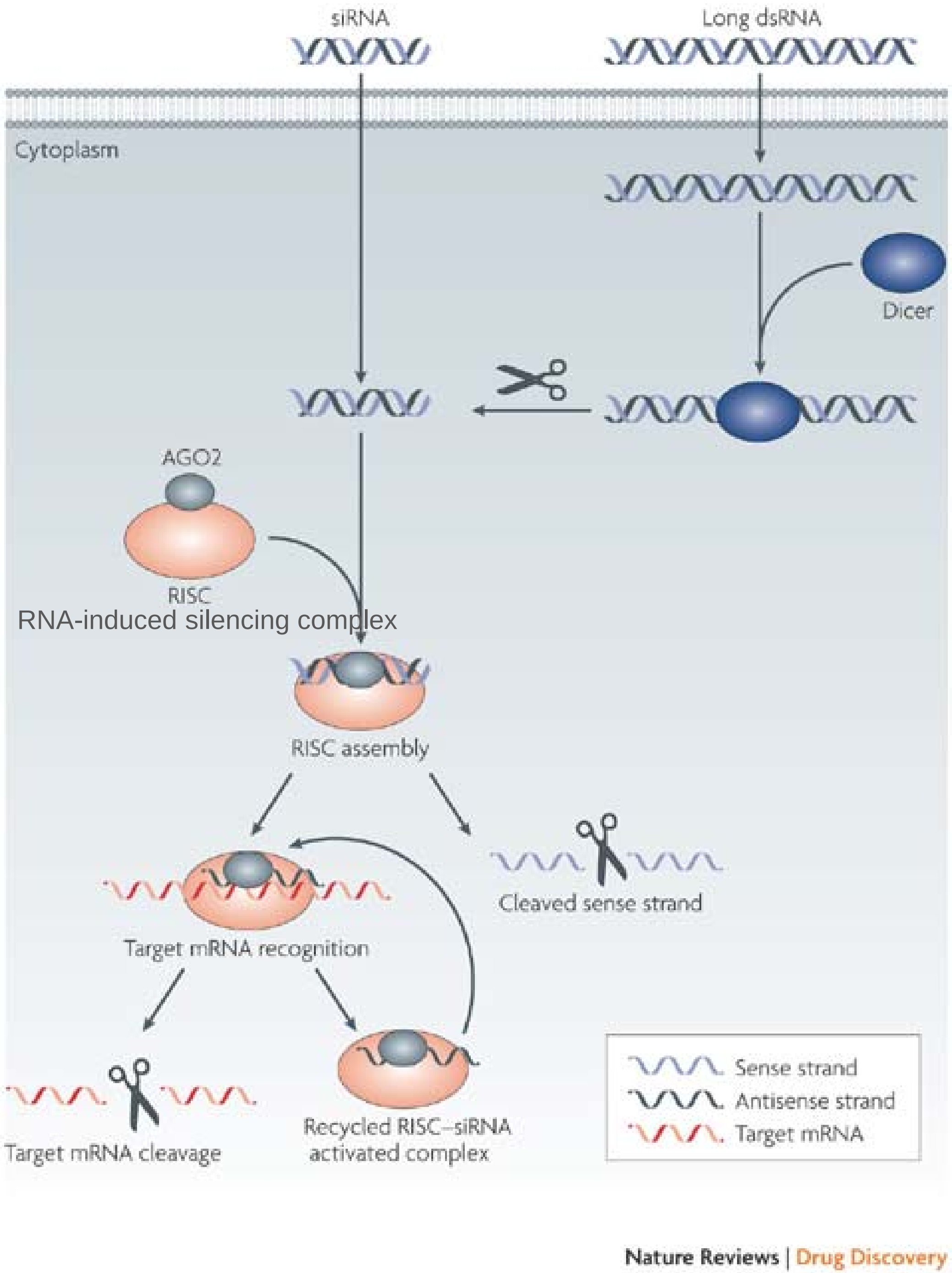
Tracking the delivery of lipid nanoparticle-mediated siRNAs

Journal club

Bei Li

16.07.2013



The mechanism of RNAi:

- Long dsRNA is introduced into the cytoplasm and cleaved into siRNA by the enzyme Dicer. Or siRNA is directly introduced into the cell.
- The siRNA is incorporated into the RISC.
- The sense strand of RNA is cleaved by AGO2.
- The activated RISC–siRNA complex seeks out, binds to complementary mRNA and degrades it.
- The activated RISC–siRNA complex can be recycled for the destruction of identical mRNA targets.

The Nobel Prize in Physiology or Medicine 2006
Andrew Z. Fire, Craig C. Mello

The Nobel Prize in Physiology or Medicine 2006

Photo: L. Cicero
Andrew Z. Fire

Photo: J. Mottern
Craig C. Mello

The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA"*

Since 1998, the discovery of RNAi, billions of dollars have been invested in the therapeutic application of gene silencing in humans.

Synthetic siRNAs becomes a promising therapeutic modality to silence disease-associated genes.

In vivo siRNA delivery

Localized delivery:

By direct instillation of siRNA into the target tissue.

- ✓ Particularly suited for the treatment of lung diseases and infections.

Systematic delivery:

By intravenous injection of delivery particles that then travel throughout the body to the target organ or tissue.

- ✓ Suited for tissues can only be reached through the systemic delivery in the bloodstream.

Several tissues are amenable to therapy:

Table 1 | **Modes of siRNA delivery and potential targets**

Mode of administration	Potential organ target	Potential disease target
Topical	Eye	Macular degeneration
	Skin	Atopic dermatitis
	Vagina	Herpes simplex virus
	Rectum	Inflammatory bowel disease
Local/direct	Lung	SARS
	Brain	Huntington's disease
	Spinal cord	Chronic pain
	Isolated tumour	Glioblastoma multiforme
Systemic	Liver	Hypercholesterolaemia
	Heart	Myocardial infarction
	Kidney	Kidney disease
	Metastasized tumours	Ewing's sarcoma

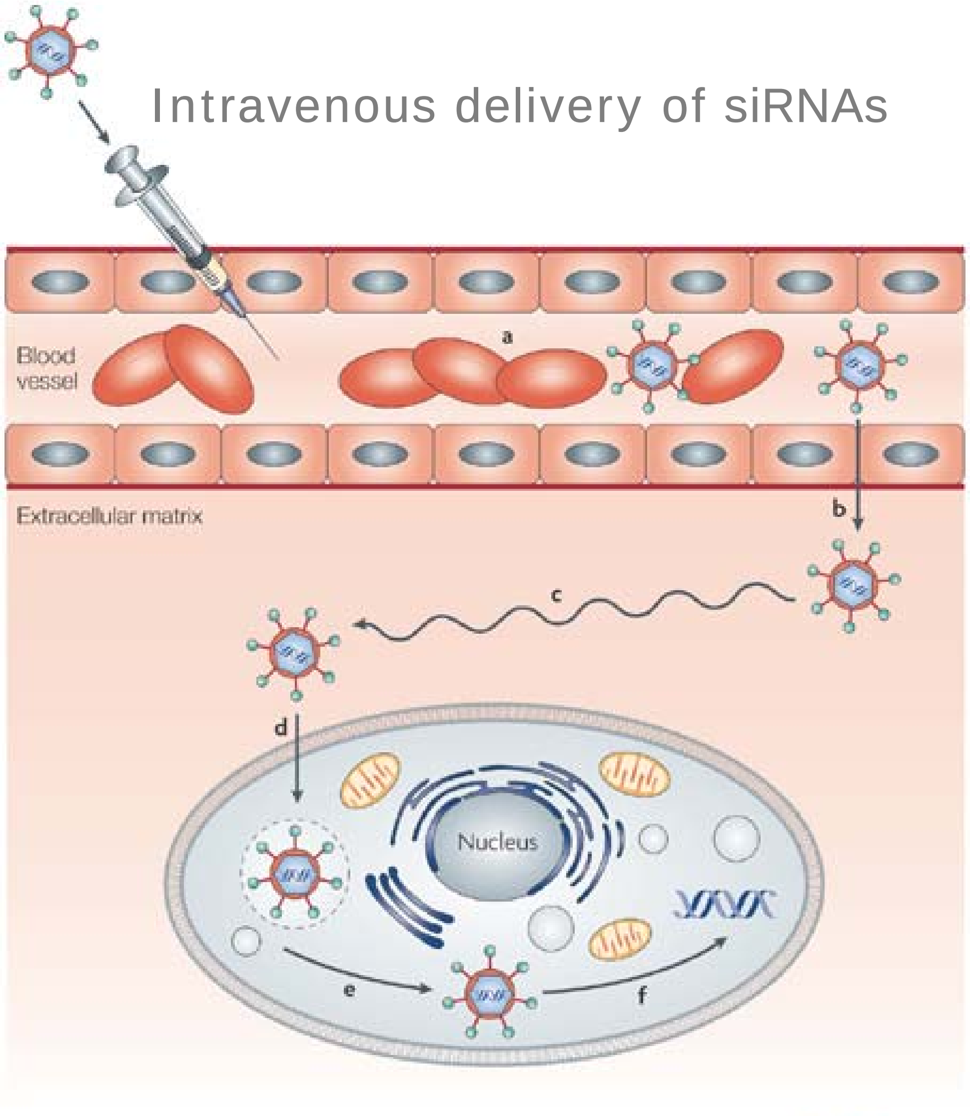
SARS, severe acute respiratory syndrome; siRNA, small interfering RNA.

Table 3 | **Current clinical trials for siRNA therapeutics**

Company	Disease	Mode of administration	Status
Allergan	Age-related macular degeneration	Topical	Phase II
Alnylam	Respiratory syncytial virus	Local/direct	Phase II
Nucleonics	Hepatitis B virus	Systemic	Phase I
Quark Pharmaceuticals/ Pfizer	Acute renal failure	Systemic	Phase I
Opko Health	Age-related macular degeneration	Topical	Phase III
Silence/Quark/Pfizer	Diabetic macular oedema	Topical	Phase II
Transderm	Pachyonychia congenita	Topical	Phase Ia/b

Delivery remains the most significant barrier to the widespread use of RNAi therapeutics in a clinical setting!

Intravenous delivery of siRNAs



Physiological barriers to the systemic delivery of siRNA nanoparticles.

<u>siRNAs have to:</u>

a) void filtration, phagocytosis and degradation in the bloodstream.

b) transported across the vascular endothelial barrier.

c) diffuse through the extracellular matrix.

d) enter target cells, typically by endocytosis.

e) escape the endosome into cytosol.

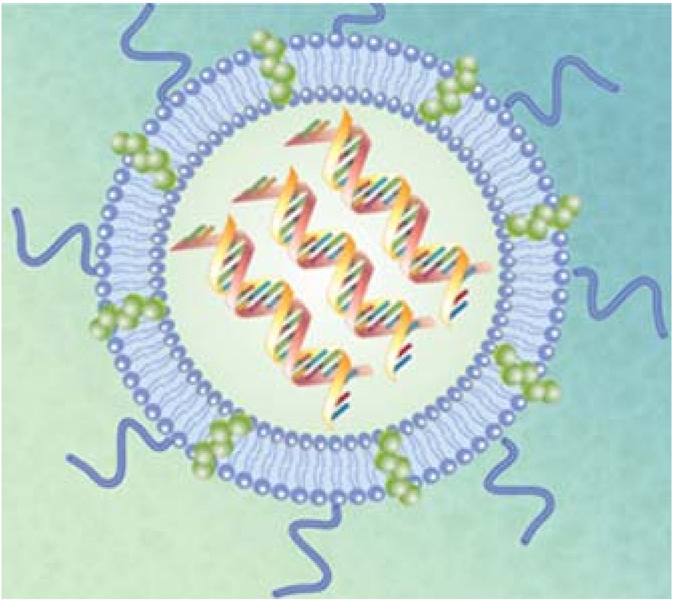
f) unpackage and release the siRNA, bind to and get loaded onto the RISC for silencing of the target gene.

Table 2 | **Selected synthetic materials for *in vivo* siRNA delivery**

Material	Model	Target	Route	Animal
Liposomes and lipids				
i-FECT	Japanese encephalitis virus (JEV) and West Nile virus (WNV)	JEV and WNV envelope	Intracranial	Mouse
Lipidoids	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, rat, monkey
	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, hamster
	Malaria	Haem oxygenase 1	Intravenous	Mouse
	Hypercholesterolaemia	PCSK9	Intravenous	Mouse, rat
LipoTrust	Liver cirrhosis	gp46	Intravenous	Rat
Oligofectamine	Herpes simplex virus 2 (HSV-2)	HSV-2-associated viral proteins UL27 and UL29	Intravaginal	Mouse
SNALP	Hepatitis B virus (HBV)	HBV	Intravenous	Mouse
	Dyslipidaemia	ApoB	Intravenous	Monkey
	Ebola (Zaire)	Polymerase L	Intravenous	Guinea pig
Cationic polymers				
Cyclodextrin	Ewing's sarcoma tumour xenograft	*EWS–FLI1*	Intravenous	Mouse
	Healthy monkey model	RRM2	Intravenous	Monkey
Dynamic PolyConjugate	Dyslipidaemia	ApoB/PPARα	Intravenous	Mouse
Poly-ethyleneimine	Glioblastoma xenograft	PTN	Intratumoral	Mouse
	Formalin-induced pain	NMDAR2B	Intrathecal	Rat
	Cervical tumour xenograft	HPV E6/E7	Intratumoral	Mouse
	Ovarian tumour xenograft	HER2	Intraperitoneal	Mouse
Small interfering RNA (siRNA) conjugates				
Cholesterol	Dyslipidaemia	ApoB	Intravenous	Mouse
	Huntington's disease	Huntingtin gene	Intrastriatal	Mouse
Fatty acids/ bile salts	Dyslipidaemia	ApoB	Intravenous	Mouse, hamster

Different systems have been developed for siRNA delivery:

- viruses
- nonviral vectors
    - liposomes
    - polycationic polymers
    - conjugates
    - nanoparticles



- Lipid nanoparticles (LNPs) are one of the advanced delivery systems for siRNAs.
- Lipids self-assemble into 60 to 80nm particles that encapsulate the siRNA molecules.

LNPs have shown:

✓ efficient gene silencing in the liver in multiple species, including nonhuman primates.

✓ robust mRNA silencing in human clinical trials.

However, the precise molecular mechanisms underlying LNP-mediated delivery of siRNAs are not yet fully understood:

- Which endocytic mechanisms are responsible for LNP uptake?
- To which endocytic compartments are LNPs transported and with which kinetics?
- Once internalized, how efficiently can siRNAs escape from endosomes?
- Are there cell-specific differences?
- How well do in vitro findings correlate with in vivo observations?

Lack of reliable methods to visualize and quantify the delivery of LNP-mediated siRNAs!

- Test siRNA delivery hypothesis.
- Gain mechanistic insights into siRNA escape.
- Develop the next generation of delivery systems for siRNA therapeutics.

Recent studies have reported some visualized methods by light microscopy that requires much higher doses of LNPs above the therapeutic range.

nature biotechnology

Image-based analysis of lipid nanoparticle–mediated siRNA delivery, intracellular trafficking and endosomal escape

Jerome Gilleron[1], William Querbes[2], Anja Zeigerer[1], Anna Borodovsky[2], Giovanni Marsico[1], Undine Schubert[1], Kevin Manygoats[1], Sarah Seifert[1], Cordula Andree[1], Martin Stöter[1], Hila Epstein-Barash[2], Ligang Zhang[2], Victor Koteliansky[2], Kevin Fitzgerald[2], Eugenio Fava[1,3], Marc Bickle[1], Yannis Kalaidzidis[1,4], Akin Akinc[2], Martin Maier[2] & Marino Zerial[1]

Received 27 March; accepted 13 May; published online 23 June 2013; doi:10.1038/nbt.2612

- ✓ Developed an analytical methodology with a combination of light and electron microscopy, quantitative image analysis and mathematical modeling;
- ✓ Visualized LNP-mediated siRNA uptake, trafficking and escape from endosomes at therapeutically relevant concentrations.

❖ Development of LNPs loaded with traceable siRNAs

- ➢ siRNAs: targrting GFP mRNA

- ➢ Labelled siRNA:
  - siRNA-alexa647
  - siRNA-gold (6-nm colloidal gold particles)

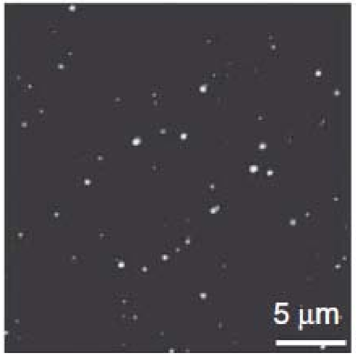
- ➢ Nanoparticles comprise:
  - ionizable lipid DLin-MC3-DMA
  - disteroylphosphatidyl choline
  - cholesterol
  - PEG-DMG

  (molar ratio of ~50:10:38.5:1.5)

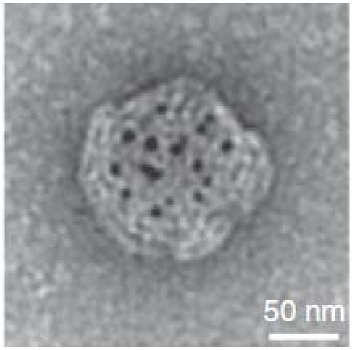
final lipid/siRNA weight ratio ~10:1

Fluorescence microscopy

siRNA-alexa647

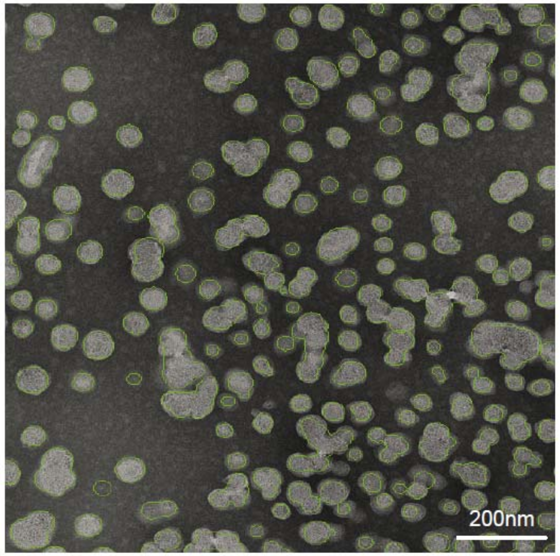


siRNA-gold

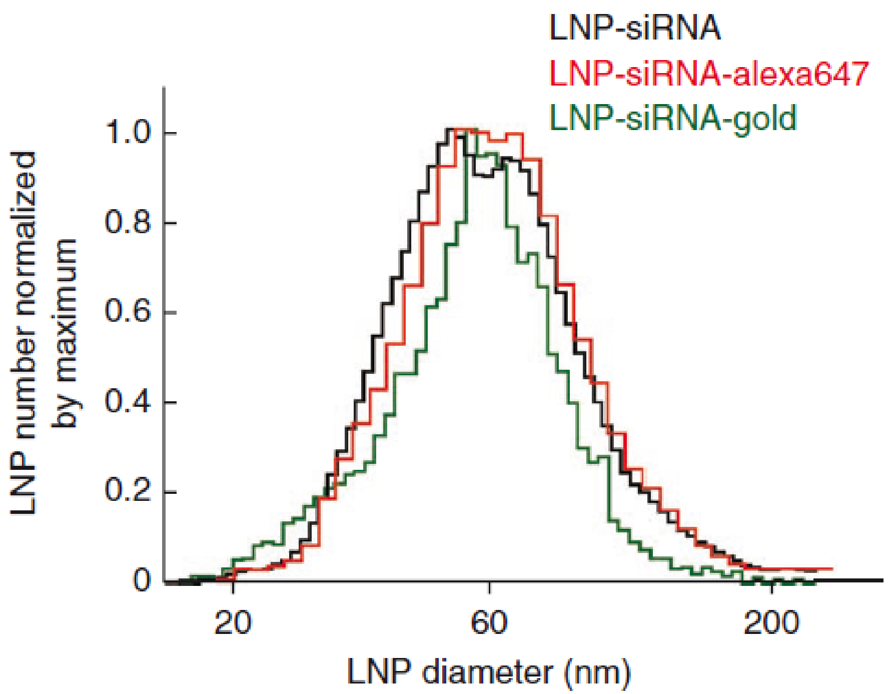


Electron microscopy (EM)

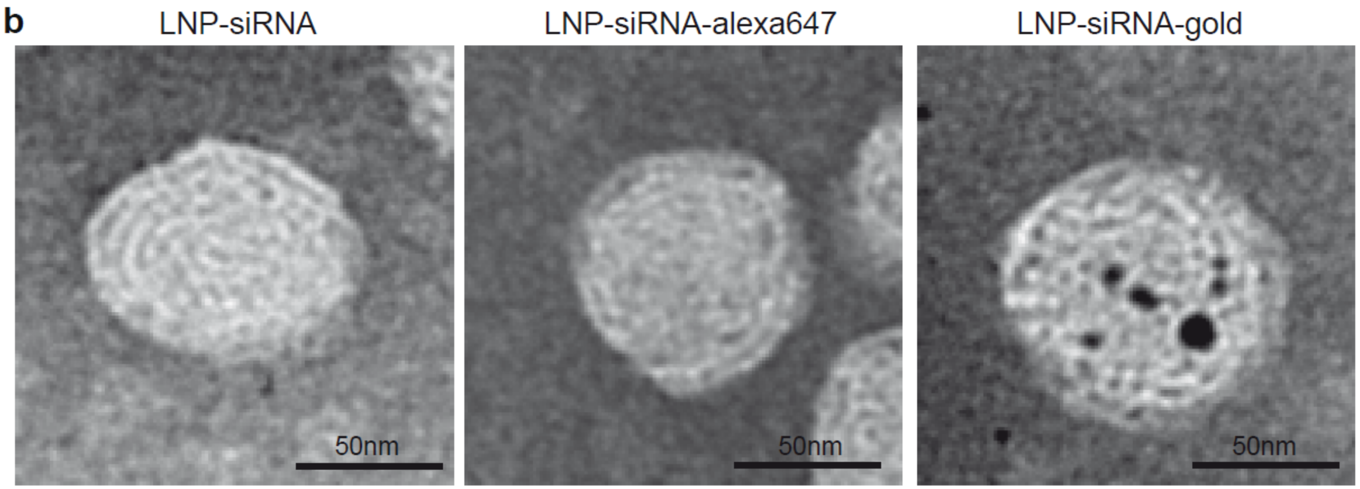
❖ Development of LNPs loaded with traceable siRNAs



Automated quantification of the preparations of the EM images.

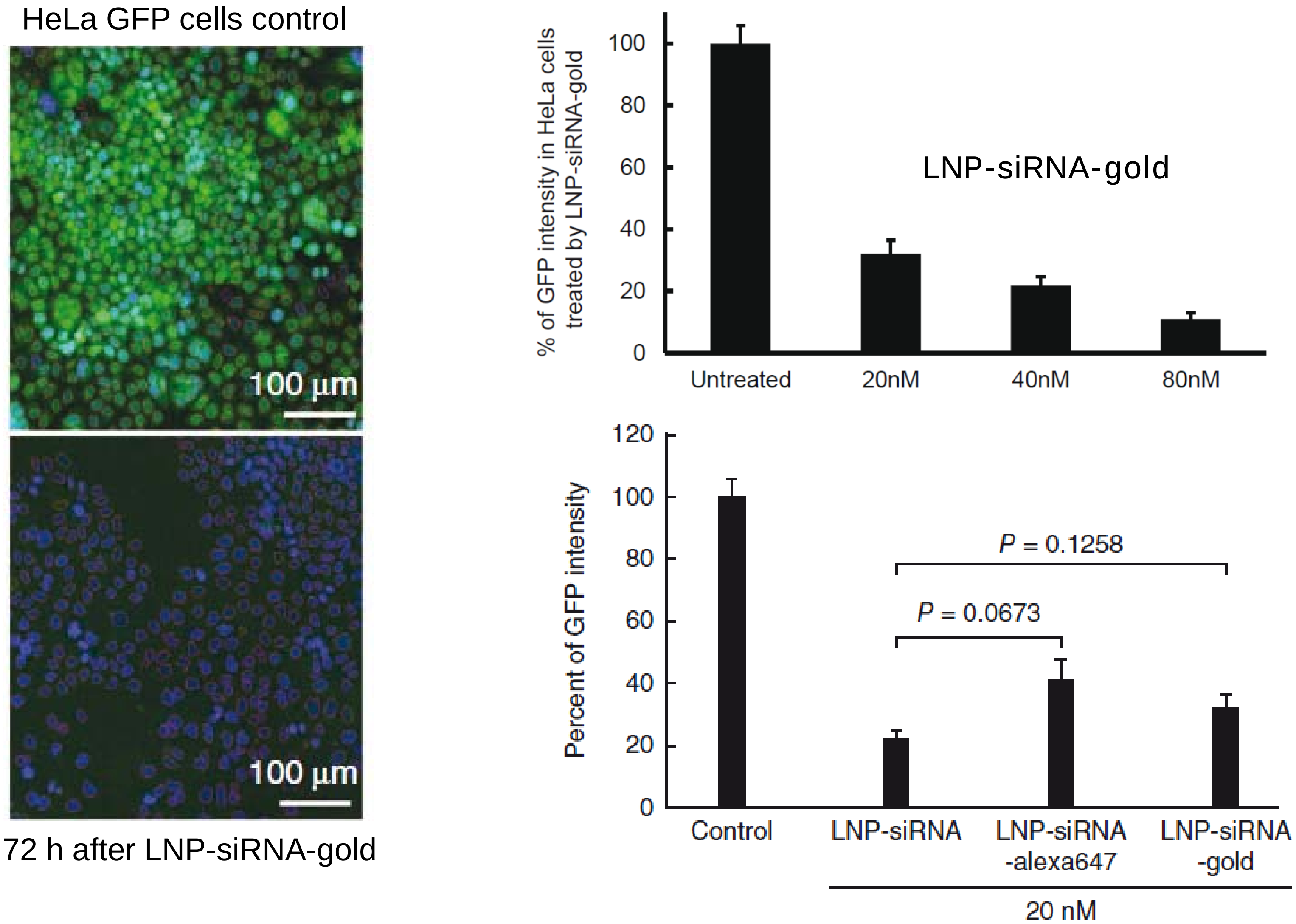


b



More than 80% had a diameter ranging from 40 to 120 nm

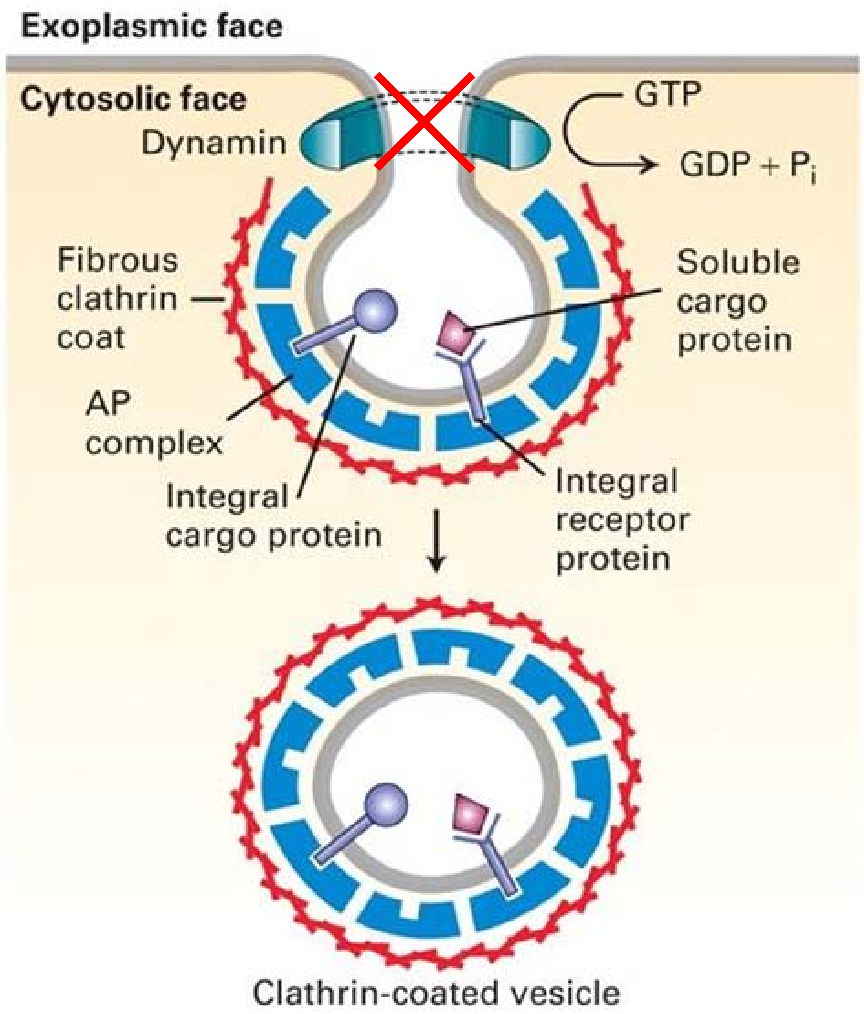
❖ Kinetics and mechanisms of LNP uptake



Quantification of GFP down regulation in HeLa GFP-expressing cells.

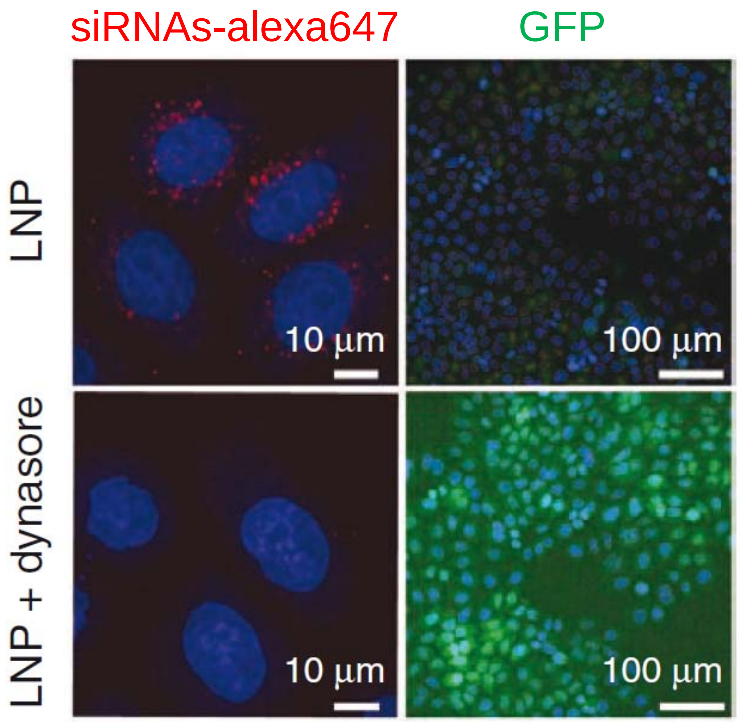
❖ Kinetics and mechanisms of LNP uptake

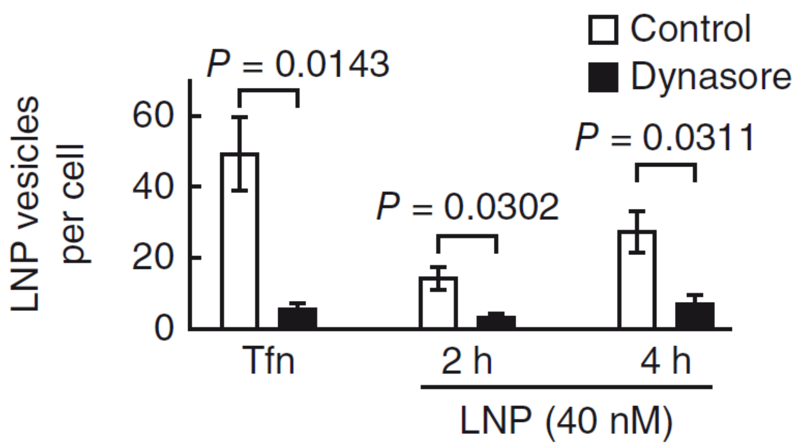
Is endocytosis required for siRNA delivery and GFP down regulation?

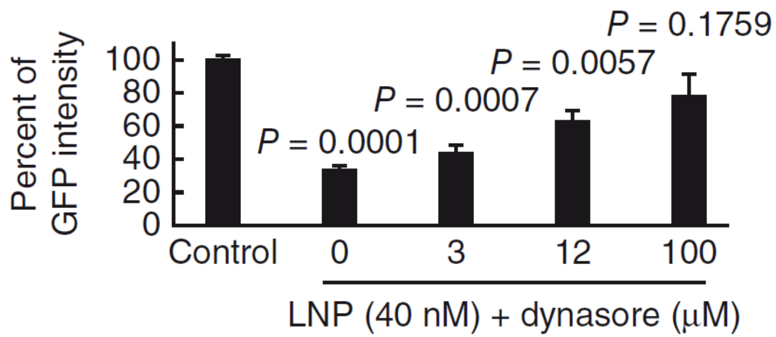


- GTPase dynamin is required for membrane fission in clathrin mediated endocytosis (CME) and other forms of internalization.
- Dynasore is a GTPase inhibitor that targets dynamin and blocks endocytosis.

❖ Kinetics and mechanisms of LNP uptake







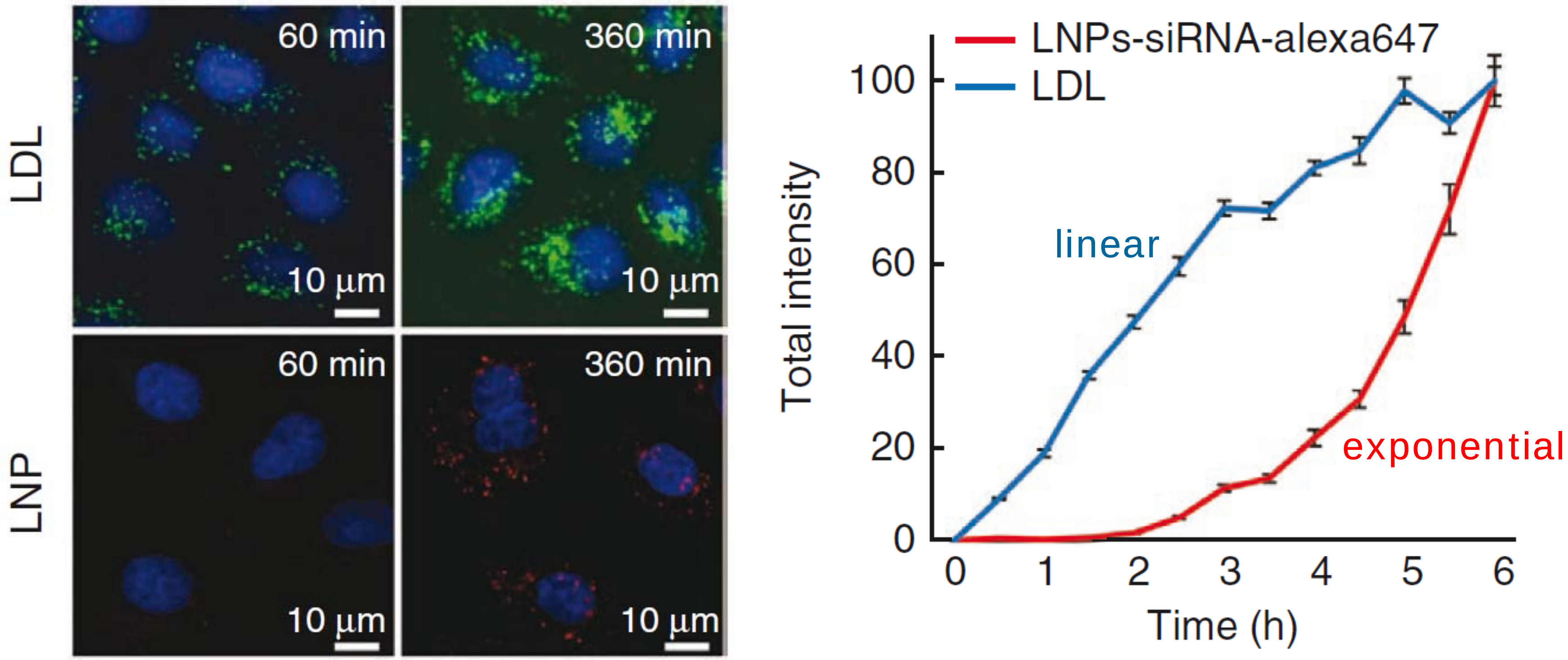
Left: uptake of LNP-siRNA alexa647
(40 nM, 4 h uptake)

Right: GFP downregulation
(40 nM, 72 h after transfection)

Dynasore treatment reduced the uptake of LNP-siRNA-alexa647 and prevented the down regulation of GFP.

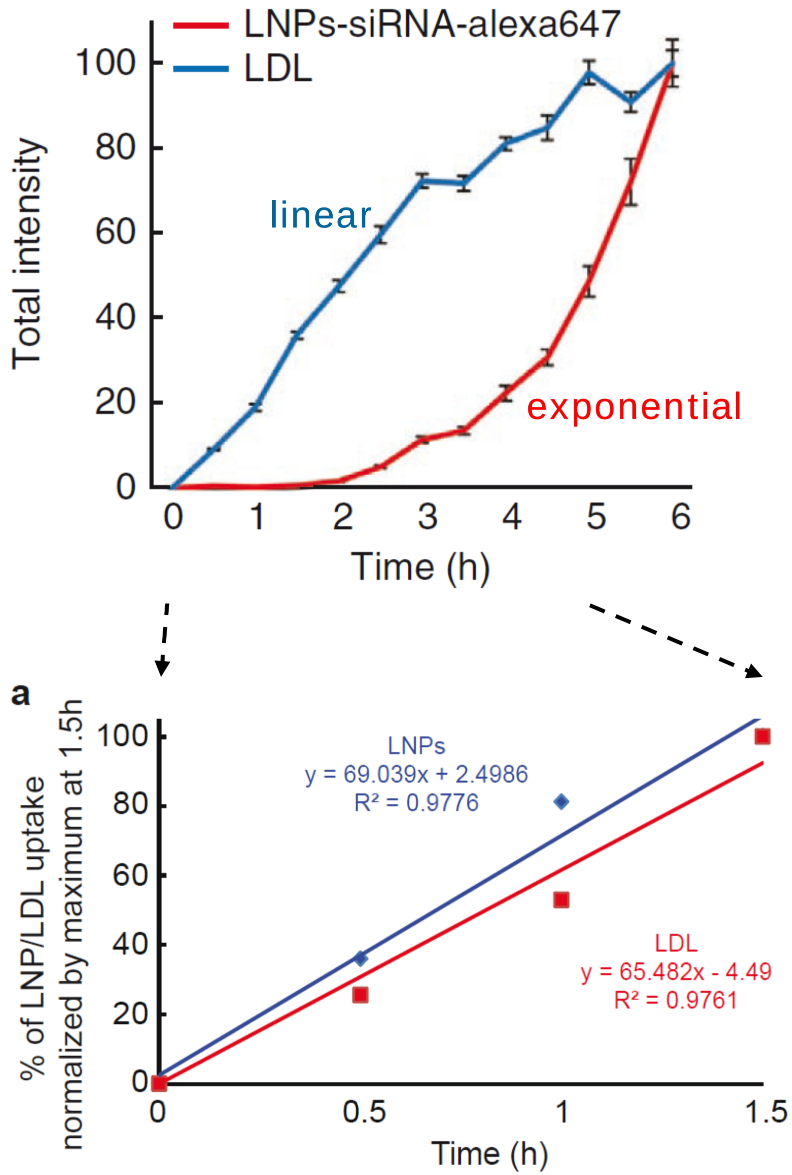
❖ Kinetics and mechanisms of LNP uptake

LNP internalization is dependent on LDLR (low-density lipoprotein receptor), which is a key regulator of CME.



Uptake kinetics of LNP-siRNA-alexa647 and LDL-488

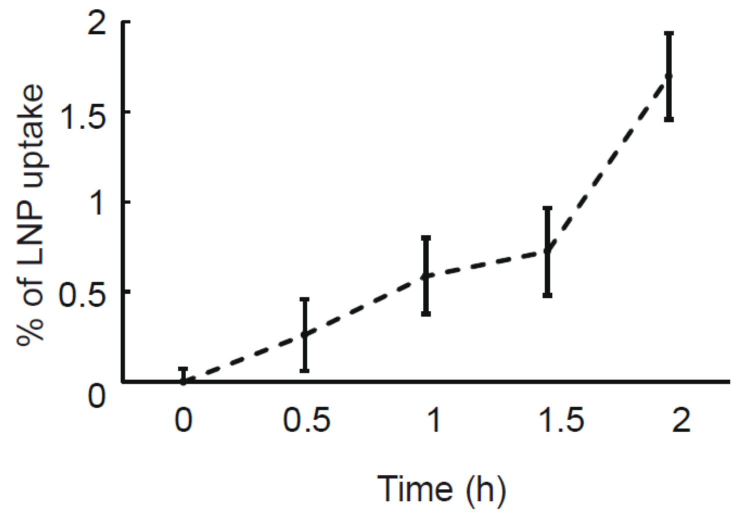
❖ Kinetics and mechanisms of LNP uptake



The uptake of LNPs display biphasic:

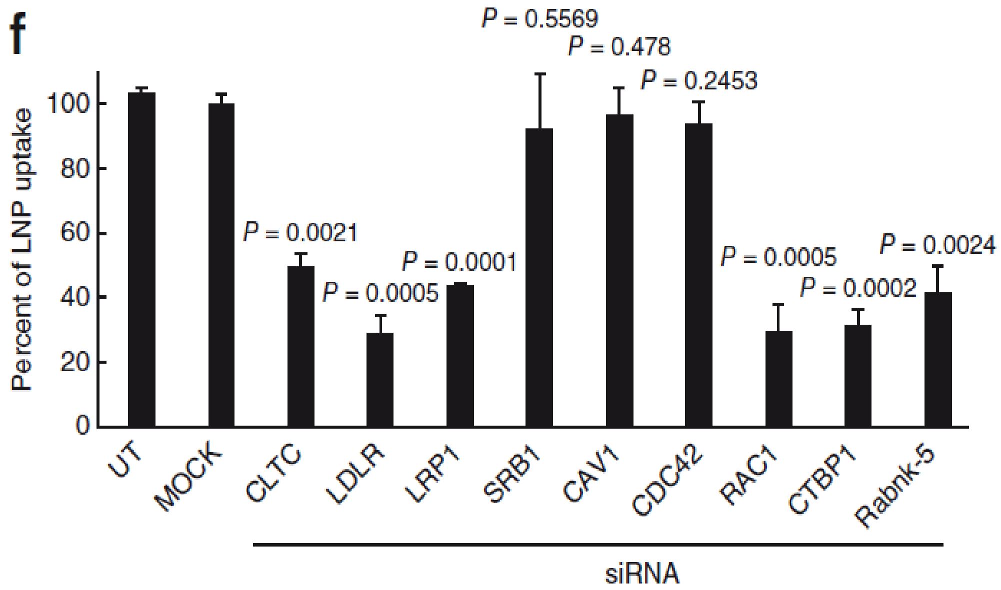
1) In the first phase (~1.5h), <1% LNPs entered cells with fast kinetics, similar to LDL endocytosis.

2) In the second phase (2–6h), 98% LNPs entered cells.



❖ Kinetics and mechanisms of LNP uptake

Which endocytic mechanisms are responsible for the internalization of the majority of LNPs that enter the cells in the second phase ?



Clathrin mediated endocytosis:

- CLTC: clathrin heavy chain
- LDLR: low-density lipoprotein receptor
- LPR1: LDL receptor-related protein 1
- SRB1: high-density lipoprotein receptor

Caveolin-mediated endocytosis:

- CAV1: Caveolin 1

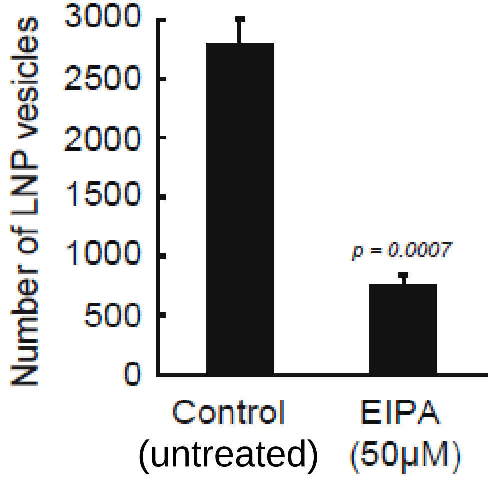
GEEC-CLIC pathways (GPI-anchored protein-enriched early endocytic compartment/clathrin-independent carriers)

- CDC42

Macropinocytosis:

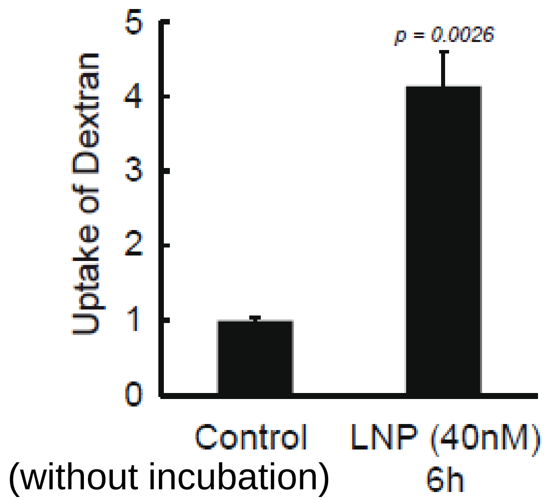
- Rac1: Ras-related C3 botulinum toxin substrate 1
- CTBP1: C-terminal binding protein 1
- Rabankyrin-5: Rab5 effector

❖ Kinetics and mechanisms of LNP uptake



EIPA: ethylisopropylamiloride, a pharmacological inhibitor of macropinocytosis.

EIPA treatment reduced LNP uptake by ~70%.

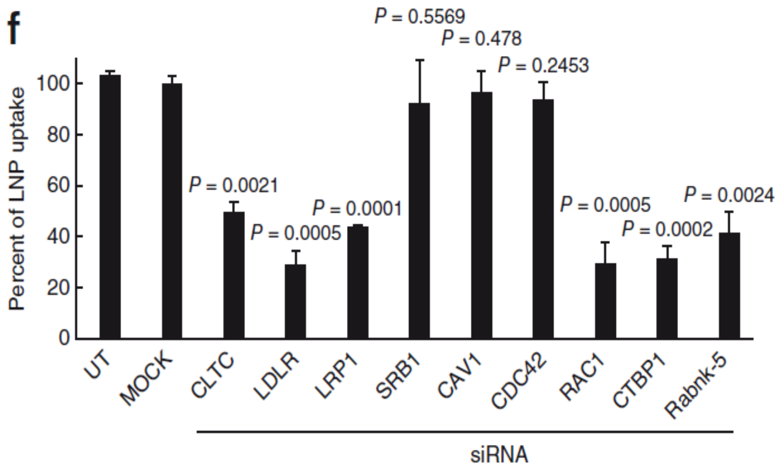


Dextran-alexa488: a fluid phase marker

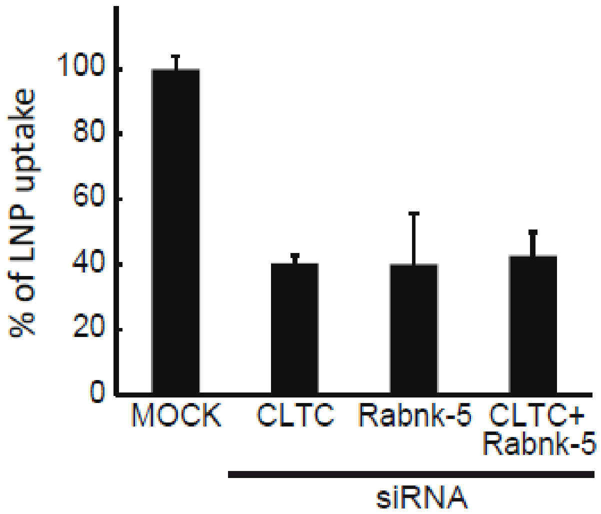
HeLa cells incubated with LNP-siRNA-alexa647 displayed an increased capacity of uptake of Dextran-alexa488.

LNPs themselves may induce macropinocytosis.

❖ Kinetics and mechanisms of LNP uptake



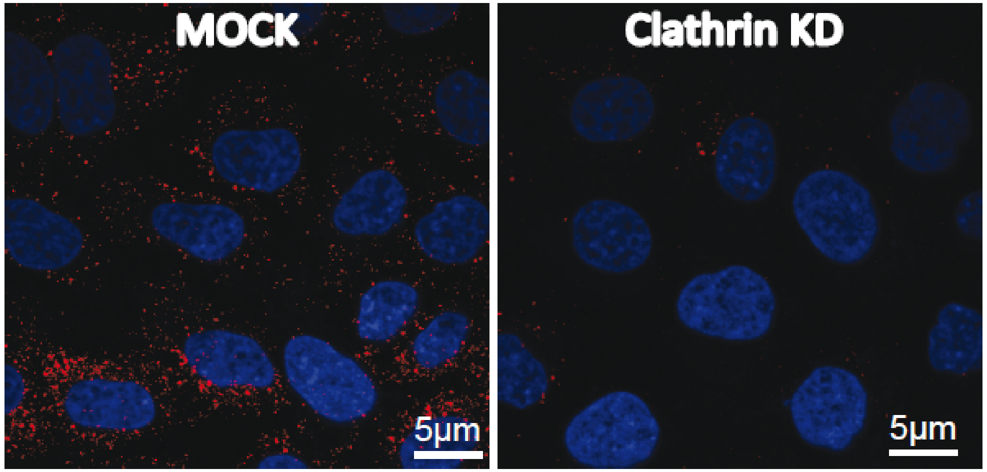
Inhibition of either CME or macropinocytosis reduced the uptake by 50–70%, suggesting that both pathways are required.



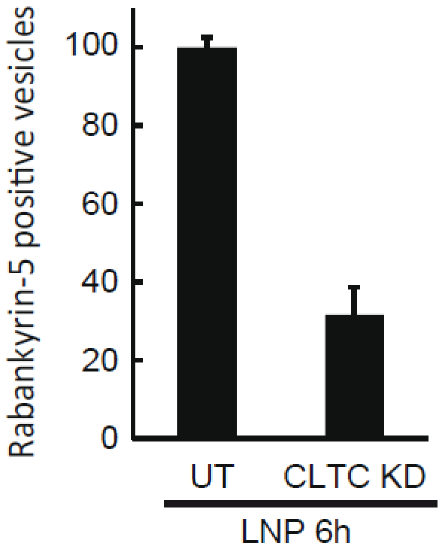
Inhibition of both CLTC and Rabankyrin-5, did not have a significant additive effect.

❖ Kinetics and mechanisms of LNP uptake

Is it possible that CME is a prerequisite for macropinocytosis activation, as it is for the entry of adenovirus?



Rabankyrin-5: immuno-labelling in HeLa cells

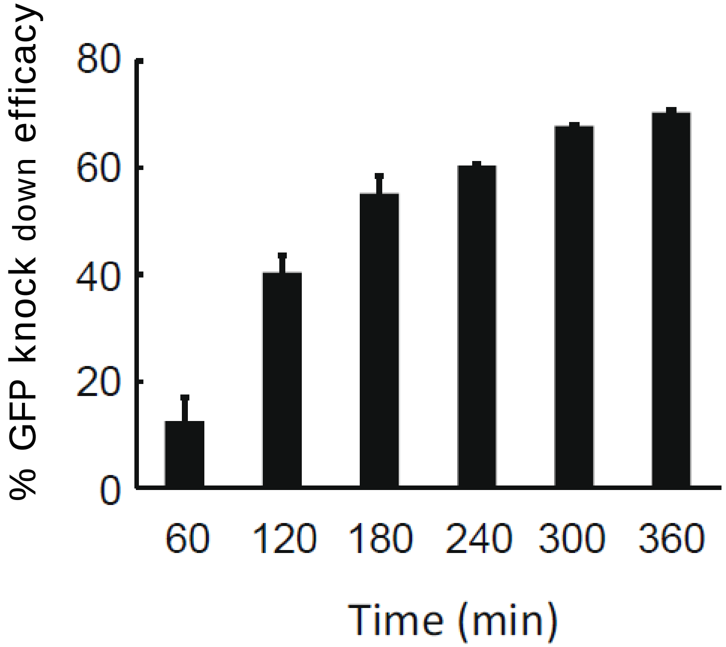


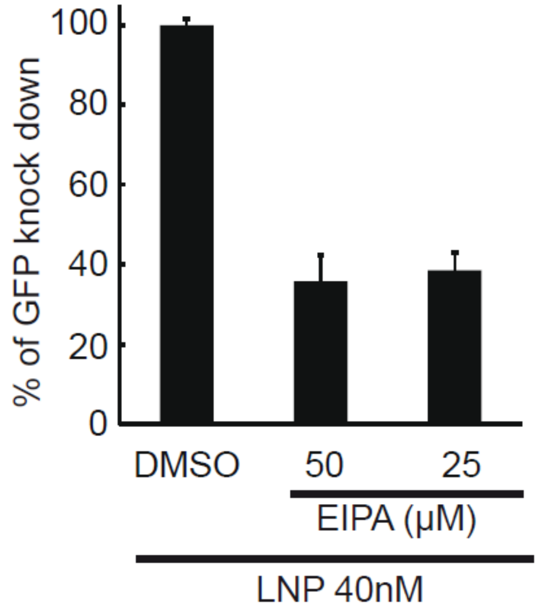
CLTC: clathrin heavy chain.
Rabankyrin-5: a regulator of macropinocytosis.

Silencing of CLTC to inhibit CME, substantially reduced the formation of Rabankyrin-5 positive vesicles upon LNP exposure.

❖ Kinetics and mechanisms of LNP uptake

What is the contribution of both pathways to gene silencing?





The first hour of uptake (by CME) resulted in ~10% of gene silencing.

The uptake from 2h on (when macropinocytosis is induced) resulted in ~90% of gene silencing.

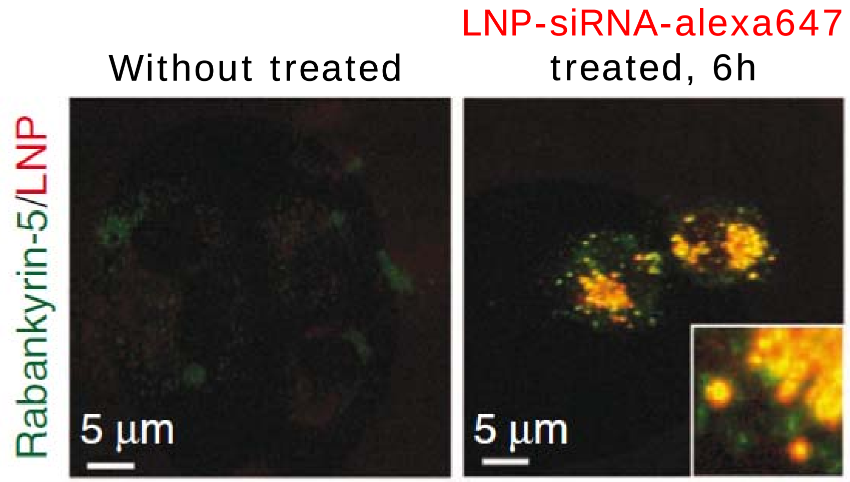
EIPA (inhibitor of macropinocytosis) reduced more than 60% the efficacy of silencing.

❖ Kinetics and mechanisms of LNP uptake

Key points:

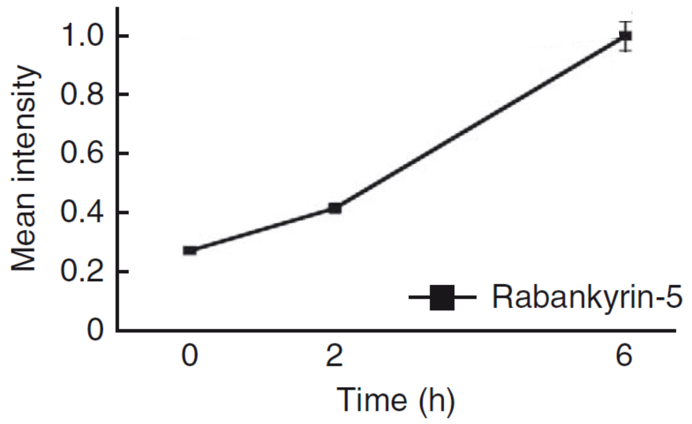
- ❑ Both CME and macropinocytosis contribute to LNP uptake and gene silencing.
- ❑ Macropinocytosis is induced following CME and is quantitatively the major delivery mechanism.

❖ Recruitment of Rabankyrin-5 during LNP trafficking

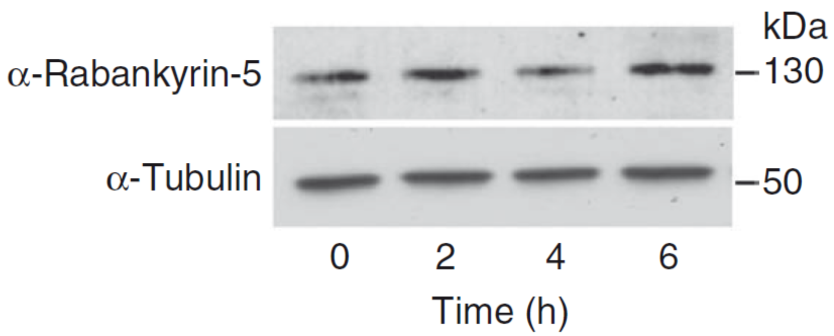


HeLa cells were stained with Rabankyrin-5

~70% co-localization of LNPs & Rabankyrin-5.



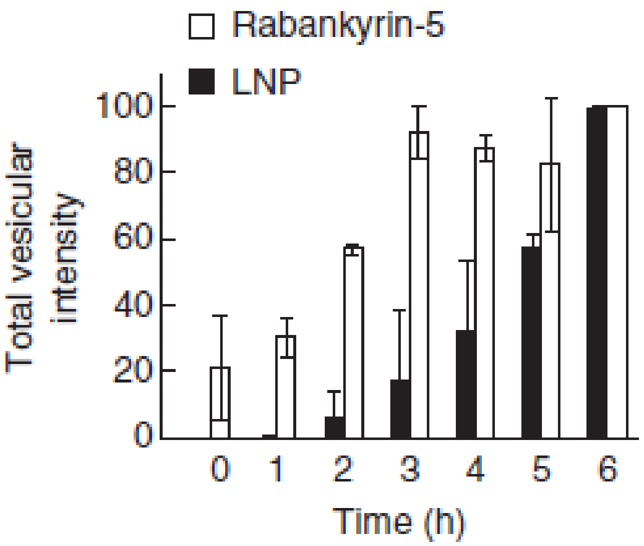
Rabankyrin-5 on membranes was progressively increased during the time course.

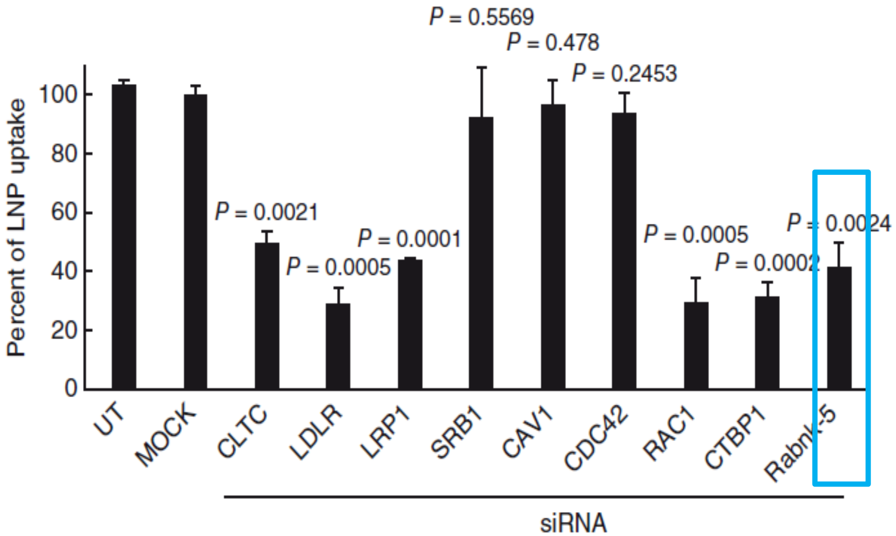


The expression level of Rabankyrin-5 protein was not increased during LNP uptake.

❖ Recruitment of Rabankyrin-5 during LNP trafficking

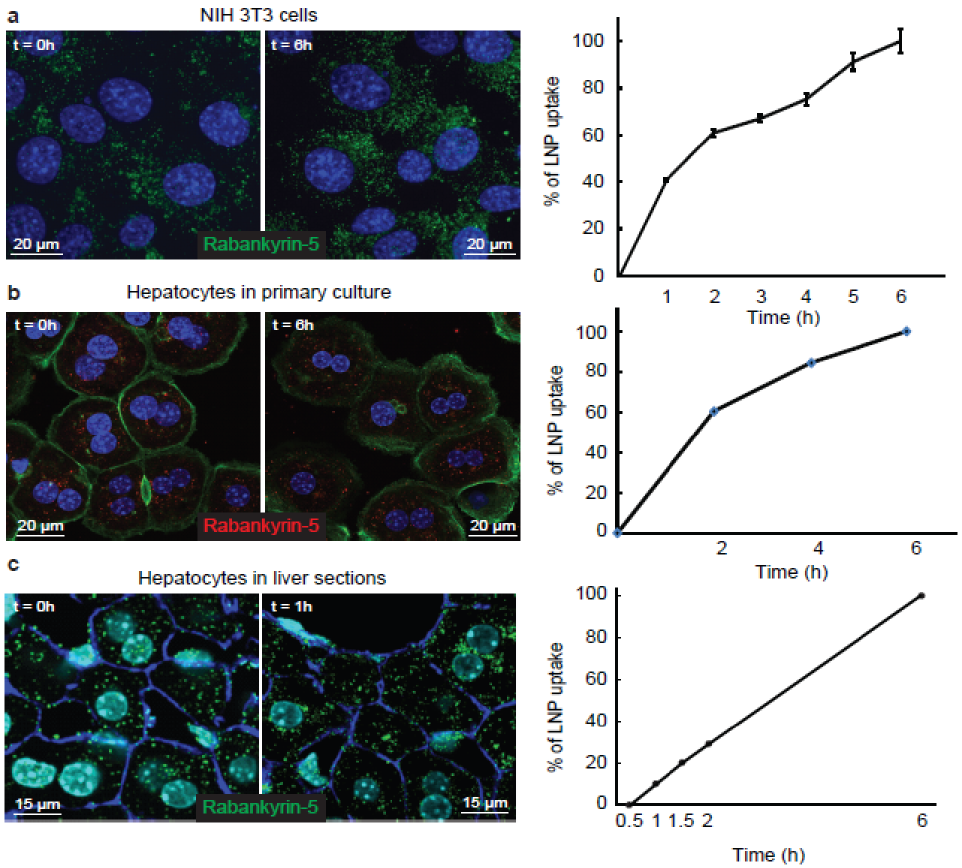
Could the increased membrane recruitment of Rabankyrin-5 account for LNP uptake?



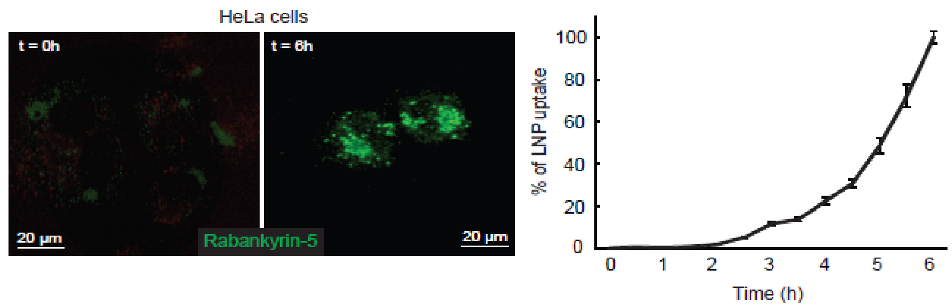


1. The kinetics of membrane recruitment of Rabankyrin-5 paralleled those of LNP uptake.
2. Down regulation of Rabankyrin-5 led to a ~60% reduction in LNP uptake.

❖ Recruitment of Rabankyrin-5 during LNP trafficking

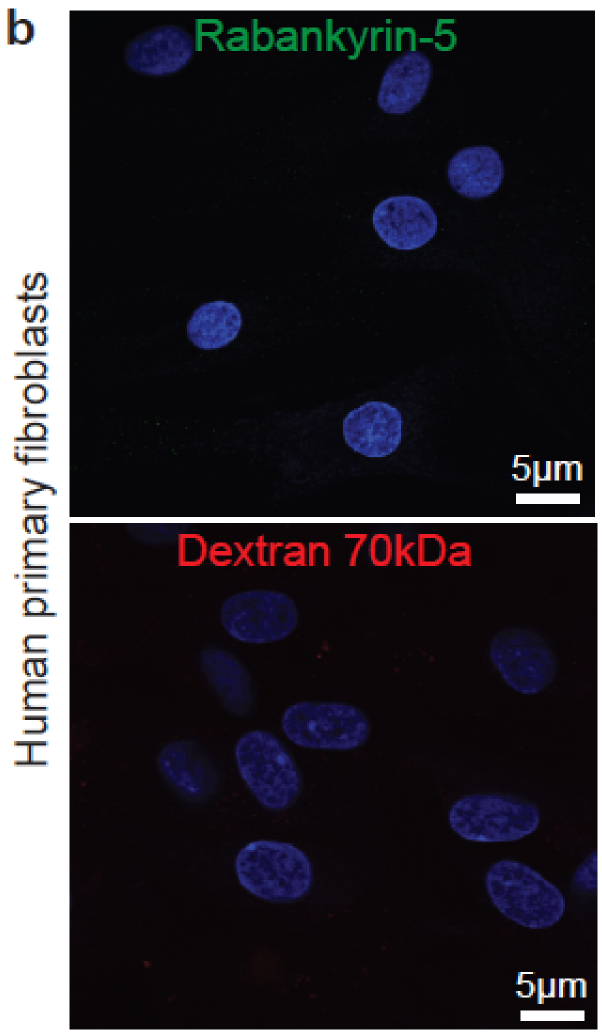


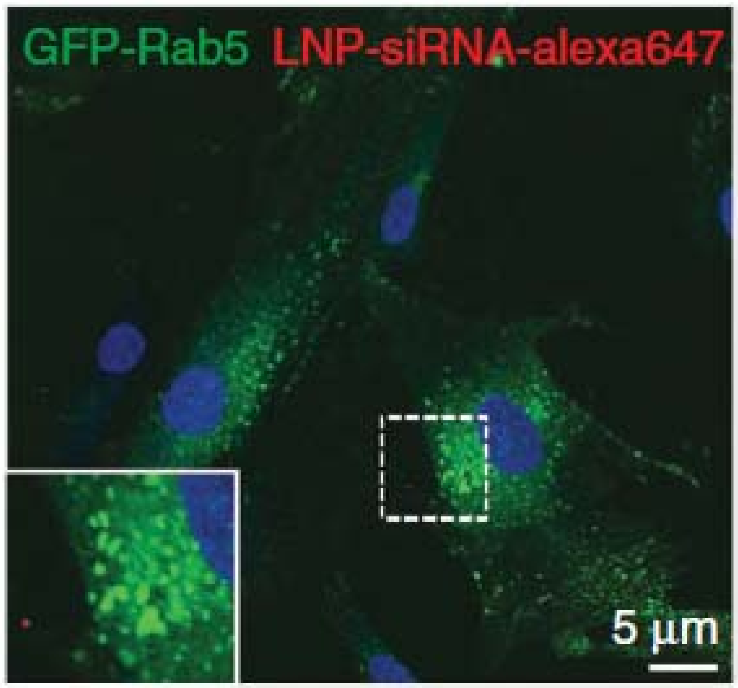
3. a) The uptake of LNPs was constitutive (linear) in cells that contained Rabankyrin-5 positive vesicles.



3. b) The uptake of LNPs was triggered (exponential) in HeLa cells that have very few Rabankyrin-5 positive vesicles under normal conditions.

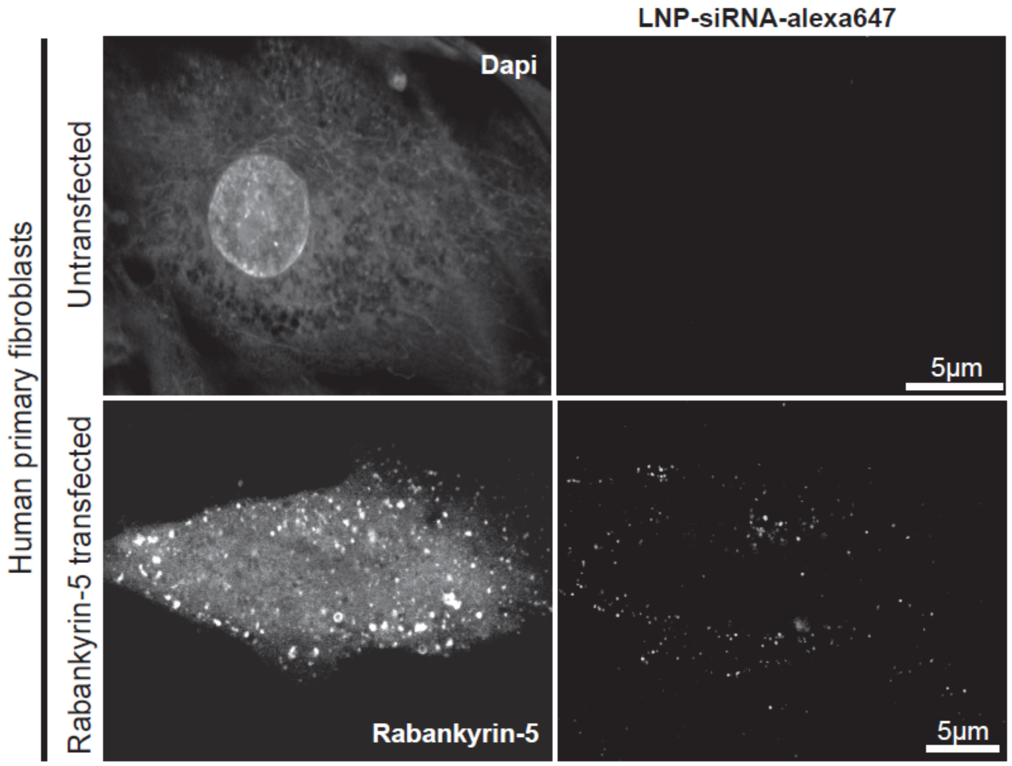
❖ Recruitment of Rabankyrin-5 during LNP trafficking

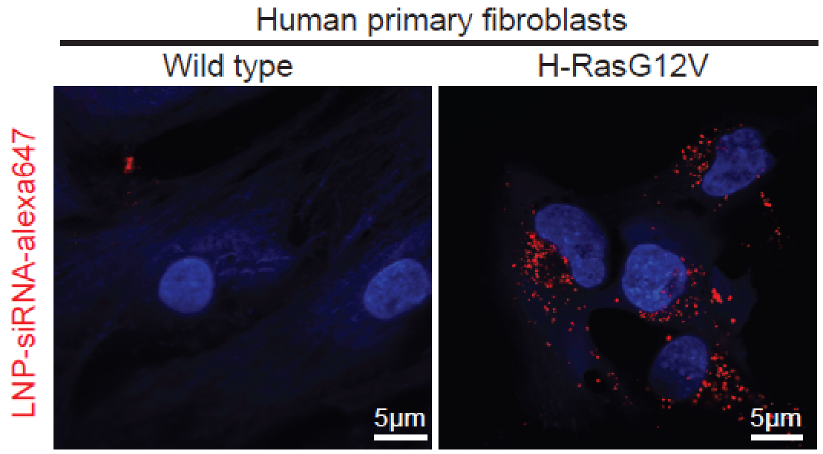


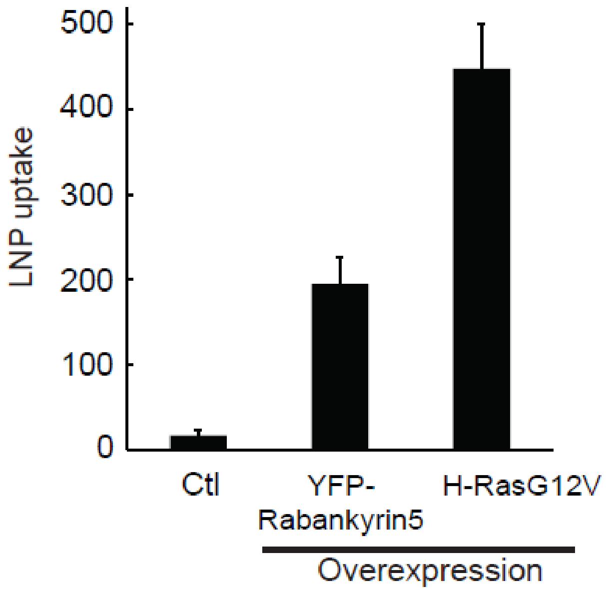


4. Human primary fibroblasts that contained few Rabankyrin-5 positive vesicles and poorly endocytosed dextran exhibited almost undetectable LNP uptake.

❖ Recruitment of Rabankyrin-5 during LNP trafficking







5. Overexpression of Rabankyrin-5 or H-RasG12V, to activate macropinocytosis, induced an increase in LNP uptake.

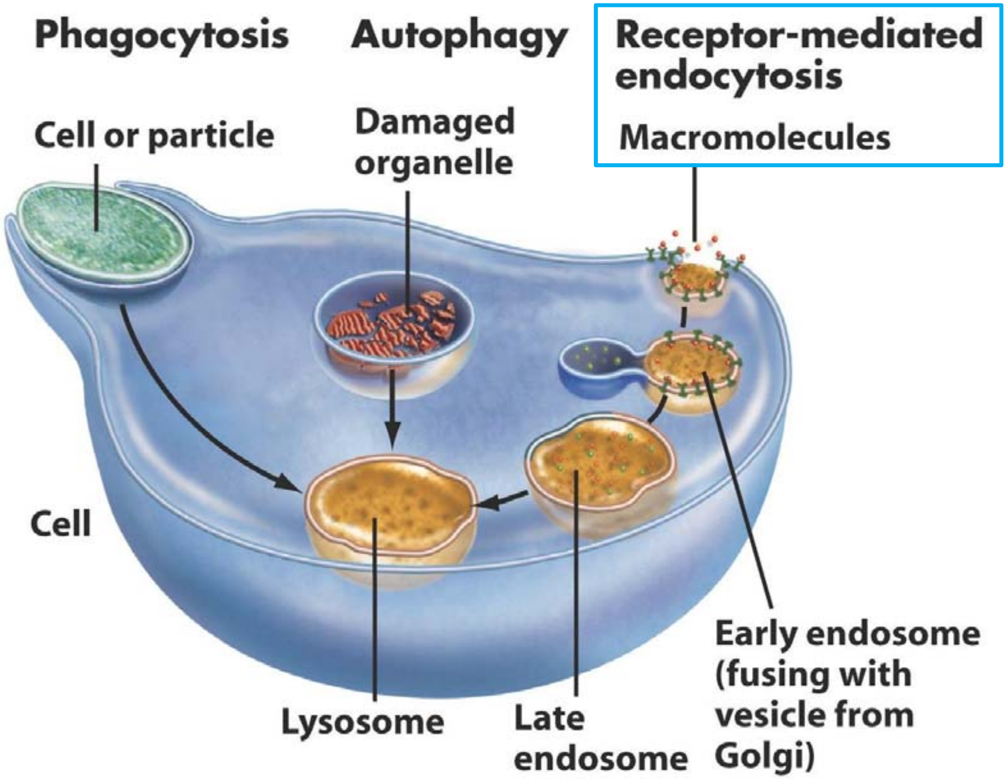
❖ Recruitment of Rabankyrin-5 during LNP trafficking

Key points:

- ❑ LNP delivery is cell type–specific.
- ❑ LNPs can trigger their own uptake by a Rabankyrin-5 dependent process.

❖ Biogenesis and maturation of LNP-containing organelles

What is the intracellular fate of LNP-containing compartments?

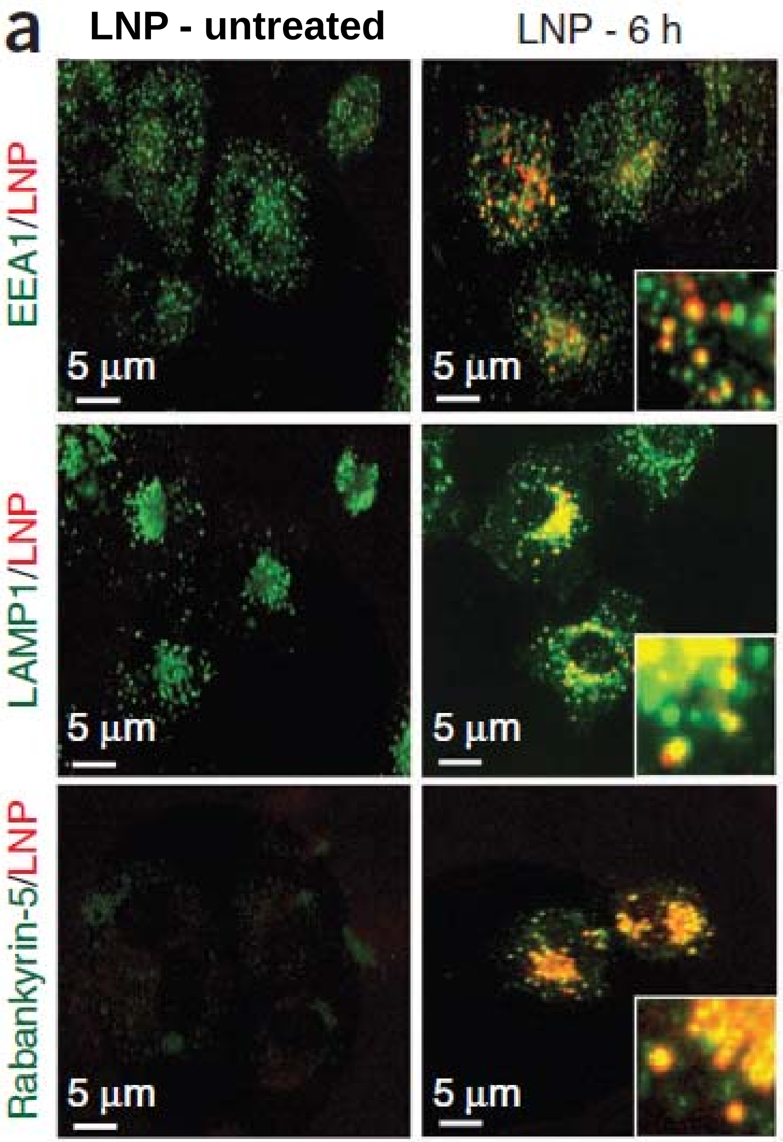


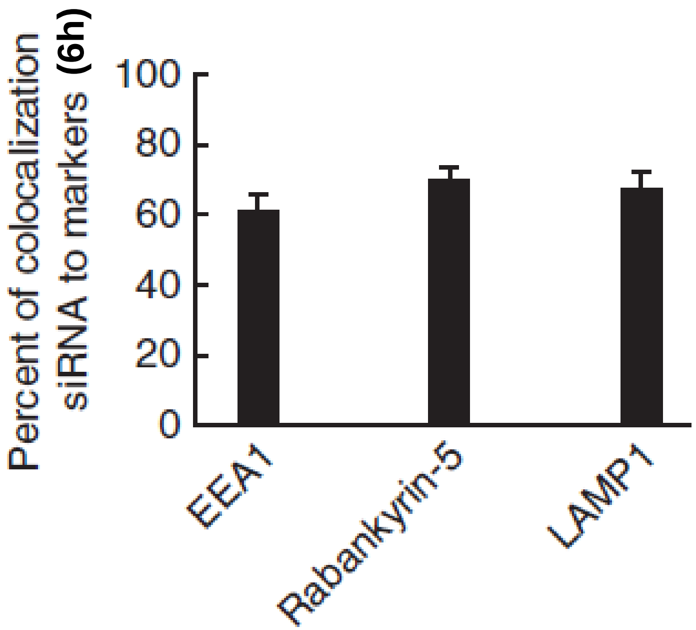
Specific markers:

- EEA1: early endosome antigen 1, for early endosomes.
- LAMP1: lysosomal-associated membrane protein 1, for late endosomes and lysosomes.

Upon internalization, cargo is sequentially transported through early endosomes, late endosomes and lysosomes.

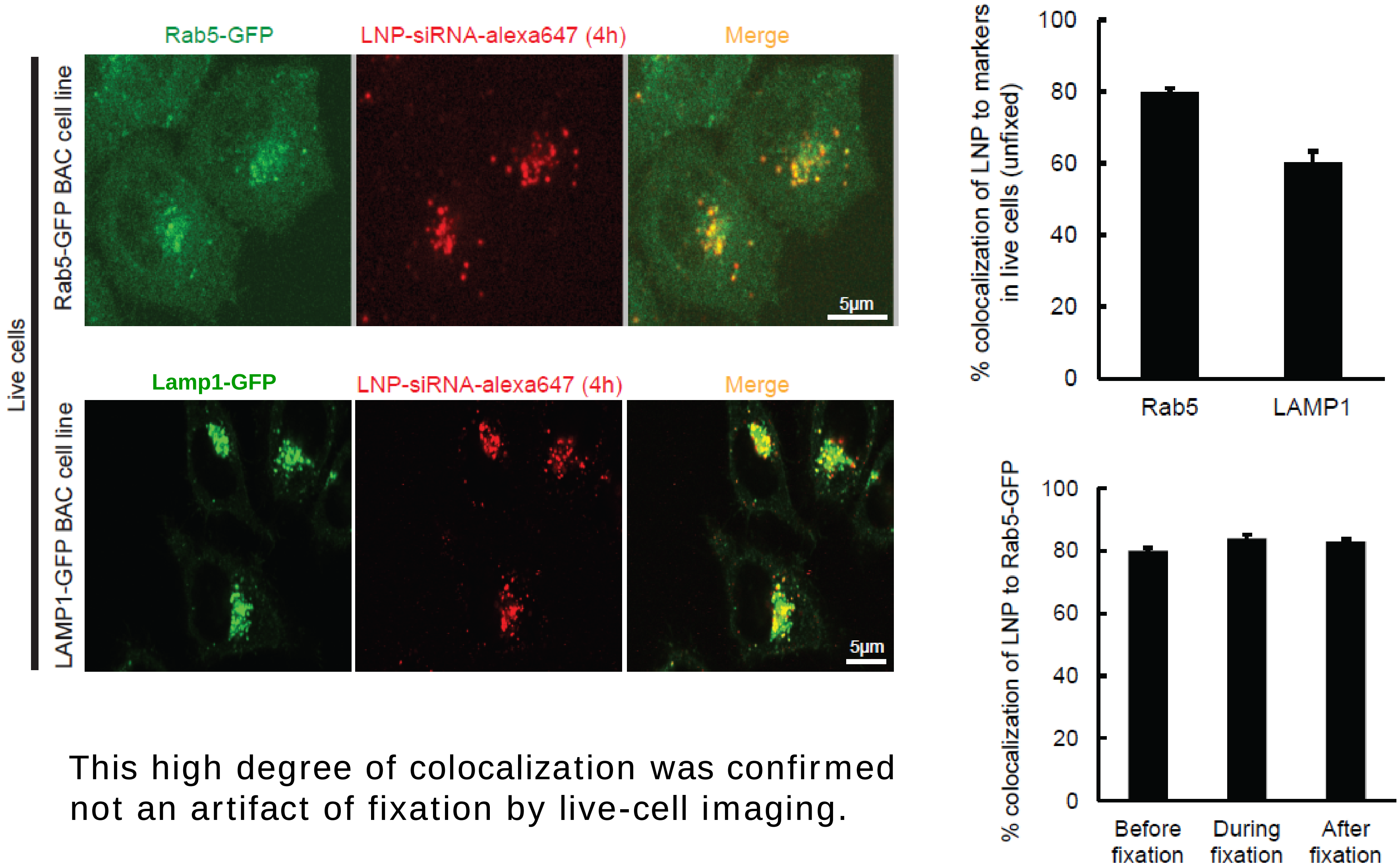
❖ Biogenesis and maturation of LNP-containing organelles_in vitro





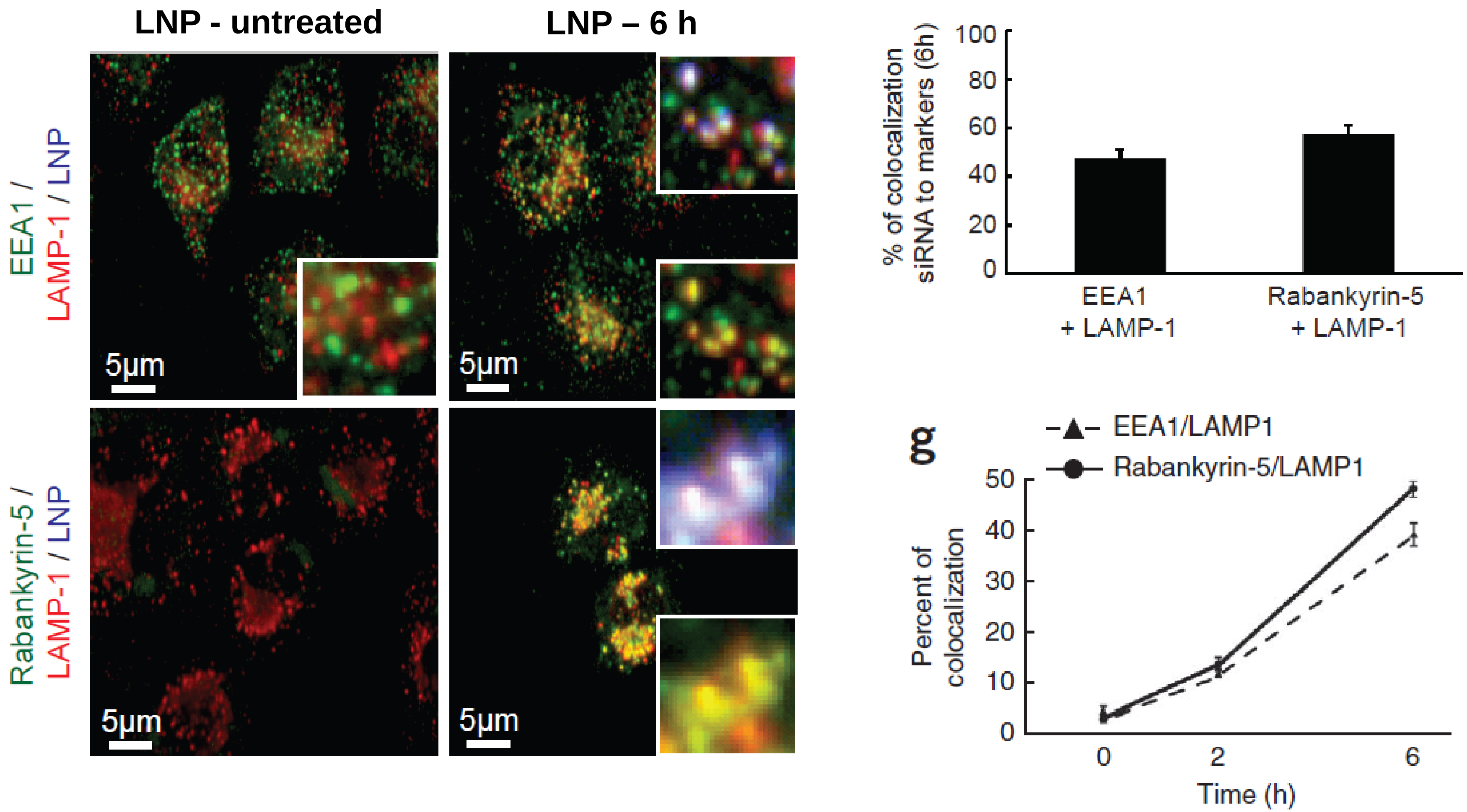
~70% co-localization of LNPs with all three markers was obtained after 6 h incubation.

❖ Biogenesis and maturation of LNP-containing organelles_in vitro



This high degree of colocalization was confirmed not an artifact of fixation by live-cell imaging.

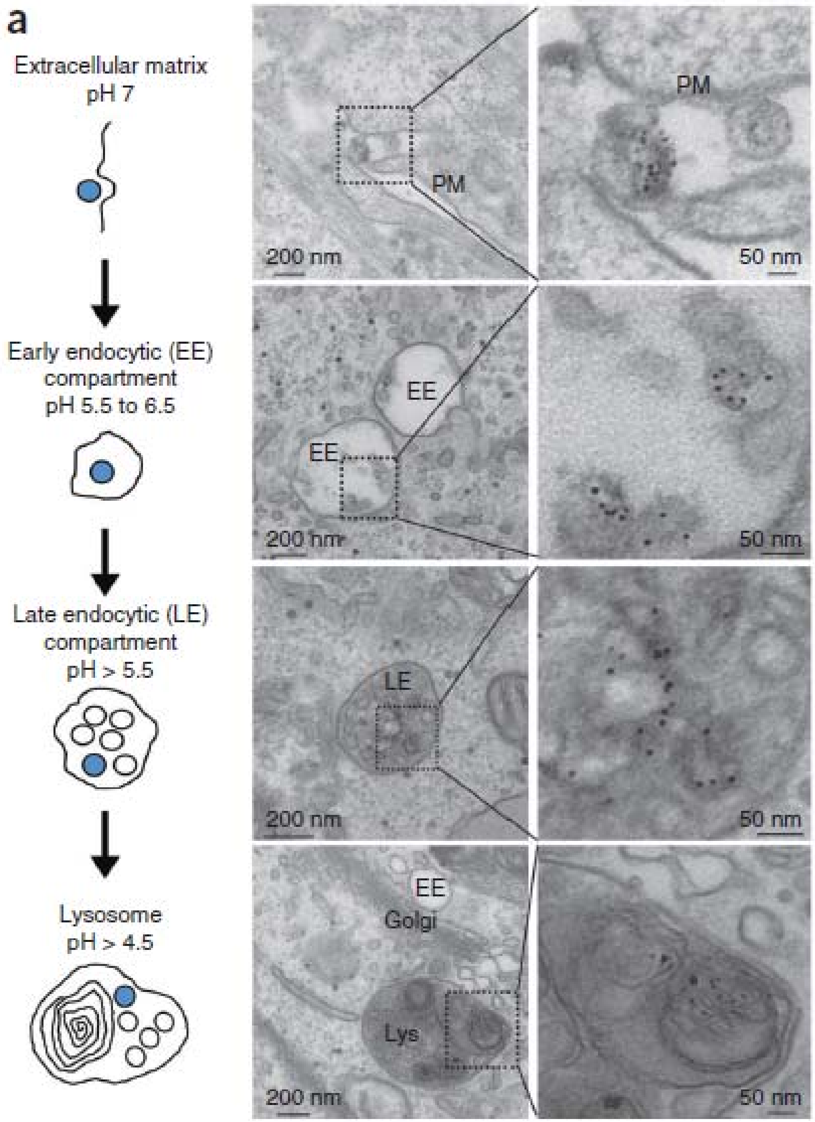
❖ Biogenesis and maturation of LNP-containing organelles_in vitro



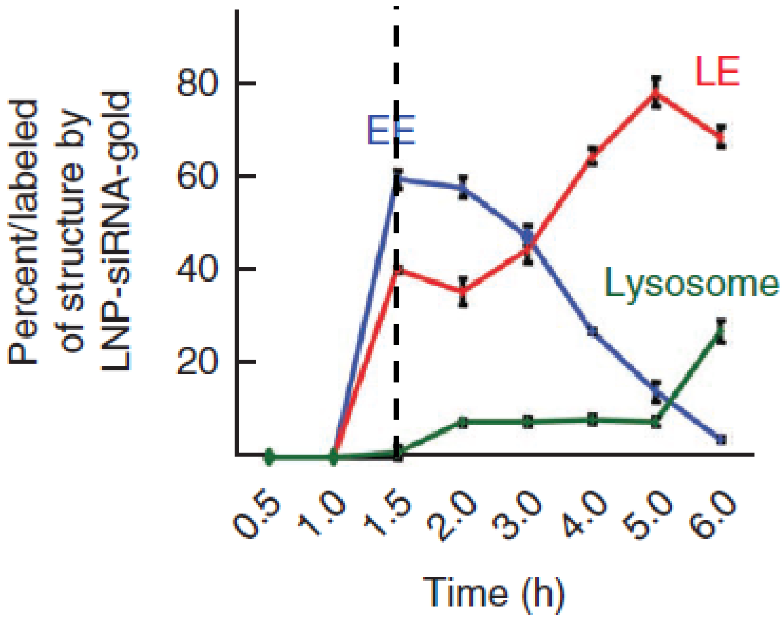
High co-localization was observed between EEA1 and LAMP1, and between Rabankyrin-5 and LAMP1.

LNPs generate a hybrid compartment containing simultaneously early and late endocytic markers.

❖ Biogenesis and maturation of LNP-containing organelles_in vitro

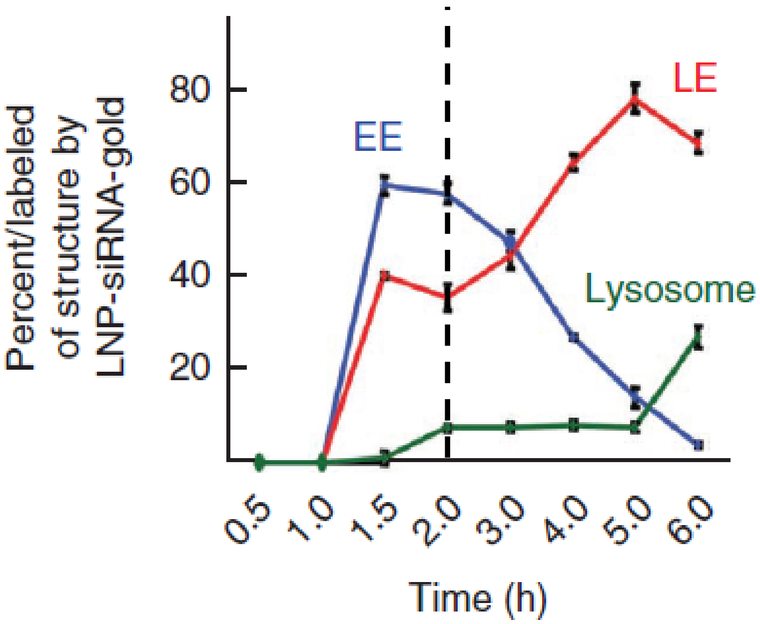


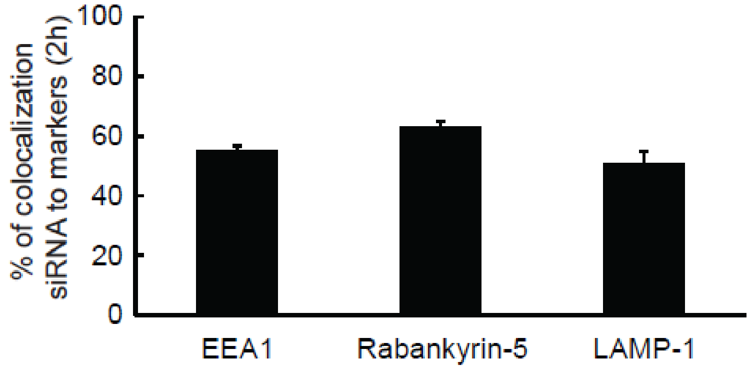
EM: morphological features at the ultrastructural level in HeLa cells.

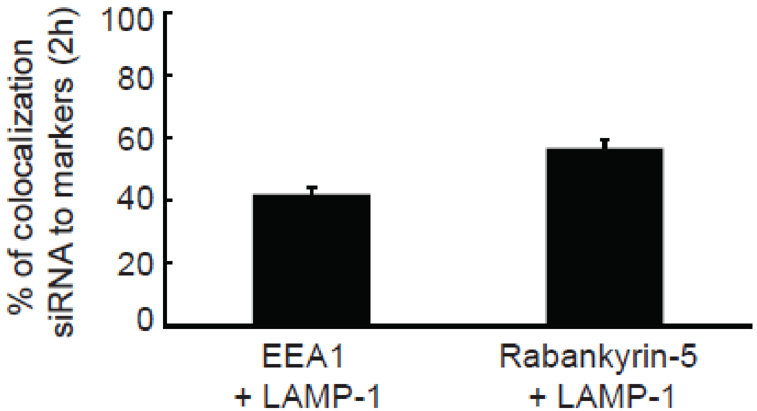


In the early phase of uptake (**1.5 h**), ~60% of LNP-siRNA-gold particles were found in EE compartment and 40% particles were found in LE compartment.

❖ Biogenesis and maturation of LNP-containing organelles_in vitro



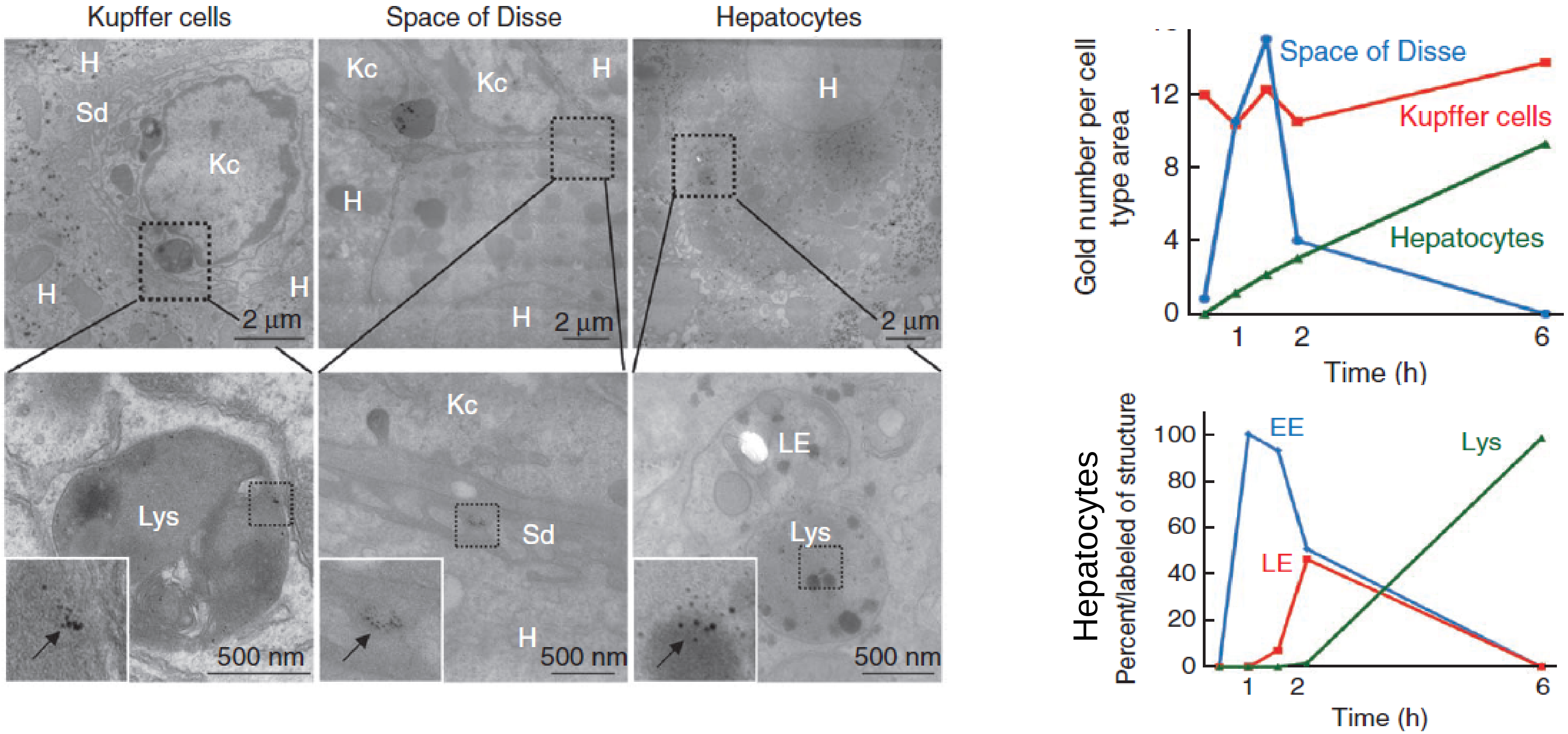




Interestingly,
the LNP-siRNA-gold particles were detected within lysosomes only **6h** after exposure.

The late endosome/lysosome marker LAMP1 had already co-localized with LNP-siRNA-alexa647 at **2h**.

❖ Bioavailability and intracellular trafficking of LNPs_in vivo

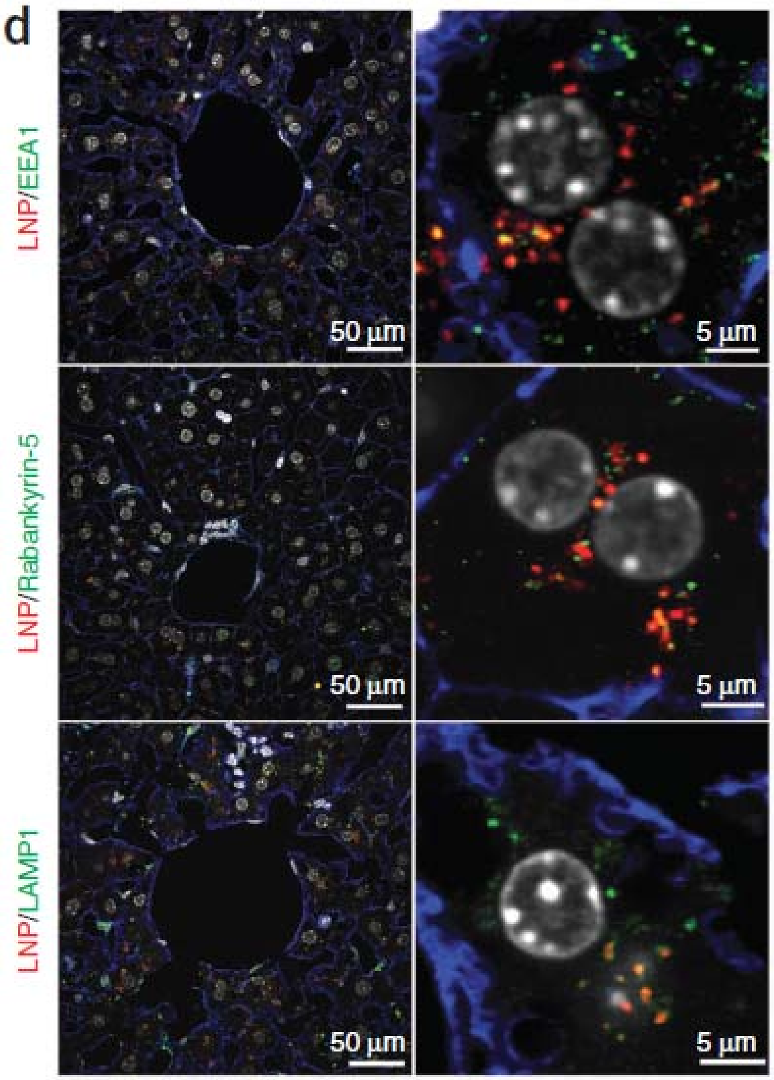


In the endosomes and lysosomes of Kupffer cells (resident macrophages of the liver), LNPs were detected 30min after tail vein injection.

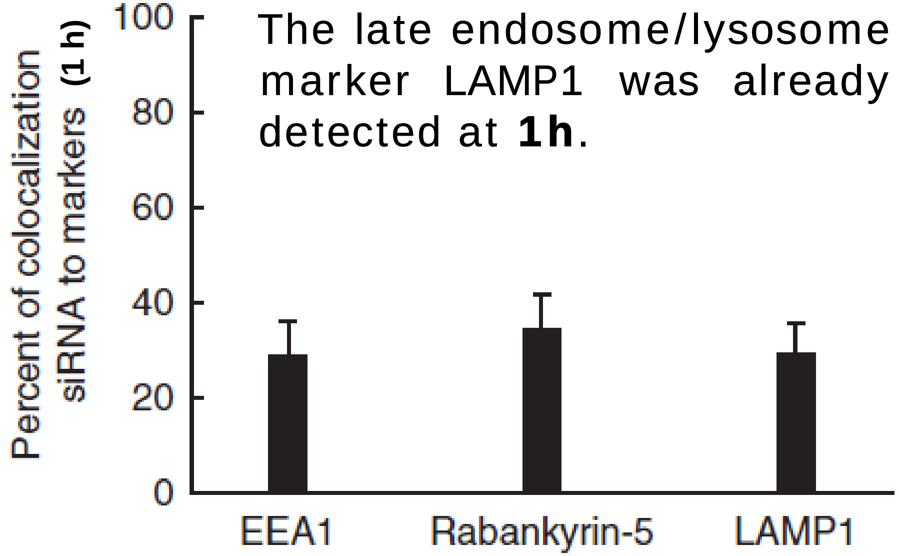
In the space of Disse, the LNPs were detected 1h after injection, concomitant with the onset of LNP uptake by hepatocytes.

In hepatocytes, LNPs were found in early and late endocytic compartments at **2h** and in lysosomes at **6h**.

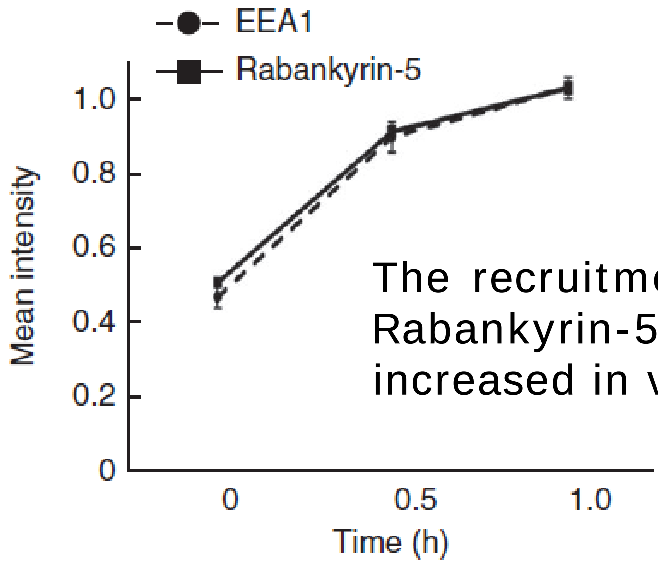
❖ Bioavailability and intracellular trafficking of LNPs_in vivo



Liver sections from mice injected with LNP-siRNA-alexa647



The late endosome/lysosome marker LAMP1 was already detected at **1h**.



The recruitment of Rabankyrin-5 was increased in vivo.

❖ Bioavailability and intracellular trafficking of LNPs

Key points:

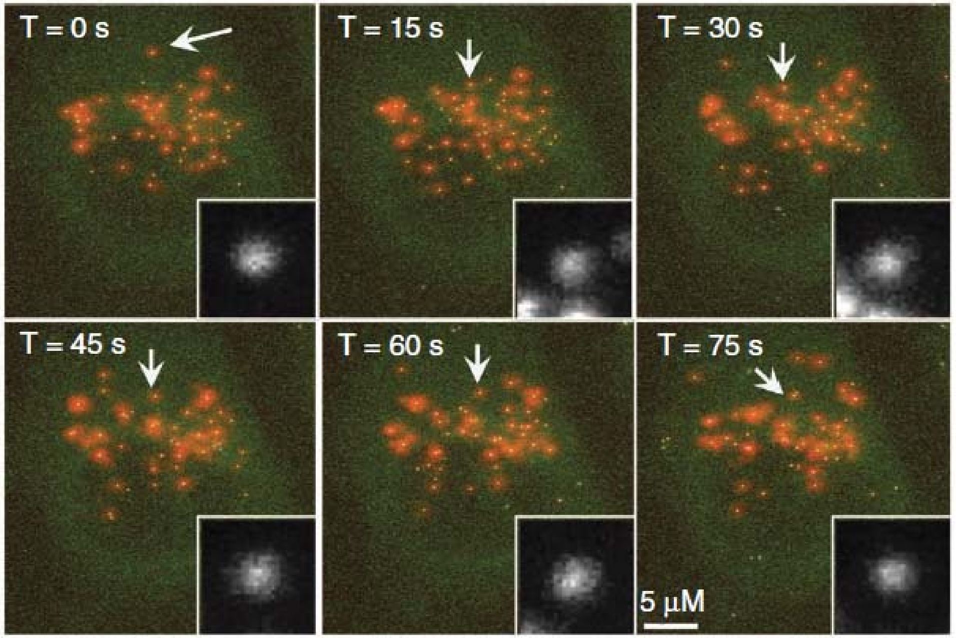
- siRNAs delivered with LNPs accumulate in a compartment having biochemical and morphological characteristics of both early and late endosomes.

- The hybrid early-late endosomal compartment matures slowly, thus the transport of LNPs into lysosomes is delayed.

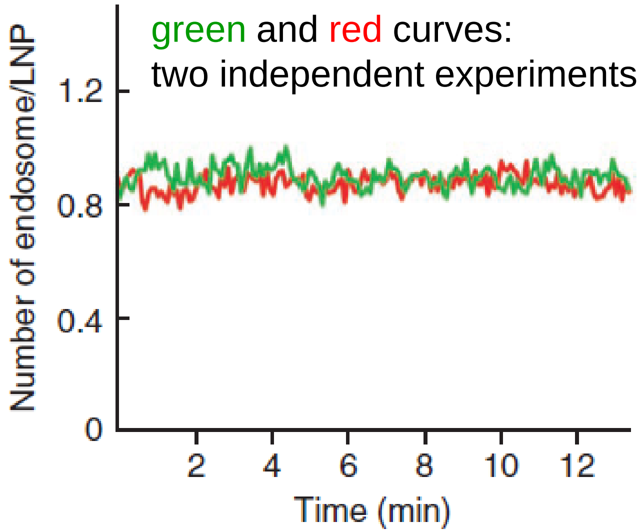
- The consistency between the data obtained in HeLa cells in vitro and hepatocytes in vivo suggests that the formation of Rabankyrin-5 positive structures is a key feature of LNP uptake and intracellular trafficking.

❖ Quantification of siRNA escape

LNPs at therapeutic doses are not massively released not due to bursting of individual endosomes or permeabilization of the limiting membrane of endosomal compartments.



Time-lapse confocal fluorescence microscopy analysis of LNP-siRNA-alexa647



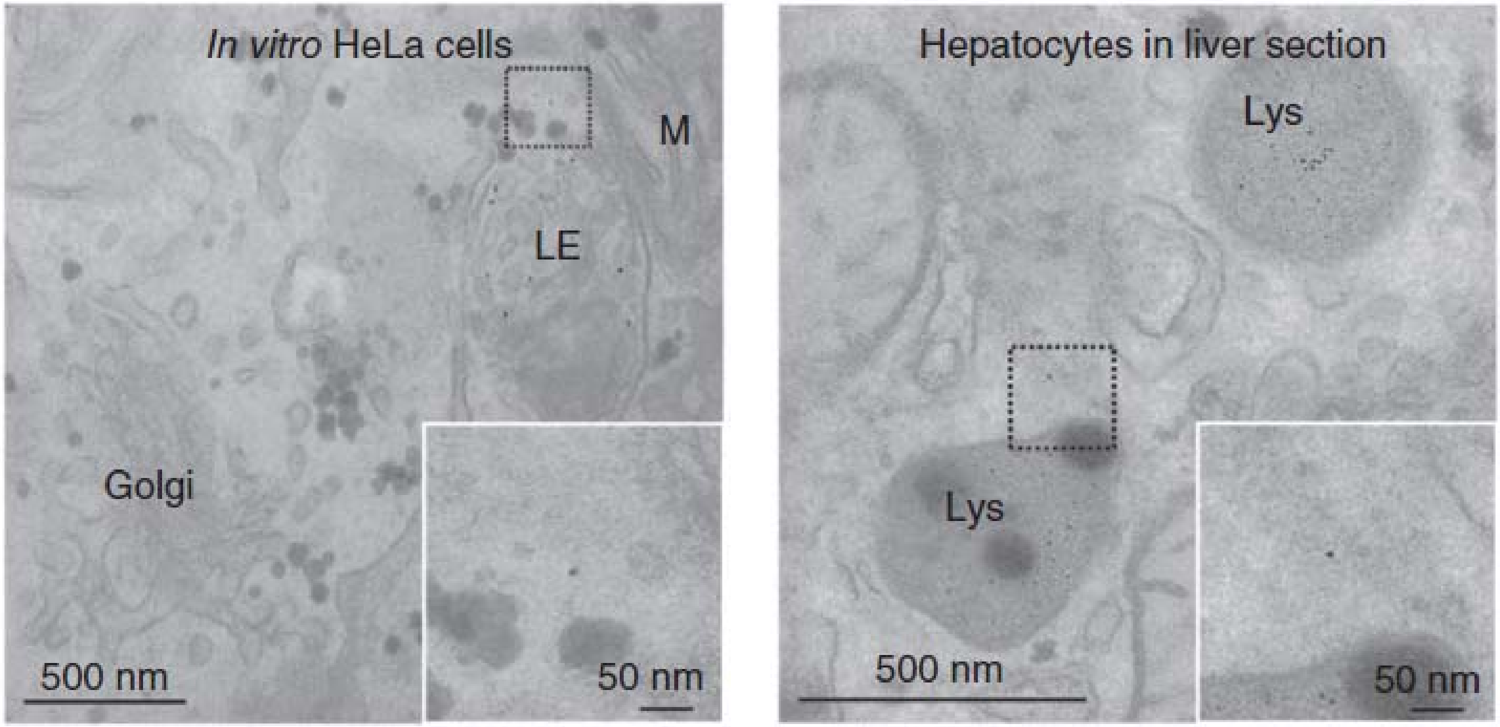
Quantification of the number of endosomes containing LNPs.

The vesicular compartments accumulating siRNAs were stable over long periods of time.

The compartments' number and content did not significantly vary during the time course.

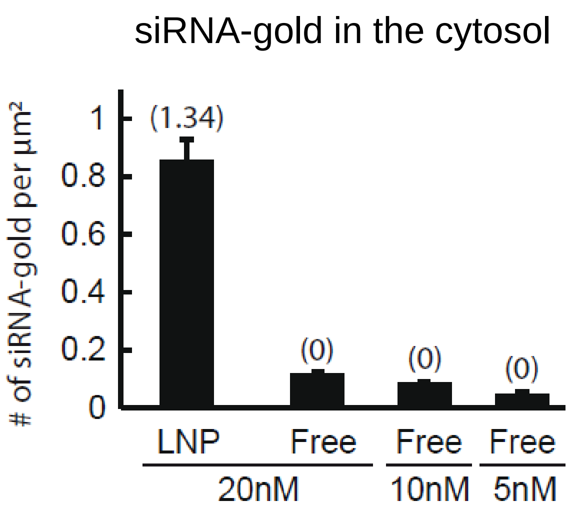
❖ Quantification of siRNA escape

More likely, the release is restricted to a limited number of siRNAs escaping from multiple endosomal compartments and undetectable by standard fluorescence microscopy methods.

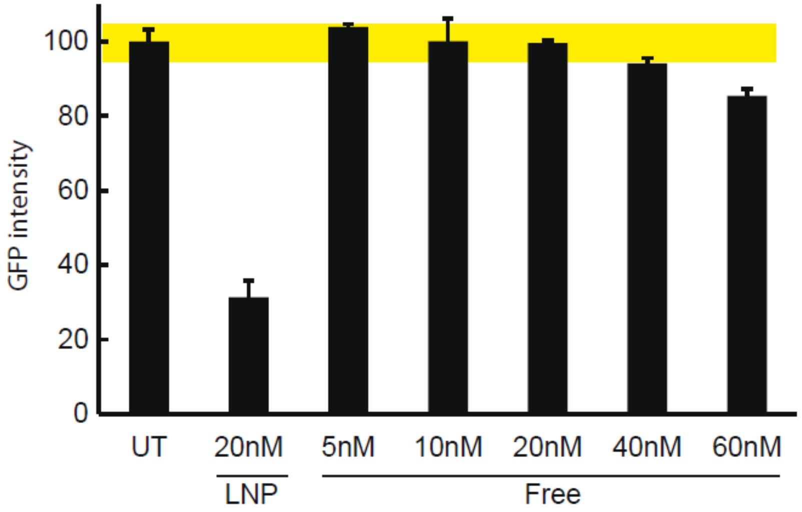


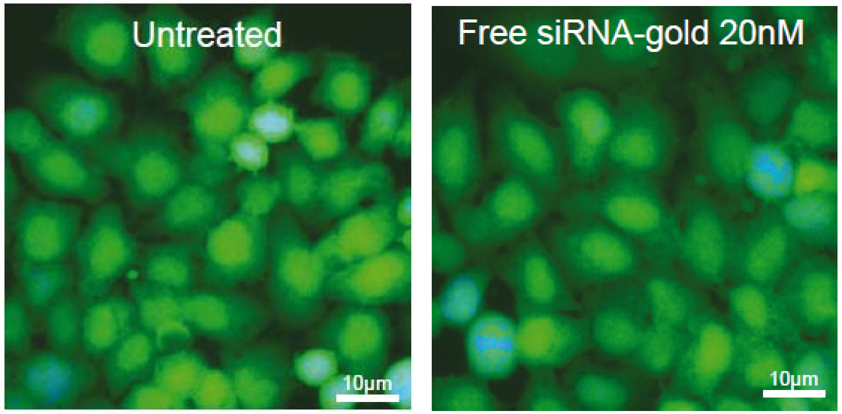
EM: directly visualize siRNA-gold particles released into the cytosol.

❖ Quantification of siRNA escape



Naked siRNA-gold conjugates did not reach the cytosol.



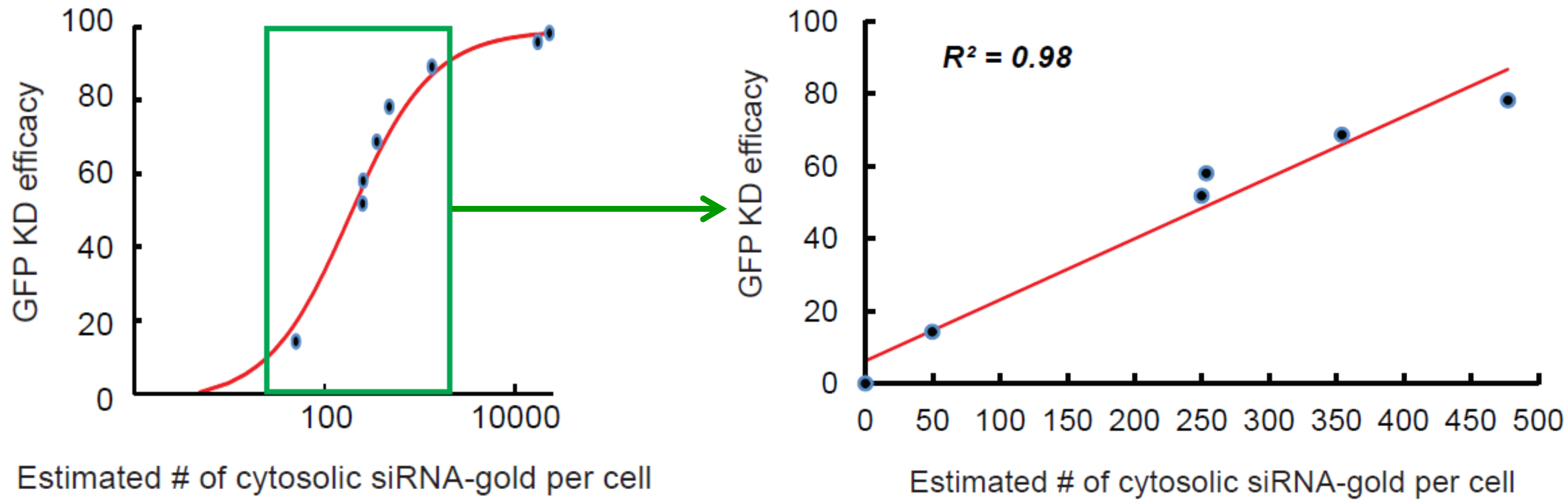


Naked siRNA-gold conjugates did not silence GFP under the same conditions.

❖ Quantification of siRNA escape

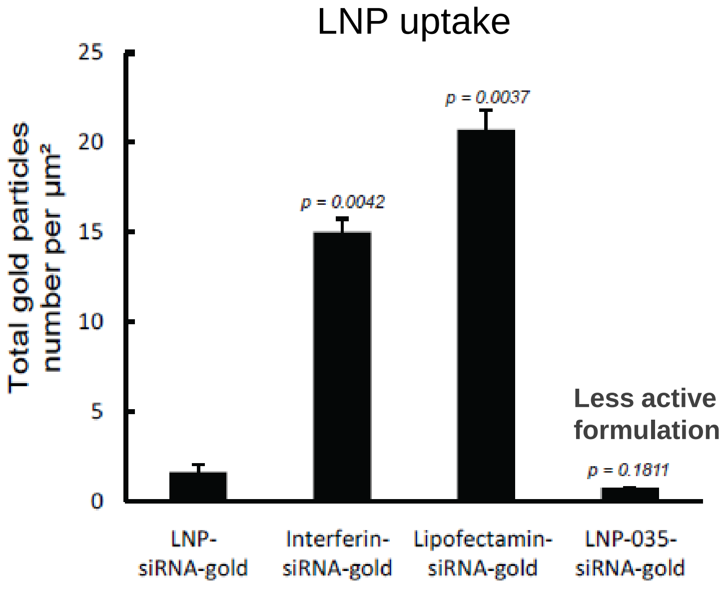
In HeLa cells, **1.34 ± 0.08%** of siRNA-gold escaped endosomes, reaching 249.8 ± 10.87 siRNA-gold per cell after 6h.

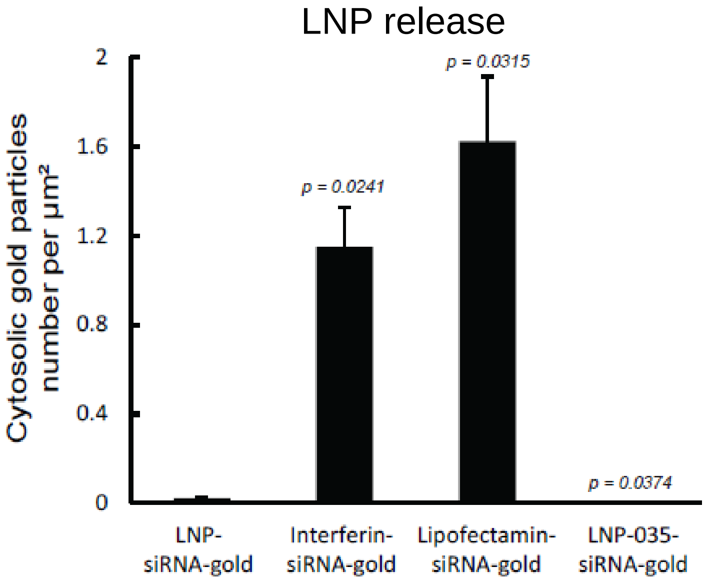
In hepatocytes in vivo, **1.66 ± 0.07%** of siRNA-gold escaped endosomes, reaching 186.05 ± 10.51 siRNA-gold per cell after 6h.

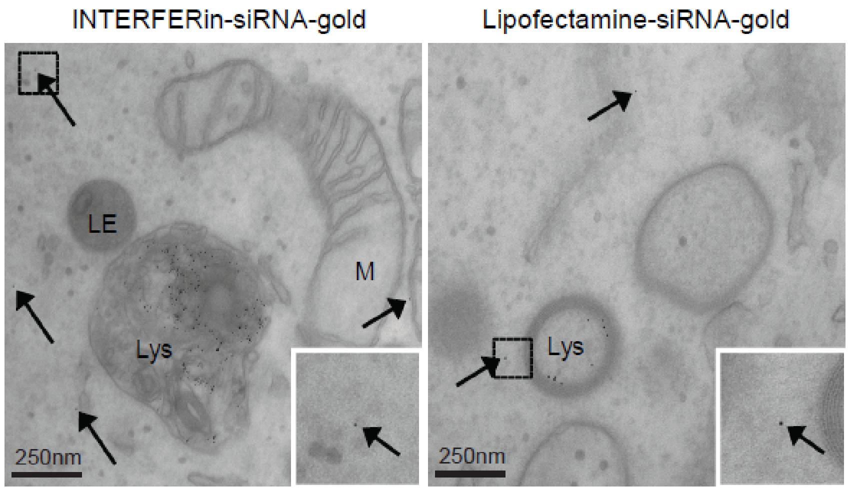


GFP silencing is highly correlated with the number of cytosolic siRNA-gold per cell in the linear phase of the curve (before the saturation phase).

❖ Quantification of siRNA escape





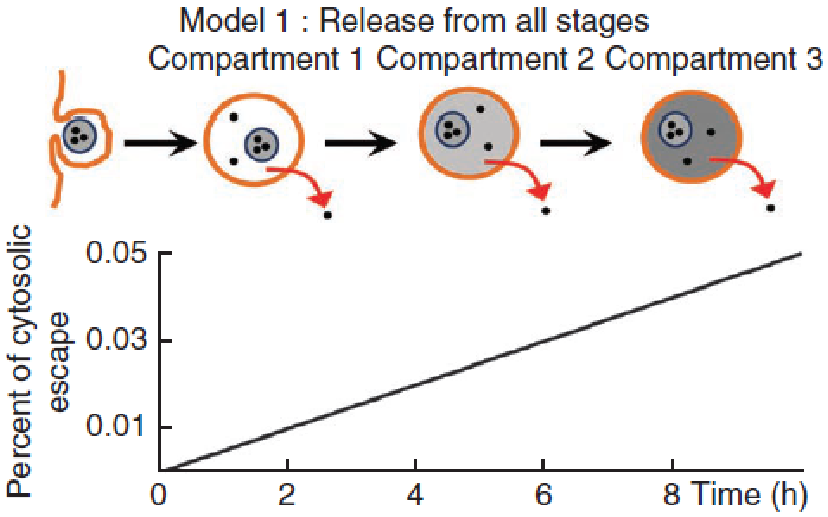


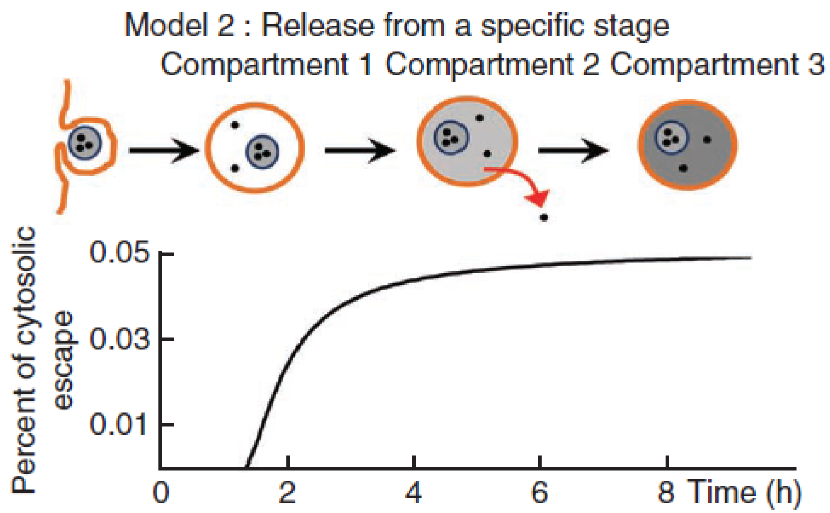
Efficient uptake did not necessarily result in efficient gene silencing.

The escape of siRNA from endosomes into the cytosol was a rate-limiting step.

❖ Quantification of siRNA escape

From which endosomal compartment do the siRNAs escape?

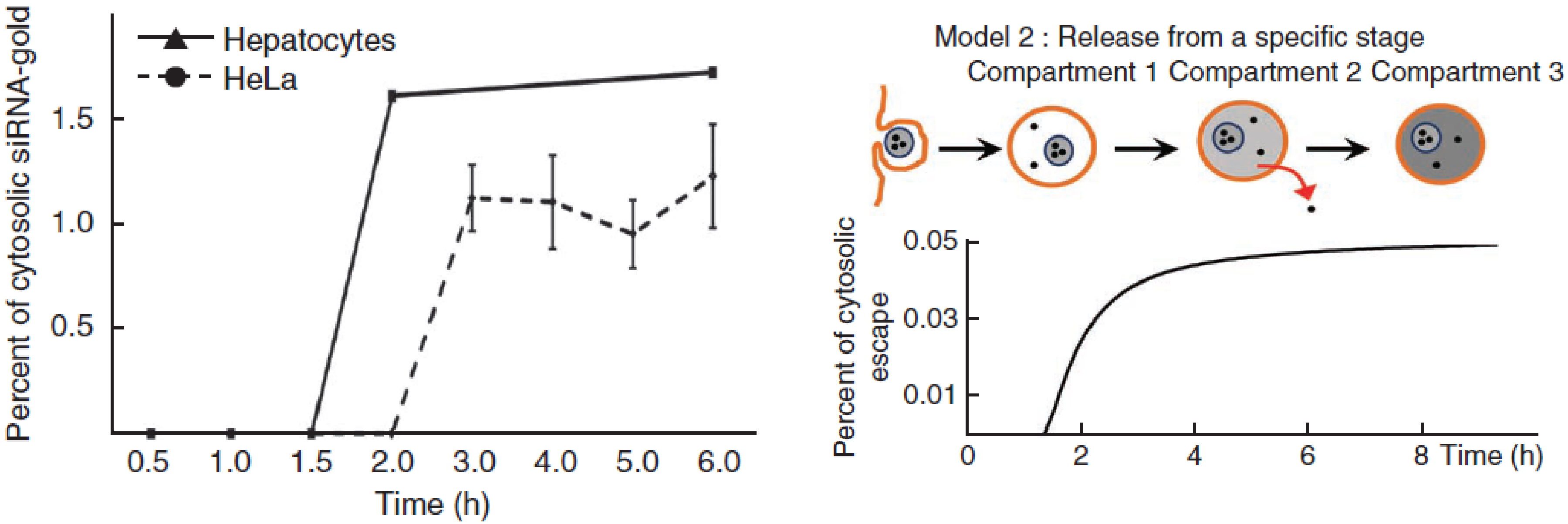




Model 1: If the release of siRNAs occurrs at all endosomal stages of LNP trafficking, cytosolic escape would be predicted to follow linear kinetics.

Model 2: If the release occurrs within a specific endosomal compartment, cytosolic escape should follow sigmoidal kinetics.

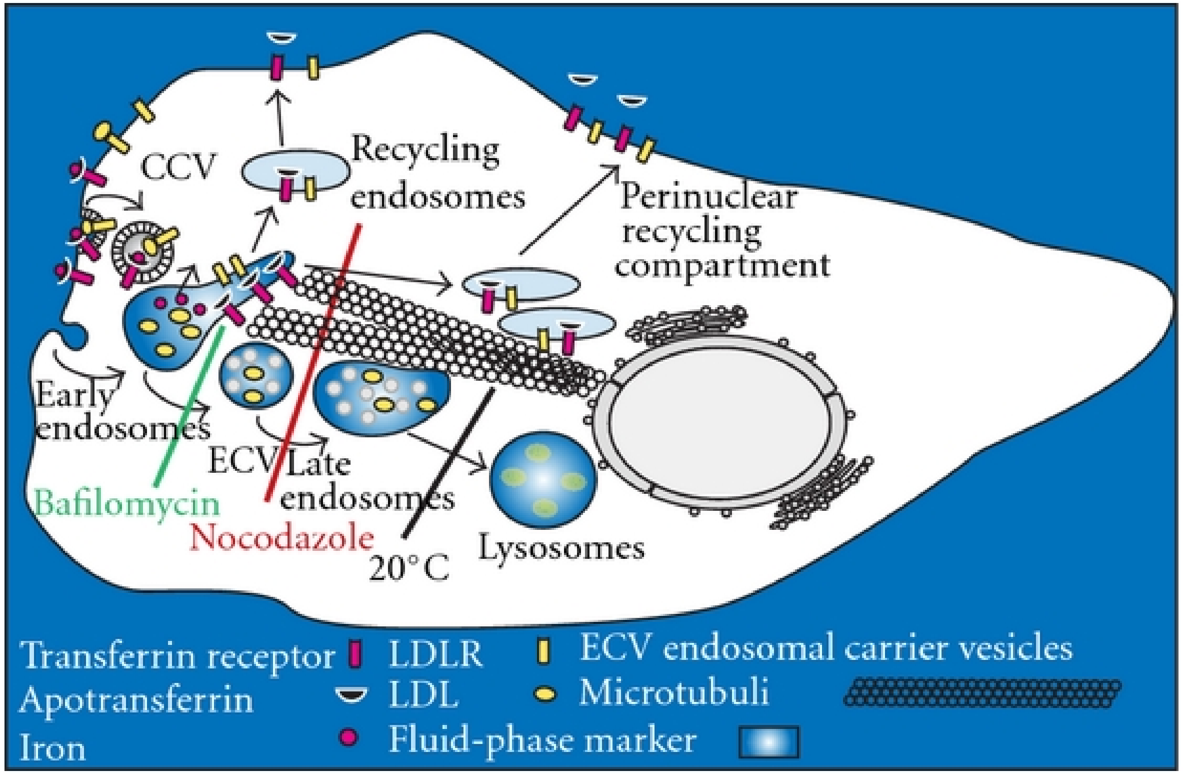
❖ Quantification of siRNA escape



In vitro and in vivo quantification of the ratio of cytosolic and total siRNA-gold indicated that the release of siRNA-gold followed sigmoidal kinetics.

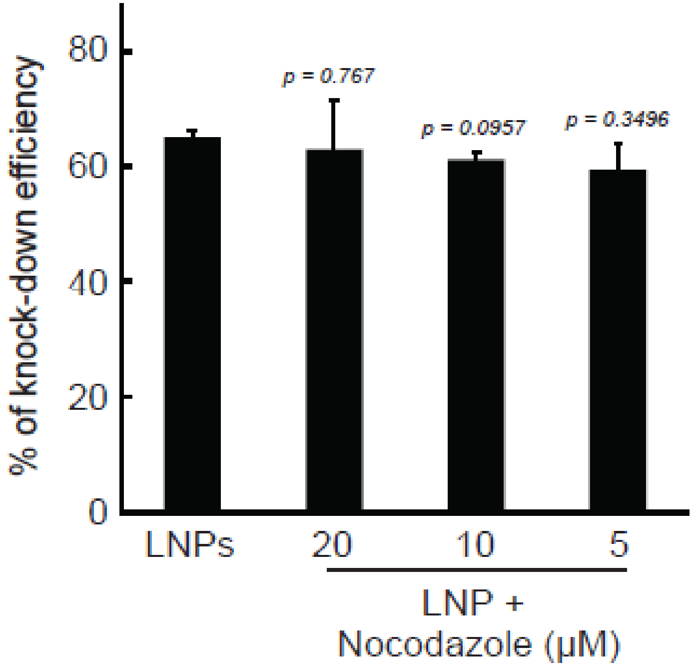
siRNA escape occurs only at a specific stage of the endosomal trafficking of LNPs.

❖ Quantification of siRNA escape



- Nocodazole: leads to accumulation of cargo in ECV.
- Bafilomycin: blocks endosomal acidification and inhibits progression of early to late endosomes.

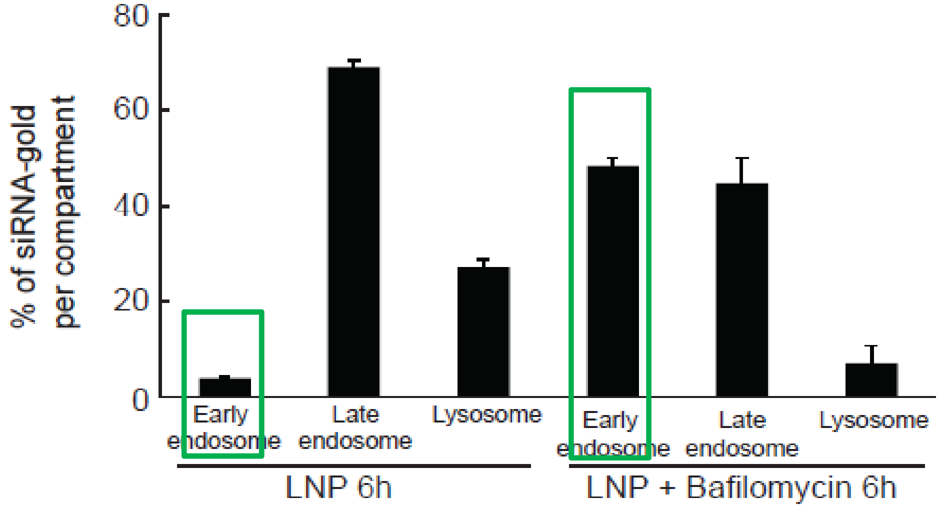
❖ Quantification of siRNA escape

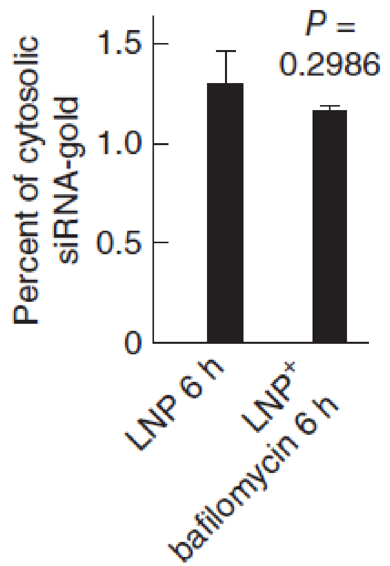


Nocodazole treatment did not inhibit GFP down regulation,

meaning that siRNA release occurred before late endosomes stage.

❖ Quantification of siRNA escape





Bafilomycin treatment inhibited the delivery of LNP from early to late endosomes,

but did not alter the efficiency of release of siRNA-gold,

meaning that the siRNA release mainly occurred in a moderately acidic, early endocytic compartment.

❖ Quantification of siRNA escape

Key points:

- ❑ The escape of siRNA from endosomes into cytosol is a key rate-limiting step in siRNA efficiency.
- ❑ Only a small fraction (1–2%) of siRNAs are released from endosomes.
- ❑ Release of siRNAs mainly occurs in a moderately acidic, early endocytic compartment.


Thank
you
for
Attention