

**Investigating the Role of Dullard and TMEM-188 in Lipid Droplet Biogenesis
in mammalian cells**

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Abstract

Yeast proteins Nem1-Spo7 colocalize to lipid droplet (LD) formation site in the endoplasmic reticulum (ER). Previous research has determined that the mammalian orthologs of these two proteins are Dullard and TMEM188. Although these two proteins have been recognized as orthologs of the yeast proteins, minimal research has been conducted to determine if these two proteins colocalize and have a similar function to the yeast Nem1-Spo7 complex in regard to LD biogenesis. After cloning the proteins of interest and visualizing them in the ER, we determined that Dullard does localize to LDs in the ER. Our research has achieved the first step in proving the Dullard-TMEM188 complex has a similar function in LD biogenesis as its yeast counterparts. Future research aims to discover the colocalization of the two mammalian proteins to LDs and mutate these proteins to determine the effects on LD formation to prove the functional significance of these two proteins. Finally, these findings will play a role in diseases involving LDs, such as obesity, diabetes, and lipodystrophy.

Introduction

Lipid droplets (LD) are fat storage molecules formed in the endoplasmic reticulum (ER) and serve a myriad of functions. These functions include energy metabolism, membrane synthesis, and the creation of lipid-derivative molecules such as hormones and bile salts. LDs are essential to life and are present in all eukaryotic cells and numerous prokaryotic cell species (Pol et al., 2014, JCB). The scientific study of LDs has increased dramatically in the past years due to the links between LD accumulation and specific pathologies such as diabetes and arteriosclerosis (Pol et al., 2014, JCB). Although the mechanism of LD formation is still under investigation, a network of proteins works together in yeast to form LDs.

Prior research has shown that Nem1, a regulator of diglycerol production, colocalizes with lipid droplet biogenesis sites in yeast's ER (Choudhary et al., 2020, JCB). Further, Nem1, in association with other proteins such as Fld1, Spo7, Pah1, and Seipin, is necessary to form LDs in the ER properly (Choudhary et al., 2020, JCB). This occurs by Nem1-Spo7 complex formation, which dephosphorylates phosphatidate phosphatase Pah1 and activates it. The activated Pah1 forms diacylglycerol (DAG) from phosphatidic acid (PA) and aids in the proper lipid droplet formation pathway in yeast (Han et al., 2011, JBC).

Since discovering the complex nature of LD formation in yeast, the orthologs of these two proteins in mammalian cells have been investigated. The mammalian protein Dullard has a conserved catalytic domain of DXDX(T/V), which allows the protein to perform similar functions to the yeast protein Nem1 (Kim et al., 2007, PNAS). It was found that Dullard localized to similar nuclear envelope domains and had similar substrate preferences in this paper. Most importantly, Dullard dephosphorylates the mammalian PA phosphatase known as lipin, and the Dullard protein functionally rescued Nem1 knockout yeast cells. Thus, Dullard is the mammalian ortholog of yeast protein Nem1.

A similar study was performed to determine the mammalian ortholog of the yeast protein Spo7. The TMEM188 protein was determined to be the Spo7 mammalian ortholog that complexes with Dullard to dephosphorylate lipin. Homology was confirmed using HHpred after discovering TMEM188 had conserved region motifs concentrated in non-hydrophobic amino acids to Spo7. (Han et al., 2011, JBC). Further, the Dullard-TMEM188 complex rescued the knockout phenotype of Nem1-Spo7 in yeast, proving the homology between the two complexes (Han et al., 2011, JBC).

This study investigates Dullard and TMEM188 in mammalian cells to determine if their functions resemble that of yeast proteins Nem1-Spo7 and determine the role these two proteins play in mammalian lipid droplet formation. Based on the LD biogenesis research in yeast, we believe the mammalian orthologs will exhibit similar traits. Therefore, the mammalian orthologs of Nem1 and Spo7, Dullard and TMEM188, respectively, will colocalize at lipid droplet biogenesis sites and are essential for lipid droplet biogenesis.

Methods

Dullard and TMEM188 were tagged at the C-terminus with fluorescence proteins GFP or mApple by cloning in N1-GFP and N1-mApple vectors. To do that, Dullard and TMEM188 were first amplified using Q-5 polymerase PCR sequencing. Then each amplified gene was run on a gel electrophoresis to confirm the PCR. Following confirmation, gel cleaning of the gene product was completed using the ZYMOCLEAN Gel DNA Recovery kit protocol. Next, the Dullard and TMEM188 cDNA were inserted in the EcoRI digested N1-GFP and N1-Apple plasmids using New England Biolabs (NEB) HiFi DNA Assembly Master Mix. For all HiFi DNA assembly reactions, 5ul of HiFi was mixed with 4ul of plasmid and 1ul of DNA. For plasmid construction, all PCRs were performed using Q-5 High Fidelity DNA polymerase, and restriction enzymes were from NEB. Dullard-GFP, Dullard-Apple, TMEM188-GFP, and TMEM188-Apple constructs were confirmed by PCR and sequencing.

Following HiFi DNA Assembly, 5ul of reaction was transformed into chemically competent E.coli cells (NEB). All plates throughout the experiments were LB kanamycin plates and were spread with 200ul of E.coli DNA mixture following transformation. Once clones were generated using the New England Biolabs protocol, the presence of the gene of interest was

confirmed using MyTaq DNA Polymerase PCR. Following the MyTaq PCR, gel electrophoresis was performed to determine the band size of the PCR product to determine if the gene of interest was properly cloned into the vector. Once positive results were confirmed, the product was sent to Eurofins for sequencing to determine the proper insertion of the gene of interest.

Results

Dullard and TMEM188-cloned in N1-GFP and N1-mApple vectors.

Cloning of Dullard and TMEM188 gene was performed in N1-GFP and N1-mApple vectors respectively in hopes of visualizing the protein complex Dullard-TMEM188 in location to lipid droplets in the endoplasmic reticulum. The vectors were digested with the BamHI restriction enzyme to linearize them. Constructs including Dullard-N1-GFP and TMEM188-N1-mApple were made and confirmed using PCR sequencing. The products were then transfected into live COS7 cells to visualize this protein complex's spatial and temporal localization. Upon viewing the images, it was clear that the proteins were not expressed correctly. As a result, we investigated the sequence of the plasmids and found that the BamHI restriction digestion caused a shift of the open reading frame of Dullard-GFP and TMEM188-mApple by one nucleotide base, resulting in a defect in the expression of Dullard-N1-GFP and TMEM188-N1-mApple gene products. As a result, we repeated the cloning by digestion of plasmids using the EcoRI restriction enzyme that would result in in-frame cloning of Dullard and TMEM188. N1-GFP and N1-mApple were digested using EcoRI, ran on a gel electrophoresis to confirm the product, and then gel cleaned and used in the HiFi DNA assembly. After many rounds of cloning, we were able to clone Dullard in the N1-GFP vector. To confirm the cloning of the Dullard gene, a MyTaq DNA polymerase PCR reaction was

performed. PCR was completed, and gel electrophoresis to confirm the identity of Dullard in the clone was performed. After a positive gel result (fig. 1), Dullard-N1-GFP cloning was confirmed by sequencing at Eurofins.

The exact process was done with TMEM188-N1-GFP and N1-mApple. However, we have yet to successfully clone TMEM188 in either the N1-GFP or N1-mApple vector. Thus, we focused on Dullard-GFP and its localization to LD in the ER.

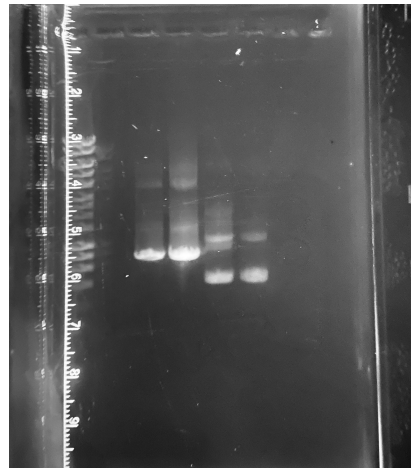


Figure 1.

Dullard-GFP MyTaq confirmation gel electrophoresis

Lane 1: ladder, Lane 3&4:Dullard-GFP, Lane 5&6:TMEM188-mApple

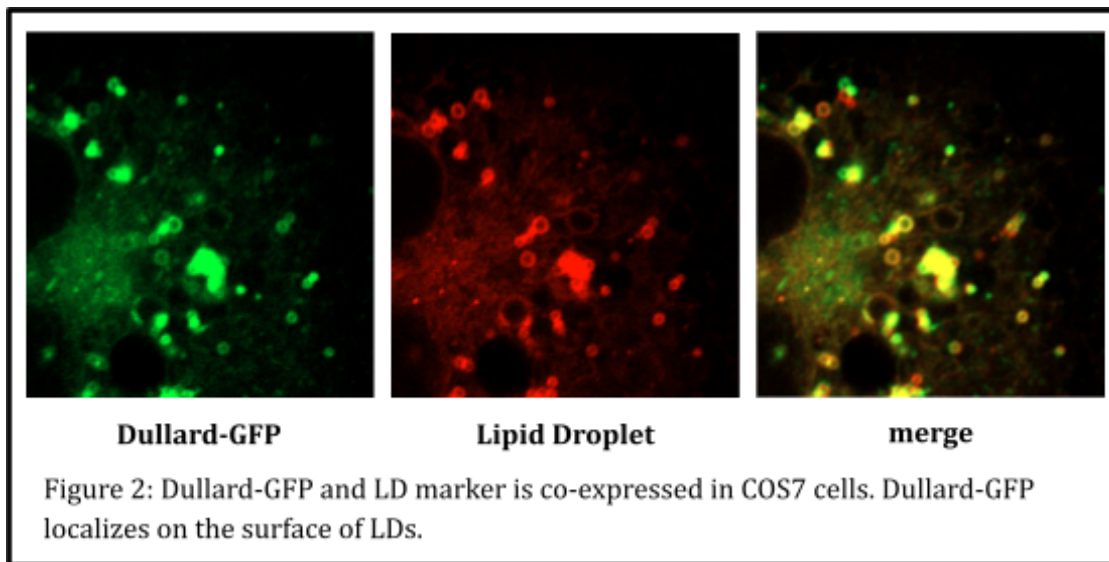
Primers: CMV-Forward, CMV-Reverse

Positive: Dullard-GFP, Negative: TMEM188-mApple

Spatial and Temporal Localization of Dullard-N1-GFP in the Endoplasmic Reticulum

After successfully cloning Dullard into the N1-GFP vector, the construct was transfected into the COS7 live cells to visualize the spatial-temporal dynamics of the proteins to lipid droplet biogenesis sites. A protein known to localize to the LD surface was used to visualize the LDs and the Dullard protein interaction. After viewing the images on the microscope [fig. 2], it was

determined that Dullard protein localizes to the ER and on the surface of the lipid droplets. The location of Dullard on the lipid droplets confirms the prediction that Dullard will mimic its Nem1 counterpart in yeast and likely have a similar function with respect to lipid droplet biogenesis.



Discussion

In this study, we have demonstrated that Dullard localizes to lipid droplet sites in the endoplasmic reticulum. The result was confirmed using microscopy to determine the localization of the protein and lipid droplets. Unfortunately, due to time constraints and errors, Dullard's counterpart TMEM188 was unable to be cloned effectively and visualized similarly. Therefore, we cannot conclude that the Dullard-TMEM188 complex acts and localizes comparably to that of Nem1-Spo7 in yeast cells. We are still looking into why we could not clone TMEM188 effectively and plan to continue the experiment. Although we could not effectively clone the Spo7 homolog, the finding that Dullard does localize to the lipid droplet sites is the first step in discovering the role of Dullard in lipid droplet biogenesis in mammalian cells.

In future research projects, the cloning of TMEM188 will continue in the N1-mApple vector to visualize the TMEM188-Dullard protein complex together in the endoplasmic reticulum. The hope is to determine where the protein complex localizes and its localization to LDs. Once the complex is cloned and visualized in the cell, next, we will determine the abundance and size of lipid droplets in HeLa cells after 1) overexpression of Dullard-GFP or TMEM188-mApple, 2) by generating the point mutations in these proteins that are observed in patients, 3) by knockdown using siRNA targeting Dullard and TMEM188 transcripts.

The overarching future goal of this project is to determine the LD biogenesis pathway in mammalian cells to determine specific links to human diseases. The implications of our preliminary findings and future research endeavors are likely to find links in the LD biogenesis pathway to human diseases such as diabetes, obesity, and lipodystrophy. Our hope is this can lead to clinical and pharmacological advances that can aid in the health of humans suffering from such conditions.

Conclusion

In conclusion, we determined that Dullard localizes in the ER membrane and on the surface of the lipid droplet. The localization was determined by amplifying the gene of interest, digesting restriction enzymes, building primers, and transforming the gene into E.coli cells. Once Dullard was confirmed to be present in the transformed cells, it was transfected into COS7 cells for visualization under the microscope. A protein known to localize to the LD surface was used to visualize the LDs and Dullard tagged with N1-GFP allowed for the clear visualization of Dullard localizing to the lipid droplets in a ring-like fashion. Future research aims at discovering the colocalization of the Dullard-TMEM188 complex, mutating the respective proteins, and

determine the effects on lipid droplets. Finally, future studies will aid in discovering the LD biogenesis pathway and hopefully lead to advances in human healthcare.

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