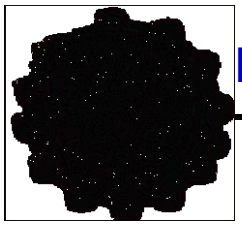
Interactions of HIV-1 Gag Protein with RNA

Alan Rein



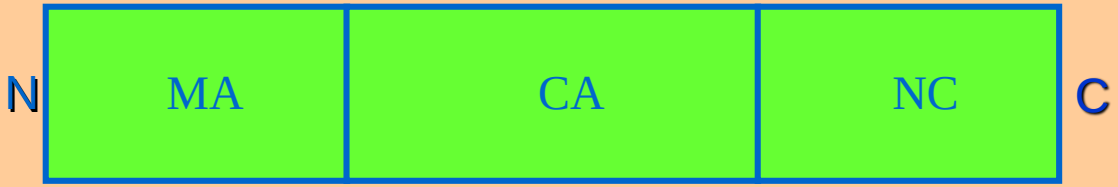
HIV Dynamics and Replication Program

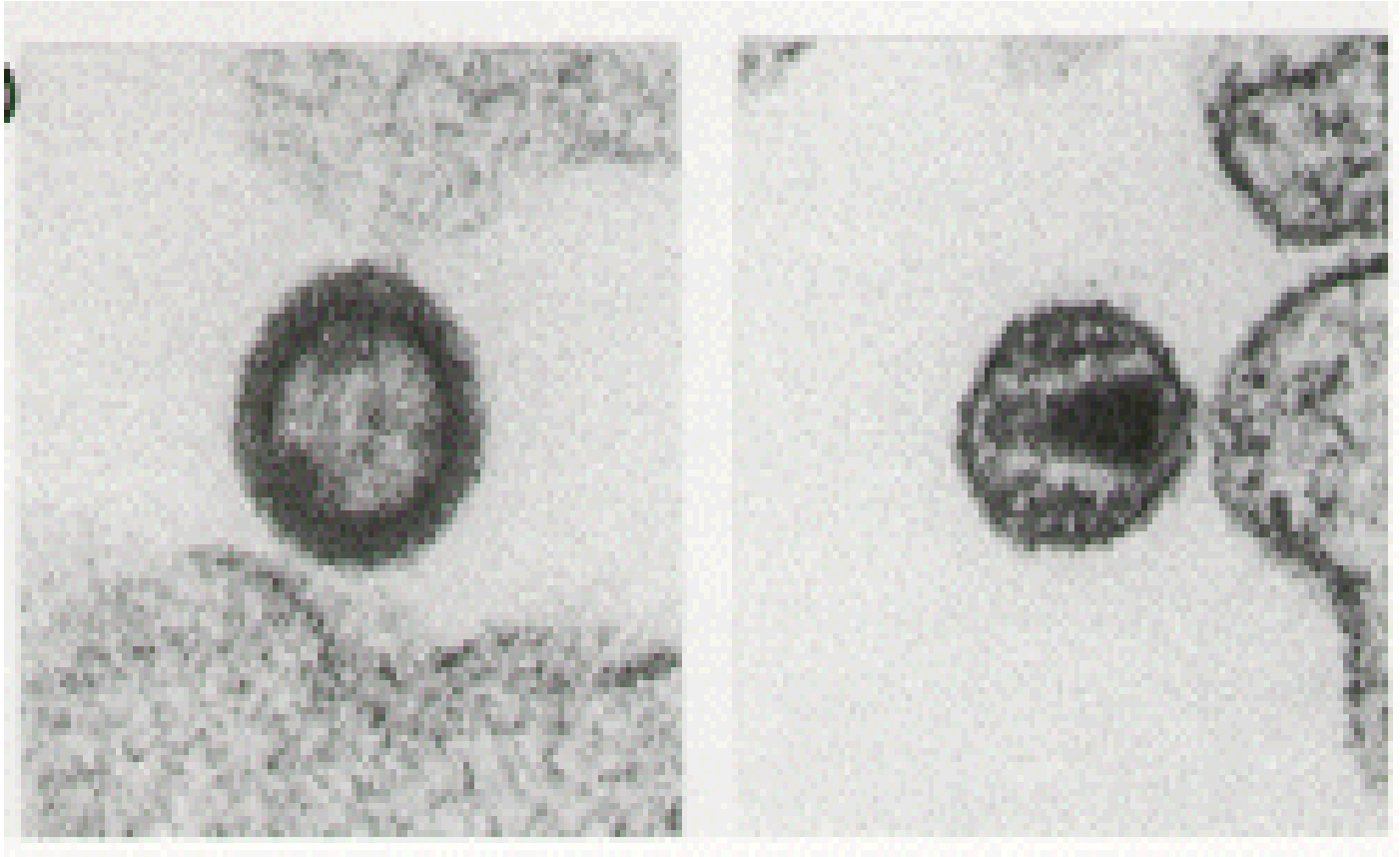
Some Basic Facts about Retrovirus Assembly

1. Expression of the Gag protein in a mammalian cell is sufficient for production and release of virus particles.

Some Basic Facts about Retrovirus Assembly

2. After the particle is released from the cell, Gag is cleaved into at least 3 cleavage products in virus maturation, termed matrix (MA), capsid (CA), and nucleocapsid (NC)

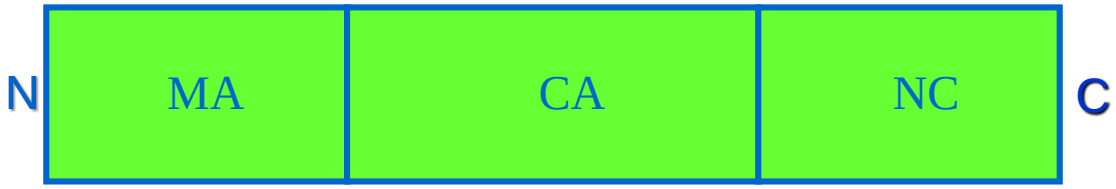




Immature Mature

Maturation brings about a global change in the structure of the virus particle.

Some Basic Facts about Retrovirus Assembly

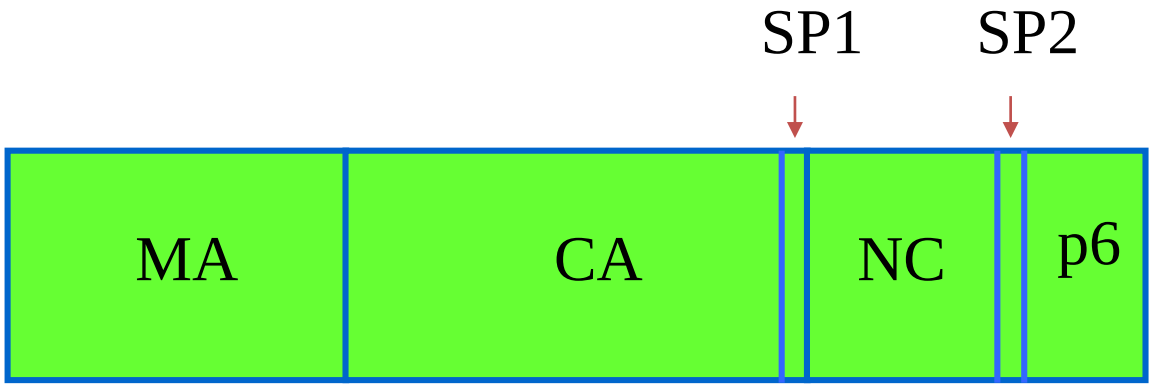


To a first approximation, the MA domain functions in interactions of Gag with the plasma membrane of the virus-producing cell (but it also binds RNA).

The CA domain does most if not all of the protein-protein interaction in assembly of the virus particle.

The NC domain does much of the interaction of Gag with RNA. It contains 2 zinc fingers that are crucial in the interactions with RNA.

A More Detailed Map of HIV-1 Gag



Interactions of Gag with RNAs

Gag interacts with RNAs in 3 distinct ways, all important for virus replication:

- **As a nucleic acid chaperone**
- **In constructing the virus particle**
- **Selecting the genomic RNA for incorporation into the particle**

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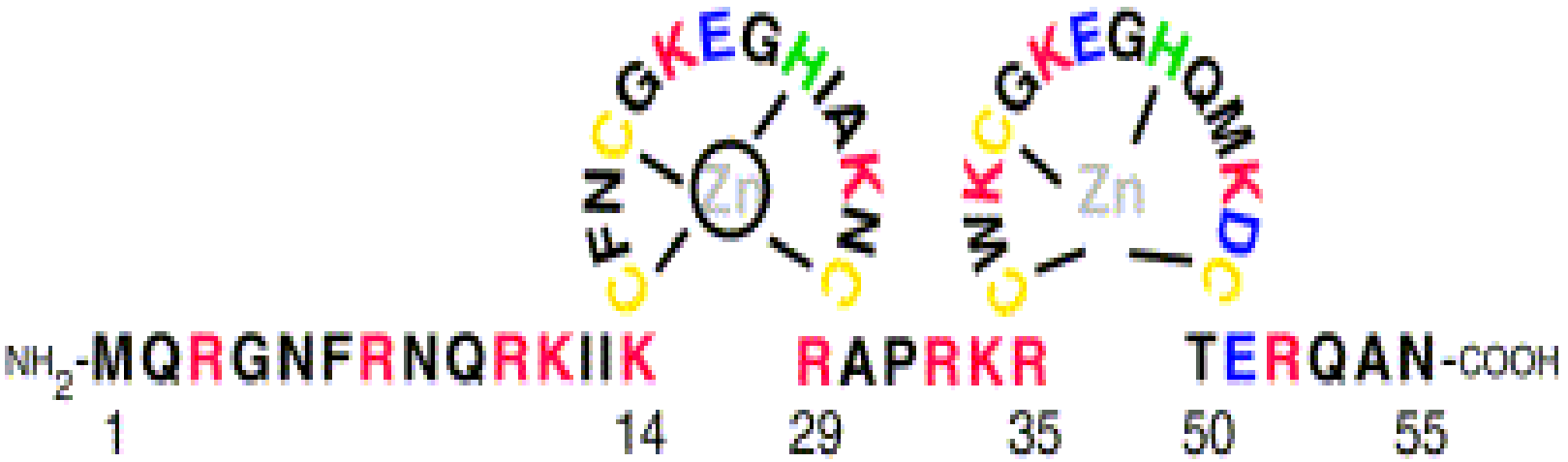
What is a Nucleic Acid Chaperone?

Just like an enzyme, a nucleic acid chaperone catalyzes the rearrangement of nucleic acids into the most thermodynamically favorable configuration…in general, the configuration with the maximal number of base-pairs. No ATP is involved.

HIV-1 NC protein is a well-studied nucleic acid chaperone.

HIV-1 NC is only 55 aa's. It is quite basic and contains 2 zinc fingers.



NA chaperones essentially promote “breathing” of NA’s, transiently breaking existing base-pairs and thus enabling NA strands to find new base-pairing partners.

Mechanism of NC's Chaperone Activity

3 properties of NC all seem to contribute to its activity:

- **It is a polycation, helping to bring NA molecules close together**
- **It is a weak destabilizer of base-pairs**
- **It binds to NA's with very rapid on-rates and off-rates**

K. Musier-Forsyth, I. Rouzina, M. Williams

The chaperone activity of NC is crucial during reverse transcription, which involves several “strand transfer” (ie, annealing) steps.

Gag is also a chaperone, presumably *via* its NC domain.

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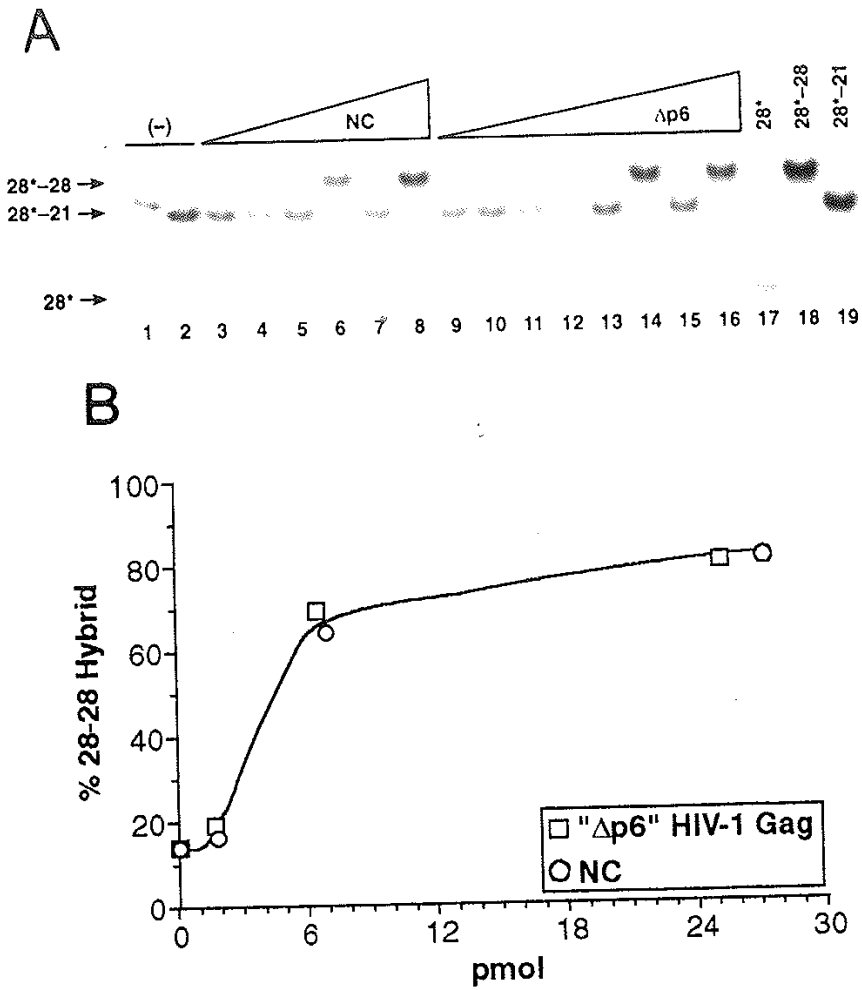


Feng et al., 1999

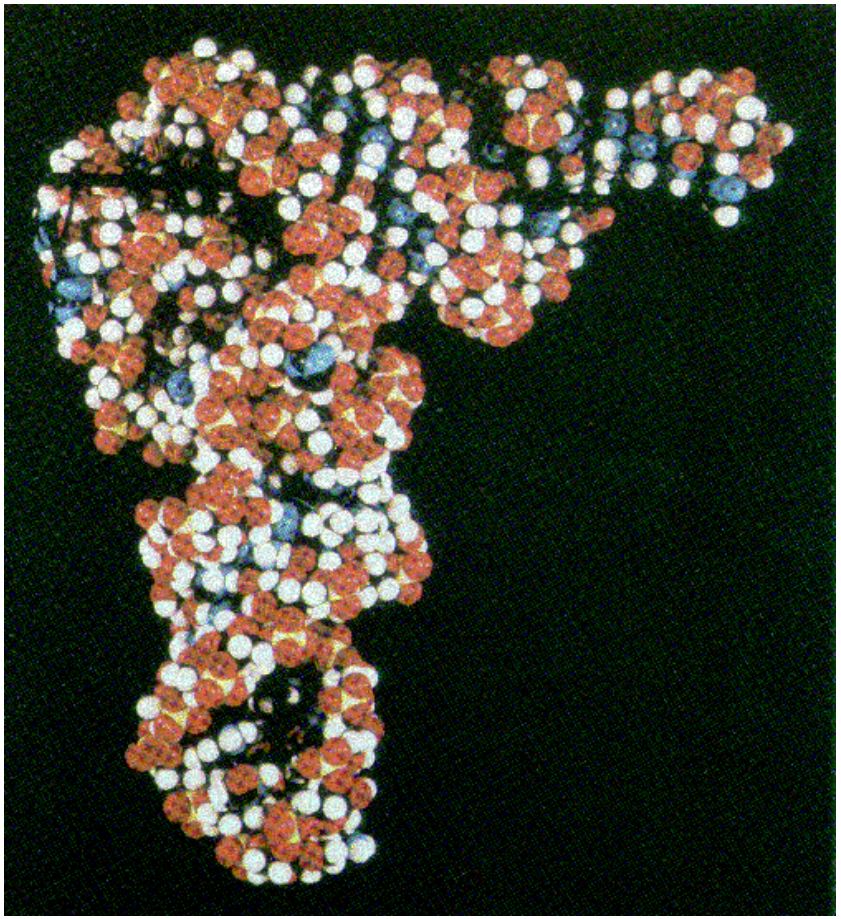
Gag is also a chaperone, presumably *via* its NC domain.

And—crucial for DNA synthesis: it anneals tRNA to an 18-base complementary stretch on viral RNA, where it will serve as primer for synthesis of viral DNA

tRNA

tRNAs are highly structured, compact molecules which play an essential role in protein synthesis.

A large fraction of their bases are paired intramolecularly.



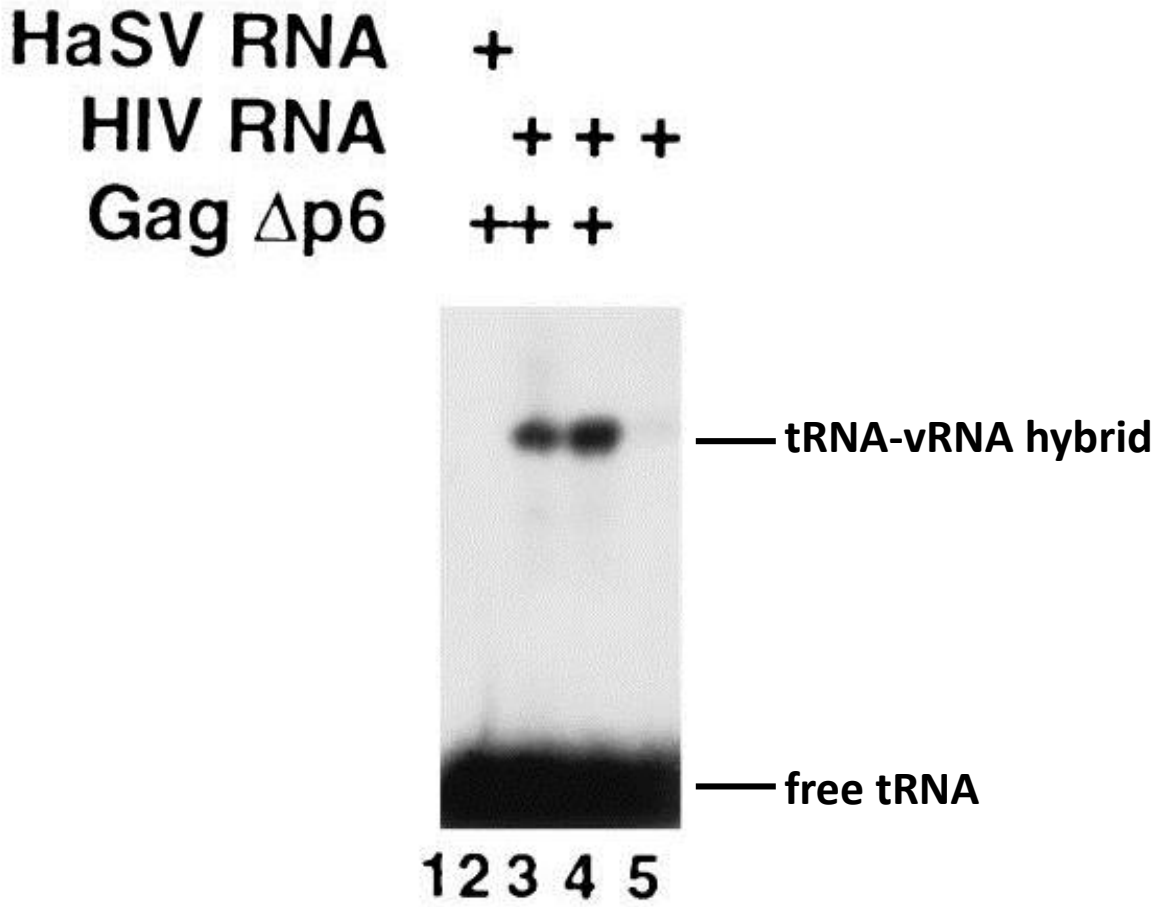
tRNA

- Therefore, many pre-existing base-pairs within the tRNA must be broken before tRNA bases can be paired with bases in the viral RNA.

- In the lab, we break pre-existing base-pairs by heating the RNA.

- But retroviruses do it at 37°C!

Annealing of tRNA to viral RNA



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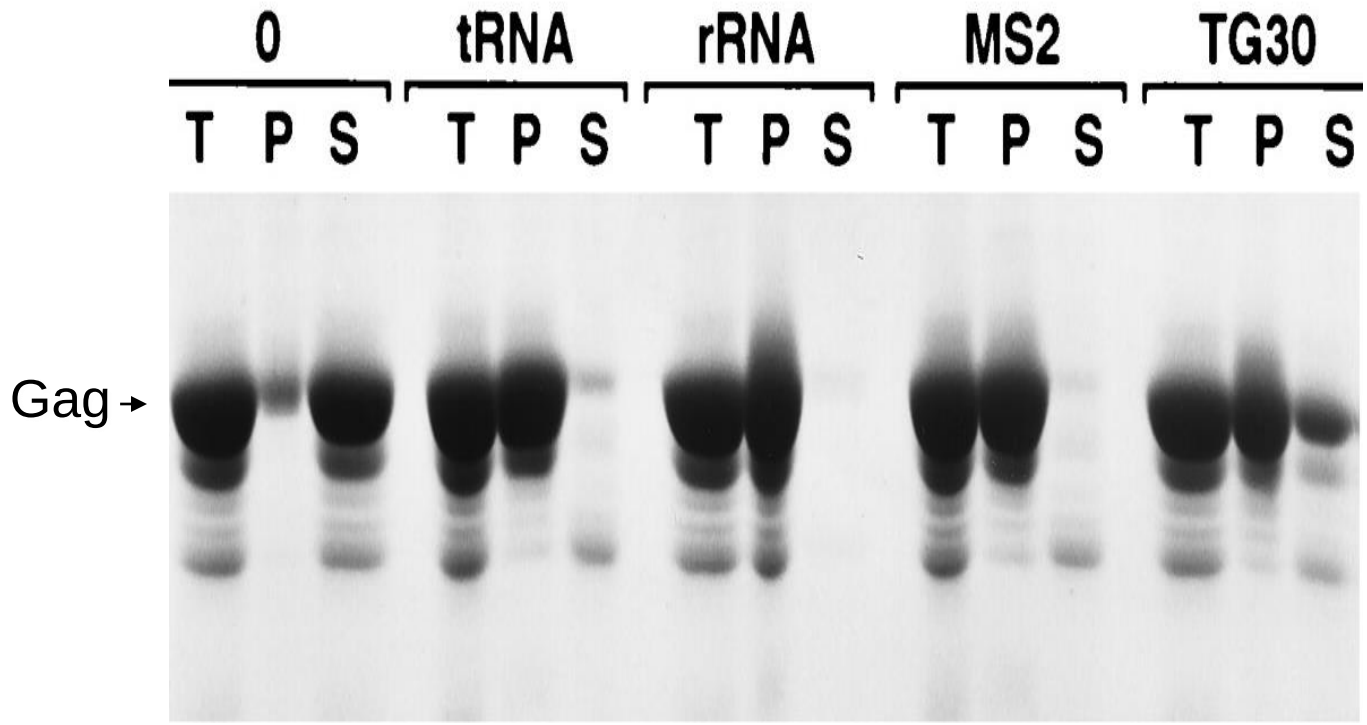
Efficient *in vitro* assembly by HIV-1 Gag protein requires nucleic acid



"Standard Assembly Conditions":
HIV-1 Gag at 20 μM in 0.1M NaCl

T, total; P, pellet; S, supernatant

Campbell & Rein, 1999

We have worked for years to try to understand how NA contributes to VLP assembly.

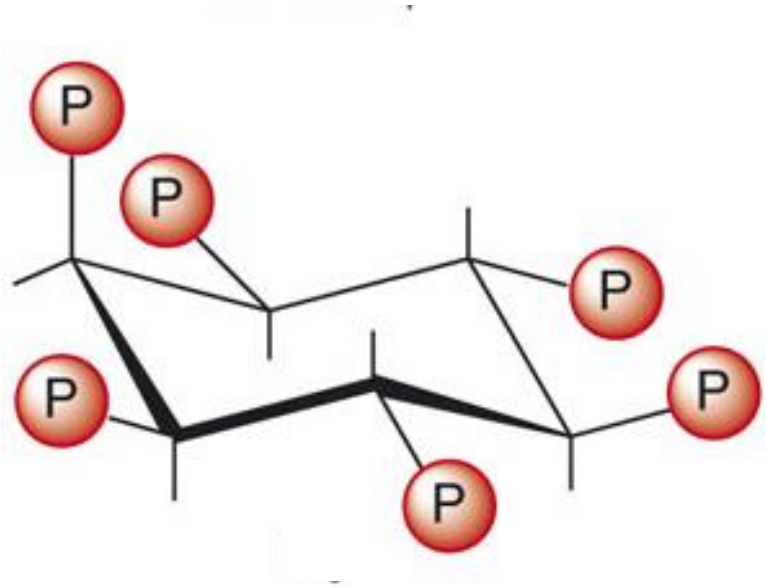
These studies included analysis of assembly by Gag protein in which the NC domain had been replaced by a leucine zipper (dimerizing) domain.

These studies imply that Gag decides to assemble when 2 or more Gag molecules are brought into close proximity at their C-termini.

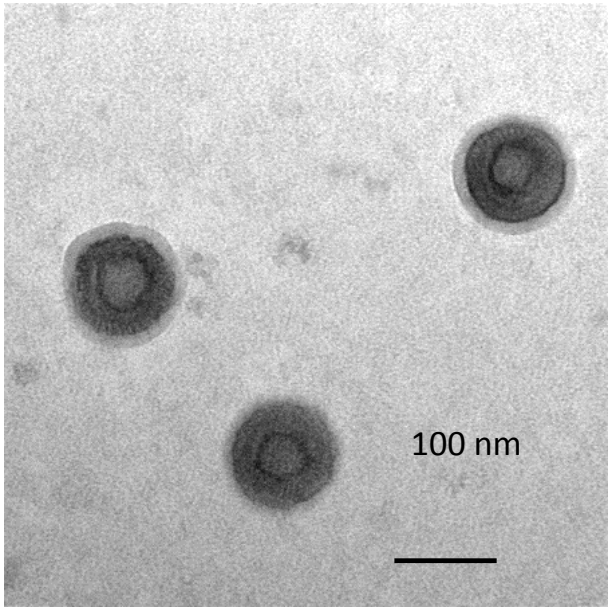
This juxtaposition induces a conformational change in SP1 (between CA and NC domains), which we suggest leads to further changes in the CA domain and exposure of new interfaces for Gag-Gag interaction leading to particle assembly.

An alternative cofactor for *In Vitro* assembly of VLPs

We have recently found that assembly can also be induced by adding IP6 to Gag *in vitro*.



Inositol hexakisphosphate (IP6)

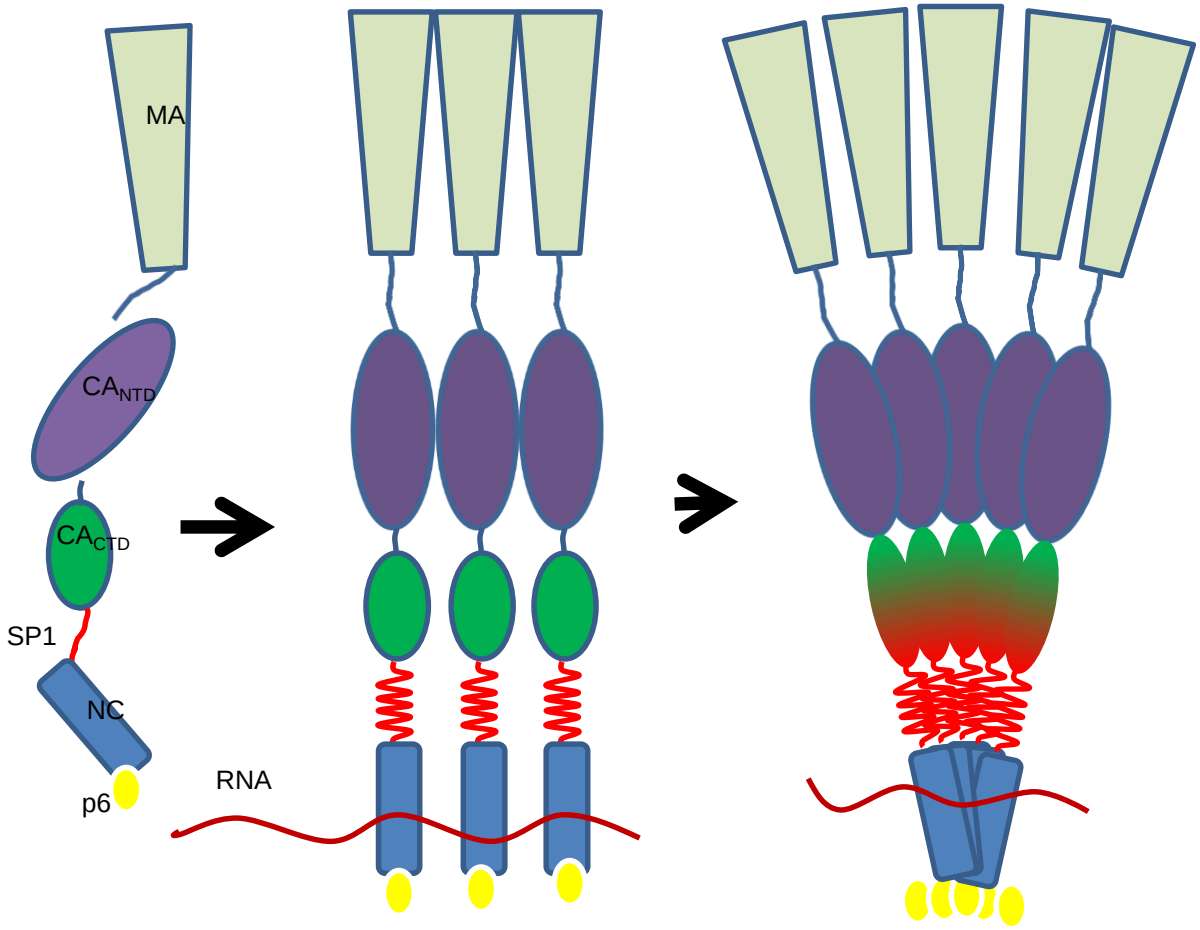


Thus we know 3 ways to induce Gag to assemble:

--add NA
--add IP6, another highly charged polyanion
--replace the NC domain with a dimerizing domain (the leucine zipper)

We believe that all of these agents are acting by bringing Gags together and flipping a switch within SP1.

Gag is Ready to Assemble when the SP1 Switch is Flipped



Datta et al., 2011, 2016

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The Problem:

RNA in Retrovirus Particles

When Gag is expressed in mammalian cells in the absence of vRNA, it still assembles efficiently.

The particles released from these cells contain normal amounts of RNA.

The RNA in these particles is cellular mRNA.

Muriaux et al., PNAS 2001; Rulli et al., JV 2007; Comas-Garcia et al., Viruses 2016

Genomic RNA is selectively packaged because it contains a "packaging signal", or "ψ"

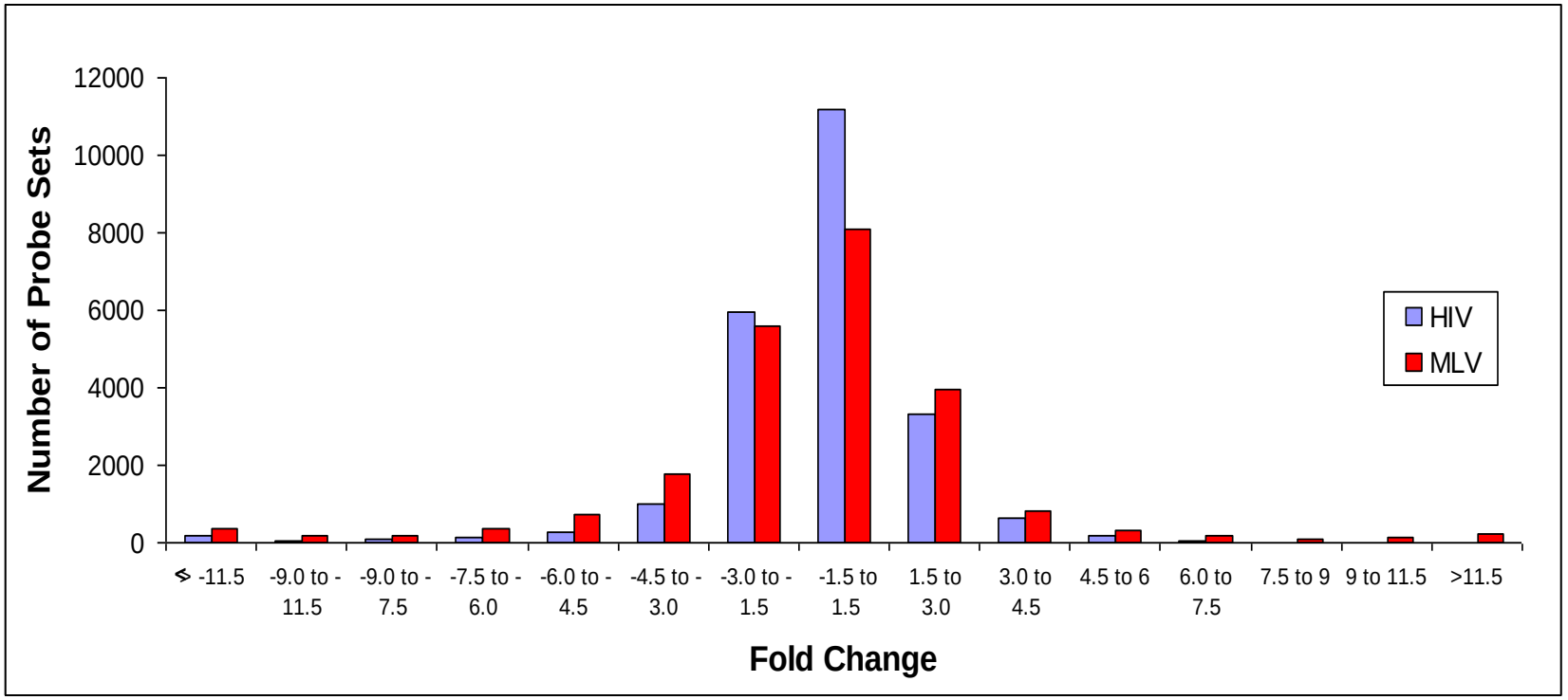
Packaging signal or Ψ

- **When ψ+ RNA is present in a virus-producing cell, it is selected for packaging with very high fidelity, although it is surrounded by a vast excess of cellular RNAs.**

- **In the absence of ψ+ RNA, particle assembly is still efficient, and cellular mRNAs are packaged in the place of gRNA.**

- **There is very little selectivity in the packaging of cellular mRNAs.**

Encapsidation of Cellular mRNAs

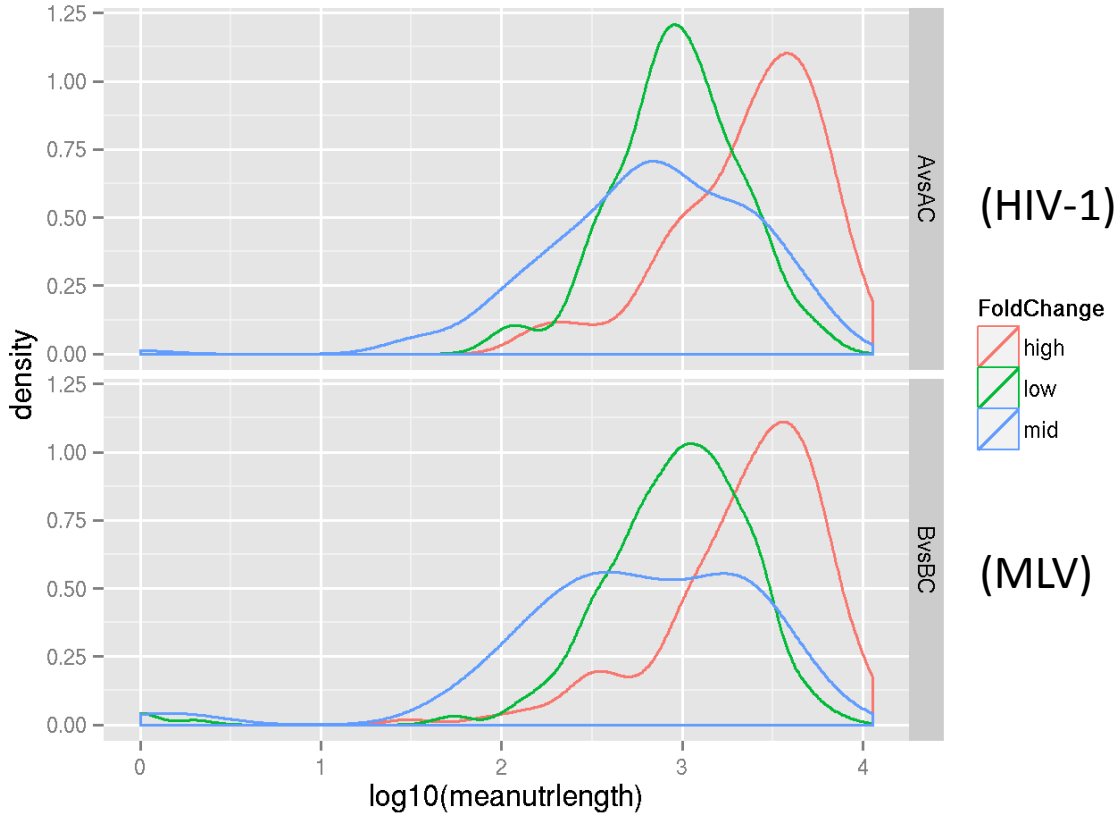


We found that the vast majority of mRNAs were packaged unselectively: that is, they were represented in the virions simply in proportion to their representation in the virus-producing cells.

Rulli et al., 2007

Encapsidation of Cellular mRNAs

Selectively packaged mRNAs tend to have long 3' UTRs.



(HIV-1)

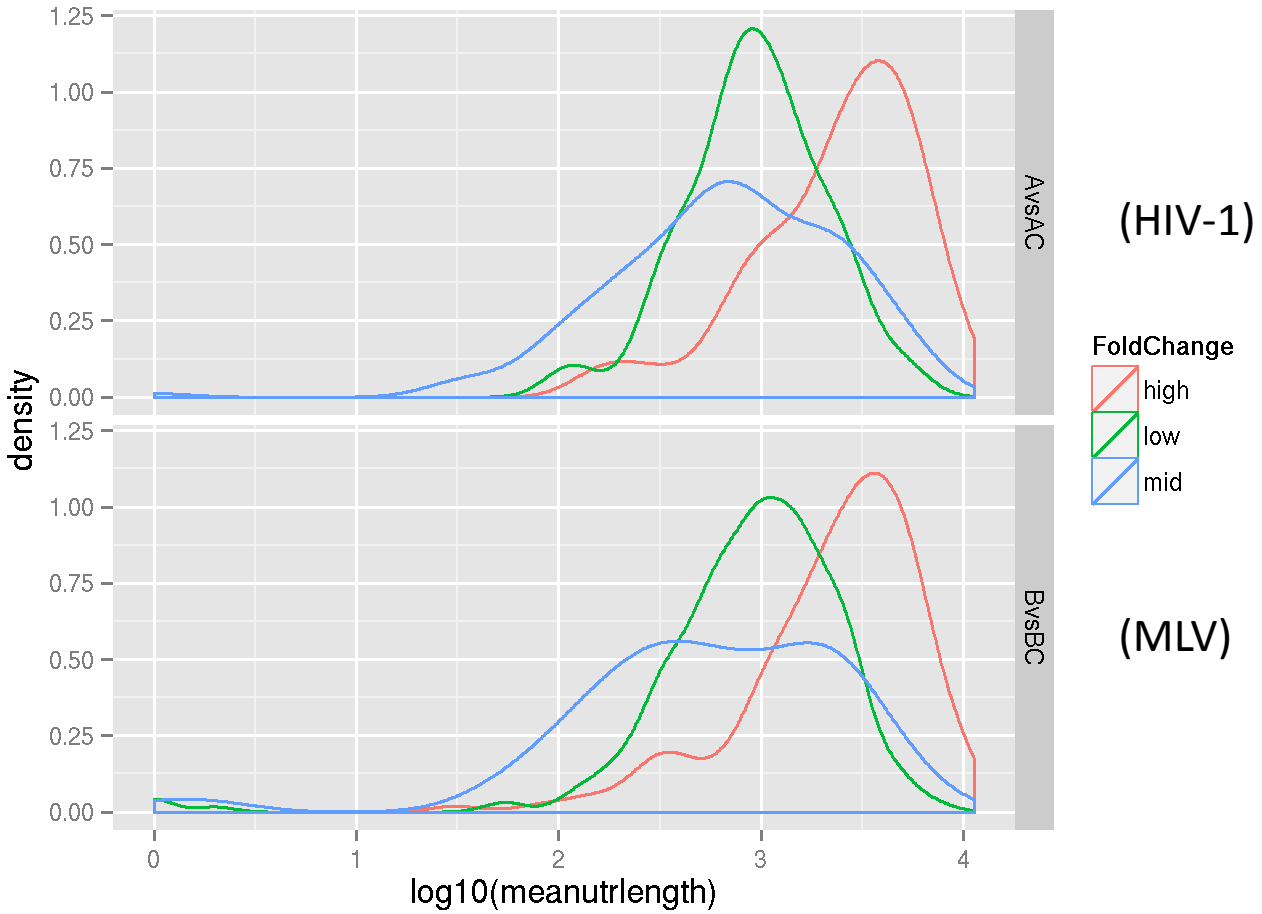
(MLV)

(1000 mRNAs with the highest, average, and lowest fold-changes were selected and their UTR lengths are plotted. P value for this correlation is ~ 10^{-16})

Comas-Garcia et al., *Viruses*, 2016

Encapsidation of Cellular mRNAs

Selectively packaged mRNAs tend to have long 3' UTRs. Presumably a long 3' UTR is a stretch of naked RNA, not occupied by ribosomes, to which Gag can bind.



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How is Genomic RNA Selected for Encapsidation?

Thus, vRNA is in competition with a very large excess of cellular mRNA for incorporation into the assembling virion. Ψ confers an advantage in this competition.

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How does this work? What is the nature of the advantage conferred by ψ in the competition?

How is Genomic RNA Selected for Encapsidation?

We have measured the binding affinity of Gag for ψ-containing and control RNAs.

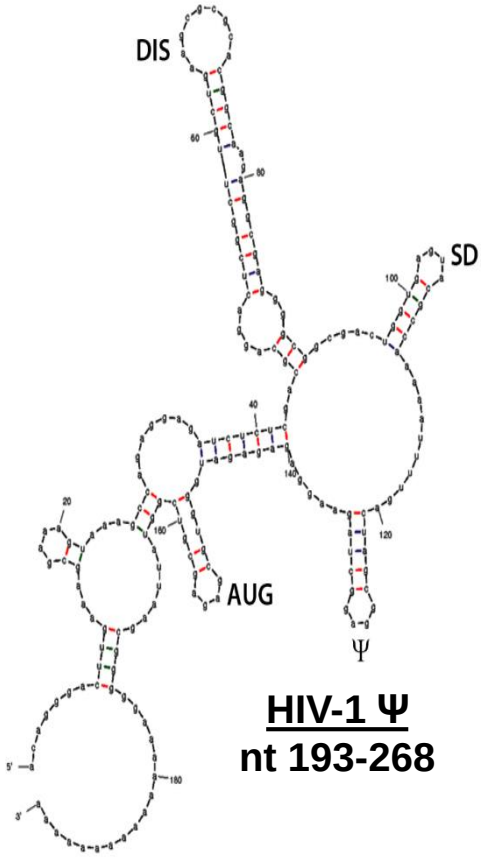
This is not trivial: it must be done under conditions where the Gag-RNA complexes do not assemble into virus-like particles.

We have used a fluorescence correlation spectroscopy setup for these measurements, although the readout was not D but quenching of the Cy5 fluorophore at the 3' end of the RNA.

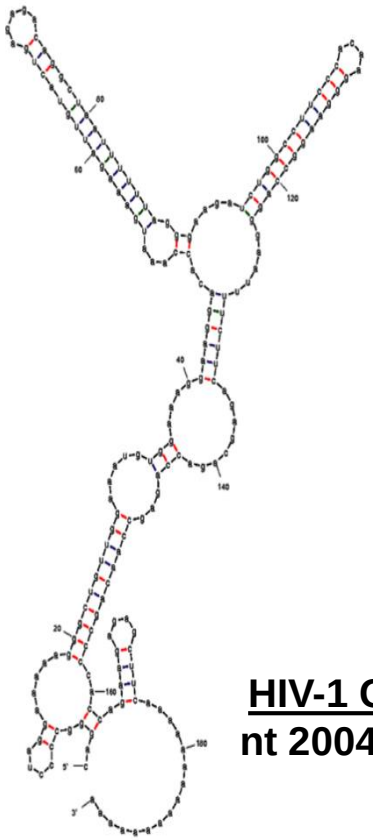
Comas-Garcia et al., *eLife*, in press

RNAs Analyzed

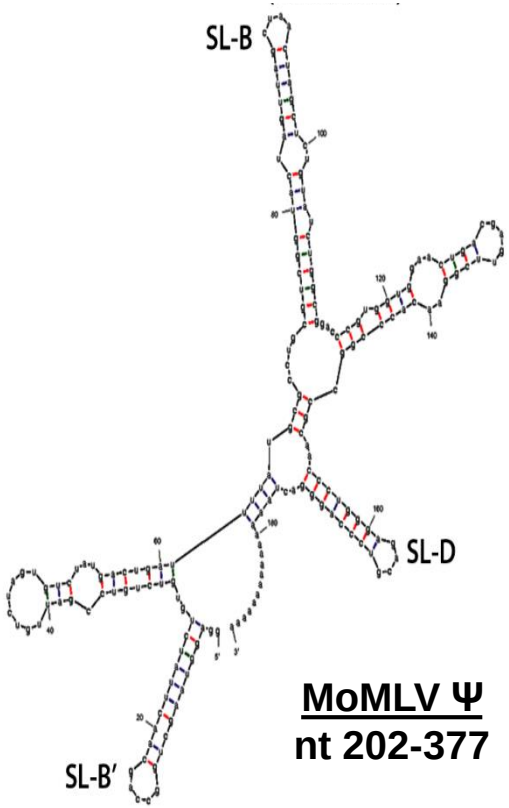
RNAs of 175 nts: HIV Ψ (monomeric & dimeric), HIV "GRPE", and MoMLV Ψ; all with Cy5 at 3' end



HIV-1 Ψ
nt 193-268

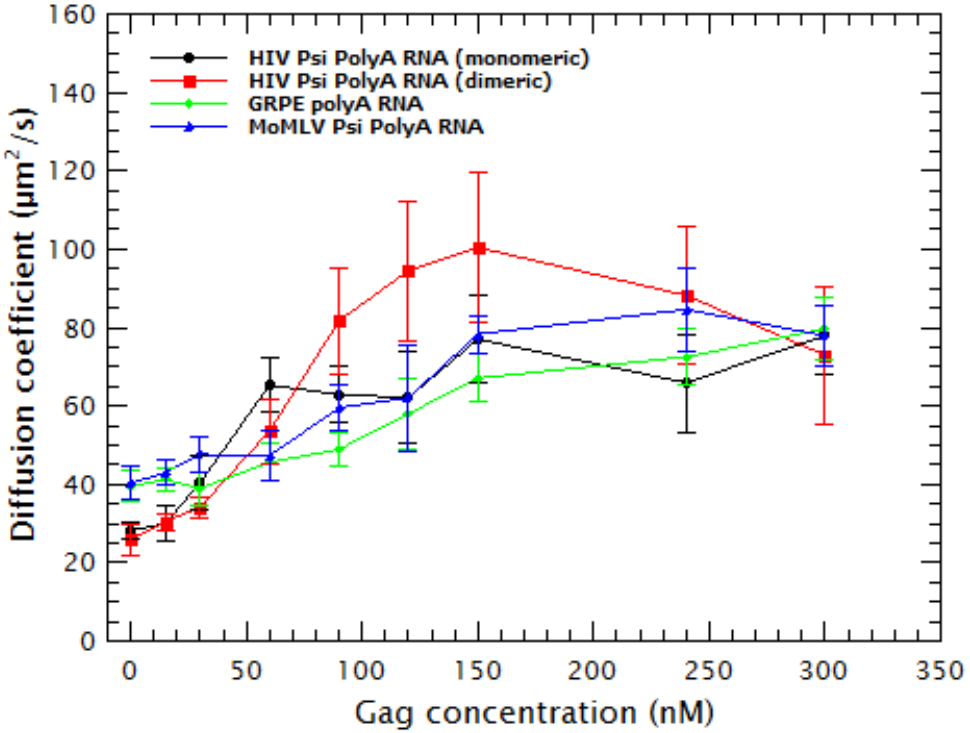


HIV-1 GRPE
nt 2004-2179



MoMLV Ψ
nt 202-377

Binding of Gag to RNA Collapses the RNA

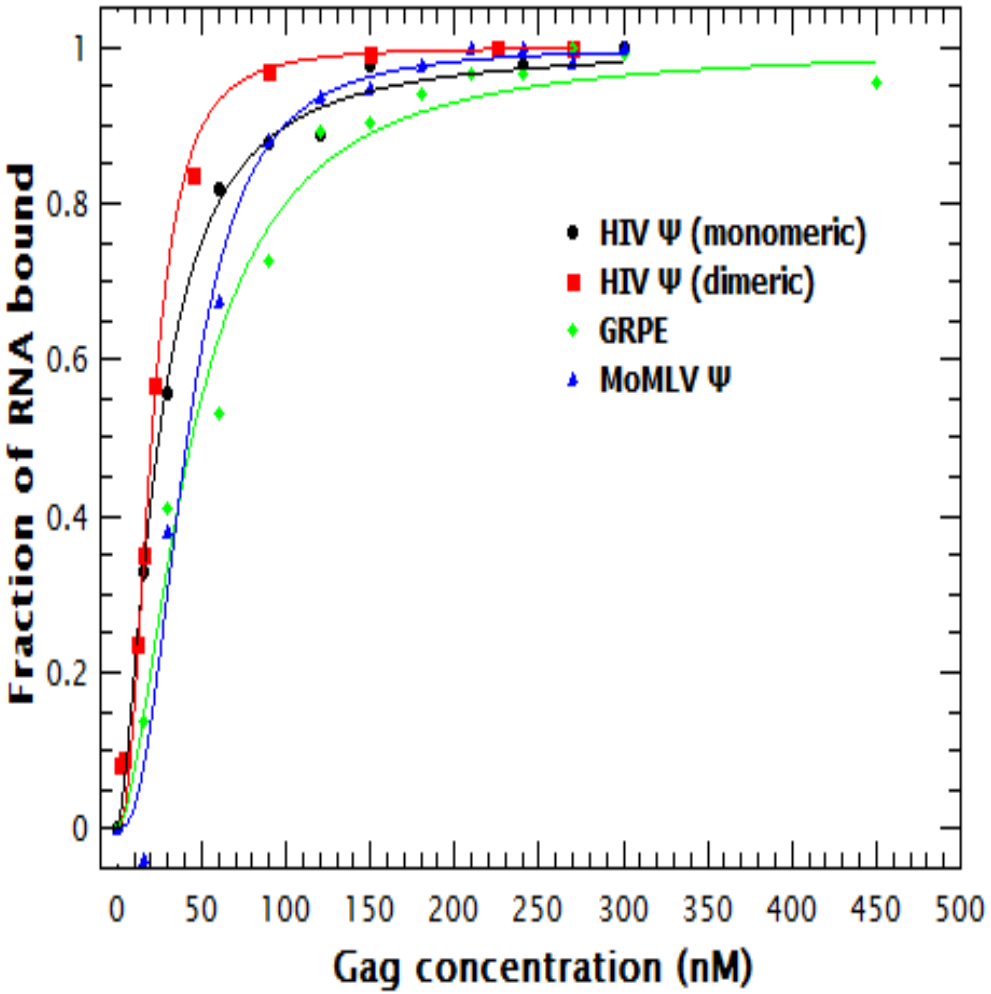


RNAs at 15 nM

0.2M NaCl
5 mM $MgCl_2$
20 mM Tris pH 7.5
1 μM $ZnCl_2$
1 mM βME

- **Binding of Gag to RNA condenses the RNA, increasing its rate of diffusion**
- **This has been seen before with capsid proteins of other viruses**

Binding of Gag to Ψ and GRPE RNAs is Almost Indistinguishable

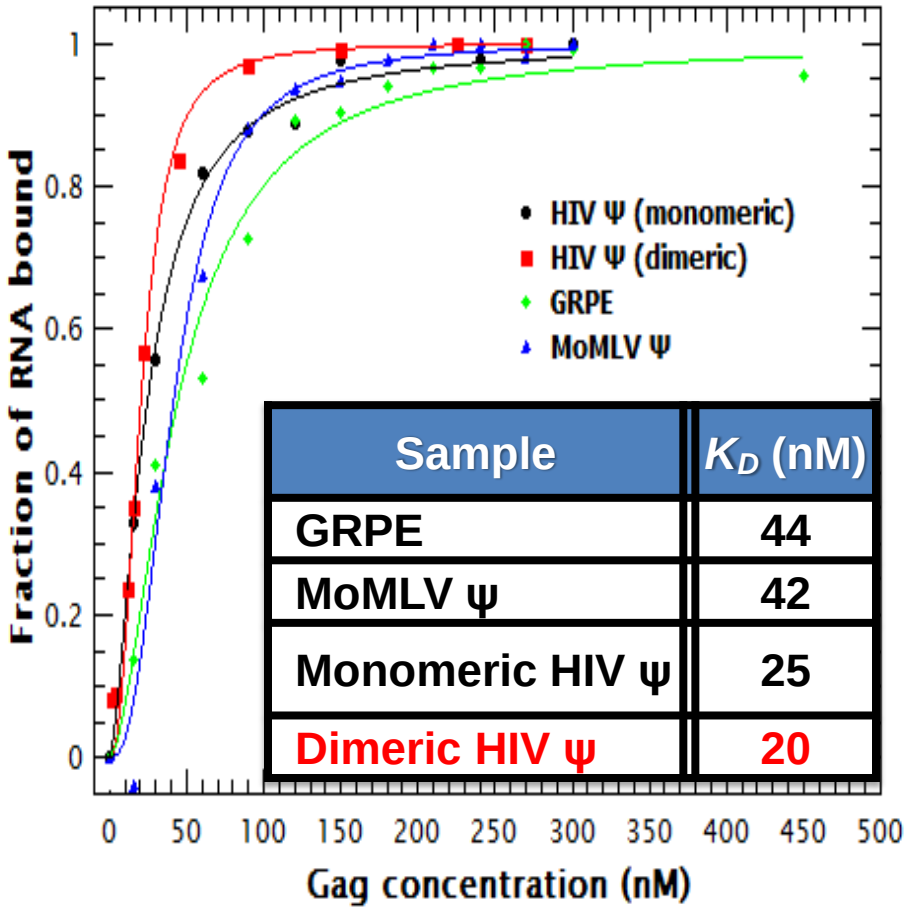


RNAs at 15 nM

0.2M NaCl
5 mM $MgCl_2$
20 mM Tris pH 7.5
1 µM $ZnCl_2$
1 mM βME

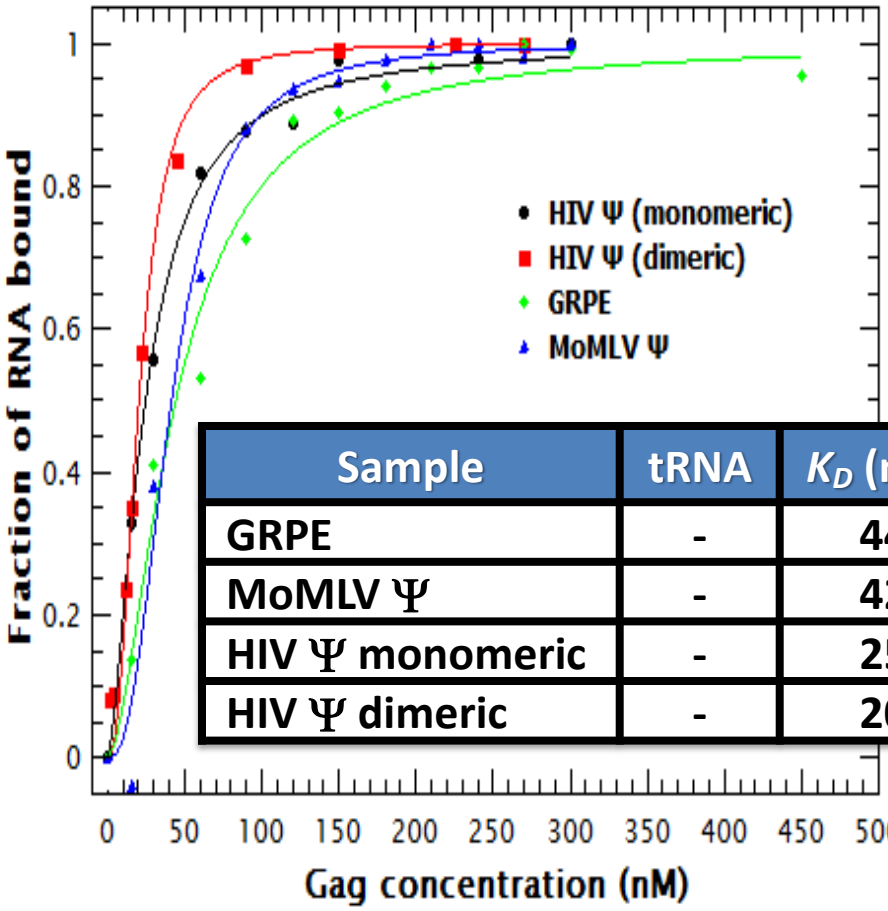
Binding determined by quenching of the Cy5 on the 3' end of the RNA

…And the Difference in Affinities Is Certainly Not Enough to Explain Selective Packaging



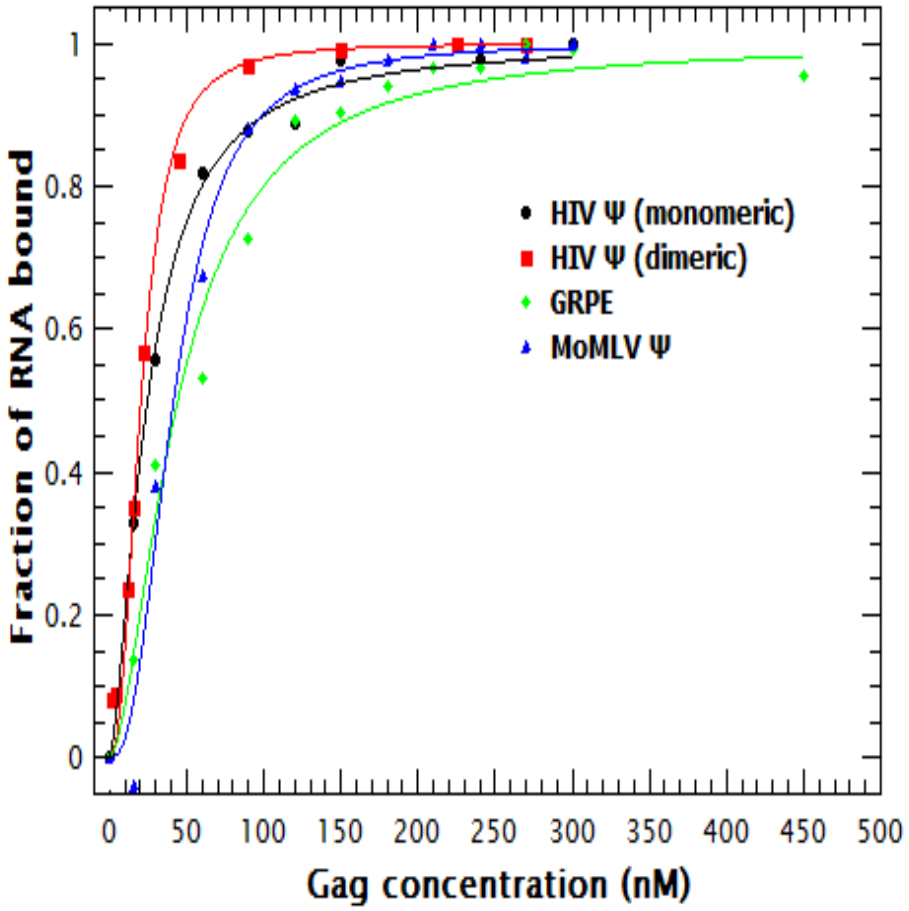
Sample	K_D (nM)
GRPE	44
MoMLV ψ	42
Monomeric HIV ψ	25
Dimeric HIV ψ	20

Binding of Gag to RNAs is Cooperative. This Would Probably Be Expected but Has Never Been Documented.



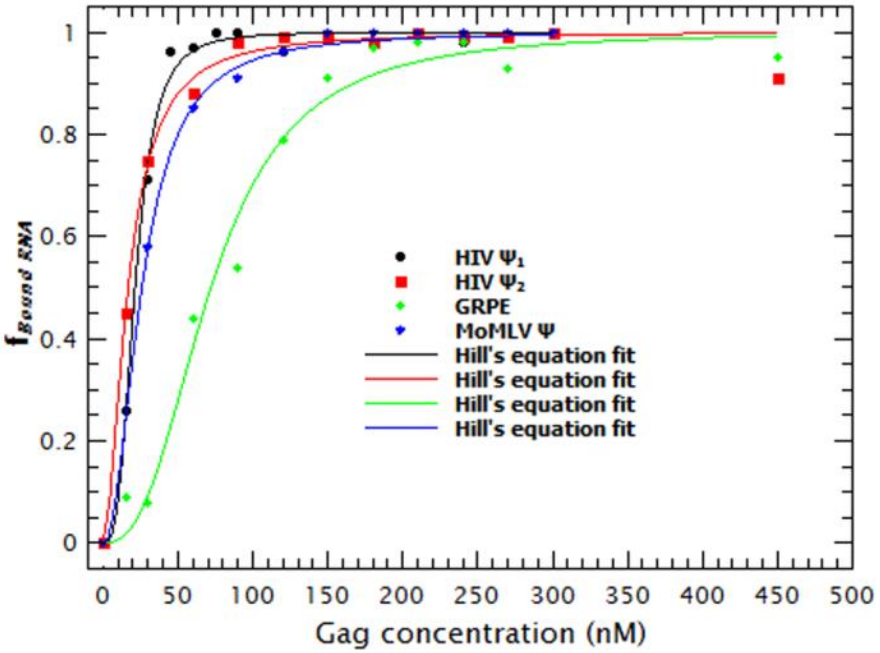
Sample	tRNA	K_D (nM)	n_H
GRPE	-	44	1.7
MoMLV Ψ	-	42	2.5
HIV Ψ monomeric	-	25	1.6
HIV Ψ dimeric	-	20	2.0

But Binding To ψ and GRPE Are Not As Similar As They Appear

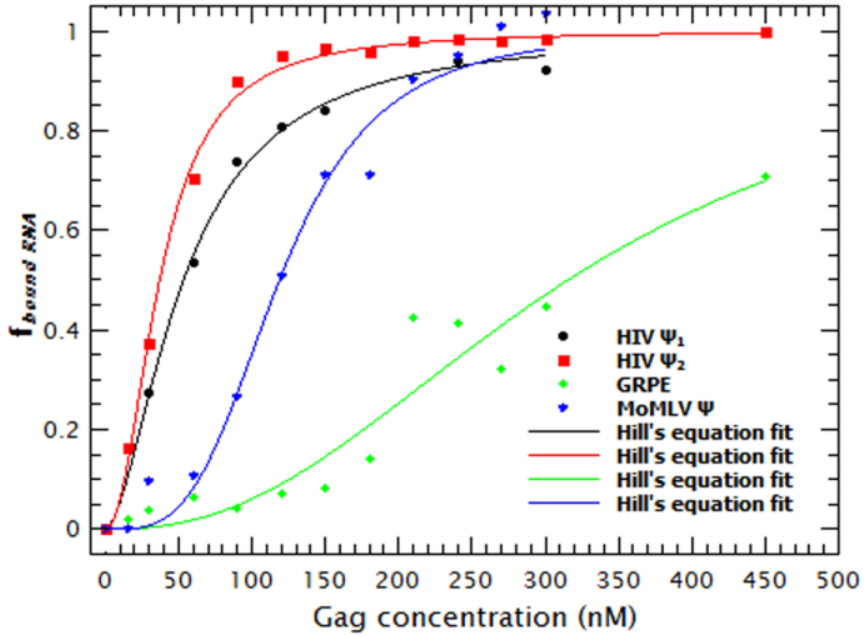


Addition of a competitor RNA reveals **binding specificity**…

no tRNA



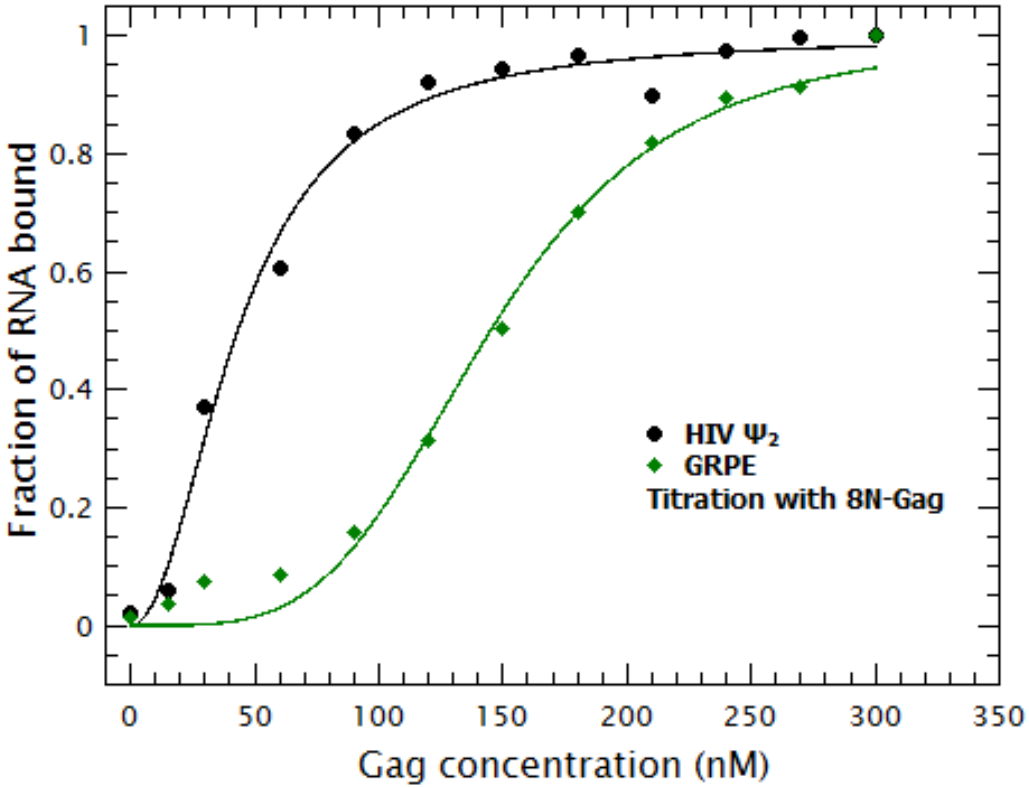
+ 50X excess tRNA



Sample	tRNA	K_D (nM)	n_H
GRPE	-	44	1.7
MoMLV Ψ	-	42	2.5
HIV Ψ monomeric	-	25	1.6
HIV Ψ dimeric	-	20	2.0

Sample	tRNA	K_D (nM)	n_H
GRPE	+	≈ 315	2.4
MoMLV Ψ	+	119	3.5
HIV Ψ monomeric	+	53	1.7
HIV Ψ dimeric	+	37	2.1

Use of “8N” Gag, with Reduced Positive Charges in the MA Domain, also Reveals Binding Specificity



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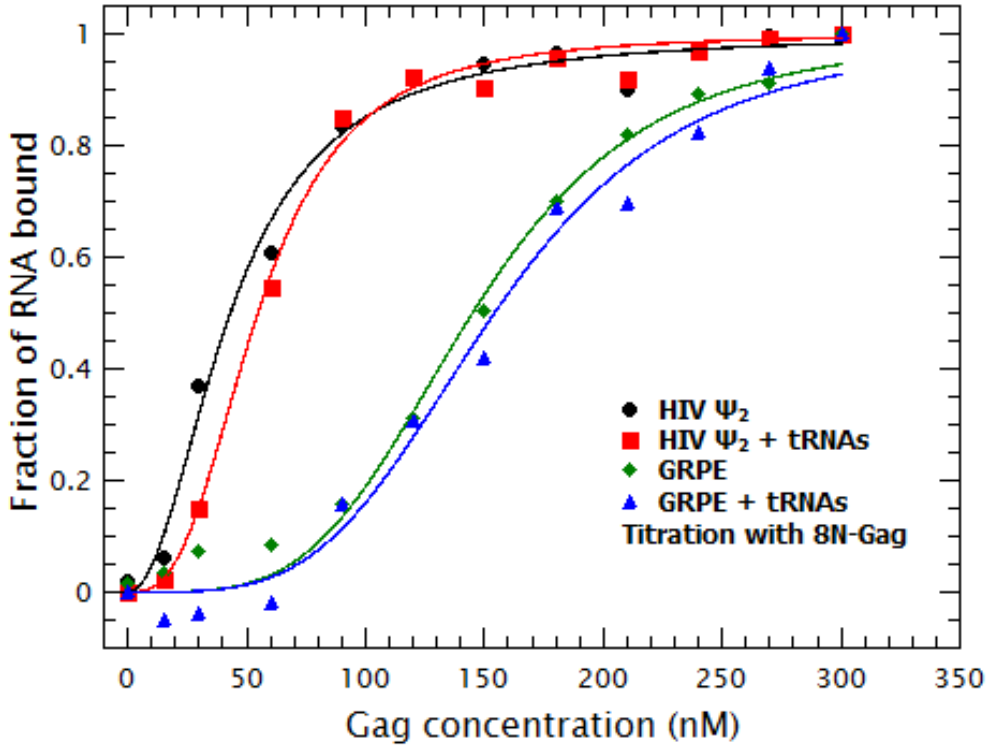
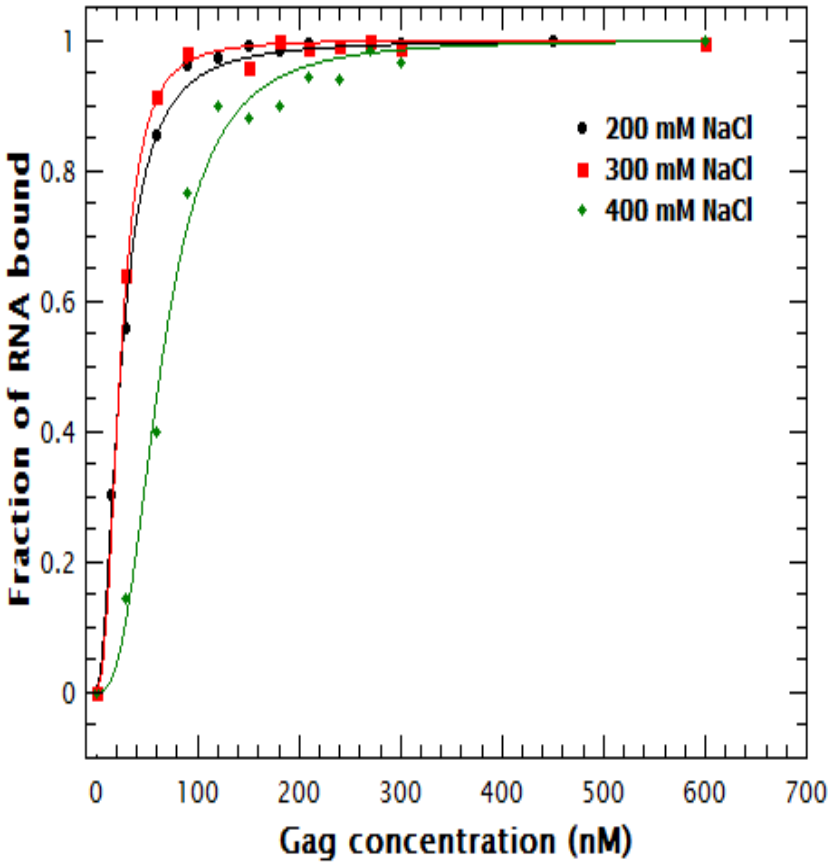


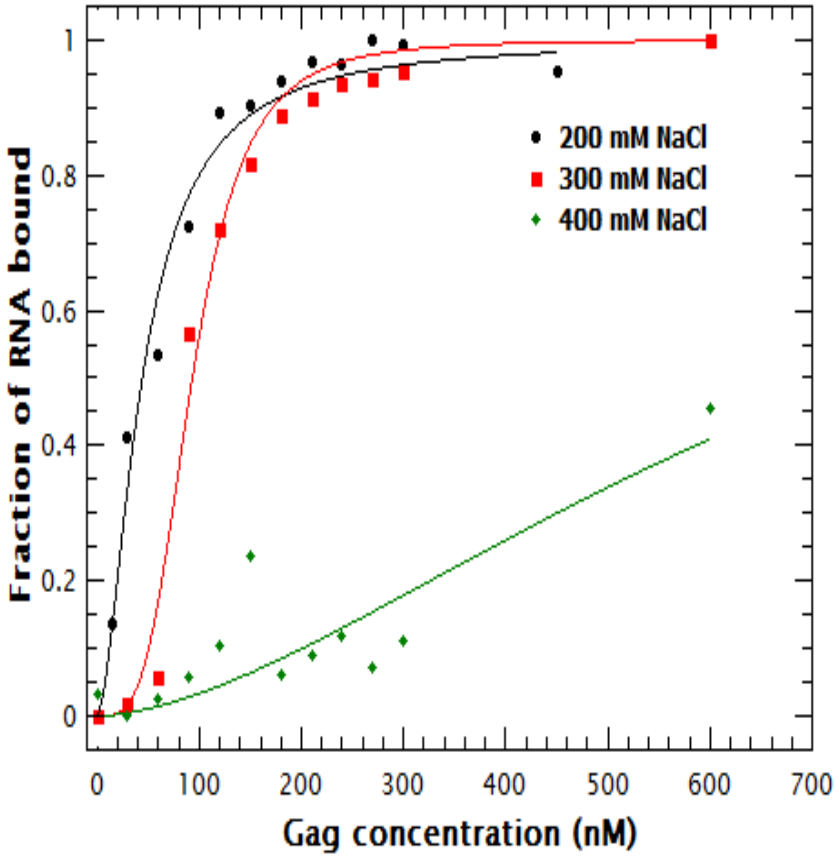
…in fact addition of tRNA has no effect on apparent K_d's of 8N Gag. This suggests the binding of tRNA can all be attributed to the MA domain.

Binding to ψ is Far More Salt-Resistant Than Binding to GRPE

HIV Ψ_2 (dimeric)



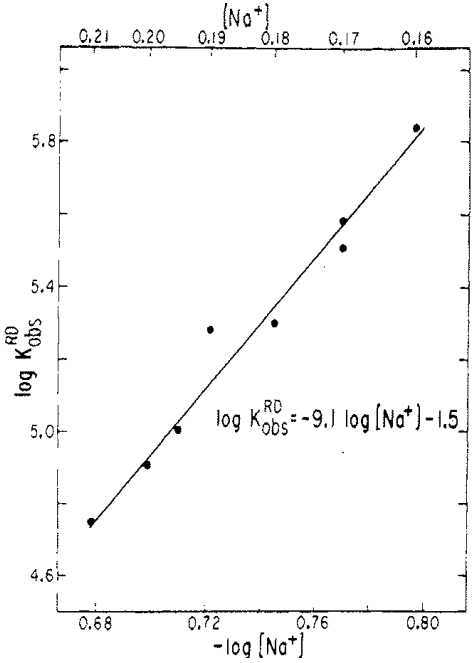
GRPE



(as originally found using fluorescence anisotropy by Webb et al., RNA, 2013)

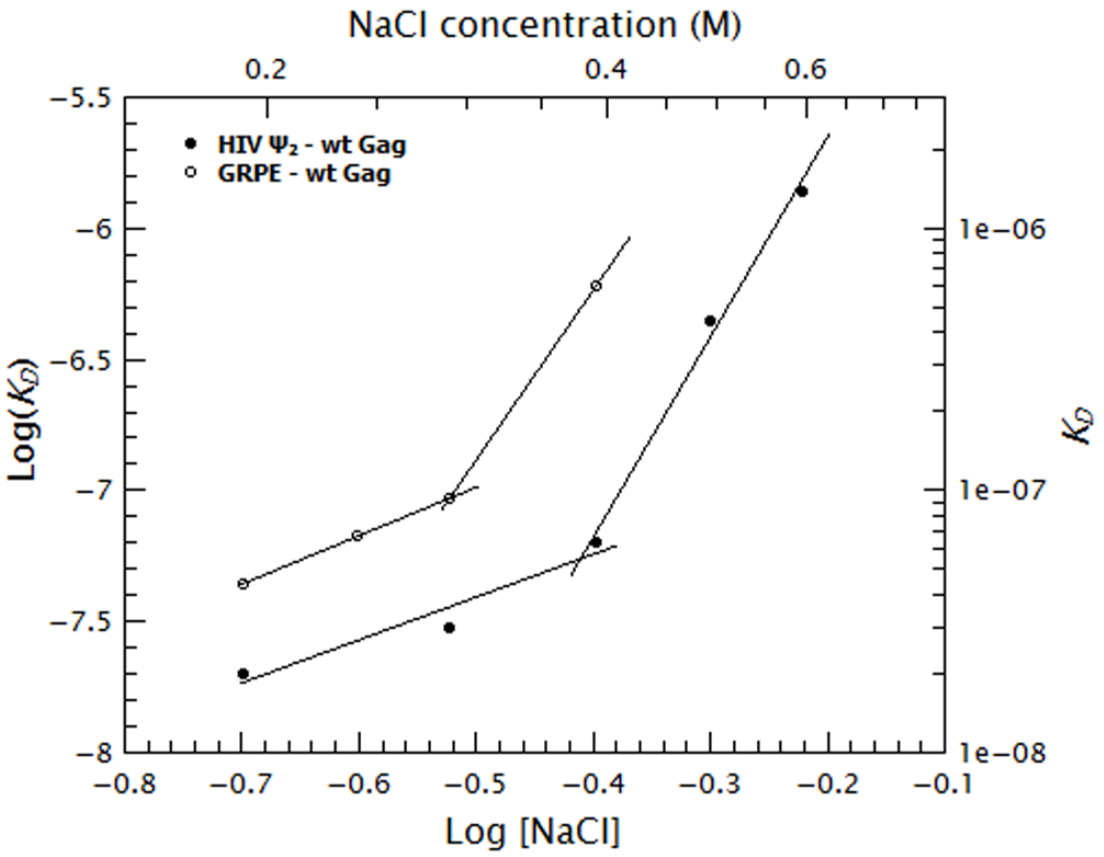
Obviously, the electrostatic interaction between proteins and nucleic acids is attenuated by increasing the ionic strength.

As shown in classic work from Record & Lohman, a plot of log[K_d] *vs.* log[Na^+] is a straight line; the slope of the line represents the number of Na^+ ions displaced by binding of one protein molecule to the nucleic acid.



deHaseth, Lohman, Record, 1977

But in the Case of Binding of Gag to RNA, We do Not Get a Straight Line



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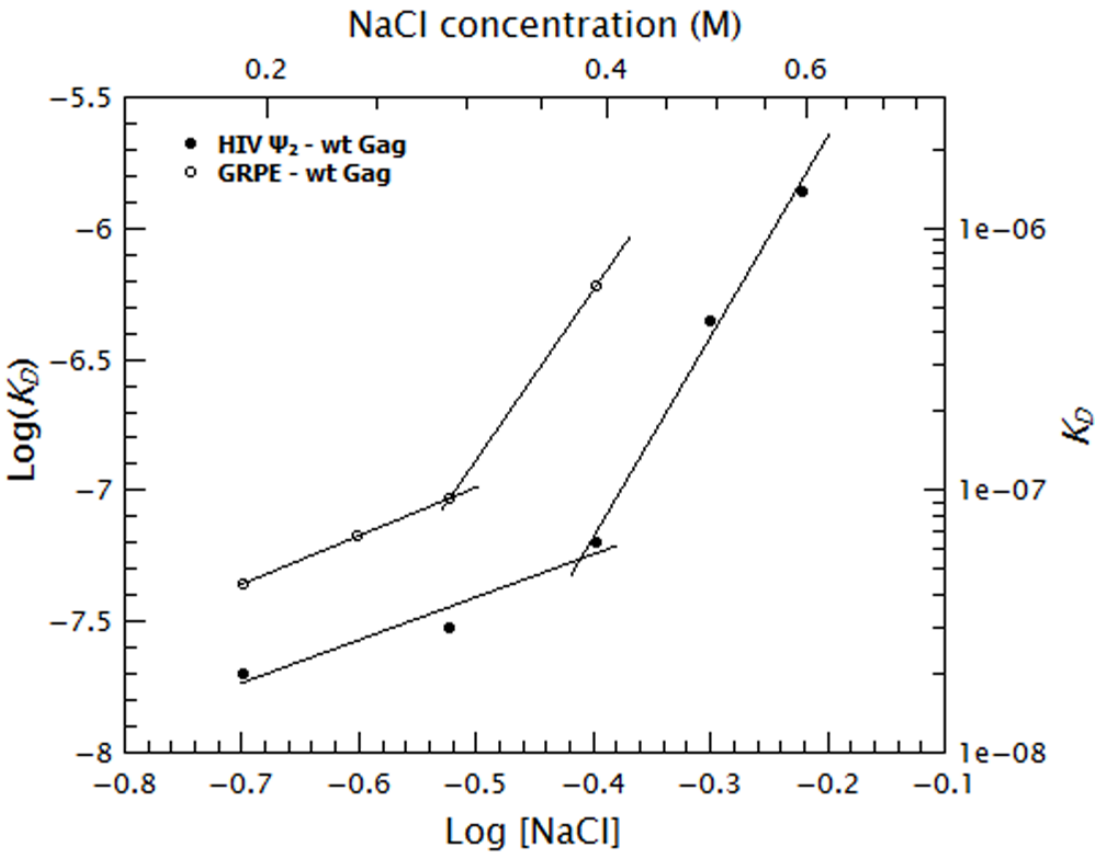

It is particularly surprising that the curve is concave, not convex

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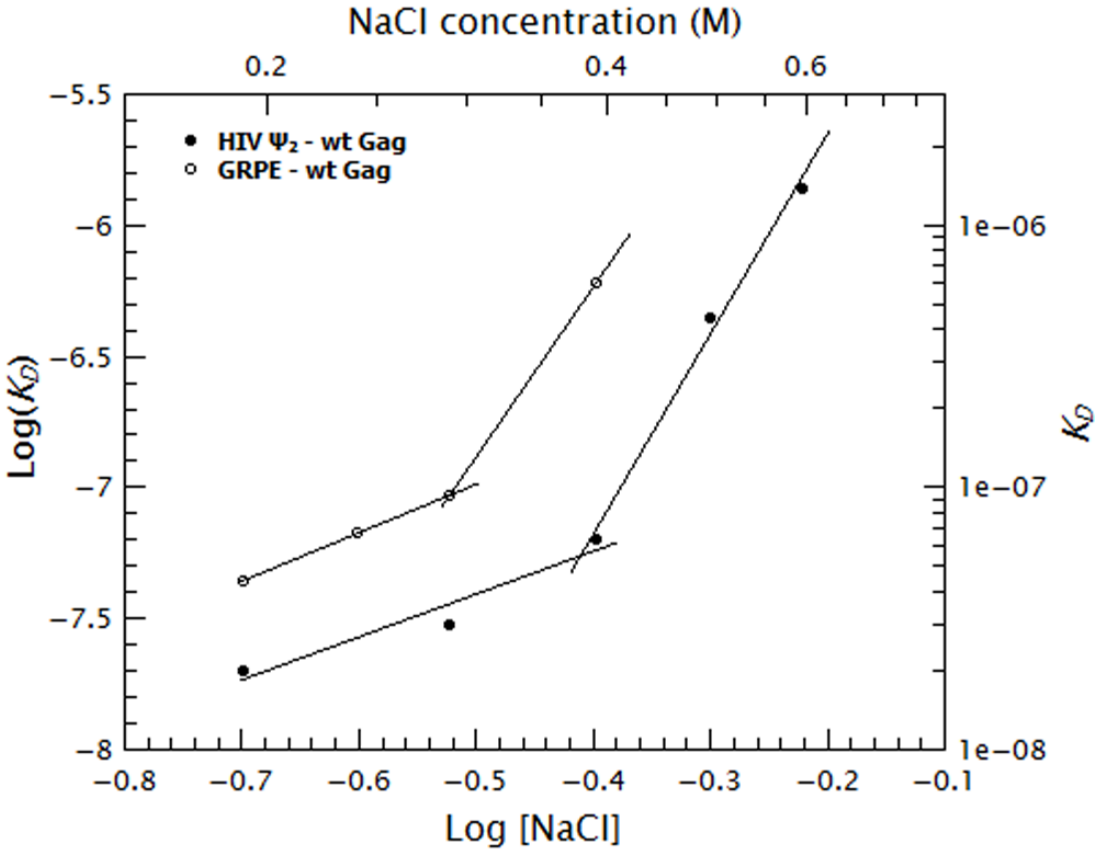
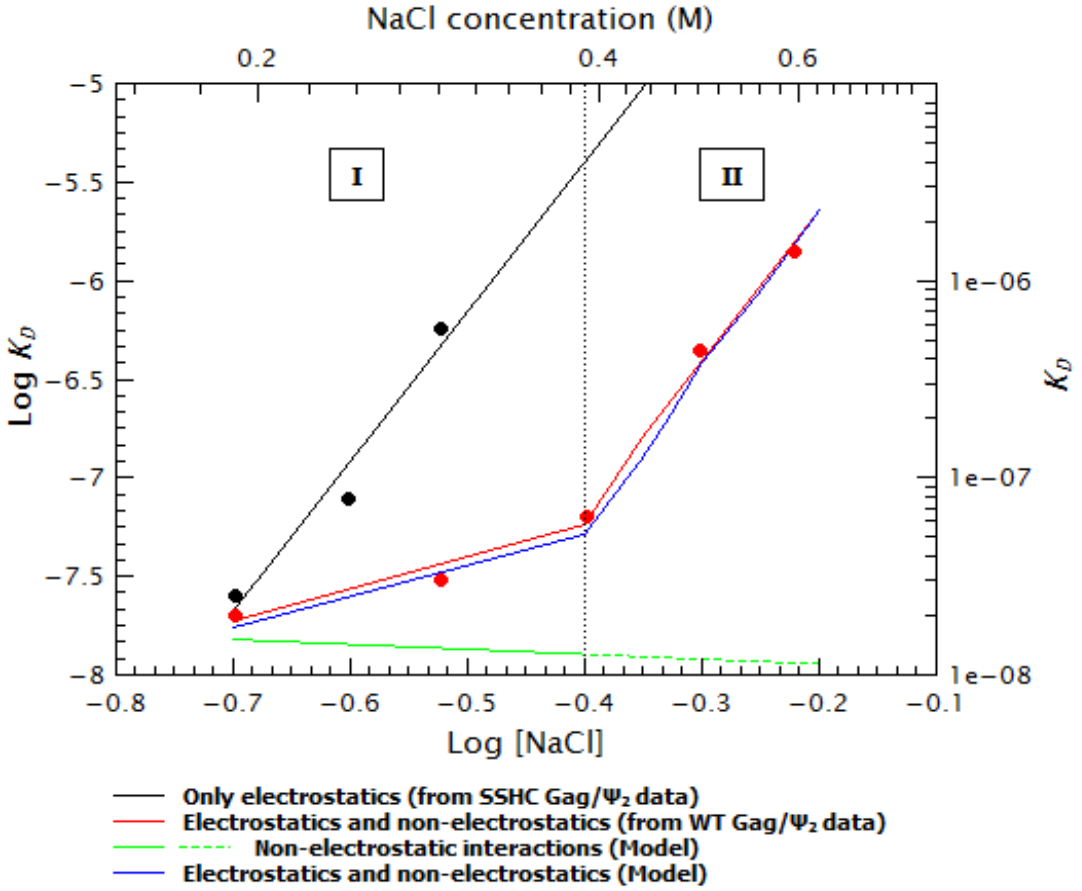


This result is quite surprising. It suggests that changing the salt concentration changes the RNA-binding properties of Gag.

Mauricio has Shown that this Concave Curve Can Be Modeled from the Data, with the Assumption that Non-Electrostatic Interactions Gradually Decline as the Salt is Increased



Gag is in monomer-dimer equilibrium in solution.

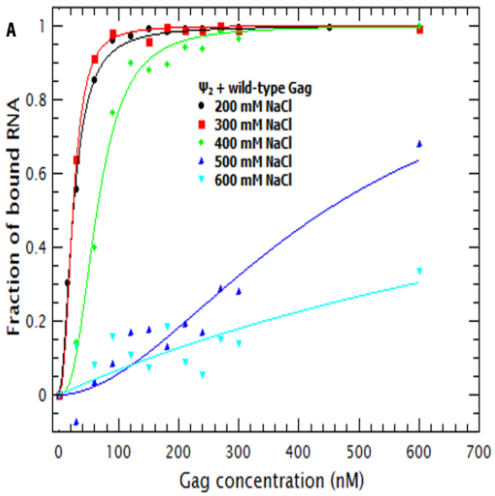
The dimer interface is within the CA domain; "WM" is a point mutant at that interface that is defective in dimerization.

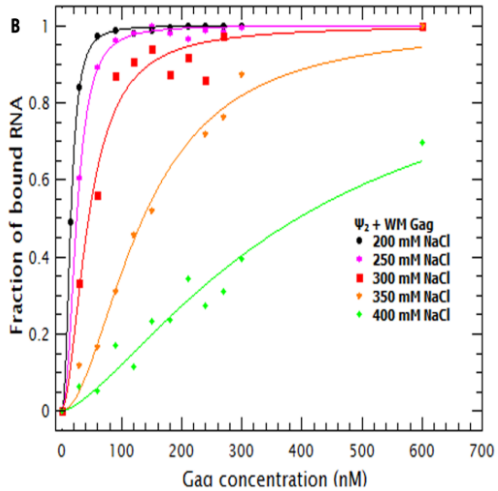
Remarkably, this Mutant has Mostly Lost its Ability to Bind the GRPE Control RNA

WT Gag

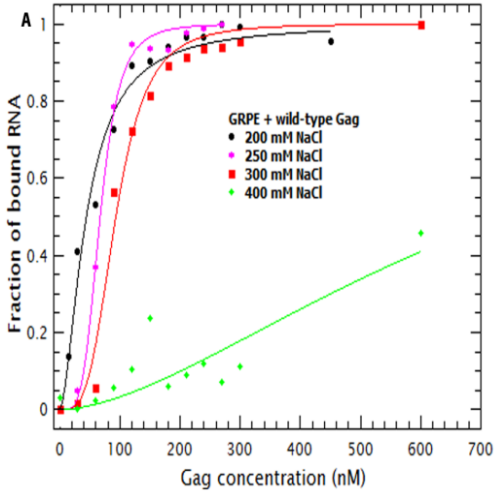
WM Mutant Gag

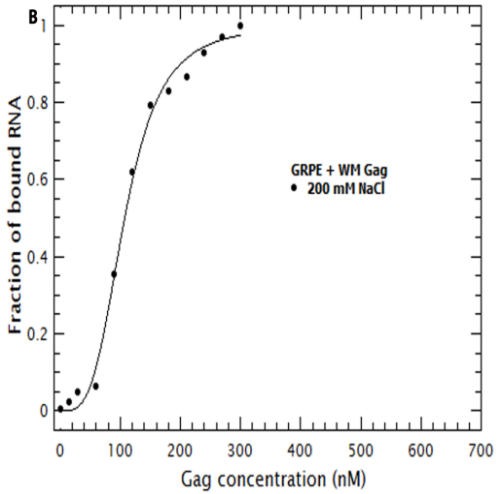
ψ



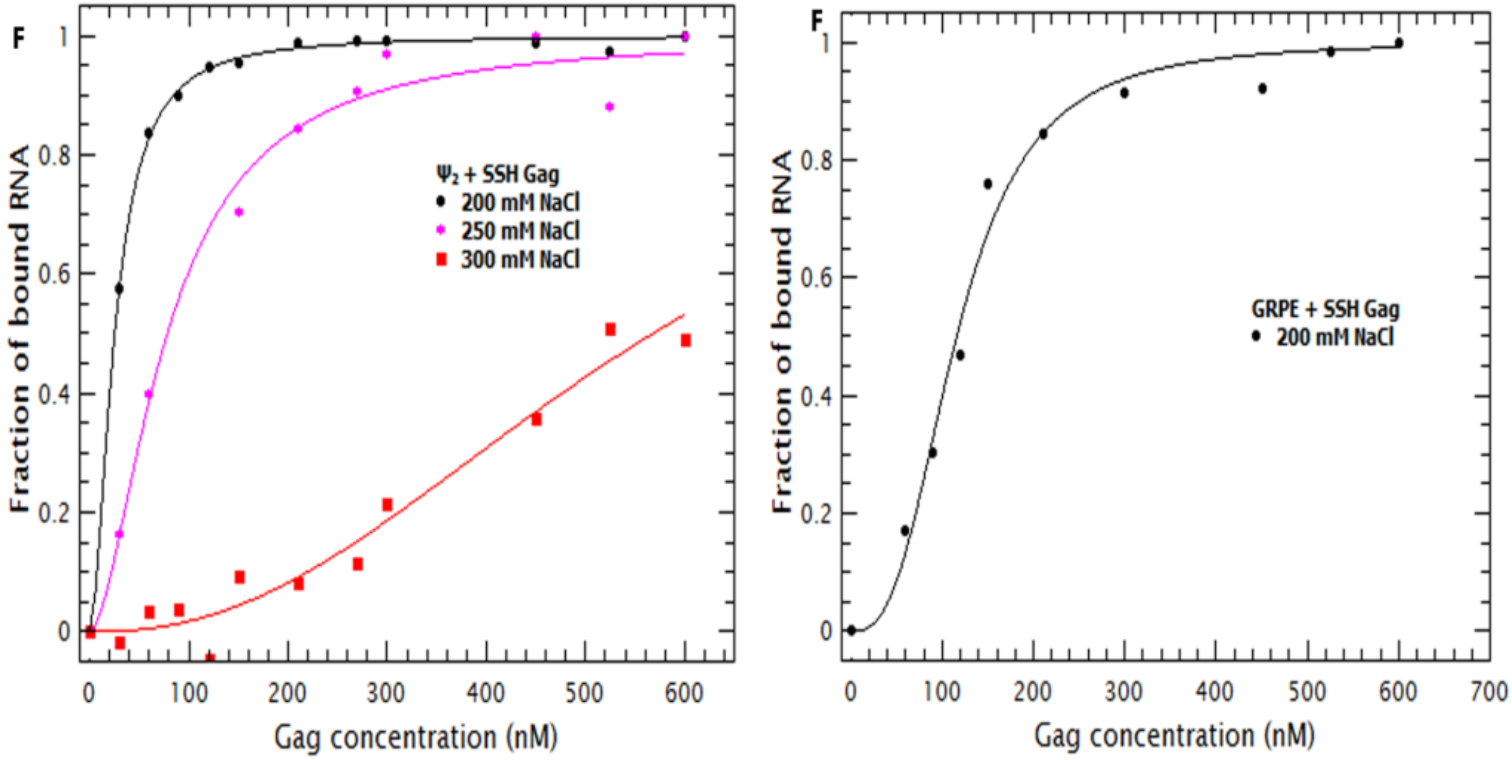


GRPE



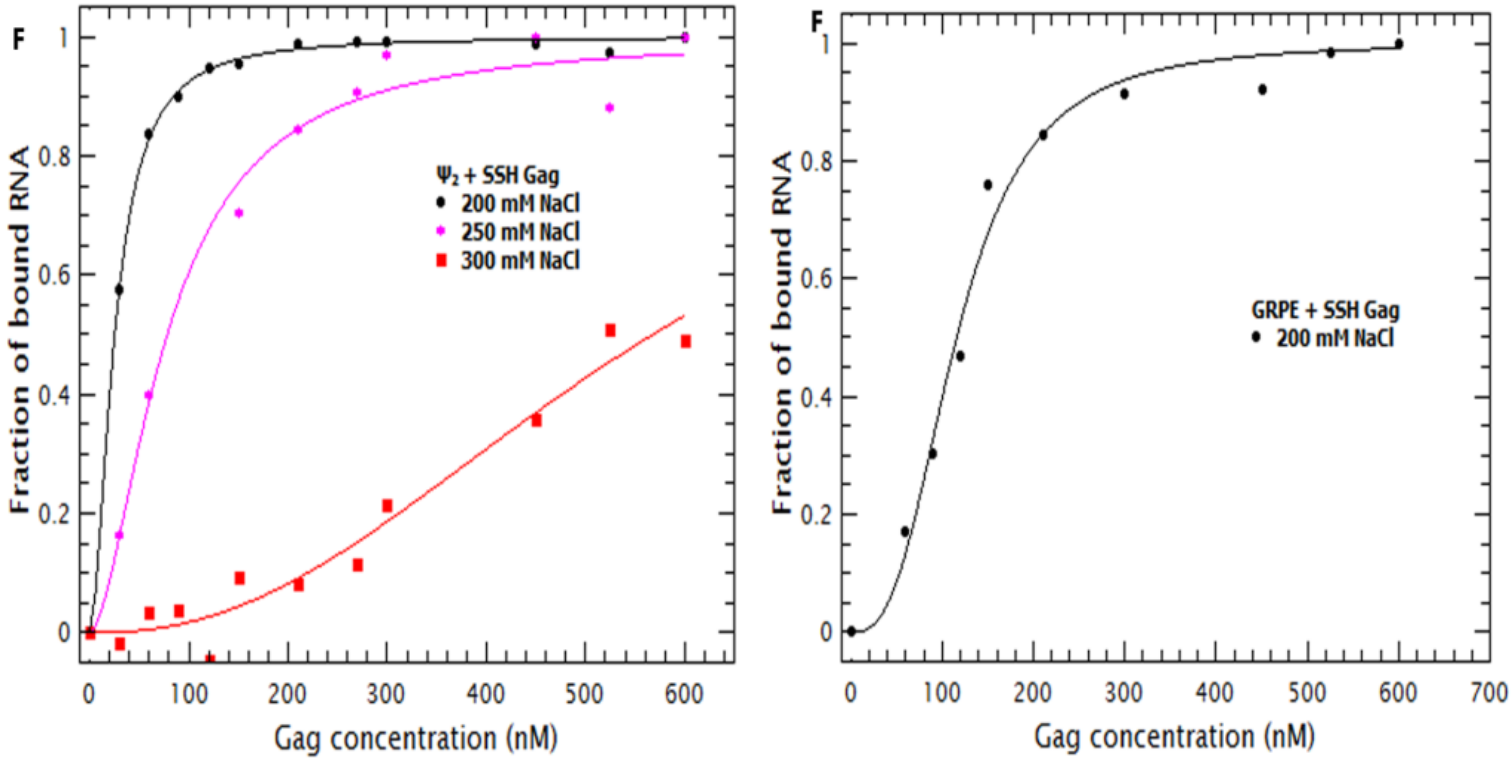


The Specific, Salt-Resistant (ie, Non-electrostatic) Binding Of Gag to ψ Can Be Almost Entirely Attributed to the Zinc Fingers in NC



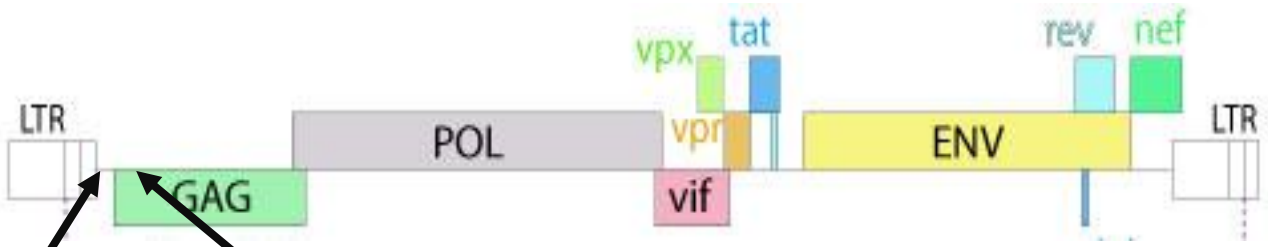
("SSH" Gag has the zinc-chelating cysteines in NC replaced with serines and thus no longer has the zinc fingers.)

The Specific, Salt-Resistant (ie, Non-electrostatic) Binding Of Gag to ψ Can Be Almost Entirely Attributed to the Zinc Fingers in NC*



(* *in vivo*, zinc finger mutants assemble OK but fail to package vRNA)

What is ψ??



~9.7 kb

Ψ is located somewhere at the 5'-UTR and beginning of the Gag ORF.

~0.6 kb

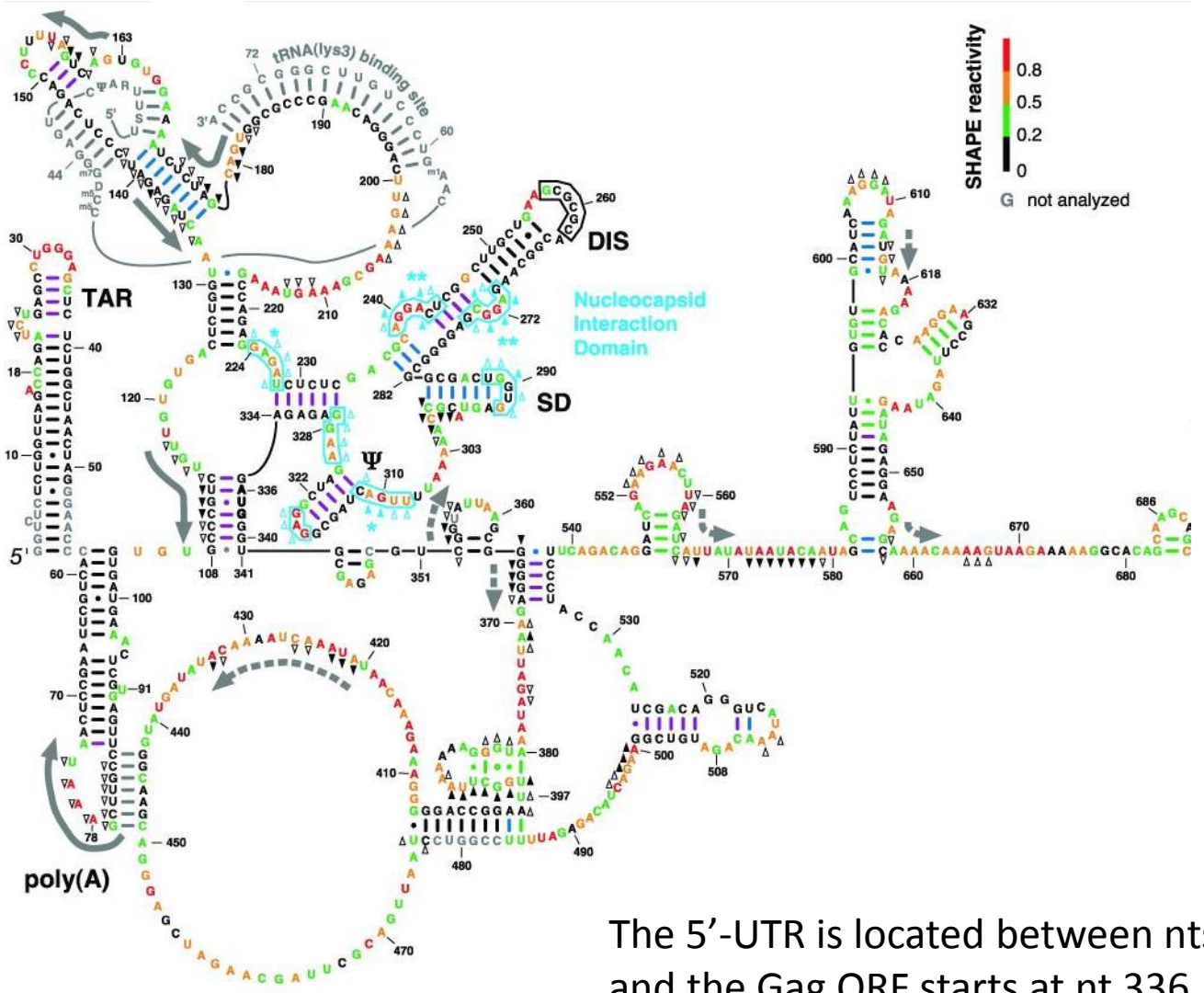
The 5’-UTR is a complicated case

- The 5’-UTR is highly structured and regulates:

1. Transcription.
2. Translation.
3. Splicing.
4. RNA packaging.
5. Binding of the tRNA that primes reverse transcription.

- Therefore, studying how Ψ controls selective packaging of the gRNA during a viral infection is extremely complicated.

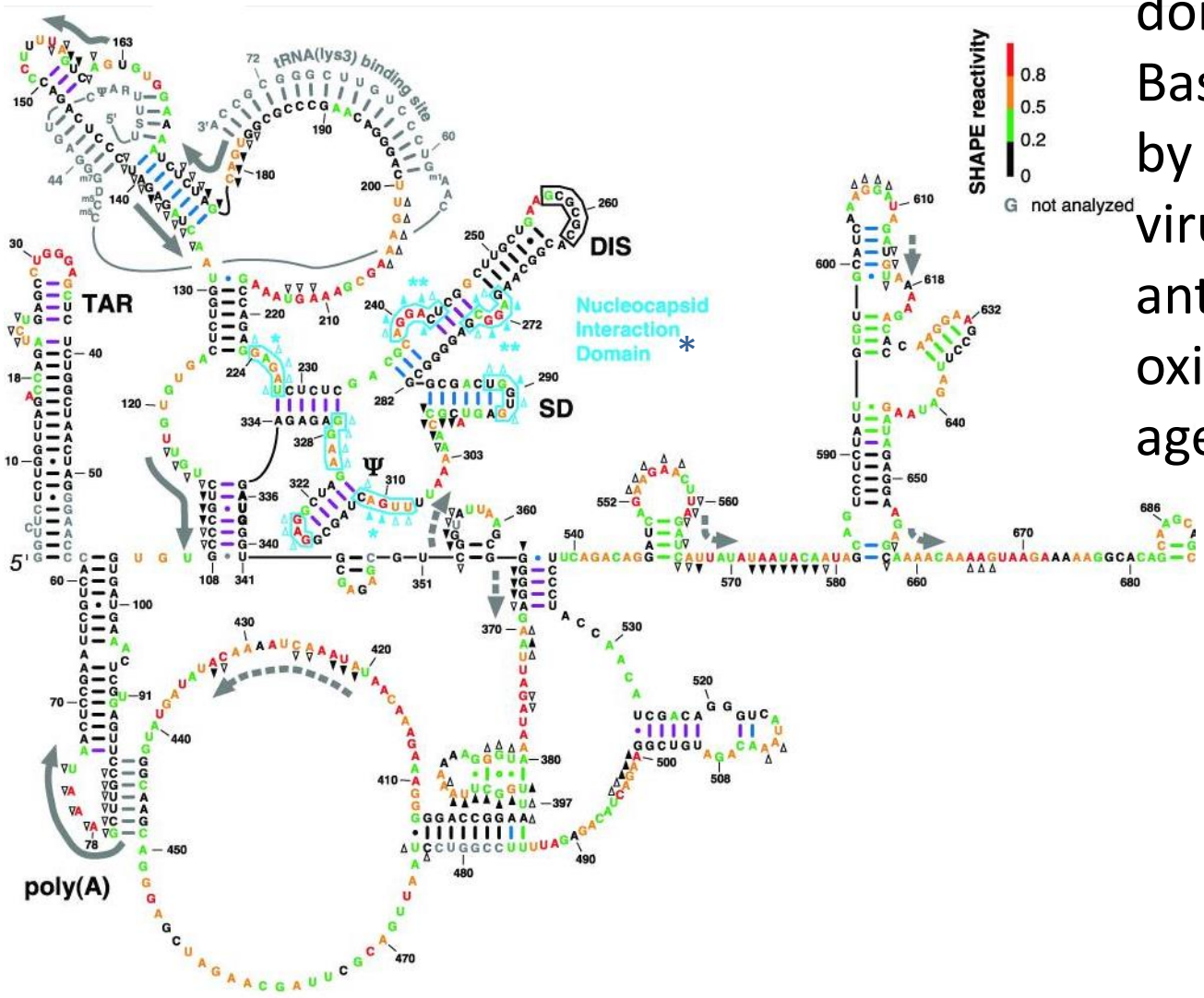
Secondary Structure of 5’ End of vRNA



The 5’-UTR is located between nts 1 and 335 and the Gag ORF starts at nt 336

- Wilkinson, K.A. Plos Biol 2008; 6(6):e96

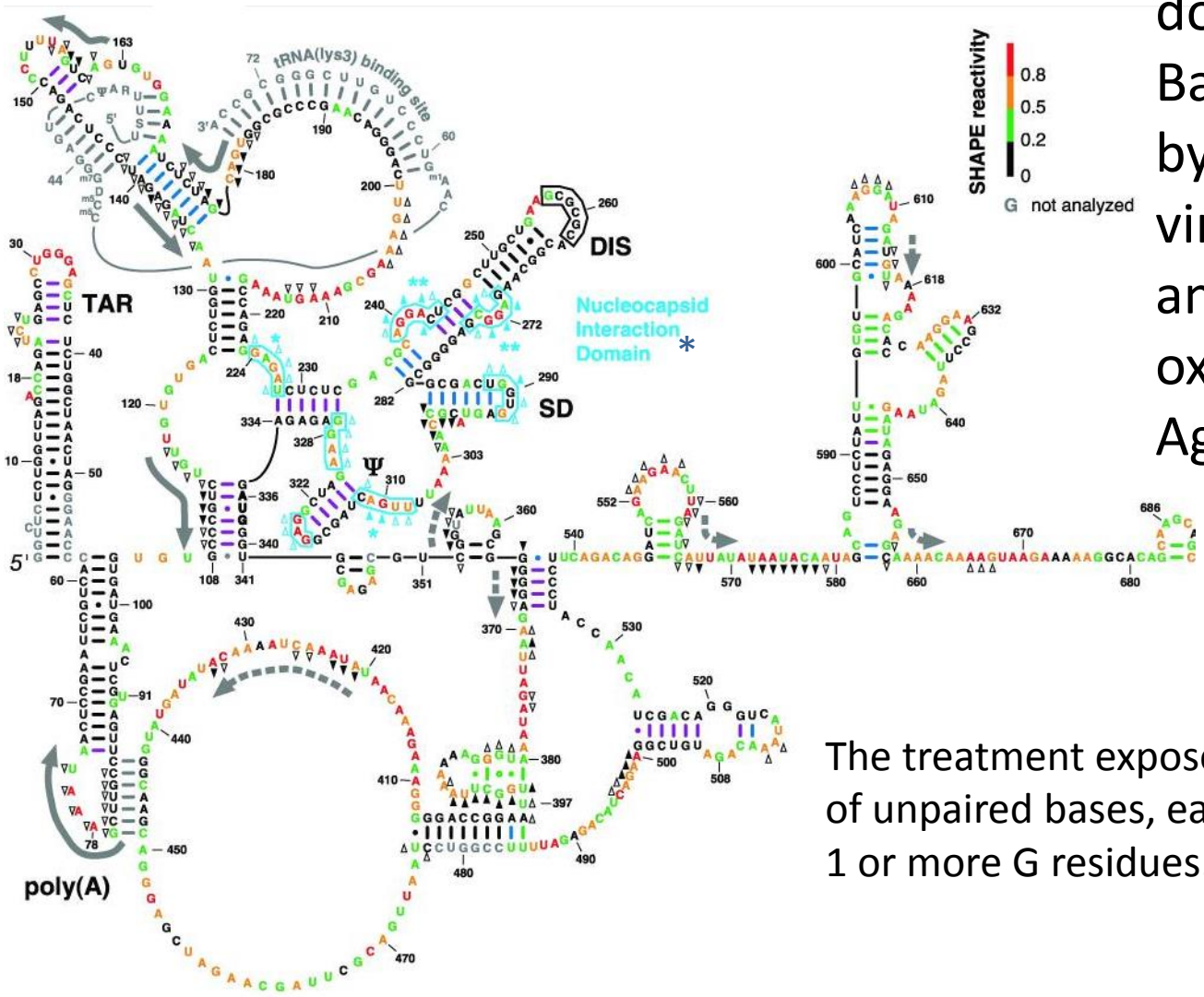
Secondary Structure of 5’ End of vRNA



*“NC Interaction domain”: Bases exposed by treating virus with anti-NC oxidizing agent

- Wilkinson, K.A. Plos Biol 2008; 6(6):e96

Secondary Structure of 5’ End of vRNA



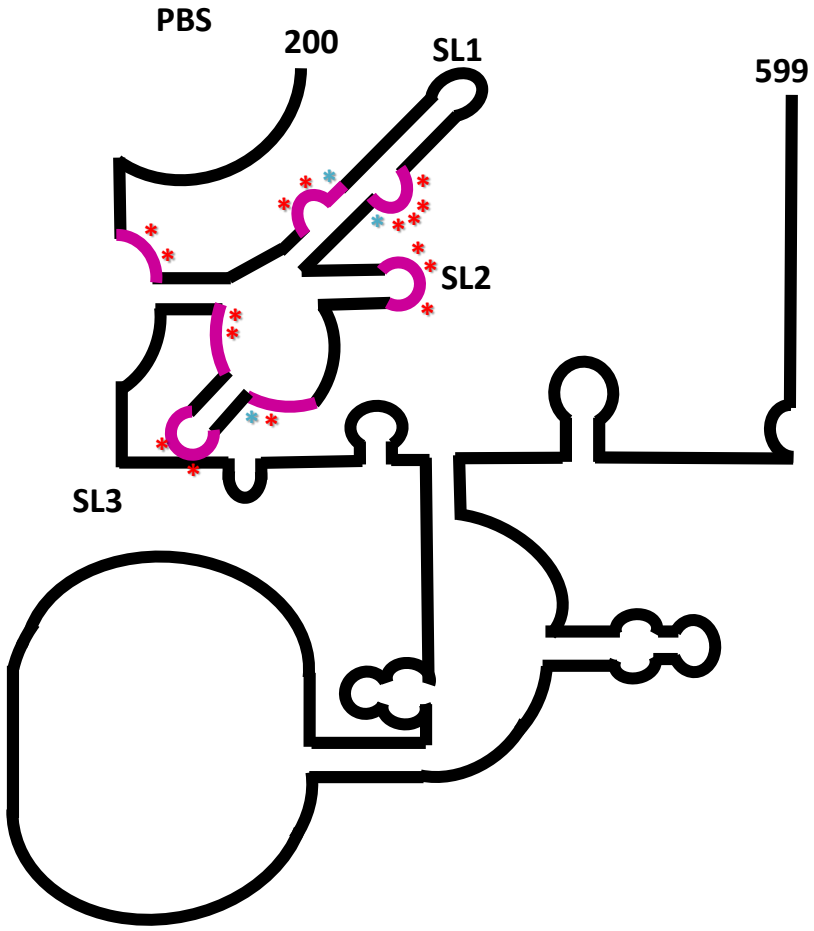
“NC Interaction domain”: Bases exposed by treating virus with anti-NC oxidizing Agent

The treatment exposed 7 tiny clusters of unpaired bases, each including 1 or more G residues.

- Wilkinson, K.A. Plos Biol 2008; 6(6):e96

Unpaired G's and C's

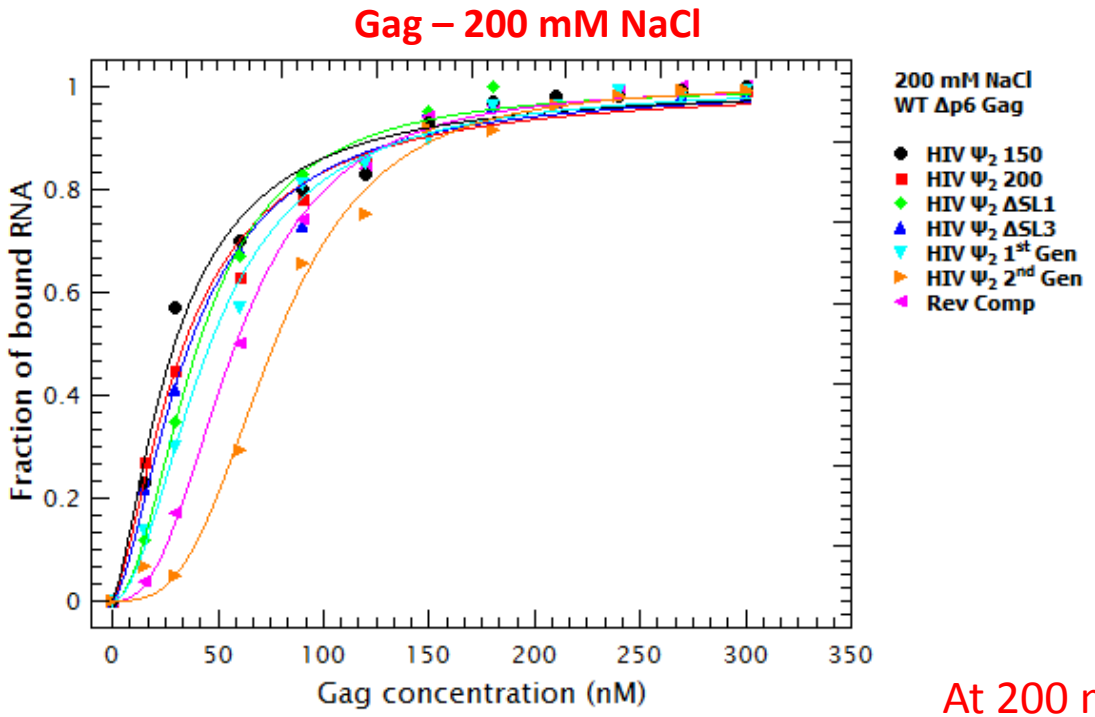
HIV-1 Ψ_2 Multiple Binding Site Mutant (MBSM) 1st Generation (200-599)



- The unpaired **G**'s and **C**'s that were proposed by Kevin Weeks to interact with the NC domain in the immature virions were mutated to A's.

All RNAs are 400-nts long, 3'-labeled with Cy5 and thermally annealed to promote RNA dimerization.

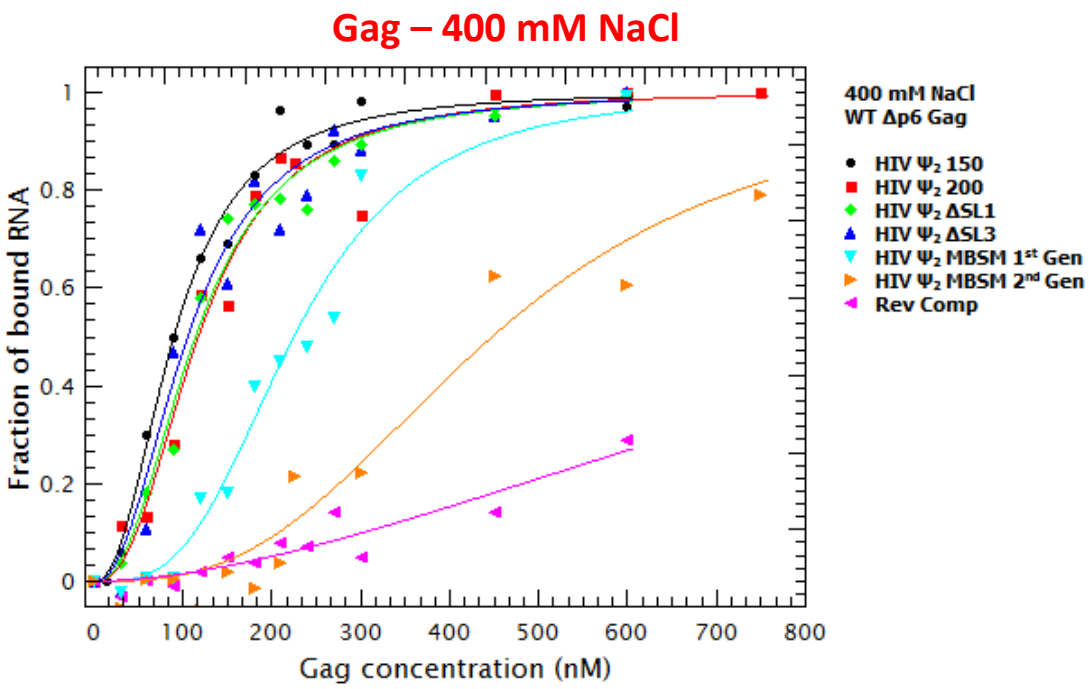
Gag binds well to the mutant RNA at 200 mM NaCl



RNA	K_D (nM)	n_H
	200 mM NaCl	
HIV Ψ_2 150	31	1.6
HIV Ψ_2 200	32	1.5
HIV Ψ_1 ΔSL1	47	2.5
HIV Ψ_2 ΔSL3	35	1.6
HIV Ψ_2 MBSM 1st Gen	48	1.8
HIV Ψ_2 MBSM 2nd Gen	77	3.1
Rev Comp	58	2.5

At 200 mM NaCl non-specific interactions mask specific binding (almost all K_D*s* are very similar to each other).

But mutating those unpaired G's weakens binding of Gag at 0.4M NaCl



RNA	200 mM NaCl K_D (nM)	400 mM NaCl K_D (nM)
HIV Ψ_2 150	31	90
HIV Ψ_2 200	32	118
HIV Ψ_1 ΔSL1	47	115
HIV Ψ_2 ΔSL3	35	106
HIV Ψ_2 MBSM 1st Gen	48	→ 241
HIV Ψ_2 MBSM 2nd Gen	77	→ 448
Rev Comp	58	1,070

Summary and Conclusions

- **Gag is a nucleic acid chaperone.**

- **Gag uses cooperative binding to RNA to bring ≥ 2 Gag molecules close together; this triggers assembly.**

- **Gag packages ψ-containing RNA with high selectivity if it is present in the cell; otherwise it packages mRNAs with very little selectivity.**

Summary and Conclusions

- **Gag binds with very similar, very high affinity to all RNAs tested in 0.2M NaCl. THEREFORE, high affinity cannot explain the selective packaging of vRNA.**

- **This binding is the sum of specific and nonspecific interactions.**

- **Specificity for ψ was revealed when mutant Gags were used, or when a nonspecific competing RNA was present, or when the salt concentration was raised to ~0.4M.**

- **Properties of the mutant Gags showed that the nonspecific binding was largely attributable to the MA domain. Gag-Gag interaction also makes a major contribution to nonspecific binding.**

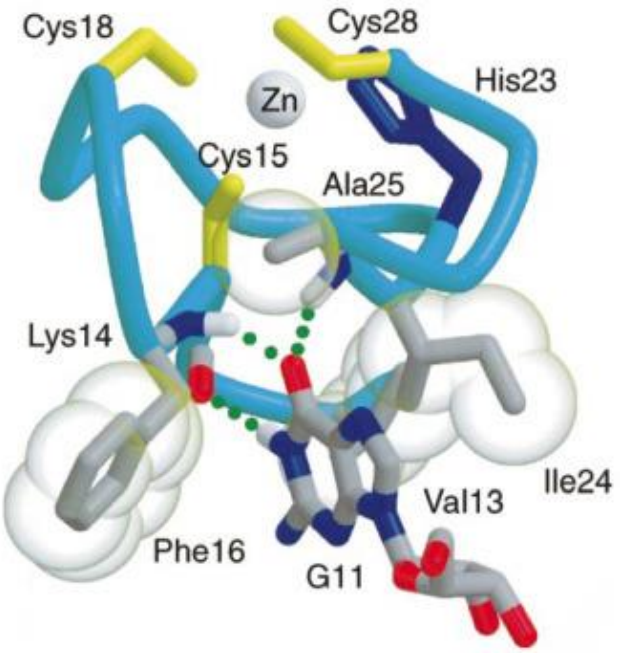
Summary and Conclusions

- **The salt-resistant binding of Gag to ψ apparently requires the unpaired G's in the "nucleocapsid interaction domain" in the 5' UTR.**

Speculative Remarks

- **Michael Summers years ago determined by NMR the structures of complexes between NC and specific stem-loops within the 5' UTR.**

He found that NC binds well to unpaired G's and that hydrophobic residues within the zinc fingers stack with the G's in these complexes.



Amarasinghe et al., JMB 2000

Speculative Remarks

- **We propose that the key to selective packaging is in the efficiency of nucleation of particle assembly.**

- **As the immature particle is a hexameric lattice of Gags, perhaps when the NC domains of 6 Gags each bind to one of the little stretches of unpaired G's, assembly is initiated. This can also occur on other RNAs, but we propose it happens faster/more efficiently on these sequences within ψ.**

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