Small Molecule Modulators of INAVA Cytosolic Condensate and Cell-Cell Junction Assemblies

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Re: JCB manuscript #202007177

Dr. Wayne I Lencer
Boston Children's Hospital Harvard Medical School
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Dear Dr. Lencer,

Thank you for submitting your manuscript entitled "Small Molecule Modulators of INAVA Cytosolic Condensate and Cell-Cell Membrane Junction Assemblies" and for your patience as the peer review time took longer than we strive for at JCB. Please accept our sincere apologies for the delay. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while the reviewers find that your study provides potentially interesting molecular insight into IBD, both reviewers have concerns that the nature and behavior of the INAVA condensates need to be characterized in greater detail. Therefore, in revising please carefully attend to all of the reviewers' constructive comments with the addition of the requested experimental data.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Judith Frydman, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The manuscript entitled "Small Molecule Modulators of INAVA Cytosolic Condensate and Cell-Cell Membrane Junction Assemblies" by Chang, Luong et al., describes a novel property of the inflammatory bowel disease risk gene INAVA (Innate Immune Activator): the ability to form, in response to the inflammatory cytokine IL-1b, biomolecular condensates that contain ubiquitin and the E3-ligase bTrCP2. The authors show that these cytoplasmic foci form via liquid-liquid phase separation. Using epithelial HCT8 cells stably expressing INAVA-GFP the authors screened for molecules that promote puncta formation and puncta disassembly. The authors identified inhibitors of the molecular chaperone Hsp90, proteasome inhibitors and H2O2, which causes oxidative stress resulting in protein oxidation and aggregation, as inducers of INAVA puncta formation. Since the INAVA puncta colocalized with polyubiquitinated proteins and with the E3 protein ligase bTrCP2, the authors proposed a role for the relocalization of INAVA from the membrane to cytoplasmic puncta in the regulation of cellular proteostasis. This conclusion warrants further investigations, as detailed below. It is unclear whether the cytoplasmic foci formed upon Hsp90 or proteasome inhibition or following treatment with H2O2 are biomolecular condensates that "age", as suggested by the authors, or rather protein aggregates that are transported with time to the perinuclear
aggresome.

Major comments:

- "Condensates are also described to undergo a process called maturation where their dynamic highly mobile nature changes over time and they become more static and less fluid (10)". The authors refer to the paper by Lin et al (2015), who characterized the maturation over time of RNA-containing droplets using recombinant proteins in vitro and showed that the stabilization was due to the formation of amyloid-like fibers. When studied in the cellular context, most of these condensates, including for example RNA-containing stress granules, are highly dynamic and rapidly disassemble with time. Maturation of stress granules or other types of condensates into an "amyloid-like" state has been described in cells, but generally this is linked to either the presence of mutated RNA-binding proteins that acquire increased aggregation propensity or to failure of the protein quality control (PMID: 30893049; PMID: 29804830; PMID: 28377462). Is the conversion into an amyloid-like state also occurring for the less mobile INAVA condensates? It would be important to understand whether these newly described INAVA condensates spontaneously convert from a liquid-like state into an amyloid-like state, using for example amyloid-specific dies (e.g. amylo-glo). This is particularly important considering that: 1) the authors previously implicated INAVA condensates in protein ubiquitination and they show that INAVA foci colocalize with polyubiquitinated proteins (FK2) upon inhibition of the proteasome or Hsp90; 2) the accumulation of polyubiquitinated proteins within other types of condensates, such as e.g. nucleoli and PML bodies, has been associated with their conversion into an amyloid-like state (PMID: 27720612; PMID: 31296649; PMID: 31271238). Does inhibition of the proteasome or Hsp90 promote the conversion of INAVA foci into an amyloid-like state?

In addition, what is the % of old puncta that do not disassemble and persist with time? Or do they all disappear over 180 min (Fig 1A)? A better characterization/definition of these "young" and "old" foci should be provided.

- The authors show that five Hsp90 inhibitors, used at nM concentrations, as well as the two proteasome inhibitors MG132 and Bortezomib or H2O2 induced INAVA foci formation. Under these conditions, the appearance of these foci occurred much faster compared to IL-1b and the foci were more irregular in shape and did not resolve in time. Moreover, these foci colocalized with the polyubiquitinated protein marker FK2. Based on these findings the authors conclude that "The functions annotated for the compounds inducing INAVA condensates imply that INAVA responds to changes in cellular proteostasis" and "These studies showed the predicted results: all newly identified agonists induced the release of INAVA from lateral membranes of Caco2BBe cells and the formation of large cytosolic condensates (Figure 3H). Thus, INAVA appears to act as a sensor and effector of cellular proteostasis - we hypothesize with its sensing activity localized to lateral (plasma) membranes, and its effector activity localized to cytosolic condensates containing machinery for targeted protein ubiquitination."

The finding that all the identified agonists induced the release of INAVA from the lateral membranes and promoted its phase separation in the cytoplasm and the finding that INAVA-GFP puncta colocalize with FK2 and the E3-ubiquitin-ligase βTrCP2 do not necessary imply a function for these foci in proteostasis. First, the large INAVA-GFP perinuclear puncta observed after 4 hrs of treatment with the Hsp90 inhibitors or MG132 (Fig 3E) are reminiscent of aggresomes, which typically form when the cellular capacity to degrade misfolded proteins is exceeded, such as it occurs upon proteasome inhibition. In addition, similar to the INAVA-GFP perinuclear foci, aggresomes colocalize with polyubiquitinated proteins. Aggresomes are localized at the MTOC, colocalize with HDAC6 and are encaged by intermediate filament proteins. It would be important to verify whether the INAVA-GFP perinuclear puncta induced by Hsp90 or proteasome inhibition are
located at the MTOC and colocalize with HDAC6 and intermediate filament proteins.

Second, does INAVA interact with Hsp90 and is its stability depending on functional Hsp90? In other words, the authors should test whether INAVA is a client of Hsp90 and whether prolonged treatment with the Hsp90 inhibitors leads to the progressive proteasome-mediated degradation of INAVA, as it occurs for several Hsp90 known clients. It is possible that short-term treatment of the cells (up to a few hrs) with the Hsp90 inhibitors leads to the accumulation of INAVA-GFP in cytoplasmic puncta/aggresomes, while prolonged treatment will lead to their progressive clearance by the proteasome and/or autophagy. These aspects should be verified. Moreover, is INAVA itself ubiquitinated upon Hsp90 inhibition or proteasome inhibition? And would polyUb-INAVA rather than other polyubiquitinated proteins be the major component of the FK2-positive/INAVA-positive foci observed upon Hsp90 inhibition or proteasome inhibition? These experiments are important to understand the link between INAVA-GFP relocalization to cytoplasmic condensates/puncta and their proposed role in proteostasis. At this stage the authors cannot use the data obtained under inhibition of Hsp90 or proteasome to ascribe a role in proteostasis to the IL-1b induced INAVA condensates.

- Concerning the control experiment performed by overexpression of myc-βTrCP2 alone, the authors concluded that "When over expressed on its own, myc-βTrCP2 did not form cytosolic puncta again indicating dependence on INAVA for condensate formation (Supplemental Figure 3H)." An alternative possible explanation is that, upon Hsp90 or proteasome inhibition, myc-βTrCP2 participates in targeting unfolded and/or polyubiquitinated INAVA-GFP to aggresomes, similar to what was previously found for e.g. CHIP, another E3 ligase and iNOS (see PMID: 18955503).

- In Fig 4A-B the authors show that the inhibitors of p38 and mTOR strongly decreased IL-1b-induced INAVA condensate formation, while having a milder effect on H2O2-induced INAVA condensates. They correlate the lower sensitivity of H2O2-induced condensates to their lower mobility. The authors then show that translation inhibitors promote the disassembly of IL-1b induced INAVA condensates and search for other agents able to resolve the less mobile "older" H2O2-induced condensates. They find that the protein translation inhibitors cycloheximide, emetine, as well as the MELK inhibitor OTSSP167, efficiently antagonize INAVA-condensate formation (Supplemental Fig 4.1 and 4.2). Intriguingly, like cycloheximide and anisomycin, mTOR kinase inhibitors attenuate general protein synthesis. Moreover, MELK phosphorylates eIF4B, similar to mTORC1/p70 S6K, sustaining protein synthesis (PMID: 27528663; PMID: 23105104). Thus, inhibition of MELK also affects protein synthesis. Considering that: 1) phase separation is concentration dependent; 2) the pool of INAVA that is found inside the IL-1b-induced condensates is highly mobile and "in equilibrium" with the surrounding cytoplasm (FRAP data shown in Fig 1), a decrease in the overall levels of INAVA-GFP might contribute to the progressive disassembly of the condensates. Can the authors comment on this aspect?

- Having found chemical compounds able to promote the disassembly of INAVA condensates, the authors then test their efficacy on stress granules containing FUS or FMR1 and FXR1, whose conversion into a less mobile state has been linked to neurodegenerative diseases. Pretreatment of the cells with the MELK inhibitor OTSSP167, the mTOR inhibitor INK128, but not with the p38 inhibitor SB203580 for 30 minutes reduced the % of cells with SGs induced by arsenate or MG132 (Figure 5A-E). Based on these data the authors conclude that "the disassembly, or degradation, of mature biomolecular condensates of different compositions seen in human disease may be similar to the mechanism of dissolution for INAVA condensates". First, to substantiate their findings the authors should test whether addition of the inhibitors only during the recovery phase after stress (and not before and during the acute stress) accelerates SG dissolution. Second, while the
composition of SGs has been well-established by different groups using microscopy, mass spectrometry and biochemistry, the composition of these newly reported INAVA condensates is still only partly known. Moreover, SG assembly and disassembly are known to be in equilibrium with polysomes and P-bodies; this is still unclear for the INAVA condensates. In light of these observations, I would avoid statements that generalize the mechanisms of assembly/disassembly of different types of condensates.

- In the literature SGs that fail to dissolve are referred to as "aberrant" or "pathological". The mechanisms leading to SG conversion from a dynamic into a "pathological" state are under investigation and include e.g. the recruitment inside SGs of mutated, ALS and FTD-linked, RBPs or defective functionality of the protein quality control (e.g. Hsp70 inhibition, VCP inhibition or mutations), as mentioned earlier. The authors refer to the "old" INAVA condensates to as pathological condensates, although it is unclear whether the mechanisms responsible for the hardening of the INAVA condensates are similar to those identified for SGs; moreover the "pathological" consequences of the hardening of INAVA condensates are unclear. I recommend carefully rephrasing the definition of "pathological" condensates throughout the manuscript.

- "The validated list includes three functionally related sets of compounds. The top-ranked "hit" was the inhibitor of protein translation cycloheximide, followed closely by emetine. Both have been shown before to antagonize other molecular condensates, thus implicating RNA, the ribosome, and nascent protein translation in condensate formation or maintenance (17-19)." This is another example of a general statement that applies specifically to RNA-containing granule, like SGs, but that cannot be directly generalized to INAVA condensates. It is well-established that SG formation occurs after polysome disassembly, when RNAs are released in massive amounts (PMID: 25013173; PMID: 32302572); conversely, treatment of the cells with cycloheximide and emetine, which stabilize the polysomes by freezing ribosomes on translating mRNAs, antagonize SG assembly and actively dissolve them (PMID: 11121440). Are similar mechanisms regulating INAVA condensate assembly/disassembly? Do INAVA condensate contain RNA and RBPs? The text should be carefully revised. Concerning the reference cited (17-19), these should be verified; Meriin et al., 2012 refers to aggresomes; the other two references are specific for SGs (Mokas et al 2009; Thomas et al 2009) and cannot be used to generalize the implication of RNA and ribosomes in condensate formation. This because different mechanisms regulate the formation of different types of condensates: e.g. PML nuclear bodies assemble through SUMO/SIM interactions, while mRNA concentration regulates SGs and P-bodies assembly (PMID: 27374333).

- In the last part of the paper the authors show that inhibition of the RhoA kinase (ROCK) led to the recruitment of INAVA to cell-cell contact sites and decreased the formation of cytoplasmic INAVA foci induced by IL-1b. Since overexpression of the catalytically inactive mutant ARNO-E156K also reduced INAVA foci formation upon addition of IL-1b, the authors conclude that the two functions of INAVA at the lateral membranes and in the cytoplasm compete with each other's. Is ARNO recruited to cytosolic INAVA condensates induced by IL-1b or Hsp90 inhibition or proteasome inhibition? Co-staining of INAVA and ARNO should be shown under the various conditions tested.

- "upon cell stress, INAVA assembles with the promiscuous E3-ligase bTrCP2, conjugated ubiquitin, and likely other yet-to-be-identified factors, to compartmentalize and regulate protein ubiquitination". The authors did not formally test whether INAVA regulates protein ubiquitination and thus cannot come to this conclusion.

- The study relies on the overexpression of INAVA-GFP. Do the authors have evidence that INAVA forms condensates also when expressed at endogenous levels?
TRAF6 is the ubiquitin E3 ligase required for IL-1β signal transduction. How can the author reconcile the fact that TRAF6 is absent from INAVA condensates with their proposed role in protein compartmentalization and ubiquitination? The authors propose that "not all INAVA-enhanced protein ubiquitination reactions (in particular those involved in inflammatory signaling) appear to occur within condensate structures." I wonder whether the INAVA condensates observed upon inhibition of Hsp90 or proteasome represent protein aggregates/pre-aggresomes rather than protein ubiquitination sites.

- "While INAVA promotes protein ubiquitination within the condensates, the condensates themselves are in turn targeted for degradation via autophagy- and/or proteasome-dependent mechanisms." What is the experimental evidence for this statement? The authors did not test whether inhibition of autophagy leads to the accumulation of INAVA condensates. While the authors focus on the role in the "cellular proteome" of the inhibitors that affect INAVA condensate dynamics, they do not take into consideration the fact that INAVA condensate dynamics might be regulated by phosphorylation. In fact, INAVA is predicted to be highly phosphorylated and phosphorylation has been implicated in the regulation of condensate dynamics (e.g. PMID: 31439799; PMID: 28790177). It is tempting to speculate that inhibition of MAPK p38α and mTOR kinases could regulate INAVA phase separation via phosphorylation. I understand that this aspect cannot be experimentally verified at this stage; however, it would be important to discuss this possibility.

- "Thus, the p38α and mTOR pathways appear to act by regulating the degradation or disassembly of INAVA condensates." Degradation and disassembly imply two different fates: in the first case, the protein is degraded either via the proteasome or autophagy; in the second case, the protein is relocated to other subcellular locations, such as e.g. the cytoplasm or the nucleus or, in this specific case, to the membrane. The authors should carefully revise their terminology.

Minor comments:

- The authors report that "the human alternative short splice-isoform of INAVA, which lacks the N-terminal region adjacent to the CUPID domain, does not assemble into puncta when fused to GFP, or function to amplify IL-1b signal transduction (8)." Deletion mutants of GFP-INAVA are already available and published by the authors (8). The authors should provide representative images showing the ability or not of these deletion mutants to form puncta in response to IL-1b or to Hsp90 and proteasome inhibitors. Moreover, does the N-terminal domain of INAVA contain a "prion-like" domain or an intrinsically disordered domain? It would be useful to provide this information using available programs (e.g. D2P2: http://d2p2.pro/; Pi-Pi predictor; PLAAC: http://plaac.wi.mit.edu).

- The kinetic of INAVA-GFP puncta upon IL-1b treatment is missing from Fig 3C. Given the fact that these foci generally disappear after 60 min, it would be good to show the time-course in presence of the inhibitors also at later time-points (see Fig 1A-C).

- Representative pictures of Fig 6, panels H and F should be provided.
hereditable risk factor for chronic inflammatory bowel disease (IBD). In epithelial cells, INAVA associates with the lateral membrane where it interacts with the GTP-exchange factor cytohesin-2 (ARNO) to promote the integrity of cell-cell junctions through F-actin assembly. Notably, upon inflammatory signaling through IL-1Beta, INAVA relocates from the lateral membrane where it accumulates in microscopically detectable cytosolic foci. In these cytosolic assemblages, INAVA enhances the activity of the E3-ubiquitin ligase TRAF6 to promote ubiquitylation of its targets required for downstream inflammatory signaling.

In this study, performed by Chang and colleagues, an analysis was conducted to characterize the cytosolic INAVA foci which arise as a consequence of immune signaling through IL-1Beta. The authors find that these cytosolic foci possess the physical characteristics of phase separated assemblies in vivo and sequestration of INAVA into these cytosolic foci serves to inhibit its activity at the lateral membrane leading to compromised epithelial barrier function and enhanced downstream ubiquitin signaling. To further understand the physiological relevance of INAVAs redistribution upon immune signaling, the authors go on to conduct high-throughput small molecule screens to identify factors which promote or inhibit INAVA condensate formation.

While the observations presented in this manuscript lead to a greater understanding of the molecular underpinnings of IBD, several points should be added and clarified before considering this manuscript for publication in JCB.

Major and minor points:

(1) A major finding in this manuscript is the observation that INAVA assembles into phase separated condensates upon IL-1Beta signaling. In Figure 1D, the authors nicely document the fusion of INAVA condensates over time. In Figure 1E, using FRAP, the authors demonstrate that newly formed condensates are liquid-like based on the reported recovery profiles and the 'older' condensates are less mobile. While these experiments are well executed, it would be helpful to see if INAVA behaves this way in vitro. If possible, can the authors do a similar type of analysis with purified INAVA-GFP or a labelled version of this protein?

(2) The assembly of biomolecular condensates in vivo can be inhibited using 1,6-Hexandioliol. Can the authors demonstrate that this is also the case for INAVA-GFP foci formed following IL-1Beta signaling?

(3) The large-scale screen performed in Figure 2 led to the identification of a number of compounds which promote INAVA-GFP condensate formation. Among the small molecules identified are proteasome inhibitors and compounds which lead to ROS generation. It is well documented that these types of chemically induced stresses also drive stress granule formation. To get a better sense of the cellular distribution of INAVA-GFP, can the authors evaluate whether or not INAVA-GFP colocalizes with stress granules, p-bodies or other phase separated compartments?

(4) The authors previously reported that INAVA promotes or amplifies ubiquitination through TRAF6. Using immunostaining, they go on to demonstrate that INAVA-GFP condensates contain ubiquitin and/or ubiquitylated proteins and the promiscuous E3-ubiquitin ligase BetaTrCP2. However, from these observations, it is not entirely clear how, if at all, INAVA-GFP condensate formation influences ubiquitylation of proteins and ubiquitin signaling in general. Can the authors more clearly show whether these two events are related (i.e. does INAVA-GFP condensate formation correlate with increased ubiquitination of substrates and does inhibition of INAVA condensate formation lead to a reduction in substrate ubiquitylation?)

(5) The authors present data showing that inhibitors of MAPK p38alpha and mTOR lead to the dissolution of INAVA-GFP condensates. On the basis of these observed findings, the authors come to the ambiguous conclusion that activation of MAPK or mTOR pathways - which cause an upregulation in proteasome activity or autophagy respectively - drive either the disassembly or degradation of INAVA condensates. However, the authors do not go on to distinguish between
these possible outcomes. To this point, can the authors evaluate the fate of INAVA-GFP condensates following IL-1Beta signaling. Are they degraded or disassembled? Based on the images presented in figure 4E (IL-1beta + INK128 or SB203580), it looks as though these condensates are likely dissolved based on the soluble fluoresce signal. However, this should be formally tested.

(6) In the second section of the results, the authors state, "Biomolecular condensates operate in key physiologic cellular processes" and only provide a single reference. Can the authors please insert the appropriate references here?
March 31, 2021

Re: JCB manuscript #202007177

Dear Dr. Frydman,

We would like to thank you and the reviewers for your interest in our work, and for the opportunity to submit a revised manuscript. This was a bit delayed due to the epidemic.

We have addressed each of the Referee’s comments, conducted new experiments, and made revisions that improve the manuscript.

Each of the reviewer’s comments was most helpful and influenced our thinking. As such, the manuscript is restructured to incorporate the new experiments and for clarity of interpretation and presentation.

The major conclusions were fully supported by the new experiments and remain largely unaltered. The new ideas that emerged from are that: the young INAVA condensates mature into structures with some but not all features associated with aggresomes; and that resolution of the INAVA condensates can occur by degradation or disassembly - depending on the agonist that induced condensate formation (presumably due to the different structures of the condensate). We also conducted new experiments that directly link puncta/condensate assembly with regulation of cellular proteostasis.

The new studies address the essential concern raised by both Referees: “that the nature and behavior of the INAVA condensates need to be characterized in greater detail”.

The new experiments and our responses to the Reviewers concerns are detailed in the letter below.

In what follows, reviewer comments are reiterated in BOLD text, and our responses in BLUE text. Locations in the manuscript where points are addressed are delineated in GRAY text, except where major restructuring occurred, and these sections are denoted by reference to the Figure or manuscript section and page numbers.

The Figures were updated to address the results of the new studies by additions to Figs 1, and 3 to 5, and Fig 8 and Supplemental Figs 1, 3, 5, and 6; and Figures 1, and 3 to 6 were revised and re-ordered in places to enhance clarity.

Total character count is now 39871.

We hope you will find the revised paper worthy for publication.

Best regards,

Wayne Lencer, Denis Chang, and Phi Luong

**Major points identified by Reviewer #1:**

“Condensates are also described to undergo a process called maturation .... Is the conversion into an amyloid-like state also occurring for the less mobile INAVA
condensates? It would be important to understand whether these newly described INAVA condensates spontaneously convert from a liquid-like state into an amyloid-like state, using for example amyloid-specific dyes (e.g. amylo-glo). Does inhibition of the proteasome or Hsp90 promote the conversion of INAVA foci into an amyloid-like state? In addition, what is the % of old puncta that do not disassemble and persist with time? Or do they all disappear over 180 min (Fig 1A)? A better characterization/definition of these “young” and “old” foci should be provided.

And related comment:

- TRAF6 is the ubiquitin E3 ligase required for IL-1β signal transduction. How can the author reconcile the fact that TRAF6 is absent from INAVA condensates with their proposed role in protein compartmentalization and ubiquitination? The authors propose that "not all INAVA-enhanced protein ubiquitination reactions (in particular those involved in inflammatory signaling) appear to occur within condensate structures." I wonder whether the INAVA condensates observed upon inhibition of Hsp90 or proteasome represent protein aggregates/pre-aggresomes rather than protein ubiquitination sites.

These were excellent and most helpful points.

We conducted new experiments to characterize the time course and nature of the maturing condensates – see new Figures 3F,G; Supplemental Figures 3.1F,G,H; and Supplemental Figures 3.2 all panels, and Supplemental Fig 5.2D.

The results show (text from revised manuscript):

The older puncta imaged 90 or 120 minutes after IL-1β, H₂O₂, or Ganetespib treatments colocalized with vimentin, a feature suggesting the maturation of condensates to aggresomes (Kopito, 2000). Vimentin colocalization was less apparent in the younger puncta (Figure 3F and Supplemental Figure 3.1F). Review of the BioGRID protein interaction database (https://thebiogrid.org/) suggests that INAVA associates with the dynein-dynactin complex (and thus microtubules), also consistent with maturation of INAVA condensates to aggresomes (Supplemental Figure 3.1G). None of the mature INAVA puncta induced by IL-1β, H₂O₂, Ganetespib, or MG132, however, stained for the presence of amyloid (Supplemental Figure 3.1H), and the mature puncta induced by IL-1β or H₂O₂ did not colocalize with the stress granule proteins G3BP1 or EDC4 (also a P-body protein) (Figure 3G and Supplemental Figure 3.2A), or with HDAC6 (Supplemental Figure 3.2B). Thus, in several cases, maturation of INAVA condensates appeared to take on some features, but not all that typify aggresomes (Kopito, 2000).

And in Discussion with respect to TRAF6 and IL-1β signaling:

The cytosolic condensates, however, do appear to be required for INAVA’s effect on IL-1β-induced signal transduction, though early non-visible forms of condensates may be involved, as suggested by the circumstantial evidence that enhanced IL-1β-induced TRAF6 signaling and puncta formation require the long isoform of INAVA. The short INAVA-S isoform, which cannot form cytosolic puncta, is inactive for both functions (Figures 1D and 4C). How the N-terminal region of INAVA enables condensate assembly, however, remains unknown. The region is predicted to be unstructured and amenable for condensate assembly (Banani et al., 2017; Lyon...
et al., 2020) (Supplemental Figure 1A); and the CUPID and C-terminal domains also score as unstructured regions. Still, it is possible a third site of function for INAVA exists for amplification of IL-1β-dependent inflammatory responses: one that occurs in the cytosol prior to condensate formation and competes with INAVA-ARNO binding on lateral membranes. This is further evidenced by our observations that TRAF6 fails to colocalize with detectable INAVA condensates induced by IL-1β and that down regulation of condensates by the p38α and mTOR inhibitors did not significantly affect NF-κB signaling. Thus, not all INAVA-enhanced protein ubiquitination reactions (in particular those involved in inflammatory signaling) may occur within condensate structures, though it remains possible INAVA-TRAF6 signaling condensates may assemble differently and be too small for detection by our methods as discussed above.

The finding that all the identified agonists induced the release of INAVA from the lateral membranes and promoted its phase separation in the cytoplasm and the finding that INAVA-GFP puncta colocalize with FK2 and the E3-ubiquitin-ligase βTrCP2 do not necessary imply a function for these foci in proteostasis. ..... First, the large INAVA-GFP perinuclear puncta observed after 4 hrs of treatment with the Hsp90 inhibitors or MG132 (Fig 3E) are reminiscent of aggresomes, .... It would be important to verify whether the INAVA-GFP perinuclear puncta induced by Hsp90 or proteasome inhibition are located at the MTOC and colocalize with HDAC6 and intermediate filament proteins.

Please see response above and new experiments as indicated.

.... the authors should test whether INAVA is a client of Hsp90 and whether prolonged treatment with the Hsp90 inhibitors leads to the progressive proteasome-mediated degradation of INAVA. ....

This experiment was performed (Supplemental Figure 3.2 panels D and E). It shows prolonged treatment with HSP90 inhibitors causes degradation of INAVA – implying that INAVA is a direct client of HSP90.

Given the rapid action of HSP90 inhibitors on the induction of INAVA condensates – we also had speculated that INAVA might be stabilized in conformation on the lateral membrane by binding HSP90 – and that release from HSP90 is the event that induces release from the membrane into the cytosol for condensate formation. This mechanism of action, among others, will be pursued in subsequent studies.

.... would polyUb-INAVA rather than other polyubiquitinated proteins be the major component of the FK2-positive/INAVA-positive foci observed upon Hsp90 inhibition or proteasome inhibition?

We consider it likely that INAVA enhances ubiquitination of itself – but we have not tested this directly. We also hypothesize that poly-ubiquitin chains may form the scaffold upon which INAVA condensates are built. It is also possible, dysfunctional ribosomes may be involved given the proteomic studies on INAVA associated proteins in the Xavier lab (Mohanan et al., 2018). But these remain ideas to be tested experimentally. And see response immediately below.

.... At this stage the authors cannot use the data obtained under inhibition of Hsp90 or proteasome to ascribe a role in proteostasis to the IL-1b induced INAVA condensates.

.... "upon cell stress, INAVA assembles with the promiscuous E3-ligase βTrCP2, conjugated ubiquitin, and likely other yet-to-be-identified factors, to compartmentalize
and regulate protein ubiquitination". The authors did not formally test whether INAVA regulates protein ubiquitination and thus cannot come to this conclusion.

This idea is more central to our hypothesis. We addressed it directly with new studies shown in Figure 4C – text from manuscript (shown below) explains the experiment and results.

To more directly test the idea that INAVA condensates may function in the regulation of cellular proteostasis, we measured total protein ubiquitination and its correlation with puncta formation. We studied HEK293T cells expressing HA-tagged ubiquitin and the long or short isoforms of INAVA-GFP, or a mutant form of INAVA (termed C/A) that contains substitution of a conserved cysteine for alanine at a site in the CUPID domain surrounded by hydrophobic residues. We hypothesized this region may form an active enzymatic site underlying INAVA function. We found that H2O2 broadly induced protein ubiquitination, but only in HEK293T cells expressing the long isoform of INAVA - the isoform that assembles into cytosolic puncta (Figure 4C). Cells expressing the short isoform, which do not form puncta, or cells expressing INAVA with the putative inactivating C/A substitution, showed no evidence for enhanced protein ubiquitination. These results correlate INAVA puncta formation with protein ubiquitination induced by cell stress (ROS). While INAVA may itself be a substrate for the ubiquitination reactions, the broad band of ubiquitinated proteins detected in cells after H2O2 treatment, spanning a wide range of apparent molecular weights, suggests INAVA promotes ubiquitination of many proteins other than itself.

Concerning the control experiment performed by overexpression of myc-βTrCP2 alone, the authors concluded that "When over expressed on its own, myc-βTrCP2 did not form cytosolic puncta again indicating dependence on INAVA for condensate formation (Supplemental Figure 3H)." An alternative possible explanation is that, upon Hsp90 or proteasome inhibition, myc-βTrCP2 participates in targeting unfolded and/or polyubiquitinated INAVA-GFP to aggresomes, similar to what was previously found for e.g. CHIP, another E3 ligase and iNOS (see PMID: 18955503).

Thank you. This possibility has been incorporated into our interpretation.

When overexpressed on its own, however, myc-βTrCP2 did not form cytosolic puncta (Supplemental Figure 4C). This result implicates dependence on INAVA as a structural or recruiting element for condensate assembly with βTrCP2, or perhaps INAVA provides substrate for ubiquitination by βTrCP2, thus enabling puncta assembly on ubiquitin chain scaffolds (Sha et al., 2009), or both.

In Fig 4A-B the authors show that the inhibitors of p38 and mTOR strongly decreased IL-1b-induced INAVA condensate formation, while having a milder effect on H2O2-induced INAVA condensates. ..... They find that the protein translation inhibitors cycloheximide, emetine, as well as the MELK inhibitor OTSSP167, efficiently antagonize INAVA-condensate formation (Supplemental Fig 4.1 and 4.2). Intriguingly, like cycloheximide and anisomycin, mTOR kinase inhibitors attenuate general protein synthesis. Moreover, MELK phosphorylates eIF4B, similar to mTORC1/p70 S6K, sustaining protein synthesis (PMID: 27528663; PMID: 23105104). Thus, inhibition of MELK also affects protein synthesis. Considering that: 1) phase separation is concentration dependent; 2) the pool of INAVA that is found inside the IL-1b-induced condensates is highly mobile and "in equilibrium" with the surrounding cytoplasm (FRAP data shown in Fig 1), a decrease in
the overall levels of INAVA-GFP might contribute to the progressive disassembly of the condensates. Can the authors comment on this aspect?

Experiments were performed to test these ideas – Supplemental Figure 5.2D. Our interpretation is stated in text of manuscript below.

As both the mTOR and MELK inhibitors can affect protein synthesis (Dennis et al., 2012; Wang et al., 2016), we next tested whether they blocked INAVA condensate formation by decreasing INAVA expression and thus its effective cytosolic concentration (Alberti, 2017; Banani et al., 2017; Boeynaems et al., 2018). We found that all three inhibitors – INK128 (mTOR), OTSSP167 (MELK), and cycloheximide – with or without IL-1β, had no effect on INAVA-GFP expression as measured by western blot (Supplemental Figures 5.1E and 5.2D). Thus, the level of INAVA expression cannot explain the mechanism(s) of puncta formation or resolution.

….. the authors then test their efficacy on stress granules containing FUS or FMR1 and FXR1, … Based on these data the authors conclude that “the disassembly, or degradation, of mature biomolecular condensates of different compositions seen in human disease may be similar to the mechanism of dissolution for INAVA condensates”. First, to substantiate their findings the authors should test whether addition of the inhibitors only during the recovery phase after stress (and not before and during the acute stress) accelerates SG dissolution. …

This experiment was performed (Supplemental Figure 6). In contrast to INAVA condensates induced by IL-1β and H2O2, the inhibitors had no effect on the time course of resolution for FUS condensates induced by arsenate or FMR1 and FXR1 condensates induced by MG132 (proteasome inhibitor).

Moreover, SG assembly and disassembly are known to be in equilibrium with polysomes and P-bodies; this is still unclear for the INAVA condensates. In light of these observations, I would avoid statements that generalize the mechanisms of assembly/disassembly of different types of condensates.

This point is well taken. Thank you. The manuscript was revised accordingly.

We next tested these compounds on condensates formed by proteins implicated in human disease (Alberti and Dormann, 2019; Bureau et al., 2017; Elbaum-Garfinkle, 2019; Takata et al., 2017) – as modeled in HeLa cells containing FUS-GFP (as transgene), or FMR1 and FXR1 (endogenously expressed). Condensates containing these molecules were induced using sodium arsenate or the proteasome inhibitor MG132. We found that both INK128 and OTSSP167, but not SB203580, antagonized FUS-GFP, FMR1 and FXR1 stress-induced condensate formation (Figures 6A-E). If applied after their formation, however, neither INK128 nor OTSSP167 enhanced puncta resolution (Supplemental Figure 6). Thus, INK128 and OTSSP167 appear to act by preventing formation of FUS-GFP, FMR1, and FXR1 condensates - rather than by enhancing their disassembly or degradation.

….. I recommend carefully rephrasing the definition of "pathological" condensates throughout the manuscript.

Done. Thank you

….. It is well-established that SG formation occurs after polysome disassembly, when RNAs are released in massive amounts (PMID: 25013173; PMID: 32302572); conversely,
treatment of the cells with cycloheximide and emetine, which stabilize the polysomes by freezing ribosomes on translating mRNAs, antagonize SG assembly and actively dissolve them (PMID: 11121440). Are similar mechanisms regulating INAVA condensate assembly/disassembly? Do INAVA condensate contain RNA and RBPs? The text should be carefully revised.

Based on the proteomic study performed by the Xavier Lab (Mohanan et al., 2018) (and evidenced by the BioGRID data base (Supplemental Figure 3.1G)), we believe it likely that INAVA condensates will be found to contain ribosomal proteins. We do not yet know if frozen polysomes, or polyubiquitin chains provide the scaffold for INAVA condensate formation. This is our next goal - to elucidate the components of the different INAVA condensates.

Concerning the reference cited (17-19), these should be verified; Merin et al., 2012 refers to aggresomes; the other two references are specific for SGs (Mokas et al. 2009; Thomas et al. 2009) and cannot be used to generalize the implication of RNA and ribosomes in condensate formation. This because different mechanisms regulate the formation of different types of condensates: e.g. PML nuclear bodies assemble through SUMO/SIM interactions, while mRNA concentration regulates SGs and P-bodies assembly (PMID: 27374333).

Thank you. This has been corrected.

- In the last part of the paper the authors show that inhibition of the RhoA kinase (ROCK) led to the recruitment of INAVA to cell-cell contact sites .... the authors conclude that the two functions of INAVA at the lateral membranes and in the cytoplasm compete with each other's. Is ARNO recruited to cytosolic INAVA condensates induced by IL-1b or Hsp90 inhibition or proteasome inhibition? Co-staining of INAVA and ARNO should be shown under the various conditions tested.

We do not find ARNO in the condensates. In a new experiment, however, we found over-expression of ARNO recruits INAVA to lateral membranes and reduces the number of cytosolic puncta (Figure 7H) – this is consistent with our model that the two functions are competing and separated by intracellular location. We point out again that ARNO binds the CUPID domain, that also scores as an intrinsically disordered region. Thus, ARNO may act by stabilizing the structure of this domain thus inhibiting condensate assembly.

- The study relies on the overexpression of INAVA-GFP. Do the authors have evidence that INAVA forms condensates also when expressed at endogenous levels?

We have not studied endogenous INAVA – but we do point out that the short isoform of INAVA (INAVA-S) does not form condensates, even when over expressed (Luong et al., 2018).

- "While INAVA promotes protein ubiquitination within the condensates, the condensates themselves are in turn targeted for degradation via autophagy- and/or proteasome-dependent mechanisms." What is the experimental evidence for this statement? The authors did not test whether inhibition of autophagy leads to the accumulation of INAVA condensates.

This is a good point. Thank you. The manuscript was revised accordingly. This statement was removed.
..... (the authors)... do not take into consideration the fact that INAVA condensate dynamics might be regulated by phosphorylation. In fact, INAVA is predicted to be highly phosphorylated and phosphorylation has been implicated in the regulation of condensate dynamics (e.g. PMID: 31439799; PMID: 28790177). It is tempting to speculate that inhibition of MAPK p38α and mTOR kinases could regulate INAVA phase separation via phosphorylation. I understand that this aspect cannot be experimentally verified at this stage; however, it would be important to discuss this possibility.

This is another good point. Thank you. The manuscript was revised accordingly.

..... The MAPK p38α is also a serine/threonine kinase. It is expressed in all cell types and operates widely in different cellular processes including inflammatory signaling (Broom et al., 2009; Coulthard et al., 2009). Post-translational phosphorylation of INAVA by these kinases may explain their mechanism of action in puncta resolution, but this has not yet been formally tested. ....

"Thus, the p38α and mTOR pathways appear to act by regulating the degradation or disassembly of INAVA condensates." Degradation and disassembly imply two different fates: in the first case, the protein is degraded either via the proteasome or autophagy; in the second case, the protein is relocalized to other subcellular locations, such as e.g. the cytoplasm or the nucleus or, in this specific case, to the membrane. The authors should carefully revise their terminology.

We have revised text wherever appropriate – And we indirectly addressed these ideas by examining INAVA expression before and after condensate formation and resolution. In the case of IL-1β-induced condensates and resolution, INAVA remains stably expressed, implicating resolution by disassembly. In the cases of HSP90 inhibitor- and H₂O₂- induced condensates, we find loss of INAVA expression after condensate resolution (or maturation) implicating resolution by degradation (Supplemental Figure 3.2D).

- The authors report that "the human alternative short splice-isoform of INAVA, which lacks the N-terminal region adjacent to the CUPID domain, does not assemble into puncta when fused to GFP, or function to amplify IL-1β signal transduction (8)." ..... The authors should provide representative images showing the ability or not of these deletion mutants to form puncta in response to IL-1β or to Hsp90 and proteasome inhibitors.

Please see newly added Figure 1D which shows this result - and also our earlier publication Luong eLife 2018.

Moreover, does the N-terminal domain of INAVA contain a "prion-like" domain or an intrinsically disordered domain? It would be useful to provide this information using available programs (e.g. D2P2: http://d2p2.pro/; Pi-Pi predictor; PLAAC: http://plaac.wi.mit.edu).

All regions of INAVA score as disordered, not just the N-terminal domain (Supplemental Figure 1A). There are no amyloid-like β-strand features, and we do not find amyloid staining as assessed by amylo (Supplemental Figure 3.1H). The N-terminal domain is not enriched in glutamine or asparagine as typified in prion-like proteins.

- The kinetic of INAVA-GFP puncta upon IL-1β treatment is missing from Fig 3C.
This was fixed – thank you

Given the fact that these foci generally disappear after 60 min, it would be good to show the time-course in presence of the inhibitors also at later time-points (see Fig 1A-C).

If we understand this comment correctly, the time courses are now shown in Supplemental Figure 3.2C.

- Representative pictures of Fig 6, panels H and F should be provided.

Done. Thank you.

**Major points identified by Reviewer #2:**

A major finding in this manuscript is the observation that INAVA assembles into phase separated condensates upon IL-1Beta signaling .... While these experiments are well executed, it would be helpful to see if INAVA behaves this way in vitro. If possible, can the authors do a similar type of analysis with purified INAVA-GFP or a labelled version of this protein?

The idea for this experiment is very good but under the circumstances (COVID-19) these studies remain beyond the scope of the current study. We believe the evidence for condensate formation presented is already compelling. In addition, we do not yet know the scaffolding molecule for INAVA condensates and the assembly of this reaction in vitro needs that component. This is an experiment we have planned to identify components of the condensates – but it is beyond the scope of this report.

The assembly of biomolecular condensates in vivo can be inhibited using 1,6-Hexandiol. Can the authors demonstrate that this is also the case for INAVA-GFP foci formed following IL-1Beta signaling?

We performed this study and found 1,6-Hexandiol amplifies – not reduces – condensate formation (Supplemental Figure 5.1 panels F and G). This is further evidence the INAVA condensates are not stress granules (see comment below).

The large-scale screen performed in Figure 2 led to the identification of a number of compounds which promote INAVA-GFP condensate formation. Among the small molecules identified are proteasome inhibitors and compounds which lead to ROS generation. It is well documented that these types of chemically induced stresses also drive stress granule formation. To get a better sense of the cellular distribution of INAVA-GFP, can the authors evaluate whether or not INAVA-GFP colocalizes with stress granules, p-bodies or other phase separated compartments?

These studies were performed and show no evidence that the INAVA condensates are stress granules, P-bodies, or amyloid. Figure 3G; Supplemental Figures 3.1H, and 3.2A and 3.2B.

The authors previously reported that INAVA promotes or amplifies ubiquitination through TRAF6. Using immunostaining, they go on to demonstrate that INAVA-GFP condensates
contain ubiquitin and/or ubiquitylated proteins and the promiscuous E3-ubiquitin ligase BetaTrCP2. However, from these observations, it is not entirely clear how, if at all, INAVA-GFP condensate formation influences ubiquitylation of proteins and ubiquitin signaling in general. Can the authors more clearly show whether these two events are related (ie. does INAVA-GFP condensate formation correlate with increased ubiquitination of substrates and does inhibition of INAVA condensate formation lead to a reduction in substrate ubiquitylation?)

This idea was queried by Referee 1 as well, and it is a central component of our hypothesis. We addressed it directly with new studies shown in Figure 4C as introduced above – the results show strong circumstantial evidence that INAVA condensates broadly amplify protein ubiquitination in live cells. The study used the short INAVA isoform that cannot form condensates as control – and also an INAVA isoform with point mutation in the CUPID domain we predicted would inactivate CUPID function as an additional control. The results are further described in text from manuscript (below).

To more directly test the idea that INAVA condensates may function in the regulation of cellular proteostasis, we measured total protein ubiquitination and its correlation with puncta formation. We studied HEK293T cells expressing HA-tagged ubiquitin and the long or short isoforms of INAVA-GFP, or a mutant form of INAVA (termed C/A) that contains substitution of a conserved cysteine for alanine at a site in the CUPID domain surrounded by hydrophobic residues. We hypothesized this region may form an active enzymatic site underlying INAVA function. We found that H$_2$O$_2$ broadly induced protein ubiquitination, but only in HEK293T cells expressing the long isoform of INAVA - the isoform that assembles into cytosolic puncta (Figure 4C). Cells expressing the short isoform, which do not form puncta, or cells expressing INAVA with the putative inactivating C/A substitution, showed no evidence for enhanced protein ubiquitination. These results correlate INAVA puncta formation with protein ubiquitination induced by cell stress (ROS). While INAVA may itself be a substrate for the ubiquitination reactions, the broad band of ubiquitinated proteins detected in cells after H$_2$O$_2$ treatment, spanning a wide range of apparent molecular weights, suggests INAVA promotes ubiquitination of many proteins other than itself.

….. can the authors evaluate the fate of INAVA-GFP condensates following IL-1Beta signaling. Are they degraded or disassembled? Based on the images presented in figure 4E (IL-1beta + INK128 or SB203580), it looks as though these condensates are likely dissolved based on the soluble fluoresce signal. However, this should be formally tested.

As written above for Referee 1: We indirectly address these ideas by examining INAVA expression before and after condensate formation and condensate resolution. In the case of IL-1β-induced condensates, INAVA remains stably expressed after condensate resolution, implicating resolution by disassembly. In contrast, in the cases of HSP90 inhibitor- and H$_2$O$_2$- induced condensates, we find loss of INAVA expression after condensate resolution implicating resolution by degradation (Supplemental Figure 3.2G).

In the second section of the results, the authors state, "Biomolecular condensates operate in key physiologic cellular processes" and only provide a single reference. Can the authors please insert the appropriate references here?

Done. – thank you.
May 11, 2021

RE: JCB Manuscript #202007177R

Dr. Wayne I Lencer
Boston Children's Hospital Harvard Medical School
Pediatrics
Boston Children's Hospital, Enders 609
300 Longwood Avenue
Boston, MA 02115

Dear Dr. Lencer:

Thank you for submitting your revised manuscript entitled "Small Molecule Modulators of INAVA Cytosolic Condensate and Cell-Cell Membrane Junction Assemblies". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision you must also address the concerns of reviewer #1 with text edits where requested. However, as overall we think you have sufficiently addressed the essential experimental points from the first round of review further experimental data is not required.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. * Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.*

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so,
how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Therefore please consider combining some of your current supplemental data and be sure to correct the call out in the tax to reflect this change. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."
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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRedit nomenclature.

B. FINAL FILES:

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor
Reviewer #1 (Comments to the Authors (Required)):

The authors have performed additional experiments to better characterize the INAVA condensates. Yet, there is still some unclarity that needs to be further addressed by the authors.

- Figure 3 and Supplementary Figure 3: "Puncta fully resolved after compound removal in cells treated with IL-1β, H2O2 or Ganetespib - implicating physiologic relevance (Supplemental Figure 3.2C). The puncta induced by MG132, however, did not resolve. The mature puncta induced by continuous HSP90 inhibitor, MG132, and H2O2 treatments were in all cases associated with loss of INAVA expression - consistent with resolution by puncta degradation, even for the puncta induced by MG132 (Supplemental Figures 3.2D and 3.2E)." This paragraph and the data are a bit confusing to me. In the methods the authors specify that "INAVA wildtype and mutant C141A long isoforms and INAVA-S (short isoform) and INAVA-S and FUS were cloned into pLVXEf1a-AcGFP-N1 (Clontech) to generate INAVA-GFP, INAVA-GFP (C141A) INAVA-S-GFP and FUS-GFP, respectively. HA tagged INAVA was cloned into pLVXPURO (Clontech)." Thus, it seems that GFP-INAVA and HA-INAVA are encoded by two different constructs. In Supplemental Figure 3.2D and E the authors use the anti-HA antibody to detect INAVA: "anti-HA measures HA tagged present in INAVA-GFP and actin was used as a loading control". However, all microscopy images show only INAVA-GFP (and never HA-INAVA). Importantly, Figure 3E shows formation of large INAVA-GFP foci upon treatment with MG132 for 2 hrs and of an aggresome-like structure upon treatment with MG132 for 4 hrs. How can the authors reconcile this result with the decrease in total HA levels after 2 hrs of treatment with MG132 shown in Supplemental Figure 3.2D? Does HA-INAVA colocalize with GFP-INAVA in the condensates? Alternatively, the aggregated INAVA species that accumulate upon proteasome inhibition (as shown in Figure 3E) might become insoluble to mild detergent or to 2% SDS; therefore, this fraction might not be detected by western blotting, leading to potential data misinterpretation. In case these species are degraded also in presence of MG132, one would expect to recover them using lysosome inhibitors. This aspect should be carefully addressed.

Finally, treatment with the Hsp90 inhibitors clearly decreases the levels of HA-INAVA. What is the fate of INAVA upon Hsp90 inhibition? Is it degraded by the proteasome (similar to what has been previously shown for other Hsp90 clients)? To claim that INAVA is a client of Hsp90 and that the INAVA puncta are degraded upon Hsp90 inhibition, the authors should co-treat the cells with the Hsp90 inhibitor and MG132 to test whether this blocks INAVA degradation. Ideally, they also should show interaction between INAVA and Hsp90. The author should carefully address these points and better explain the methodology and the data interpretation.

The last sentence of this paragraph is also a bit confusing: "Thus overall, depending on the stimulus, the mature INAVA condensates varied in their gross morphologies, in their time of onset after agonist exposure, and when and if they resolved - suggesting different structural compositions and mechanisms underlying their assembly and resolution." How can the author sustain that there are different types of assembly and disassembly mechanisms? Are rather the data suggesting that INAVA might become unstable and aggregate upon Hsp90 inhibition? Figure 3D clearly shows that Hsp90 inhibition and MG132 treatments lead to the formation of larger puncta. Is INAVA-GFP still highly mobile within the puncta that form upon Hsp90 and proteasome inhibition? FRAP experiments should be performed to compare mobility of INAVA-GFP inside
puncta formed upon treatment with Hsp90 or proteasome inhibition to firmly conclude about their nature and fate (similar to what shown in Supplemental Figure 5.2A).

- Is the short INAVA-S isoform also a client of Hsp90? And is it rapidly degraded upon Hsp90 inhibition? Additional experiments using this mutant (which does not undergo phase separation) may help to dissect how binding to Hsp90 and phase separation influence INAVA localization and stability. Interestingly, a recent paper showed that the kinase DYRK3 is a client of Hsp90 and that DYRK3 accumulates inside condensates in absence of Hsp90 (Mediani et al., 2021; PMID: 33738926). DYRK3 undergoes liquid-liquid phase separation and is recruited to several types of membraneless organelles such as stress granules and splicing speckles (Rai et al., 2018; PMID: 29973724). Upon Hsp90 inhibition, targeting of DYRK3 to condensates such as nuclear speckles increased, similar to enhanced condensate formation by INAVA-GFP. In their paper, Mediani et al. suggested that Hsp90 binding to DYRK3 may antagonize its phase separation. I wonder whether a similar mechanism may apply here to INAVA.

Other comments:
- Supplemental Figure 3.1H: it would be good to include a positive control for detection by amylo-glo of amyloid-like structures (e.g. transcriptional stress is known to induce A-body formation; see Audas et al., PMID: 27720612).

- Figure 4: It would be important to show also by microscopy the distribution of HA-ubiquitin in cells expressing the different forms of INAVA (WT, short and C/A) untreated and treated with H2O2. This will be more informative than the blotting per se, especially considering that the authors suggest a role for INAVA phase-separation in proteostasis.

- Vimentin staining is quite odd. One would expect a filamentous-like staining in the cytoplasmic, with some staining in the nucleus. The authors show mainly intranuclear vimentin staining and lack of filaments (e.g. Figure 3F).

Reviewer #2 (Comments to the Authors (Required)):

In the revised version of their manuscript, Lencer and colleagues have satisfied the major points put forth in my initial assessment of their study pertaining to IL-1β induced INAVA condensate formation in epithelial cells. Importantly, a major criticism, also raised by Referee #1, concerned whether ubiquitylation and the formation of INAVA condensates are at all linked - a point which seems important to their general hypothesis regarding the function of the described biomolecular assemblies. The authors have now established a connection between these processes showing that INAVA condensate formation correlates with extensive protein ubiquitination as evidenced by data presented in Figure 4C. Intriguingly, their data suggest that a wide range of substrates depend upon INAVA condensate formation for their ubiquitylation.

In addition, the authors have made attempts to address several basic points regarding the fate and localization of INAVA assemblies. Of importance to their main hypothesis, they now show that IL-1β induced INAVA assemblies undergo dissolution which is consistent with their idea that these structures behave like phase separated, biomolecular condensates. While they also report that those INAVA assemblies formed via oxidative stress or Hsp90 inhibition appear to be degraded, this does not detract from their conclusions as it is known that phase separated assemblies, such as stress granules, can possess varying material states depending on the type of stress, thereby influencing their fate. The authors also go on to show that INAVA puncta are a novel type of
assembly that do not coalesce with stress granules or processing bodies. Along these lines, while it would be interesting to see whether INAVA condensates can assemble using purified protein in vitro (a point which should be followed up in future studies), their evidence for phase separation in vivo is nevertheless compelling and satisfactory for the study at hand. Considering the overall improvements made to the revised manuscript, including improvements in data quality and presentation, I recommend this paper for publication in the Journal of Cell Biology.
May 24, 2021

Re: JCB manuscript #202007177

Dear Dr. Nunnari and Lindsey Hollander

Thank you for your interest in publishing our work.

We upload here all materials requested and note that we have responded to the queries of Referee 1 as requested by altering the text of the manuscript to:

1) Clarify that all studies except for Fig 4C were performed with HCT8 cells expressing INAVA-HA-GFP – so the immunoblot and imaging studies of Figs 3E and Supplemental Fig 2M (newly revised manuscript) were performed on cells expressing the same protein.
2) Clarify the comment about vimentin staining
3) Addition of text describing that cells studied in Fig 4C were expressing GFP-INAVA (fused at the N-terminus and lacking the HA-tag)
4) And statement of the uncertainty in evidence that INAVA may be a direct client of the HSP90 chaperone.

Thank you.

Best regards,

Wayne I. Lencer, MD