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THE SYNTHESIS AND APPLICATION OF (2,2-METHOXYETHOXY)(2-NITROBENZYL) 2,4-DICHOLOPHENOXYACETATE AS A CAGED AUXIN IN THE AUXIN-INDUCIBLE DEGRADATION (AID) SYSTEM IN *C. ELEGANS*

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ABSTRACT: Controlling protein degradation pathways can be a useful tool when manipulating target proteins in cell signaling pathways. Techniques for protein abundance manipulation have been developed and are an important part in biochemical research, however these techniques are limiting in their abilities and environments. It has been shown that in the presence of plant hormone auxins, modified TIR1 F-box protein promotes ubiquitylation of auxin inducible degrons (AID). We synthesized and used a plant hormone based auxin to try to induce protein degradation of fluorescently tagged AID proteins using photoirradiation in *Caenorhabdtis elegans*. We were able to synthesize our caged auxin through a standard grignard reagent reaction and fisher esterification reaction. Then, we were able to show that a similar based caged auxin can become uncaged upon photolysis with UV illumination. Unfortunately, we couldn't demonstrate spatial and temporal control during intracellular uncaging of the auxin in our model organism *C. elegan*s. We should have seen decreased fluorescence in our fluorescently tagged target proteins upon UV photoirradiation of our caged auxin. This auxin inducible degradation system in *C. elegan*s has a lot of potential for future research, and could yield extremely useful techniques for developments of manipulating degradation pathways.

INTRODUCTION: Protein degradation is an important part in cell regulation, cell signaling and modifying protein concentrations[7]. The diversity of protein half-lives is vast and can vary from a couple minutes to a couple months. Degradation of ubiquitinated proteins in eukaryotes is primarily carried out by large multi-protein complexes in the 26S proteasome[8]. In nature, in vivo protein degradation occurs as a regulatory mechanism for gene expression. However, a variety of tools have been developed to modify and track degron tagged protein expression in biological systems. Many regulatory protein expression techniques originate at the DNA level in gene expression or at the RNA level when using RNA interference methods[9, 10]. However these approaches are limited in their effect because they are indirect and can result in long depletion times for proteins with long half-lives[9]. Additionally, these techniques are not easily reversible in nature, and can induce other unrelated side effects in pleiotropic genes[9]. In order to fully control protein degradation we took a more direct approach. Techniques with more precise spatial and temporal control of protein abundance such as light-mediation[2], small molecule protein stabilization[4], temperature regulation[5], and protein protein interactions[6] are better at modifying protein degradation in the model organism, *Caenorhabdtis elegans*[9]. In this paper, we used membrane permeable small molecules, auxins, to induce degradation of Auxin Inducible Degrons (AID).

Plants have evolved a unique feature where specific types of plant hormones called auxins promote rapid degradation of IAA family transcription factors in the SCF E3 ligase specific ubiquitylation pathway[13]. Other eukaryotes lack the ability to produce auxins, but share SCF E3 ligase in the ubiquitylation pathway[14]. SCF E3 ligase complex is made up of four subunits,

Rbx1, Cul1, Skp1 and a variable F box protein[9]. SCF F box modified TIR1 ligase has been shown to induce ubiquitylation of AID tagged proteins in the presence of auxins[10] in plants. Although orthologs of AID and TIR1 only exist in plants, SCF TIR1 complex can form due to the high degree of proteasomal conservation of Skp 1 in eukaryotes[10].

Caging our auxin is an important tool in this investigation because it allows us to see precise physiological responses to the auxin and track timed responses of AID degradation[12]. Caged molecules are bioactive molecules with an inactivating functional group attached to it that can only be reactivated when photocleaved by UV illumination in a UV lamp at 350-360 nm[12]. Because we decided to cage our auxin, we can uncage our auxin intracellularly and have better spatial and temporal control over auxin reactivity and concentration. The last step to this investigation involves the use of fluorescence proteins. Fluorescence proteins are an extremely useful and popular tool in biological research and can be engineered to emit multiple colors. Fluorescence proteins are genetically fused to target proteins, which causes the cell to express the target protein with an attached fluorescence protein[14]. In order to measure the rate of degradation of AID, we genetically engineered AID to express green fluorescence protein (GFP), which emits green light at 488-507 nm[16], so we could visually see the depletion of AID tagged proteins over time.

The goal of this investigation was to synthesize a new caged auxin, (2,2-methoxyethoxy)(2-nitrobenzyl) 2,4-dichlorophenoxyacetate, using a simple grignard and esterification reaction, to add to the library of effective auxins. We could then analyze the kinetic properties of the uncaging reaction by measuring the absorbance versus time post UV illumination. By measuring the absorbance, we can calculate the rate constant to know the concentration of the remaining starting material in our auxin after the ester photocleavage. Then, we could use our synthetic auxin to track the rate of degradation of AID, intracellularly in *C. elegans*, by measuring the fluorescence of GFP tagged AID. As a control group, we fused a similar fluorescent protein, mRuby which emits red light at 558-605 nm[15], to TIR1 which won't get degraded and therefore the absorbance will stay the same. We can compare the fluorescence data of GFP versus mRuby to see if GFP tagged AID decreased in the presence of our auxin over time. This functional assay has potential to be an extremely useful tool in real life application where AID can be fused to knock out proteins. Our investigation demonstrates the versatility of this system for knocking out proteins in *C. elegans*.

RESULTS AND DISCUSSION: Even though auxins are a plant hormone, it can be utilized in *C. elegans* as an important tool in the ubiquitylation pathway. We use the auxin to insure that there aren't any off target effects. Using a ligand that doesn't have a wildtype biological role in the *C. elegans,* will enable properly targeted experiments.

Synthesis of 2-nitrobenzyl alcohol

To make the alcohol, we prepared our own Grignard reagent by adding Phenylmagnesium chloride to 1-bromo-2-(2-methoxyethoxy)ethane, an alkyl halide. The resulting Grignard reagent was then added to the carbonyl carbon of the aldehyde (2-nitrobenzaldehyde) to synthesize an alcohol. During the separatory processes, we left the stopcock on the separatory funnel open on accident and lost a third of our product, so the crude alcohol product weighed 507 milligrams. After rotovap and purifying our product via column chromatography, the purified reaction yielded 0.398 mg of a dark yellow colored translucent liquid, which was then analyzed via TLC and ^{1}H NMR. The percent yield was 55%. During TLC analysis, we found that there were different R_f values when comparing our starting aldehyde and the crude product indicating that we had made our desired product. In the ^{1}H NMR, there was no aldehyde peak which signifies the synthesis of our alcohol. Looking at our peaks we may have a lot of impurities but we certainly got the aromatic peak around 7.5-8.0 ppm region. There was an OH peak at 2.7 ppm. Ethyl Acetate peaks at 4.2, 2.0, and 1.25 ppm, which were responsible for some of the irregular peaks in our ^{1}H NMR. This reaction concludes the synthesis of our uncaged auxin.

Synthesis of (2,2-methoxyethoxy)(2-nitrobenzyl) 2,4-dichlorophenoxyacetate

We then performed an esterification reaction to add our caging group to our auxin. We used the prepared alcohol with a carboxylic acid (2,4-dichlorophenoxyacetic acid) for this reaction. We carried out this synthesis by adding DCC and DMAP to yield the final reaction product. When we added the 2,4-dichlorophenoxyacetic acid, DCC, and DMAP to the alcohol, the color changed to a milky light yellow. After the reaction was complete, we carried out the extraction step, the bottom layer was our organic layer and the top layer was the water. This was due to the reason that dichloromethane (DCM) is more dense than water. After Rotavap, the residue was purified by silica gel column chromatography to give the caged auxin. But during the process of silica gel column chromatography, the TLC plates revealed a small spot that aligned with our crude sample. The purified ester weighed 0.023 grams. The percent yield was at 3% for our final ester, possible factors may be that some side reactions occurred, the presence of anhydrous sodium sulfate, and maybe the reaction was not given enough time to reach completion. When analyzing the ^{1}H NMR for our final ester product, we were unsure if we formed our product or not. The key piece of data that would determine the formation of our desired product is the hydrogen (H5) bound to the carbon most adjacent to the ester and phenyl where our peg chain extends. This hydrogen is unique to our final product, and we wouldn't have seen it in any other previous ^{1}H NMR spectra. The problem was that we still had a lot of impurities in the 4.0 to 5.5 ppm region, which was where we should've seen that triplet. Another piece of data from the ^{1}H NMR that questioned the formation of our target molecule was in the aromatic region, 6.5-8.5 ppm. There we saw eight unique peaks signifying the eight unique aromatic hydrogen. However, there should've only been seven unique peaks. Also, there were two singlets, but our molecule should only have one singlet in that region. We concluded that our ^{1}H NMR sample had a lot of impurities, and we cannot give strong evidence that we made our desired product except the OH peak and aromatic peaks. Since we only had 0.023 g of our final product, we discarded it and proceeded on with the Indole-3-acetic acid provided in lab.

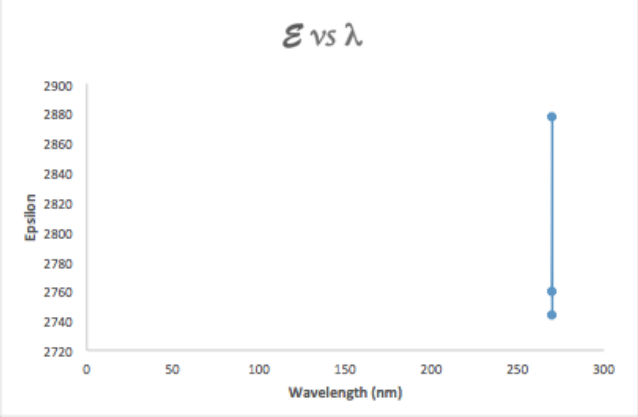

Figure 1. The maximum molar absorptivity was 2877.6 L mol^{-1} cm^{-1} and the minimum absorptivity was 2744 L mol^{-1} cm^{-1} at 270 nm. The molar absorptivity was calculated using the Beer lambert equation A= elc, where e (epsilon) is the molar absorptivity, l is the path length of the cuvette which for us was 1 cm, and c is the auxin concentration.

UV-vis Spectrophotometer and Kinetics of the caged auxin

The kinetics part of the research was to utilize the UV-vis spectrophotometer to quantify the rate constant (k) for the UV-induced cleavage of the caged auxin. We found a concentration at 0.25 mM where the absorbance maximum was <1. Beer's law was used to plot this spectrum of Epsilon vs. Wavelength (**Figure 1**) above. The maximum molar absorptivity was 2877.6 L mol^{-1} cm^{-1} and the minimum absorptivity was 2744 L mol^{-1} cm^{-1}. Then with this UV-vis data, we were able to identify the wavelength where there was the greatest change in Epsilon upon cleavage. So we designed a protocol to measure the rate constant for the cleavage by collecting 10 data points at different irradiation times (**Figure 2**).

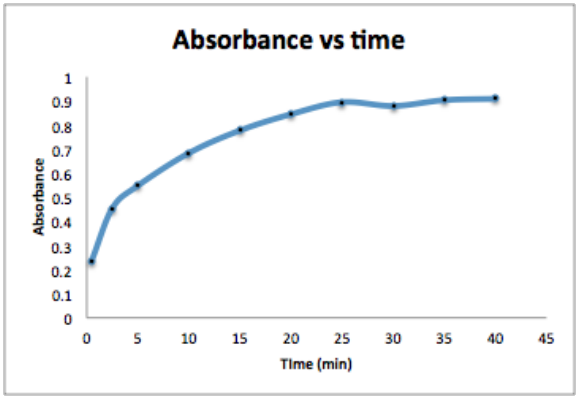

Figure 2. Graph of Absorbance vs. Time of caged-auxin. Concentration at 0.25 mmol of caged auxin at 250-500 nm using standard cuvette (1.8 mL of solution). The maximum absorbance values were recorded at 270 nm. UV-vis were taken every 30 seconds to try and get as much data as possible, however, some of this data was inconsistent. As the UV exposure time increased, the absorbance also increased.

For the UV kinetics, the absorbance increases because the product absorbs more than the starting material and the graph levels off because the reaction is under the first order kinetics. In which the reaction rate is directly proportional to the reactant concentration.

With regards to our light kinetics reaction, we were able to collect earlier time points to make our absorption vs time graph more accurate. And with the data, we calculated for the natural log of initial absorbance over absorbance (**Figure 3**).

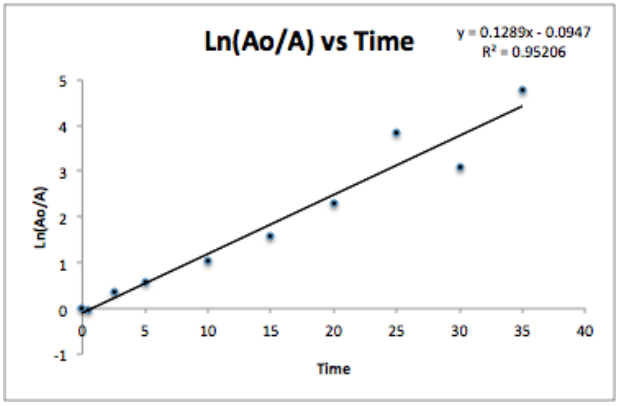

Figure 3. Graph of natural log of initial absorbance over absorbance vs. time. A linear trendline was fitted. The slope represents the rate constant, k, which is 0.1289 min^{-1} and the R^2 value is 0.95206 signifying a decent fit line. The rate constant was calculated to see the concentration of the remaining starting material in our auxin after the ester cleavage. We took the inverse of the rate law for easier interpretation. We plotted Ln((Af-A)/(Af-Ai)) which gave us a positive linear sloping trendline.

Gel Electrophoresis/PCR

Gel electrophoresis is a standard lab procedure used to separate DNA fragments by size (length of the base pairs). During this process an electrical field is used to move the negatively charged DNA to the positive electrode through an agarose gel matrix. DNA fragments that are smaller and shorter migrate through the gel more quickly and further in distance. Gel electrophoresis was performed to confirm the presence of the AID-GFP gene. The samples of the DNA were amplified in the PCR machine. Comparing the band of our DNA sample with those from the DNA ladder, the PCR product did not show up in the gel. Our first thought was that the *C.elegans* did not have the sequence that we genetically engineered into it due to the lack of any band. The gel electrophoresis showed that the *C.elegans* was negative for the GFP-AID gene, and the sample did not appear in the gel. However, there are various, more plausible, reasons to why it may not have worked, we might have loaded the gel improperly or there might have been a technical issue with the primers.

Determination of the extent auxin uncaging by esterases in the worms/fluorescence

Next, we evaluated the intracellular auxin concentration by light-controlled manipulation. We used our synthetic auxin to track the rate of the degradation of GFP tagged AID, intracellularly in *C. elegans*, by measuring the fluorescence of the GFP at 488-507 nm[16]. The list of our controls and the auxin concentration experiments are shown in Table 1.

Controls	Indole expeirments
Buffer only	1.0 mM
1 mM indole-M9 buffer (10% DMSO)	0.75 mM
1 mM Napth-M9 buffer (10% DMSO)	0.50 mM
10 % DMSO + M9 buffer	0.33 mM

Table 1. List of controls and auxin concentrations.

Although our auxin molecules are caged and should not cause protein degradation without UV irradiation, it is still possible that some uncaging may occur due to the hydrolysis by naturally occuring cellular esterases in the worms. The first designed experiment, using the 5 samples above, was used to determine the intensity of natural esterase hydrolysis reaction without UV illumination. We used this experiment as a baseline to understand the rate of natural intracellular uncaging reactions. When using UV illumination, we should have seen much higher yields of AID-GFP degradation. However, we found that worms with and without UV illumination, had the same trends in fluorescence activity. We compared the fluorescence data of GFP versus mRuby to see if the GFP tagged AID would decrease in the presence of our auxin over time, and we saw that they both increased. From **figure 4**, we graphed GFP/mRuby ratios over the course of 4 hours at different auxin concentrations. We needed to use two fluorescence points, so that we could compare the relative degradation of proteins in this pathway. The controls were used as a method to ensure that GFP is degrading significantly faster in relation to other protein in this pathway. The fluorescence ratio fluctuated greatly, but the final values seemed to increase. This signifies a couple possibilities: 1. The esterase in the worms and/or UV illumination were unable to cleave the caging group 2. Unforeseen upregulation of GFP-AID could have altered our results. 3. Our caged auxin never diffused across the cell membrane due to differences in polarity 4. The steric forces of our uncaged auxin lowered its affinity to bind to TIR1 5. We used too low of a concentration of auxin for any significant phenotypic effect.

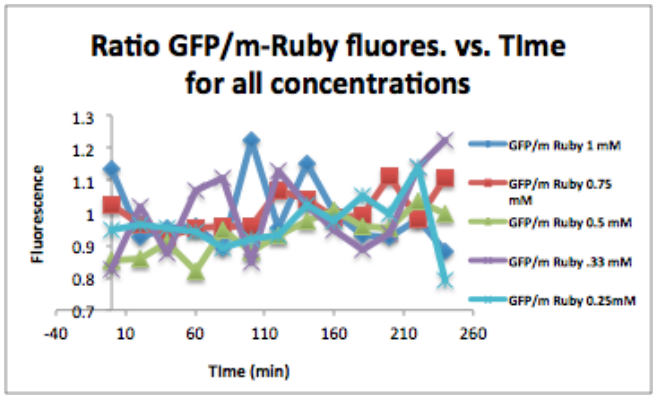

Figure 4. Fluorescence ratio of **GFP/m-Ruby** versus Time in C.elegans. The auxin concentrations at the right read 1 mM, 0.75 mM, 0.50 mM, 0.33 mM and 0.25 mM. The highest fluorescence signal is at 1mM auxin concentration and 0.33 mM has signal at the lowest.

CONCLUSION: Although we were unsuccessful in our biological assay, we were able to produce some of our caged auxin product. After purification and ^{1}H NMR analyses, we determined that it is possible that we synthesized (2,2-methoxyethoxy)(2-nitrobenzyl) 2,4-dichlorophenoxyacetate, although it had a lot of solvent impurities in it which made most of the integration values hard to read. With regards to the kinetics portion of this investigation, we were able to show that the uncaging reaction was a direct effect of the UV illumination. When translating this reaction to C. elegans we were unable to prove that UV illumination catalyzed auxin uncaging, and therefore unable to prove that our synthetic auxin, (2,2-methoxyethoxy)(2-nitrobenzyl) 2,4-dichlorophenoxyacetate, was able to promote degradation in the E3 ubiquitylation pathway. Instead of seeing the GFP/mRuby ratio fluorescence decrease, we saw that it had increased which signified that the relative amount of GFP compared to mRuby, contrary to our prediction, increased over time. This may have been because AID wasn't properly genetically engineered into the *C. elegans* genome, the concentration of our caged auxin were too low, the uncaged auxin was

unable to bind to TIR1 due to steric forces, the caged auxin did not diffuse through the cell membrane or the uncaging reaction did not work intracellularly. Although our GFP degradation in *C. elegans* did not work, this functional assay has potential to be an extremely useful tool in real life application where AID can be fused to knock out proteins. With more time and persistence, our investigation demonstrates an intelligent, potential and versatile tool for future research in protein knock out technology in *C. elegans*.

EXPERIMENTAL:

Synthesis of (2,2-methoxyethoxy)(2-nitrobenzyl) 2,4-dichlorophenoxyacetate

A solution of 1-bromo-2-2-(methoxyethoxy)ethane (0.6729 mL) was added to 50 mL Round bottom flask (RBF). 5 mL of dry THF was also added and the mixture was cooled to -40^{o}C with a bath of dry ice and acetonitrile. The atmosphere was evacuated and flushed with N_2 on the Schlenk line. Then to a different RBF still under N_2, PhMgCl (151 mg) was dissolved in dry THF (~5 mL) and added dropwise to make our Grignard reagent. The mixture was stirred at -40^{o}C for 30 minutes. Then 2-nitrobenzaldehyde (152 mg) was added dropwise into the reaction. Continuously at -40^{o}C, the mixture was stirred for 40 mins and monitored by TLC for completion. Then the reaction was quenched with HCl and extracted with EtOAc (100 mL) for three times. Then the organic layer was washed with saturated NH_4Cl solution and brine (NaCl). After drying it over Na_2SO_4, the layer was concentrated in rotary evaporator. Thin-Layer Chromatography (TLC) was performed using 4:6 Hexane-EtOAc as the solvent system to detect any impurities and to check if the product was made. Co-spot the TLC plate with the aldehyde diluted. 1H NMR sample was made and run. The residue from the Rotovap was then purified by silica gel column chromatography using (8:2) Hexane:EtOAC as the solvent system. For the silica gel column chromatography, to a clean column, add cotton, small layer of sand, and dry silica. Flow solvent through to compress the silica by using air. Once all is compact, add a small layer of sand then add more solvent and flow it through. Once the solvent gets to the sand, add the alcohol sample that's dissolved in minimum amount of DCM, with a glass pipet slowly and directly in the mid of buret. Collected sample was put in new RBF and concentrated in rotary evaporator to give 2-nitrobenzyl. (398 mg, yield 55%) as yellow liquid. ^{l}H NMR ^{l}H NMR (300MHz, CDCl3) δ=7.92 (1H, d), 7.7 (1H, d), 7.65 (1H, t), 7.5 (1H, t), 3.75 (?H, m), 3.65 (?H, m), 7.18 (?H, t), 3.45 (?H, s), 2.75 (?H, d), 1.7 (2H, m), 1.68 (2H, m) 1.5 (2H, m), 1.47 (2H, m), 1.33 (1H, m), 1.25 (2H, m), 1.2 (2H, m), 0.9 (1H, m), 0.88 (2H, d)

The alcohol (2 mmol, 398 mg, 1 eq.) and N,N'-dicyclohexylcarbodiimide DCC (619 mg, 2 mmol, 1.5 equ.) was added to the solution of 2,4-dichlorophenoxyacetic acid (2 mmol, 887 mg, 2 eq.) and 4-dimethylaminopyridine DMAP (391 mg, 2 mmol, 1.6 eq.) in DCM (5 mL). The mixture was stirred for 2 hours at room temperature. Then poured the reaction mixture into the saturated NH_4Cl (10 mL) and extracted with EtOAc three times (30 mL each) with the separatory funnel. Then wash the EtOAc layer with brine about 10 mL and dry over the anhydrous Na_2SO_4. Concentrated in Rotary Evaporator. The residue was purified by silica gel column chromatography (Hex:EtOAc 8:2 system) to give DMPNB-caged auxin. (23 mg, Yield, 3%) ^{l}H NMR ^{l}H NMR (300MHz, CDCl3) δ 8.15 (1H, d), 7.6 (3H, m), 7.57 (2H, m), 7.5 (2H, m), 7.45 (1H, d), 7.25 (1H, d), 6.75 (1H, d), 5.45 (2H, s), 5.0 (1H t), 4.75 (1H, s),4.44 (4H, m), 4.3 (4H, m), 2.3 (?H, m), 1.75-1.25 (?H, m), 1.0 (?H, m), 0.25 (?H, m) 3.01

UV-vis Cleavage

50 mL of 1X Phosphate buffered saline (PBS) made in conical tube by adding 5 mL of 10X PBS and 45 mL of H_2O. Into a 40 mL conical centrifuge tube, 10 mg of the Indole was added to 32.15 mL of PBS. 0.3215 mL of 1% DMSO solution was added to increase solubility. The 0.5 mM concentration was put under the UV irradiation for 30 minutes then put into the UV-vis spectrophotometer. Then the process was repeated with a 15 minute time interval adjustments under the UV lamp. Then more dilute sample was prepared by using 5 mL of 0.5X concentrated solution and 5 mL of the 10X PBS and 45 mL of H_2O was put into a 50 mL conical centrifuge tube creating a 0.25 mM

solution. This concentration was also put under UV irradiation for 15 minutes and then put under the UV-vis spectrophotometer. Then 10 data points were collected at different irradiation times, this time UV spectrum was acquired at a faster time interval for every 30 second interval for the first three data points and then two minutes time interval for the other seven data points.

Gel Electrophoresis- *C. elegans* Single Worm PCR

5 microliters of the lysis buffer was pipetted into a 200 microliter tube cap. Then 1-3 worms were picked and placed in the cap of the tube. Closed the tube and flicked it to get the worms to the bottom of the tube. Froze the worms in liquid nitrogen that incubates at 65 degrees celsius in thermocycler for 10 minutes. The PCR machine was used to carry out the lysis of the worm and to release the genomic DNA. The 200 microliter tube was heated to 65 degrees for 90 minutes and inactivated proteinase K by heating to 95 degrees for 15 minutes. Then 2 microliters of each primer, primers 1 and 2, was added along with 40 microliters of PCR supermix. The mixture (10 microliters) was then pipetted into lane 4 of the gel well.

GFP degradation in *C. elegans*

From the plates of c.elegans the TA flooded using 1.5 mL of M9 buffer, pipetted 0.75 mL of buffer on top of the plate and gently agitated it to life up the worms. Then the plate was tipped and the worm solution was pipetted into microcentrifuge tube. The worms were settled to the bottom of the tube. Then removed the buffer from top and reduced the volume to ~0.6 mL using a plastic pipet. This will essentially concentrate down the worms.

Into a 384-well plate, five experiments were designed and ran. For each of the experiment, 10 microliter of the auxin solution and 50 microliters of the worm solution was utilized. Five auxin concentrations, 1 mM, 0.75 mM, 0.5 mM, 0.33 mM, 0.25 mM was calculated to carry out the experiment. Each concentration was made by using the dilution equation. ($\mathbf{M_1V_1 = M_2V_2}$ **).** The final volume was set to 60 microliters and the designed auxin concentrations were diluted down from 60 mM of the auxin concentration. For example, for 0.75 mM concentration, 0.75 microliters of (0.0233 mg Auxin in 1 mL DMSO stock solution), 5.4 microliters of straight DMSO, and 3.85 microliters of the M9 buffer was added to a small bile. Because a total of 10 microliter auxin solution was needed, everything was multiplied by 3 to make it into a greater amount. 10 microliter of the auxin solution was first pipetted into the appropriate wells, and the TA added in the 50 microliters of worm solution to each well. After all the solutions were in the plate, the TA collected fluorescence data on a plate reader in the Heemstra lab.

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