QMRC of Morelli et al

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To see the latest version check https://medewitt.github.io/bio720/qmrc-morelli.pdf.

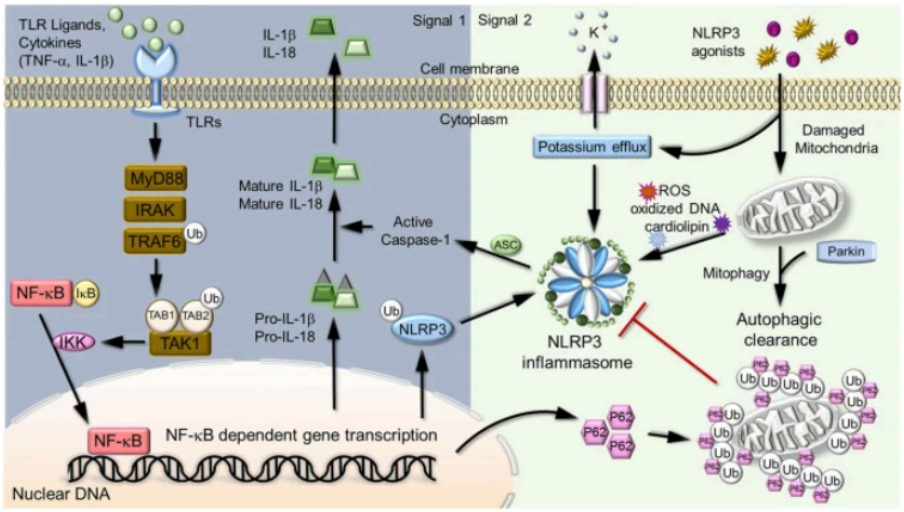
1 Questions

1.1 How do cells "sense" when they are infected with a virus? How do they "turn on" interferon gene expression?

Cells utilizes a series of approaches. One of the approaches is the through pathogen-associated molecular patterns which recognize particular foreign protein patterns and then initiate autocrine and paracrine signalling responses. These approaches include the existance of toll-like receptors (TLRs), RIG-1-like receptors, and nucleotide-binding organization triggers. These then upregulate expression of chemochines, cytokines, and interferons.

1.2 What was already known about regulation of the transcription factor NF-kB?

NF-kB is known to regulate innate immunity, adaptive immunity, cell differentiation, proliferation, and survival. NF-kB is known to mediate inflatmoty responses and is typically sequestered in the cytoplasm. When not activated it is inhibited by the IkB; however in response to stress the IkB is phorspohalated and triggers ubiquitin mediated degratation freeing the NF-kB to go to the nucleos to induce cytokine, chemokine, responses.



1.3 What was already known about the cellular targets of rotavirus NSP1?

NSP1 is known to target b-TrCP, IRF3/5/7/9, MAVS, and/or TNF receptorassociated factor 2 (TRAF2) to try to regulate host immune response. However, the C-terminius is highly variable and there was some though that the RING domain near the N terminus may act as an ubiquitin ligase.

1.4 What was the overall objective of this study?

The objective of this study is to show that NSP1 can inhibit host interferon response through the blocking of the release of NF-kB. Furthermore, this study shows that the mechanism is likely through the preservation of the bTrCp which disallows the turnover of the IkB disallowing the NF-kB to reach the nucleous to generate an innate immune response

2 Definitions

Autocrine signaling is a form of signaling in which a cell secretes a hormone or a chemical substance that binds to the receptors on the same cell, leading to functional changes in the cell (Liang et al 10.4161/cbt.26717)

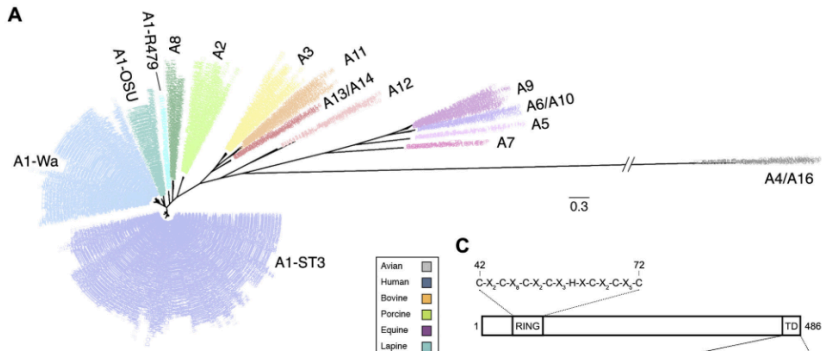
Paracrine signaling is a form of cell signaling in which the target cell is near the signal-releasing cell. (Liang et al 10.4161/cbt.26717)

3 Figure 1

FIG 1 Human and porcine RVA strains conserve a PDL motif within the NSP1 C terminus. (A and B) Maximum likelihood phylogenetic trees, assembled from 556 RVA NSP1 sequences collected globally since 1958. Branches are colored according to genotype. (A) Radial topology illustrates distances between the 16 RVA NSP1 genotypes shown. Genotype A1 resolves into four subgroups. The scale bar indicates phylogenetic distance (changes per site). (B) A circular cladogram shows phylogenetic relationships between NSP1 sequences isolated from

different species. The inner ring is colored according to host species. The outer ring is colored according to the presence (dark red) or absence (white) of a C-terminal PDL motif (DSGxS). NSP1 sequences used in this study are indicated. (C, top) Arrangement of key NSP1 domains. The consensus sequence of the RING domain is shown. (Bottom) Alignment of NSP1 C termini into three major groups. The PDL motif and N-terminal lysine residue are shaded in grey. TD, targeting domain. (D) Alignment of viral PDL motifs and phosphodegrons from known targets of beta-TrCP, shaded as in panel C

3.1 Panel A



3.1.a Q

Where are the genetic differences in the differential immune antagonism observed in the NSP1 protein?

3.1.b M

Phylogentic analysis of the ORFs from NSP1 in genbank using an unrooted tree generated from maximum likelihood approaches.

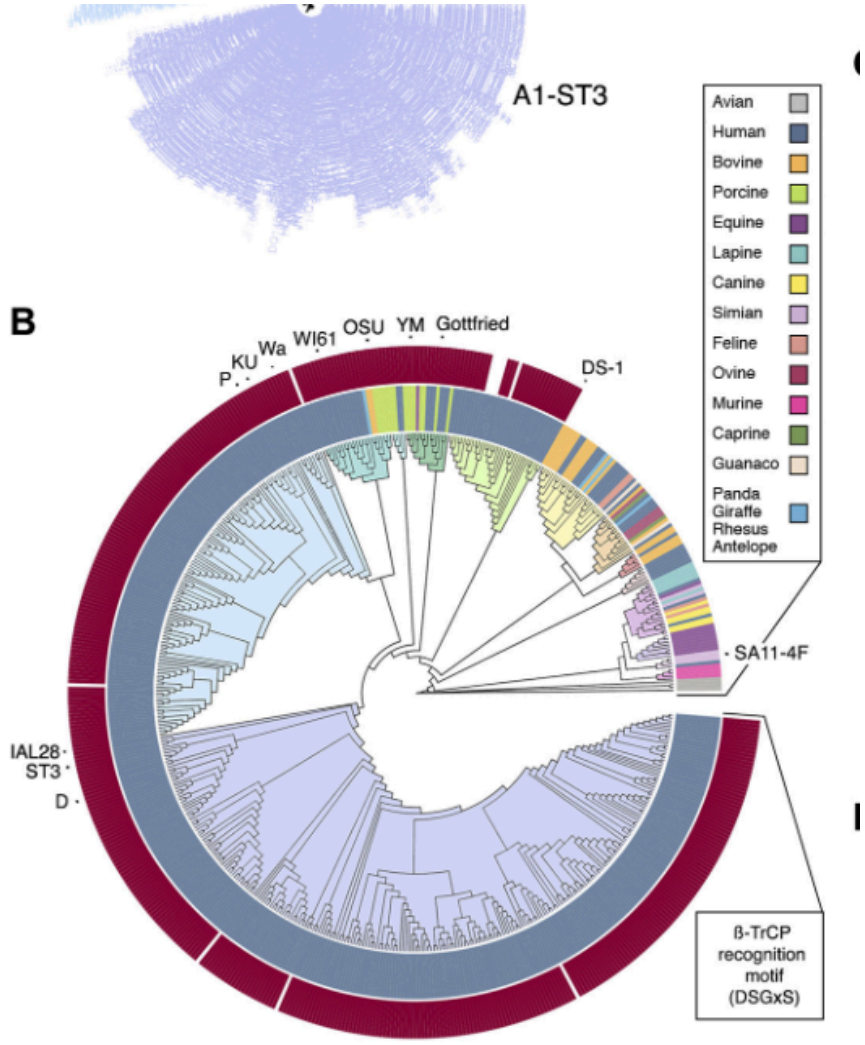
3.1.c R

Genotype A has 4 major groups/clusters

3.1.d C

There are four clusters. I agree with this finding. NV recombination may make this tree a little challenging? I don't know.

3.2 Panel B



3.2.a Q

Are these observed phylogenetic differences related to species alternate life -histories?

3.2.b M

Create a cladogram to examine the origins of the sequences (i.e., host species) given the phylogeny

3.2.c R

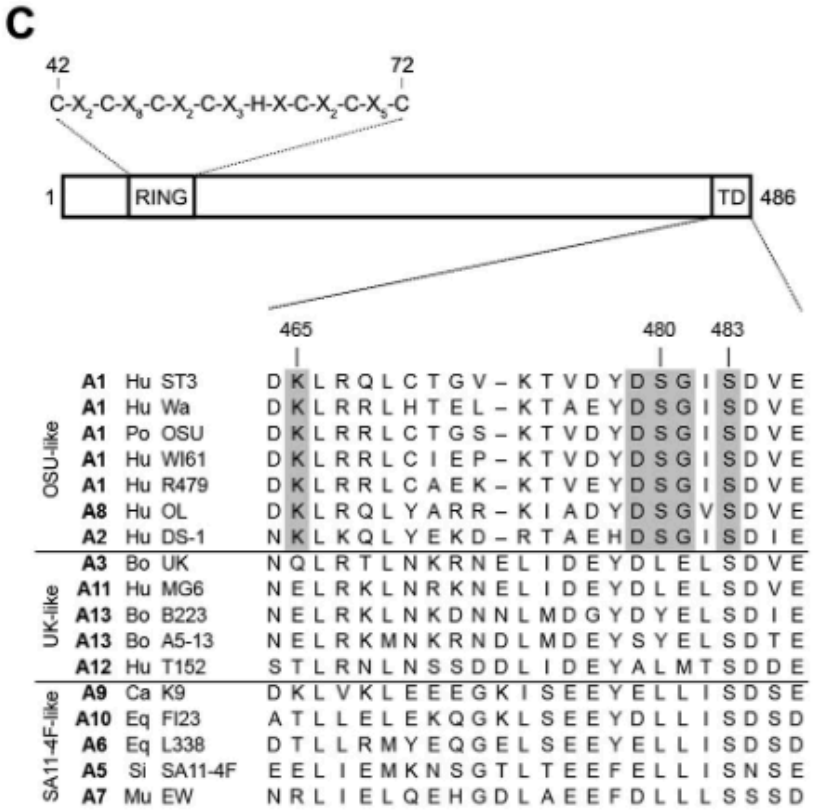
Yes, there are some noted differences by species.

3.2.d C

I agree with this finding but I worry about sampling bias.

Types appeared to separate into their phylogenetic history.

3.3 Panel C



3.3.a Q

Are the sequences in the domains of interest conserved across species in details?

3.3.b M

Examine the consensus sequences among the two domains of interest for NSP1 by host-species.

3.3.c R

NSP1 sequences used in this study are indicated. The sequences appear to be highly conserved across the species in the OSU group (porcine) and to some extent the human one.

3.3.d C

All full-length RVA NSP1 proteins are thought to utilize an N-terminal RING domain to interact with a cellular E2 ubiquitin-conjugating enzyme and a C-terminal sequence element to provide binding specificity for a host innate immune target

3.4 Panel D

3.4.a Q

Are the PDL sequences conserved within the human sequences?

3.4.b M

Review the sequences.

3.4.c R

Alignment of viral PDL motifs and phosphodegrons from known targets of *beta*-TrCP shown with high degree of conservation except maybe VAC.

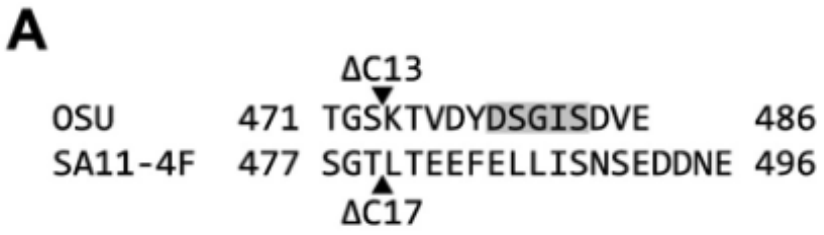
3.4.d C

Yes, it appears that there is some alignment.

4 Figure 2

OSU NSP1 requires an intact C terminus to target ?-TrCP for degradation. (A) Alignment of the C termini from OSU and SA11-4F NSP1 proteins. Arrowheads indicate the OSU-? C13 and SA11-4F-?C17 (SA11-5S) truncation mutants. The PDL motif is shaded in grey. (B) HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with medium ? 25 ng/ml TNF-?. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity. Data (mean ? SD from one of three experiments performed in triplicate) were analyzed by two-way ANOVA (pairwise as indicated) using Tukey's multiple comparisons test. (C) HEK293T cells cotransfected with NSP1 and FLAG-?-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of ?-TrCP is expressed as a percentage of ?-TrCP in OSU-?C13-transfected cells. Data (mean ? SD from three independent transfections) were analyzed pairwise with OSU. ns, not significant (P ? 0.05); **, P ? 0.01;** *, P ? 0.001. See also Fig. S1 to S4 in the supplemental material.

4.1 Panel A



4.1.a Q

Can we make a mutant with a truncated PDL motif?

4.1.b M

MAke a truncation mutant

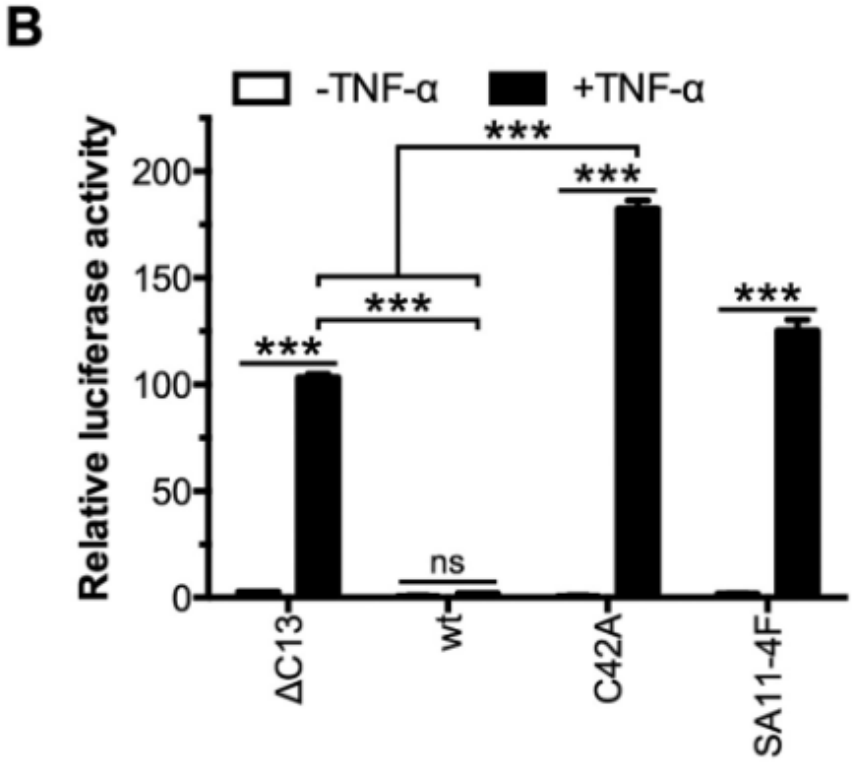
4.1.c R

A truncation mutant was made.

4.1.d C

This figure just shows that the delta C truncation mutant was made.

4.2 Panel B



4.2.a Q

With the truncation of the C-termininus domain, will we see the immunoregulatory features?

4.2.b M

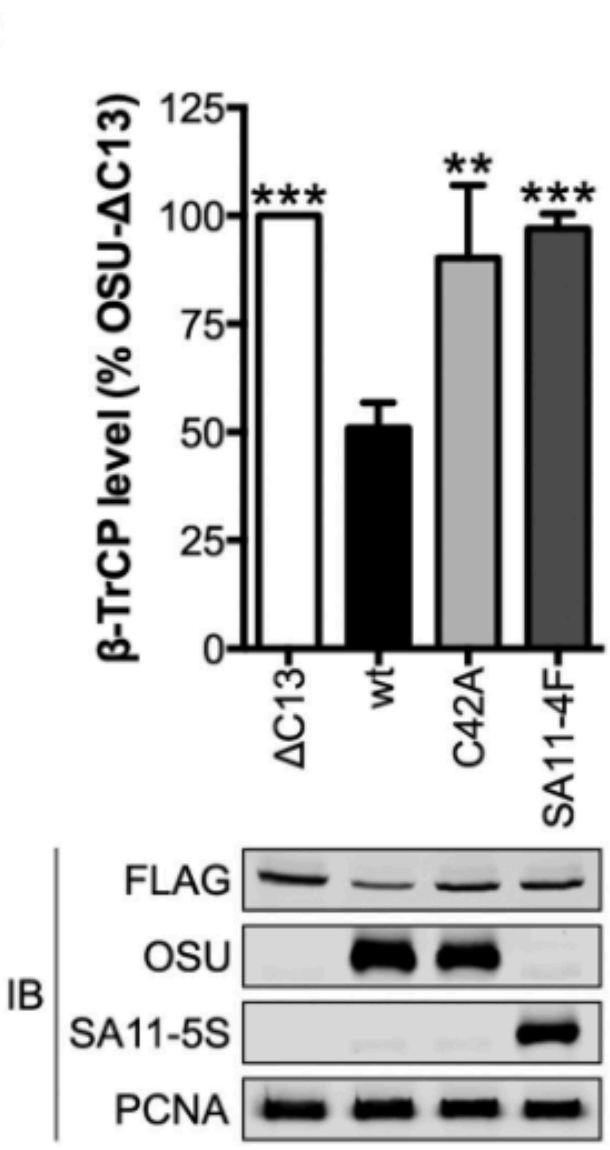
HEK cells were transfected with NSP1 anf NFkB with a luciferase reporter. The NSP1 mutant was transfected. Activated with TNF-alpha. Thus if NfkB is expressed, we will see it with the luciferase reporter with higher level indicating that the expected antiviral response is taking place.

4.2.c R

OSU NSP1 blocked activation (wt) while truncation mutant did not in the presence of TNF alpha. This mutant was also seen in both the OSU-C42A (point mutant predicted to disrupt the N-terminal RING domain) and SA11-4F (which mediates the degradation of IRFs but not [?]-TrCP)

4.2.d C

4.3 Panel C



4.3.a Q

What is the role of the PDL motif in NSP1 mediation of beta TrCP?

4.3.b M

HEK cells transfected with NSP1 and FLAG-beta Trcp assayed by quantitative immunoblotting. The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells.

4.3.c R

50% lower in cells with the wild type vs the mutants.

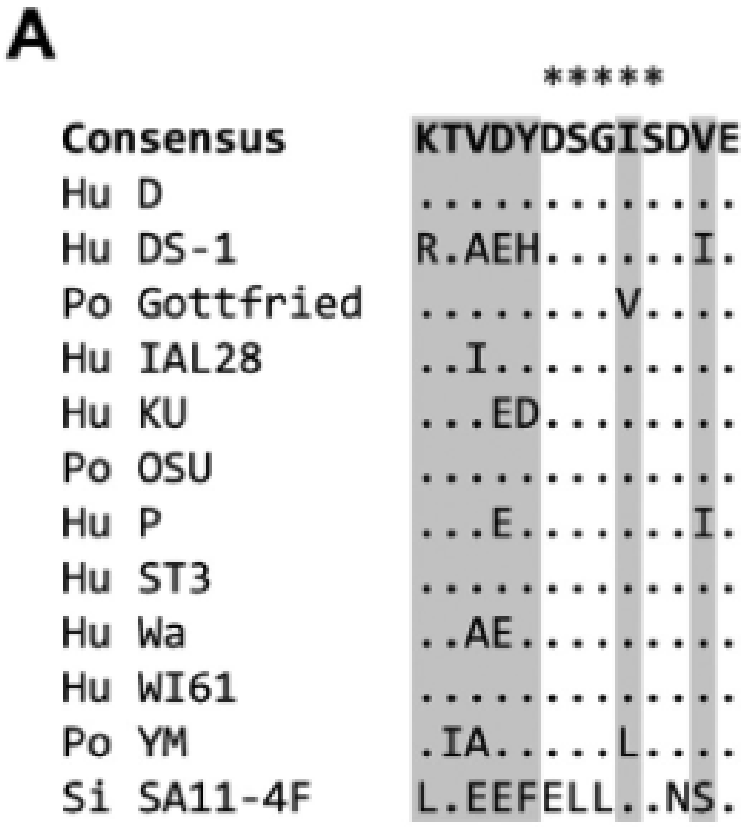
4.3.d C

This shows again that the intact C-terminus appears to be important.

5 Figure 3

Human and porcine RVA NSP1 proteins conserve NF-B antagonist activity. (A) Alignment of the C termini from OSU, related RVA, and SA11-4F NSP1 proteins. The last four residues of SA11-4F NSP1 (DDNE) are not shown. Sites of variability in the consensus sequence (excluding SA11-4F) are shaded in gray, dots indicate positions of identity, and asterisks indicate the PDL motif. Hu, human; Po, porcine; Si, simian. (B) HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity. Data (mean [?] SD from one of three experiments performed in triplicate) were analyzed by two-way ANOVA (pairwise wt/[?]C NSP1) using Sidak's multiple comparisons test. (C) HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). For each NSP1, the level of [?]-TrCP is expressed as a percentage of [?]-TrCP in cells cotransfected with the corresponding [?]C mutant. Data (mean [?] SD) are from three independent transfections. ***, P [?] 0.001. See also Fig. S5 and S6 in the supplemental material

5.1 Panel A



5.1.a Q

Do human and porcine NSP1 proteins conserve the observed NGkB atangonist activity as visualized through their sequence?

5.1.b M

Review the consensus alignment of the C termini from OSU, related RVA, and SA11-4F NSP1 protein

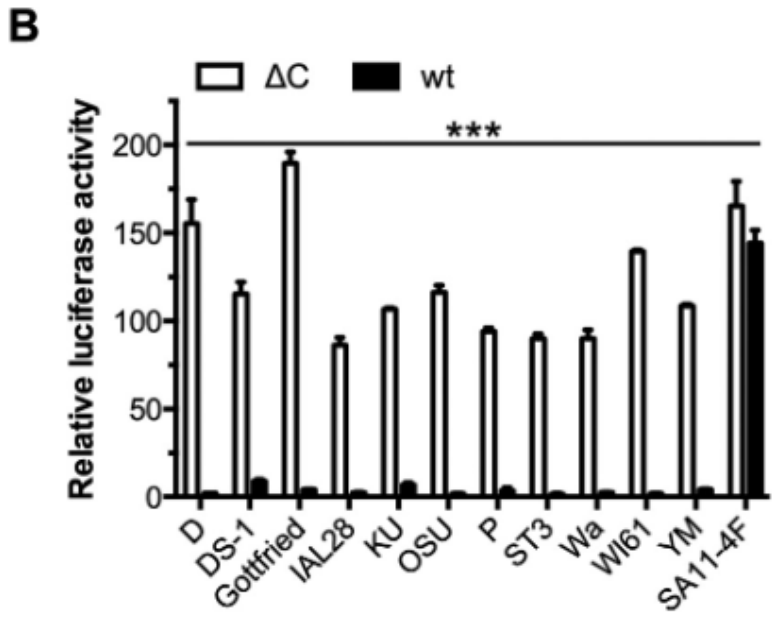
5.1.c R

Yes, there appears to be some sites of variability more so in the Simian motif than in the human and porcine motifs.

5.1.d C

Yes, it looks like there is some level of conservation.

5.2 Panel B



5.2.a Q

Is the observed C-terminus activity experiment with the mutant repeatable amongst the different consensus strains?

5.2.b M

HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. This was done for both the wild-type and the truncation mutant. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity.

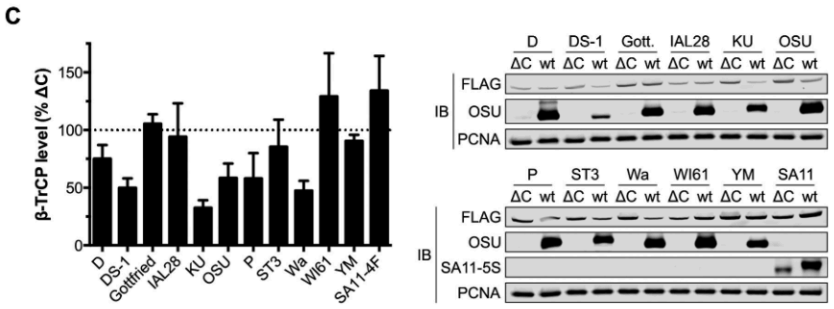
5.2.c R

In human and porcine strains, we see a tremendous and significant difference in NFkB suppression vs the wild-type. It appears that both the wild-type and the mutant in the simian strain did not have any real differences.

5.2.d C

This figure shows some level of species conservation.

5.3 Panel C



5.3.a Q

Is the mechanism of the antongistic activity through the betaTrCp and is this conserved through the different species?

5.3.b M

HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). For each NSP1, the level of [?]-TrCP is expressed as a percentage of [?]-TrCP in cells cotransfected with the corresponding deltaC mutant.

5.3.c R

Variable levels of beta-TrCp degradation are seen across the different strains.

5.3.d C

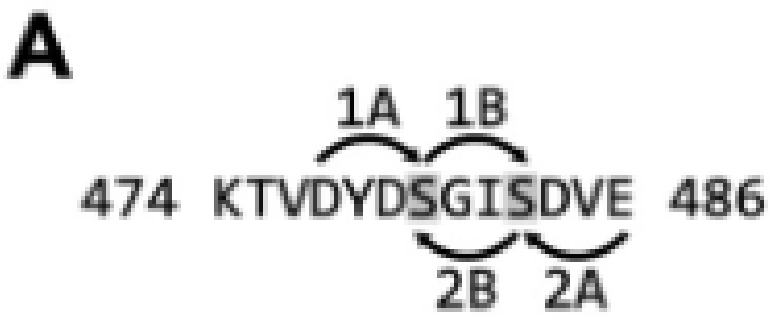
This group of OSU-like NSP1 proteins did not universally conserve [?]-TrCP degradation activity, despite all members having a C-terminal PDL motif and potently blocking NF-B activation

6 Figure 4

The serine residues of the PDL motif are required for NSP1 function. (A) Model for phosphorylation of the PDL motif of OSU NSP1 by CKI or CKII. Arrows point from the priming residue to the site of serine phosphorylation (shaded). 1A and -B, CKI; 2A and -B, CKII. (B and C) HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity. Data (mean [?] SD from one of three experiments performed in triplicate) were analyzed pairwise with OSU. (D) HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells. Data (mean [?] SD from three independent transfections) were analyzed pairwise with OSU. (E) HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin.

Immunoblots represent 3% clarified wholecell lysate (WCL) and 10% eluted protein. ns, not significant (P ? 0.05); , *P ? 0.05;* ***, P ? 0.01;*** , P ? 0.001

6.1 Panel A



6.1.a Q

Which serine residues are required for PDL activity?

6.1.b M

A review of the sequence of the NSP1

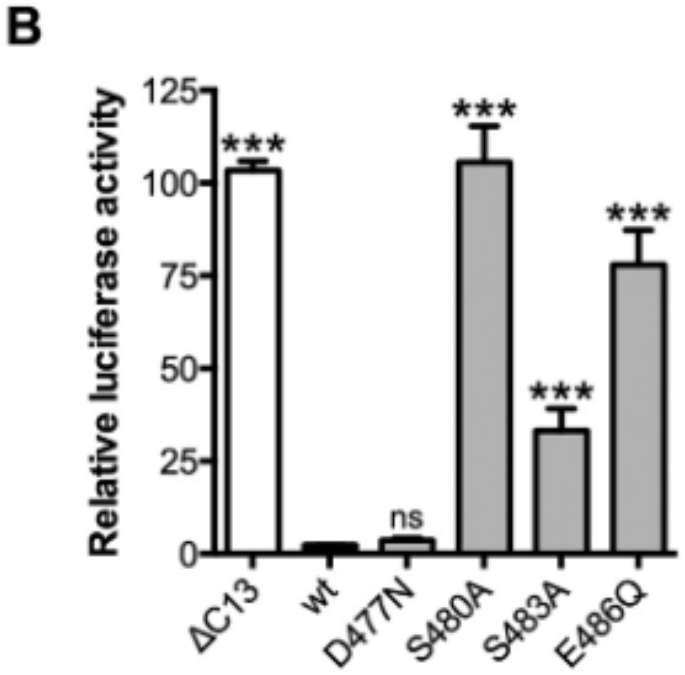
6.1.c R

HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-?. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity.

6.1.d C

No conclusions yet. This piece sets the hypothesis.

6.2 Panel B



6.2.a Q

Is the C-terminus the driver of the activity or will individual protein residue changes be enough to change the anti-NFkB antagonism?

6.2.b M

HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity.

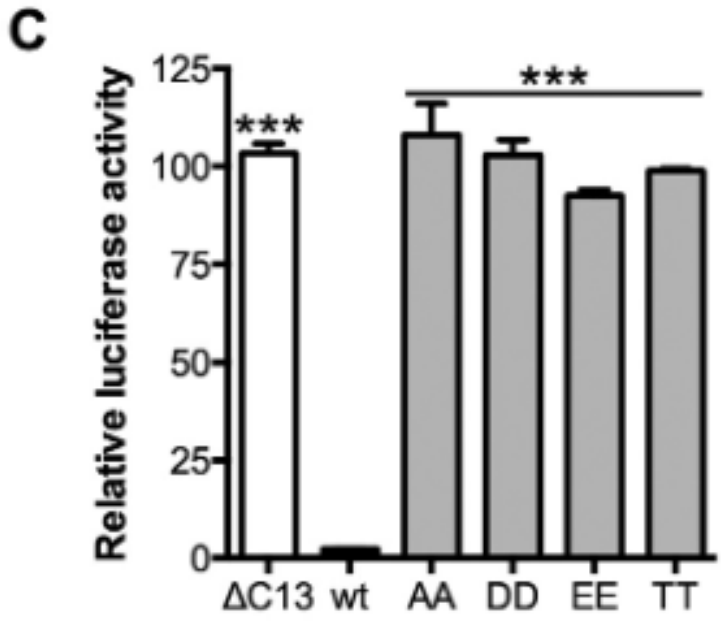
6.2.c R

All of the point mutations (e.g., S480A) resulted in a decreased amount of suppression compared to wild-type, though there was some quantitative variaiton.

6.2.d C

It appears that the entirity of this motif needs to be conserved in this position in order to have the antagonism compared to wild-type. This suggests that all of the amino acides in this position need to be present to affect this outcome.

6.3 Panel C



6.3.a Q

Is serine a strict need at these positions?

6.3.b M

HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-?. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity.

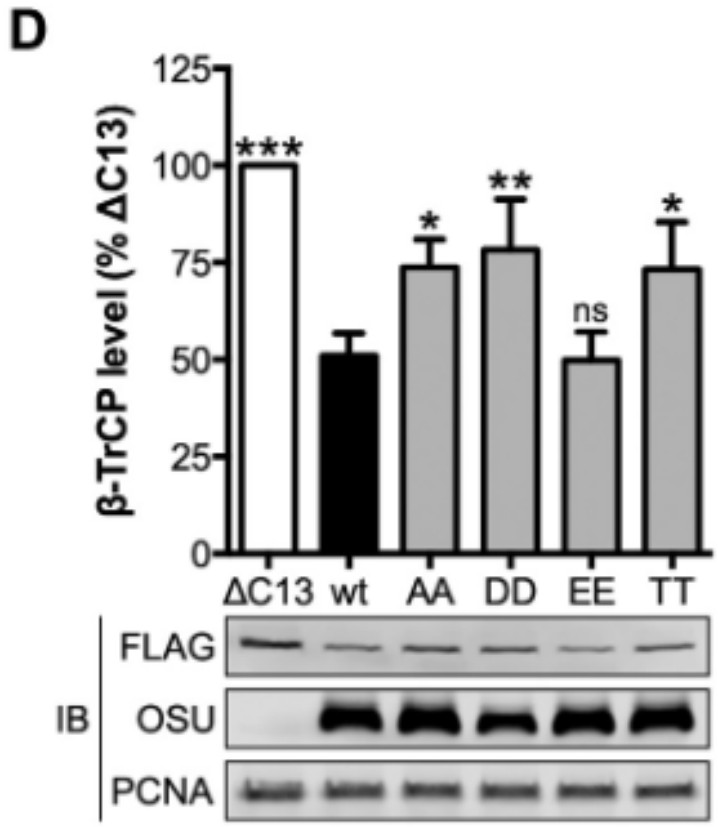
6.3.c R

All of the point mutations resulted in a decreased amount of suppression compared to wild-type, though there was some quantitative variaiton.

6.3.d C

There appears to be a strict requirement for serine at these positions for the antagonism to occur.

6.4 Panel D



6.4.a Q

Do these amino acid substitutions have the same impact on beta TrCP levels?

6.4.b M

HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells.

6.4.c R

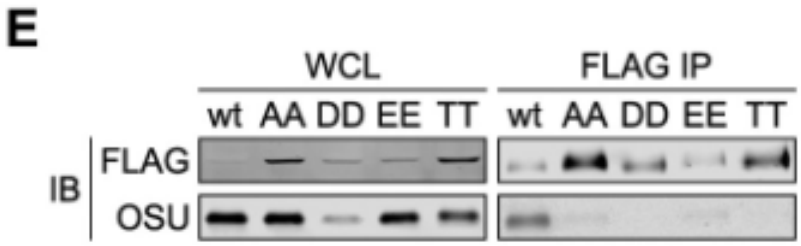
OSU and OSU-EE NSP1 were functionally equivalent in this assay, while OSU-AA, -DD, and -TT NSP1 proteins also mediated a modest degree of [?]-TrCP degradation.

6.4.d C

This suggested that transient interaction of the NSP1 RING domain with the E2 subunit of the SCF[?]-TrCP complex may have been sufficient to stabilize a likely weak interaction between the mutated PDL motif and the [?]-TrCP subunit. An electronegative sequence might sufficiently sta-

bilize the likely transient interaction required by NSP1 to polyubiqutinate [?]-TrCP. This makes sense given the substitution patterns we are observing with some

6.5 Panel E



6.5.a Q

Are the NSP molecules able to bind to the beta TrCP with these AA substitutions?

6.5.b M

HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin. Immunoblots represent 3% clarified wholecell lysate (WCL) and 10% eluted protein.

6.5.c R

WCL is whole cell lysate. There is poor binding to the OSU.

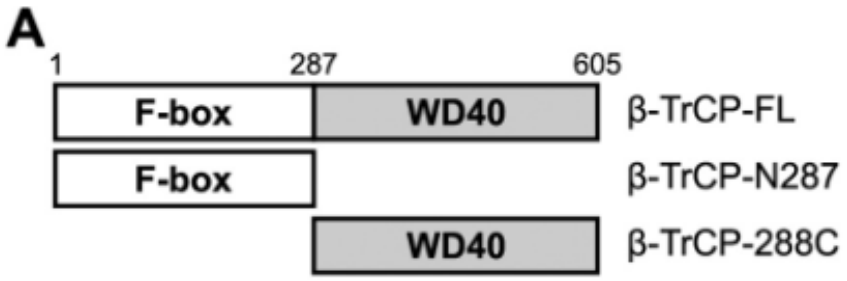
6.5.d C

FLAG immunoprecipitation of [?]-TrCP:NSP1 complexes from cotransfected HEK293T cells demonstrated that the serine residues were critical for this interaction

7 Figure 5

NSP1 targets the phosphodegron-binding pocket of [?]-TrCP. (A) Diagram of [?]-TrCP domain organization and truncations. (B) HEK293T cells cotransfected with OSU NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells. Data (mean [?] SD) are from three independent transfections. (C) Structure of the [?]-TrCP WD40 domain (green) and associated [?]-catenin phosphodegron peptide (yellow) (12). R510 is colored purple. (D) HEK293T cells cotransfected with OSU NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells. Data (mean [?] SD) are from three independent transfections. (E) HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin. Immunoblots represent 3% clarified whole-cell lysate (WCL) and 10% eluted protein

7.1 Panel A



7.1.a Q

What does the truncation mutant look like?

7.1.b M

Create the mutant.

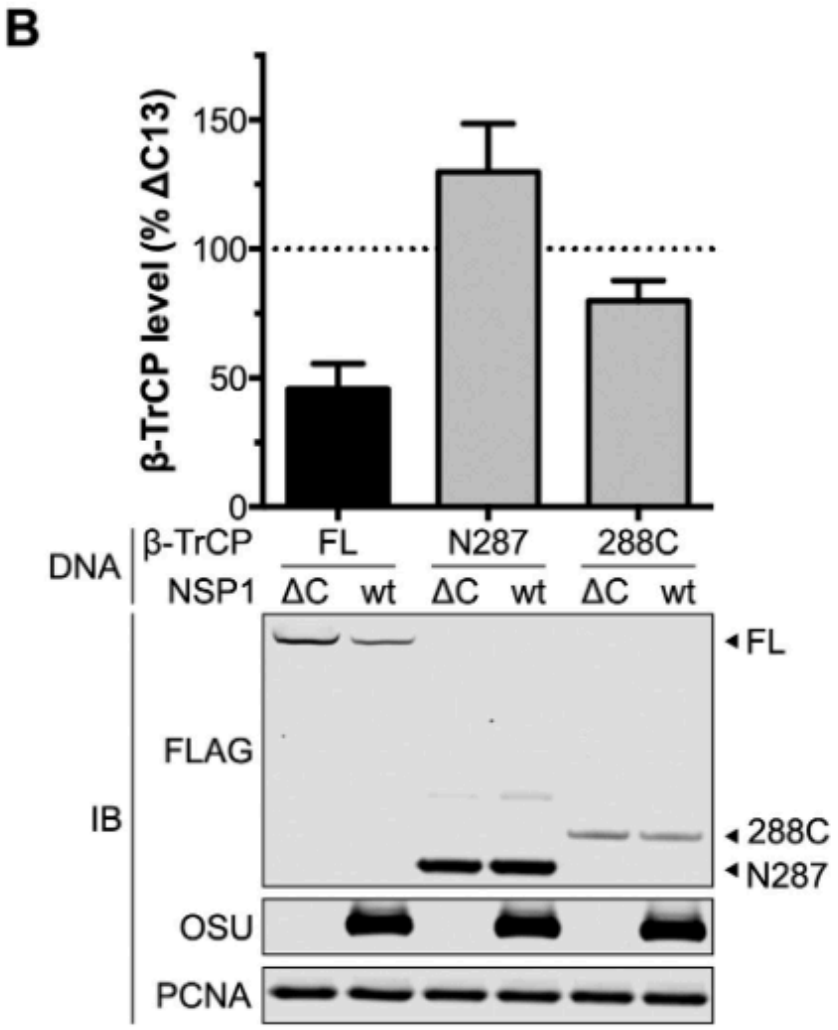
7.1.c R

Mutant created.

7.1.d C

Diagram of [?]-TrCP domain organization and truncations.

7.2 Panel B



7.2.a Q

Does the NSP1 continue to recognize the appropriate site on beta TrCP for degradation?

7.2.b M

EK293T cells cotransfected with OSU NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells.

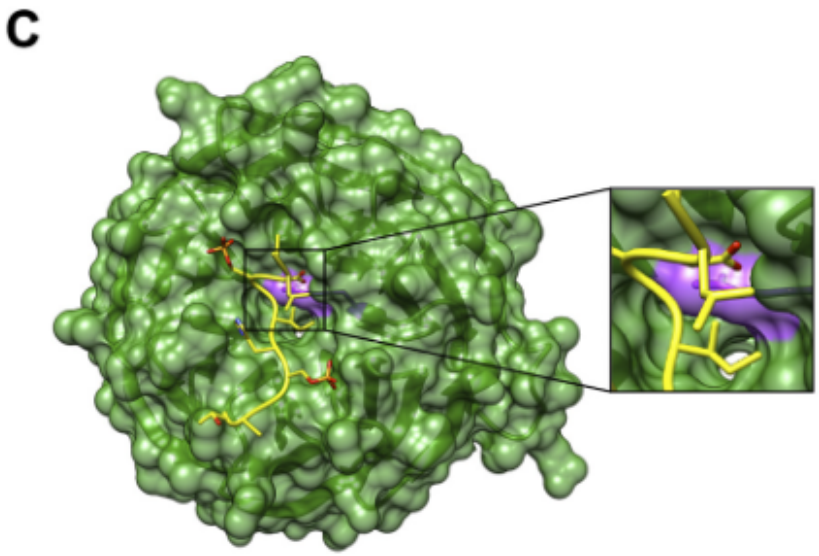
7.2.c R

NSP1 mediated the degradation of [?]-TrCP-288C, although less efficiently than it did full-length [?]-TrCP.

7.2.d C

This suggested preferential recognition of the full-length protein, perhaps in the context of the SCF[?]-TrCP complex.

7.3 Panel C



7.3.a Q

What is the structure of the NSP region of interest?

7.3.b M

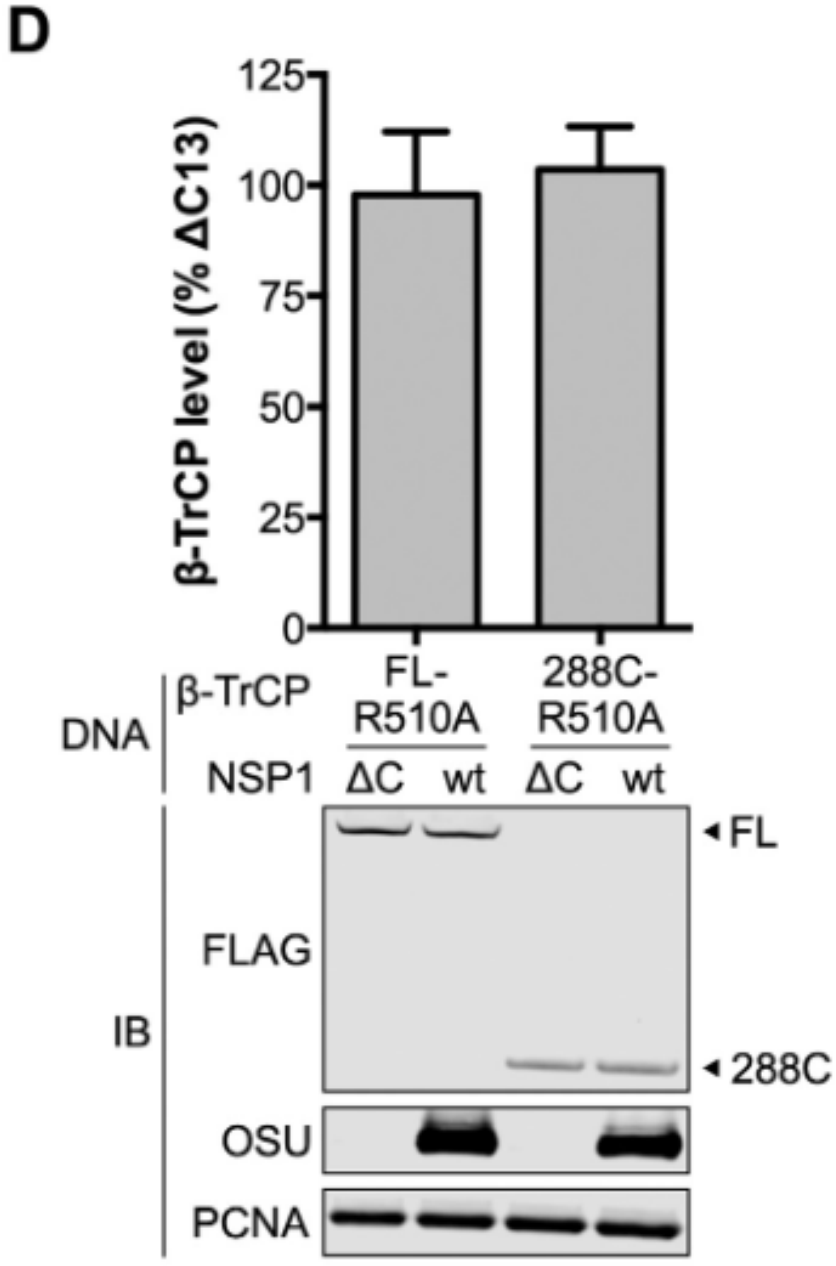
NA

7.3.c R

NA ### C

Aspartic acid, which almost invariably marks the phosphodegron start, forms the most extensive network of contacts, in particular with residues R510 and Y524

7.4 Panel D



7.4.a Q

Do modiciations in the binding pocket alter the degradation of TcCP mutants (e.g., is this process very specific to the sequence)?

7.4.b M

The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in OSU-[?]C13-transfected cells.

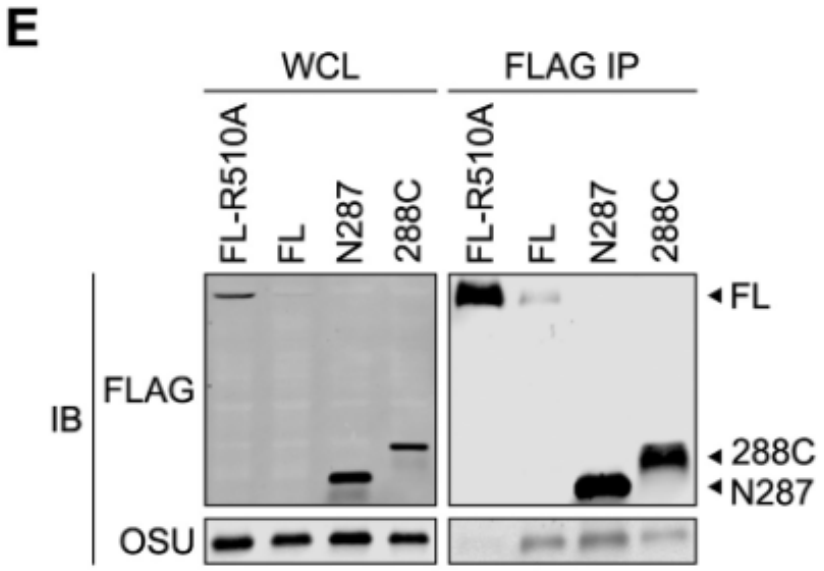
7.4.c R

OSU NSP1 could not mediate the degradation of either [?]-TrCP-R510A or [?]-TrCP-288CR510A,

7.4.d C

Indicates that NSP1 targets the binding pocket used by [?]-TrCP to interact with cellular phosphodegrons

7.5 Panel E



7.5.a Q

Doe oes the phosphodegrons region need to be intact for the degradation of the TcCP?

7.5.b M

HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin. Immunoblots represent 3% clarified whole-cell lysate (WCL) and 10% eluted protein.

7.5.c R

FLAG-[?]TrCP-R510A also failed to coimmunoprecipitate OSU NSP1

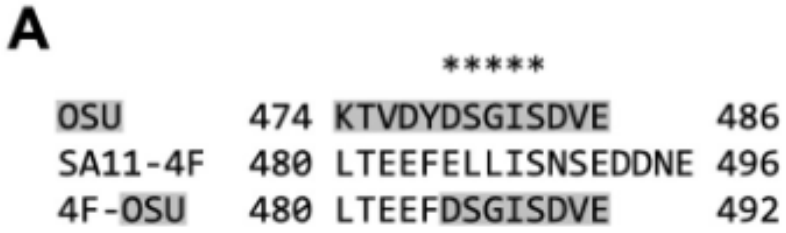
7.5.d C

[?]-TrCP recognizes cellular phosphodegrons and the NSP1 PDL motif in a similar manner.

8 Figure 6

The PDL motif is the minimum sequence required by NSP1 to target [?]-TrCP. (A) Alignment of the C termini from OSU, SA11-4F, and 4F-OSU NSP1. Residues from OSU NSP1 are shaded in gray, and asterisks indicate the PDL motif. (B) HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity. Data (mean [?] SD from one of three experiments performed in triplicate) were analyzed pairwise with SA114F. (C) HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in SA11-5Stransfected cells. Data (mean [?] SD from three independent transfections) were analyzed pairwise with SA11-4F. (D) HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin. Immunoblots represent 3% clarified whole-cell lysate (WCL) and 10% eluted protein. ns, not significant (P [?] 0.05); , *P [?] 0.05;* ***, P [?] 0.01;*** , P [?] 0.001.

8.1 Panel A



8.1.a Q

What is the shared sequence?

8.1.b M

Review the sequence?

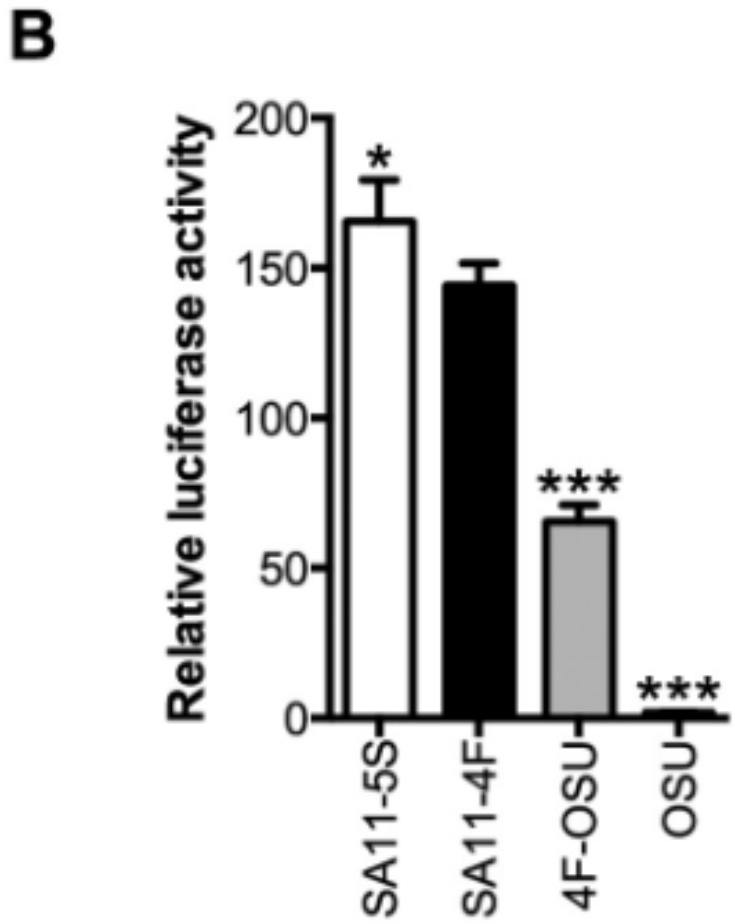
8.1.c R

Alignment of the C termini from OSU, SA11-4F, and 4F-OSU NSP1. Residues from OSU NSP1 are shaded in gray, and asterisks indicate the PDL motif.

8.1.d C

OSU seems to have the same sequence.

8.2 Panel B



8.2.a Q

What is the minimum sequence needed to target degradation?

8.2.b M

HEK293T cells were cotransfected with NSP1 and NF-B firefly and HSV-tk Renilla luciferase reporters. At 24 h p.t., cells were stimulated for 4 h with 25 ng/ml TNF-[?]. Relative luciferase activity was calculated by normalizing firefly to Renilla luciferase activity.

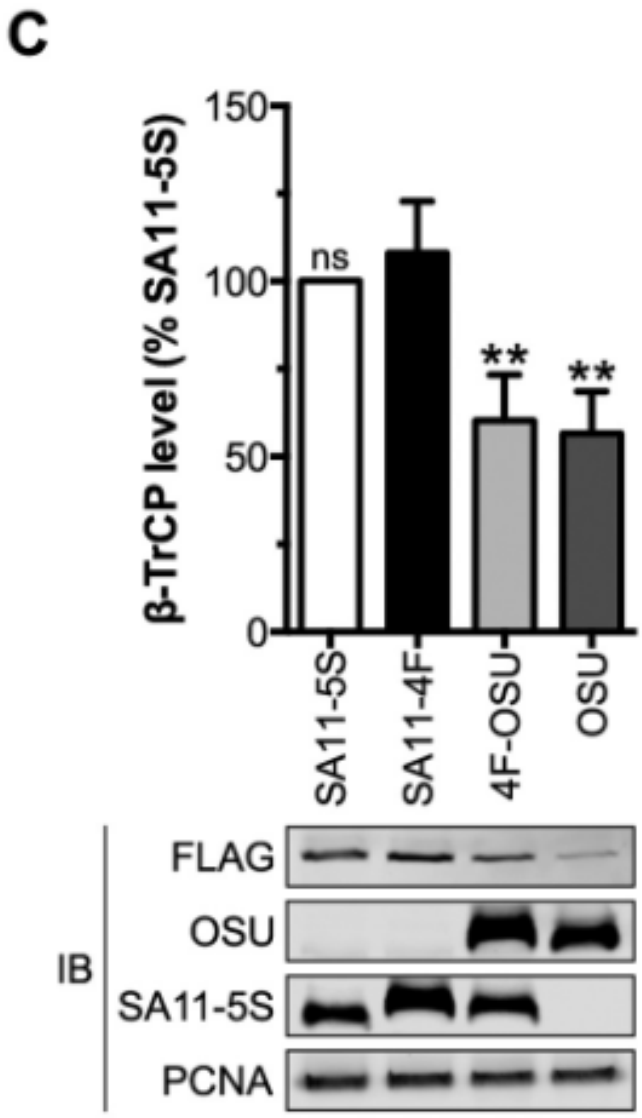
8.2.c R

4F-OSU lacked the potency of OSU NSP1

8.2.d C

Indicates that regions outside the C-terminal PDL motif likely contribute to NF-B antagonism by OSU NSP1 and are not fully captured by SA11-4F NSP1

8.3 Panel C



8.3.a Q

What is the effect of those sequence variants on beta TrCP degradation?

8.3.b M

HEK293T cells cotransfected with NSP1 and FLAG-[?]-TrCP were assayed 24 h p.t. by quantitative immunoblotting (IB) (normalized to PCNA). The level of [?]-TrCP is expressed as a percentage of [?]-TrCP in SA11-5Stransfected cells.

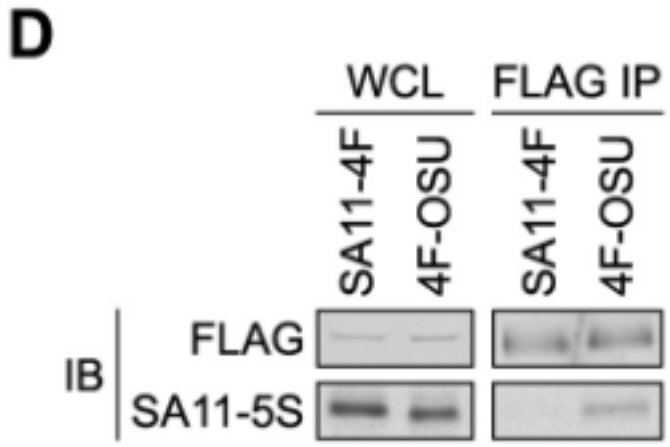
8.3.c R

The 4F-OSU chimera was able to induce [?]-TrCP turnover comparable to that of OSU NSP1.

8.3.d C

This finding suggests that the PDL motif, when paired with an intact RING domain, is sufficient to target SCF[?]-TrCP and that SA11-4F and OSU NSP1 proteins conserve an architecture and mechanism similar enough to permit this transfer of functionality.

8.4 Panel D



8.4.a Q

IS the 5 residue motif sufficient for beta Tc-CP degradation?

8.4.b M

HEK293T cells cotransfected with NSP1 and [?]-TrCP were immunoprecipitated (IP) with anti-FLAG resin. Immunoblots represent 3% clarified whole-cell lysate (WCL) and 10% eluted protein.

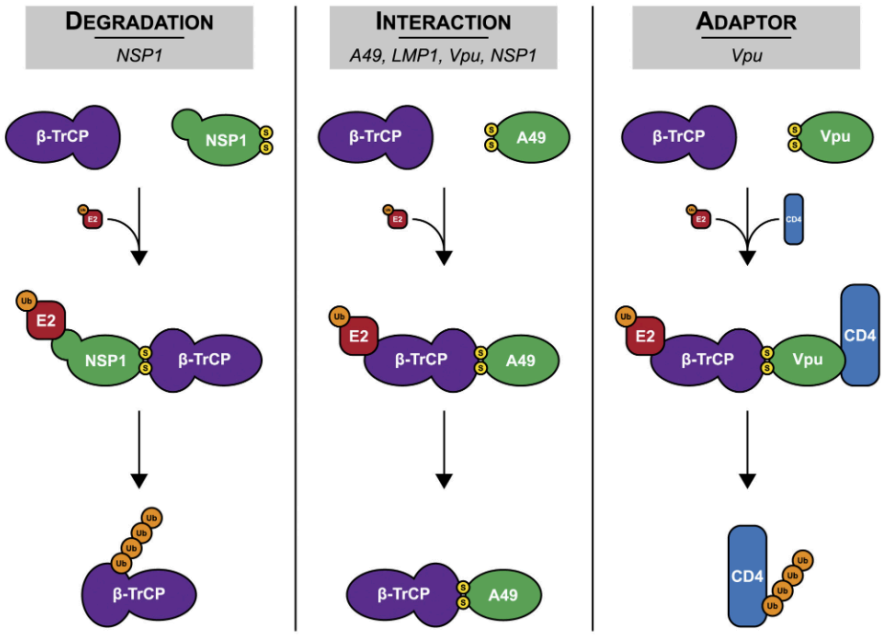
8.4.c R

Based on this blot the 5 protein motify results in similar profiles to the wild-type indicating that the wild-type functionality is recovered.

8.4.d C

9 Figure 7

Diverse functions of viral PDL motif-containing proteins. The models depict how RV NSP1, EBV LMP1, HIV-1 Vpu, and VACV A49 disrupt [?]-TrCP activity. These viral proteins use a PDL motif to (i) induce the turnover of [?]-TrCP (degradation), (ii) block [?]-TrCP from recognizing cellular phosphodegrons (interaction), or (iii) induce the turnover of cellular proteins by bridging [?]-TrCP to these targets (adaptor). Yellow, serine residues of the PDL motif; orange, ubiquitin.



9.1.a Q

What is the overall mechanism for what we observe?

9.1.b M

Diagram.

9.1.c R

There are three different proposed mechanisms

1. Degradation of bTrCP which degrades that which degrades the inhibitor
2. Blocking
3. Bridging

9.1.d C

The bridging is the from the literature and I am hestitant to comment on it, but we seem to have good evidence of the degradation approach.