

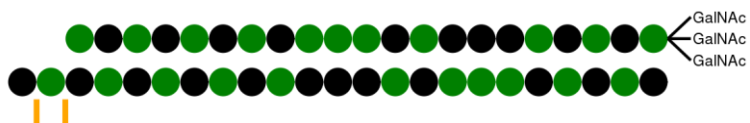
# Evidence for an Intracellular Depot that Contributes to the Extended Duration of Activity of GalNAc-siRNA Conjugates

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**OTS – 9/28/20**

# Extended Duration of Activity by ESC Conjugates

Stabilized template chemistry on the same target sequence shows longer duration in humans\*

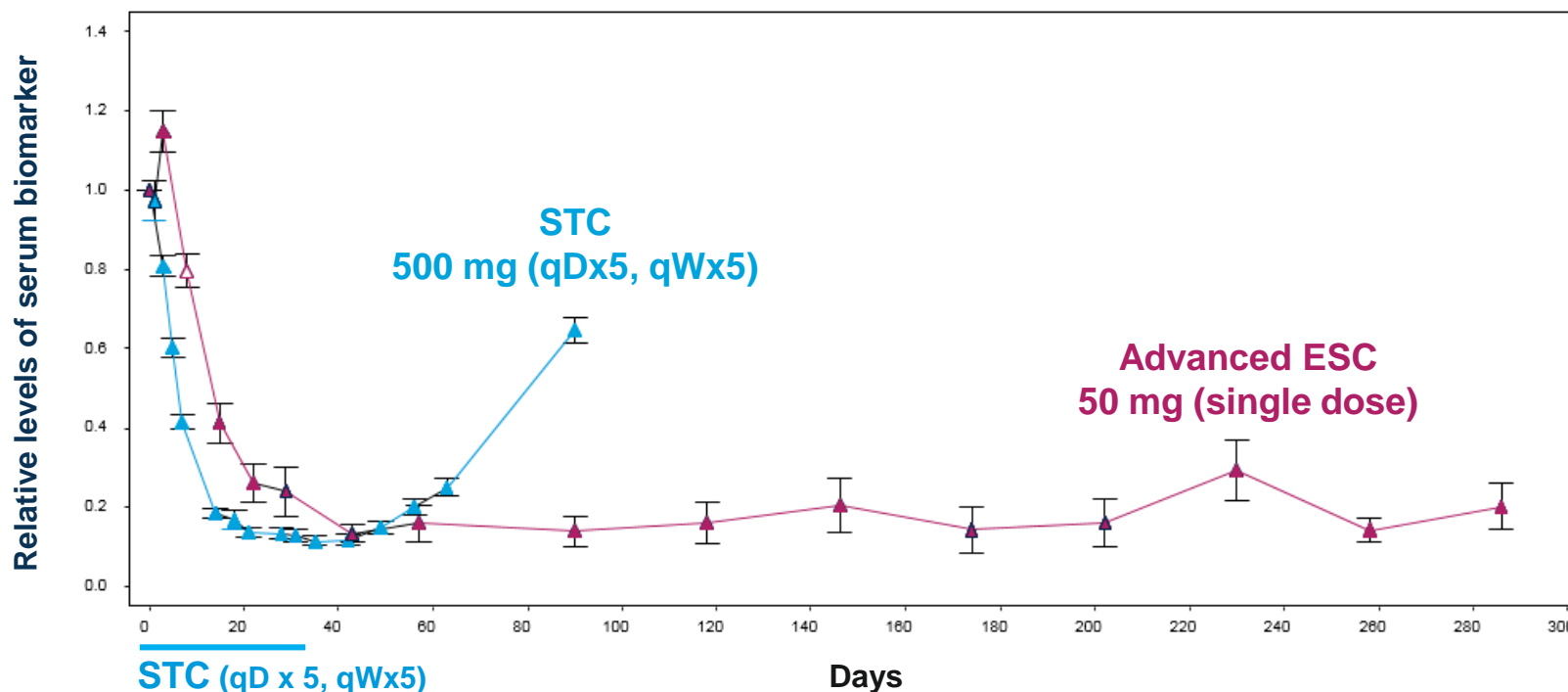
**STC: Standard Template Chemistry**



**Advanced ESC: Enhanced Stability Chemistry**



● 2'-Fluoro    ● 2'-OMe    | PS- Phosphorothioate



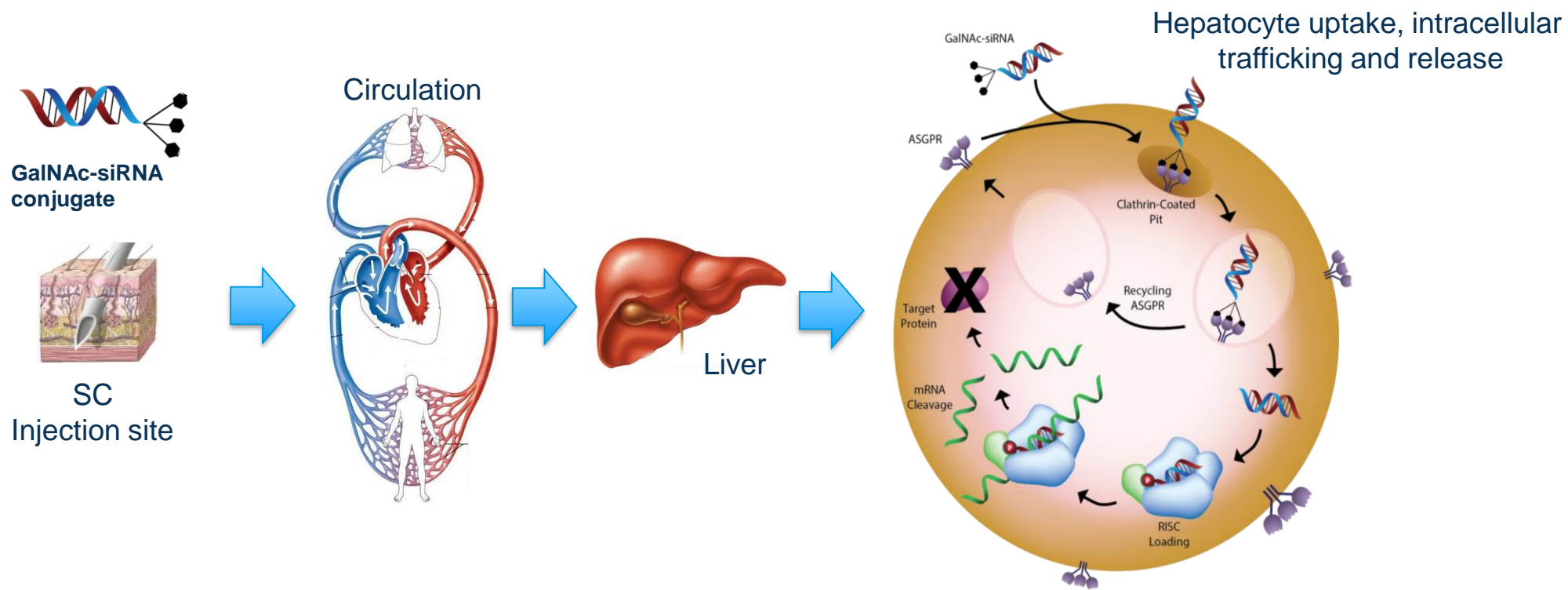
STC (qD x 5, qWx5)

Advanced ESC (Single dose)

- Extended duration also seen in preclinical species

\*Phase 1 data in healthy volunteers from separate studies

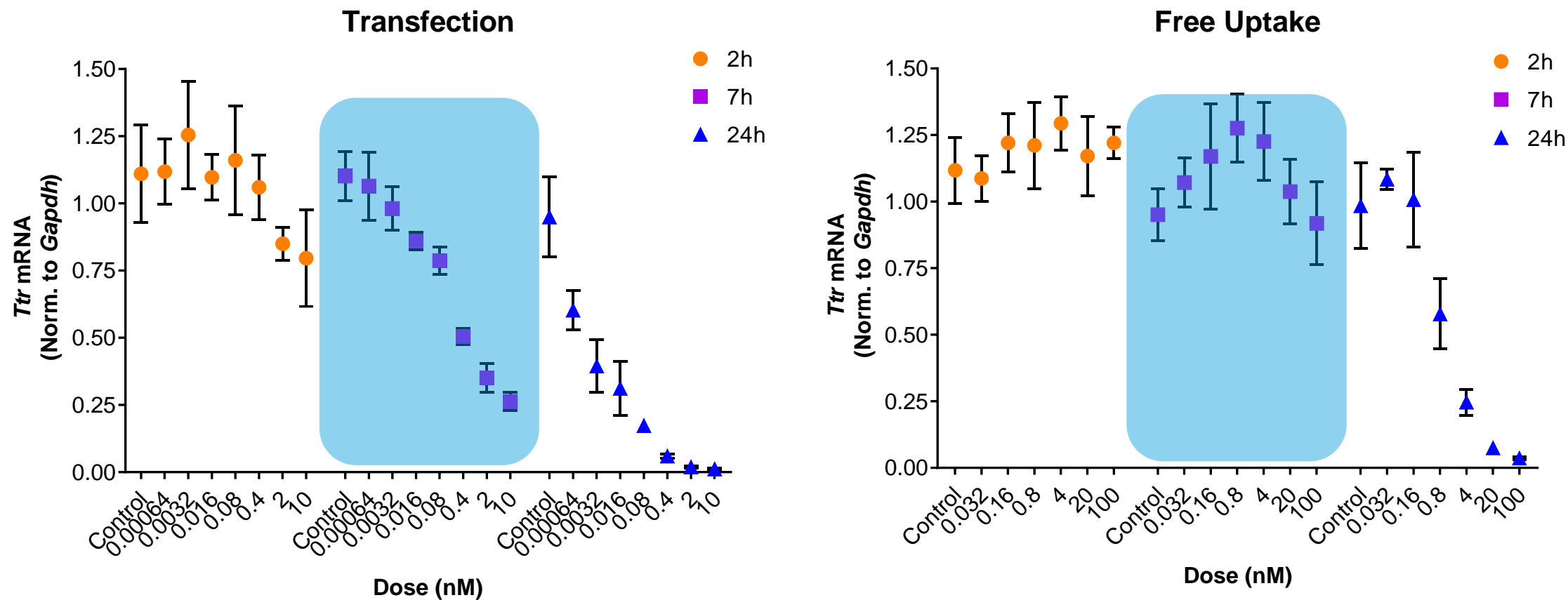
# Exploring Possible Reasons for the Extended Duration of Effect



- Sustained release of conjugate from SC injection site to liver?
- Increased half-life of siRNA-loaded RISC?
- Continuous supply of siRNA from an intracellular depot?

# Target mRNA Knockdown Delayed Following ASGPR-Mediated Uptake

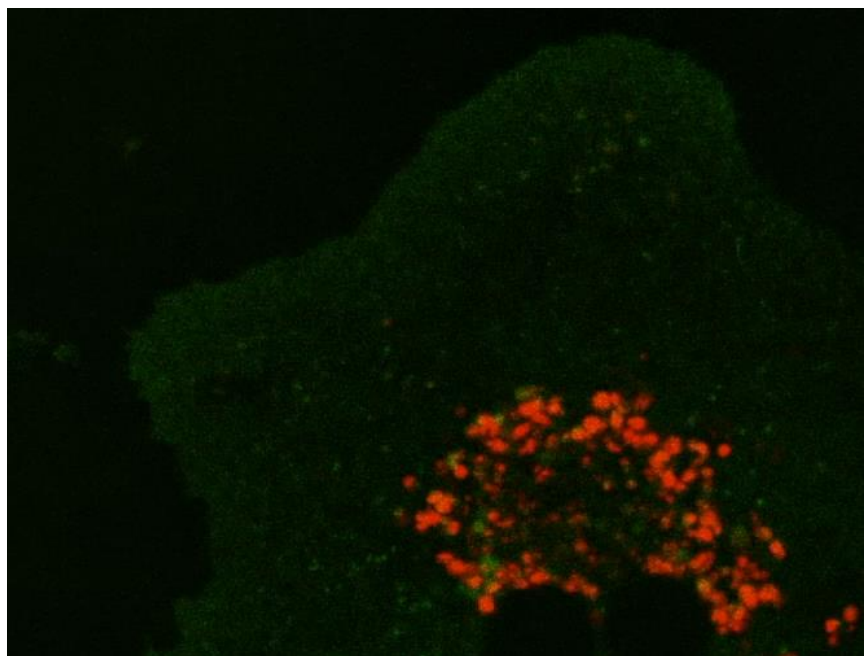
Advanced ESC siRNA shows a delay in activity following free uptake into primary hepatocytes



# GalNAc-siRNAs Accumulate in Acidic Lysosomes

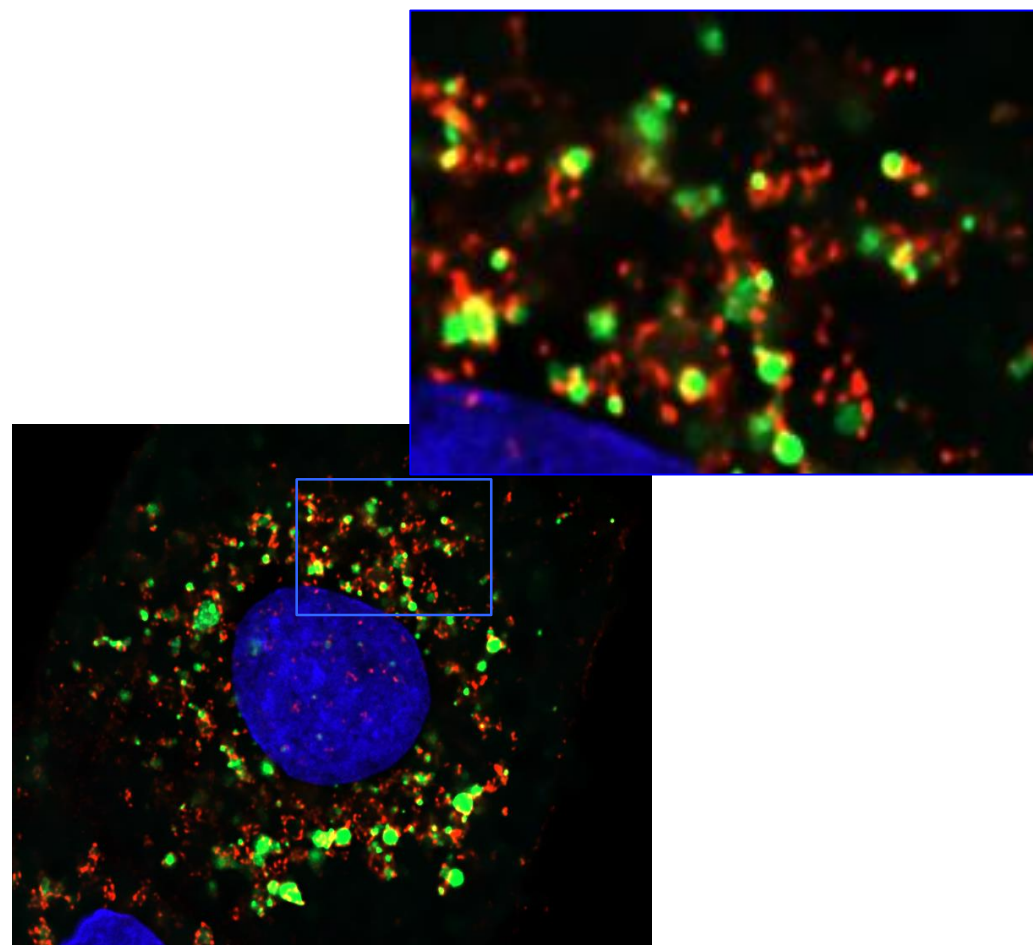
## Rat primary hepatocytes (live cell imaging)

- siRNA (10 nM free uptake, 1 min after dose)
- Lysosomes (LysoTracker)
- 30 minutes real time



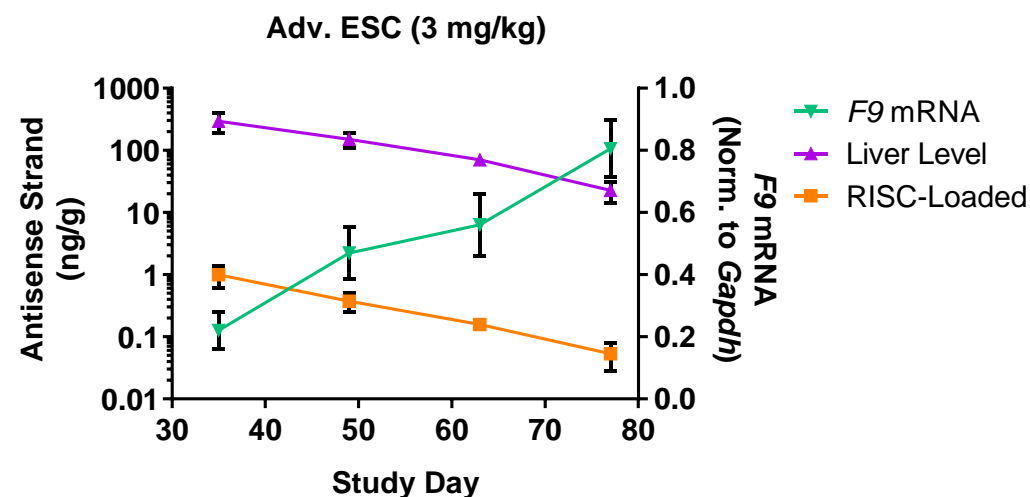
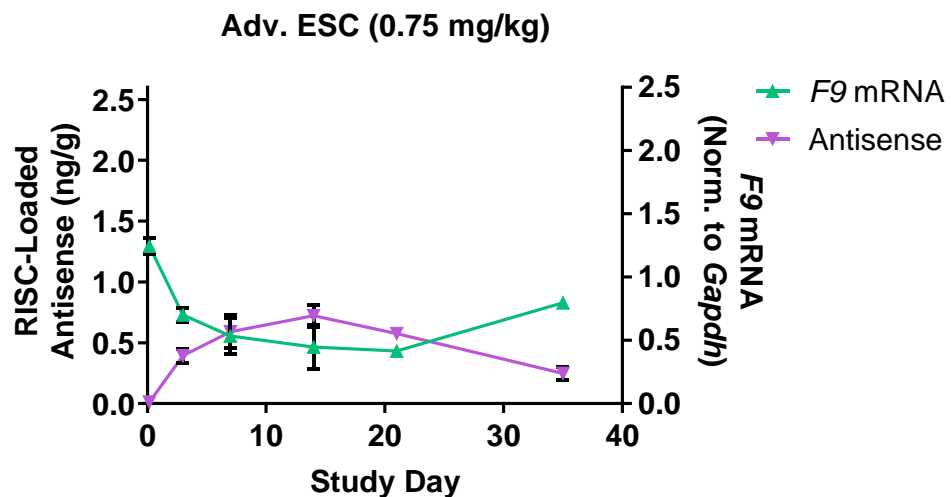
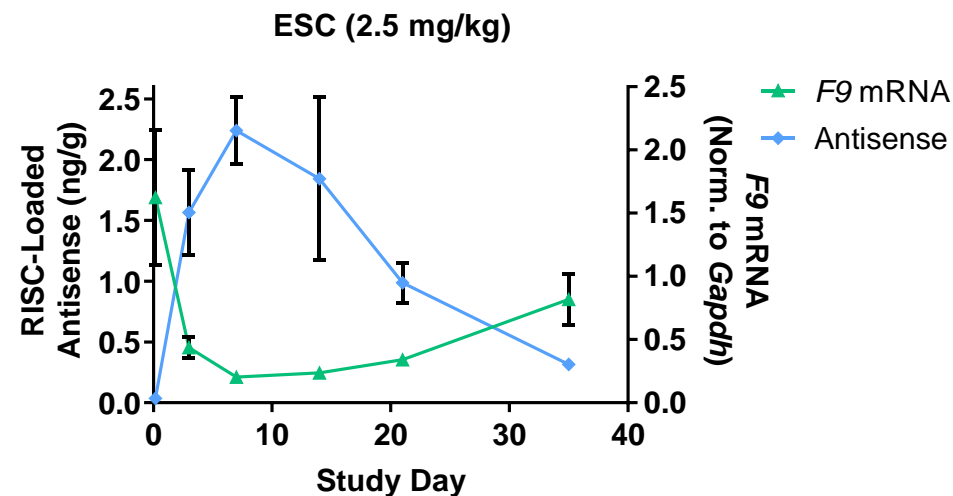
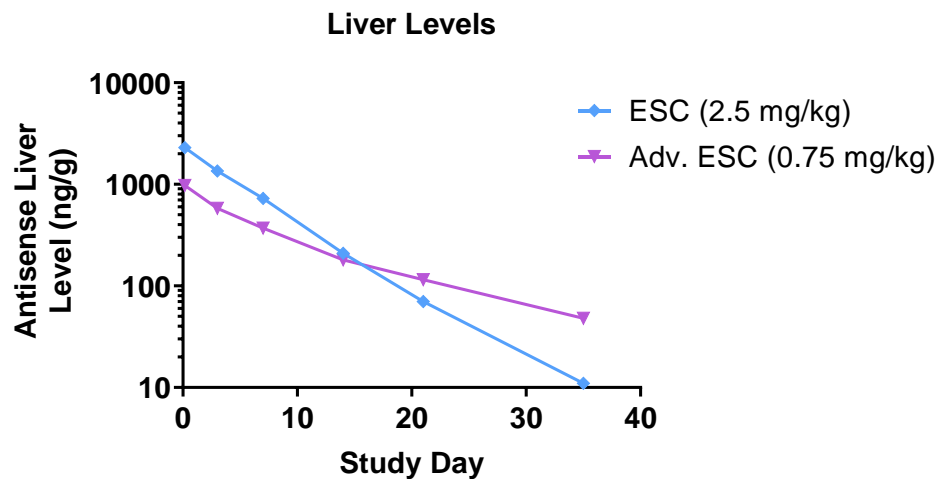
## Rat primary hepatocytes (fixed cells)

- siRNA (10 nM free uptake, 48 hours post-dose)
- Lysosomes (LAMP1 antibody)



# RISC Loading Correlates with Target mRNA Knockdown

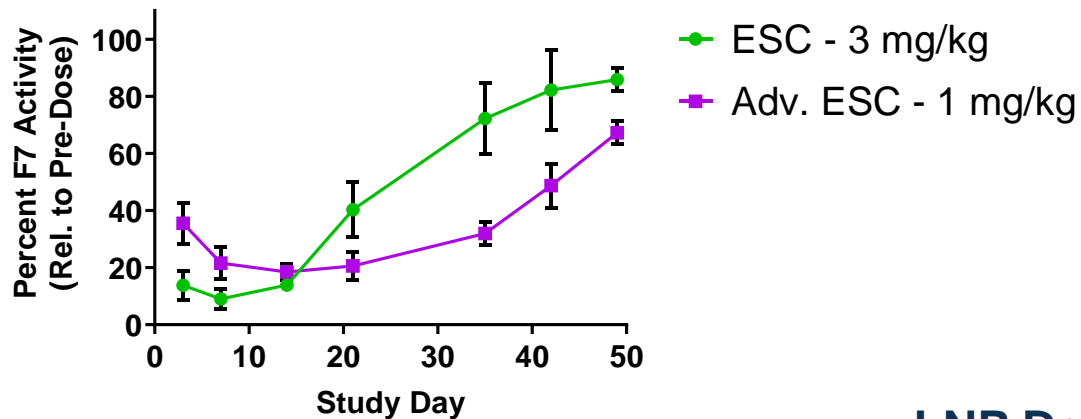
RISC loaded siRNA is a small fraction of total liver siRNA



# siRNA Duration is Dependent on Delivery Modality and Stability

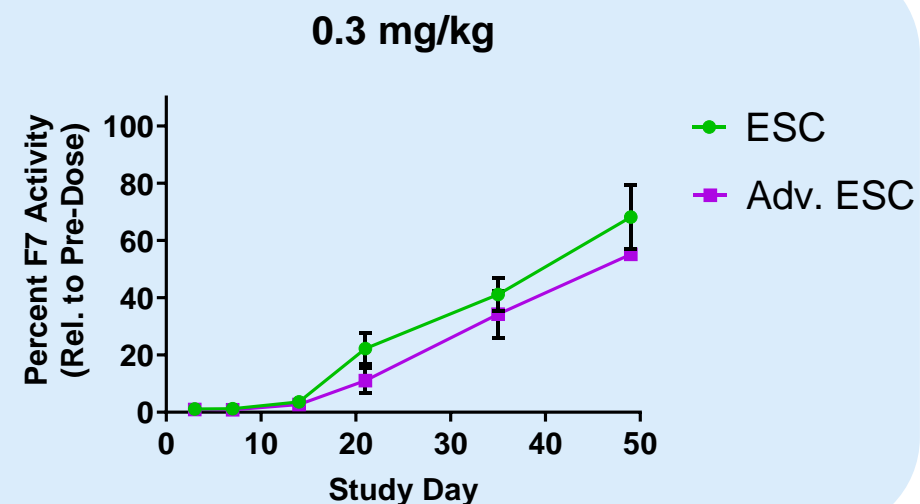
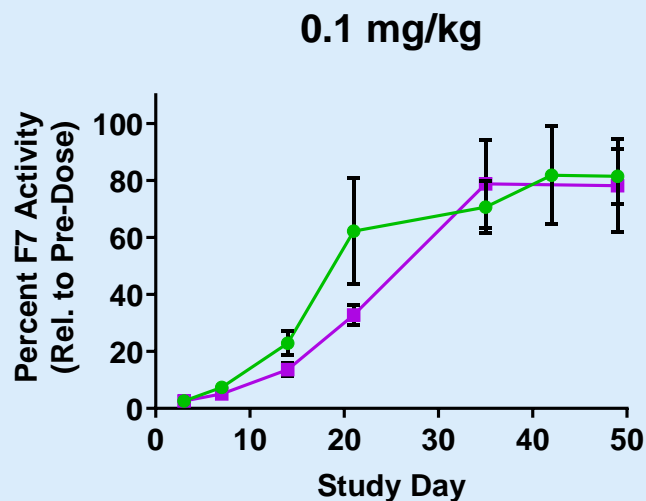
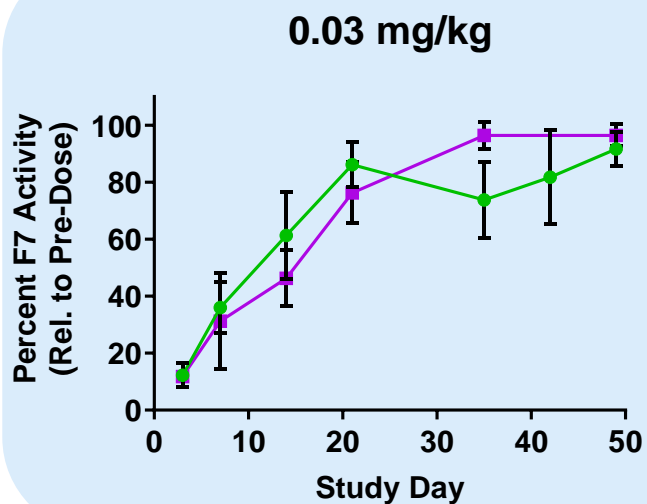
Template designs of different stability have similar activity profiles following LNP delivery

## SC Delivery



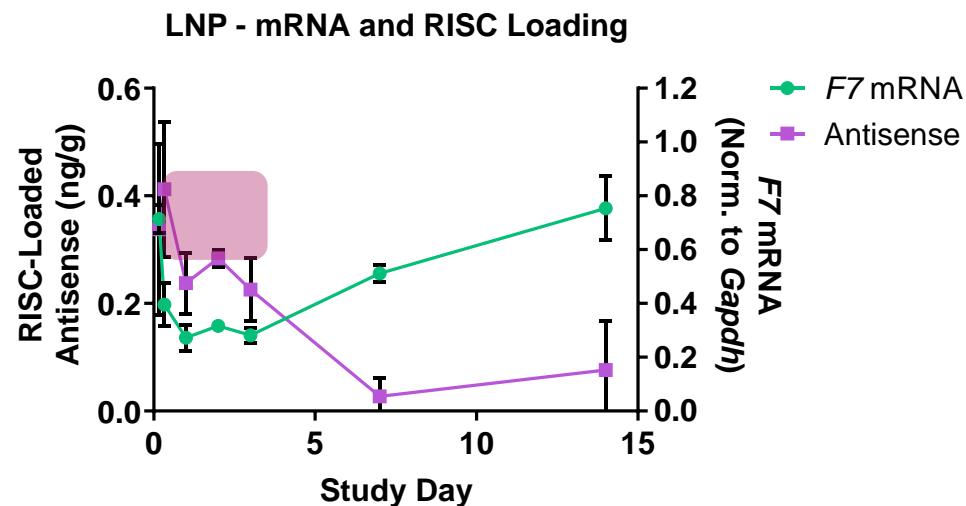
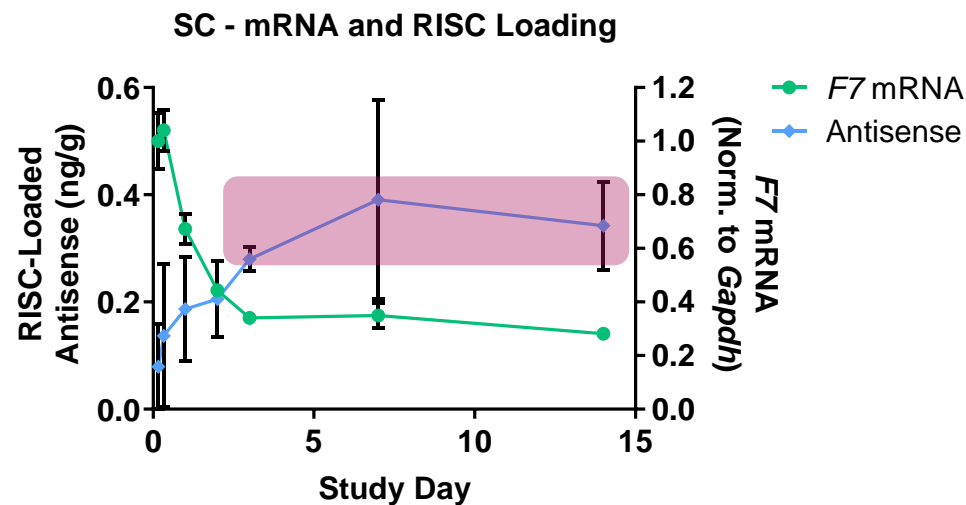
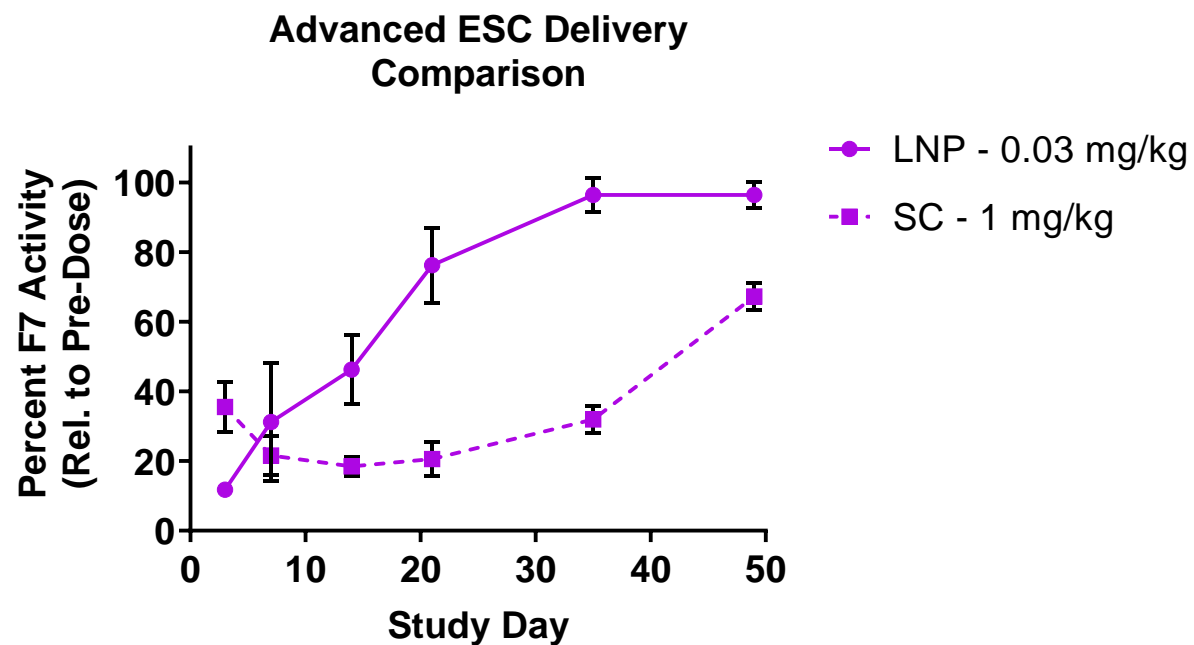
- Chemical stability is critical for GalNAc-siRNA delivered by SC dosing
- LNP delivery allows siRNAs with different stability to show equivalent potency

## LNP Delivery



# RISC Loading is Sustained Following GalNAc-siRNA Delivery

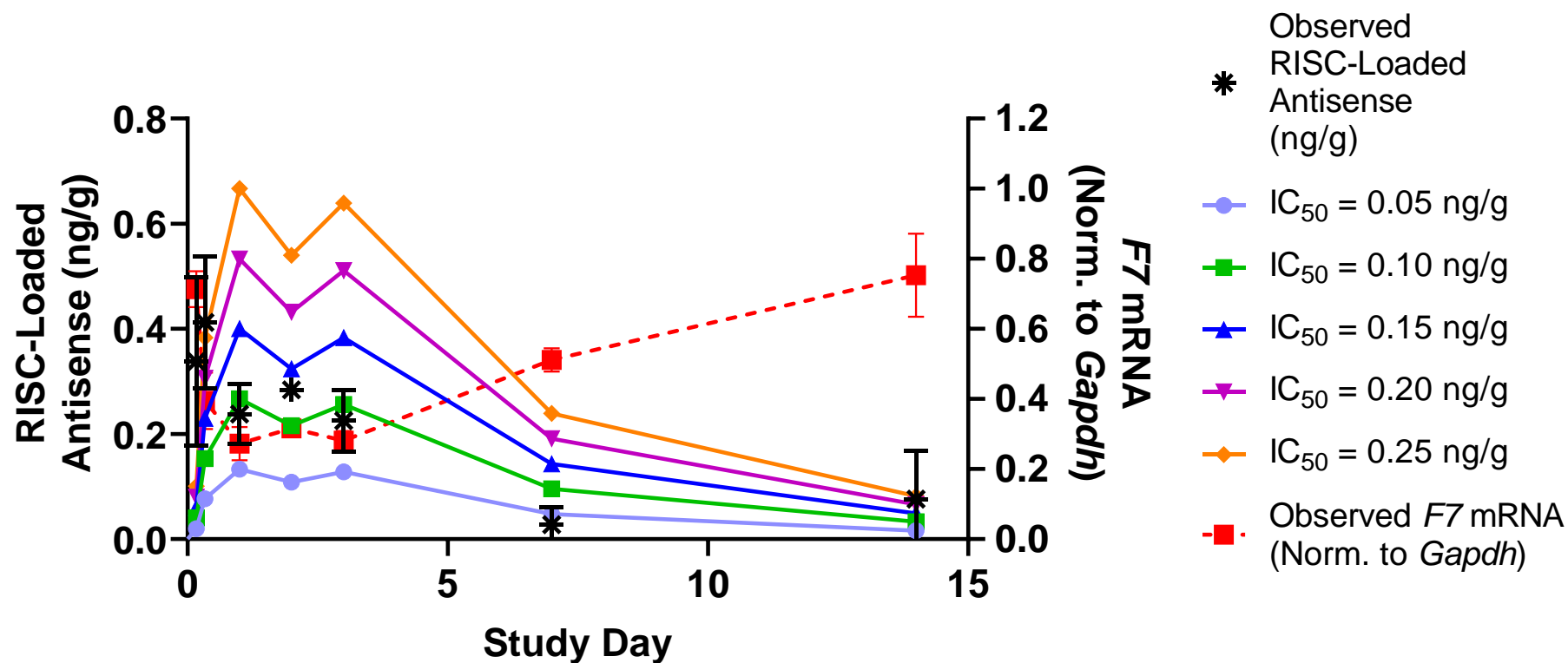
When matched for maximum knockdown, LNP-delivered siRNA does not sustain RISC loading





# The Half-Life of Loaded RISC is Approximately Four Days

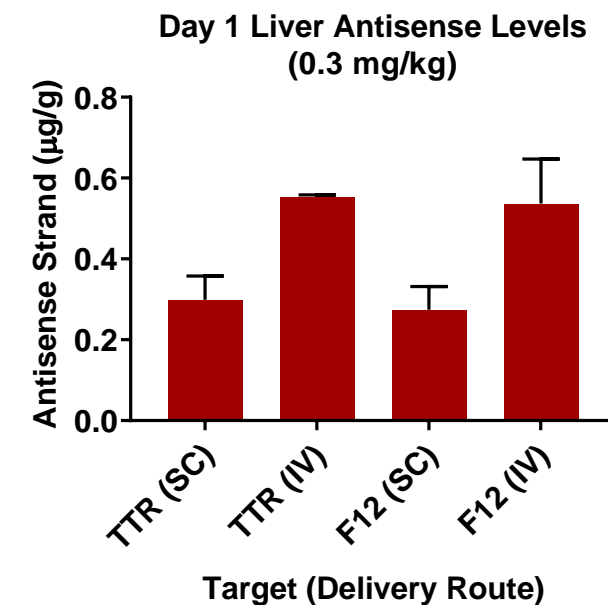
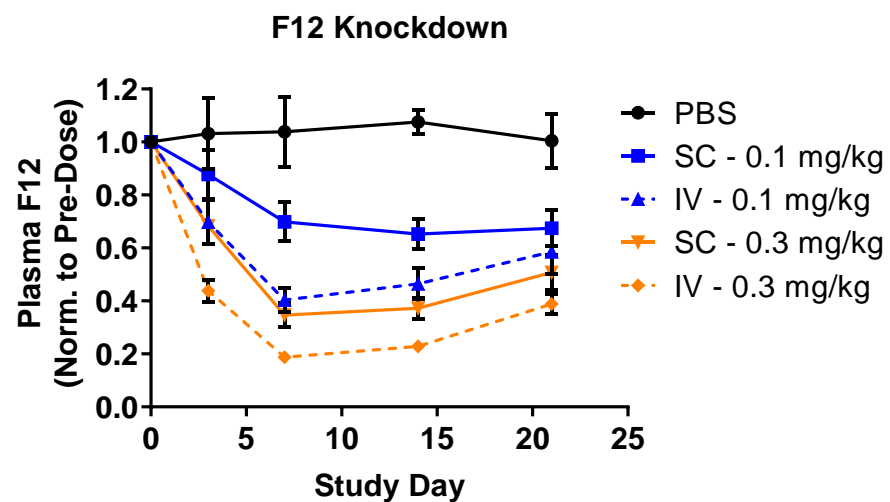
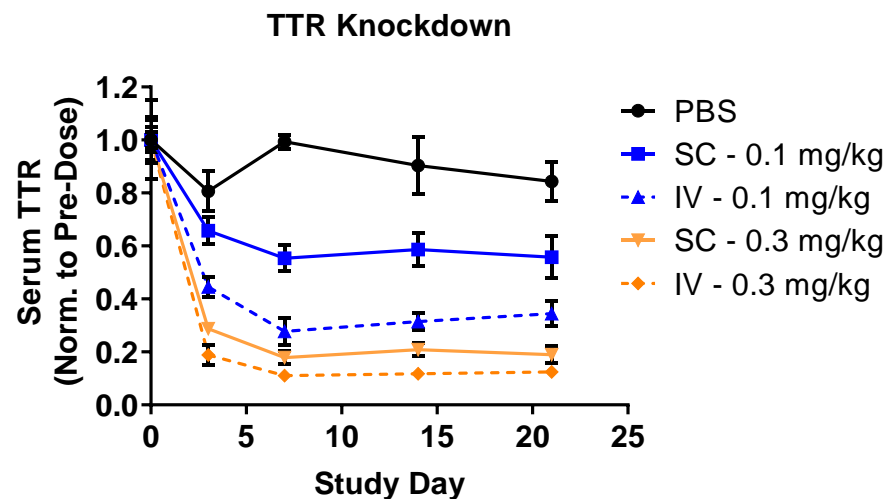
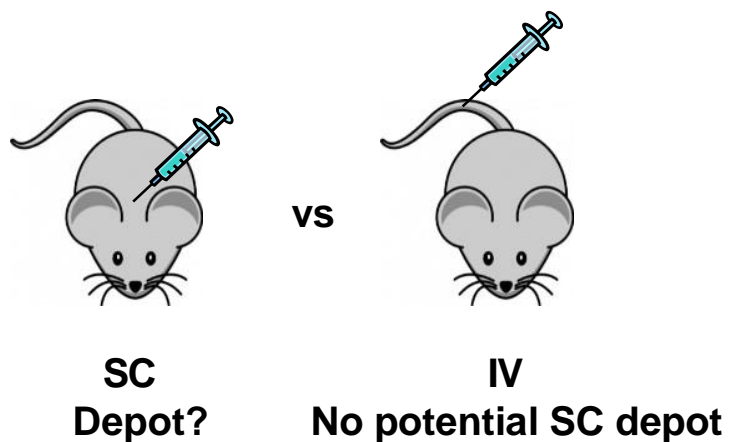
The long duration of action is not explained by the half-life of loaded RISC



- Calculated siRNA loaded RISC half-life is comparable to previous studies (~5-10 days)
- The RISC half-life is the same for ESC and Advanced ESC designs, suggesting these two template designs are equivalent in stability and activity once loaded into RISC

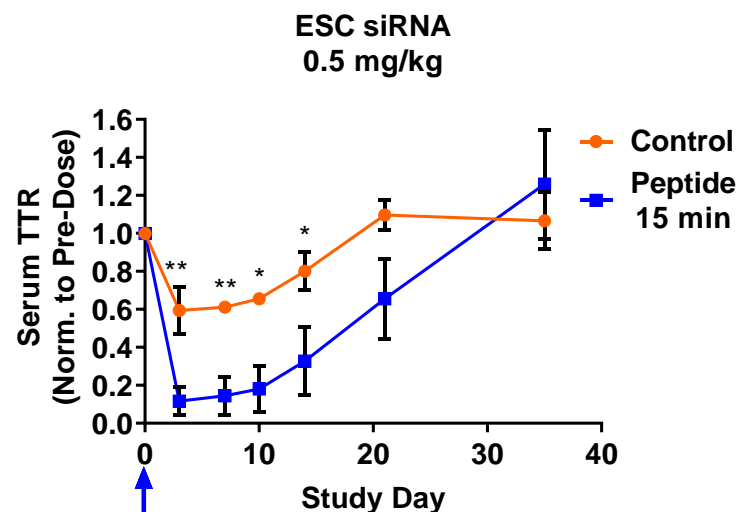
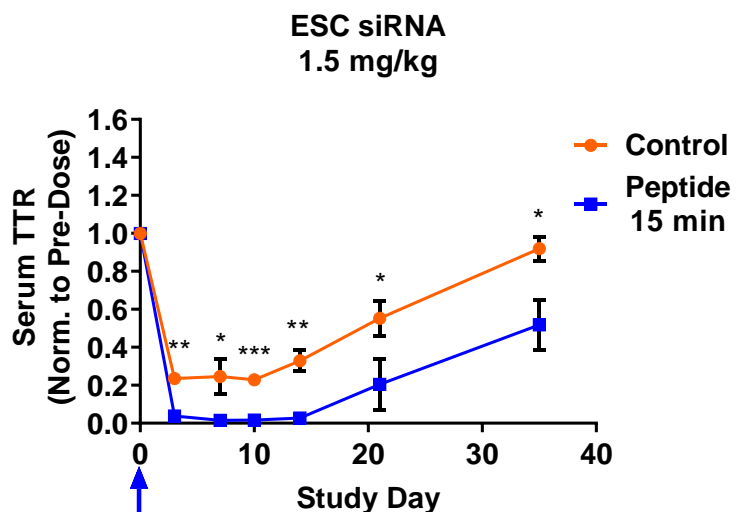
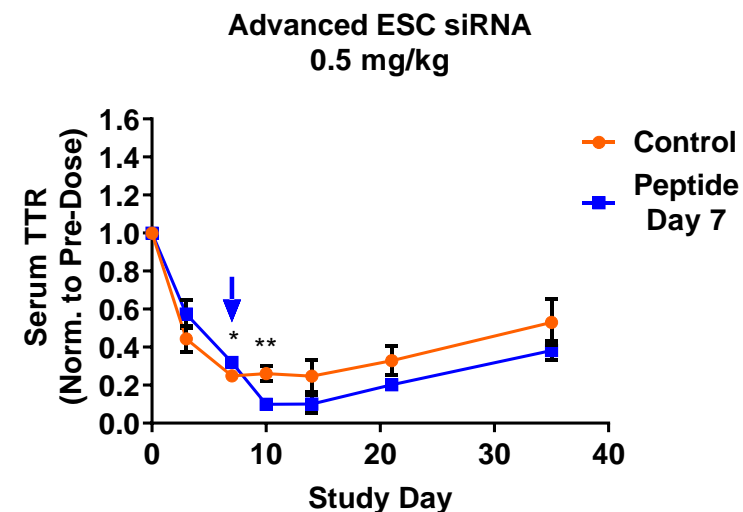
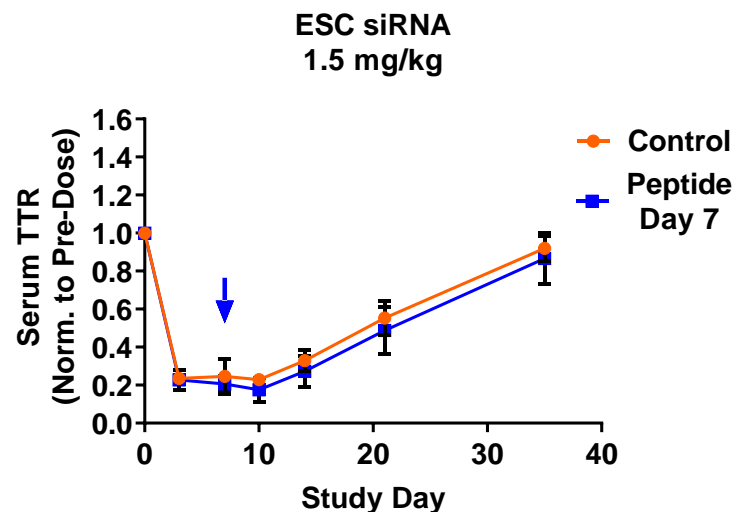
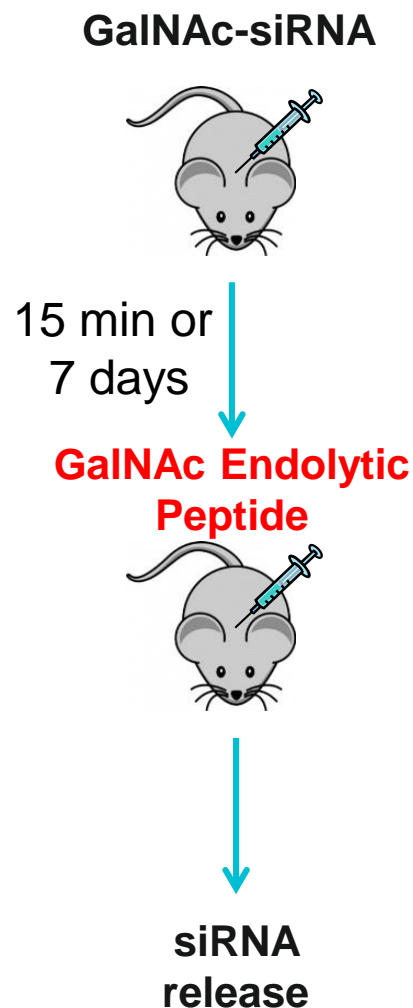
# The Subcutaneous Site of Injection is not a Depot for siRNA

At low doses IV administration of GalNAc-siRNA is superior to SC dosing



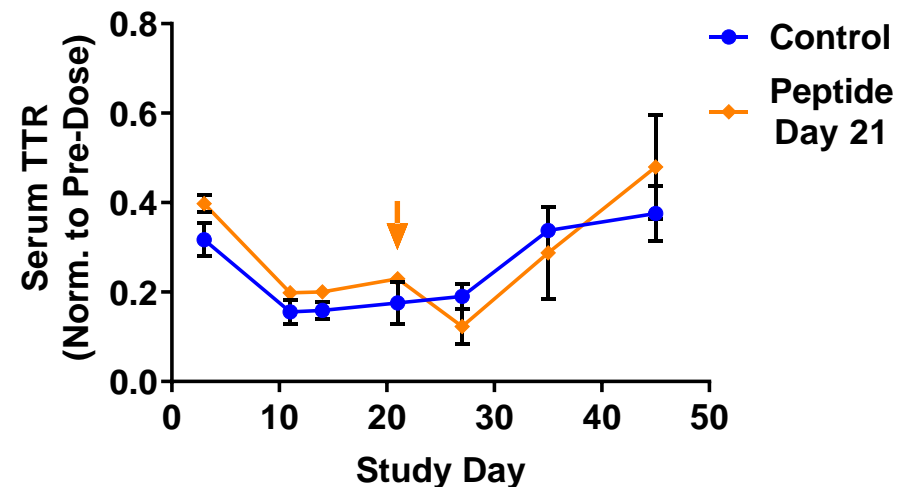
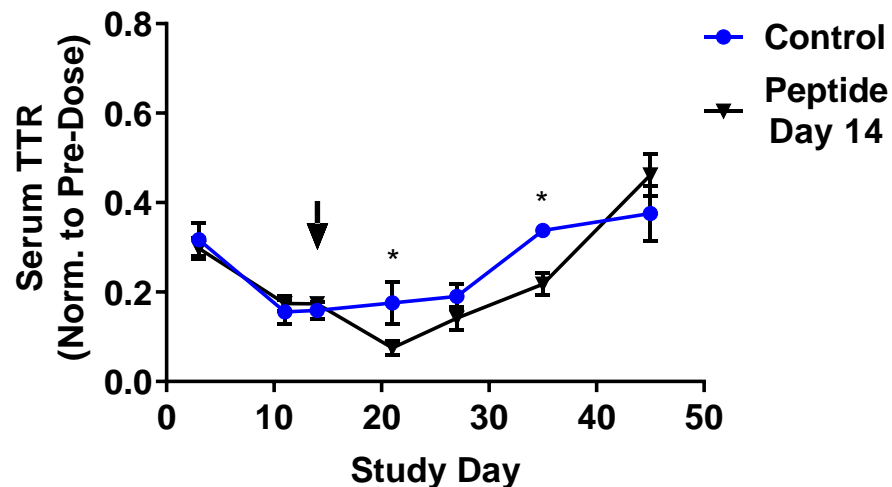
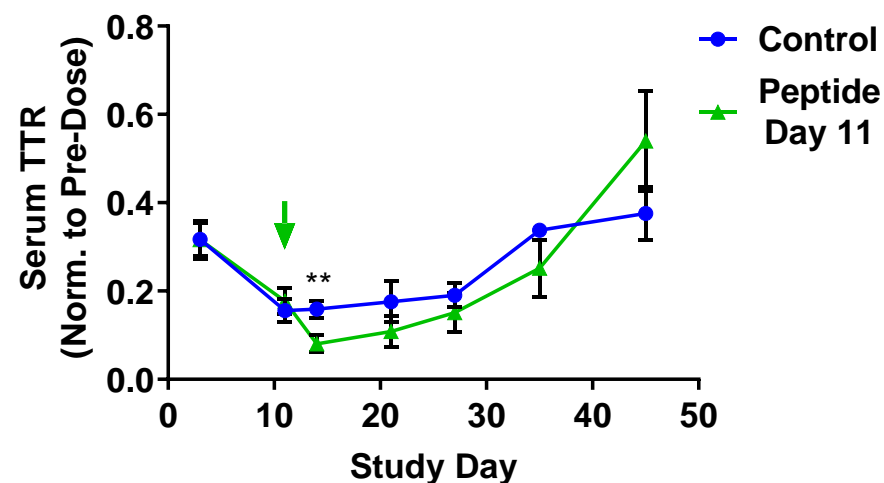
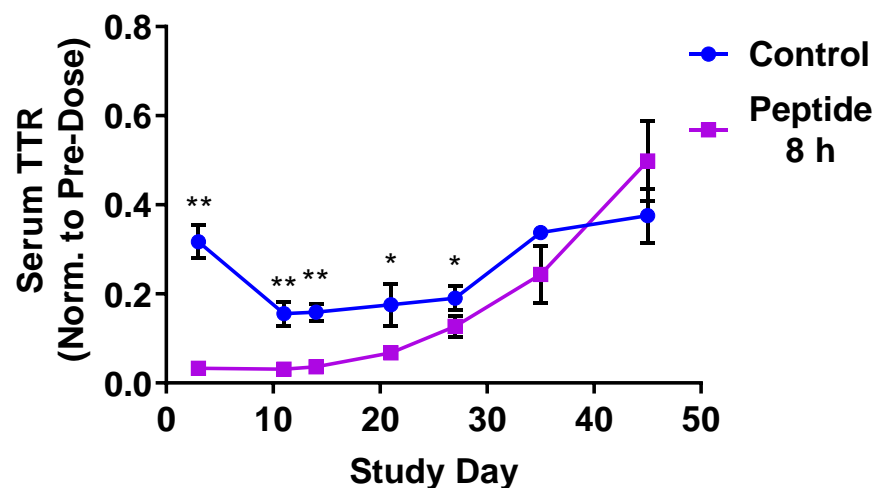
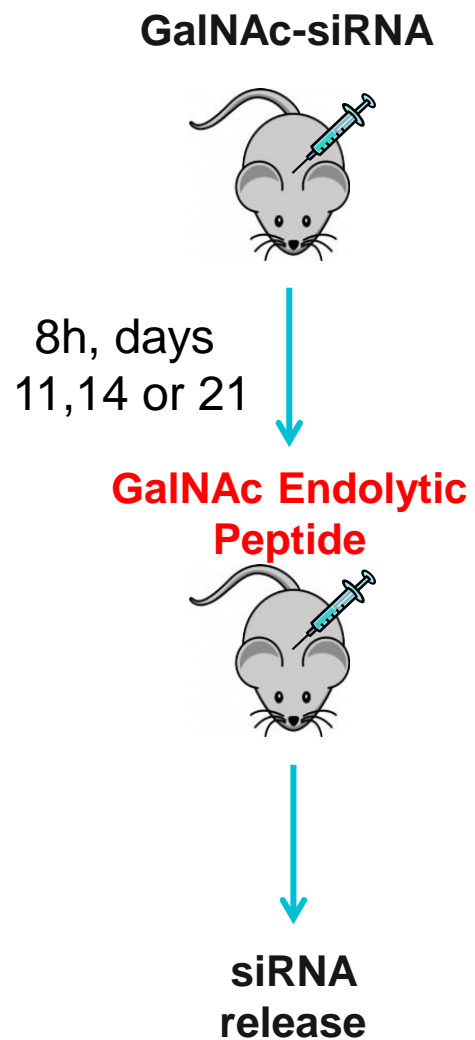
# An Endolytic GalNAc-Peptide Releases Functional siRNA into RISC

More stable Advanced ESC design shows the benefit at later time points



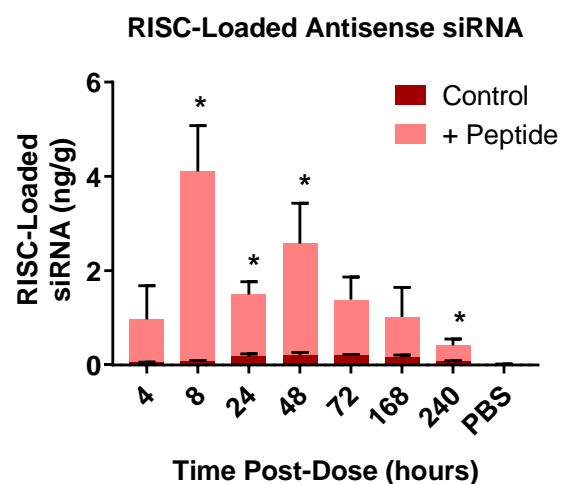
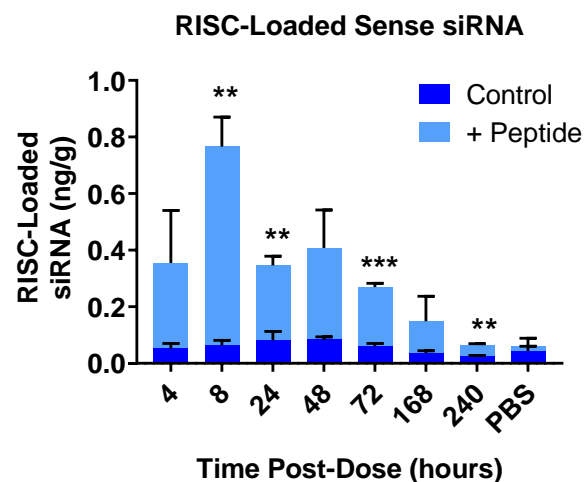
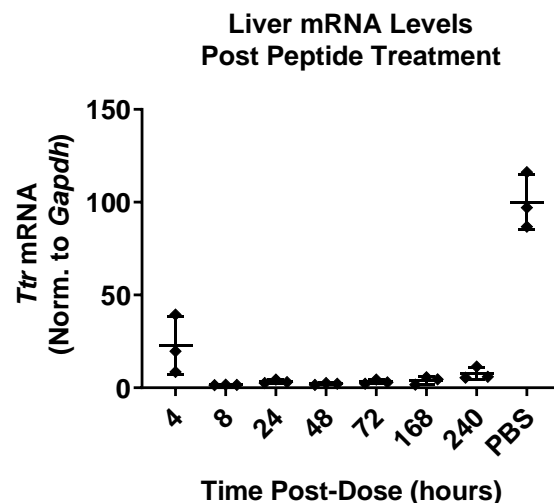
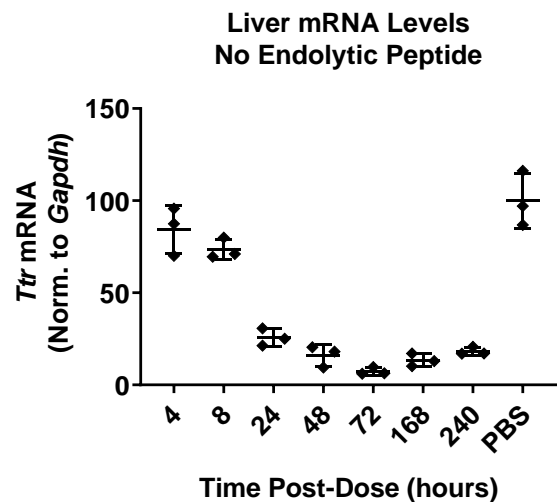
# GalNAc-Peptide Releases Functional siRNA Through Day 21

Chemically-stabilized siRNA survives for weeks in harsh acidic compartments



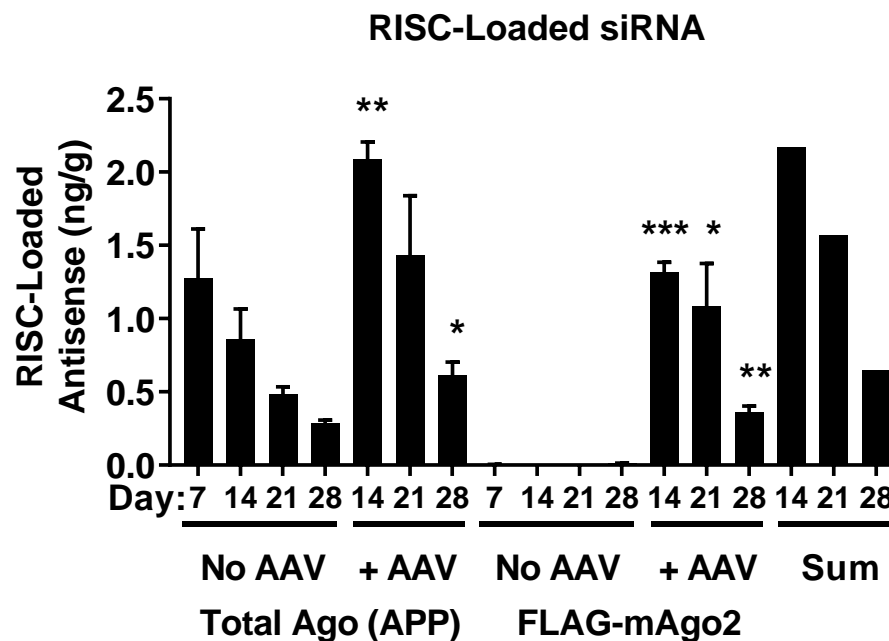
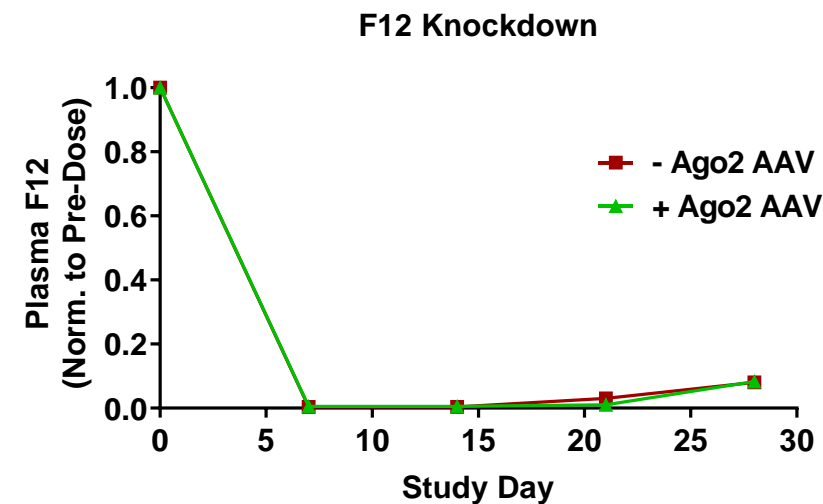
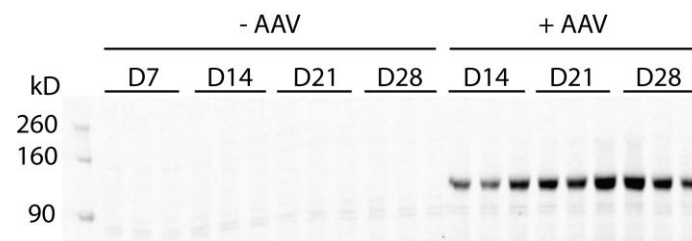
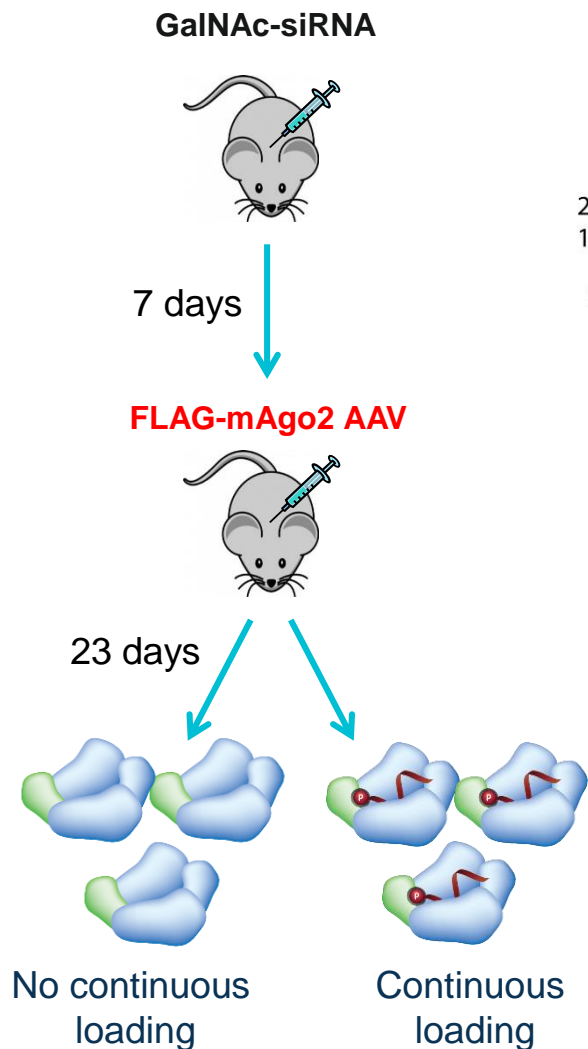
# RISC Loading is Increased Following GalNAc-Peptide Dosing

Functional siRNA is liberated and loaded into RISC after endolysosomal disruption

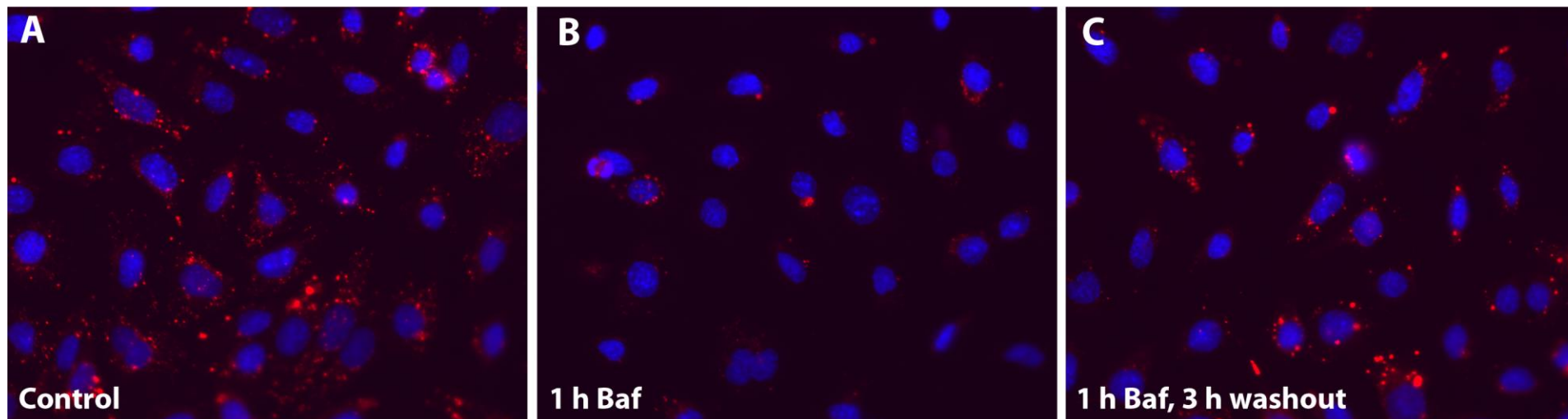
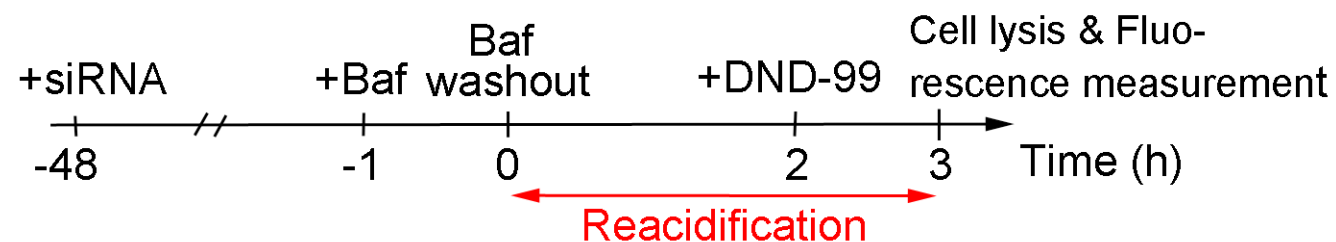


- Endolytic GalNAc-peptide dosed 15 minutes after siRNA
- Significant increases in RISC loading seen after 8 hours following peptide administration
- RISC loading may act as an additional reservoir for siRNA activity, dictated by the half-life of ~4 days

# siRNA is Continuously Loaded into RISC Over Time



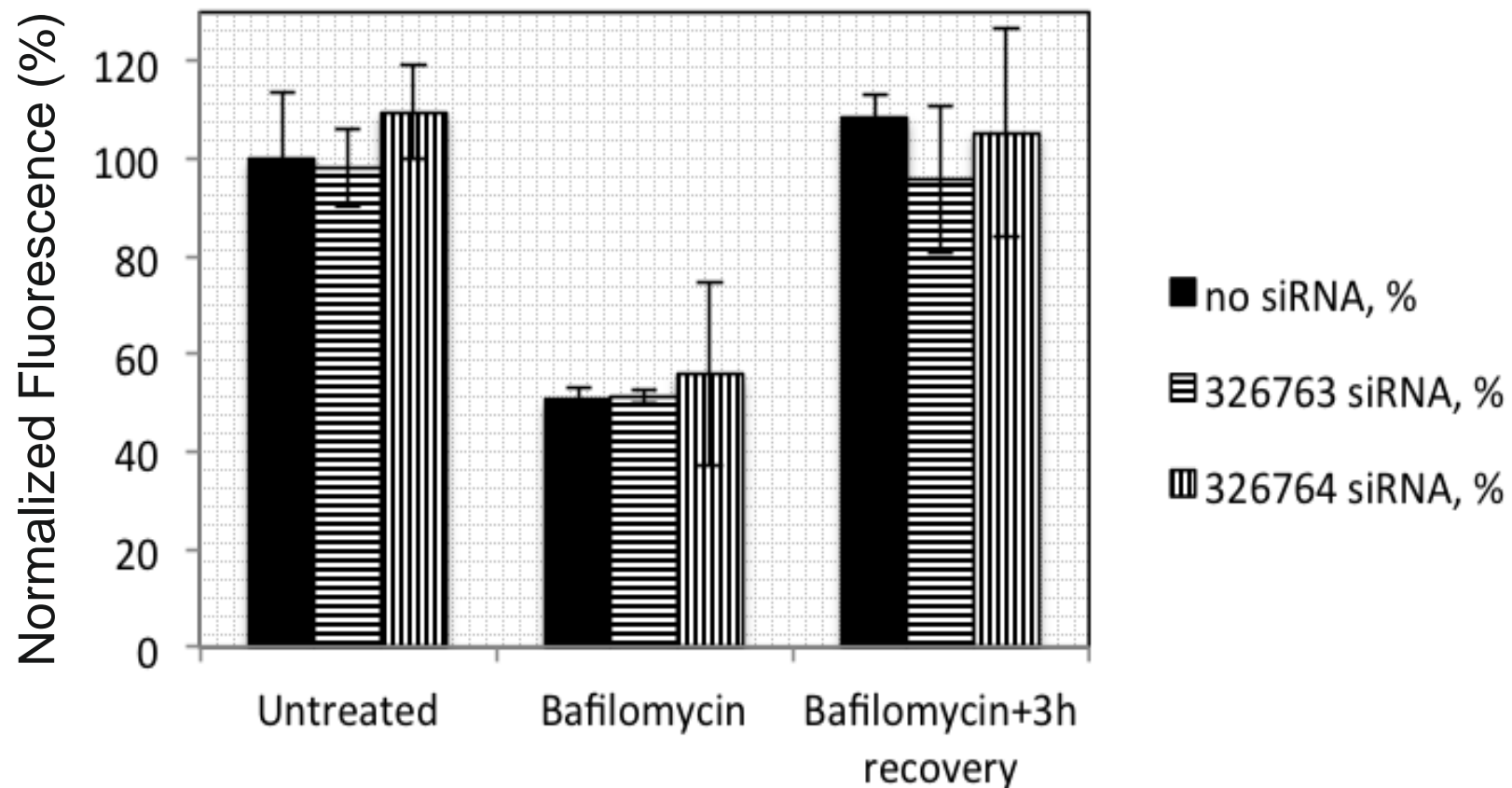
# Highly Specific and Potent Vacuolar-ATPase Inhibitor Bafilomycin Reversibly Blocks Intra-Vesicular Acidification at Low Concentrations (100 nM)



- Acidic vesicles are labeled with lysosomotropic red-fluorescent dye LysoTracker Red DND-99 (mouse cortical collecting duct cell line M-1 is shown)
- This dye is freely permeant across cell membranes at neutral pH, but becomes protonated and trapped in acidic compartments

# siRNAs do not Perturb Reacidification in Primary Rat Hepatocytes

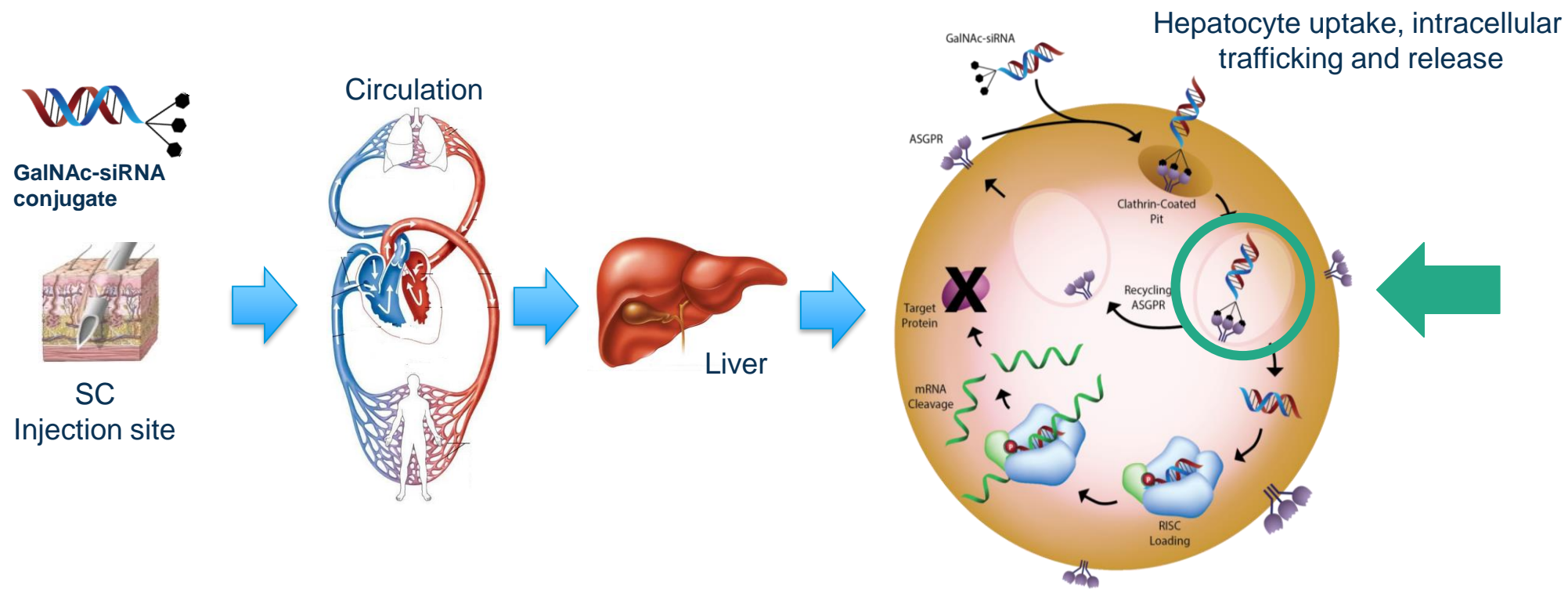
Lysosomal pH recovery following V-ATPase disruption is not affected by the presence of siRNA



- siRNAs were delivered under free uptake conditions at 100 nM for 48 h in primary rat hepatocytes. Bafilomycin was used at 150 nM for 1 h before washout.



# An Intracellular Depot for Conjugate Extended Duration of Effect



- Sustained release of conjugate from SC injection site to liver
- Increased half-life of siRNA-loaded RISC
- Continuous supply of siRNA from an intracellular depot

## Conclusions

- Optimization of siRNA template to enhance metabolic stability increases potency and duration
- GalNAc-siRNA conjugates are rapidly internalized but have a delayed onset of action
- siRNA conjugates accumulate in acidic intracellular compartments in primary rat hepatocytes
  - Lysosome reacidification is not impacted by siRNAs
- The half-life of siRNA-loaded RISC in mice is approximately 4 days
- Sustained RISC loading achieved following GalNAc-siRNA delivery compared with LNP delivery
- Functional siRNA can be released from acidic compartments up to three weeks post-dose
- Ectopically expressed tagged Ago2 continues to load siRNA weeks after dosing

# Acknowledgments



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