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Defining Protein Interactions: Ankle Link Proteins of Stereocilia in Hair Cells

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Ankle Link Proteins of Stereocilia in Hair Cells

by

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Abstract

Hearing loss presenting without any other defects is referred to as nonsyndromic, whereas hearing loss in combination with one or more defects is referred to as syndromic hearing loss. Usher syndrome, one example of syndromic hearing loss, is a genetic disorder that affects both hearing and vision. There are several genes associated with Usher syndrome. The genes *GPR98* and *USH2A* are critical for ankle link formation and when mutated result in deafness. Our lab (Barr-Gillespie Lab at OHSU) is interested in further characterizing the protein-protein interactions required to form the ankle links. This includes two genes (*PDZD7* and *MYO7A*) known to be important for stereocilia link formation, and a candidate member of the ankle link complex (*ANKRD24*). Co-IPs were performed to determine if ANKRD24, MYO7A and PDZD7 interact with previously characterized ankle link proteins GPR98 or USH2A. Based on results from this experiment, no definitive interaction was found between ANKRD24, MYO7A and PDZD7 with GPR98.

Introduction

The ear is divided into three main parts, the external ear, the middle ear, and the inner ear. Sound waves are collected by the external ear and travel down the ear canal, where they vibrate the eardrum. The eardrum articulates with the bones of the middle ear and mechanically transmits the sound waves to the inner ear. The sensory hair cells of the inner ear are contained within the cochlea and are responsible for the conversion of sound waves into neural signals. These signals are propagated to the brain via the auditory nerve and are ultimately perceived as sound.

The sensory hair cells are further categorized into inner and outer hair cells based on morphological and physiological characteristics. One of the most striking features of these cells is the hair-like bundles of stereocilia that project from their surfaces. The stereocilia are deflected in response to sound waves received from the environment and gated ion channel(s) found at their tips open in response to this mechanical stimulus. This physiological process, referred to as mechanotransduction, allows cations to enter the hair cells and triggers a receptor potential. Transduction channels are cation selective and depolarization activates neurotransmitter release at the base of the hair cell, conveying the hair cell's excitation to the central nervous system (Gillespie & Müller, 2009).

The receptor potential is an electrical signal that allows the hair cell to pass auditory stimuli received by the ear, to the brain via the auditory nerve fibers that connect to the bottom of hair cells. Hair cells do not fire action potentials; instead they display graded receptor potentials, where the change in cell potential from the resting level is proportional to the stimulus.

Genetic conditions, noise exposure or hair cell toxic drugs like some antibiotics can damage hairs cells, which lead to hearing loss. Hair cells do not regenerate in mammals so these insults cause permanent damage, leaving limited therapeutic options like hearing aids or cochlear implants available to patients (Nadol, 1993).

Mutations in the genes required for stereocilia link formation results in syndromic hearing loss

Hearing loss presenting without any other defects is referred to as nonsyndromic, whereas hearing loss in combination with one or more defects is referred to as syndromic hearing loss. Usher syndrome, one example of syndromic hearing loss, is a genetic disorder that affects both hearing and vision. There are three types: type 1, type 2 and type 3 (Williams, 2015). Type 1 causes deafness at birth with decreased night vision as a child. Type 2 causes moderate to severe hearing loss at birth with decreased night vision in the teens. Finally, type 3 causes progressive hearing loss in childhood with night vision problems beginning in the teens.

There are several genes associated with Usher syndrome, one of which is *USH2A. USH2A* encodes for the protein usherin, an important component of the basement membrane and plays a role in ear development. *MYO7A* is another Usher syndrome related gene, encoding for the protein myosin 7a; myosins are mechanochemical proteins (Sakai et al., 2015). These protein products have been shown to have interactions with each other and play an important role in maintaining the stereocilia (Ahmed et al., 2003).

Normal development of stereocilia is required for hearing function

A complex series of cellular events are required for the successful development of the stereocilia. One developmental requirement is the establishment of a series of linkages that connects a single stereocilium to its adjacent stereocilia. A subset of these links, which are localized to the base of the stereocilia, are referred to as ankle links and are required for normal hearing function. The genes *GPR98* and *USH2A* are critical for ankle link formation and, when mutated, result in deafness. Our lab (Barr-Gillespie Lab at OHSU) is interested in further characterizing the protein-protein interactions required to form the ankle links. This includes two genes (*PDZD7* and *MYO7A*) known to be important for stereocilia link formation and a candidate member of the ankle link complex (*ANKRD24*). It is suggested that MYO7A conveys proteins of the ankle link complex to stereocilia (Michalski et al., 2007).

PDZD7 is a paralog (genes related by duplication within a genome) of the scaffolding proteins harmonin and whirlin, which are associated with Usher syndrome. Mutations in *PDZD7* increase the severity of Usher type II syndrome, caused by mutations

in *USH2A* and *GPR98* (Zou et al., 2013). Usherin and GPR98 form hair cell stereocilia ankle-links. Immunofluorescence and by overexpression of tagged proteins in rat and mouse hair cells showed that PDZD7 localizes to the ankle-link region, overlapping with usherin, whirlin, and GPR98 (Grati et al., 2012).

Mouse models are used for to study hearing loss in depth

Transgenic mice are genetically modified mice and are commonly used for research as models for human diseases. Genetically modified mice are a useful tool for genetic manipulation, as their tissues and organs are similar to humans. A common type of genetically modified mouse is a knockout mouse, where a single gene is removed.
It is suggested that proteins encoded by the *Ush2a* gene assembles into the ankle link complex at the base of the stereocilia. Mice with mutations in the *Ush2a* gene have been critical in showing that USH2A proteins play a role in the ankle link complex. Loss of individual *Ush2a* gene expression can lead to different morphologies and functions. Mutant mice subjected to auditory brainstem response tests had significant threshold elevations in frequency range, relative to wild-type control mice. The gradient of hearing impairment in three USH mutant mice correlated with the degrees of ankle link complex disruption and stereociliary bundle defects (Zou et al., 2015). Knockout or abnormal expression of the *Ush2a*, *Gpr98*, *Whrn*, or *Pdzd7* gene causes disorganization and gradual degeneration of hair bundles in mice (Chen et al., 2014).

Determining protein interactions

One technique essential for testing interactions between proteins is immunoprecipitation (IP). IP is a technique used to isolate a protein complex from a cellular lysate and relies on the use of antibodies (specific for the protein of interest) to selectively capture the targeted protein. IP of intact protein complexes, such as those containing and antigen with bound proteins or ligands, is called co-immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein that is thought to be part of a larger complex of proteins. By targeting this known protein with an antibody, it can also pull down the entire protein complex out of solution. As a result, one can see which proteins are interacting with each other and are members of a

complex. This works when the proteins involved in the complex bind to each other tightly, allowing to pull down multiple proteins of the complex out. The first experimental procedure required for performing a Co-IP is to express the targeted proteins by transfection. A transfection is when a plasmid (an assembly of nucleic acids) is introduced into cells. The plasmid has the genetic information needed to encode the target protein and a tag attached to it. The tag aids in the visualization and purification of the protein.

Protein interactions are observed by performing a western blot. Western blots use gel electrophoresis to separate denatured proteins by length. The proteins are then transferred to a membrane, where they are stained with antibodies that are specific with that target protein (Towbin et al., 1979). For a western blot, samples can be taken from whole tissue or from cell culture. Solid tissues can be broken down and homogenized, while cells can be lysed via detergents. Afterwards, proteins are separated by gel electrophoresis, using a polyacrylamide gel buffers containing SDS (sodium dodecyl sulfate). Because proteins become covered in the negatively charged SDS, they move towards the positively charged electrode through the gel. Next, the proteins are moved from the gel onto a membrane made of polyvinylidene difluoride (PVDF). This transfer is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF. The membrane is then probed for the protein of interest, with an antibody. Finally, the western blot is ready for detection of probes that are bound to the protein of interest.

LCK

LCK is a protein tyrosine kinase that contains the amino acid sequence GCVCSSNPE. This sequence targets LCK, or another other protein containing the sequence, to bind to the plasma membrane (Klapisz et al., 1999).

Methods/Procedure

General cell culture and maintenance

HEK cells (human embryonic kidney 293) were grown in 1X DMEM growth media containing 10% FBS and 1% Pen-strep. Cells were incubated at 37°C with 5% carbon dioxide.

Plasmid preparation

The 140 amino acid intracellular c-terminal side of GPR98 was used with the amino acid sequence:
wmlvlfvifnslqglyvfvvyfilhnqtccpmkasytvemnghpgpstafftpgsgippageinkstqnlinameevpsdwerssfqqtsqaspdlktspqngasfpssggygpgsliadeesqefddlifalktgaglsvsdnesgqgsqeggtltdsqivelrripiadthl (GenBank AAL30812.1). This construct was engineered to contain the LCK tag, followed by the attachment of two flexible linker MYC tags, then with the 140 amino acid end of GPR98 on the c-terminal side.

Transfections

Western blot and Co-IP:
HEK cells were transfected at 40-50% confluency, using the Qiagene Effectine Kit. Single transfections included: 400 ng GPR98-STREP, 400 ng PDZD7-GFP, 400 ng MY07A-GFP and 800 ng ANKRD24-GFP. Co-transfections consisted of 400 ng GPR98-STREP and 400 ng PDZD7-GFP, 400 ng GPR98-STREP and 400 ng MYO7A-GFP, and 400 ng GPR98-STREP and 800 ng ANKRD24-GFP. Cells were incubated at 37°C overnight and prepared for IP the following day. When this experiment was repeated a second time, transfections were performed in duplicates.
Immunofluorescence Assay:
HEK cells were transfected at 40-50% confluency, using the Qiagene Effectine Kit. Single transfections consisted of 500 ng LCK-2xMYC-GPR98 and 500 ng MY07A-GFP. Co-transfections consisted 500 ng LCK-2xMYC-GPR98 and 500 ng MY07A-GFP. Cells were incubated at 37°C overnight.

Generating HEK cell Lysates

After 24 hours of transfection, cells were washed with 1x PBS and dislodged in 300 μl of lysis buffer (When this experimented was repeated a second time, 500 μl of lysis buffer was used instead). The contents were transferred to a 1.5 ml tube and incubated on ice for 30 minutes. After incubation, the cells were spun down at 12,000 x g for 10 minutes and the soluble fraction was saved (about 250 μl for the IP and 50 μl for the western blot). When this experiment was repeated, samples were sonicated at 30% for 3 pulses of 15 seconds each, after incubating on ice for 30 minutes. Additionally, 100 μl of the supernatant was saved for the western blot while 900 μl was saved for the IP instead.

Western blot:

5 μl of lysate (from the 50 μl fraction) of each sample were added to a tube containing 2.5 μl of sample buffer, 1 μl of reducing agent and 1.5 μl water for a total of 10 μl and samples were incubated at 95°C for 10 minutes. During the repeat of this experiment, 100 μl of 2x sample buffer was added to the samples instead.

Co-IP:

10 μl of Strep-Tactin beads were used per sample and beads were washed 5 times with 1x PBS. After the final wash, 100 μl of 1x PBS was added to the beads and they were distributed to each sample and incubated overnight at 4°C with end rocking motion. After the overnight incubation, samples were spun down at 3,000 x g for 3 minutes, the supernatant was removed and 250 μl of lysis buffer was added. Samples rotated at 4°C for 5 minutes, spun down again at 3,000 x g for 3 minutes, and this was repeated for a total of 4 washes. After the final wash, the supernatant was removed and 40 μl of sample buffer was added to each tube (10 μl sample buffer + 4 μl reducing agent + 26 μl H_2O = 40 μl sample buffer). The IPs were incubated at 95°C for 10 minutes, spun down 3,000 x g for 3 minutes and the supernatant was removed. During the repeat experiment, 200 μl of 1x sample buffer was added to the samples instead.

GFP Blot: 26 μl of lysate was added to a tube containing 10 μl of sample buffer, 4 μl of reducing agent and 0 μl water for a total of 40 μl and samples were incubated at 95°C for 10 minutes.

Strep Blot: 5 μl of lysate was added to a tube containing 2.5 μl of sample buffer, 1 μl of reducing agent and 1.5 μl water for a total of 10 μl and samples were incubated at 95°C for 10 minutes.

Running The Gels

4-12% Bis-Tris gels were run for 50 minutes using MOPS running buffer and 10 μl of each sample was loaded on the gel, except for the GFP blot containing strep-tactin beads, which was 40 μl. During the repeat experiment, 20 μl of each sample was loaded instead.

Transferring gel to membrane

After the 50 minutes, gels were soaked in transfer buffer for 15 minutes (100 ml methanol + 100 ml 10x transfer buffer + 800 ml water = 1L transfer buffer) while PVDF membrane was soaked in 100% methanol for 2 minutes and then in transfer buffer for 5 minutes. The transfer apparatus was set up for 1.5 hours at 60 volts.

Blocking and adding antibodies

After the transfer, the membrane was rinsed in water and incubated in block for 1 hour with ECL Prime Blocking Agent. After an hour, the blot incubated overnight in a 1:500 dilution (diluted in block) of the primary antibody (GFP or Strep-Tactin). The following morning, the blot was washed 4 times for 5 minutes each, in 1x PBS + 0.1% tween and then incubated for 1 hour in a 1:10,00 dilution (diluted in block) of secondary antibody goat-anti-mouse IgG. Finally the blots were washed 4 times for 5 minutes each, with 1x PBS + 0.1% tween.

Imaging the blot

The Amersham ECL Prime Western Blot Detection Reagent was used and 500 μl of reagent A with 500 μl of reagent B was added to each blot and incubated for 30 seconds. Following incubation, the blots were imaged with production of light from a chemiluminescence reaction and the light was detected by a CCD camera.

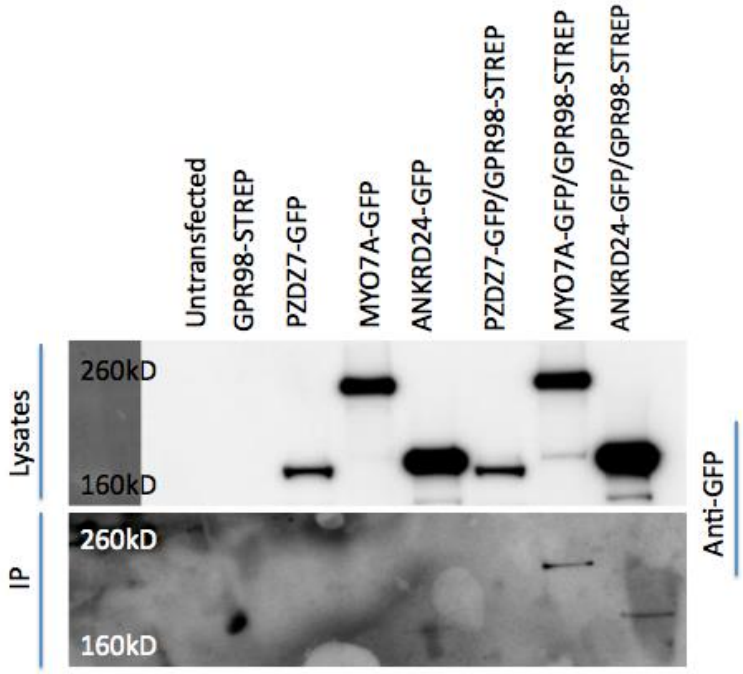
Immunofluorescence Assay

After about 24 hours of transfection, cells were washed with 1x PBS and fixed in 4% PFA for 30 minutes. Following the PFA, cells were washed 3 times in 1x PBS and

permeabilized for 10 minutes. Cells were washed 3 times again in 1x PBS and blocked for 1 hour. After 1 hour, cells were treated with a 1:500 dilution (in block) of the MYC antibody for 2 hours. They were then washed 3 times in 1x PBS and treated with a 1:500 dilution of secondary antibody (donkey-anti-mouse IgG Alexa Fluor® 647) for 1 hour. Finally cells were washed 3 times in 1x PBS and mounted for imaging.

Data

A.)



B.)

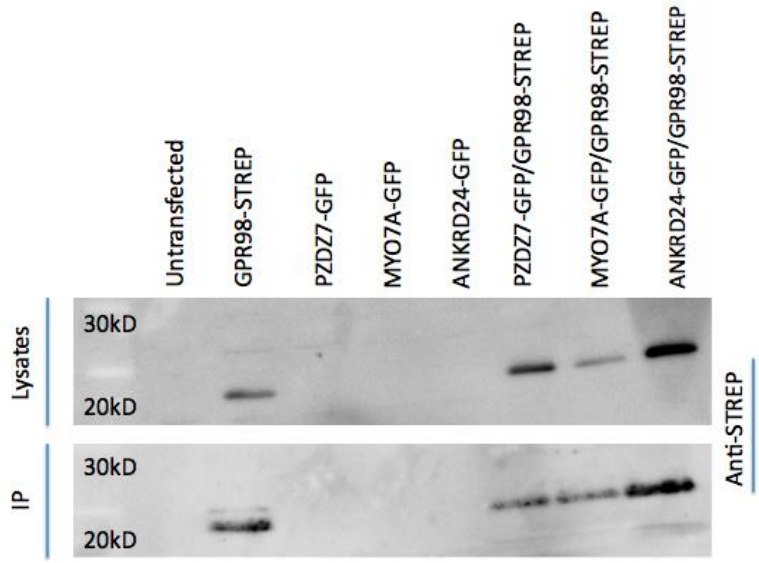
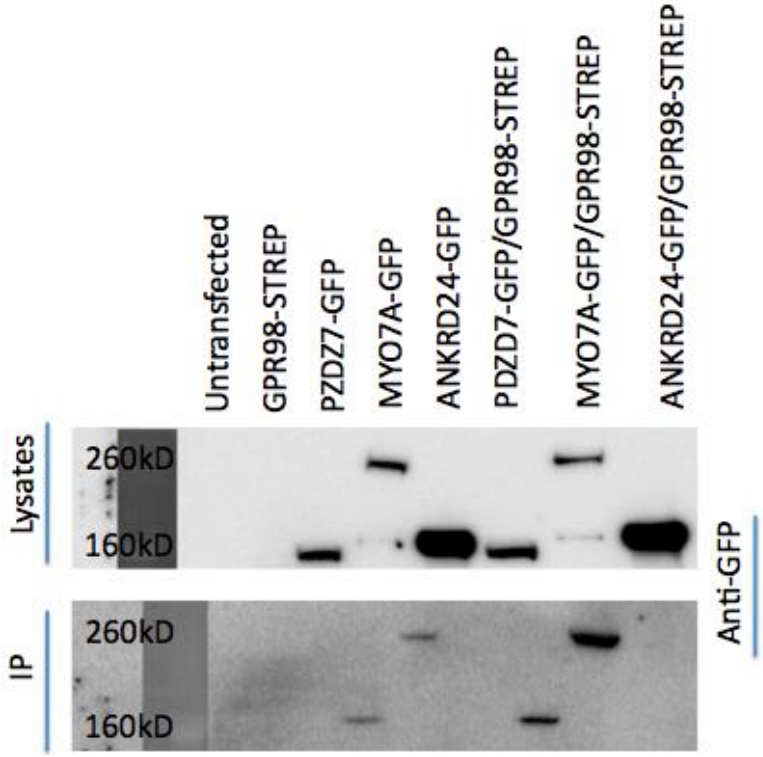


Figure 1: A.) GFP blot of lysates and IP from the first experiment B.) Strep blot of lysates and IP from the first experiment

A.)



B.)

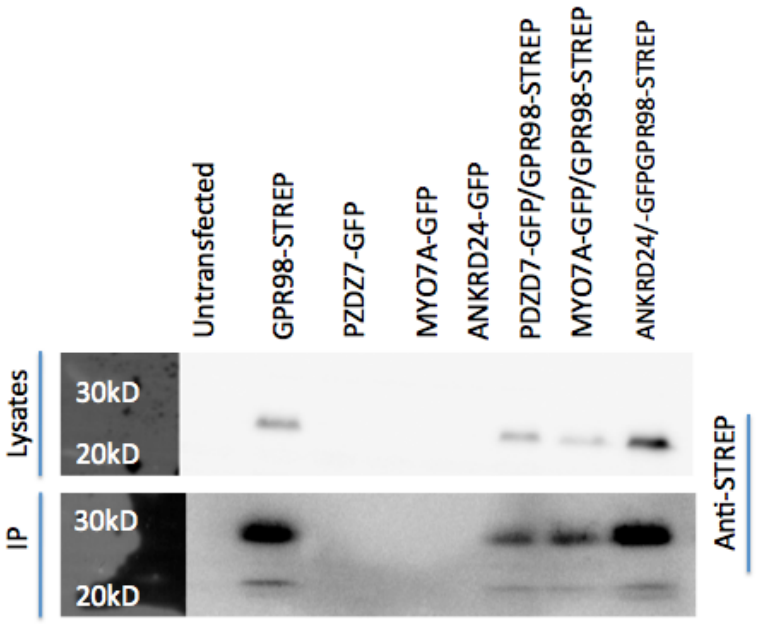


Figure 2: A.) GFP blot of lysates and IP from the second experiment with modified protocol B.) Strep blot of lysates and IP from the second experiment with modified protocol

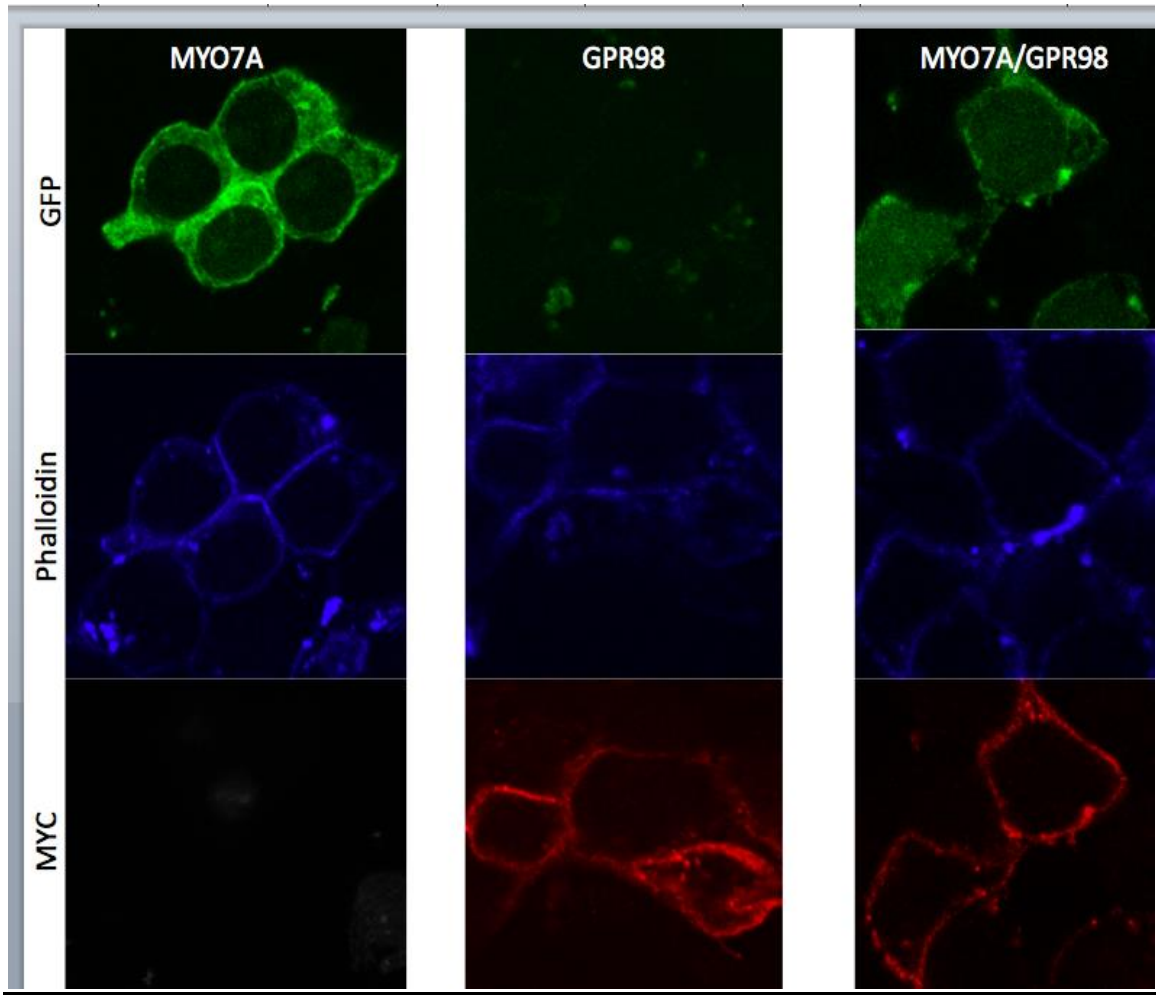


Figure 3: Microscopy images of immunofluorescence assay with MYO7A-GFP and LCK-2xMYC-GPR98

Figure 1a shows a band pulled down with a similar size to MYO7A, under the lane containing the co-transfection of MYO7A with GPR98. There was also a band under the lane containing the co-transfection of ANKRD24 with GPR98, with a size similar to ANKRD24. Moreover, there was a band with a similar size in the lane containing the ANKRD24 single transfection, but at a lower intensity. There was no band was detected under the co-transfection of PDZD7 and GPR98.

Figure 1a also shows a western blot of the samples probed with a GFP antibody and constructs containing a GFP tag (MYO7A, PDZD7 and ANKRD24) were detected for the single transfection, as well as for co-transfections with GPR98.

In figure 1b, the same blot was done (as in figure 1a) but a strep antibody was used instead. The constructs containing the strep antibody (GPR98) was detected for both the single transfection and co-transfection.

Western blots of the samples with the GFP antibody and strep antibody, figures 2a and 2b respectively, yielded the same results from the first experiment (figure 1a and 1b). Moreover, the IP blot with strep antibody also produced the same result from the first experiment. However the IP blot with the GFP antibody (figure 2a) gave confounding results from the first experiment. There was no band under the co-transfection of ANKRD24 with GPR98, but there was a band in the lane with the co-transfection of both MYO7A with GPR98 and PDZD7 with GPR98. Although the first experiment showed a band with the co-transfection of MYO7A with GPR98, it did not show one with PDZD7 and GPR98. Moreover, there were bands under the single transfections of both MYO7A and PDZD7.

Figure 3 shows that MYO7A remains in the cytoplasm after co-transfection with LCK-2xMYC-GPR98.

Discussion

Usher syndrome, a genetic disorder, is an example of syndromic hearing loss that affects both hearing and vision. There are several genes associated with usher syndrome. Co-IPs were performed to determine if ANKRD24, MYO7A and PDZD7 interact with previously characterized ankle link proteins GPR98 or USH2A.

Prior to performing the IP experiments, GPR98 and USH2A constructs (containing a double strep tag) were transfected in HEK cells. A western blot was run of the lysates to test the strep antibody and GPR98 was detected, but USH2A was not. When the blot was repeated but again USH2A was not detected; as a result, the IP experiment focused on interactions with GPR98 only.

Results from the first experiment (figure 1a) showed a potential interaction between MYO7A and GPR98. Because the samples were prepared with strep-tactin beads (which GPR98 would bind to) and the blot was detected with a GFP antibody (which would detect MYO7A), a band would entail a possible interaction between the two proteins. There was also a band under the lane containing the co-transfection of ANKRD24 and GPR98, with a size similar to ANKRD24. However there was a band with another similar size in the lane containing the ANKRD24 single transfection. If there was an interaction between ANKRD24 and GPR98, a band is predicted to appear in the co-transfection lane only. Because the intensity of the band in the co-transfection lane was higher than that of the band in the single ANKRD24 transfection, this experiment must be repeated under more stringent wash conditions to reduce the background. There was no band detected under the co-transfection of PDZD7 and GPR98.

When the experiment was repeated with a modified protocol to yield a more concentrated amount of elute for the IP, results from the blot suggested that GPR98 and MYO7A may not interact. There was no band under the co-transfection of ANKRD24 with GPR98, but there was also a band in the lane with the co-transfection of both MYO7A with GPR98 and PDZD7 with GPR98. Although the first experiment showed a band with the co-transfection of MYO7A and GPR98, it did not show one with PDZD7 and GPR98. Moreover, there were bands under the single transfections of both MYO7A

and PDZD7. This may have been due to MYO7A and PDZD7 binding nonspecifically to the strep-tactin beads. Again, this experiment must be repeated under more stringent wash conditions to reduce the background.

Because the first experiment gave results suggesting a possible interaction between MYO7A and GPR98, a transfection was performed with only MYO7A and GPR98 constructs. LCK targets protein binding to bind to the plasma membrane and it was predicted that MYO7A would be induced to the membrane, in a co-transfection with GPR98. Results showed that MYO7A remained fairly cytoplasmic and did not appear to have moved towards the membrane. This may suggest no interaction between MYO7A and GPR98, however this can also depend on the relative levels of GPR98 and MYO7A expressed. If there is a greater concentration of MYO7A than GPR98, it could be difficult to see a small fraction of MYO7A moving to the membrane. This experiment should be repeated with varying concentrations of each construct, in order to verify results.

Throughout the experiment, there was room for multiple sources of error. During the transfections, the wrong concentration of plasmids may have been used, or cells could have been transfected with the wrong plasmid. During preparation of lysates, incorrect amounts of lysis buffer or sample buffer could have been added due to pipette errors, leading to variable amounts of each sample and affecting final concentrations. During the western blot, the gels may have not had enough time to run and during the membrane transfer, the voltage or duration of time may have been too high or too low. Finally, the blots may have been detected with the wrong antibody.

Based on results from this experiment, no definitive interaction was found between ANKRD24, MYO7A and PDZD7 with GPR98, however experiments need to be repeated multiple times to verify results.

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