UNIVERSITY OF CALIFORNIA SAN DIEGO

Functional Impact of Regulated Expression on Yeast Two-Hybrid

A thesis submitted in partial satisfaction of the requirements
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in

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by

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The thesis of Jesus Hernandez is approved, and it is acceptable in quality and form for publication of microfilm and electronically.

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ABSTRACT OF THE THESIS

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Traditional use of the yeast two-hybrid (Y2H) system is primarily for identifying unknown protein interactors to proteins of interest. Alternatively, Y2H can be adapted to make quantitative measurements of interaction strength, but there are some obstacles. For example, unequal expression of the proteins of interest will lead to false comparisons and using slightly toxic proteins can decrease cell viability. To alleviate this issue, we sought to control expression by implementing an inducible *CUP1* promoter design using the major nuclear export factor,

Nxf1, as bait. We observed that induction increased expression levels above that of the traditionally used constitutive *ADH1* promoter in the pGBK vector. Furthermore, we showed that *CUP1* driven expression levels are titratable and dependent on copper concentrations. Lastly, our findings suggest that we could quantify the changes in growth and therefore interaction strength using a liquid growth assay. Thus, this modified assay has the potential to allow for comparisons of protein interaction strength using the Y2H system.

INTRODUCTION

To ask how allelic differences can affect protein-protein interactions (PPI), we had the goal of quantitatively measuring interactions using a regulated system. PPIs are crucial for understanding the mechanisms behind biological processes. The connections between proteins dictate pathways that are central for cellular function. To elucidate these networks, PPIs have been explored through different approaches that each have their own advantages. One particular approach, the yeast two-hybrid (Y2H) system is an *in vivo* assay that relies on the GAL4 protein, encoding a DNA binding domain and transcriptional activator domain, for testing PPIs (Fields and Song, 1989). A hybrid protein must be created such that there is a fusion between a bait protein and the DNA binding domain, and a fusion between a prey protein and the activation domain. When the bait and prey proteins associate together, it reconstitutes a functional GAL4 protein, allowing GAL4 to bind to and activate reporter genes (Chien et al., 1991; Fields and Song, 1989). Established extensions of this system to fit specific needs include the yeast one-hybrid to detect DNA-protein interactions (Ouwerkerk and Meijer, 2001), the yeast three-hybrid to detect small molecules that link two proteins (Licitra and Liu, 1996), and mammalian two-hybrid systems, which adopt this split reporter approach in mammalian cells (Luo et al., 1997). Traditional uses of the Y2H assay were primarily for identifying unknown protein interactors to a certain molecule. With many variants and ease of adaptability, here we use the Y2H as a liquid growth assay to test known protein-protein interactions and potentially measure the interaction strength in a quantitative manner.

Most available Y2H systems use a strong constitutive promoter to drive high-level expression, but regulated expression might be beneficial for some applications. The classical

GAL4-based Y2H system most commonly utilizes the *ADH1* promoter, which is constitutively expressed. An alternative to the GAL4-based system is the LexA-based two hybrid system which utilizes a unique DNA binding domain, the prokaryotic LexA protein, under control of an *ADH1* promoter or alternatively a galactose inducible promoter, *GAL1* (Clontech, 2009). While constitutive expression from the GAL4-based system may be adequate for most applications, substituting the promoter can allow for more selective control over expression while also increasing expression levels. Outside of galactose induction, commonly used regulated promoters in yeast include the inorganic phosphate repressed *PHO5* promoter, the methionine repressed *MET25* promoter, and the copper induced *CUP1* promoter (Da Silva and Srikrishnan, 2012). The copper metallothionein gene, *CUP1*, functions in copper detoxification and is regulated by copper ions (Butt et al., 1984). Here, we use the *CUP1* promoter to drive expression since it can be easily activated with the addition of copper ions to the media (Thiele and Hamer, 1986). We demonstrate that replacing the *ADH1* promoter in the common yeast two-hybrid vector pGBK with the *CUP1* promoter allows titratable expression of functional bait proteins for regulated yeast two-hybrid assays. This may have application for bait proteins that are mildly toxic under constitutive conditions or for quantitative comparisons of affinity between bait proteins with different steady-state levels when expressed in yeast.

To test regulated expression in Y2H, we aimed to use the nuclear export factor 1, *Nxf1*, as bait of interest in our assay. Traditionally, *Nxf1* functions as a cellular factor that mediates mRNA export (Grüter et al., 1998) with RNA shuttling occurring through physical association of the polyadenylated RNA (Katahira et al., 1999; Segref et al., 1997). Nxf1 is a 619 amino acid protein with domains including an RNA binding domain (RBD), a leucine rich repeat (LRR) domain, a nuclear transport factor 2 (NTF2)-like domain, and a ubiquitin associated domain

(UBA) (Braun, 1999; Katahira et al., 1999; Suyama et al., 2000). It is worth noting that a subset of retroviruses has been shown to recruit *Nxf1* and similar transport machinery as host cofactors in viral export (Grüter et al., 1998; Pasquinelli et al., 1997; Sakuma et al., 2014). Furthermore, Nxf1 forms a heterodimeric complex with an RNA export associated cofactor, Nxt1 (Katahira et al., 1999), that enhances nucleoporin binding and has a role in stable mRNA export (Wiegand et al., 2002). This Nxf1-Nxt1 complex has shown significant affinity for CTE containing viral RNAs, demonstrating recruitment without the traditional use of viral adaptor molecules (Kang et al., 2000).

As the prey of interest for Y2H, we narrowed down the list of candidate protein interactors, which include Rae1, eIF4A3, Nup62, and Nup98, based on their roles in RNA export and RNA associated activities. Nup62 and Nup98, which are nuclear pore complex (NPC) components and bind to the C-terminal UBA domain of Nxf1, have been demonstrated to participate in gene regulation in the NPC (Kalverda et al., 2010). Rae1 has been shown to participate in RNA export (Pritchard et al., 2000) and binds to both a segment of the NTF2-like domain and N-terminal domain of Nxf1 (Blevins et al., 2003). Additionally, eIF4A3 has been shown to be recruited by mRNA during splicing (Ferraiuolo et al., 2004) with the binding site on Nxf1 currently unmapped. While we had hoped to test all candidate proteins, our work will measure the interaction strength and potential interaction differences between the Nxf1 alleles and Nup62.

The relationship between viral elements and Nxf1 is notable because an allele of *Nxf1* can act on mutations caused by a subset of transposable elements (TE). Initially described by Barbara McClintock, TEs are mobile DNA segments that were found to move within maize genomes and influenced expression of genes near the location of these segments (McClintock, 1950).

Importantly, TEs have a high prevalence in the human and mouse genome and are studied because of their resulting mutations (Consortium et al., 2002; Lander et al., 2001). A well-studied subset of endogenous retrovirus (ERV) TEs, known as intracisternal-A particle (IAP), cause insertion mutations that most commonly result in aberrant RNA splicing, but are not limited to premature termination, alternative polyadenylation, and aberrant transcription (Floyd et al., 2003; Hamilton et al., 1997; Maksakova et al., 2006). Accordingly, transposable elements have a large role in the function of host transcription.

More specifically, we wanted to test the *Nxf1*^{CAST} allele, which is a natural variant of *Nxf1* that functionally acts as a genetic modifier of mutations caused by endogenous retroviral IAPs (Floyd et al., 2003). Altogether, *Nxf1*^{CAST} suppressed 20 unique mutations induced by IAPs in mice (Concepcion et al., 2009, 2015; Floyd et al., 2003). Suppressed intronic mutations included full-length and several deletions classes of IAP that spanned a range of genomic locations and resulted in a variety of expression phenotypes. The molecular basis of the suppression included an increase in correctly spliced wildtype RNA along with a decrease in IAP-dependent mutant RNA (Floyd et al., 2003). When the IAP mutation is suppressed, we see a decrease in aberrantly spliced RNA products due to correct processing of the pre-mRNA and splicing around the IAP. However, this mechanism of suppression has not been characterized.

Ultimately, we wanted to ask what interaction differences occur between the allelic forms of Nxf1. The *Nxf1*^{B6} and *Nxf1*^{CAST} alleles appear to be expressed at equivalent steady-state mRNA and protein levels but differ by two amino acid polymorphisms: S48P and E610G (Floyd et al., 2003). When the E610G polymorphism was edited into endogenous *Nxf1*^{B6}, the edited E610G allele performed the same modifying activity as the *Nxf1*^{CAST} allele (Concepcion et al., 2015). This demonstrated that 610 glycine is the key polymorphism for *Nxf1*^{CAST} modifier

activity. The difference in amino acid sequence but not expression level suggested that the mechanism by which the *Nxf1*^{CAST} allele suppresses should include a difference in interaction strength with one or more binding partner. Nxf1^{CAST} and Nxf1^{B6} protein would likely interact to a different degree with binding partners, such as partners that could influence RNA processing. While many interacting proteins bind to different domains of Nxf1, a narrower subset have been mapped to bind the UBA-like c-terminal domain (also called Tap_C) containing position 610 (Bachi et al., 2000; Kang and Cullen, 1999; Katahira et al., 1999). As such, we wanted to test whether position 610 allelic differences changed relative interaction strengths.

To quantitatively measure interaction strength, we had to address a few prominent problems that interfered with analysis. A crucial issue for quantification being that unequal expression of different bait proteins can interfere with comparisons across measurements. We have observed that the alleles of *Nxf1*, which differ only by an E610G polymorphism, consistently expressed protein at unequal levels in yeast. This issue arises since the Nxf1-Nxt1 binding complex is conserved in yeast as the homologs Mex67p and Mtr2p, respectively (Katahira et al., 1999). The conservation of the binding network can negatively impact expression levels, as it is plausible for the endogenous yeast machinery to influence exogenous Nxf1. As Nxf1 is drawn away, the total Nxf1 protein available for interaction with binding partners may be reduced. In theory, this limit on protein abundance can interfere with the Y2H assay, however it is unknown. Next, there is the issue of expressing slightly toxicity bait constructs. By regulating when expression occurs, we might be able to induce bait constructs without overly harming the cell. Therefore, we wanted to express bait genes in a regulated manner to circumvent possible issues. Here we constructed an inducible system to activate expression of bait constructs with the addition of exogenous copper. This was accomplished with

the inducible yeast *CUP1* promoter in place of the constitutive *ADH1* promoter. We expect that by controlling expression levels, we can mitigate any toxic effects of Nxf1 on yeast cells and equalize bait protein levels for comparative proteomics.

The purpose of this present study is to modify the Y2H assay to gain an understanding of the potential protein-protein interaction mechanism that allows $Nxf1^{CAST}$ to perform alternative RNA processing. First, we established a functional adaptation to a yeast-two hybrid expression system by incorporating a copper inducible promoter to regulate expression. We demonstrated that using a *CUP1* promoter increased protein levels above standard levels seen with the *ADH1* promoter used in the classical Y2H system. Additionally, we showed that expression is titratable, with an approximate dosage response dependent on the copper concentration used allowing for the potential of equal expression levels. Then, through the adapted liquid growth assay, we examined how a known interactor responds to the alleles of Nxf1 and suggest that we can quantify interaction strength with our modified Y2H assay.

METHODS

Site saturation mutagenesis and plasmid construction:

The pGBKT7 empty vector harboring the GAL4 DNA-binding domain was used for cloning bait constructs. The pGADT7 empty vector harboring the GAL4 activating domain was used for cloning prey constructs. Both vector backbones were produced for the Yeast Matchmaker Two-Hybrid Systems (Clontech Laboratories, Inc). The following bait constructs were cloned into pGBKT7: full length *Nxf1* (mouse Nxf1 amino acids 1-619) and truncated c-terminal fragments (mouse Nxf1 amino acids 373-619), *Lamin* (human lamin C amino acids 66-230), and *p53* (mouse p53 amino acids 72-390). The following prey constructs were cloned into pGADT7: full length mouse *Nup62* (mouse Nup62 amino acids 1-527) and *Large-T* (SV40 large-T antigen amino acids 84-708).

The c-terminal *Nxf1*^{B6} fragment (amino acids 373-619), containing the NTF2-like and UBA domains, was amplified from full length mouse *Nxf1*^{B6} cDNA. To create a subset of Nxf1 single amino acid variants at position 610, site saturated mutagenesis was performed with primers containing NNN at each residue of interest. Two PCR fragments were amplified with Phusion and contained 16 bp overlapping ends. The unpurified fragments were treated with 1 µL of *Dpn*I, heat inactivated *Dpn*I, and ligated to digested pGBKT7 vector using NEBuilder HiFi DNA Assembly. The reaction mix of the plasmid library was transformed into NEB 5-alpha bacterial cells using the standard High Efficiency protocol, PCR screened, with a subset of positive clones sequenced to determine the spread of amino acid variant. This method was used to create the C-terminal *Nxf1*^{CAST} and *Nxf1*^{B6} fragments (amino acids 373-619). Following standard restriction digest cloning techniques, full length *Nxf1* consructs were constructed by

cloning the N-terminal *Nxf1* fragment (amino acids 1-372) upstream of C-terminal *Nxf1* fragments.

As previously described, the *CUP1* promoter sequence used is 430 bp (Butt et al., 1984). The promoter sequence was modified to alter the *Bam*HI site to GATC and alter the *Nde*I site to CATAaG. The sequence was synthesized as a gBlock Gene Fragment (IDT), amplified with Phusion, PCR purified to remove the polymerase, and digested. The pGBKT7 vector was digested to remove the 705 bp *ADH1* promoter, and then clone in the *CUP1* promoter.

Yeast stock:

Yeast strains were obtained as a part of the Yeast Matchmaker Two-Hybrid Systems (Clontech Laboratories, Inc). The yeast strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2 : : GAL1UAS-GAL1TATA-HIS3, MEL1 GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ)* was used to express bait plasmids in single-transformed yeast and both the bait and prey plasmids in co-transformed yeast. The yeast strains Y187 (*MAT*α*, ura3-52*, *his*3*-200, ade 2-101*, *trp 1-901, leu 2-3*, *112*, *gal4*Δ, *met–*, *gal80*Δ, *URA3 : : GAL1*UAS-*GAL1*TATA-*lacZ, MEL1*) was used to express prey plasmids (don't need since I don't have any data using just Y187).

Competent yeast cells were grown in 2x YPD media (Fisher Scientific, DF0427-17-6) supplemented with 80 mg/L of adenine hemisulfate, harvested, and frozen down essentially as described (Gietz and Schiestl, 2007). The competent yeast were transformed with 200 ng of plasmid for single-transformants and 200 ng of each plasmid for co-transformants using the LiAc/SS carrier DNA/PEG method as essentially described (Gietz and Schiestl, 2007). The transformed yeast was spread onto media containing 1.8% agarose, minimal SD base media

(Takara Bio, 630411), and a composition of every essential amino acid except tryptophan (SD/-Trp) (630413), leucine (SD/-Leu) (630414), or tryptophan and leucine (SD/-Trp/-Leu) (630417). This selected for the plasmids depending on the type of transformation and was incubated at 30°C for 3-5 days.

Western blot assay:

Yeast liquid cultures to be used for protein extraction were grown essentially as described in the Clontech Yeast Protocols Handbook (Clontech, 2009). A single yeast colony (<2 months old) containing bait or prey or both plasmids was picked into 5 mL of selective medium. Cultures were incubated overnight for approximately 18-22 hours at 30°C and 220rpm until saturation. Overnight culture was added to fresh selective medium to create an OD_{600} = ~0.2-0.3 in 7 mL total. To obtain mid-log phase of OD_{600} = ~0.45-0.65, the new cultures were incubated for approximately 3 hours at 30°C and 220rpm. For vectors with a *CUP1* promoter, $CuSO_4 \cdot 5H_2O$ (Ricca Chemical, 2330-16) was added to induce expression during the 3-hour incubation. $CuSO_4 \cdot 5H_2O$ was added the directly to the liquid cultures at the specified concentrations. After incubation, 5 mL of chilled liquid culture was harvested by transferring to prechilled tubes, washing twice with ice-cold H2O, and pelleting at 1000xg and 4-10°C for 5 minutes. The harvested yeast was stored at -80°C until used for protein extraction.

Yeast protein extraction was performed using an NaOH and SDS sample buffer boiling method essentially as described (Kushnirov, 2000). Yeast cells were resuspended in 0.2M NaOH and incubated for 5 mins at room temperature. The cells were pelleted and resuspended in SDS sample buffer (0.06M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue) supplemented with protease inhibitor (1% Millipore Sigma P8340, 1%

PMSF), incubated at 100°C for 3 minutes, and pelleted with the lysate retained. The volume of NaOH and SDS sample buffer used was dependent on the final OD_{600} measurement before harvesting to account for variability in growth. The protein extractions were stored at -20°C until used in western blots.

7 µL of protein extract was ran on Laemmli SDS-PAGE gels and then transferred onto nitrocellulose membranes (Bio-Rad, 1620112). Membranes were stained with Ponceau-S for initial visualization. Bait proteins were C-Myc tagged and detected with mouse anti-C-Myc monoclonal antibody (Invitrogen, MA1-980) and IR-680 conjugated donkey anti-mouse secondary antibody (LI-COR, 926-68072). Blots were imaged using a LI-COR Odyssey CLx imaging system. To detect total protein as a loading control, blots were re-probed with anti-phosphoprotein antibody cocktail (Millipore Sigma, P3430 and P3300) and IR-800 conjugated secondary antibody. Blot images were quantified using ImageJ and data points were corrected using protein signals normalized to total protein.

Quantitative liquid growth yeast two-hybrid assay:

A single co-transformed colony (<2 months old) containing both bait and prey plasmids was picked into 2-3 mL of SD/-Trp/-Leu liquid media. Cultures were incubated overnight for approximately 18-22 hours at 30°C and 220rpm until saturation. The overnight cultures were diluted into fresh SD/-Trp/-Leu media to a constant optical density, then split evenly into SD/-Trp/-Leu/-His media to create an OD_{600} = ~0.13. $CuSO_4 \cdot 5H_2O$ was added directly to the yeast in SD/-Trp/-Leu/-His media at the specified concentrations. 950 µL of sample was then added to a 24-well flat bottom plate following a matrix format. The plate was incubated at 30°C with rocking in a chamber humidified with the same type liquid media to offset evaporation. The

samples were resuspended before measuring the OD_{613} every hour over approximately 40 hours. The optical density measurements were taken using a Tecan i-control Infinite 200 plate reader.

RESULTS

Unequal expression of Nxf1^{B6} and Nxf1^{CAST}

Previous studies have shown that endogenous mouse *Nxf1*^{B6} and *Nxf1*^{CAST} alleles have equal steady state mRNA and protein expression (Concepcion et al., 2015). This suggested that the mechanism of *Nxf1*^{CAST} suppression could be PPI related. Our goal was to determine what effects the allelic forms of *Nxf1* had on interaction to associated proteins. Therefore, all of the work was done using a Y2H vector system.

In a previous test when using the standard pGBK-*ADH1* vector, we observed that transformed yeast with the C-terminal *Nxf1*^{B6} allele expressed at a lower level than the C-terminal *Nxf1*^{CAST} allele (data not shown). We hypothesized that since we used truncated fragments of *Nxf1* containing amino acids 373-619, the unincluded domains were potentially important for stable expression. However, implementing the full length *Nxf1*^{B6} and *Nxf1*^{CAST} alleles did not correct the expression complication. Normalized Western blot data confirmed that there is a consistent expression difference, with Nxf1^{B6} being expressed at lower levels than Nxf1^{CAST} (Fig. 1C). To make a fair comparison between alleles, we aimed to alleviate the issue of equalizing protein levels by controlling expression.

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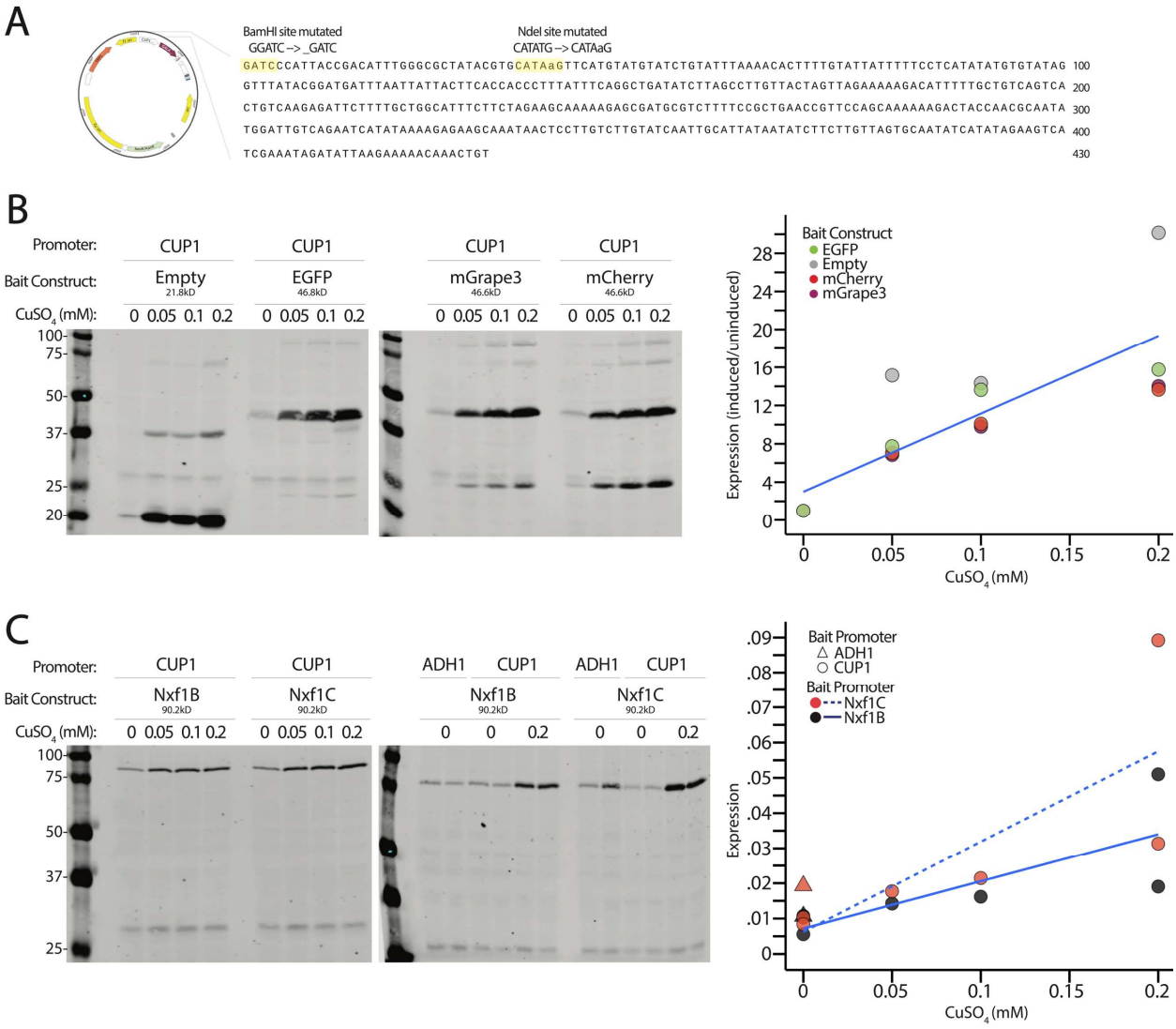


Figure 1. CUP1 promoter is dosage sensitive to copper ion concentrations.

(A) *CUP1* promoter design. The final 430 bp promoter sequence included a base pair deletion to the *BamH*I site and a base pair mutation to the *Nde*I site to allow for cloning within the vector. **(B)** The *CUP1* promoter increased expression in response to an increase in $CuSO_4$ concentration. Western blot data showed a change in protein expression as $CuSO_4$ concentrations increased from 0 (uninduced) to 0.05, 0.1, and 0.2 mM (induced). Normalized expression levels were plotted relative to 0mM $CuSO_4$ (uninduced) and a best fit line was overlayed. **(C)** The *CUP1* promoter increased expression levels above that from the *ADH1* promoter; Nxf1C consistently reaches higher expression levels than Nxf1B. Western blot data showed a difference in expression when using an induced *CUP1* promoter compared to the *ADH1* promoter, and an expression difference between Nxf1B and Nxf1C. Normalized expression levels were plotted as raw values and a best fit line was plotted only for CUP1 driven Nxf1B and Nxf1C (Nxf1B, $Nxf1^{B6}$; Nxf1C, $Nxf1^{CAST}$).

Increased CUP1 driven expression

The classical Y2H GAL4 system relies on a 700 bp *ADH1* promoter that is suggested to have high, constitutive expression (Clontech, 2009). To examine how a copper inducible promoter design would respond in a Y2H system, we designed the *CUP1* promoter to replace the *ADH1* promoter in the commonly used pGBK vector (Fig. 1A).

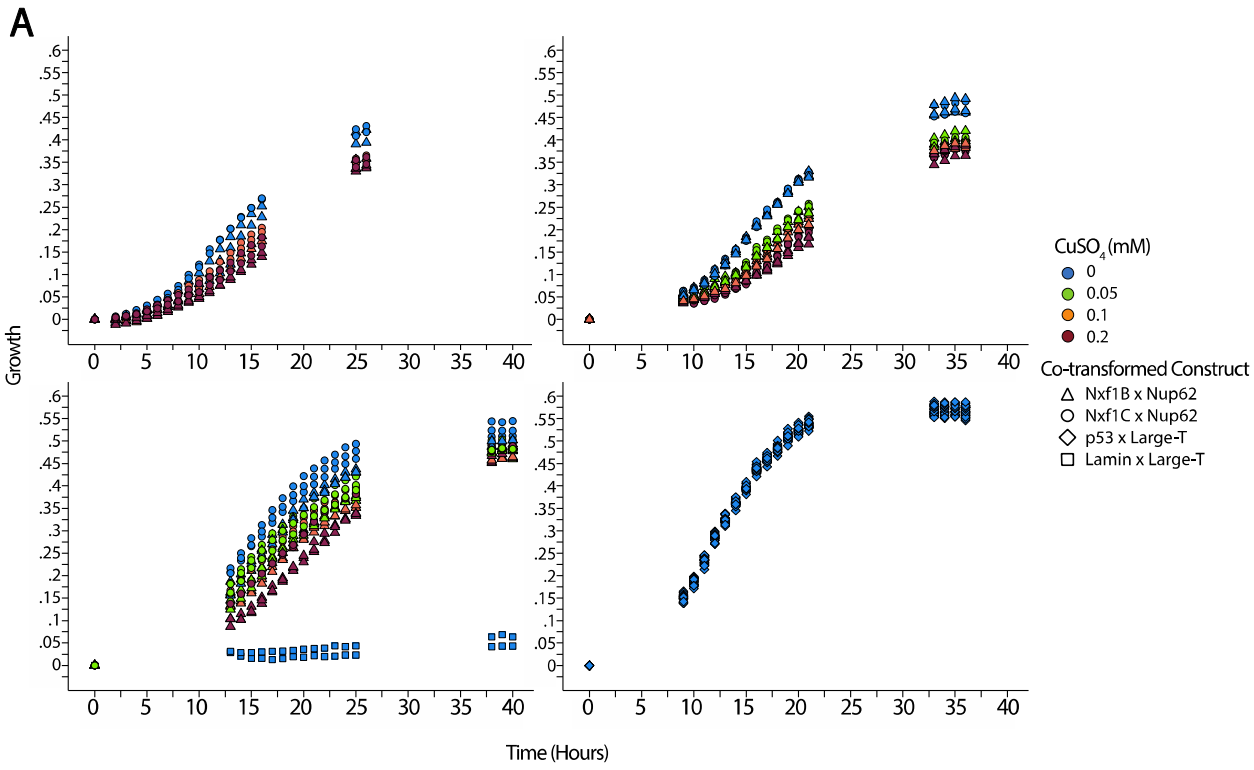
To evaluate expression, we initially sought to measure light intensity of the following fluorescent reporter genes: EGFP, mCherry, and mGrape3. All fluorescent reporters formed a corresponding protein of the correct size on Western blots (Fig. 1B-C). However, we were not able to detect fluorescence in colonies grown on solid media using filtered light at the appropriate wavelength (data not shown). One possible explanation could be an issue of improper folding due to the proximity of the proteins to the epitope tag.

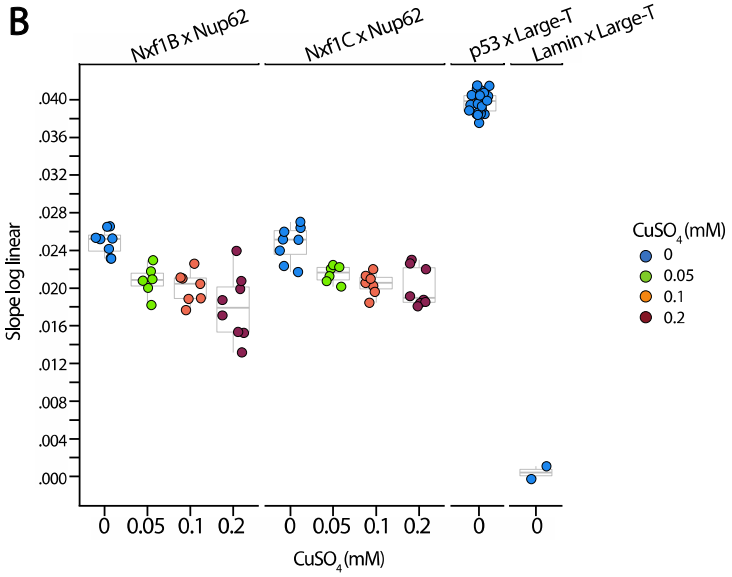
Previous work has shown that there is an optimal copper ion concentration for *CUP1* driven expression before expression decreases when oversaturated with copper (Butt et al., 1984). Knowing that copper is toxic in excess (Labbé et al., 1997), we determined viable $CuSO_4$ concentrations using a modest range of concentrations: 0.05 mM, 0.1 mM, and 0.2 mM. From normalized Western blot data, we showed that maximum *CUP1* driven expression was greater than *ADH1* driven expression (Fig. 1C). Then, from the range of concentrations, we showed that expression levels are dependent on the copper concentration used, where expression increased as the $CuSO_4$ concentration increased (Fig. 1B). Relative to uninduced expression, EGFP, mCherry, and mGrape reached a greater level of expression with 0.2 mM $CuSO_4$ (Fig. 1B). Similarly, Nxf1B and Nxf1C also reached a greater level of expression using 0.2mM $CuSO_4$ (Fig. 1C). Our results indicate that the *CUP1* promoter is a suitable replacement to the *ADH1* promoter; with

the *CUP1* promoter, we obtained greater maximum expression levels and titratable expression dependent on the concentration of $CuSO_4$ used.

Figure 2. Y2H growth rate decreases at higher bait expression levels.

(A) Growth curves of liquid Y2H assays over time. Yeast co-transformed with either *Nxf1*B x *Nup62*, *Nxf1*C x *Nup62*, *p53* x *Large-T* (positive interaction control), or *Lamin* x *Large-T* (negative interaction control) were cultured in SD/-Trp/-Leu/-His media to select for interaction in a 24-well plate. Samples were grown at 30°C with rocking and measured at OD_{613} in hourly intervals for approximately 40 hrs (Nxf1C, $Nxf1^{CAST}$; Nxf1B, $Nxf1^{B6}$). **(B)** Quantitative measurements of the growth rate derived from the growth curves in (A). The slope during the log linear growth phase from (A) was utilized to calculate the growth rate of each sample. The growth rates showed a gradual declined for *Nxf1*B x *Nup62* and *Nxf1*C x *Nup62* as bait expression increased.


A
Growth
Time (Hours)
CuSO4 (mM)
0
0.05
0.1
0.2
Co-transformed Construct
Nxf1B x Nup62
Nxf1C x Nup62
p53 x Large-T
Lamin x Large-T


B
Nxf1B x Nup62
Nxf1C x Nup62
p53 x Large-T
Lamin x Large-T
Slope log linear
CuSO4 (mM)
0
0.05
0.1
0.2

Y2H quantification using liquid growth assay

To establish if the Y2H system can be adapted to utilize the *CUP1* expression construct, we first tested how $CuSO_4$ induction would work on solid media. We obtained growth of co-transformed yeast with a *CUP1* expressing bait construct and associated prey protein; however, solid media presented a difficult challenge for measuring PPIs (data not shown).

To determine more accurately the changes in PPI strength, we implemented a liquid growth assay in a 24-well flatbottom format. The liquid assay allowed us to more easily measure growth over time to create a normalized growth curve. Since the Y2H system relies on PPIs for growth, we used the growth rate as a proxy for measuring interaction strength. Our assumption was that stronger interactions will grow at a faster rate. Therefore, we measured the slope of the logarithmic growth phase to possibly quantify growth rate. The growth rate was a useful readout for examining potential quantifiable measurements, rather than the traditional Y2H which produced a 'yes or no' interaction readout.

Interestingly, when testing *CUP1* driven $Nxf1^{B6}$ or $Nxf1^{CAST}$ as bait co-transformed with *Nup62* as prey, we observed that the growth rate decreased as the $CuSO_4$ concentration increased (Fig. 2A-B). The decrease in growth rate was the opposite trend of what one might speculate from increasing expression. We suspect that overexpression of a mildly toxic protein can compound the toxic effects which is why we see a decrease in growth rate. The positive and negative interaction control, *p53* x *Large-T* and *Lamin* x *Large-T* respectively, grew accordingly so we can be confident in the auxotrophic selection and interactions occurring. Altogether, our findings imply that the growth rate could be measured and eventually provide a quantification of PPI strength even though we saw a decrease in growth rate when overexpressing *Nxf1*.

DISCUSSION

Overview

Measuring PPI strength through a yeast-two hybrid assay exhibited many challenges. When expressing bait constructs in yeast, we had the issue of unequal expression from the *ADH1* promoter. Furthermore, the current system does not allow for quantification of the PPI strength. To mitigate issues, we employed an inducible promoter and proposed a way to quantify PPIs. We showed that induced *CUP1* driven expression reached higher expression levels than with an *ADH1* promoter and that expression can be precisely controlled with the copper concentration used. Then, using the Y2H as a liquid growth assay, our findings imply that the growth rate and thus interaction can be quantified, where growth rate in dependent on how robustly the bait and prey interact.

Comparing PPIs across constructs

One of our original goals was to normalize the expression differences between $Nxf1^{B6}$ and $Nxf1^{CAST}$ to eventually make a comparison of PPI strength. Our results suggests that $Nxf1^{B6}$ at 0.1 mM $CuSO_4$ and $Nxf1^{CAST}$ at 0.05 mM $CuSO_4$ have similar levels of bait expression (Fig. 1C). If we assume that expression levels are equated at their corresponding copper ion concentration, then we can make a fair comparison of PPI strength because the only factor that should be influencing the growth rate is interaction strength. When we look at the Y2H growth rate for *Nxf1*^{B6} x *Nup62* at 0.1 mM $CuSO_4$ and *Nxf1*^{CAST} x *Nup62* at 0.05 mM $CuSO_4$, we see that *Nxf1*^{B6} and *Nxf1*^{CAST} do not display a detectable difference in PPI strength (Fig. 2B). While

residue 610 is important for *Nxf1*^{CAST} function (Concepcion et al., 2015), our results do not indicate a strong PPI related mechanism for the suppressive function of *Nxf1*^{CAST}.

Overexpression causes a decline in growth rate

There are multiple reasons why we would see a decline in growth rate for higher copper ion concentrations, primarily that overexpression of a mildly toxic construct to the Y2H assay can have the unfortunate effect of increased toxicity. If we were to control how long *Nxf1* is overexpressed, we might be able to circumvent the issue of prolonged expression while still having abundant protein expression to activate the Y2H assay. This might be achievable if we later removed the $CuSO_4$ from the media or transferred the yeast into new media without $CuSO_4$.

Limitations and future direction

These modifications set the groundwork for making comparisons of PPIs strength with the Y2H. While Y2H has been heavily exploited to identify unknown protein interactor, it does not commonly provide quantitative measurements of interaction. Here, we outline a few parameters that should be met before this can be more easily accomplished. Equalizing expression of the bait proteins of interest can be readily achieved, but the range of *CUP1* driven induction must first be tested on the proteins of interest. This is because induction levels appear to be dependent on the protein being expressed. Furthermore, in our hands we noticed variability in expression levels from experiment to experiment. This could be caused by variability in how the samples were handled or due to a low sample size. Finally, expressing toxic proteins only compounds the toxicity. Overall, our work provides insight to making useful modification to the Y2H assay and provides a basis for quantitative and comparative analysis of PPIs

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