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A proteomics approach identifies novel resident zebrafish Balbiani body proteins Cirbpa and Cirbpb

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Abstract

The Balbiani body (Bb) is the first marker of polarity in vertebrate oocytes. The Bb is a conserved structure found in diverse animals including insects, fish, amphibians, and mammals. During early zebrafish oogenesis, the Bb assembles as a transient aggregate of mRNA, proteins, and membrane-bound organelles at the presumptive vegetal side of the oocyte. As the early oocyte develops, the Bb appears to grow slowly, until at the end of stage I of oogenesis it disassembles and deposits its cargo of localized mRNAs and proteins. In fish and frogs, this cargo includes the germ plasm as well as gene products required to specify dorsal tissues of the future embryo. We demonstrate that the Bb is a stable, solid structure that forms a size exclusion barrier similar to other biological hydrogels. Despite its central role in oocyte polarity, little is known about the mechanism behind the Bb's action. Analysis of the few known protein components of the Bb is insufficient to explain how the Bb assembles, translocates, and disassembles. We isolated Bbs from zebrafish oocytes and performed mass spectrometry to define the Bb proteome. We successfully identified 77 proteins associated with the Bb sample, including known Bb proteins and novel RNA-binding proteins. In particular, we identified Cirbpa and Cirbpb, which have both an RNA-binding domain and a predicted self-aggregation domain. In stage I oocytes, Cirbpa and Cirbpb localize to the Bb rather than the nucleus (as in somatic cells), indicating that they may have a specialized function in the germ line. Both the RNA-binding domain and the self-aggregation domain are sufficient to localize to the Bb, suggesting that Cirbpa and Cirbpb interact with more than just their mRNA targets within the Bb. We propose that Cirbp proteins crosslink mRNA cargo and proteinaceous components of the Bb as it grows. Beyond Cirbpa and Cirbpb, our proteomics dataset presents many candidates for further study, making it a valuable resource for building a comprehensive mechanism for Bb function at a protein level.

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Keywords

Oocyte development; Balbiani body; membraneless organelles; proteomics; liquid-solid phase separation; hydrogel; CIRBP

Introduction:

Oogenesis primes the fertilized egg for embryonic development. In zebrafish and *Xenopus* embryos, asymmetry of the oocyte is essential for determining the fertilization site, germ cell specification, and establishing the anterior-posterior and dorsal-ventral axes (Berois, Arezo et al. 2011, Escobar-Aguirre, Elkouby et al. 2017, Jamieson-Lucy and Mullins 2019a). During mouse oogenesis, the oocyte is also organized into distinct cytoplasmic domains, which are important for fertilization and oocyte maturation (Kotani, Yasuda et al. 2013, Takahashi, Kotani et al. 2014), like in fish and frogs. A conserved oocyte structure that acts as one of the first markers of oocyte asymmetry is the Balbiani body (Bb) (Oh and Houston 2017). The Bb is observed in a wide variety of organisms from insects to fish, mice, and humans (Hertig 1968, Pepling, Wilhelm et al. 2007). It is comprised of a membraneless aggregate, containing a variety of membrane-bound organelles such as mitochondria and endoplasmic reticulum, ribonucleoprotein particles, and an electron dense substance called nuage (Bradley, Kloc et al. 2001, Kloc, Dougherty et al. 2002, Kloc, Jedrzejowska et al. 2014). In fish and frogs, the Bb carries maternal mRNAs to the prospective vegetal pole, where it disassembles, releasing its cargo at the cortex where it is tethered until the start of embryonic development (Howley and Ho 2000, Houston 2013). These maternal mRNAs are important for axial patterning and generating the germ plasm (Houston and King 2000, Houston and King 2000, Marlow 2010, Ge, Grotjahn et al. 2014).

Understanding membraneless organelles such as the Bb can give us insights into a wide variety of cellular processes. Other membraneless organelles include stress granules and P-bodies in mammalian cells, which function in mRNA sequestration and processing during stress (Protter and Parker 2016, Luo, Na et al. 2018), and P granules in C elegans, which act in germ cell determination (Marnik and Updike 2019). To separate from the cytoplasm, these membraneless organelles undergo phase separation with P granules undergoing a liquid to liquid phase transition (Brangwynne, Eckmann et al. 2009, Khong, Jain et al. 2018, Schuster, Reed et al. 2018). Other membraneless structures form dense, stable proteinaceous aggregates within the cytoplasm. This type of aggregate is of clinical significance because of their similarity to non-functional, detrimental structures found in degenerative diseases such as ALS and Alzheimer's (Blennow, de Leon et al. 2006, Ramesh and Pandey 2017). The Bb shares structural qualities with these aggregates; it is comprised in part of amyloid-like fibrils, similar to those that form the hallmark plaques of Alzheimer's disease (Boke, Ruer et al. 2016). However, the Bb differs importantly from these structures: it is a functional component of oogenesis and disassembles naturally (Wallace and Selman 1990). This makes the Bb an attractive model for studying membraneless organelles and other aggregates.

To identify novel components of the Bb, we isolated Bbs and subjected them to proteomics analysis to identify the protein content. We identified four previously known Bb-resident

proteins, validating the approach. Among the 77 proteins identified, we focused on the Cold Inducible RNA-Binding Proteins A and B (Cirbpa and Cirbpb), because they stood out among our candidates for having predicted self-aggregation properties as well as RNA-binding ability. We speculate that they may play a functional role within the Bb.

Results:

The Balbiani body is a robust, stable structure

We found that the Balbiani body (Bb) is a stable structure that grows slowly over the course of oocyte development. As the oocyte progresses through stage I, its size steadily increases, and we can therefore use oocyte diameter as a proxy for its development over time. When we isolated oocytes and compared Bb diameter to the whole oocyte diameter, we observed that Bb size increased along with oocyte size in an approximately linear fashion (Figure 1A). Many membraneless organelles employ dynamic liquid-liquid phase separation mechanisms (Hyman and Simons 2012), but the apparent slow growth of the Bb indicates a more stable, solid structure. This is consistent with Xenopus FRAP experiments which show that mRNA and proteins do not move quickly within the Bb (Chang, Torres et al. 2004, Boke, Ruer et al. 2016). Anecdotally, we noticed that when crushed against a glass dish or slide, the Bb smears as if it is made of putty. To demonstrate its solid nature experimentally, we isolated Bbs from stage I oocytes and placed them in PBS, using the membrane dye $DiOC_6$ to visualize them. Even over the course of two hours, we observed no change in the overall shape of the Bb (Figure 1B). By contrast, the material in liquid-liquid phase-separated droplets dissipates when disturbed (Brangwynne, Eckmann et al. 2009).

These results led us to consider that the Bb behaves more like a hydrogel than a droplet. Hydrogels such as the nuclear pore complex (Bonner 1975) and associated P granules in *C elegans* (Updike, Hachey et al. 2011) are capable of forming size exclusion barriers, where nonspecific molecules over a certain size cannot penetrate the structure. In the Bb, even specific cargo mRNAs can take upwards of 24 hours to fully penetrate into the structure (Chang, Torres et al. 2004); we hypothesized that a general size-exclusion barrier may prevent the uptake of non-specific molecules into the Bb. We tested if the Bb forms a size exclusion barrier by injecting differently sized fluorescently-labeled dextran molecules into stage I oocytes. To introduce small molecules such as fluorescent dextran or synthetic mRNA into the oocyte, we needed to develop a new technique. One-cell stage embryos are approximately 500 μm in diameter, while stage I oocytes range from 50 to 100 μm in diameter, five to ten times smaller than embryos and a thousand-fold smaller in volume. We adapted the zebrafish embryo injection method to a much smaller scale and were able to successfully inject and image live oocytes by embedding them in a thin layer of agarose (Figure 1C) (Kobayashi et al., 2021).

We found that the Bb did not exclude 4.4 kD dextran molecules. As the size increased to 40, 70, and 155 kD, we found that the Bb partially excluded the larger dextrans after a two-hour incubation (Figure 1D). This implies that the Bb structure may exclude non-specific molecules. However, unlike the nuclear pore complex where molecules under 40 kD can enter the nucleus and anything larger than 40 kD is excluded, the Bb only partially excluded

large molecules. This may be because the Bb is a heterogeneous structure with many protein components contributing to a hydrogel-like material.

The stable, solid nature of the Bb makes it a good candidate for isolation and analysis compared to other membraneless organelles. We next took advantage of this property to investigate the protein components of the Bb.

Generating a more complete zebrafish Balbiani body proteome

Despite the Bb's importance to oogenesis and utility as a model for membraneless organelles, the protein components of the zebrafish Bb have not previously been characterized. Proteomics studies in Xenopus have identified a number of Bb proteins, however, those results included very high levels of yolk proteins and hemoglobin, not expected in stage I oocytes. In these previous studies, the only validated Bb protein was the Xenopus homolog of the previously known zebrafish resident Bb protein Bucky ball (Xvelo) (Bontems, Stein et al. 2009, Heim, Hartung et al. 2014, Boke, Ruer et al. 2016). Less than a dozen resident Bb proteins have been validated across all model organisms, but given the Bb's complex function throughout development, we expected many more functional protein components. To gain a more complete view of Bb resident proteins and better understand the mechanisms of formation, growth, and disassembly of the Bb, we set out to identify the zebrafish Bb proteome. Using zebrafish as a model, grants us access to a library of genetic tools that we can use in the future to study the functions of the Bb proteins identified.

We isolated Bbs from zebrafish stage I oocytes as described (Elkouby and Mullins 2017, (Jamieson-Lucy and Mullins 2019b)) and performed proteomics. We collected the Bbs stained with $DiOC_6$ and analyzed them by LC/MS mass spectrometry (Figure 2A, Table 1). This generated a proteomics dataset of over 200 proteins, with 77 replicated hits found over the course of five independent experiments (Supplemental Table 1).

Importantly, we found several previously identified resident Bb proteins, validating our approach, as well as proteins from mitochondria and ER, organelles that are abundant within the Bb. Also present were ribosomal components and RNA-binding proteins. STRING analysis showed that our Bb protein set was significantly enriched for predicted interactions compared to the whole genome (Figure 2B). The top GO terms for the replicated proteomics dataset are related to RNA-binding and translation (Figure 2C), which conforms to expectations of the Bb as an RNA-rich structure that functions in the regulation of maternal mRNAs. We found significant overlap between proteins in our Bb proteome and those found in stress granules, P bodies, and the Xenopus Bb (Figure 2D–E) (Boke, Ruer et al. 2016, Youn, Dyakov et al. 2019). For example, our lab previously discovered Bucky ball (Buc) as an essential protein for formation of the Bb (Dosch, Wagner et al. 2004, Marlow and Mullins, 2008, Bontems, Stein et al. 2009), and it is a known Bb component that was present both in our proteomics dataset and in the Xenopus Bb (Boke, Ruer et al. 2016). Lsm14b, Ddx6, Cirbpa and Cirbpb are additional examples of proteins shared between the zebrafish, Xenopus, and stress granule/P-body proteomes (Weston and Sommerville 2006, Pepling, Wilhelm et al. 2007).

We then narrowed our focus to the 77 proteins that were present in at least two of five proteomics experiments. To ensure that we were not simply identifying the most abundant proteins in the oocytes, we compared the number of peptide hits for each protein to mRNA expression level in oocytes at a similar stage as an estimate for protein abundance. Expression data were generated by performing RNAseq on stage I oocytes between 40 and 70 μm in diameter and their associated follicle cells to compare to our Bb data. Oocytes of this size have formed a mature Bb but not started the Bb disassembly process. We found that our proteomics dataset included both high and low expression genes, and several very highly expressed genes in the oocyte had only a few peptide hits, supporting that our proteomics work identified genuine Bb components and is not simply a recapitulation of the whole oocyte proteome (Figure 2F). This set of zebrafish Bb proteins will be a useful resource for future studies of the Bb.

Cirbpa and Cirbpb are RNA-binding proteins with predicted self-aggregation properties

With an interest in pursuing further proteins contributing to the amyloid like structure within the Bb, we performed a search for predicted self-aggregating properties among our proteomic hits. We used a primary sequence-based algorithm PLAAC (Prion-Like Amino Acid Composition) to search our proteomics data set for proteins with predicted self-aggregating or prion-like domains (PrLDs). This search returned three proteins with presumptive PrLDs. One result was the known Bb protein Buc, with a predicted PrLD at its N-terminus (Figure 3A). Buc is essential for Bb formation, and has been shown to form aggregates in vitro that are dependent on its self-aggregating N-terminus (Marlow and Mullins, 2008, Bontems, Stein et al. 2009, Boke, Ruer et al. 2016). The two other results were Cold Inducible RNA-Binding Proteins A and B (Cirbpa and Cirbpb), with predicted PrLDs at the C-terminus (Figure 3A). These proteins are teleost paralogs and are 67.5% identical at the amino acid level, and have identical domain structures.

The Cirbp RNA-binding proteins contain a predicted N-terminal RNA Recognition Motif (RRM) domain and a disordered C-terminal region. Cirbp's RNA-binding specificity is predicted to be relatively low; analysis of its mRNA targets in the mouse testis reveal a preference for uracil repeats, but no specific sequence motif (Xia, Zheng et al. 2012). Cirbp proteins are highly expressed in the gonads, particularly in the testis in response to cold stress (Nishiyama, Danno et al. 1998). In cells stressed by cold or other insults, Cirbp expression increases, and the protein shuttles from its usual location in the nucleus to stress granules in the cytoplasm (De Leeuw, Zhang et al. 2007, Liao, Tong et al. 2017). Like the Bb, stress granules are membraneless, RNA-rich structures, and have been shown to have a phase-separated liquid-like outer layer and a more solid aggregate core (Wheeler, Jain et al. 2017).

To further strengthen our prediction that Cirbpa and b may have self-aggregating properties, we compared their sequences to both Buc and RNA-binding protein Fused in Sarcoma/ Translocated in Sarcoma (FUS), a well-characterized RNA-binding and aggregating protein that forms amyloid-like plaques in degenerative diseases including Amyotrophic Lateral Sclerosis (ALS) (Baloh 2012, Lu, Lim et al. 2017, Lee, Ghosh et al. 2020). As expected, the PLAAC algorithm predicted that FUS contains large PrLDs (Figure 3A).

Another characteristic of self-aggregating proteins is the presence of disordered regions (Schuster, Dignon et al. 2020). Disordered domains within proteins have no fixed tertiary structure unless induced by interacting with another protein or molecule. As many as one third of all proteins have significant stretches of disordered sequence, which correlates with functions such as RNA/DNA binding and protein-protein interactions (Shimizu and Toh 2009). Conversely, highly ordered proteins with very stable tertiary structures are correlated with enzymatic activity and receptor proteins. We used FoldIndex to predict disordered versus ordered regions in the primary sequences of Cirbpa, Cirbpb, Buc and FUS. We found that Cirbpa was predicted to be 80.5% disordered, and Cirbpb 99.5% (Figure 3B). The largest disordered regions spanned the entire C-terminus for both proteins, consistent with the N-terminus containing a conserved RRM domain. The Buc amino acid sequence is 70.4% disordered, while FUS is 90.5% (Figure 3B).

An essential feature of the FUS protein to form amyloid-like aggregates is a series of serine/glycine-tyrosine-serine/glycine repeats ([S/G]Y[S/G]) (Kato, Han et al. 2012). Cirbpa and Cirbpb also have [S/G]Y[S/G] repeats in their PrLDs (Figure 3C). Cirbpa has five copies of this motif, and Cirbpb has eight. When adjusted for protein length, this is a similar frequency to the repeats in the FUS protein (FUS ratio repeats:amino acid 1:25, Cirbpa 1:37, Cirbpb 1:25). Conversely, Buc does not contain this motif anywhere in its sequence, and neither did any other candidates in our proteomics dataset. This made Cirbpa and Cirbpb attractive candidates for further validation and functional study. We hypothesized that Cirbpa and Cirbpb bind to maternal mRNAs via their N-terminus RRM domain, while their C-terminus consists of a disordered tail with aggregating properties that may function in interacting with other proteins or forming amyloid-like structures or fibrils that contribute to the Bb (Figure 3D).

Cirbpa and Cirbpb localize to the Balbiani body *in vivo*

The first step in our further study of Cirbp was to examine its behavior in vivo. We generated Cirbpa-Venus and Cirbpb-Venus constructs to inject into living cells. We injected mRNA encoding the tagged Cirbp fusion proteins into live oocytes and observed their localization. When injected as a positive mRNA control, Venus-Buc localizes to the Bb as expected (Figure 4A), and a negative control of Venus alone did not show enrichment in the Bb (Figure 4D).

Both Cirbpa-Venus and Cirbpb-Venus localized to the Bb, validating our proteomics results and indicating that they are genuine Bb components *in vivo* (Figure 4B, C). In addition to fluorescence within the Bb, we also observed that the exogenous proteins spontaneously formed smaller granules within the cytoplasm. This may be an artifact of overexpression, especially in the case of Buc, which endogenously localizes exclusively to the Bb at this stage. It is of interest, however, if components of the Bb such as Buc and Cirbp are able to spontaneously nucleate smaller aggregates that could contribute to the growth of the Bb over the course of oocyte development.

Interestingly, we observed no nuclear localization of Cirbpa-Venus or Cirbpb-Venus in injected stage I oocytes. This was unexpected, given that Cirbp is a nuclear protein in mammalian cells (De Leeuw, Zhang et al. 2007). When we injected one-cell stage zebrafish

embryos with mRNA encoding zebrafish Cirbpa-Venus, in contrast, it localized to the nucleus (Figure 5A). Cirbp's nuclear localization is mediated by a nonclassical nuclear localization signal (Bourgeois, Hutten et al. 2020), which must be disabled in the oocyte, allowing for its unique localization pattern in stage I oocytes compared to other cells. This localization is also stage-specific; in stage II oocytes, after Bb disassembly, Cirbpa-Venus and Cirbpb-Venus are ubiquitous throughout the cytoplasm and also present within the nucleus (Figure 5B). The protein also no longer forms granules in the cytoplasm. In contrast, stage II oocytes injected with Venus-Buc have fluorescence arranged asymmetrically along the cortex, recapitulating endogenous localization (Figure 5B). Oocytes do not have time to progress from stage I to stage II between injection and imaging, indicating that exogenous Buc can correctly dock at the cortex without first localizing to the Bb.

Both the RRM domain and PrLD are sufficient to enrich Cirbpa within the Balbiani body

Finally we examined the role of Cirbpa's RRM and PrLD domains in Bb localization. We hypothesized that the PrLD of Cirbpa functions to localize the protein to the Bb, while the RRM domain binds maternal mRNA targets that are carried by the Bb. To test functional roles of each domain within Cirbpa, we generated Venus tagged truncation constructs and examined their localization in stage I oocytes. The first construct deletes the carboxy terminus of the protein (Cirbpa$^{\Delta 84-185}$-Venus), removing the entire PrLD and leaving only the RRM. The second removes the amino-terminal RRM (Cirbpa$^{\Delta 1-83}$-Venus) and leaves the full PrLD intact. We predicted that the PrLD would be necessary and sufficient for Bb localization. We injected oocytes with Cirbpa$^{\Delta 1-83}$-Venus and found that the PrLD can localize to the Bb without the RRM domain, as expected. However, when we injected Cirbpa$^{\Delta 84-185}$-Venus mRNA into stage I oocytes, we were surprised that the RRM alone was sufficient to enrich the protein within the Bb, showing that the PrLD is not an absolute requirement (Figure 5C). This experiment shows that both the RRM and the PrLD can associate with the Bb.

Discussion:

We identified a large number of candidate resident Bb proteins, including proteins known to be present in the Bb of other organisms, as well as proteins previously not found. Some of these proteins may be specific to zebrafish or may not have been detected yet in other organisms. Our Bb proteomics runs were limited by the number of Bbs that could be collected at one time (~100–200/collection) and were not exhaustive. Some known Bb localized proteins, such as Rbpms2/Hermes and Macf1, were not identified in our proteome, consistent with a non-exhaustive Bb proteome identification. However, we found many candidate proteins shared between the Bb and other ribonuclear protein aggregate structures, such as stress granules and P bodies (Figure 2D,E). Moreover, some candidate proteins, such as Vasa, Eif4enif1, Hspd1, Cirbpa, and Cirbpb, were also found enriched in an embryo germ plasm (Buc-GFP pulldown) proteomics study (Krishnakumar, Riemer et al. 2018). Further functional studies of these candidate proteins may lead to shared mechanisms across membraneless organelles.

We found that the Bb proteome is highly enriched for RNA-binding proteins, consistent with the large number of RNAs known to localize to the Bb. More surprisingly, we found many ribosomal subunits and proteins involved in translational control, which raises the still-unanswered question of if the Bb is translationally active or quiescent. Aggregates like stress granules sequester and translationally suppress mRNA transcripts, so we are inclined to speculate that mRNAs in the Bb are similarly translationally repressed. However, if ribosomes are present or even enriched, that might not be the case.

Of the proteins we identified in our proteomics, Cirbpa and b stood out because of their RNA-binding ability and predicted self-aggregating properties. The Bb stains positively for amyloid-like fibrils (Boke, Ruer et al. 2016), and we find that the Bb forms a robust, solid hydrogel-like structure within the cytoplasm. Our results support the hypothesis that Cirbpa and b have a stage I oocyte-specific function. Cirbpa and Cirbpb can localize to the Bb, validating our proteomics results. They do not localize to the nucleus as in somatic cells, and can spontaneously form granules in the oocyte. However, their localization patterns are unique compared to Buc in stage II oocytes, indicating that they likely function differently in the oocyte from Buc. In particular, the transition from associated with Buc in the Bb to no longer associated with Buc at the oocyte cortex suggests a change in Cirbp during Bb disassembly. Any functional insights into Bb disassembly are of interest, as understanding how the oocyte naturally dismantles a large protein aggregate may provide avenues for studying how to reverse deleterious aggregates in human disease.

Beyond their function in the cold stress response, Cirbps are involved in a variety of cellular and disease processes. They mitigate damage from hypoxia by inhibiting apoptosis, and are classified as proto-oncogenes due to this apoptosis-suppressing function; they also have been implicated in pro-inflammatory pathways, and are neuroprotective post CNS injury. Cirbp is also a component of stress granules, and can spontaneously form phase-separated liquid-liquid droplets in vitro (Bourgeois, Hutten et al. 2020). Since zebrafish Cirbpa and Cirbpb are also predicted to self-aggregate in addition to bind RNA (Figure 3D), there may be shared mechanisms between the two structures.

In the Bb, we found that both the RRM and the PrLD of Cirbp can localize to the Bb independently. We hypothesize that the RRM domain may bind to the enriched mRNA within the Bb and therefore becoming enriched itself. It is important to note that PrLDs do not all function similarly in vivo and are not necessarily interchangeable. In Xenopus, when the PrLD of Xvelo, the Buc ortholog, was replaced by the PrLD of the prion-like protein FUS, it failed to localize to the Bb (Boke, Ruer et al. 2016). Here we show that the PrLD of Cirbp is sufficient to localize to the Bb; however, whether it could substitute for the Buc PrLD will require further studies. Our results open up the possibility of multivalent interactions where each Cirbpa protein has multiple binding partners, and together the PrLD and RRM integrate mRNA cargo into the Bb or stabilize the Bb by crosslinking mRNAs to the amyloid-like aggregates within the structure. In our model, Cirbp spontaneously self-aggregates via the PrLD tail, while the RRM binds to mRNA cargo. As the Bb grows, Cirbp, Buc, and other protein components of the Bb form a fibrous tangle around mRNA, mitochondria and other smaller organelles (Figure 6). By validating Cirbpa and Cirbpb

as Bb resident proteins, we added another link between the Bb and other membraneless organelles, and an avenue into further functional studies of the Bb in the zebrafish.

Methods

Animal Husbandry:

Adult zebrafish were kept at 28° C in a 13-hr light/11-hr dark cycle. All experiments and animal husbandry were done in compliance with NIH guidelines and those of the University of Pennsylvania and were approved by the University of Pennsylvania IACUC. The Tübingen (TU) strain was used as wild type. Ovaries were dissected from juvenile female zebrafish (approximately 8 weeks post fertilization, standard length 15–21 mm) as described (Elkouby and Mullins 2017).

Size quantification:

Stage I oocytes were isolated (Elkouby and Mullins 2017) and stained with $DiOC_6$ at a concentration of 20 nM for 1 hr followed by two 30 min washes with L-15 media, then imaged by confocal microscopy. Image stacks were sectioned using MATLAB to generate outlines of the oocyte and Bb. A 3D ellipsoid was fit to the outlines, and the lengths of the principal axes were averaged to obtain a value for the oocyte and Bb diameter.

Balbiani body collection and proteomics:

Bbs were collected as described in (Jamieson-Lucy and Mullins 2019b). Briefly, we dissociated the ovaries to release single oocytes by incubating with digestive enzymes (Collagenase I, Collagenase II and Hyaluronidase) and manual separation. Bbs were visualized using $DiOC_6$, then the oocytes where lysed into a protease inhibitor cocktail by passing them multiple times through a 30-gauge needle, freeing the labeled Bbs. The Bbs were then collected using a microinjection needle and transferred to a minimal volume of protease inhibitor cocktail and stored at −80 °C until nanoLC/nanospray/MS/MS. The University of Pennsylvania proteomics core facility performed the analysis and data were acquired with Xcalibur 2.0 (ThermoFisher) and analyzed with Scaffold 3 (Proteome Software) software package. Cutoffs: Peptide p-value: <95%; Protein p-value <99.0%.

RNAseq:

Whole adult zebrafish ovaries were dissociated using the same process as described in the Bb proteomics. Oocytes were then separated by size to isolate different stages, and their mRNA extracted (Elkouby and Mullins 2017). After RNA extraction, samples were treated with oligo(dT) beads to enrich for poly(A)+ RNA, according to the manufacturer protocol. RNA-seq libraries were prepared using strand-specific TruSeq Illumina adapters and sequenced by the Yale Center for Genome Analysis. For record keeping and bioinformatics analysis, sample annotations were stored in LabxDB (Vejnar and Giraldez 2020). The “import_ensembl” tool from the FONtools (Vejnar) was used (a) to import Ensembl release 78 (Howe, Achuthan et al. 2020), (b) to create an index for the Zv9 zebrafish genome, and (c) to generate FON1 files containing genes (or “metagenes”) that concatenates the isoforms of each gene together using the “--method union” option of the “fon_transform” tool (Vejnar). Raw reads were mapped onto zebrafish genome Zv9 using STAR (Dobin, Davis

et al. 2012) with default parameters. Read counts per gene were computed by summing the total number of reads overlapping at least 10 nucleotides the gene annotation. Reads mapping to multiple loci accounted for 1 divided by the number of loci to which the read was mapped to. Counts were normalized to RPKM using gene length and the total number of reads mapped to the zebrafish genome.

Protein primary sequence and network analysis:

Primary protein sequences were analyzed using Foldindex (Prilusky, Felder et al. 2005) to predict protein order and disordered sequences, and PLAAC (Prion-Like Amino Acid Composition) (Lancaster, Nutter-Upham et al. 2014) to identify possible self-aggregating domains. The STRING network (Szklarczyk, Gable et al. 2019) was generated using the subset of the 77 replicated protein hits which could be identified by the *Danio rerio* STRING v11 dataset.

Oocyte injections:

Starting with juvenile ovaries with few late-stage oocytes, we used forceps to manually remove oocytes larger than stage II. Oocyte injections were performed as described (Kobayashi et al, 2021). The oocytes were embedded in a thin layer of low-melt agarose to immobilize them, and microinjected with a minimal volume of either dextran (final concentration 4 mg/ml) or mRNA. mRNA for injection was generated using the SP6 MMessage Machine kit (ThermoFisher Science AM1340). After injection, oocytes were incubated overnight in L-15 media and then stained with either Mitotracker red at a concentration of 100 nM or $DiOC_6$ at a concentration of 20 nM for 1 hr followed by two 30 min washes with L-15 media. The layer of agarose was then pressed against a cover slip and imaged using confocal microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The solid membraneless oocyte Balbiani body aggregate as a size exclusion barrier
- Balbiani body isolation, collection, and proteomics reveals 77+ proteins
- Cold Inducible RNA Binding Protein validated as Balbiani body resident protein
- Cirbp Prion-like domain and RRM independently localize to the Balbiani body

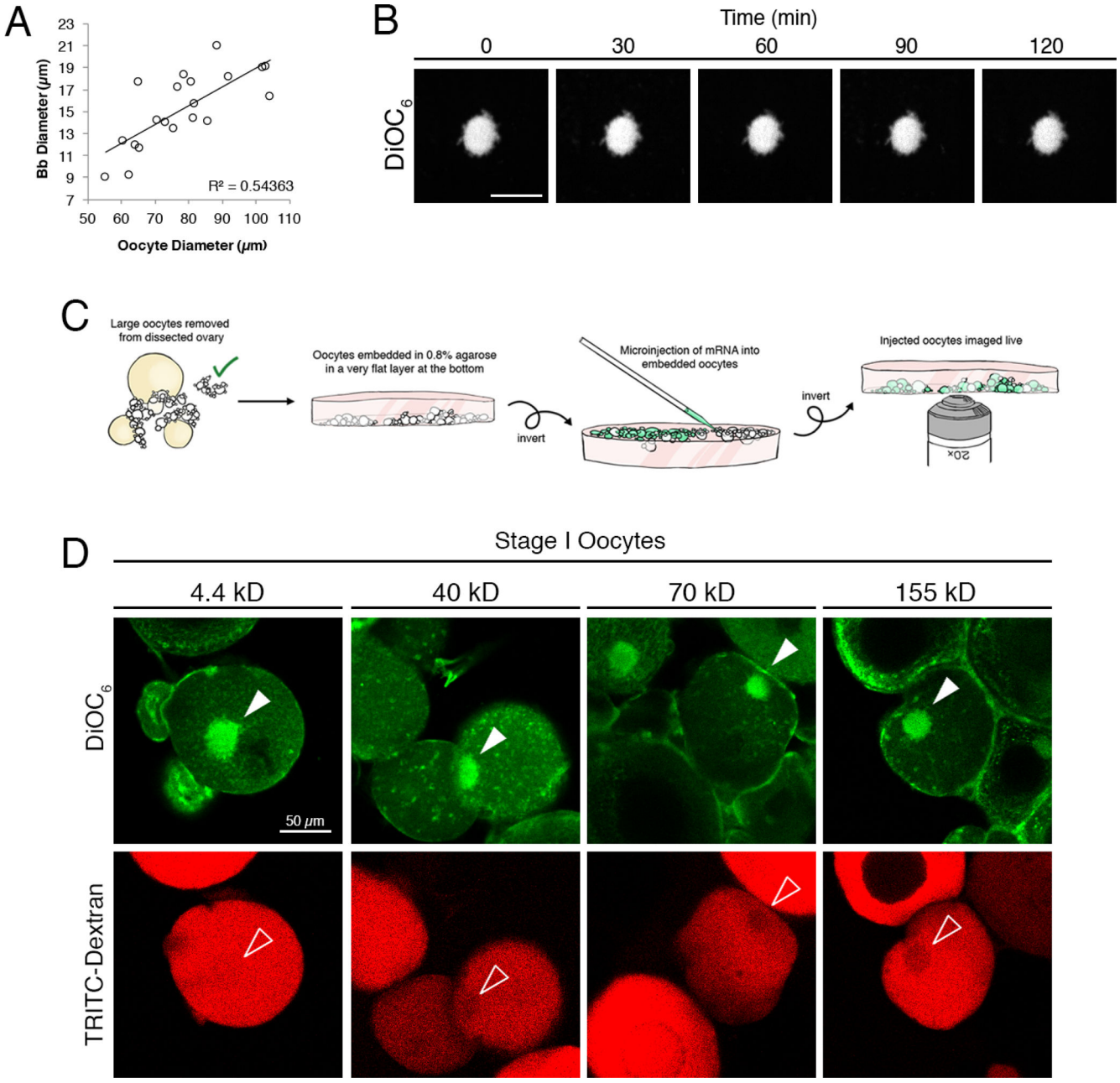

Figure 1: The Balbiani body exhibits hydrogel-like properties.

a) Bb diameter plotted against oocyte diameter. b) Time course of isolated Bbs in PBS (scale bar: 50 μm. n=6 Bbs). c) Schematic depicting injection technique. d) Live stage I oocytes injected with TRITC-labeled dextran and incubated for 2 hours before imaging for DiOC6 and TRITC (4.4 kD: n=12 oocytes, 40 kD: n=9, 70 kD: n=17, 155 kD: n=16 oocytes). Solid arrows: Bb; open arrows: Bb position.

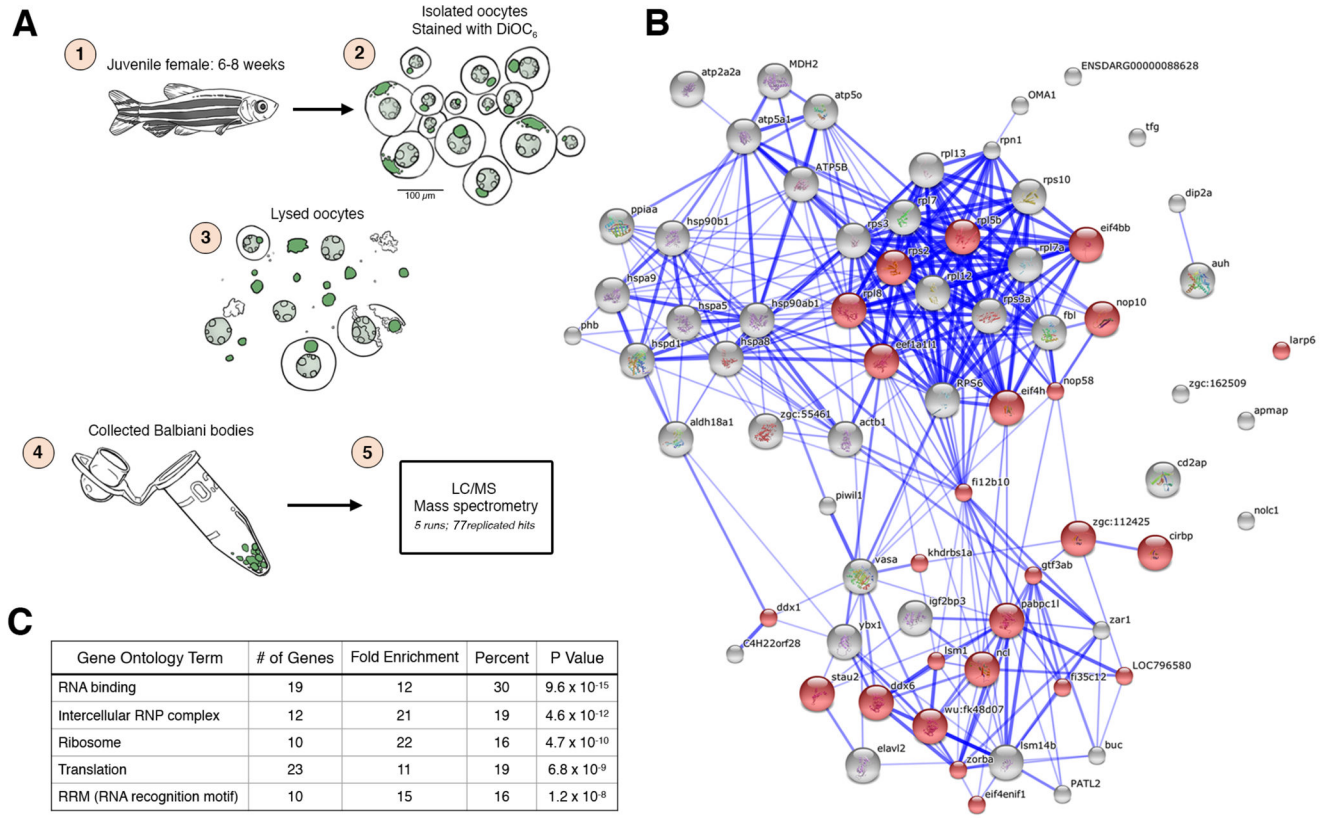

Figure 2. Mass spectrometry identifies novel Balbiani body resident candidate proteins and reveals similarities to other membraneless organelles.

a) Experimental setup: stage I oocytes are harvested from juvenile zebrafish ovaries, stained with $DiOC_6$, and lysed to release Bbs. Bbs are collected and sent for LC/MS mass spectrometry. 5 independent experiments yielded 217 total identified proteins and 77 replicated protein hits (present in at least 2 experiments). b) STRING network showing predicted interactions between Balbiani body proteins. Heavier lines between proteins indicate more evidence of a relationship between the two proteins. Proteins with RNA-binding properties are highlighted in red. c) Gene ontology (GO) for replicated hits. Fold enrichment: enrichment over background frequency of GO term in full Danio rerio genome. Percent: percent of analyzed Bb proteomic hits annotated with GO term. d) All identified

proteins compared to previously identified Bb proteins and known stress granule/p body proteome, and e) the reported Xenopus Bb proteome. f) Peptide count (total) for all replicated proteins plotted against RPKM from RNAseq of early stage I oocytes (40–70 μm).

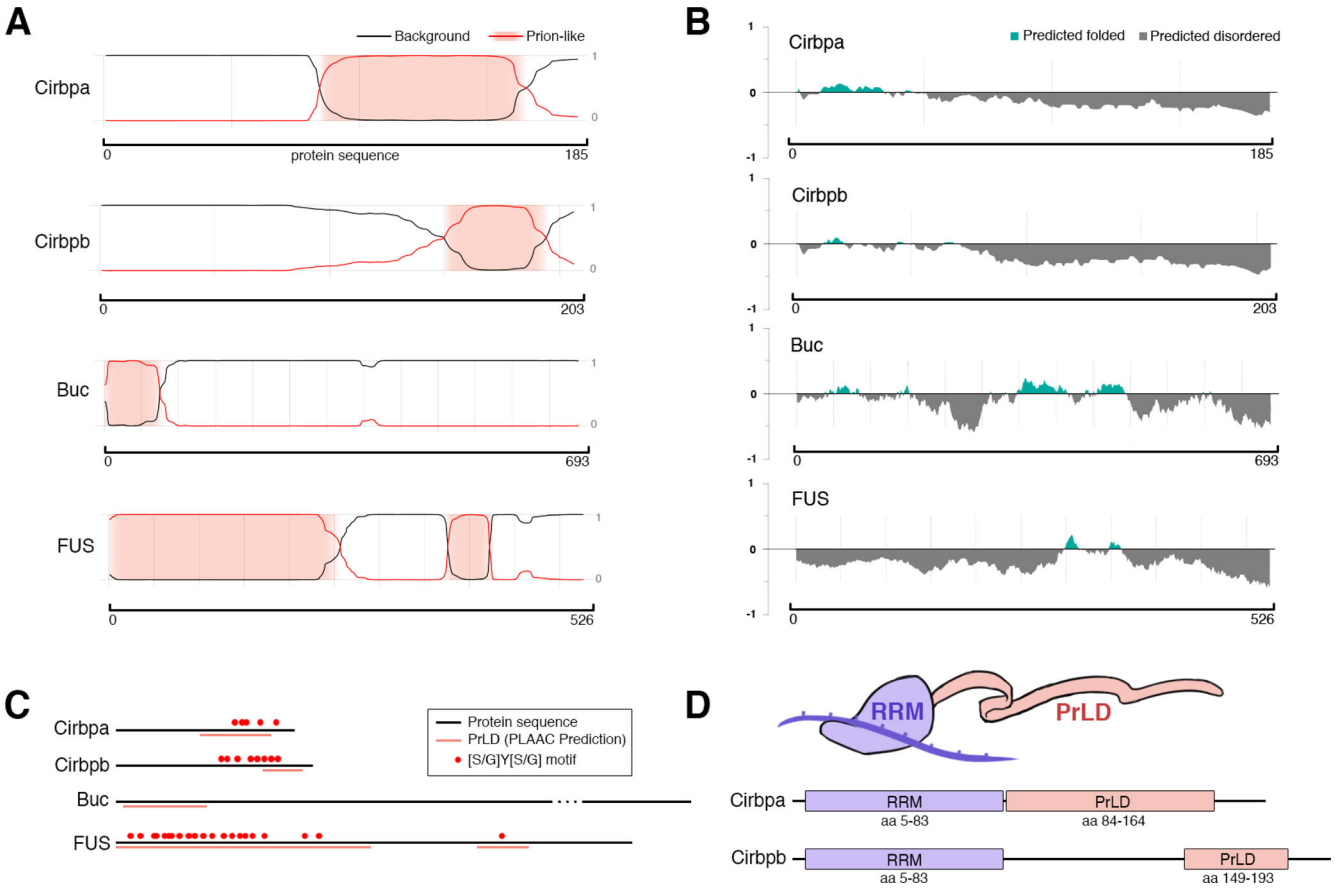

Figure 3. Amino acid sequence-based analysis shows Cirbpa and b have predicted self-aggregating properties similar to Buc and FUS.

a) PLAAC results predicting prion-like self aggregation domains. Peach shading indicates predicted self-aggregating sequence. b) FoldIndex disorder predictions. c) Comparison of [S/G]Y[S/G] repeats between Cirbpa, Cirbpb, Buc and FUS. d) Domain structure of Cirbpa and Cirbpb.

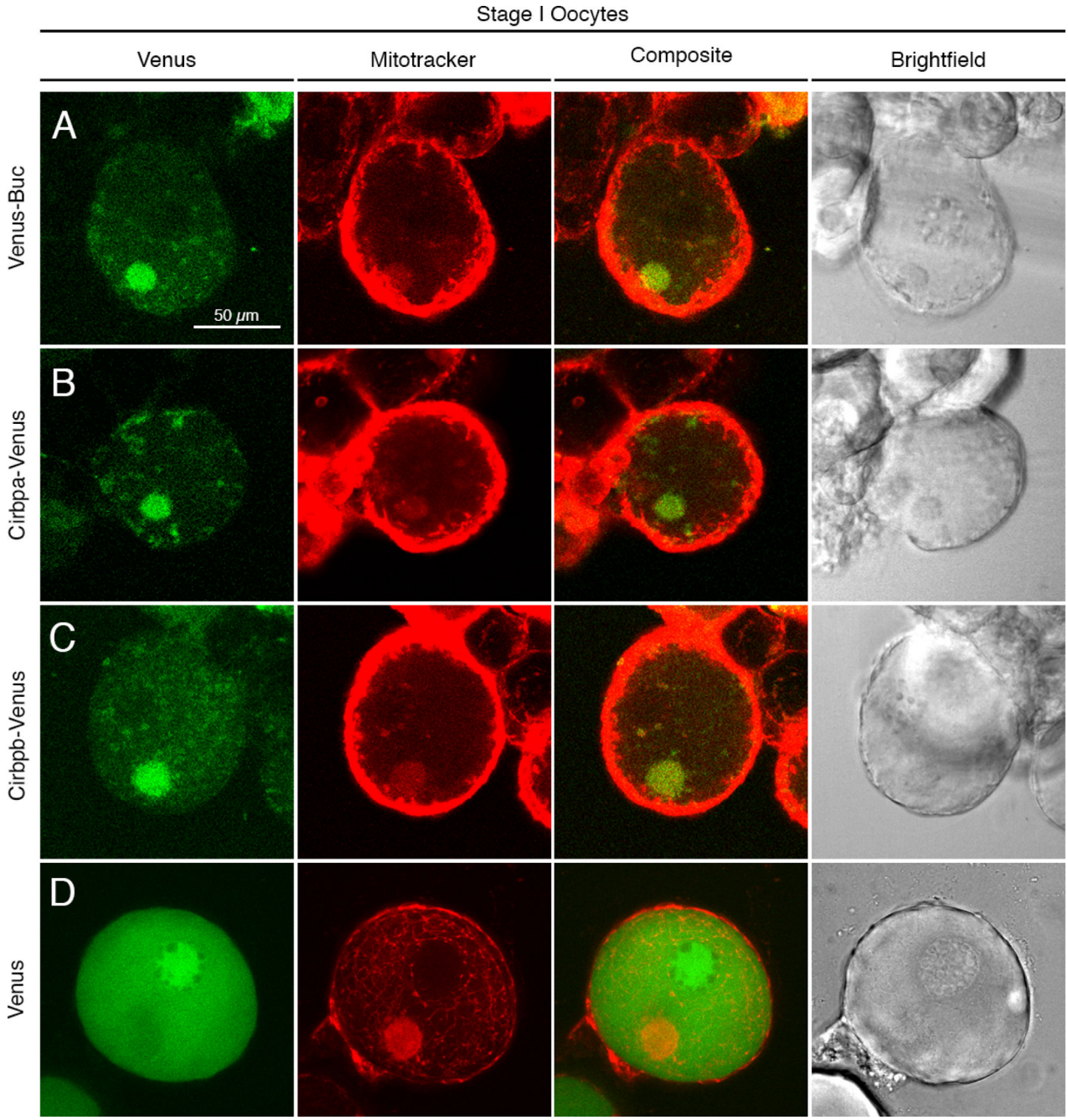

Figure 4. Cirbpa and Cirbpb localize to the Bb *in vivo*.

a-d) Stage I oocytes injected with a) Buc-Venus (n=15 oocytes) b) Cirbpa-Venus (n=9 oocytes), c) Cirbpb-Venus (n=13 oocytes) or d) Venus (n=6) (green) and stained with mitotracker (red). The Bb is visible in brightfield images. All results representative of at least 2 experiments.

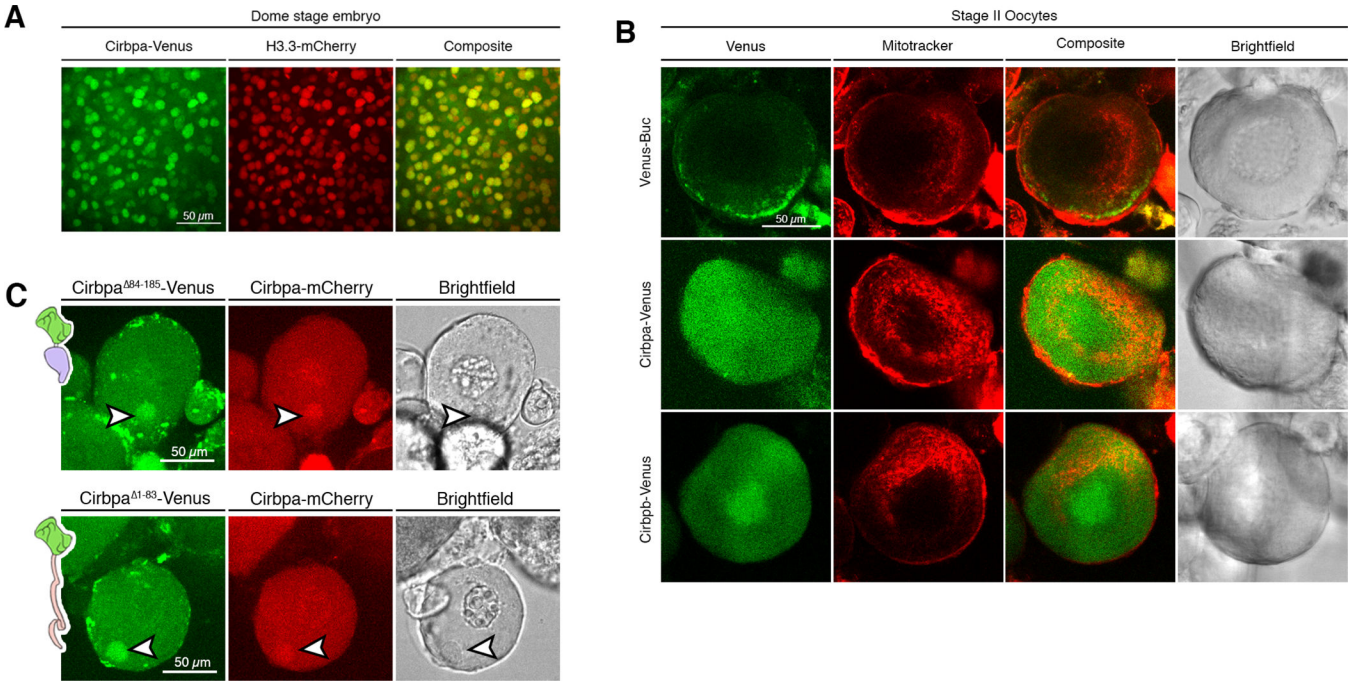

Figure 5. Oocyte and stage-specific localization of Cirbp by its RRM and PrLD.

a) Dome stage embryo (animal z-slice) injected at the 1-cell stage with mRNA encoding Cirbpa-Venus (green) and H3.3-mCherry to label the nucleus (red) (n=6 embryos). b) Stage II oocytes injected with mRNA encoding Buc-Venus (n=13 oocytes), Cirbpa-Venus (n=3 oocytes), or Cirbpb-Venus (n=3 oocytes) (green) and stained with mitotracker (red). All results representative of at least 2 experiments. c) Stage I oocytes co-injected with Cirbpa$^{\Delta 84-185}$-Venus (RRM, lacking the carboxy-terminal PrLD) or Cirbpa$^{\Delta 1-83}$-Venus (PrLD, lacking the amino-terminal RRM) (green) and Cirbpa-mCherry (red). A white arrowhead denotes the Bb.

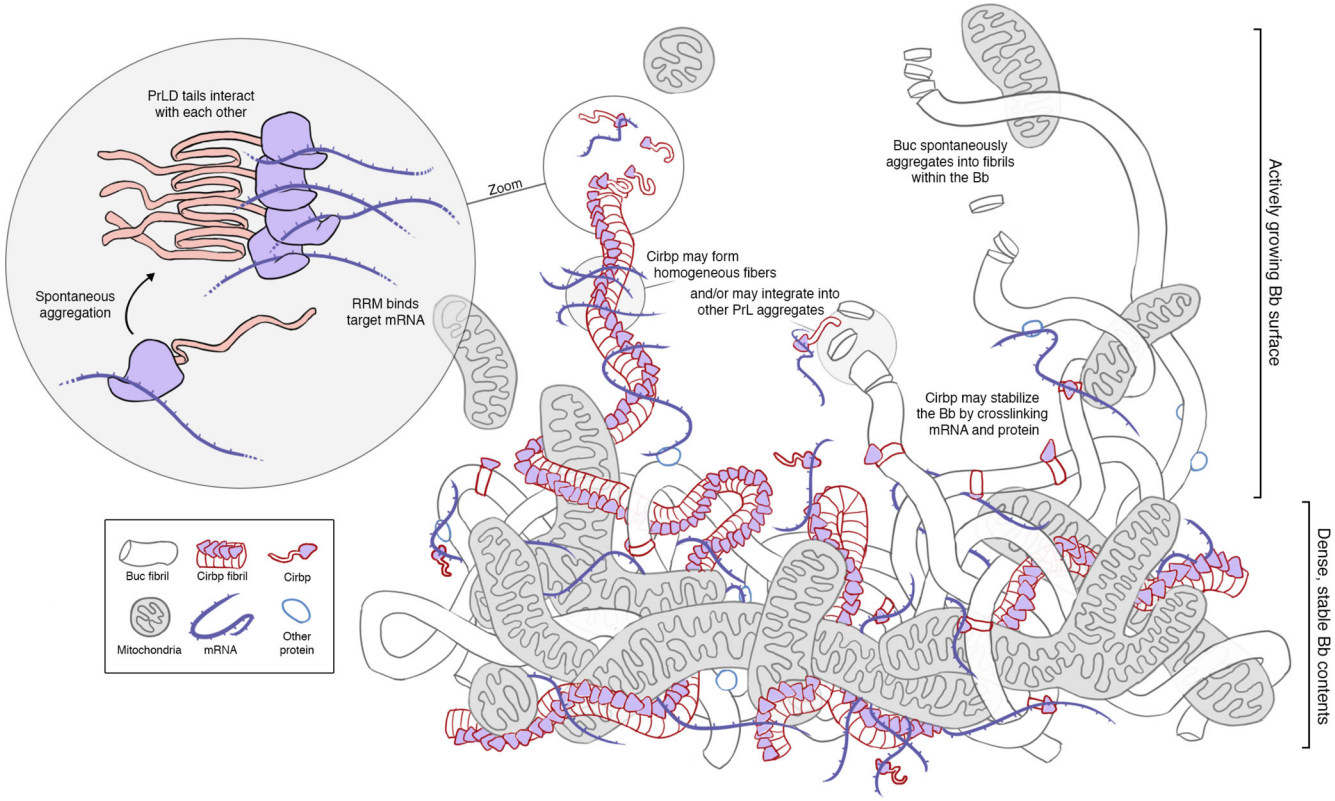

Figure 6. Schematic depicting a model of Cirbp's integration into the Balbiani body.

Hypothetical model for how Cirbp may self-aggregate into fibers and contribute to the hydrogel structure of the Bb. Cirbp may form homogenous or heterogeneous fibrils while binding to Bb mRNAs. Mitochondria reside within the structure, which solidifies into a densely cross-linked network.

Table 1.

Top 25 identified proteins by total peptide count.

Gene Name	Gene Symbol	Also known as	Peptide count	Granule association	Function	Phenotype	Reference
LSM family member 14B	Lsm14b *	Rap55	109	Balbiani body (mouse, fly)	Translation control and mRNA binding activity	-	(Cai-Lian, Qi-Wen et al. 2010)
DEAD (Asp-Glu-Ala-Asp) box helicase 6	Ddx6 *	p54a	86	Balbiani body, p bodies	Translation suppression and mRNA degradation	Morpholino causes tail defect in zebrafish	(Ayache, Bénard et al. 2015, Huang, Ku et al. 2017)
Elongation factor 1-alpha (si:dkey-37o8.1)	Eef1a	Ef-1 alpha	74	Promotes aggresome formation	Translational elongation	Mutants implicated in neurological disorders in mammals	(Meriin, Zaarur et al. 2012, Idigo, Soares et al. 2020)
poly(A) binding protein cytoplasmic 1-like	Pabpc1l	ePAB	70	Stress granules	mRNA binding and post-trancriptional regulation	Mouse mutant females are infertile	(Friend, Brook et al. 2012, Kobayashi, Winslow et al. 2012)
Zygote arrest 1	Zar1	-	68	Granules in 2-cell mouse embryo	Required for oocyte development	In zebrafish oocytes apoptose and adult mutants become all male	(Miao, Yuan et al. 2017)
Y-box binding protein 1	Ybx1	Yb1, Nsep1	66	Stress granules	Cold shock response and mRNA regulation	Zebrafish maternal effect mutant: embryos display cleavage defects and fail to undergo MZT	(Kumari, Gilligan et al. 2013, Sun, Yan et al. 2018)
Insulin-like growth factor 2 mRNA binding protein 3	Igf2bp3	Vg1-RBP	62	mRNA vegetally localized in zebrafish oocytes	mRNA transport and localization	Maternal effect causes severe developmental defects in zebrafish	(Ren, Lin et al. 2020)
Eukaryotic translation initiation factor 4E nuclear import factor 1	Eif4enif1	4E-T	61	P bodies	Translation factor shuttling	-	(Ayache, Bénard et al. 2015)
Si:dkey-208k4.2 protein (P43 5S RNA-binding protein, medaka)	42Sp43	Thesaurin-b	44	Not found in Xenopus Balbiani body	Zinc-finger, RNP storage particle component	-	(Joho, Darby et al. 1990, Schneider, Dabauvalle et al. 2010)
Cytoplasmic polyadenylation element binding protein 1b	Cpeb1b	Zorba	33	Stress granules	mRNA binding and post-transcriptional regulation, Dazl regulator	Mutations associated with human ovarian deficiency	(Wilczynska, Aigueperse et al. 2005, Sousa Martins, Liu et al. 2016)
Heat shock protein 5	Hspa5	BiP, Grp78	30	-	Protein folding and assembly	Zebrafish mutant is lethal	(Amsterdam, Nissen et al. 2004, Moreno and Tiffany-Castiglioni 2015)
Cold inducible RNA-binding protein a	Cirbpa *	-	28	Stress granules	Cold shock response and mRNA regulation	Mouse mutant males have imparied spermatogonia proliferation, otherwise fertile	(De Leeuw, Zhang et al. 2007, Masuda, Itoh et al. 2012)

Gene Name	Gene Symbol	Also known as	Peptide count	Granule association	Function	Phenotype	Reference
Heat shock protein 90alpha1.2	Hsp90aa1.2	Cb820	27	-	Protein folding and assembly	Zebrafish mutant has PGC migration defects	(Du, Li et al. 2008, Pfeiffer, Tarbashevich et al. 2018)
ATP synthase F1 subunit alpha	Atp5fa1	Atp5a1	25	-	ATP synthesis	-	(Jonckheere, Renkema et al. 2013)
Cold inducible RNA-binding protein b	Cirbpb *	-	25	Stress granules	Cold shock response and mRNA regulation	Mouse mutant female produce morphologically normal oocytes but embryos fail to complete MZT	(De Leeuw, Zhang et al. 2007, Masuda, Itoh et al. 2012)
Piwi-like RNA-mediated gene silencing 1	Piwil1	Ziwi	22	Nuage, Germ granules	germ cell development and piRNA metabolic process	Loss of Ziwi Triggers Germ Cell Apoptosis	(Houwing, Kamminga et al. 2007)
Poly(A) binding protein nuclear 1-like	Pabpn1l	-	20	Nuclear aggregates	mRNA binding and post-transcriptional regulation	Mouse mutant female produce morphologically normal oocytes but embryos fail to complete MZT	(Zhao, Zhu et al. 2020)
Heat shock 60 protein 1	Hspd1	Hsp60	20	-	Protein folding and assembly	Zebrafish mutant has regeneration defects	(Pei, Tanaka et al. 2016)
PAT1 homolog2	Patl2	-	20	P bodies	Translational repression	Mutations in mice and humans cause female infertility	(Nakamura, Tanaka et al. 2010, Ozgur, Chekulaeva et al. 2010, Chen, Zhang et al. 2017)
ATP synthase F1 subunit beta	Atp5f1b	-	19	-	ATP synthesis	-	(Jonckheere, Renkema et al. 2013)
Bucky ball	Buc *	-	18	Balbiani body	Balbiani body formation, oocyte polarity	Zebrafish mutant lacks Balbiani body, AV polarity defects	(Marlow and Mullins, 2008, Bontems, Stein et al. 2009)
Elongation factor 1-alpha (eef1a1b)	Eef1a	Ef-1 alpha	18	Promotes aggresome formation	Translational elongation	Mutants implicated in neurological disorders in mammals	(Meriin, Zaarur et al. 2012, Idigo, Soares et al. 2020)
ELAV like neuron-specific RNA binding protein 2	Elavl2	HuB	17	Germ granules	mRNA binding and post-transcriptional regulation	suggested to be involved in germ-cell maintenance in Xenopus	(Mickoleit, Banisch et al. 2011, Wiszniak, Dredge et al. 2011)
Elongation factor 1-alpha (eef1a1l1)	Eef1a	Ef-1 alpha	17	Promotes aggresome formation	Translational elongation	Mutants implicated in neurological disorders in mammals	(Meriin, Zaarur et al. 2012, Idigo, Soares et al. 2020)
Malate dehydrogenase 2, NAD (mitochondrial)	Mdh2	-	17	-	carbohydrate metabolic process and malate metabolic process	Human ortholog(s) of this gene implicated in developmental and epileptic encephalopathy 51	(Gebriel, Prabhudesai et al. 2014, Ait-El-Mkadem, Dayem-Quere et al. 2017)

An * indicates that the protein has been validated as a Bb component.