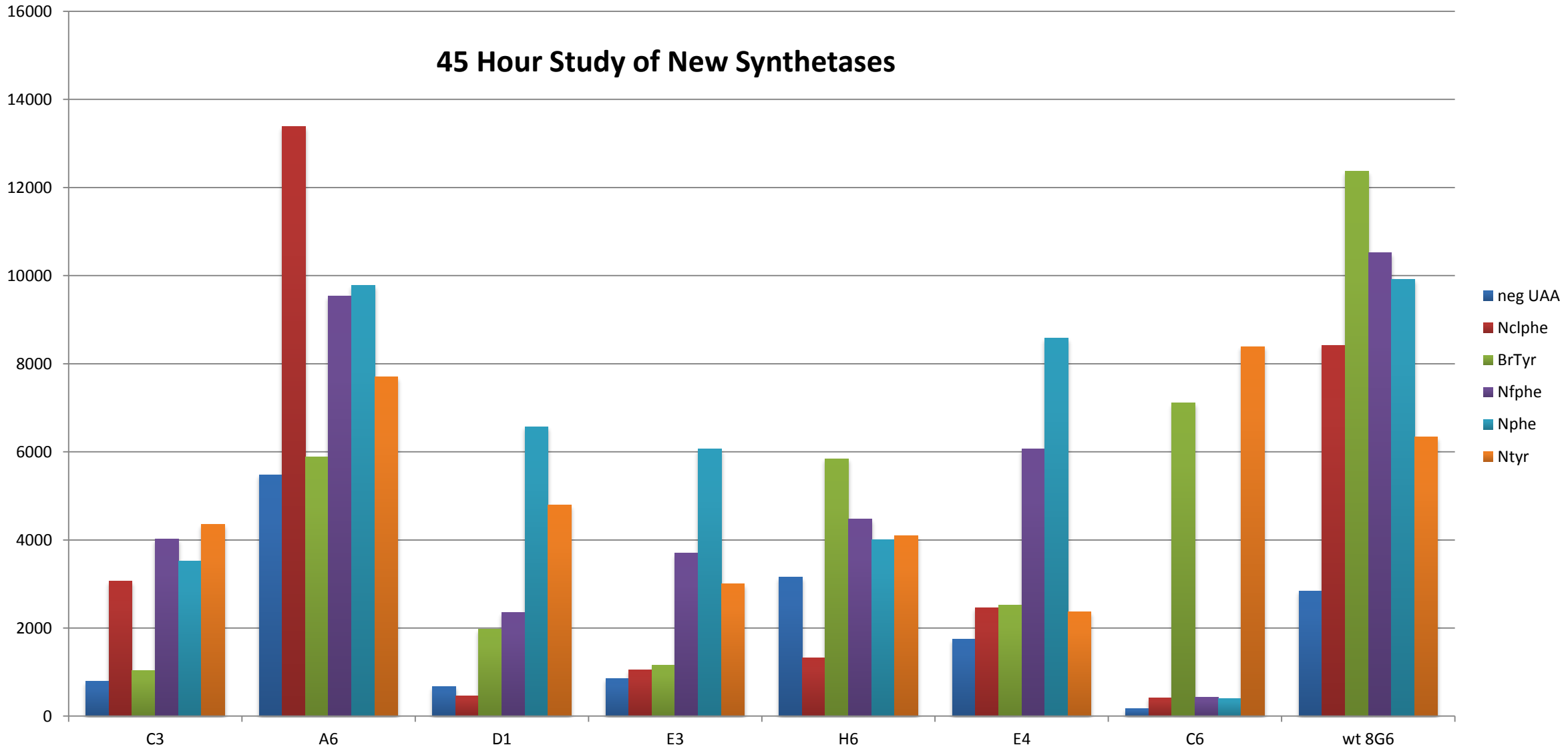


Figure: A permissivity study focusing on several tyrosine and phenylalanine analogues in GFP(green fluorescence protein); the horizontal axis shows the synthetases being screened.

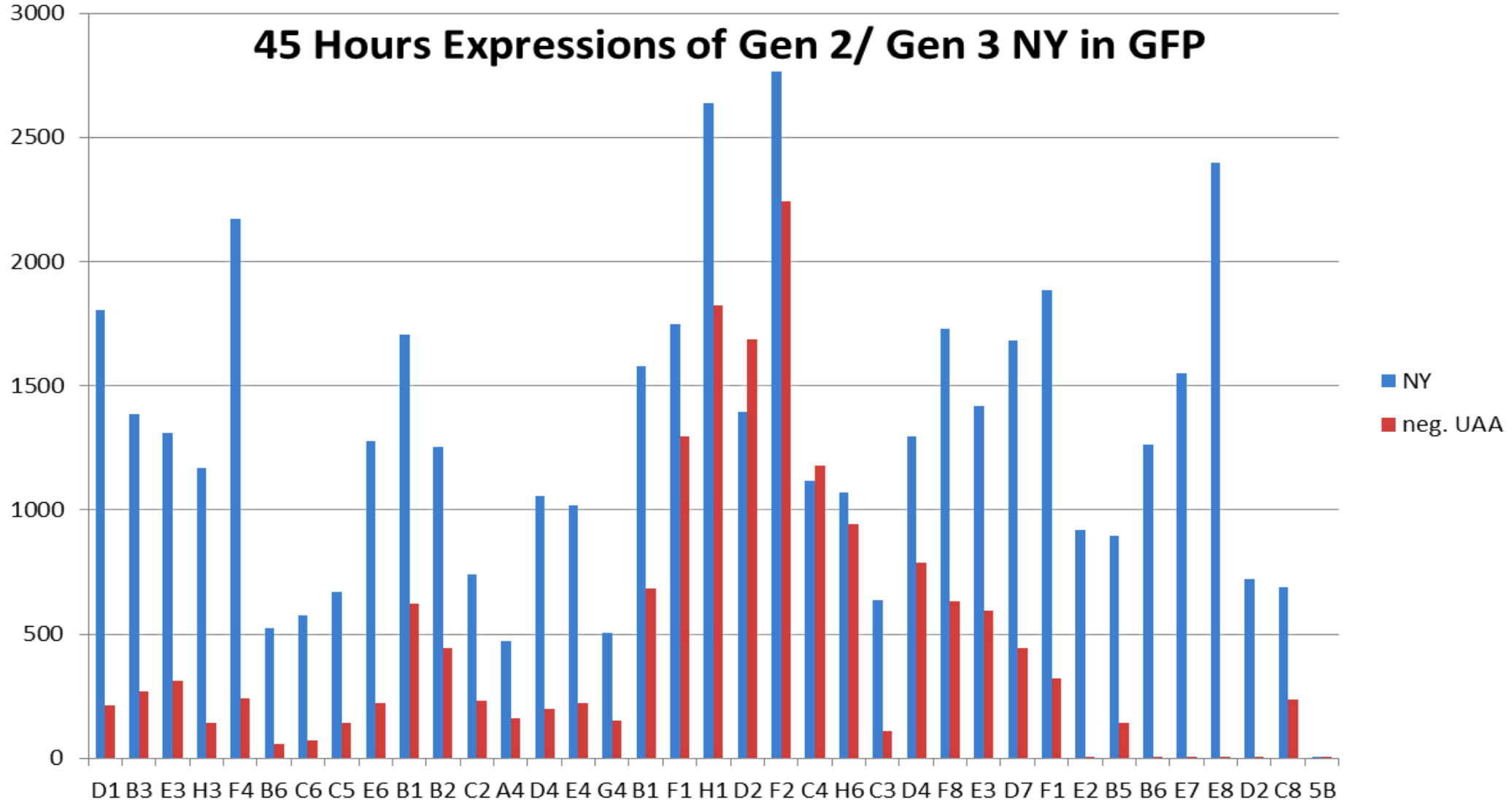
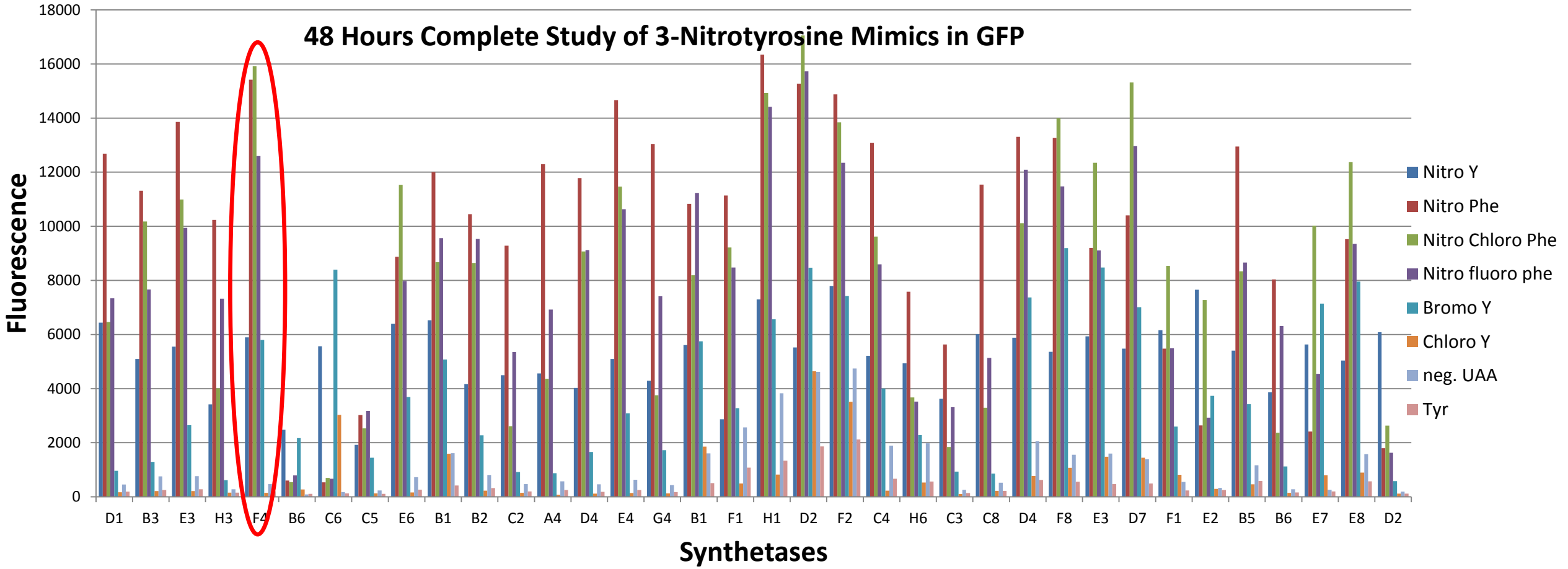


Figure: A fidelity study focusing on nitrotyrosine in GFP(green fluorescence protein); the horizontal axis shows the many synthetases being screened

Selection of a permissive synthetase for Hsp90

Which will best allow for the incorporation of available ncAAs into Hsp90?



Once a synthetase has been selected based on GFP studies, it can be moved into the proper plasmid for expression of nonnatural Hsp90

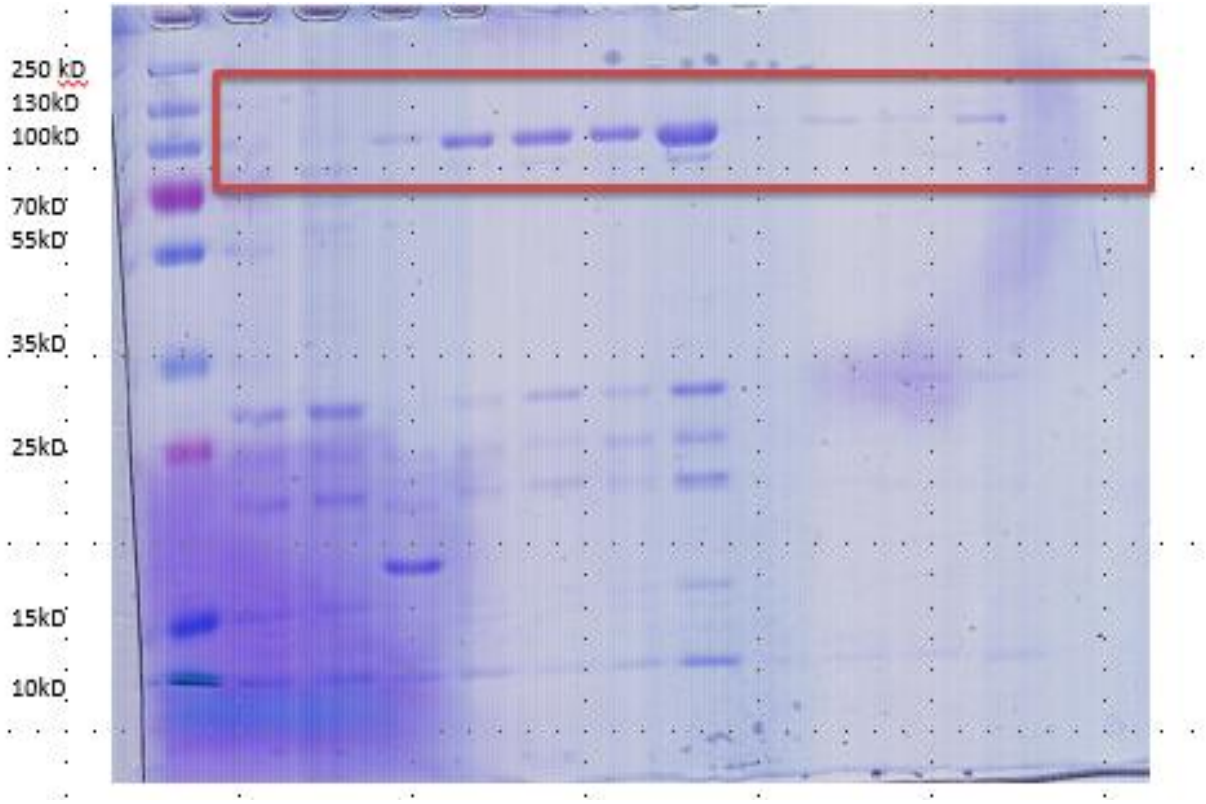
Nonnatural Hsp90 Purification and Gel Analysis

Confirmation of successful protein expression

ÄKTA Purification

- Expressed protein is lysed and filtered in a special buffer
- Run through the AKTA protein purification program, on a 5mL Nickel NTA affinity column.

SDS Polyacrylamide Gel Electrophoresis



Hsp90 (90kDa) +nitrotyrosine at sites 33 and 56

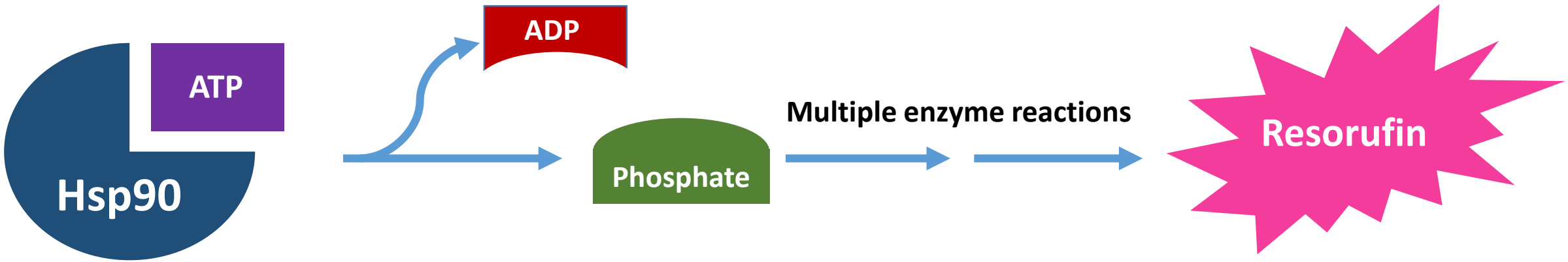
ATPase assay – background

How can Hsp90's hydrolysis of ATP be measured?

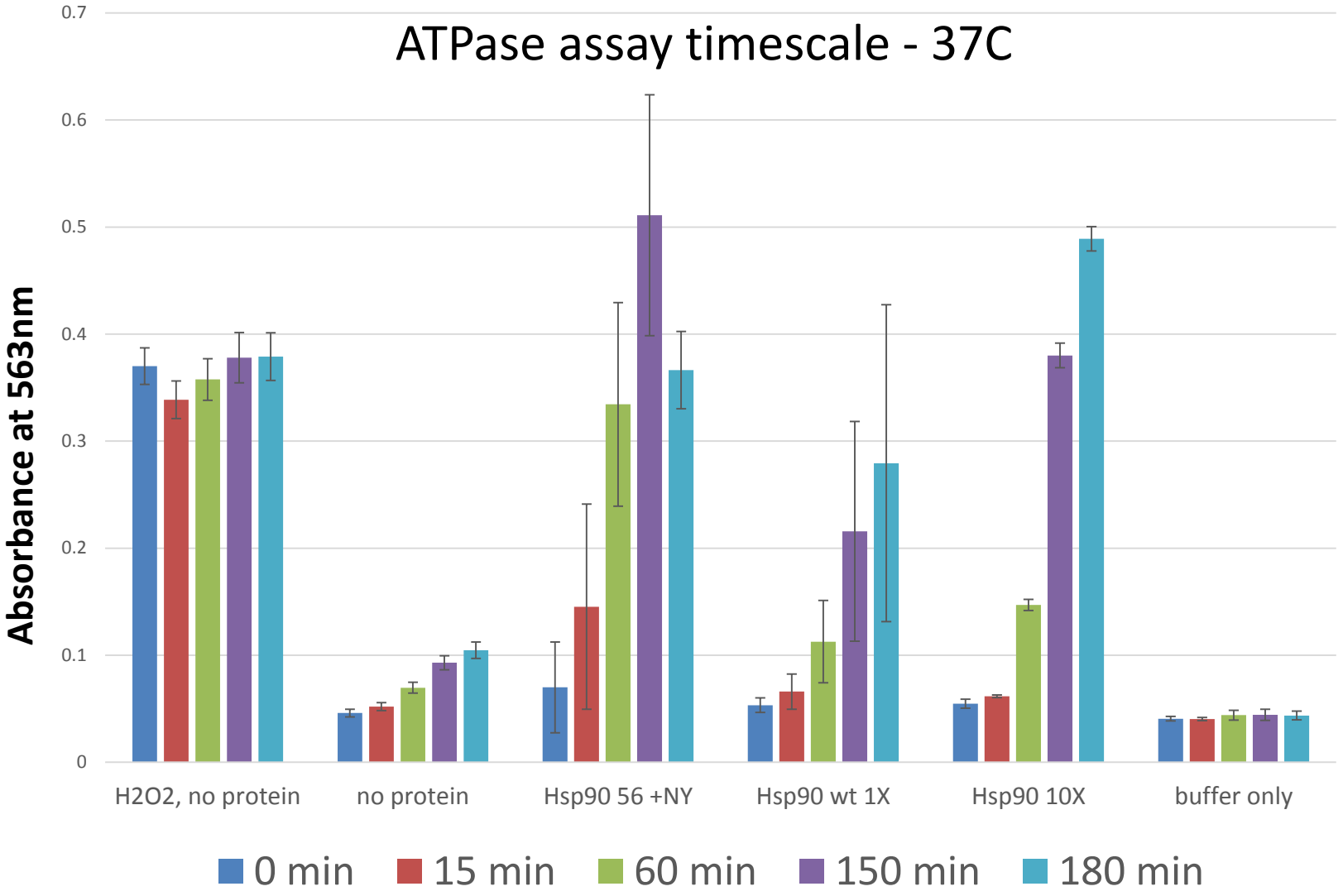
- Recall that much of Hsp90's activity is dependent on its ability to hydrolyze **ATP** into **ADP** and **phosphate**

- An optimized assay was performed with **Hsp90 wildtype and Hsp90 with a nitrotyrosine incorporated into an active site residue**

- The assay measures the absorbance of **resorufin**, an end product of a reaction which starts with ATP breakdown



ATPase assay results



- At near-physiological temperature and over time, Hsp90 is able to hydrolyze more ATP
- Hsp90 + nitrotyrosine may hydrolyze ATP better than wildtype Hsp90
- This could mean that a nitro group on a tyrosine within the active site helps ATP bind tighter

Summary and conclusions

A recap of my research

- In oxidative stress, Hsp90 is nitrated at several tyrosine residues, causing motor neuron death associated with neurodegenerative disease
- The F4 synthetase was characterized and selected as the most effective in incorporating ncAAs into Hsp90
- Nitrotyrosine and its analogues were successfully incorporated into Hsp90 using characterized synthetases. Protein expression was observed via purification and gel analysis.
- An ATPase assay was successfully set up and carried out, revealing that Hsp90 with nitrotyrosine may hydrolyze ATP more efficiently than its wildtype counterpart

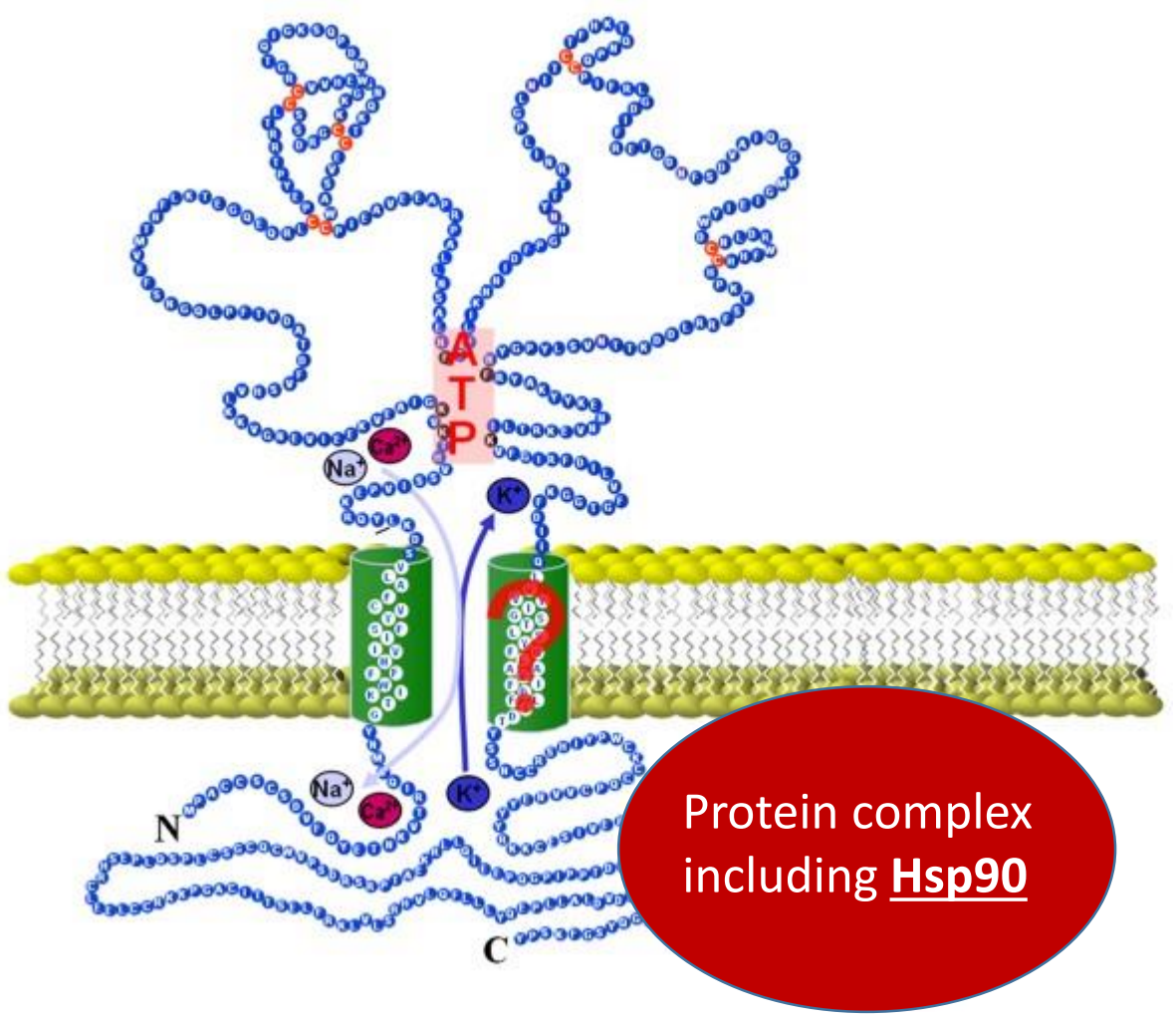
Future: Hsp90 and the P2X7 receptor

A mysterious protein-receptor interaction

Nitration of Hsp90 stimulates P2X7 receptor-dependent activation of the Fas pathway → **cell death**

Hsp90 is also known to associate with and regulate the P2X7 receptor in normal cellular conditions

Using the tool of *site specific incorporation* to make non-natural Hsp90 we can investigate these phenomena!



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- References

Franco, Maria C., Yaozu Ye, and Ryan A. Mehl. "Nitration of Hsp90 Induces Cell Death."*Proceedings of the National Academy of Science* (2012). Print.