JCB Journal of Cell Biology

REPLY

Reply to "Phosphorylation of G3BP1-S149 does not influence stress granule assembly"

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In this issue, Panas et al. (2019. *J. Cell Biol.* https://doi.org/10.1083/jcb.201801214) challenge the data published in the Tourrière et al. (2003. *J. Cell Biol.* https://doi.org/10.1083/jcb.200212128) paper on the role of G3BP phosphorylation in stress granule (SG) assembly. This reply addresses that letter and suggests that more work is needed to understand the role of this modification in SG formation.

In this issue, Panas et al. publish a refutation article putting into question the impact of G3BP phosphorylation on its S149 residue in G3BP-mediated assembly of stress granules (SGs) that we originally reported in Tourrière et al. (2003). They identified additional mutations that were accidently introduced in two plasmids expressing pEGFP-G3BP1 phosphomutant isoforms, which we originally generated and shared with numerous groups, including the authors. Indeed, they identified an alanine to threonine replacement at position 54 in the pEGFP-C1-G3BP1-S149A mutant and a serine to proline substitution at position 99 in pEGFP-C1-G3BP1-S149E. Therefore, they constructed a new set of G3BP1 mutant isoforms with a specific single mutation (A54T, S99P, S149A, and S149E) to dissect the effects of each site on SG assembly. They found that a construct containing only S149A or S149E comparably nucleates and rescues SGs in U2OS-WT and ΔΔG3BP1/2 U2OS cells. In contrast, the introduction of a single S99P mutation in G3BP1 alters the protein's ability to nucleate or rescue SGs, equivalent to the S99P-S149E double mutant. As this finding impacts other reports using these plasmids to investigate the functional effects of S149 phosphorylation, we apologize for this accidental mutation that has escaped our notice and would like to thank and acknowledge the work done by Panas et al. (2019) to advise our community.

However, we respectfully disagree with one of the main conclusions of the letter and we think that the data presented are not enough to definitively exclude the role of S149 phosphorylation in modulating G3BP-mediated SG assembly. In our paper (Tourrière et al., 2003), the S149E mutation, which expresses the phosphomimetic isoform of G3BP, was used as an additional argument that the phosphorylation of S149 is important for G3BP multimerization and SG assembly. First, as stated in the letter by Panas et al. (2019) as well as in numerous reports, G3BP is indeed phosphorylated on its S149 and S232 residues. Second, we showed that arsenite treatment diminished the phosphorylation of S149 of endogenous G3BP by 50%, using in vivo labeling and immunoprecipitation of phosphorylated G3BP. In their letter, Panas et al. (2019) have used mass spectrometry (MS) analysis to quantify the phosphorylation status of S149. Due to the high content of acidic residues surrounding S149 leading to poor recovery, it is difficult if not impossible to have a rigorous quantification of phosphorylated G3BP. Hence, in our opinion, MS analysis alone may not be sufficient to precisely measure the phosphorylation status of S149. If the authors would like to make this point, they should use stable isotope labeling using amino acids in cell culture (SILAC) in combination with MS analysis to quantitively determine the impact of stress on G3BP phosphorylation. Third, we showed that the acidic domain of G3BP spanning from position 138 to 334 can efficiently compete with SG induced by arsenite (Tourrière et al., 2003). This domain contains S149 but not S99.

Additionally, our results have been confirmed by previous studies such as Kwon et al. (2007). In that study, the authors immunoprecipitated endogenous G3BP and used an anti-phosphoserine antibody to show that arsenite treatment reduced the level of phosphorylation of G3BP. They also showed that the reduction in G3BP phosphorylation level enhances the interaction of G3BP with HDAC6. This observation is also consistent with the fact that the G3BP-S149E mutant has less affinity with HDAC6.

Panas et al. (2019) state that S99P is instable, leading to less efficient interaction with protein partners. This is also difficult to reconcile with the fact that S149A and S149E have the same affinity for Sirt6 (Jedrusik-Bode et al., 2013), whereas they have different stability due to the S99P mutation. If S99P should destabilize G3BP, as suggested by Panas et al. (2019), this mutation should be expected to decrease the binding of G3BP to Sirt6. In

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addition, Jedrusik-Bode et al. (2013) have used an antibody against G3BP to demonstrate that Sirt6 influences the phosphorylation of G3BP on Ser149 and this correlates with SG assembly. Again, we do not see how the mutant S149E can be misleading and would change the conclusion of Jedrusik-Bode et al. (2013).

To summarize, we are grateful to Panas et al. (2019) for reporting the two accidental mutations and apologize to the community for any inconvenience. However, we believe that the jury is still out regarding the role of S149 phosphorylation on G3BP-mediated SG assembly. Much more work is needed before excluding a role of this modification in SG formation.

Acknowledgments

The authors declare no competing financial interests.

Submitted: 14 May 2019
Accepted: 14 May 2019

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