PROTEIN CHARACTERIZATION OF HUMAN YPEL2 AND YPEL HOMOLOG YEAST MOH1

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY
ÇAĞLA ECE OLGUN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

AUGUST 2018

Approval of the Thesis:

PROTEIN CHARACTERIZATION OF HUMAN YPEL2 AND YPEL HOMOLOG YEAST MOH1

submitted by **ÇAĞLA ECE OLGUN** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences**_____

Prof. Dr. Orhan Adalı
Head of Department, **Biological Sciences** _____

Prof. Dr. Mesut Muyan
Supervisor, **Biological Sciences Dept., METU** _____

Examining Committee Members:

Prof. Dr. A. Elif Erson-Bensan
Biological Sciences Dept., METU _____

Prof. Dr. Mesut Muyan
Biological Sciences Dept., METU _____

Asst. Prof. Dr. Erkan KİRİŞ
Biological Sciences Dept., METU _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Biological Sciences Dept., METU _____

Asst. Prof. Dr. Murat Cevher
Molecular Biology And Genetics Dept., Bilkent University _____

Date: 03.09.2018

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Çağla Ece Olgun

Signature:

ABSTRACT

PROTEIN CHARACTERIZATION OF HUMAN YPEL2 AND YPEL HOMOLOG YEAST MOH1

Olgun, Çağla Ece

M.S., Department of Molecular Biology and Genetics

Supervisor: Prof. Dr. Mesut Muyan

August 2018, 55 pages

17ß-estradiol (E2), the main circulating estrogen hormone, has an important role in the physiological and pathophysiological regulation of many tissues and organs including breast tissue. Regulation of cell proliferation, differentiation and death in target tissues is mediated by E2. The estrogen receptor (ER), a transcription factor, provides the lasting effect of E2 on cells via regulation of targeting gene expression.

Previous microarray and gene expression studies in our laboratory reveal that YPEL2, which is one of the members of YPEL gene family, is an estrogen responsive gene. For understanding function of YPEL2, over-expression systems in COS7 and MCF7 were used. However, over-expression of any YPEL family gene leads to a nuclear membrane disassembly, DNA leakage and then rapid cell death. This prevented us to examine the functional features of YPEL2 in mammalian cells. In addition, the members of the YPEL gene family proteins share a remarkably high amino acid sequence homology. This high sequence homology also prevented us to assess the function of YPEL proteins by decreasing the amount of any YPEL protein using siRNA approaches. Therefore, to understand function and mechanistic aspects of YPEL2, new approaches were needed.

In cells, protein functions within the context of a dynamically changing network of interacting protein partners. Thus, the identification of protein partners of protein of interest gives important information about the function of protein. In this study, to understand function of YPEL2, we used BioID system through which putative protein partners of protein of interest are defined. Putative interacting protein partners of YPEL2 were then analyzed with gene annotation tool, DAVID. According to gene clusters generated through GOTERM Biological process analysis, possible protein partners are clustered in different biological processes such as RNA processing and ribosome biogenesis. Also, our results suggest that a group of protein partners of YPEL2 is involved in the formation of stress granules.

That the YPEL gene family is conserved through yeast to human with high amino acid sequence homology suggests conserved functions for Ypel proteins. In yeast there is one homolog of YPEL gene family: MOH1, on which there is a few information on structure and function. In addition to BioID studies, to assess a function to YPEL2, we wanted to generate a cell model for YPEL proteins using yeast. To establish this model, we initially wanted to characterize the functional feature of MOH1; we reasoned that a better understanding functional futures of the yeast YPEL homolog of MOH1 could provide an important experimental system to characterize YPEL2 functions. Using yeast strains, we found that the deletion of MOH1 causes a decrease in cell survival when cells are grown under nutritional depletion stress. Also, when survival of wild type and *moh1*Δ cells were compared under different stress conditions, survival patterns of cells differed according to stress conditions. Through these results, we conclude that MOH1 is an important factor in stress responses of cells to different stress conditions and that MOH1 affects cellular survival differently dependent upon stress conditions.

Keywords: *YPEL2*, *MOH1*, BioID, yeast, stress

ÖZ

İNSAN YPEL2 PROTEİNİ VE MAYADAKİ YPEL HOMOLOĞU MOH1 PROTEİNİNİN KARAKTERİZASYONU

Olgun, Çağla Ece
Yüksek Lisans, Moleküler Biyoloji ve Genetik Bölümü
Tez Danışmanı: Prof. Dr. Mesut Muyan
Ağustos 2018, 55 sayfa

Dolaşımdaki ana östrojen hormonu 17β-östradiyol (E2), meme dokusu da dahil olmak üzere birçok organ ve dokunun fizyolojik ve patofizyolojik olarak düzenlenmesinde önemli bir role sahiptir. Hedef dokulardaki hücre çoğalması, farklılaşması ve ölümünün düzenlenmesi E2 üzerinden gerçekleştirilir. Bir transkripiyon faktörü olan östrojen reseptörü, E2'nin hücre içindeki kalıcı etkilerin, hedef genlerin ifadeleri düzenleyerek sağlar.

Laboratuvarımızda daha önceden gerçekleştirilen mikrodizin ve gen ifadesi çalışmaları yüksek derecede korunmuş olan YPEL gen ailesinin üyesi *YPEL2*'nin bir östrojen yanıt geni olduğunu göstermiştir. *YPEL2*'nin fonksiyonunun anlaşılması için genin fazla ifade edilmesi (over-expression) çalışmaları COS7 ve MCF7 hücrelerinde yapılmıştır. Ancak, YPEL gen ailesinin genlerinden herhangi birinin fazla ifade edilmesi hücre çekirdek zarının bozulup DNA'nın sitoplazmaya geçmesine ve ardından hızlı hücre ölümüne yol açtığı gözlemlenmiştir. Bu nedenle fonksiyonel genin fazla ifadesi çalışmaları memeli hücrelerde devam ettirilememiştir. Buna ek olarak, YPEL gen ailesi çok yüksek amino asit sekans homolojisi göstermektedir. Bu yüksek homoloji, siRNA yaklaşımını kullanarak YPEL sentezini susturulmasına engel olmaktadtır. Dolayısıyla YPEL2'nin fonksiyonunu tayin edebilmek için yeni yaklaşımlara ihtiyaç vardır.

Hücre içinde proteinler dinamik etkileşim ağları içinde fonksiyonlarını gerçekleştirirler. Protein-protein etkileşimlerinin tanımlanması, ilgilenilen proteinin fonksiyonu hakkında önemli bilgiler verir. Bu nedenle yakınlık bağımlı biyotin ekleme yaklaşımını kullanarak Ypel2'nin olası protein partnerlerini tanımlandı. Yaptığımız *in silico* analizlerle Ypel2'nin olası protein partnerlerinin RNA süreçleri ve ribozom biyogenezi gibi farklı biyolojik süreç kümelerinde yer aldığı belirlendi. Ayrıca, çalışmalarımız, Ypel2'nin protein partnerlerinin stres granüllerinin oluşumunda yer aldığını da göstermektedir.

YPEL gen ailesi mayadan insana kadar yüksek bir amino asit sekans homolojisiyle korunarak gelmiştir. Bu korunma ortak bir fonksiyona işaret eder. Mayada YPEL gene ailesinin tek bir homoloğu bulunmaktadır: MOH1. BioID çalışmalarına ek olarak, Ypel2'ye fonksiyon tayin edebilmek için mayada yeni bir sistem geliştirildi. Bu yeni sistemi kullanarak mayada Moh1'ın fonksiyonel özelliklerini bulmanın Ypel2'nin fonksiyonu hakkında da bilgi verebileceğini öngörmekteyiz. Çalışmalarımıza göre *MOH1* geninin silinmesi suda 14 gün yaşam sonucunda hücrenin hayatta kalma oranını düşürmektedir. Ayrıca H_2SO_4 ve H_2O_2 gibi farklı stres indükleyicilerinin varlığında MOH1 geni silinmiş ve silinmemiş hücrelerde farklı stres tepkileri gözlemledik. Bu sonuçlardan yola çıkarak MOH1'in hücrenin strese tepkisinde önemli bir rolü olduğunu ve farklı stres koşullarında MOH1'in hücre yaşamına etkisinin farklı olduğunu bulguladık.

Anahtar kelimeler: *YPEL2, MOH2,* BioID, maya, stres

ACKNOWLEGMENTS

I would like to thank firstly my advisor Prof. Dr. Mesut Muyan for his guidance and encouragement during my research. I am grateful for the opportunity he presented that has launched me on the path of becoming an independent researcher.

I would like to thank members of my thesis committee; Prof. Dr. Ayşe Elif Erson Bensan, Asst. Prof. Dr. Erkan Kiriş, Assoc. Prof. Dr. Çağdaş Devrim Son and Asst. Prof. Dr. Murat Cevher for the time and effort they invested in my educational and scientific journey and critical reading of my thesis.

I would like to thank Assoc. Prof. Dr. Çağdaş Devrim Son and Asst. Prof. Dr. Nihal Terzi Çizmecioğlu for their valuable suggestions and helpful discussions. Also, I thank to Dr. Cory Dunn and his student Emel Akdoğan for welcoming me to their laboratory as a visitor and teaching me some of the yeast techniques used in this study.

I cannot thank enough to my labmates for their friendship and moral and physical supports. I thank to Burcu Karakaya for never leaving me alone in tough times and Gizem Kars for not only enlightening discussions but also our inside jokes. I would like to express my thanks to Gamze Ayaz for all her academic teachings and guidance, and most importantly her friendship during all these three years; Pelin Yaşar for not only experimental and academic advising but also our precious conversations; Negin Razizadeh for her kind heart and listening to me tirelessly for long times. I also thank to Gizem Turan for her helping hands in my experiments and her smiling face. Lastly I would like to thank Kerim Yavuz for his excellent jokes and our cheerful little talks.

I wish to express my deep appreciation to my dearest lifelong friends, Onur Demir the one with dark humor and Ayşegül Özman the one with extraordinary laugh. I cannot express enough thanks to you for your love, support and your patience. Also, I would like to thank Fahriye Banu Demirkılınç and Hüner Aydın for their support and solidarity. It is my privilege to have you in my life.

I cannot find appropriate words to express my gratitude to my parents for always being there for me and their endless love and support. I wish to thank my older sister Melike Sığla Olgun, in particular, for her never-ending love, support and tolerance to me. Without her, I could not be the person that I am today. Also, I wish to thank my brother-in-law Atilla Orgunmat for his kind talks and moral support like my own brother.

I would like to also thank TUBITAK, whose generous support through the TUBITAK-KBAG 117Z213 grant allowed me to carry out studies on the development of a yeast model for YPEL2.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

CHAPTER 1

INTRODUCTION

1.1 Estrogen Signaling

Estrogen receptors (ERs) are nuclear hormone receptors that regulate transcriptional activities in cells [1]. Estrogens modulate the activities of ERs. Estrogens are classified into three groups: estrone, estradiol (E2) and estriol [2]. In blood circulation, 17β-estradiol (E2) is main hormone that regulates cellular growth, differentiation and function of a broad range of target tissues and also contributes to the initiation and development of target tissue malignancies [2], [3].

Estrogen receptor α and β (ERα and ERβ) are transcription factors encoded by unique genes located on different chromosomes, chromosome 6 and chromosome 14 respectively. These estrogen receptor subtypes are expressed in the same as well as in different tissues and expression levels vary according to tissue types [4]. Although they have differences in amino acid sequences which are responsible for subtle subtype-specific features, they share high degree of structural and functional characteristics. Similar to other nuclear hormone receptors, ERs have six different functional domains: A/B, C, D, E and F. The A/B domain contains an activation function, referred to as the AF1, through interaction with co-regulators. The C domain, called the DNA binding domain (DBD, is responsible for binding to specific DNA sequences critical

for regulation of target gene expression. The D domain is the hinge region that includes a nuclear localization signal. The E/F domain at the C-terminus of ERs, also known as ligand binding domain (LBD), is critical for the receptor dimerization and the activation function 2 (AF2) [5], [6].

Dimerization of estrogen receptors occurs immediately after synthesis and this dimerization process is independent of E2. In the presence of E2, surfaces for protein-protein interactions are generated through structural reorganization of ERs' LBD. These reorganizations lead to the conversion of the inactive ER to functionally active form, through which genomic and non-genomic events are carried out [7].

While the regulation of gene expression in target tissues is provided via genomic E2-ER signaling pathway, activation of downstream signaling pathways arises as a result of non-genomic E2-ER signaling [8]. In genomic E2-ER signaling, there are two different mechanisms: the estrogen response element (ERE)-dependent and ERE-independent signaling pathway. As the name implies, in the ERE-dependent signaling pathway, the E2-ER complex binds to DNA directly through ERE. However, there is no direct binding of E2-ER to DNA in ERE-independent signaling pathway. Instead, the E2-ER complex interacts with other transcription factors, such as activator protein 1 (AP-1) and stimulatory protein 1 (SP-1) already bound to their cognate response elements [7], [8].

1.2 Human Yippee-like (YPEL) gene family

Human YPEL gene family (YPEL1 through 5) was firstly identified by Hosono *et. al.* in 2004 during comprehensive sequence analysis. Members of YPEL gene family are found on different chromosomes: *YPEL1* on 22q11.2, *YPEL2* on 17q23.2, *YPEL3* on 16p11.2, *YPEL4* on 11q12.1 and *YPEL5* on 2q23.1 [10].

Members of the Ypel family share high amino acid sequence similarity. Among human Ypel family members, Ypel1 through 4 have high amino acid sequence homology; between 83.2 – 96.6%. Ypel5, on the other hand, has the lowest amino acid sequence homology with other human Ypel family members; 43.8 – 49.5 % [10] (Figure 1). According to the study of Hosono *et. al*., 68 different species have 100 YPEL family genes. For example, African green monkey, mouse and human have 5 different Ypel family members, whereas there is only one homolog in yeast. In addition to these species, insects, fishes, birds, plants and protozoans also have Ypel family members [10]. This evolutionary conservation suggests that Ypel proteins play critical roles in cellular functions.

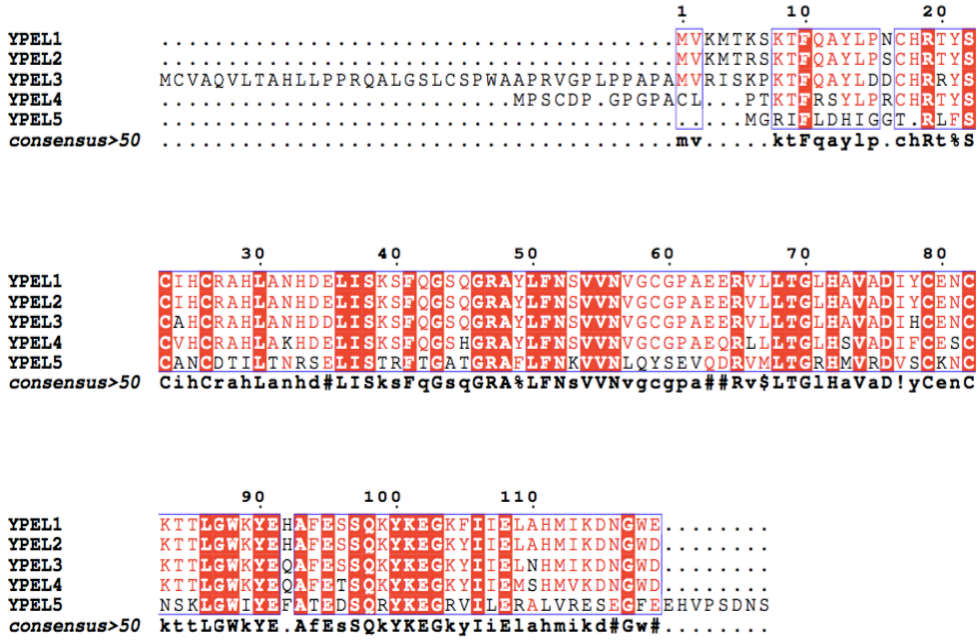

Figure 1. Amino acid sequence alignment of YPEL family members constructed via ESPript 3.x [11]

Through PCR analysis with cDNA libraries obtained from distinct tissues and organs, Hosono *et. al.* [10] generated expression profiles of YPEL genes. They found that the members of YPEL family genes are expressed in a broad range of tissues and organs, except *YPEL1*. Expression of *YPEL1* is only observed in

the fetal brain and testis. While *YPEL2* is expressed in the heart, kidney, lung, pancreas, placenta, skeletal muscle, leukocyte, prostate, spleen, testis, fetal brain, fetal heart, fetal kidney, fetal liver, fetal lung, fetal skeletal muscle and fetal spleen, *YPEL3* and *YPEL5* are expressed in all tissues observed. *YPEL4* expression is seen in the brain, ovary, colon, placenta, spleen, testis, small intestine, lung, fetal spleen and bone marrow, fetal lung, fetal liver, fetal heart and fetal brain [10].

To identify cellular localization of YPEL gene family members in human, Hosono *et. al.* generated two different antibodies [10]. The C termini of Ypel1 and Ypel5 were used for the generation antibodies. Immunocytochemistry performed with antibody generated against Ypel1 showed that the antibody recognizes Ypel1-4. Further studies indicated that Ypel1-4 localize to the nucleus and as a dense spot surrounding the nucleus in COS-7 cells. These results, when merged with anti-gamma tubulin staining, as an indicator for centrosome, suggested that some of these Ypel1-4 proteins were found at centrosome during interphase. Moreover, it appeared that the localization of Ypel1-4 change during cell cycle of COS-7 cells: they were found on or close to mitotic apparatus instead of centrosome during mitotic phase. Immunocytochemistry studies with anti-Ypel5 antibody indicated that during interphase Ypel5 were found at same localization with the other members. However, during mitotic phase, Ypel5 was shown to be at the mitotic spindle [10].

Previous studies conducted in our laboratory suggested that *YPEL2* and *YPEL3* are estrogen responsive genes. Both of these genes were repressed by E2 in a time dependent manner [9]. In these studies, it was shown that *YPEL2* and the other members of YPEL gene family are endogenously expressed in African green monkey kidney fibroblast-like cell line (COS7) with low protein levels [9]. Studies directed at the understanding the functions of these proteins used

initially an over-expression system in MCF7 and COS7 cells. It was found that the overexpression of YPEL genes family leads to nuclear membrane damage; leakage of DNA into the cytoplasm followed by a rapid cellular death. This prevented functional analysis of Ypel proteins in mammalian cells [9]. Our findings together with virtually no study in the literature about other members of the family necessitated other experimental approaches to assess the function(s) of Ypel proteins.

1.3 Yeast Yippee-like protein: MOH1

Ypel proteins share high homology at amino acid level as a result of highly conserved sequence similarity. Functional or mechanistic studies on any Ypel proteins through simple knock-out experiments with siRNA is difficult because of this high degree of sequence conservation. Although we attempted with several siRNA design against individual Ypel proteins or with common sequences for the entire Ypel family, we failed in altering the synthesis of the proteins (our unpublished data). In addition, as mentioned in Section 1.2, the over-expression of *YPEL* genes leads to DNA diffusion into cytoplasm and cell death [9]. All of these reasons led us to develop new approaches to reveal the functional and mechanistic characteristics of *YPEL2*.

Because YPEL gene family has one homolog in yeast, MOH1, yeast can be used to establish a new model to better understand functional features of Ypel2. We envisioned that through the characterization of Moh1 protein, we could study the functions of Ypel2, in particular and Ypel proteins in general, in cellular processes. Similar to its eukaryotic homologs, Moh1 is a protein with unknown function in yeast as well. However, there are several studies which provide preliminary findings about Moh1.

In a study where regulation of N-myristoylation in *S. cerevisiae* was examined, it was shown that myristoyl-CoA:protein *N*-myristoyltransferase (Nmt1p) leads deficiency in protein N-myristoylation and the loss of colony forming unit (CFU) during transition of cells from growth in rich media to growth in nutrition deprivation media (stationary phase). Because Nmt substrates might affect stationary stage survival, known and putative Nmt substrates were searched in yeast. Moh1 was predicted to be a Nmt1p substrate and was identified as N-myristoylated protein due to presence of N-myristoylation motif. To mimic the effect of Nmt1p mutation, putative Nmt1p substrates were deleted individually. In twelve deletion strains, there was a similar effect with Nmt1p mutation and the loss of colony forming unit. Δ*MOH1* strain was one of these twelve strains and the deletion of *MOH1* caused 10^4-fold change in CFU in water after 25 days. In addition to this observation, octapeptides derived from Moh1 N-terminus were myristoylated after incubation with Nmt1p, which also proved that Moh1 is a Nmt substrate [12].

In a genome-wide expression analyses of *S. cerevisiae* under sugar-induced osmotic stress, the expression levels of 589 genes were changed with high sugar concentrations. *MOH1* was identified as one of the 346 up-regulated genes [13].

Martinez *et. al. s*tudied on gene expression in and exit from stationary phase in *S. cerevisiae* to better explain how survival of cell occurs during this process. Gene expression profiles of cells were comparatively analyzed when cells were in stationary state (nutrient depletion) and in rich medium after nutrient depletion. Stationary phase (SP)-expressed genes defined according to their abundance in stationary phase and in rich medium (refeeding after stationary phase). SP-expressed mRNAs were abundant during nutrient depletion; however, their abundance decreased by refeeding of cells. According to this study, *MOH1* was one of the 107 SP-expressed gene because its mRNA

abundance decreases after 5-10 min of re-feeding. To understand whether these SP-expressed genes are also SP-essential genes, or not, mutant strains with deletions in SP-expressed genes was observed after temperature shift (from 30°C to 37°C). *MOH1*-deleted mutant strain lost viability after 16 days at 37°C [14].

In another study, the possible function of Moh1 was examined through comparison of the wild-type (WT) strain and a mutant strain in which *MOH1* is deleted [15]. According to this study, when these strains were induced with apoptotic factors, such as UV radiation, DNA-damaging drug, hyperosmotic shock and heat shock, *MOH1*-deleted mutant strain was more resistant to cytotoxicity of these factors than the WT-strain. In addition, transformant strains with the WT-*MOH1* or with expression vectors bearing the individual YPEL gene family members were produced to see whether these transformants recapitulate the effects of the WT-strain under lethal UV-radiation. Compared to other YPEL family members, Ypel5 had more sensitivity to UV radiation, which showed similar sensitivity to the WT-strain. When apoptotic signatures of the WT-strain and transformants with the WT-*MOH1* and *YPEL5* bearing plasmids were compared with apoptotic signatures of MOH1-deleted strain under UV-radiation, it was found that apoptotic events were observed at a lesser level in *MOH1*-deleted strain than in other strains. This study suggested that Moh1 has a role in mitochondria-dependent apoptosis caused by DNA damage and this function conserved at the human Ypel5 [15].

1.4. Aim of the study

Proteins function within the context of dynamically changing network of interacting protein partners. Thus, the identification of protein partners of a protein gives important information about the potential functions and mechanistic features of the protein. Our previous results that the overexpression of Ypel protein family of proteins in mammalian cells causes

nuclear membrane disassembly leading to DNA leakage into the cytoplasm suggest that Ypel proteins could be an important contributor to the structural/functional integrity of nuclear membrane. To assess this prediction, we wanted to utilize the recently introduced proximity dependent biotin labelling (BioID) approach, a versatile and an effective system to identify the interaction partners of a test protein in cellular environment. We foresee that the identification of Ypel2 protein partners could allow us to begin to effectively study functional features of the protein in cellular context.

In human, there are five different *YPEL* genes with a high amino acid homology as a result of high degree of sequence identity. This renders difficulty in studying the functional features of each YPEL in human cell lines. Because of this reason, the establishment of a new model system was necessary to study Ypel2 protein function specifically. The conservation of Yippee-like proteins from yeast to human together with high amino acid sequence homologies suggests also conserved function features of Yippee-like proteins. In yeast, there is only one homolog of YPEL gene family, MOH1. Yeast cells are an important model system for the study of eukaryotic cell biology as it offers an efficient and easy means of dissecting the gene function in physiological processes using genetic and molecular biology tools. By using yeast as a model system, a better understanding of functional features of Moh1 can provide a background with which new experimental approaches would be developed to assess the role of Ypel2 and other Ypel proteins in cellular events.

The aim of this study is therefore directed at a better understanding of Yepl2 functions through the identification of possible Ypel2 interactors in mammalian cells and the establishment of a yeast model system by dissecting the functions of the YPEL homolog of yeast Moh1.

CHAPTER 2

MATERIALS AND METHODS

2.1. Proximity-dependent biotin labelling (BioID)

2.1.1. Cell lines and growth conditions

For BioID studies, African green monkey kidney fibroblast-like cell line COS7 were used. COS7 cell line was a kind gift from Asst. Prof. Özgür Şahin (Bilkent University, Ankara, Turkey).

COS7 cells were grown in high glucose Dulbecco's modified eagle medium (DMEM) without phenol red (Lonza, Belgium, BE12-917F). Medium was supplemented with 8% fetal bovine serum (Merck, Germany, S0115), 1% penicillin streptomycin (Lonza, Belgium, BE17-602E) and 2 mM L-glutamine (Lonza, Belgium, BE17-605E).

Cells incubated in humidified incubator with 5% CO_2 at 37°C. During growth, passaging or refreshing of cells were done every 2-3 days. Cells were maintained maximum 6 weeks in the incubator.

2.1.2. Cloning of YPEL2 into BioID plasmids

For BioID studies, pcDNA3.1 MCS BirA*(R118G)HA plasmid were purchased from Addgene, USA. In our lab, for previous studies, flag tagged YPEL2 (flag-YPEL2) bearing pBS-KS(-) plasmid were constructed [9]. For

cloning of flag-YPEL2 into pcDNA3.1 MCS BirA*(R118G)HA, pBS-KS(-)_flag-YPEL2 plasmid was double digested with XhoI (New England Biolabs, USA, R0146) and BamHI (New England Biolabs, USA, R0136) restriction enzymes. Flag-YPEL2 DNA fragment was extracted from gel by using Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, USA, D4007). The extracted flag-YPEL2 DNA fragment was treated with T4 DNA polymerase (Thermo Scientific, USA, EP0061) to form blunt ends at the DNA. After T4 DNA polymerase treatment, this construct was digested with EcoRI(New England Biolabs, USA, R0101) restriction enzyme. The digested flag-YPEL2 DNA fragment were purified by using QIAquick PCR Purification kit (Qiagen, Germany, 28106)

To digest pcDNA3.1 MCS BirA*(R118G)HA plasmid, firstly plasmid was cut with NheI (New England Biolabs, USA, R0131) and T4 DNA polymerase (Thermo Scientific, USA, EP0061) treatment was applied. After that, plasmid was digested with EcoRI (New England Biolabs, USA, R0101). Digested plasmid was treated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, USA, EF0654) to prevent plasmid from self ligation. The digested pcDNA3.1 MCS BirA*(R118G)HA plasmid were purified by using QIAquick PCR Purification kit (Qiagen, Germany, 28106)

The Flag-YPEL2 DNA fragment was ligated with 50 ng of the digested pcDNA3.1 MCS BirA*(R118G)HA plasmid with 6:1 insert:plasmid ratio. Ligation was performed with Rapid Ligation Kit (Thermo Scientific, USA, K1422) at room temperature for 15 minutes. Ligation mixture was transformed into chemically competent *E.coli XL1-blue* cells. After transformation, single colonies were selected into 5 ml LB-Ampicilin medium and grown overnight. From these cultures, plasmid isolation was done with QIAprep Spin Miniprep Kit (Qiagen, Germany, 27106). Constructs were sequenced to ensure that sequences were correct.

2.1.3. Fluorescence Microscopy

2.1.3.1. Transfection

For ICC studies, COS7 cells, $1.5x10^4$ cells/well, were seeded onto 12-well cell culture plates in which round coverslips had been placed. Transfection was carried out after 48 hour after seeding by using pcDNA3.1 MCS BirA*(R118G)HA empty vector (EV) and pcDNA3.1 MCS BirA*(R118G)HA-flag-YPEL2 plasmids. For each construct, there were 2 wells: one for biotin addition (plus-biotin) and the other for no biotin addition (minus-biotin). 1 µg of DNA was used for transfection. DNA mixed with DMEM high glucose medium. 2 µl of Turbofect *in vitro* transfection reagent was then added onto DNA-DMEM mixture. Transfection mixture was incubated at room temperature for 30 minutes to form transfection complex. During incubation, medium was removed and fresh complete growth medium was added onto cells. After incubation, transfection reagent was added onto cells drop-wise. After 4 hours from transfection, medium was refreshed.

2.1.3.2. Biotin and ATP addition

20 hour after transfection, biotin (Sigma, Germany, B4639) and ATP (Adenosine 5'-triphosphate disodium salt hydrate, Sigma, Germany, A2383) were added into fresh complete growth medium. This medium with biotin and ATP were used to refresh cells in plus-biotin wells. Plus-biotin medium included 50 µM of biotin and 1 mM of ATP as final concentration. Medium in minus-biotin wells were refreshed with fresh complete growth medium.

2.1.3.2. Immunocytochemistry

After 14 hours from biotin addition, medium on the cells was removed. Cells washed with PBS three times. For fixation of cells, 3.7 % formaldehyde was used. Cells incubated with formaldehyde for 30 minutes at room temperature. After incubation, formaldehyde was removed. To be sure that all of the remaining fixative was removed, cells were washed three times with PBS. 0.4 % Triton-X was added onto cells to permeabilize them and 10 minutes-incubation at room temperature was done. After three PBS washes, 10% bovine serum albumin (BSA) in PBS was added onto cells for Flag-M2 antibody (Sigma, Germany, F1804) or anti-biotin antibody (Abcam, USA, ab53494). Cells were incubated with 10% BSA for 1 hour at room temperature with gentle agitation for blocking. BSA containing PBS was removed after 1 hour and primary antibody solutions were added sequentially. The Flag-M2 antibody was used as 1:250 in 3% BSA-PBS and the anti-biotin antibody was used as 1:100 in 3% BSA-PBS. After incubation with primary antibody solutions for 2 hours at room temperature, primary antibody solutions were removed and cells were washed with PBS three times. For the Flag-M2 antibody, an Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (Abcam, USA, 150113) was used as 1:1000 in 3% BSA-PBS. For the biotin antibody, an Alexa Fluor® 647 conjugated goat anti-rabbit secondary antibody (Abcam, USA, ab150083) was used as 1:1000 in 3% BSA-PBS. Secondary antibody incubations were done for 30 minutes at room temperature in the dark. After secondary antibody incubations, cells were washed with 3 times with PBS. Coverslips were mounted onto glass slides with a drop of Fluoroshield Mounting Medium with DAPI (Abcam, USA, ab104139). DAPI in the hardening medium labels nuclei. Edges of coverslips were sealed with nail polish. Slides were dried in the dark. For imaging, fluorescence microscopy (Leica) was used in the laboratory of Assoc. Prof. Dr. Çağdaş Son (METU, Biological Sciences).

2.1.4. Mass Spectrometry Analysis

2.1.4.1. Transfection

For mass spectrometry analysis, COS7 cells, as $75x10^4$ cells/dish, were seeded onto 10 cm-dish culture dishes. For each construct, four dishes were used. Transfection was done with pcDNA3.1 MCS BirA*(R118G)HA empty vector and the flag-YPEL2 cDNA bearing pcDNA3.1 MCS BirA*(R118G)HA. For control, four dishes were not transfected with any of these plasmids (untransfected control). 20 µg DNA was diluted into 2 ml DMEM high glucose medium. 40 µl of Turbofect *in vitro* transfection reagent added into DNA-DMEM mixture. Transfection mixture were incubated for 30 minutes at room temperature. After incubation, this mixture was added dropwise onto fresh medium which covers cells. 4 hours after transfection, medium was changed with fresh complete growth medium.

2.1.4.2. Biotin and ATP addition

Biotin and ATP addition was done as described in Section 2.1.3.2.

2.1.4.3. Protein isolation and Streptavidin Affinity Capture of Biotinylated Proteins

14 hours after biotin and ATP addition, cells were trypsinized, collected in medium and centrifuged at 500g for 6 minutes. Cell pellet was then washed two times with PBS. After, cells were collected, they were lysed at room temperature in lysis buffer (50 mM Tris, pH=7.4; 500 nm NaCl; 0.4% SDS; 5 mM EDTA; 2 % Triton-X; 1mM DTT; 1x Protease inhibitor). Cells in lysis buffer were actively sonicated for 7.5 minutes with 5 seconds on and 10 seconds off pulse cycles. Cell lysates, after sonication, was centrifuged at 14000 rpm for 15 minutes and supernatant was collected. Protein concentration measurement was done with Quick Start Bradford Protein assay (Bio-Rad, USA, 500-0201).

3 mg of protein from each sample was incubated with Streptavidin Magnetic Beads (New England Biolabs, USA, S1420S) overnight on rotator. Beads were collected with magnetic separator and washed twice with Wash Buffer 1 (2% SDS in dH_2O) for 10 minutes. Then, beads were washed once with Wash Buffer 2 (2% deoxycholate; 1% Triton-X; 50 mM NaCl; 50 mM HEPES, pH=7.5; 1mM EDTA) for 10 minutes, once with Wash Buffer 3 (0.5% NP-40; 0.5% deoxycholate; 1% Triton-X; 500 mM NaCl; 1 mM EDTA) for 10 minutes and once with Wash Buffer 4 (50 mM Tris, pH=7.4; 50 mM NaCl) for 30 minutes. All washing steps were done at room temperature. After washing, some of the sample was spared for western blot analysis and the remaining was send to Koç University for Mass Spectrometry analysis.

Samples for western blot analysis was processed to separate proteins from streptavidin magnetic beads. For elution of proteins, 500 µM biotin containing Laemmli buffer were used. Laemmli buffer was added onto bead-protein mixture and incubated at 95°C for 5 minutes. After incubation proteins were collected and beads were discarded by the aid of magnetic separator.

2.1.4.4. Western Blot

For Western blot analysis, 50 µg of total protein was loaded onto 12 % of SDS-PAGE gel. Before samples were loaded onto the gel, they were incubated with 6x Laemmli buffer at 95°C for 5 minutes for denaturation. Proteins on the gel were transferred onto PVDF membrane (Roche, Switzerland) through wet transfer technique for 65 minutes at constant 100 V voltage. The membrane was blocked with 5 % skim milk for in 0.05 % TBS-T (Tris buffered saline-tween) for 1 hour at room temperature for an anti-biotin antibody (Abcam, USA, ab53494). After blocking, the anti-biotin antibody was used at 1:200 dilution in 5% skim milk in 0.05 % TBS-T. The membrane was incubated with the anti-biotin antibody for 1 hour at room temperature. Three PBS washes was done and then the secondary antibody, goat anti-rabbit-HRP (Santa Cruz

Biotechnology, USA) was added onto membrane at 1:2500 dilution in 5% skim-milk in 0.05 % TBS-T. After 1hour of the secondary antibody incubation, the membrane was washed with PBS three times. For visualization, the enhanced chemiluminescence (ECL) kit (Clarity Western ECL Substrate, Bio-Rad, USA, 1708280) was used. The membrane incubated with ECL substrate for 5 minutes in the dark and visualization was carried out with ChemiDoc^{TM} MP system (Bio-Rad, USA). Images were analyzed with Image Lab 5.1 (BioRad, USA).

In addition to the anti-biotin antibody, S-14 Ypel antibody was also used at 1:100 dilution in 5% skim milk containing 0.05 % TBS-T. As the secondary antibody, the same secondary antibody mentioned above was used with same dilution.

2.1.4.4. Mass Spectrometry analysis for protein identification

Proteins on the streptavidin magnetic beads were sent to Koç University, İstanbul, Turkey for mass spectrometry analysis. Samples on beads subjected to trypsin digestions. Using peptide cleanup C18 StageTips, peptides were then isolated and concentration were measured. For peptide analysis, LC-MS/MS on Orbitap based mass spectrometer was used. Identification of proteins was done by using Proteome Discover 1.4 software.

2.2. Establishment of yeast model

2.2.1. Cell strains and maintenance

We used BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) strain as the wild type (WT) strain and also its isogenic knockout mutant strain (*moh1*Δ) in this study. Both of these strains were purchased from Dharmacon, UK.

Cell strains were grown in yeast extract-peptone-dextrose (YPD) medium containing 1% yeast extract (Sigma, Germany, 70161), 2% dextrose (Sigma, Germany, 49159) and 2% peptone (Sigma, Germany, 912489). For the selection of strains, we used a synthetic complex medium without uracil (SC-URA) that contains 0.67% yeast nitrogen based without amino acids (Sigma, Germany, Y0626), 2% dextrose and 0.192% as well as a yeast synthetic drop-out medium supplements without uracil, YPD with 5-FOA (1 mg/ml 5-FOA (Zymo Research, USA, F9001). For spot tests, we added appropriate amounts sulfuric acid (Merck, Germany, 1.00713) and hydrogen peroxide (Sigma, Germany, H1009) into medium.

All liquid cultures were incubated at 30°C with reciprocal shaking (180 rpm). Solid cultures were also incubated at 30°C.

2.2.2. Cloning of URA3, MOH1 and YPEL2 into pBS-KS(-) vector with upstream and downstream homology arms

For changing *KanR* marker gene in *moh1*Δ-By4741 cell strain with *URA3* marker gene, *URA3* were cloned into pBS-KS(-) plasmid with homology arms. First of all, the upstream (UPS) and downstream (DNS) homology arms of URA3 were cloned into plasmid. UPS and DNS homology arm of URA3 constructs were prepared by polymerase chain reaction (PCR) in which *moh1*Δ-By4741 genomic DNA was used as the template. The UPS homology arm of URA3 were amplified with 500 nM URA3 UPS_XhoI_FP and URA3 UPS_NheI_REP primers by using Phusion High-Fidelity DNA Polymerase

(Thermo Scientific, USA, F530) with GC buffer and 200 μM of each dNTP. PCR were performed as follows: 2 minute initial denaturation at 98°C; followed by a 35 cycle with 30 second denaturation at 98°C, 30 second annealing at 60°C , 30 second extension at 72°C and 10 minutes final extension at 72°C; and infinite hold at 4°C. To amplify the DNS homology arm of URA3 and URA3 gene, the same PCR conditions were used with different primers: URA3 DNS_EcorI_FP and URA3 DNS_BamHI_REP for homology arm, and URA3_NheI_FP and URA3_EcoRI_REP for URA3 gene. After PCR, all PCR products were purified with QIAquick PCR Purification kit (Qiagen, Germany, 28106). The PCR purified UPS homology arm and pBS-KS(-) plasmid were digested with XhoI (New England Biolabs, USA, R0146) and NheI (New England Biolabs, USA, R0131) restriction enzymes. Ligation, transformation and plasmid isolation were done as described Section 2.1.2. The UPS homology arm of URA3 bearing pBS-KS(-) plasmid were used to clone the URA3 gene. For cloning of the PCR amplified the URA3 DNA as well as the URA3 plasmid were digested with NheI (New England Biolabs, USA, R0131) and EcoRI (New England Biolabs, USA, R0101). After ligation and transformation, plasmids were isolated and the UPS homology arm and the URA3 bearing pBS-KS(-) plasmid were used to clone the DNS homology arm of URA3. The PCR amplified DNS homology arm and the pBS-KS(-)_UPS homology arm_URA3 plasmid were double digested with EcoRI (New England Biolabs, USA, R0101) and BamHI (New England Biolabs, USA, R0136) restriction enzymes. Ligation, transformation and plasmid isolation were performed and plasmids bearing the UPS homology arm, URA3 and DNS homology arm were sent to sequencing to ensure sequence fidelity.

To clone the flag-MOH1 and flag-YPEL2 cDNAs with the UPS and DNS homology arm of MOH1 pBS-KS(-)_flag-YPEL2 [9] plasmid was used. The UPS and DNS homology arms were amplified with PCR. PCR conditions were same with PCR mentioned above. To amplify UPS homology arm of MOH1 UPS_XhoI_FP and UPS_NcoI_REP primers were used while for the

amplification of the DNS homology arm of MOH1, DNS_EcoRI_FP and DNS_BamHI_REP were used. For PCRs, the WT-BY4741 genomic DNA was used as template. Firstly, the UPS homology arm of MOH1 were cloned into pBS-KS(-)_flag-YPEL2 plasmid. For this purpose, both PCR product and plasmid were digested with XhoI (New England Biolabs, USA, R0146) and NcoI (New England Biolabs, USA, R0193) restriction enzymes. After ligation and transformation were performed, plasmid isolation was done and the pBS-KS(-)_flag-YPEL2 bearing the UPS homology arm of MOH1 plasmid were obtained. This plasmid was used to clone the DNS homology arm of MOH1. The Plasmid and DNS homology arm of MOH1were cut with EcoRI (New England Biolabs, USA, R0101) and BamHI (New England Biolabs, USA, R0136). After digestion, ligation, transformation and plasmid isolation were performed as described above. Through this process, pBS-KS(-) with the UPS homology arm, flag-YPEL2 and DNS homology arm was constructed.

To clone the flag-MOH1, the *MOH1* gene was amplified with XnmetN_MOH1_FP and MOH1_EpolyaB_REP primers by the same PCR conditions described above. The PCR amplified MOH1 and UPS and DNS homology arm in the pBS-KS(-)_flag-YPEL2 plasmid were digested with NheI (New England Biolabs, USA, R0131) and EcoRI (New England Biolabs, USA, R0101) restriction enzymes. Ligation, transformation and plasmid isolation were done. Plasmid was sequenced to be sure that sequences were correct.

2.2.3. Insertion of the *URA3*, flag-*MOH1* and flag-*YPEL1-5* genes into the yeast genome

To replace the KanMX4 marker with the URA3 marker in the *moh1*Δ-BY4741 strain by homologous recombination, PCR was performed with primers URA3 UPS_XhoI_FP and URA3 DNS_BamHI_REP and the pBS-KS(-)_UPS_URA3_DNS plasmid was used as template. PCR was performed as described above. PCR product, which contains the URA3 gene with the homology arms of KanR, was used for transformation into the *moh1*Δ-BY4741

strain. For transformation, single colony was selected from the *moh1*Δ-BY4741 strain solid culture and grown overnight at 30°C with shaking at 180 rpm in 5 ml YPD. This overnight culture was sub-cultured into 50 ml of fresh YPD at 1:100 dilution. Subculture was grown for 4-6 hours. OD_{600} was measured on the hour. When OD_{600} is ~0.5, cells were pelleted at 4000 rpm for 5 minutes and cell pellet washed with sterile distilled water. After washing, cells were re-suspended in 1 ml of 0.1 M LiAc solution and then pelleted at 12000 rpm for 10 seconds. Cell pellet re-suspended in 400 µl of 0.1 LiAc solution. Before cell harvesting, 2 mg/ml single stranded salmon sperm DNA (Sigma, Germany, D1626) were boiled at 98°C for 10 minutes and then placed on the ice at least 10 minutes. 240 µl of 50% (w/v) PEG (Sigma, Germany, 202444), 36 µl of 1 M Liac, 50 µl of 2 mg/ml single stranded salmon sperm DNA, 34 µl of PCR product and 50 µl of cell suspension were mixed in this order and vortexed 2 minutes. Transformation mix were incubated at 30°C for 30 minutes and then 42°C for 20 minutes. After incubation, transformation mixture was centrifuged at 7000 rpm for 15 minutes. Cell pellet were re-suspended in 400 µl of sterile distilled water and 200 µl of this cell suspension was plated onto SC-URA Agar plates for selection. Plates were incubated at 30°C for 2-3 days.

The Flag-MOH1 or the flag-YPEL2 cDNA was inserted into *moh1*Δ-BY4741 cells into the place of URA3 selection marker. For insertion, the flag-*MOH1* with homology arms and flag-*YPEL2* with homology arms were amplified by PCR in which pBS-KS(-)_UPS_flagYPEL2_DNS or pBS-KS(-)_UPS_flagMOH1_DNS was used as the template. PCR conditions were the same with PCR as described in Section 2.2.2. We used UPS_XhoI_FP and UPS_BamHI_REP primers. Transformation of PCR products into yeast strains was performed as described above. In this transformation instead of purchased *moh1*Δ-BY4741 strain, *moh1*Δ-BY4741 strain with URA3 marker was used. After transformation, we employed counter selection approach to select transformants. For this purpose, cells were seeded onto YPD-Agar plates and

incubated at 30°C for 24 hours. After 24-hour-incubation, replica plating was performed onto YPD-5-FOA plates and these plates were incubated at 30°C for

2-3 days. Because the protein product of *URA3* gene is toxic when 5-FOA is present [16], transformation was accepted as successful if colonies grow on YPD-5-FOA agar plates.

2.2.4. Genomic DNA isolation

Single colony was selected into 5 ml of YPD from strain from which genomic DNA (gDNA) is isolated. Cells were grown overnight at 30°C with shaking at 180 rpm. Overnight culture was centrifuged at 4000 rpm for 5 minutes. Cell pellet was dissolved into yeast lysis buffer (10 mM Tris, pH=8.0; 2% Triton X-100, 1% SDS, 100 mM NaCl, 1mM EDTA). Cell suspension was taken into an Eppendorf tube. 200 µl of 25:24:1 phenol:chloroform:isoamyl alcohol (Amresco, Canada, AIK169) and 300 mg of acid-washed glass beads (Sigma, Germany, G9268) were added onto cell suspension. This mixture was vortexed for 4 minutes. After vortexing, 200 µl of Tris-EDTA (TE) buffer was added into the mixture and centrifuged at 6500 rpm for 2 minutes. Aqueous phase was transferred into a fresh 2 ml eppendorf tube and mixed with 1 ml of 100 % EtOH by inversion. After EtOH addition, the mixture was centrifuged at 12000 rpm for 2 minutes. Supernatant was discarded, pellet was re-suspended in 400 µl of TE buffer. Then, 1 ml of 100% EtOH and 14 µl of 3 M ammonium acetate were added. Genomic DNA was pelleted at 12000 rpm for 2 minutes. Supernatant was discarded and pellet was left for air dry approximately for 15 minutes. After air dry, DNA was re-suspended in 100 µl of Milli-Q water.

2.2.5. Control for transformation of URA3, flag-MOH1 and flag-YPEL2

To check whether or not intended gene insertions into the yeast genome were successful, PCR was performed. We used two different primer sets in different combinations to check whether URA3 was inserted in the place of KanR gene.

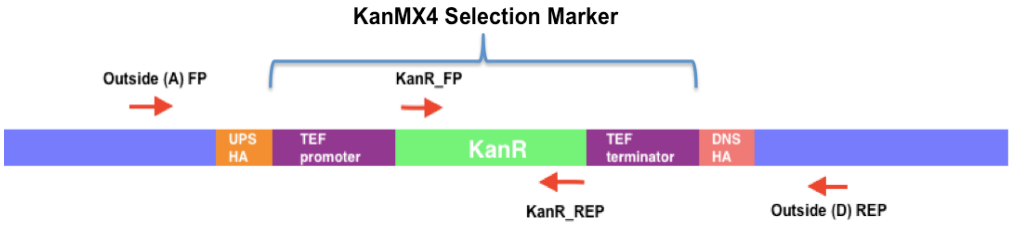

Figure 2. Schematic representation of the KanMX4 selection marker and the placement of primers used for PCR in the *moh1*ΔBY4741 genome

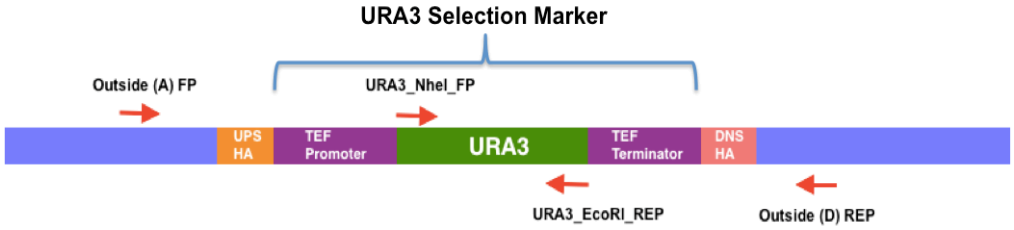

Figure 3. Schematic representation of the URA3 selection marker and the placement of primers used for PCR in the *moh1*ΔBY4741 genome

PCR reaction was performed by using Taq DNA Polymerase (5 U/µl) (Thermo Scientific, USA, EP0402) with Taq buffer with KCl, 200 µM final concentration of each dNTP, 2 mM final concentration of $MgCl_2$ and 500 nm final concentration of each primer. PCR were performed as follows: 3 minutes of an initial denaturation at 98°C; 30 cycles of 30 second denaturation at 98°C, 30 second annealing at 60°C, 30 second extension at 72°C; 10 minute final extension at 72°C and the infinite hold at 4°C. To control URA3 insertion, four different primer combinations were used: (1) URA3_NheI_FP & URA3_EcoRI_REP, (2) URA3_NheI_FP and Outside (D) REP, (3) KanR_FP & KanR_REP and (4) KanR_FP and outside (D) REP.

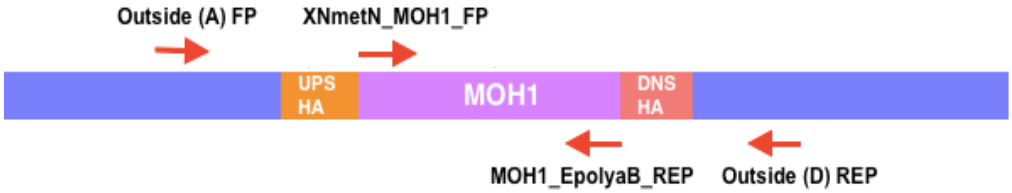

Figure 4. Schematic representation of *MOH1* and the placement of primers used for PCR in the WT-BY4741 genome

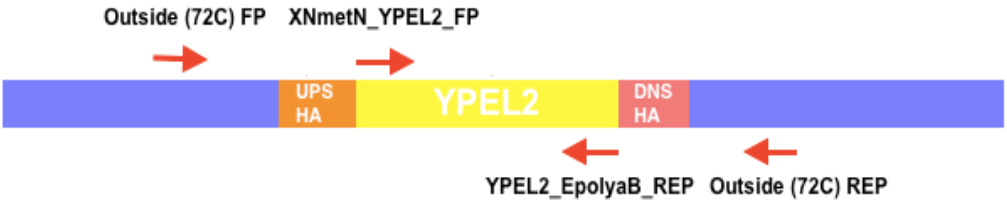

Figure 5. Schematic representation of *YPEL2* and the placement of primers used for PCR in the WT-BY4741 genome

To control the replacement of URA3 selection marker with *MOH1*, PCR was performed with conditions described above. In this PCR, 5 different primer combinations were used: (1) XNmetN_MOH1_FP and MOH1_EpolyaB_REP, (2) Outside (A) FP and MOH1_EpolyaB_REP, (3) outside (A) FP and outside (D) REP, (4) URA3_NheI_FP & URA3_EcoRI_REP, and (5) URA3_NheI_FP and Outside (D) REP.

To control the YPEL2 cDNA insertion into the genome, PCRs were performed with five different primer combinations: (1) XNmetN_YPEL2_FP and YPEL2_EpolyaB_REP, (2) XnmetN_YPEL2_FP and outside (72C) REP, (3) outside (72C) FP and outside (72C) REP, (4) URA3_NheI_FP & URA3_EcoRI_REP, and (5) URA3_NheI_FP and Outside (D) REP. For primer combination 1, 2 and 3, two-step reaction performed with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA, F530) was as

follows: 2 minute initial denaturation at 98°C; 35 cycles of 30 second denaturation at 98°C, 1 minute annealing and extension at 72°C; 10 minute final extension at 72°C; and the infinite hold at 4°C.

After PCR, 5 µl of 20 µl PCR products were run onto 1% agarose gel at 100 V.

2.2.6. Growth under nutrient stress (Growth in water)

Single colonies of the WT-BY4741, moh1Δ-BY4741, moh1Δ-BY4741+flag-MOH1 and moh1Δ-BY4741+flag-YPEL2 strains were grown in 5 ml of YPD overnight at 30°C with shaking at 180 rpm. Cells were counted under light microscope and $50x10^6$ cells were sub-cultured into 20 ml of YPD. Cells were grown at 30°C with shaking at 180 rpm for one week. After one week of incubation, cells were pelleted at 4000g for 5 minutes and washed with 1 M of sorbitol (D-Sorbitol, Sigma, Germany, S1876) twice and with sterile distilled water once. Cells were taken into 20 ml of sterile distilled water and incubated at 30°C with shaking at 180 rpm. Aliquots from these water cultures were taken every second days and 4 µl of 10-fold serial dilutions from each aliquot were spotted onto YPD-agar plates. Cells were incubated at 30°C for 2 days. Visualization was done with ChemiDoc™ MP system (Bio-Rad, USA).

2.2.7. Stress induction and spot tests

For stress induction, we used YPD-Agar medium. After autoclaving and cooling to ~55°C, we added 0.22% H_2SO_4 or 3.25 mM H_2O_2 as stress inducer into YPD-agar medium. Medium was then poured into petri dishes to form "stress agar plates".

For spot tests, single colonies were selected from the WT-BY4741, moh1Δ-BY4741, moh1Δ-BY4741+flag-MOH1 and moh1Δ-BY4741+flag-YPEL2 strains. Single colonies were grown in 5 ml of YPD overnight at 30°C with shaking at 180 rpm. After overnight growth, subcultures were taken from each

culture into 20 ml of YPD at 1:100 dilution. Cells were grown at 30°C with shaking at 180 rpm at least 4 hours until cells reached to a log phase growth (i.e. OD_{600} should be ~0.5). Then, 1 ml of aliquots were taken from each culture and cells were counted on Neubaurer haemocytometer. For spot test, starting cell concentration was $2.5x10^6$ cells/ml. 4 µl of 10-fold serial dilutions from these cultures were spotted onto YPD-Agar plates with stress inducers. After spotting were completed, plates were left to dry at least 1 hour. Plates were then incubated at 30°C for 40 hours. After incubation, images of spots were taken with ChemiDoc™ MP system (Bio-Rad, USA).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Proximity-dependent biotin labelling (BioID)

The proximity-dependent biotin labelling system (BioID) is used to identify proteins that interact with a set of protein partners in a spatiotemporal manner to execute their functions. In BioID, "prey" protein is expressed as a fused protein to the promiscuous mutant *E.coli* biotin ligase (BirA*) which tags proteins with biotin. BioID-mediated biotin tagging occurs at three protein populations: (i) directly interacting proteins, (ii) indirectly interacting proteins and (iii) vicinal proteins. These tagged proteins are captured with streptavidin magnetic beads and their identities are determined with a mass spectrometry analysis (Figure 6).

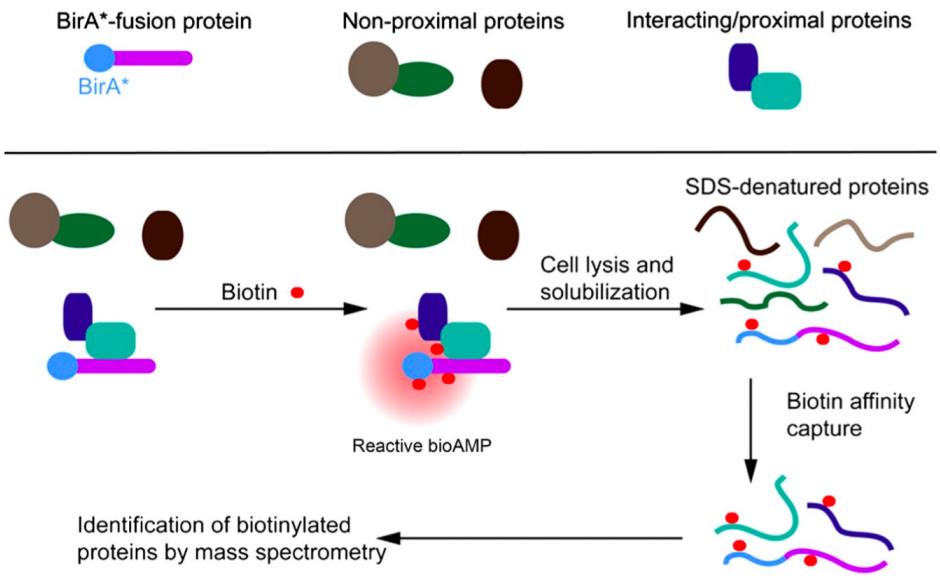

Figure 6. Schematic representation of the working principle of BioID [17]

Since proteins exert their functions in a dynamically changing environment of interacting protein partners, we anticipated that the identification of protein partners critical for YPEL2 functions could be an important prelude for the delineation of YPEL functions in mammalian cells. To accomplish this, we generated an expression vector bearing a cDNA that encodes for BirA*(R118G)HA-FlagYPEL2 fusion protein. To ensure that vector synthesizes a functional protein, we carried out immunocytochemistry and western blot analysis in transfected COS7 cells.

COS7 cells were transiently transfected with pcDNA3.1 vector bearing BirA*(R118G)HA genetically fused to none as control (Figure B.2) or the flag-YPEL2 cDNA. ICC was performed with both the flag (Figure 7.1b and 7.2b) and biotin (Figure 7.1c and 7.2c) antibodies. The Flag antibody was used to detect specifically YPEL2 and the biotin antibody was used to detect biotinylated proteins. To stain nuclei, DAPI was used (Figure 7.a). To ensure that the synthesized fusion protein is functional, we also treated cells without or with Biotin (50 µM).

3.1.1. Immunocytochemistry (ICC)

We observed that in both biotin plus and biotin minus cells, BirA*-Ypel2 protein was synthesized as assessed with the flag antibody. Previously we observed that endogenously synthesized YPEL2 or overexpressed Flag-YPEL2 is localized in and periphery of the nucleus [9]. Similarly, BirA*-Ypel2 protein was also detected in and around nuclei of the transfected cells (Figure 7) This indicates that intracellular localization of Ypel2 did not change when fused with BirA* protein. Moreover, we detected biotin antibody staining only in cells treated with Biotin (Biotin+) that shows an overlapping staining observed with the Flag antibody. This indicates that exogenously added biotin is required for the detection of biotinylated proteins. This finding also allowed us to confidently eliminate the possibility of detecting endogenously biotinylated proteins.

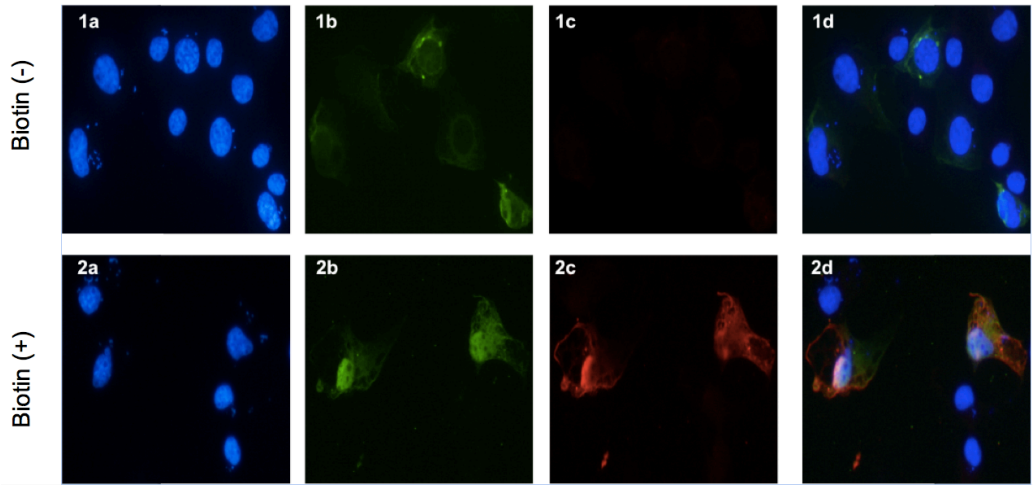

Figure 7. Immunocytochemistry of transient transfected COS7 cells with Flag and Biotin Antibody. COS7 cells were seeded on coverslips in 12-well cell culture plates and grown for 48 hours. Cells were then transiently transfected with pcDNA3.1 bearing the BirA*(R118G)HA-flag-YPEL2 cDNA. 20 hour after transfection, cells were treated without (Biotin -) or with 50 µM biotin and 1 mM ATP. 14 hours after biotin addition, cells were fixed with 3.7% formaldehyde and permeabilized with 0.4% Triton-X. Blocking was done with 10% bovine serum albumin (BSA) in PBS for 1 hour. After blocking, cells were incubated with the a Biotin antibody (1:100 in 3% BSA-PBS) for two hours and flag-M2 antibody (1:250 in 3% BSA-PBS) for two hours, sequentially. After primary antibody incubation, cells were incubated with Alexa Fluor® 647 conjugated goat anti-rabbit secondary antibody (red channel emission; 1:1000 in 3% BSA-PBS) for the biotin antibody for 30 minutes and an Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (green channel emission; 1:1000 in 3% BSA-PBS) for the Flag-M2 antibody for 30 minutes, sequentially. Nuclei was detected through staining with DAPI (blue channel emission). a. DAPI staining, b. Flag-M2 antibody, c. Biotin antibody, d. Merge (Overlapping images taken by different emission channels.) Similar results were obtained with two independent experiments.

3.1.2. Western Blot

Our ICC experiment results suggest that BirA*-Ypel2 protein is synthesized in transiently transfected COS7 cells and could be functional to biotinylate proteins. To assess this further, we carried out western blot analysis using cells transfected without or with the expression vector bearing BirA*(R118G)HA-flag-YPEL2 cDNA in the absence or presence of Biotin as described in the

section of 3.1.1. Cells extracts were then subjected to precipitation using streptavidin magnetic beads. Precipitated proteins were separated with SDS-PAGE and the gel was subjected to WB analysis using the biotin antibody (Figure 8A). Also, for further confirmation of WB results, the Flag-Ypel2-BirA* fusion protein was detected with S-14 Ypel2 antibody (Figure 8B).

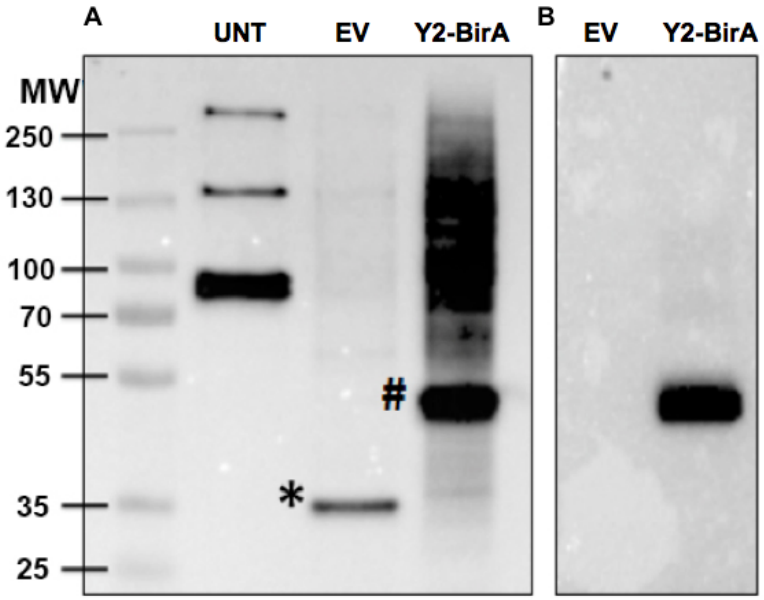

Figure 8. Western Blot Analysis of transiently transfected COS7 cells. Cells grown for 48 hours in 10-cm dish were transfected with pcDNA3.1 MCS BirA*(R118G)HA empty vector (EV) or pcDNA3.1 MCS BirA*(R118G)HA-flag-YPEL2 (Y2-BirA). We also used cells were not transfected as control (UNT). 20 hours after transfection, 50 µM biotin and 1 mM ATP were added onto cells. Cells were maintained an additional 14 hours and then subjected to protein isolation. 3 mg of protein for each sample was incubated with streptavidin conjugated magnetic beads to precipitate biotinylated proteins. Beads were collected by the aid of magnetic separator and proteins were separated from beads with Laemmli buffer and heat. 50 µg of protein from each sample was then loaded onto 12% SDS-PAGE. A. Biotin antibody was used to detect precipitated biotin-tagged proteins. B. A YPEL antibody was used to detect precipitated biotin-tagged Ypel protein. MW: Molecular weight marker. Similar results were obtained from two independent experiments. *: indicates endogenously self-biotinylated BirA*, 35 kDa #: indicates the self-biotinylated flag-Ypel2-BirA* fusion protein, 49 kDa.

Molecular weight of the BirA* protein is 35 kDa protein and of the flag-Ypel2 is 15 kDa. When the flag-Ypel2-BirA protein was synthesized as fusion, the expected molecular mass is ~50 kDa. As seen in Figure A, there were no protein with expected molecular weight (~50 kDa) in precipitated proteins isolated from untransfected or empty vector transfected COS7 cells. When, precipitated proteins isolated from the BirA*(R118G)HA-flag-YPEL2 cDNA bearing pcDNA3.1 transfected COS7 cells were examined with the biotin or S-14 Ypel antibody, a band that correspond to the expected size of the proteins is observed. Moreover, we also observed many biotinylated proteins with varying molecular sizes only in cells synthesizing the flag-Ypel2-BirA* fusion protein. These results showed that transfected COS7 cells synthesize a functional flag-Ypel2-BirA protein.

Biotinylation capacity of flag-Ypel2-BirA fusion protein was also assessed with WB analyses. In figure 8A., biotinylated proteins were detected by the biotin antibody. When untransfected and EV transfected cells were examined, there were a few bands in un-transfected cells, one of which corresponds to the self-biotinylated BirA protein. On the other hand, biotinylated proteins were seen as a smear in cells synthesizing the flag-Ypel2-BirA protein. These results indicate that the flag-Ypel2-BirA fusion is a functional protein that could be used for the identification of putative interacting protein partners of YPEL2.

3.1.3. Mass Spectrometry Analysis

After we were certain that BioID system was working, samples were subjected to mass spectrometry analysis at the Koç University. Mass spectrometry analyses revealed a number of proteins from three different samples: (1) Untransfected COS7 cells (UNT group), (2) pcDNA3.1 MCS BirA*(R118G)HA empty vector transfected COS7 cells (EV group) and (3) flag-YPEL2 bearing pcDNA3.1 MCS BirA*(R118G)HA transfected COS7 (YPEL2 group) cells. The reason for using biotin added but untransfected or

empty vector transfected cells was to increase the likelihood of specific protein detection labelled with the flag-Ypel2-BirA fusion protein. When two biological replicas of mass spectrometry analysis results were combined, there are 110 proteins which were revealed from COS7 cells transfected with the flag-Ypel2-BirA cDNA bearing pcDNA3.1 MCS BirA*(R118G)HA transfected COS7 cells (Figure 9.a). To identify proteins specifically labelled biotinylated proteins, data from each sample were compared and the common proteins from each sample (UNT group, EV group and YPEL2 group) were defined. Proteins that were not shared with UNT group and EV group were defined as the YPEL2 group specific proteins. These YPEL2 group specific proteins were then analyzed with a gene-annotation enrichment tool, DAVID (Figure 9.b). According to the GOTERM-Biological Process analysis, putative interacting protein partners of Ypel2 were found in clusters related to RNA processing, ribosome biogenesis, cell cycle, protein folding, microtubule-based processes and regulation of translation and transcription.

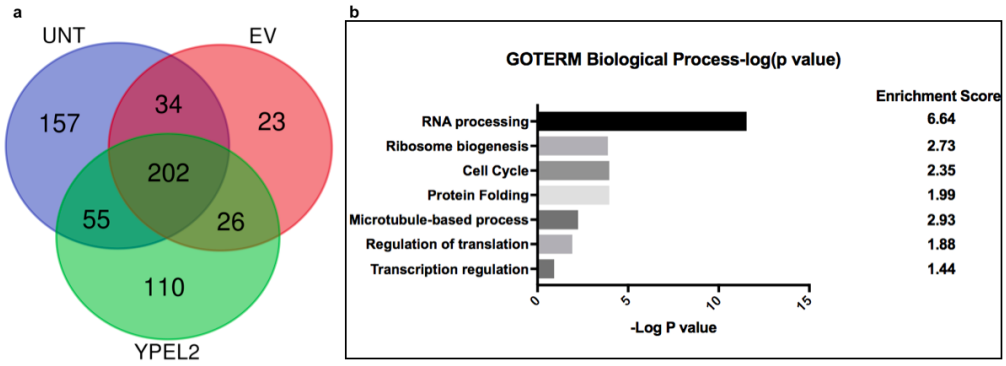

Figure 9. Mass Spectrometry Results a. Venn diagram that represents number of proteins revealed from each sample as a result of the mass spectrometry analysis. Results represent the combination of two biological replicates of mass spectrometry analyses. UNT: Proteins from untransfected COS7 cells, EV: Proteins from pcDNA3.1 MCS BirA*(R118G)HA empty vector transfected COS7 cells, YPEL2: Proteins from the flag-YPEL2 cDNA bearing pcDNA3.1 MCS BirA*(R118G)HA transfected COS7 cells b. Graphical representation of DAVID GOTERM-Biological Process analysis of YPEL2 group specific proteins revealed from mass spectrometry analysis.

Since BioID system is an exploratory in nature, interactions between YPEL2 and a protein detected with mass spectrometry must be confirmed with other approaches. Through BioID, we were able to detect possible interacting partners of Ypel2. Using co-immunoprecipitation, co-localization and mammalian-two-hybrid approaches, we will assess the validity of this possibility.

Nevertheless, our results suggest that the putative protein partners of Ypel2 in COS7 cells include proteins that involve in stress granules which are dense aggregates of the cytosol composed of proteins and RNAs that appear in response to stress.

3.2. Establishment of a yeast model

The YPEL gene family members have highly conserved amino acid sequences. Due to this high homology, assessing the functional feature for any Ypel protein has proved to be difficult using approaches to decrease the synthesis of proteins by siRNA or augment the synthesis by overexpression, the latter which leads to immediate cell death. All of these problems necessitated the use of a system that allows us studying the function of individual YPEL family members, especially the estrogen responsive gene product YPEL2. For this reason, we chose yeast as the model organism, because yeast have only one homolog of YPEL: MOH1.

For deciphering the function of a gene and its protein product(s), the deletion of or expression suppression of the gene are one of the most widely used approaches. In yeast, homologous recombination provides an easy and a powerful tool for gene deletion, particularly PCR-mediated targeted gene deletion/replacement [18]. Homologous recombination in yeast occurs between the identical or mostly identical sequences [19]. Any DNA fragment can be amplified with homology arms, which are identical with upstream and

downstream sequences of the gene targeted to be deleted/replaced with DNA fragment generated with PCR approaches. When DNA fragment with these homology arms is transformed in yeast cells, homologous recombination occurs and gene of interest is replaced with this DNA fragment (Figure 10) [18].

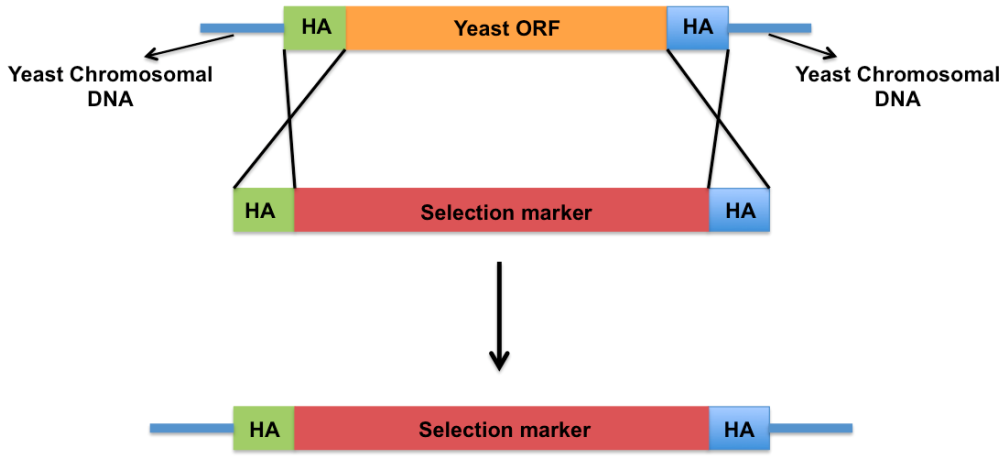

Figure 10. Schematic representation of homologous recombination (HA: Homology arm)

By comparing WT strains and isogenic mutants of these WTs, information about the function of the deleted gene and its protein product can be obtained. Based on this and to establish a yeast model system, in order to initially characterize the function of *MOH1* protein product, we used the WT-BY4741 and *moh1*Δ-BY4741 yeast strains. These strains are MATa *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0.* Using these strains, we also generated two additional strains. One of them had the flag tagged *MOH1* and the other had the flag tagged *YPEL2* in the genome at the locus of *MOH1* to facilitate the detection with ICC and/or WB, immunoprecipitation of the tagged proteins. The flag-MOH1 or the flag-YPEL2 cDNA were inserted into *moh1*Δ-BY4741 genome through PCR-mediated gene replacement strategy. To carry out this, we

initially used a counter selection strategy with 5-FOA. This was necessary, because there was no way for selection of transformed cells if we directly replaced KanMX4 marker with flag tagged genes by homologous recombination. As mentioned in Section 2.2.3, 5-FOA induces toxicity when binds to the protein product of *URA3* [16]. To insert flag tagged genes, firstly the *URA3* gene was replaced with *KanR* as the KanMX4 selection marker and then flag-tagged genes were inserted into the *moh1*Δ-BY4741 genome with the URA3 selection marker.

For homologous recombination to occur, the engineered DNA fragment must carry, homology arms. For homologous recombination, sufficient length of homology arms is suggested to be 40-60 nucleotides. However, extending length of homology arms up to 200 nucleotides increases homologous recombination efficiency greatly [18]. Our attempts for homologous recombination with short homology arms (45 nucleotides) were not successful. Because of this, we decided to use long homology arms which are longer than 200 nucleotides. For this purpose, before insertion into genome, the *URA3*, flag-*MOH1* and flag-*YPEL2* ORF with 5' and 3' long homology arms were cloned into pBS-KS(-) vector.

3.2.1. Controlling the insertion of URA3, flag-MOH1 and flag-YPEL2 into the yeast genome

To study the function of *MOH1* and *YPEL2* in yeast, the flag tagged ORFs of each gene were inserted into the yeast genome. For this purpose, firstly, the *URA3* ORF were inserted into the *moh1*Δ-By4741 genome by replaced with *KanR* as the KanMX4 marker. Through this replacement, the URA3 selection marker cassette was generated in the *moh1*Δ-By4741 genome. After transformation into cells, colonies were screened to obtain successful transformed cells. For this purpose, the deletion of the *KanR* gene in the new strain was confirmed by PCR with different primer sets. To understand that the

KanR gene was successfully deleted, two different sets of primers were used (Figure 2). If the replacement successfully occurred, there should be no amplification by PCR with primers specific to *KanR*. To control the replacement success, we also used two distinct primer sets: one set for *URA3* and one set for genome to ensure that *URA3* was not inserted someplace else in the genome.

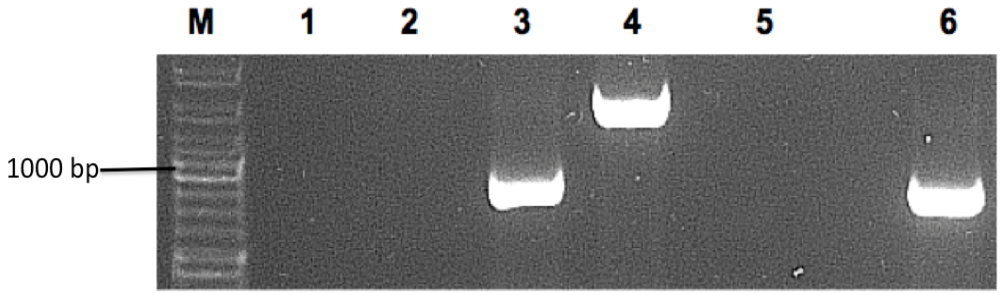

Figure 11. PCR products to control genomic insertion of URA3. M: DNA marker; 1: PCR with KanR FP and KanR REP; 2: PCR with KanR FP and Outside (D) REP; 3: PCR with URA3_NheI_FP and URA3_EcoRI_REP, 823 bp; 4: PCR with URA3_NheI_FP and Outside (D) REP; 5: No template control; 6: Positive control (URA3_NheI_FP and URA3_EcoRI_REP primers, p426GPD plasmid as template), 823 bp.

As seen in figure 11, we detected no PCR product in the 1st and 2nd lane. This showed that, *KanR* were successfully deleted from the genome. In the 3rd and 4th lane, there were PCR products with expected molecular sizes. The Band in 3rd lane confirms the successful insertion of *URA3* and the band in 4th lane confirmed that *URA3* was inserted into the expected locus in the genome.

After controls for *URA3* were carried out, the flag-*MOH1* cDNA or the flag-*YPEL2* cDNA was inserted into the *moh1*Δ-By4741 strain carrying the URA3 selection marker. For the insertion of these two ORFs, the URA3 marker was completely deleted from the genome. Colonies were then screened for

successful transformants. To control colonies with PCR, the same approach was used as in *URA3* replacement. In figure 12, the flag-MOH1 ORF and in figure 13, the flag-YPEL2 ORF insertion was examined.

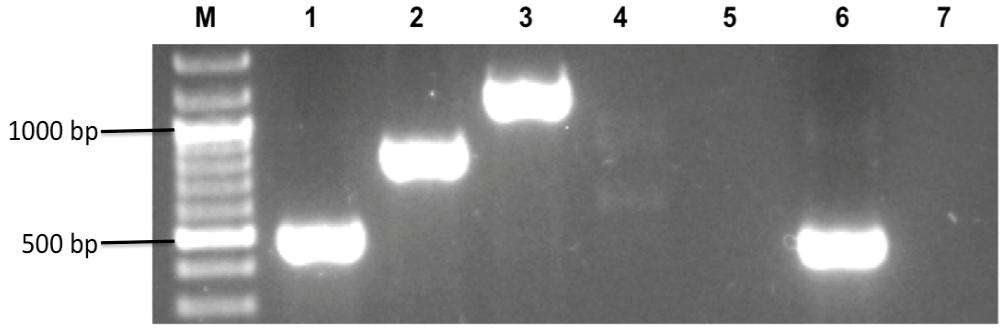

Figure 12. PCR products to control genomic insertion of flag-MOH1. M: DNA marker; 1: XNmetN_MOH1_FP and MOH1_EpolyaB_REP, 484 bp; 2: Outside (A) FP and MOH1_EpolyaB_REP, 836 bp; 3: outside (A) FP and outside (D) REP, 1149 bp; 4: URA3_NheI_FP & URA3_EcoRI_REP; 5: URA3_NheI_FP and Outside (D) REP; 6: Positive control (XNmetN_MOH1_FP and MOH1_EpolyaB_REP primers, WT-BY4741 gDNA as template), 484 bp; 7: No template control

Lanes 1, 2 and 3 showed the successful insertion of the flag-*MOH1* ORF at the expected locus in the *moh1*Δ-By4741 genome. In the 4th and 5th lanes, there was no PCR product, which was an expected result for a successful deletion. Primers used in the flag-*MOH1* ORF insertion control PCRs were represented in Figure 3 and Figure 4.

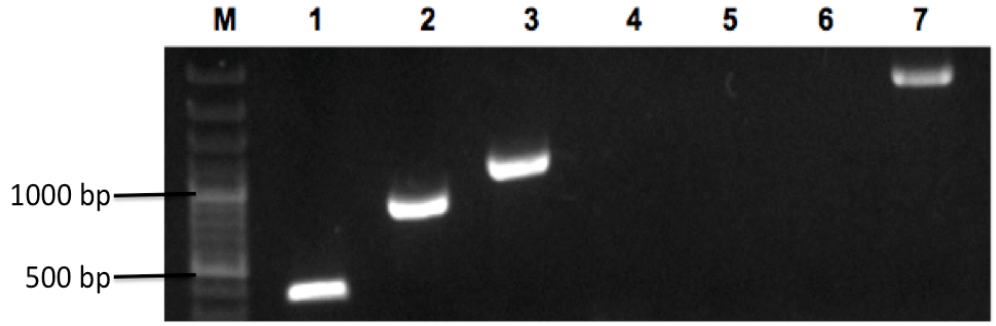

Figure 13. PCR products to control genomic insertion of flag-MOH1. M: DNA marker; 1: XNmetN_YPEL2_FP and YPEL2_EpolyaB_REP, 425 bp; 2: XnmetN_YPEL2_FP and outside (72C) REP, 744 bp; 3: outside (72C) FP and outside (72C) REP, 1154 bp; 4: URA3_NheI_FP & URA3_EcoRI_REP; 5: URA3_NheI_FP and Outside (D) REP; 6: No template control; 7: Positive control (outside (72C) FP and outside (72C) REP; *moh1*Δ-BY4741 gDNA as template)

As mentioned above, the same controls for insertion of the flag-*YPEL2* ORF were done with YPEL2 specific primers, genome specific primers and URA3 specific primers (Figure 3 and Figure 5). PCR products in 1st, 2nd and 3rd lanes indicated the successful insertion of the flag-YPEL2 at the genomic locus; the absence of PCR products in 4th and 5th lanes showed the complete deletion of the URA3 selection marker (Figure 13).

According to these results, we confidentially state that the flag-*MOH1* or the flag-*YPEL2* ORF was successfully inserted into the *moh1*Δ-By4741 genome in the place of the URA3 selection marker.

3.2.2. Growth under nutrient stress (Growth in water)

Ashrafi et. al. showed that Moh1 is an N-myristoylated protein and its deletion leads to decrease in cell forming unit (CFU) when mutants were grown in water up to 25 days [12]. To understand the role of MOH1 and its protein product in yeast, firstly we decided to confirm this studies results. For this purpose, growth assay in water were performed as mentioned in Section 2.2.6.

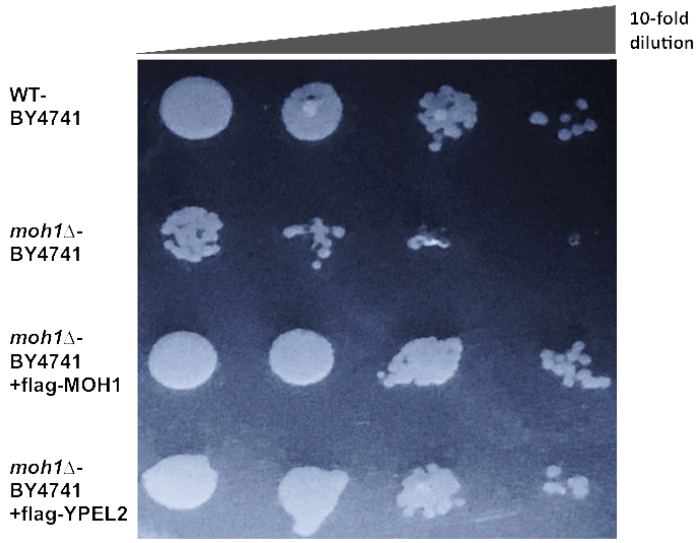

Figure 14. Spot test assay for growth in water at 14th day. 50x10^6 cells from overnight culture of each strain were sub-cultured in 20 ml YPD and cells were grown for one week at 30°C with gentle shaking. After 1 week, cells were washed with 1M sorbitol and sterile water and taken into 20 ml of sterile distilled water. Every second days, aliquots were taken from each culture. 4 µl of 10-fold serial dilutions from these aliquots were spotted onto YPD plates. Plates were incubated at 30°C for 2 days. Similar results were obtained from two independent experiments.

Results of growth assay in water were presented in figure 14. As figure shows, after 14-days-growth in water, the *moh1*Δ-BY4741 cell strain was more susceptible to cell death when compared with the WT-BY4741, the flag-MOH1 ORF or the flag-YPEL2 ORF inserted *moh1*Δ-BY4741 strains. Also, these results imply that the insertion of the flag-MOH1 or flag-YPEL2 ORF

into the *moh1*Δ-BY4741 genome provides similar effect as seen in WT-BY4741 cells. When cells were grown in water, they were exposed to nutrient depletion. For any cell type, nutrient depletion causes stress induction and thus cells respond to stress [20]. According to these results, it could be concluded that Moh1 has a role in stress response in yeast and in the absence of Moh1, the survival rate of cells decreases.

3.2.3. Stress induction and spot tests

To further investigate whether Moh1 has a role in stress response in yeast, we decided to use different stress inducer and observed cellular viability of different strains under these stress conditions. For this purpose, H_2SO_4 and H_2O_2 were used as the main stress inducers during these experiments (Figure 15). In addition to these stress inducers, other inducers like NaN_3 were used (Figure C.1 in appendix). The amount of stress inducers was determined through dose response experiments. For these experiments, stress agar plates were prepared by using different amount of stress inducer and spot tests were performed on these plates (Data shown in Appendix.). The minimal amount of stress inducer that causes maximal yeast response was then chosen as the experimental amount.

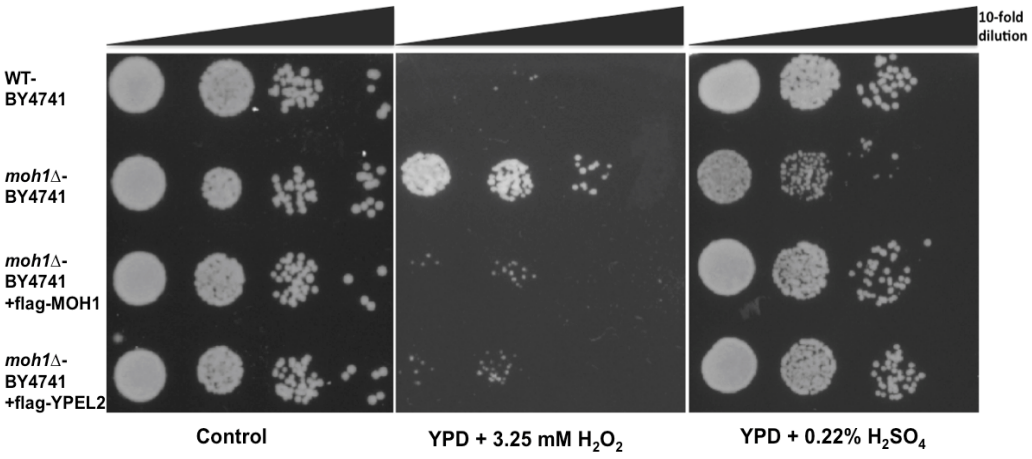

Figure 15. Spot tests for different stress inducers. Sub-cultures were taken from overnight cultures of each strain and grown until they reached to log phase. After that, cells were counted and $2.5x10^6$ cells were taken from each sample into 1 ml distilled water. 10-fold serial dilutions were done for each culture. 4 µl of these serial dilutions were spotted onto plates: Control YPD, YPD with 3.25 mM H_2O_2 and YPD with 0.22% H_2SO_4. Similar results were obtained from at least 3 independent experiments.

The treatment of cells with H_2O_2 leads cells to respond to it as an oxidative stress and H_2SO_4 leads cells to low acidic stress. In these two different stress conditions, *moh1*Δ-BY4741 cells produced different responses. Under the oxidative stress, *moh1*Δ-BY4741 cells were more resistant and survival rates of them were higher than the other strains we used. On the other hand, under the acidic stress, *moh1*Δ-BY4741 cells lost their resistance against the stress and the survival rate of WT- BY4741 cells was higher than the survival rate of *moh1*Δ-BY4741 cells.

According to these results, we concluded that Moh1 has a role in stress response of cells. Based on type of stress and response that cell gives, the effect of Moh1 on survival rate differed.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

In cells, proteins function within the context of dynamically changing network of interacting protein partners. Thus, protein-protein interactions reveal important information about the functional features and mechanistic aspects of the interested protein. BioID is an important system through which protein interactome of protein of interest can be defined. By using BioID system, we here attempted to define putative protein partners of Ypel2.

1. We conclude that Ypel2 fused with BirA* protein is functional. The cellular localization of Ypel2 did not change when it was produced as a fusion protein. By using this fusion protein, putative protein partners of Ypel2 can be biotinylated and defined with mass spectrometry analysis.
2. Results from mass spectrometry analyses reveal a number of proteins that could be putative protein partners of Ypel2. Confirmation of interactions between some of these proteins and Ypel2 could provide important information about functions of Ypel2 in cells in particular and Ypel proteins in general. For this purpose, by using different approaches like co-immunoprecipitation, we will confirm interacting partners of Ypel2. Through this, protein interactome network of Ypel2, and then functional features of Ypel2, can be better understood.
3. However, it must be noted that we over-expressed Ypel2-BirA* fusion protein in the COS7 cells. Over-expression could have led to the

detection of false-positive putative protein partners. In addition, we obtained results at 18 hour biotin labelling. This could lead to overrepresentation of the protein partners with long half-life and underestimatation of those with short half-life. It is therefore imperative that a time-course of labelling studies be carried out. This could involve a more robust labelling together with a controlled amount of synthesis of fusion proteins using, for example, an inducible expression system.

Studying Ypel2 in mammalian systems is difficult because of the high amino acid sequence homology between YPEL family proteins. In addition to this, over-synthesis of any YPEL family proteins leads to nuclear membrane disassembly, DNA leakage to cytoplasm and cell death. By necessity, we decided to use yeast as a model, in which only one YPEL family homolog, MOH1, is present. By using yeast as a model system, we conclude that:

1. The deletion of *MOH1* leads to a decrease in survival of cells when yeasts were grown in water for at least 14 days.
2. MOH1 has an important role in cellular stress response. Under different stress conditions (H_2O_2, H_2SO_4, NaN_3), *moh1*ΔBY4741 cells represented different patterns for survival. In the case of H_2SO_4 and NaN_3, WT-BY4741 cells were more resistant to stress; whereas under H_2O_2 stress, *moh1*ΔBY4741 cells were more resistant to stress condition than the wild-type cells.

To understand the function of Moh1,

1. Currently, we are performing western blot and qPCR analyses to see whether or not there is a change in protein and mRNA levels under different stress conditions.
2. We plan to study the importance of myristoylation of MOH1. Ashrafi *et. al.* showed that MOH1 is an N-myristoylated protein [12]. Firstly, we aim to confirm the myristoylation of MOH1. Then, we will check

whether myristoylation of MOH1 is important or not during stress responses.

We aimed to define protein partners of MOH1 by using TurboID system. The working principle of TurboID system is the same with BioID. However, in TurboID the biotin ligase has a higher catalytic activity when compared with BirA* alone or fused with a protein of interest [21]. For this purpose, we have started the cloning of the flag-MOH1 ORF into TurboID vectors.

REFERENCES

[1] L. Björnström and M. Sjöberg, "Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes," *Mol. Endocrinol.*, 2005.

[2] P. Yaşar, G. Ayaz, and M. Muyan, "Estradiol-Estrogen Receptor α Mediates the Expression of the CXXC5 Gene through the Estrogen Response Element-Dependent Signaling Pathway," *Sci. Rep.*, vol. 6, 2016.

[3] J. M. Hall, J. F. Couse, and K. S. Korach, "The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling," *Journal of Biological Chemistry*. 2001.

[4] J. Huang, X. Li, R. Hilf, R. a Bambara, and M. Muyan, "Molecular basis of therapeutic strategies for breast cancer.," *Curr. Drug Targets. Immune. Endocr. Metabol. Disord.*, 2005.

[5] H.-R. Lee, T.-H. Kim, and K.-C. Choi, "Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse," *Lab. Anim. Res.*, 2012.

[6] P. Ascenzi, A. Bocedi, and M. Marino, "Structure-function relationship of estrogen receptor α and β: Impact on human health," *Molecular Aspects of Medicine*. 2006.

[7] S. L. Nott *et al.*, "Genomic responses from the estrogen-responsive element-dependent signaling pathway mediated by estrogen receptor α are required to elicit cellular alterations," *J. Biol. Chem.*, 2009.

[8] P. Vrtačnik, B. Ostanek, S. Mencej-Bedrač, and J. Marc, "The many faces of estrogen signaling," *Biochemia Medica*. 2014.

[9] G. Gupur, “Cloning and Initial Protein Characterization of an estrogen responsive gene: YPEL2,” Middle East Technical University, 2014.

[10] K. Hosono, T. Sasaki, S. Minoshima, and N. Shimizu, “Identification and characterization of a novel gene family YPEL in a wide spectrum of eukaryotic species,” *Gene*, 2004.

[11] X. Robert and P. Gouet, “Deciphering key features in protein structures with the new ENDscript server,” *Nucleic Acids Res.*, 2014.

[12] K. Ashrafi, T. A. Farazi, and J. I. Gordon, “A role for Saccharomyces cerevisiae fatty acid activation protein 4 in regulating protein N-myristoylation during entry into stationary phase,” *J. Biol. Chem.*, 1998.

[13] D. J. Erasmus, G. K. Van Der Merwe, and H. J. J. Van Vuuren, “Genome-wide expression analyses: Metabolic adaptation of Saccharomyces cerevisiae to high sugar stress,” *FEMS Yeast Res.*, 2003.

[14] M. J. Martinez *et al.*, “Genomic analysis of stationary-phase and exit in Saccharomyces cerevisiae: gene expression and identification of novel essential genes.,” *Mol. Biol. Cell*, 2004.

[15] J. Y. Lee, D. Y. Jun, J. E. Park, G. H. Kwon, J. S. Kim, and Y. H. Kim, “Pro-apoptotic role of the human YPEL5 gene identified by functional complementation of a yeast moh1Δ mutation,” *J. Microbiol. Biotechnol.*, 2017.

[16] J. D. Boeke, F. La Croute, and G. R. Fink, “A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance,” *MGG Mol. Gen. Genet.*, 1984.

[17] K. J. Roux, D. I. Kim, M. Raida, and B. Burke, “A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells,” *J. Cell Biol.*, 2012.

[18] M. Gray and S. M. Honigberg, “Effect of chromosomal locus, GC content and length of homology on PCR-mediated targeted gene replacement in Saccharomyces.,” *Nucleic Acids Res.*, 2001.

[19] C. L. Inc and U. S. E. Only, “Yeast Protocols Handbook,” *Yeast*, 2008.

[20] J. R. Broach, “Nutritional control of growth and development in yeast,” *Genetics*. 2012.

[21] T. C. Branon *et al.*, “Directed evolution of TurboID for efficient proximity labeling in living cells and organisms,” *bioRxiv*, 2017.

APPENDIX A

PRIMERS

Table A.1. List of primers used in this study

Primer Name	Sequence (5'-3')
URA3 UPS_XhoI_FP	CGCATCTCGAGCATTTGCATCCATACATTTTGATGGCCGC
URA3 UPS_NheI_REP	CGCATGGCTAGCGGTTGTTTATGTTCGGATGTGATGTGAGAACTG
URA3 DNS_EcoRI_FP	CGCATGAATTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAG
URA3 DNS_BamHI_REP	CGCATGGGATCCACATACGATTGACGCATGATATTA
URA3_NheI_FP	CGCATGCTAGCATGTCGAAAGCTACATATAAGGAACGTGCTGC
URA3_EcoRI_REP	CGCATGGAATTCGTTTTGCTGGCCGCATCTTCTC
UPS_XhoI_FP	CGCATCTCGAGGACAGAACTCTGTCCTACTTTATC
UPS_NcoI_REP	CGCATGCCATGGTTTCTTCTACAGTAAGATAAGCTTCT
DNS_EcoRI_FP	CGCATGAATTCTGATGTCTTCCTTTGTCTGCTATCTAGCACCTCT
DNS_BamHI_REP	CGCATGGGATCCGGCTACTTGAAAACAACTGGAC
XNmetN_YPEL2_FP	CGCATCTCGAGACCATGGCTAGCGTGAAGATGACAAGATCGAAG

Table A.1. (continued)

YPEL2_EpolyaB_REP	CGCATGGATCCTTTATTAGAATTCGTCC CAGCCATTGTC
XnmetN_MOH1_FP	CGCATCTCGAGACCATGGCTAGCGGATT GCGTTACTCCATATATAT
MOH1_EpolyaB_REP	CGCATGGGATCCTTTATTAGAATTCAGT ACATTTACAAATGTTTTTC
KanR_FP	CTCGCGATAATGTCGGGCAATCA
KanR_REP	ATCCTGGTATCGGTCTGCGATTC
Outside (A)_FP	TACTGTACTTTGCTGACTTGCATTC
Outside (D)_REP	ACATAATCTTTGGGCGTATTACAAC
Outside (72C)_FP	GCTGTGGTTCGAGTCTGTCGCACGCCGT GTAATGT
Outside (72C)_REP	AGACTCGTTCAGGGCATAAGTCAATGGC CCTGT

APPENDIX B

IMMUNOCYTOCHEMISTRY OF UNTRANSFECTED AND TRANSIENTLY TRANSFECTED COS7 CELLS

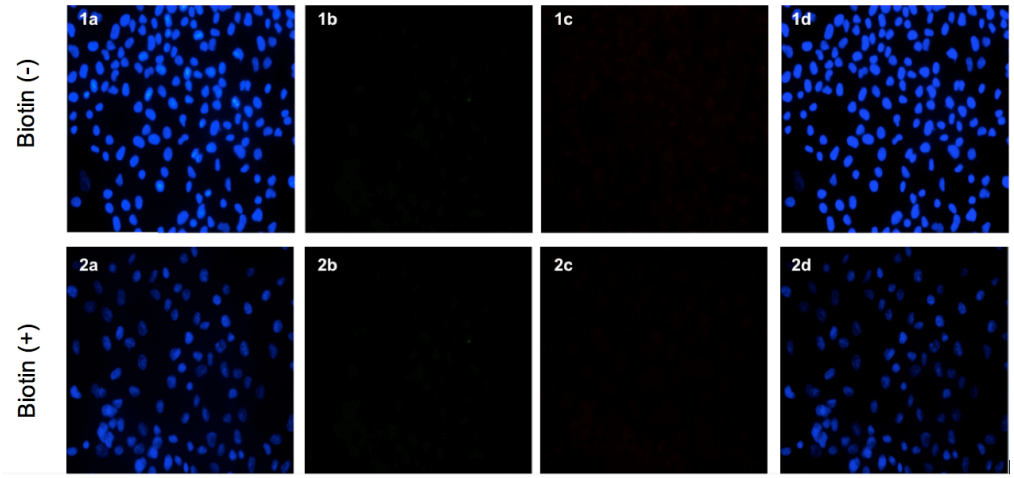

Figure B.1. Immunocytochemistry of untransfected COS7 cells with Flag M2 and Anti-biotin antibody. COS7 cells were grown on coverslips in 12-well culture plates for 48 hours. 48 hours after seeding, cells were refreshed with fresh complete media and grown for additional 20 hours. After 20 hours, 50 µM biotin and 1 mM ATP onto cells in biotin-plus wells. Cells in biotin-minus wells were refreshed only. 14 hours after biotin addition, fixation of cells were done by 3.7% formaldehyde. Fixed cells were permeabilized with 0.4% Triton-X. For blocking, 10% BSA in PBS was used for 1 hour. After blocking, cells were treated with biotin antibody (1:100 in 3% BSA-PBS) for 2 hours and the Flag M2 antibody (1:250 in 3% BSA-PBS) for 2 hours, sequentially. After that, an Alexa Fluor® 647 conjugated goat anti-rabbit secondary antibody (red channel emission; 1:1000 in 3% BSA-PBS) for the biotin antibody and an Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (green channel emission; 1:1000 in 3% BSA-PBS) for the Flag-M2 antibody were used. Incubation time for each secondary antibody was 30 minutes and incubations were done sequentially. Nuclei detection was done by DAPI staining (blue channel emission). a. DAPI staining, b. Flag-M2 antibody, c. Biotin antibody, d. Merge (Overlapping images taken by different emission channels). Similar results were obtained with two independent experiments.

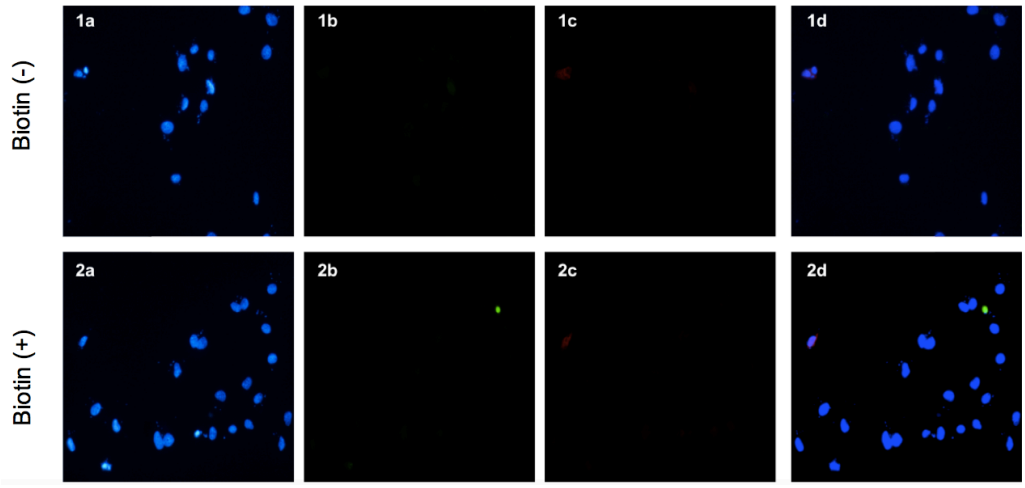

Figure B.2. Immunocytochemistry of COS7 transiently transfected with an expression vector bearing BirA* cDNA. COS7 cells were seeded onto coverslips in 12-well culture plates. After growth for 48 hours, cells were transiently transfected with pcDNA3.1 MCS BirA*(R118G) vector. 20 hours after transfection, 50µM biotin and 1mM ATP were added onto cells in biotin-plus wells. Cells in biotin-minus wells were refreshed. After 14 hour from biotin addition, cells were fixed with 3.7% formaldehyde. For permeabilization, 0.4% Triton-X was used. For blocking, cells were incubated with 10% BSA in PBS for 1 hour. After blocking, the biotin antibody (1:100 in 3% BSA-PBS) and the Flag M2 antibody were used as primary antibody. Primary antibody incubation was done for 2 hours for each antibody, sequentially. As a secondary antibody, an Alexa Fluor® 647 conjugated goat anti-rabbit secondary antibody (red channel emission; 1:1000 in 3% BSA-PBS) for the biotin antibody for 30 minutes and an Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (green channel emission; 1:1000 in 3% BSA-PBS) for the Flag-M2 antibody for 30 minutes was used. Nuclei were stained with DAPI (blue channel emission.) a. DAPI staining, b. Flag-M2 antibody, c. Biotin antibody, d. Merge (Overlapping images taken by different emission channels.) Similar results were obtained with two independent experiments.

APPENDIX C

SPOT TESTS TO DETERMINE AMOUNT OF STRESS INDUCERS

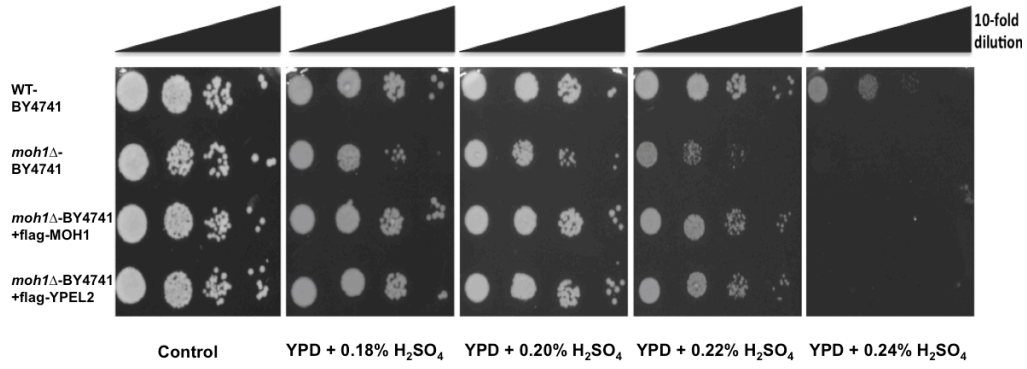

Figure C.1. Spot test to determine amount of H_2SO_4. Single colony was selected for each sample and grown overnight. Subcultures were prepared by using overnight cultures of each sample and grown until they reach log phase. When cultures reach the log phase, cells in each subculture were counted. $2.5x10_6$ cells from each culture were taken into 1 ml distilled water. 10-fold serial dilutions were applied for each sample. After serial dilutions, 4 µl of sample were taken from each serial dilutions and spotted onto stress agar plates with different concentration of H_2SO_4. Same procedure were carried out to determine proper amount of H_2O_2 and NaN_3.

APPENDIX D

SPOT TEST WITH NaN_3 INDUCED CELLS

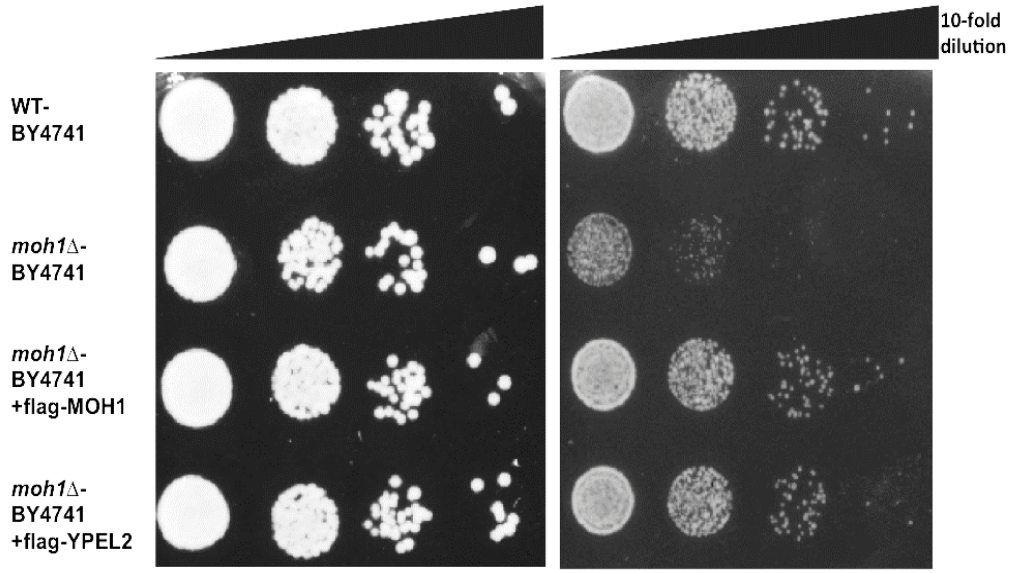

Figure D.1. Spot test for NaN_3 induced cells. Single colonies were selected for each strain. Colonies were grown overnight. From each overnight culture, sub-cultures were taken. Subcultures were grown for 4-6 hours until they reached log phase. When cultures were log phase, cell counts were done. From each culture $2.5x10^6$ cells were taken into 1 ml distilled water. Each sample was serially diluted 10-fold and 4 µl of these serial dilutions was used for spotting onto the plates: YPD as control, YPD with 0.4 mM NaN_3. Similar results were obtained from at least 3 independent experiments.