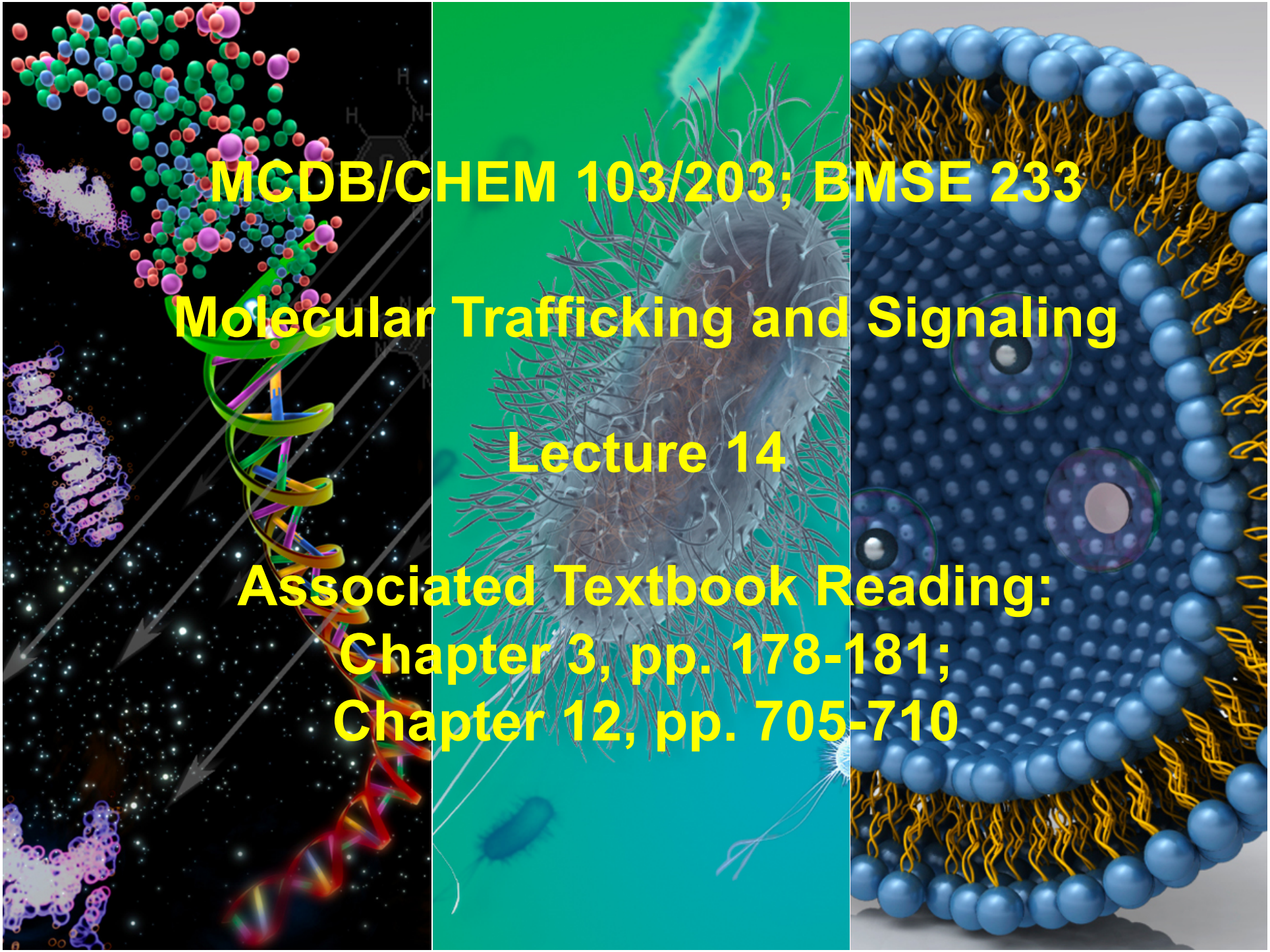


MCDB/CHEM 103/203; BMSE 233

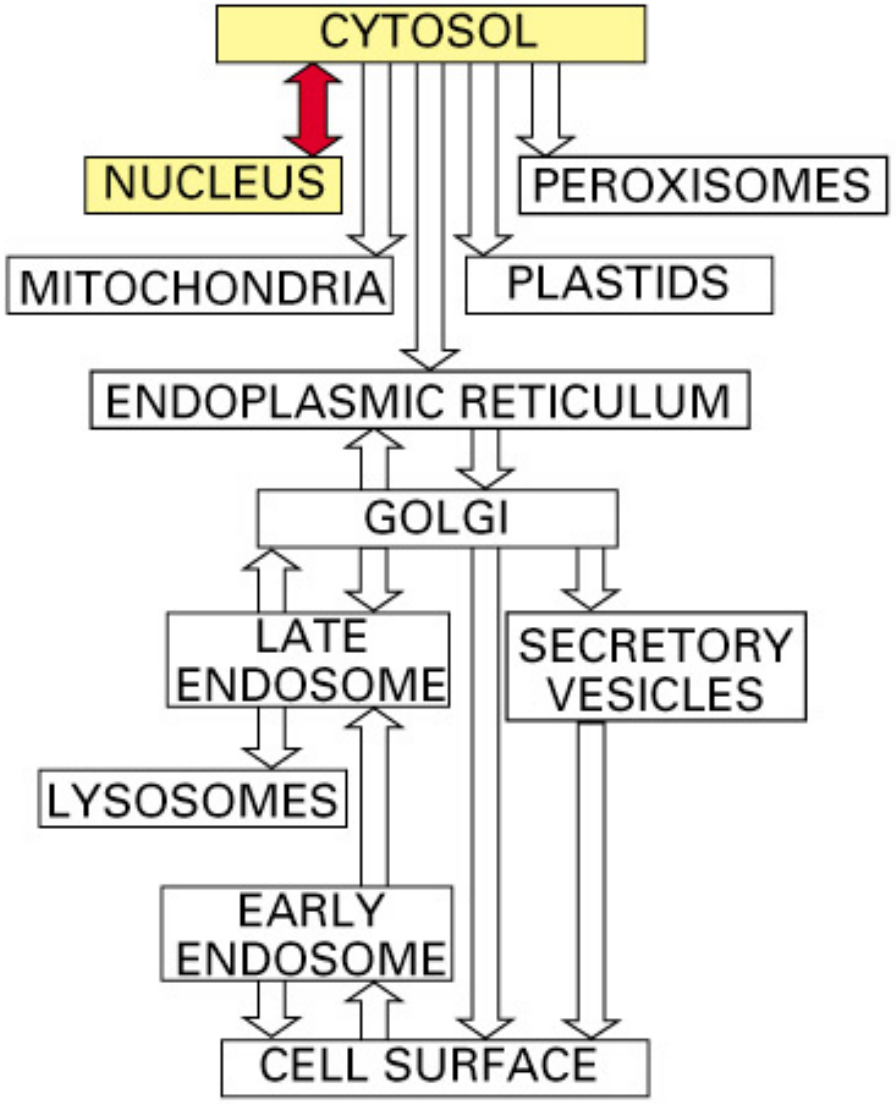
Molecular Trafficking and Signaling

Lecture 14

Associated Textbook Reading:
Chapter 3, pp. 178-181;
Chapter 12, pp. 705-710

Cytoplasm-Nucleus Transport

Cytoplasm-Nucleus Transport



general features:

gated transported via NPC

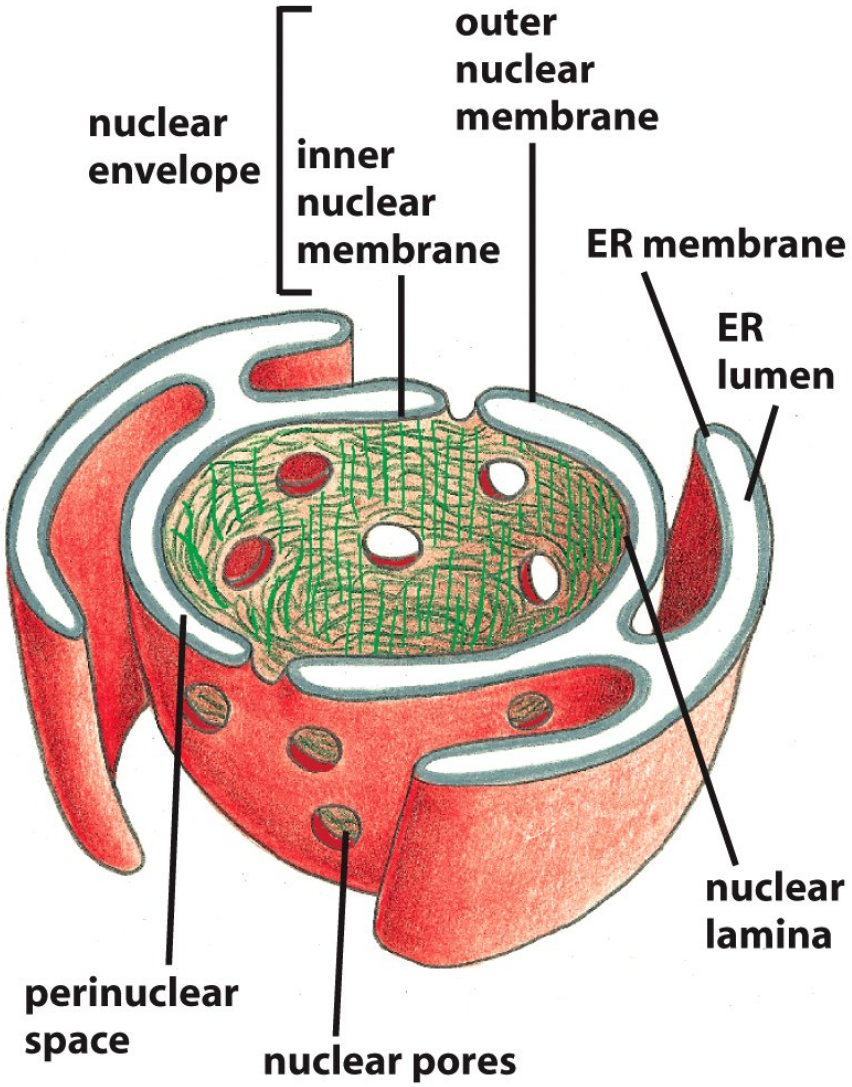
bi-directional transport (histones (in), ribosomes (out))

both import and export signal sequences are present

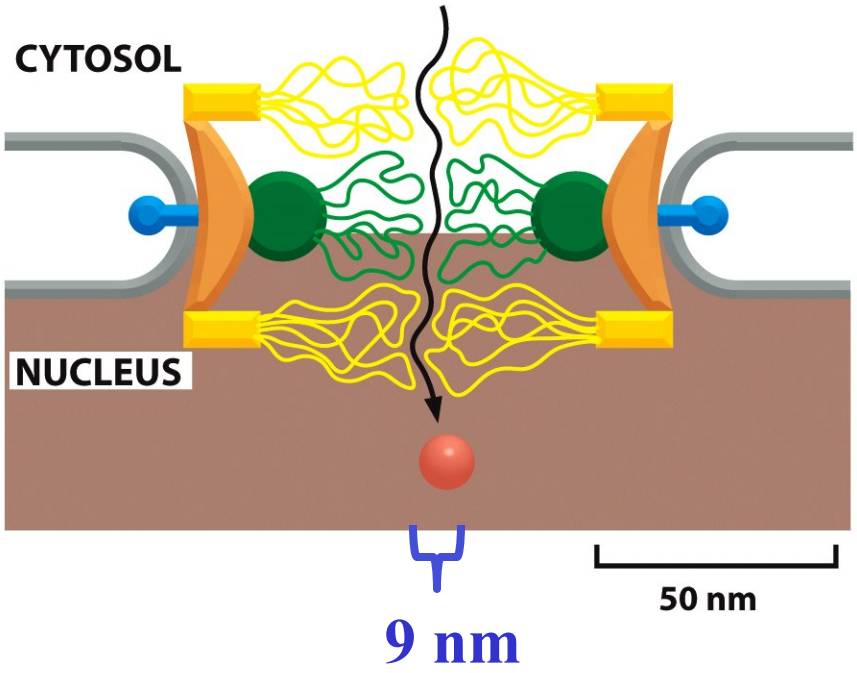
Post-translational import

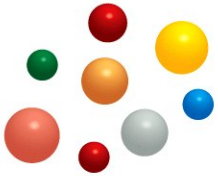
imported as folded proteins

Cytoplasm-Nucleus Transport



Passive Transport of Small Molecules through the NPC





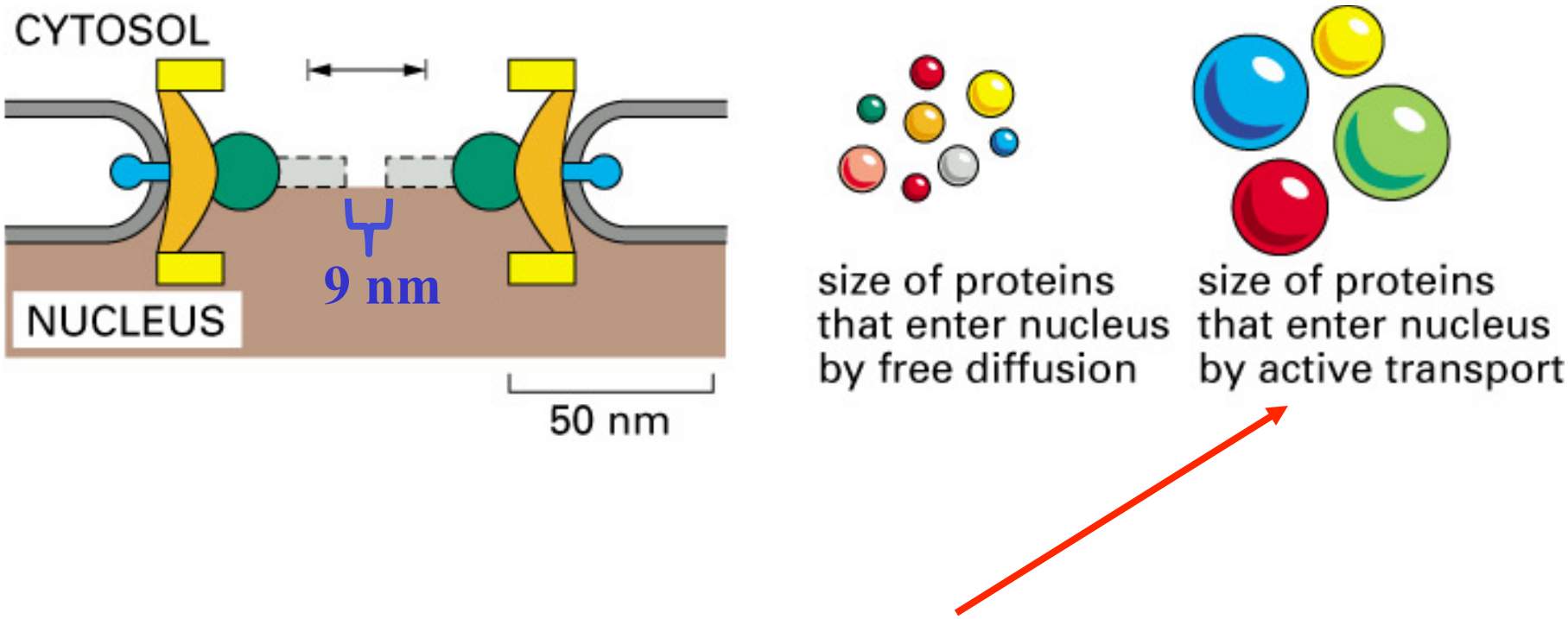
size of molecules
that enter nucleus
by free diffusion

water, ions,
small metabolites,
small proteins (<60 kD)

pore size estimated from
free diffusion experiments

Protein larger than 60 kD can be found in the nucleus. How are these transported?

Transport of a Ribosome by the NPC



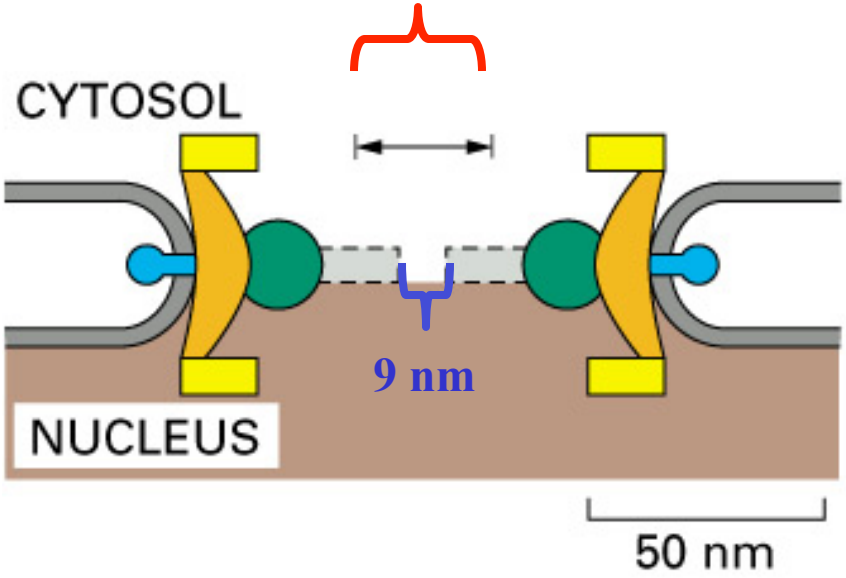
For example, ribosome assembly takes places in the nucleus but the assembled ribosome needs to be in the cytosol to translate mRNA. Thus, a completely assembled ribosome composed of many proteins and RNAs (>20 nm, >1000 kD) must go through the NPC to enter the cytosol.

How does this happen given that the pore size of free diffusion is 9 nm?

Transport Through the Nuclear Pore Complex

Thus NPC functions as a diaphragm with adjustable pore size

The large pore size of NPC during the active transport can allow the large protein complex such as a ribosome to go through



water, ions, small metabolites, small proteins (<60 kD)

protein or protein complex (>60 kD)

size of proteins that enter nucleus by free diffusion

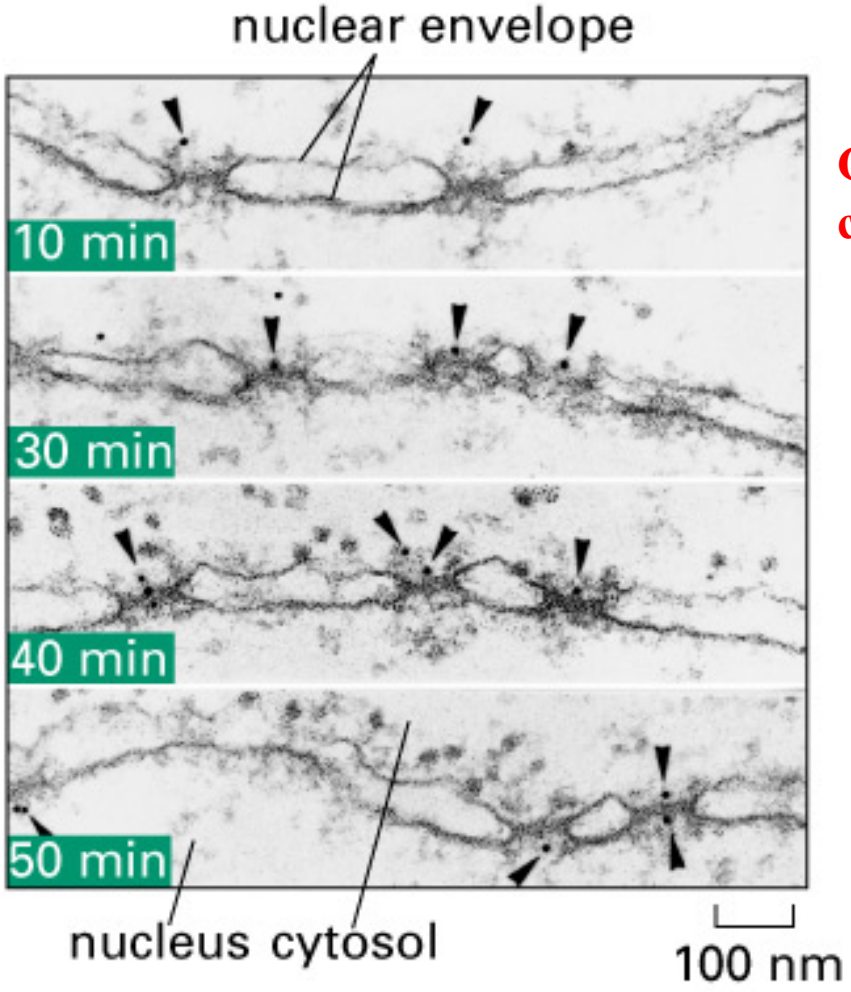
size of proteins that enter nucleus by active transport

passive diffusion no energy required

active transport energy required

Evidence of Active Transport by the Nuclear Pore Complex

Gold particles coated with a peptide containing a nuclear localization signal injected into a cell (gold is electron dense allowing the visualization (arrows) by electron microscopy)



Gold particles first attached to the cytoplasmic side of a NPC

Gold particle then moved into the NPC

Gold particle has appeared in the nucleus

Features of NLS (nuclear localization/import signal)

One or two continous stretches of amino acids enriched with positive charge residues

No absolute sequence consensus

May be located anywhere within a protein

In addition to the NLS, a NES (nuclear export signal) also exists

Features of NES (nuclear export signals)

One stretch of amino acids enriched with Leucine

No absolute sequence consensus

May be located anywhere within a protein

How to identify a transport signal on a cargo protein?

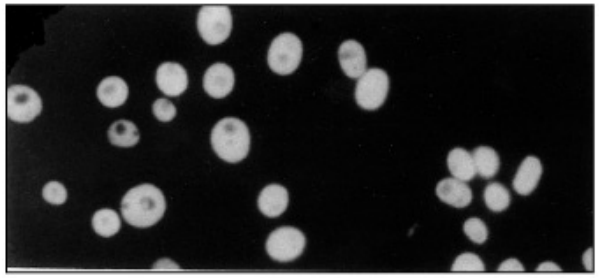
How to identify the transport machinery?

The same principles to be discussed can be used for other transport events

How was the NLS identified?

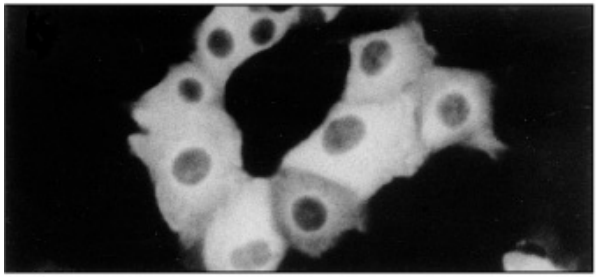
(A) LOCALIZATION OF T-ANTIGEN CONTAINING ITS NORMAL NUCLEAR IMPORT SIGNAL

Pro–Pro–Lys–Lys–Lys–Arg–Lys–Val–



(B) LOCALIZATION OF T-ANTIGEN CONTAINING A MUTATED NUCLEAR IMPORT SIGNAL

Pro–Pro–Lys–Thr–Lys–Arg–Lys–Val–



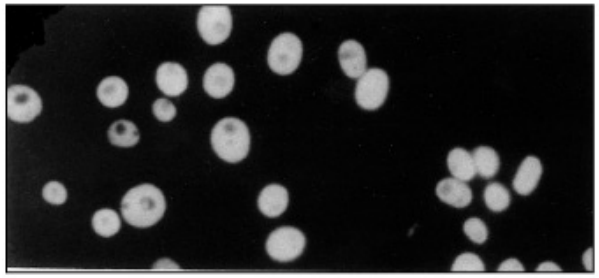
Mutational analysis initially demonstrates that KKKRK is necessary for the nuclear localization of T-antigens

Can you conclude that KKKRK is an NLS?

How was the NLS identified?

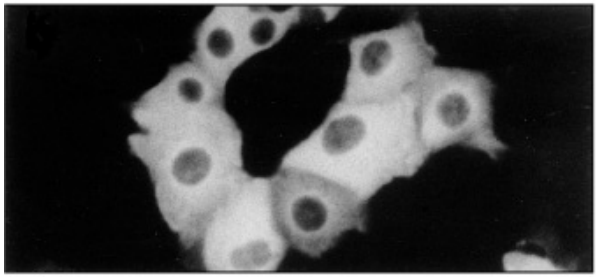
(A) LOCALIZATION OF T-ANTIGEN CONTAINING ITS NORMAL NUCLEAR IMPORT SIGNAL

Pro–Pro–Lys–Lys–Lys–Arg–Lys–Val–



(B) LOCALIZATION OF T-ANTIGEN CONTAINING A MUTATED NUCLEAR IMPORT SIGNAL

Pro–Pro–Lys–Thr–Lys–Arg–Lys–Val–



Mutational analysis initially demonstrates that KKKRK is necessary for the nuclear localization of T-antigens

Can you conclude that KKKRK is an NLS?

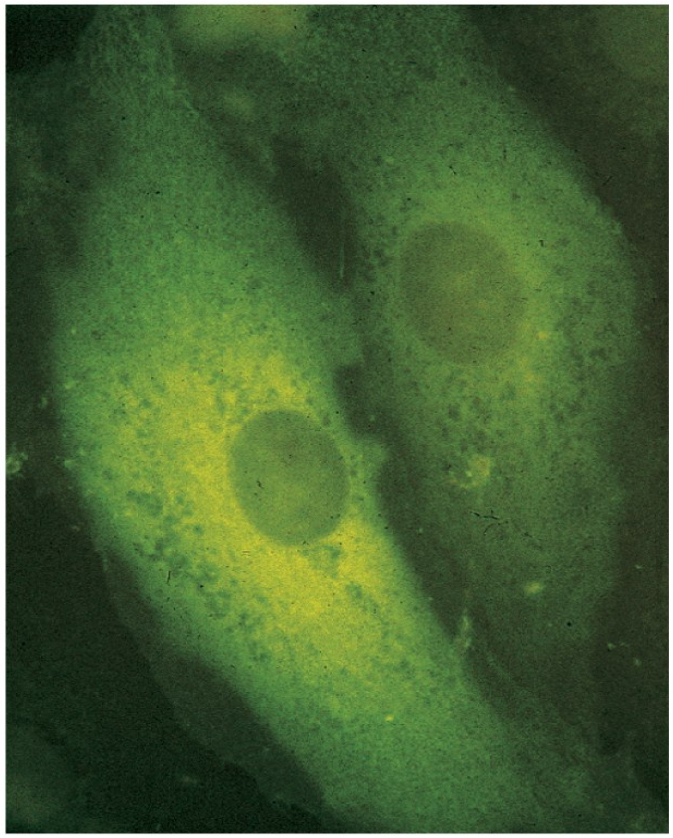
No. there are many other possibilities. For example, KKKRK may be required for the folding of the cargo protein.

How to show if KKKRK is sufficient?

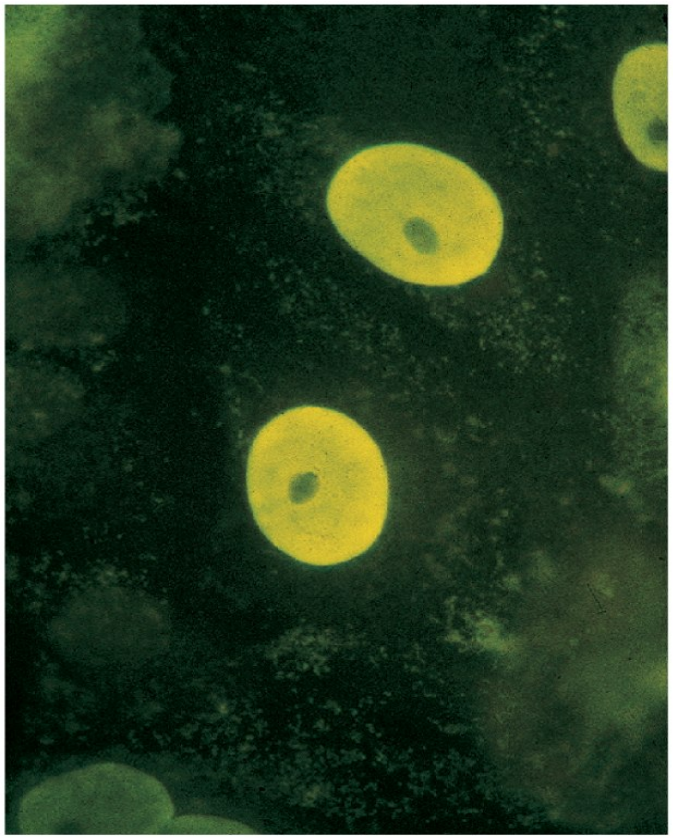
Demonstration of sufficiency of KKKRK as a NLS

The easiest way is to gene recombinant DNA approaches to fuse the KKKRK Sequence to a cytoplasmic protein and to test whether the fusion protein can be targeted to the nucleus after transfection (transfection is a gene transfer process)

(a) **pyruvate kinase**



(b) **pyruvate kinase – KKKRK fusion**



Since KKKRK is both necessary and sufficient, we can conclude that it is a NLS

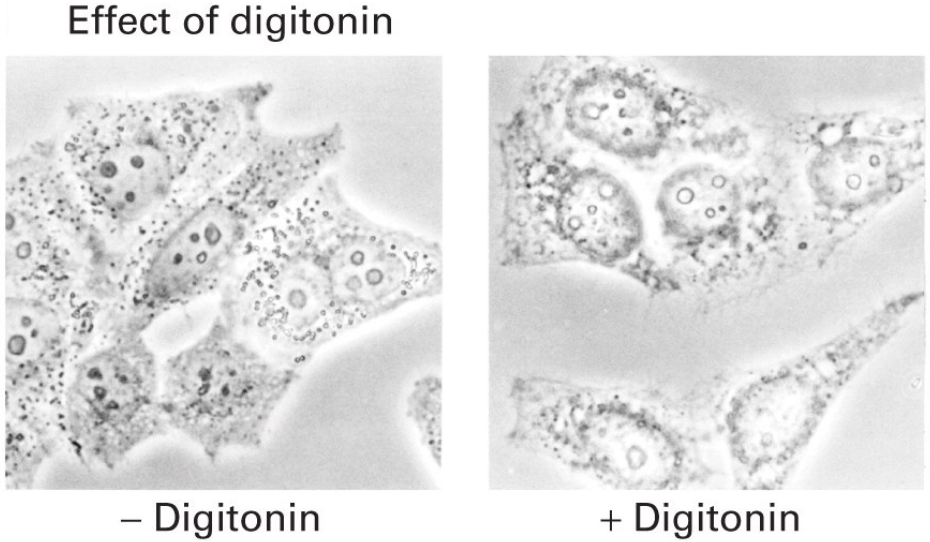
How to identify a transport signal on a cargo protein?

(DNA transfection approach; to define sufficiency & necessity)

How to identify the transport machinery?

The same principles to be discussed can be used for other transport events

Nuclear Transport is Abolished After the Permeabilization of the Plasma Membrane



+ digitonin

Digitonin makes holes selectively on the plasma membrane and leaves the intracellular membranes intact

↓

Cytoplasmic content is lost but not most nuclear proteins (since nuclear membrane remains intact).

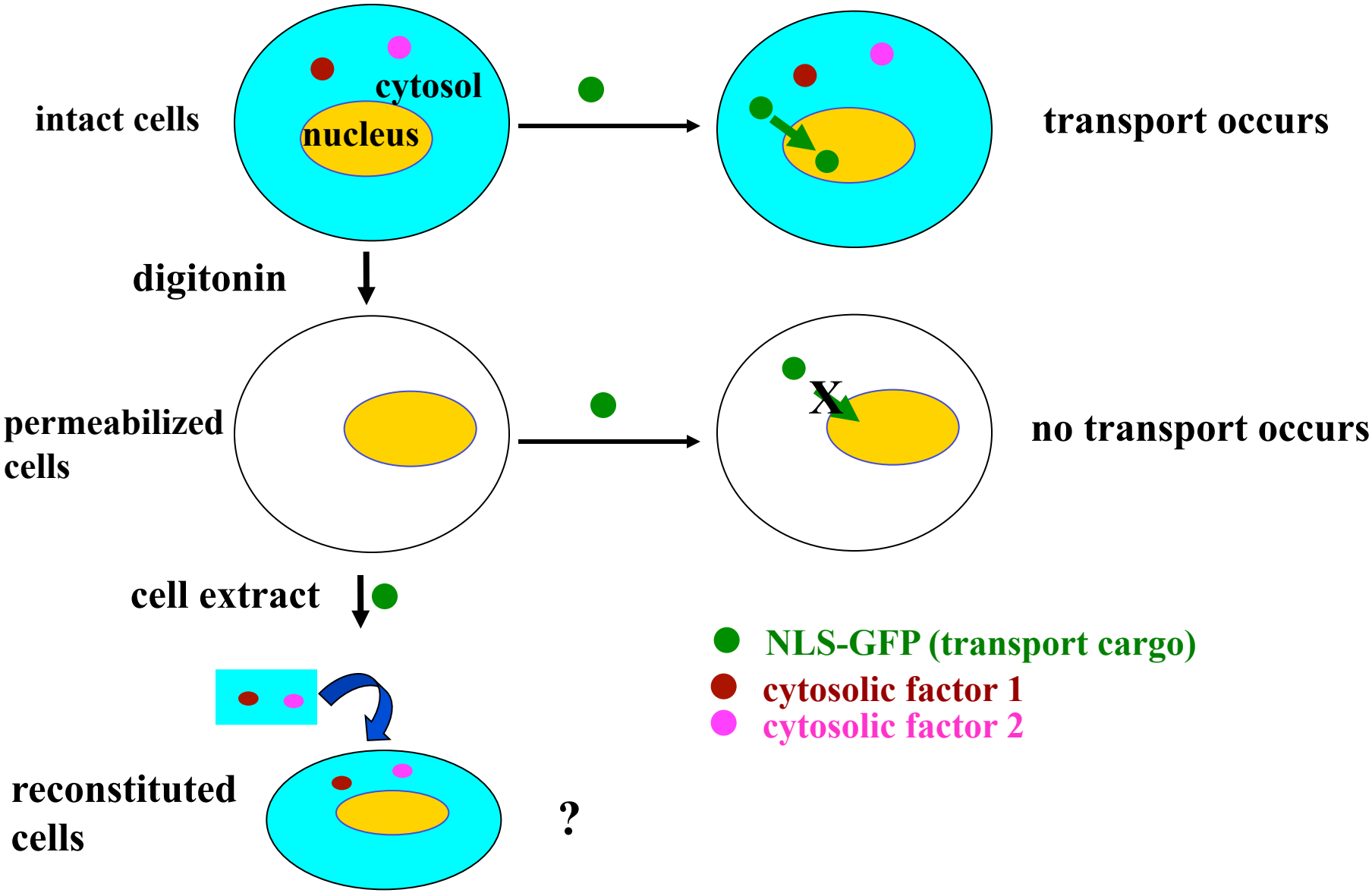
↓

1. Add GFP-NLS fusion (>60 kDa)
2. Incubate
(to allow transport to occur)
3. wash to remove cytoplasmic fusion
(to examine nuclear import)

No nuclear transport can be detected (as shown by the lack of green fluorescence in the nucleus)

The above experiment suggests that cytosolic factors might be required for the nuclear transport, but there are other explanations as well.

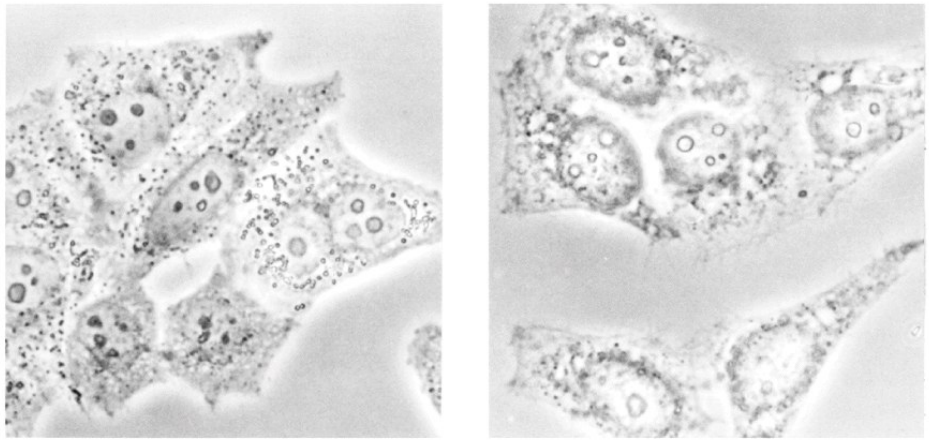
In vitro reconstitution of the nuclear transport



cytosolic factors include importins, exportins, Ran, NTF2 (discussed soon)

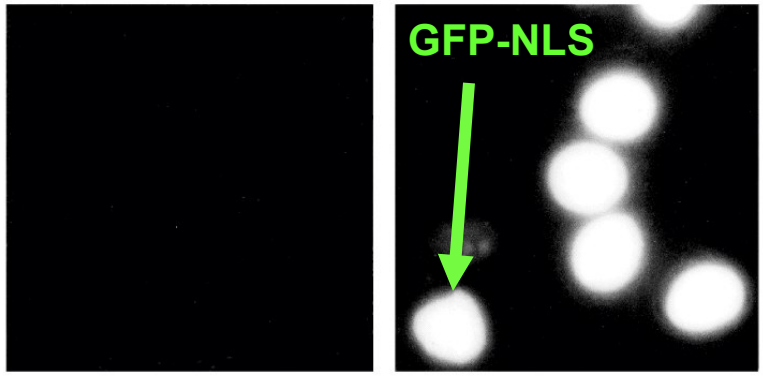
Nuclear Transport of Permeabilized Cells can be Restored After the Addition of Cytosol Extracts

(a) Effect of digitonin



– Digitonin + Digitonin

(b) Nuclear import by permeabilized cells



– Lysate + Lysate

+ digitonin

Digitonin makes holes selectively on the plasma membrane and leaves the intracellular membranes intact

↓

Cytoplasmic content is lost but not most nuclear proteins (since nuclear membrane remains intact).

↓

1. Add GFP-NLS fusion (>60 kDa)
2. Incubate in the presence of cytosol extract (providing the missing cytosolic factors)
3. wash to remove cytoplasmic fusion

↓

nuclear transport resumes

The above experiment shows that cytosolic factors are required for nuclear transport

How to identify a transport signal on a cargo protein?

(DNA transfection approach; to define sufficiency & necessity)

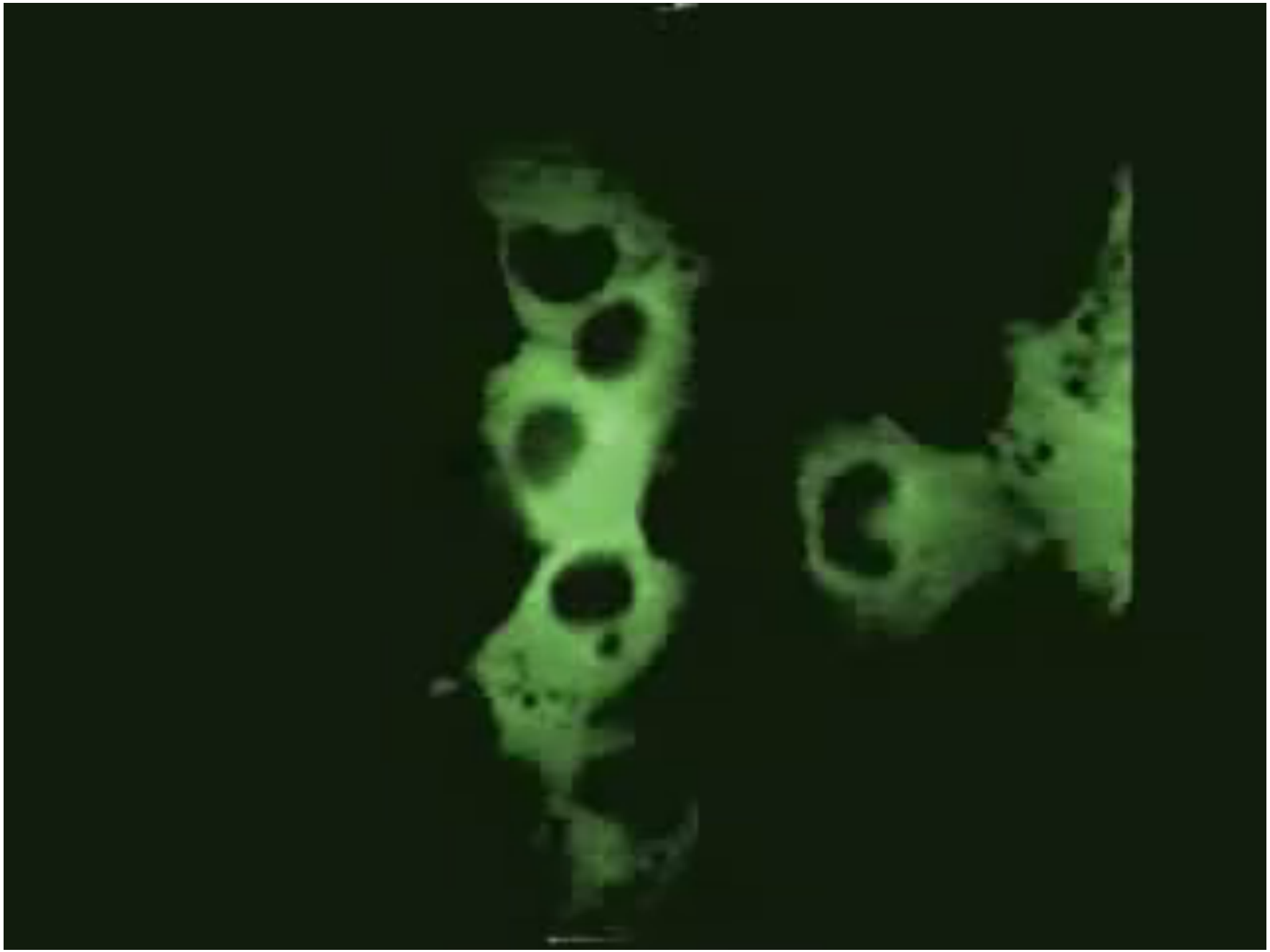
How to identify the transport machinery?

(*In vitro* reconstitution is also a common method to investigate a cellular process such as protein transport)

(Alternatively, genetic approaches can be used to study the transport mechanism)

The same principles to be discussed can be used for other transport events

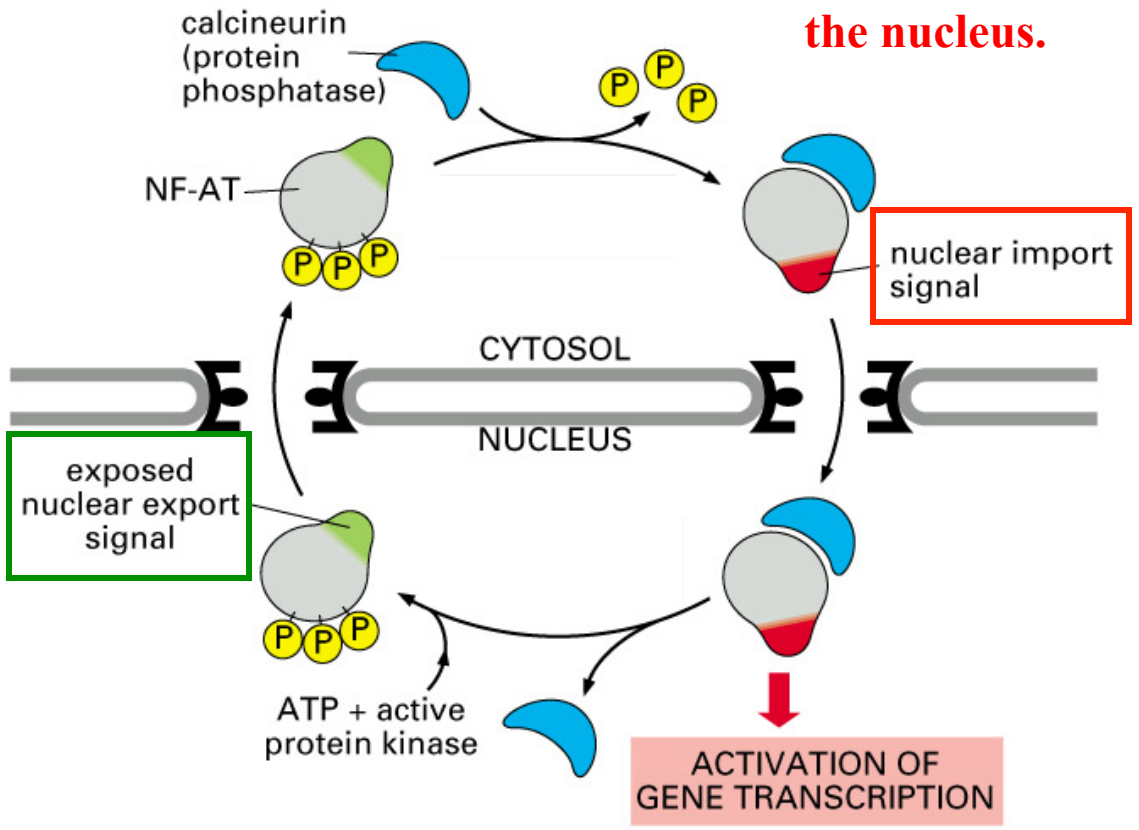
Regulation of NF-AT Nuclear Import by Ca^{2+} : movie



Example of Regulation of Nuclear Transport

NF-AT (nuclear factor of activated T cells) is a gene regulatory protein required for the proper immune response. NF-AT stays in the cytosol but moves into the nucleus upon the activation of T-cell. NF-AT contains both a NLS and a NES.

NLS allows NF-AT to enter the nucleus.

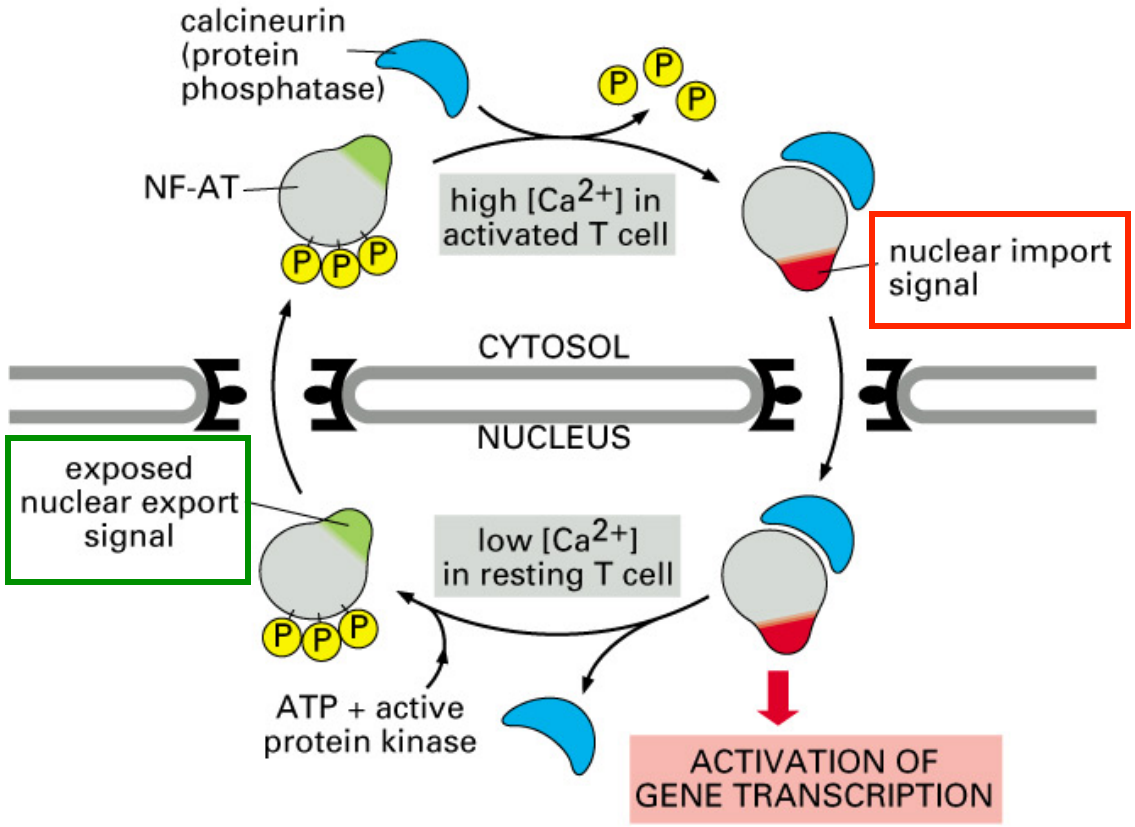


NES allows NF-AT to exit the nucleus.

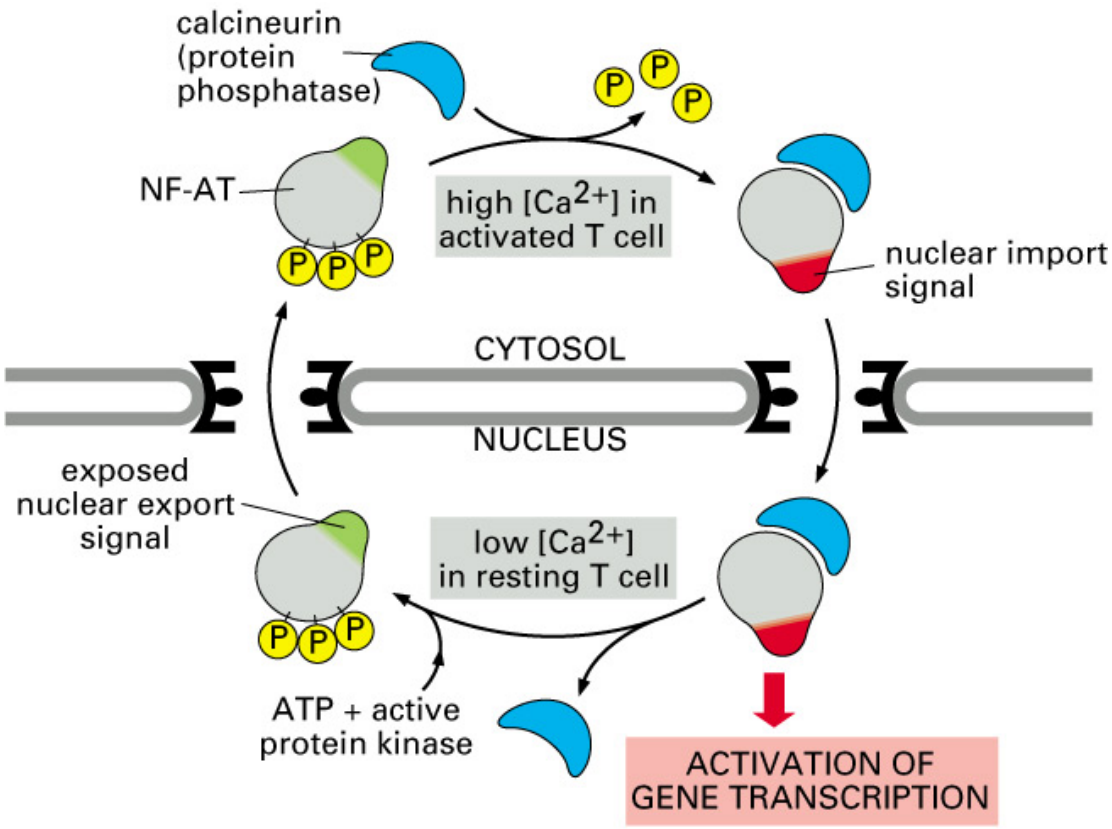
Regulatory Mechanism of Nuclear Transport Involving NF-AT

T-cell activation (an immunologic event) causes an increase of intracellular Ca $^{2+}$ concentration which in turn activates calcineurin (a protein phosphatase).

Active calcineurin can then regulate NLS and NES functions by phosphorylation/ dephosphorylation.



Regulatory Mechanism of Nuclear Transport Involving NF-AT



The phosphorylation of NF-AT masks the NLS and exposes the NES (staying in the cytosol)

increased Ca2+ in a stimulated T-cell cytoplasm activates calcineurin

activated calcineurin binds to NF-AT and dephosphorylates it

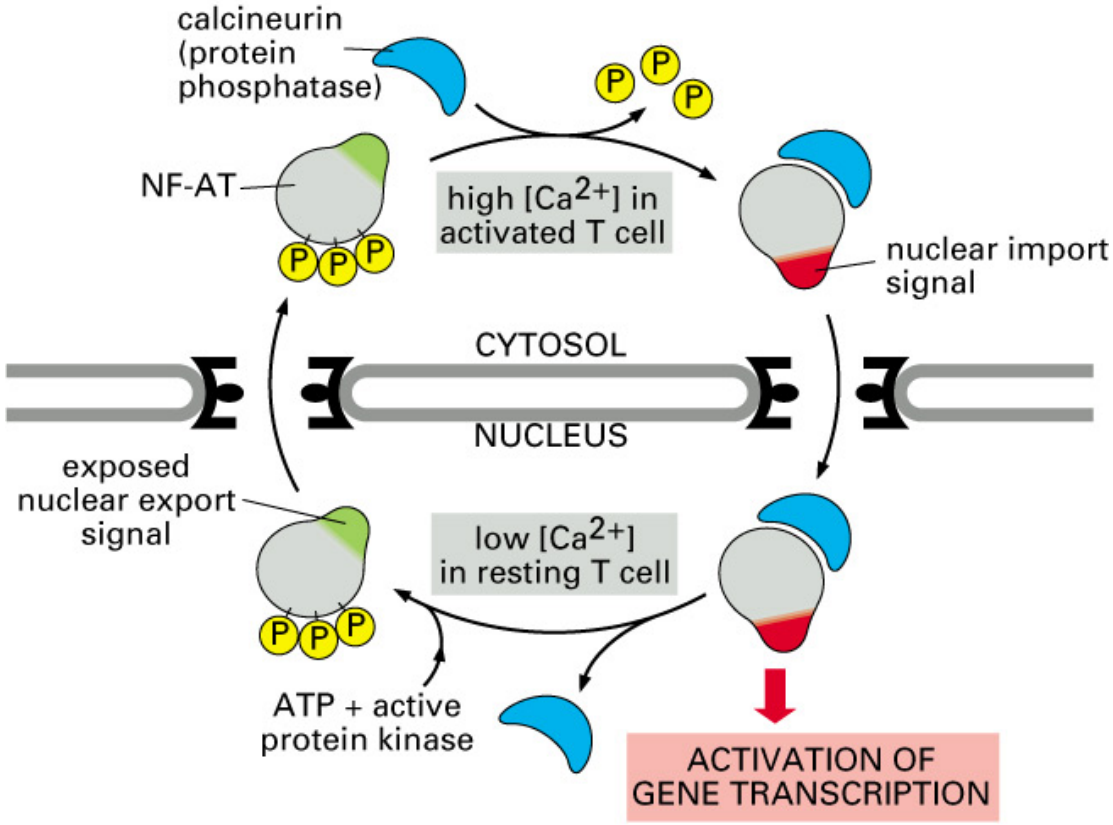
NES is masked and NLS exposed

NF-AT moves into the nucleus

NF-AT alters gene transcription in cells of the activated immune system

Calcineurin is a major target of immunosuppressive drugs for transplant rejection and autoimmune disease.

Regulatory Mechanism of Nuclear Transport Involving NF-AT



When T cell is no longer stimulated, cytoplasmic Ca2+ conc. is decreased

Decreased Ca2+ leads to the inactivation of calcineurin

Inactive calcineurin dissociates from NF-AT and NF-AT becomes mostly phosphorylated and cytosolic

NES exposed and NLS masked (moving into the cytosol)

NF-AT moves out of the nucleus of a stimulated T cell

Several Concepts in Regulation of Cellular Processes:

protein conformational changes

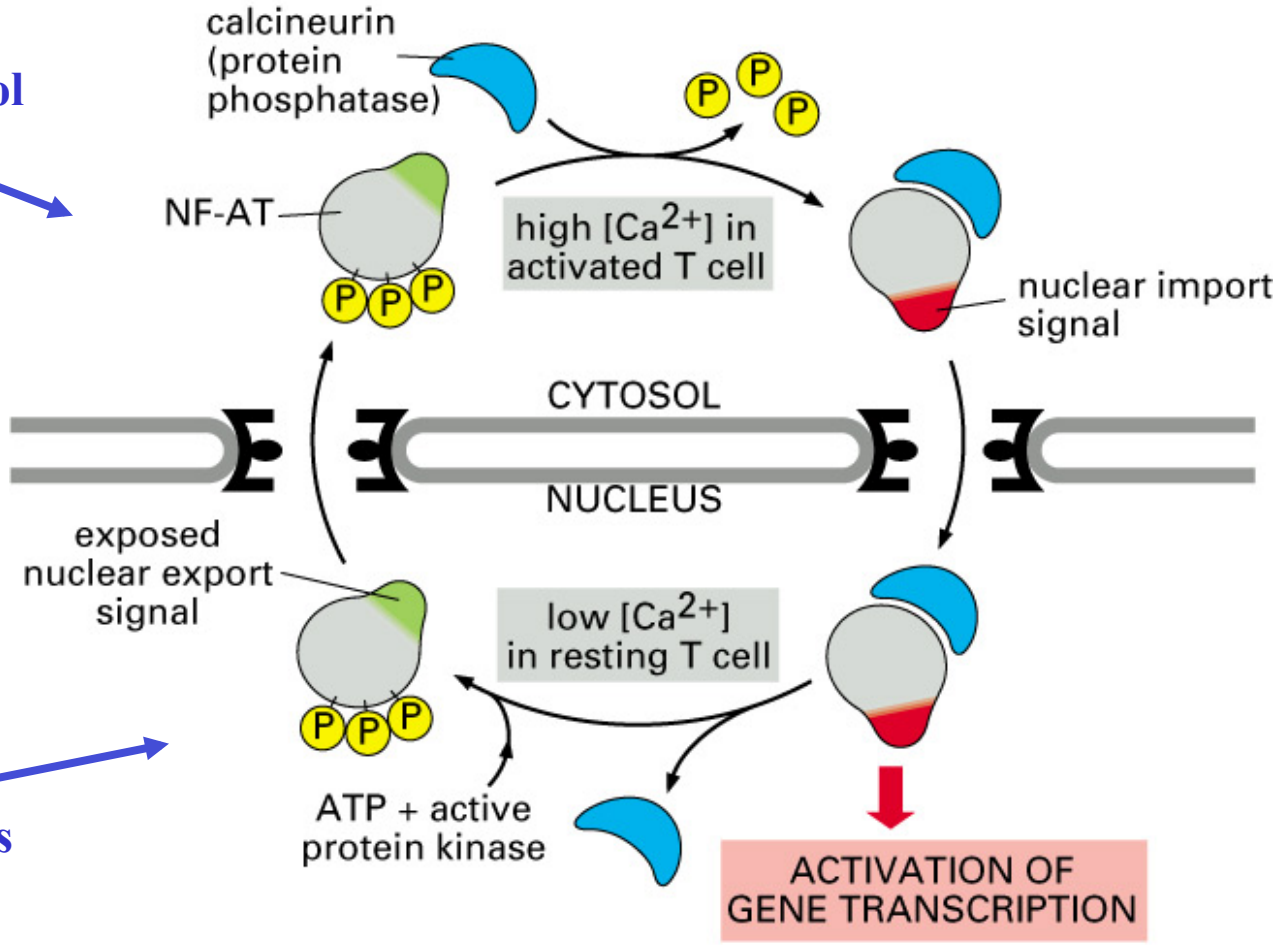
Ca2+ as a signaling molecule

Regulation of [Ca2+]

[Ca2+] between the cytosol and the nucleus

steady-state localization of a protein ≠ site of its action

NF-AT resides in the cytosol of resting T cells

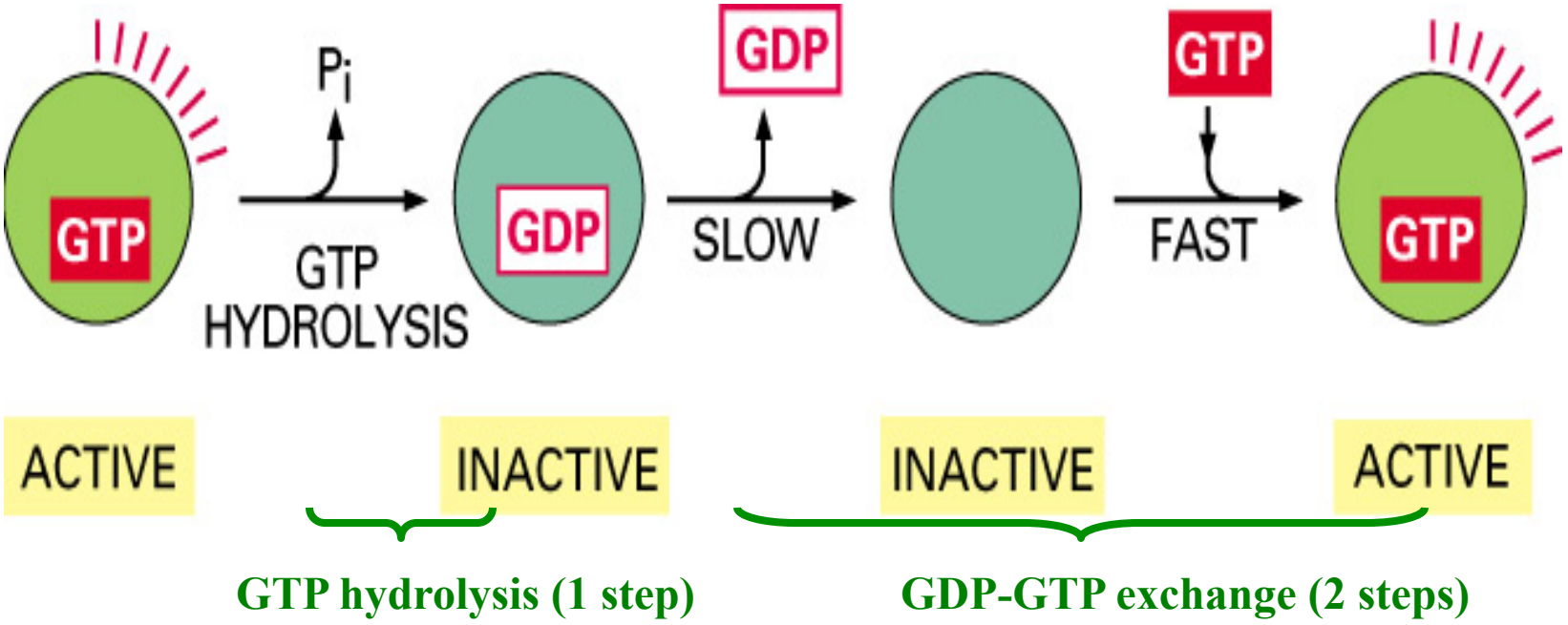


NF-AT functions in the nucleus of stimulated T cells

What is the machinery mediating nuclear transport?

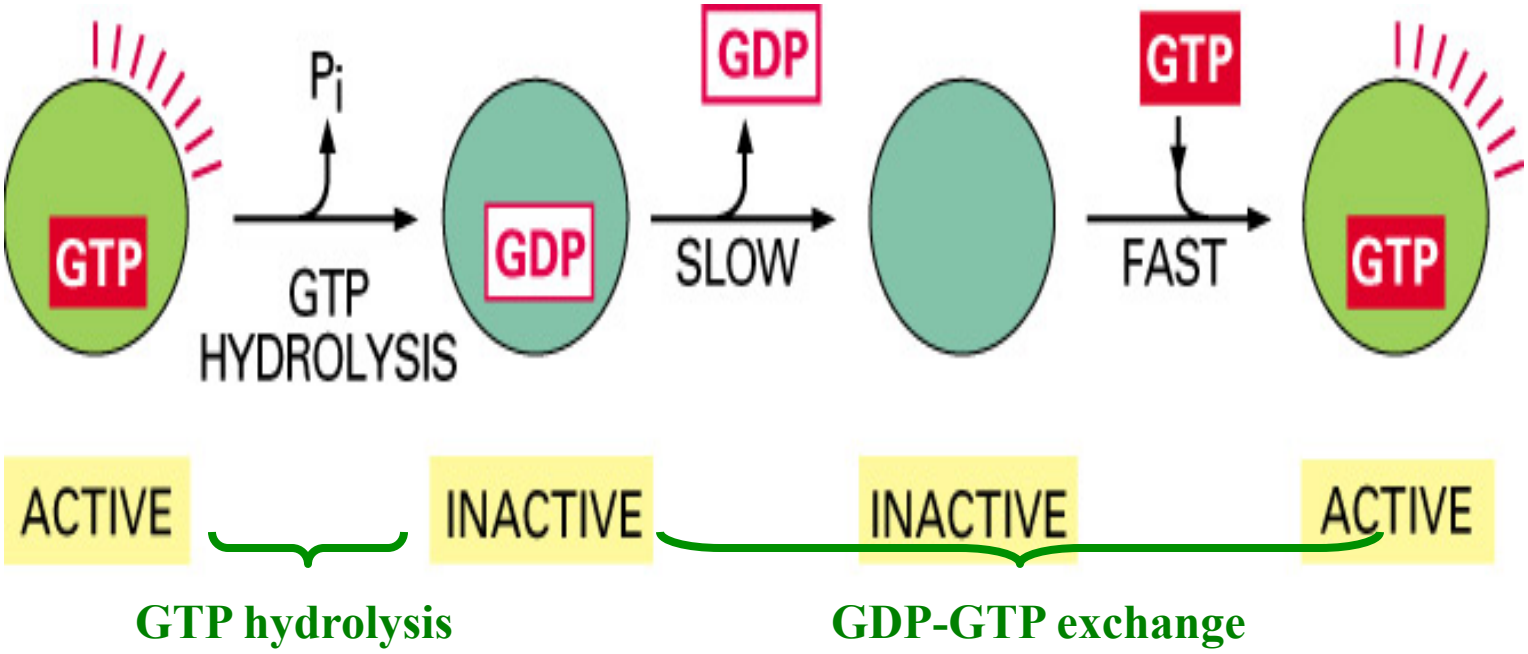
Small GTPases Often Function as Major Regulators of Transport

A GTPase is a protein which can bind and hydrolyze GTP.
It is present in two forms, GTP-bound (the active form) and GDP-bound (the inactive form)
The conversion of two forms is a 3-step process as shown below.



Small GTPases involved in protein transport work by controlling the assembly and disassembly of the transport machinery at specific compartments

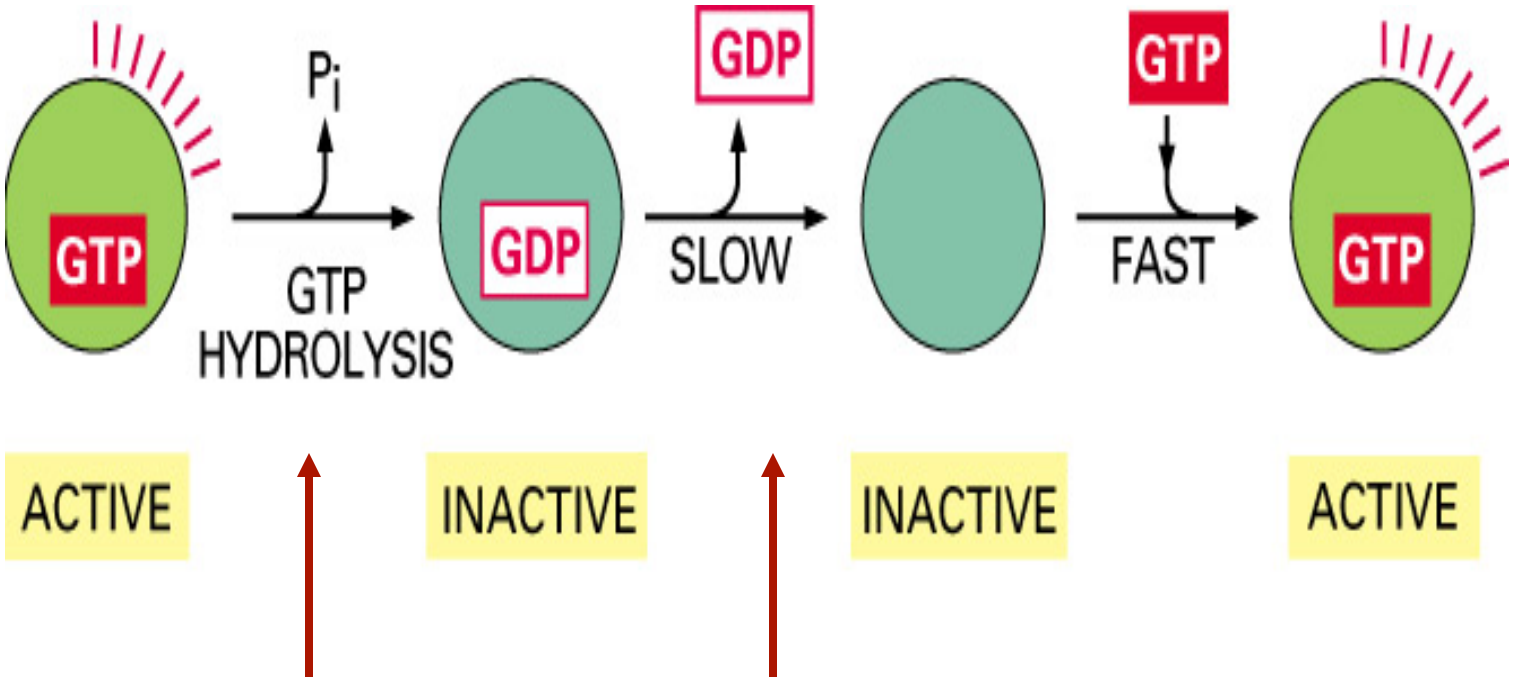
Two Families of GTPases: Large and Small GTPases
(each consisting of many members)



Small monomeric GTPases are well known to mediate intracellular protein transport and signaling.

Large GTPases (heterotrimeric G proteins, ex. Gαβγ) primarily mediate signaling on the plasma membrane.

The 3 Steps of a GTPase Cycle are not Kinetically Equal



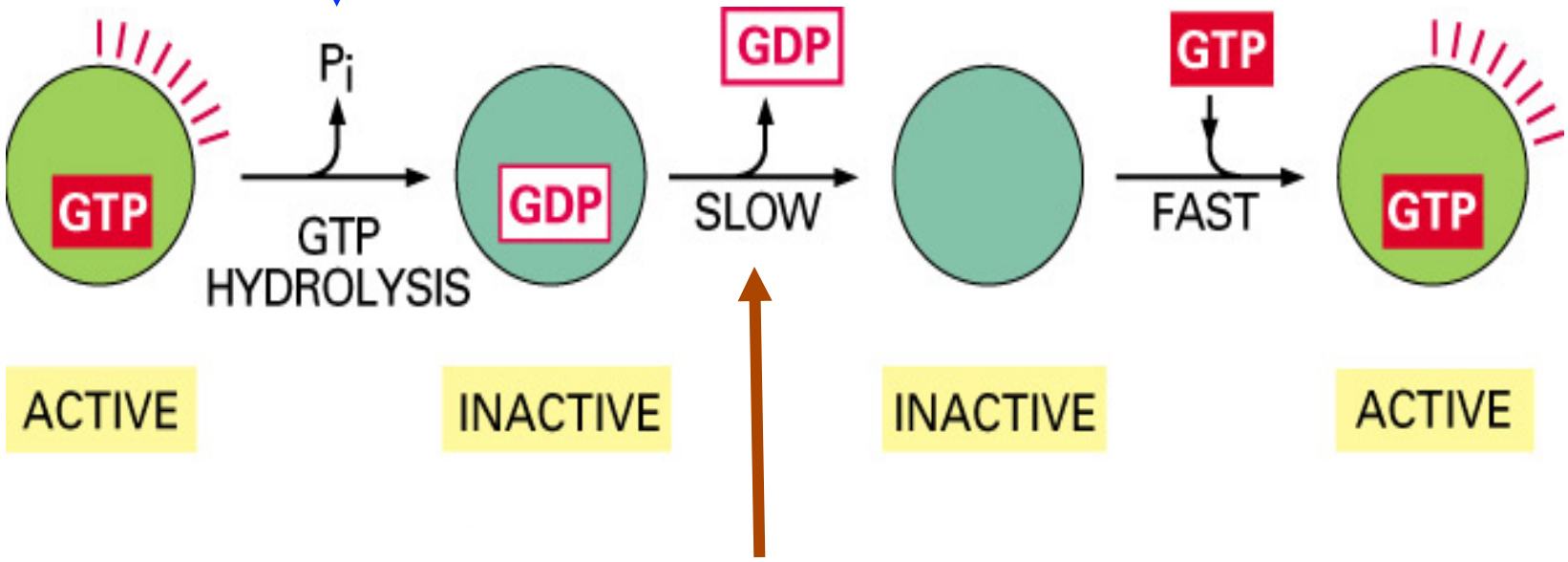
The intrinsic activity of a GTPase to hydrolyze GTP can be low or high

The intrinsic GDP dissociation rate of most GTPases is usually slow

GAP, GEF, and GDI Regulate a Small GTPase by Controlling its GTP/GDP Cycle

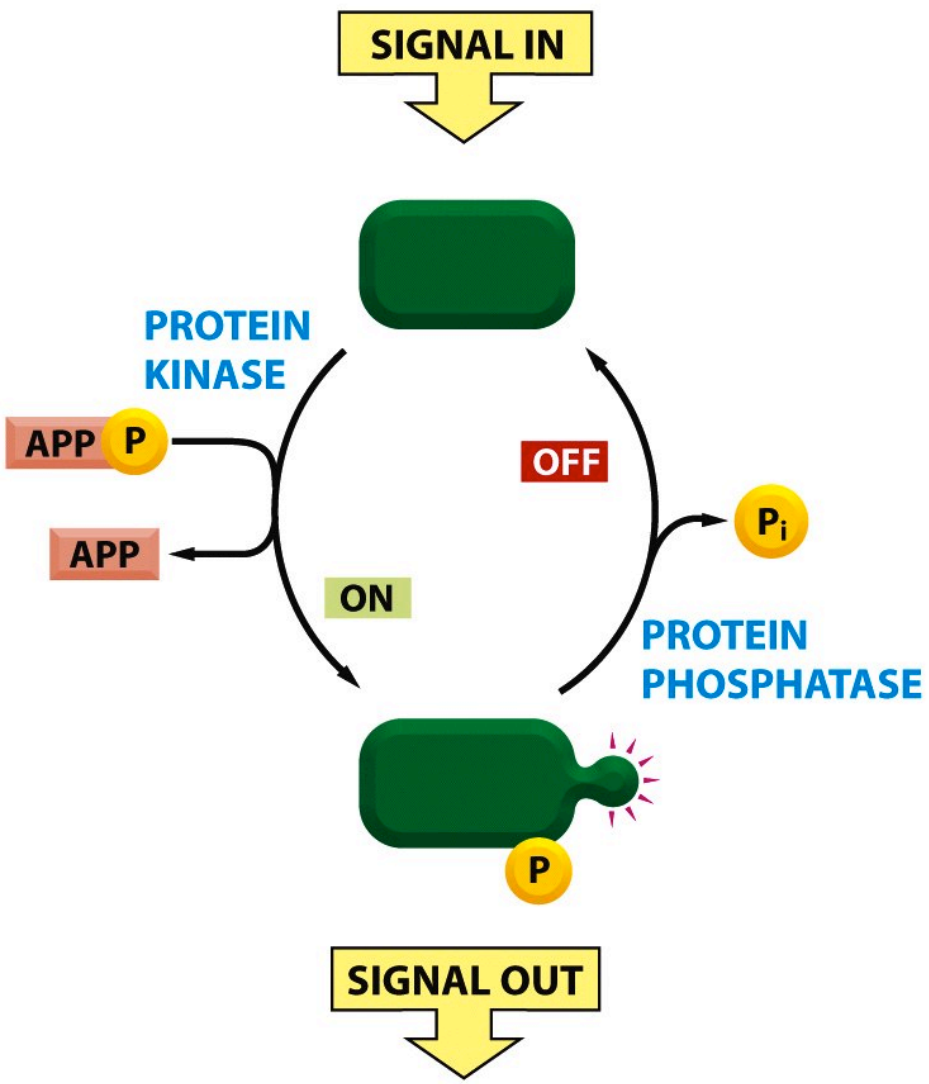
GAP (GTPase activating protein)
It increases the rate of GTP-GDP hydrolysis and thus inactivates a GTPase

GEF (Guanine nucleotide exchange factor)
It increases the rate of GDP-GTP exchange and thus activates a GTPase

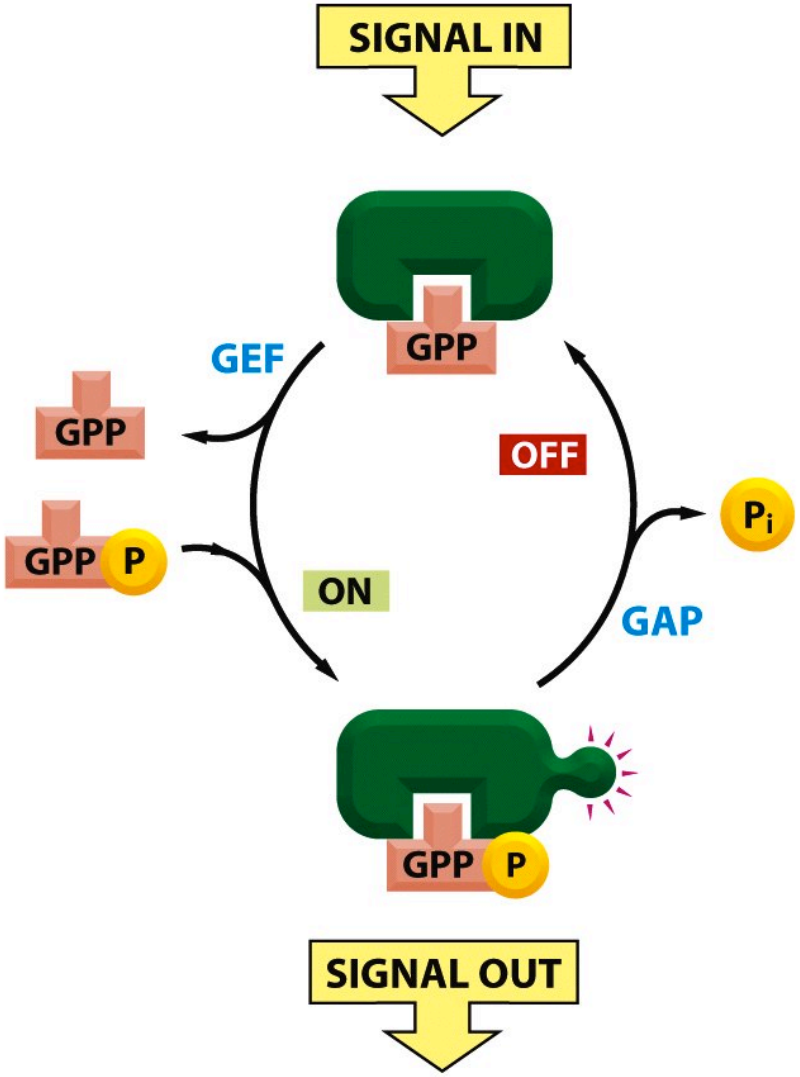


GDI (GDP-dissociation inhibitor)
It inhibits the rate of GDP dissociation

For different GTPases, there are different GAPs, GEFs and GDIs



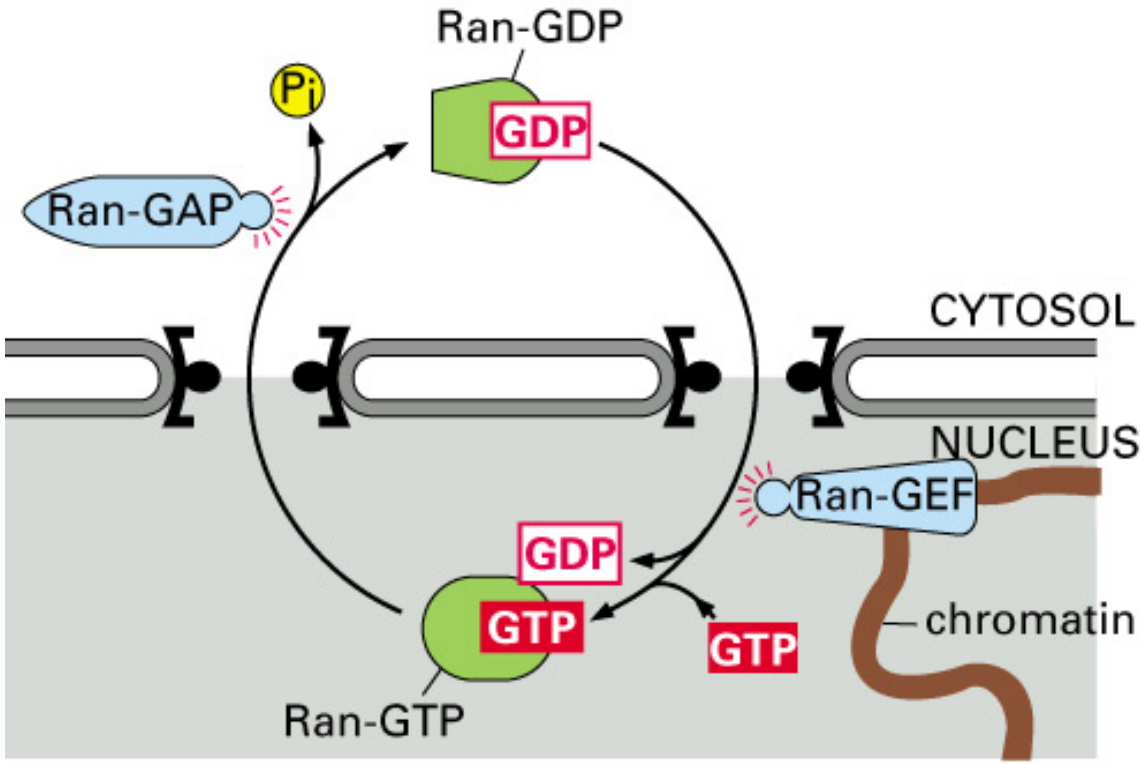
SIGNALING BY PHOSPHORYLATED PROTEIN



SIGNALING BY GTP-BINDING PROTEIN

Ran GTPase Controls the Bi-Directional Nuclear Transport

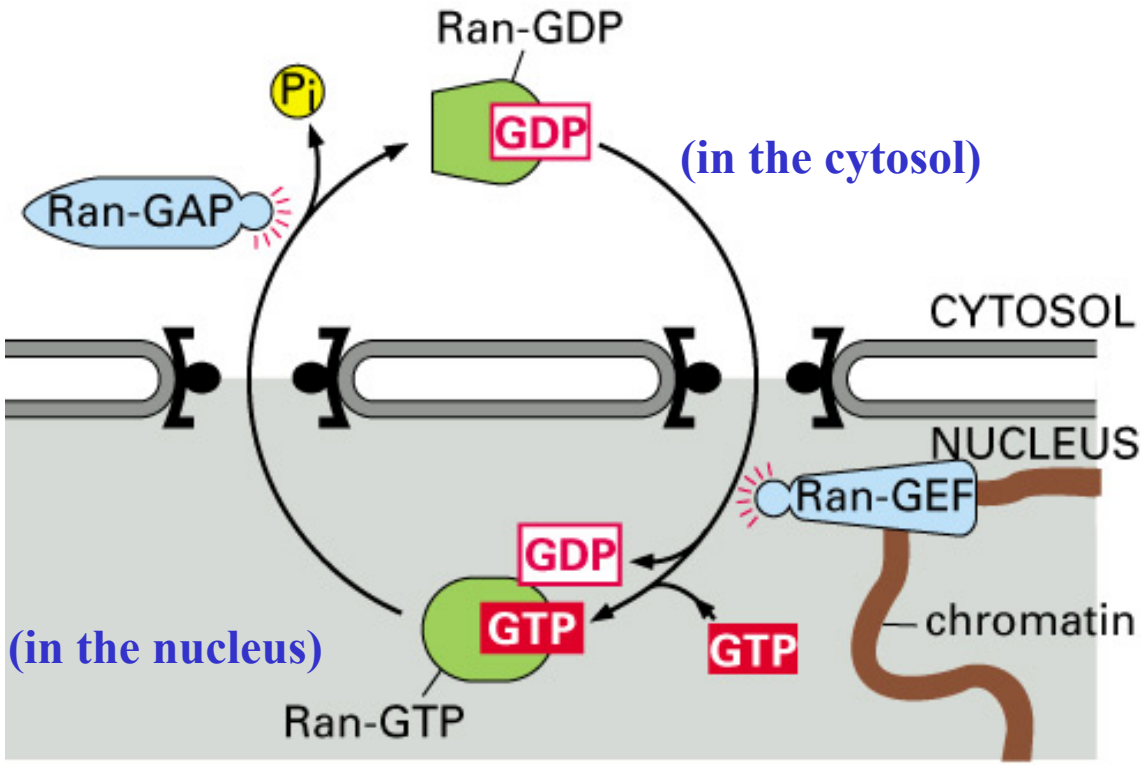
Nuclear transport is bi-directional
The Ran GTPase is known to mediate transport in both directions



Ran-GDP is mainly found in the cytosol and Ran-GTP is mainly found in the nucleus.

How can one protein (Ran) mediate transport in both directions?

GAPs and GEFs of Ran GTPase are Differentially Localized



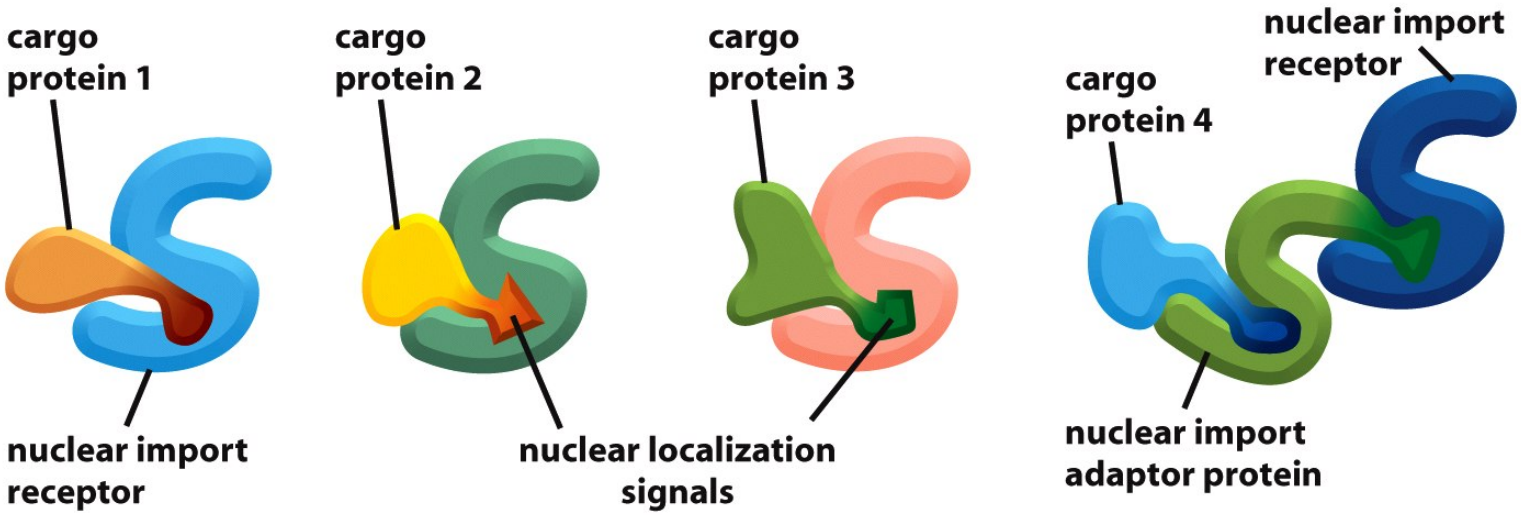
Ran-GAP is mainly found in the cytosol and Ran-GEF is mainly found in the nucleus.

Why are Ran-GAP and Ran-GFP differentially localized?
Binding to Ran-GEF (nuclear chromatin) or Ran-GAP (cytoplasmic side of membrane)

Importins (nuclear import receptors recognizing the NLS)
Exportins (nuclear export receptors recognizing the NES)

Different NLS and NES signals are recognized by different importins and exportins

Importins and exportins can interact with cargo proteins directly or indirectly (by an adaptor)

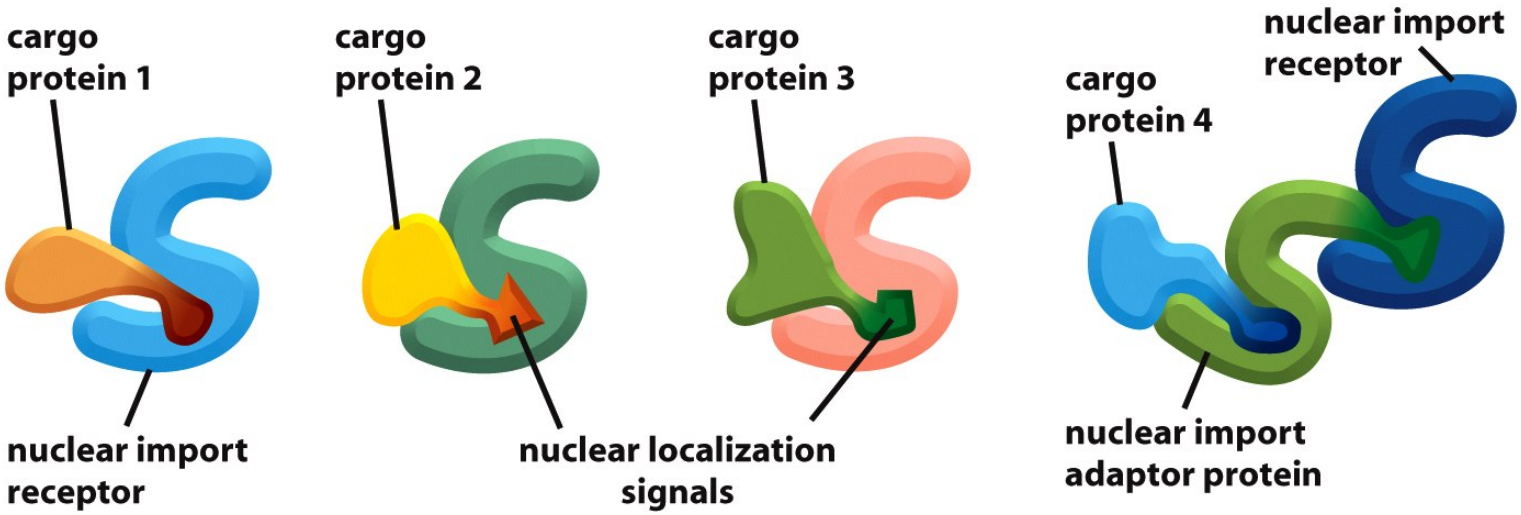


Importins and Exportins

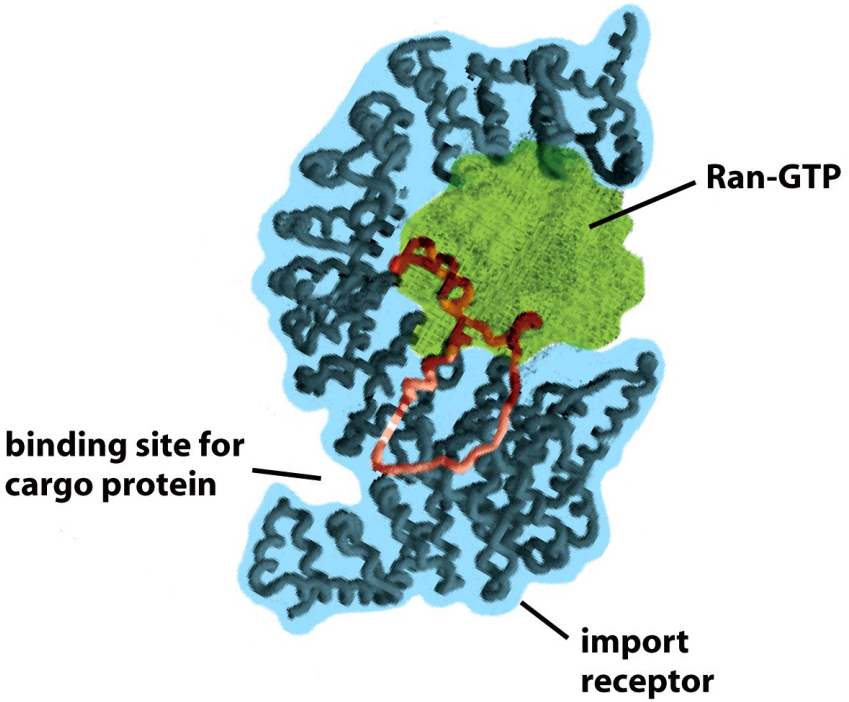
Importins recognize cargo in the cytosol and release them in the nucleus

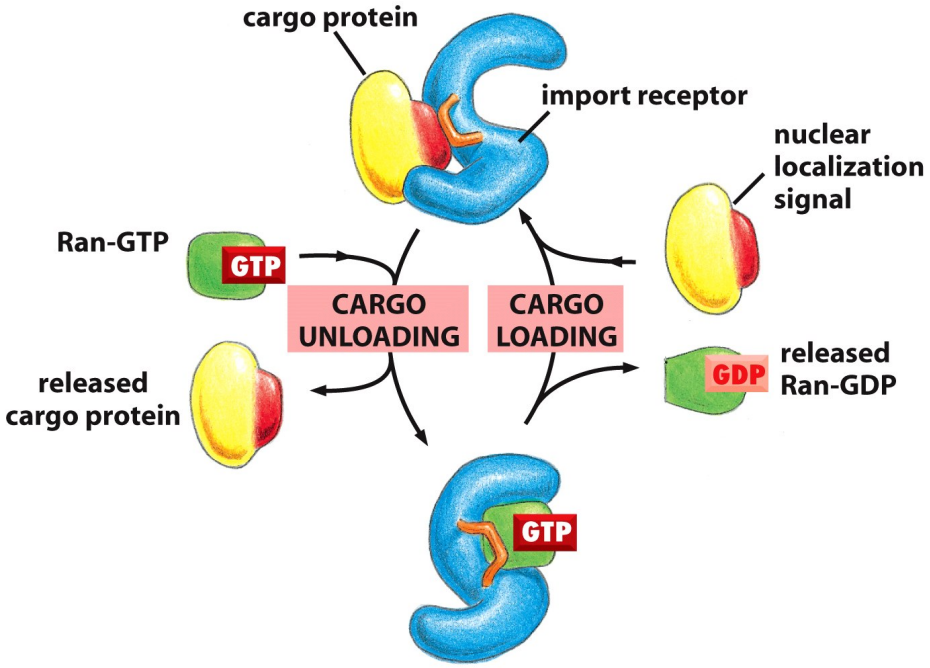
Exportins recognize cargo in the nucleus and release them in the cytosol

cargo ⟷ importins or exportins ⟷ nucleoporins (NPC components)



Importins and Exportins



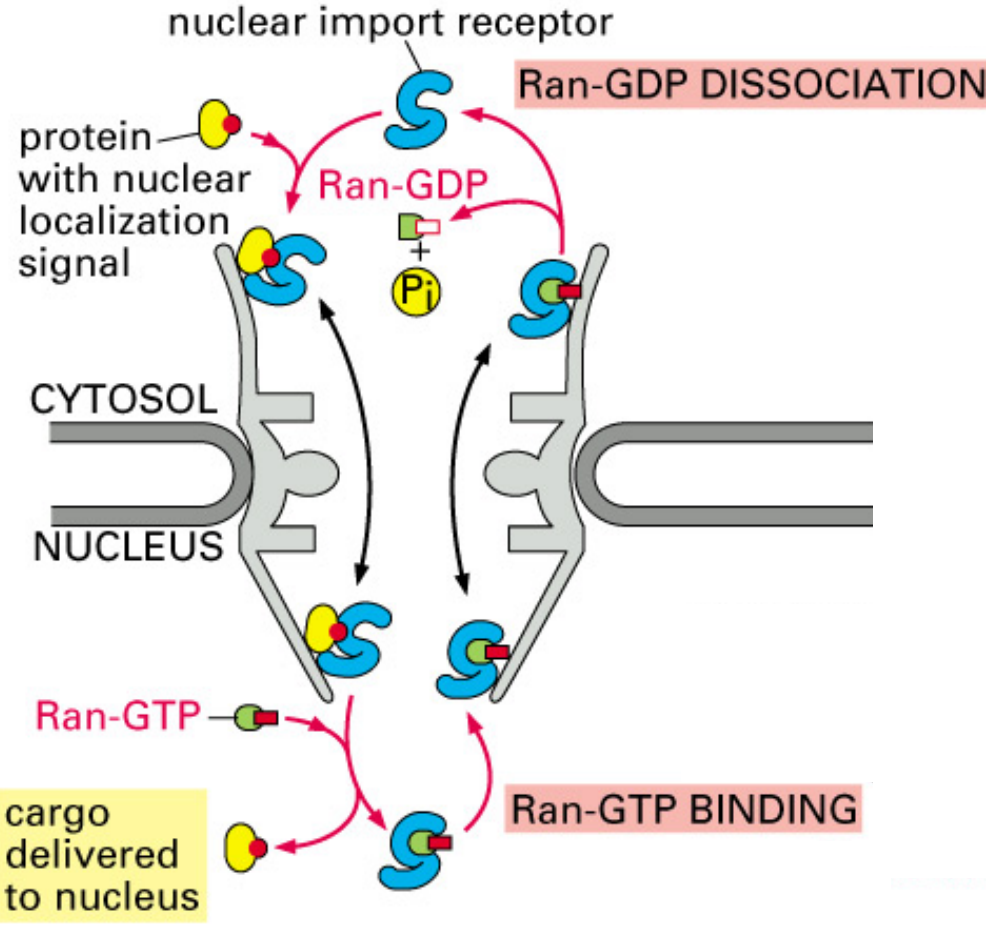


Ran-GTP binding causes release of nuclear cargo

How Does the Differential Distribution of Ran-GDP and Ran-GTP Determine the Transport Direction During Nuclear Import?

For nuclear import:
The cargo-importin complex must form in the cytosol but break up in the nucleus

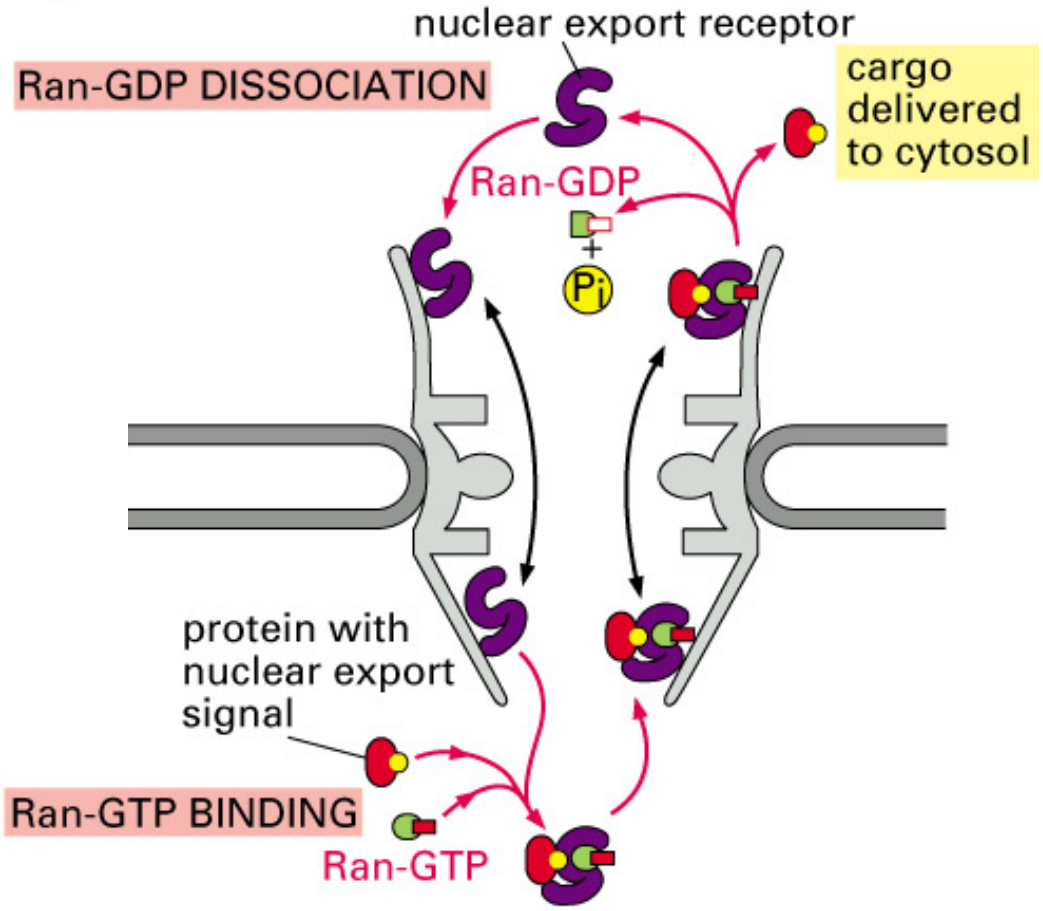
This is achieved by a switch of the bi-molecular interaction between importin, cargo, Ran-GDP & Ran-GTP



bi-molecular interaction: importin-Ran-GTP > importin-cargo > importin-Ran-GDP
(one can view the Ran-GTP as the active form)

In the cytosol, importin prefers cargo over Ran-GDP (leading to cargo binding)
In the nucleus, importin prefers Ran-GTP over cargo (leading to cargo release)

How Does the Differential Distribution of Ran-GDP and Ran-GTP Determine the Transport Direction During Nuclear Export?

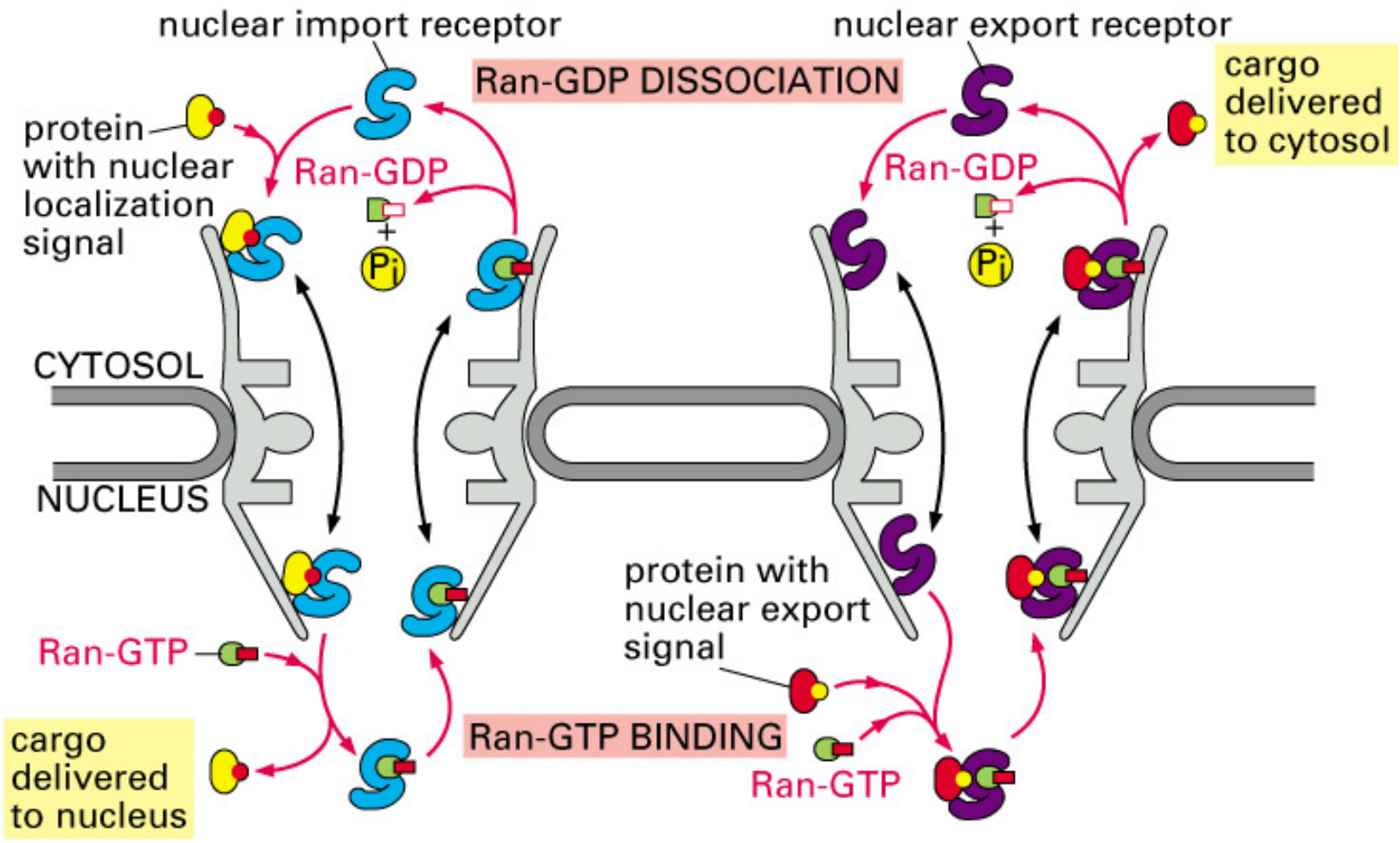


For nuclear export:
The cargo-exportin complex must form in the nucleus but break up in the cytosol

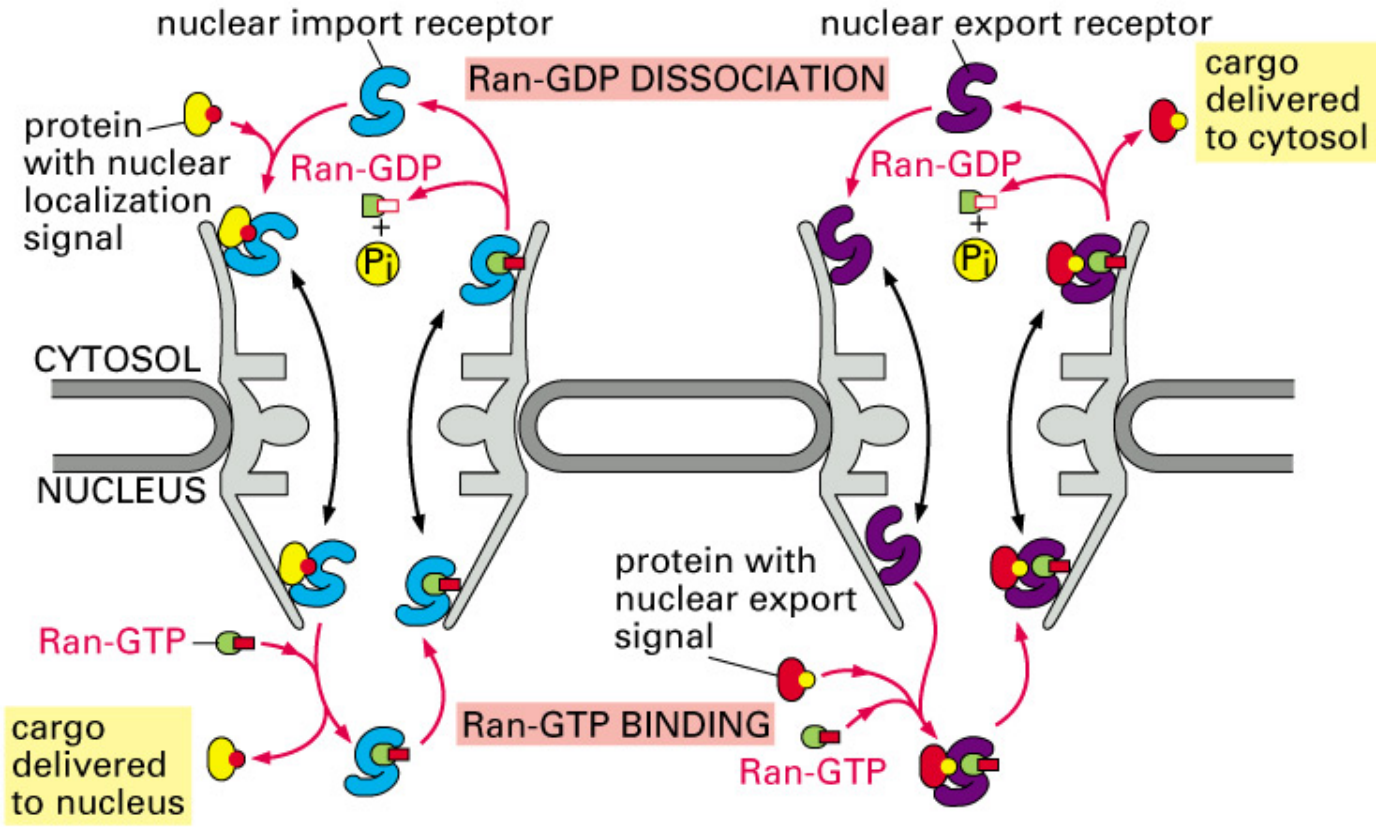
This is achieved by a switch of the tri-molecular interaction between exportin, cargo, RanGDP & RanGTP (bi-molecular interaction involving exportin is unstable)

exportin-cargo-RanGTP is stable but exportin-cargo-RanGDP is unstable
RanGTP promotes the cargo binding to the exportins in the nucleus
RanGDP promotes the cargo release from exportins in the cytosol

Is There Any Problem with This Mechanism?



For Each Import or Export Cycle, RanGTP is Consumed in the Nucleus and RanGDP is Accumulated in the Cytosol.



If not corrected, all the Ran would end up in the cytosol in their GDP form. As a result, the transport will eventually stop.

NTF2 (Nuclear Transport Factor 2) Recycles Ran-GDP to the Nucleus

NTF2 (not shown) forms a stable complex w/ Ran-GDP but not Ran-GTP