Studies on the function and target-RNA recognition of RNA-binding proteins essential for mouse germ cell differentiation

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Introduction

In animals that reproduce through sexual reproduction, the formation of functional gametes is crucial in order to pass along genetic information to the next generation. RNA-binding proteins play major roles in regulating correct germ cell development. These proteins generally make functional complexes; however, what kind of protein structures are needed to form these complexes and bind specific RNA are largely unknown. One family of RNA-binding proteins that is essential for germ cell development is NANOS. I focused on NANOS2, which is male-specific and required for male-type differentiation of germ cells (Suzuki et al., 2008; Tsuda et al., 2003). It has a conserved zinc finger domain and similar N-terminal, which are required for interaction with its partner, DND1 (Suzuki et al., 2015) and the CCR4-NOT deadenylation complex (Suzuki et al., 2012), respectively. NANOS2 and DND1 binding is necessary to recruit target mRNA. Once the mRNA is bound, NANOS2, DND1, CCR4-NOT, and other proteins form P-bodies, which are granules for RNA degradation/storage (Aizer et al., 2014; Kedersha et al., 2005). Binding of DND1 and NANOS is required for P-body formation (Suzuki et al., 2015).

The formation of P-bodies and targeting of mRNA to them by NANOS2 are key events in male differentiation, and NANOS2-DND1 complexes are essential for P-body formation. Currently, no crystal structure is available for DND1, and although we tried several methods to purify recombinant DND1 to solve its structure, they were unsuccessful because DND1 is highly insoluble. As such biochemical methods failed, a different method is needed to reveal how DND1 and NANOS2 interact and bind mRNA. In addition, how the mRNA targets are selected and what happens to them inside P-bodies are unanswered

questions. Therefore, I decided to use live imaging techniques to examine the interactions among DND1, NANOS2, and mRNA, and the subsequent formation of P-bodies. Using fluorescently labelled proteins, the formation of P-bodies, their dynamics and the interactions of RNA binding proteins can be visualized in cultured cells and germ cells. The purpose of this study was to clarify how NANOS2 and DND1 contribute to target-RNA binding and the formation of P-bodies. In addition, the effects of mutant proteins on P-body formation and mRNA recruitment were investigated.

Materials and Methods

Cell culture

HEK-293T, NIH3T3 and ciN2D13T3 cells were cultured in DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin at 37ºC in 5% CO_2.

Western Blotting

CiN2D13T3 cells were lysed in lysis buffer. After centrifugation, supernatants were boiled in 2x sample buffer for 5 min and run on gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred to PVDF (Immobilon) membranes. Membranes were blocked in 5% skim milk/ PBST for 1 hr at RT. They were then incubated with the following primary antibodies overnight at 4°C: anti-DAZL, anti-HA and anti-FLAG-HRP. After washing 3 times with PBST, membranes were incubated with HRP-conjugated secondary antibodies for 1 hr at RT. Protein bands were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific).

mRNA imaging using dCas13

dLwaCas13a–NF and the Cas13 guide expression vector were purchased from Addgene. The targeting guide for *ACTB* was created following the report by Abudayyeh et al. As dazl is germ cell-specific, *Dazl* with its 3'UTR was cloned into the pcDNA3.1 expression vector and used to assess guide specificity. Several targeting guides for *Dazl* were created following the same procedure as for the ACTB guide. To image *ACTB,* HEK-293T cells were transfected with dLwaCas13a–NF, the *ACTB* guide and DDX6-mcherry constructs using PEI. Forty-eight hours after transfection, stress granule formation was induced by culturing the cells in medium with 400 μM sodium arsenite for 1 hour and imaged using a 100x lens at 37°C in 5% CO_2 with the FV1200 confocal microscope. To image *Dazl,* NIH3T3 cells were transfected with dLwaCas13a–NF and a *Dazl* guide with or without the Dazl-3'UTR construct using Lipofectamine LTX + Plus. Forty-eight hours after transfection, cells were imaged using a 100x lens at 37°C in 5% CO_2 with the FV1200 confocal microscope. Z-stack images were compiled to create 3D time-lapse animations using the included software.

Fluorescence live imaging

For 4-color live imaging, BFP2, tagRFP and mi670 fluorescent proteins were used. tagRFP and mi670 were gifts from Dr. Wada at Fukushima Medical University. BFP2 was added to the N-terminal of NANOS2 and del-N-NANOS2. tagRFP was added to the C-terminal of DND1, DND1R98A and DDX6. mi670 was added to the C-terminal of DCP1a and DDX6.

All constructs were created using the InFusion system and inserted into the pcDNA3.1 expression vector. NIH3T3 cells were transfected with constructs for the dCas13 system and a combination of BFP2-, tag-RFP- and mi670-tagged proteins using lipofectamine LTX + Plus. Live imaging was performed 24-48 hours after transfection on either the Olympus FV1200 confocal microscope or Deltavision Ultra.

Immunostaining and RNA visualization by ViewRNA

After removing the culture medium, cells were fixed with 4% paraformaldehyde/PBS for 15 min at RT. They were then permeabilized with 0.3% Triton-100 for 15 min at RT. Cells were blocked with 3% skim milk/PBST for 30 min and then incubated overnight with the following primary antibodies: mouse anti-NANOS2, mouse anti-CNOT1, rat anti-HA, rabbit-anti-Rck and chicken anti-GFP. After washing with PBST, cells were incubated with secondary antibodies conjugated with Alexa Fluor 405, 488, 594 and 647 for 30 min at RT. DNA was stained with DAPI. For ViewRNA staining of *Dazl* mRNA, the ViewRNA Cell Plus kit was used following the manufacturer's instructions with the Dazl probe set. P-bodies were stained using anti-Rck. For ViewRNA and immunostaining, cells were imaged using Olympus FV1200 confocal microscope. Super-resolution microscopy was performed using the Olympus IXplore SpinSR microscope.

Statistical Analysis

For the colocalization analysis, Coloc (a package for colocalisation analyses, https://cran.r-project.org/web/packages/coloc/vignettes/vignette.html) was applied to Z-stack images of

cells. The presented correlation coefficients are the r-values after Costes thresholding. The cytofluorograms are a scatterplot of the spatial localization and intensity of pixels in 2 channels. The diagonal line in the middle of the plot is the intensity ratio.

Results

Evaluation of the dCas13 system for live imaging of mRNA

To assess the feasibility of the dCas13 imaging system, I first observed the movement of human beta-actin (ACTB) mRNA into stress granules, which are another type of RNP granule that store transcripts under stress conditions (Kedersha et al., 2005; Buchan et al., 2009). HEK-293T cells were transfected with dCas13, ACTB guide and DDX6-mcherry constructs referring to the method reported by Abudayyeh et al. After culturing the cells with arsenite to induce stress, I visualized mRNA localization to stress granules. As negative controls, cells transfected without a guide and with a non-targeting guide were imaged. This dCas13 construct inhibits its own transcription when not bound to a target, therefore, without a guide, only nuclear expression or no GFP expression is observed. On the other hand, with the ACTB guide, the GFP signals representing *ACTB* mRNA made pools within the cytoplasm, and DDX6-mcherry-positive stress granules formed around these pools. When cells were not transfected with the guide, these beta-actin RNA pools were not observed. These results were similar to those reported in in the original paper, confirming that this system works well.

Next, I moved to applying this system to a specific NANOS2-target mRNA, *Dazl.* As it was previously reported that NANOS2 binds the *Dazl* 3'UTR (Kato et al., 2016), I designed several guides in the N-terminal region to avoid Cas13 interference. *Dazl* is also germ cell

specific, which made guide assessment simpler. In order to test their specificity, NIH3T3 cells were transfected with each guide with or without *Dazl*. Guide 1 resulted in GFP signal both with and without *Dazl*, indicating that other mRNAs were targeted. Guide #2 resulted in GFP signal only when *Dazl* was present with limited background and I employed this guide in all experiments thereafter.

NANOS2 and DND1 alter *Dazl* localization

The current hypothesis for the mechanism of mRNA regulation by NANOS2 is that upon its expression, it binds DND1 and targets specific mRNA to P-bodies to be possibly degraded. I therefore first performed time lapse imaging of *Dazl* localization before and after the induction of NANOS2 and DND1. NIH3T3 cells that express NANOS2 and DND1 upon doxycycline (Dox) addition were created in our lab. Once Dox was added, the number of P-bodies increased along with the increasing amount of *Dazl* based on the strong GFP accumulation in and around P-body foci. By 6 hours after doxycycline addition, both NANOS2 and DND1 are expressed and large P-bodies with a characteristic *Dazl* localization pattern were observed. Most *Dazl* had accumulated around P-bodies, suggesting that it was being actively targeted by NANOS2 and DND1.

For further confirmation of *Dazl* targeting to P-bodies, I employed the ViewRNA system to stain *Dazl* mRNA in ciN2D13T3 cells. Similar to the live imaging results, without NANOS2 and DND1, most *Dazl* was in the cytoplasm and not colocalized with DDX6 (r=0.21). After adding Dox, the localization pattern shifted, and more *Dazl* was localized to P-bodies, with a colocalization coefficient of r= 0.52. Based on these results,

Dazl is targeted to P-bodies when NANOS2 and DND1 are expressed, which was expected.

Individual roles of DND1 and NANOS2

Based on the live imaging experiments, *Dazl* is actively recruited to P-bodies when NANOS2 and DND1 are present. However, the major remaining question is which RNA-binding protein is actually binding *Dazl*: NANOS2 or DND1. Using super resolution microscopy of immunostained cells, NANOS2, DND1 and *Dazl* exhibit a stacking pattern in P-bodies consistent with the condensation of phase separation, making it unclear what is associated with what at a given time point. Thus to address the above question, live imaging of the individual proteins involved is necessary. I took advantage of the variety of fluorescent proteins currently available and created a 4-color protein set of NANOS2, DND1, *Dazl* and P-body marker. I created BFP2-tagged NANOS2, tagRFP-tagged DND1 and mi670-tagged DCP1a, with *Dazl* mRNA being marked by GFP-tagged dCas13. Using this combination to represent wild-type, I performed 4-color live imaging using wild-type and mutant proteins. By this analysis, I was able to visualize the roles of each protein in the process to P-body recruitment.

Assessment of the role of CNOT1 binding by NANOS2

Based on the results of live imaging using the individual proteins and mutant DND1, I hypothesized that the recruitment of CNOT1 by NANOS2 is what enables the localization of target mRNA to P-bodies. NANOS2 has a CNOT1 binding motif in its N-terminal, and if this N-terminal is deleted, CNOT1 cannot be bound, and mice carrying such mutant NANOS2

were sterile (Suzuki et al., 2012). I thus used BFP2-tagged NANOS2 lacking the N-terminal (del-N-NANOS2) to evaluate *Dazl* localization when CNOT1 is not recruited.

Discussion

The formation of P-bodies in germ cells by NANOS2 and DND1 plays an essential role in the progression of male germ cell differentiation. However, until now, no study has actually investigated the individual roles of these proteins or their effects on mRNA via live imaging. Being RNA-binding proteins, the lack of a system to easily visualize RNA has made their functional analysis difficult. As the purpose of this study was to differentiate the individual roles of NANOS2 and DND1 during this process of mRNA regulation through the formation of P-bodies, I needed a method to visualize RNA in real time alongside the individual proteins. I used NIH3T3 cells because they naturally have P-bodies, meaning the required machinery is present. By adding NANOS2 and DND1, the same phenotypes observed in germ cells are observed, P-body localization and cell cycle arrest, demonstrating that this *in vitro* system well reproduces germ cells. After transfecting the cells with the dCas13 system, I was able to visualize the targeting of *Dazl* to P-bodies. This led to the next question, which RNA-binding protein is actually binding *Dazl.* Based on the time-lapse imaging, I created a model of the events leading to mRNA regulation by NANOS2 and DND1. Using the dCas13, these events excluding CNOT1 recruitment were able to be visualized in real time. Recent technological advances have made it possible to image RNA and proteins together. Although there are other systems, such as the MS2-MCP system, for imaging RNA, they each have disadvantages, especially regarding signal strength. By employing this novel system, I was able to

distinguish the roles of two germ cell-specific proteins that function as a complex.