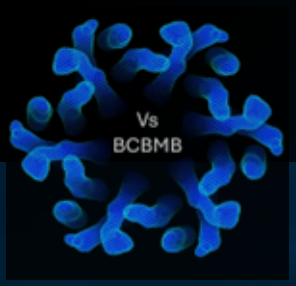


Advanced Biochemistry

MEMBRANE REMODELLING
A Case Study

The chapter on lipids and membranes introduced a LOT of facts and detail….

….to the point where it is easy to forget that all of this information is generated by actual biomedical, biophysical, or cell biological research

This slide collection is composed from slides I used for invited lectures on research that was ongoing in my laboratories at Yale and Northwestern.

The results shown in this presentation were deemed transformational for the field at the time….but I do not blame you if you are not interested.

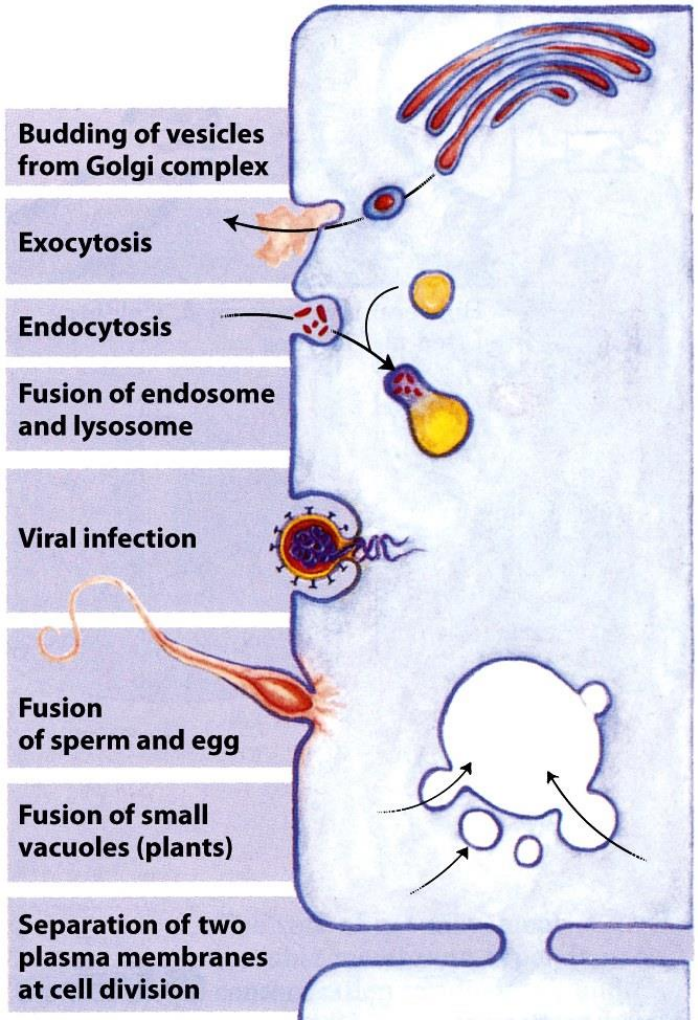
In this case, just skip this entire handout – it will not negatively impact your study of any of the other chapters

Bending Boundaries

Using Electron Cryomicroscopy to Visualize Mechanisms of Membrane Remodeling

Vinzenz Unger, Dept Molecular Biosciences; Chemistry of Life Processes Institute
Northwestern University

Membrane Remodeling - A Crucial Process in Cell Biology



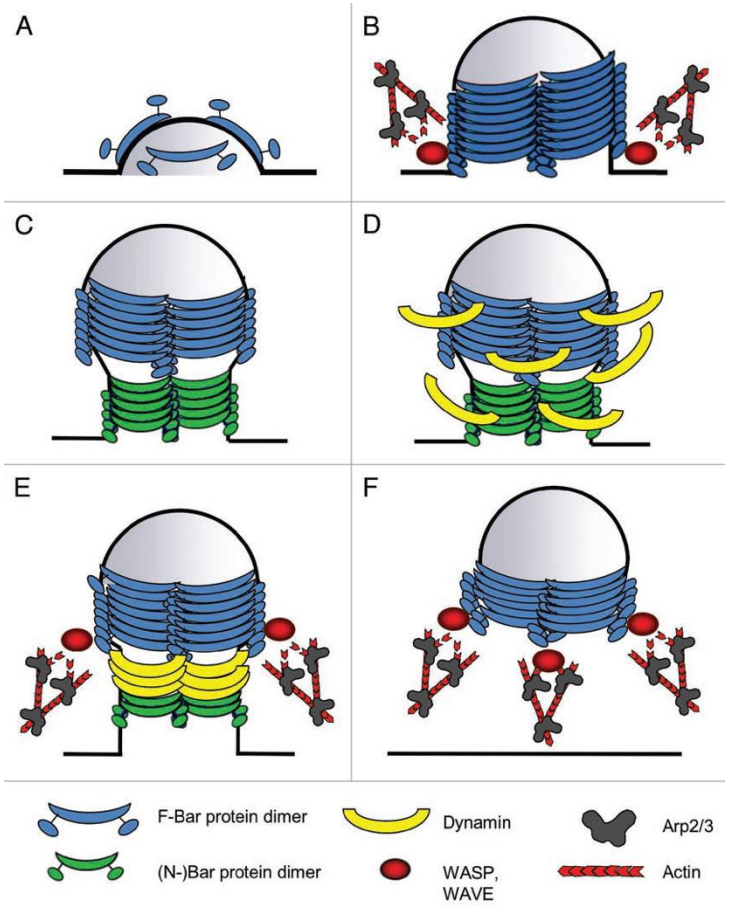
This starting point will look familiar to you if you worked through the LIPIDS and MEMBRANES Chapter

A few examples for processes that require remodeling:

- Vesicle transport
- Exocytosis
- Endocytosis
- Cell migration
- Cell division
- Viral entry and exit

Common to all these processes:
membranes need to be bent (= low curvature → high curvature)

Endocytosis – The Active Vesicular Uptake of External Materials and Substances Through – is Routinely Covered in Introductory Cell Biology Courses



Fricke et al (2014)
https://www.tandfonline.com/doi/full/10.4161/cib.3.2.10521

If you have never heard about it, here is a link to quickly inform yourself about the big picture

ENDOCYTOSIS

For this presentation, we will use endocytosis and example for a process that requires membrane remodeling = changing the shape/curvature of membranes.

In this case, reshaping the membrane leads to the formation of a vesicle that is "pinched off" and contains the material the cell wants to take up

The cartoon to the left summarizes key steps and mechanisms that occur during vesicle formation …

…and as you will discover ….

….a good part of this cartoon is informed by the results of the research my lab did back then …

Meet The Fearless

You are probably "sick" of seeing my face because it is all over the chapters. So you won't see it here anywhere. Though, the person I DO want feature with a picture is

Adam Frost
was a graduate student in my lab at Yale. He is the person that did all the structural and computational work. Without him – none of structural work would have happened.
All in all, it took Adam ~4 years to arrive at the major results that are presented here.

After holding faculty positions in Utah and at UCSF, Adam now works at Altos Labs

Also important to note: Adam did not work in isolation, but was supported by critical collaborative efforts with the following

Ed Egelman (UVa)**, Bridget Carragher** (NRAMM)**, Pietro De Camilli** (Yale), **Rushika Perera** (Yale, Purdue), **Aurelien Roux** (Yale, U of Geneva)**,**

Funding: NIDA, NIGMS, Epilepsy Foundation, DFG, Cancer Society

Membrane Tubule Formation by the BAR Domain Superfamily

Fixed and contrast enhanced thin slices of cells overexpressing proteins known to be involved in membrane curvature sensing and remodeling revealed numerous tubular invaginations that were extending from the cell surface.

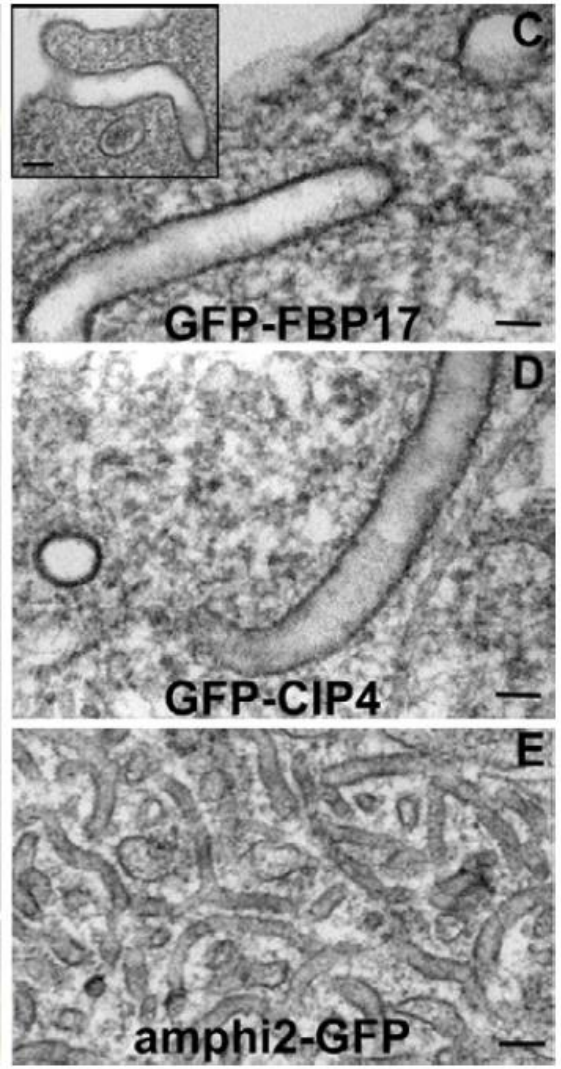
The top two panels show the morphology of these "tubes" for two "F-BAR" proteins (BAR=*bin, amphiphysin, rvs)*

- FBP17 (formin binding protein 17) and
- CIP4 (*cdc24* interacting protein 4 = **c**ell **d**ivision **c**ycle 24 interacting protein 4) .

The bottom panel shows the outcome of over-expressing a BAR domain named amphiphysin protein from a different subfamily, called N-BAR proteins [you will understand shortly what sets them apart]

GFP: green fluorescent protein

The proteins were fused to GFP to allow visualization of these assemblies in living cells (= independent approach to verify that the electron micrographs are showing something real)



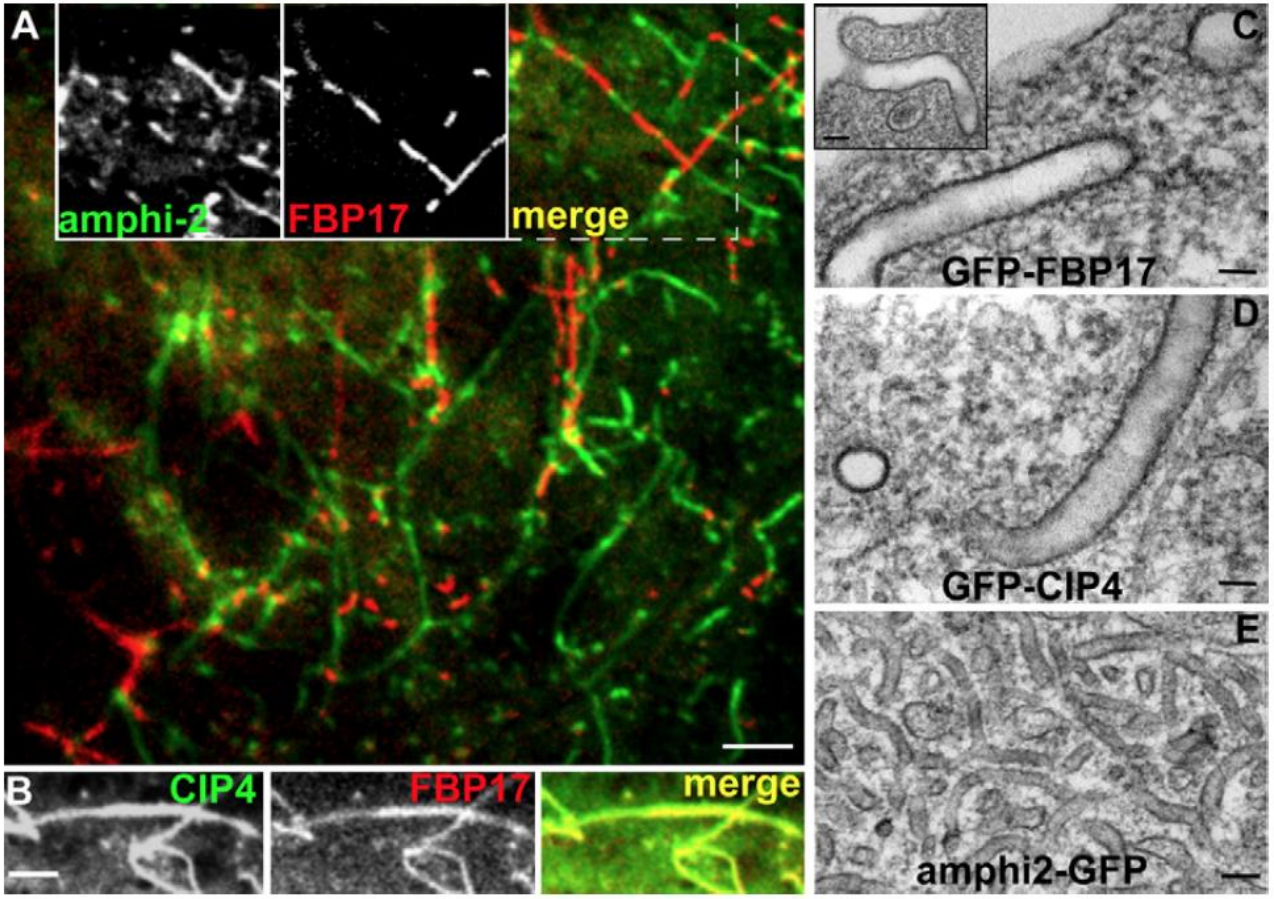
Frost etal (2008) Cell 132:807-17 DOI: 10.1016/j.cell.2007.12.041

Membrane Tubule Formation by the BAR Domain Superfamily

The live cell fluorescence imaging confirms that the BAR proteins cause extensive tubulation of membranes

The fluorescence images also revealed something in addition: when two BAR proteins are co-expressed, some tubules are decorated simultaneously by both proteins …. **BUT**

…and that was previously unknown…. the N-BAR and F-BAR coatings are mutually exclusive (alternating green-red segments = these two protein types can not form a "mixed coat" (while two F-BAR proteins don't mind collaborating)

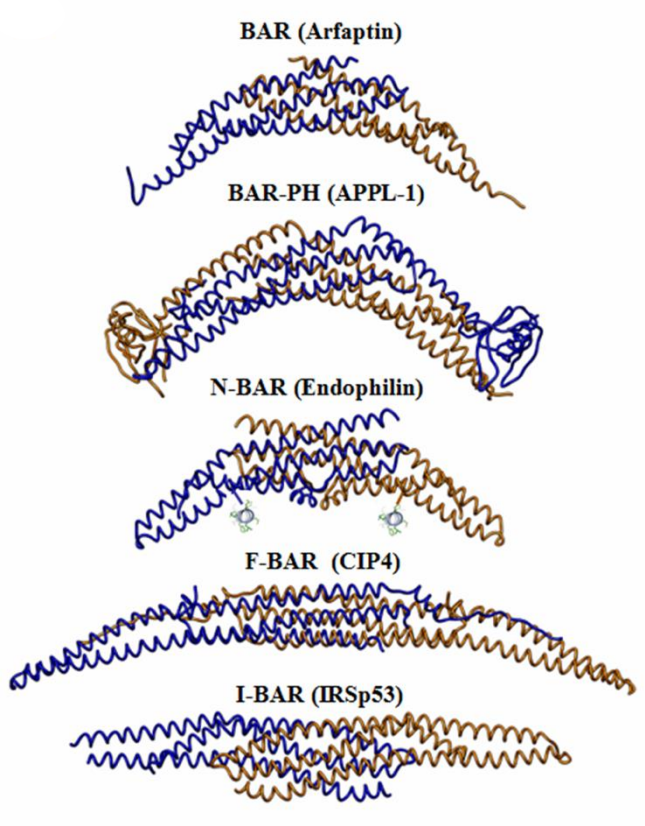


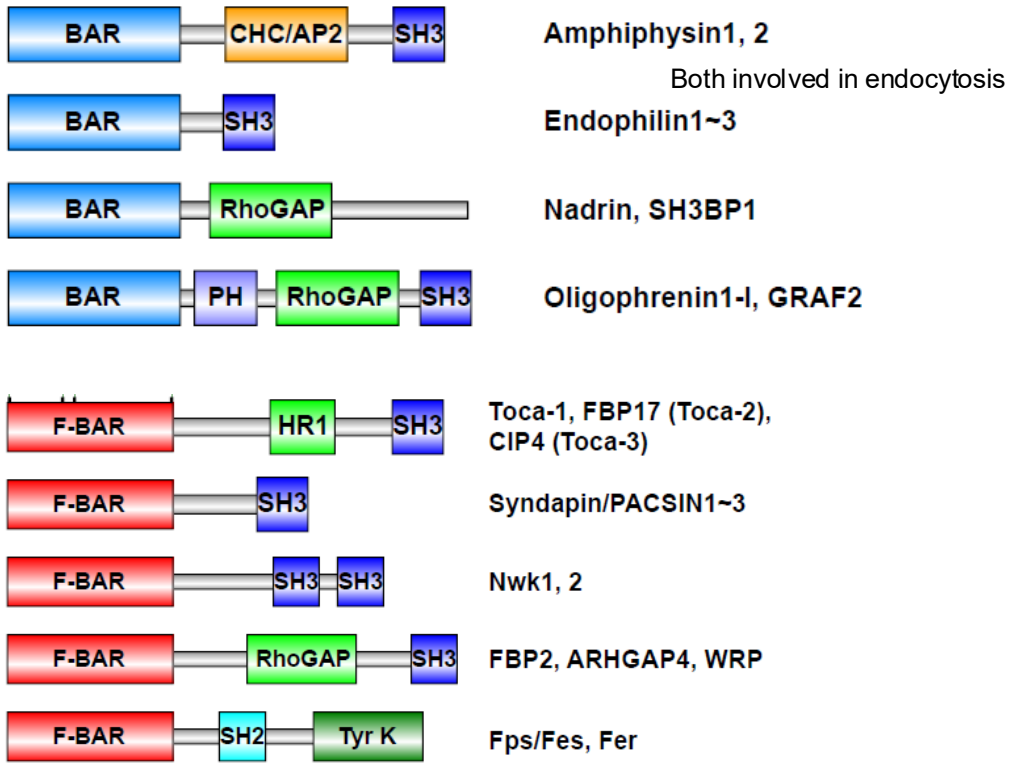
Frost etal (2008) Cell 132:807-17 DOI: 10.1016/j.cell.2007.12.041

The BAR Domain Superfamily

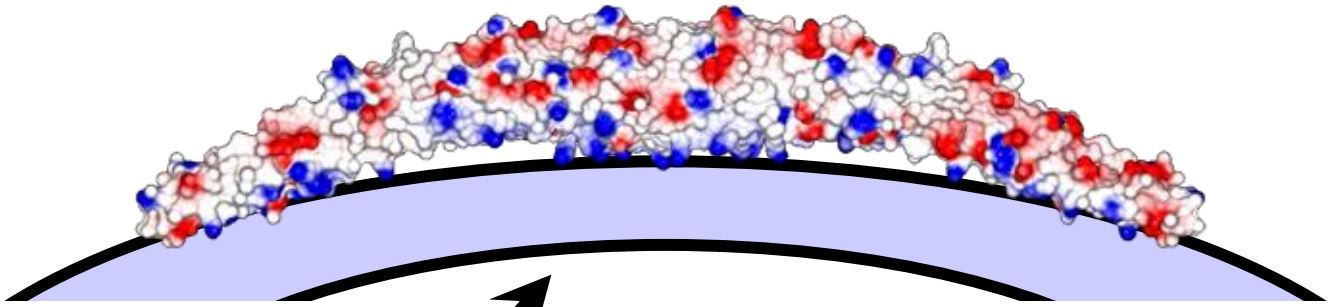
At the time these experiments were done, structural biologists had already worked out a lot about the structure of the BAR domains that are responsible for remodeling the membrane in processes like endocytosis.…

Short Summary: BAR-domains are "banana shaped" dimeric proteins. Based on their degree of curvature and presence of certain additional structural features, BAR domains are grouped into different families: N-BAR have a more pronounced arch and carry small "amphipathic" helical segments that are absent from the shallower F-BAR domains, and I-BAR domains that have an inverted arch





Just looking at the structures suggested two different mechanisms by which BAR domains could achieve changes in membrane curvature:

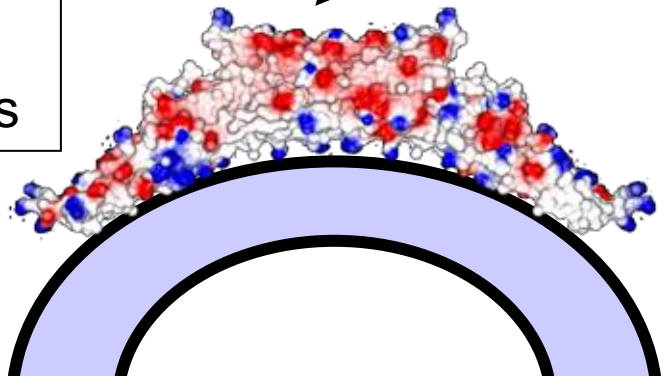


F-BAR Domains

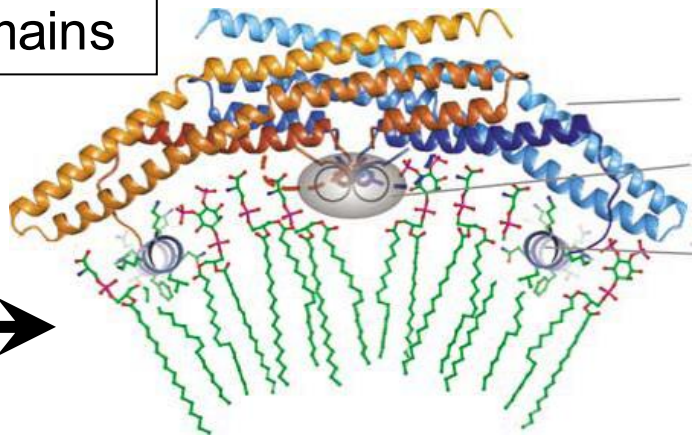
Shape-Based "Scaffolds" : Stabilize/Detect Cognate Degrees of Curvature?

Amphipathic "Wedges" : Induce Bilayer Buckling?

"Classical" BAR Domains

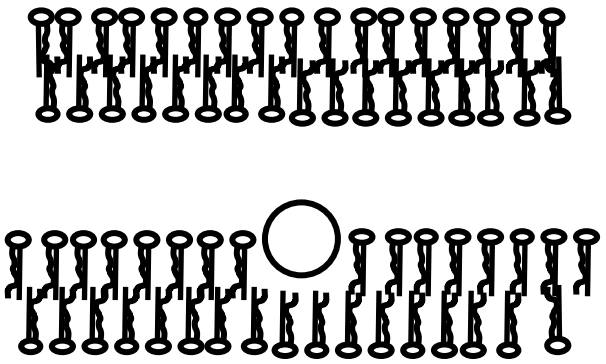


N-BAR Domains



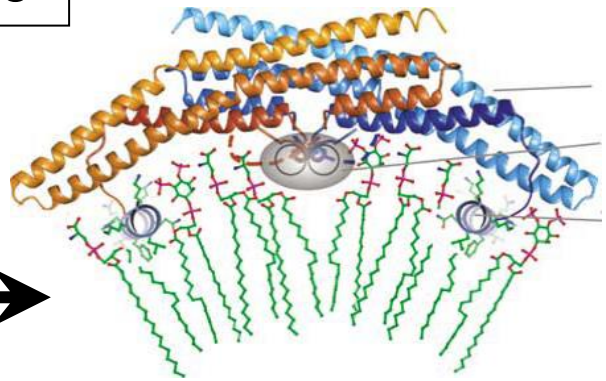
Just looking at the structures suggested two different mechanisms by which BAR domains could achieve changes in membrane curvature:

This "wedging" may not be intuitive to you …. so here is a brief explanation



Amphipathic "Wedges" : Induce Bilayer Buckling?

N-BAR Domains

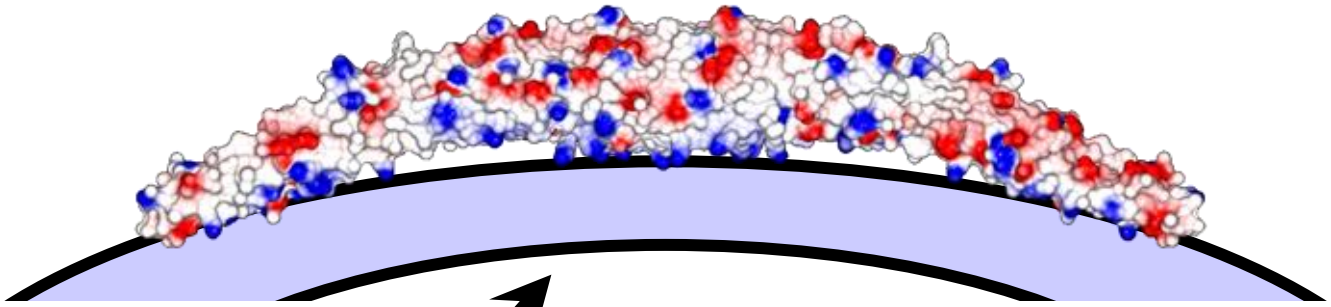


Insertion of a wedging protein piece into just one Leaflet laterally pushes headgroups away from the insertion point (increases the "area size" of this leaflet")

→ If the bilayer was a flat sheet with open edges…this would make some hydrophobic chains be completely exposed to water ….

→ …the bilayer avoids that by "buckling" (upwards in this situation) because then the "pathlength" to cover in the upper leaflet increases compared to the lower leaflet = the inserted protein basically "makes up" for some of the lipids and allows the lipid molecules at the edges to get back into "register"

Just looking at the structures suggested two different mechanisms by which BAR domains could achieve changes in membrane curvature:



F-BAR Domains

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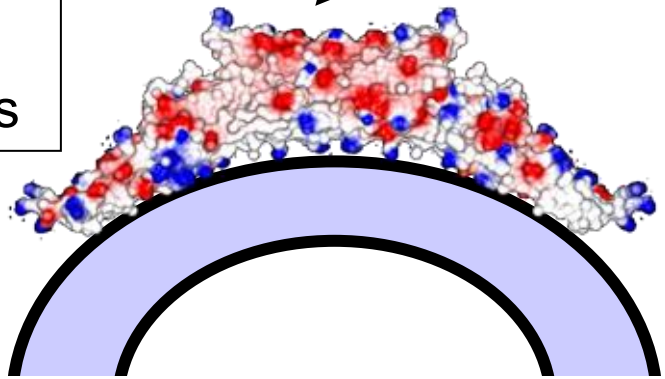
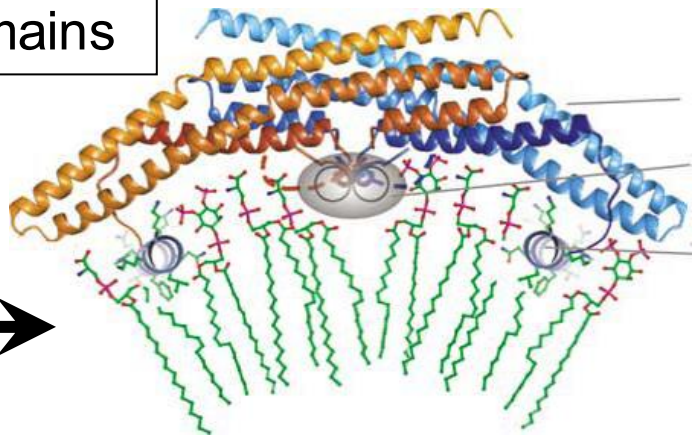
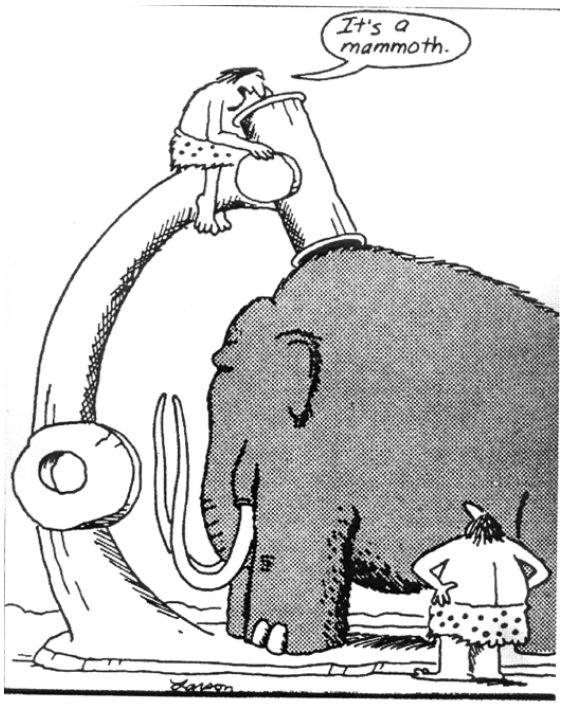


N-BAR Domains



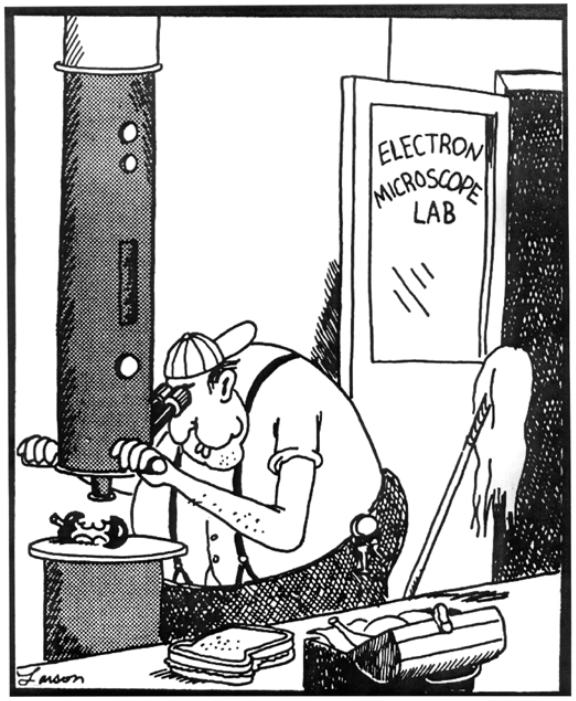
How Can We Test These Hypotheses Directly?

"It is very easy to answer many of these fundamental biological questions; you just *look at the thing!* <…> Make the microscope one hundred times more powerful, and many problems of biology would be made very much easier." *Richard Feynman: There's Plenty of Room at the Bottom, Dec 29th 1959*



Early microscope

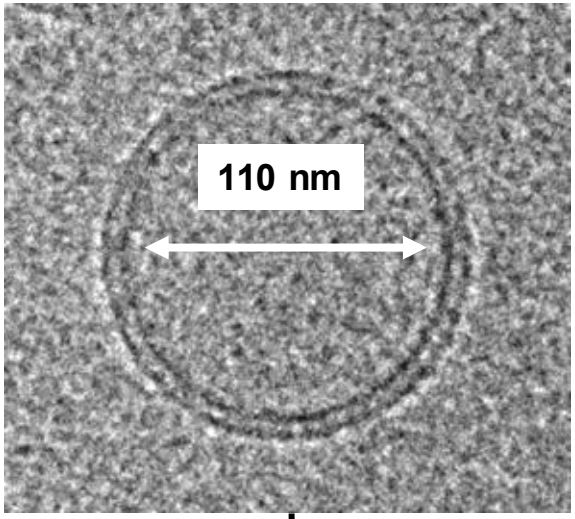
Coarse



EM 1960-2005(ish)
7-30Å resolution,
near atomic (= better than 4Å) in only a handful of cases

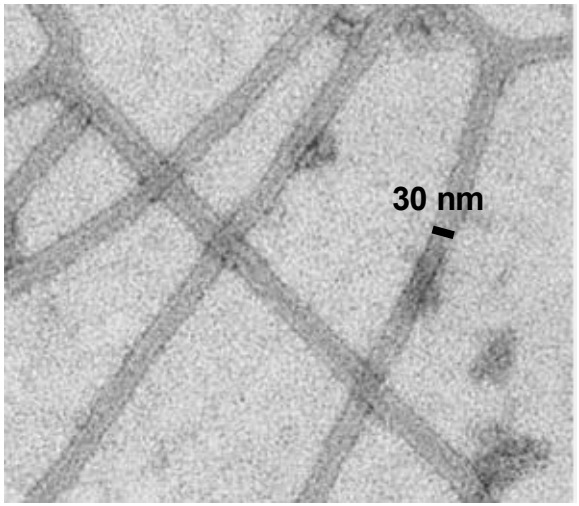
EM since ~2010
new detectors for recording images have pushed resolutions to near atomic range almost routinely

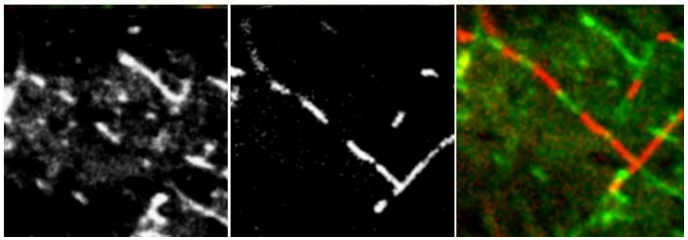
If we want to use electron microscopic imaging to determine the structure of the protein coats on the membrane surface…will the structures of tubules we generate in the test tube (*in vitro)* be meaningful?

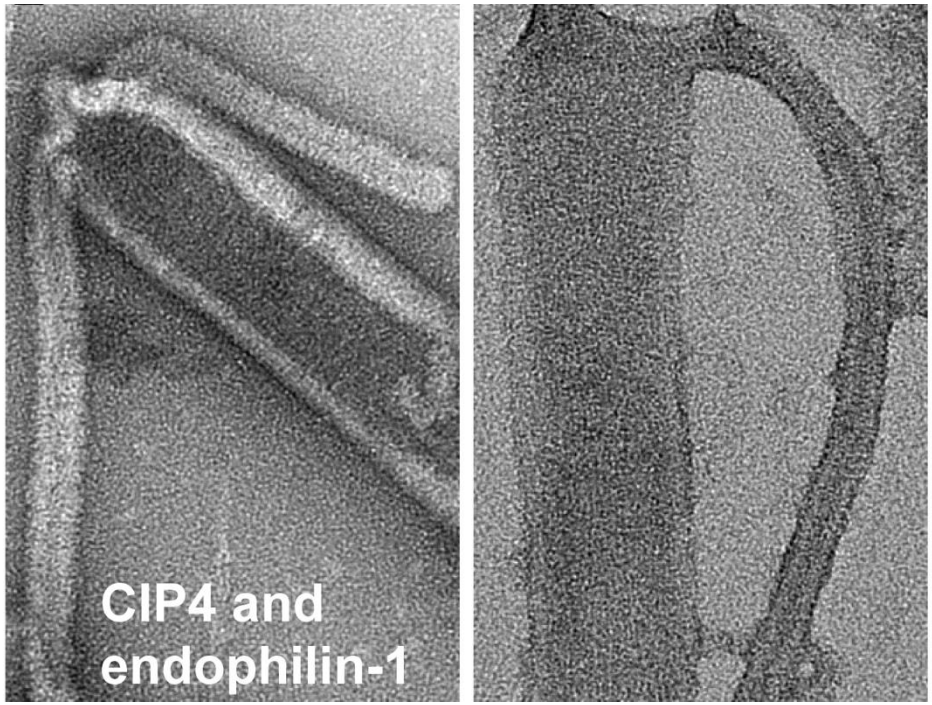


take suspension of liposomes, incubate with isolated protein, examine in electron microscope

BAR





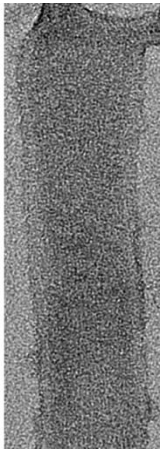


Initial analysis on contrast enhanced samples suggested that the *in vitro* activity of the purified BAR proteins reproduces morphologies and behavior observed in living cells

= F-BAR make wide tubes, N-BAR make narrow tubes

If present simultaneously, the coats formed by the two types of BAR proteins are mutually exclusive

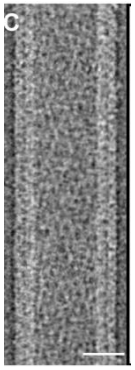
Structure Determination Requires Resolution that Is Higher Than What be Accomplished with Contrast Enhancing Stains



While the preliminary results obtained from stained samples were very encouraging, to see individual molecules and to resolve their structure + how they relate to the membrane surface requires a switch in approach

→ switch to Electron-Cryomicroscopy

The major difference is that the sample is not covered and dried up in the presence of a heavy metal salt as shown in the image ot the left (uranylacetate = generates very good contrast and makes the samples insensitive to the electron beam).



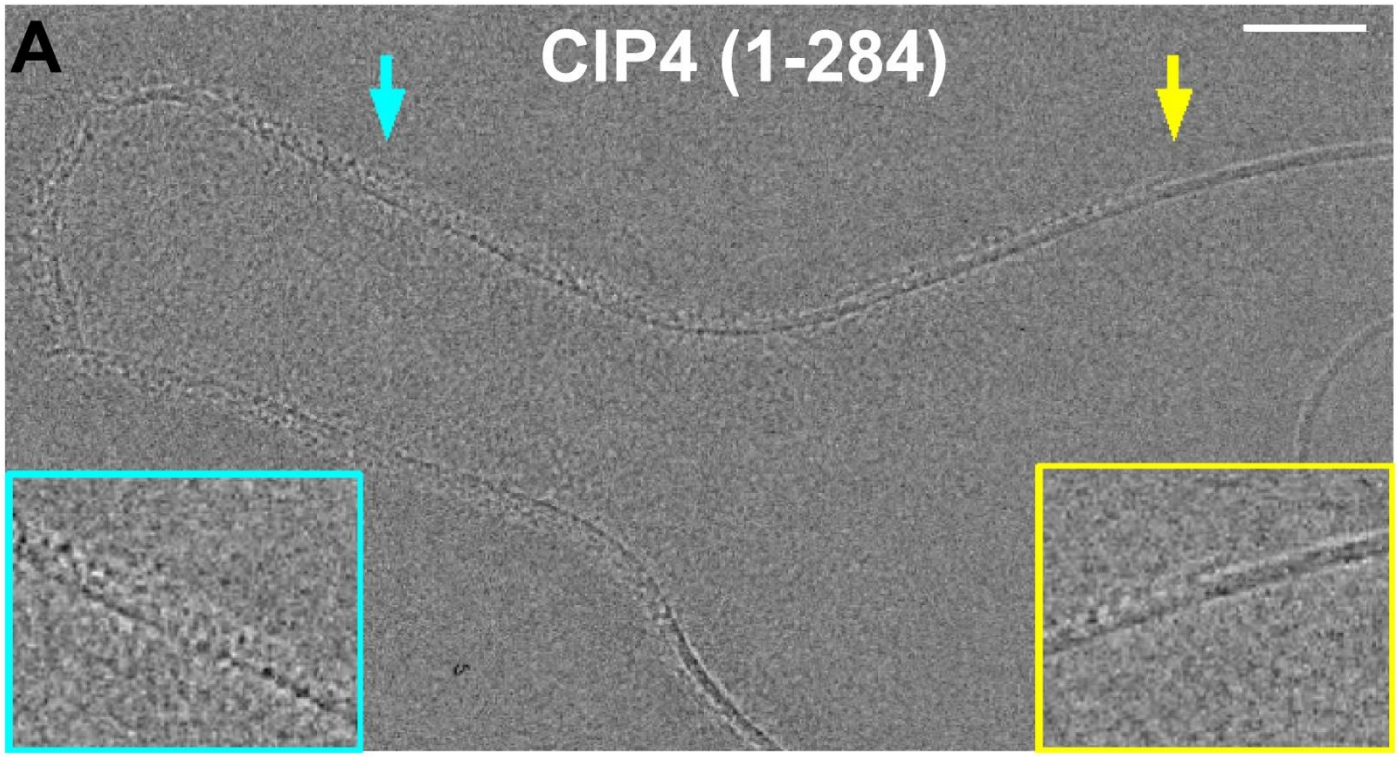
Instead, the sample is applied to an
electron microscope grid in the buffer that was used to generate the sample (= an aqueous buffer)
→ then the grid is plunged into liquid ethane to rapidly freeze everything in place.

Once frozen, the grid is transferred onto a special sample holder that allows keeping the sample at -175˚C (=frozen) and transferred into the microscope.

In this approach, the rapid freezing preserves the molecular structure, and the water turns into a transparent, amorphous ice layer (solid state of water without the crystalline structure of ice cubes in your freezer).

Prepared this way – you can retrieve true molecular and (today) even near atomic detail …but you can take an image only once because the lack of the contrast enhancing heavy metal stain renders the biological material very sensitive to the radiation of the electron beam. Even very short exposures, using very low brightness of the electron beam, will destroy all useful structural information ➔ you have to put the information together by analyzing many images and combine their data to overcome the large degree of noise in each of the individual images (you may remember slides 2-4 from the "What Do You See" Chapter)

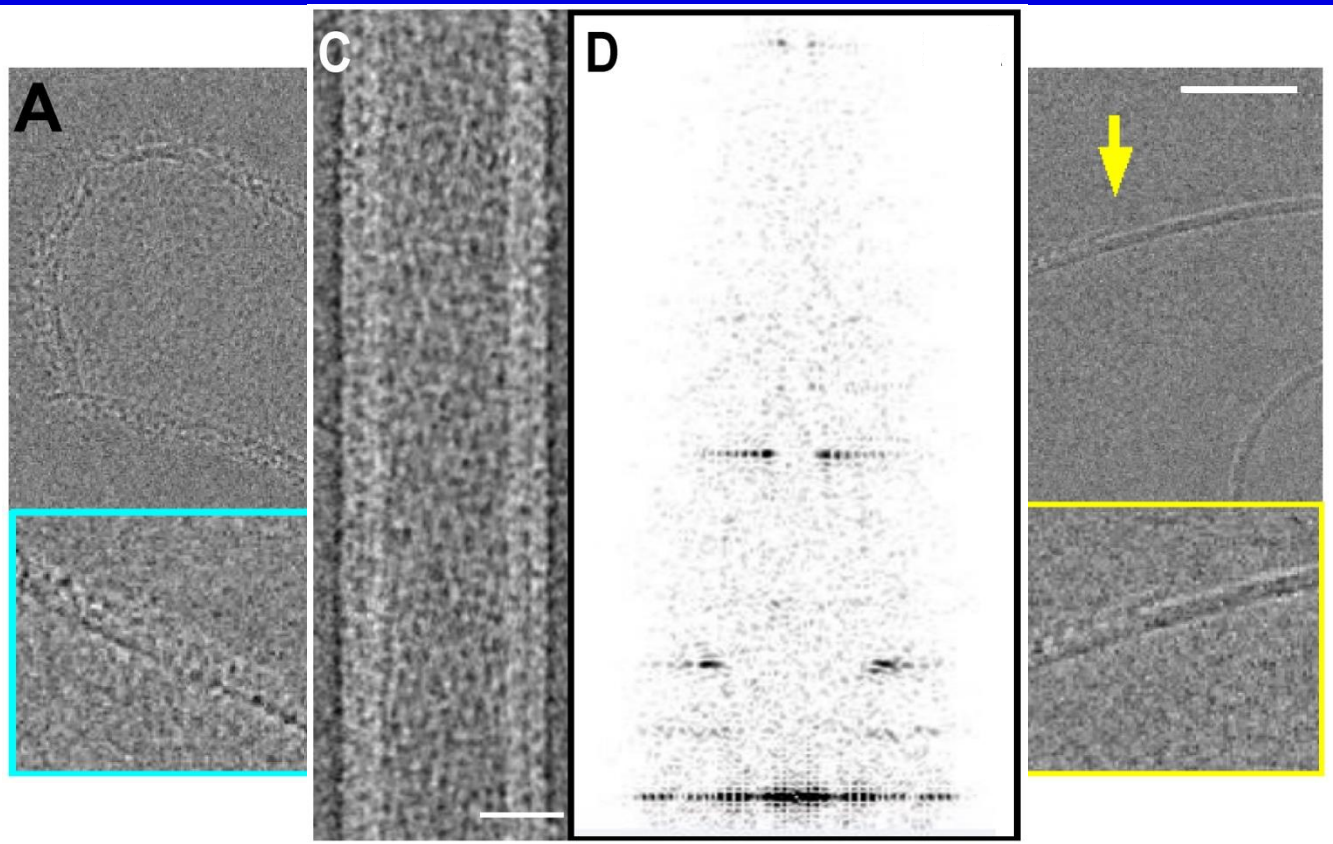
Direct Visualization of Tubule Formation



This image of an unstained, frozen hydrated sample captured a snapshot of a liposome in the process of being tubulated

- To the right of the yellow arrow you see the "naked" membrane surface with no protein molecules discernable
- To the left of the yellow arrow it looks like a "smudging tool" was applied to blurr the surface of the bilayer ==> some protein is accumulating here, but …. The surface still seems to be flat!!!! (this was puzzling until we found out why at a later stage … and by accident)
- Finally, in the region where tubulation occurred you can see a "thick" layer of protein" that is covering the surface

Direct Visualization of Tubule Formation



Looking at these images, things got REALLY exciting when Adam took pictures of just tubular regions at a larger magnification (C), and then calculated what is called a "Fourier Transform" of the images of the tubes (D).

The Fourier Transform is the calculated diffraction pattern of the image … and tells you if anything in this image is "regular" (like a protein coat would be…presumably). If it is, you will either see sharp diffraction spots (if it is a flat, 2D-array of things like in "What Do You See, Slides 2-4), or streaked out lines like here. Without doing anything… just seeing this diffraction patterns told us that the hollow cylindrical shape of the membrane tubules was intact and that the protein covered the membrane surface by forming a coat with helical symmetry …. Using the information in this pattern should allow us to calculate the structure of the coat!!!!

Direct Visualization of Tubule Formation

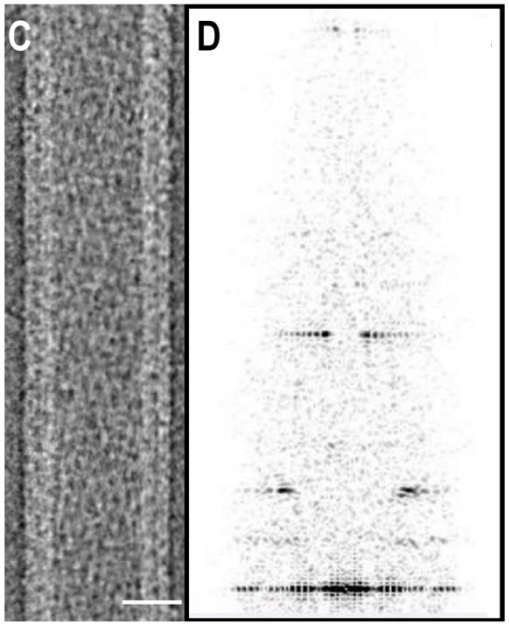
Every expert on the planet thought it would be a matter of hours to calculate the structure …. but, every expert shortly started wringing their hands saying that this particular case is special and so tricky that it cannot be solved ……

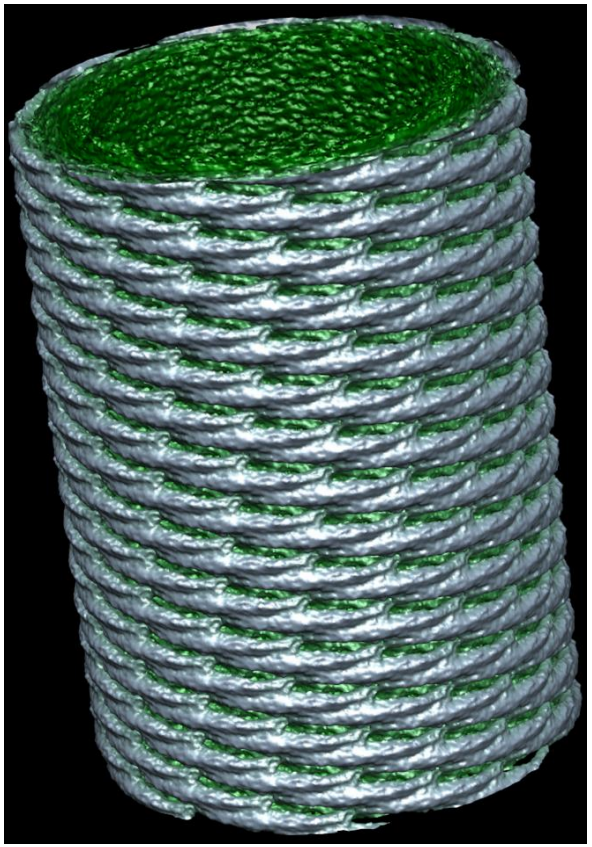
not the answer we hoped to hear ….

….and that is when Adam got creative by adapting existing methods to deal with this particular sample ….

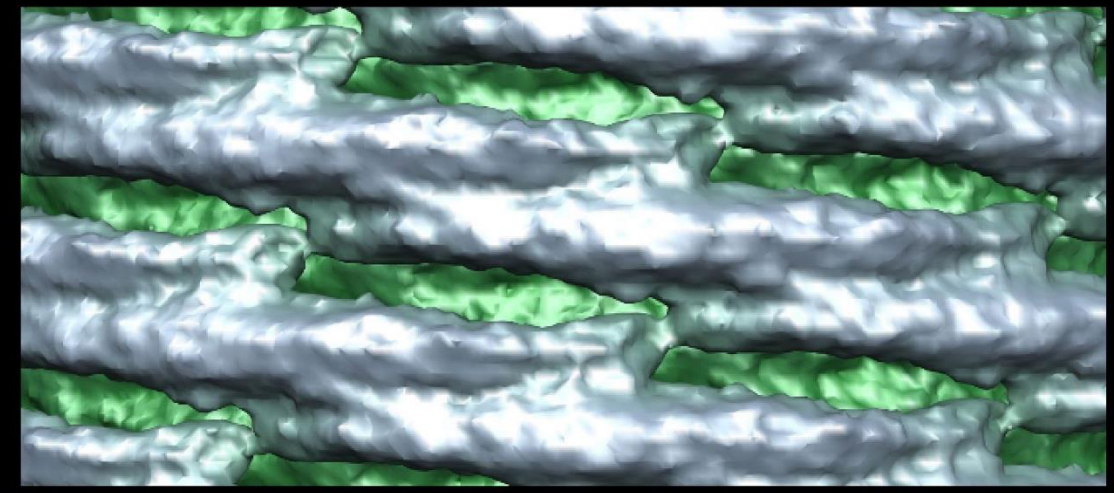
…..which, **a few years later** yielded the structure that was deemed a transformational breakthrough in this field because for the first time – people could see how the individual molecules arrange, and how they support the high curvature of the membrane ….

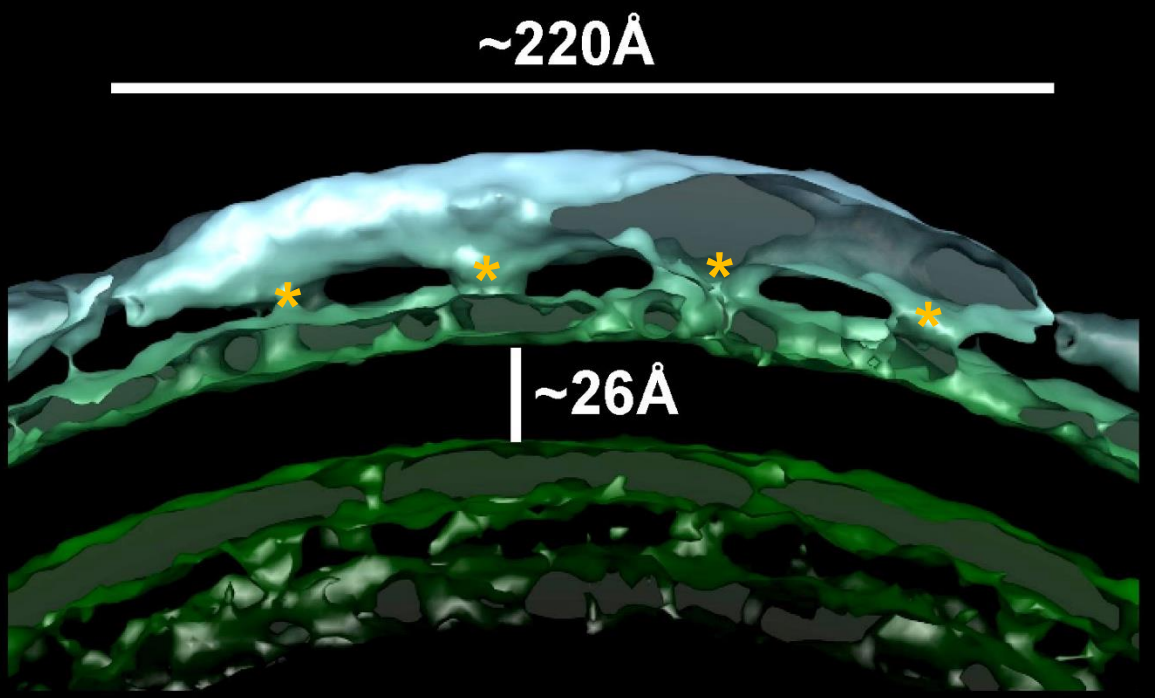
Direct Visualization of Tubule Formation





Iterative Helical Single Particle Reconstruction





Each of the silvery objects is one F-BAR domain dimer.

Seen from above the membrane, you can tell how the spiral staircase ascends and how adjacent dimers interact extensively along a lateral surface as you step up from one to the next dimer

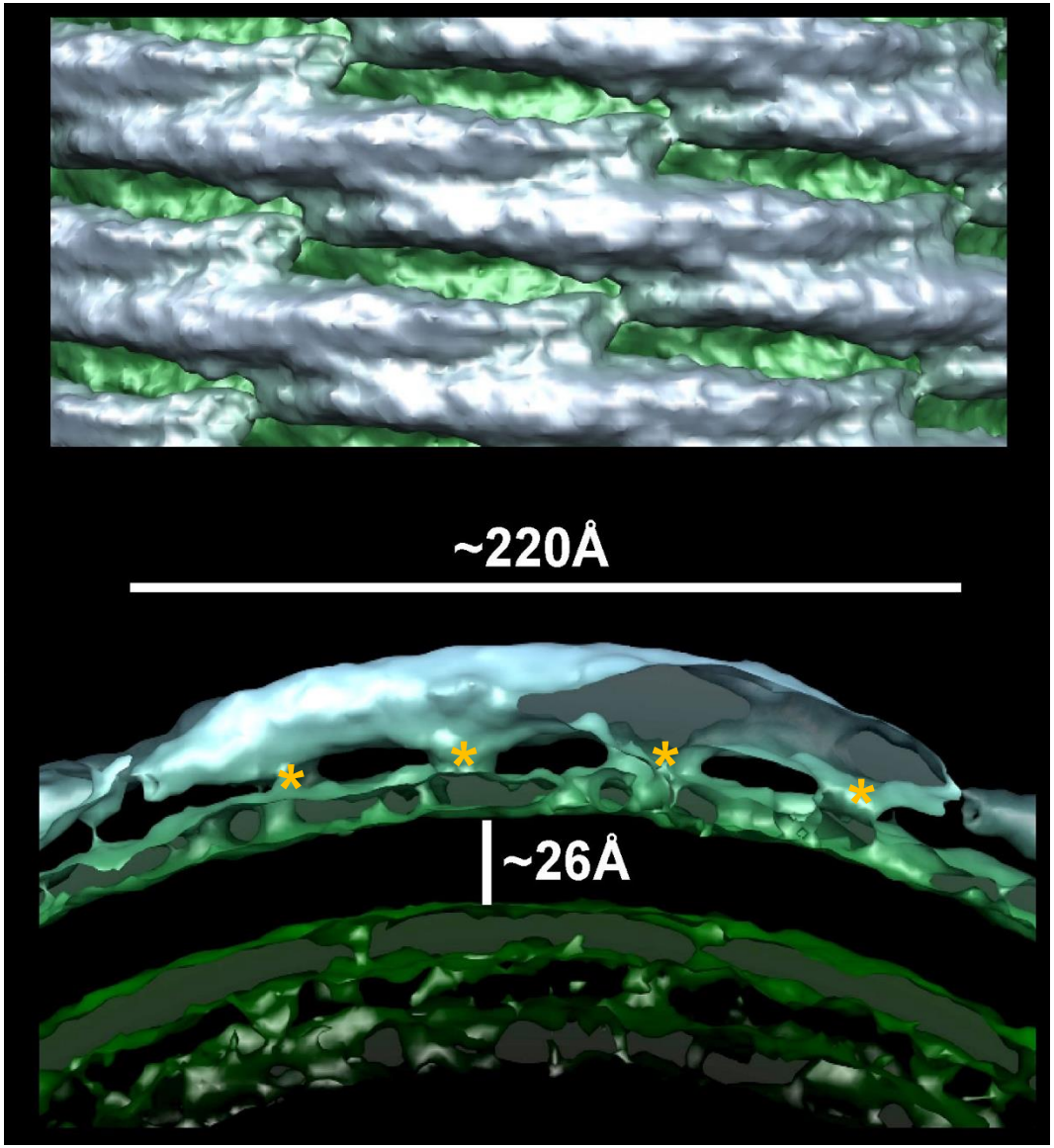
Seen from the side, you recognize how the F-BAR domain "cradles" the surface of the membrane ….

You also can see the hydrophobic core and the two membrane surface layers

…a first unexpected finding was that the F-BAR domain seems to have "attachments points" that are directly touching the membrane surface (*)

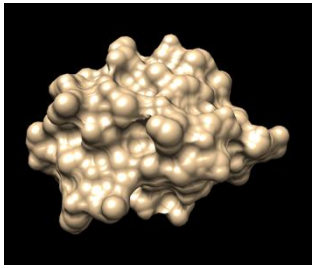
Frost et al (2008), Cell

Iterative Helical Single Particle Reconstruction



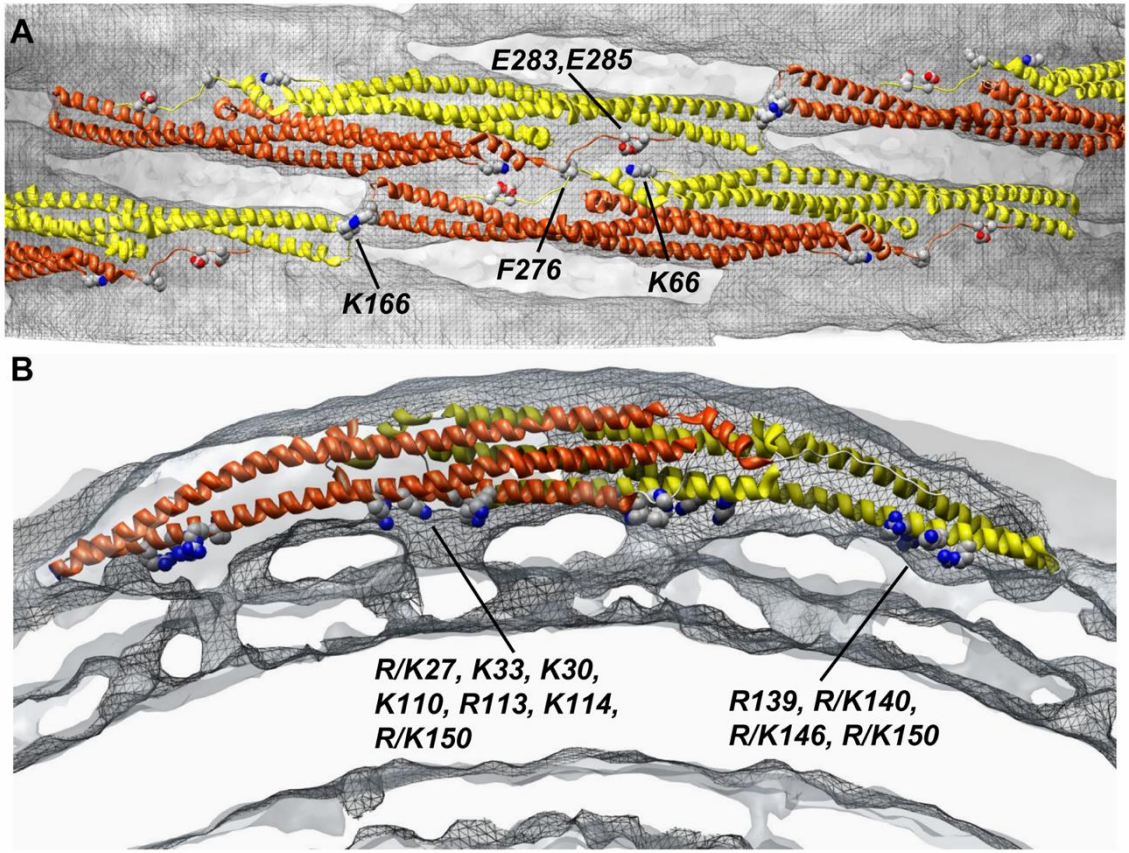
If you worked through the "Fundamentals – PROTEINS" Chapter, you will recognize that what is shown in these renderings is the molecular surface of the F-BAR protein domains

As a reminder, here is a small picture of the Atx1 surface that was shown and discussed in the Fundamentals Chapter



Just like in the case of Atx1, the inside of the F-BAR domains seems to be empty … which you know is not true … however….in this case, the reconstruction of the molecules did not have enough resolution to allow visualizing the atomic detail ….
to get there we have to "cheat" by placing the known high-resolution structure (determined in the absence of membranes by different methods) into the experimentally determined molecular envelope of the molecules as they are engaged to the surface

A Coat Composed of Shaped-Based Scaffolds



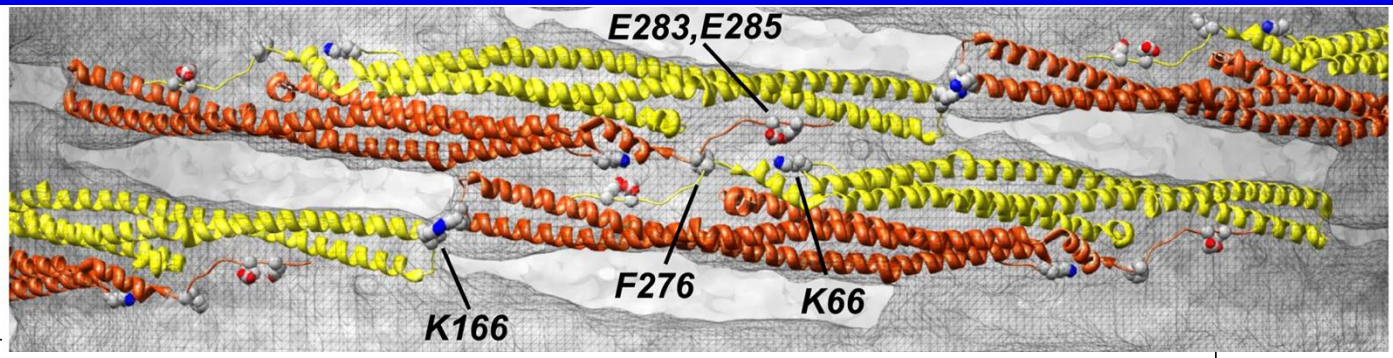
The atomic models fit well into the envelope without any need for adjustments to the atomic structures. This provides confidence that the envelopes are reliable and that binding to the membrane does not induce significant changes to the F-BAR domain shape

Beyond that, fitting the atomic structures into the envelope provides new insights. The modeling predicts

- defined interactions between amino acid sidechains
  - along the lateral interface (E283,E285 & K66) (F276),
  - and the tips of molecules where K166 seems involved

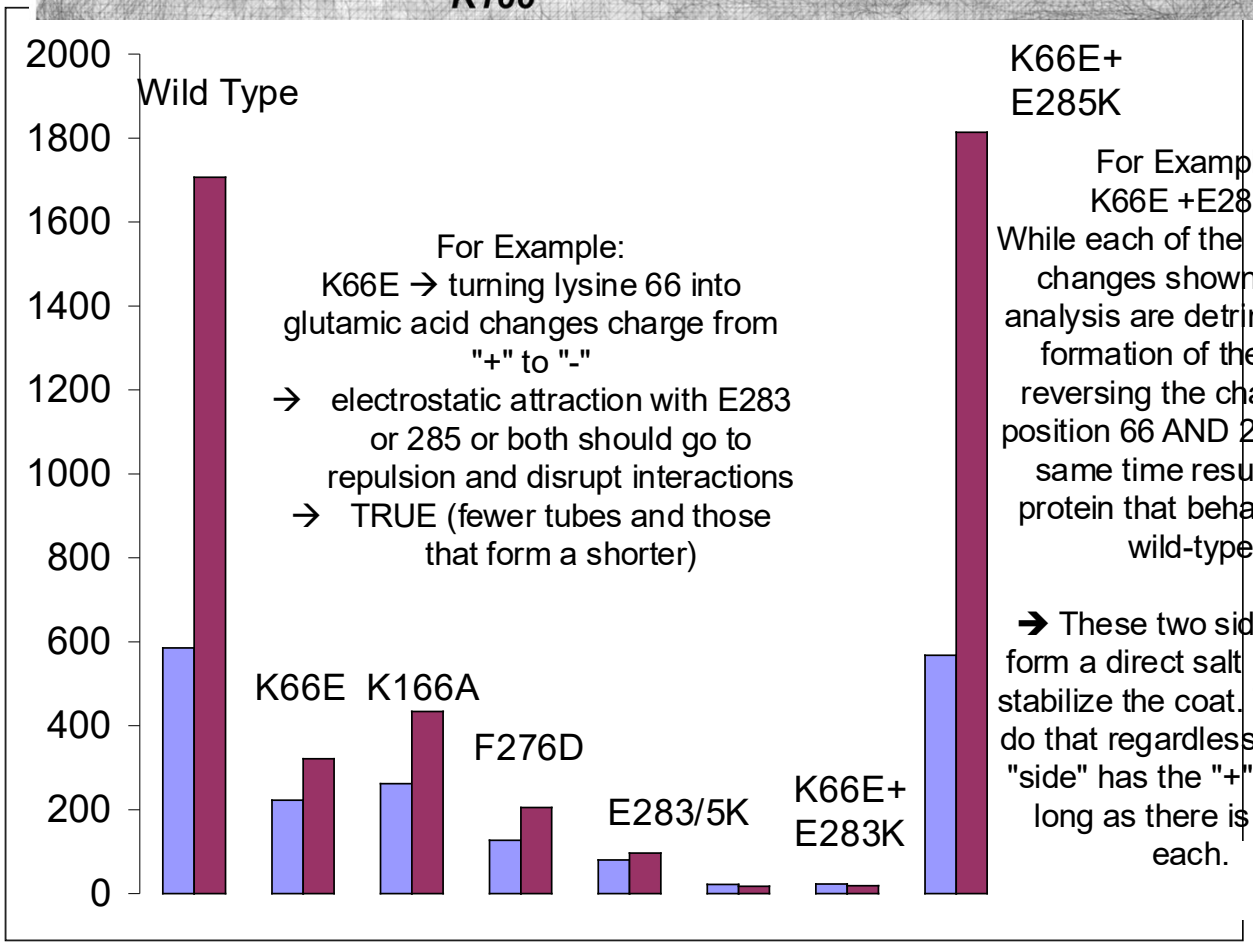
Intriguingly, clusters of positively charged lysine (K) and arginine (R) sidechains coincide with the locations where the envelope directly contacts the membrane surface (* on previous slide).
This immediately suggests that the sidechains are not "tucked against" the dimer like in the atomic structure (that was determined in the absence of a membrane), but are "extended" to interact with negatively charged lipid headgroups on the membrane, thus attaching the molecule to the membrane

If the model is "accurate", then we should be able to verify the predicted interactions by generating mutant proteins and study their behavior

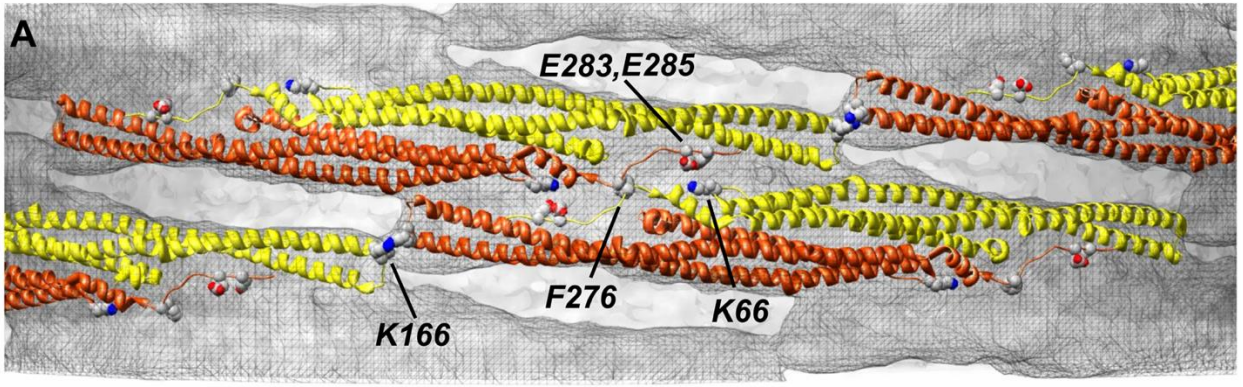


Total #

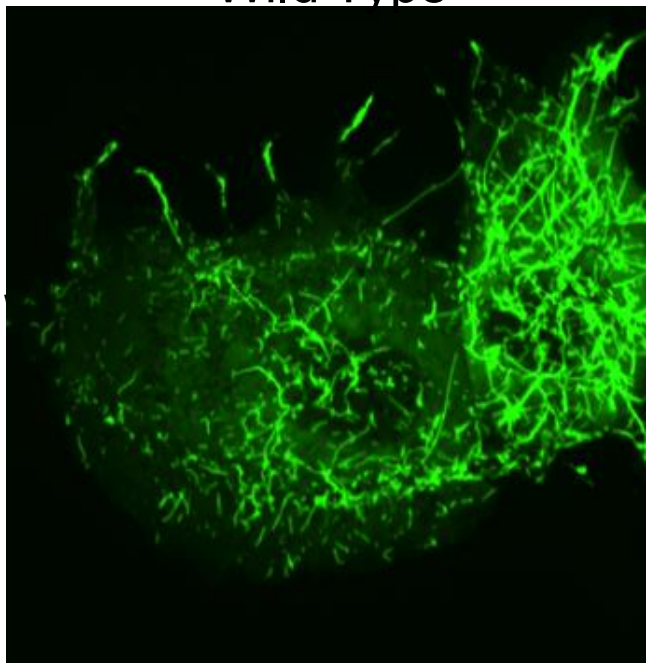
Total Length µm



Encouraged by the good agreement of predicted molecular interactions with behavior of mutant proteins *in vitro, the ultimate question is: does any of this hold up inside a living cell? Short answer is: YES*



Wild Type

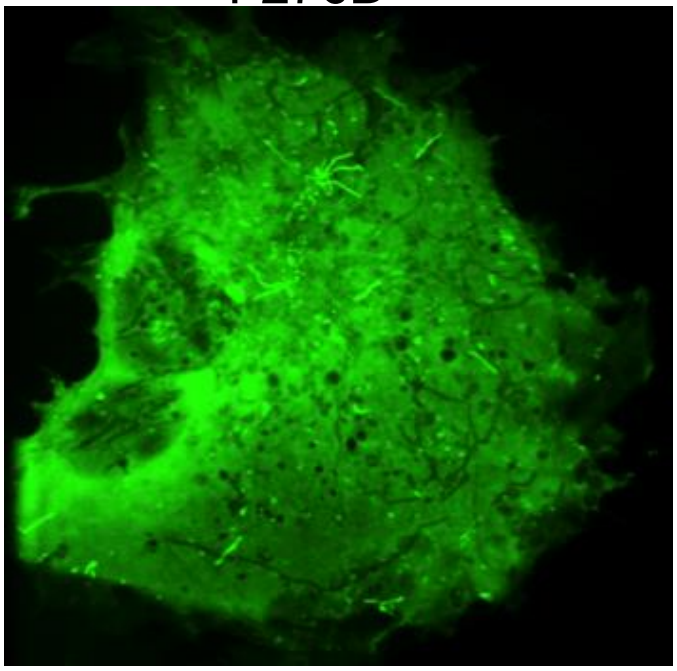


Green fluorescent versions of the wild type protein cause robust tubulation *in vivo*

And

In agreement with the model, turning the hydrophobic contact site F276 in the lateral interaction interface into an electrostatic repulsion abolishes tubulation *in vivo* = further support for the model

F276D



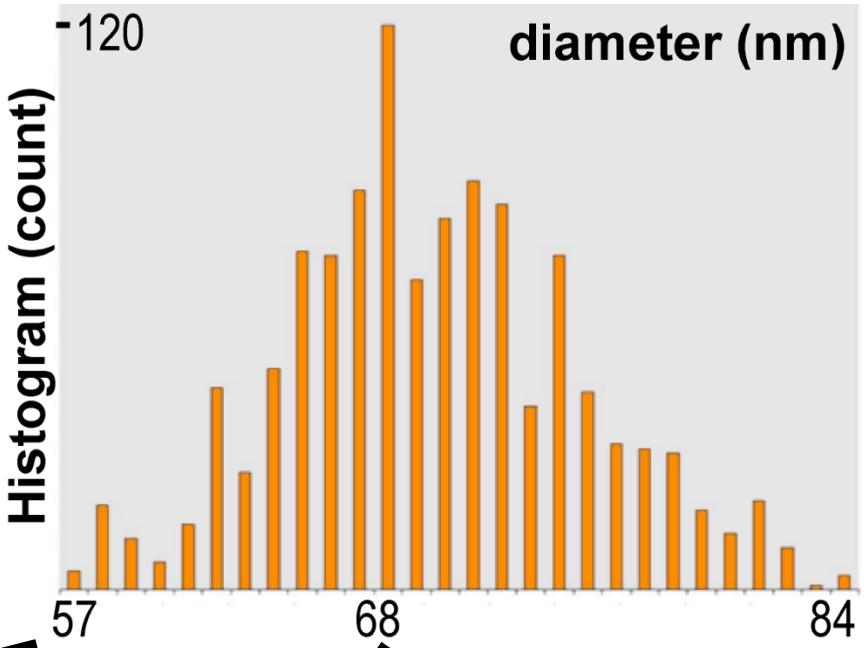
Going Even Further: analysis of tubes formed by wild-type and select mutant proteins provided an explanation for the observed variability in tube diameters

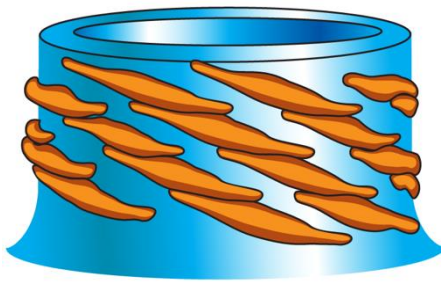
The histogram shows that tubulation using wild type protein creates tubes with a distribution of different diameters

How is this possible??

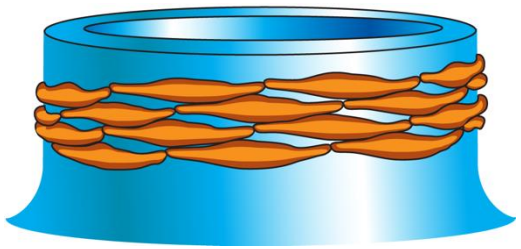
Three hypothetical mechanisms:
The "banana-shaped dimer" can adopt a more pronounced curvature (= narrower tubes)
or
The dimer lattice tilts with respect to the tube long axis ("chinese handcuff")

or a little bit of both

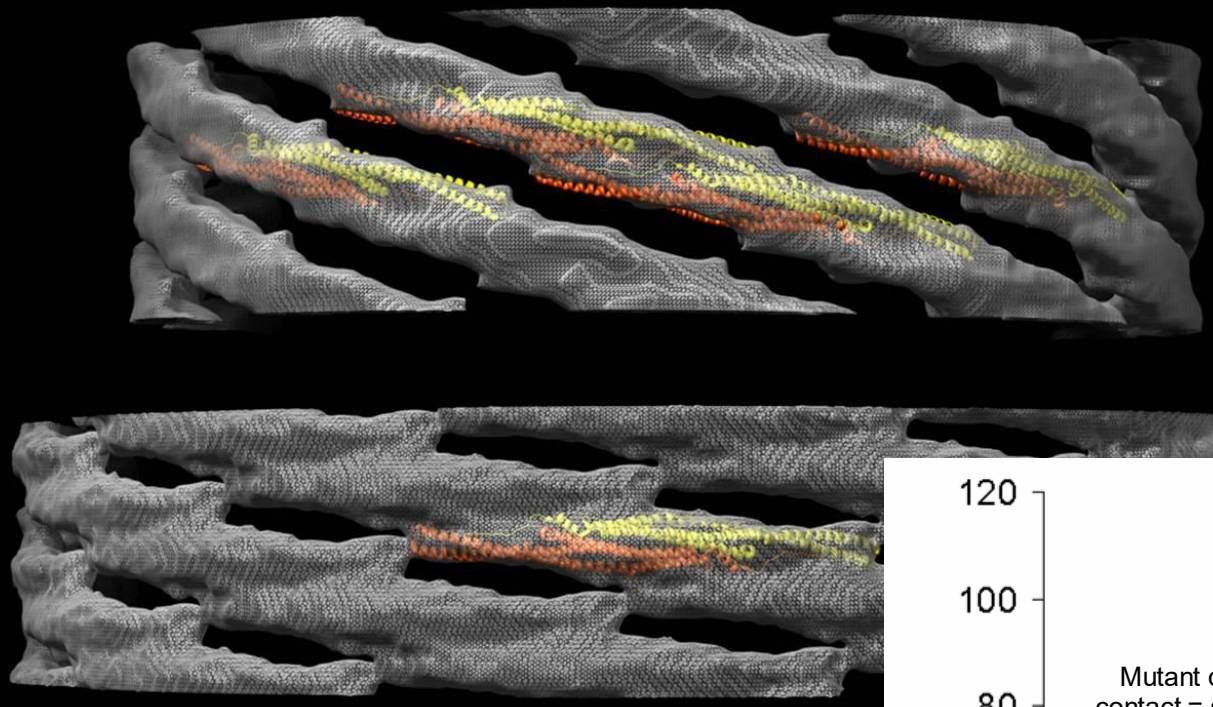


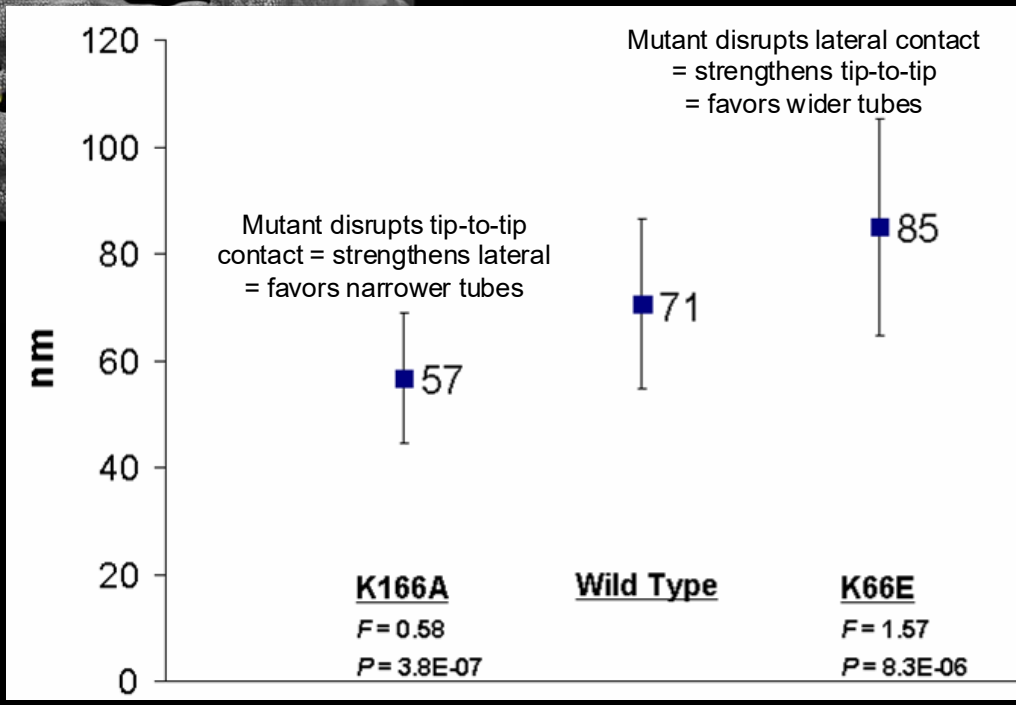


OR ???



F-BARs: A Molecular Version of “Chinese Handcuffs”

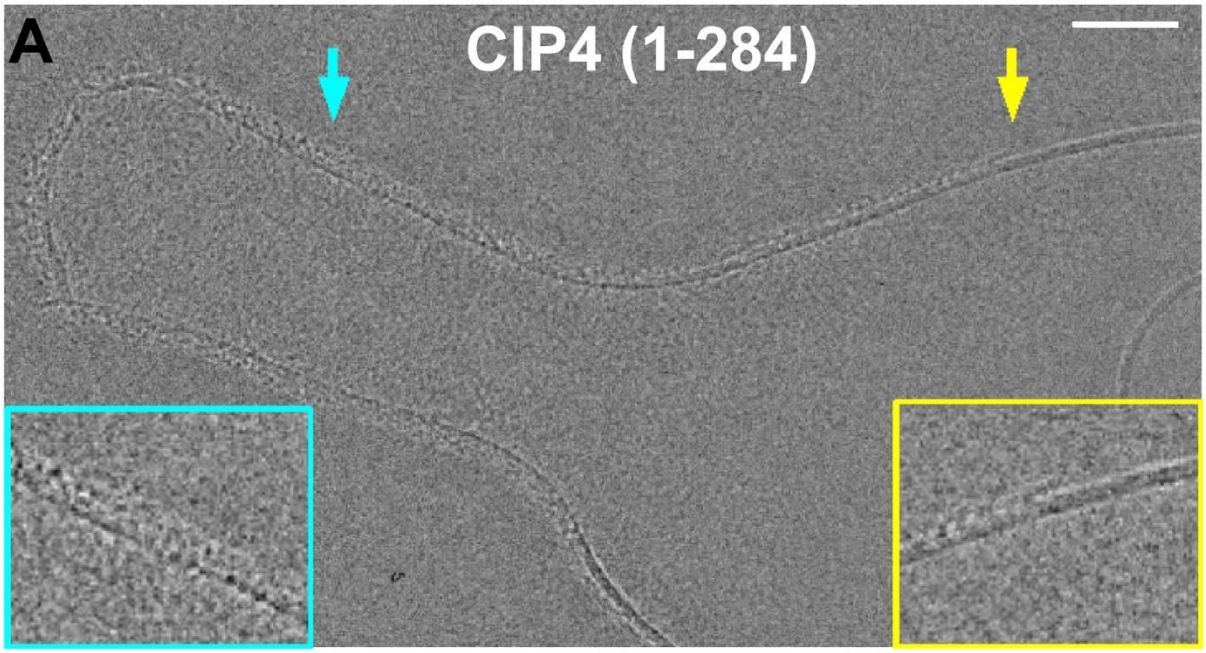




Breaking Completely New Grounds

Up to this point, all the predictions of the "pseudo atomic" model held up when tested through *in vitro* and *in vivo* experiments.

For the first time in this field, the model directly visualized the "scaffolding mechanism" for F-BAR domain induced curvature generation and provided molecular scale mechanistic explanations for how F-BAR domains sustain protein coats that are observed on the surface of highly curved membrane tubules.



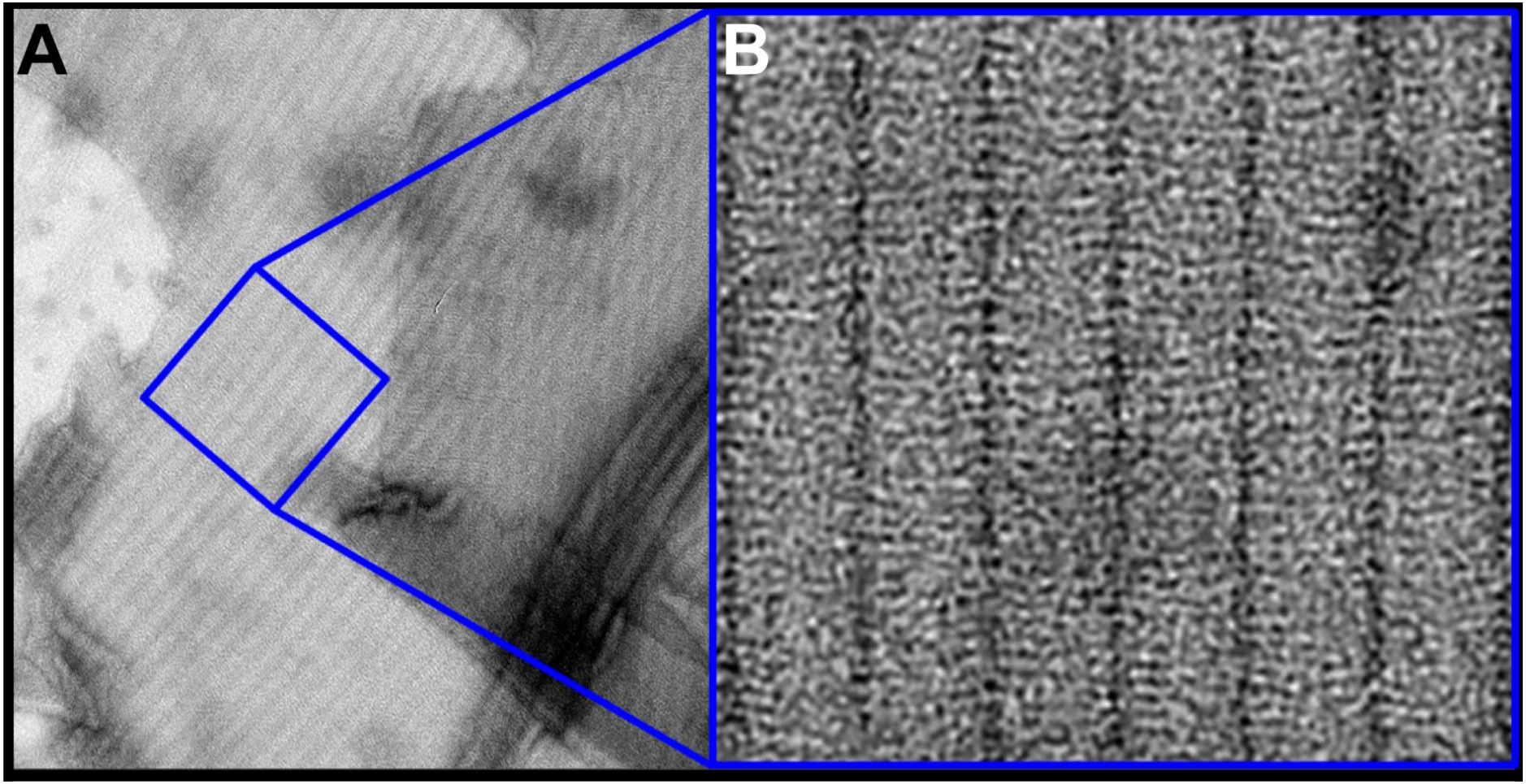
However, the studies so far did not tell us how exactly the protein **causes the deformation** of the membranes.

The initial picture was "weird" in that there is that region just left to the yellow arrow where protein seems to accumulate without curvature being changed …. if only …..we could get a look at what the structure looks like in these regions…!!!

DISCOVERY

….and by accident we/Adam did….

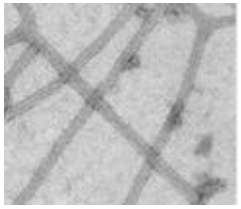
….one day he left a tubulation reaction overnight in the fridge ….
checking it out next morning by making a quick "contrast enhanced" sample he observed THIS instead of the tubular structures that formed at warmer temperatures

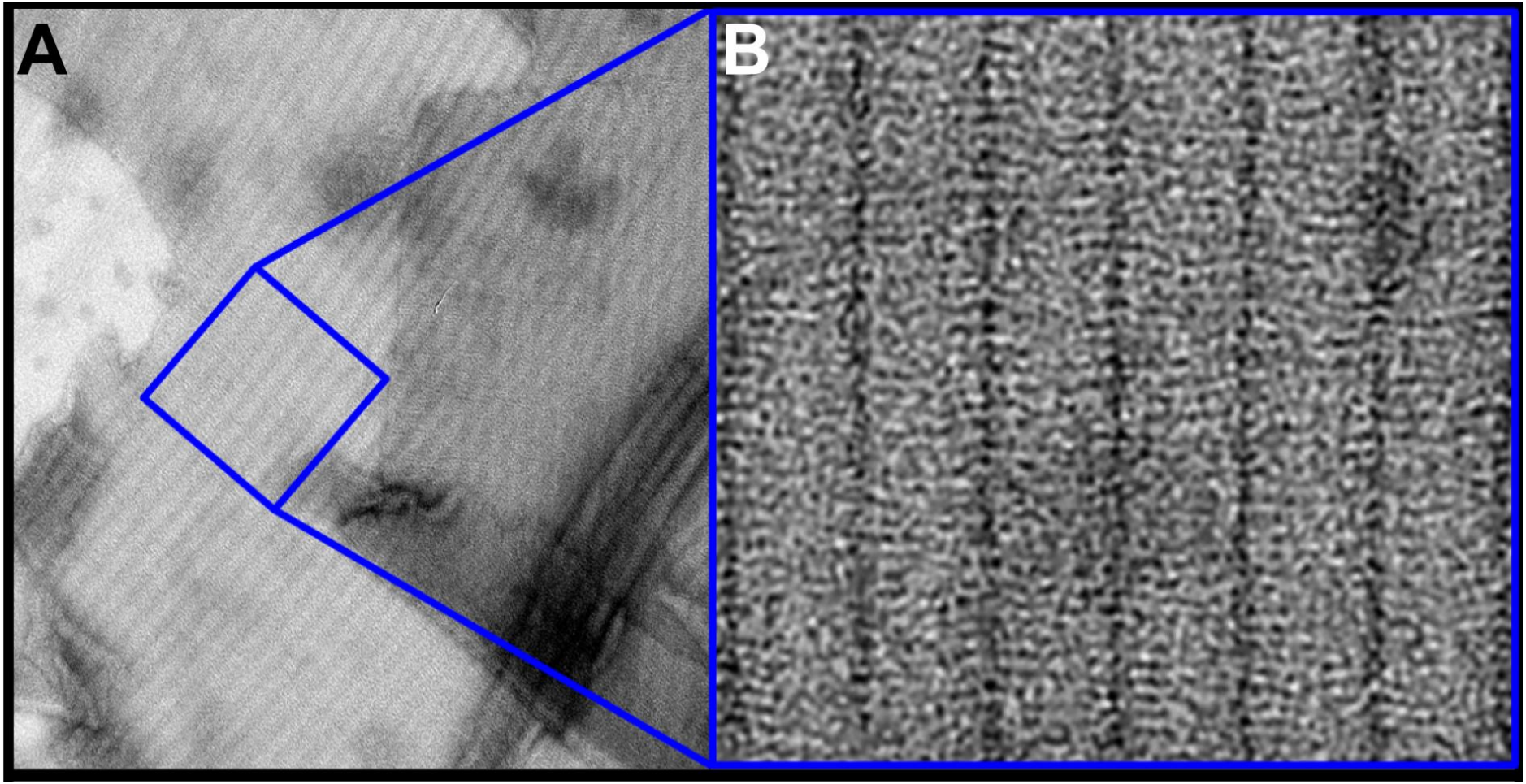


F-BARs Adsorb onto Flat, Rigid Membranes

….you don't have to be experienced to see that this pattern looks completely different … instead of the tubes (small picture to the right)_ … you observe large sheets with striations that look like a "pajama fabric" … or = at a higher magnification - cookies stacked up in a cookie tray

….somehow the molecules were bound to a **FLAT** surface ….

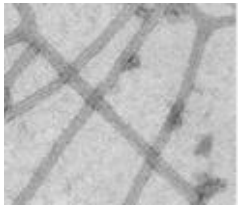


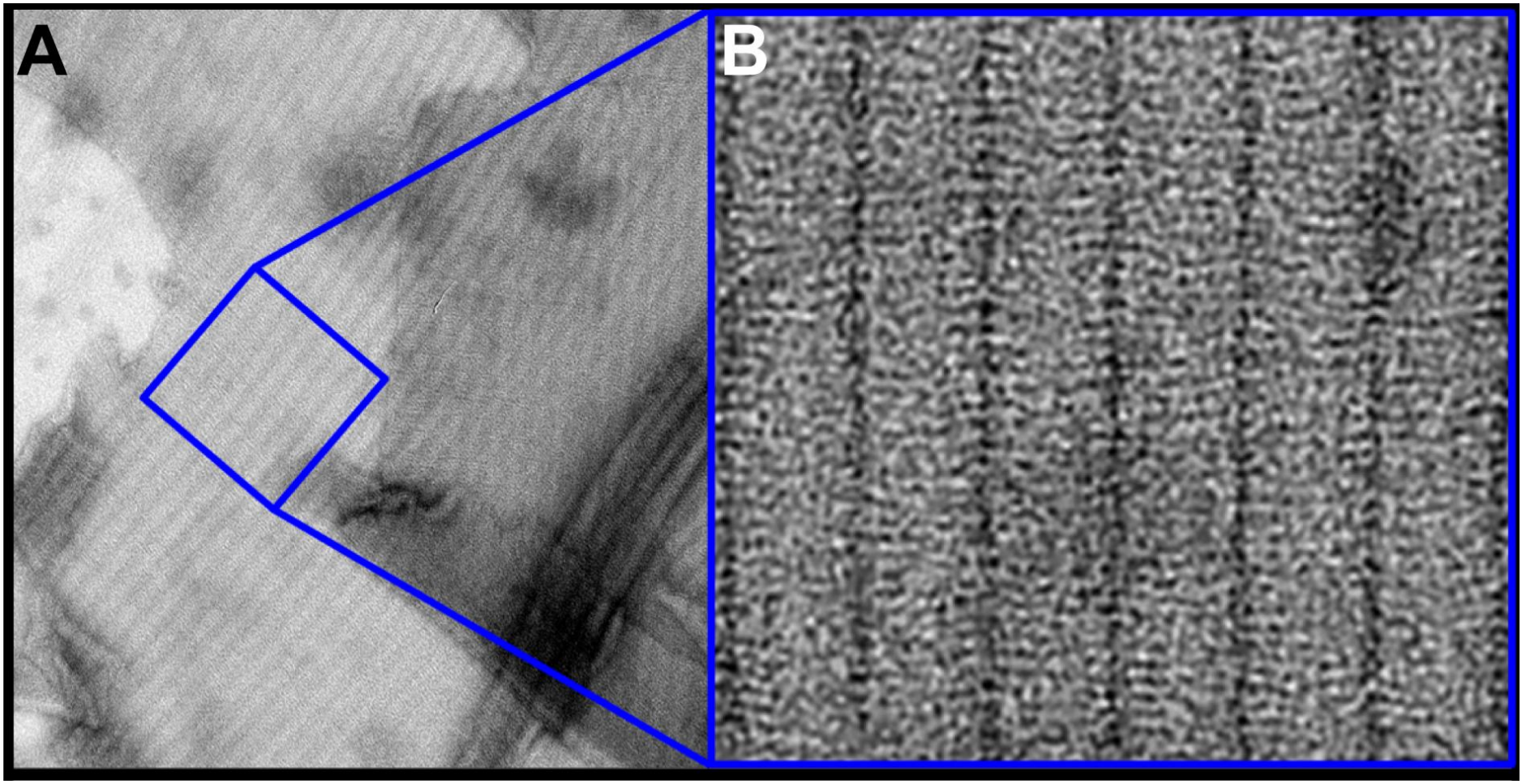


F-BARs Adsorb onto Flat, Rigid Membranes

….how could that be? ….

well … it turned out that 4˚C was below the phase transition temperature for the lipid mix Adam used to generate the samples = the membranes were rigid (look back at the LIPD chapter, slides 60-63 to refresh your memory) = they somehow could bind the protein, but could not bend to turn into tubes

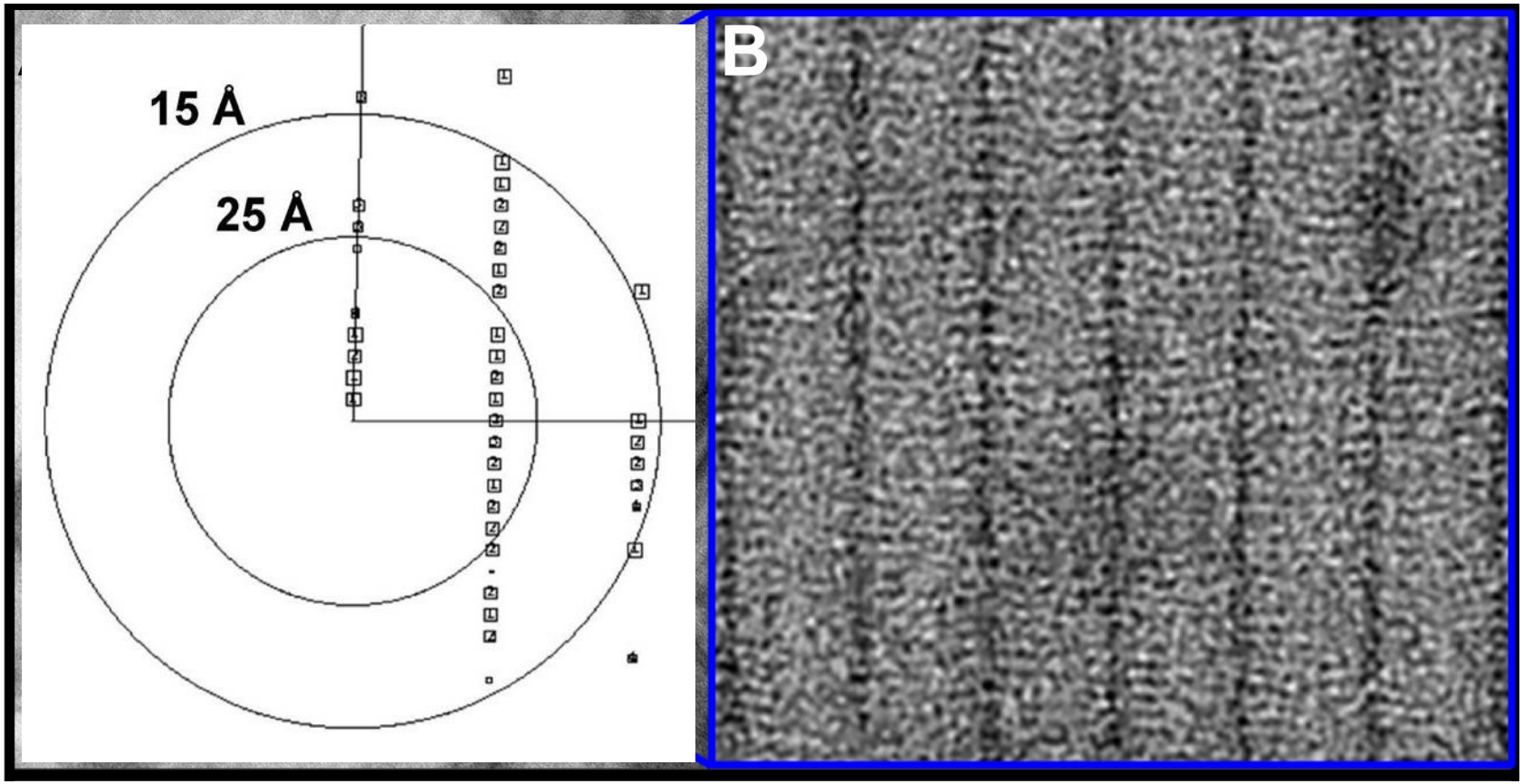




F-BARs Adsorb onto Flat, Rigid Membranes

…in other words … this could be an intermediate stage in membrane remodeling where the system was "arrested" just prior to curvature being induced by the protein –
Question is …. can one use these images of the "flat" state to see how the protein is bound to it?
Short answer is …. YES!

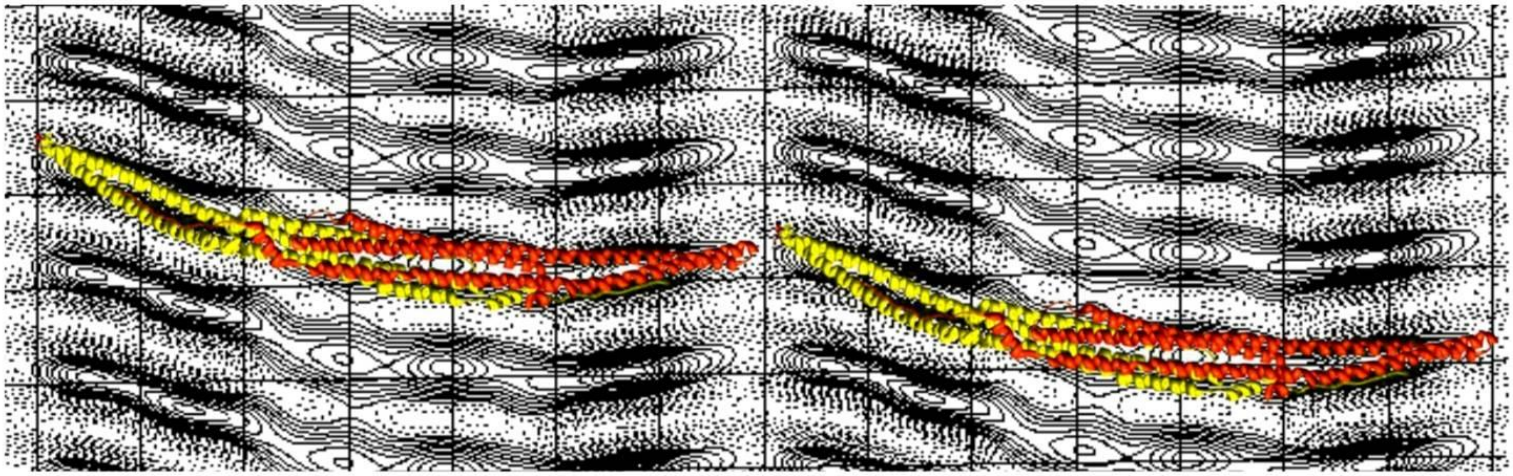
Calculation of the Fourier Transform showed defined diffraction spots …
….this is 2D-crystalline/ordered array of the protein on a flat membrane surface!



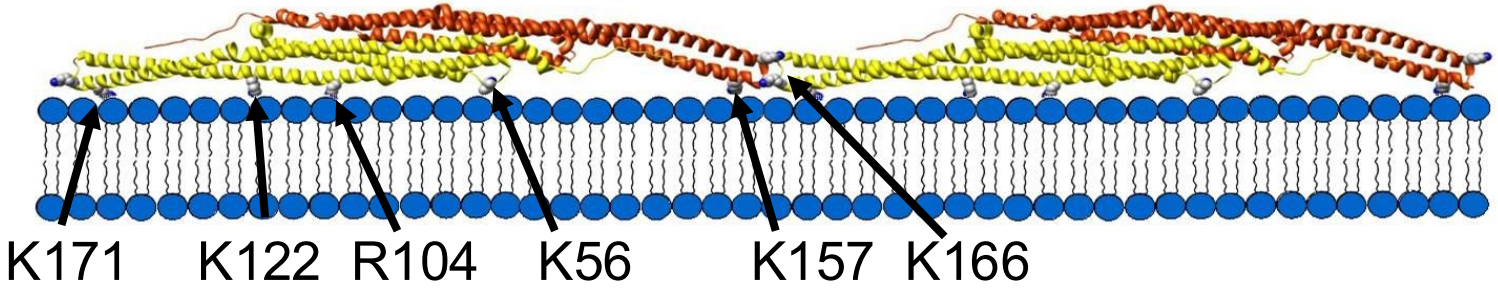
F-BARs Bind Flat Membranes Via a Surface Other than Their Concave Face

Calculating how this looked like in "real space" gave this picture…..showing the outline of the molecules lying on the membrane surface. Despite trying … this sample was so tricky that we could not generate a full 3D structure…but even these simple "projection maps" allowed us to get a rough estimate for how the molecule might fit into the densities we observed.

PERPENDICULAR TO BILAYER SURFACE



PARALLEL TO BILAYER SURFACE



Scrutinizing the model revealed a whole slew of positively charged amino acid sidechains that were NOT part of the concave/curved dimer surface

➔ These newly identified players all were on a "flat" surface on the side of the dimer, suggesting that these amino acid sidechains are responsible for the initial engagement of dimers with the low curvature membranes

F-BARs Bind Flat Membranes Via a Surface Other than Their Concave Face

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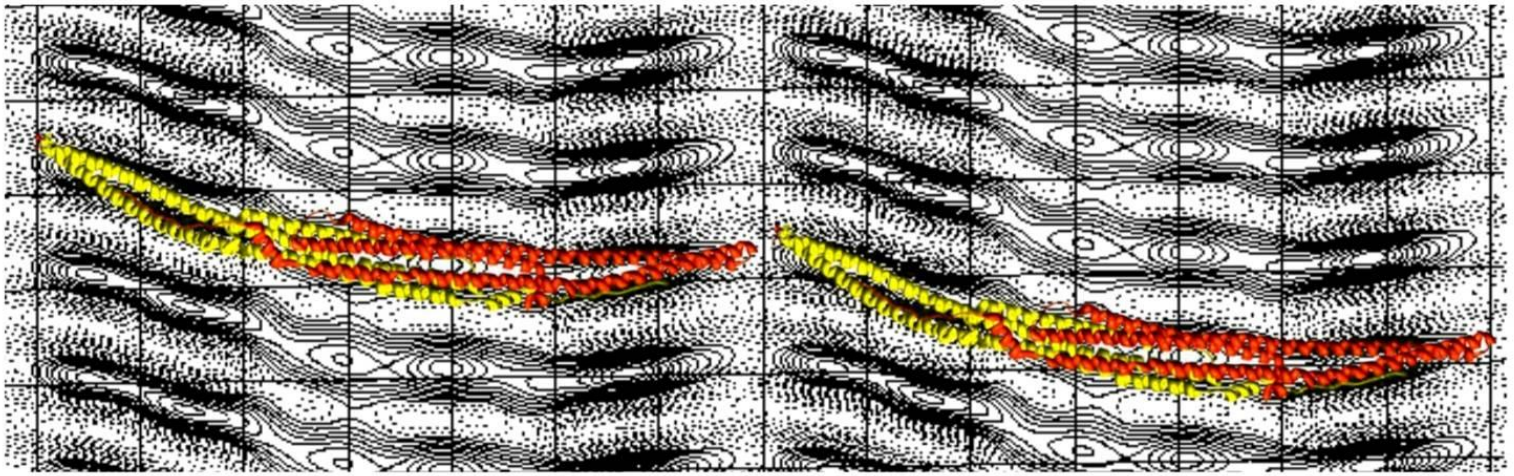
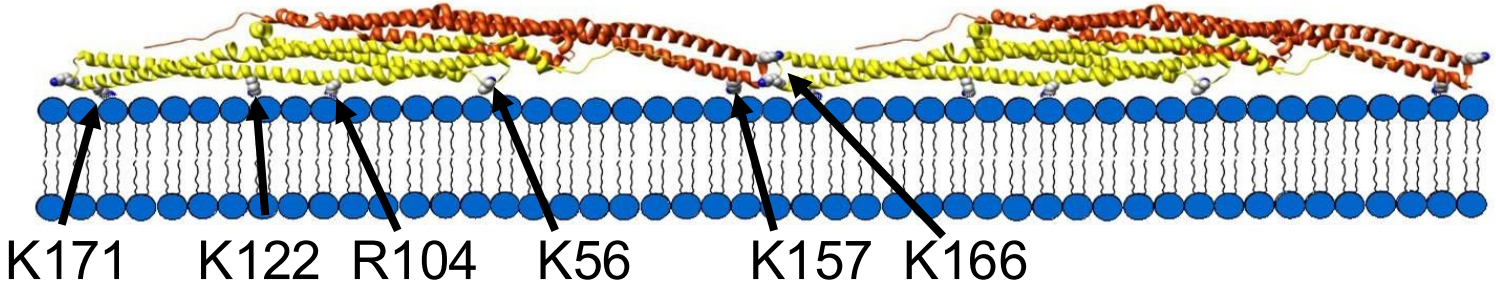
➔Mutational analysis provided further support for this idea and allowed Adam to propose an actual (and first) mechanistic model for how F-BAR domains generate curvature in membranes …..

PERPENDICULAR TO BILAYER SURFACE



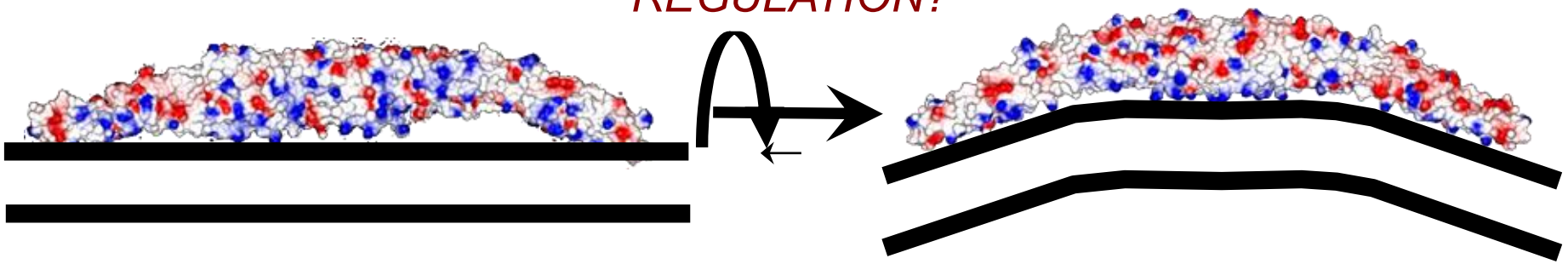
PARALLEL TO BILAYER SURFACE



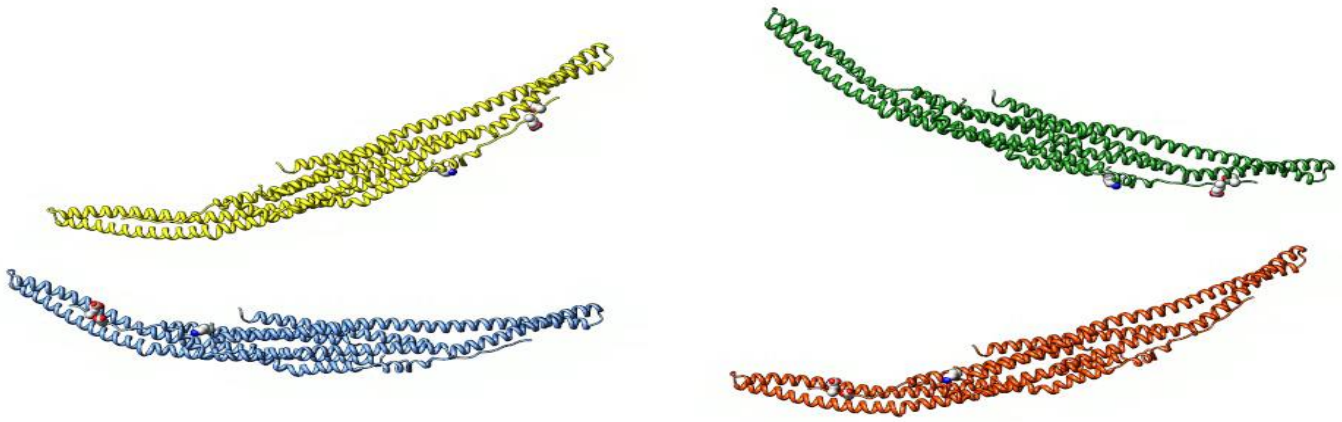
Regulated Self-Assembly of the F-BAR Coat

View Parallel with the Bilayer, Induction of Local Curvature via a ~55° rotation

REGULATION?



View Perpendicular to the Bilayer, Formation of Lateral Contacts (watch the short animation to get an appreciation how rotation of the dimers causes the coat to assemble and the membrane to deform)



The very simple short clip shows you the principal steps of the process, starting with four dimers bound to a flat surface, then rotating to expose the concave surface to the membrane and sliding into register to form the coat. In reality, the rotation and alignment occur simultaneously …

If you worked through this presentation

THANK YOU

I hope you enjoyed this story and that it gave you an appreciation for how biomedical/biophysical research generates the data that over time find their way into textbooks

If you would like to read the full research paper, you can find it here:

Frost etal (2008) Cell 132:807-17 DOI: 10.1016/j.cell.2007.12.041

And a second study, describing the structure of a N-BAR coat bound to membranes

Mim etal (2012) Cell 149(1):137-45. doi: 10.1016/j.cell.2012.01.048.