THE EMBO JOURNAL

Manuscript EMBO-2011-80095

# ARF6 GTPase protects the post-mitotic midbody from 14-3-3- mediated disintegration

Nimesh Joseph, Andrea Hutterer, Ina Poser, Masanori Mishima

*Corresponding author: Masanori Mishima, University of Warwick*

**Review timeline:**

| | |
|---|---|
| Submission date: | 09 November 2011 |
| Editorial Decision: | 07 December 2011 |
| Accepted: | 18 April 2012 |

## 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.)

1st Editorial Decision 07 December 2011

Thank you for submitting your manuscript on ARF6 and 14-3-3 regulation at the midbody for our consideration. We have now received the comments of two expert referees, which you will find copied below. Both referees consider your study interesting in principle but raise a number of concerns that would need to be addressed prior to eventual publication. One common major point is the request to visualize microtubules to clarify the phenotypic interpretation and better specify the relative timing of abscission events. Related to this is referee 1's concern with the definition/analysis/conclusions regarding 'midbody disintegration', which will need clarification. I notice that these concerns may simply reflect confusion with the terms 'midbody' and 'Fleming body' but this should be clarified; while EM analysis may not be essential for this, validation of disintegration by visualization of another Fleming body component in addition to MKLP1 (e.g. Cep55) would helpful.

Should you be able to satisfactorily address these main issues as well as the several more specific points raised in the reports, then we should be happy to consider a revised manuscript further for publication. In this respect, please note that it is our policy to allow a single round of major revision only, making it important to diligently and comprehensively answer to all the points at this stage; should you have any questions or concerns regarding the reports, please do not hesitate to get back to me for further clarifications or consultations.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you

 

foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

With best regards,
Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is a well-written manuscript that focuses on the role of Arf6 during cytokinesis. While the accumulation of Arf6 at the midbody has been described almost ten years ago, the role and significance of Arf6 during cell division remain controversial. Thus, this is a timely manuscript that proposes a novel idea about the role of Arf6 in regulating midbody stability. At first half of the manuscript authors builds on previously published work showing that MKLP1 (part of the centralspindlin complex) directly interacts with Arf6 and 14-3-3. In a nice set of data, authors convincingly demonstrate that 14-3-3 and Arf6 form a mutually exclusive complex with MKLP1. Dr. Mishima and colleagues then go on and map Arf6 binding domain within MKLP1 and identify the MKLP1 mutations which block binding to Arf6 without having any effect on 14-3-3 binding. These mutations then allow them convincingly demonstrate that Arf6 binding to the midoby is required for cytokinesis.
In the second half of the manuscript Dr. Mishima and colleagues attempt to determine the functional significance of Arf6 recruitment to the midbody. Unfortunately, this part of the manuscript is much less developed and most of the author conclusions about Arf6 inhibiting 14-3-3-induced midbody disintegration is not supported by shown data. For one thing, the only measurement of midbody "disintegration" is the presence of MKLP1 at the midbody. Midbody is a very complex structure that consist from many cross-liked and inter-digitated microtubules that are embedded in midbody "matrix". Thus, other midbody markers, such as tubulin, need to be analyzed before any conclusions can be reached. It is also not very clear what authors mean by "disintegration". Many published papers have shown that midbody is very stable structure that is actually inherited by one of the daughter cells and needs to be degraded by autophagy. Yet, in this manuscript authors seem to suggest that midbody simply "disintegrates". This, disintegration needs to be clearly demonstrated. Perhaps by EM to see the changes in microtubule reorganization. Or at very least by time-laspe imaging of GFP-tagged tubulin. How this "disintegration" fits with autophagy-dependent midbody degradation is also unclear. These questions need to be addressed before this manuscript is ready for publication to make sure that authors study midbosy "disintegration" rather then simple MKLP1 relocation away from the midbody. Manuscript also has several smaller but still significant questions regarding experimental design (see below):

1) In figure 2, GST-only controls need to be shown.

2) Does Arf6 or 14-3-3 binding influence Cyk4 binding to MKLP1. Since Cyk4 recruitment to the midbody by MKLP1 is essential for cytokinesis, it is important to show that Arf6 does not block centralspindlin complex formation.

3) Figure 4. Similarly, do MKLP1 mutations that inhibit Arf6 binding have any effect on Cyk4 binding? If yes, that may be the reason that these mutations cannot fully rescue MKLP1 knock-down.

4) Figure 6. The change in midbdoy life shown in part D due to large variation is not very convincing.

 

Referee #2 (Remarks to the Author):

Accumulation of higher-order clusters of the centralspindlin complex at the central spindle/midbody is important for targeting of many essential abscission factors. A previous study by the same authors showed that centralspindlin clustering is inhibited by the 14-3-3 protein, which binds to phosphorylated MKLP1. In this manuscript, Joseph et al. report a new regulator of centralspindlin, the endosomal GTPase ARF6, which antagonizes 14-3-3 by competitive binding to MKLP1 and thereby stabilizes the midbody. They establish a separation-of-function point mutant MKLP1, which they use to demonstrate the importance of ARF6-mediated stabilization of the midbody for successful abscission. The regulation of abscission is poorly understood and this study by Joseph et al. provides important new insights. Most conclusions in this paper are well supported by high-quality data and the results are interesting for a broad readership. The authors, however, need to provide more precise measurements of abscission timing as a reference for the interpretation of their kinetic localization data.

Major points:

The manuscript provides extensive live imaging data and quantitative kinetic measurements of protein abundance and co-localization at the midbody. What is missing, however, is a reliable reference time point for abscission, which is known to be quite variable in different cell lines / strains. It is essential for proper interpretation of the data to discriminate pre-abscission midbodies from post-abscission midbody remnants, which still contain high levels of MKLP1. The phase contrast images shown in Fig. 5 do not provide sufficient detail to reliably determine the abscission time point. The authors therefore need to include a detailed kinetic analysis of abscission timing in unperturbed control cells (e.g., using GFP-labeled midbody-microtubule disassembly as an assay). These experiments should be performed with the same cell type and experimental conditions as for their localization measurements.

The authors also need to clarify the relative timing of abscission, ARF6 accumulation at the midbody, and Aurora B-dependent phosphorylation. This experiment could be performed, for example, by statistical analysis of fixed cell populations stained for pS708-MKLP1, ARF6, and a reference staining for microtubules to discriminate pre- and post-abscission stages.

p 5.: The authors state that Aurora B activity is undetectable 30 min after anaphase onset, referencing Fuller et al. (Nature, 2008). This observation by Fuller et al. was based on a cytoplasmic FRET biosensor, which may not respond to locally confined pools of Aurora B. Another study by Steigemann et al. (Cell, 2009) reports that Aurora B remains active at the midbody until abscission. This is also suggested by data reported by the Mishima laboratory in a previous publication (Douglas et al., Current Biol, 2010), which shows that even though an Aurora B-dependent phosphorylation on S708 of MKLP1 generally drops significantly during telophase progression (Fig. 3A), a midbody-localized pool pS708-MKLP1 persists until late telophase (Fig. 3B). The authors should consider the possibility of simultaneous action of ARF6 and Aurora B in regulating MKLP1 also during late stages of cytokinesis in their introduction/discussion/model.

1st Revision – authors' response 16 April 2012

 

Point-by-point response to Referees' comments

*Referee #1 (Remarks to the Author):*

*This is a well-written manuscript that focuses on the role of Arf6 during cytokinesis. While the accumulation of Arf6 at the midbody has been described almost ten years ago, the role and significance of Arf6 during cell division remain controversial. Thus, this is a timely manuscript that proposes a novel idea about the role of Arf6 in regulating midbody stability. At first half of the manuscript authors builds on previously published work showing that MKLP1 (part of the centralspindlin complex) directly interacts with Arf6 and 14-3-3. In a nice set of data, authors convincingly demonstrate that 14-3-3 and Arf6 form a mutually exclusive complex with MKLP1. Dr. Mishima and colleagues then go on and map Arf6 binding domain within MKLP1 and identify the MKLP1 mutations which block binding to Arf6 without having any effect on 14-3-3 binding. These mutations then allow them convincingly demonstrate that Arf6 binding to the midoby is required for cytokinesis.*

We greatly appreciate that Referee #1 recognises the importance, timeliness and novelty of our study on the role of ARF6 in regulation of the stability of the midbody.

*In the second half of the manuscript Dr. Mishima and colleagues attempt to determine the functional significance of Arf6 recruitment to the midbody. Unfortunately, this part of the manuscript is much less developed and most of the author conclusions about Arf6 inhibiting 14-3-3-induced midbody disintegration is not supported by shown data. For one thing, the only measurement of midbody "disintegration" is the presence of MKLP1 at the midbody. Midbody is a very complex structure that consist from many cross-liked and inter-digitated microtubules that are embedded in midbody "matrix". Thus, other midbody markers, such as tubulin, need to be analyzed before any conclusions can be reached. It is also not very clear what authors mean by "disintegration". Many published papers have shown that midbody is very stable structure that is actually inherited by one of the daughter cells and needs to be degraded by autophagy. Yet, in this manuscript authors seem to suggest that midbody simply "disintegrates". This, disintegration needs to be clearly demonstrated. Perhaps by EM to see the changes in microtubule reorganization. Or at very least by time-laspe imaging of GFP-tagged tubulin. How this "disintegration" fits with autophagy-dependent midbody degradation is also unclear. These questions need to be addressed before this manuscript is ready for publication to make sure that authors study midbosy "disintegration" rather then simple MKLP1 relocation away from the midbody.*

We appreciate this comment and accept that we need to clarify whether the disappearance of the MKLP1 merely reflects the loss of MKLP1 from the midbody, leaving an almost intact midbody behind, or it is associated with the disintegration of the whole midbody.

Although we could not make EM observations, we performed additional knock-down and rescue assays in the HeLa cells that are stably expressing other fluorescently-tagged midbody components, as suggested by Referee #1. There was no residual mCherry-tubulin signal remained on the midbody (or on the midbody remnant) when the EGFP signal of the ARF6-binding defective V786A mutant of MKLP1 was lost (new Figure 6A). We confirmed that this is not due to the faster photobleaching of mCherry compared to EGFP since the same thing (no residual tubulin signal) was observed with the combination of mCherry-MKLP1 V786A and the EGFP-tubulin (new Figure 6B). Furthermore, we observed GFP-tagged CEP55 expressed from its native promoter on a bacmid (Poser

2009). CEP55 is a late midbody protein, which starts to accumulate about 15 min after midbody formation and reaches a plateau at 70 to 85 min. CEP55 works as an adaptor linking between centralspindlin and ESCRT machineries. Similarly to tubulin, when MKLP1 V786A was lost from the midbody, CEP55 signal at the midbody was lost as well (not shown). We also observed the fragmentation of the CEP55 accumulation at the midbody into multiple smaller pieces in an almost identical manner to that observed for the mutant MKLP1 (new Figure 6C). These observations strongly support our view that disappearance of the MKLP1 mutant from the midbody reflects the disintegration of the whole of the Flemming body or the midbody remnant.

We totally agree with the view of this reviewer that, in the wild type situation, the central dense region of the midbody (Flemming body and the midbody remnants) is a very stable structure inherited by one of the daughter cells, which needs a special mechanism such as autophagy for removal unless it is released into the extracellular space. Our new insight based on the current observations is that this stability is ensured by an active regulatory mechanism. Abrogation of this mechanism then provides another possible route for the removal of the midbody remnant.

*Manuscript also has several smaller but still significant questions regarding experimental design (see below):*

*1) In figure 2, GST-only controls need to be shown.*
We have now added the GST-only controls to this figure.

*2) Does Arf6 or 14-3-3 binding influence Cyk4 binding to MKLP1. Since Cyk4 recruitment to the midbody by MKLP1 is essential for cytokinesis, it is important to show that Arf6 does not block centralspindlin complex formation.*

We agree with Referee #1 that the possible effects of ARF6 or 14-3-3 binding on the binding between CYK4 and MKLP1 should be excluded since all the known functions of MKLP1 and CYK4 during cytokinesis depends on the formation of the centralspindlin complex. We examined the possible effect of 14-3-3 binding on the binding of MKLP1 and CYK4 in our previous paper. 14-3-3 co-immunoprecipitates with both MKLP1 and CYK4 (MgcRacGAP) from the crude lysate (Douglas 2010 Figure 2D), indicating that 14-3-3 can bind the centralspindlin holocomplex. When the interaction between MKLP1 and 14-3-3 was enhanced by an Aurora kinase inhibitor, ZM447439, the amount of the co-immunoprecipitated CYK4 showed an increase, rather than a decrease, equivalent to that observed for MKLP1 (Douglas 2010 Figure 3F). This indicates that 14-3-3 does not affect the formation of centralspindlin complex.

As for the possible effect of ARF6 on the centralspindlin complex formation, we repeated the experiments in Figure 2B and confirmed that CYK4 was also pulled-down from the crude extract by GST-ARF6. The response of the pulled-down CYK4 to the Aurora kinase inhibitor and the 14-3-3 as a competitor was very similar to that of MKLP1. This confirms that ARF6 does not block centralspindlin complex formation. Figure 2B has now been updated and the text modified to point out that the ARF6-binding does not affect the MKLP1-CYK4 interaction within centralspindlin.

*3) Figure 4. Similarly, do MKLP1 mutations that inhibit Arf6 binding have any effect on Cyk4 binding? If yes, that may be the reason that these mutations cannot fully rescue MKLP1 knock-down.*

To address this point, we examined the co-immunoprecipitation between the endogenous CYK4 and the exogenously expressed EGFP-MKLP1 wild-type or the mutants. All forms of EGFP-MKLP1 specifically co-immunoprecipitated CYK4. Although there was a slight variation in the amount of the immunoprecipitated EGFP-MKLP1s, the variation in the amounts of co-immunoprecipitated CYK4 followed a very similar pattern to that of the different EGFP-MKLP1 constructs on the GFP-trap beads. These data are now presented in Supplementary Figure S4.

Experimental results presented here in response to the points 2) and 3) are consistent with our knowledge that CYK4 binds to the long neck region and the following N-terminal part of the coiled coil stalk of MKLP1 and that the C-terminal half of MKLP1, which covers the Aurora B phosphorylation/14-3-3 binding motif and the ARF6-binding site, is not required for the centralspindlin complex formation.

*4) Figure 6. The change in midbdoy life shown in part D due to large variation is not very convincing.*
We agree that the variance of the midbody life is not very small. However, analysis by Wilcoxon rank sum test showed p-value of 0.024 for comparison between the V786A mutant and the S710A & V786A double mutant, indicating that the effect of the inhibition of 14-3-3 binding by S710A mutation is statistically significant ($p < 0.05$). We appreciate Referee #1 for making us realise that we had failed to describe the statistical method. We have now modified the corresponding figure legend.

*Referee #2 (Remarks to the Author):*

*Accumulation of higher-order clusters of the centralspindlin complex at the central spindle/ midbody is important for targeting of many essential abscission factors. A previous study by the same authors showed that centralspindlin clustering is inhibited by the 14-3-3 protein, which binds to phosphorylated MKLP1. In this manuscript, Joseph et al. report a new regulator of centralspindlin, the endosomal GTPase ARF6, which antagonizes 14-3-3 by competitive binding to MKLP1 and thereby stabilizes the midbody. They establish a separation-of-function point mutant MKLP1, which they use to demonstrate the importance of ARF6-mediated stabilization of the midbody for successful abscission. The regulation of abscission is poorly understood and this study by Joseph et al. provides important new insights. Most conclusions in this paper are well supported by high-quality data and the results are interesting for a broad readership.*

We appreciate that Referee #2 agrees on the importance of our study.

*The authors, however, need to provide more precise measurements of abscission timing as a reference for the interpretation of their kinetic localization data.*

*Major points:*

*The manuscript provides extensive live imaging data and quantitative kinetic measurements of protein abundance and co-localization at the midbody. What is missing, however, is a reliable reference time point for abscission, which is known to be quite variable in different cell lines / strains. It is essential for proper interpretation of the data to discriminate pre-abscission midbodies from post-abscission midbody remnants, which still contain high levels of MKLP1. The phase contrast images shown in Fig. 5 do not provide*

*sufficient detail to reliably determine the abscission time point. The authors therefore need to include a detailed kinetic analysis of abscission timing in unperturbed control cells (e.g., using GFP-labeled midbody-microtubule disassembly as an assay). These experiments should be performed with the same cell type and experimental conditions as for their localization measurements.*

We agree with Referee #2 on the importance of discriminating between pre-abscission midbodies and post-abscission midbody remnants for the better interpretation of our data. However, precise determination of abscission timing is not a trivial issue especially in a cell type such as HeLa Kyoto cells that we used in this study, in which good cell-to-matrix adhesion is maintained during division and the daughter cells tend to stay close to each other. The laboratory of Dr. Daniel Gerlich has established an excellent assay to determine the timing of abscission by monitoring the continuity of cytoplasm between the daughter cells using photo-activatable GFP (PA-GFP assay). Unfortunately, this assay cannot be easily incorporated in our knock-down and rescue assays. However, Gerlich and colleagues also reported that abscission determined by the PA-GFP assay occurs almost instantaneously after the disassembly of the microtubules on either side of the Flemming body (simply called midbody in their papers). Thus, following the suggestion by Referee #2, we carefully examined the microtubule disassembly in the HeLa Kyoto cells that are stably expressing GFP-tubulin and compared its timing with the behaviour of the Flemming body (Supplementary Figure S1).

As expected, we observed that the first disassembly of the midbody microtubules occurs around 70 min after the midbody formation consistently with the reports from the Gerlich laboratory. In contrast, however, we found a large variability in the behaviour of the Flemming body. In some cells, the Flemming body migrated away from the border between the daughter cells immediately after the disassembly of microtubules (Figure S1A). In other cells, the Flemming body remained at the border showing back and forth movement between the two daughter cells for several hours before it migrated from the border to the more central cell cortex of one of the two daughters (Figure S1B). Unfortunately, with the current resolution of the images, it was difficult to clearly determine whether the Flemming body is kept connected to both the daughter cells via thin bridges during this long period.

This wide variety in the abscission timing monitored by the morphology of the intercellular bridge is consistent with the previous observation in HeLa cells (90 min to 6 h after the midbody formation, Piel et al 2001). Delayed abscission or prolonged connection could be a consequence of a persistent chromosome bridge (Steigemann et al 2009). However, considering its relatively low frequency (5% in HeLa cells, Steigemann et al 2009), chromosome bridging cannot explain all the cases of very late abscission. A more consistent explanation would be that the PA-GFP assay and the morphological separation might be monitoring two distinct steps of the abscission.

In theory, the discontinuity of the daughter cytoplasms can happen by narrowing the channel without irreversible scission of the plasma membrane. While the closure of the channel by constriction of the cortical ESCRT filaments happens in a stereotypical timing that is tightly coupled with the microtubule disassembly (Guizetti et al 2011), the final separation including the change of the topology of the plasma membrane might need additional biochemical or mechanical steps that occur later rather gradually (or perhaps sporadically). Very late furrow regression (for example, ~2.5 h after the midbody formation in Figure 5C) in the MKLP1 mutant cells indicates that the integrity of the midbody (or Flemming body) has to be maintained until the cells complete those steps that are required to ensure the irreversible separation of daughters.

In summary, there is a limitation in our methods of determination of abscission timing and there could be two situations that would be detected as “not separated yet” by bright field observation but could be determined to be “separated” by the PA-GFP assay: 1) actually the daughters have completely been separated but the midbody remnant remains at the border and 2) the daughters are still connected with a single continuous plasma membrane even though the channel between the two daughters is closed. Our method cannot discriminate between these possibilities. Thus, measuring the abscission timing using this criterion should be interpreted as placing an upper limit on the timing of abscission because it is possible that abscission has already occurred at the point of migration of the midbody remnant away from the border. We modified the main text to clarify this point.

*The authors also need to clarify the relative timing of abscission, ARF6 accumulation at the midbody, and Aurora B-dependent phosphorylation. This experiment could be performed, for example, by statistical analysis of fixed cell populations stained for pS708-MKLP1, ARF6, and a reference staining for microtubules to discriminate pre- and post-abscission stages.*

In the context of the necessity of the protection of centralspindlin from dissociation by 14-3-3, we think it is more important to know when the potential 14-3-3-binding form, S710-monophosphorylated form (pS710), appears following the gradual decrease of Aurora B signal at the midbody. As suggested by Referee #2, we performed the statistical analysis of the cells fixed at different time points and stained for pS710 and tubulin after the release from arrest at metaphase. To compare the result with other live-imaging data, we also observed the division of the cells released from the arrest in time lapse and determined the average timing of the midbody formation (i.e. completion of furrow ingression). As shown in Supplementary Figure S2, pS710 signal showed a gradual increase from 0 to 135 min after midbody formation while the intensity of microtubules showed a slightly more rapid decrease. This is consistent with the patterns of decrease of Aurora B signal from the midbody (and our previous observation that Aurora B inhibition increases pS710 phosphorylation) and appearance of the ARF6 on the Flemming body, which starts around 50 min and reaches plateau around 150 min, acting as a protector from 14-3-3.

*p 5.: The authors state that Aurora B activity is undetectable 30 min after anaphase onset, referencing Fuller et al. (Nature, 2008). This observation by Fuller et al. was based on a cytoplasmic FRET biosensor, which may not respond to locally confined pools of Aurora B. Another study by Steigemann et al. (Cell, 2009) reports that Aurora B remains active at the midbody until abscission. This is also suggested by data reported by the Mishima laboratory in a previous publication (Douglas et al., Current Biol, 2010), which shows that even though an Aurora B-dependent phosphorylation on S708 of MKLP1 generally drops significantly during telophase progression (Fig. 3A), a midbody-localized pool pS708-MKLP1 persists until late telophase (Fig. 3B). The authors should consider the possibility of simultaneous action of ARF6 and Aurora B in regulating MKLP1 also during late stages of cytokinesis in their introduction/discussion/model.*

We greatly appreciate this comment and agree with Referee #2 that there could be a time window in which the simultaneous action of ARF6 and Aurora B regulates the stability of the Flemming body. The scheme in the original Figure 7 was not precise in that the role of Aurora B phosphorylation was shown limited to anaphase. We amended this point and modified the main text.

Acceptance letter 18 April 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal!

Before we will able to send you a formal letter of acceptance, there is just one minor thing I need to ask you for, a brief 'author contribution' statement to be included at the end of the manuscript text. Therefore, please send us a Word file (which we will need in any case for production purposes) with the amended manuscript text including this statement.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

---

Referee #2

(Remarks to the Author)
The authors have satisfactorily addressed all concerns and the manuscript should be published in EMBO Journal.

 