IDENTIFYING MUCOLIPIN INTERACTORS USING THE SPLIT-UBIQUITIN YEAST TWO-HYBRID SYSTEM

By

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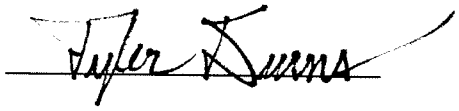

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Abstract:

While much is known about lysosomes, the proteins and mechanisms that participate in their biogenesis are still somewhat ambiguous. Mutations in the *MCOLN1* gene have been shown to lead to an accumulation of large vacuoles resembling hybrid organelles of late endosomes and lysosomes. Experimental evidence has revealed that the ML proteins, encoded by the *MCOLN* genes, are involved in this pathway. To give better indication of their function, I sought to identify proteins that physically associate with ML1 and ML2 using the Split-Ubiquitin Yeast Two-Hybrid system. This system has previously been proved to be successful in identifying transmembrane protein interactors. This screen yielded numerous candidate interactors that are currently being confirmed by co-localization and co-immunoprecipitation assays. These candidate interactors include trafficking regulators and channel proteins, suggesting regulation of channel and transport activities of ML proteins.

Introduction:

Endocytosis is a vesicular-mediated process by which materials such as macromolecules are internalized into a cell from its exterior (1). It is a process critical to the survival and function of cells given its role in nutrient uptake, receptor downregulation, growth, and differentiation. This process begins with the invagination of the plasma membrane and formation of vesicles that fuse to form a membrane-bound compartment called an early endosome. From early endosomes, molecules are sent back to the plasma membrane, sent to the Golgi Apparatus, or allowed to progress through the endocytic pathway to the "late endosome". Lysosomes are formed from late endosomes and are acidic organelles that contain degradation enzymes.

While the function of lysosmes is understood, little is known about their biogenesis. Among the proteins believed to be responsible in the formation of lysosomes are the mucolipin proteins (ML1, ML2, and ML3) (2). Mucolipins are six-pass transmembrane proteins that belong to the transient receptor potential (TRP) cation channel superfamily. The localization of the mucolipin proteins has been extensively studied. However localization can only sites of action but not function. Mucolipins have been found to associate with themselves and with each other (3). Mutations in *MCOLN1*, the gene that encodes ML1, result in defective lysosomal biogenesis, leading to the formation of enlarged vacuoles that resemble a hybrid of late endosomes and lysosomes. Loss of ML1 ultimately leads to cell death in some tissues (4).

I carried out a screen to identify proteins that interact with the mucolipins. Detection of interactors will aid in identifying mechanisms of action of mucolipins. The large screen was designed using cDNA libraries from mouse heart and kidney, using an extensive process where they were transformed into yeast, prioritized, isolated, and again transformed into yeast for

retesting; the staple of this screen being the Split-Ubiquitin Yeast Two-Hybrid system. This screen identified several candidate proteins that associate with ML1 and ML2 and that will shed light on their functions in the endocytic pathway.

Materials and Methods:

Step-wise Process of the Screen- NubG fused cDNA libraries are transformed into yeast containing the ML-Cub expressing plasmids. The yeast are then streaked onto selection plates (missing the amino acids Leucine, Tryptophan, Adenine, and Histidine). Potential interactors are then grown in minimal media that is missing the same amino acids. The total DNA is isolated, and transformed into *Escherichia coli* to isolate the NubG plasmid. The purified NubG plasmids are transformed again into the ML-Cub expressing yeast and the interactions are confirmed on -Leu-Trp-His-Ade selection plates. Confirmed interactors are sent to sequencing.

Split-Ubiquitin Yeast Two-Hybrid Summary- The Split-Ubiquitin Yeast Two-Hybrid system is based on the knowledge that ubiquitin can be separated into two pieces, allowed to assemble when in close proximity, and still function. Functioning of ubiquitin entails its ability to recruit proteases to cleave various materials, in this case, releasing a transcription factor that translocates to the nucleus resulting in the expression of several amino acid biosynthetic enzymes.

This system tests for the interaction between two proteins. The protein of interest is know as the "Bait" (either mouse mucolipin-1 or mucolipin-2) and has the ubiquitin CUB domain fused to it. Attached to the CUB domain is a transcription factor, LexA-VP16; Lex A binds to the promoter and VP16 recruits RNA Polymerase. Attached to the "Prey" proteins is

the NubG domain of ubiquitin. These "Prey" proteins are the potential interactors from the cDNA libraries. The bait protein-expressing plasmid also carries the *LEU2* gene, whereas the prey protein-expressing plasmid also carries the *TRP1* gene. The NMY51 yeast strain used for the assays has the genotype *MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2:: (lexAop)4-HIS3 ura3:: (lexAop)8 -lacZ-ade2:: (lexAop)8- ADE2GAL4*. Therefore, the yeast carrying both plasmids is able to grow in media lacking Leucine and Tryptophan. When the two proteins interact the CUB and NubG domains reconstitute Ubiquitin. Ubiquitin proteases then cleave off the LexA-VP16 transcription factor that moves into the nucleus, resulting in expression of Histidine and Adenine biosynthetic enzymes. Transcription and expression are seen by growth of the yeast in -Leu-Trp-Ade-His medium. If the proteins do not interact, the NubG and CUB domains do not bind, ubiquitin does not assemble, and no growth is seen on media lacking Histidine and Adenine.

Amplification of cDNA Library- Three different cDNA libraries were tested against ML1 and ML2: mouse heart x-NubG cDNA (from Dual Systems), mouse heart NubG-x (from Igor Stagljar), and mouse kidney NubG-x (from Dual Systems originally, gift from Math Cuajungco). Amplification of cDNA was done with XL10-Gold Ultra-competent Cells (Stratagene, #200314). Transformations were made to give several million colonies, and done as the protocol from the Dual Systems kit designated. Colonies were scraped and mixed. Aliquots were saved at -80° in YPAD with 15% glycerol. Cultures were incubated in a 37° shaker for 3-4 hours in 2XYT+Amp. DNA preps were done using Qiagen Maxi-prep kits.

Yeast Transformation- Several colonies of yeast containing the CUB plasmid (mucolipin protein of interest) are streaked into ~ 1' x 0.5' patches, onto SC-Leu plates and allowed to grow for

several days at 30°. Each patch equates for one transformation of one plasmid. The yeast is scraped into a microcentrifuge tube containing 1 ml of 0.1 M lithium acetate. The solution is spun at 13000 rpm for ten seconds. The supernatant is discarded and the yeast are resuspended in 50 ul of 0.1 M lithium acetate. Each transformant tube contains 50 ul of the yeast precipitate/lithium acetate solution, 2 ul of DNA, and 0.5 ml of poly ethylene glycol solution (8 ml 50% PEG, 1 ml 10x TE, and 1 ml 1.0 M lithium acetate). This mixture is vortexed and left at room temperature for 24 hours. After the 24-hour period, the transformant mixtures are centrifuged at 13000 rpm for 30 seconds. The supernatant is discarded and the precipitate is resuspended in 150 ul of 0.9% NaCl and plated onto a –Leu-Trp plate. The plate is left at 30° for 4-7 days until yeast colonies appear.

Streaking onto Selection Plates- Potential interactors were streaked onto SC-Leu-Trp plates. Streaking was done to allow for individual colonies to grow. For controls we used Fur4 and Ost1. Fur4 is located at the plasma membrane and Ost1 is located in the Endoplasmic Reticulum. The normal Nub domain normally contains an Isoleucine at position 13; this NubI domain binds very efficiently to the CUB domain. However, when a Glycine is in position 13, this NubG has conformation change that reduces the efficiency of its interaction with the CUB domain. Therefore NubI fusions of Fur4 and Ost1 are used as positive controls, and NubG fusions as negative controls. The use of controls from separate organelles also helped give indication of localization of ML-CUB proteins in yeast. pPR3-N expresses NubG and is another negative control that indicates background growth. Three colonies were streaked onto selection plates (-Leu-Trp-His-Ade and 10 mM concentration of 3-Amino-1,2,4-triazole) for each test. Growth was recorded on days 3 and 4.

Selection in Liquid Media- Yeast were grown in liquid selection media (-Leu-Trp-His-Ade and 10 mM concentration of 3-Amino-1,2,4-triazole to eliminate background plasmids). Liquid cultures were shaken for 3-5 days at room temperature; yeast were then grown on selection plates for 3-4 days.

Yeast Prep- To transfer the DNA from yeast to bacteria, we performed Yeast Preps. Yeast Preps were based off the protocol provided by the Zymo Research (Zymoprep I Yeast Plasmid Miniprep Kit). Our procedure was identical until the final steps. Each yeast prep was washed twice with 1 ml of 70% ethanol to remove the salt residue before resuspension in ddH_2O.

E. coli Transformation- *E. coli* transformations were done using electroporation. Electro-compotent cells were thawed on ice, and 1.0 ul of yeast-prep DNA was added and put in a chilled cuvette. These were pulsed at 2.5 kV, 200 Ω, and 2.5 uF. 1 ml of SOC was immediately added, and the culture transferred to a 37° and allowed to grow for 1 hr. After one hour of incubation, cultures were plated onto LB-Amp Plates. Plates were incubated at 37° for 24 hours.

Minipreps- One *E. coli* colony was placed into a sterile, glass tube containing 3 ml of 2XYT and 3 ul of Ampicillin (100 ug/ul) and allowed to shake at 37° for 24 hours. 1 ml of the culture was placed into a microcentrifuge tube and the procedure provided by the Qiagen was followed.

Making Electro-competent Cells- XL1Blue cells were grown overnight in a 10 ml culture of LB or 2XYT. This was added to 400 ml of LB or 2XYT and allowed to grow until the OD_{550} reached 0.5. The culture was then put into several centrifuge bottles and placed on ice for 1 hour. It was then spun at 5000 rpm for 10 minutes and the supernatant was discarded. The cells then underwent three washes with ice-cold water. The fourth and final wash was with 15% glycerol in

YPAD. Cells were then resusupended in 15% glycerol in YPAD. Aliquots of 50 ul were stored in tubes and immediately frozen at -80 °.

Results:

I. ML1 Cub + X-NubG

Total colonies screened: 12,530,700
Candidate interactors found: 36
Proteins identified: 26

Table 1:

Protein	Accession #	# of Times Isolated	Notes
FERM domain containing 5 (gene: Frmd5)	CAQ12956	1	B41, transmembrane
Retrovirus-related Env polyprotein from Fv-4 locus	P11370	1	Coiled-coil, transmembrane
vacuolar protein sorting 4b (VPS4b)	NP_033216	1	MIT, AAA
Btbd2 protein	AAI15684	1	BACK
open reading frame 61, isoform CRA_c (membrane)	EDL31617	1	4 transmembrane
Transmembrane Protein 163 (Cation Efflux Motif)	NP_082411	1	6 transmembrane
ERGIC and golgi 3	NP_079792	1	2 transmembrane
runt related transcription factor 2	EDL23422	1	coiled-coil
Ubc protein (Ubiquitin Suprfamily)	AAH25894	1	4 ubiquitin
voltage-gated sodium channel type V alpha	NP_067519	2	IQ, 19 transmembrane
Gja1 protein (has connexin superfamily domain)	AAH55375	1	CNX, 3 transmembrane

protocadherin beta 20	NP_444375	1	5 cadherin, 1transmembrane
protocadherin gamma subfamily A2 (has Ca2+ binding domain)	NP_291063	1	6 cadherin, transmembrane
ATPase, Ca++ transporting, slow twitch 2 isoform b	NP_033852	10	Cation_ATPase_N, 6 transmembrane
mCG10343, isoform CRA_b (mitochondrial carrier protein)	EDL21527	1	2 transmembrane
retinoic acid induced 17, isoform CRA_d	EAW54643	1	none
mCG133388, isoform CRA_e	EDL10122	1	6 cadherin, transmembrane
isoform CRA_b	EDL15349	1	14 EGF, transmembrane... overlapping domains leads to four representations of protein
isoform CRA_c	EDK97851	1	EGF, EGF_CA
unnamed protein product-1	BAE21227	1	3 transmembrane
similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 3	XP_001476773	1	Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain
phosphatidylinositol 4-phosphate 5-kinase type 1-beta	BAA13031	1	PIPKc
plexin	BAC97985	1	Sema, 2 PSI, 3 IPT, transmembrane
Zmiz1 protein	AAH58646	1	none
Yip1 interacting factor homolog B isoform 2	NP_001103671	1	5 transmembrane
Clk2-Scamp3 protein	ACC61069	1	serine/threonine protein kinases, catalytic domain, 4 transmembrane

II. ML1 Cub + NubG-X

Total colonies screened: 23,796,600
Candidate interactors found: 13
Proteins identified: 8

Table 2:

Protein	Accession #	# of Times Isolated	Notes
unnamed protein product	BAE30441	1	6 transmembrane
surfeit gene 4	NP_035642	1	5 transmembrane
lysosomal-associated protein transmembrane 4B	NP_277056	1	4 transmembrane
ATP synthase F0 subunit 6	YP_002381175	1	6 transmembrane
NADH dehydrogenase subunit 1	YP_002791042	4	7 transmembrane
NADH dehydrogenase subunit 5	AAY96699	2	16 transmembrane
peroxisomal membrane protein 2	NP_033019	2	3 transmembrane
Peroxisomal biogenesis factor 16 (PEX16)	Q91XC9	1	none

III. ML2 Cub + NubG-X

Total colonies screened: 23,796,600
Candidate interactors found: 61
Proteins identified: 13

Table 3:

Protein	Accession #	# of Times Isolated	Notes
unnamed protein product	BAE38069	1	7 transmembrane
cytochrome c oxidase subunit I	YP_001686700	3	12 transmembrane
NADH dehydrogenase subunit 1	NP_904328	29	7 transmembrane

unnamed protein product	CAA24090	1	7 transmembrane
ATP synthase F0 subunit 6	NP_904333	9	6 transmembrane
leptin receptor overlapping transcript-like 1	NP_080885	4	4 transmembrane
unnamed protein product	BAE20704	5	6 transmembrane
lysosomal-associated protein transmembrane 4B	NP_277056	2	4 transmembrane
unnamed protein product	BAB24089	1	4 transmembrane
NADH dehydrogenase subunit 4L	NP_904336 NP_008116	3	2 transmembrane
LOC67509	NP_080485 XP_912122 XP_978435	1	transmembrane
unnamed protein product	CAA24080	1	7 transmembrane
cytochrome b	BAG67077	1	6 transmembrane

IV. ML2 Cub + X-NubG

Total colonies screened: 14,728,800
Candidate interactors found: -
Proteins identified: -

No useful data obtained due to an undetermined error.

Discussion:

The Spit-Ubiquitin Yeast Two-Hybrid System is efficient in that it allows for the testing of potential candidate interactors of transmembrane protein using full-length constructs and without their targeting to the nucleus. This, in contrast to the traditional Yeast Two-Hybrid System, where proteins are artificially targeted into the nucleus (5). Among the limitations of the system is that mammalian proteins are not always correctly modified in yeast, which can lead to false results. Proteins may also interact when they are co-expressed in yeast cells, when they

may not be expressed in the same mammalian tissues or subcellular compartments. This system also gives no indication of the length of interaction (candidate proteins may interact transiently or for an extended period). As such, other assays should be utilized to confirm results from Yeast Two-Hybrid assays, including co-localization and co-immunoprecipitation studies.

The Spit-Ubiquitin Yeast Two-Hybrid screens with ML1 and ML2 are likely identifying true interactors. Indeed, other students in the Fares lab have confirmed that the endoplasmic reticulum-golgi intermediate compartment protein (ERG1c) co-immunoprecipitates with ML1 and ML2. ERG1c is known to help mediate trafficking between the ER and Golgi Apparatus. This protein is believed to allow for the modification of proteins that are destined for transport from the ER (6). Thus, it is possible that ERG1c regulates the transport of ML1 and ML2 from the ER to the Golgi Apparatus and lysosomes.

The screen identified several other proteins that that localize to endosomes/lysosomes and/or have defined roles in endocytic transport. These include phosphatidylinositol 4-phosphate 5-kinase type I-beta (PIP5 kinase), VPS4b, and lysosomal-associated transmembrane protein 4b (LAP4b). PIP5 kinase has been connected to vacuolar protein sorting and endosome function (7). VPS4b is known to associate with the endosomal compartments, and is involved in intracellular protein trafficking; it has an ATPase activity that releases endosomal sorting complex required for transport (ESCRT) complexes from endosomes (8). LAP4b localizes to lysosomes, though its roles in this compartment have not been elucidated (9).

In this screen we found several proteins that suggest some of the observed channel activities of the ML proteins may be modulated by their interactions with other channel proteins. These include the voltage-gated sodium channel type V alpha and Transmembrane Protein 163 Cation Efflux Motif. ML1 may also associate with ion pumps, for example the ATPase, Ca++

transporting, slow twitch 2 isoform b.

Future studies include testing the specificity of the identified interactors with different ML proteins via the Split-Ubiquitin Yeast Two-Hybrid system. Intreactions will also be confirmed using co-localization and co-immunoprecipitation assays. Eventually the Fares lab hopes to decipher how protein interactors assist mucolipin-dependent membrane trafficking.

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