Review of: "Classical swine fever virus NS5A inhibits NF-κB signaling by targeting NEMO"

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The authors manuscript reports that 'Classical swine fever virus NS5A inhibits NF-κB signalling by targeting NEMO' by inhibiting K63-ubiquitination of NEMO (for unknown purposes) yet enhances K27-linked ubiquitination (driving degradation?). NS5A had been previous shown to inhibit NFkB activation *e.g.* from Poly I:C stimulation so it makes sense. Since these likely use different E3 ligase complexes it's hard to see what the mechanism is here and what the purpose is in K63 ubi inhibition. There's a lot of data presented in the manuscript some reasonably-controlled some poorly. I think overall there's a promising story in there, but it could be improved with additional controls. Issues and observations are listed below.

One immediate observation: It's odd they rarely see decreases in NEMO levels when co-transfected with NS5A outside **FIG 6** when they are trying to make that point. Was this effect highly variable?

More clarity needed in Figures in terms of what is being done . For example: Figure 8 shows a His pull-down, but it's not mentioned what is His tagged (presumably NEMO?)

Language: Needs a native English- speaker to revise, some errors seen throughout, *e.g.*

26 'Previous report' - 'a previous report' or previous reports'
33 - 'ubiquitin assay' -> 'a ubiquitin assay'

Unusual spacing inserts *e.g.* line 96 'In unstimulated cells, [long space] NFkB…'

Mol weight indicators overlapping in FIGs need to be fixed.

MS data: The authors describe that LC-MS was used to arrive at the observation, can they show the LC-MS data to highlight where NEMO sits in the hierarchy of hits? (*e.g.* volcano plots). Did any E3 ligase components show up that might explain the mechanism of K48/K27 ubi differences that underly the model of activity?

FIG 1A

Convincing strong interaction with endogenous NEMO. Blot for NEMO somewhat sharply cut off at the bottom, increase blot visibility above and below

FIG 1B

NEMO only control. In my experience, overexpressed NEMO can be 'sticky' so always important to show NEMO alone to check co-IP conditions are good. Although they do address it somewhat in 1C including IgG controls

FIG 1C

Odd the dramatic decrease in NEMO levels when infected with CSFV is not seen at all in the lysate control.

FIG 2

Always include full length NEMO in these truncations analyses as a positive control. It's essential.

They could have exploited the truncations analysis a bit more with additional NEMO truncations. Some of the truncations seem peculiar, why truncation mid CC1 and mid CC2? Only clear interaction is with delN3 Is the interaction between the ZF and CC1/2? Would it interact with ZF plus full CC2 and lacking CC1 entirely? Interesting they see some stickiness of NEMO truncations (we found the same thing)

Blot splicing in FIG2B WB: His lane between lanes 4/5? If so indicate where the splicing was done with a line.

FIG 3

Here they do use full length NS5A as a positive control (which only raises the question stronger of why they didn't do the same in FIG 2/NEMO truncation experiments).

It looks like they're all interacting except mutant 2 (and there is a weaker band for this truncation) so they conclude the interaction occurs in region 125-250. In scenarios like this it's very important to show NS5A truncations without NEMO to determine if they're interacting with the beads.

FIG 4

Always nice to see a truncation that doesn't interact with the suggested target also not inhibit the pathway in a different assay (TNFa driven kB-luc expression). This is always helpful validating evidence (assuming the truncation isn’t simply folding improperly).

Not generally advisable to do transient transfection with overall population readouts (only when all cells

are co-transfected with the same plasmids) but they do also see some effect with TNFa stimulation and IFNa expression.

FIG 5

Pretty convincing inverse correlation of NS5A and NEMO levels when both are co-transfected. Decreases in endogenous less convincing. This could be improved by transient transfection of NS5A and confocal for endogenous NEMO as different levels of NS5A expression in cells can be correlated with proportional decreases in endogenous NEMO. However, this observation is confirmed by MG132 titration in **FIG 6**

FIG 7

Should consistently show controls without ubiquitin

Not a strong difference in NEMO Ub-HA in A. More convincing in the IPs (B.)

FIG 8

A positive control for something that is K48-ubiuitinated would have helped here to demonstrate the effect/construct is working. However, **FIG 9** more convincingly demonstrates the effect is K27-linked ubiquitination

FIG 9

Control for NEMO with the different HA Ubi constructs would be helpful, not just when co-expressed with NS5A.

FIG 10

Reasonable evidence it inhibits K63-linked ubiquitination in the IP