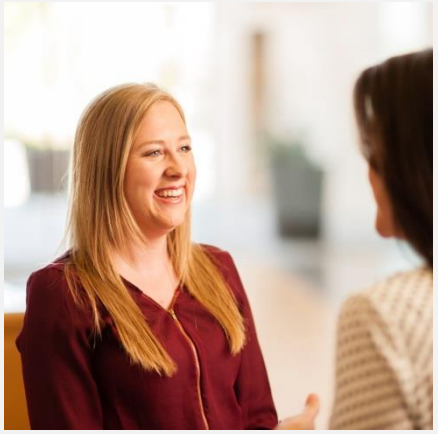


Antisense Technology
Past, Present & Future

Stanley T. Crooke, MD, PhD
Chairman of the Board, Chief Executive Officer

IONIS® PHARMACEUTICALS

Antisense Technology: Acknowledgements

- All Ions Past and Present
- Key Ionis Leaders
- Ionis Collaborators
- Ionis Partners
- Ionis Investors
- Ionis Patients

Antisense Technology

- The notion
  - Create oligonucleotide drugs designed to bind to target RNA via Watson-Crick hybridization
  - Agnostic as to post binding mechanisms

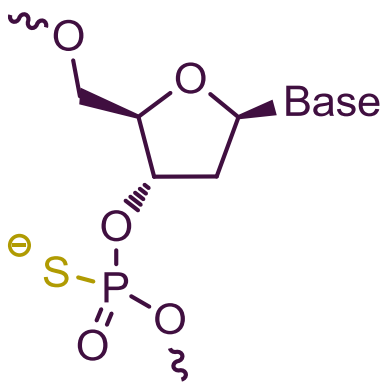
[*] Zamecnik, P.C. and M.L. Stephenson, Proceedings of the National Academy of Science USA, 1978. **75**(1): 280–284.

Ionis Chemistry Evolution

First Generation Antisense Chemistry
through the Conjugation Revolution

Chemistry Evolution – Early Generations

Gen 1



1st Generation Chemistry

Phosphorothioate P=S

- Adds stability; every other day dosing
- Improves distribution to tissues

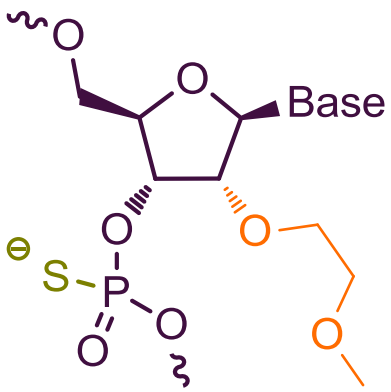
Administration

I.V., enema, intravitreal

First Antisense Drug Approved by FDA

From Beginning to Present Day

Chemistry Evolution – Early Generations



First Systemic Antisense Drug Approved by FDA

Latest Antisense Drug Approved by FDA

SPINRAZA™
(nusinersen) injection
12 mg/5 mL

2nd Generation Chemistry

MOE Gapmer

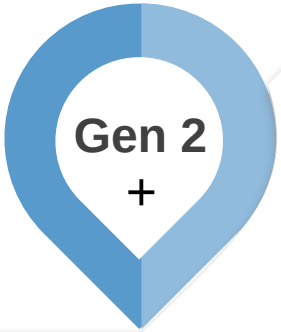
- Increases potency; 200 mg/kg/wk dosing
- Increases stability; weekly dosing
- Reduces pro-inflammatory effects and non-specific toxicities

Administration

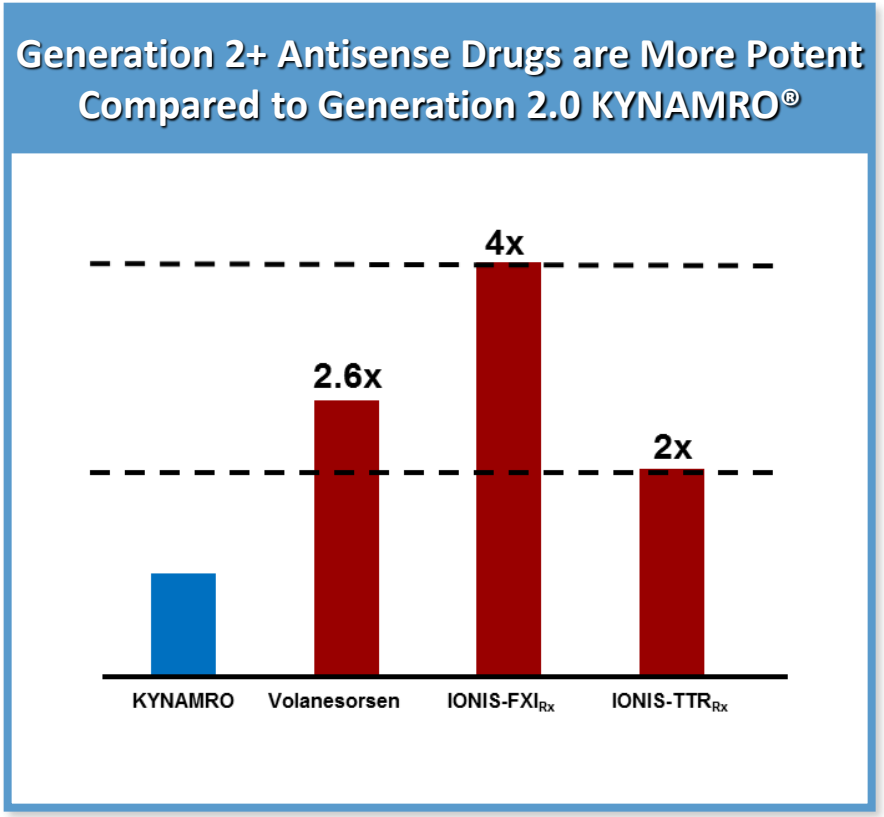
Sub Q, I.V., inhalation, topical, intrathecal

From Beginning to Present Day

Chemistry Evolution – Early Generations



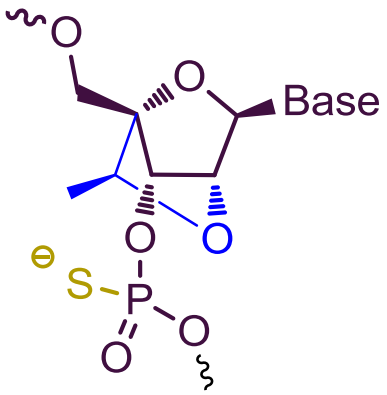
- Optimized screening
- Further improved potency and reduced side-effects of Gen 2 class
- Better tolerated



*Potency derived from ED_{50} after 4 weeks of treatment; compared to KYNAMRO® Phase 1 studies

From Beginning to Present Day

Chemistry Evolution – Robust Generation



Gen 2.5

Generation 2.5 Chemistry

cEt Containing Gapmer

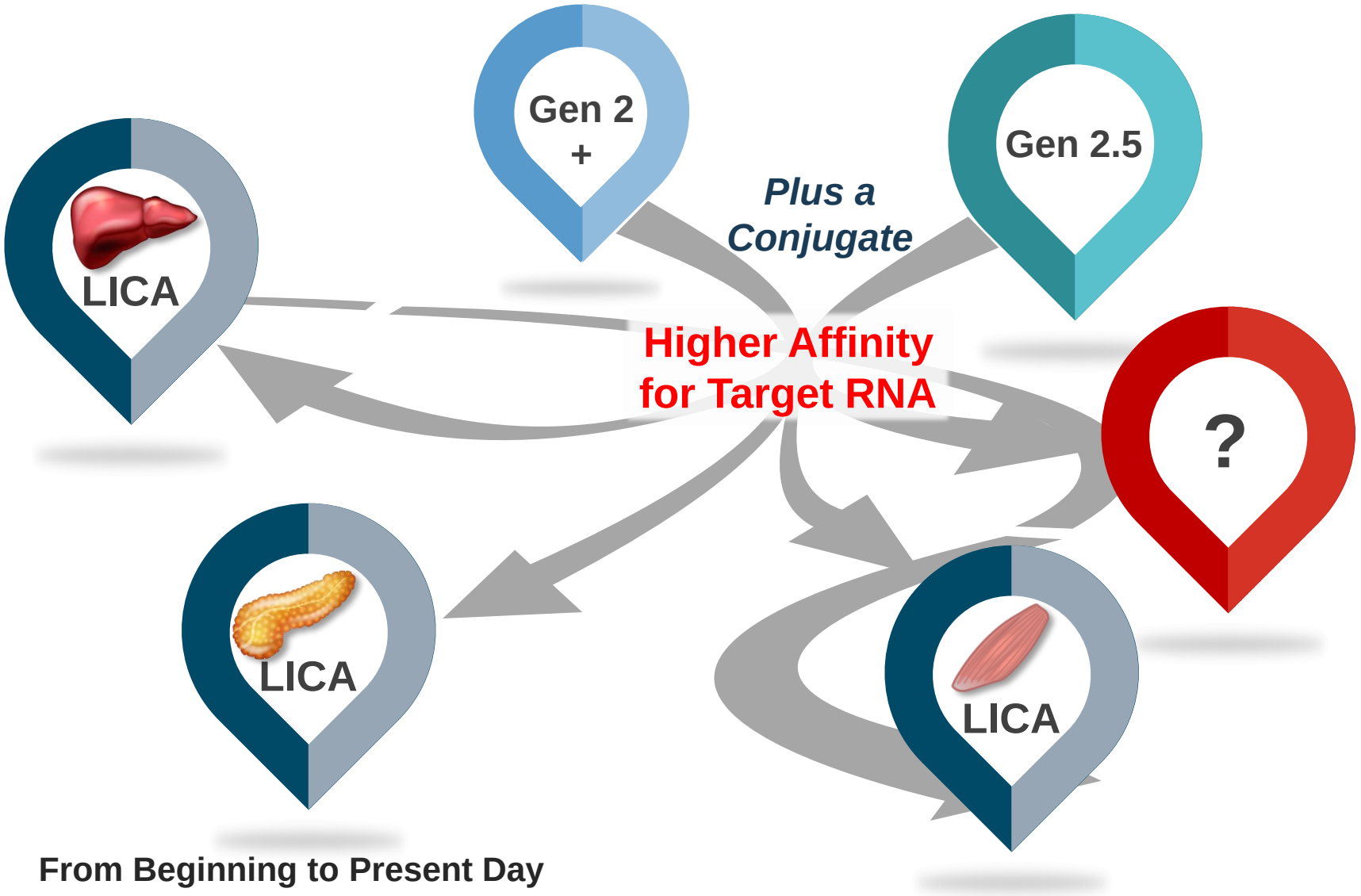
- 10 fold increase in potency via higher affinity for target RNA
- Expands range of targets and tissues

Administration

As previous + the potential for oral

From Beg... to Present Day

Chemistry Evolution – Power of Conjugations



Chemistry Evolution – Power of Conjugations

Gen 2

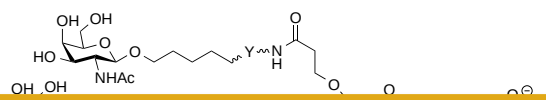
Gen 2.5

LICA targeting Liver

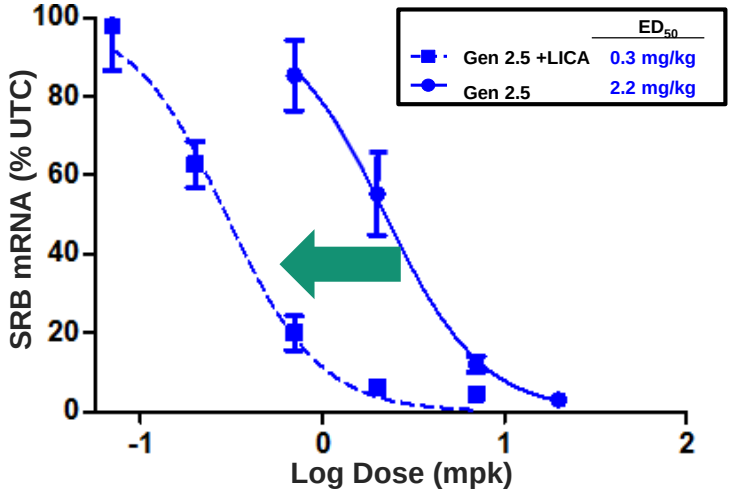
Gen 2+ and Gen 2.5

- Increases distribution to hepatocytes
- Improvements in potency and therapeutic index
- Improved patient convenience with smaller volumes
- Less frequent dosing regiments

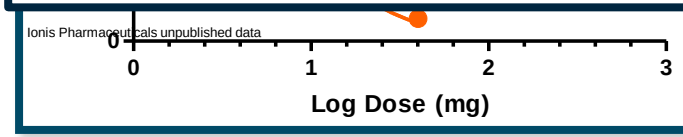
N-Acetylgalactosamine (GalNAc) Conjugate



Comparison of Dose-response Curves in Preclinical Studies



Generation 2.5 + LICA has the potential to broaden addressable patient populations



Ionis Pharmaceuticals unpublished data

From Beginning to Pres

Chemistry Evolution – Power of Conjugations

Gen 2

Gen 2.5

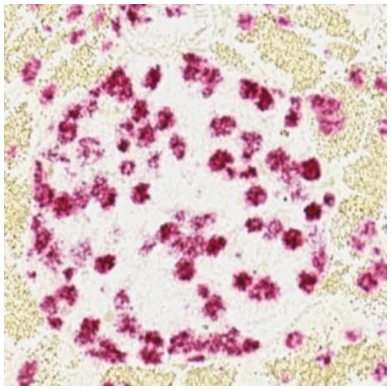
LICA

LICA

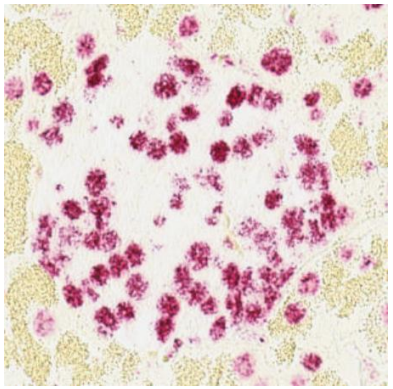
LICA targeting Pancreas (Glip1)

Gen 2+ and Gen 2.5

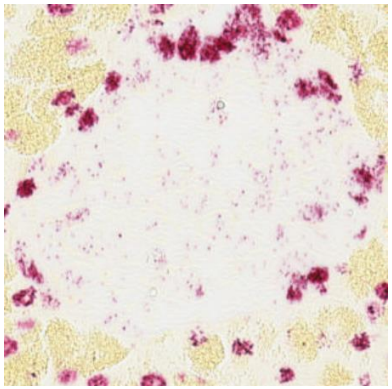
- Improves uptake in pancreas beta cells



Saline



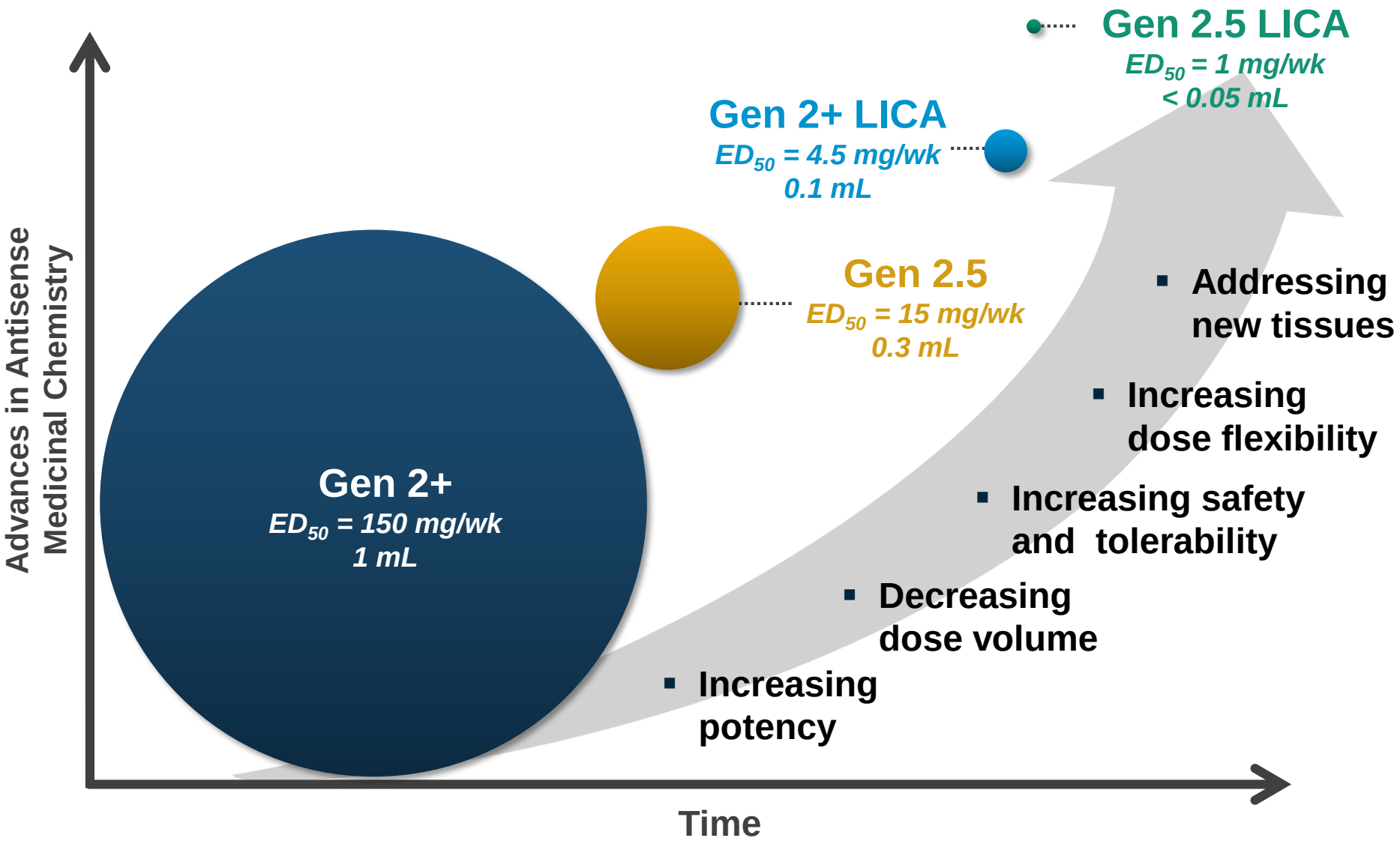
Gen 2.5 ASO



Gen 2.5 + LICA ASO

From Beginning to Present Day

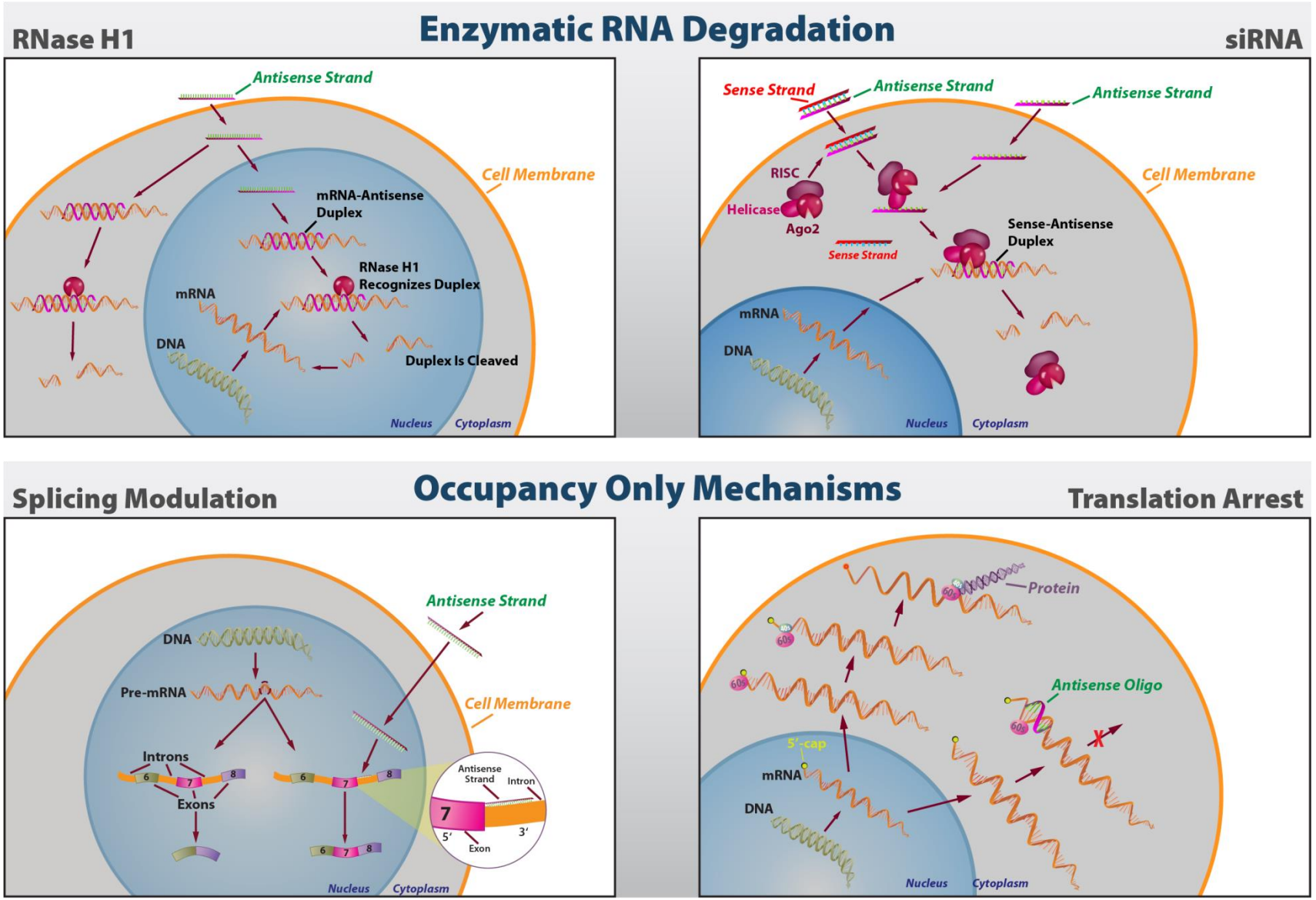
Advances in Our Chemistry Substantially Improve the Utility of Antisense Drugs



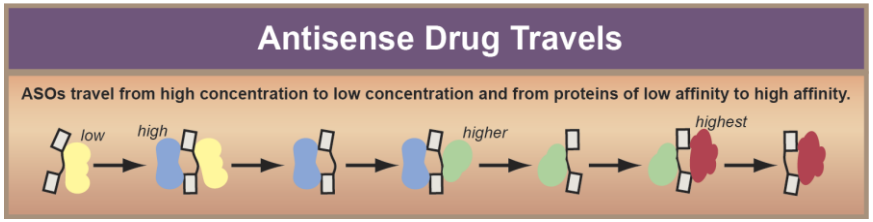
ED_{50}'s and dose volumes are representative of liver targets

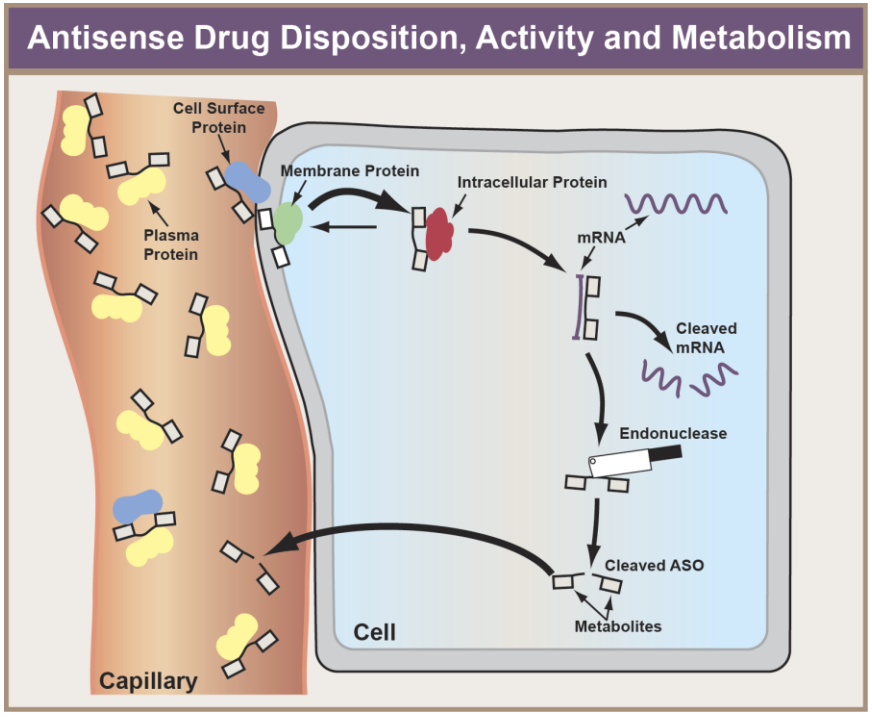
The Evolution of Antisense Mechanisms of Action

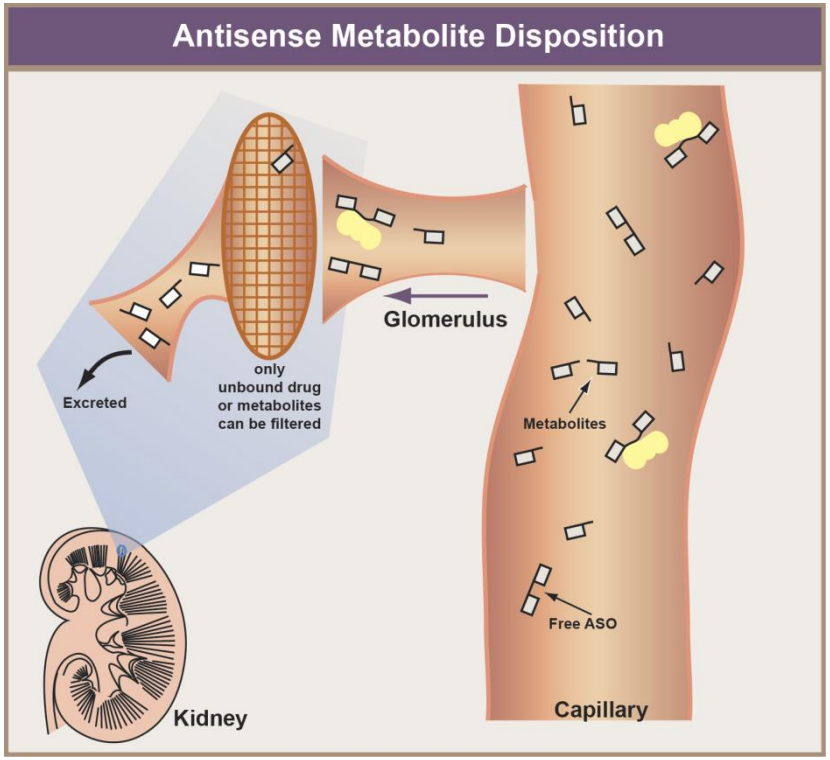
Antisense Mechanisms [Simplified]



Cartoon of ASO distribution, metabolism and excretion







Levin, A.A., Yu, R.Z., Geary, R.S. Basic Principles of the Pharmacokinetics of Antisense Oligonucleotide Drugs. In Antisense Drug Technology: Principles, Strategies, and Applications, Second Edition, Crooke, ST. (ed), Baco Raton, FL, *CRC Press*, Chapter7,183-215, 2008.

Toxicological Properties (2’ MOE chimeras)

- Class generic effects characterized and understood
- Mechanisms of most adverse events understood
- Pro inflammatory induced tolerability issues largely resolved
- Interactions of specific ASOs with specific disease populations can not be extrapolated
  - Prudent clinical trials in disease populations still required

Large Safety Database Provides Evidence of Good Safety Profile of 2nd Generation Antisense Drugs

- Large clinical safety database (Ionis safety experience)
  - >6,000 patients treated with Ionis 2nd Generation antisense drugs (iv/sc)
    - Doses: 0.6-15 mg/kg
  - >3,000 patients in the integrated safety database
- Integrated safety database:
  - No platform generic liver or renal toxicities identified
  - No platform generic platelet toxicities identified
  - No platform safety issues identified in clinical studies for the following systems:
    - Cardiac, CNS, muscle, hematology, liver, kidney
  - No clinically significant drug-drug interactions observed
    - Lack of P450 metabolism interactions
    - Lack of major transporter interactions

Crooke, S.T., et al, Mol Ther 24(10):1771-1782, 2016; Crooke, S.T., et al, NAT, FastTrack Article, February 1, 2017.

Antisense Today

A Landmark Advance in the Treatment of Spinal Muscular Atrophy

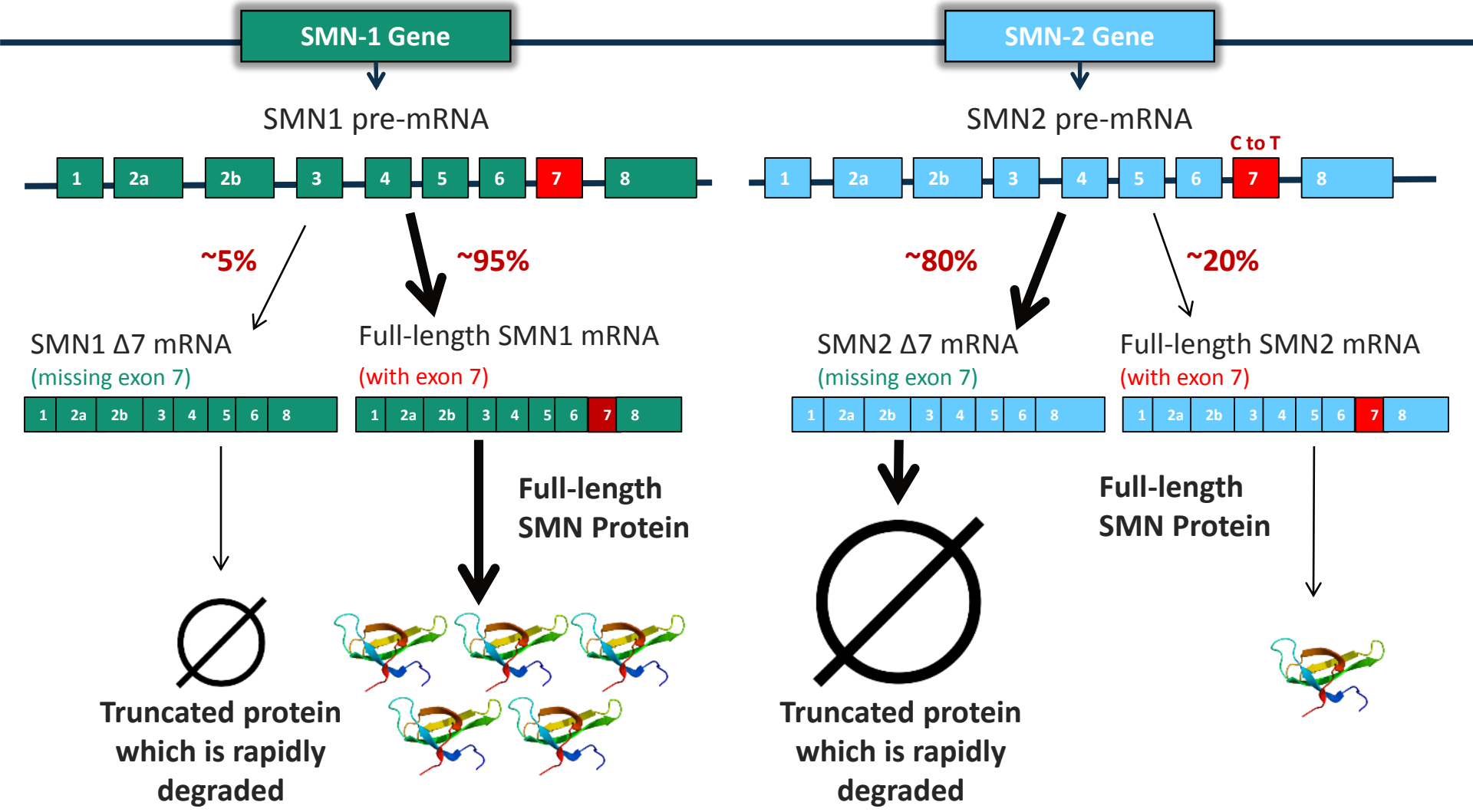
SPINRAZA™ (nusinersen) injection 12 mg/5 mL

Approved in U.S. to treat pediatric and adult patients with spinal muscular atrophy

For important prescribing and safety information, please refer to: www.spinraza.com

Normal Humans Have Both SMN1 and SMN2 Genes Due to Gene Duplication

Both Genes Undergo Alternative Splicing of Exon 7



SPIRNAZA Demonstrated Benefit Across a Broad Range of Patients with SMA in Multiple Clinical Studies

Pre-symptomatic Infants with SMA[1]	- All infants in study were alive and event free - Most infants demonstrated improvements in muscle function scores - Most infants achieved motor milestones at the appropriate age
Infantile-onset SMA	- Increased event-free survival vs. natural history - Most infants demonstrated improvements in motor function scores - Most infants achieved new motor milestones - Improvements in scores continue with longer treatment
Later-onset SMA	- Many children demonstrated improvements in motor function scores - Some children gained or regained the ability to walk - Improvements in scores continue with longer treatment

Bertini, E. (2016, October 8). Nusinersen in Pre-symptomatic Infants With Spinal Muscular Atrophy (SMA): Interim Efficacy and Safety Results From the Phase 2 NURTURE Study, Granada, Spain.

Cameron’s Progress

Continuous Growth Driven by Our Advanced Pipeline of Innovative Drugs

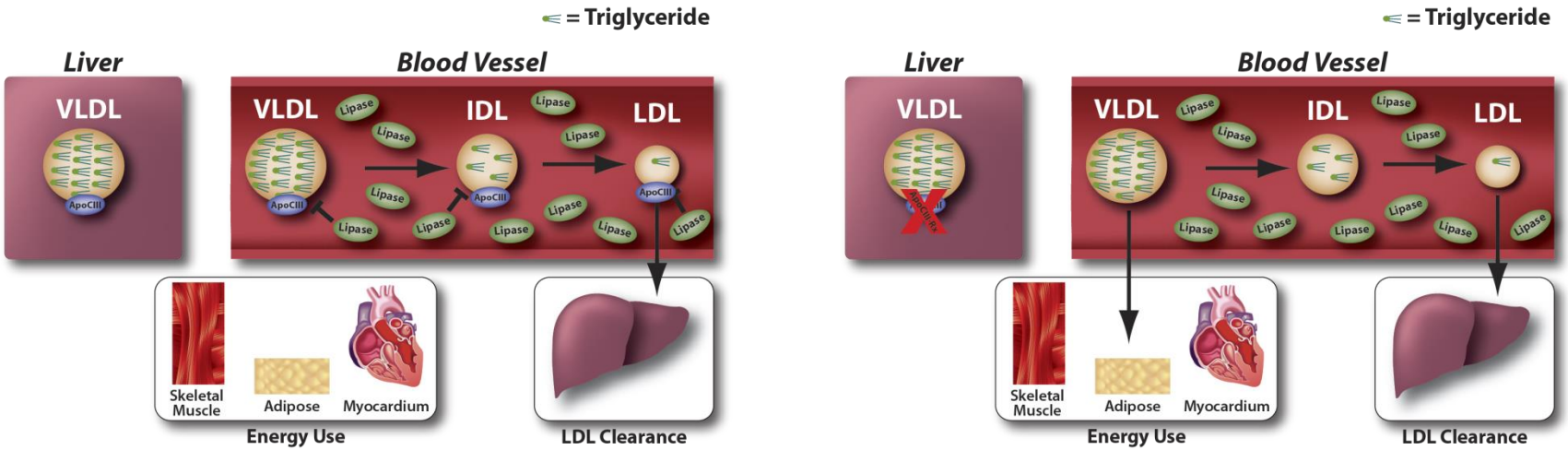
Volanesorsen

For Familial Chylomicronemia Syndrome (FCS) and Familial Partial Lipodystrophy (FPL)

AKCEA™ THERAPEUTICS

A subsidiary of Ionis Pharmaceuticals, Inc.

ApoC-III Inhibits Clearance of Triglycerides from Blood



- ApoC-III is a glycoprotein synthesized principally in the liver that plays a key role in determining serum triglyceride levels
  - Inhibits clearance of triglycerides from the blood
  - Inhibits lipoprotein lipase (LPL) and hepatic lipase (HL) – two enzymes in blood that promote TG clearance
  - Reduces liver clearance of lipoproteins
  - Promotes VLDL formation and secretion from the liver

Phase 3 Program: Volanesorsen Demonstrated Consistent, Robust TG-lowering

Volanesorsen-treated	COMPASS All Patients (n = 75)	COMPASS FCS Subset (n = 5)	APPROACH (n = 33)
Mean % Change in TGs from Baseline	-71% (*p < 0.0001*)	-73%	-77% (*p < 0.0001*)
Mean Absolute Change in TGs from Baseline	-869 mg/dL	-1511 mg/dL	-1712 mg/dL
Achieved TGs <500 mg/dL (%)	59 (82%)*	3 (60%)	15 (50.0%)**

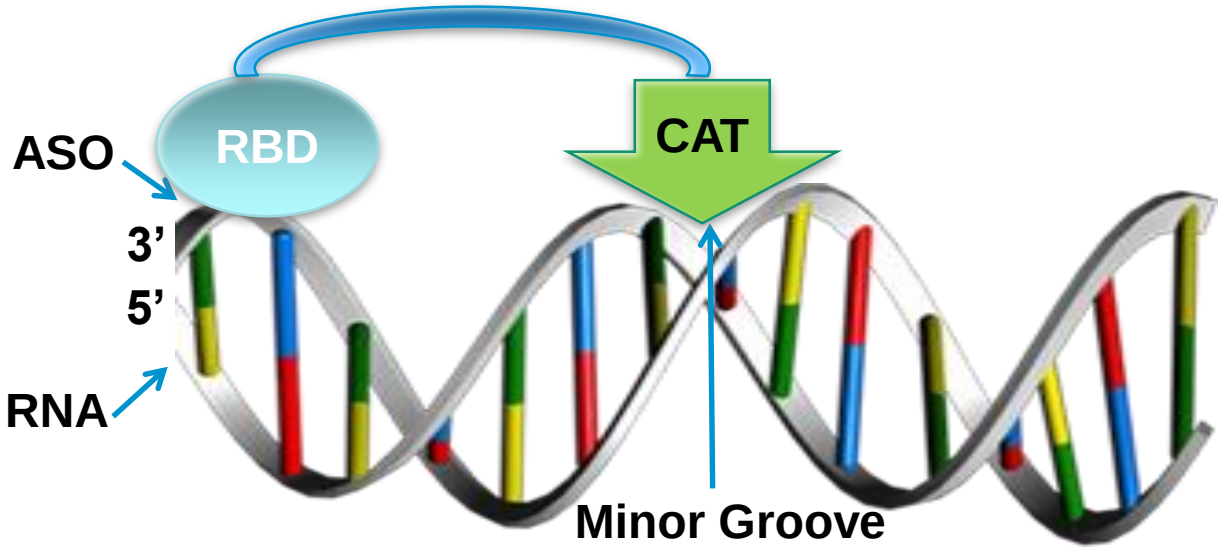
*n = 72; Data includes all patients with triglycerides ≥500 mg/dL at baseline

**n = 30; Data includes all patients with triglycerides ≥750 mg/dL at baseline

Crooke Group

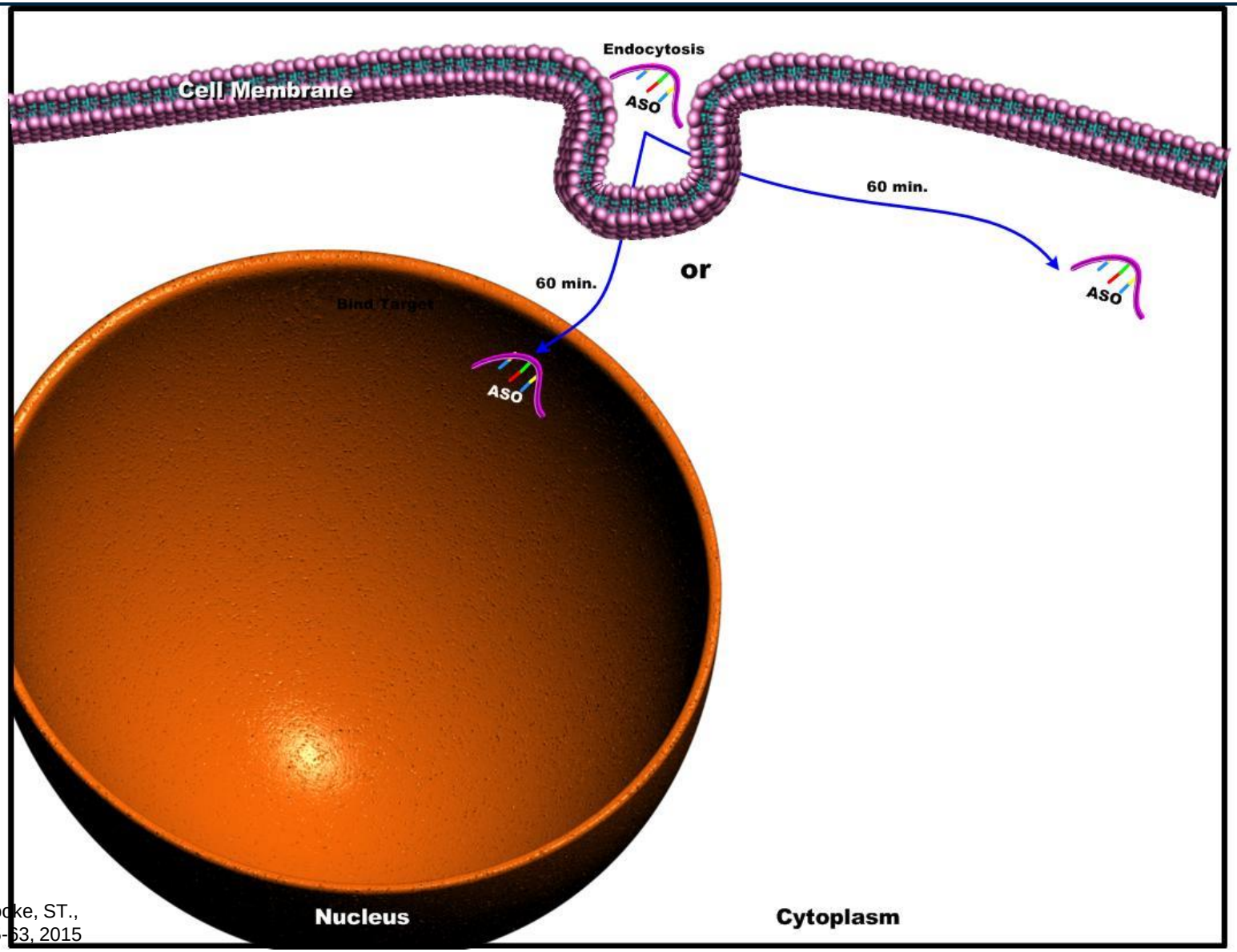
- Xia-hua Liang
- Wen Shen
- Tim Vickers
- Shiyu Wang
- Jeff Bailey
- Josh Nichols
- Hong Sun
- Cheryl Li De Hoyos

Model of RNase H1 Interacting with ASO/RNA Heteroduplex



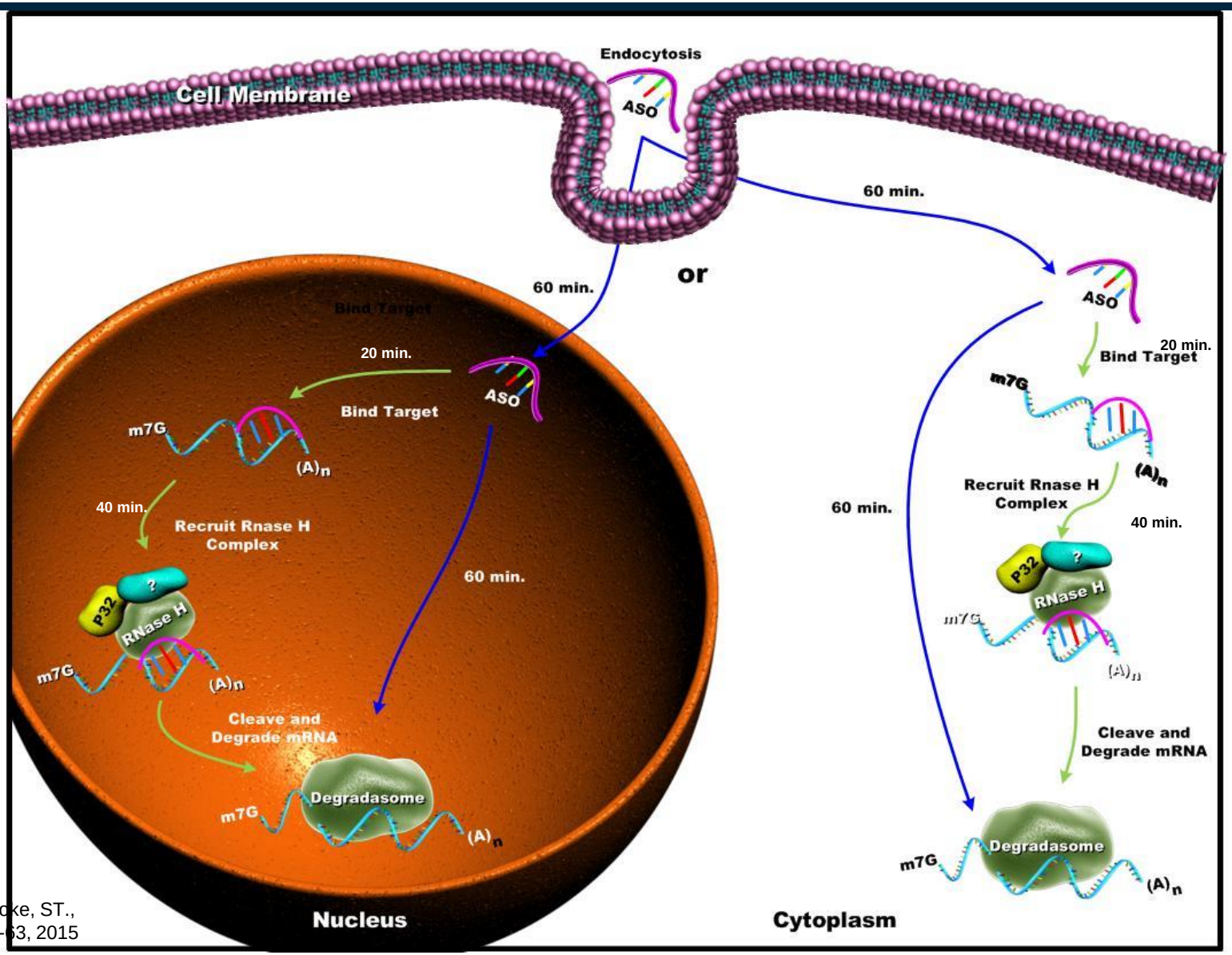
Lima, WF, et al, NAR 44(11):5299-312, 2016; Wu, H, et al, PLos One 8(8):e71006, 2013; Lima, WF, et al, Mol Pharmacol 71:83-91, 2007; Lima, WF, et al, Mol Pharmacol 71:73-82, 2007; Lima, WF, et al, Antisense Drug Technology: Principles, Strategies, and Applications, 2nd Edition, 47-74. CRC Press 2007; Lima, WF, et al, J Biol Chem 279:36317-36326, 2004; Wu, H, et al., J Biol Chem 279:17181-9, 2004; Lima, WF, et al, J Biol Chem 278:49860-49867, 2003; Lima, WF, et al, J Biol Chem 278:14906-14912, 2003; Wu, H., et al, J Biol Chem 276:23547-23553, 2001; Miraglia, L., et al, Antisense and Nuc Acid Drug Dev 10:453-461, 2000; Wu, H, et al, J Biol Chem 274:28270-28278, 1999; Wu, H, et al, Antisense and Nuc Acid Drug Dev 8:53-61, 1998; Lima, WF, et al, J Biol Chem 272:18191-18199, 1997; Lima, WF, et al, Biochemistry 36:390-398, 1997.

ASO Transit and RNase H1 Recruitment (Estimates)



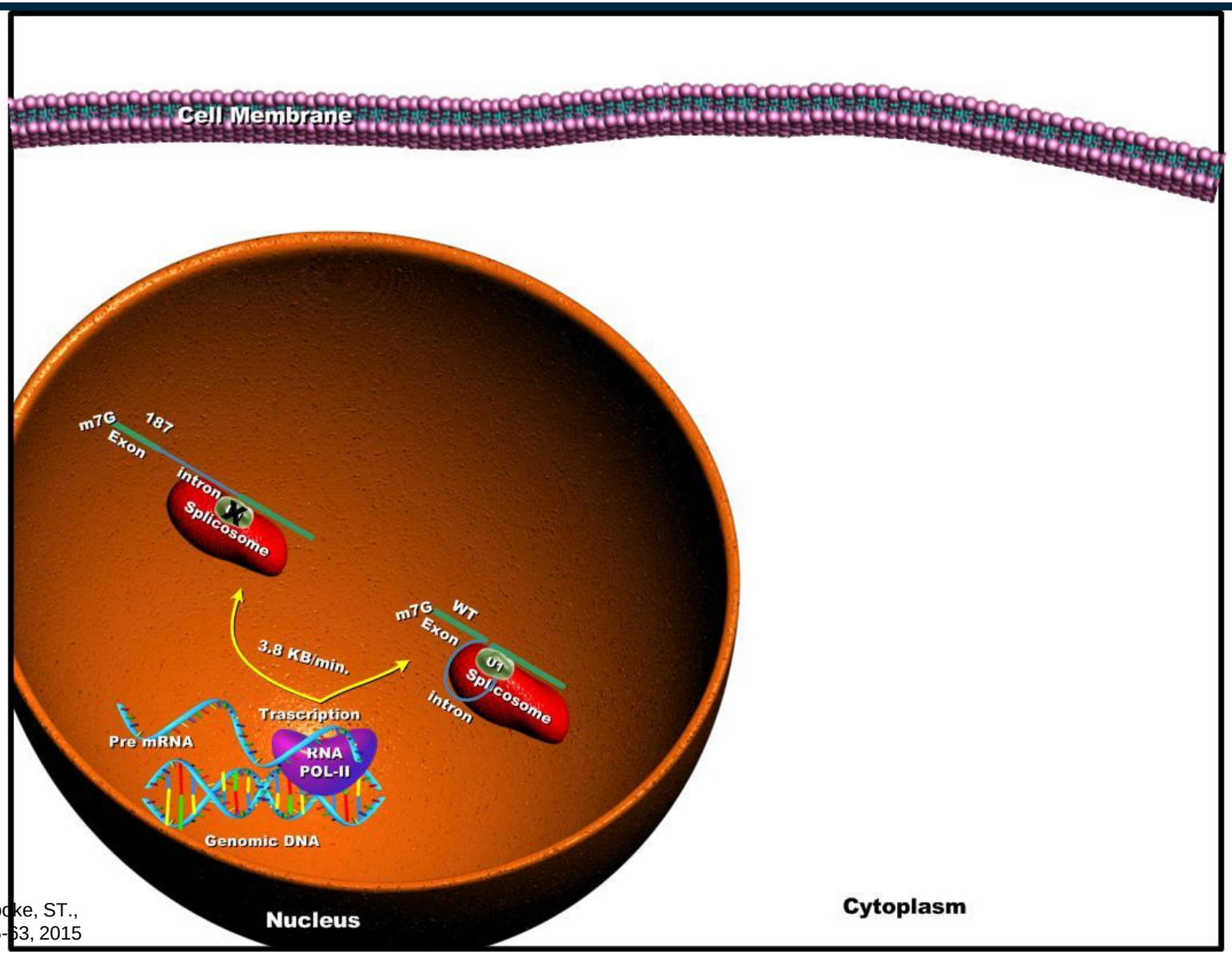
Vickers, T., & Crooke, ST.,
NAR 43(18):8955-63, 2015

ASO Transit and RNase H1 Recruitment (Estimates)



Vickers, T., & Crooke, ST.,
NAR 43(18):8955-63, 2015

RNA Transcription and Processing Rates



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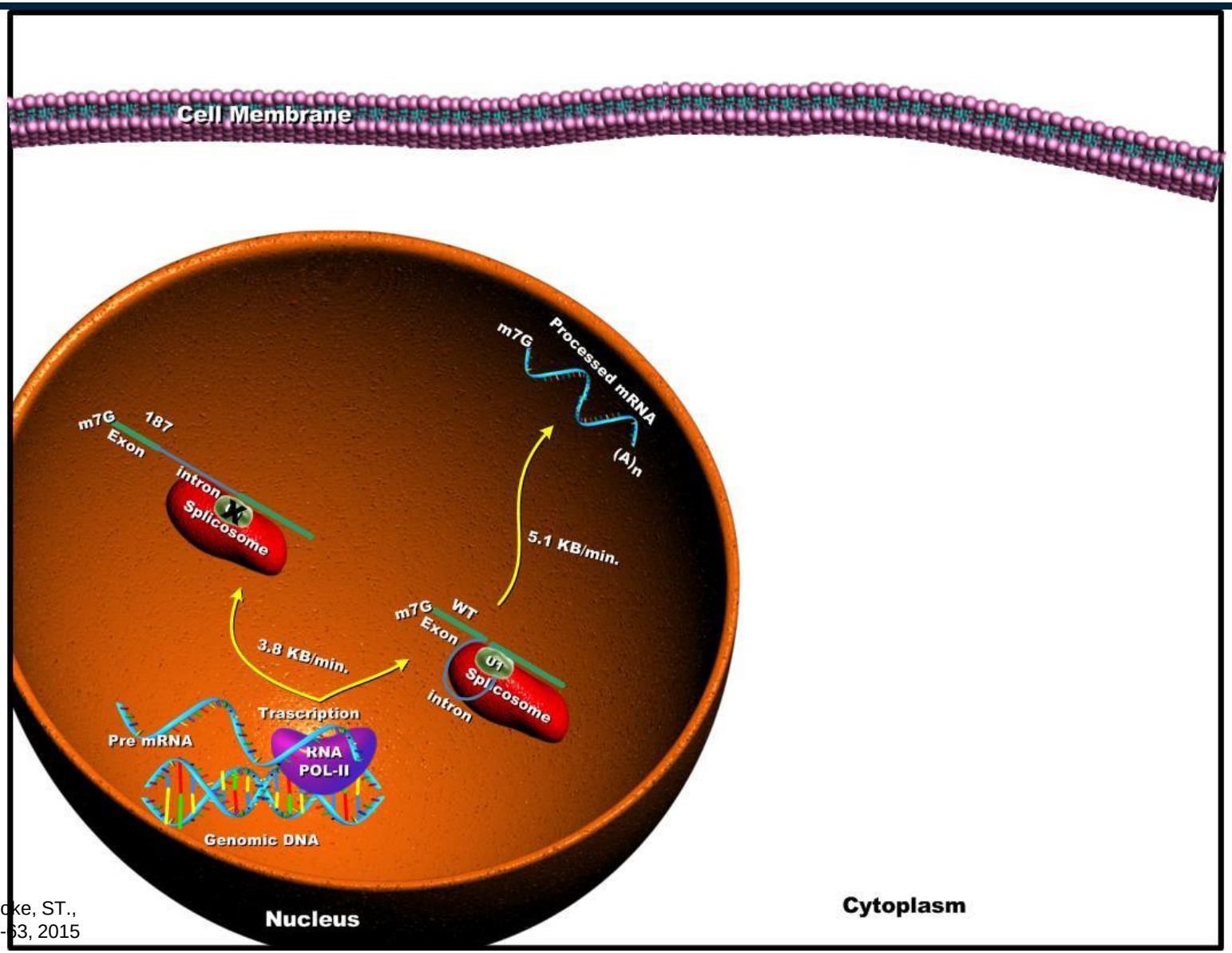
RNA Transcription and Processing Rates



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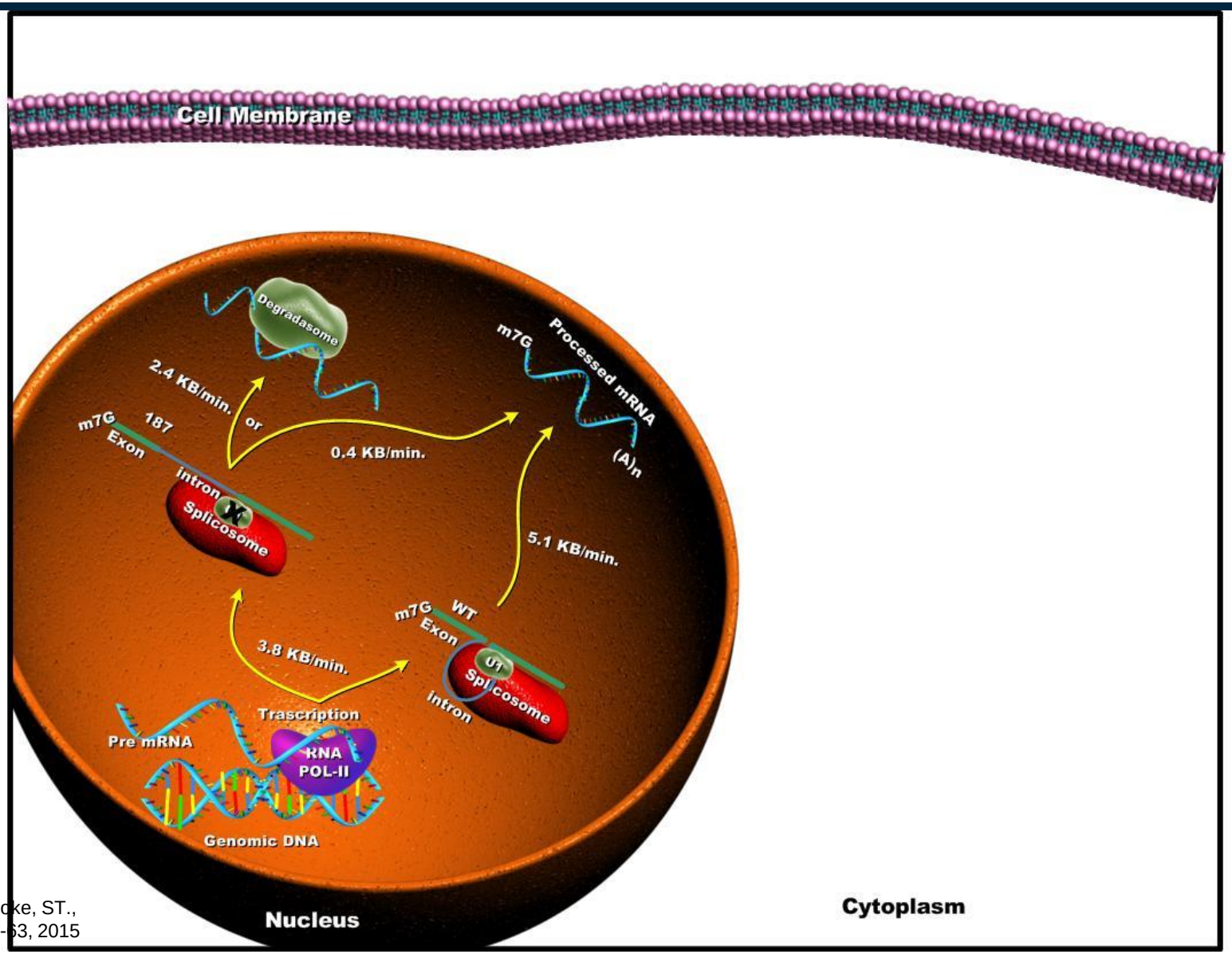
RNA Transcription and Processing Rates



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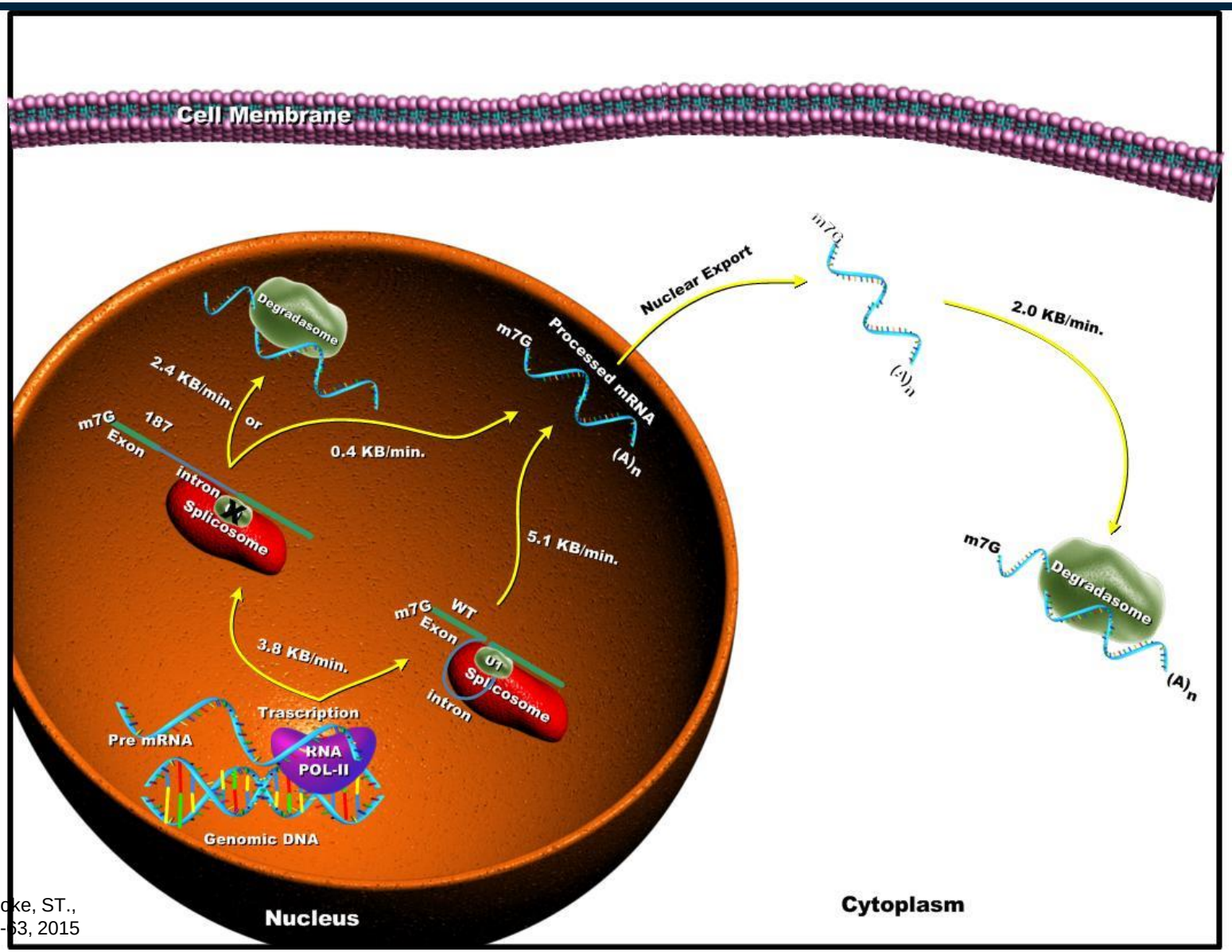
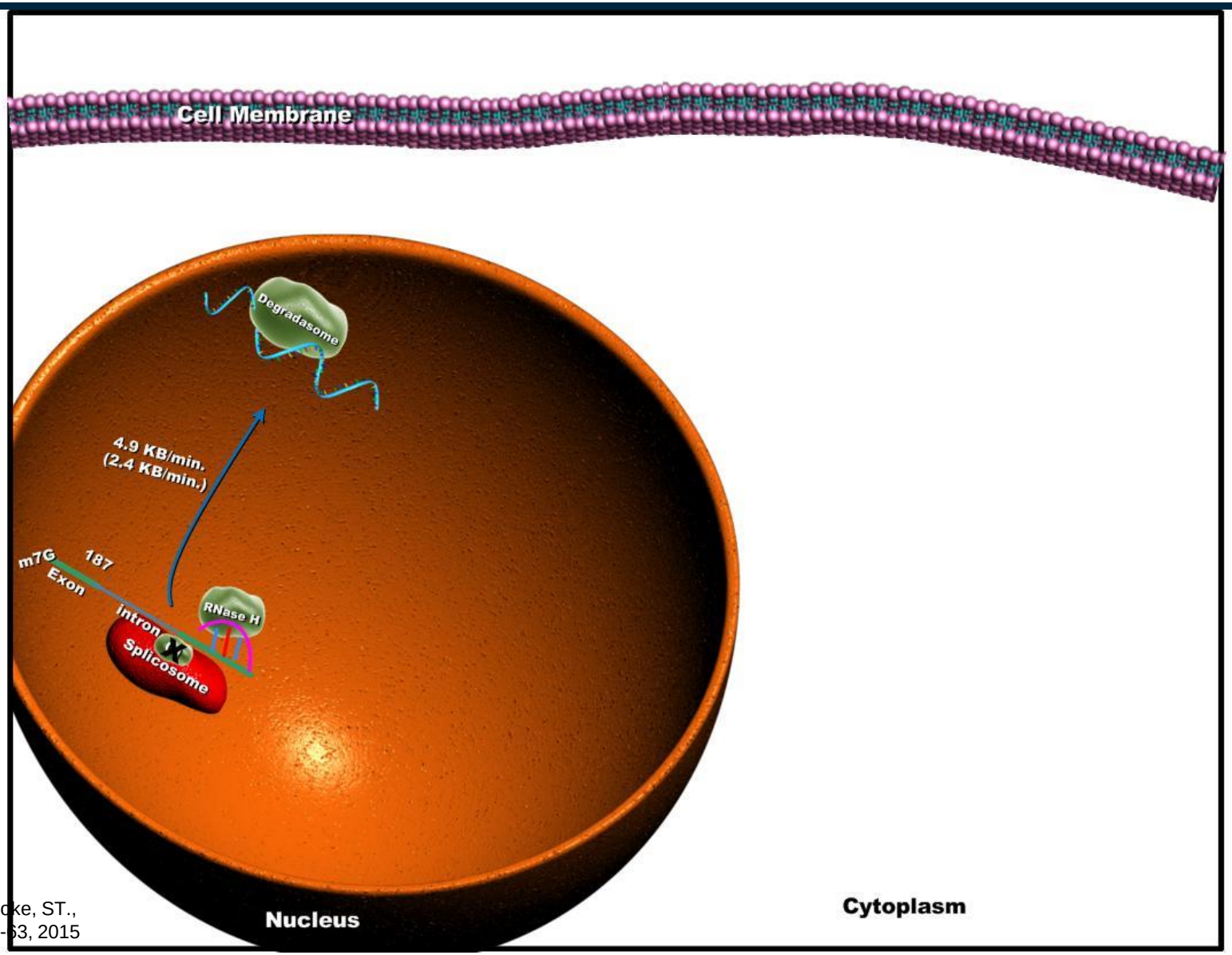
RNA Transcription and Processing Rates



Vickers, T., & Crooke, ST.,
NAR 43(18):8955-63, 2015

RNase H1 ASOs Approximately Double Natural Degradation Rates



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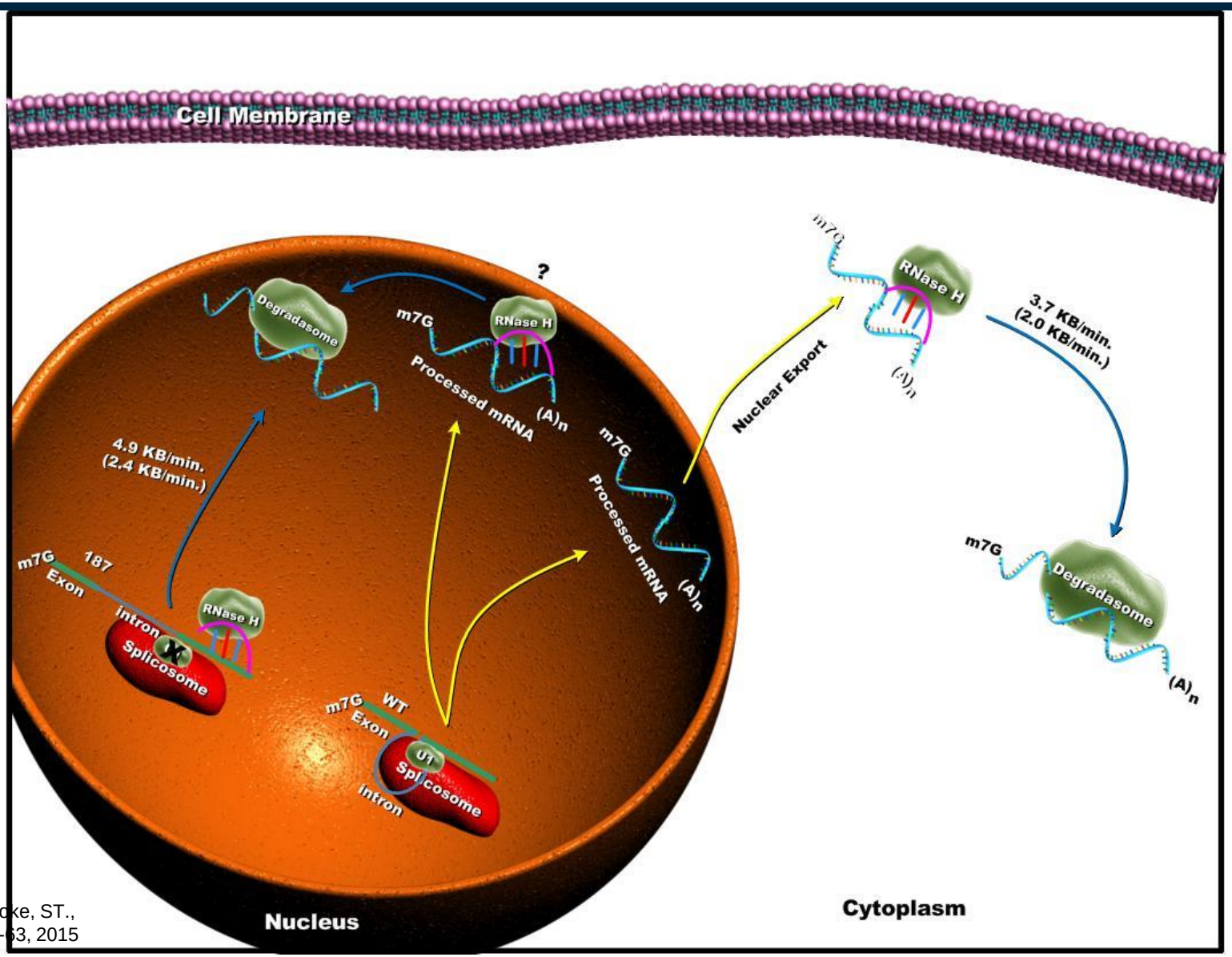
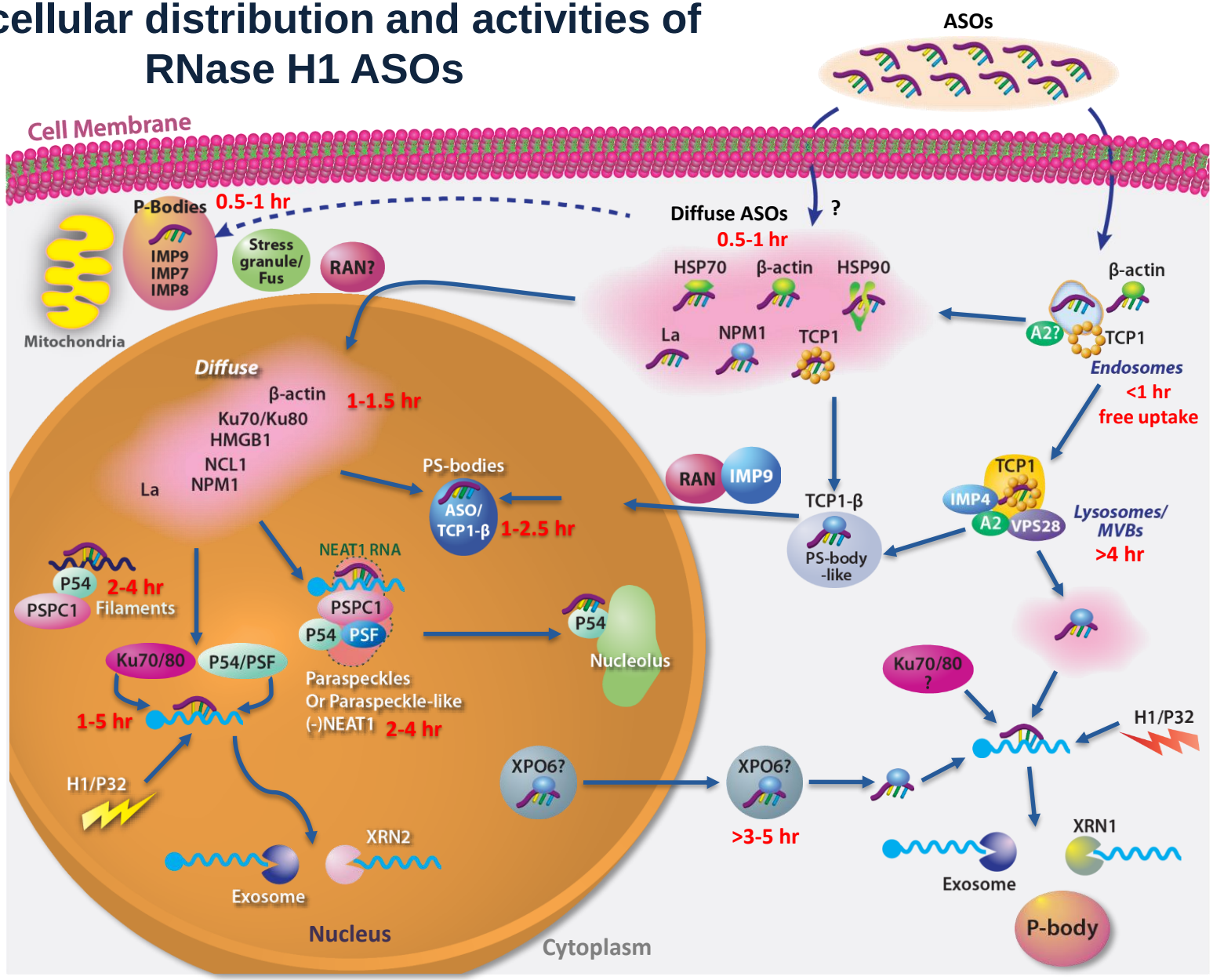
RNase H1 ASOs Approximately Double Natural Degradation Rates



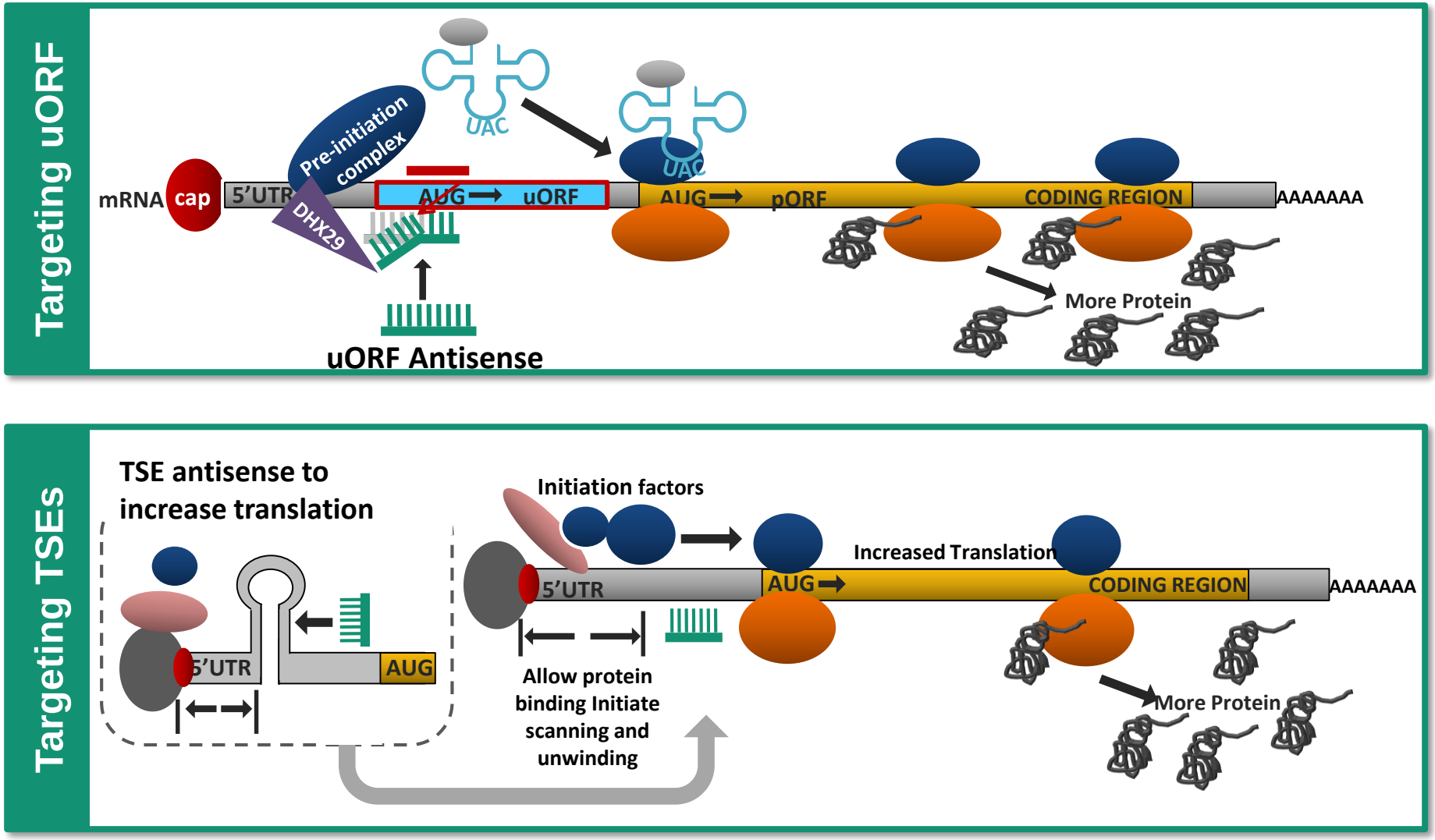
Vickers, T., & Crooke, ST.,
NAR 43(18):8955-63, 2015

Subcellular distribution and activities of RNase H1 ASOs



Wu, et al, PLos One 8:8 e71006, 2013; Lima, et al, PLos One 9(7) e101752, 2014; Vickers and Crooke, PLos One 9 e108625, 2014;
Vickers, et al, PLos One 9 e110615, 2014. Liang, et al, NAR 42(12):7819-32, 2014; Shen, et al, NAR 42(13):8648-62, 2015; Liang, et al, NAR 43(5):2927-45, 2015.

New Antisense Mechanisms to Increase Target Protein Levels Broaden our Potential to Treat Disease



Liang, X.H, et al, Nat Biotech 34(8):875-880, 2016; Liang, X.H., et al, NAR, in press.

Antisense Tomorrow

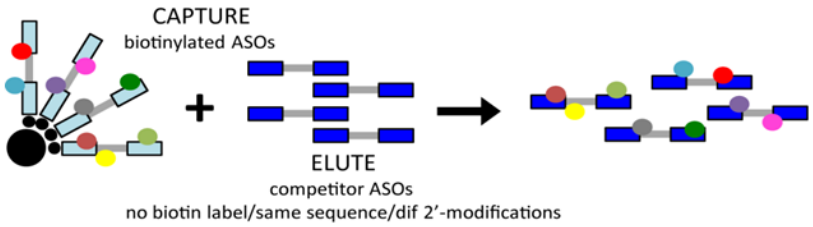
To Understand the Molecular Mechanisms of ASOs, We Must Now Consider Two Codes

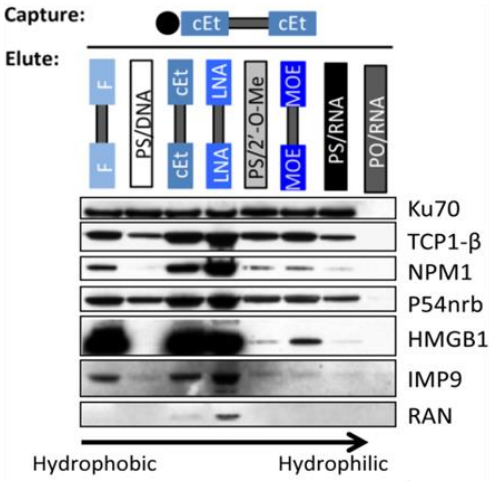
- **Nucleic acid**
  - Oligonucleotides
    - Sequence
    - Charge
    - Phosphorothioates
    - 2’ modifications
    - Orientation of 2’ modified wings
    - Structure
      - Duplexes
      - G quartets, et al
      - Lattice works
  - RNA
    - Sequence
    - Atypical bases (A to I editing, for example)
    - Structure
    - 2’ modifications
    - Base modifications
    - RNase H1 site and sequence preferences
    - Protein binding sites

- **Amino acid**
  - Oligonucleotide
    - Phosphorothioates
      - Number
      - Placement
    - Charge
    - 2’ modifications
      - Hydrophobicity
      - Number
      - Orientation (5’ or 3’)
    - Sequence
    - Base modifications
    - Pendant groups (conjugates)?
  - Protein
    - Domains
    - Structures
    - Charge
    - Hydrophobicity
    - Modifications?
      - Acylation
      - Phosphorylation
      - Glycosylation
      - Lipidation
      - Ubiquitinylation

Proteins Determine the Fate of PS-ASOs both *in vitro* and *in vivo*

More than 50 Intracellular ASO Binding Proteins have been Identified and Characterized





- Protein reduction **increases** ASO activity (14)
- Protein reduction **reduces** ASO activity (6),
- Protein reduction has **no effect on** ASO activity **(19).**

➢ Uncolored proteins are not characterized.

Δ = Proteins that affect ASO localization

Nucleic acid binding proteins (30)

Protein		Feature
CArG binding factor		RNA binding
DHX30		RNA binding
EIF2S2		RNA binding
eIF4H		RNA binding
GRSF		RNA binding
HMGB1		DNA binding
hnRNP D1Like		RNA binding
hnRNPA1		RNA binding
hnRNPA2		RNA binding
hnRNPF		RNA binding
hnRNPH1		RNA binding
hnRNPK		RNA binding
hnRNPQ		RNA binding
hnRNPU		RNA binding
hnRNPUL		RNA binding
ILF2		RNA binding
ILF3		RNA binding
KHSRP		RNA binding
Ku70		DNA binding
Ku80		DNA binding
La/SSB	Δ	RNA binding
NCL		RNA binding
NPM1	Δ	RNA binding
P54nrb	Δ	RNA/DNA binding
PC4/Sub1		DNA binding
PSF	Δ	RNA/DNA binding
PSPC1	Δ	RNA binding
RHA		RNA binding
RNF163/ZNF9		DNA binding
YBX1 protein		RNA binding

Chaperon proteins (11)

Proteins	Feature
GRP78/Bip	hs protein
HSC70	hs protein
HSP90-AA1	hs protein
Hsp90-AB	hs protein
HSPA1L	hs protein
TCP1-alpha	hs protein
TCP1-beta	hs protein
TCP1-delta	hs protein
TCP1-episilon	hs protein
TCP1-gamma	hs protein
TCP1-Theta	hs protein

Other proteins (17)

Proteins		Feature
ACLY		Enzyme
Albumin		Secreted
Annexin A2	Δ	Membrane binding
ATAD3A		Membrane
FTCD/58K	Δ	Enzyme
IMP9	Δ	Transport
JKTPB1 delta 6		hnNRP like
KCTD12		Membrane receptor
LRPPRC		Transport/Transcription
NARS		tRNA synthase
NDKA		Enzyme
RAN	Δ	Transport
SHMT2		Enzyme
Thymidylate kinase		Enzyme
VARS		tRNA synthase
β-actin (ACTB)		structure
β-tubulin (TUBB2C)		structure

Liang et al, NAR 42(12):7819-32, 2014; Shen et al, NAR 42(13):8648-62, 2014; Liang et al, NAR 43(5):2927-45, 2014; Shen et al, NAR 43(6) 2015.

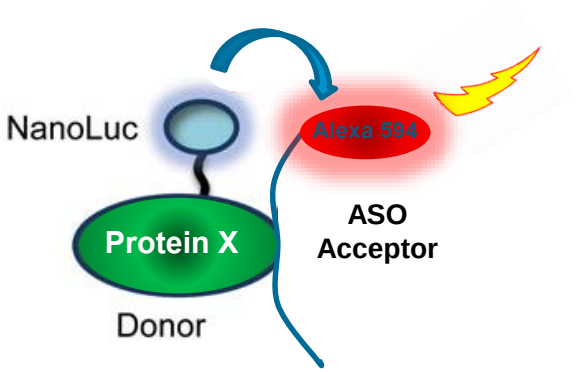
Proteins Appear to Direct ASO Subcellular Localization

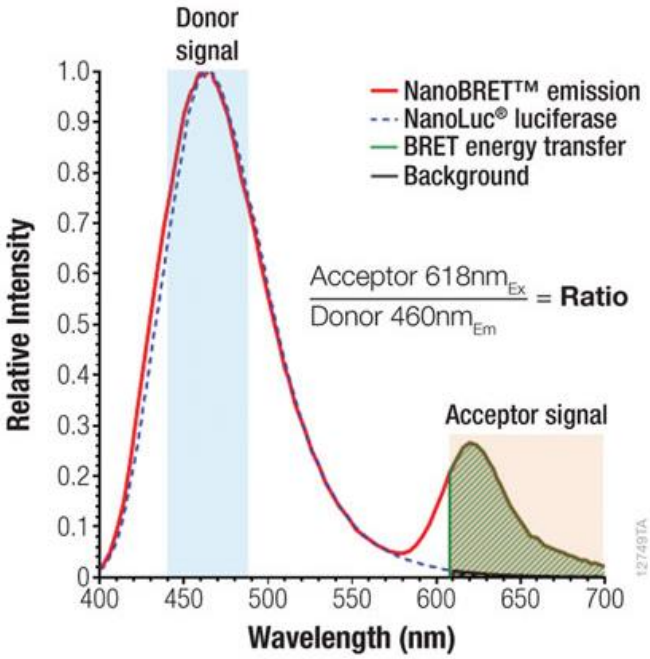
- In all subcellular sites in which ASOs accumulate identified to date, interactions with specific proteins appear to be essential
- Many proteins that enhance activity appear to do so by altering subcellular localization
  - Nuclear import/export determined by importins/exportins
  - Golgi trafficking defined by Golgi-58k
  - Endosome trafficking defined by several proteins
  - Even diffuse ASOs seem to be associated with specific proteins
- Preliminary SAR suggests that, in the long term, ASO design may support increased therapeutic index by altering subcellular localization
- Several proteins reduce ASO activity by competing with RNase H1 for binding to duplex
  - Ku70
  - Ku80
  - hnRNPs
- Several reduce activity by altering subcellular localization
  - VPA28
  - TS6101
  - RAB7
  - RAB9

Crooke, ST., et al, 2016, Nat. Biotech, in Press; Liang, XH., et al, NAR, 44(8):3892-907, 2016; Liang, XH., et al, NAR, 43:2927-2945, 2015; Vickers, T., & Crooke, ST., PLosOne Aug 2016 ; Shen, W., et al, NAR, 43(9):4569-4578, 2015.

ASO NanoBRET Assay Review

- NanoBRET (Bioluminescence Resonance Energy Transfer) relies on the energy from a NanoLuc luciferase tagged protein to act as a BRET donor
  - **NanoLuc Luciferase**
    - 100 fold brighter than other luciferases
    - Very small (19kD)
    - High physical stability
    - Large Acceptor/Donor spectral separation. Up to 10-fold improvement of dynamic range over traditional BRET (Rluc/YFP)
- Light energy is transferred to a fluorescently tagged ASO (Alexa 594) acting as a BRET acceptor
- This energy transfer is moderated by the proximity of the two partners
- Allows quantitative measurement of interactions between a protein and ASO *in vitro* and in intact cells
- No protein purification required
- Homogenous assay, no washing





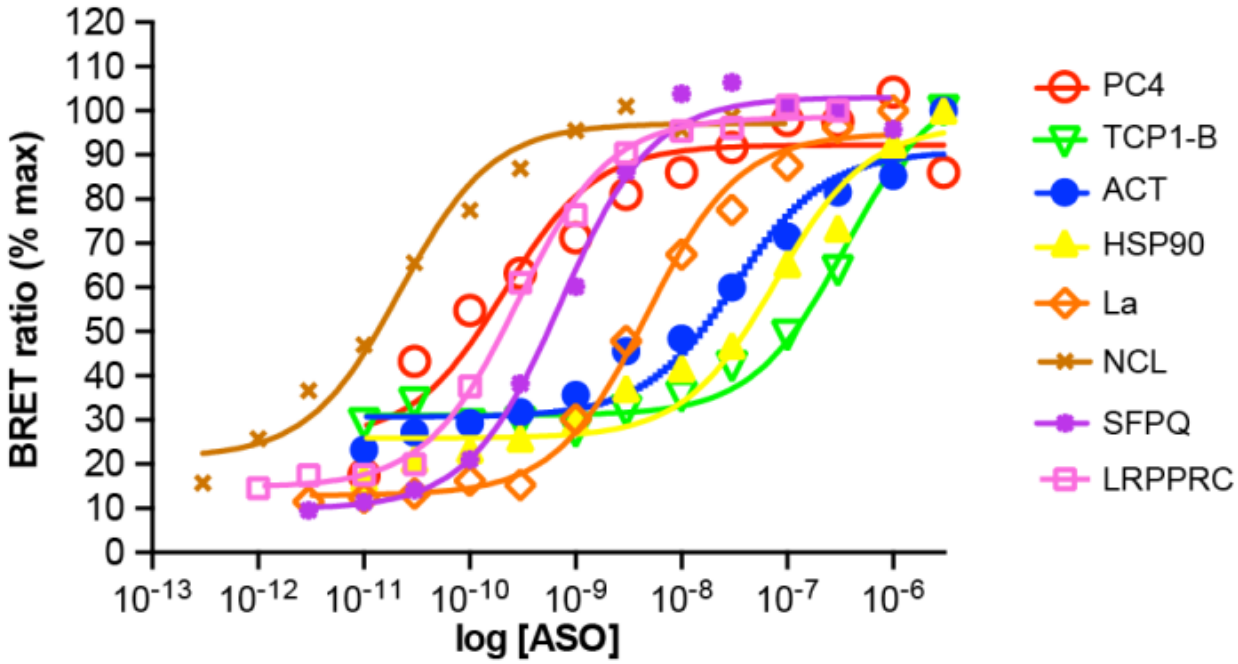
Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nat. Biotech, in press, 2016.

BRET Assay Summary and Conclusions

- Rapid throughput assay from cell lysates and immunoprecipitates can generate:
  - Kd
  - Bmax
  - Relative Kd's
  - Relative binding distances and orientation
- Does not require protein purification or denaturation
  - Mutant proteins can be rapidly generated and expressed
- Ratiometric assay normalizes for well to well variation in luminescent intensity; highly reproducible
- Supports evaluation of native protein/ASO interactions
- Supports evaluation of protein complex/ASO interactions
- Supports evaluation of high affinity/abundance proteins in intact (DIG permeabilized) cells.
- Limitations:
  - ASO NanoBRET assay does not work in live cells, even for high abundance/affinity proteins
  - Amplitude of BRET signal is limiting for large proteins

Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nature Biotech 35:230-237, 2017.

PS-ASO Binding Affinities for Proteins Vary by Over 4 Orders of Magnitude



	PC4	TCP1-B	ACT	HSP90	La	NCL	SFPQ	LRPPRC
K_D (nm)	0.21 ± 0.09	348.7 ± 57.35	30.48 ± 9.29	77.76 ± 22.46	4.74 ± 0.69	0.022 ± 0.004	0.71 ± 0.11	0.28 ± 0.03

Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nature Biotech 35:230-237, 2017.

2’ Modifications have Significant Effects on Affinity of Proteins for PS-ASOs

Protein	NLuc	Domains	size (kD)	K_D Fl (nm)	**K_D cEt** (nm)	K_D MOE (nm)
SSBP1	N/C	1-DBD	17	0.1	**0.5**	0.07
FUS	C	1-RBD	52.7	0.12	**0.6**	1.8
PC4	N	1-DBD	14.4	0.21	**1.1**	6.1
RPL11	N		20	0.6	**1.3**	3.7
NCL (RBD 1-4)	N/C	4-RBD	39	0.002	**1.7**	0.009
P54	N/C	2-RBD	54	0.8	**2**	13
Ku70	C	2-DBD	69.9	3	**4**	15
RNAse H1	N/C	1-HBD	32	2	**5**	2
RPL5	N		34.4	9.8	**17.4**	15.7
La	N/C	2-RBD	46.8	0.9	**43**	308
HSP90 (mid)	C		47	98	**43**	167
Staufen	C	3-RBD	55		**100**	
TCP1-B	N		57	189	**113**	398
ACTB	N		42	28	**295**	252
NMP1	N/C	1-DBD	28.4	>1000	**>1000**	>1000
ANXA2	C	1-RBD	38	>1000	**>1000**	>1000
XRN2	C		109	ND	**ND**	ND

- **Many proteins have greater affinity for hydrophobic 2’ mods**

•More than 2 orders of magnitude difference in affinity between 2’ mods for certain proteins

Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nature Biotech 35:230-237, 2017.

Backbone Modifications can have Profound Effects on PS-ASO Protein Interactions

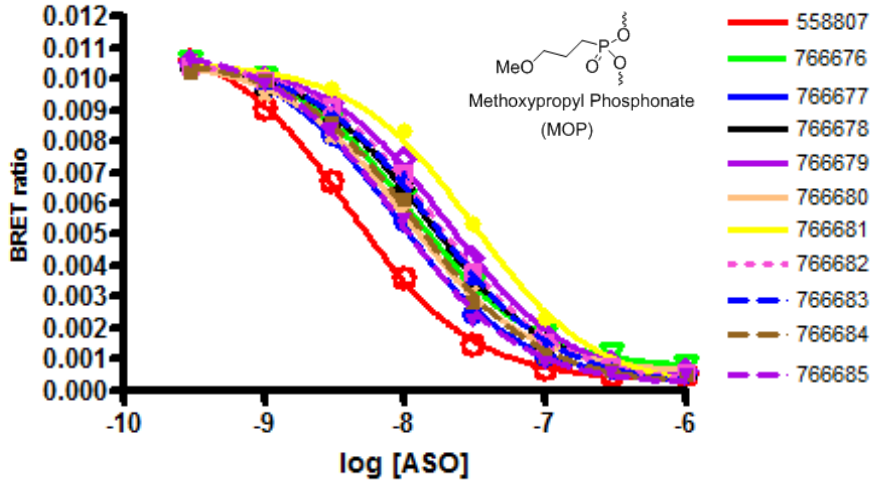
(MOP substituted cEt gap-mers *vs* nucleolin)

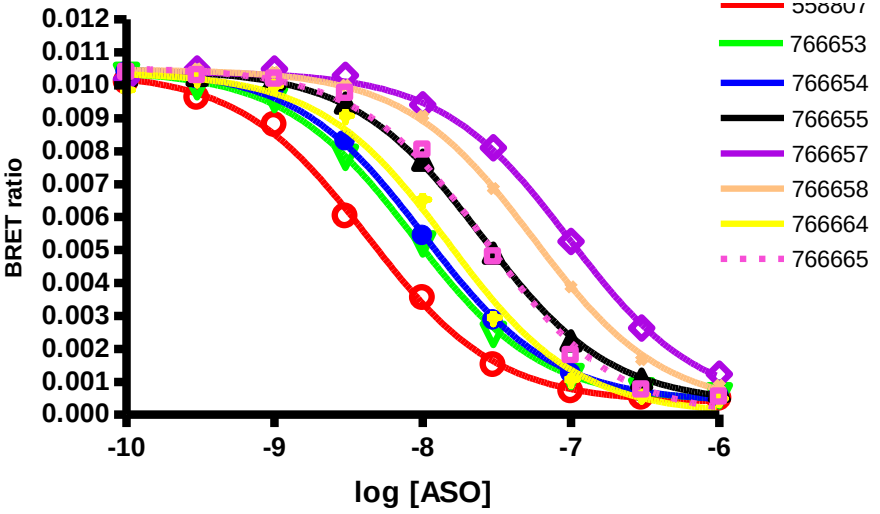
OTS 2016

Ionis #	Sequence 5' → 3'
558807	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766676	Gks mCks Aks Tdx Gds Tds Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766677	Gks mCks Aks Tds Gdx Tds Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766678	Gks mCks Aks Tds Gds Tdx Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766679	Gks mCks Aks Tds Gds Tds Tdx mCds Tds mCds Ads mCds Ads Tks Tks Ak
766680	Gks mCks Aks Tds Gds Tds Tds mCdx Tds mCds Ads mCds Ads Tks Tks Ak
766681	Gks mCks Aks Tds Gds Tds Tds mCds Tdx mCds Ads mCds Ads Tks Tks Ak
766682	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCdx Ads mCds Ads Tks Tks Ak
766683	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Adx mCds Ads Tks Tks Ak
766684	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Ads mCdx Ads Tks Tks Ak
766685	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Ads mCds Adx Tks Tks Ak

Ionis #	Sequence 5' → 3'
558807	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766653	Gks mCks Aks Tdx Gdx Tds Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766654	Gks mCks Aks Tds Gdx Tdx Tds mCds Tds mCds Ads mCds Ads Tks Tks Ak
766655	Gks mCks Aks Tds Gds Tdx Tdx mCds Tds mCds Ads mCds Ads Tks Tks Ak
766666	Gks mCks Aks Tds Gds Tds Tdx mCdx Tds mCds Ads mCds Ads Tks Tks Ak
766657	Gks mCks Aks Tds Gds Tds Tds mCdx Tdx mCds Ads mCds Ads Tks Tks Ak
766658	Gks mCks Aks Tds Gds Tds Tds mCds Tdx mCdx Ads mCds Ads Tks Tks Ak
766659	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCdx Adx mCds Ads Tks Tks Ak
766665	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Adx mCdx Ads Tks Tks Ak
766664	Gks mCks Aks Tds Gds Tds Tds mCds Tds mCds Ads mCdx Adx Tks Tks Ak

mC:5 methyl cytosine, k: cEt, d: deoxy, x: MOP





Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nature Biotech 35:230-237, 2017.

PS-ASO Protein Interactions
Structure Activity Relationships

PS-ASOs

- Phosphorothioate
  - Number (minimum 9-10)
  - Placement
- The hydrophobicity of the 2’ group (3-4 orders of magnitude)
  - Promiscuity of protein binding
  - Types of proteins
  - Polarity
  - Domains bound
  - 2’F causes degradation of paraspeckel proteins
- Sequence matters to some proteins
  - 3 orders of magnitude
  - A few motifs emerging
    - GGG for La and P54

Proteins

- Some proteins are relatively promiscuous
  - Bind on 2’ modifications equally well
- Most are sensitive to
  - 2’ modifications
  - Sequence
  - Polarity of 2’ modifications
- RNA binding domains are prominent
  - Other domains also involved
- Nucleolin and SSB1 have highest affinity
- Nucleolin binding correlates with potency
- Stoichiometry varies from 1-3 ASOs per protein molecule
  - Number of RNA binding domains does not correlate

New Medicinal Chemical Oportunities

- Key proteins enhance or limit PS-ASO activity identified
- Key proteins and pathways to productive uptake identified
- Key proteins that determine subcellular distribution identified
- Very modest, essentially 1-2 nucleotide changes, can selectively alter interactions with key proteins
- Sequence, 2’ modifications and PS content and new protein-centered modifications can be used to optimize therapeutic index

Conclusions

- Antisense Today
  - Broad activity in many tissues
  - Multiple routes of administration
  - Acceptable safety
  - Improved tolerability
  - Multiple mechanisms
  - New chemistries enhance performance
  - Now possible to selectively increase translation of specific proteins (Agonist like activity)

- Antisense Tomorrow
  - Driven by advances in understanding the molecular events leading to activity
  - Focus on understanding the amino acid code
  - Broad new horizons for medicinal chemistry

Thank You

SMA – a severe rare disease caused by homozygous loss of function of the SMN1 gene

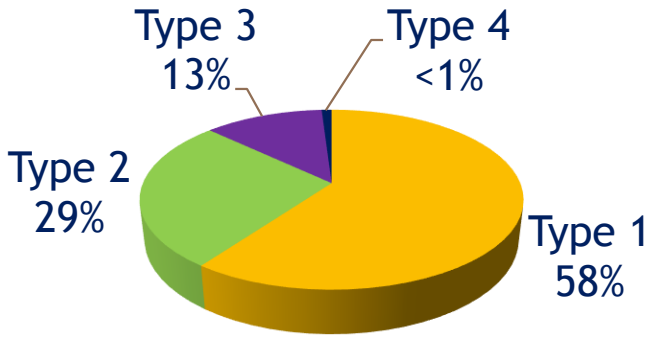
Type 1
- Most severe form of the disease
- Age of symptom onset <6 months
- Never able to sit
- Very short life expectancy
- Most have two SMN2 genes

Type 2
- Age of symptom onset >6 months
- Able to sit or stand, but not walk
- Muscle weakness/skeletal deformities
- Shortened life expectancy
- Most have 3-4 SMN2 genes

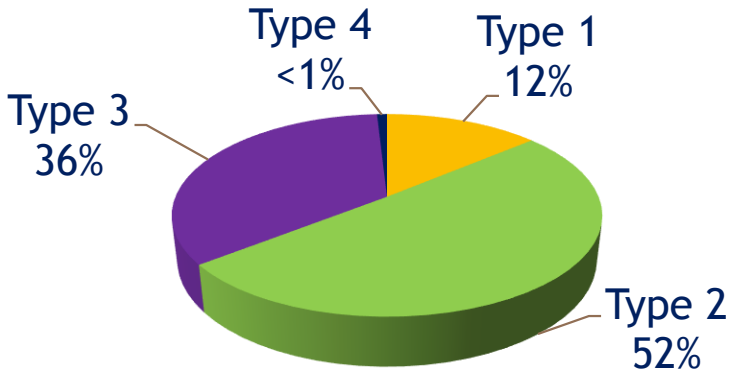
Type 3
- Age of symptom onset >6 months
- Able to walk with difficulty
- Muscle weakness/skeletal deformities
- Close to normal life expectancy
- Most have 3-4 SMN2 genes

Type 4
- Adult onset

Incidence of SMA Types*



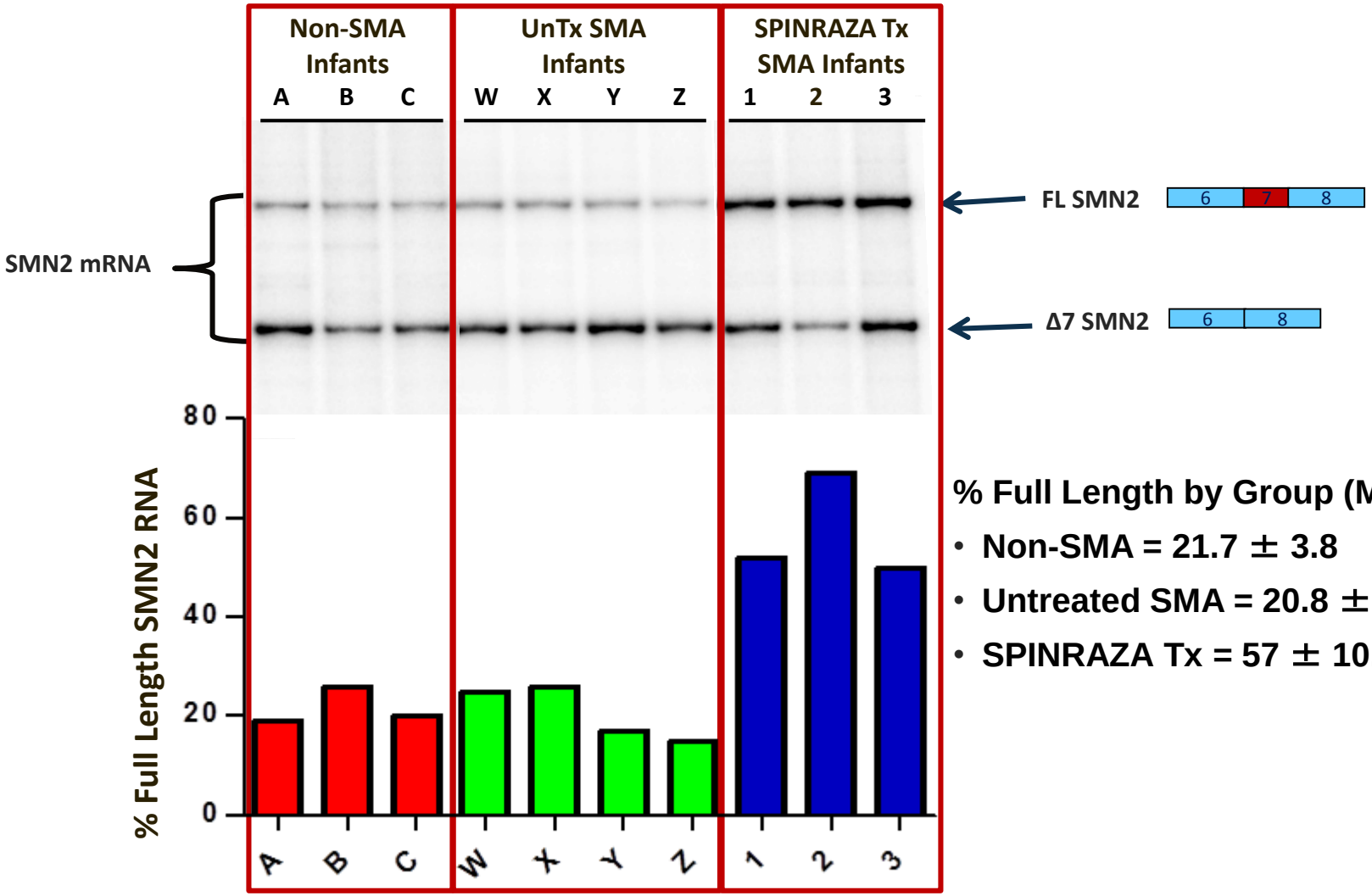
Prevalence of SMA Types**



*Ogino et al. Eur J Hum Genet 2004 Dec;12(12):1015-23; **SMA Foundation estimates (www.smafoundation.org)

Greater Amount of Full Length SMN2 mRNA is Observed in Thoracic Spinal Cord Tissue Analyzed from SPINRAZA-treated SMA Infants Compared to Non-SMA Infants and Untreated SMA Infants

Semi-Quantitative RT-PCR Analysis



% Full Length by Group (Mean ± SD)

- **Non-SMA = 21.7 ± 3.8**
- **Untreated SMA = 20.8 ± 5.6**
- **SPINRAZA Tx = 57 ± 10.4**

1989
Why antisense?

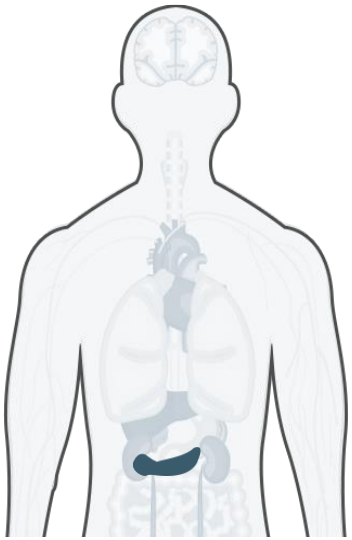
- Potential to be a revolutionary platform for drug discovery
- Best opportunity for quantum increase in specificity
- Only direct route from the genome to the patient
- Best opportunity to improve the productivity of the industry
  - Research efficiencies
  - Fewer development failures
  - Development efficiencies
- A pharmacological opportunity

Addressing a Broad Spectrum of Diseases

From Common Diseases Affecting Millions…

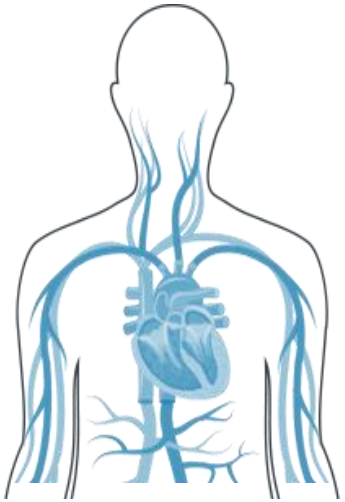
Type 2 Diabetes

IONIS-GCGR$_{Rx}$



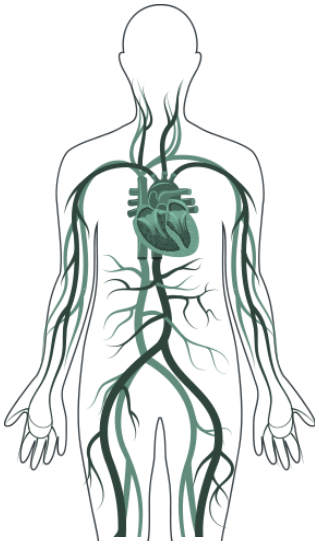
Cardiovascular Disease

ACKEA-APO(a)-L$_{Rx}$



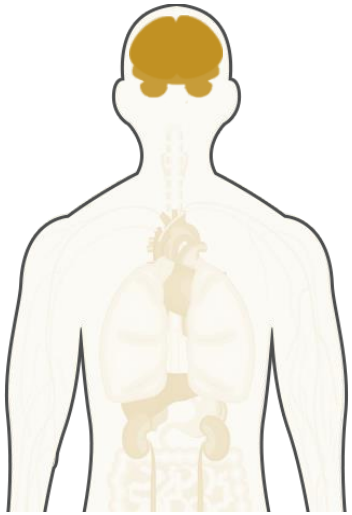
Clotting Disorders

IONIS-FXI$_{Rx}$



Parkinson's and Alzheimer's Disease

IONIS-BIIB4$_{Rx}$
IONIS-BIIB6$_{Rx}$

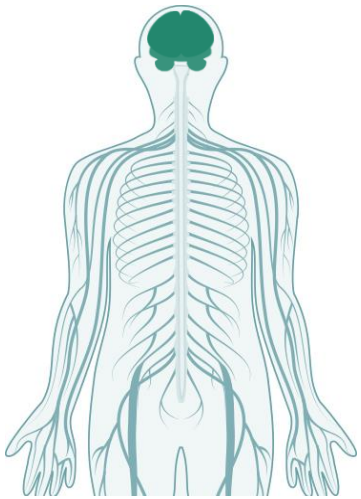


Addressing a Broad Spectrum of Diseases

… to Inadequately Treated Rare Diseases

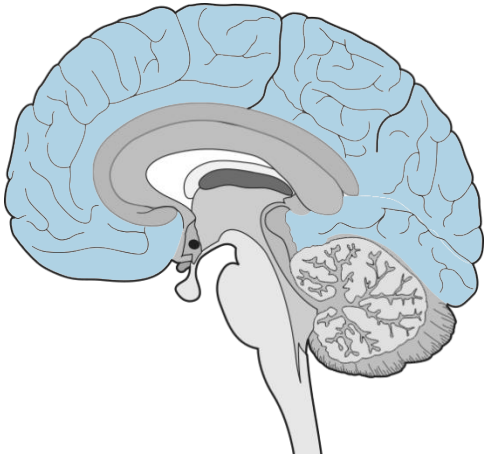
Amyotrophic Lateral Sclerosis

IONIS-SOD1$_{Rx}$



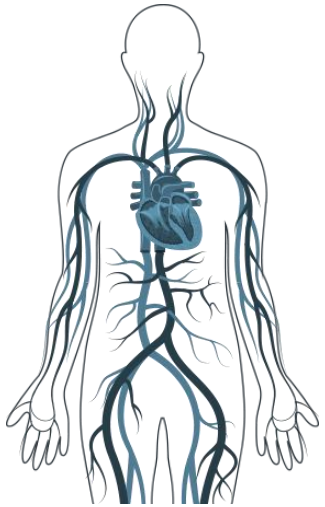
Huntington's Disease

IONIS-HTT$_{Rx}$



Beta-thalassemia

IONIS-TMPRSS6-L$_{Rx}$



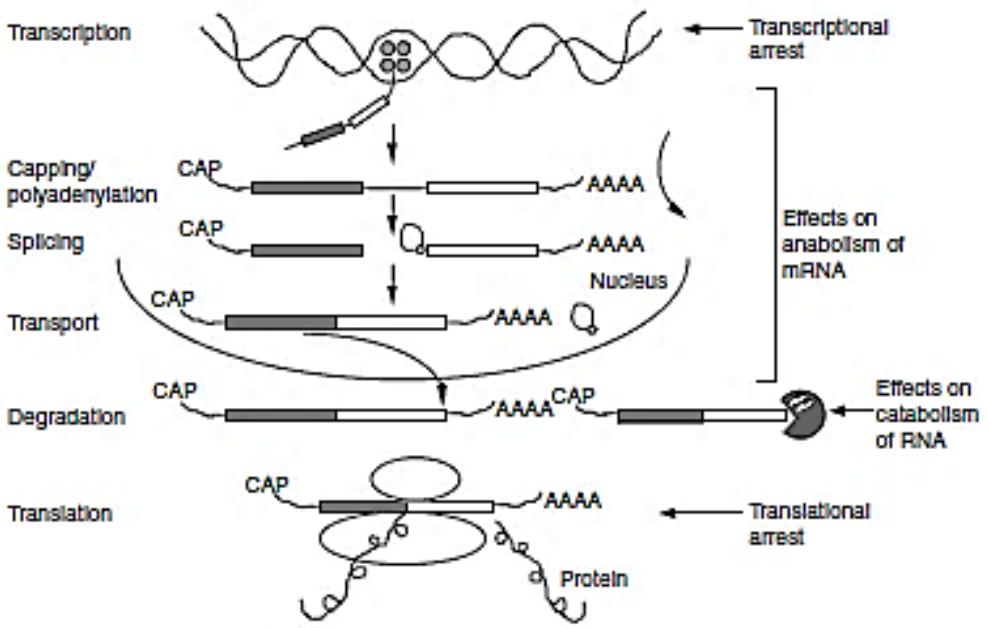
Key early decisions in the evolution of antisense technology

- Define the opportunities and challenges in pharmacological terms
- Select phosphorothioates as first generation chemistry
  - Stability
  - Protein binding [Kd for albumin ~140mM]
- Screen multiple sites in target RNAs
  - Advances in screening continue to generate better drugs
- Select RNase H1 based antisense as key mechanism
- Invest broadly in oligonucleotide medicinal chemistry
- Characterize pharmacokinetics in vivo
  - In culture, many cell lines rapidly lose nucleic acid uptake phenotype
  - New methods
  - Multiple species, organ, suborgans
- Develop multiple methods to prove mechanism
- Understand mechanisms of adverse events
- Advance manufacturing and analytical chemistry
- Commit to core antisense research

ASOs alter the intermediary metabolism of RNA

RATES MATTER

- What is the rate limiting step in the intermediary metabolism of the RNA?
- Steps in ASO drug action are all dramatically slower than for small molecules



Antisense mechanisms shown to result in pharmacological activities and antagonist-like effect

- Occupancy only-mediated mechanisms
  - Modulation of RNA spicing
  - Translation arrest
  - Disruption of necessary RNA structures
    - RNA binding protein sites
    - Enhanced degradation because of decipher of 3’ UTR structures
- Occupancy-activated RNA degradation
  - RNase H1
  - Ago2

Today We Have a Pipeline Filled with Potentially Transformative Generation 2+ Antisense Drugs

The NEW ENGLAND JOURNAL of MEDICINE

Targeting APOC3 in the Familial Chylomicronemia Syndrome

THE LANCET

Antisense therapy targeting apolipoprotein(a): a randomised, double-blind, placebo-controlled phase 1 study

The NEW ENGLAND JOURNAL of MEDICINE

Factor XI Antisense Oligonucleotide for Prevention of Venous Thrombosis

The NEW ENGLAND JOURNAL of MEDICINE

Antisense Inhibition of Apolipoprotein C-III in Patients with Hypertriglyceridemia

Observations relevant to the RNase H1 mechanism

- **RNase H1 is limiting**
  - Extent
  - Rate
- **Multiple factors have been shown to be irrelevant**
  - RNA copy number (1 – 100,000)
  - Transcription rate
  - RNA half-life (except for very short half-life RNAs eg., cMyc)

Safety Database Demonstrates that Serious Platelet Declines is Not a 2nd Generation Class Effect

Post-Baseline Platelet Count in Completed Randomized, Controlled Clinical Studies

			Antisense Drug Dose, mg/week					
Confirmed, n (%)	Placebo (N=597)	ASO Total (N=1,516)	≤ 75 (N=152)	>75-175 (N=279)	>175-275 (N=776)	>275-375 (N=111)	>375-475 (N=168)	>475 (N=30)
N	566	1,416	144	260	720	107	156	29
≥ 75 to <100 K/mm^3	0 (0.0%)	6 (0.4%)	0 (0.0%)	0 (0.0%)	3 (0.4%)	0 (0.0%)	3 (1.9%)	0 (0.0%)
≥ 50 to <75 K/mm^3	2 (0.4%)	2 (0.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (1.3%)	0 (0.0%)
≥ 25 to <50 K/mm^3	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
< 25 K/mm^3	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

* Number of subjects with normal baseline platelet counts and with post baseline values
Majority of patients dosed for 3-6 months
Does not include patients in anticancer studies

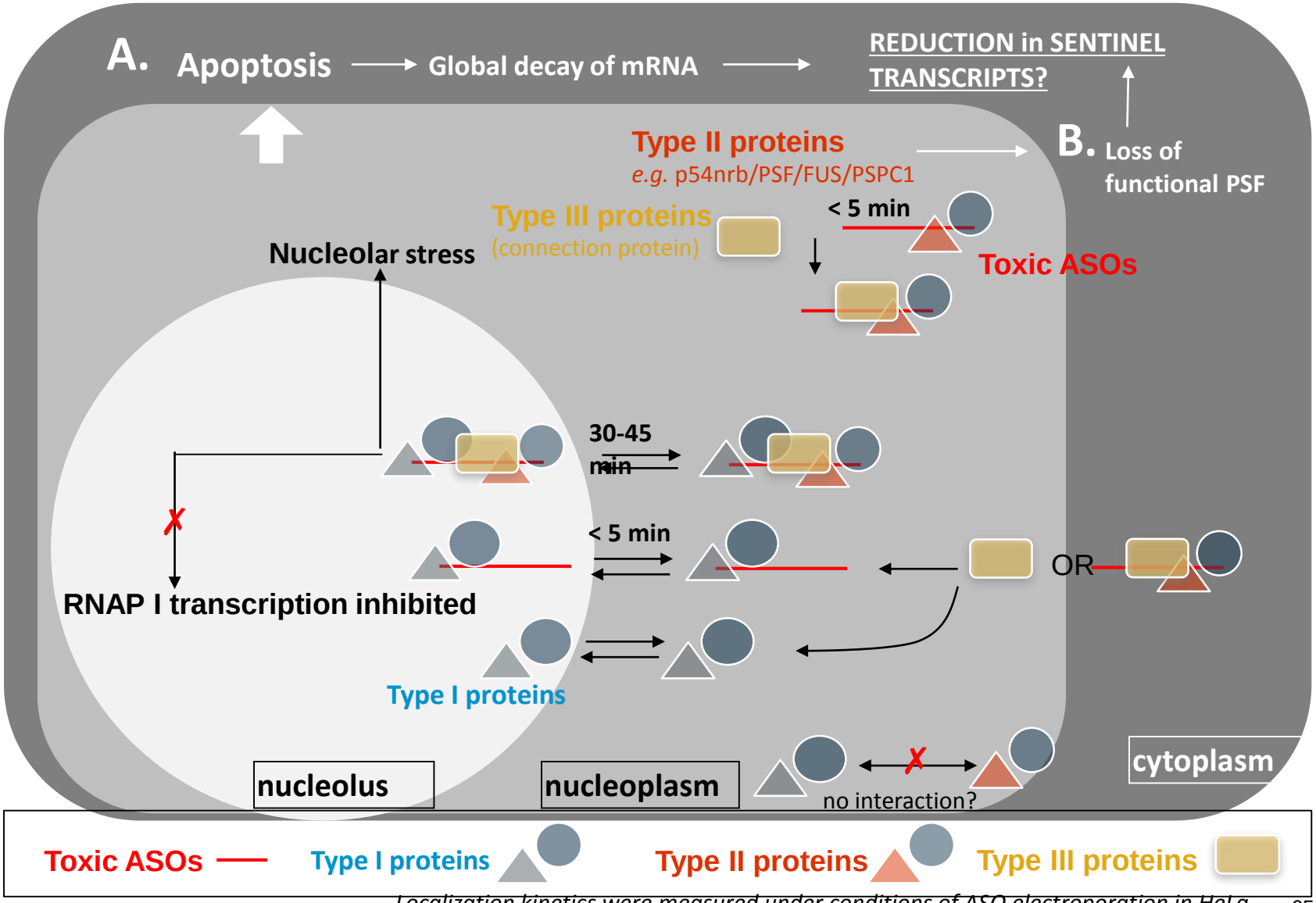
Crooke, ST., et al, Mol. Ther. 2016.136, doi: 10.1038/mt
add NAT brenda platlet NAT in press .

Factors that Influence the On and Off-target Activity of RNase H1 ASOs

Factor	On-target	Off-target
Watson and Crick hybridization	Δ = 1 – 2X	2 – 10X↓
Higher Order structure of target mRNA	1 – >100X↓	2 – 40X↓
mRNA binding proteins	1 – >30X↓	10 – >100X↓
Human RNase H1 activity	Δ = 1 – 3X	>10X↓
Multiple ASO binding sites on mRNA (3 – 9 sites)	3 – 10X↑	10 – 100X↑
ASO/mRNA binding proteins (RNase H competitors)	2 – 10X↓	Variable
Productive ASO binding proteins (Enhance hybridization, localize ASO to productive compartments)	40 - 60%↑	Unknown
Unproductive ASO binding proteins (Inhibit hybridization, sequester ASO in non-productive compartments)	40 - 60%↓	Unknown

A Path to Toxicity

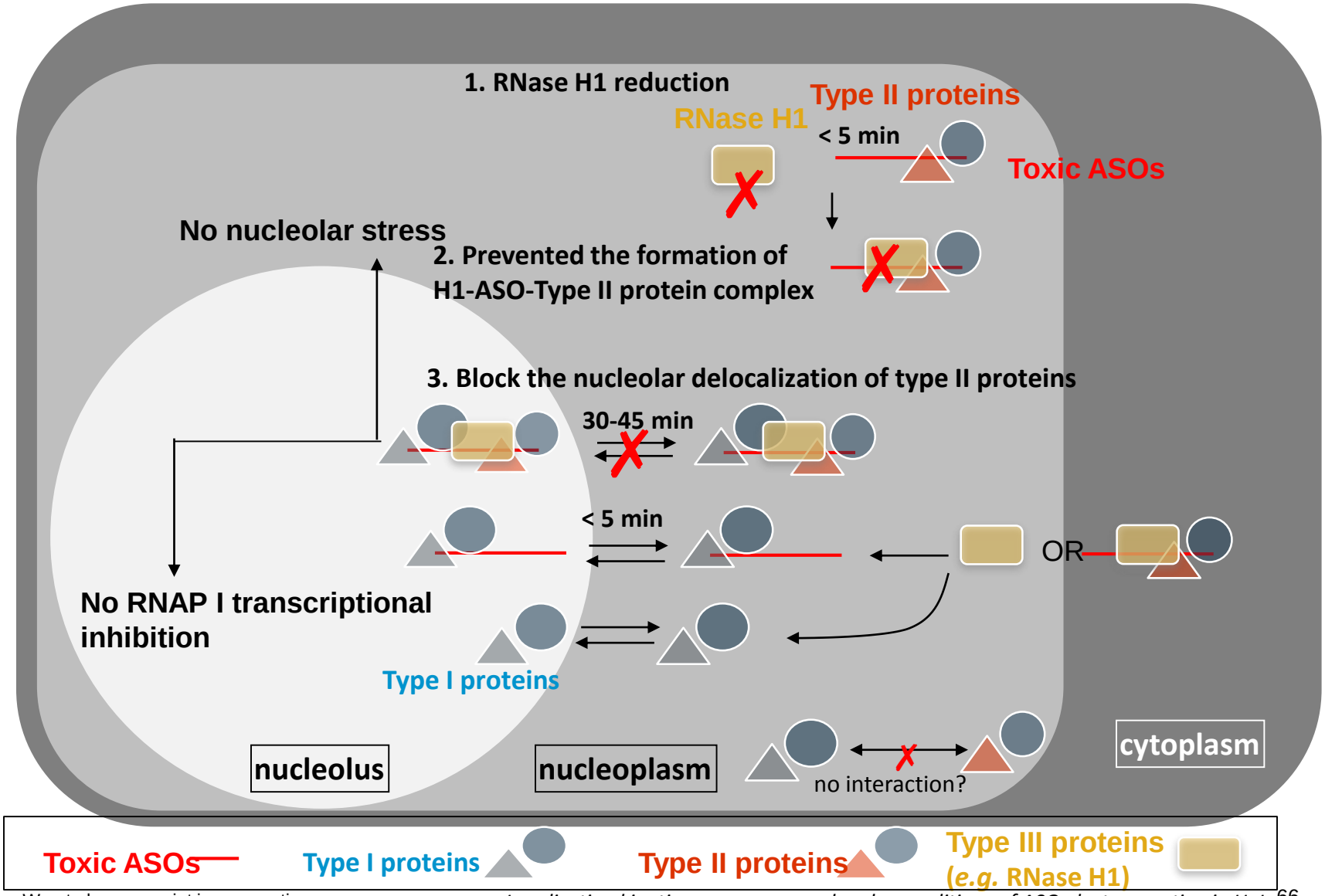
Nucleolar recruitment of cellular proteins (e.g. p54nrb/PSF) by toxic ASOs



Localization kinetics were measured under conditions of ASO electroporation in HeLa

A Path to Toxicity

RNase H1 (a type III protein) reduction blocks toxicity because it is needed to delocalize type II proteins

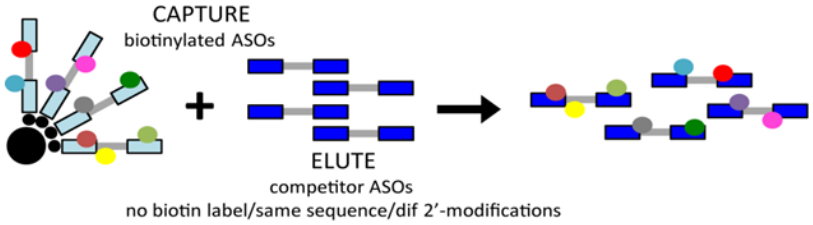


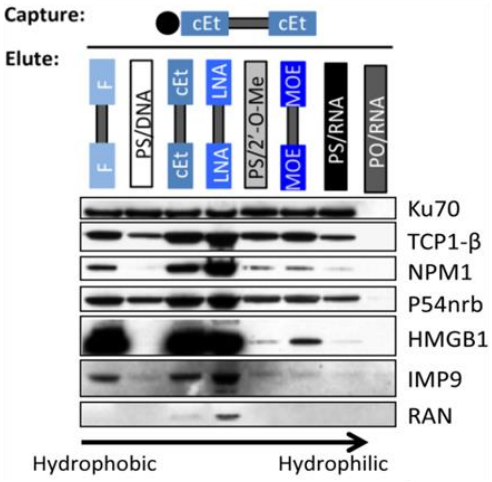
Shen, W., et al, manuscript in preparation

Localization kinetics were measured under conditions of ASO electroporation in HeLa

More than 50 Intracellular ASO Binding Proteins have been Identified and Characterized

OTS 2016





- Protein reduction **increases** ASO activity (14)
- Protein reduction **reduces** ASO activity (6),
- Protein reduction has **no effect on** ASO activity **(19).**

➢ Uncolored proteins are not characterized.

Δ = Proteins that affect ASO localization

Nucleic acid binding proteins (30)

Protein	Feature
CArG binding factor	RNA binding
DHX30	RNA binding
EIF2S2	RNA binding
eIF4H	RNA binding
GRSF	RNA binding
HMGB1	DNA binding
hnRNP D1Like	RNA binding
hnRNPA1	RNA binding
hnRNPA2	RNA binding
hnRNPF	RNA binding
hnRNPH1	RNA binding
hnRNPK	RNA binding
hnRNPQ	RNA binding
hnRNPU	RNA binding
hnRNPUL	RNA binding
ILF2	RNA binding
ILF3	RNA binding
KHSRP	RNA binding
Ku70	DNA binding
Ku80	DNA binding
La/SSB Δ	RNA binding
NCL	RNA binding
NPM1 Δ	RNA binding
P54nrb Δ	RNA/DNA binding
PC4/Sub1	DNA binding
PSF Δ	RNA/DNA binding
PSPC1 Δ	RNA binding
RHA	RNA binding
RNF163/ZNF9	DNA binding
YBX1 protein	RNA binding

Chaperon proteins (11)

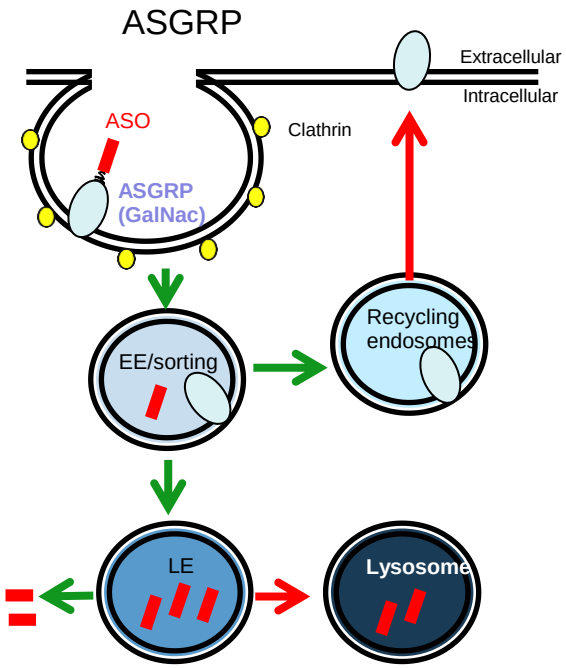
Proteins	Feature
GRP78/Bip	hs protein
HSC70	hs protein
HSP90-AA1	hs protein
Hsp90-AB	hs protein
HSPA1L	hs protein
TCP1-alpha	hs protein
TCP1-beta	hs protein
TCP1-delta	hs protein
TCP1-episilon	hs protein
TCP1-gamma	hs protein
TCP1-Theta	hs protein

Other proteins (17)

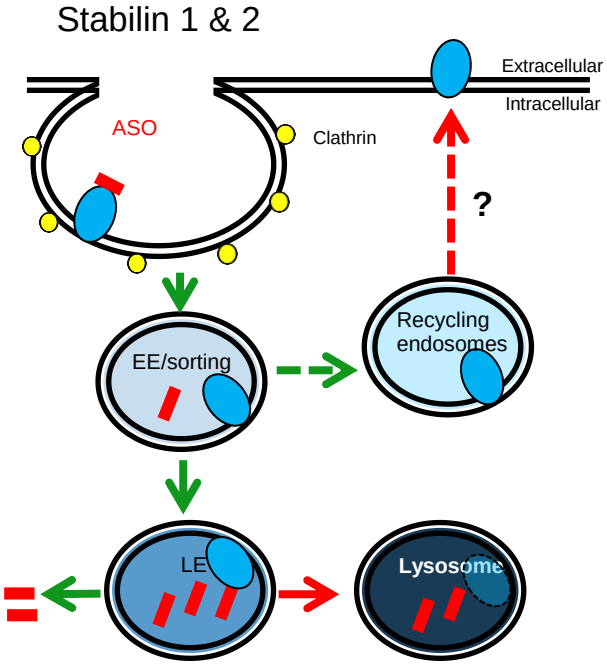
Proteins	Feature
ACLY	Enzyme
Albumin	Secreted
Annexin A2 Δ	Membrane binding
ATAD3A	Membrane
FTCD/58K Δ	Enzyme
IMP9 Δ	Transport
JKTPB1 delta 6	hnNRP like
KCTD12	Membrane receptor
LRPPRC	Transport/Transcription
NARS	tRNA synthase
NDKA	Enzyme
RAN Δ	Transport
SHMT2	Enzyme
Thymidylate kinase	Enzyme
VARS	tRNA synthase
β-actin (ACTB)	structure
β-tubulin (TUBB2C)	structure

Liang et al, NAR. 42(12):7819-32, 2014; Shen et al, NAR 42(13):8648-62, 2014; Liang et al, NAR 43(5):2927-45, 2014; Shen et al, NAR 43(6) 2015.

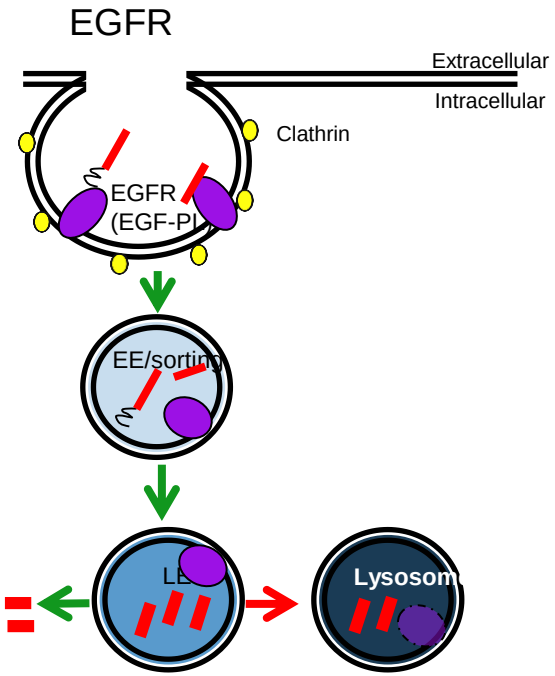
Receptor Mediated Productive Uptake Pathways



Prakash TP, et al.,
Nucleic Acids Res. 2014;42(13):8796-807.



Miller CM, et al.,
Nucleic Acids Res. 2016;44(6):2782-94.



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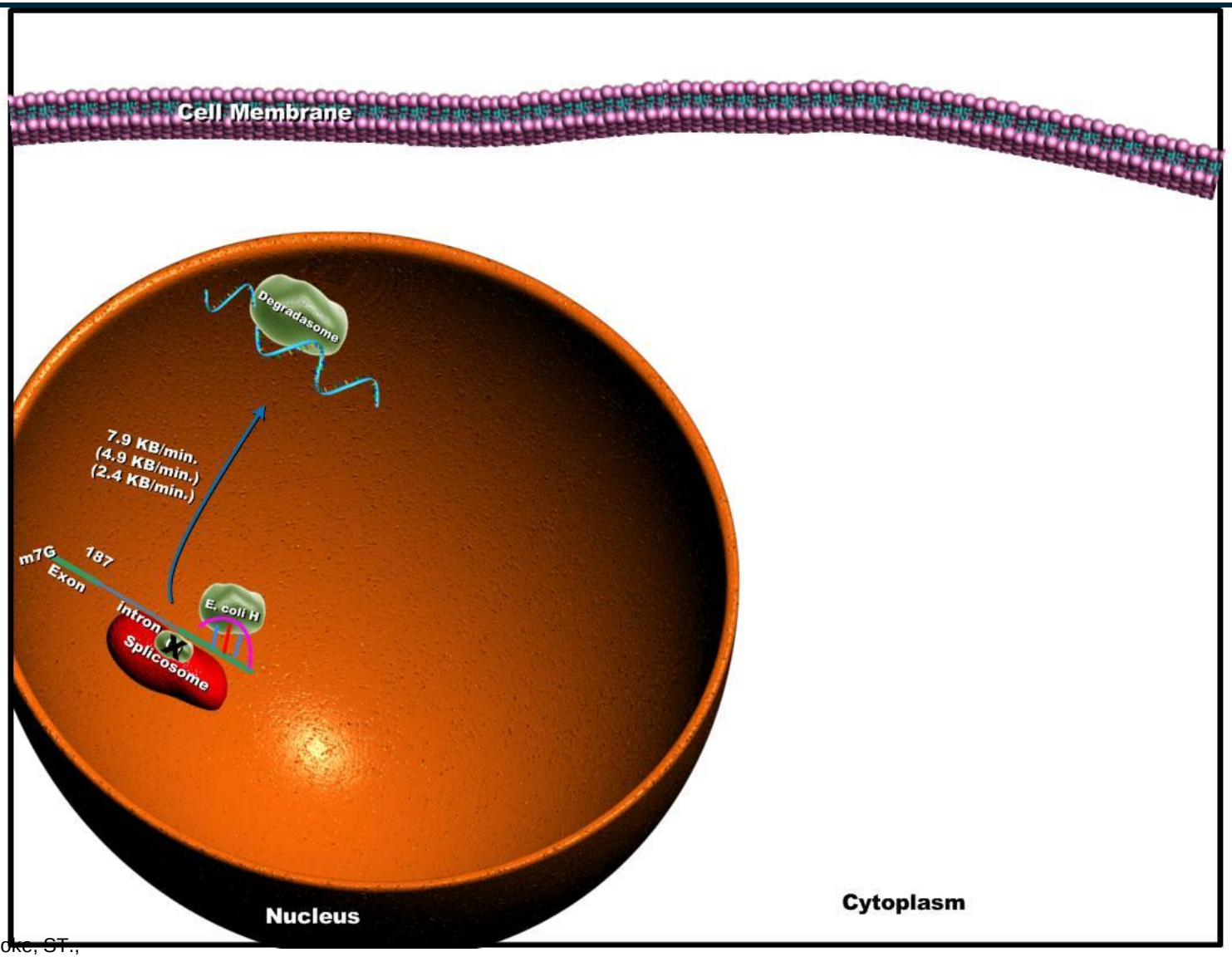
Pharm Res. 1996;13(1):57-61.
-Juliano RL., et al.,
J. Drug Target. 2013; 221(1): 27-43.
-Wang, S., et al, Nucleic Acid Res
-Wang, S., et al., submitted

SPINRAZA: Only Drug Approved for the Treatment of SMA

Indications and Usage

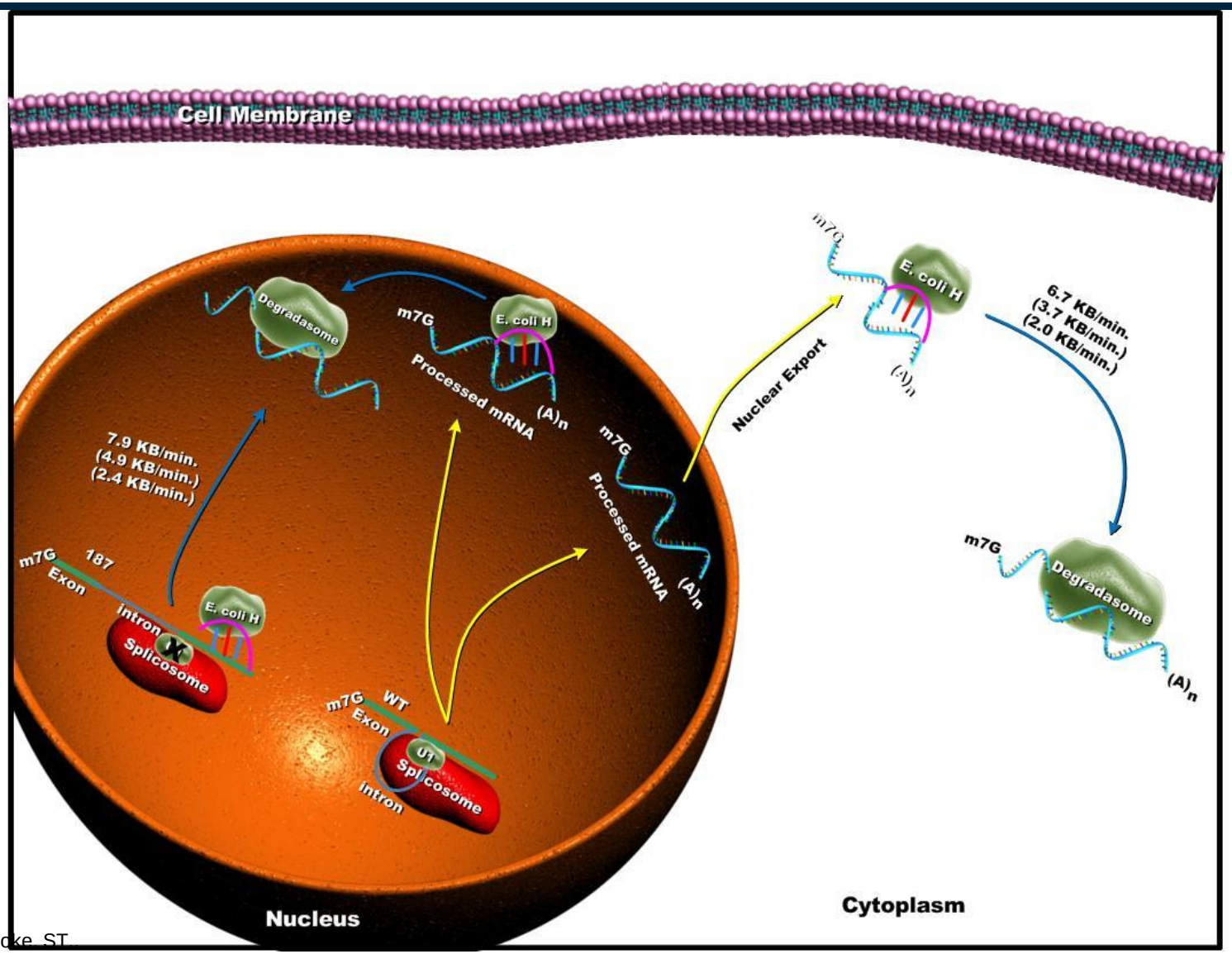
SPINRAZA is a survival motor neuron-2 (SMN2)-directed antisense oligonucleotide indicated for the treatment of spinal muscular atrophy (SMA) in pediatric and adult patients

Over Expression of e.Coli RNase H1 Confirms Once Again That Human RNase H1 Levels are Limiting



Vickers, T., & Crooke, ST.,
NAR 43(18):8955-63, 2015

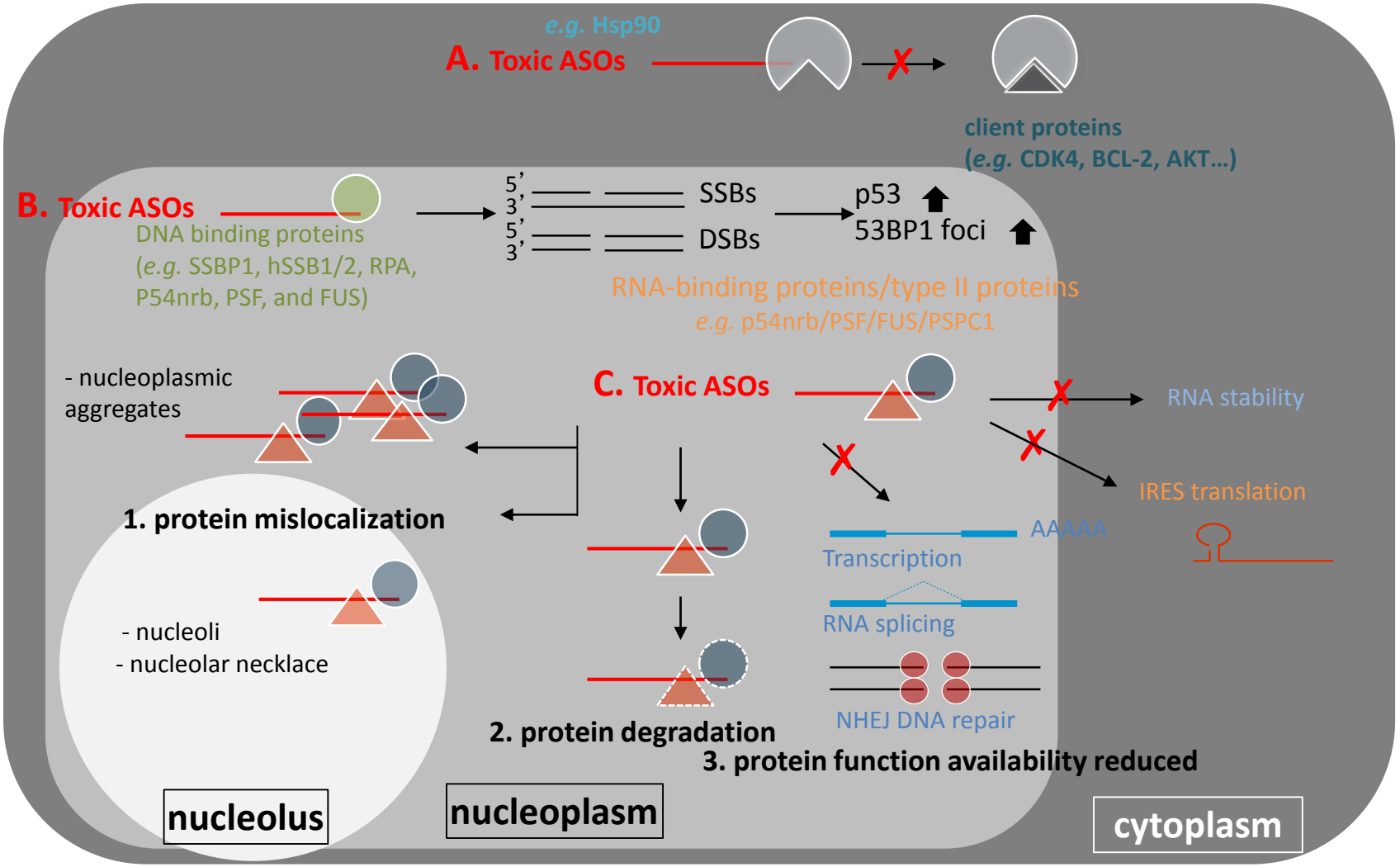
Over Expression of e.Coli RNase H1 Confirms Once Again That Human RNase H1 Levels are Limiting



Vickers, T., & Crooke, ST.
NAR 43(18):8955-63, 2015

A Path to Toxicity

Interactions between toxic ASOs and cellular proteins can affect multiple cellular pathways and lead to toxicity



Shen, W., et al, manuscript in preparation

The RNase H1 Mechanisms: General Observations

- RNase H1 is limiting for RNase H1 ASOs
  - Extent
  - Rate
- Multiple factors have been shown to be irrelevant
  - RNA copy number (1 – 100,000)
  - Transcription rate
  - RNA half-life (except for very short half-life RNAs, e.g. cMyc)
- Limits of the RNase H1 cellular processes

RNA Degradation Rates			
Intrinsic Cellular RNA Degradation rates (kb/min/cell)		RNase H1 ASO-Induced RNA degradation rates (kb/min/cell)	
Nucleus	Cytoplasm	Nucleus	Cytoplasm
2.7	2.0	7.9-11.0	3.7-7.0

Liang, L, et al. Nat Biotechnol 34(8):875-80, 2016; Crooke, ST, et al. Nature Biotech in press, 2016; Wang, S., et al. Nucleic Acid Res Jul 4. pii: gkw595. 2016; Lima, WF, et al. Nucleic Acid Res 44(11):5299-312, 2016; Lima, WF, et al, Nucleic Acid Res 44(7):3351-63, 2016; Vickers, TA, and Crooke, ST. Nucleic Acid Res 43(18):8955-63, 2015; Vickers, TA, et al. PLos One 9(10):e110615, 2014; Vickers, T and Crooke, ST. PLos One 9(10):e108625, 2014; Lima, W, et al. PLos One (7)9:e101752, 2014; Wu, H, et al. PLos One 8(8):e71006, 2013; Lima, WF, et al. The RNase H Mechanism. In Antisense Drug Technology:Principles, Strategies, and Applications, Second Edition, Crooke, ST. (ed) Baco Raton, FL, Chapter 2, 47-74, 2008.

New Antisense Mechanisms Selectively Increase Target Protein Levels

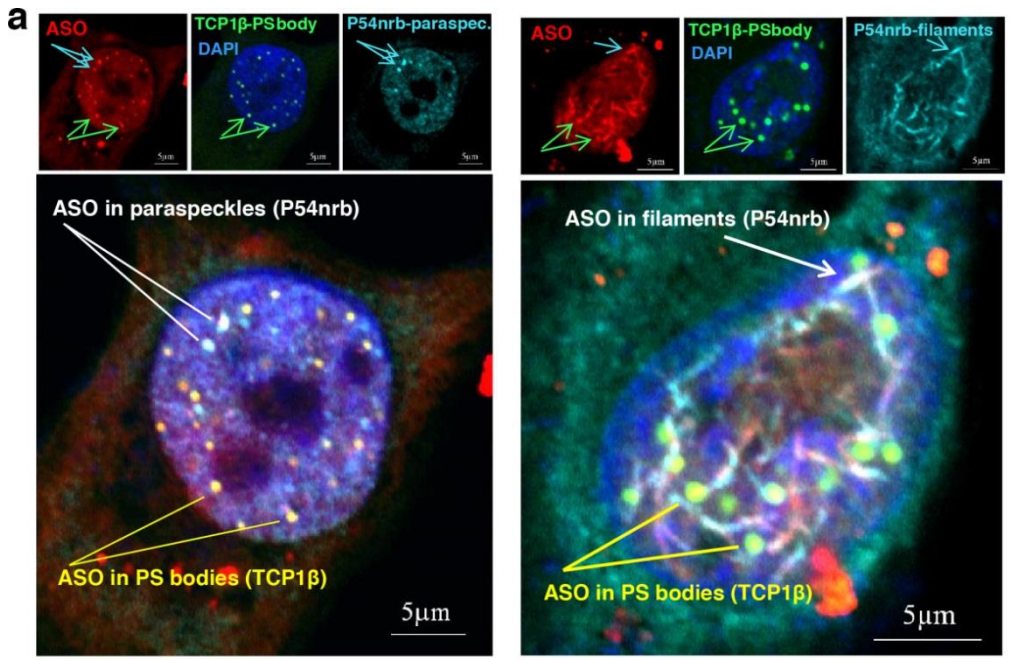
Many, if not most, mRNAs regulate their own translation

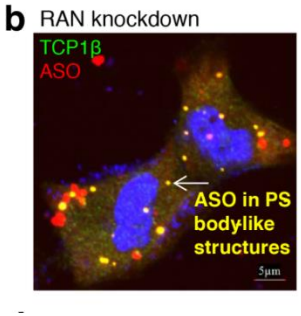
- Sequence and structural motifs throughout mRNAs appear to play key roles in regulating translation

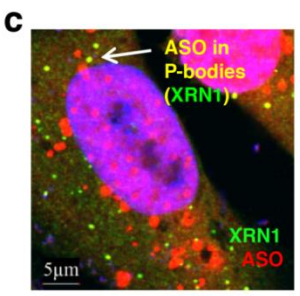
Targeting upstream RNA elements provides a novel approach to increase translation of specific proteins

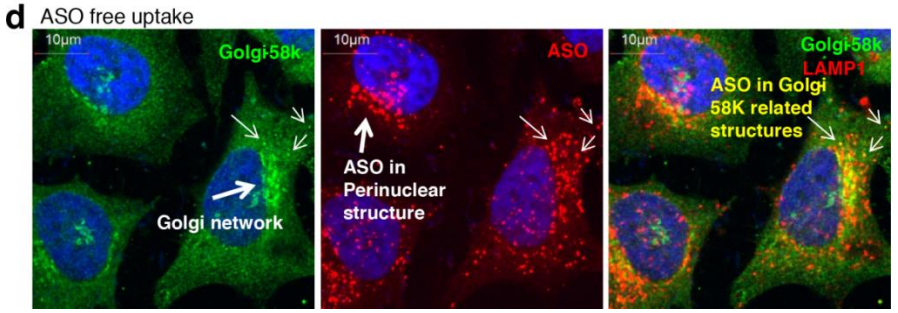
- Targeting upstream open reading frames (uORFs) and translation suppression elements (TSEs) with antisense increases functional proteins through unique mechanisms
- Together these mechanisms down regulate translation of 70-90% of mRNA transcripts in the cell

Some sites of subcellular localization of PS ASOs





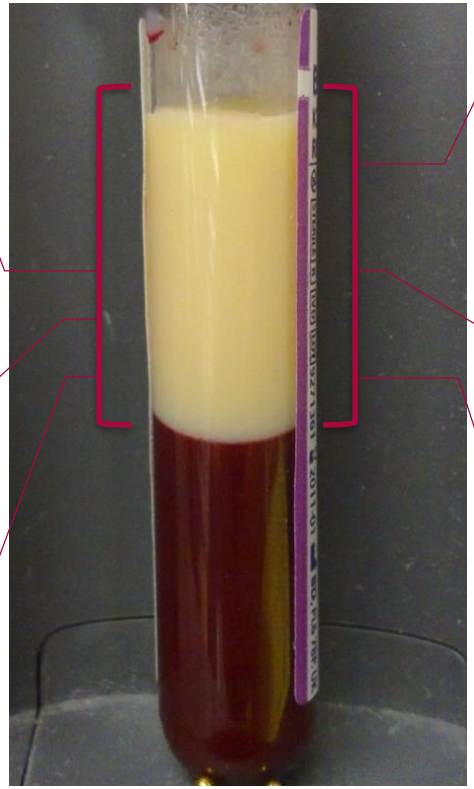




Liang et al, NAR 42(12):7819-32, 2014; Shen et al, NAR 42(13):8648-62, 2014; Liang et al, NAR 43(5):2927-45, 2014; Shen et al, NAR 43(6) 2015; Liang et al, NAR 2014;42(12):7819-32; Shen et al, NAR 2014;42(13):8648-62; Liang, et al, in preparation; Crooke, ST., et al, 2016 Nat. Biotech, in press.

FCS is a Life-threatening Disease with Multiple Severe Daily Manifestations

Extreme Triglyceride Accumulation Forms Fat Layer in Blood



Physical Manifestations
- Acute pancreatitis
- Diabetes
- Fat deposits under skin
- Abdominal pain
- GI disturbances

Neurological symptoms
- Cognitive impairment
- Memory loss
- Mild dementia
- Confusion
- Fatigue

Financial burden
- Days missed from work
- ER visits
- Hospitalization

Emotional burden
- Anxiety
- Depression
- Guilt
- Social isolation

Severe dietary restrictions
- ~10g of total fat daily

Severe quality of life burden on patients and their families

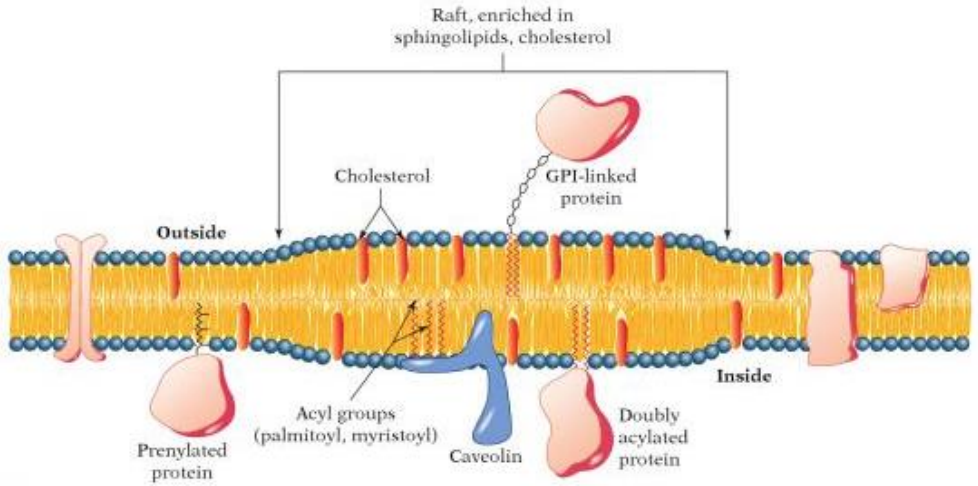
Data-based Testable Molecular Models for all Facets of ASO Activities

Membrane Lipid Composition Can Affect ASO Uptake/Activity

Major membrane components

- Sterols (cholesterol is the major sterol)
- Fatty acids
  - 12-24 carbons
  - Saturated or unsaturated
- Positively charged phospholipids
  - Phosphatidylcholine
  - Sphingolipids
- Negatively charged phospholipids
  - Phosphotidyl inositol
  - Phosphadidyl ethanolamine
  - Phosphotidy serine
  - Amdiolipin
- Integral membrane proteins
- Membrane associated proteins

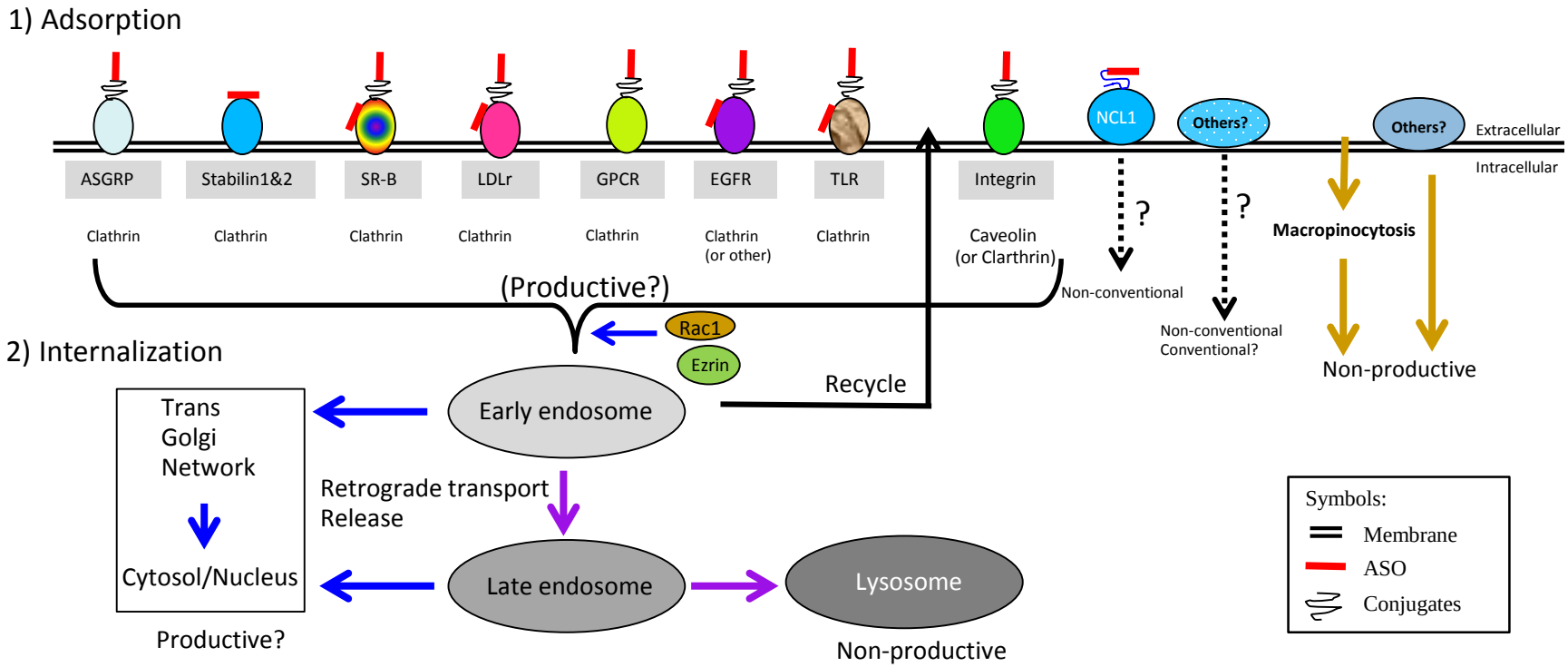
Lipid rafts



- **Signaling lipids such as free fatty acids and their derivative species like ceramide generally facilitate ASO potency**
- **Structural lipids such as phosphatidylcholine (PC) and phosphatidylserine (PS) play minor roles in ASO activity**

Wang, S., et al, Submitted.

ASO adsorption and uptake

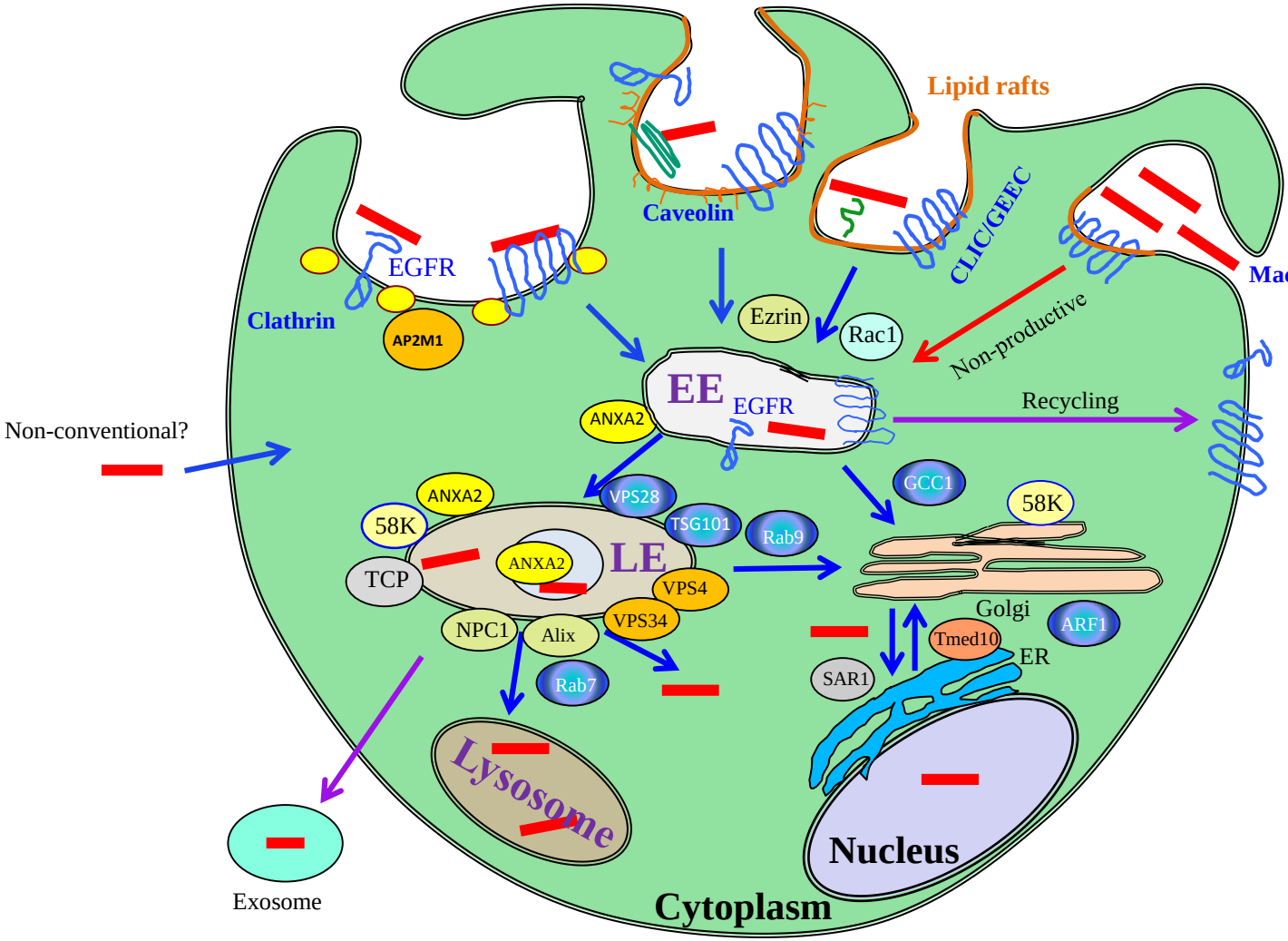


→ **ASOs taken up via receptor-mediated pathways: productive**

→ **ASOs taken up via macropinocytosis: unproductive**

Crooke, ST., et al, 2016 Nat.Biotech, in press; Wang, S., et al, Submitted.

Model of ASO Uptake and Subcellular Transport



Non-productive

Reduction:

VPS28	GCC1
TSG101	Rab9
Rab7	ARF1

increased ASO activity

Productive

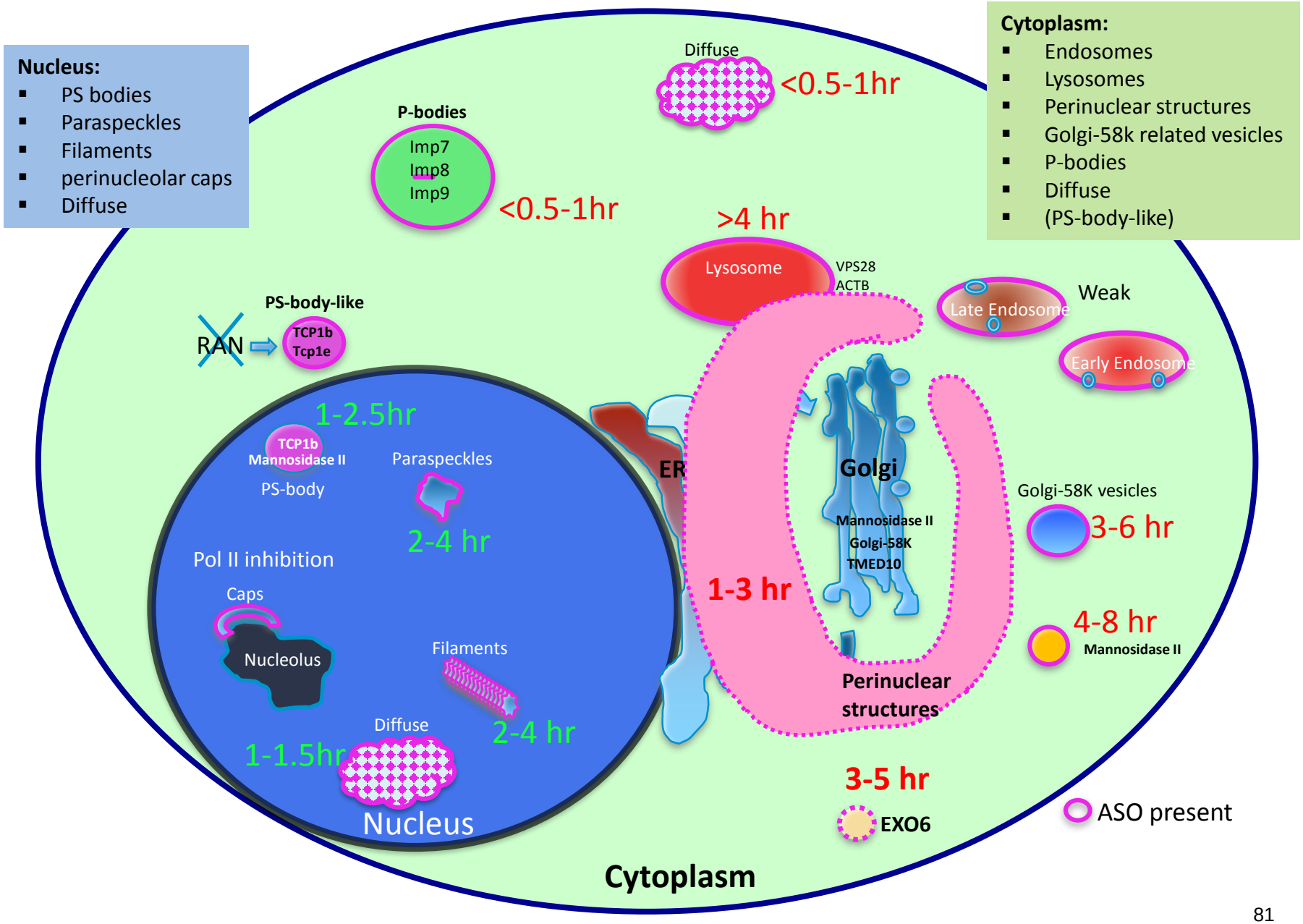
Reduction:

ANXA2	VPS4
58K	VPS34
Alix	SAR1
Rac1	TMED10
Ezrin	EGFR

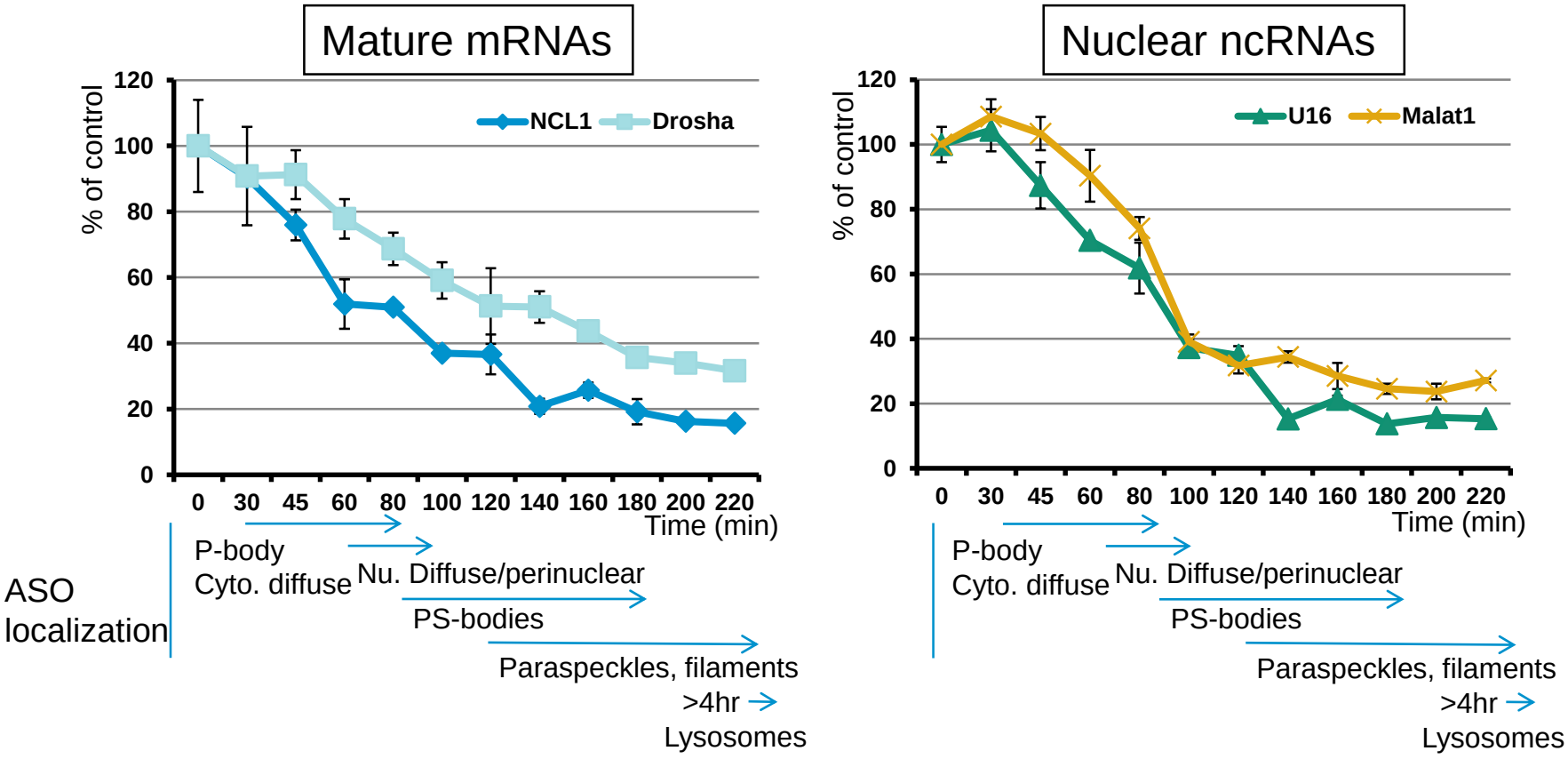
reduced activity

Koller E. et al., NAR, 2011. 39(11):4795-807; Wagenaar TR, et al., NAR, 2015;43(2):1204-15; Crooke ST., et al., Nat. Biotech., 2017, in press; Wang S. et al., NAR, 2016; 44(15):7314-30.

ASO subcellular localization upon transfection



The Kinetics of ASO Activity Upon Transfection



Kinetics: activity correlates with P-body/nuclear localization of ASOs

Crooke, ST., et al, 2016 Nat. Biotech, in press.

A Path to Toxicity

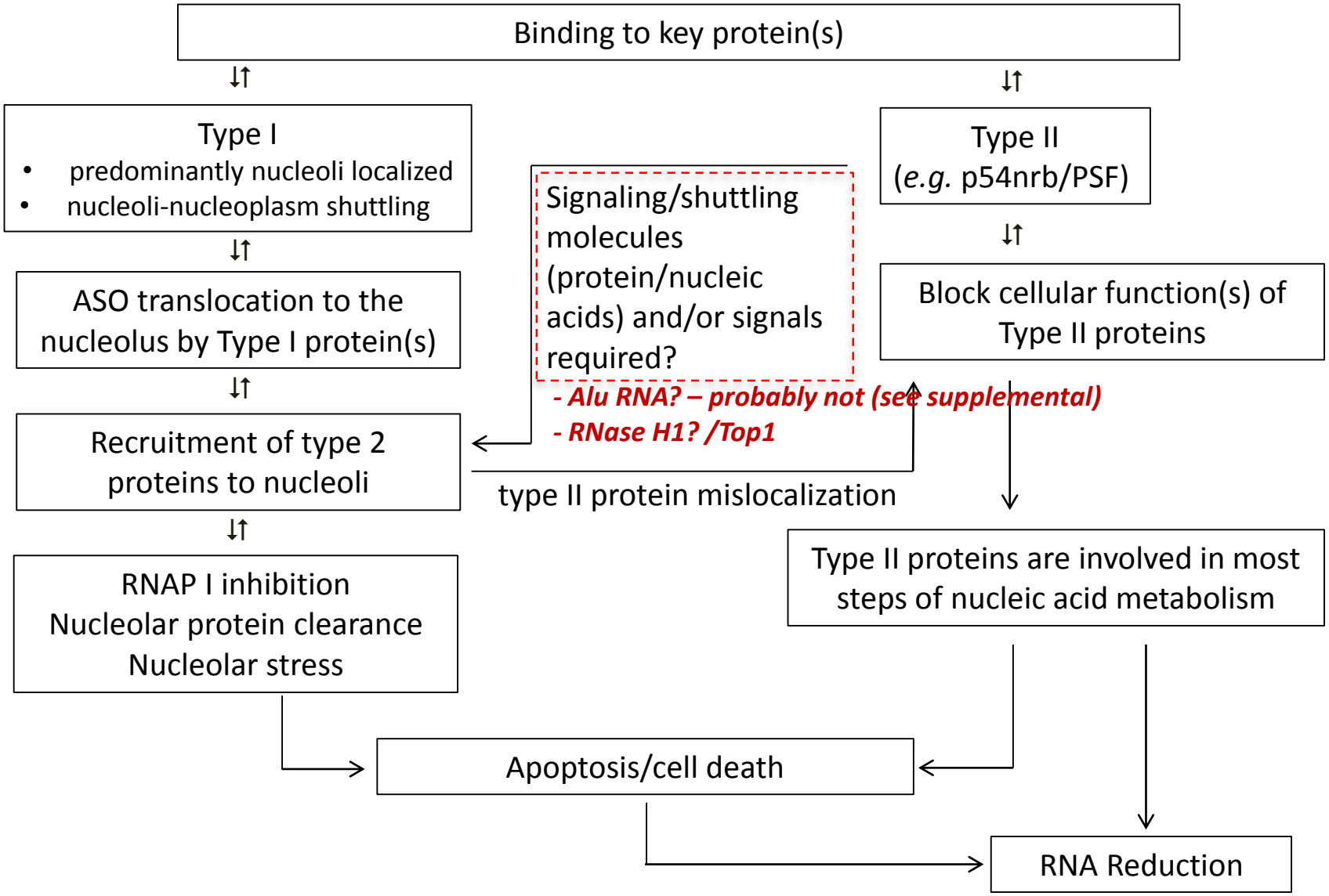
ASO-Protein Interactions Contribute to the Cyto- and Hepato- Toxic Potentials of PS-ASOs Candidate Proteins

Type	Candidates	Characteristics	Measurements
I	Nucleolar proteins: NCL1*	• predominant nucleolar localization • can shuttle dynamically: nucleolus-nucleoplasm • responsible for ASO localization to nucleoli	• loss of ASO localization to nucleoli upon type I protein depletion
II	p54nrb/PSF/ PSPC1/FUS	• preferential association with toxic PS-ASOs • mislocalization to nucleoli due to protein-ASO interaction	• nucleolar localization in the presence of toxic ASOs
III	RNase H1* /Top1*	• connecting type I and II proteins • localize to and dynamically shuttle between nucleolus and nucleoplasm	• loss of nucleolar localization of type II proteins upon type III protein depletion • nucleolar localization of ASOs retained in type III protein depleted cells

** speculated candidate proteins*

Shen, W., et al, manuscript in preparation.

A Path to Toxicity



Shen et al, manuscript in preparation.

The RNase H1 mechanism: an example of the molecular details now understood

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- Lima, W.F., Wu, H. and Crooke, S.T. The RNase H Mechanism. *In Antisense Drug Technology: Principles, Strategies, and Applications*, Second Edition, Crooke, ST. (ed) Baco Raton, FL, Chapter 2, 47-74, 2008.
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The RNase H1 mechanism: an example of the molecular details now understood

- Lima, W.F., Rose, J.B., Nichols, J.G., Wu, H., Migawa, M.T., Wyrzykiewicz, T.K., Vasquez, G., Swayze E.E. and Crooke, S.T. The positional influence of the helical geometry of the heteroduplex substrate on human RNase H1 catalysis. *Mol Pharmacol* 71:73-82, 2007.
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- Miraglia, L., Watt, A.T., Graham, M.J. and Crooke, S.T. Variations in mRNA content have no effect on the potency of antisense oligonucleotides. *Antisense and Nuc Acid Drug Dev* 10:453-461, 2000.

PS-ASO Protein Interactions
General Observations

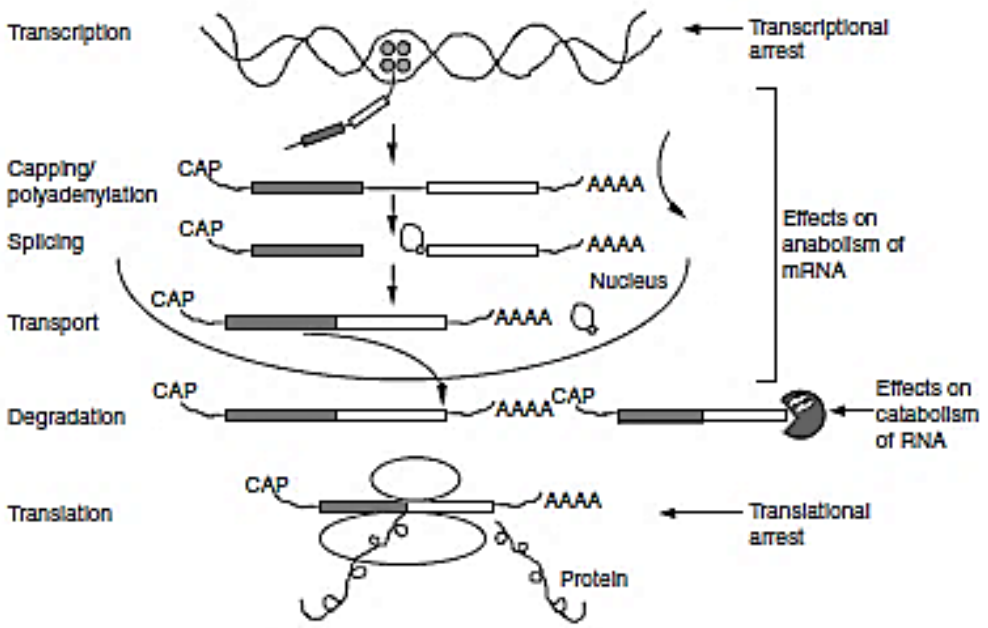
- Cell surface proteins determine whether PS-ASOs enter "productive" uptake pathways
- A limited number of cellular proteins bind PS-ASOs
- Protein migration is responsible for PS-ASO migration in cells
- Proteins can inhibit ASO activity
  - Competition with RNase H1, eg. Ku 20/80, splicing factors
  - Other mechanisms (Hsp90)
- Proteins can enhance PS-ASO activity
  - Cellular localization
  - Facilitation of hybridization?
- Proteins are responsible for the active import and export of PS-ASOs to the nucleolus
- Nucleolin shuttles PS-ASOs in and out of the nucleolus

Vickers, T., Crooke, ST., PLoS One 11(8) 1-17, 2016; Crooke, ST., et al, Nat. Biotech, in press, 2016.

ASOs alter the intermediary metabolism of RNA

RATES MATTER

- What is the rate limiting step in the intermediary metabolism of the RNA?
- Steps in ASO drug action are all dramatically slower than for small molecules



Antisense drug action can be rationalized by traditional receptor theory and pharmacological principals