DYNAMIC PROTEIN INTERACTION NETWORK OF YPEL2

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

BY

GİZEM TURAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

SEPTEMBER 2020

Approval of the thesis:

DYNAMIC PROTEIN INTERACTION NETWORK OF YPEL2

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ABSTRACT

DYNAMIC PROTEIN INTERACTION NETWORK OF YPEL2

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September 2020, 63 pages

Estrogen hormones, especially 17β-estradiol (E2), play critical roles in the physiological and pathophysiological regulation of many organs and tissues, including breast tissue. E2 regulates the proliferation, differentiation, and death of target cells by binding to the estrogen receptor (ER). ER is a transcription factor that regulates gene expression involved in the manifestation of the cellular phenotype. In previous microarray studies conducted in our laboratory for the determination of genes regulated by E2-ER signaling pathways, it was found that the YPEL2 gene is an ER target gene and the expression of this gene is regulated by the direct binding of E2-ER to DNA. YPEL2 is a member of the highly conserved eukaryotic Yippee-like gene family, including YPEL1, 3, 4, and 5. High-level homologies among members of YPEL proteins in eukaryotes indicate common and fundamental functions. To our knowledge, no study is yet conducted on the YPEL2 gene or protein. However, studies on the other members of the YPEL family suggest that Ypel proteins located at the peri-nucleus and in the nucleus participate in the modulation of cellular proliferation, senescence, and apoptosis by regulating cell cycle phases. Due to the high degree of homology among Ypel proteins, we predict that YPEL2 is also involved in cellular proliferation and death mediated by E2-ER signaling. Cellular proteins function within the context of a dynamically changing

network of interacting protein partners. The identification of protein partners of YPEL2 could provide important information on the action mechanisms of the protein. Therefore, we aim to identify the interacting protein partners of YPEL2 using an inducible TurboID approach to generate dynamic protein interaction networks in a time-dependent manner and to verify some of these interactions in cell models synthesizing protein partners endogenously or ectopically with various protein-interaction assays.

Keywords: *YPEL2,* Protein Partners, TurboID, Estrogen, Estrogen Receptor

ÖZ

YPEL2 PROTEİNİN DİNAMİK PROTEİN ETKİLEŞİM AĞININ TANIMLANMASI VE ÖNDOĞRULANMASI

Turan, Gizem
Yüksek Lisans, Biyoloji
Tez Yöneticisi: Prof. Dr. Mesut Muyan

Eylül 2020, 63 sayfa

Östrojen hormonları, özellikle 17β-estradiol (E2), meme dokusu dahil olmak üzere birçok organ ve dokunun fizyolojik ve pato-fizyolojik düzenlenmesinde rol oynar. E2, hücresel fenotipin tezahüründe yer alan gen ifadelerini düzenleyen transkripsiyon faktörü olan östrojen reseptörüne (ER) bağlanarak hedef hücrelerin çoğalmasını, farklılaşmasını ve ölümünü düzenler. Daha önce laboratuvarımızda yapılan ERE-bağımlı ve ERE-bağımsız sinyal yolaklarının düzenledikleri genlerin tanısına yönelik mikrodizin çalışmalarında, YPEL2 geninin bir ER hedef geni olduğunu bulgulanmış ve bu genin ifadesinin ERE-bağımlı sinyal yolağı aracılığı düzenlendiği gözlemlenmiştir. YPEL2, YPEL1, 3, 4 ve 5'i de içeren yüksek oranda korunmuş ökaryotik Yippee benzeri gen ailesinin bir üyesidir. Ökaryotlarda YPEL proteinleri üyeleri arasındaki yüksek dereceli homolojiler ortak ve temel fonksiyonlara işaret eder. YPEL2 gen ya da proteini üzerine yapılmış bir çalışma yoktur. Ancak, YPEL ailesinin diğer üyeleri ile yapılan sınırlı sayıda çalışmalarda, peri-nükleer ve nükleer proteinler olarak tanımlanan YPEL proteinlerinin hücre çoğalması, yaşlanması ve ölümünü hücre döngüsüne katılarak düzenledikleri önerilmiştir.YPEL proteinleri arasındaki yüksek düzeyde homoloji nedeniyle, YPEL2 proteinin de E2-ER-tarafından düzenlenen hücre çoğalması ve ölümünde

önemli bir rol oynadığı öngörmekteyiz. Proteinler işlevlerini dinamik protein etkileşim ağları içinde yerine getirdikleri için, protein partnerlerinin belirlenmesi YPEL2 proteininin işlev mekanizmasına yönelik önemli bilgiler sağlayacaktır. Yakınlık-bağımlı biyotin ekleme (TurboID) yaklaşımı, proteinler arasında zaman-ve-alansal ilişkileri *in cellula* tanımlamakta kullanılmaktadır. Bu nedenle amacımız, YPEL2'nin olası protein partnerlerini dinamik TurboID yaklaşımıyla tanımlamak; tanımlanan proteinler aracılığıyla YPEL2'nin dinamik protein etkileşim ağı portresini *in silico* oluşturmak; olası ağ protein partnerlerinden seçilecek proteinlerle YPEL2 arasındaki etkileşimi ektopik ve endojen protein partnerleri senteze eden model hücrelerde çeşitli protein etkileşim yaklaşımlarıyla doğrulamaktır.

Anahtar Kelimeler: *YPEL2,* Protein Partnerleri, TurboID, Östrojen, Östrojen Receptörü

To all the woman who chase their dreams

ACKNOWLEDGMENTS

I would like to thank firstly my supervisor Prof. Dr. Mesut Muyan for his help, support, and guidance throughout my work. 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 my thesis committee members Assoc. Prof. Dr. Nurcan Tunçbağ and Prof. Dr. Çetin Kocaefe for their kind interest and critical contributions.

I would like to thank my laboratory mates for their help, support, and friendship. I thank to Pelin Yaşar for not only academic advising but also counseling about life experiences and Çağla Ece Olgun for shedding light on my challenging YPEL2 journey. I would like to express my thanks to Gamze Ayaz for all her academic teachings and guidance, and most importantly her friendship; Gizem Kars and Kerim Yavuz for their fun friendship and support during these three years; Öykü Deniz Demiralay for her helping hands in my intense experiments. Lastly ı would like to thanks Negin Razizadeh and Burcu Karakaya for their kind heart and precious friendship.

I do not know how to express my gratitude to my parents İsmet and Hatice and my lovely brother Hakan. They have been by my side in every moment of my life. Without them, none of the work I have done and will have any real meaning.

I wish to express my deep appreciation to my dearest friend Çetin Berçin Memiş for being at my side all my hard times.

I would like to thank TUBITAK (117Z213) for supporting this study.

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INTRODUCTION

1.1 Estrogen-Estrogen Receptor Signaling

Estrogens are steroid hormones and play important roles in the dynamic regulation of physiological functions of many tissues and organs [1]. There are three estrogen hormones; estrone, estradiol (E2), and estriol. 17β-estradiol (E2) is the most important estrogen hormone in the circulation. E2 has a critical effect on both physiological and pathophysiological regulation of breast tissue, especially in the initiation and development of breast cancer [2]. The effect of E2 on mammary epithelial cells is regulated by the ligand-dependent transcription factor estrogen receptors (ER) ERα and ERβ. Although ERα and ERβ are encoded from different genes on different chromosomes, they share structural domains that show a high degree of functional features [3]. According to tissue types, ERs exhibit distinct expression patterns. For example, ERα is predominantly expressed in the breast, uterus, cervix, and vagina; ERβ is expressed in the testis, lung, spleen, hypothalamus, and thymus [4].

ERα and ERβ have six structural domains: A to F (from N-terminus to C-terminus), responsible for different functions. The A/B domain interacts with the co-regulatory proteins and plays a role in the transcriptional activity of ER. The C domain is the DNA binding domain responsible for the binding of the receptor to DNA. The D domain has a nuclear localization signal and assists the post-translational modification of ERs. The E/F domain also called the ligand-binding domain (LBD) is responsible for dimerization and ligand-dependent transactivation function of the receptor [5][6].

ERs upon synthesis are dimerized and translocated to the nucleus independent of ligand binding. The binding of E2 causes ER to switch from an inactive form to active form by changing the structural organization of the LBD [7].

E2-ER can interact with DNA through two different mechanisms: estrogen response element (ERE)-dependent and ERE-independent pathways. In the ERE-dependent signaling pathway, E2-ER recognizes and binds DNA through a specific sequence of DNA, estrogen-responsive element (ERE). In the ERE-independent signaling pathway, E2-ER interacts with a transcription factor already bound to the cognate regulatory regions of DNA [7].

1.1 Yippee-like (YPEL) gene family

The Yippee protein was first described in a yeast binary hybrid system as the protein that interacts with the *Hyalophora cecropia* (giant silk moth) hemolin protein. The Yippee protein contains a zinc-finger motif [8]. In 2004, the existence of genes encoding similar proteins (Yippe-like) and located on different chromosomes was identified in many species, including the human, and it was found that a eukaryotic gene family was preserved in the evolutionary process [9]. The human YPEL1 gene as a mouse Ypel1 homolog has been identified in the 22q11.2 chromosome region. Subsequent studies led to the discovery of human paralogs on four different chromosomes, called YPEL2 (17q23.2), YPEL3 (16p11.2), YPEL4 (11q12.1), and YPEL5 (2q23.1). YPEL1 is expressed only in testes and fetal brain; 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; YPEL4 has been observed in the brain, lung, placenta, colon, ovary, small intestine, spleen, testis, fetal brain, fetal heart, fetal liver, fetal lung, fetal spleen, and bone marrow. YPEL3 and YPEL5 gene expressions are seen in all tissues tested [9].

As a result of studying the amino-acid sequence similarities between human and mouse orthologs and paralogs of the YPEL gene family, it was found that there is a

high degree of conservation both within and between species. While there is a similarity between 83.2% and 96.6% between human paralogs from Ypel1 to Ypel4, this rate is 43.8% in Ypel5, which has the lowest similarity. Analyses with other species revealed that 100 YPEL family genes were found in 68 species, including mammals, birds, amphibians, fish, insects, nematodes, protozoa, plants, and fungi. In these species, it has been shown that the amino-acids cysteine and histidine are highly conserved in the proteins that make up the Ypel family, and a common consensus sequence has been obtained. According to this, YPEL has a consensus sequence of 86 amino acids: C-X2-C-X19-G-X3-L-X5-N-X13-G-X8-C-X2-C-X4-GWXY-X10-K-X6-E. In this consensus sequence, the number of non-consensus amino-acids identified as X is the same for all species studied [9]. These findings indicate that the amino-acid sequences of Ypel proteins in all species are highly conserved and predict a fundamental function for Ypel proteins in cells. Mammals such as humans, monkeys, mice have five Ypel homologs, birds and fish have four, amphibia have three, nematodes, insects, and procordates have two Ypel homologs, whereas yeast has one Ypel gene homolog.

Studies on YPEL family genes and proteins are very limited. Craniofacial development is the result of integrated events organized with the participation of many tissues. Microdeletions observed in the human 22q11.2 chromosome region lead to symptoms such as Di George syndrome, Velocardiofacial syndrome, and craniofacial malformations, heart defects, thymus and parathyroid gland hypoplasia [10]. Since the human YPEL1 gene is located on chromosome 22q11.2 and the mouse ortholog is located in the region of chromosome 16, it is predicted that YPEL1 is involved in craniofacial development [10] [11]. Indeed, a 2001 study showed that the mouse Ypel1 gene plays a role in craniofacial development [12]. In another study in which Ypel1 gene expression in zebrafish embryos was reduced with morpholino oligo, it was found that Ypel1 participated in craniofacial development [13]. It has been suggested that Ypel1 affects craniofacial development by participating in epithelial cell differentiation processes [12].

Ypel3 was first described in 2003 as a small unstable apoptotic protein (SUAP) in mouse myeloid precursor cell lines [14]. In 2009, in microarray studies performed on ER-positive MCF7 cells derived from breast adenocarcinoma, it was found that SUAP was a gene activated by p53 and led to the redefinition of SUAP as the YPEL3 gene [15]. Studies on HCT116 cells derived from colorectal carcinoma and U2OS cells derived from osteosarcoma have also reported that YPEL3 gene expression increases in response to DNA damage, and this increase suppresses cell growth and causes cell aging and apoptosis. Similarly, it has been found that E2 plays a role in cell aging by suppressing YPEL3 gene expression in MCF7 cells through ER [16]. In studies conducted with clinical samples, it has been observed that YPEL3 gene expression is suppressed in colon, lung, and ovarian cancers compared to normal tissues [16] [17]. As a possible epigenetic mechanism, it is thought that YPEL3 gene expression decreases as a result of methylation of CpG islands in the promoter region [16].

In a study on Ypel4, it was found that the Ypel4 protein interacts with the major vault protein (MVP) and it was suggested that this interaction participates in the regulation of the activities of kinases involved in the signaling pathways of mammalian cells [18]. Apart from that, it has been suggested that Ypel4, which is synthesized ectopically, increases cell proliferation in response to Angiotensin II and K^+ ions in HAC15 cells derived from human adrenocortical carcinoma [19].

In pioneering studies conducted in 2000 in COS7 cells derived from African green monkey kidney fibroblast-like cells with antibodies developed against the Ypel5 protein, it was observed that the Ypel5 protein was localized in the cell nucleus, the nucleolus, centrosome, and mitotic apparatus depending on the cell cycle. Based on these observations, it has been suggested that Ypel5 participates in mitotic cell division. In line with these findings, it has been observed that the reduction of Ypel5 synthesis by siRNA approaches suppresses cell proliferation by prolonging the G1 and G2 + M cell cycle stages in COS7 cells [20]. In another

study, it was observed that the high-level of synthesis through over-expression of the YPEL5 gene by transfections in HeLa cells derived from cervical adenocarcinoma leads to rapid cell death [21]. In addition, the chimeric Ypel5-PPP1CB or PPP1CB-Ypel5 protein formed by Ypel5 and a serine/threonine-protein phosphatase PP1-beta-catalytic subunit (PPP1CB) has been reported to play a role in mature B-cell leukemia [22].

All of these limited numbers of studies suggest that YPEL family proteins are involved in cell proliferation, aging, and death in response to various stimuli in cells. To our knowledge, there is no study conducted on the YPEL2 gene or protein. Due to the high level of conservation in nucleotide and amino-acid sequences, we predict that the E2-ER responsive YPEL2 gene protein could contribute to crucial cell events such as E2-regulated cell proliferation and death. Gene amplification leading to high-level gene expression is observed in many tumors, including breast tumors [23]. 17q23 amplicon, including a large number of candidate oncogenes with YPEL2, is highly expressed in more than 40% of breast tumors. Interestingly, among these amplicon genes, only YPEL2 gene expression does not show copy number-dependent expression [24]. Moreover, it has been suggested that single and combination polymorphic gene variations in oncogenes including the YPEL2 gene in the 17q23 amplicon are associated with breast cancer risk [25]. These findings suggest that YPEL2 may also play a role in breast cancer ontology as well.

Although its significance is unknown, it has been observed that YPEL2 and YPEL3 expressions increase in chronic liver C virus infections as well [26].

1.2 Aim of This Study

In our previous and ongoing studies to test the prediction that YPEL2, which is an E2-ER responsive gene, plays a key role in cell proliferation and death regulated by E2-ER, we found that the YPEL2 protein causes cell death by disrupting nuclear

membrane integrity [27]. But we do not know the mechanism of how YPEL2 performs its functions. Since proteins fulfill their tasks in dynamic protein interaction networks, determining protein partners can provide important information about the mechanism of action of the YPEL2 protein. We aim here to elucidate this mechanism by identifying possible protein partners of YPEL2 with dynamic proximity-dependent biotinylation (TurboID) approach, which has been effectively used to define spatio-temporal protein interactions, and by preliminary verifying the protein interactions in model cells synthesizing endogenously or ectopically introduced protein partners.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Growth Conditions

COS7 (African green monkey kidney fibroblast) described previously (10.1016/S0303-7207(01)00493-2) were maintained in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium without phenol red (DMEM, BI, 01-053-1A) supplemented with 10% fetal bovine serum (Biological Industries, USA, 04-007-1A), %1 L-glutamine (BI, 03-020-1B) and 1% Penicillin/Streptomycin (BI, 03-031-1B). During doxycycline-dependent induction of transgenes, cells were seeded in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium without phenol red containing 10% fetal bovine serum without Doxycycline to prevent unwanted induction of transgenes (Tet-free FBS, Biowest, S181T), %1 L-glutamine and 1% Penicillin/Streptomycin. Cells were refreshed every three days and incubated in cell culture humidified incubator with 5% CO_2 at 37°C.

2.2 Cloning

2.2.1 Cloning of YPEL2 and TurboID in pcDNA

pBS-KS(-) bearing the YPEL2 cDNA and pcDNA that contain 3x-Flag tag plasmids were constructed in our laboratory. YPEL2 was amplified with cloning primers that contain XhoI and BamHI restriction enzyme sites. Primers used for cloning PCR are shown in Table A.1 (Appendix A). The amplified YPEL2 fragments and pcDNA-3x-Flag empty vector were double digested with XhoI (New England Biolabs, USA, R0146L) and BamHI (New England Biolabs, USA, R0136) restriction enzymes. The digested plasmid was treated with FastAP Thermosensitive Alkaline Phosphatase

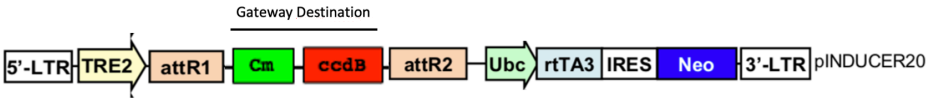
(Thermo Scientific, USA, EF0651) to prevent the plasmid from self-ligating. The digested products were run on 1% agarose gel and expected size of DNA fragments cut and extracted from gel by using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, 740609.50). The insert and the vector were ligated with a 3:1 insert:vector molar ratio at room temperature for 15 minutes by using the Rapid DNA Ligation Kit (Thermo Scientific, USA, K1422). The ligation mixture was transformed into chemically-competent XL1-Blue cells. 16h after the transformation, single colonies grown on LB agar ampicillin plates were picked, put into 4 ml LB-Ampicillin medium, and incubated on an orbital shaker for overnight. Colonies were screened for the presence of expected inserts by colony PCRs using gene-specific cloning primers. Plasmids were isolated from one colony containing the expected insert size and used for further studies.

For TurboID studies, 3x-HA-TurboID-NLS-pcDNA3 (Plasmid #107171) were purchased from Addgene, USA. For cloning of Turbo HA into pcDNA3.1 empty vector and at the C-terminus of 3x-Flag-YPEL2 in pcDNA 3.1, Turbo-HA was amplified from the 3x-HA-TurboID-NLS-pcDNA3 plasmid by using specific cloning primers. The HA tag sequence was contained within the reverse primer. Primers used for cloning PCR are shown in Table A.1 (Appendix A). pcDNA 3.1 empty vector, previously cloned pcDNA-3x-Flag-YPEL2 vector and amplified Turbo HA PCR fragment were double digested with BamHI (New England Biolabs, USA, R3136L) and KpnI (New England Biolabs, USA, R01425) restriction enzymes. Digested plasmids were treated with FastAP Thermosensitive Alkaline Phosphatase for 30 minutes. Samples were run on 1% agarose gel and the expected size of DNA fragments was cut and extracted from gel by using NucleoSpin Gel and PCR Clean-up Kit. Subsequent ligation and transformation steps were carried out as described in the cloning of YPEL2 into the pcDNA 3x-Flag EV vector. Screened positive plasmids were then sent for sequencing to ensure the fidelity of sequences.

2.2.2 Multiple Cloning Site insertion on Gateway Destination of pINDUCER20

For inducible expression, an inducible promoter bearing vector; pINDUCER20 (Plasmid #44012) which were purchased from Addgene, USA was used. pINDUCER20 was modified by inserting Multiple Cloning Site (MCS) on gateway destination sequences for easy cloning of the gene of interest as an inducible cassette system. A reverse primer designed with multiple restriction enzyme cut sites (Xho1, Bstz17I, NheI, NsiI, MluI, PmeI, BclI, and SalI) and PCR fragments were generated with BstAP1-MCS-SalI. PCR fragment was digested with BstAP1 (New England Biolabs, USA, R06545) and SalI (New England Biolabs, USA, R3138L). The vector was double digested with BstAP1 (New England Biolabs, USA, R06545S) and XhoI (New England Biolabs, USA, R0146L). The digested plasmid was treated with FastAP Thermosensitive Alkaline Phosphatase to prevent plasmid from self-ligation. Digested products were run onto a 1% agarose gel and the expected size of DNA fragments was cut and extracted from gel by using NucleoSpin Gel and PCR Clean-up Kit. Since XhoI and SalI have compatible cohesive ends, PCR fragments were ligated with a digested vector with 6:1 insert:vector molar ratio at room temperature for 15 minutes by using the Rapid DNA Ligation Kit. The ligation mixture was transformed into chemically induced competent XL1-Blue cells. After 16h, single colonies grown on LB agar ampicillin plates were picked and placed into 4 ml LB-Ampicillin medium and incubated overnight. From these overnight grown cultures, plasmids were isolated by using ZymoPURE Plasmid Miniprep Kit (Zymo Research, USA, D4211). Plasmids were then sent for sequencing to be sure sequences are correct.

pINDUCER20 (Addgene, Plasmid #44012)



pINDUCER20 with Multiple Cloning Site

Figure 1. pINDUCER20-MCS vector construction.

2.2.3 Cloning of YPEL2 and Turbo cDNA into the Inducible Promoter Bearing pINDUCER20 vector.

3x-Flag-YPEL2-Turbo-HA and Turbo-HA bearing pcDNA 3.1(-) plasmids were constructed previously. For cloning of 3x-Flag-YPEL2-Turbo-HA and Turbo-HA (EV) into pINDUCER20, new cloning primers were designed, shown in Table X (Appendix). The 3x-Flag-YPEL2-Turbo-HA and the Turbo-HA sequences were amplified with specific cloning primers. The 3x-Flag-YPEL2-Turbo-HA PCR fragment and the pINDUCER20 empty vector were double digested with Bstz17I (New England Biolabs, USA, R3594S) and MluI (New England Biolabs, USA, R3198L); The Turbo-HA PCR fragment and the pINDUCER20 empty vector were double digested with XhoI and MluI restriction enzymes. The digested vectors were treated with FastAP Thermosensitive Alkaline Phosphatase to prevent plasmid from recircularization. The ligation and transformation steps were carried out as described in section 2.2.2.

2.2.4 Cloning of Putative Protein Partners of YPEL2 into pcDNA-HA and pcDNA 3x-Flag Empty Vectors

For the cloning of cDNAs of the ELAVL1, ADSS, and EEF1D, the NCBI database was used to design two sets of primers for each gene. The first set was composed of the 5' and 3' primers at the untranslated region and the second set of primers as the nested primers, which anneal to a more internal region of cDNAs than the first set, contained restriction enzyme sites. The first run of PCR was performed with the first set of primers by using the COS7 cDNA library. Then, the products from the first reaction were used as a template to carried out the second run of PCR with the second set of primers. Second PCR products were run on 1% agarose gel, excised and purified by using NucleoSpin Gel and PCR Clean-up Kit.

The HA or the 3x-Flag tag bearing pcDNA 3.1 (-) empty vector had been already designed in our laboratory. Amplified PCR products, the pcDNA-HA, and pcDNA 3x-Flag empty vectors were double digested with appropriate restriction enzymes. Digested vectors were treated with FastAP Thermosensitive Alkaline Phosphatase for 30 minutes. After 30 minutes, all samples run on 1% agarose gel, and expected DNA sizes were purified by using NucleoSpin Gel and PCR Clean-up Kit. Subsequent ligation and transformation steps were carried out as described in section 2.2.2.

2.3 TurboID and BioID Comparison

2.3.1 Transfection

For western blot analysis, $6x10^4$ COS7 cells/well were seeded into 6-well tissue culture plates. After 48 hours, transfection of the pcDNA-3x-Flag-YPEL2-BirA-HA or the pcDNA-3x-Flag-YPEL2-Turbo-HA were carried out. The pcDNA-3x-Flag-YPEL2-BirA-HA was previously generated in our laboratory. For each construct,

there were 4 groups: plus-biotin and minus-biotin groups with 3 and 14-hour biotinylation durations. The transfection mixture contains 2 µg DNA, 200 µL phenol red-free DMEM high glucose medium, and 4 µL Turbofect *in vitro* transfection reagent (Thermo Scientific, USA, R0532) per well. After 30 minutes of incubation at room temperature, the transfection mixture was added drop-wise onto cells with a refreshed growth medium.

2.3.2 Biotin and ATP Addition

24 hours after transfection, 50 µM biotin (Sigma, Germany, B4639), and 1 mM ATP (Adenosine 5'-triphosphate disodium salt hydrate, Sigma, Germany, A2383) were added into the fresh complete growth medium. The mixture was then added onto the plus-biotin cell group. Minus-biotin wells were only refreshed with the complete growth medium.

2.3.3 Protein Isolation

3 and 14 hours from the addition of biotin-ATP, cells were collected and washed with cold PBS. Cells were lysed in 50 µl lysis buffer (50mM Tris, pH: 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 2% Triton-X, 1 mM DTT, 1x protease inhibitor). After collecting cell extracts in lysis buffer, extracts were sonicated for 5-minute active sonication with 10 seconds ON and 15 seconds OFF cycles. Extracts were then centrifugated at 7500rpm for 15 minutes at 4°C. Supernatants were collected and protein concentration was measured by using Quick Start Bradford Protein assay (Bio-Rad, USA, 5000205).

2.3.4 Western Blot

50 µg of total protein was denatured in 6x Laemni buffer that contains 30% B-mercaptoethanol at 95^{o}C for 5 minutes and loaded into 10% SDS-PAGE gel. Proteins

were then transferred onto a PVDF membrane (Advansta, WesternBright™ PVDF-CL, L- 08008-001) through a wet transfer system for 70 minutes at 100 V. After protein transfer from the gel to a membrane, the membrane was blocked with 5% Skim milk in 0.05 Tris Buffered Saline-Tween (TBS-T) for 1 hour. The membrane was then incubated with the anti-biotin antibody (1:200 in 5% skim milk in 0.05 % TBS-T, Abcam, USA, ab53494) for 1 hour at room temperature. After primary antibody incubation, the membrane was washed three times with 0.05 TBS-T for 5 minutes each. The secondary antibody, goat anti-rabbit-HRP (1:4000 dilution in 5% skim-milk in 0.05 % TBS-T, Santa Cruz Biotechnology, USA), was added onto the membrane and incubated for 1 hour at room temperature. For imaging, the membrane incubated with WesternBright ECL substrate (Advansta, K-12045-D50) in 1:1 luminol-enhancer reagent:peroxide reagent ratio in the dark for 2 minutes. ChemiDoc™ MP system (Bio-Rad, USA) was used for imaging the membrane, and Image Lab 5.1 (BioRad, USA) program was used for analyzing the image results.

2.4 Inducible System Optimization

2.4.1 Dox Dose-Response Optimization

2.4.1.1 Transfection

COS-7 cells, $6x10^4$ cells/well, were seeded into 6-well tissue culture plates for 48h. Cells were then transfected with pINDUCER20-3x-Flag-YPEL2-Turbo-HA or pINDUCER20-Turbo-HA empty vector (EV). The transfection mixture was prepared as indicated in section 2.3.1 and added onto the cells after 30 minutes.

2.4.1.2 Dox Induction

24 hours after transfection, doxycycline (Dox, Doxycycline Hyclate, Applichem, Germany, A2951) were added onto the cells at final concentrations of 1000ng/ml,

100ng/ml, 10ng/ml, 5ng/ml, 2.5 ng/ml, 1 ng/ml and only 0.1% DMSO (AppliChem, USA, A3672,0259) as vehicle control. pINDUCER20 empty vector transfected cells were only treated with 1000 ng/ml Dox or 0.1% DMSO. Cells were maintained for 24 hours until protein isolation.

2.4.1.3 Total Protein Isolation

RIPA Buffer (50mM Tris (pH:8), 1% Triton-X, 0.5% Sodium Dioxychalate, 150mM NaCl (pH:7.4), 0.1% SDS, 1mM EDTA) was used for cell lysis and total protein isolation. Cells were trypsinized, collected with medium, and washed with 1X PBS once. Cells were pelleted by centrifugation at 300g for 5 minutes. Cell pellets were dissolved with 100 µl RIPA Buffer which contains freshly added 1X protease inhibitor (Roche, 11 873 580 001). Cells were kept on ice for 30 minutes. Cell lysates in RIPA Buffer were actively sonicated for 1 minute with 10 seconds ON and 15 seconds OFF cycles. Lysates were then centrifugated at 14000g for 20 min at 4 °C. Supernatants were collected into new tubes. Protein concentrations were measured by using Quick Start Bradford Protein assay.

2.4.1.4 Western Blot

For western blot analysis, a total of 75 µg protein were denatured in 6x Laemni buffer that contains 30% B-mercaptoethanol at 95^{o}C for 5 min. Proteins were loaded to 10% SDS gel and ran approximately 90 min at 100V. Proteins were then transferred onto a PVDF membrane through a wet transfer system for 70 minutes at 100 V. The membrane was blocked with 5% skim milk in 0.1 Tris Buffered Saline-Tween (TBS-T) overnight at 4°C. After blocking, the membrane was incubated with a Flag-M2 (Sigma, USA, F1834) or anti-HA (Abcam, USA, ab9110) antibody for 1 hour at room temperature. The Flag-M2 antibody was diluted at 1:1000 in 5% Skim milk in 0.1% TBS-T and the anti-HA antibody was diluted at 1:1000 in 5% Skim milk in 0.1% TBS-T. After the primary antibody incubation, the membrane was washed

three times with 0.1 TBS-T for 5 minutes each. An HRP-conjugated goat anti-mouse secondary antibody (Advansta, USA, R-05071-500) was used for the Flag-M2 antibody. An HRP-conjugated goat anti-rabbit secondary antibody (Advansta, USA, R-05072-500) was used for the anti-HA antibody at 1:5000 dilution in 5% Skim milk in 0.1% TBS-T. The membrane was incubated with the secondary antibody for 1 hour at room temperature and washed three times with 0.1% TBS-T for 5 minutes each. The membrane incubated with WesternBright ECL substrate kit in 1:1 luminol-enhancer reagent:peroxide reagent ratio in the dark for 2 minutes and imaged with ChemiDoc^{TM} MP system (Bio-Rad, USA).

2.4.2 Induced Expression Time-Course Optimization

2.4.2.1 Transfection

For immunocytochemistry, COS7 cells, 1.5x10^4 cells/well, were seeded onto12-well cell culture plates which contain sterile 15 mm round coverslips. 48 hours later, the transfection of pINDUCER20-3x-Flag-YPEL2-Turbo-HA or pINDUCER20-Turbo-HA was carried out. The transfection mixture contains 1 ug DNA, 100 µL phenol red-free DMEM high glucose medium, and 2 µL Turbofect *in vitro* transfection reagent per well. Transfection mixtures were added onto cells by drop-wise. Four hours later, the spent medium was refreshed with a fresh growth medium.

2.4.2.2 Dox Induction

24 hours after the transfection, cells were treated without 0.1% DMSO as vehicle control or with 1 ng/ml, 10 ng/ml or 100 ng/ml of doxycycline to induce the induction of 3x-Flag-YPEL2-Turbo-HA or Turbo-HA expression.

2.4.2.3 Immunocytochemistry

24, 36, or 48 hours (indicated for each experiment) after the biotin addition, the medium was aspirated and cells were washed with PBS three times. Cells were then fixed with 3.7% formaldehyde for 30 minutes and then permeabilized with 0.4% Triton-X for 10 minutes. For the prevention of non-specific protein interactions, cells were incubated with %10 BSA in PBS for 1 hour. The primary antibody Flag-M2 (1:250 in 3% BSA-PBS, Sigma Aldrich, Germany, F-1804) and Lamin B1 (1:200 in 3% BSA-PBS, Abcam, USA, ab16048) was added on cells transfected with pINDUCER20-3x-Flag-YPEL2-Turbo-HA. The anti-HA (1:500 in 3% PBS, Abcam, ab9119) and Lamin A (1:200 in 2% NGS, ab8980, Abcam, USA) antibodies were added on the cells transfected with pINDUCER20-Turbo-HA for 2 hours with gentle agitation. After primary antibody incubation, antibody solutions were removed and cells were washed with PBS three times. In labeling with two different primary antibodies, antibodies were used sequentially. Secondary antibody incubations were carried out for 30 minutes for each antibody in the dark. An Alexa Fluor® 488 conjugated goat anti-mouse (1:1000 in 3% BSA, Abcam, USA, ab150113) secondary antibody is used for the Flag-M2 antibody; an Alexa Fluor® 647 conjugated goat anti-rabbit (1:250 in 3% BSA, Abcam, USA, ab150077) secondary antibody was used for the Lamin B1 antibody; an Alexa Fluor® 488 conjugated goat anti-rabbit (1:1000 in 2% NGS, Abcam, USA, ab150077) secondary antibody was used for the HA antibody and Alexa Fluor® 647 conjugated goat anti-mouse (1:250 in 2% NGS, Abcam, USA, ab150113) secondary antibody was used for the Lamin A antibody. After several PBS washes, coverslips were mounted onto the glass slides with Fluoroshield Mounting Medium with DAPI (Abcam, USA, ab104139) for nucleus staining. Slide edges were sealed with transparent nail polish and were left to dry in the dark. Imaging was carried out with a Nikon Eclipse 50i Fluorescence Microscope in the laboratory of Prof. Dr. Rengül Atalay (METU, Bioinformatics).

2.5 TurboID Optimizations

2.5.1 Transfection

COS7 cells, $6x10^4$ cells/well, were seeded in 6-well tissue culture plates and 48 hours later, transfection of the pINDUCER20-3x-Flag-YPEL2-Turbo-HA vector or the pINDUCER20-Turbo-HA vector was carried out. The growth medium was refreshed after 4 hours of transfection.

2.5.2 Dox Induction

24 hours from transfection, cells were treated with 10 ng/ml of DOX for the induction of transgene expressions.

2.5.3 Biotin and ATP Addition

After 24 hours from induction of expression, biotin and ATP (Adenosine 5'-triphosphate disodium salt hydrate) were added onto cells. Three different biotin-ATP concentrations were used in this experiment; 50 µM biotin, and 1 mM ATP; 100 µM biotin, and 2 mM ATP, 250 µM biotin, and 2 mM ATP.

2.5.4 Protein Isolation

Cells were collected at three different time-points; 30 minutes, 1 hour and 3 hours. Cells were washed with cold PBS and cell lysis was carried out as described in section 2.3.3.

2.5.5 Western Blot

A total 50 µg protein was loaded onto 10% SDS PAGE gel after denatured in 6x Laemni buffer at 95^{o}C for 5 minutes. Proteins were then transferred from gel to PVDF membrane and incubated with 5% skim-milk blocking solution in 0.05 % TBS-T (Tris-buffered saline-tween) overnight at 4^{o}C. After blocking, the membrane was incubated with a 1:200 diluted anti-biotin antibody for 1 hour at room temperature. After three TBS-T washes, a horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (Advansta, USA, R-05072-500) was added onto the membrane at 1:4000 dilution for 1 hour. After three 0,05 % TBS-T washes, the membrane was developed by using WesternBright ECL substrate in 1:1 luminol-enhancer reagent:peroxide reagent ratio in the dark for 2 minutes for visualization with the ChemiDoc^{TM} MP system (Bio-Rad, USA).

2.6 Proximity Dependent Biotinylation (TurboID) Assay

2.6.1 Transfection

For TurboID assay, 75x10^{4} cells COS7 cells were seeded onto 10 cm^{2} tissue culture dishes. 48 hours after, transfection was carried out.

In this experiment, there were four biotinylation times: 1 hour, 3 hours, 6 hours, and 16 hours. Also, there were three groups which are 3x-Flag-YPEL2-Turbo-HA or Turbo-HA transfected and untransfected cells. Each experiment was carried out as two biological replicates. Transfection mixture contains 12 ug DNA, 1200 µL phenol red-free DMEM high glucose medium, and 24 µL Turbofect *in vitro* transfection reagent per dishes. Transfection mixtures were prepared and incubated for 30 minutes at room temperature. After 30 minutes, mixtures were added onto cells by dropwise with a fresh medium that contains Tet-free FBS. After 4 hours, the spent medium was refreshed.

2.6.2 Dox Induction

24 hours after transfection, cells were treated with 10 ng/ml of doxycycline for the induction of expression of the 3x-Flag-YPEL2-Turbo-HA or the Turbo HA transgene.

2.6.3 Biotin and ATP addition

24 hours after Dox induction, 50 µM biotin and 1 mM ATP (Adenosine 5'-triphosphate disodium salt hydrate) were added into the fresh growth medium and the medium was placed onto the cells.

2.6.4 Protein Isolation and Streptavidin Affinity Capture of Biotinylated Proteins

Cells were collected at 1 hour, 3 hours, 6 hours, and 16 hours after biotin addition. Cells were washed with cold PBS and then lysed in 400 µl lysis buffer. Cell lysis protocol was carried out as described in section 2.3.3. After lysis, protein concentrations were measured by using Quick Start Bradford Protein assay, and 6 mg of protein sample was incubated with 500 µl Streptavidin magnetic beads (NEB S1420S) overnight. Beads were collected by using Magna GrIP Rack and washed twice with 400 µl Wash Buffer 1 (2% SDS in dH_2O) for 10 minutes. Then, beads were washed once with 400 µl Wash Buffer 2 (2% deoxycholate; 1% Triton-X; 50 mM NaCl; 50 mM HEPES, pH=7.5; 1mM EDTA), Wash Buffer 3 (0.5% NP-40; 0.5% deoxycholate; 1% Triton-X; 500 mM NaCl; 1 mM EDTA) for 10 minutes followed with Wash Buffer 4 (50 mM Tris, pH=7.4; 50 mM NaCl) for 30 minutes. 10% of the bead-protein mixture was aliquoted for western blot analysis and the remaining sample (90%) was sent to Koç University for mass spectrometry analysis.

2.6.5 Western Blot

For western blot analysis, proteins were eluted from beads by adding 40 µl of Laemmli-DTT sample buffer containing 500 nM Biotin and boiling at 95°C for 10 minutes. Then, the total volume was loaded onto a 10% SDS-PAGE gel. Subsequent western blot analysis steps were carried out as described in section 2.3.4.

2.6.6 Mass Spectrometry analysis for protein identification

Proteins bound to beads were sent to Koç University Cell Proteomics Laboratory, Istanbul, Turkey for mass spectrometry analysis. Trypsin was used to digest proteins bound to beads. Peptide purification and concentration were carried out by using C18 StageTip (Thermo-Fisher). Peptides were then analyzed with LC-MS/MS mass spectrometer (Thermo- Fisher). Proteome Discoverer 1.4 software (Thermo-Fisher) to identify proteins.

2.7 Co-immunoprecipitation (Co-IP)

Mass spectrometry results showed that YPEL2 putatively interacts with a number of proteins at different time points. Among these interacting partners, ELAV like RNA Binding Protein 1 (ELAVL1), Adenylosuccinate Synthase (ADSS), Eukaryotic Translation Elongation Factor 1 Delta (EEF1D), Sequestosome1 (SQSTM1), TAR DNA Binding Protein (TARDBP) and TATA-Box Binding Protein Associated Factor 15 (TAF15) were selected based on their functional properties and being common in different biotinylation time points to verify that they are YPEL2 interactors. For co-immunoprecipitation assays PCR amplified YPEL2, ELAVL1, ADSS, EEF1D, SQSTM1, TARDBP or TAF15 cDNA were cloned into a pcDNA 3.1(-) vector bearing in-frame sequences at the 5'-end of the multiple cloning site that encode for an amino-terminally located 3xFlag or HA tag by the use of NheI and EcoRI or NheI and BamHI restriction enzymes.

2.7.1 Western Blot

2.7.1.1 Transfection

For Co-IP experiments, COS7 cells, $6x10^4$ cells/well, were seeded onto 6-well tissue culture plates. Cells were transfected with expression vectors bearing the 3xFlag tagged-YPEL2 cDNA and/or the HA-tagged vector bearing -ELAVL1, -ADSS, -EEF1D, -SQSTM1, -TARDBP and -TAF15 cDNA for 48 hours. For co-transfection, the transfection mixture contains total of 2 ug DNA per well, 1 ug from each vector.

2.7.1.2 Total Protein Isolation and Immunoprecipitation

36 hours after transfection, cells were collected and lysed with M-PER total protein Extraction Reagent (Thermo Scientific, 78501) that contained fresh 1X PhosSTOP (Roche, 04906837001) and 1X Protease inhibitor cocktail (Roche, 11 873 580 001). Protein concentrations were measured with Bradford Protein Assay. For Co-IP, there were three groups for each transfected protein; input, IgG control, and Immunoprecipitated group (IP). Total protein concentrations of IgG and IP group was equal amounts. In these experimental series, 500 µg total protein was equally divided for IgG and IP. For input, 10% of the total protein sample was separated for protein analysis. 500 µg total protein lysates was incubated with 25 µl Protein A and G conjugated magnetic beads at 4°C for 1 hour to remove the non-specific proteins. After 1 hour, the magnetic field was applied to separate beads from lysates and pre-cleared lysates were transferred to a clean 1.5 ml Eppendorf tube. Then, pre-cleared lysates were incubated with the anti-HA antibody at 4°C overnight on a rotator. After antibody incubation, lysates were incubated with 25 µl Protein A and G conjugated magnetic beads at 4°C for 1 hour to collect the target protein through the anti-HA antibody. Then, beads were washed three times with 300 µl wash buffer (10 mM

HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Igepal, protease inhibitor, and phosphatase) for 5 minutes. Target protein was eluted from beads by adding 30 µl of 2x SDS Loading buffer (187.5 mM Tris pH 6.8, 6% (w/v) SDS, 30% glycerol, 150 mM DTT, 0.03% (w/v) bromophenol blue, 10% β-mercaptoethanol) and boiled at 95°C for 10 minutes. The magnetic field was applied for 30 seconds and the supernatant was loaded onto 10% SDS-PAGE gel. Western blot assay was carried out. Same experiment was applied with precipitation with Flag antibody for further confirmation of interaction between proteins.

2.7.1.3 Western Blot

Input and eluted proteins form IgG control and IP groups were loaded on 10% SDS-PAGE gel respectively. Subsequent transfer and blocking steps were carried out as described in section 2.3.4. After blocking, the membrane incubated with the Flag-M2 antibody for 1 hour at room temperature. After three washes with 0.1% TBS-T, an HRP conjugated goat anti-mouse IgG secondary antibody in blocking buffer was added on the membrane and incubated for 1 hour at room temperature. The membrane washed three times with 0.1% TBS-T for a total of 15 minutes and the membrane was incubated with WesternBright ECL in 1:1 luminol- enhancer reagent:peroxide reagent ratio for 2 minutes and imaged with ChemiDocTM MP system (Bio-Rad, 1708280).

2.7.2 Fluorescent Microscopy

2.7.2.1 Transfection

For immunocytochemistry assay, COS7 cells, $1.5x10^4$ cells/well, were seeded onto 15 mm coverslip placed 12-well tissue culture plates. After 48 hours, cells were transfected with plasmids bearing the 3xFlag-YPEL2 and/or HA-ELAVL1 by using

Turbofect transfection reagent. Transfection mixture contains a total of 1 ug DNA per well, 500 ng DNA from each plasmid.

2.7.2.2 Immunocytochemistry

36 hours after transfection, cells were washed three times with 1x PBS and fixed with 3.7% formaldehyde for 30 minutes. Then, cells were permeabilized with 0.4% Triton-X-100 for 10 minutes. Blocking was done with 10% Bovine Serum Albumin (BSA) for 1 hour. Flag (1:250, Sigma Aldrich, F-1804) and HA (1:500, Abcam, ab9119) primary antibodies were added sequentially on the cells for 2 hours for each antibody. Cells then washed three times with 1x PBS and incubated with secondary antibodies for 30 minutes for each secondary antibody. An Alexa Fluor 488-conjugated goat anti-mouse (1:1000 in 3% BSA) for the Flag antibody or an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:1000 in 3% BSA) for the HA antibody was used. After three 1x PBS washes, coverslips were mounted onto the glass slides with the Fluoroshield Mounting Medium with DAPI, and coverslip edges were sealed with transparent nail polish. Imaging was carried out with a Nikon Eclipse 50i Fluorescence Microscope in the laboratory of Prof. Dr. Rengül Atalay (METU, Bioinformatics Institute).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 BioID and TurboID Comparison

Since proteins perform their functions in a network of proteins whose identities and amounts change temporally and spatially in response to intrinsic and extrinsic stimuli, the identification of YPEL2 partners could contribute to a better understanding of the regulatory mechanisms of YPEL2-mediated cellular processes in physiology and pathophysiology. Enzyme-catalyzed proximity labeling (PL) is an effective alternative to immunoprecipitation and biochemical fractionation for proteomic analysis of macromolecular complexes, organelles, and protein interaction networks. Following its introduction, the proximity-dependent biotinylation (BioID) approach has been widely used for the identification of interacting partners of many proteins [28]. The BioID approach is based on the genetic fusion of a promiscuous labeling enzyme, a mutant *E. coli* biotin ligase enzyme, BirA*(R118G), which is defective in both self-association and DNA binding, to a protein-of-interest to biotinylate proximity proteins [29]. The addition of biotin initiates covalent tagging of endogenous proteins within a few nanometers of the promiscuous enzyme. Biotinylated proteins are then selectively isolated with biotin-affinity capture and identified with mass spectrometry (MS).

The major disadvantage of BioID is its slow kinetics. This necessitates labeling with biotin for 18–24 h to produce sufficient biotinylated material for proteomic analysis. This precludes the use of BioID for studying dynamic processes that occur on the timescale of minutes. The recent development of TurboID, which was engineered by the directed evolution of *E. coli* biotin ligase, is suggested to circumvent the slow kinetics of BioID approach by biotinylating proteins at much higher efficiencies and shorter times.

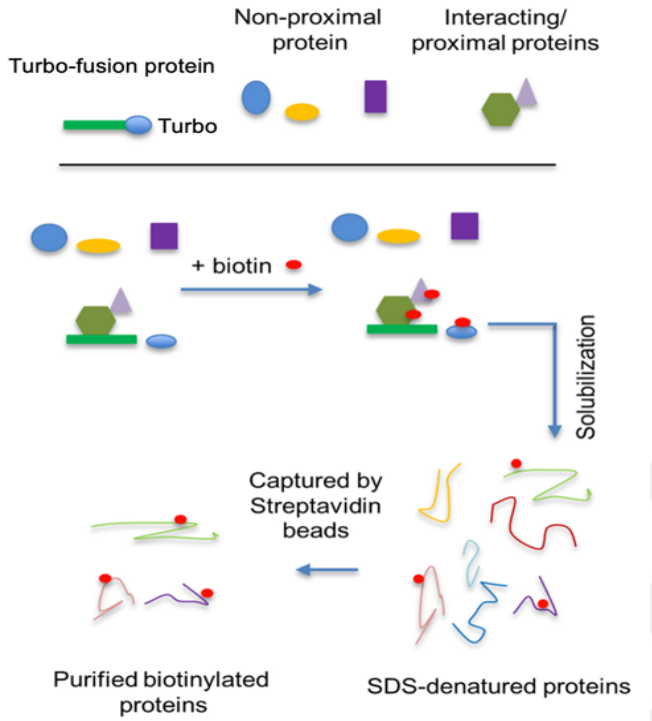


Figure 2. Schematic representation of the working principle of TurboID. Turbo-fusion protein biotinylates proximal and non-proximal proteins in the presence of biotin. Biotinylated proteins are precipitated with streptavidin magnetic beads and identified by mass spectrometry analysis [27].

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To generate the protein components of BioID and TurboID we engineered fused Flag-YPEL2-Turbo-HA and Turbo-HA constructs by cloning of the Flag-YPEL2 cDNA or the Turbo-HA cDNA (from 3x-HA-TurboID-NLS-pcDNA3 plasmid purchased from Addgene) to the pcDNA3.1(+) empty vector. To comparatively test the efficiencies of BioID and TurboID, we transiently transfected COS7 cells with the expression vector bearing BirA*(R118G)-HA or Turbo-HA cDNA as the empty vector control, or the Flag-YPEL2-BirA*(R118G)-HA or Flag-YPEL2-Turbo-HA cDNA. 24 hours after transfection, cells were treated without (as control) or with 50 μM biotin and 1 mM ATP for 3 or 14 hours. Then, cells were subjected to western blot analysis using the biotin antibody to detect biotinylated proteins (Figure 2). The molecular weight of the BirA* and Turbo proteins are about 35 kDa, and the YPEL2 fusion proteins are about 50 kDa. WB results showed that TurboID compared to BioID biotinylated the cellular protein at much higher efficiencies at an early time point. Based on these observations, we surmised that TurboID is our choice of the enzyme to elucidate the putative protein partners of YPEL2.

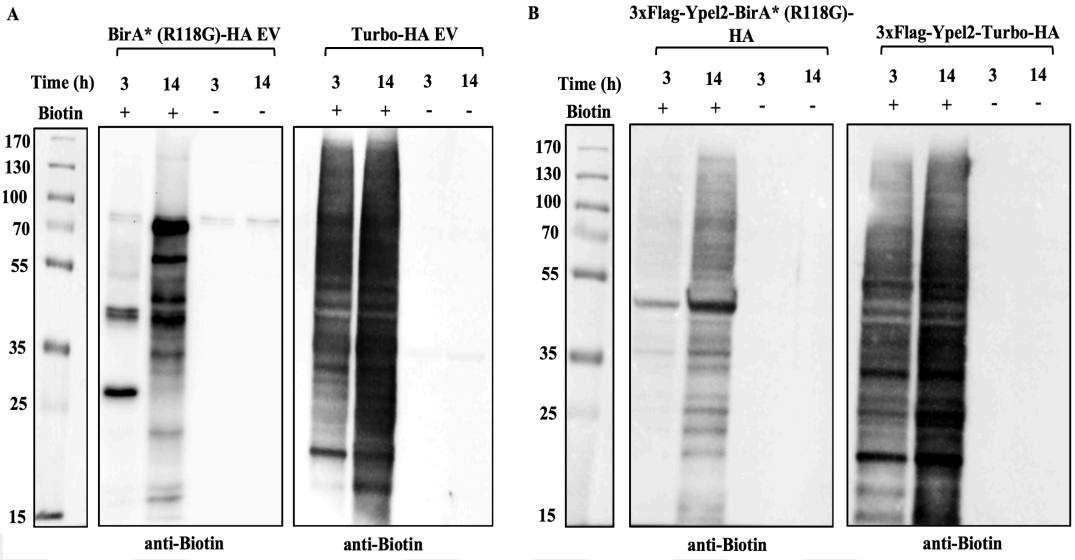


Figure 3. Comparative biotinylation activities of BirA* and Turbo proteins by WB. COS7 cells were transiently transfected with pcDNA-BirA*(R118G)-HA, pcDNA-Turbo-HA empty vectors (A), pcDNA-Flag-YPEL2-BirA*(R118G)-HA, or pcDNAFlag-YPEL2-Turbo-HA (B). 24h after transfections, cells were incubated without or with 50 μM biotin and 1 mM ATP for 3 hours or 14 hours. 50 μg protein from each sample was loaded onto %10 SDS-PAGE and the Biotin antibody was used to detect biotinylated proteins. MW indicates molecular weight markers.

3.2 Dox-Dependent Inducible System/Tetracycline (Tet) Inducible Expression

Previous studies from our laboratory [27] using COS7 cells derived from transformed African green monkey kidney fibroblast-like cells and a commercial pantropic anti-YPEL antibody (S-14; Santa Cruz Biotechnology TX, USA), which is no longer available, detect the endogenous YPEL2 as other Ypel proteins. These proteins display a diffuse intracellular localization but primarily localizes to the nuclear periphery as the LaminA, a structural component of the nuclear lamina, to localize the nuclear envelope and DAPI (4', 6-Diamidino-2-Phenylindole, Dilactate), as a nuclear counterstain, indicate (Fig 4A). This was similar to the observation of Hosono et al. [9] who identified the Ypel family proteins for the first time using COS7 cells. Since S-14 was a pantropic antibody that recognizes the entire Ypel family proteins and since we engineered recombinant proteins to bear a Flag epitope

at the amino-terminus, using both the Flag-M2 and the S-14 Ypel antibodies would also allow us to verify the intracellular location of YPEL2 protein specifically. To examine this issue, we transiently transfected COS7 cells. Surprisingly, we detected Flag-YPEL2 using the Flag-M2 antibody both inside and outside of the nucleus, whose boundary is indicated with the detection of the nuclear membrane LaminB1 protein in cells transfected with the Flag-YPEL2 cDNA (Figure 4B). The DAPI staining indicates that some of the DNA was diffused out from the nucleus into the cytoplasm in a pattern perfectly overlapping with the staining of Flag-YPEL2. Cells that were not stained with the Flag-M2 antibody as an indication of un-transfected cells within the same population showed a DAPI staining that was identical to that observed in cells transfected with the vectors bearing none (EV) or the Flag-ERα cDNA (Figure 4C). This suggests that the over-expressed YPEL2 is directly or indirectly leads to the leakage of DNA into the cytoplasm and subsequently induces acute cell death. Based on these observations we, therefore, surmised that the over-synthesis of YPEL2 in transiently transfected COS7 cells, as well as MCF7 cells (data not shown), leads to the disintegration of the nuclear membrane and leakage of the chromatin into the nucleus.

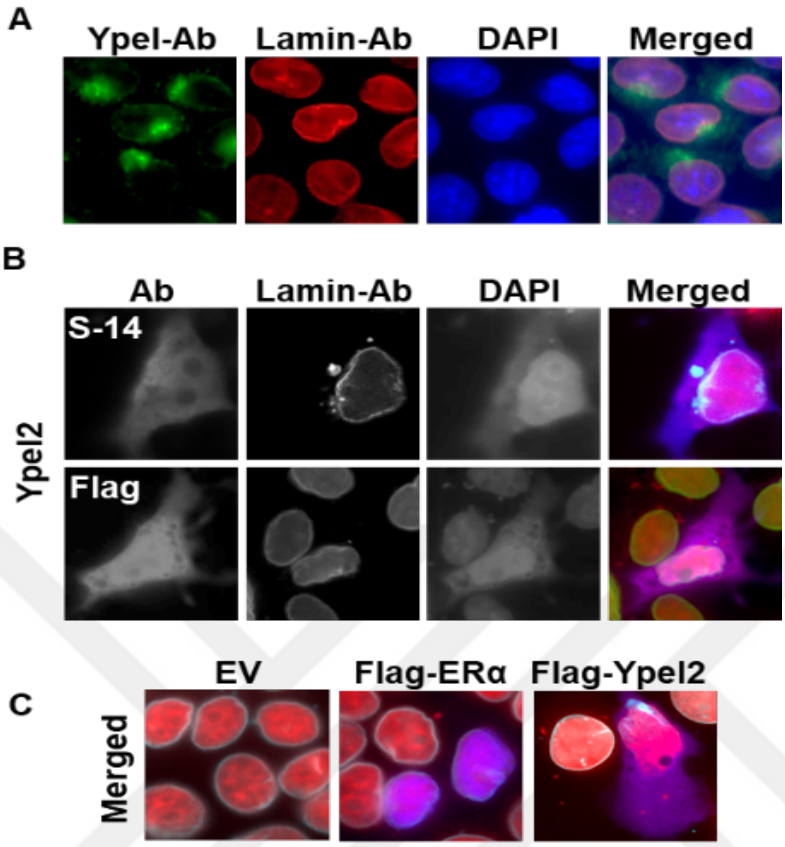


Figure 4. Immunocytochemistry of COS7 cells for YPEL2 protein. (A) endogenous YPEL2 location. COS7 cells grown on coverslips in 12-well cell culture plates were fixed with 2% paraformaldehyde, permeabilized with 0.4% Triton-X100. After blocking with 10% normal goat serum (NGS) for one hour, cells were incubated with the S-14 Ypel antibody (1:50 in 2% NGS) for two hours and then with LaminA antibody (1:200 in 2% NGS) for two hours, sequentially. An Alexa Fluor-488 conjugated goat anti-rabbit secondary antibody at 1:1000 in 2% NGS was used for the visualization of Ypel protein. An Alexa Fluor-647 conjugated goat anti-mouse secondary antibody at 1:200 in 2% NGS was used for the visualization of LaminA. Nuclei were counterstained with DAPI. Merged denotes the overlapped images taken by different emission channels. (B & C) COS7 cells were transiently transfected with pcDNA3.1 (-) bearing none (as control, empty vector: EV), a Flag-YPEL cDNA, or Flag-ERα cDNA (as the control for nuclear staining). 36 hours after transfection, cells were fixed with 2% paraformaldehyde, permeabilized with 0.4% Triton-X100. After blocking with 10% bovine serum albumin (BSA) for one hour, cells were incubated with the Flag-M2 (1:250 in 3% BSA) for two hours and then with the LaminB1 (1:200 in 3% BSA) antibodies for two hours, sequentially. An Alexa Fluor-488 conjugated goat anti-mouse and an Alexa Fluor-647 conjugated goat anti-rabbit (1:1000 and 1:200 in 3% BSA, respectively) secondary antibodies were used for the visualization of Flag-Ypel and LaminB1, respectively. Nuclei were counterstained with DAPI (Figure is taken from G. Güpür MSc Thesis, 2014).

But the problem arises when we want to study the function of YPEL2 in cells at amounts that lead to cell death. In addition, as we observed with overexpression of YPEL2 cDNA which is also valid for all ectopic transgene expression systems, a high level of synthesis of the protein could also lead to unpredictable consequences such as nonspecific protein biotinylation, and an increase in false-positive partner identification. To minimize potential experimental artifacts, we attempted to generate efficient biotinylation by using an inducible system that enables us to produce transgene, 3xFlag-YPEL2-Turbo-HA, in a controlled manner without inducing acute cell death. Here, what we want to accomplish is primarily to prevent nuclear membrane disassembly. To achieve this, we wanted 1) to establish a minimum amount of the inducer to stimulate the maximal amount of transgene expression; and 2) to optimize the process of biotinylation a) in a time- and b) biotin amount-dependent manner.

There are many inducible expression systems used in protein function studies. Tetracycline (Tet) inducible system is one of the widely used methods. In this inducible system, the transgene expression is tightly controlled by the presence of the antibiotic tetracycline (Tet) or its stabile derivative doxycycline. Also, this system provides a reversible gene expression by the withdrawal of the antibiotic. pINDUCER20-MCS is a single-vector system that provides constitutive expression of the Tet receptor rtTA3 and contains promoter sequences composed of tetracycline-response elements (tetracycline-responsive element, TRE) that regulate transgene expression. TRE is a tetracycline operator (tetO) composed of 7 repeats of a 19 nucleotides sequence. rtTA3, activated depending on Tet concentrations, associates with TREs, and triggers transgene expression.

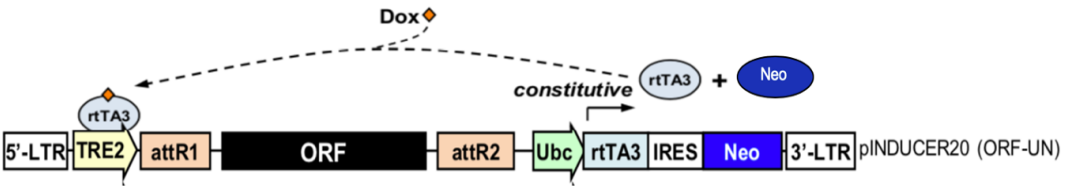


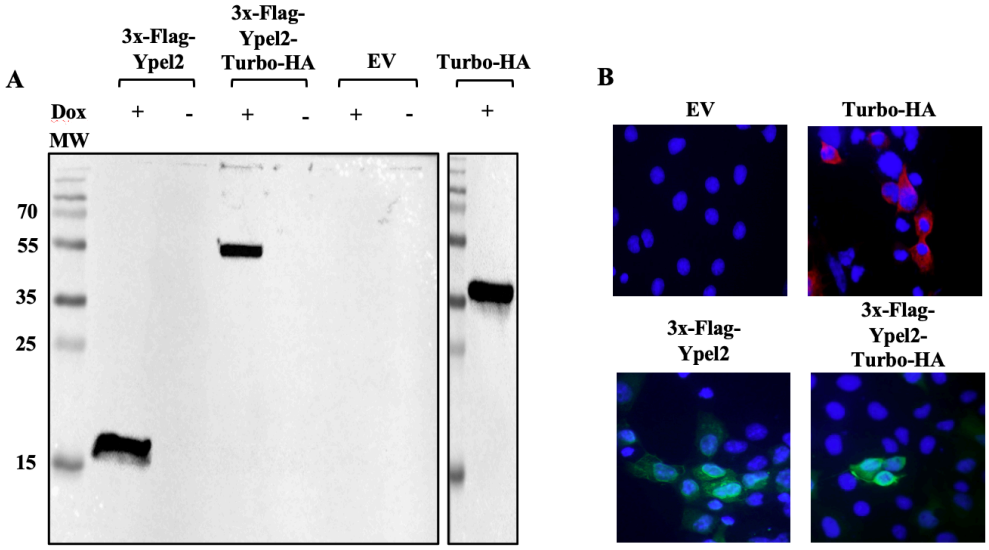
Figure 5: Working principle of pINDUCER20 vector system. pINDUCER20 vector contains transactivator protein (rtTA3), the binding site for transactivator protein (TRE2), and the gene of interest. In the presence of tet (or one of its stable analog doxycycline, Dox), transactivator protein (reverse tetracycline-controlled transactivator 3, rtTA3) binds to tet and undergoes a conformational change. This conformational change allows transactivator protein to bind transactivator response elements (TRE). This transactivator-TRE interaction turn on the transgene expression. The schema is adapted from Meerbrey et al., [30].

3.2.1 3xFlag-YPEL2, 3xFlag-YPEL2-Turbo-HA, or Turbo-HA transgene synthesis and localization in transfected COS7 cells.

After cloning, we examined the inducible expression of cloned genes in transiently transfected COS7 cells by western blot (WB) and also checked if YPEL2-Turbo fusion protein can change the subcellular localization of YPEL2 by using immunocytochemistry (ICC). We transiently transfected COS7 cells with pINDUCER20 vector without or with 3xFlag-YPEL2, 3xFlag-YPEL2-Turbo-HA, or Turbo-HA cDNA (Empty Vector, EV). We induced protein synthesis for 24 hours with a new medium containing 1 µg/ml doxycycline, the concentration of which was based on previous studies. In the WB analysis, we used the Flag antibody for the detection of 3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA and the HA antibody for the detection of Turbo-HA. The 3xFlag-YPEL2 cDNA was expected to synthesize protein with 16.8 kDa of 146 amino acids and the 3xFlag-YPEL2-Turbo-HA cDNA would synthesize a protein with a molecular mass of 53.3 kDa (MW), and Turbo-HA with a molecular mass of 37 kDa (MW). Our results show the expression of proteins with the expected molecular mass only in the presence of Dox. The Flag-M2 antibody did not detect any protein in the extracts of COS7 cells transfected with the empty vector (EV) (Figure 6A).

Our ICC results, performed with the approach used for WB described above, indicated that 3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA proteins show diffuse intracellular staining with high localization at the nuclear periphery. Also, ICC results showed that the 3xFlag-YPEL2-Turbo-HA fusion protein and Turbo-HA can efficiently biotinylate the cell proteins in the presence of biotin. Importantly, the overlap of the Flag antibody and the Biotin antibody staining indicates that 3xFlag-YPEL2-Turbo-HA biotinylates proteins where it is located (Figure 6C). These findings suggest that the Turbo-HA fused with YPEL2 protein efficiently biotinylates proteins only in the presence of biotin.

This finding suggests that the Turbo-HA when fused to YPEL2 does not alter the intracellular localization of YPEL2 as well. However, we also observed that the overexpression of YPEL2 and the YPEL2-Turbo fusion, induced with 1 µg/ml Dox for 24 hours, causes nuclear membrane disruption and also cell death. This is a clear contrast to Turbo-HA which shows cytoplasmic staining without any change in nuclear membrane integrity and cell survival (Figure 6B). These results, therefore, indicate that defects in nuclear membrane integrity and acute cell death is due to over-synthesized YPEL2 but not due to Turbo-HA alone or as a fusion to YPEL2.



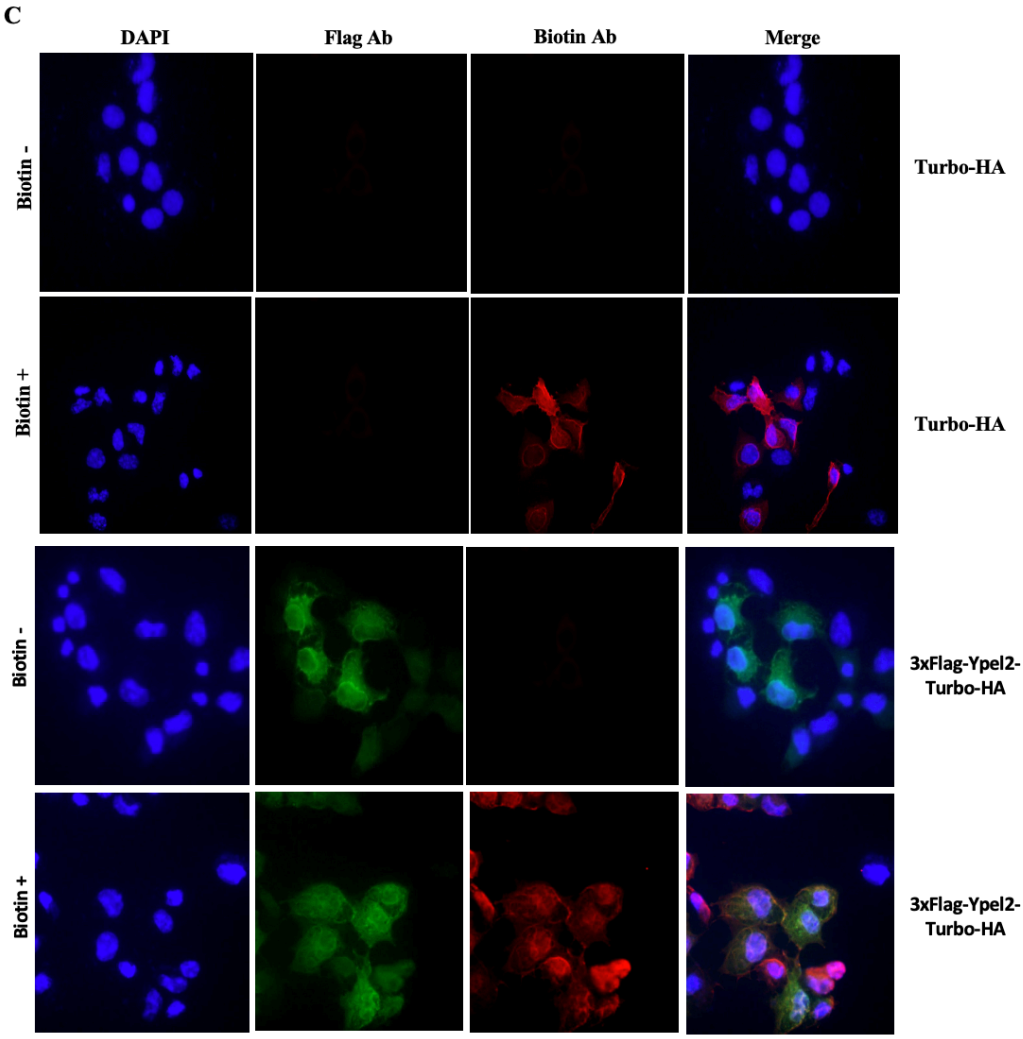


Figure 6. Transgene synthesis, location and biotinylation activity in transfected COS7 cells. (A) Cells were transfected with the pINDUCER20-MCS vector carrying no cDNA (Empty Vector, EV), or Turbo-HA, 3xFlag-YPEL2, or 3xFlag-YPEL2-Turbo-HA cDNA. 24 hours after transfection, cells were treated with 1 ug/ml doxycycline to induce protein synthesis. Cells were collected 24 hours after induction for total protein isolation. Equal amounts (75 µg) of proteins were separated in 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins transferred to the membrane were subjected to WB for determination of 3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA with Flag antibody and Turbo-HA with HA antibody. Molecular mass (MW) markers are shown as kDa. Our findings show that in transiently transfected cells, transgene synthesis is efficient only in the presence of Dox. For ICC, COS7 cells transfected with the approach defined by WB. (B) Transgene proteins synthesized for 24 hours in response to Dox were used to determine the intracellular localization of transgene proteins. Cells were stained with DAPI, the Flag, or the HA antibody followed by green fluorescent

secondary (3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA) or red fluorescent secondary (Turbo-HA) antibody. **(C)** Biotinylation capacity in the presence or absence of 50 µg / ml Biotin was observed with the Biotin antibody (red). DAPI staining (blue) indicates the nucleus. Our superimposed imaging results showed that 3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA (green) are localized at the cytoplasm and periphery of the cell nucleus, and 3xFlag-YPEL2-Turbo-HA can effectively biotinylate the cell proteins in the staining with the Biotin antibody. In the cells that synthesize Turbo-HA, it is observed that the protein is localized in the cytoplasm of the cell in the staining with the HA antibody (red).

3.2.2 Determination of the Inducible 3xFlag-YPEL2, 3xFlag-YPEL2-Turbo-HA, or Turbo-HA Synthesis by Western Blot.

As our ICC results showed that the overexpression of YPEL2 protein in response to 1 µg/ml Dox causes nuclear membrane disruptions and also cell death. Thus, alterations in cellular integrity due to the over-synthesis of YPEL2 could cause non-specific protein biotinylation. To circumvent this problem, we needed to optimize the expression levels of YPEL2 protein. For this reason, we examined the responses of transgenes to different concentrations of Dox in COS7 cells with transient transfections. COS7 cells seeded in six-well cell tissue culture plates were kept for 48 hours in media containing 10% TET-Free FBS. We transiently transfected cells with pINDUCER20 inducible expression vectors without and with the 3xFlag-YPEL2, 3xFlag-YPEL2-Turbo-HA, or Turbo-HA cDNA. 24 hours after transfection, we induced protein synthesis with a new medium containing Dox at various concentrations (0-2.5-5-10-100-1000 ng/ml). Cells were then collected for total protein isolation. Equal amounts (75 µg) of proteins were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins transferred to the membrane were subjected to WB for the determination of the 3xFlag-YPEL2 and 3xFlag-YPEL2-Turbo-HA syntheses with the Flag antibody and Turbo-HA expression with HA antibody. Molecular mass (MW) markers are shown as kDa. Our results suggest that increasing Dox concentration increases protein synthesis reaching maximal levels at 100 ng/ml Dox concentration for YPEL2 and YPEL2-Turbo protein (Figure 7).

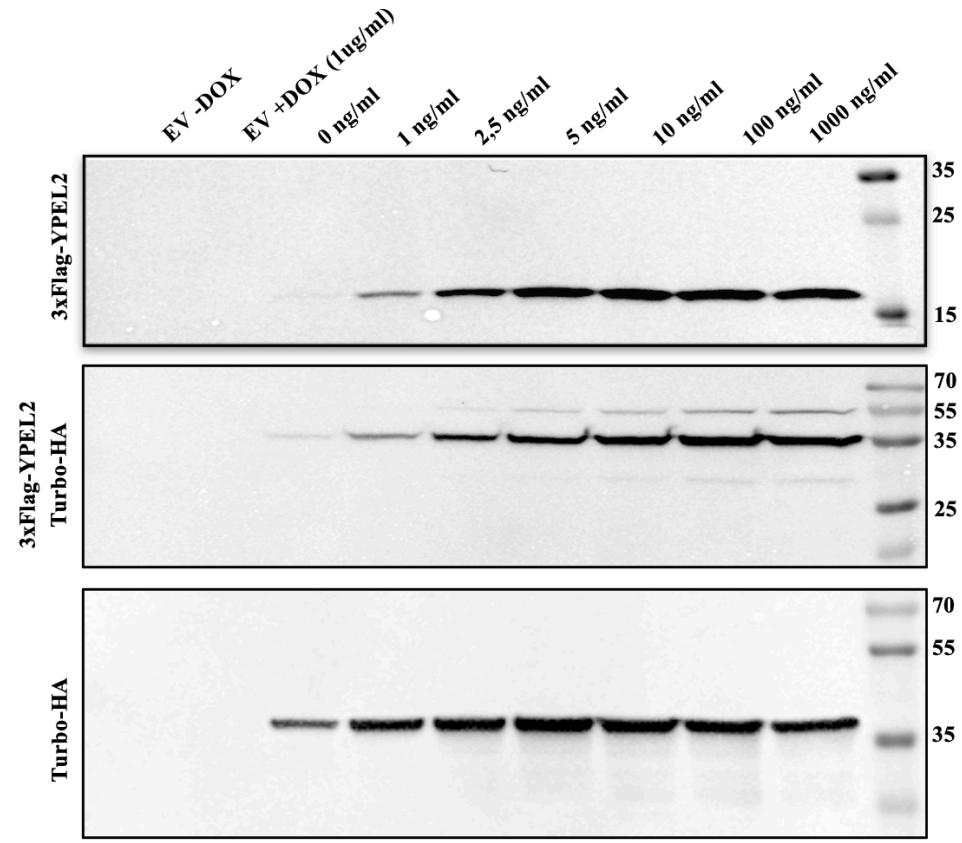


Figure 7. Transgene synthesis in response to Doxycycline in COS7 cells. Cells were transiently transfected with the pINDUCER20-MCS vector carrying no cDNA (Empty Vector, BV), 3xFlag-YPEL2, or 3xFlag-YPEL2-Turbo-HA cDNA. 24 hours after inducing protein synthesis, we collected cells with PBS. We added a 25x protease inhibitor cocktail (Roche, Germany) to RIPA lysis buffer and the extraction buffer solution, and we performed total protein isolation. Protein concentrations were measured by using Bradford Protein Assay (Bio-Rad, USA, 500-0201). We ran equal amounts (75 µg) of proteins in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated. We then transferred the proteins to the PVDF membrane. After blocking the membrane, we scanned it with the Flag antibody. After blocking the membrane, we scanned proteins with the Flag antibody and treated the membrane with a labeled secondary antibody and substrates (WesternBright ECL substrate, Advansta, CA, USA) sequentially. We imaged the chemiluminescence signal in Bio-Rad Image Processor.

3.2.3 Induced Expression Time-Course Optimization

Dox-dose response experiments indicate that Dox with increasing concentrations effectively increase the synthesis of the YPEL2 protein which reaches the maximal level at 100 ng/ml of Dox concentration. Since our aim here is to use the minimal amount of Dox to induce a level of YPEL2 synthesis that does not adversely affect the nuclear membrane integrity, we also wanted to optimize the duration of Dox induction in a Dox concentration-dependent manner. Protein synthesis induced with three different Dox concentrations (1ng/ml, 10ng/ml, and 100ng/ml) for 24, 36, and 48 hours. The cells were then subjected to ICC with LaminB1 to observe nuclear membrane integrity and with Flag antibodies (Figure 8). The results suggested that 1 ng/ml Dox induction led to a low amount of YPEL2 synthesis even in 48 hours and we surmised that this amount of protein would be insufficient for protein biotinylation. Whereas, 100 ng/ml dox induction resulted in high levels of YPEL2 synthesis and disruption of nuclear membrane integrity at all three-time points. Also, 100 ng/ml dox induction causes massive cell death after 24 hours. On the other hand, 10 ng/ml Dox induction for 24 hours provided efficient YPEL2 expression without altering the nuclear membrane integrity. We, therefore, selected 10 ng/ml Dox for subsequent experiments.

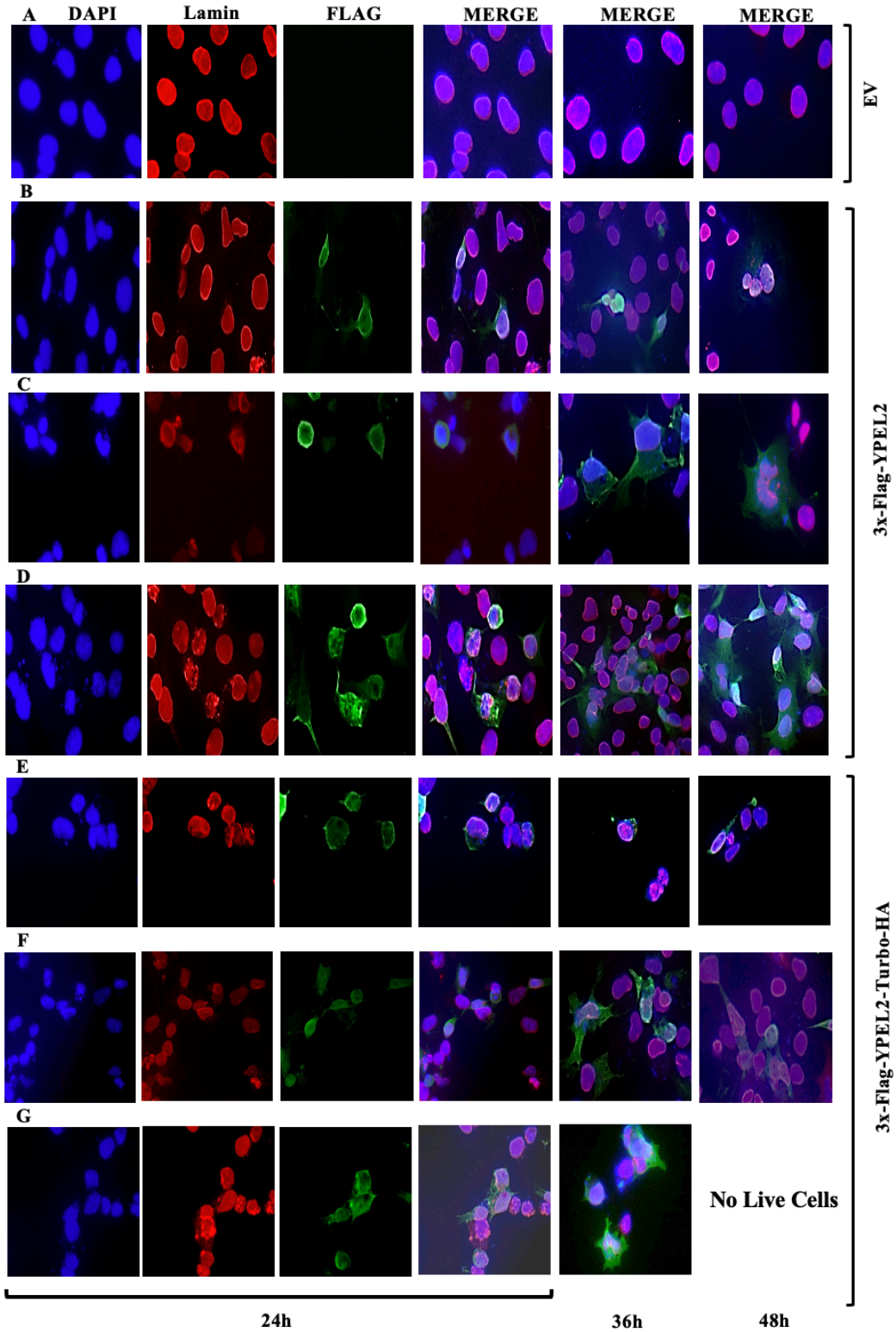


Figure 8. Time-dependent intracellular localization of transgene protein induced with different Dox concentrations in transfected COS7 cells. COS7 cells seeded in 12-well cell tissue culture plates were kept for 48 hours in media containing

10% TET-Free FBS. Then, we transiently transfected cells with pINDUCER20 inducible expression vector bearing 3xFlag-YPEL2, 3xFlag-YPEL2-Turbo-HA cDNA, or empty vector (EV) (A). 24 hours after transfection, we induced protein synthesis with three different Dox concentrations; (B) (E) 1 ng/ml, (C)(F) 10 ng/ml, (D)(G)100 ng/ml for 24,36 and 48 hours. Then, cells were washed with PBS and fixed with %3.7 formaldehyde. After fixation, cells were blocked with %10 BSA and incubated with LaminB1 and Flag primary antibodies. Nuclear membranes were detected with the LaminB1 (red) and the YPEL2 proteins were identified with the anti-flag (green) antibody. DAPI was used for nuclear staining.

3.3 Biotin Amount and Biotinylation Time Optimization

After completing the inducible system optimizations, we began TurboID optimizations. Generally, 50 µM biotin is used in the labelling of intracellular proteins and is sufficient for BioID system for 18 hours biotinylation. However, we know that TurboID has faster labeling kinetics and biotinylates proteins efficiently in a very short time, so we needed to test different concentrations of biotin amount in different time points to determine the optimum biotin amount at a specific time point.

To decide the optimal biotin concentration, we induced transfected cells with 10 ng/ml Dox for 24 hours. We then incubated the cells in a new medium containing 50 µM, 100 µM, or 250 µM of Biotin for 30 minutes, 1, and 3 hours. After each experiment period, we collected and lysed the cells. We compared the efficiencies of different biotin concentrations by WB. Our results showed that the 3xFlag-YPEL2-Turbo-HA fusion protein was able to biotinylate cell proteins efficiently starting from 30 minutes, and some protein profiles could change qualitatively and quantitatively with time (Figure 9). Increasing the amount of biotin after 30 minutes does not cause significant changes in the amount of biotinylation. The results suggest that using 50 µM of biotin over the experimental periods would effectively biotinylate cell proteins. We decided to use 50 µM biotin because increasing biotin concentration to more than needed may increase non-specific biotinylation in cells.

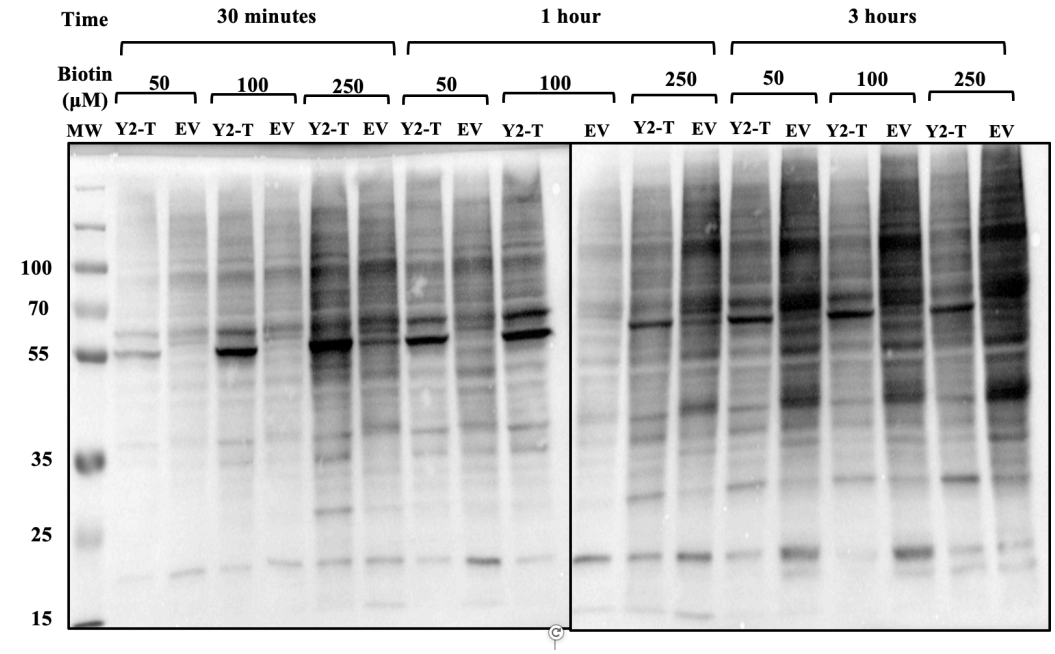


Figure 9. Determination of optimum biotin concentration for TurboID in COS7 cells. COS7 cells transfected with the pINDUCER20-MCS vector carrying 3xFlag-YPEL2-Turbo-HA (Y2-T) or Turbo-HA (EV) cDNA with 10 ng/ml doxycycline for transgene synthesis 24 hours after transfection. 24 hours after the response to the dox, the cells were incubated in a medium containing 50 µg/ml, 100 µg/ml, or 250 µg/ml biotin for 30 minutes, 1 and 3 hours. Cells collected after the experiment were lysed and separated by 10% SDS-PAGE. Samples were exposed to WB with a biotin antibody (1: 500 dilution, Abcam). Molecular mass (MW) markers are shown as kDa.

3.4 YPEL2 Associated Proteins in COS7 Cell Line

Based on our optimization studies, we selected 10ng/ml dox concentration, 50µM biotin amount, and 1, 3, 6, and 16 hours as the biotinylation time for dynamic proximity interaction network analysis by mass spectrometry. To carry out this, COS7 cells (60k/well) were plated in each well of two six-well tissue culture plates for 48 hours. Cells were then transiently transfected with pINDUCER20 empty vector (EV), pINDUCER20 Turbo-HA, or pINDUCER20 3x-Flag-YPEL2-Turbo-HA for 24 hours. Cells were then subjected to 10 ng/ml Dox for 24 hours. 24 hours later media was changed and a new medium containing 50 um biotin and 1 mM ATP for 1, 3, 6, and 16 hours were added onto the cells. At the end of a given time, cells

were lysed and biotinylated proteins in cell lysates were captured with streptavidin-conjugated magnetic beads. Protein fragments following on-bead tryptic proteolysis of the captured proteins were subjected to mass spectrometry (MS). 50 µl protein-streptavidin sample was aliquoted to test that the experiment was worked effectively by WB before we carried protein processing for MS analysis. Bound proteins in the aliquot were eluted from the streptavidin beads with Leamni-DDT sample buffer containing 500 nM D-Biotin at 98°C for 10 minutes and used for WB analysis (Appendix B).

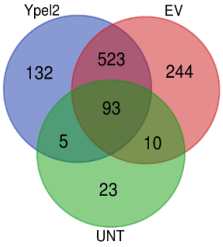
pINDUCER20-3x-Flag-YPEL2-Turbo-HA transfected COS7 cells are our test group; Turbo-HA and pINDUCER20 empty vector (EV) transfected COS7 cells were the negative control groups. Mass spectrometry identified a large number of proteins from each group conducted as two biological replicates carried out as two technical repeats. Subtractive analyses of identified proteins from the EV, Turbo-HA, and 3x-Flag-YPEL2-Turbo-HA synthesizing cells revealed 132 proximal interactors of YPEL2 for 1 hour, 106 protein for 3 hours, 81 proteins for 6 hours, and 119 for 16 hours depicted in Venn diagrams as well (Figure 10). List of all proteins identified by LC/MS-MS analysis shown in Table C.1 (Appendix C). Our findings indicate that the numbers and identities of YPEL2's possible protein partners vary with time as also depicted in Figure 11.

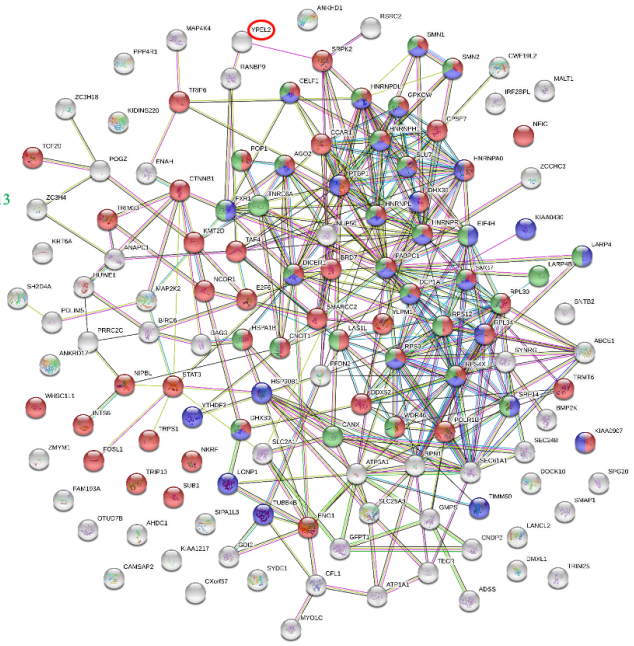
Gene ontology analyses for biological process and molecular function using the STRING software, which is a biological database source of known and predicted protein-protein interactions [31], suggest that YPEL2 interacts with proteins largely grouped in the nucleic acid metabolic processes, which can further be sub-grouped into RNA metabolic processes, RNA binding, and ribonucleoprotein complex proteins. (Figure 10). Proteins identified as RNA metabolic process proteins include PABPC1, DDX52, TAF15, TAF4, SMARCC2, TRMT6, TRIM33, CTNNB1, NCOR, and FUB1. RNA binding proteins comprise TARDBP, EEF1D, G3BP1, ELAVL1, AGFG1, SMN1, and PTBP1. Proteins involved in ribonucleoprotein complex include SQSTM1, DDX6, ATXN2L, UPF1, GNB2L1, LARP4B, and CANX.

A

COS7, Ypel2-Turbo, 1h Biotin Treatment

GO:0016070, RNA metabolic process, 57 of 3430, 7.14e-09
GO:0003723, RNA binding, 32 of 850, 8.45e-13
GO:1990904, Ribonucleoprotein complex, 31 of 770, 4.23e-13

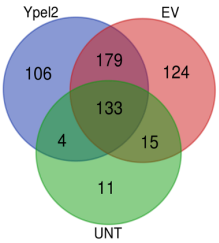


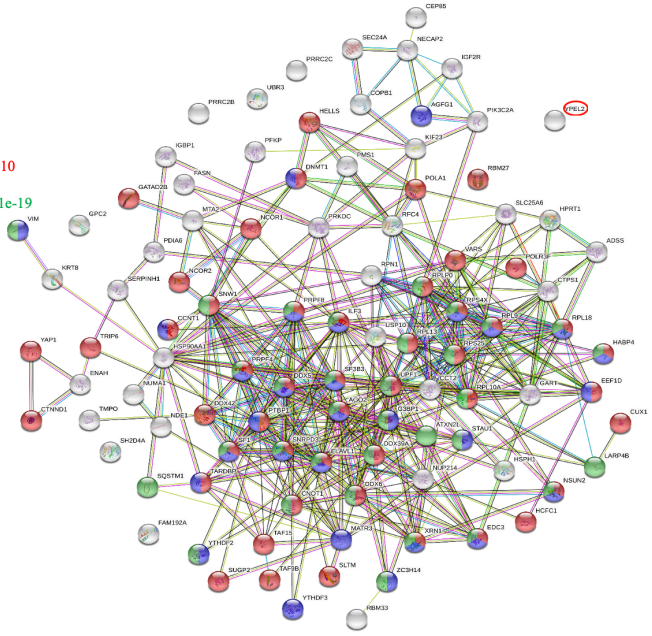


B

COS7, Ypel2-Turbo, 3h Biotin Treatment

GO:0016070, RNA metabolic process, 48 of 3430, 4.90e-10
GO:0003723, RNA binding, 29 of 850, 8.50e-14
GO:1990904, Ribonucleoprotein complex, 33 of 770, 5.01e-19





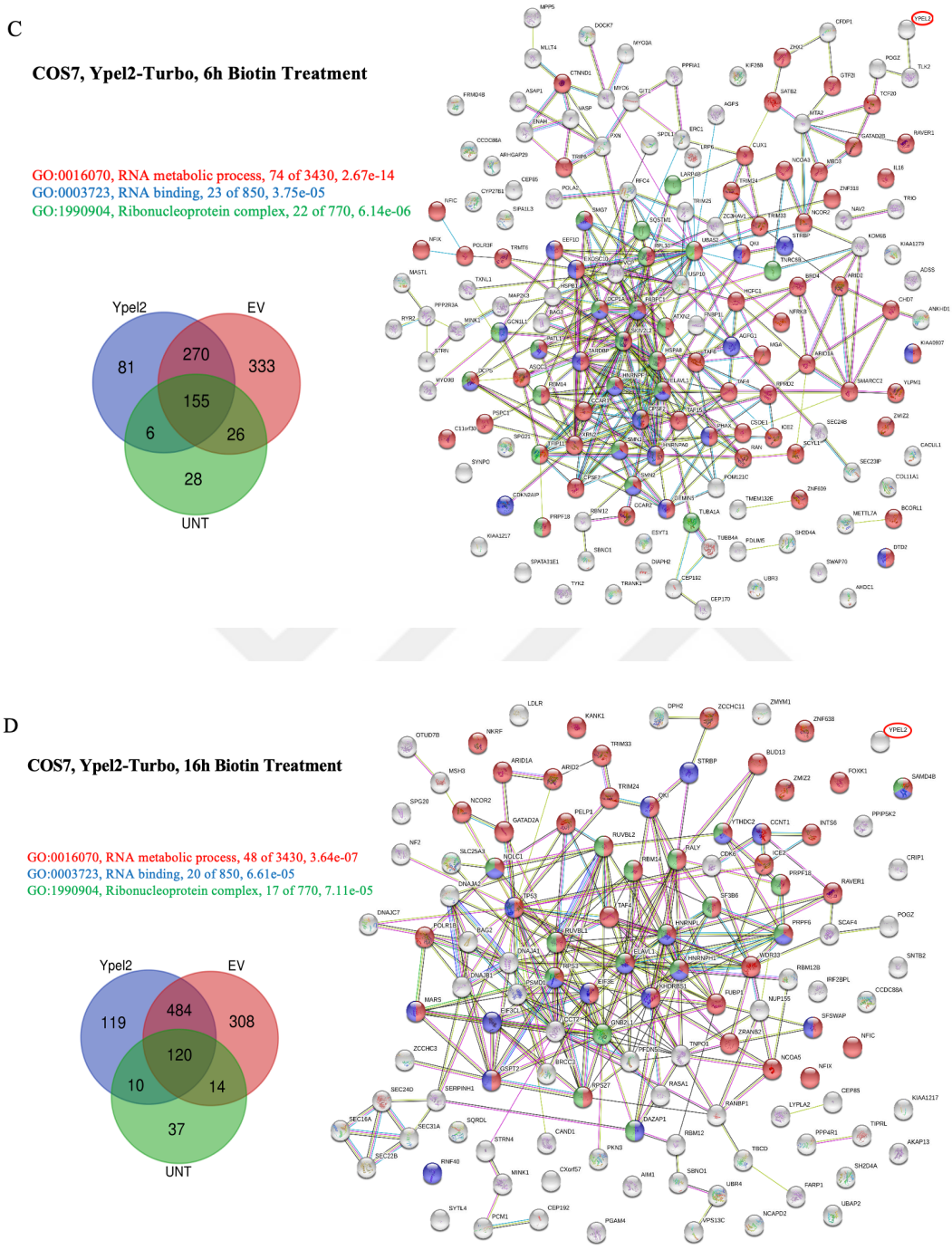


Figure 10. Summary of Mass Spectrometry Results. Display of TurboID-MS results according to their biological process and molecular functions by STRING software and Venn diagram representations of protein numbers performed for (A) 1 hour (B) 3 hours (C) 6 hours (D) 16 hours.

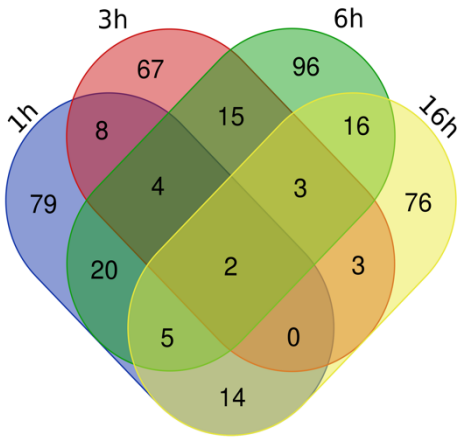


Figure 11. Venn diagram representation of YPEL2's possible protein partners in a time-dependent manner. The numbers and identities of YPEL2's possible protein partners vary with time. The intersections indicate the common protein numbers.

We observed that while the protein identities at time points change, the functional association remains largely unaltered; that is, in all-time points we tested, the putative YPEL2 interactors remain largely associated with RNA metabolic processes. Interestingly, we also observed that the number of proteins involved in gene expressions becomes more apparent at 6 hours and increases in numbers by 16 hours. This suggests that the early RNA metabolic events subsequently lead to alterations in gene expressions.

Furthermore, and importantly, the identities of some of the putative protein partners of YPEL2 appear to be associated with the stress granules (Table 1). Stress granules are cytoplasmic aggregates composed of proteins and RNA molecules and indicate mostly stalled translation initiation complexes promoting the repression of protein synthesis and usually form reversibly in response to altered cellular oxidative state, heat, or osmotic shock as well as viral infections [32]. The presence of stress granules allows cells to block or delay the activation of cell death pathways, repair the damage, and survive the stress. But sustained stress conditions eventually lead to cell death pathways including apoptosis or autophagy [32].

Our findings that the putative YPEL2 protein partners include proteins mostly involved in RNA metabolic processing and partly overlap with proteins involved in

the formation and sustaining stress granules suggest that YPEL2 is involved in stress responses and the sustained synthesis of it could lead to cell death.

Table 1. Common Proteins with Stress Granules

1h	3h	6h	16h
AGO2	AGO2	ANKHD1	AKAP13
ANKHD1	ATXN2L	BAG3	CEP85
ANKRD17	CEP85	CCAR1	DAZAP1
BAG3	CNOT1	CEP85	DNAJA1
CCAR1	CTNND1	CPSF7	EIF3E
CELF1	DDX6	CSDE1	ELAVL1
CFL1	EDC3	CTNND1	FUBP1
CNOT1	ELAVL1	DCP1A	HNRNPH1
CPSF7	G3BP1	GEMIN5	KHDRBS1
DCP1A	HABP4	HNRNPF	MARS
DHX30	HSP90AA1	HSPB1	NKRF
DHX36	ILF3	LARP4B	NOLC1
EIF4H	KIF23	NCOA3	QKI
FXR1	LARP4B	PABPC1	RACK1
GFPT1	NSUN2	PATL1	RANBP1
HNRNPDL	NUP214	PDLIM5	RBM12B
HNRNPH1	PRRC2B	PSPC1	RPS3
LARP4	PRRC2C	QKI	SAMD4B
LARP4B	PTBP1	RAN	SNTB2
MAP4K4	RFC4	SMG7	TNPO1
MARF1	SF1	SQSTM1	UBAP2
NKRF	SQSTM1	TAF15	ZCCHC3
NUP50	STAU1	TARDBP	ZNF638
PABPC1	SUGP2	TNRC6B	
PDLIM5	TAF15	TRIM25	
PRRC2C	TARDBP	TRIP6	
PTBP1	TRIP6	USP10	
SMG7	UPF1	VASP	
SMN1	USP10	YLPM1	
SNTB2	XRN1		
TNRC6A	YTHDF2		
TRIM25	YTHDF3		
TRIP6	ZC3H14		
YLPM1			
YTHDF3			
ZCCHC3			

3.5 Verification of YPEL2 interactions with putative protein partners.

In the TurboID system, although proteins are biotinylated according to direct or indirect interaction, biotinylation can occur without physical contact between proteins due to proximity. Therefore, the interaction between proteins must be

verified with other approaches. For further confirmation, we performed co-immunocytochemistry and co-immunoprecipitation assays.

Based on our TurboID-MS analyses and implications from STRING results, we chose six proteins as possible protein partners of YPEL2 for verification. The potential YPEL2 protein partners whose information we have compiled from GeneCards: The Human Gene Database database; EEF1D (eukaryotic translation elongation factor 1 delta) is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome, ADSS (adenylosuccinate synthase) plays a role in the conversion of inosine monophosphate to adenosine monophosphate, ELAVL1 (ELAV Like RNA Binding Protein 1) protein regulates mRNA stability by binding to the 3'-UTR region of mRNAs, SQSTM1 (Sequestosome 1) also known as ubiquitin-binding protein, is an autophagy receptor required for selective macroautophagy, and TARDBP (TAR DNA Binding Protein) is an RNA-binding protein that is involved in various steps of RNA biogenesis and processing, TAF15 (TATA-box binding protein associated factor 15) RNA and ssDNA-binding protein that may play specific roles during transcription initiation at distinct promoters and can enter the preinitiation complex together with the RNA polymerase II (Pol II). The common properties of the selected proteins are that they play a role in the cellular stress response and/or the formation of the stress granule complexes.

3.6 Co-immunoprecipitation (Co-IP) of YPEL2 protein with its protein partners.

To confirm the interaction between YPEL2 and ELAVL1 proteins, we performed co-immunoprecipitation experiments. We transiently transfected COS7 cells with pcDNA-HA-YPEL2 and/or pcDNA-3x-Flag-ELAVL1 expression vectors. After 36 hours, the immunoprecipitation was carried out with the nuclear extracts of transfected COS7 cells by using the HA antibody together with the A and G magnetic beads. We also performed an immunocytochemistry assay using the Flag antibody

to assess the co-localization. The Co-IP coupled WB analysis was used to verify the interaction between YPEL2 and ELAVL1 proteins by using the Flag (HA for immunoprecipitation with Flag antibody) antibody. Results suggested that there is an interaction between these two proteins (Figure 12).

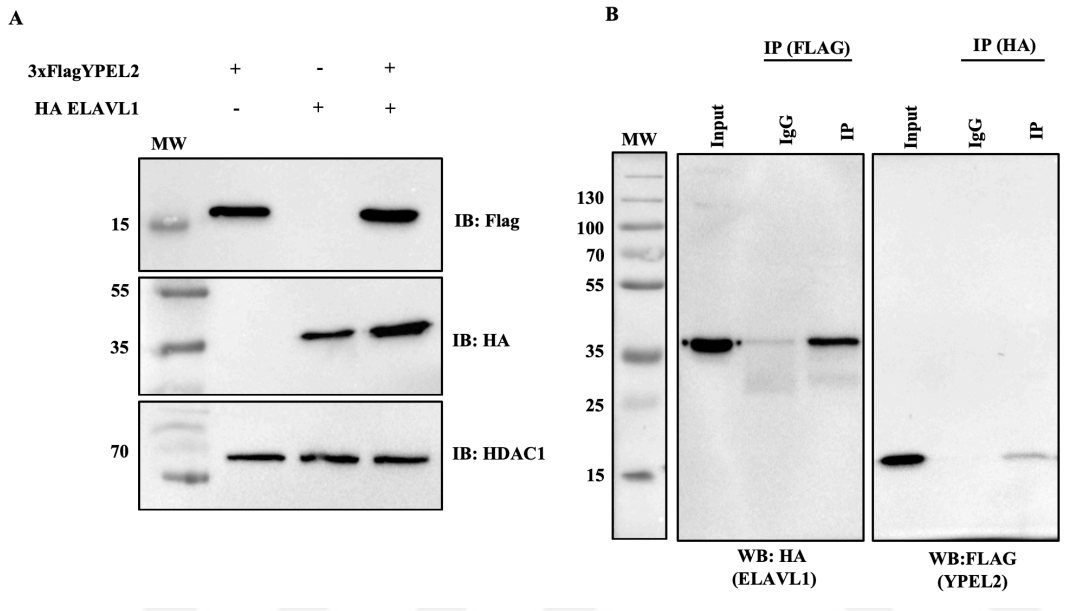


Figure 12. Interaction of YPEL2 and ELAVL1 in COS7 cells. Protein synthesis of indicated constructs was also verified by western blot using either the HA or the Flag antibody. HDAC1 was used as a loading control (A). COS7 cells transiently co-transfected with pcDNA-3x-Flag-YPEL2 and pcDNA-HA-ELAVL1, and were subjected to Co-IP with HA or Flag antibodies separately, also isotype-matched IgG for the control group. Precipitated protein was detected with Flag or HA antibodies in WB. 50 µg total protein extract was used for input and 500 µg protein was used for Co-IP (B). MW shows molecular weights in kDa.

3.7 Subcellular Localization of YPEL2 Protein Partners

After the verification that ELAVL1 and YPEL2 proteins are interacting partners, I wanted to examine, as a preliminary observation, their intracellular localization with immunocytochemistry. COS7 cells were transiently transfected with the pcDNA-3x-Flag-YPEL2 and/or pcDNA-HA-ELAVL1 vectors for 24 hours. The Flag antibody (green) was used to detect YPEL2, and the HA antibody (red) was used to identify

ELAVL1 (Figure 13). YPEL2 showed a diffused cellular location; whereas, ELAV1 was primarily localized to the nucleus and showed partial localization in the cytoplasm. Both proteins showed an overlapping pattern and importantly, two proteins overlap at prominent knots in the cytoplasm that could be attributed to stress granules.

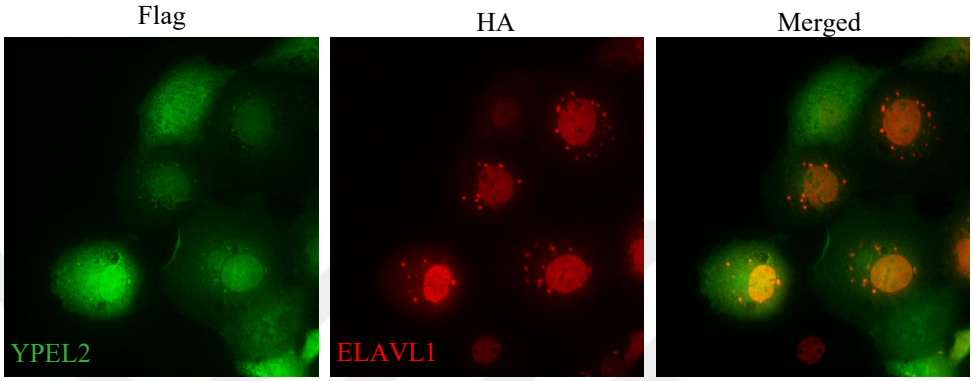


Figure 13. Immunocytochemisrty of subcellular localization of the YPEL2 protein and its protein partner ELAVL1. COS7 cells were transiently transfected with the pcDNA-3x-Flag-YPEL2 and the pcDNA-HA-ELAVL1 vectors for 24 hours and stained with the Flag or the HA antibody. The nucleus was stained with DAPI (data not shown).

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

As proteins carry out their functions in dynamic protein interaction networks, determining protein partners will provide important information about the functions of the YPEL2 protein. The proximity-dependent biotinylation (BioID) approach has been effectively used to define time-and-spatial relationships between proteins in cells. Over-expression of YPEL2 protein causes the nuclear membrane disassembly, DNA leakage to the cytoplasm, and cell death. This necessitated the development of an inducible system for the expression of YPEL2 to permit an effective functional analysis of YPEL2 without causing eventual cell death. Based on our results:

1. We conclude that TurboID is more effective than BioID for determining protein partners of YPEL2 protein. To identify the protein partners of YPEL2, we used the TurboID approach.
2. We generated an inducible system that expresses a functional TurboID fused YPEL2 without causing cell death.
3. Mass spectrometry analyses identified many potential YPEL2 interacting proteins labeled with biotin for 1 hour, 3 hours, 6 hours, and 16 hours. The majority of early time proteins are involved in the RNA metabolic processes and are known proteins that are involved in the formation and maintenance of stress granules. We, therefore, predict that YPEL2 is a stress response protein playing a critical role in cellular responses to stress.
4. Our preliminary studies using co-localization and Co-IP approaches suggest that YPEL2 interacts with ELAVL1.

In our ongoing experiments,

1. Since one of the consequences of the stress granule formation is the temporary suspension of protein synthesis until stress is lifted, we also plan to investigate alterations in protein synthesis using various biochemical approaches when YPEL2 synthesis is induced.
2. We also plan to generate a recombinant YPEL2 protein to assess its structural and functional features for further delineation of its role in cellular events.
3. We are also in the process of testing the interaction of YPEL2 with the putative interacting partners using inducible expression systems using co-localization, Co-IP, and Proximity Ligation Assay, which is a powerful approach that provides the detection of protein-protein interactions with high sensitivity and specificity [32].

We believe deciphering the functional features of YPEL2 would also contribute to the role of evolutionary conserved Ypel family proteins in cellular events.

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APPENDIX A

PRIMERS

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

Primer Name	Sequence (5'-3')
XhoI YPEL2 FP	CGCTATGCTCGAGGTGAAGATGACAAGATCGAAG
BamHI YPEL2 REP	CGCATGGGATCCGTCCCAGCCATTGTCCTTGATCATG
BamHI NotI BirA FP	CGCATGGGATCCGCGGCCGCAGGCGGCAGCGCTAGCAAAGACAATACTGTG
KpnI PolA HA AgeI BirA REP	CGCATGGGTACCTTTATTAAGCGTAATCTGGAACATCGTATGGGTAACCGGTGTCGGCCCTGCTGAATTCCTTTTC
pINDUCER20 BstAP1 FP	CTGATGCATCTGTTGCAACTCACAGTCTGGGGCATCAAG
pINDUCER20 MCS REP	CTGATGTCGACTGATCAGTTTAAACACGCGTATGCATGCTAGCGTATACCTCGAGTGATATCTGCAGAATTCCACCACACT
Bstz17I 3xFlag pInducer20 FP	CGCATGGTATACCCATGGAAGACTACAAAGACCAT
XhoI Turbo FP	CGCTATGCTCGAGATGGCTAGCAAAGACAATACT
MluI HA REP	CGCTATGACGCGTTTTATTAAGCGTAATCTGGAA

Table A.2. Tag sequences (DNA and aminoacid)

3xFLAG	gactacaaagaccatgacggtgattataaagatcatgacatcgactacaaagacgatgacgacaag
	DYKDHDGDYKDHDIDYKDDDDK
HA	tacccatacgatgttccagattacgct
	YPYDVPDY A

APPENDIX B

WB RESULTS OF PRECIPITATED PROTEINS BY TURBOID ASSAY

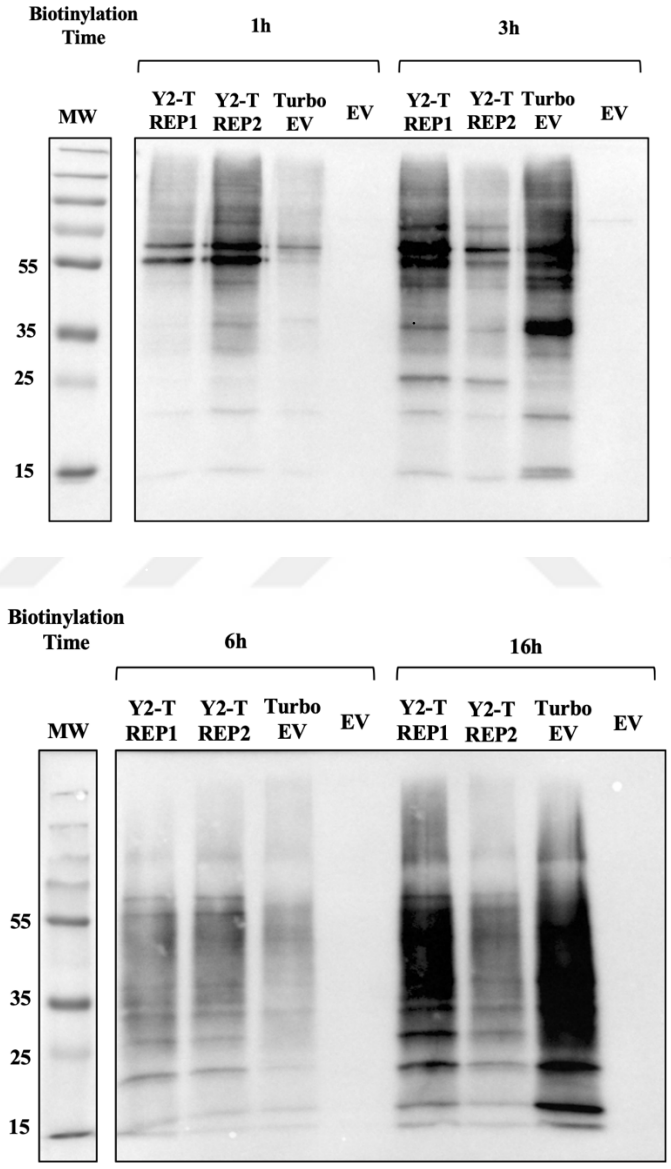

Figure B.1. WB results of precipitated proteins after 1,3,6,16 hours biotinylation time for LC/MS-MS analysis.

APPENDIX C

LIST OF PROTEINS IDENTIFIED BY LC/MS-MS ANALYSIS

Table C.1. List of proteins identified by LC/MS-MS analysis

1h	3h	6h	16h
CNOT1	CNOT1	TAF4	SBNO1
MYO1C	DDX39A	ZNF609	EIF3CL
TAF4	PIK3C2A	TXNL1	TAF4
KMT2D	PRPF4	STRN	ARID1A
HNRNPDL	CCNT1	SIPA1L3	SEC16A
WDR46	CTNND1	CTNND1	TRIM24
NKRF	NCOR1	DIAPH2	NKRF
HNRNPR	MTA2	BRD4	PPIP5K2
SIPA1L3	SEC24A	CSDE1	CCNT1
ANKRD17	STAU1	SEC24B	DNAJA2
NCOR1	HPRT1	BAG3	RNF40
E2F6	RPN1	MBD3	SEC22B
SLU7	RPLP0	TUBB4A	TIPRL
SEC24B	KRT8	HSPB1	NUP155
BAG3	HSP90AA1	HSPA8	SEC24D
MAP4K4	VIM	PABPC1	PRPF6
KRT6A	POLA1	EEF1D	SEC31A
RPN1	IGF2R	ADSS	SCAF4
ATP1A1	SLC25A6	CUX1	ZRANB2
ENO1	CTPS1	YLPM1	LYPLA2
NFIC	DDX5	TAF6	BAG2
HSPA1B	GART	VASP	LDLR

SLC2A1	PPIB	HCFC1	TP53
PABPC1	DDX6	AGFG1	NFIC
HSP90B1	DNMT1	HNRNPF	HNRNPL
HNRNPL	RPL13	RAN	MSH3
FOSL1	PTBP1	GTF2I	RASA1
CFL1	VARS	EXOSC10	RPS3
RPS12	EEF1D	TARDBP	DNAJB1
ATP5F1A	ADSS	HNRNPA0	DNAJA1
PTBP1	RPL9	SQSTM1	HNRNPH1
CANX	RFC4	TRIM25	NF2
ADSS	NUP214	USP10	RPS27
HNRNPH1	CUX1	NFIX	RANBP1
CTNNB1	TMPO	TRIP6	BRCC3
MAP2K2	MATR3	KIAA1217	CRIP1
LONP1	RPS9	AHDC1	SERPINH1
SRP14	YAP1	RPRD2	MARS
STAT3	FASN	ARID2	EIF3E
RPS9	SERPINH1	CEP85	RACK1
RPL34	HCFC1	UBR3	CCT2
YLPM1	AGFG1	TUBA1A	FOXK1
GMPS	COPB1	POGZ	SLC25A3
GDI2	PMS1	KHDC4	CDK6
FXR1	RPS13	PATL1	KHDRBS1
SUB1	SNRPD3	TLK2	AKAP13
ABCE1	RPS4X	ANKHD1	SFSWAP
SEC61A1	RPS25	CCAR1	SNTB2
RPS4X	RPL10A	RAVER1	KANK1
RPL30	IGBP1	CCAR2	NFIX
TUBB4B	CCT2	SYNPO	ZNF638

FAM193A	PRKDC	CPSF7	NOLC1
SRPK2	PFKP	ENAH	NCAPD2
SLC25A3	KIF23	SMARCC2	PCM1
GFPT1	RPL18	CEP192	ELAVL1
CAMSAP2	NSUN2	GEMIN5	CCDC88A
HNRNPA0	ILF3	PSPC1	SAMD4B
SNTB2	TARDBP	GATAD2B	ZMYM1
TRIM25	G3BP1	SMG7	UBR4
EIF4H	SQSTM1	LARP4B	KIAA1217
TRIP13	SNW1	TAF15	UBAP2
TRIP6	USP10	DCPS	TUT4
SMN2	NUMA1	DTD2	ICE2
SMN1	PDIA6	MASTL	ARID2
CWF19L2	SF3B3	PDLIM5	OTUD7B
TIMM50	SF1	DOCK7	RADX
ZMYM1	TRIP6	RBM14	CEP85
KIAA1217	ELAVL1	QKI	PKN3
AHDC1	PRRC2B	YPEL2	VPS13C
OTUD7B	HABP4	POLR3F	POGZ
NIPBL	CEP85	SH2D4A	CAND1
RADX	PRPF8	DCP1A	GATAD2A
SYDE1	ZC3H14	CPSF2	RBM12B
LARP4	UBR3	TFIP11	RAVER1
DHX30	YTHDF3	TRIM33	GSPT2
RSRC2	DDX42	TNRC6B	PELP1
POGZ	SUGP2	NCOR2	SPART
HUWE1	XRN1	NCOA3	PGAM4
YTHDF3	GPC2		MINK1
KHDC4	ENAH		ZMIZ2

ZC3H18	ATXN2L		CEP192
ANKHD1	GATAD2B		PPP4R1
CCAR1	HSPH1		TNPO1
SMAP1	LARP4B		FUBP1
SPART	TAF15		SYTL4
CPSF7	UPF1		DAZAP1
ENAH	RBM33		RBM14
TNRC6A	EDC3		QKI
SMARCC2	YPEL2		YPEL2
PPP4R1	SRSF8		STRBP
SMG7	FAM192A		PSMD1
LARP4B	POLR3F		PFDN5
CELF1	SH2D4A		DNAJC7
GPKOW	TAF9B		PRPF18
DOCK10	HELLS		DPH2
PDLIM5	NECAP2		BUD13
CNDP2	SLTM		TBCD
YPEL2	NDE1		WDR33
RANBP9	RBM27		IRF2BPL
POP1	SEPTIN9		YTHDC2
NSD3	AGO2		SH2D4A
ANAPC1	PRRC2C		POLR1B
IRF2BPL	YTHDF2		NCOA5
DHX36	NCOR2		STRN4
SH2D4A			RBM12
POLR1B			ZCCHC3
BRD7			RALY
DCP1A			INTS6
BIRC6			TRIM33

LANCL2			RUVBL2
BMP2K			RUVBL1
ZCCHC3			SF3B6
TECR			FARP1
MALT1			CRYBG1
TCF20			NCOR2
TRPS1			SQOR
PFDN2			
TRMT6			
AGO2			
NUP50			
INTS6			
KIDINS220			
SYNRG			
TRIM33			
ZC3H4			
DICER1			
DDX52			
DMXL1			
MARF1			
LAS1L			
PRRC2C			