getting into it

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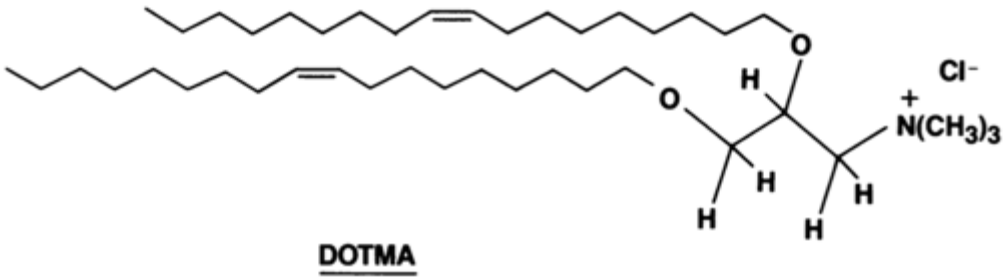
new technologies for intracellular cargo delivery

TECHNICAL JOURNAL CLUB 19TH MAY 2015

KARL FRONTZEK

INSTITUTE OF NEUROPATHOLOGY

Intracellular delivery



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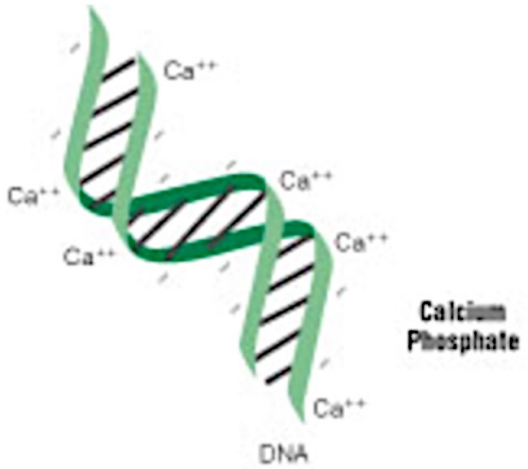
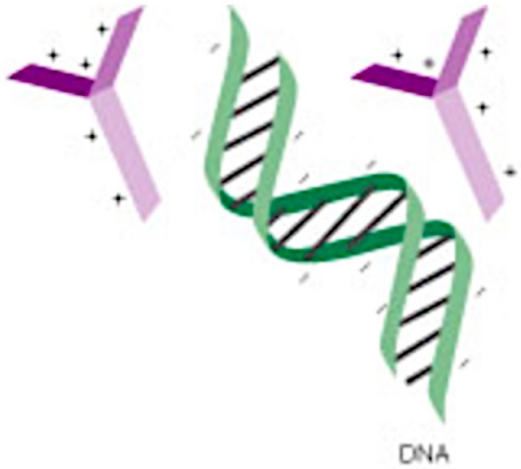


Calcium Phosphate



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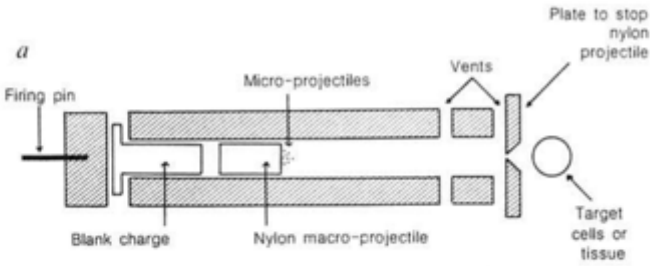
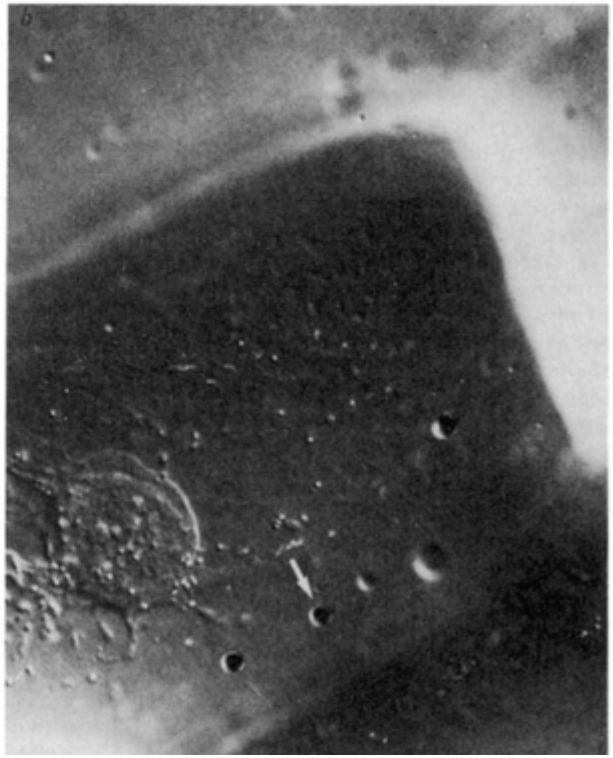
Intracellular delivery

NATURE VOL. 327 7 MAY 1987 LETTERS TO NATURE





Gene guns
TM Klein et al., Nature 1987

RESEARCH

Gene transfer into muscle by electroporation in vivo

Hiroyuki Aihara[1,2] and Jun-ichi Miyazaki[1]*

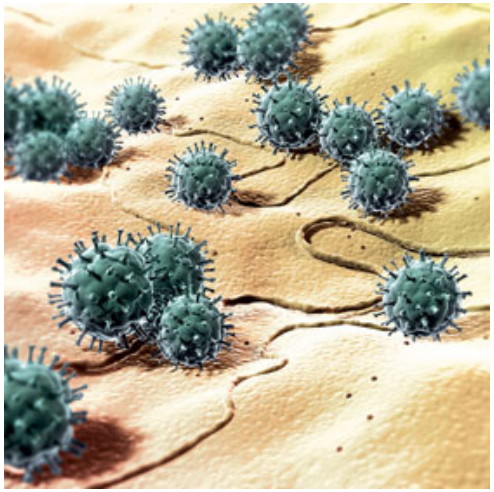
Brief Communications

Nature **418**, 290-291 (18 July 2002) | doi:10.1038/418290a

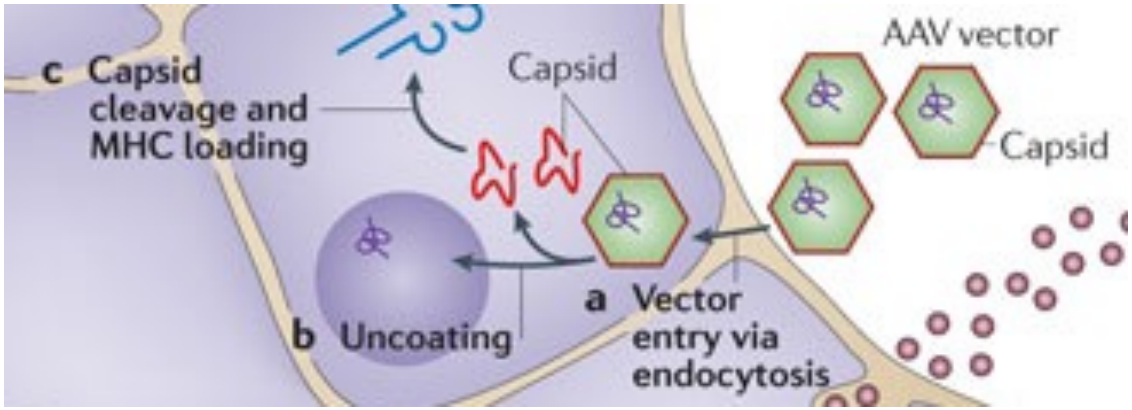
Cell biology: Targeted transfection by femtosecond laser

Uday K. Tirlapur & Karsten König

Intracellular delivery



Lentiviral packaging
www.clontech.com



Adeno-associated virus packaging
Mingozzi F & High KA, Nat Rev Genetics 2011

Intracelullar delivery of native proteins/DNA

„in vitro“ surgery

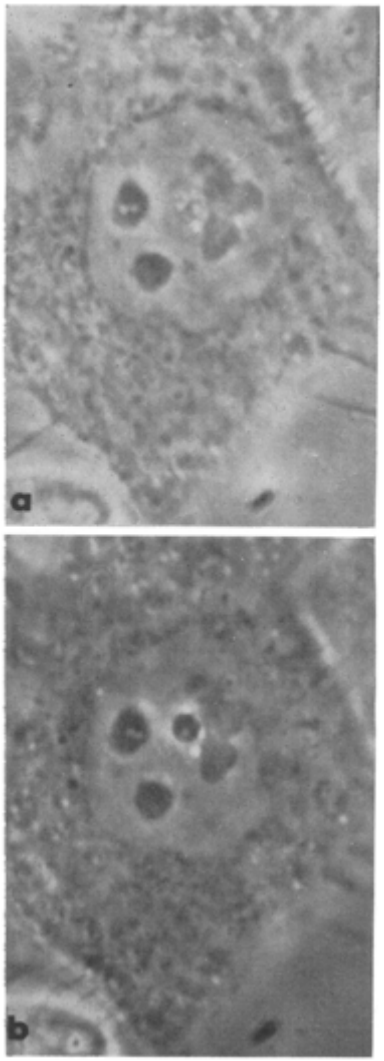
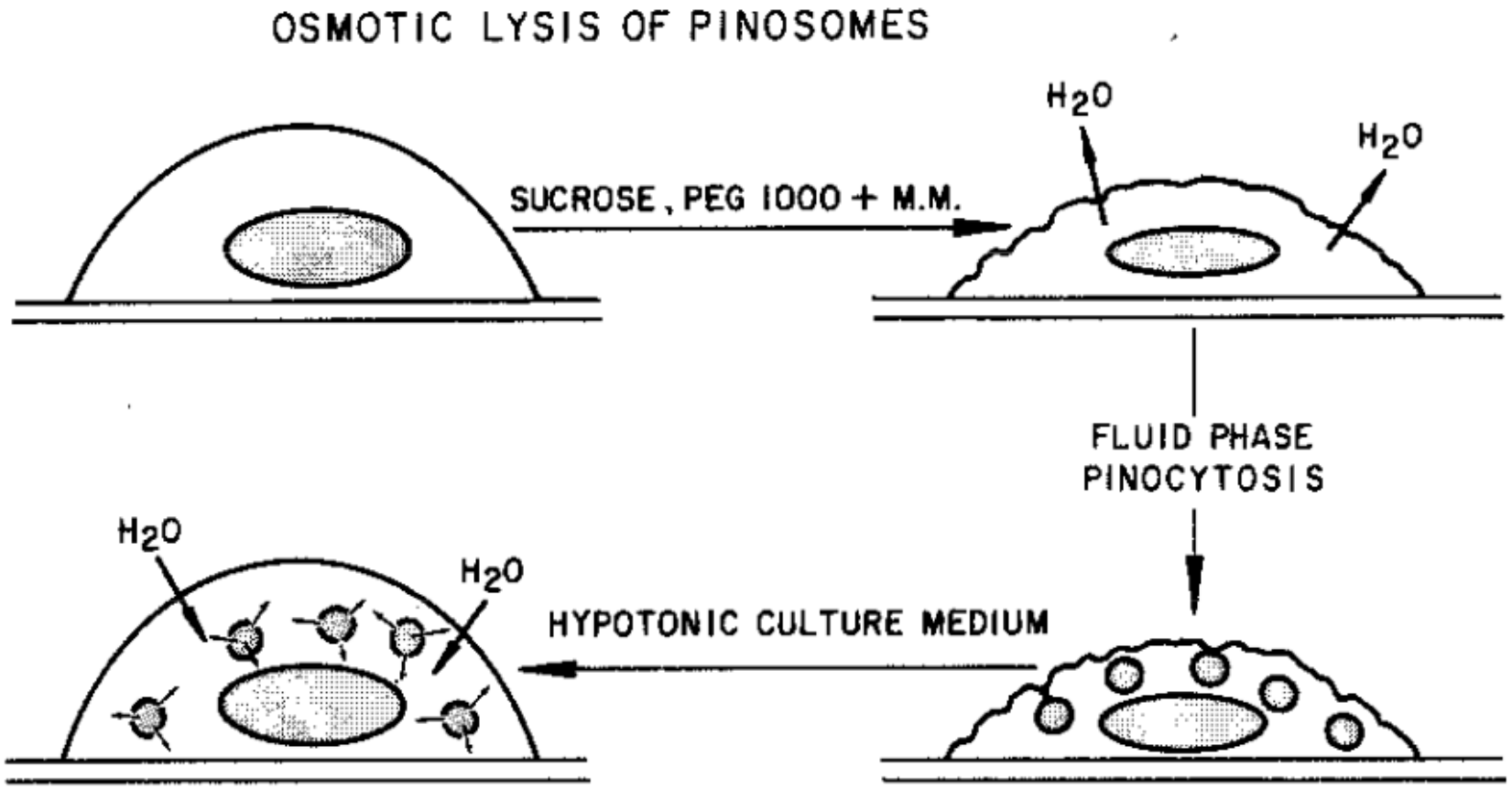


FIG. 1.

Diacumos EG et al., PNAS 1970

Intracelullar delivery of native compounds

„osmotic lysis of pinocytic vesicles“



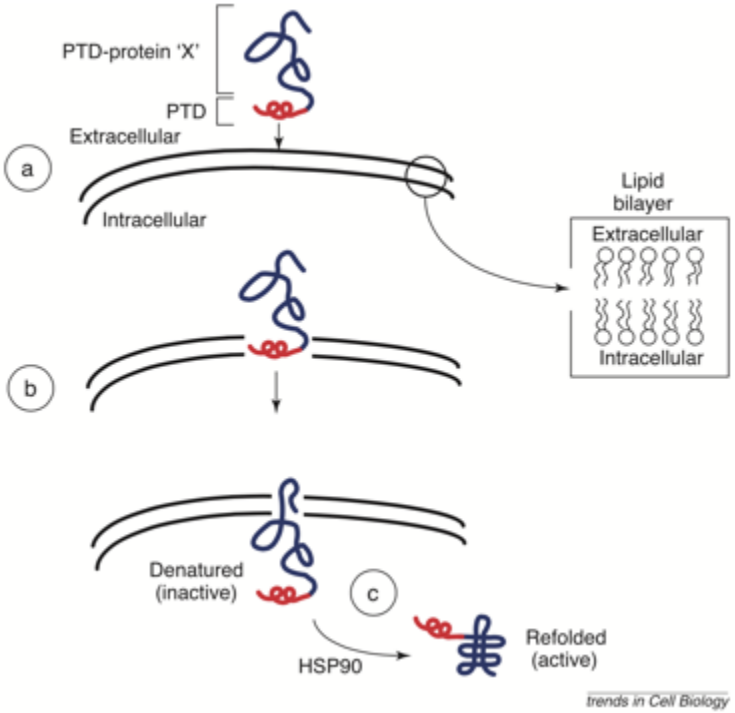
Okada CY & Rechsteiner M, Cell 1982

Intracellular delivery with fusion proteins carrying „protein-transduction domains“

TABLE 1 – AMINO ACID SEQUENCE OF CHARACTERIZED PTDS

PTD	Amino acid sequence
HIV-1 TAT	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg[a]
HSV VP22	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Glu
Antp	Arg-Gln-Iso-Lys-Iso-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys

[a]Gly-Arg-Lys-Lys-Arg-Arg is a potential nuclear localization sequence.
Abbreviation: PTD, protein-transduction domain.



Correspondence

Nature Biotechnology **19**, 713 (2001)
doi:10.1038/90741

Is VP22 nuclear homing an artifact?

Mathias Lundberg[1] & Magnus Johansson[2]

1. Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-17177 Stockholm, Sweden
2. Division of Clinical Virology F68, Karolinska Institute, Huddinge University Hospital, S-14186 Stockholm, Sweden
(e-mail: magnus.johansson@mbb.ki.se).

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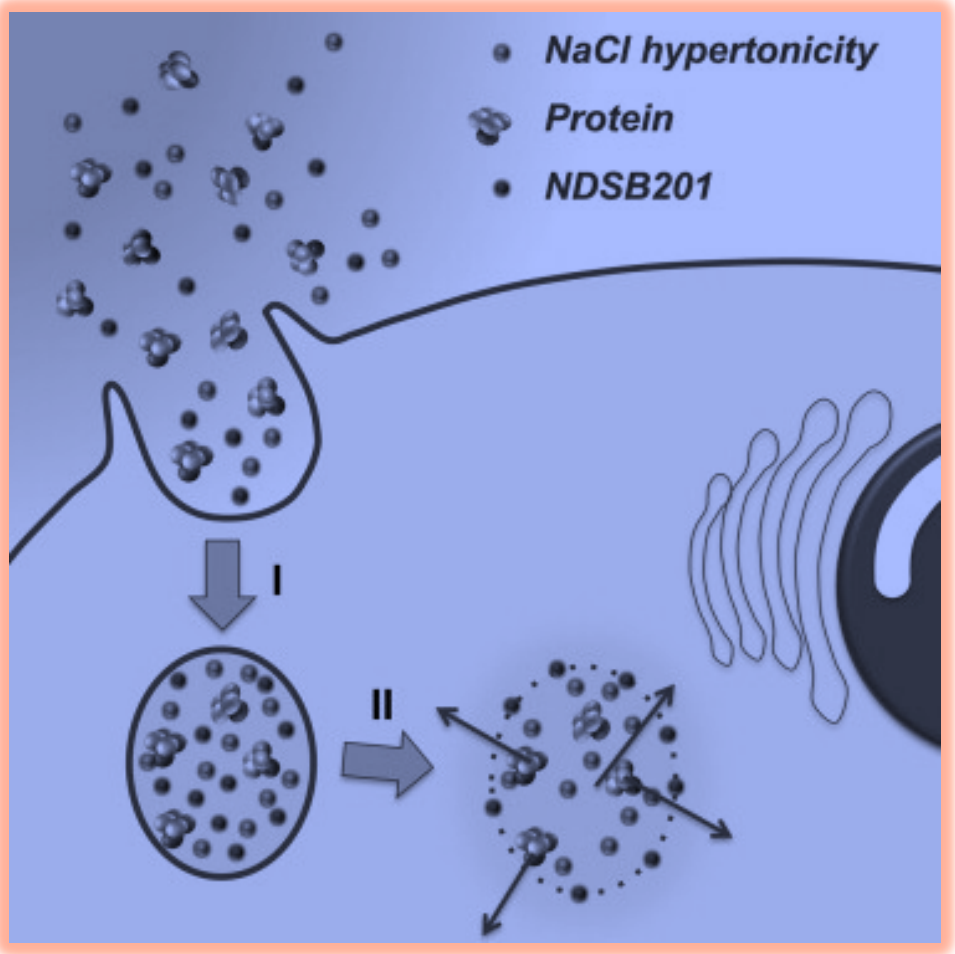
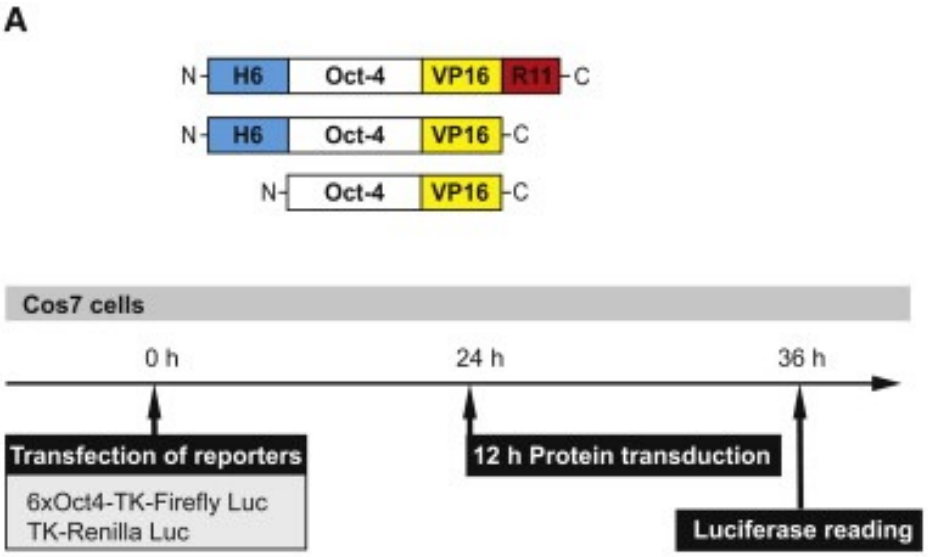
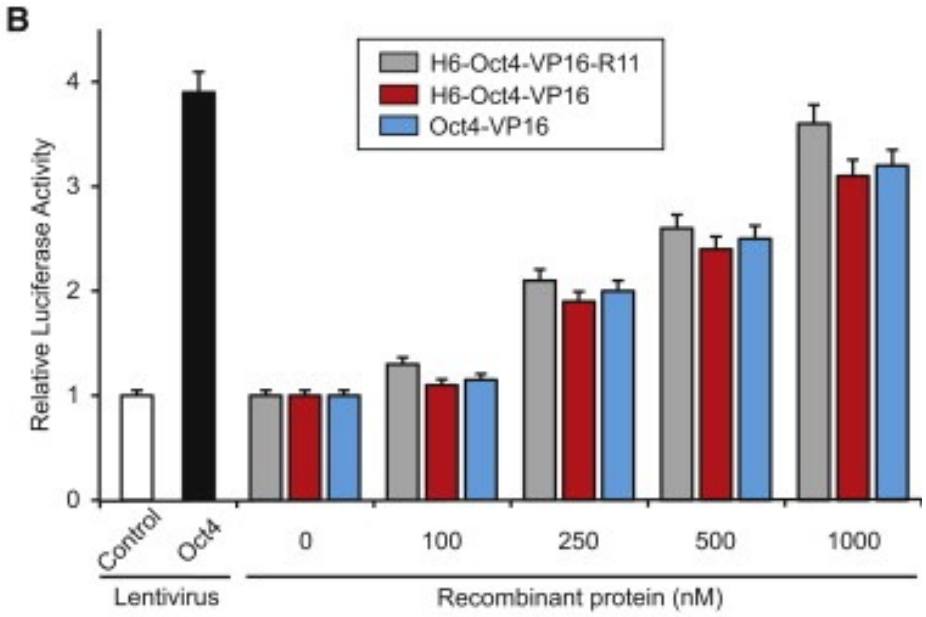


„ITOP“
INDUCED TRANSDUCTION BY OSMOCYTOSIS AND PROPANEBETAINE

D‘ASTOLFO ET AL., CELL 2015

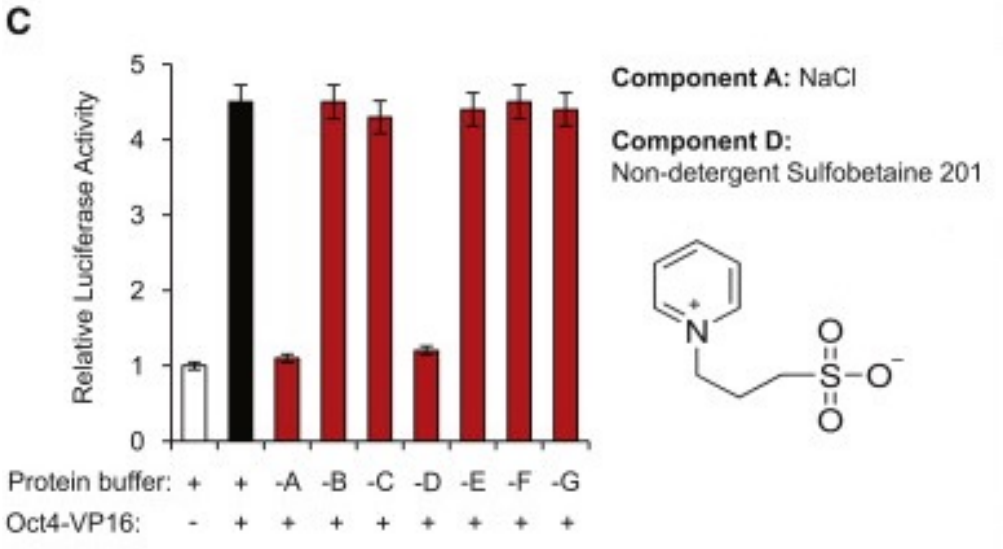
Transduction of native protein independent of a „cell-penetrating peptide“

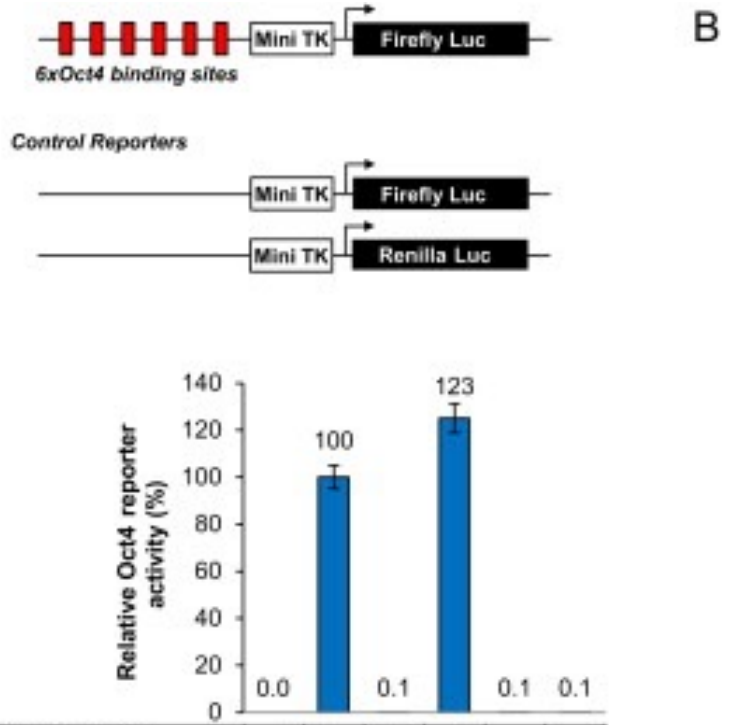




H6 = 6xHis Tag
R11 = poly-arginine CPP

Omitting NaCl and NDSB-201 from the transduction buffer prevents efficient Oct4 protein delivery



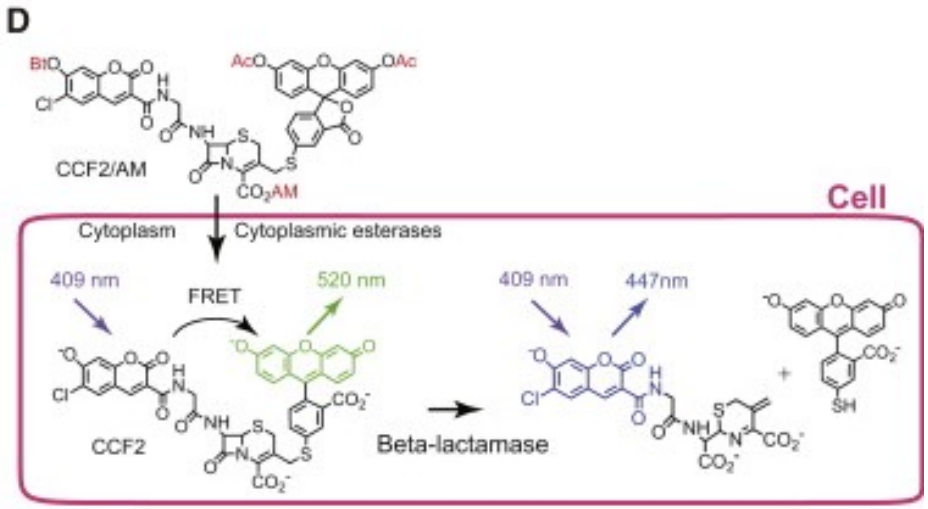


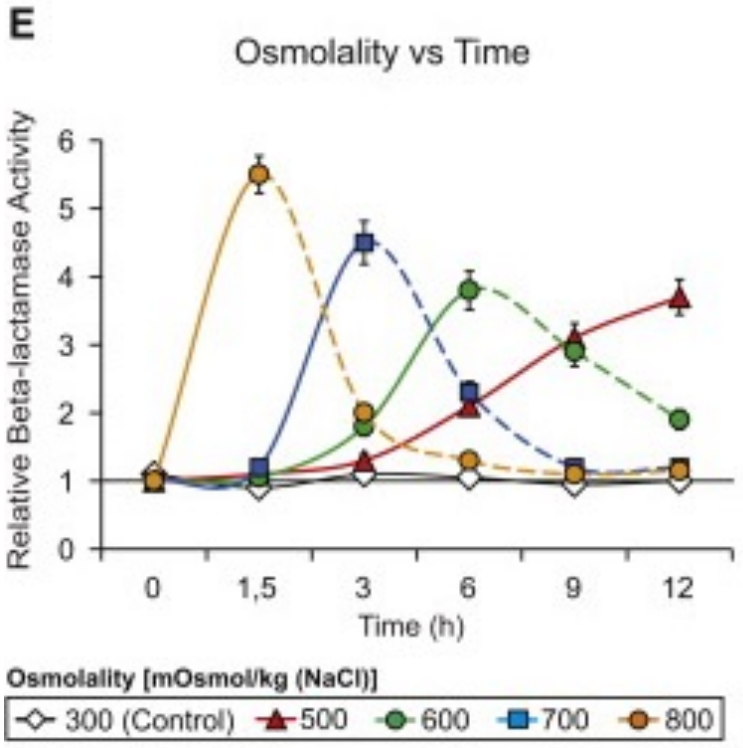
6xOct4-TK-LUC	+	+	+	+		
TK-LUC (Control)					+	+
TK-Renilla	+	+	+	+	+	+
Lenti	+					
Lenti-Oct4		+				
Protein Buffer (Control)			+		+	
Oct4-VP16 (500 nM)				+		+

NDSB-201: non-detergent Sulfobetaine 201

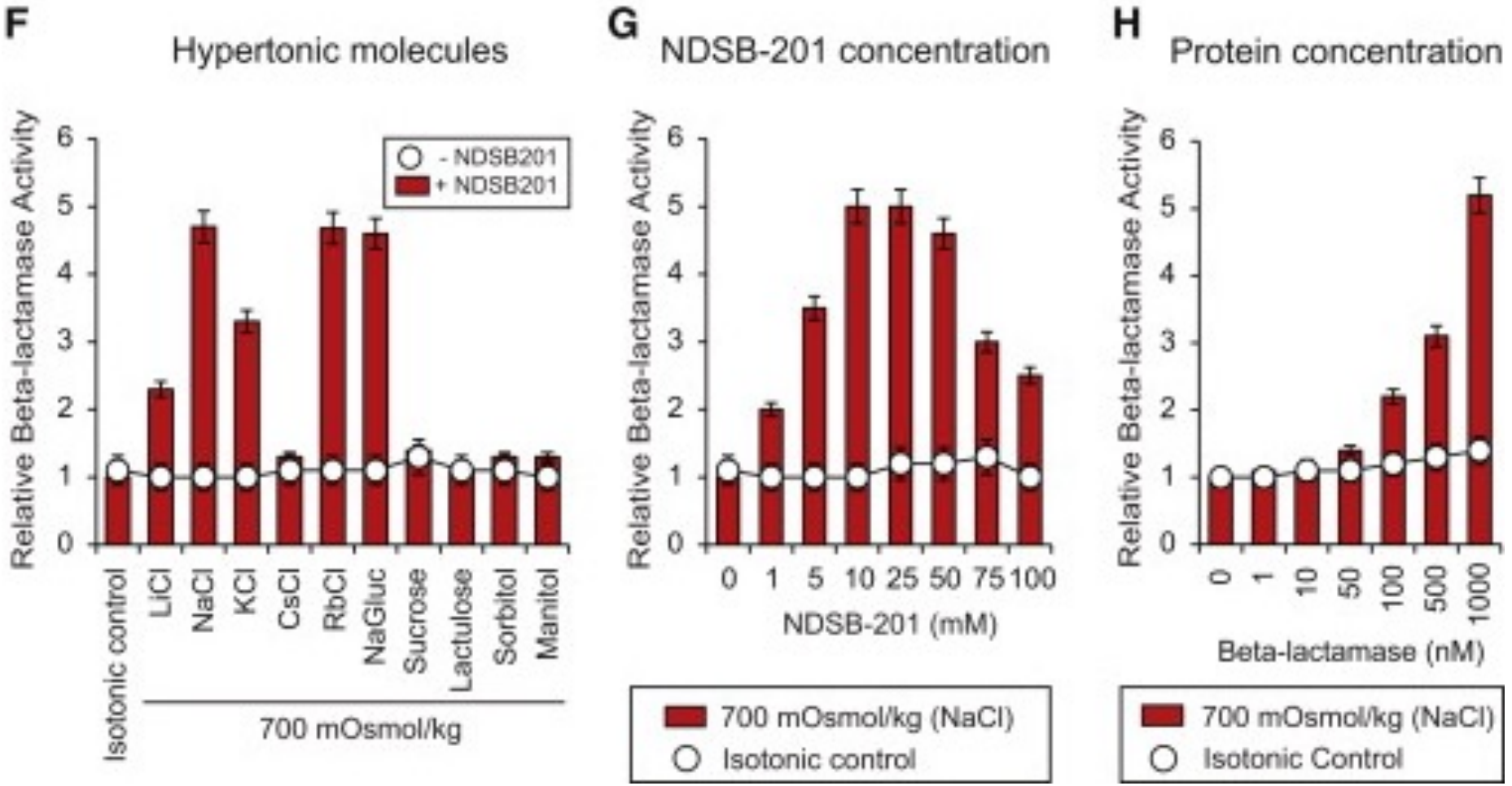
A: 1M NaCl
B: 50 mM NaH_2PO_4
C: 50 mM Tris-HCl
D: 250 mM NDSB-201
E: 100 µM 2-mercaptoethanol
F: 125 µM $MgCl_2$
G: 125 µM $ZnCl_2$

Optimal transduction time is directly proportional to NaCl-induced hyperosmolarity

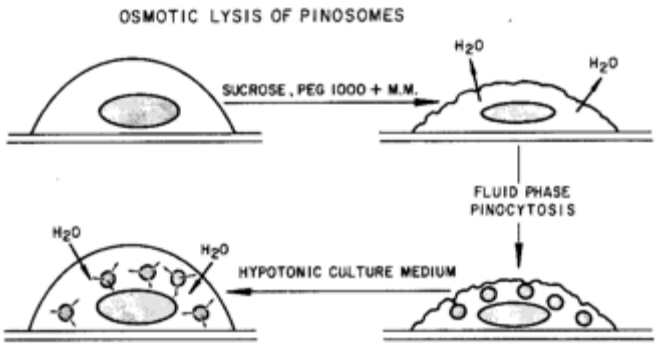




Cl^- anions are not essential for induction of hypertonicity



iTOP vs osmotic lysis of pinocytic vesicles



Okada CY & Rechsteiner M, Cell 1982

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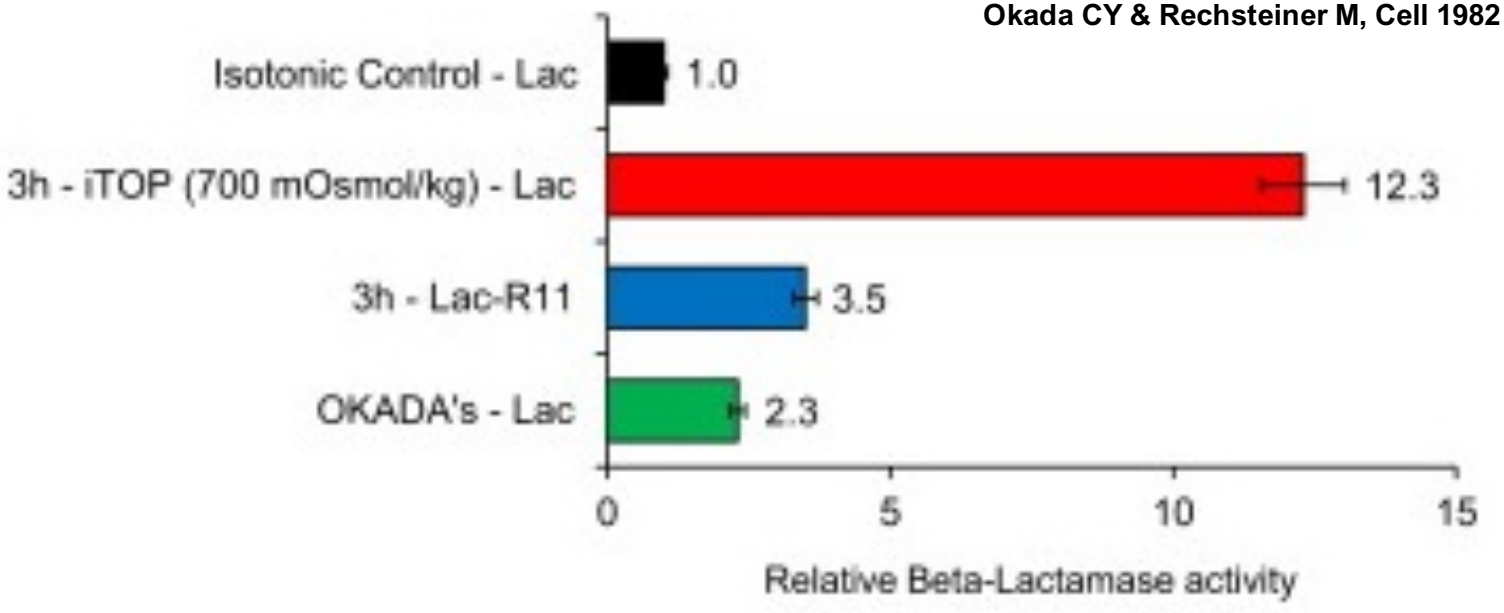


THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 273, No. 22, Issue of May 29, pp. 13645–13651, 1998
Printed in U.S.A.

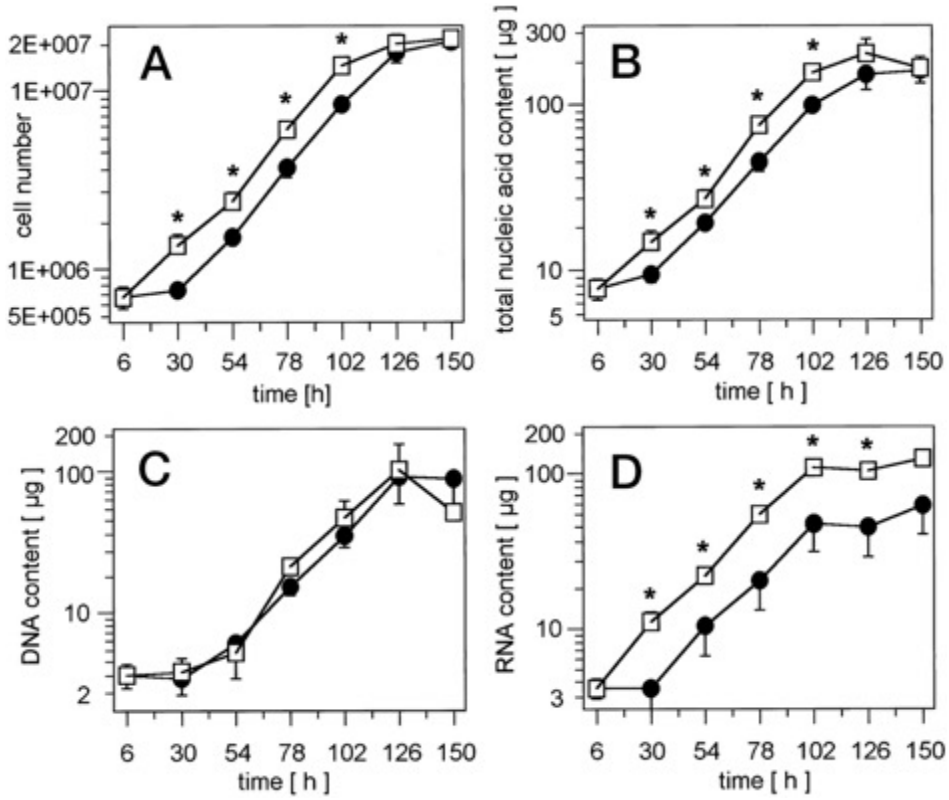
Hyperosmolality Causes Growth Arrest of Murine Kidney Cells

INDUCTION OF GADD45 AND GADD153 BY OSMOSENSING VIA STRESS-ACTIVATED PROTEIN KINASE 2*

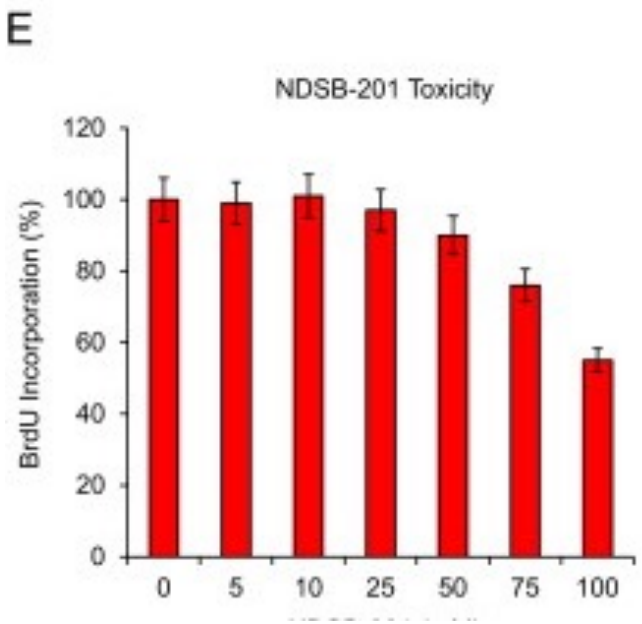
(Received for publication, January 13, 1998, and in revised form, March 16, 1998)

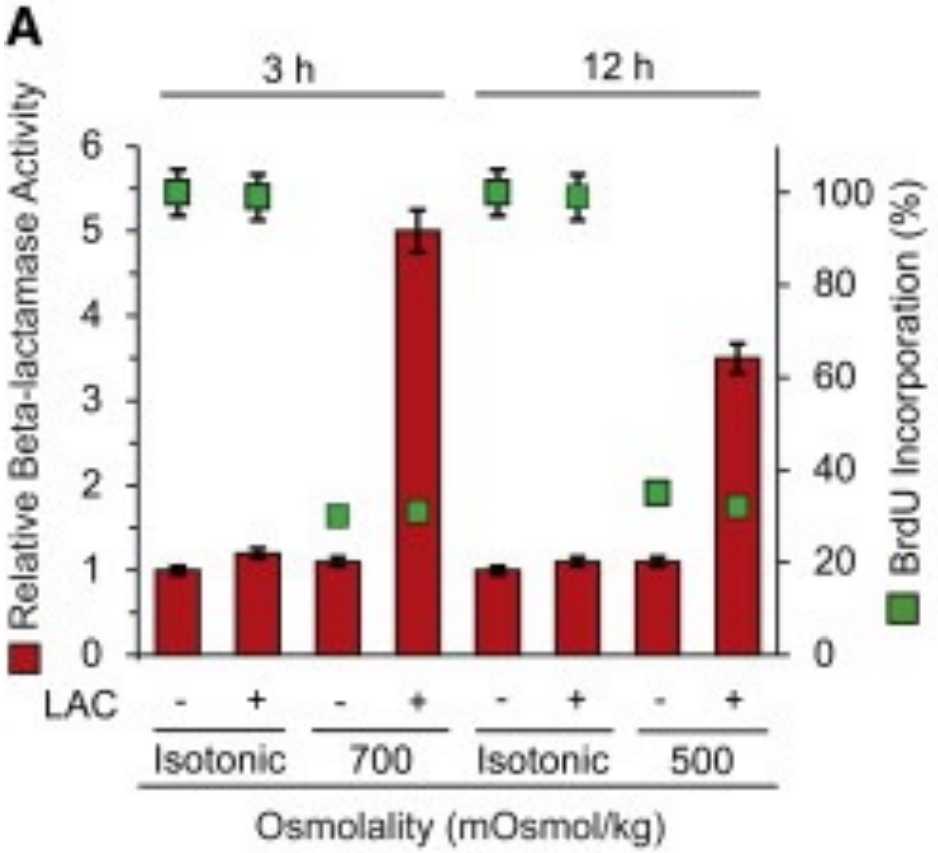
Dietmar Kültz‡, Samira Madhany§, and Maurice B. Burg

From the Laboratory of Kidney and Electrolyte Metabolism, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1603

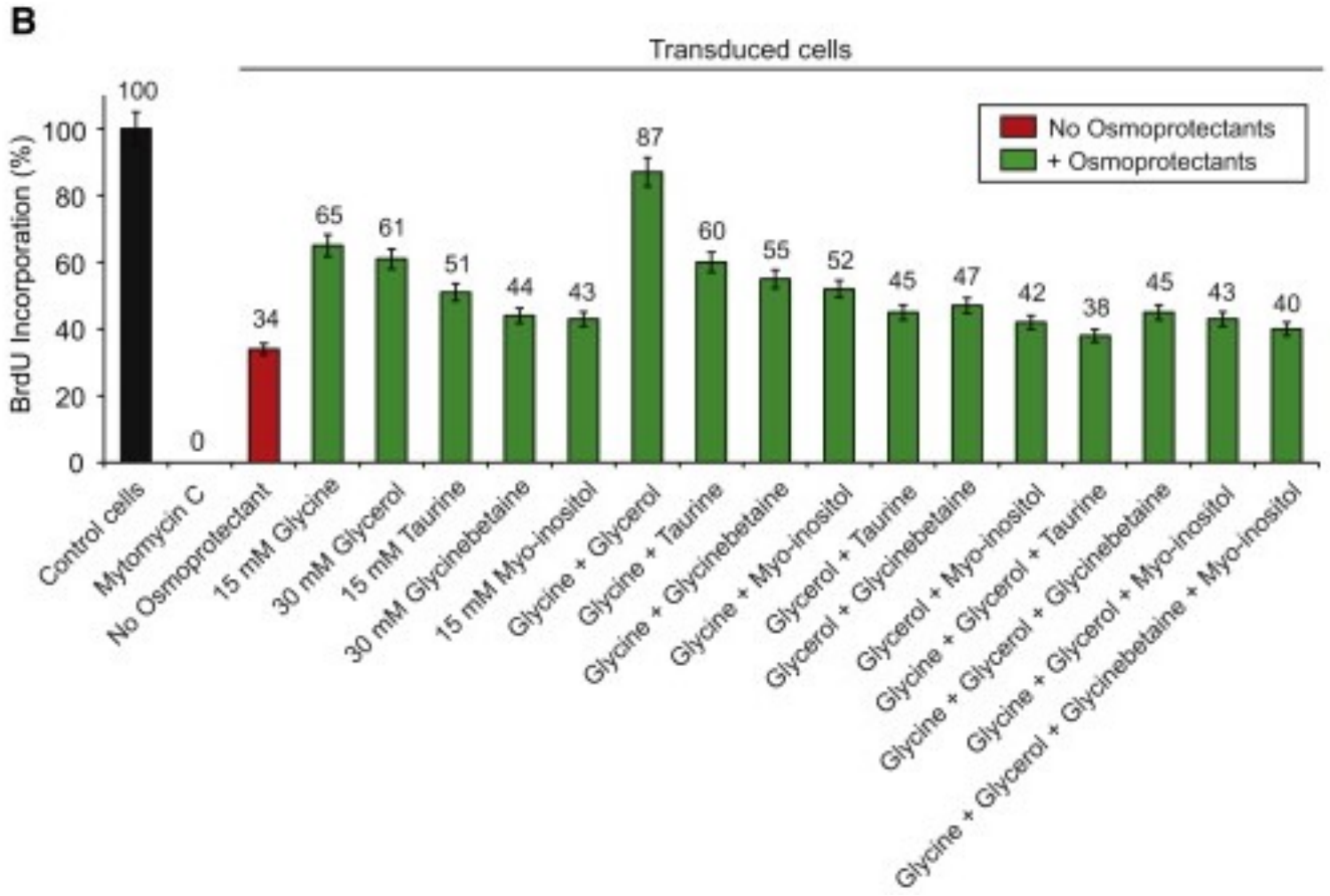


Hypertonicity induces cell-cycle inhibition

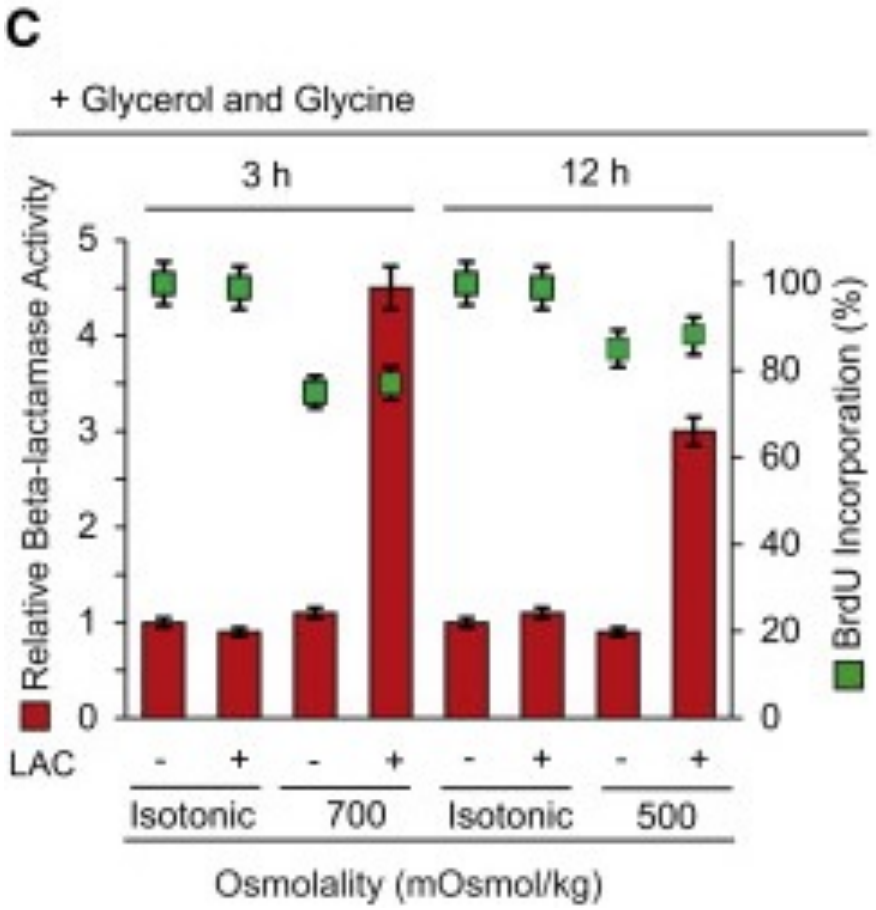


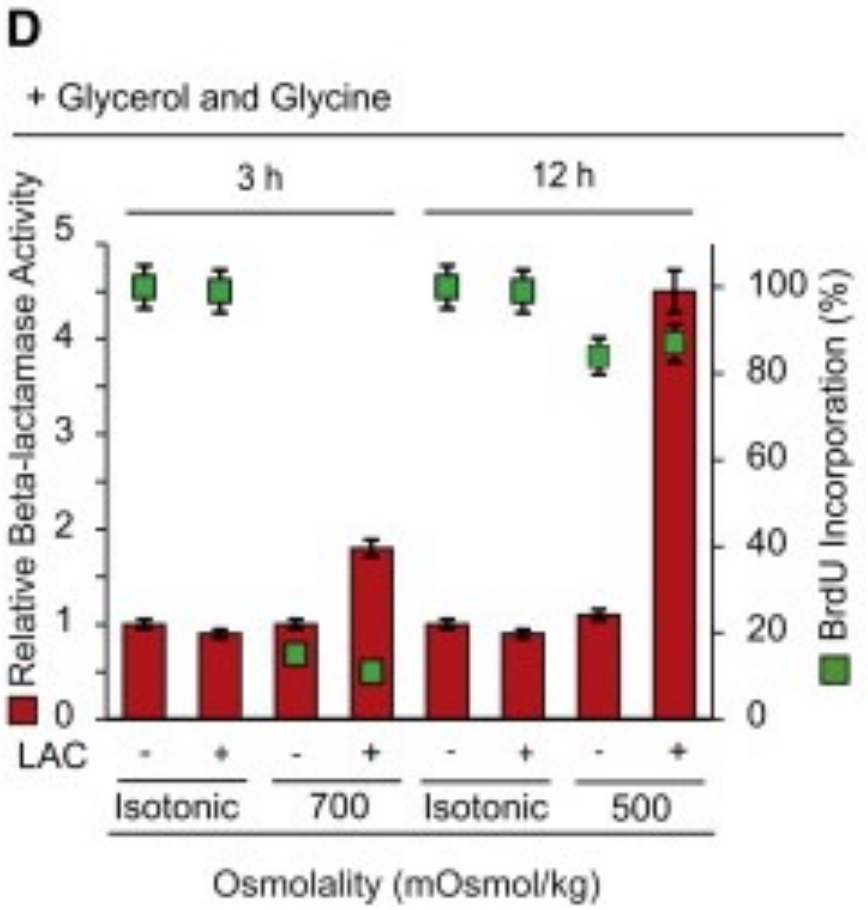


Glycerol and glycine rescue hypertonicity-induced cell-cycle inhibition

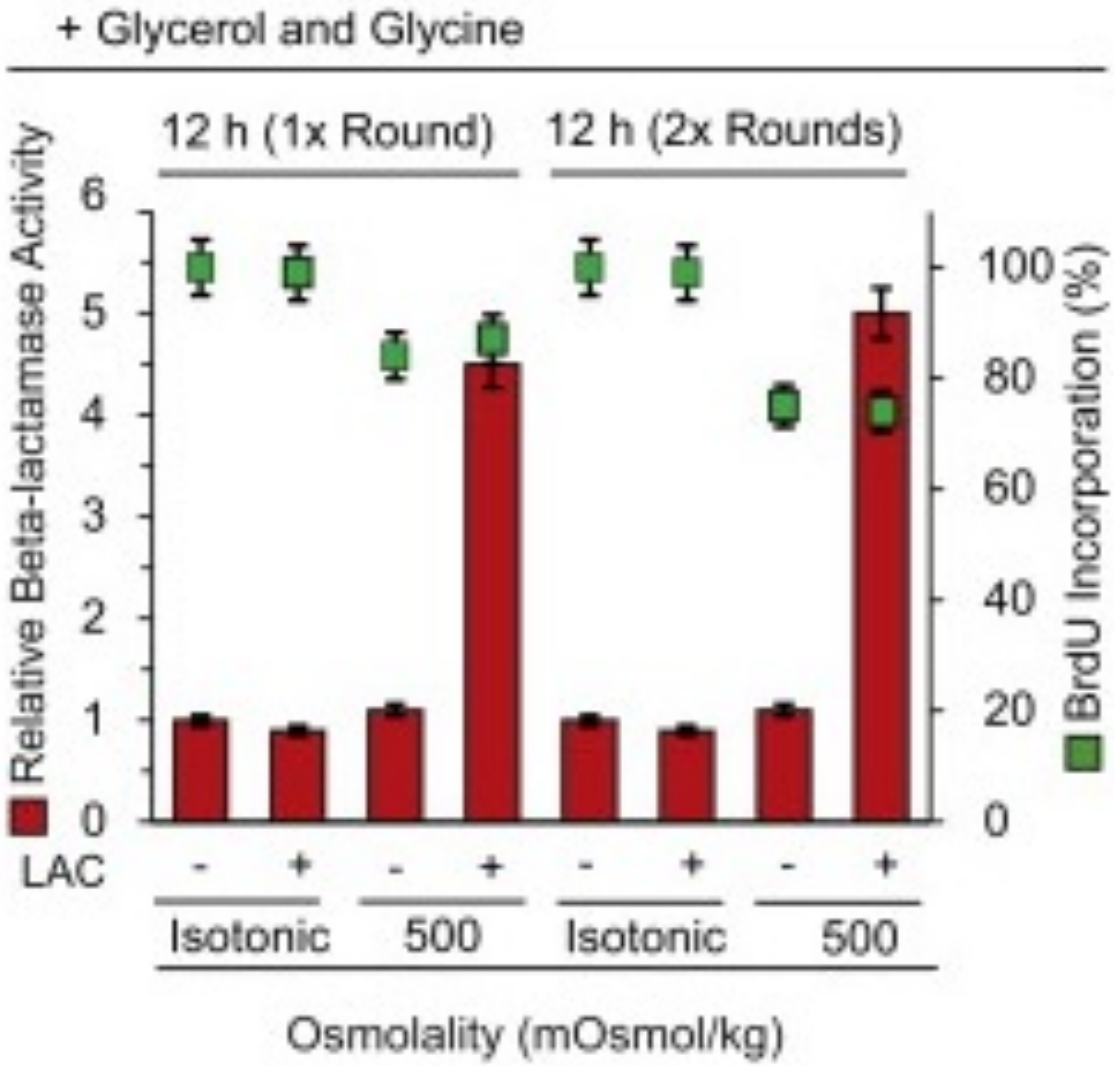


Addition of glycerol/glycine gives similar transduction efficiacies in MEFs but not in mESCs

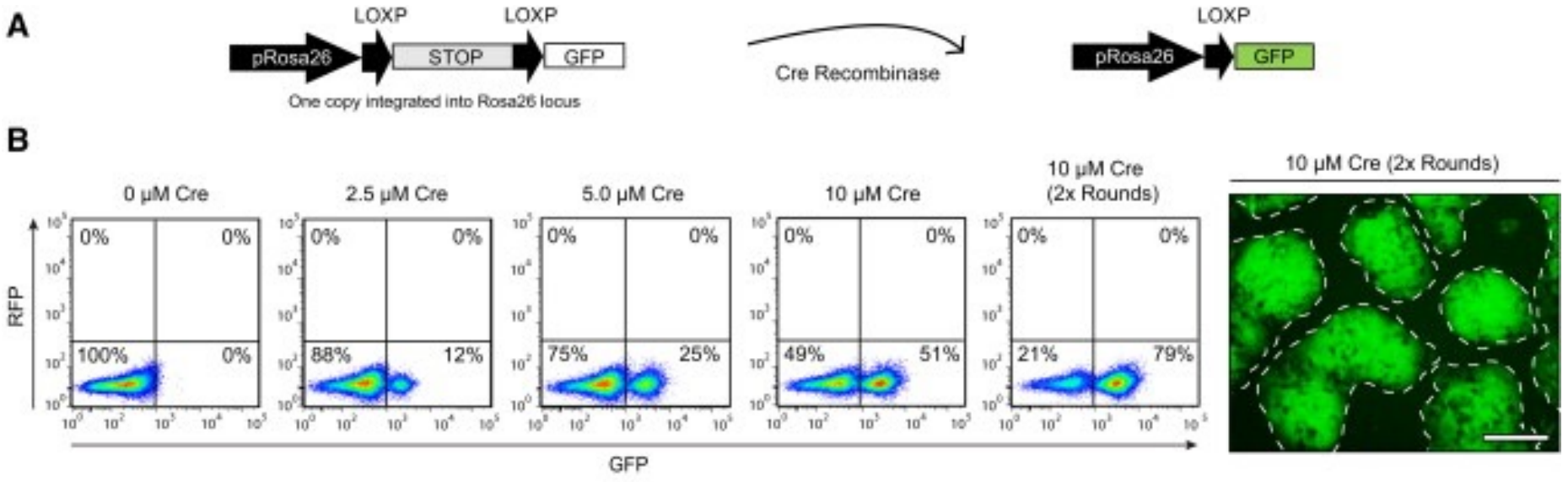




Multiple rounds of hypertonicity lead to high transduction yield and remain cell viability in sensitive cell lines (i.e. mESC)

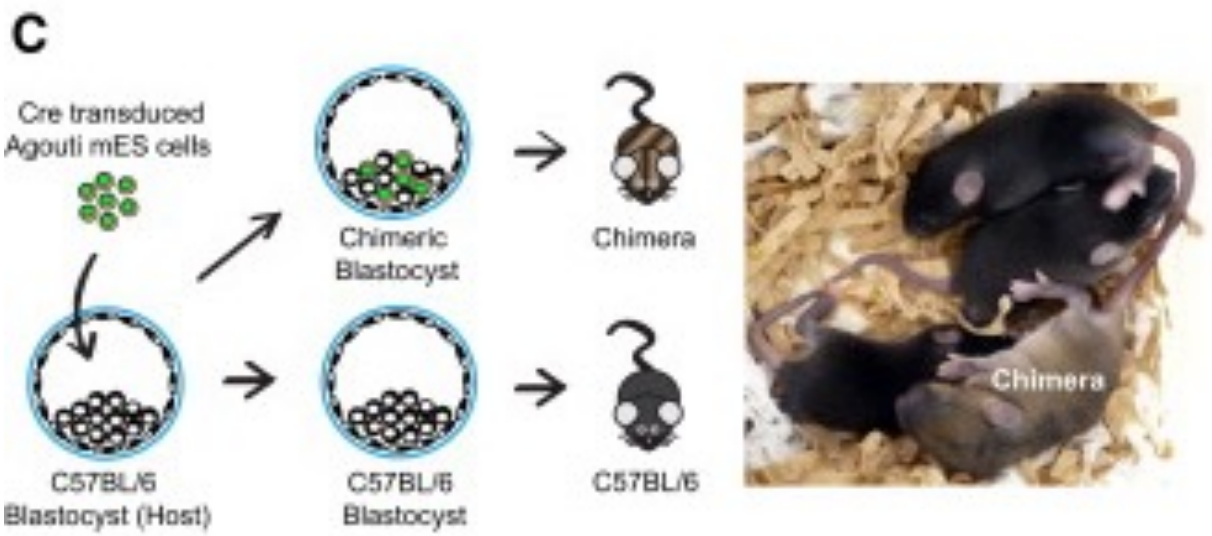


Efficient protein transduction in multiple primary cell types

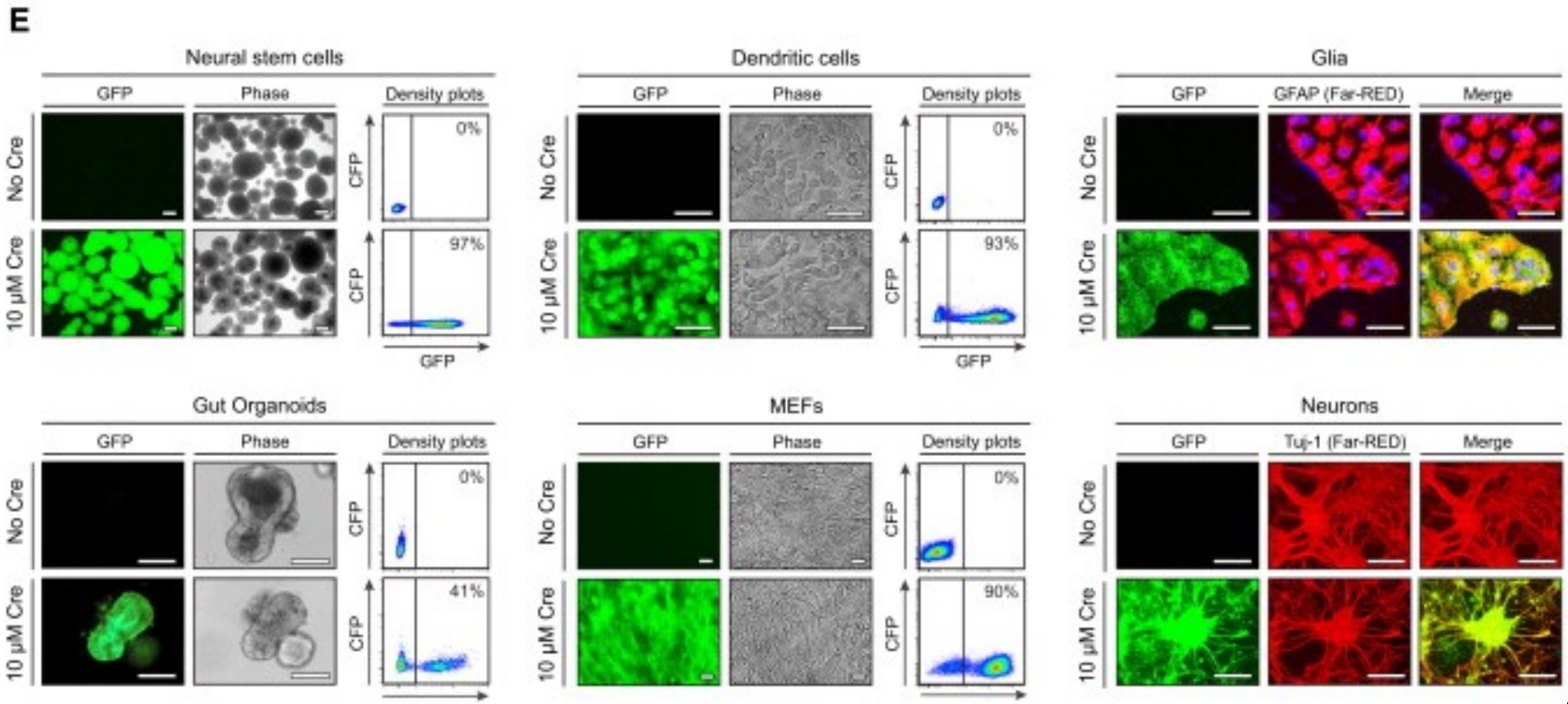


„mESCs were transduced with Cre protein at 500 mOsmol/kg for 12 hr“

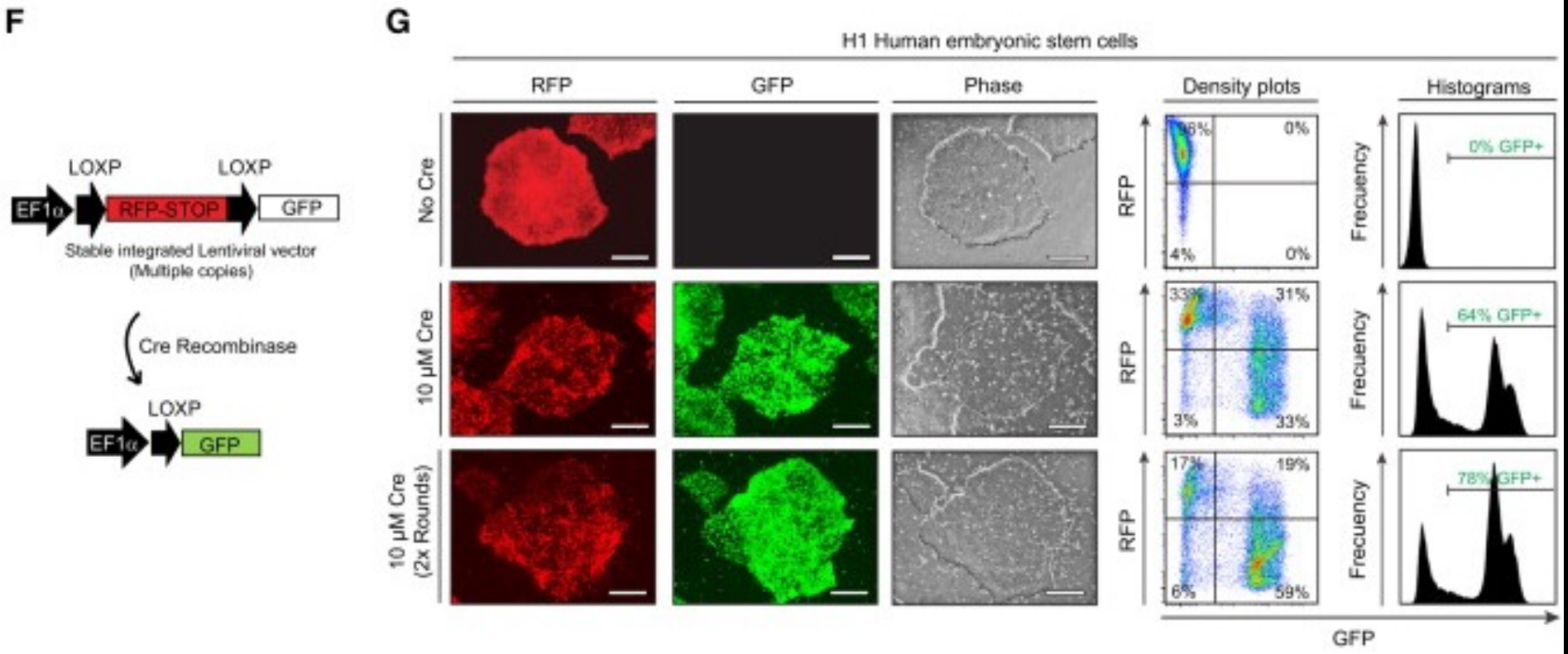
Cre protein transduction does not disrupt mESC pluripotency and Cre-transduced mESCs contribute to the germline



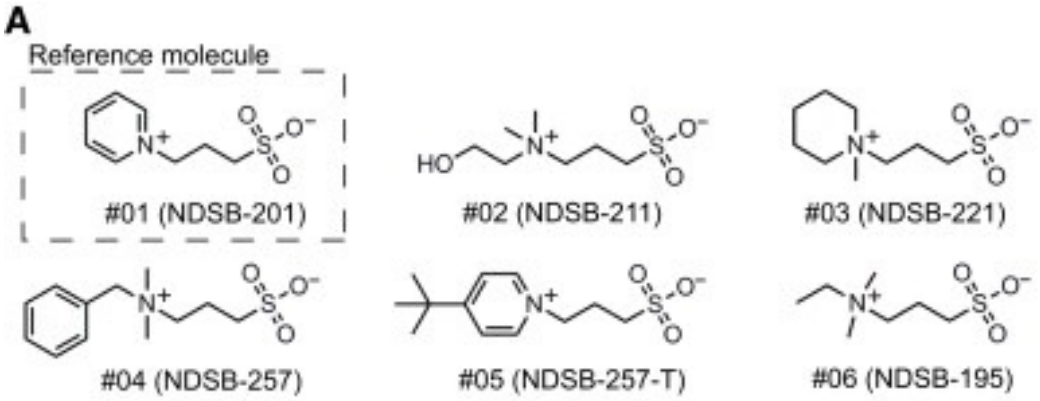
iTOP facilitates the efficient transduction of multiple primary cell lines

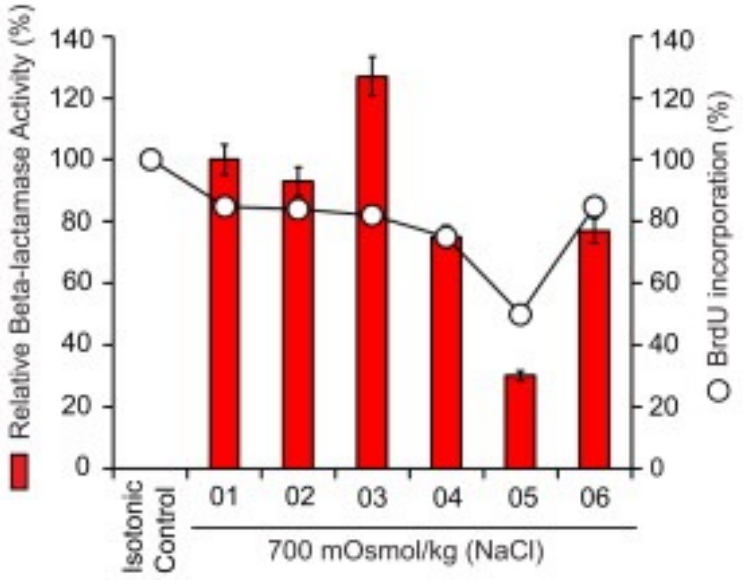


Efficient transduction with iTOP in human ESCs

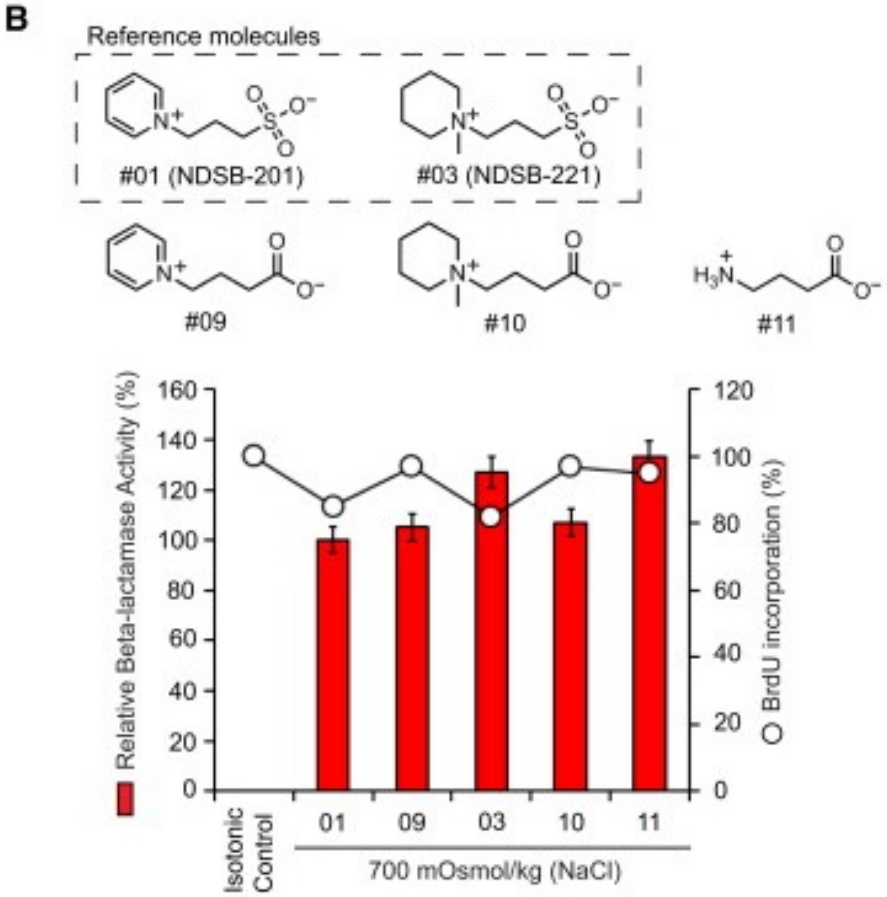


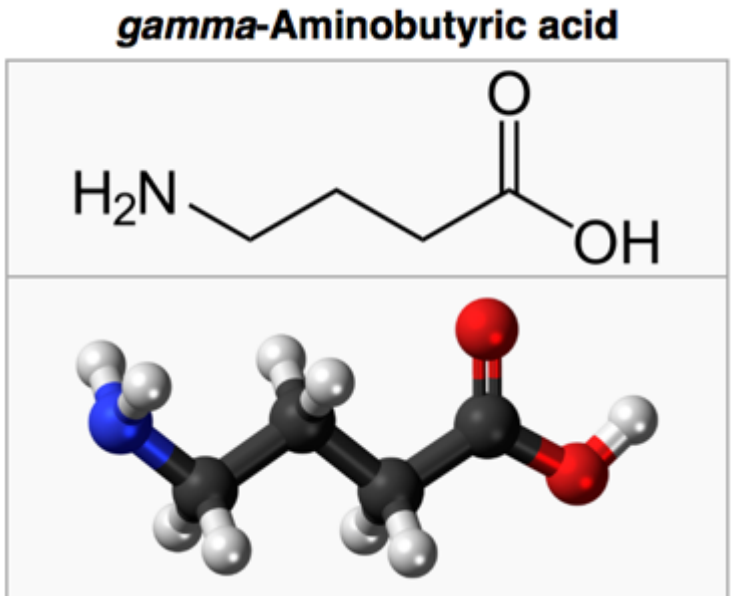
Structural Features of the Transduction Compound



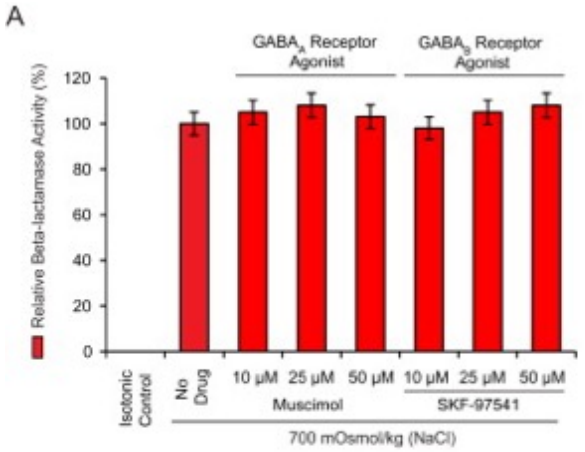


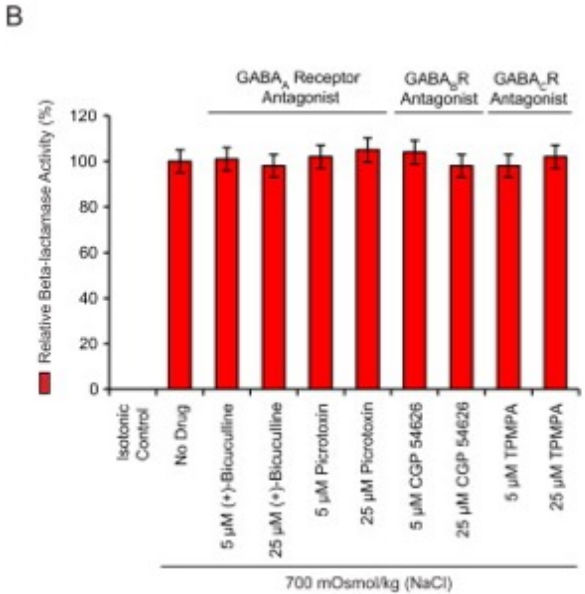
Replacing sulfonate with carboxyl residues + replacing the amine improves cell viability





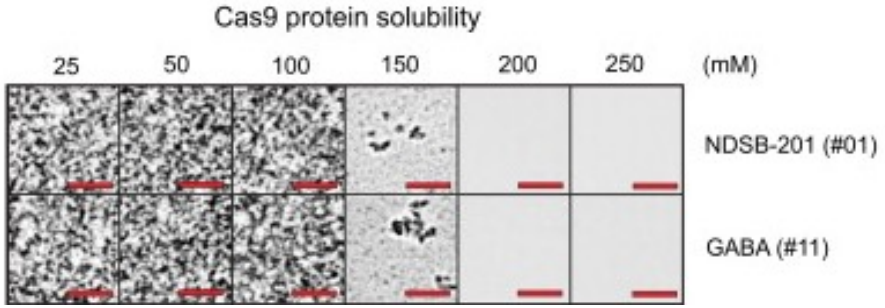
GABA-R (ant-)agonists do not impact transduction

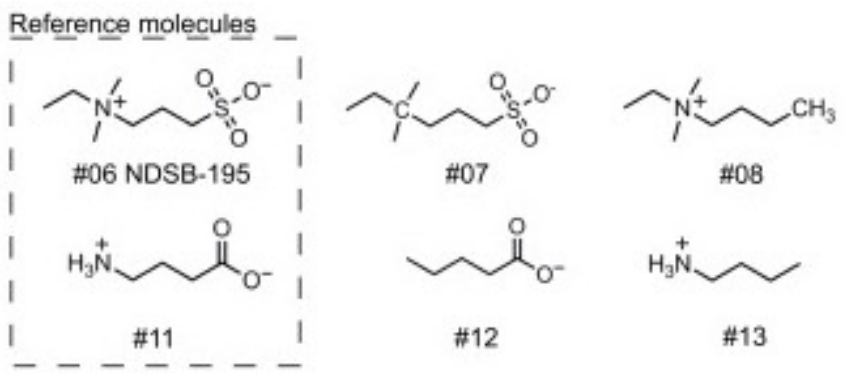


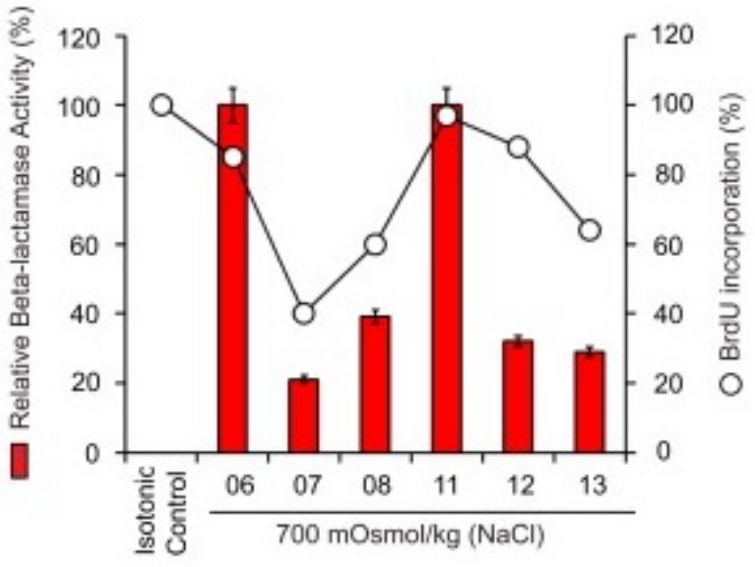


25 mM GABA needed for iTOP is 5 logs higher than concentrations needed for neurotransmission

GABA has similar properties in increasing protein solubility as NDSB-201 and both amine and carboxyl groups are needed for protein transduction



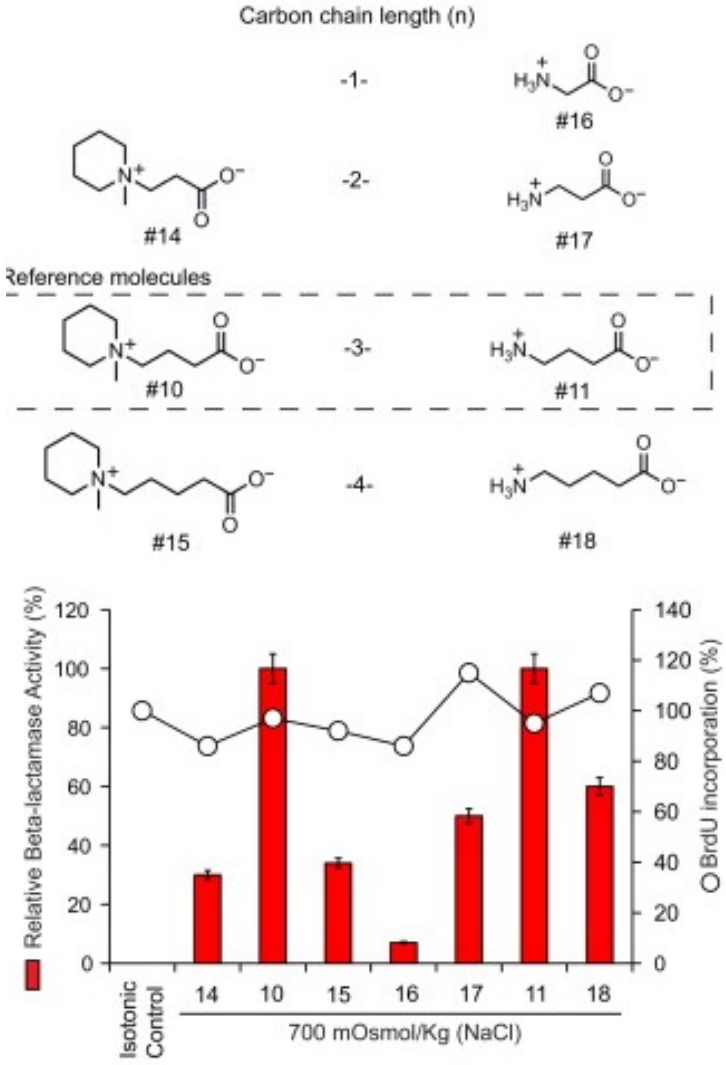




Determination of the the optimal length of the carbon chain spacer

The ideal molecule

- zwitterionic @ neutral pH with
  - (-) + charged amino group
  - (-) – charged sulfonyl/carboxyl group
- 3 carbons between N_{Rx}^{+} and $SOOO^{-}/COO^{-}$ groups

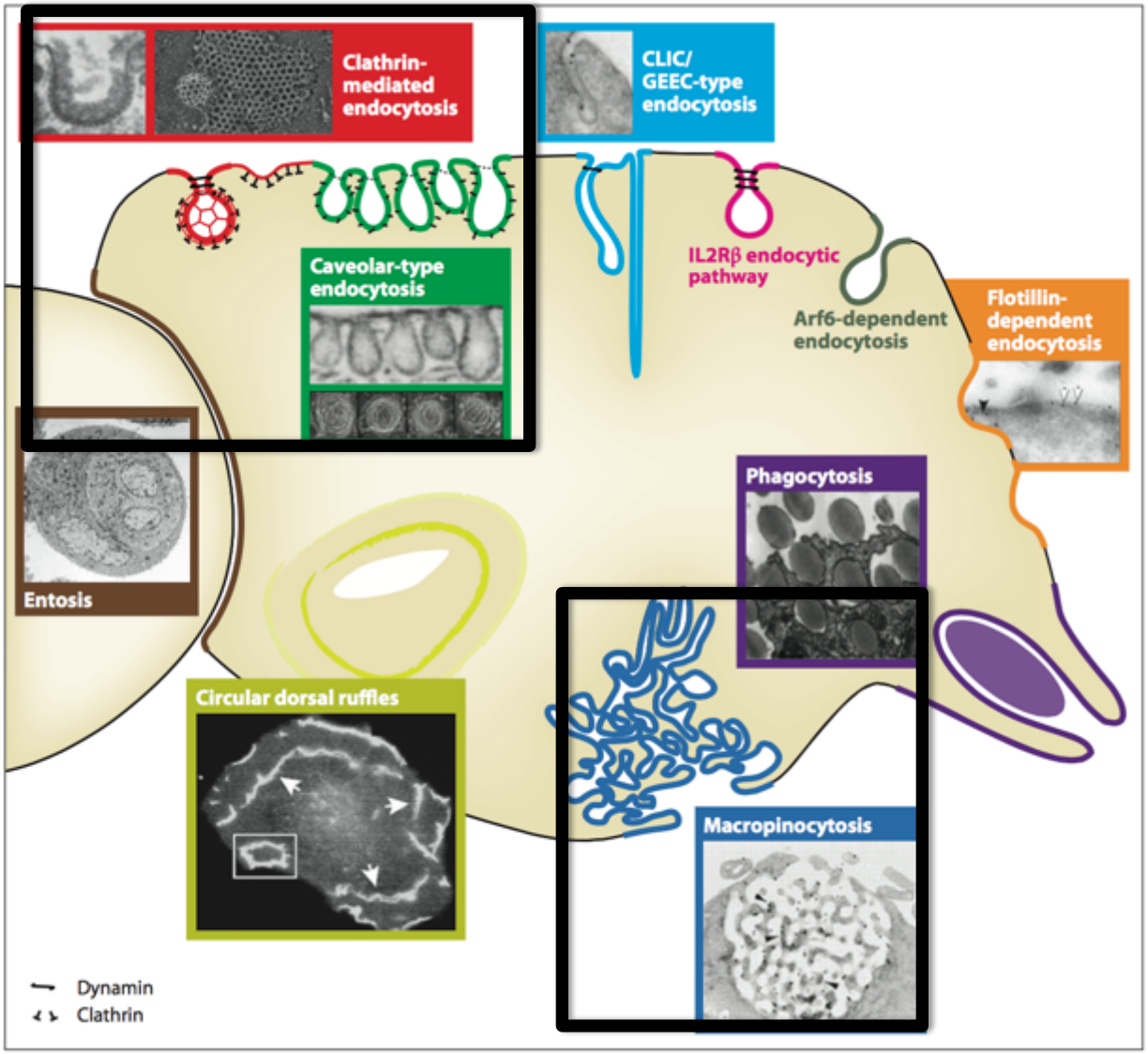


Transduction efficiacies are not confounded by differences in protein solubility

C

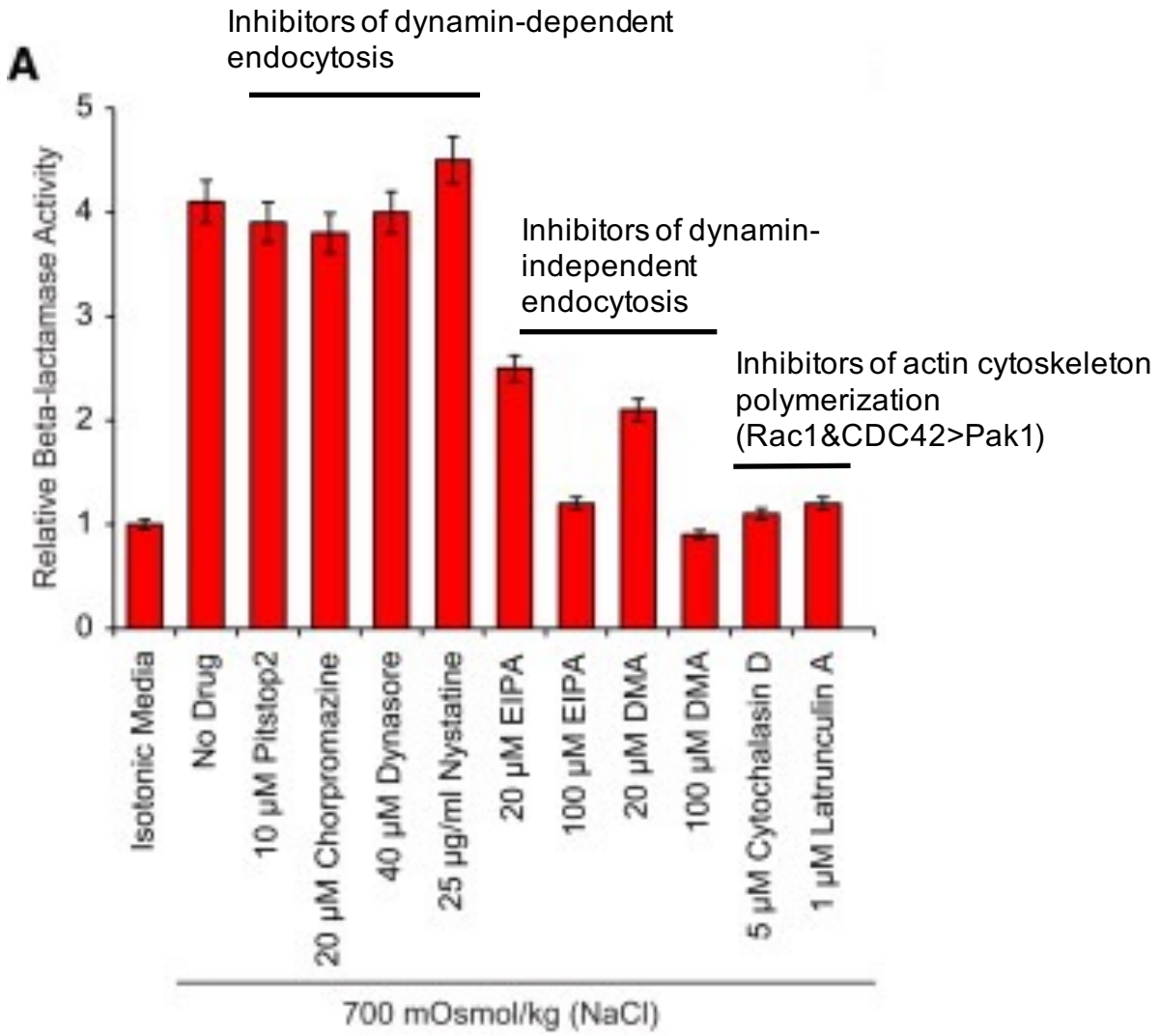
Pictures	Main components of Transduction media
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #14** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #10** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #15** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #16** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #17** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #11 (GABA)** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #18** - 1 µM Beta-lactamase
	- Culture media adjusted with NaCl to 700 mOsmol/kg - **50 mM compound #11 (GABA)** - 1 µM Beta-lactamase - 50 % Ethanol

Dissecting the Mechanism of Protein Transduction

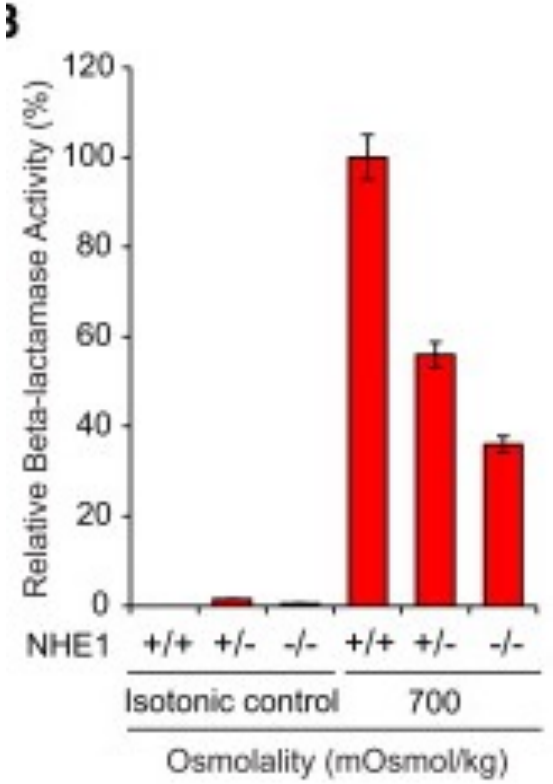


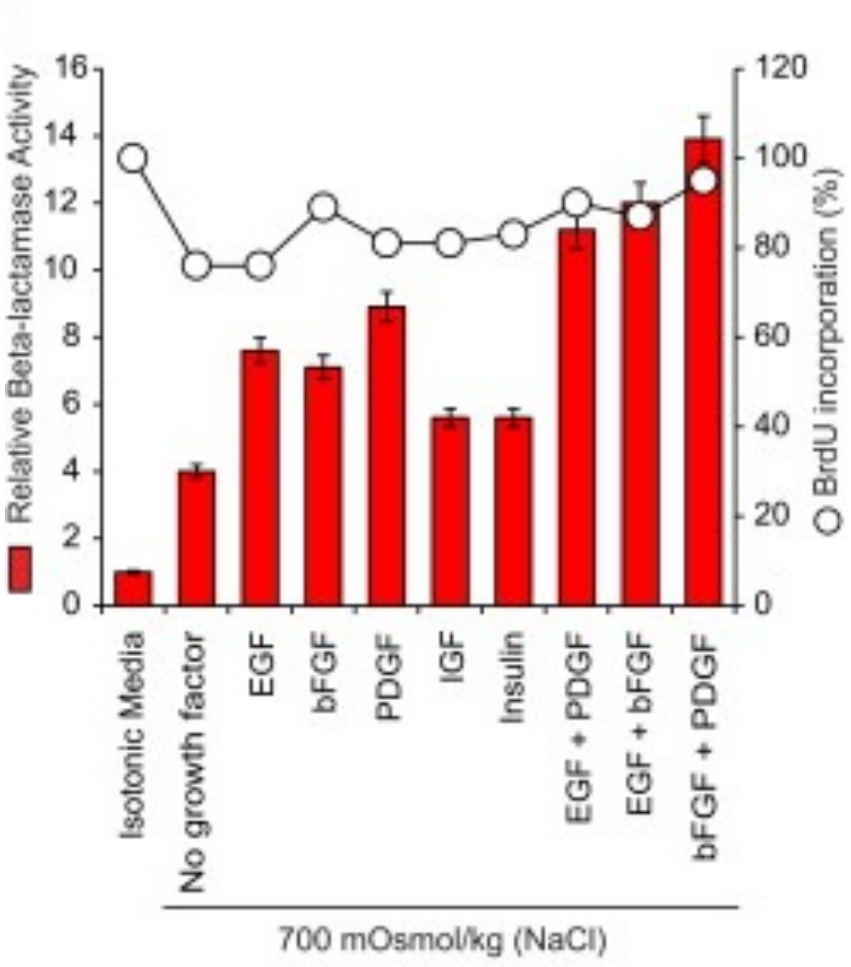
Doherty GJ & McMahon HAT
Annu Rev Biochemistry 2009

Protein transduction is mediated by macropinocytosis

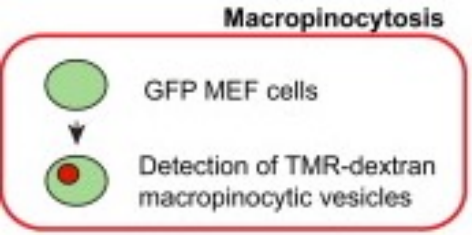


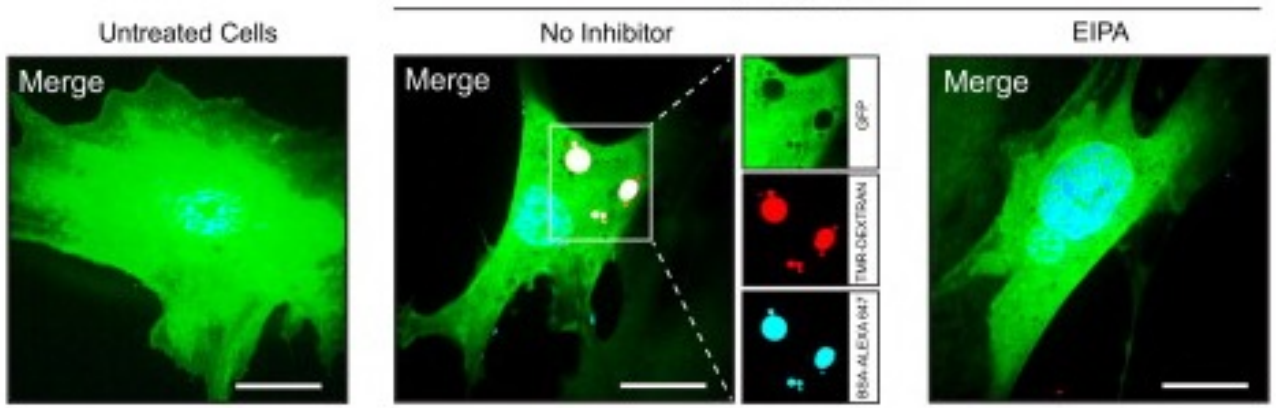
NHE1 Na+/H+ antiporter function is important for protein transduction through macropinocytosis, which is enhanced through TK-acting growth factors

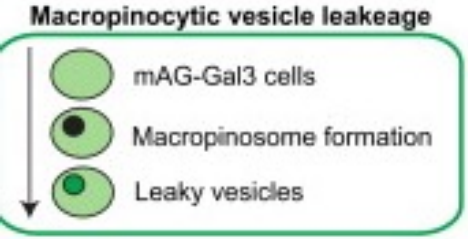


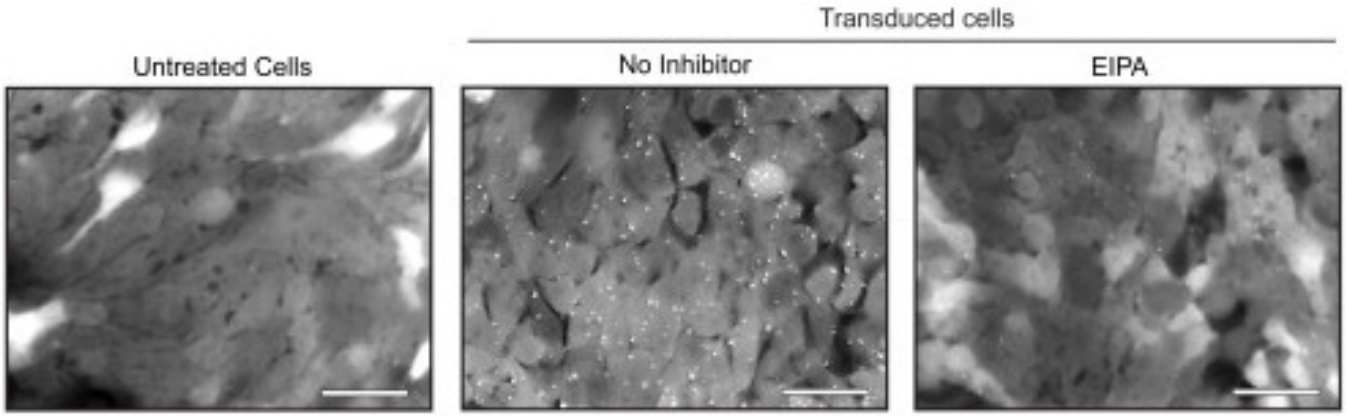


Quantifying macropinocytosis and vesicle release

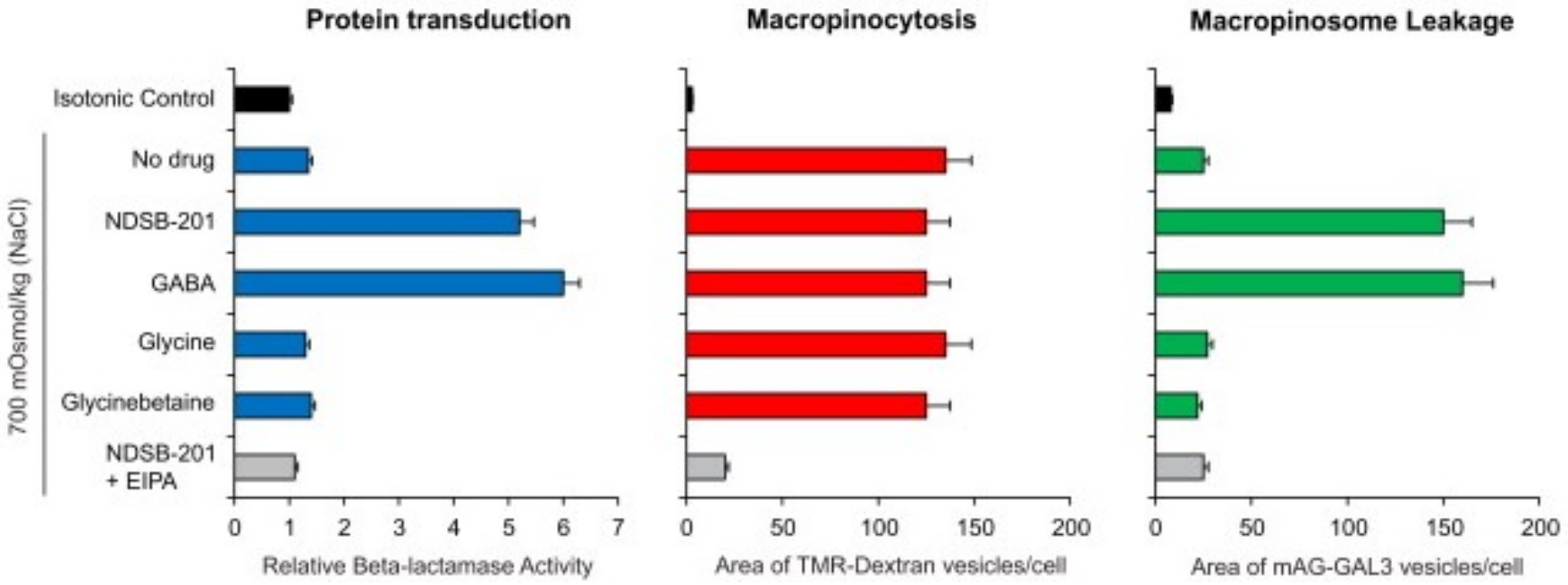




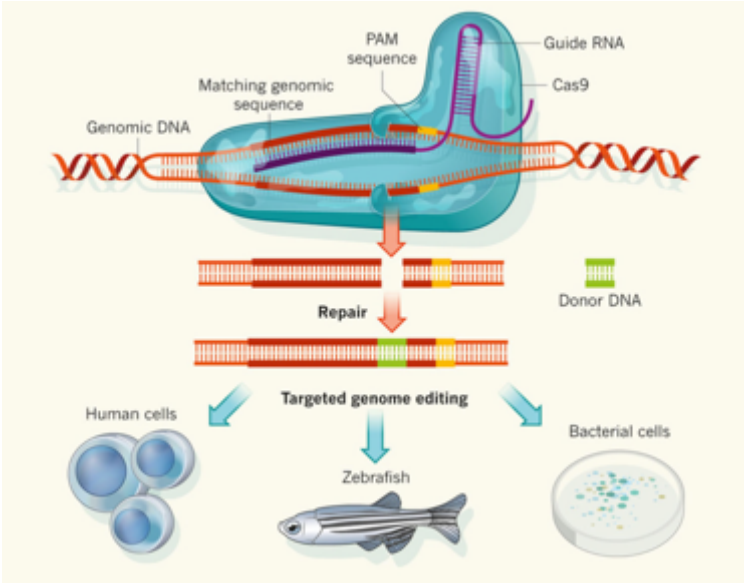




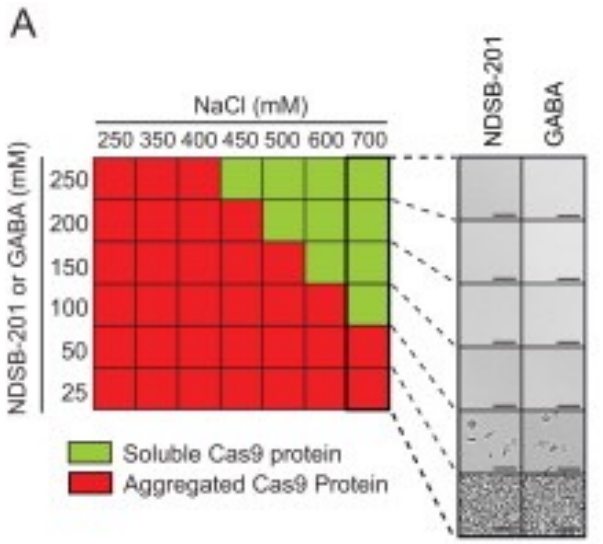
Quantifying macropinocytosis and vesicle release

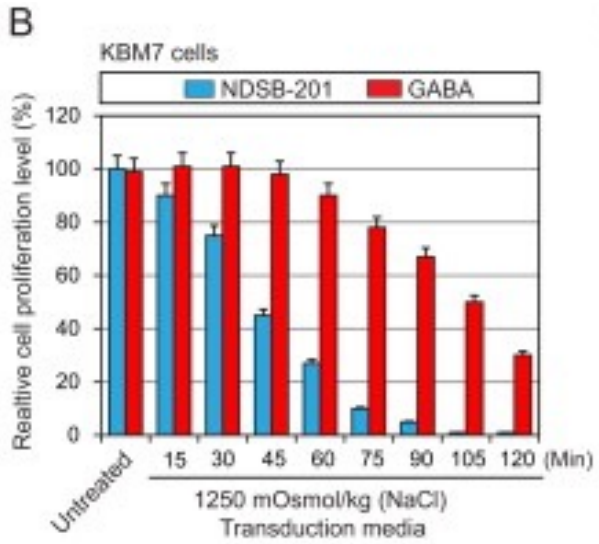


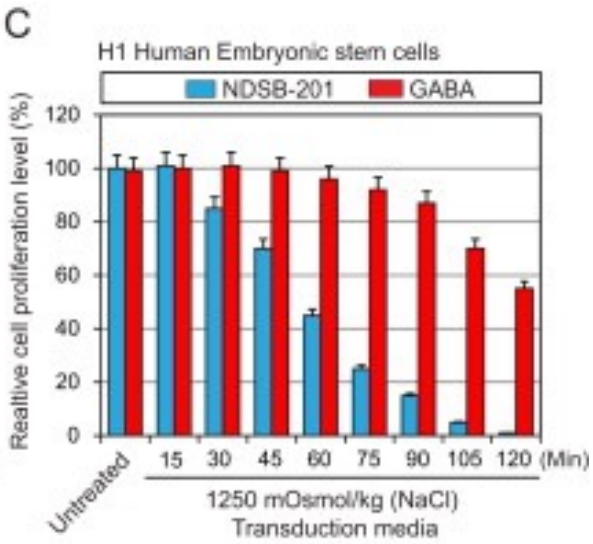
iTOP for Crispr/Cas9 in primary cells



Charpentier E & Doudna JA
Nature 2013







High salt concentrations and DNA breaks

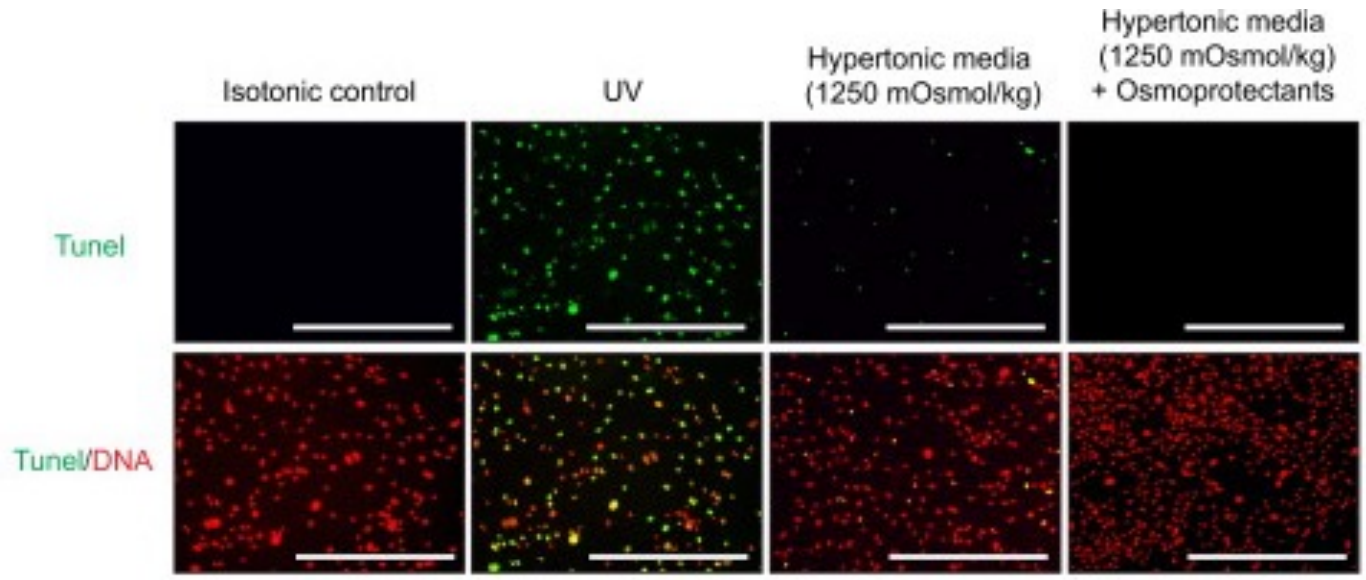
Proceedings of the National Academy of Sciences of the United States of America

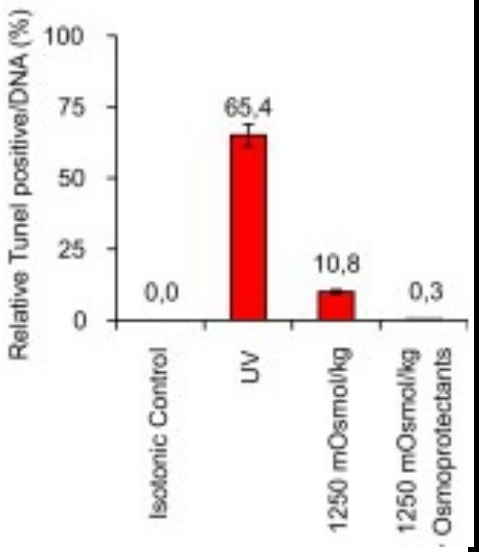
CURRENT ISSUE // ARCHIVE // NEWS & MULTIMEDIA // FOR AUTHORS // ABOUT PNAS COLLECTED

> Current Issue > vol. 108 no. 51 > Christophe E. Redon, 20281–20282, doi: 10.1073/pnas.1117713109

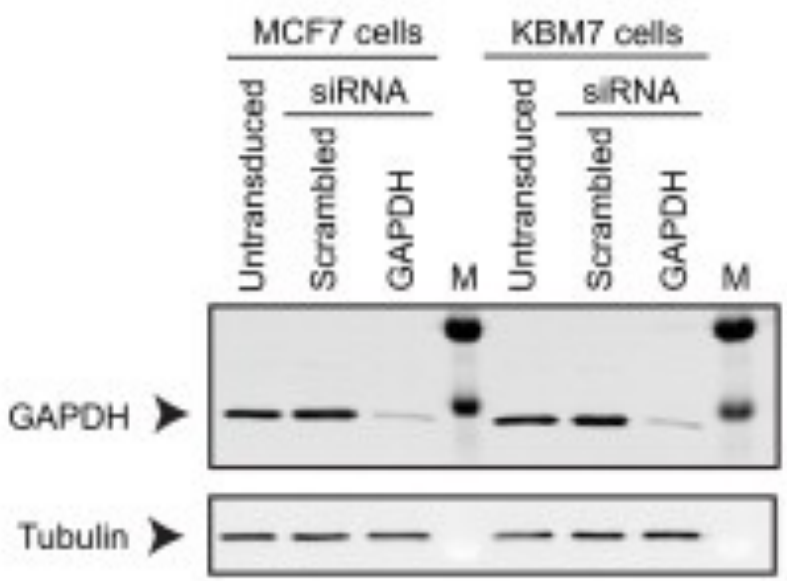
High salt and DNA double-strand breaks

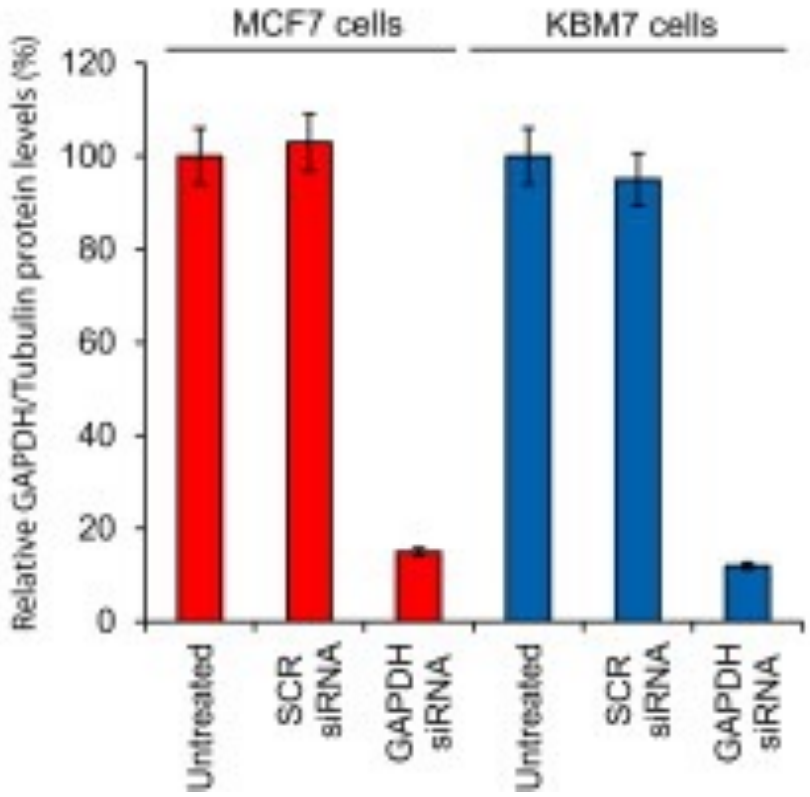
Christophe E. Redon and William M. Bonner[1]



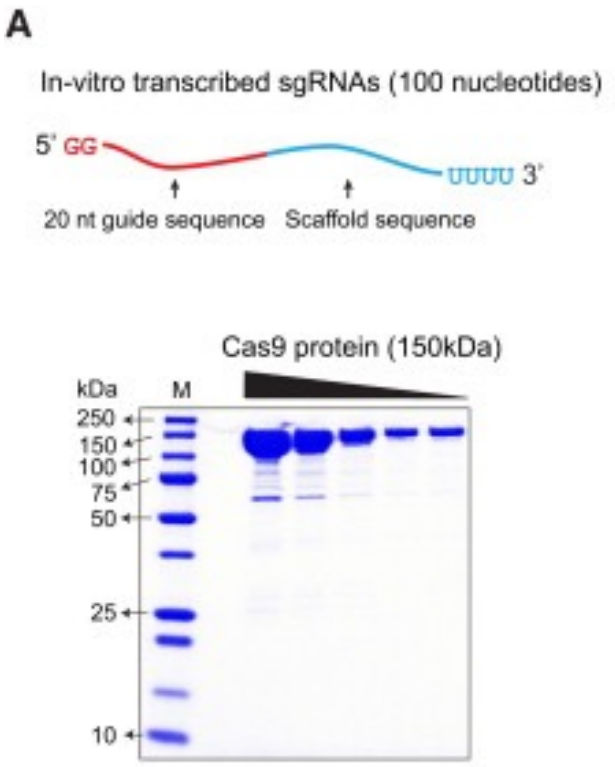


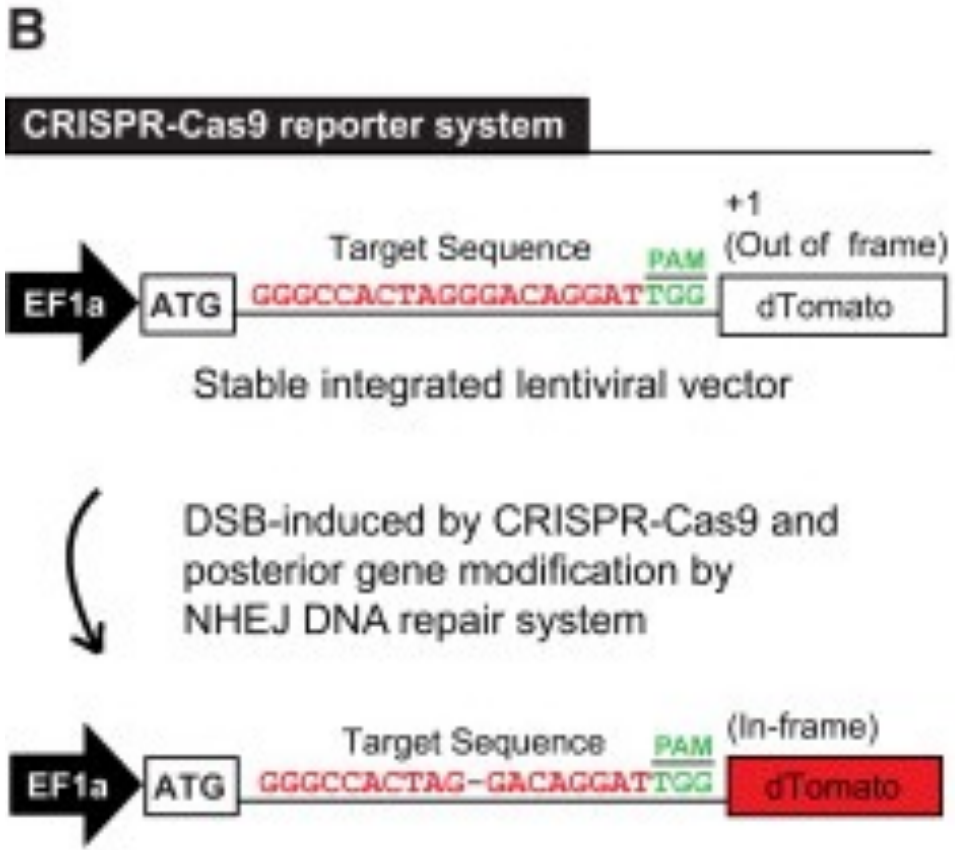
iTOP does not seem to affect (si)RNA delivery



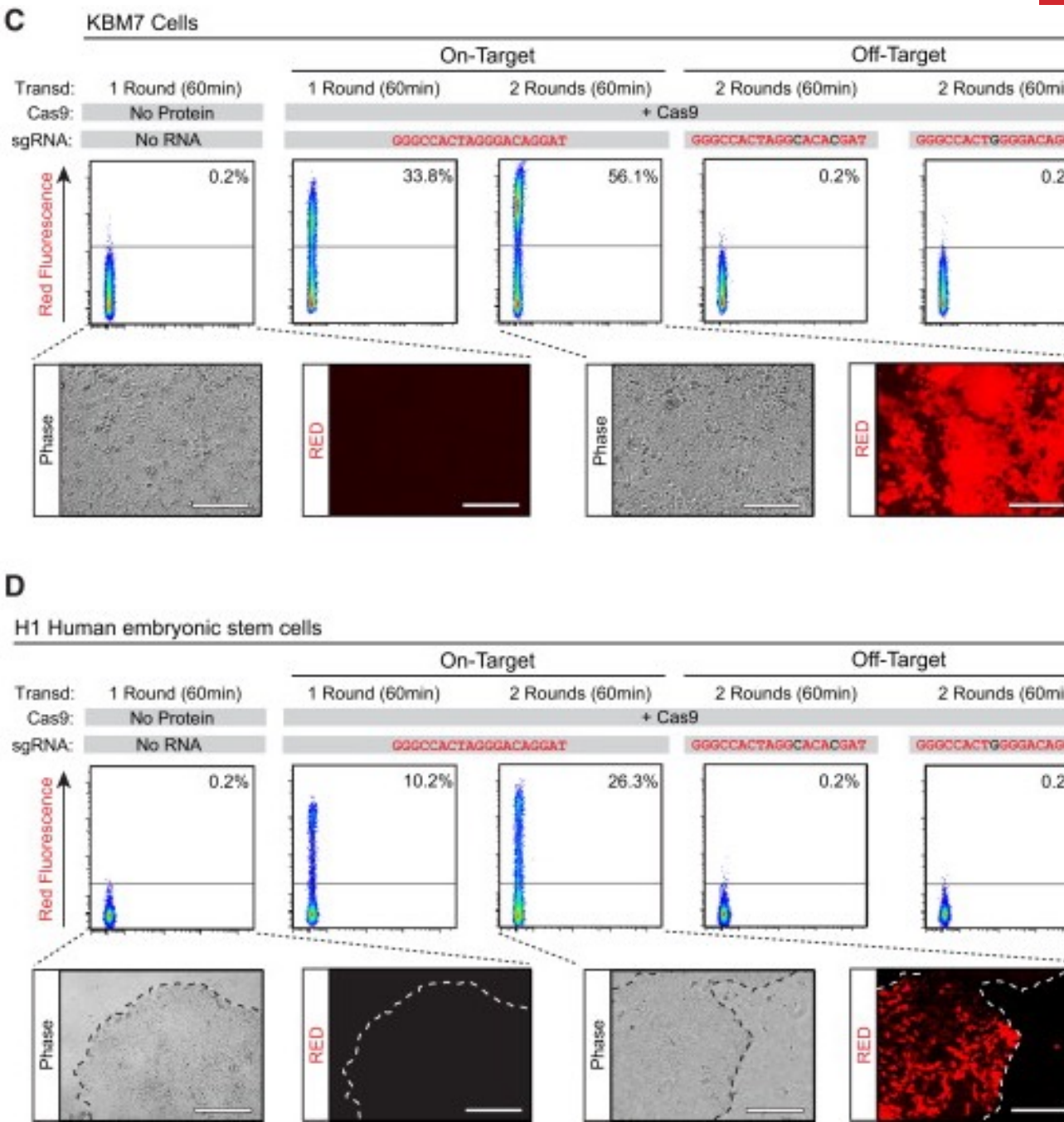


iTOP/Crispr-Cas9

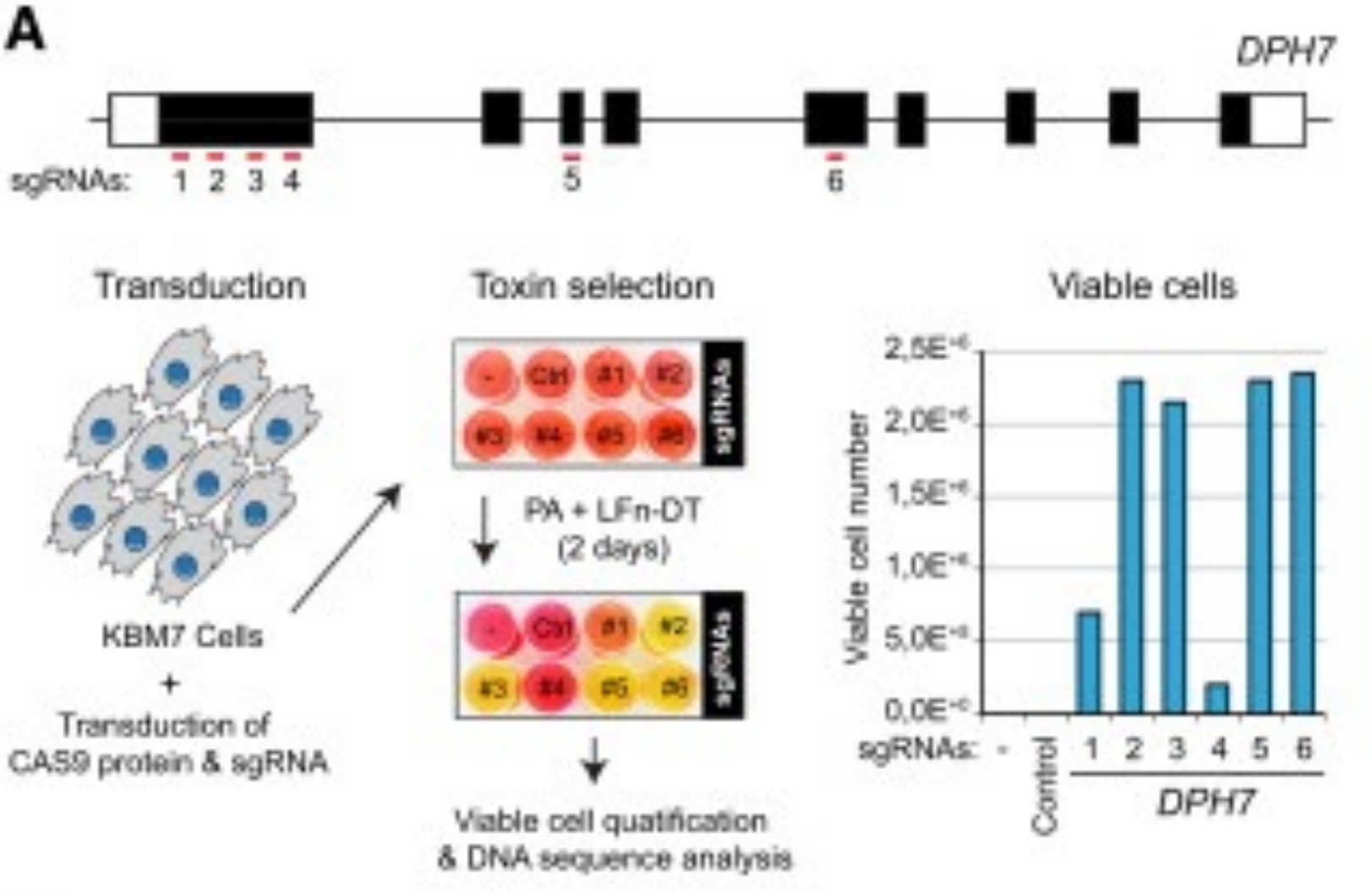




iTOP/ Crispr-Cas9

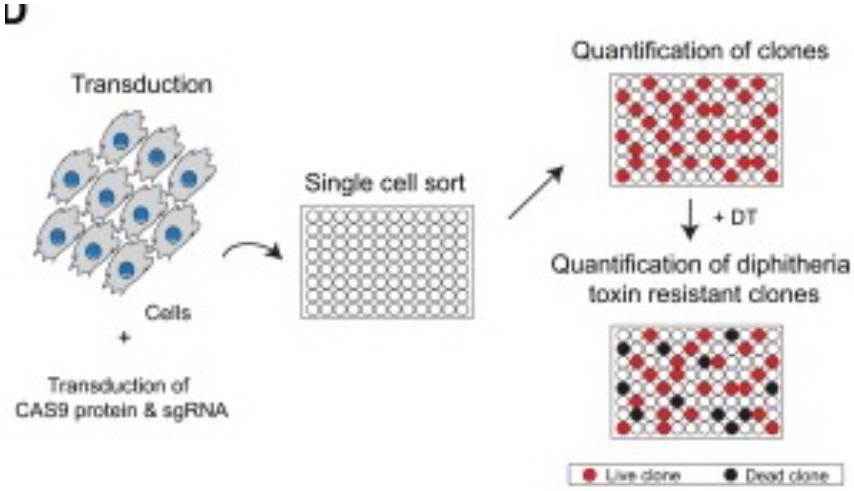


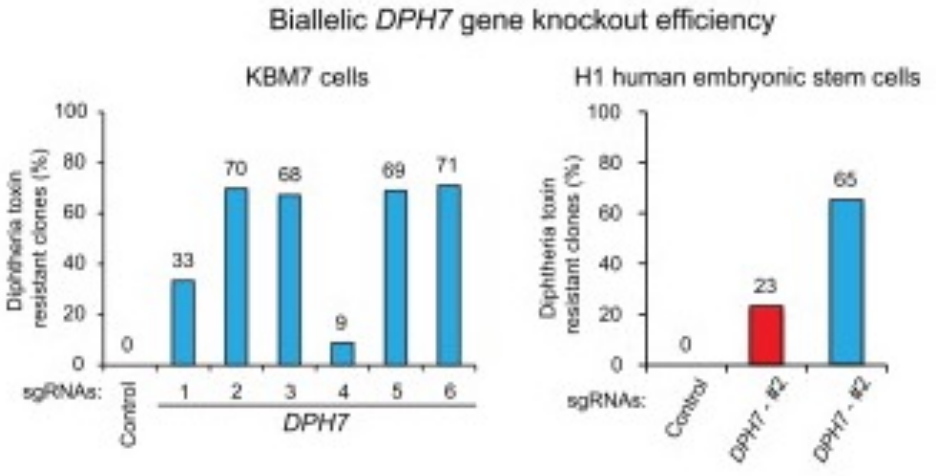
iTOP/Crispr-Cas9



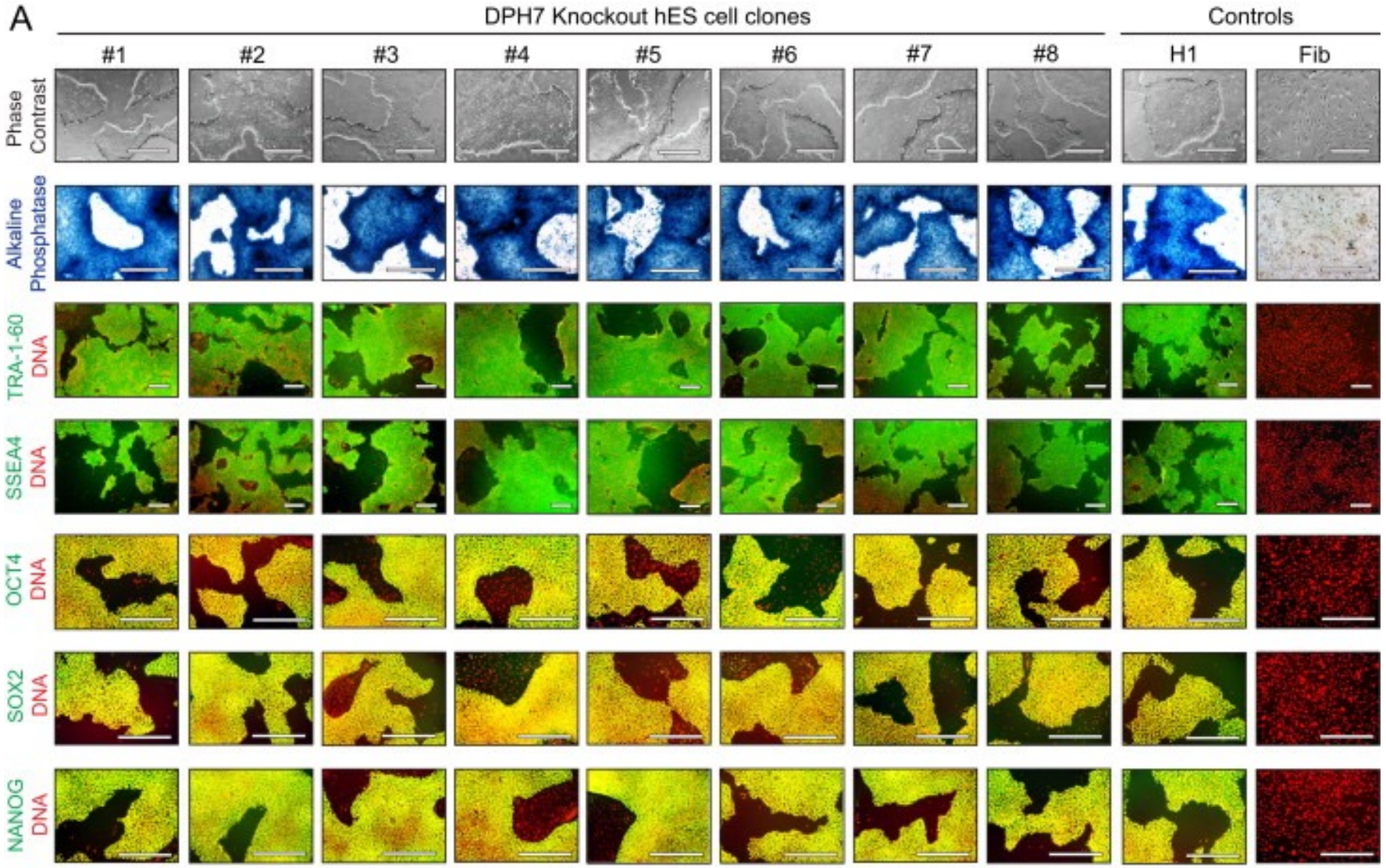
iTOP/Crispr-Cas9

Biallelic deletion efficiacy with iTOP/Crispr-Cas9

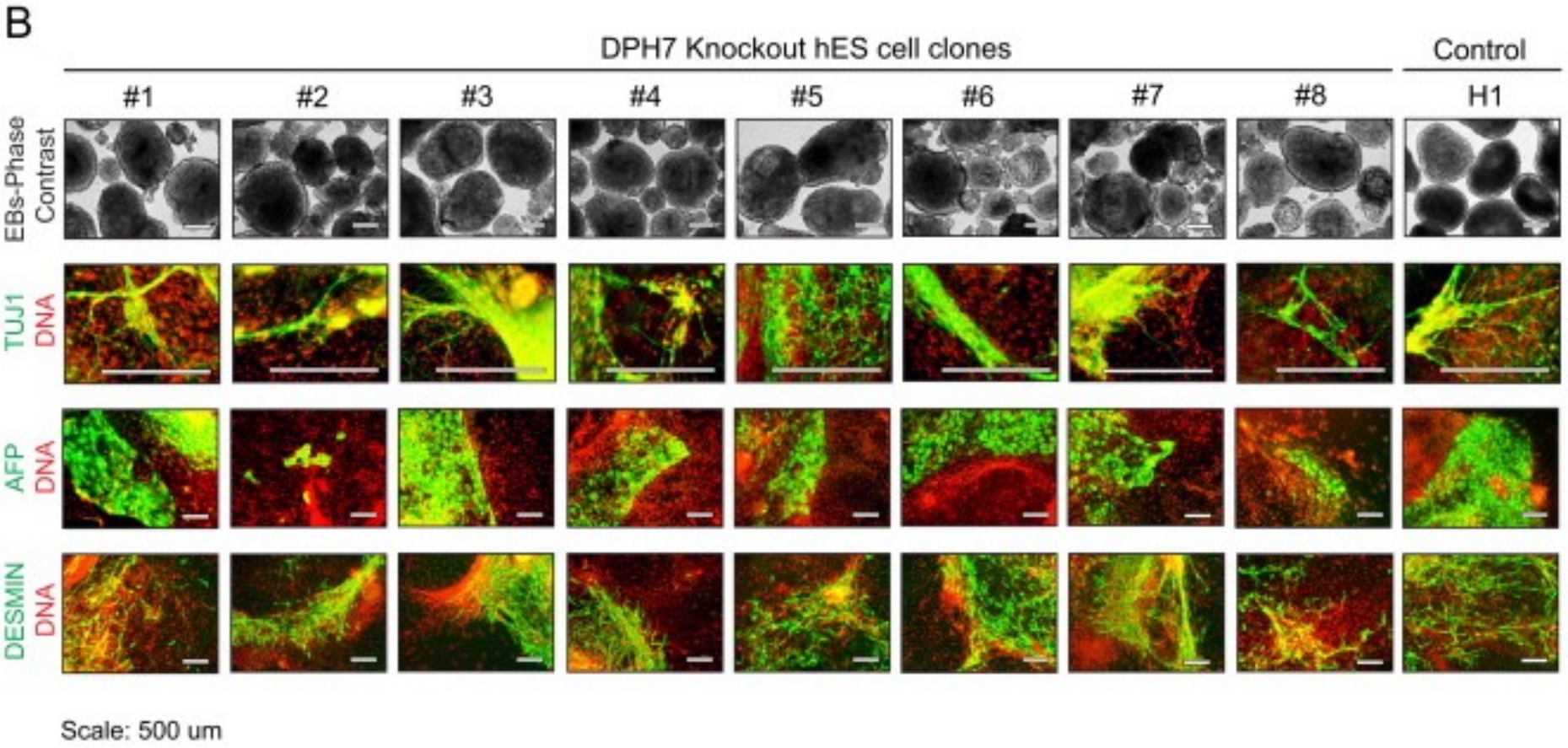




Pluripotency markers are retained in iTOP/Crispr-Cas9 hESCs



iTOP/Crispr-Cas9 treated hESCs can differentiate into cells of all 3 germ layers

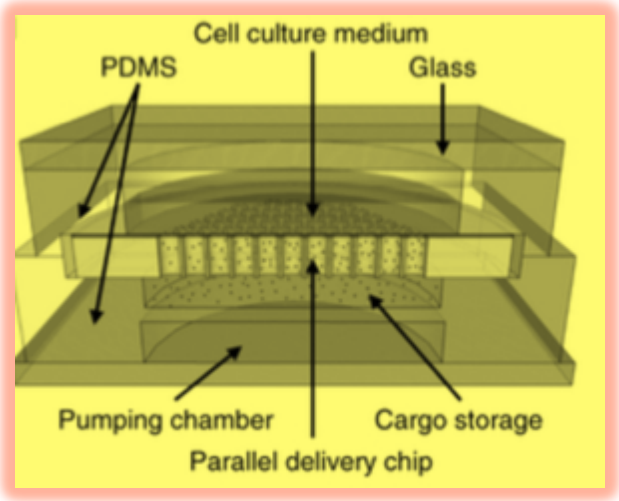


Summary/critical remarks

- **Pro:**
  - Very convenient (no biosafety issues <> Lentivirus, AAV etc), fast (building lentivirus, AAV for a couple of weeks) and cheap technique (250g of NDSB 201 around 100 sFr),
    5x transduction buffer
    - *500 mM NaCl, 25 mM NaH_2PO4, 250 mM NDSB-201, 150 mM glycerol, 75 mM glycine, 1.25 mM $MgCl_2$, 1 mM 2-mercaptoethanol at pH 8.0*
  - Very efficient with almost no cell toxicity
  - Works well with primary cell lines (<> Crispr/Cas9 not so much)
- **Contra:**
  - Only transient transfection
  - If applied more than 2 rounds > cell viability?
  - Authors claim possibily suitable for treatment of genetic disease, no such data evident from present report

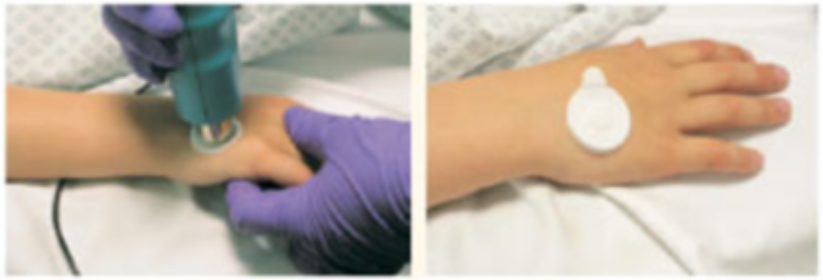
WU Y ET AL., NATURE METHODS 2015

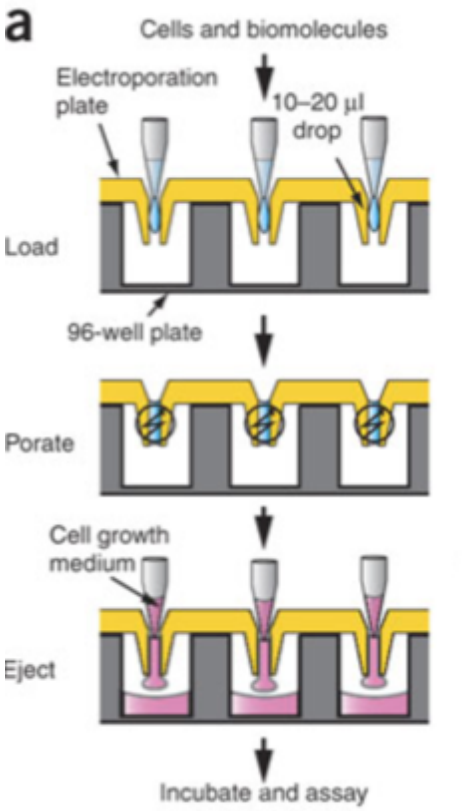
MASSIVELY PARALLEL DELIVERY OF LARGE CARGO INTO MAMMALIAN CELLS WITH LIGHT PULSES



Physical approaches to bypass endocytosis

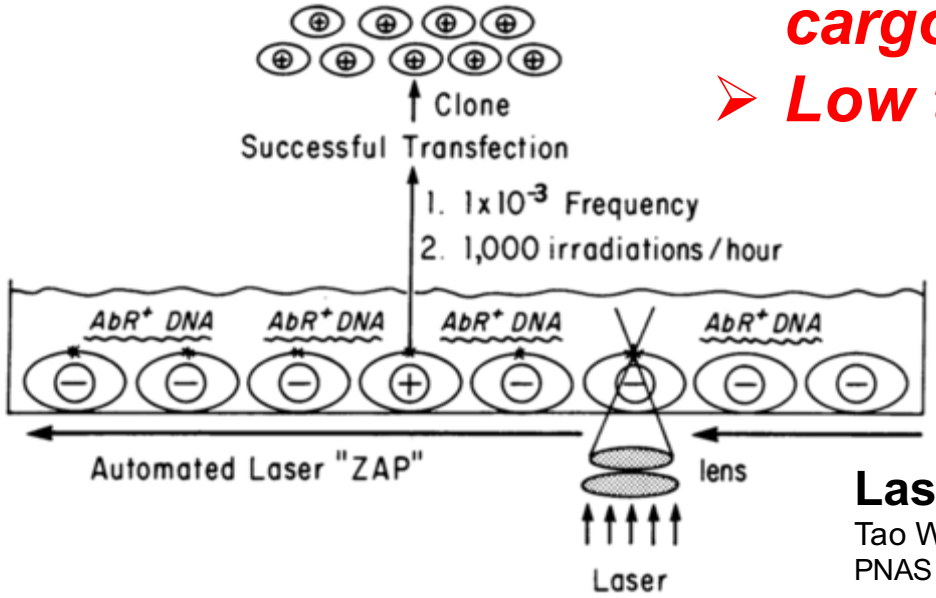
1) *Create transient pores*
2) *Cargo delivery before pores reseal*





Drop electroporation
Guignet EG & Meyer T
Nat Methods 2008

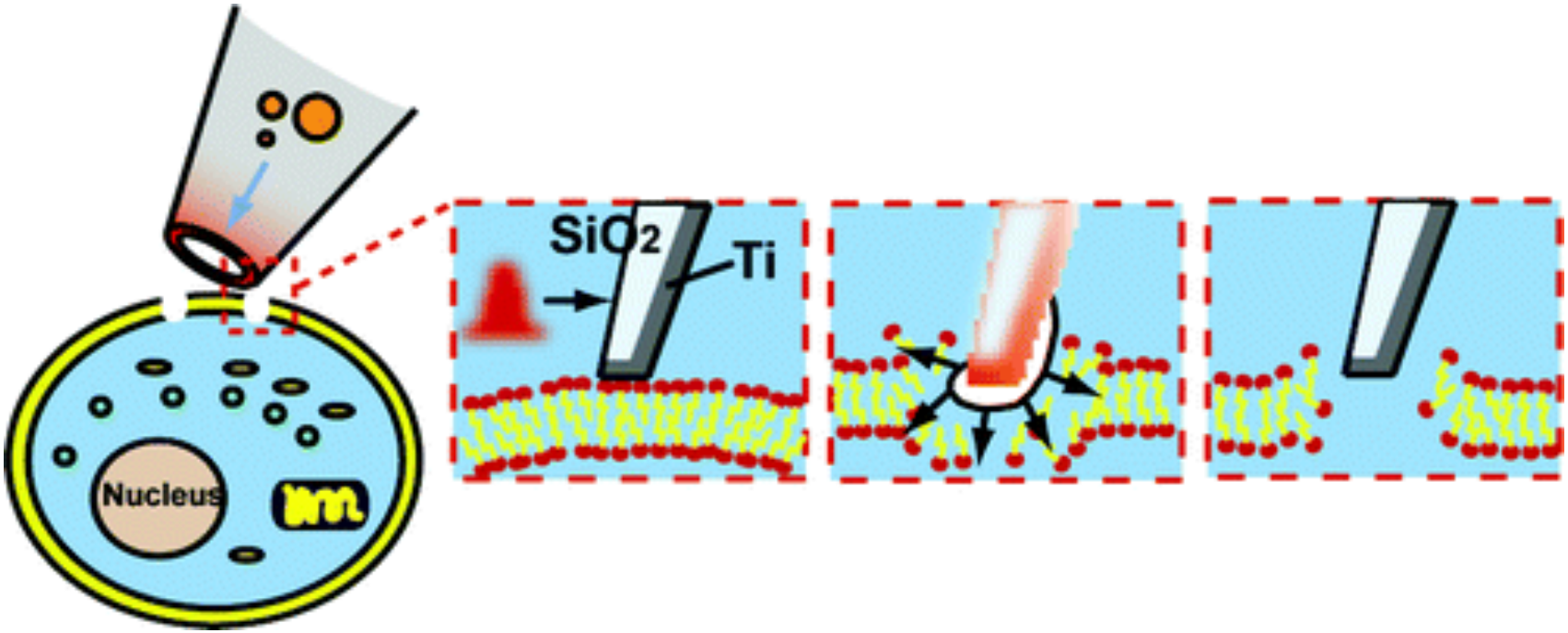
Ultrasound
Mitragotri S
Nat Rev Drug Discovery 2005



- ***Traumatic cell lysis with larger cargos***
- ***Low throughput***

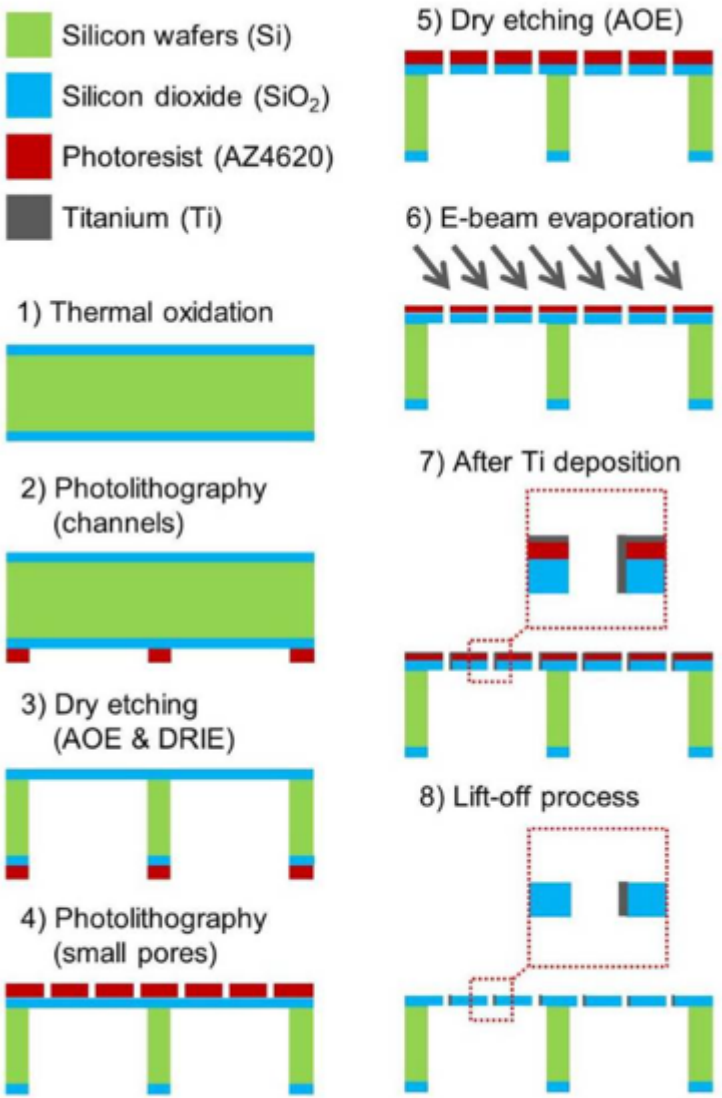
Laser micropuncture
Tao W et al.
PNAS 1987

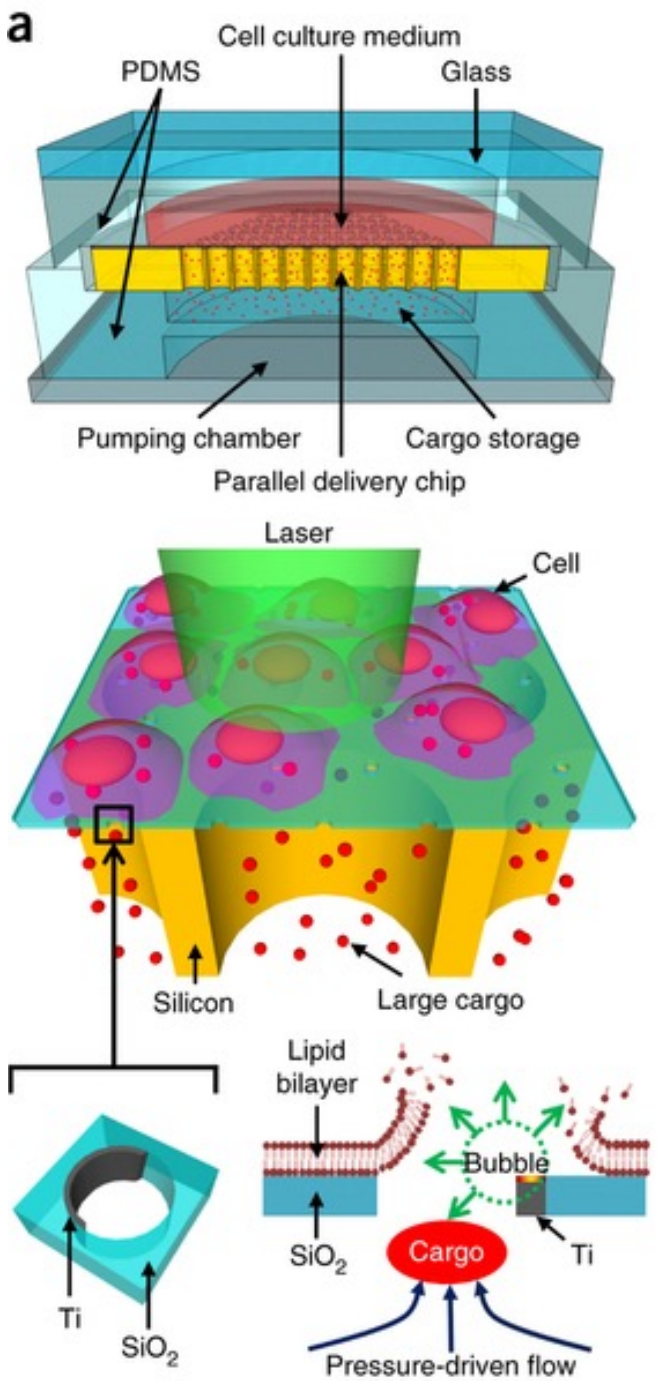
Large cargo delivery – a photothermal nanoblade



Wu T et al., Analytical Chemistry 2011

BLAST – biophotonic laser-assisted surgery tool

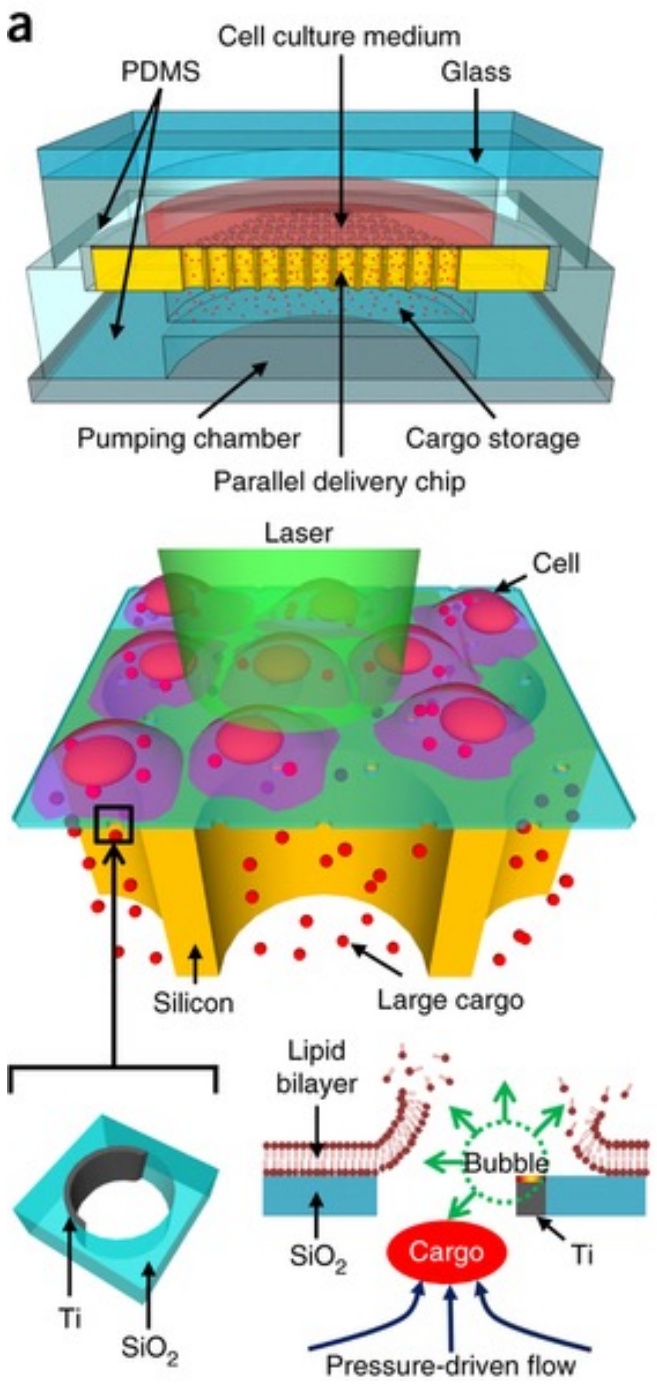




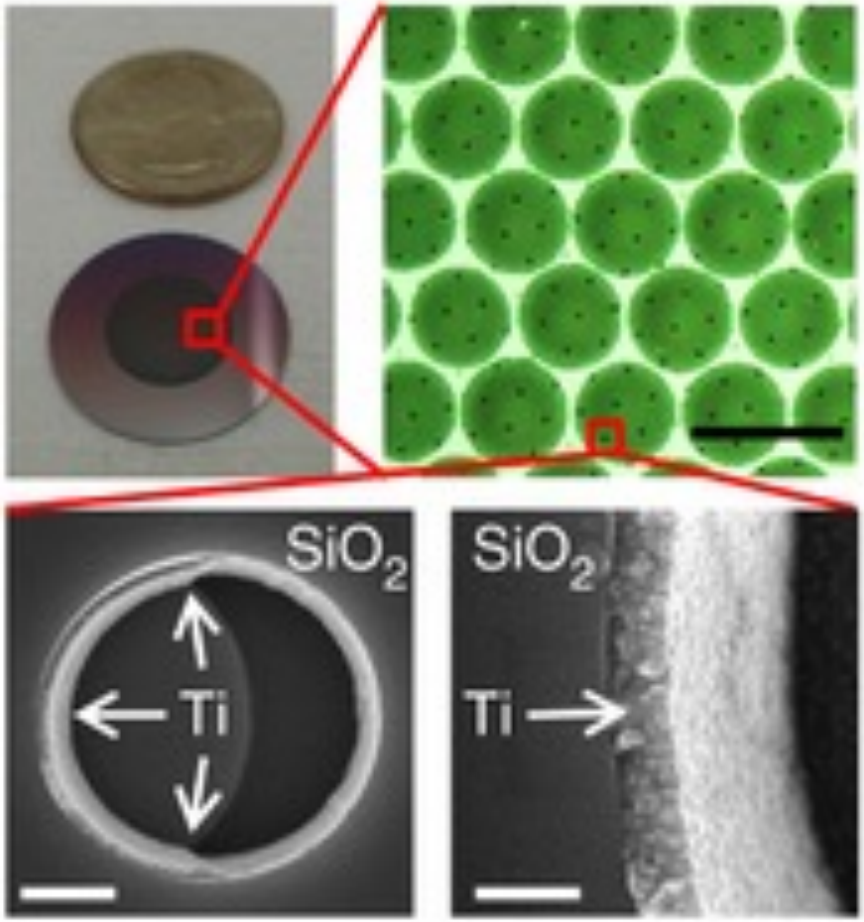
BLAST – biophotonic laser-assisted surgery tool

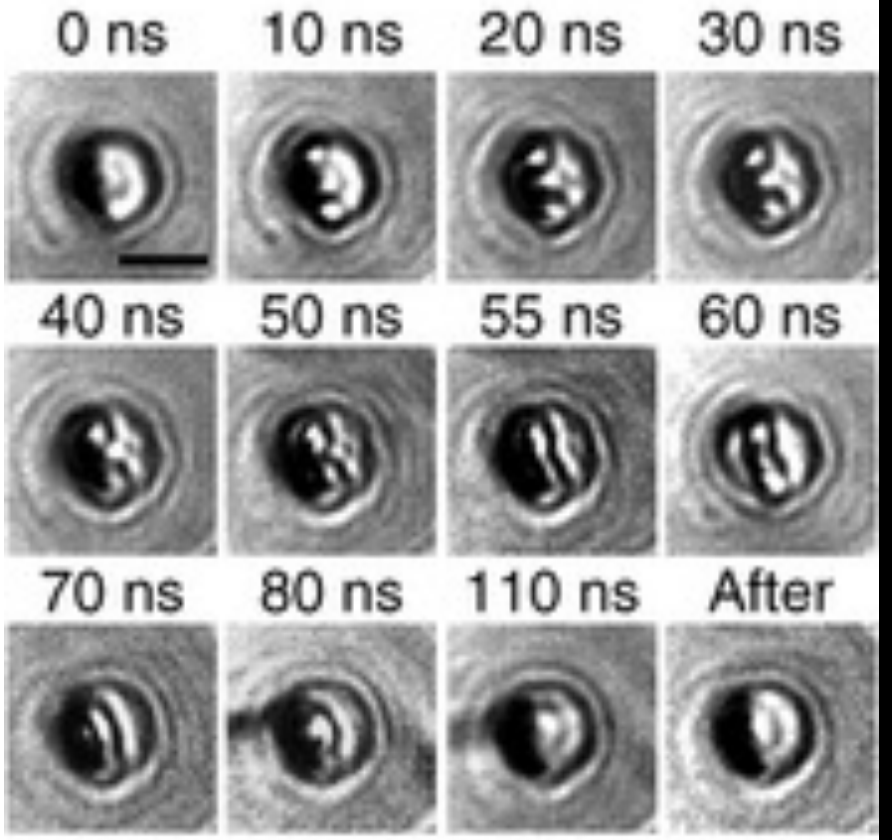
(i) Cells are cultured or made to adhere on a silicon chip
(ii) The chip is assembled with a microliter chamber loaded with the cargo to be delivered
(iii) A nanosecond-pulse laser scans rapidly across the entire chip to generate membrane pores in cells and immediately thereafter the elastic chamber is pressurized to deliver cargo through these transient pores (10s)

- **Each batch can deliver cargo into 100‘000 cells within the chip area (1cm^2)**
- **Each batch delivery takes 1 min**

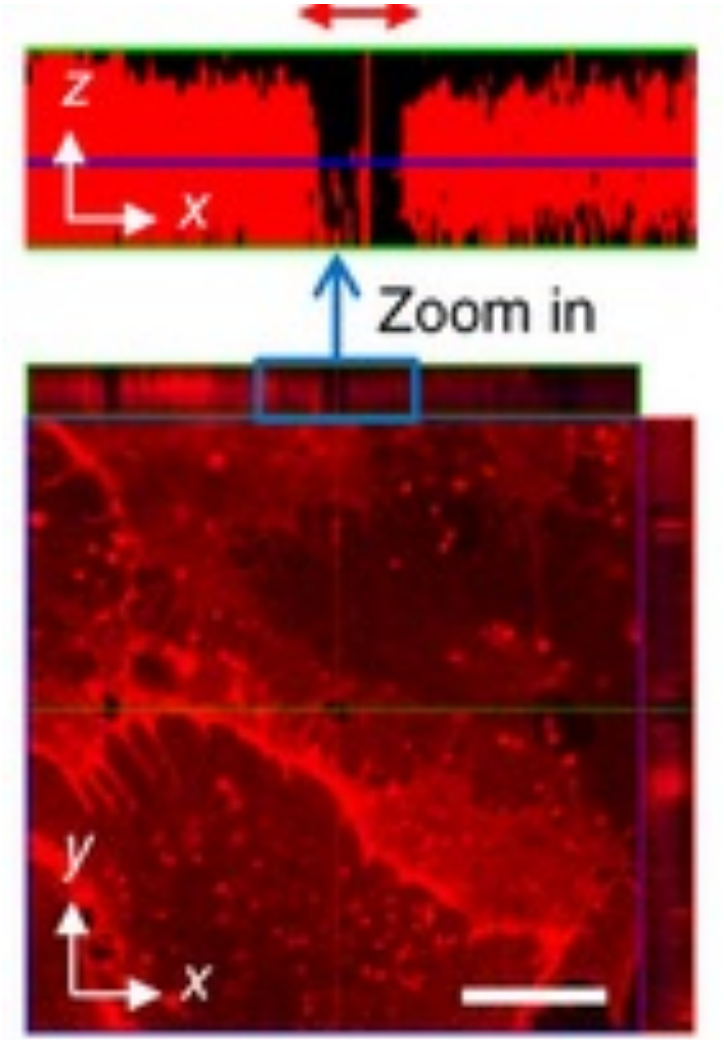


Mechanism of opening transient cell-membrane pores

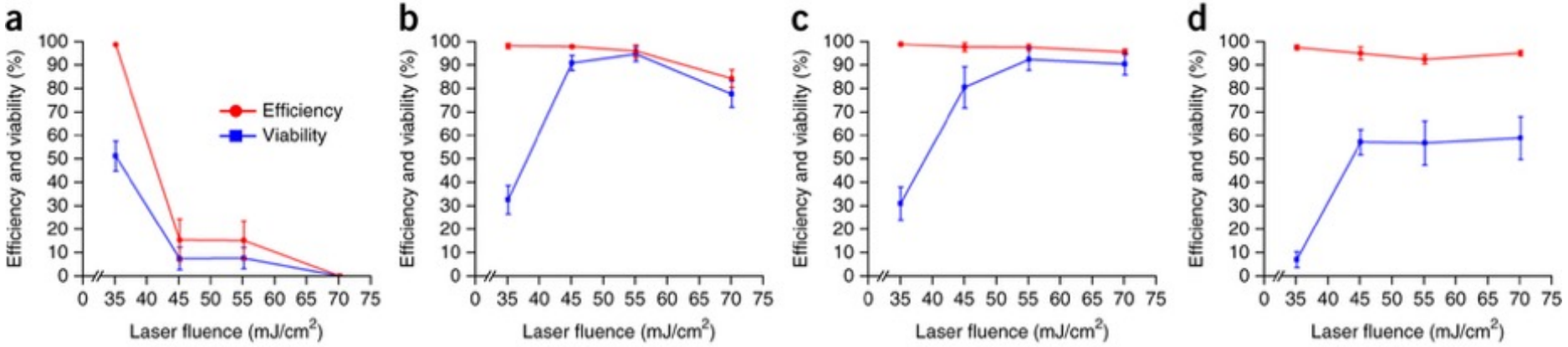




Rapid bubble bursts are highly localized



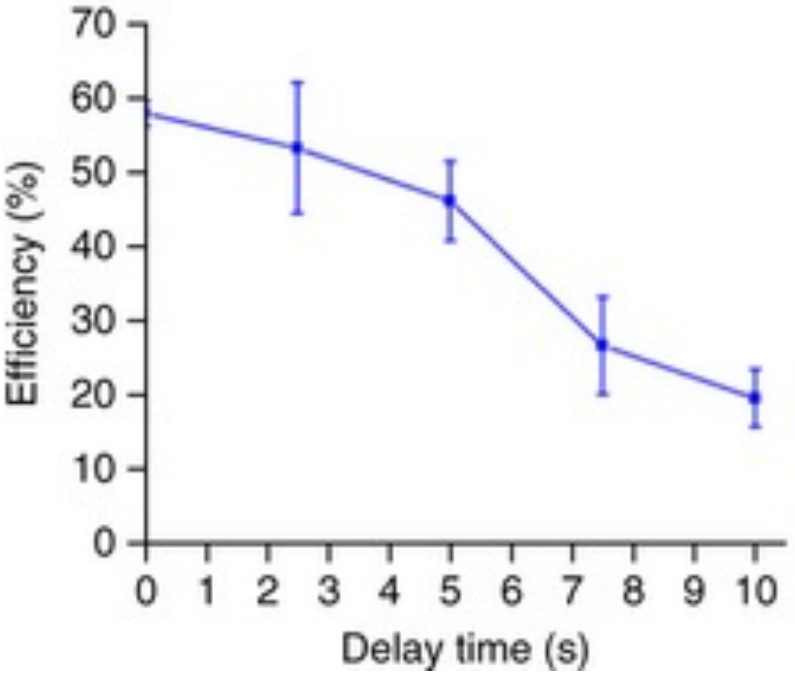
Cargo delivery efficiency and cell viability is dependent on SiO2 hole density and laser energy



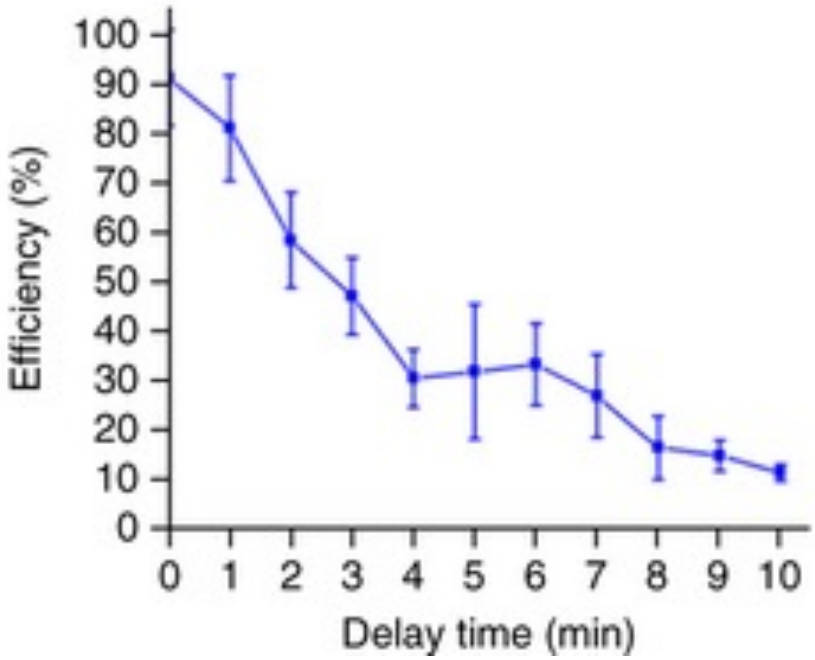
1.15 **0.28** **0.18** **0.12**

Holes/10 μm²

Larger cargo tolerates less delivery delay due to rapid pore resealing



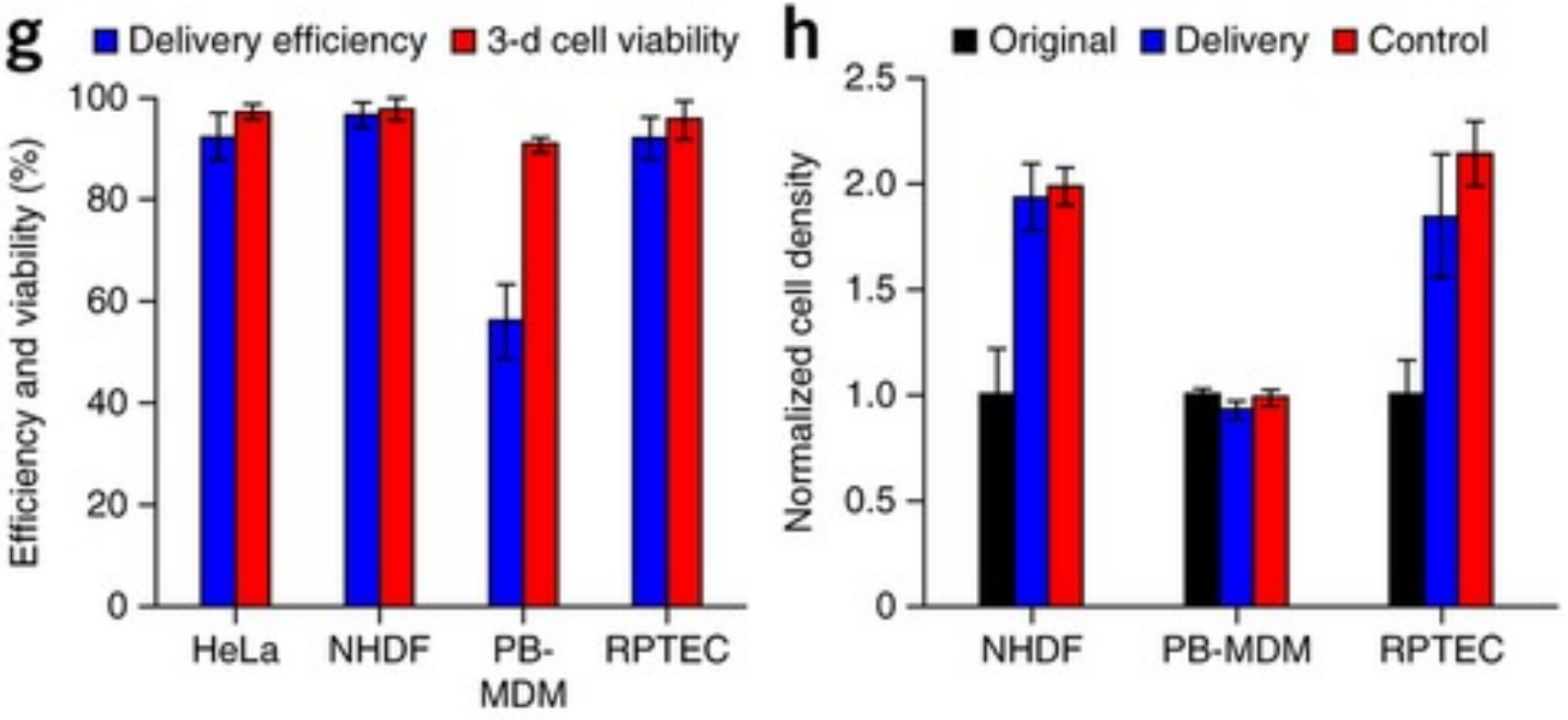
GFP-tagged F. novicida
(ca 1 μm in diameter)



Calcein
(622 Da)

> Complete membrane resealing took more than 10 minutes

Cargo delivery does not affect neither 3-day cell viability nor cell proliferation



human peripheral blood monocyte–derived macrophages (**PB-MDMs**)
primary normal human dermal fibroblasts (**NHDFs**)
human primary renal proximal tubule epithelial cells (**RPTECs**)
one cancer cell line (**HeLa**)

Efficient delivery of

a) 2 µm fluorescent polystyrene beads

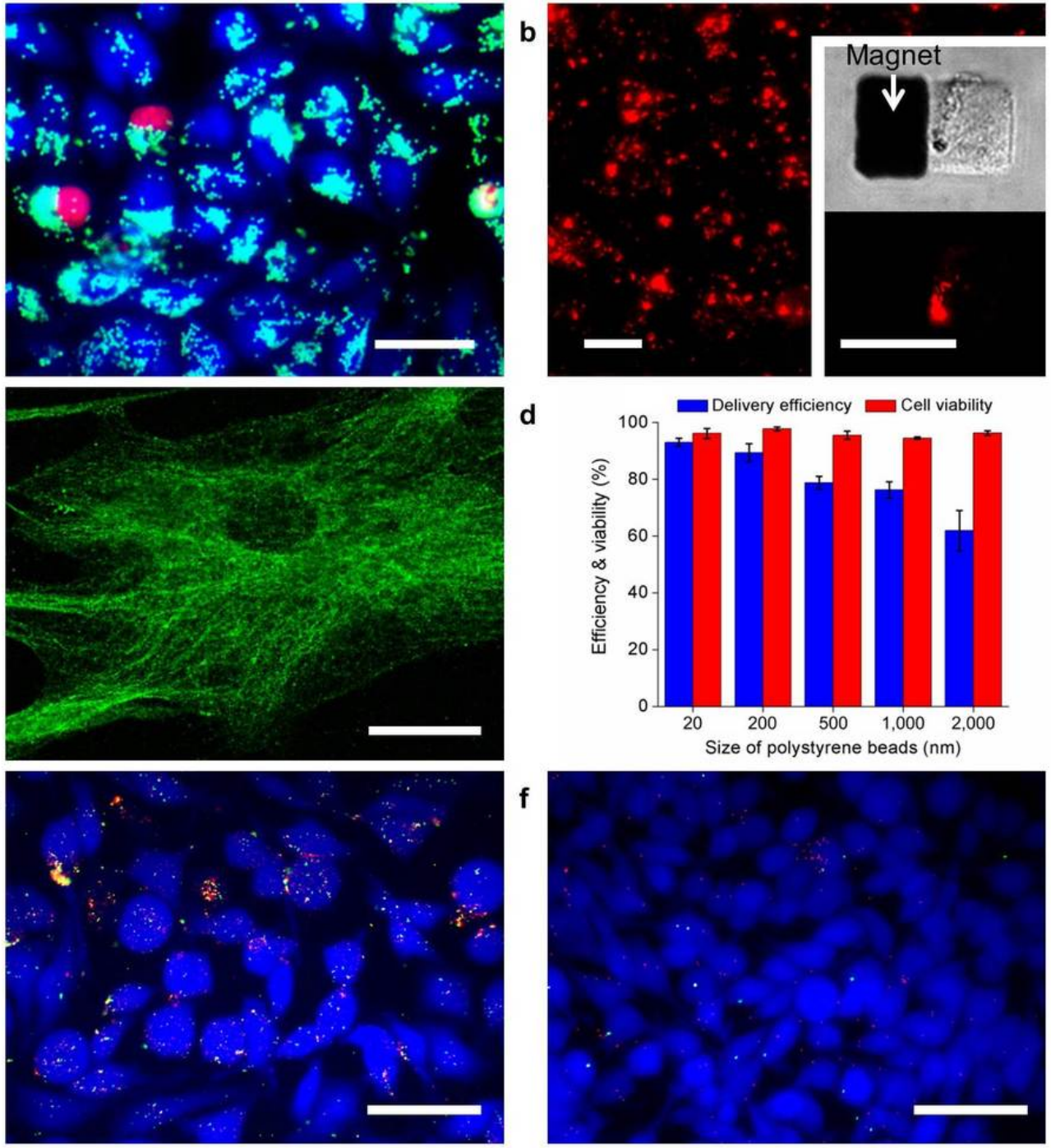
b) 200 nm magnetic beads

c) anti-tubulin AB

d) Delivery efficiency of 5 differentially sized polystyrene beads

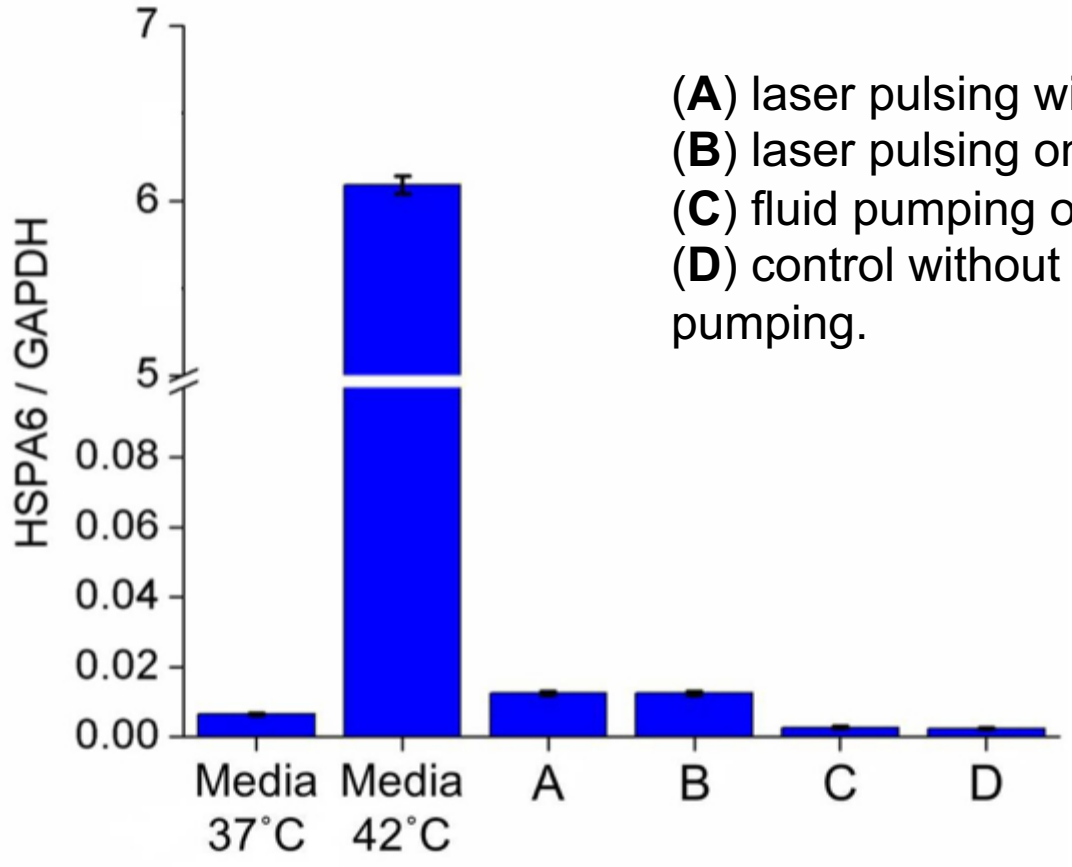
e) co-delivery of 100&600 nm beads

f) Like e) w/o laser pulsing



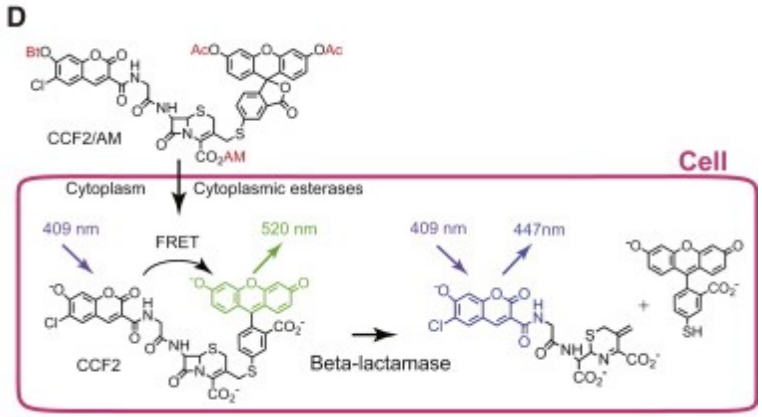
The general cell stress sensor heat-shock gene HSPA6 is not upregulated by BLAST

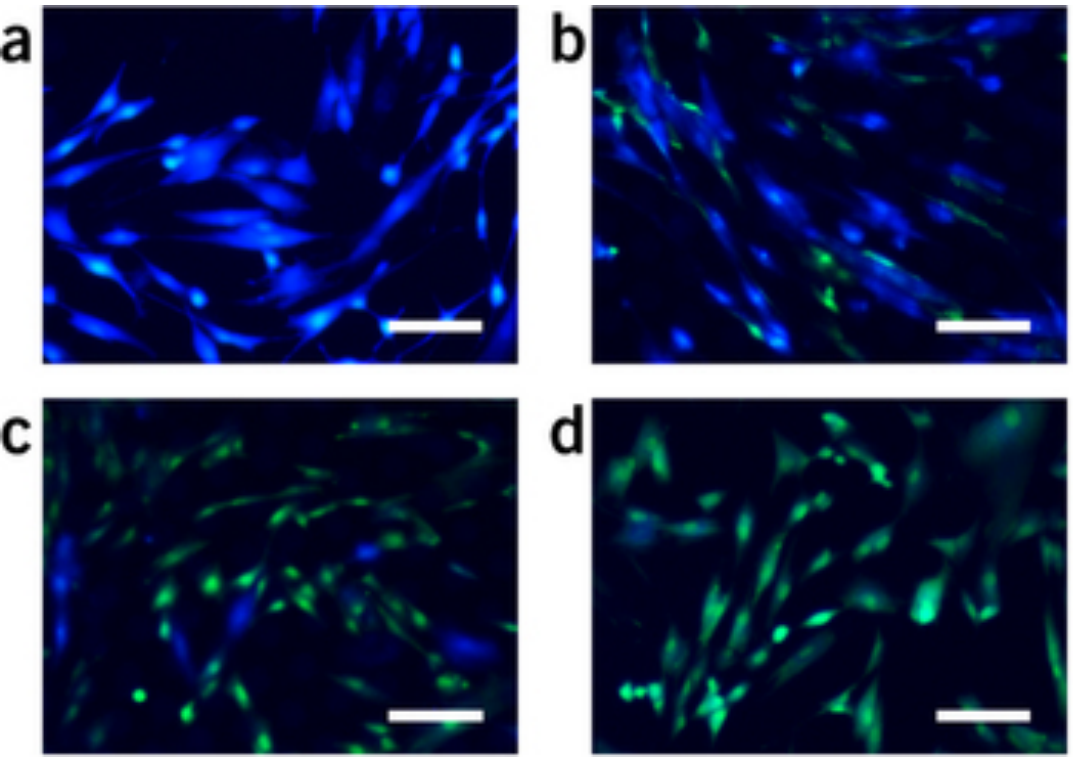
Supplementary Figure 5: Evaluation of stress levels of HeLa cells after BLAST delivery.

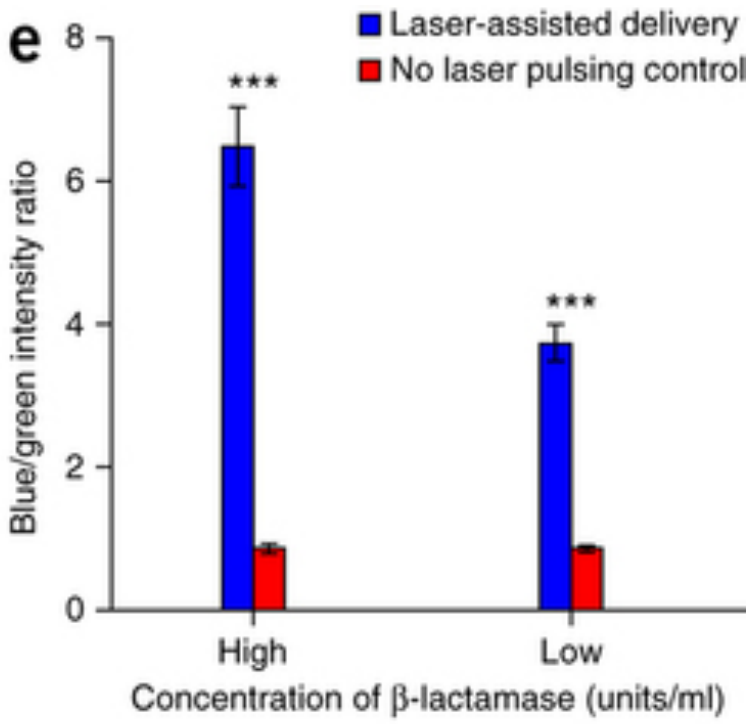


(**A**) laser pulsing with fluid pumping
(**B**) laser pulsing only
(**C**) fluid pumping only
(**D**) control without laser pulsing and fluid pumping.

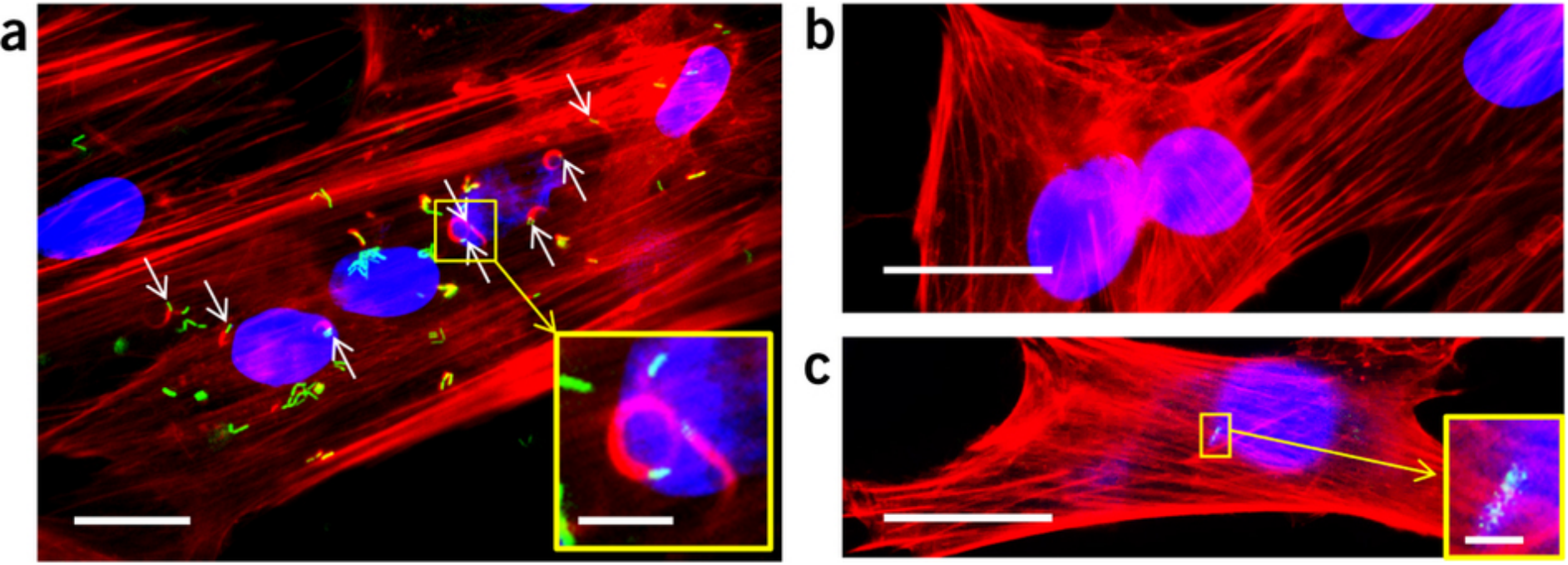
BLAST facilitates efficient cytosolic delivery



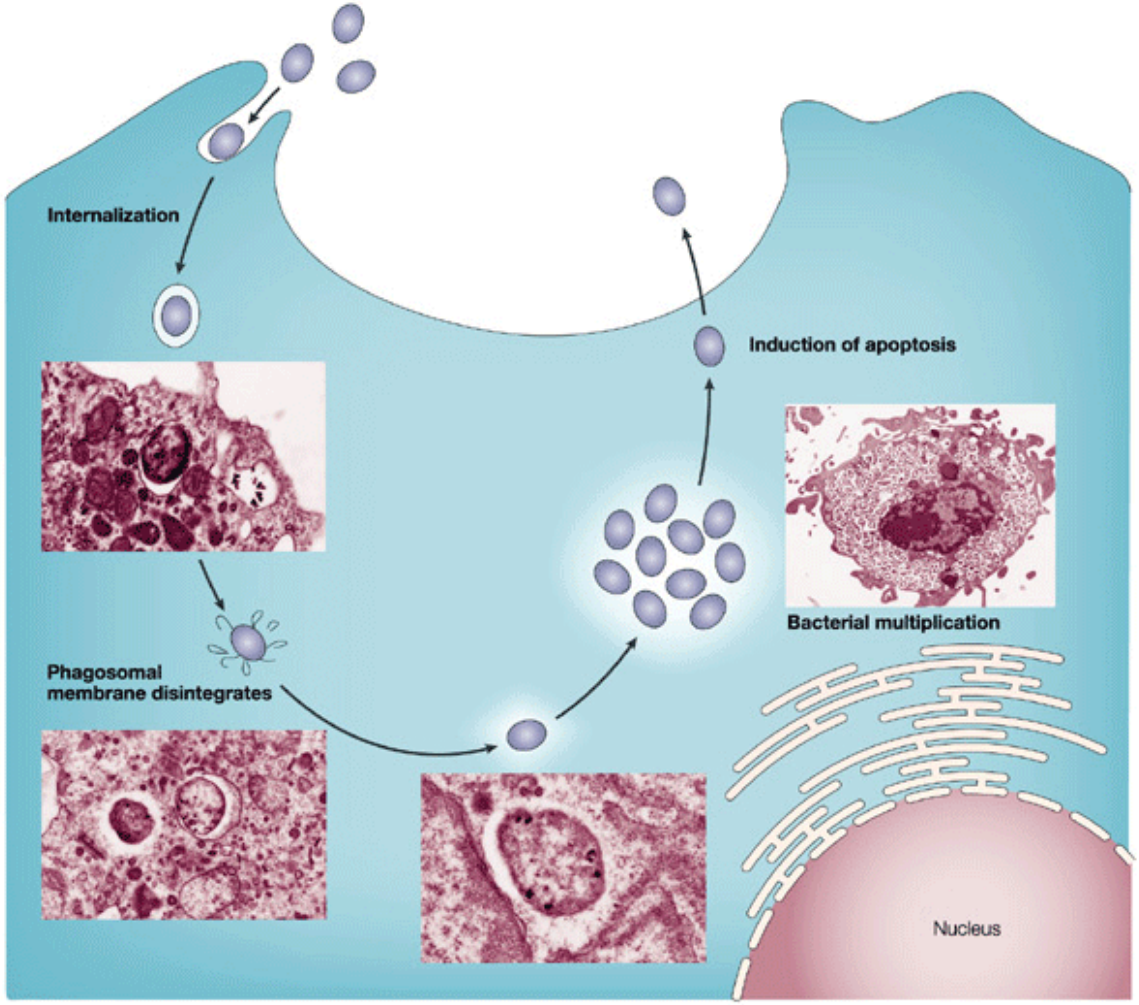




BLAST-induced uptake of Listeria monocytogenes induces actin comet tails



BLAST allows studying genes important for the replication cycle of *Franciscellae*



Oyston PCF et al.,
Nat Rev Microbiology 2004

iglC is important for cytosolic replication after phagosomal escape in F. novicida