F-actin-rich territories coordinate apoptosome assembly and caspase activation during DNA damage-induced intrinsic apoptosis

Virginia King and Kenneth Campellone

Corresponding author(s): Kenneth Campellone, University of Connecticut

Review Timeline:

Submission Date:	2022-04-07
Editorial Decision:	2022-05-20
Revision Received:	2023-03-03
Accepted:	2023-03-03

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E22-04-0119
TITLE: F-actin-rich territories coordinate apoptosome assembly and caspase activation during intrinsic apoptosis

Dear Dr. Campellone:

Our two expert reviewers disagree on the disposition of your paper. One finds it easy to read and well documented. The other feels that microscopic localization experiments are not sufficient to draw any firm conclusions about mechanisms. Under such circumstances, a third reviewer can be helpful, but finding two reviewers was challenging, so we have not yet identified another individual, who is willing to review. Rather than prolonging the review process, I am returning the paper to you now.

Under these circumstances, I am willing to give you a chance to respond to the negative review and address the points made by the positive reviewer, but you may also want to consider another home for your work.

I have a few comments of my own for you to consider:

• The results would be easier to read if the presentation were more direct. You use a conversational style with topic sentences containing the results buried in paragraphs after sometimes long historical introductions. Keep in mind that this is the results section, not the historical introduction to the work.

• The extensive documentation of your results with images and quantitative measurements is a strength, but some of the quantitative measurements seem to be redundant or overly complicated. Some simplification to focus on the main points would make the presentation more accessible.

• How might a network of actin filaments physically exclude a medium-sized protein like XIAP?

• Why are actin filaments often around the JMY spots (Figs 1A, 2A) but sometimes overlap with JMY (Fig 4E). Do the conditions differ in these experiments

• The relative sizes of the molecules in Fig 7 are far from realistic.

Sincerely,

Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Campellone,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript by King and Campellone, the authors have followed up on their recently published paper in PLOS Genetics. Herein, they demonstrate that, during etoposide-induced apoptosis, cells form F-actin-rich "territories" that contain JMY, WHAMM, and Arp2/3 proteins, as well as cytochrome c, Apaf-1, and caspases-9 and -3. They argue that these clusters mediate more effective formation of Apaf-1 apoptosomes and consequently more efficient activation of caspases-9 and -3. There are several concerns with the manuscript, but the primary overarching criticism is that the paper relies exclusively on imaging (colocalization) experiments, and there is really no data to support the idea that these F-actin-rich territories have anything at all to do with Apaf-1 oligomerization into apoptosomes, recruitment of caspase-9, or the activation of caspase-3.

Major criticisms:

1. The only intervening treatments in the study consist largely of knocking out JMY and WHAMM genes (and in some cases replacing them with mutants), but according to their previous publication this prevents MOMP and cytochrome c release, thereby making any analysis of apoptosome formation and caspase activation impossible. One wonders if the caspase-3 activity itself might be leading to the collapse of the actin network, which in turn results in the entrapment of some apoptosome components within the actin structures?

Indeed, this concept is reminiscent of previously published work, wherein caspases were found within cytoplasmic inclusions of cleaved intermediate filaments (MacFarlane et al., J. Cell Biol., 2000; Lee et al., J. Cell Biol., 2002; Dinsdale et al., Am. J. Pathol., 2004). The authors might want to repeat some of their more critical experiments in the presence of a caspase inhibitor, such as zVAD.fmk, which does not prevent Apaf-1 oligomerization into apoptosomes or the recruitment of caspase-9, but does inhibit its catalytic activity and thus its downstream activation of caspase-3 (Bratton et al., EMBO J., 2001).

2. There is no biochemical evidence whatsoever to demonstrate that actin, JMY, WHAMM, Arp2/3, etc. have any direct effects on apoptosome activity. Reconstitution assays using pure recombinant proteins would be required to confirm the authors' main assertion.

3. The authors argue that etoposide treatment leads to increased "cytosolic expression" of JMY, but this appears to be based on imaging alone. Is JMY expression really increased based on western blotting, or are the authors referring to a change in cellular localization or a change in protein confirmation that results in more prominent fluorescence?

4. The authors refer several times to "relative stoichiometric amounts" of various proteins within the actin clusters. It's unclear how they've determined the stoichiometry of any of proteins based on their assays?

5. The authors argue that 77% of Apaf-1 puncta colocalize with JMY, but to these eyes there is quite a lot of Apaf-1 that is not present within puncta - is this monomeric Apaf-1 or are some apoptosomes simply not present within the actin clusters?

Minor comment:

In the discussion, the authors state that "intracellular mechanisms underlying caspase recruitment to and activation by apoptosomes are not well characterized". Several papers have provided fairly detailed step-by-step mechanisms for this process in vitro (e.g. Wu et al., Nat. Commun., 2016). The authors might wish to highlight the evidence, which suggests that different mechanisms are at play in vivo?

Reviewer #2 (Remarks to the Author):

In this manuscript by Virginia L King and Kenneth G Campellone, the authors investigate the role of the actin nucleation factor JMY in DNA damage induced intrinsic apoptosis. They reveal by fluorescence microscopy that after etoposide induced DNA damage JMY (endogenous or GFP overexpressed) accumulates in the cytosol, which is followed by accumulation of F-actin at these sites. They termed these sites of JMY accumulation F-actin-rich territories. They further show that accumulation of actin nucleation factors is selective at the F-actin territories. Next to JMY they found the Arp2/3 complex and Cortactin as well as WHAMM and LAP-WHAMM but not N-WASP, WASH, and WAVE2 accumulated at these sites. In addition, the authors found that inhibitor proteins of JMY and WHAMM such as STRAP and Tubulin are excluded from the accumulation zones. Next to actin nucleating proteins the authors identified core apoptosome components namely cytochrome c and Apaf-1 enriched within F-actin-rich territories. Particularly, cytochrome c and to a slightly lower degree Apaf-1 showed colocalization with JMY in most cases. To test if the recruitment of apoptosome components leads to caspase activation, the authors analyzed the abundance of active cleaved caspase-3 in the F-actin-rich territories by immunofluorescence microscopy. XIAP an inhibitor of apoptosis was found at the periphery of F-actin-rich territories and not in the center. To test if JMY mediates actin polymerization, the authors performed reconstitution experiments with different JMY mutants in JMY knockout fibroblasts deficient in caspase-3 activation. In these experiments caspase-3 activation was restored when WT JMY was expressed but not JMY mutants, linking actin polymerization to caspase activation. Lastly, they show that both JMY and WHAMM are important for the assembly of F-acin-rich territories and subsequent caspase activation.

This is an interesting and very elaborated body of work. The experimental data are of high quality and the results are convincing. The text is well written making it an enjoyable read. I have a few points that should be address prior to publication.

1. The title "F-actin-rich territories coordinate apoptosome assembly and caspase activation during intrinsic apoptosis" is misleading since the authors only show etoposide induced intrinsic apoptosis. I suggest to clarify this in the title. Nevertheless, it would be worthwhile to explore experimentally if other intrinsic autophagy inducers lead to the formation of F-actin-rich territories. This would definitely broaden the impact of the manuscript.
2. Line 165-167: "Thus, in response to DNA damage, cells undergo an overall increase in JMY expression due to the formation of a juxtanuclear cluster of JMY puncta." From the fluorescence measurements I am not convinced that one can make the argument of increased expression. Is it really expression or concentration of the cytosolic pool? Do JMY accumulations form after blocking protein synthesis with for example cycloheximide?
3. Figure S3ACan the authors use a broader mitochondrial marker such as mitotracker to check for the absence of mitochondria from cytosolic cytochrome C pool in the F-actin-rich territories?
4. In Figure 3C the authors measure cyto c punta per territory. How is this done? In Figure 4A it is hard to see puncta at all. In my eyes a pearson correlation coefficient would be a more meaning full way to measure colocalization of the individual proteins.
5. In Figure 4E the authors show XIAP localization at the periphery of the F-actin-rich territories. It would be more meaning full to show caspase staining rather than JMY to see a separation of the inhibitor from the caspase pool.

RE: Manuscript #E22-04-0119
TITLE: F-actin-rich territories coordinate apoptosome assembly and caspase activation during intrinsic apoptosis

Dear Dr. Campellone:
Our two expert reviewers disagree on the disposition of your paper. One finds it easy to read and well documented. The other feels that microscopic localization experiments are not sufficient to draw any firm conclusions about mechanisms. Under such circumstances, a third reviewer can be helpful, but finding two reviewers was challenging, so we have not yet identified another individual, who is willing to review. Rather than prolonging the review process, I am returning the paper to you now.
Under these circumstances, I am willing to give you a chance to respond to the negative review and address the points made by the positive reviewer, but you may also want to consider another home for your work.
I have a few comments of my own for you to consider:

• The results would be easier to read if the presentation were more direct. You use a conversational style with topic sentences containing the results buried in paragraphs after sometimes long historical introductions. Keep in mind that this is the results section, not the historical introduction to the work.
--We have shortened the background/rationale parts of the Results, and specifically moved some of the information about the debate on JMY localization to the Introduction.

• The extensive documentation of your results with images and quantitative measurements is a strength, but some of the quantitative measurements seem to be redundant or overly complicated. Some simplification to focus on the main points would make the presentation more accessible.
--We don't disagree. But we receive questions during our talks, meetings, and posters about many of these quantifications, so our experience is that some investigators are interested in more intricate details than others. We are trying to strike a balance between the two.

• How might a network of actin filaments physically exclude a medium-sized protein like XIAP?
--We don't think that molecular size is the reason that XIAP doesn't penetrate to the center of the F-actin-rich territory. Similarly sized proteins (e.g., N-WASP, WAVE2) look diffuse inside and outside the territory. On the other hand, Cortactin, like XIAP, is found at the periphery of mature round territories. We speculate that XIAP may interact with actin filaments or F-actin-binding proteins at the periphery of the territory (lines 553-554).

• Why are actin filaments often around the JMY spots (Figs 1A, 2A) but sometimes overlap with JMY (Fig 4E). Do the conditions differ in these experiments
--We should have described this more clearly in the first submission. The F-actin-rich territories are likely in different stages of development. In the revised paper, we better describe our understanding of the formation and maturation of the cytoskeletal structures in a new panel (Figure 2A; lines 159-169).

• The relative sizes of the molecules in Fig 7 are far from realistic.
--We agree, although the diversity of components within the territory makes it difficult to depict them while maintaining clarity. Our focus is to show (i) the major players in the territory, (ii) the assembly of the apoptosome into a mature heptamer, and (iii) the accumulation of active caspase-3 within the territory. We revised the Legend to reflect that the stoichiometry and size of organelles and molecules in the model are illustrative and not necessarily drawn to scale (lines 1117-1118).

Sincerely,
Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Campellone,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.
A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).
When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.
Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.
Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.
In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link:
https://www.mbcpapers.org/cgi-bin/main.plex?el=A3o2LQj5A2iwF2I1A9ftdFlpSy3brHlcOnTFPCT6gZ

Please contact us with any questions at mboc@ascb.org.
Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript by King and Campellone, the authors have followed up on their recently

published paper in PLOS Genetics. Herein, they demonstrate that, during etoposide-induced apoptosis, cells form F-actin-rich "territories" that contain JMY, WHAMM, and Arp2/3 proteins, as well as cytochrome c, Apaf-1, and caspases-9 and -3. They argue that these clusters mediate more effective formation of Apaf-1 apoptosomes and consequently more efficient activation of caspases-9 and -3. There are several concerns with the manuscript, but the primary overarching criticism is that the paper relies exclusively on imaging (colocalization) experiments, and there is really no data to support the idea that these F-actin-rich territories have anything at all to do with Apaf-1 oligomerization into apoptosomes, recruitment of caspase-9, or the activation of caspase-3.

Major criticisms:

1. The only intervening treatments in the study consist largely of knocking out JMY and WHAMM genes (and in some cases replacing them with mutants), but according to their previous publication this prevents MOMP and cytochrome c release, thereby making any analysis of apoptosome formation and caspase activation impossible.
--According to our previous paper (King et al., PLoS Genetics, 2021), knocking out JMY (and to a lesser extent, WHAMM) delays cyto c release. That paper never claims that knocking out JMY or WHAMM prevents MOMP or cyto c release. That paper also shows that cyto c 'puncta' are a previously-unrecognized intermediate between 'mitochondrial' and 'diffuse cytosolic' cyto c localization. Knocking out JMY abolishes the formation of the cytosolic cyto c punta. The current paper shows where and when the cyto c puncta form. The genetic manipulations ("intervening treatments") in the current paper include knocking out WHAMM, JMY, both WHAMM & JMY, and rescuing JMY-KO cells with multiple GFP-JMY constructs. At the request of this reviewer, we have added some caspase inhibitor experiments (see next point).

One wonders if the caspase-3 activity itself might be leading to the collapse of the actin network, which in turn results in the entrapment of some apoptosome components within the actin structures? Indeed, this concept is reminiscent of previously published work, wherein caspases were found within cytoplasmic inclusions of cleaved intermediate filaments (MacFarlane et al., J. Cell Biol., 2000; Lee et al., J. Cell Biol., 2002; Dinsdale et al., Am. J. Pathol., 2004). The authors might want to repeat some of their more critical experiments in the presence of a caspase inhibitor, such as zVAD.fmk, which does not prevent Apaf-1 oligomerization into apoptosomes or the recruitment of caspase-9, but does inhibit its catalytic activity and thus its downstream activation of caspase-3 (Bratton et al., EMBO J., 2001).
--As shown throughout the current paper, phalloidin staining indicates that F-actin-rich territories are present in cells where the actin cytoskeleton is largely intact, not collapsed. Therefore, during the period of apoptosis that we imaged, any caspase-3 effects on actin are unlikely to entrap apoptosome components. However, based on this reviewer's suggestion, we quantified JMY puncta and F-actin-rich territory formation in cells treated with zVADfmk (broad caspase inhibitor) as well as zDEVDfmk (executioner inhibitor) (Figure S7; lines 370-380). In the presence of either inhibitor, the proportion of cells with territories did not decrease – it actually increased slightly. As we expected, the zVADfmk inhibitor also prevented caspase-3 cleavage/activation within the territory. These results are consistent with our model that JMY-driven actin assembly is responsible for territory biogenesis but inconsistent with the idea that active caspase-3 leads to the creation of such apoptosome-containing F-actin-rich territories.

2. There is no biochemical evidence whatsoever to demonstrate that actin, JMY, WHAMM, Arp2/3, etc. have any direct effects on apoptosome activity. Reconstitution assays using pure recombinant proteins would be required to confirm the authors' main assertion.

-- The Reviewer is correct in that we did not perform any biochemical experiments of apoptosome activity. But we did not make any claims that the nucleation factors have direct effects on apoptosome activity. The reviewer's assertion is different from ours. Our data indicate ("main assertion" is) that the nucleation factors create a cytoskeletal compartment where apoptosomes form and caspases are activated in intact cells – we show that the apoptosome components have a punctate localization within territories and that executioner caspase-3 is cleaved/activated within territories. Our study is a purely cell biological one. While actin-based motility of beads (1999) and the biogenesis of filopodia-like structures (2010) are good examples of cytoskeletal structures that have been reconstituted in vitro, the reconstitution of more complex F-actin rich territories (especially in conjunction with apoptosomes and caspases) is not yet feasible. The strength of our study is that the analyses take place in intact cells, not in a reductionist *in vitro* system.

3. The authors argue that etoposide treatment leads to increased "cytosolic expression" of JMY, but this appears to be based on imaging alone. Is JMY expression really increased based on western blotting, or are the authors referring to a change in cellular localization or a change in protein confirmation that results in more prominent fluorescence?
--Increased protein levels of JMY were previously shown elsewhere (Demonacos et al., 2001; Coutts et al., 2007; Coutts et al., 2009). We have added an immunoblot replicating the previous observations (Figure S3A; lines 128-132). The current paper focuses on immunofluorescence microscopy to show consistent JMY expression in the nucleus and increased JMY expression specifically in the cytoplasm (in F-actin-rich territories). We don't have any evidence that a conformational change in JMY results in more prominent recognition by the JMY antibodies.

4. The authors refer several times to "relative stoichiometric amounts" of various proteins within the actin clusters. It's unclear how they've determined the stoichiometry of any of proteins based on their assays?
--We did not refer to relative stoichiometric amounts of any "proteins". We referred to relative stoichiometric amounts of "structures" and "puncta". We do not know the absolute concentrations of JMY, cyto c, or Apaf-1 proteins in the cells or in specific regions of the cells. To our knowledge, the intracellular molar concentrations of JMY and Apaf-1 have not been defined in the literature.

5. The authors argue that 77% of Apaf-1 puncta colocalize with JMY, but to these eyes there is quite a lot of Apaf-1 that is not present within puncta - is this monomeric Apaf-1 or are some apoptosomes simply not present within the actin clusters?
--Apaf-1 staining within the territory includes a combination of diffuse staining, small puncta, and larger puncta or clusters. We presume that the diffuse signal (above background) represents cytosolic, potentially monomeric Apaf-1, whereas the punctate structures represent different sized groups of apoptosomes. In general, previously-published images of Apaf-1 in cells have not provided much quality information about its specific subcellular localization. We do not know of any antibodies that can selectively distinguish monomeric versus polymeric Apaf-1.

Minor comment:

In the discussion, the authors state that "intracellular mechanisms underlying caspase recruitment to and activation by apoptosomes are not well characterized". Several papers have provided fairly detailed step-by-step mechanisms for this process in vitro (e.g. Wu et al., Nat. Commun., 2016). The authors might wish to highlight the evidence, which suggests that different mechanisms are at play in vivo?
--We agree that the mechanisms underlying caspase association and activation by apoptosomes are very well characterized using purified proteins *in vitro*. We cite the Wu paper and several other

structural/biochemical papers (lines 61-64) as well as reviews (lines 58-59; 511-513). However, apoptosomes are not particularly well characterized in cells – this is the gap filled by our paper. We do not think that different mechanisms are at play in cells. We presume that the protein-protein interaction, multimerization, cleavage, and other biochemical mechanisms that have been determined using *in vitro* systems still hold true in cells – we do not refute this. Instead, we show where and when these things happen in the complex cytoplasmic context of intact cells.

Reviewer #2 (Remarks to the Author):

In this manuscript by Virginia L King and Kenneth G Campellone, the authors investigate the role of the actin nucleation factor JMY in DNA damage induced intrinsic apoptosis. They reveal by fluorescence microscopy that after etoposide induced DNA damage JMY (endogenous or GFP overexpressed) accumulates in the cytosol, which is followed by accumulation of F-actin at these sites. They termed these sites of JMY accumulation F-actin-rich territories. They further show that accumulation of actin nucleation factors is selective at the F-actin territories. Next to JMY they found the Arp2/3 complex and Cortactin as well as WHAMM and LAP-WHAMM but not N-WASP, WASH, and WAVE2 accumulated at these sites. In addition, the authors found that inhibitor proteins of JMY and WHAMM such as STRAP and Tubulin are excluded from the accumulation zones. Next to actin nucleating proteins the authors identified core apoptosome components namely cytochrome c and Apaf-1 enriched within F-actin-rich territories. Particularly, cytochrome c and to a slightly lower degree Apaf-1 showed colocalization with JMY in most cases. To test if the recruitment of apoptosome components leads to caspase activation, the authors analyzed the abundance of active cleaved caspase-3 in the F-actin-rich territories by immunofluorescence microscopy. XIAP an inhibitor of apoptosis was found at the periphery of F-actin-rich territories and not in the center. To test if JMY mediates actin polymerization, the authors performed reconstitution experiments with different JMY mutants in JMY knockout fibroblasts deficient in caspase-3 activation. In these experiments caspase-3 activation was restored when WT JMY was expressed but not JMY mutants, linking actin polymerization to caspase activation. Lastly, they show that both JMY and WHAMM are important for the assembly of F-acin-rich territories and subsequent caspase activation.

This is an interesting and very elaborated body of work. The experimental data are of high quality and the results are convincing. The text is well written making it an enjoyable read. I have a few points that should be address prior to publication.

1. The title "F-actin-rich territories coordinate apoptosome assembly and caspase activation during intrinsic apoptosis" is misleading since the authors only show etoposide induced intrinsic apoptosis. I suggest to clarify this in the title. Nevertheless, it would be worthwhile to explore experimentally if other intrinsic autophagy inducers lead to the formation of F-actin-rich territories. This would definitely broaden the impact of the manuscript.
--Thanks for this suggestion. We have added a new figure showing JMY/F-actin-rich territory formation during mitomycin C-induced apoptosis (Figure S2; lines 124-126). We have changed the title to "…during DNA damage-induced intrinsic apoptosis".

2. Line 165-167: "Thus, in response to DNA damage, cells undergo an overall increase in JMY expression due to the formation of a juxtanuclear cluster of JMY puncta." From the fluorescence measurements I am not convinced that one can make the argument of increased expression. Is it really expression or concentration of the cytosolic pool? Do JMY accumulations form after blocking protein synthesis with for example cycloheximide?

-- Increases in JMY protein levels following treatment of cells with DNA damaging agents were previously shown by other investigators (Demonacos et al., 2001; Coutts et al., 2007; Coutts et al., 2009). We have added a new immunoblot confirming the earlier observations (Figure S3A). Based on our fluorescence measurements, the increased JMY expression is concentrated in the cytoplasmic puncta, and not in the nucleus. This is presumably caused by protein stabilization not necessarily upregulation of expression at the transcriptional/translational level. While we haven't tested whether cycloheximide blocks JMY accumulation, the Coutts 2007 paper indicates that JMY levels are controlled by MDM2, ubiquitination, and the proteasome. The expressed JMY protein is stabilized during apoptosis.

3. Figure S3ACan the authors use a broader mitochondrial marker such as mitotracker to check for the absence of mitochondria from cytosolic cytochrome C pool in the F-actin-rich territories?
-- Based on this good suggestion, we made some interesting new observations. In the revised paper, in addition to cyto c, we show 4 mitochondrial markers: mitochondrial DNA (mtDNA), MitoTracker (membrane potential-dependent stain), AIF (intermembrane space or released), and BAX (outer membrane pore-forming protein specific to pro-apoptotic mitochondria). These data appear in a new figure (Figure 4). Within F-actin-rich territories, mitochondrial DNA was present, but MitoTracker staining was mostly diffuse, presumably due to a loss of mitochondrial membrane potential. Consistent with the idea that mitochondria are permeabilized specifically within F-actin-rich territories, BAX-positive structures were found exclusively in the territories. Finally, cyto c formed puncta within the territories, whereas AIF did not. Like cyto c (King et al., 2021), AIF was still present in mitochondria outside of the territories. These results support the model that mitochondria undergo MOMP within the territories – while AIF diffuses away, cyto c is retained and able to form puncta along with JMY and the apoptosome component Apaf-1.

4. In Figure 3C the authors measure cyto c punta per territory. How is this done? In Figure 4A it is hard to see puncta at all. In my eyes a pearson correlation coefficient would be a more meaning full way to measure colocalization of the individual proteins.
-- Admittedly, the puncta are variable in brightness, size, and number, so choosing magnified images to illustrate the diversity of structures is challenging. We have added Pearson correlation coefficients for JMY with cyto c and Apaf-1 structures within territories (Figure 5D). Coefficients were high for those factors, whereas the coefficient for JMY with mtDNA was very low (lines 290-293; 1013-1017).

5. In Figure 4E the authors show XIAP localization at the periphery of the F-actin-rich territories. It would be more meaning full to show caspase staining rather than JMY to see a separation of the inhibitor from the caspase pool.
-- This was an excellent suggestion. We moved the images of JMY/XIAP/F-actin from the previous 4E to Supplemental Figure S6. We added CleavedCasp-3/XIAP/F-actin to Figure 6E-G. The images and quantification show that XIAP found at the territory periphery generally surrounds the CleavedCasp-3 located at the territory interior.

RE: Manuscript #E22-04-0119R
TITLE: "F-actin-rich territories coordinate apoptosome assembly and caspase activation during DNA damage-induced intrinsic apoptosis"

Dear authors:

Thank you for your careful response to review. I think you have addressed the concerns raised by me and the reviewers, so I am happy to recommend publication in MBoC.

Tom Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Campellone:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Your paper is among those chosen by the Editorial Board for Highlights from MBoC. Hightlights from MBoC appears in the ASCB Newsletter and highlights the important articles from the most recent issue of MBoC.

All Highlights papers are also considered for the MBoC Paper of the Year. In order to be eligible for this award, however, the first author of the paper must be a student or postdoc. Please email me to indicate if this paper is eligible for Paper of the Year.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
