Biodegradable – Engineering the Stability of Proteins

Mario Hermann

Journal Club

11/02/2014

Experimental manipulation of proteins

DNA → RNA → Protein

Gene knockout
- Gene-targeting - mouse ES cells
- Genome editing in embryos & cell lines

Transgenes for overexpression
- native proteins
- mutant protein versions
- fusion-proteins

Artificial transcription factors
- modulation of gene expression

RNA interference
- knockdown mainly in cultured cells/slices

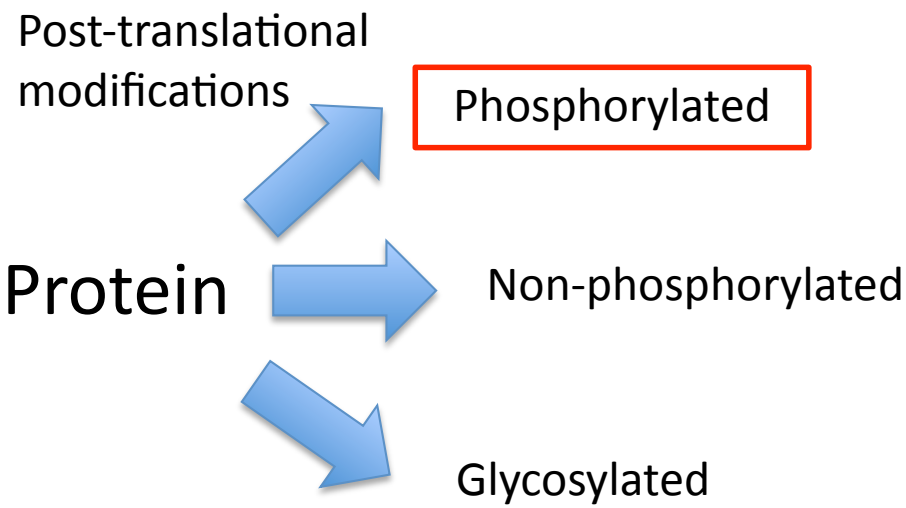
RNA aptamers/ ribozymes
- rate of translation
- splicing
- mRNA degradation

Small molecules
- inhibitors
- agonists
- modulators

Antibodies
- activating
- inhibiting
- clearing

Recombinant proteins/ synthetic peptides

Experimental manipulation of proteins

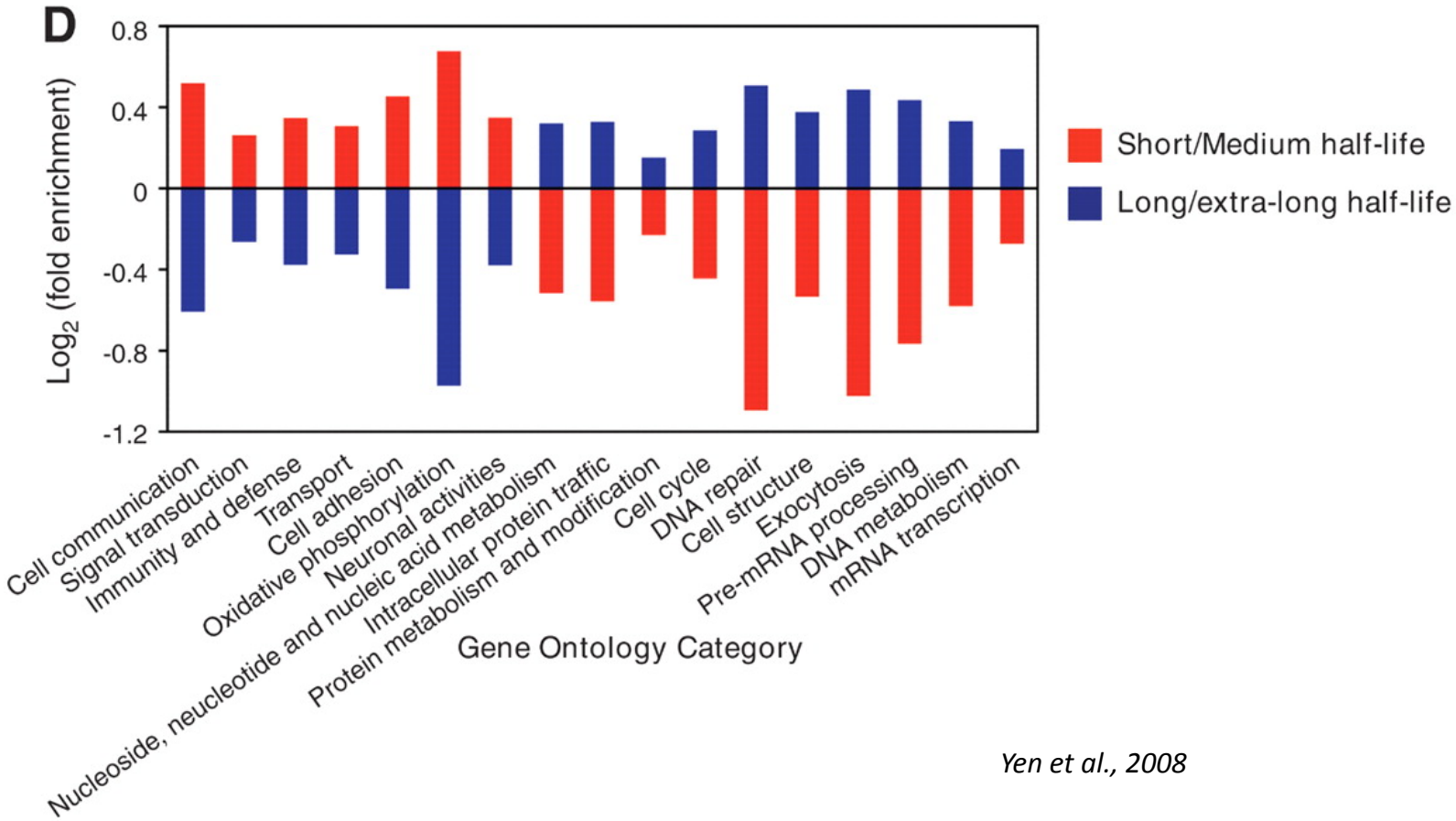


Some numbers related to protein turn-over

- Average protein half-lifes
  - Yeast (cell cycle 1.5h): 43 min
  - Mammalian cells (cell cycle 24h)
    - Dividing: 0.5 – 35h
    - Non-dividing: 43h
  - Mouse (brain, liver, blood): 3 -9 d
- Time scales of minutes to years

Toyama & Hetzer, 2013

Rate of protein turn-over correlates with protein function



Yen et al., 2008

Extremely long-lived proteins

Table 1 | **Known long-lived proteins and molecules**

Protein or molecule*	Age‡	Measure	Organism	Method
Eye lens crystallin	>70 years	Lifetime	Human	L-/D-Asp racemization
Collagen	117 years	Half-life	Human	L-/D-Asp racemization
Elastin	>78 years	Lifetime	Human	L-/D-Asp racemization
Enamel and dentine	>70 years	Lifetime	Human	L-/D-Asp racemization
Histones	223 days	Half-life	Mouse	Radio isotope pulse-labelling
	117 days	Half-life	Mouse	Radio isotope pulse-labelling
	218 days	Half-life	Rat	Radio isotope pulse-labelling
Nuclear pore proteins	>1 month	Lifetime	Worm	Radio isotope pulse-labelling
	>1 year	Lifetime	Rat	Stable isotope pulse-chase labelling and mass spectrometry
Myelin	95 days	Half-life	Rat	Radio isotope pulse-labelling
	>100 days	Half-life	Mouse	Radio isotope pulse-labelling
Myelin proteolipid protein	>100 days	Half-life	Mouse	Radio isotope pulse-labelling
REC8	Weeks	Lifetime	Mouse	Radio isotope pulse-labelling

Toyama & Hetzer, 2013

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- mRNA degradation
- splicing

Small molecule inhibitors

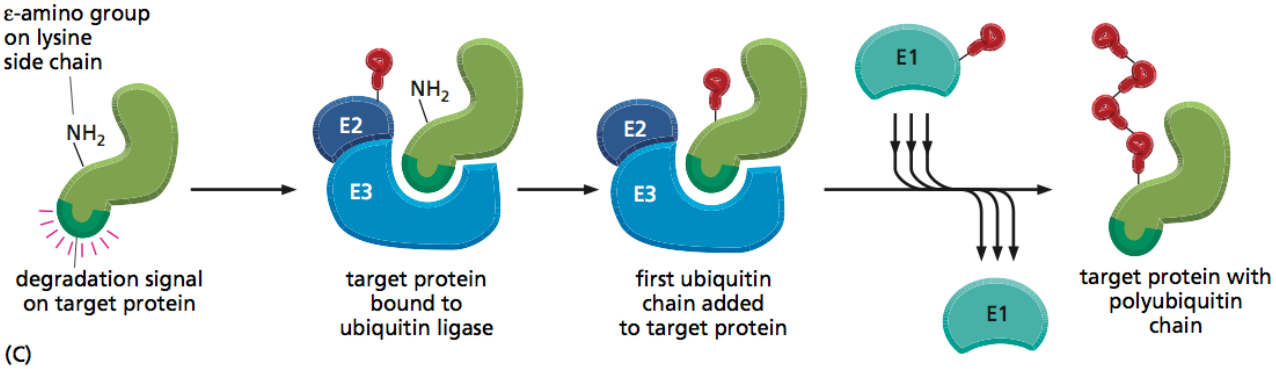
Antibodies
- activating
- inhibiting
- clearing

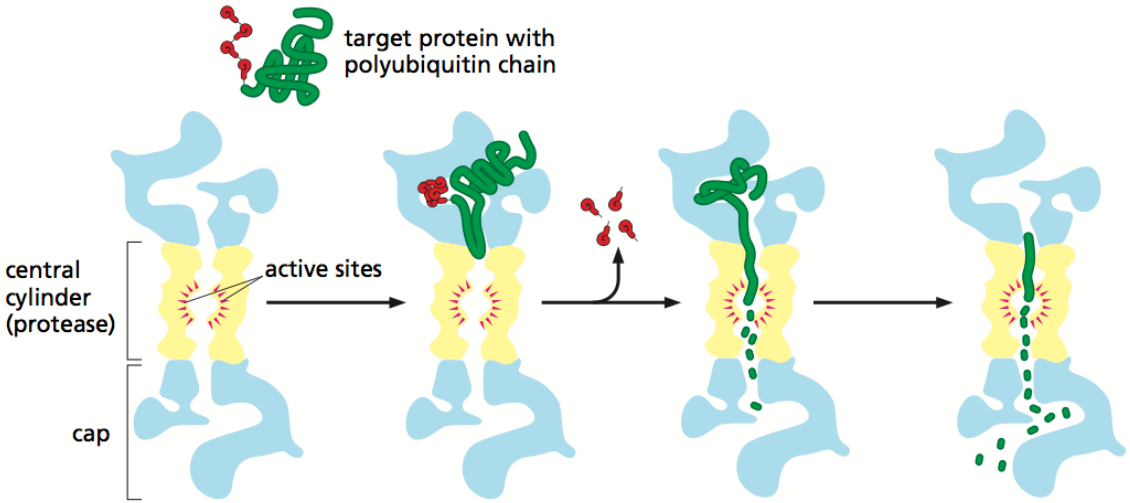
Synthetic peptides

Abundance

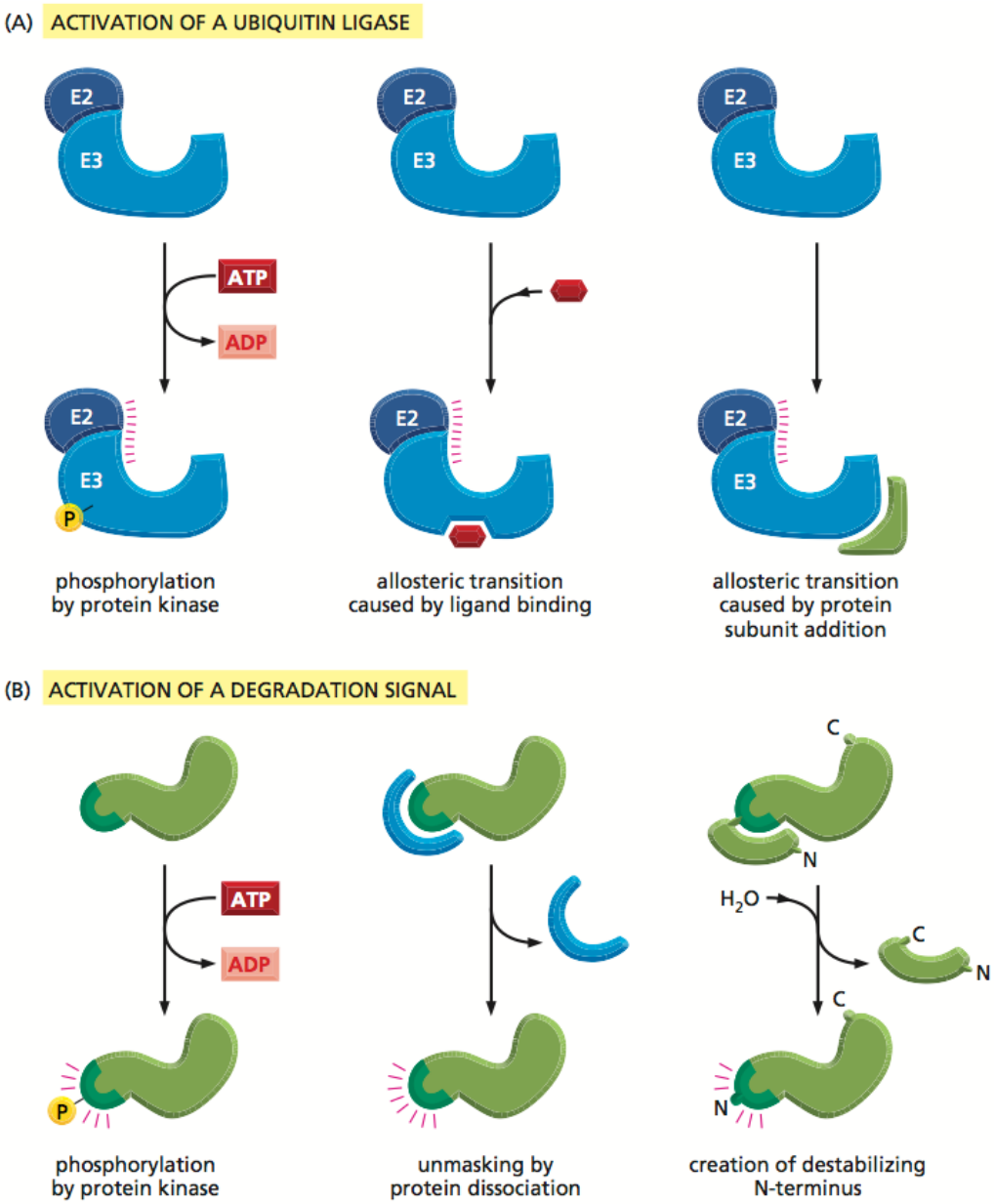
Function

Ubiquitin-proteasome protein degradation pathway



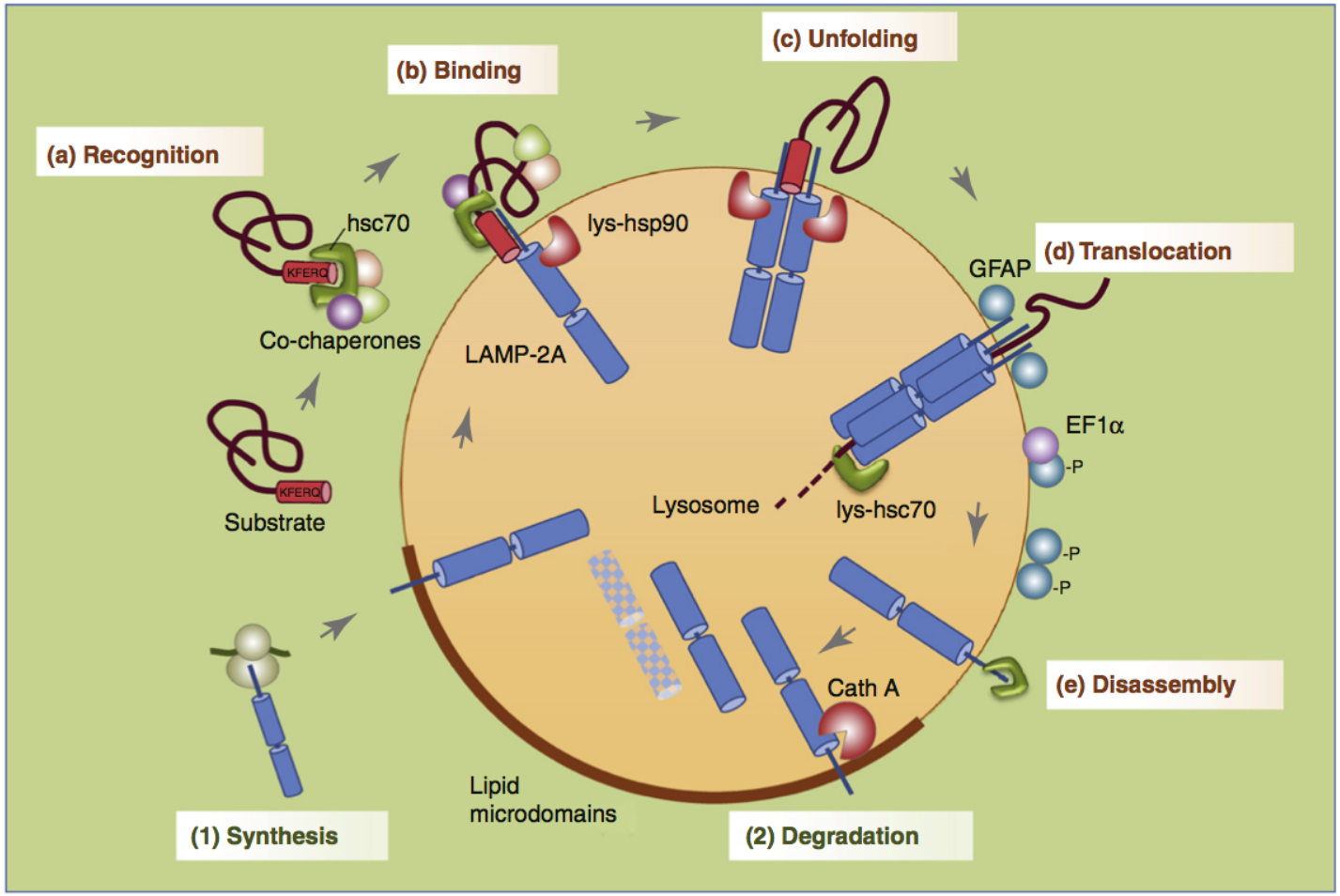


Alberts et al., Molecular Biology of the Cell, 5th Edition, 2008



Alberts et al., Molecular Biology of the Cell, 5th Edition, 2008

Chaperone-mediated autophagy - CMA



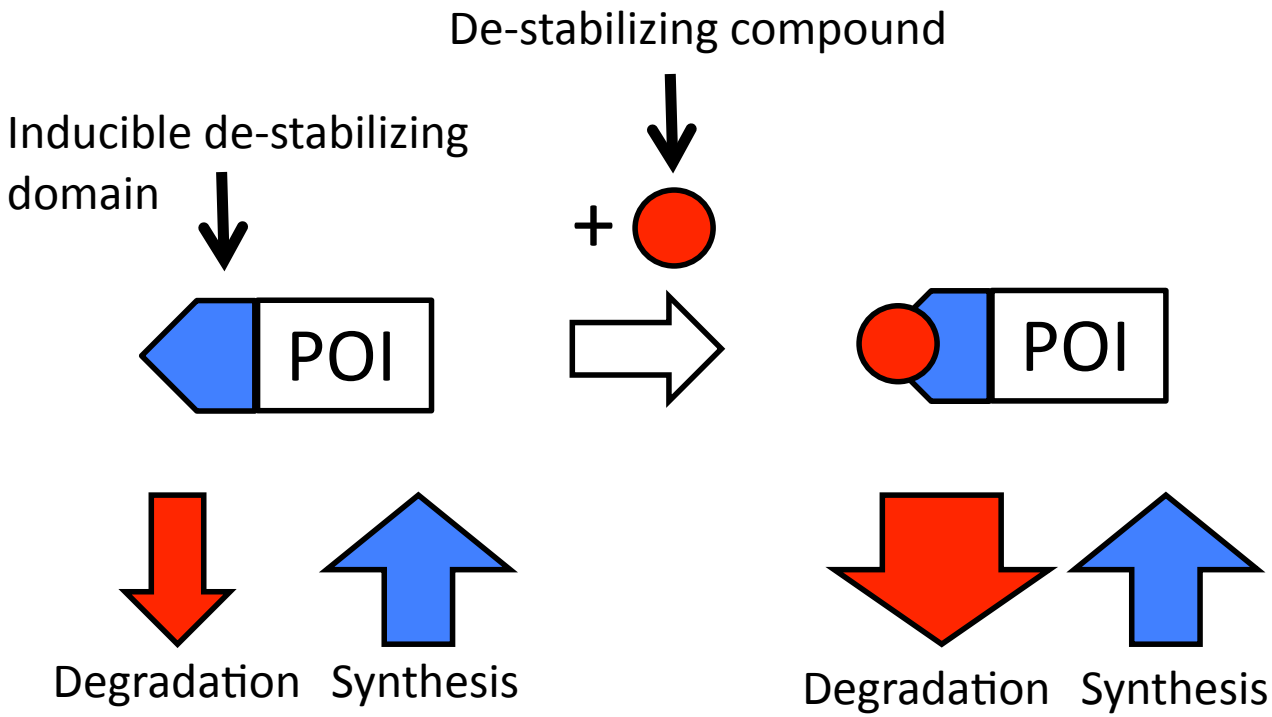
Kaushik & Cuervo, 2012

CMA-targeting motifs

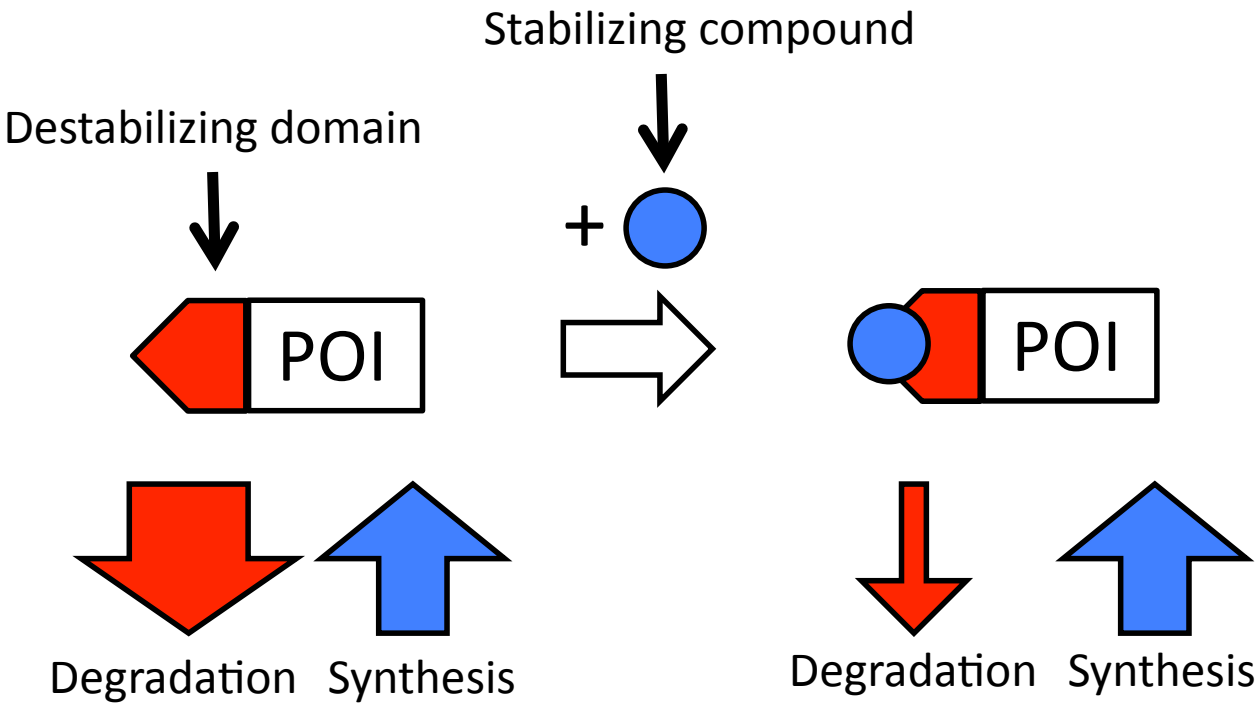
- motif based on physical properties of AA
- necessary and sufficient to target proteins for CMA
- only detected in soluble cytosolic proteins, rarely on membrane proteins
- targeting of proteins to CMA possibly regulated by
  - unfolding and exposure of motif
  - phosphorylation or acetylation can complete motif

How to experimentally direct proteins of interest towards proteasomal or lysosomal degradation?

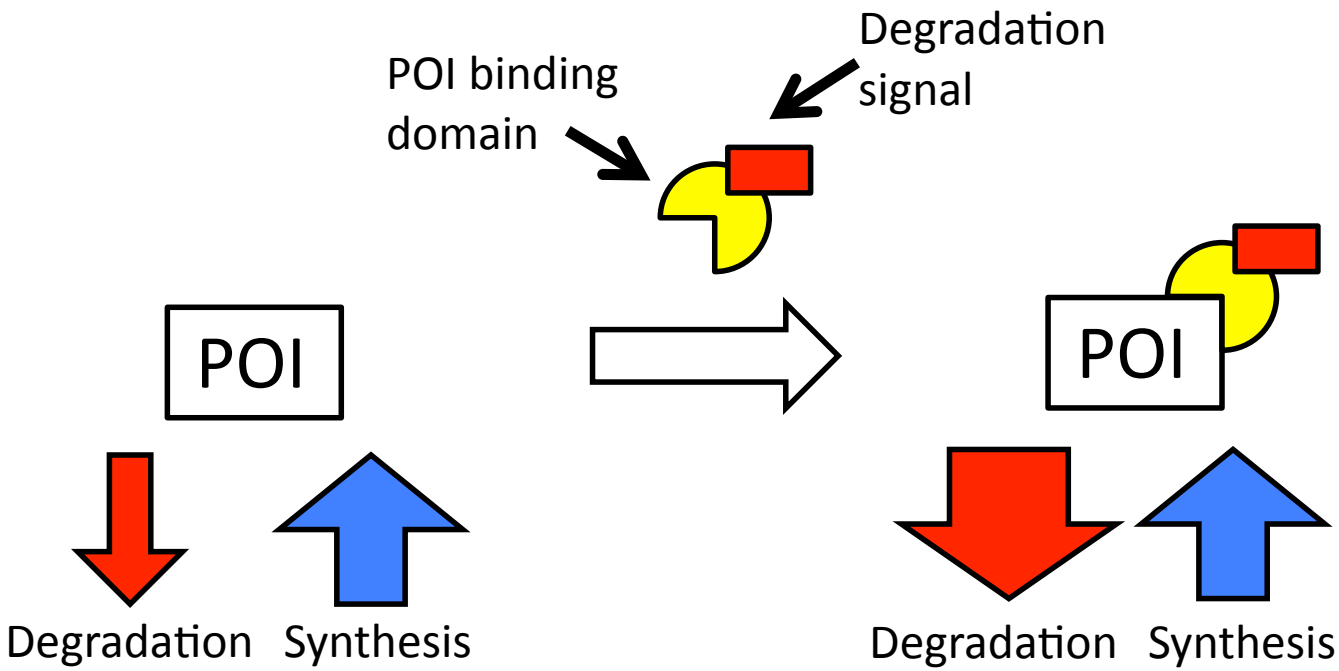
Taking control over protein degradation



Taking control over protein degradation



Taking control over protein degradation



An auxin-based degron system for the rapid depletion of proteins in nonplant cells

Kohei Nishimura[1], Tatsuo Fukagawa[2], Haruhiko Takisawa[1], Tatsuo Kakimoto[1] & Masato Kanemaki[1]

[1]Department of Biological Sciences, Graduate School of Science, Osaka University, Osaka, Japan. [2]Department of Molecular Genetics, National Institute of Genetics and The Graduate University of Advanced Studies, Shizuoka, Japan. Correspondence should be addressed to M.K. (mkanemaki@bio.sci.osaka-u.ac.jp).

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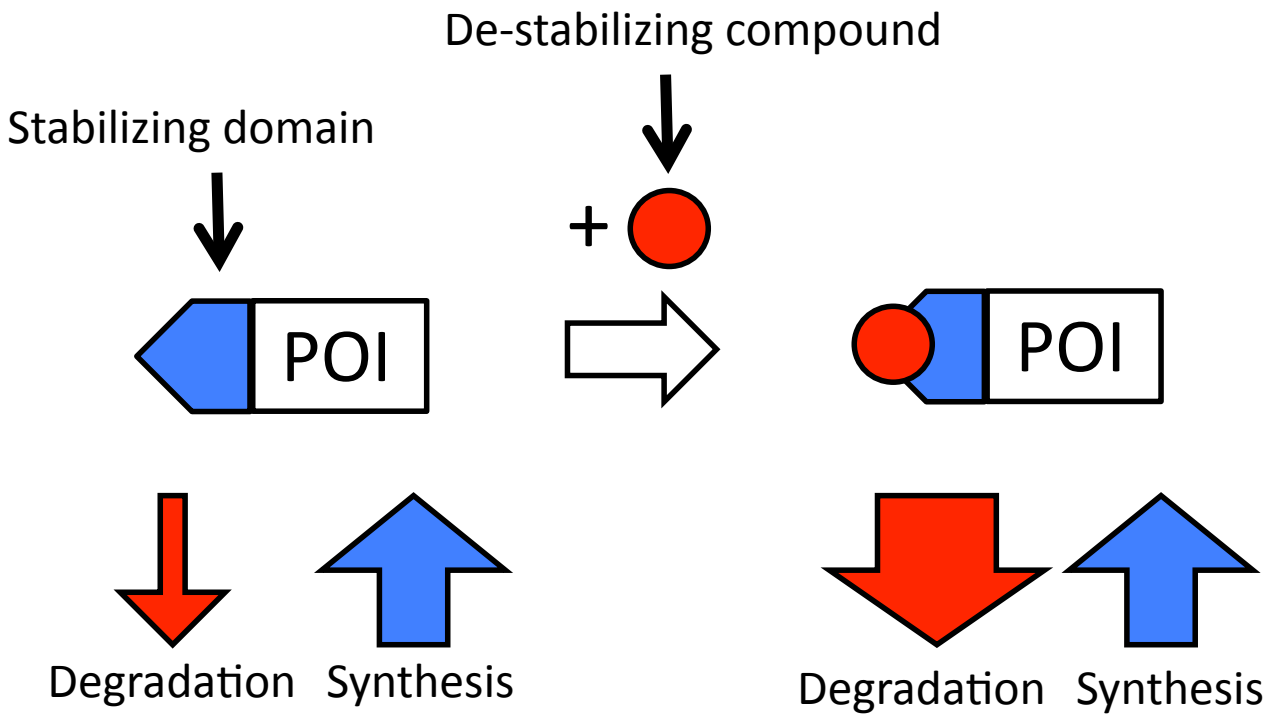
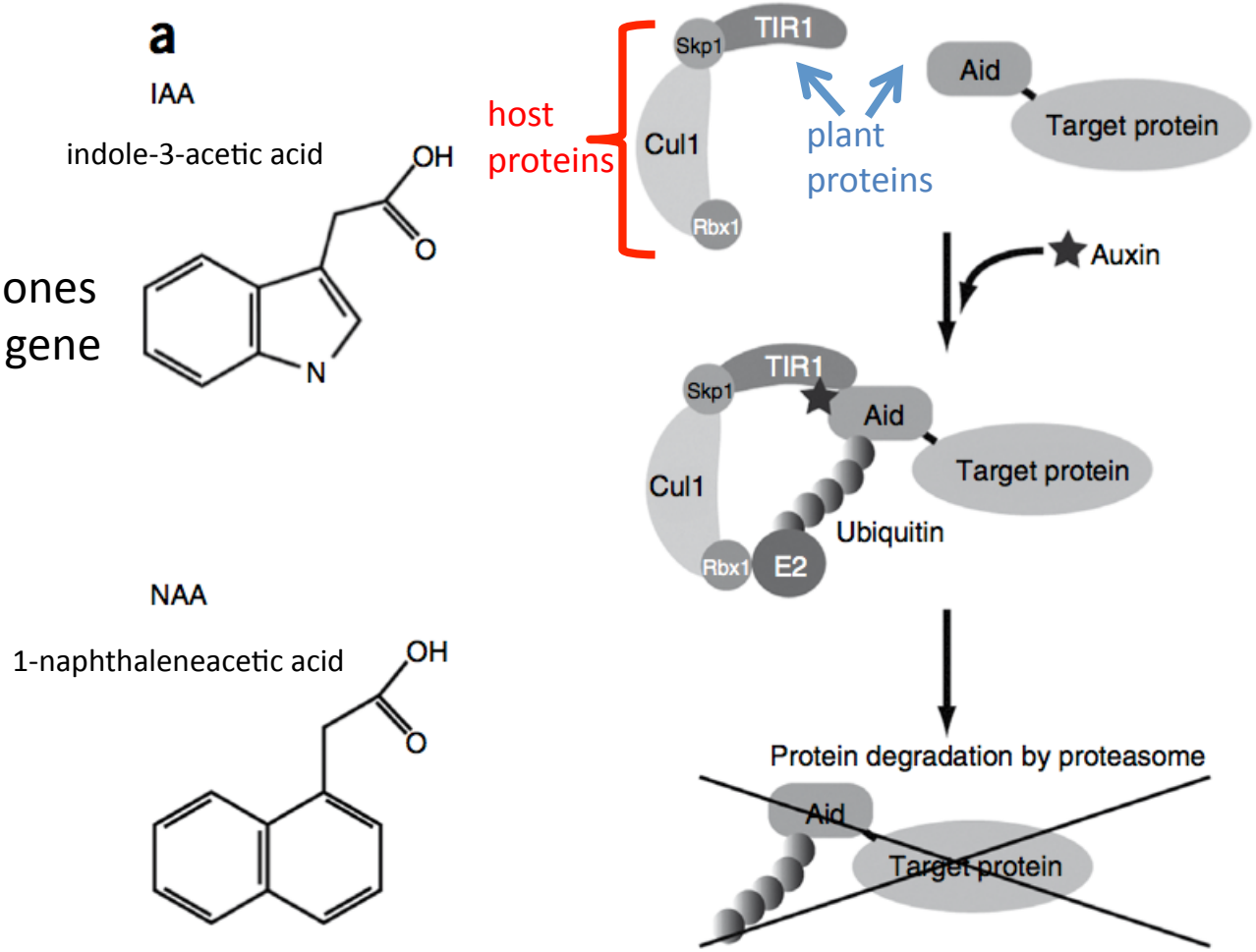


Goals of this study

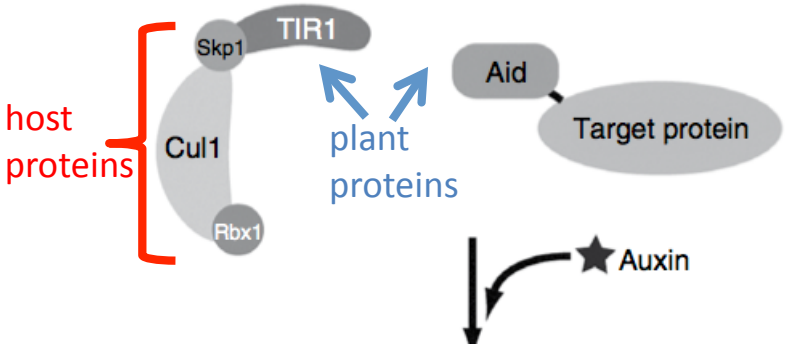
- Establish a highly efficient protein-degradation system for studying the function of cell cycle proteins

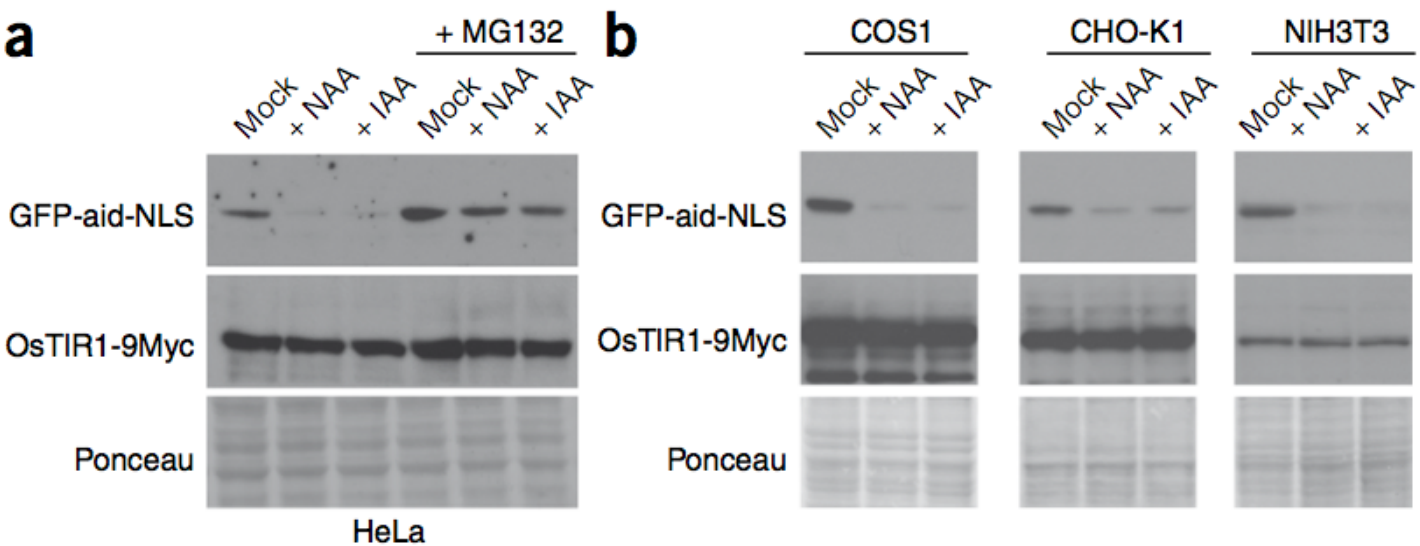
Auxin-based protein depletion

Auxins
plant hormones controlling gene expression



Auxin-based protein degradation in mammalian cells





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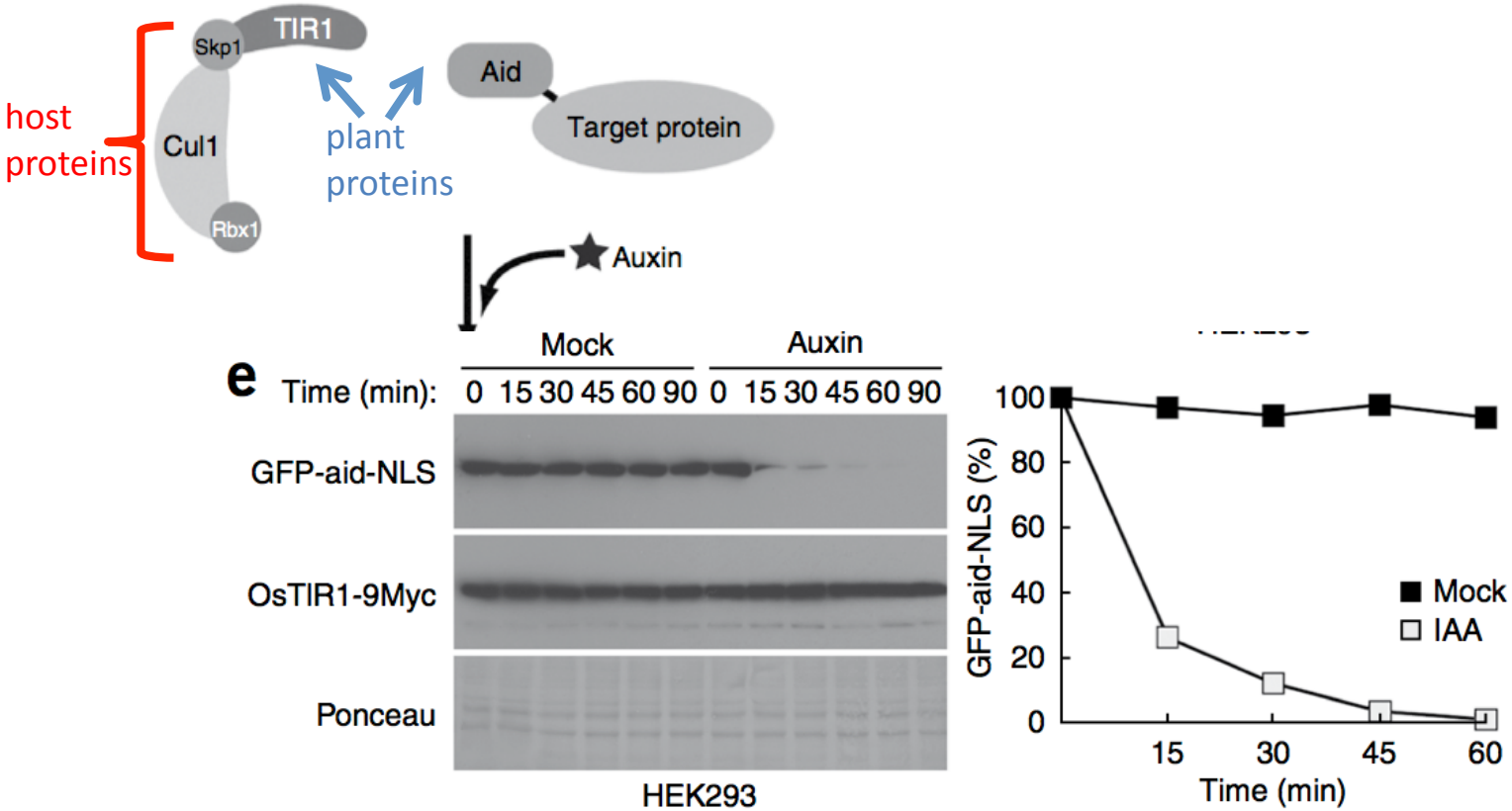
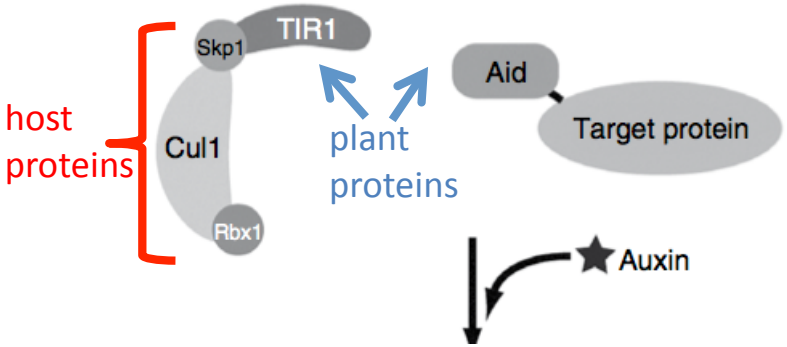
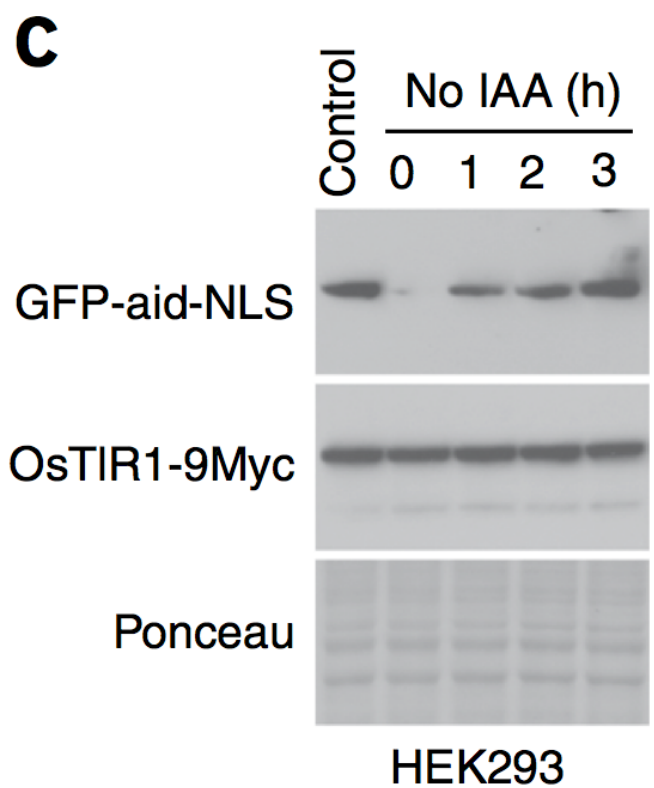


Stably expressing GFP-aid & OsTIR1

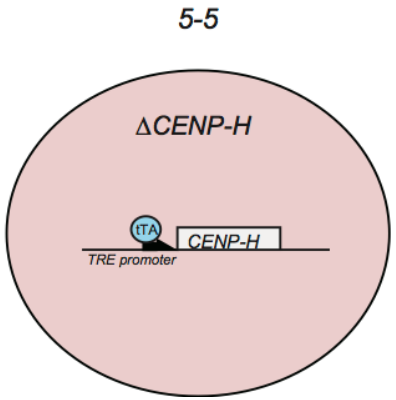
Auxin-based protein degradation in mammalian cells





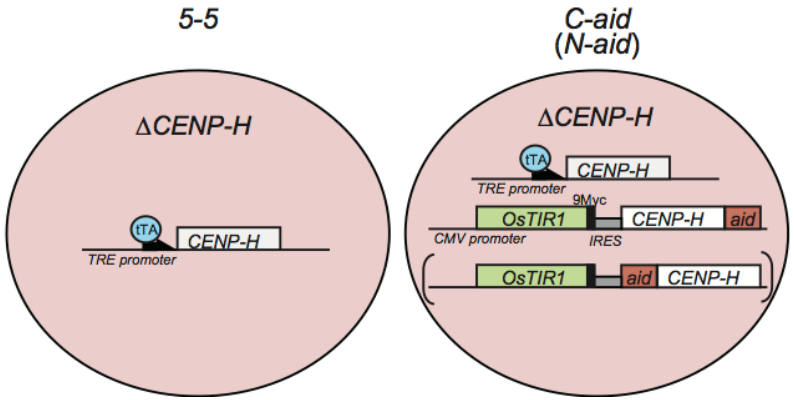
Stably expressing GFP-aid & OsTIR1
2h pre-treatment with auxin

Rapid depletion of the kinetochore protein CENP-H



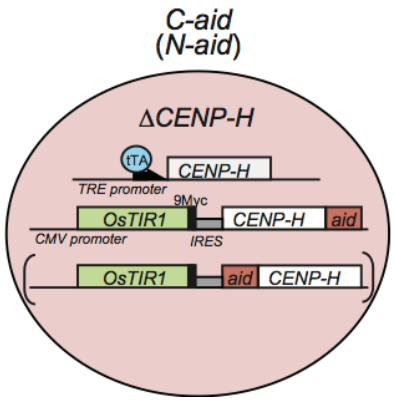
Addition of tetracycline abolishes CENP-H expression

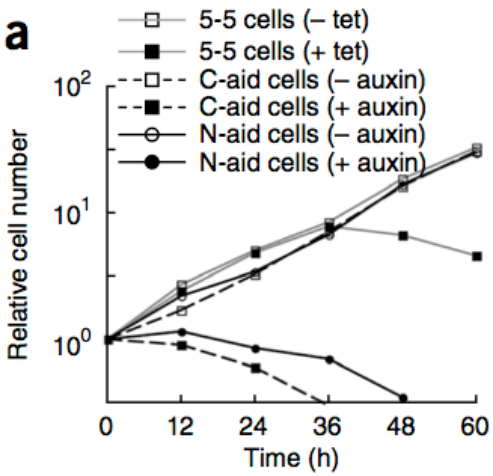
Rapid depletion of the kinetochore protein CENP-H

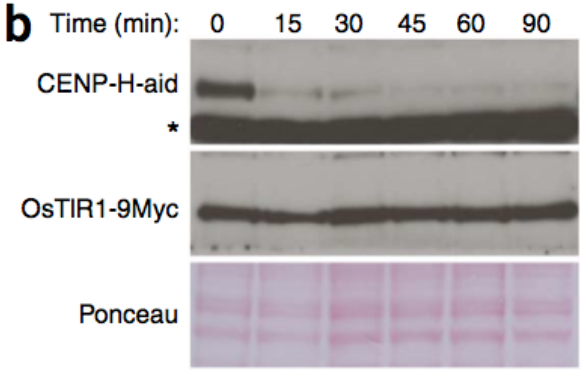


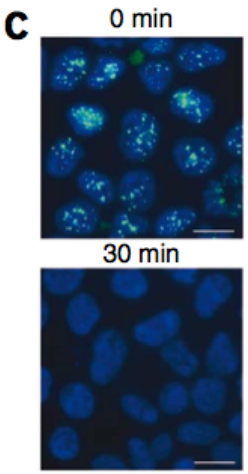
1. Addition of tetracycline abolishes CENP-H expression
2. Addition of auxin depletes CENP-H-aid protein

Rapid depletion of the kinetochore protein CENP-H

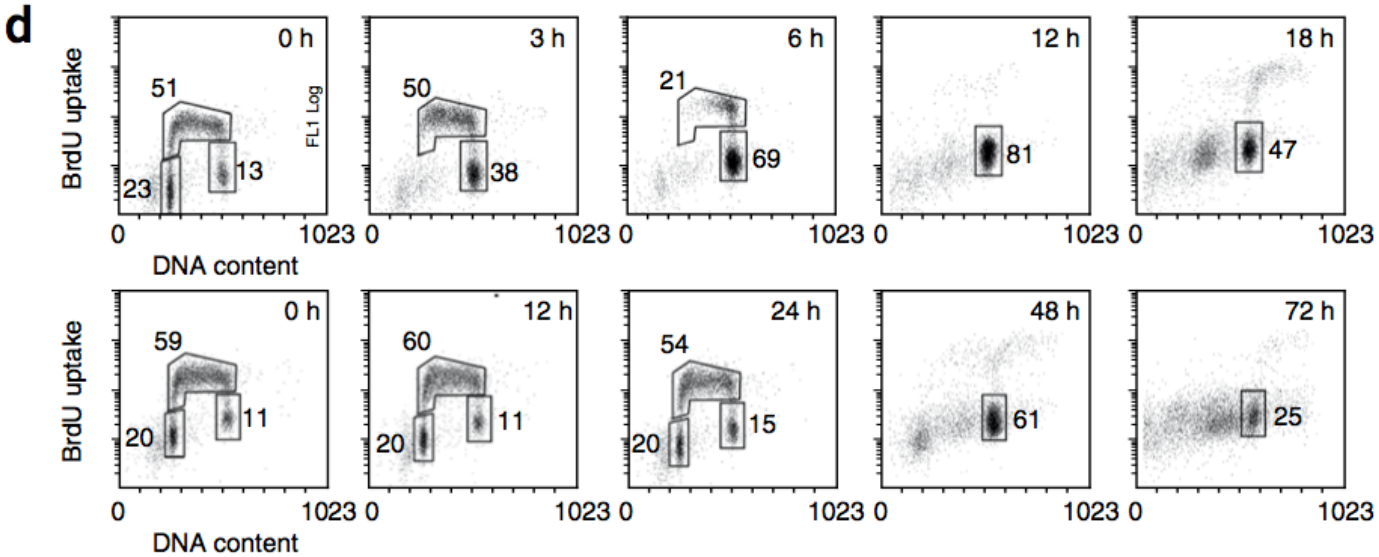








Cell cycle arrest in G2-M phase



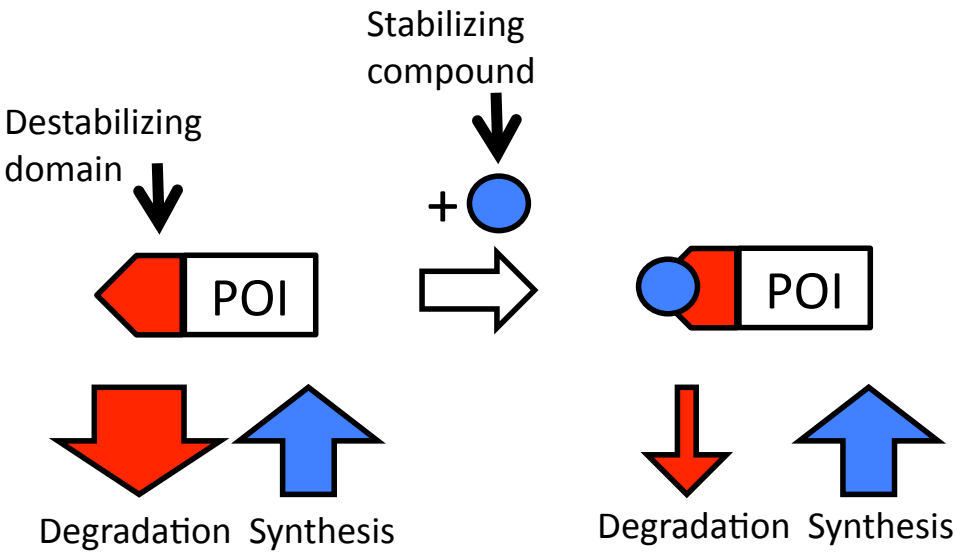
Summary

! Auxin-based protein depletion as fast as 15 min after auxin application in cultured cells

? How fast/efficient is the system in intact animals

Inducible control of gene expression with destabilized Cre

Richard Sando III[1,2], Karsten Baumgaertel[1], Simon Pieraut[1], Nina Torabi-Rander[1], Thomas J Wandless[3], Mark Mayford[1] & Anton Maximov[1]



[1]Department of Molecular and Cellular Neuroscience, The Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, California, USA. [2]The Kellogg School of Science and Technology, The Scripps Research Institute, La Jolla, California, USA. [3]Department of Chemical and Systems Biology, Stanford University, Stanford, California, USA. Correspondence should be addressed to A.M. (amaximov@scripps.edu).

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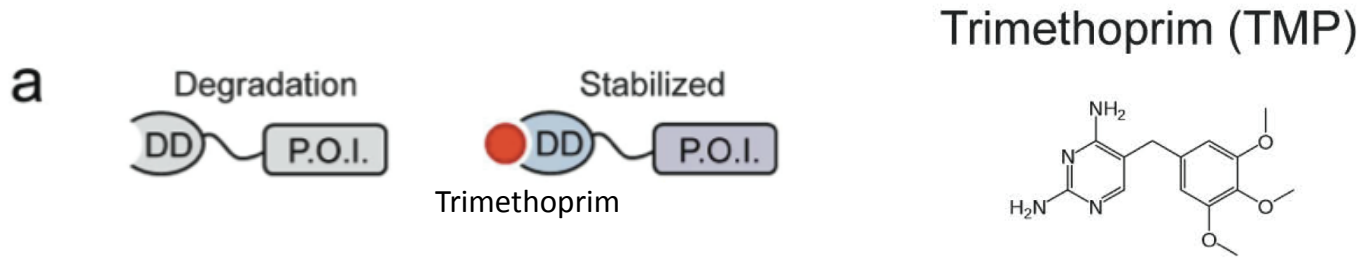
Goals of this study

- Establish a genetic approach for rapid *in vivo* silencing of synaptic transmission in the mouse brain

- Evaluate the potential of this approach for modifying bahavioral responses

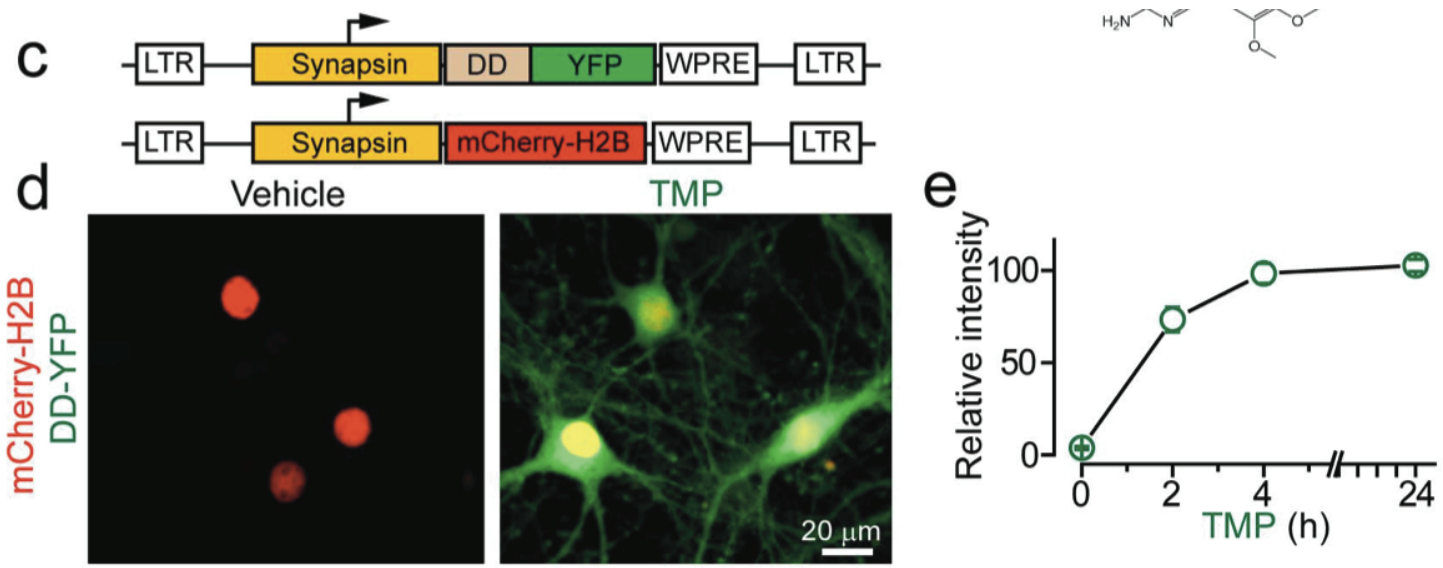
Trimethoprim-mediated protein stabilization



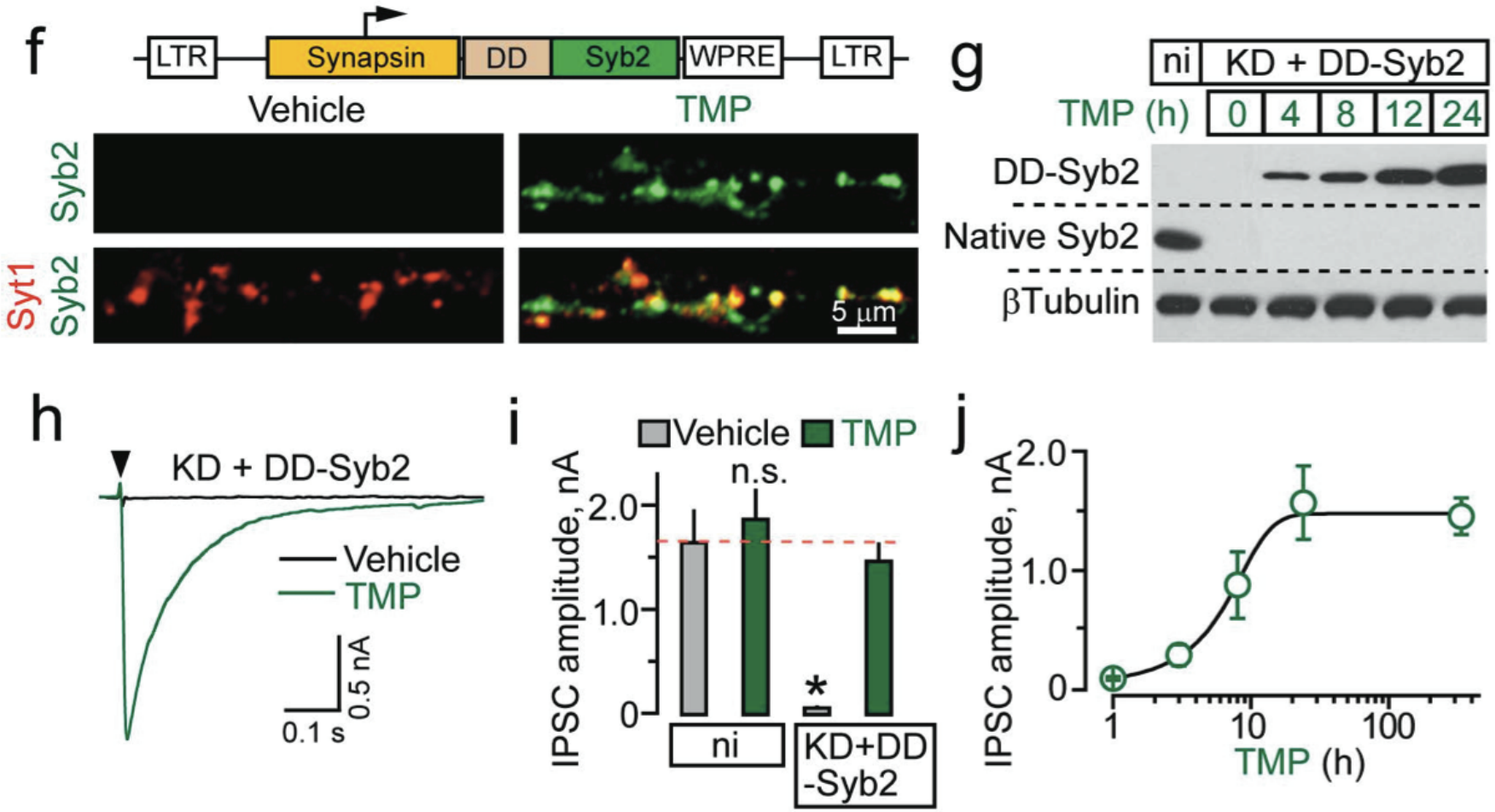
- DD = mutant destabilized *E.coli* dihydrofolate reductase – *Iwamoto et al., 2010*
  - Fused to N-terminus of proteins of interest
- Trimethoprim (TMP) specifically inhibits *E.coli* but not mammalian dihydrofolate reductase
  - Commercially available, low cost
  - Oral application, crosses blood-brain-barrier (rats)
  - After i.p. injection **peaks after 10 min** in mouse brain, **declines below detection limit after 30 min**

Stabilization of YFP in cell culture

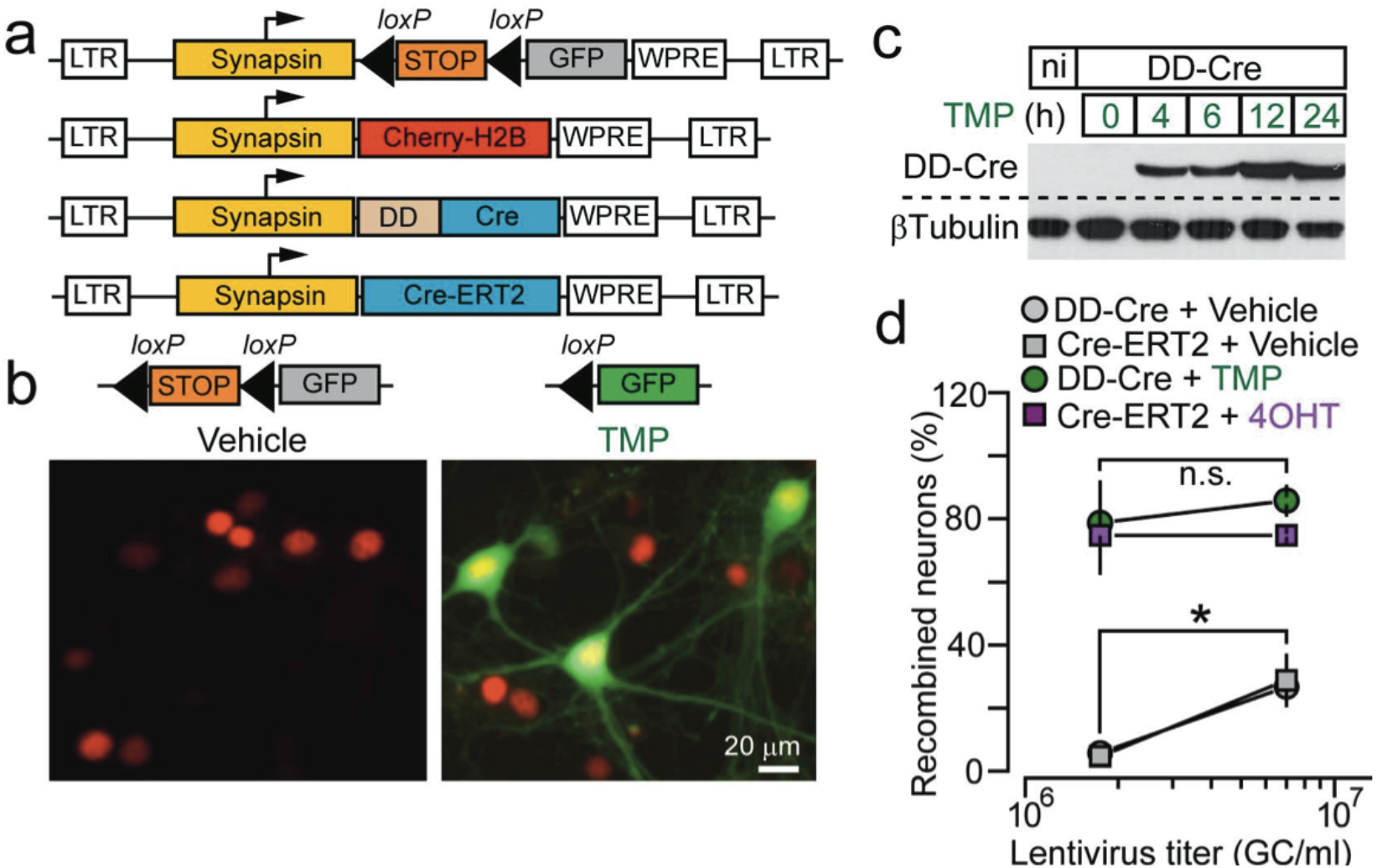
- Mixed cortical cultures infected with lentiviruses
- Continuous TMP treatment



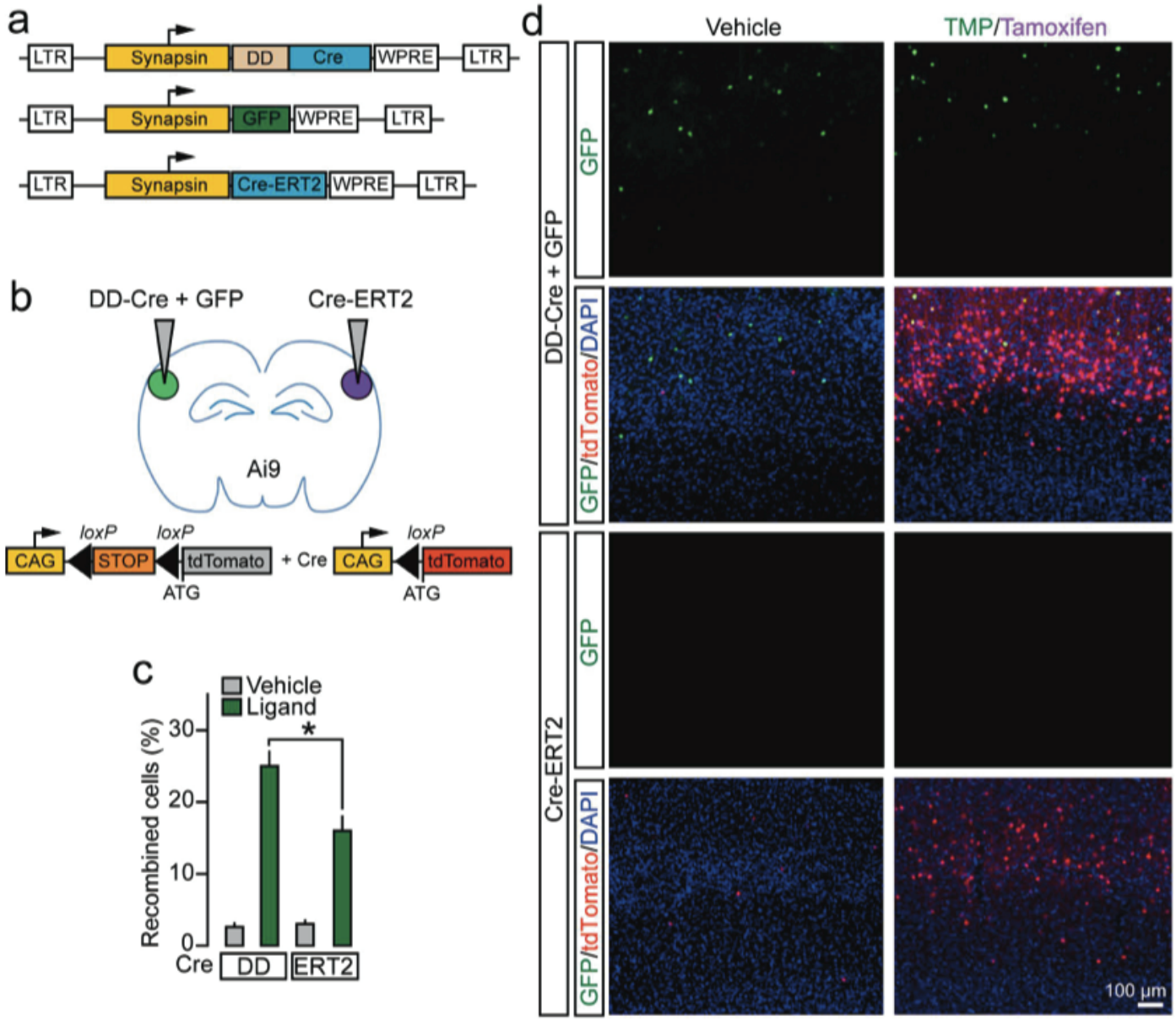
Stabilization of synaptic vesicle SNARE protein Syb2



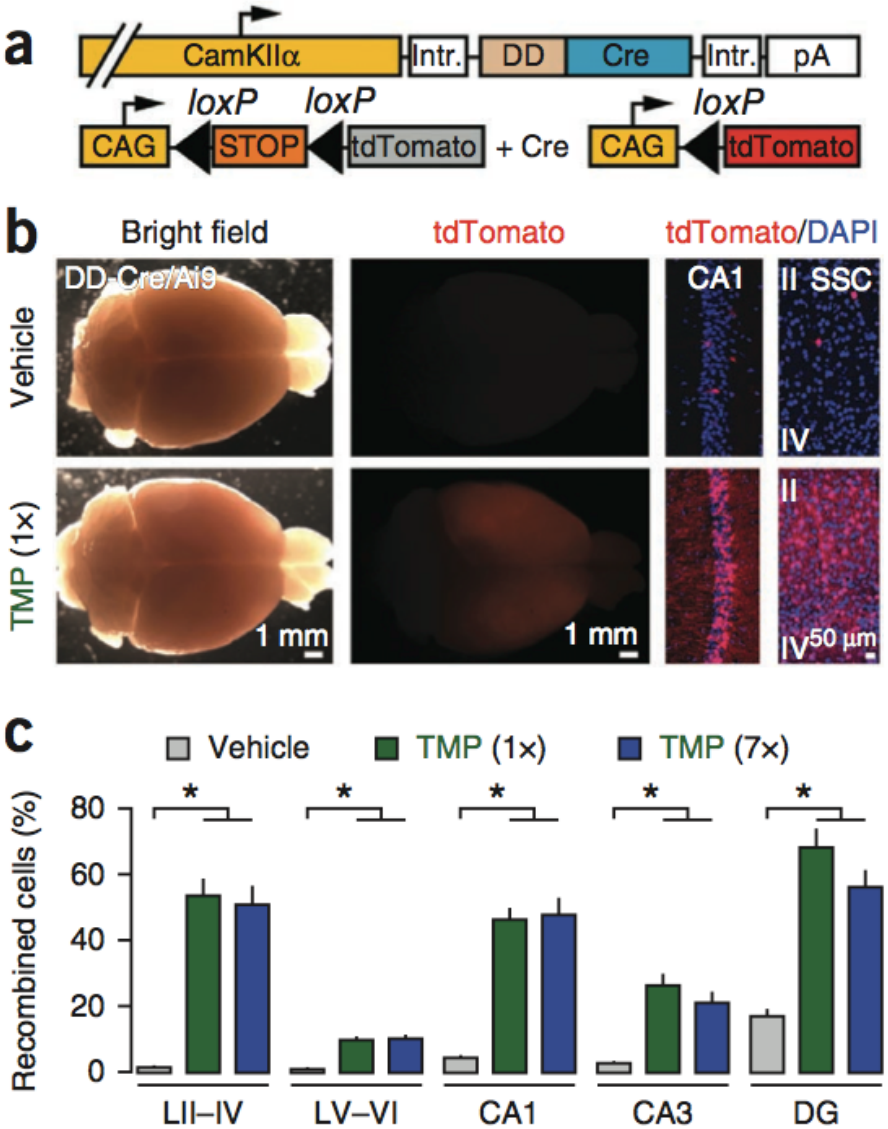
Stabilization of Cre in cell culture



DD-Cre in intact mice with viral delivery

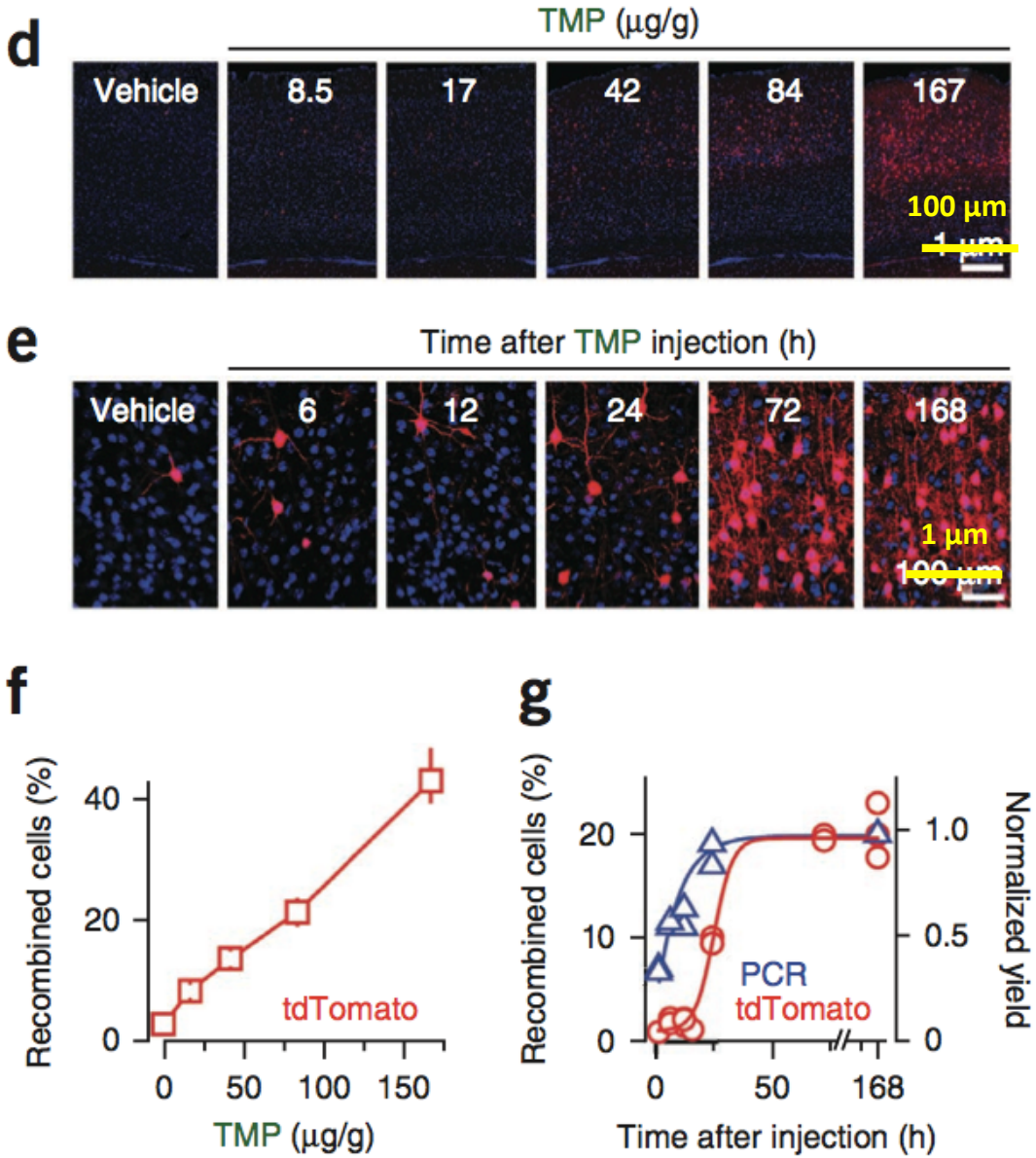


DD-Cre transgenic mice

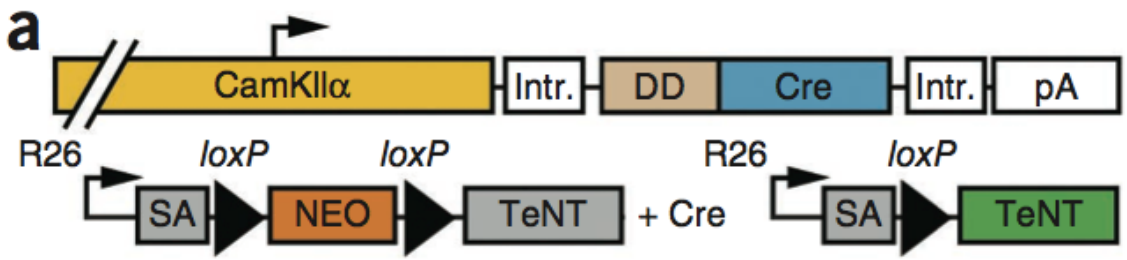


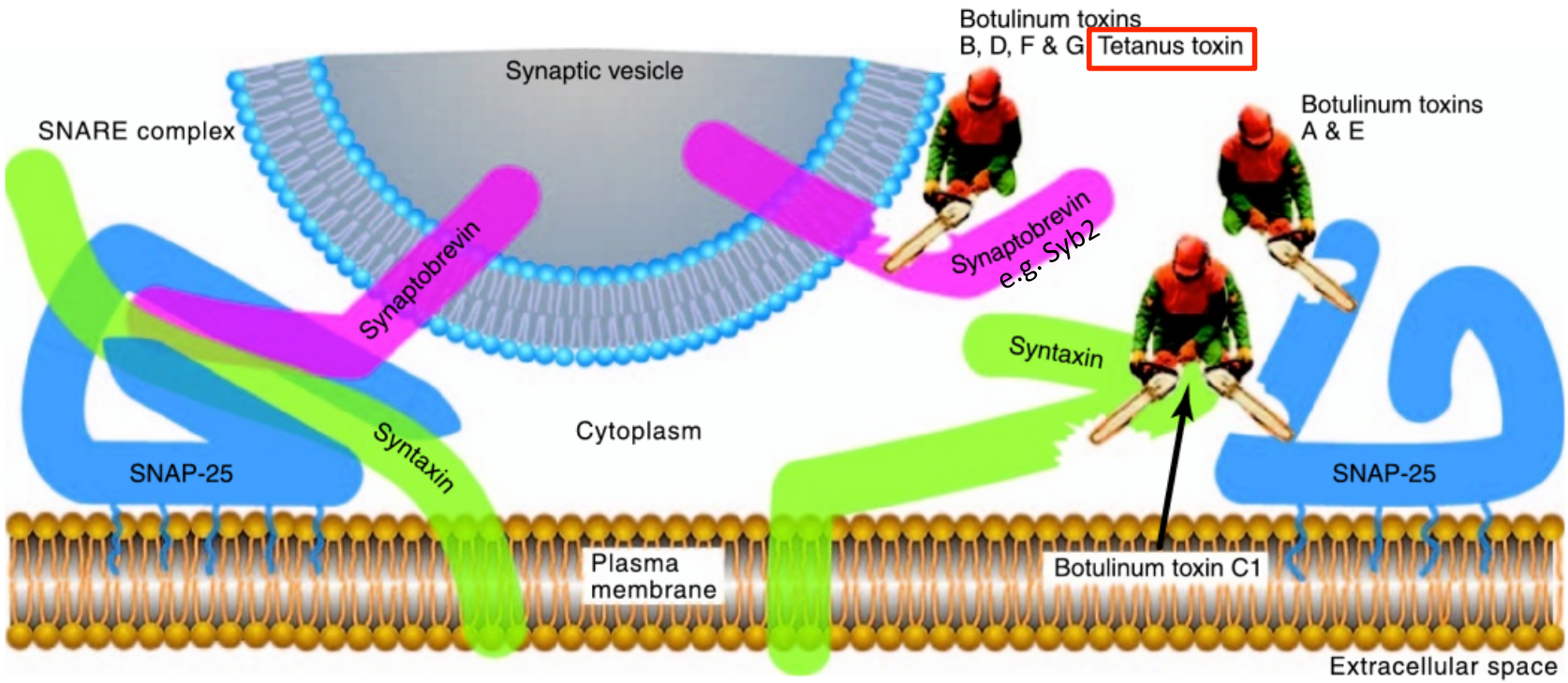
- P30 mice treated with
  - 1 dose 170 µg/g TMP
  - 7 doses 170g µg/g TMP
- Histology P37
- Percentage of recombined cell relative to DAPI positive cells

DD-Cre - timing and dose-dependency



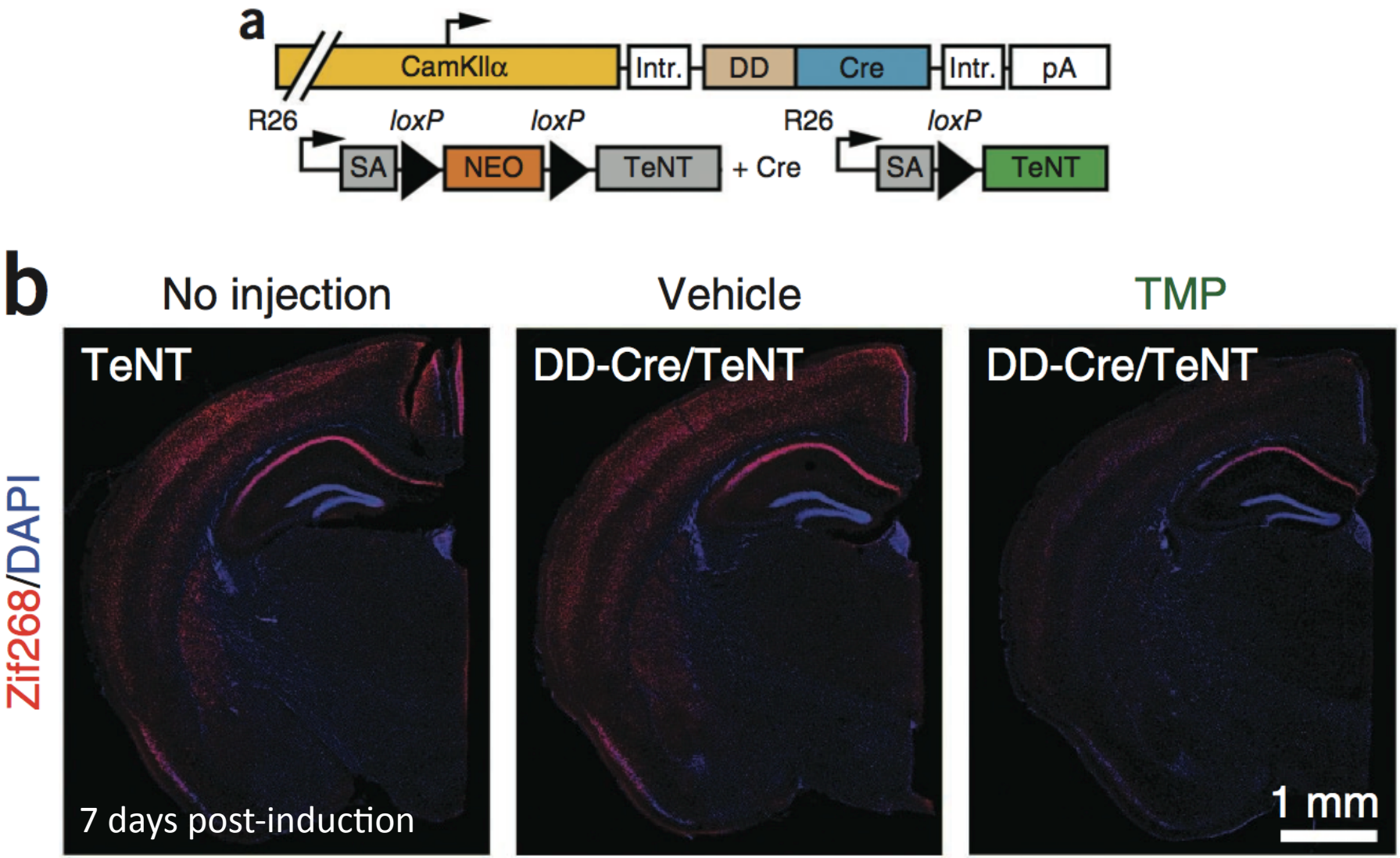
DD-Cre-mediated synaptic silencing





From Molecules to Networks, 2nd edition, 2009

DD-Cre-mediated synaptic silencing



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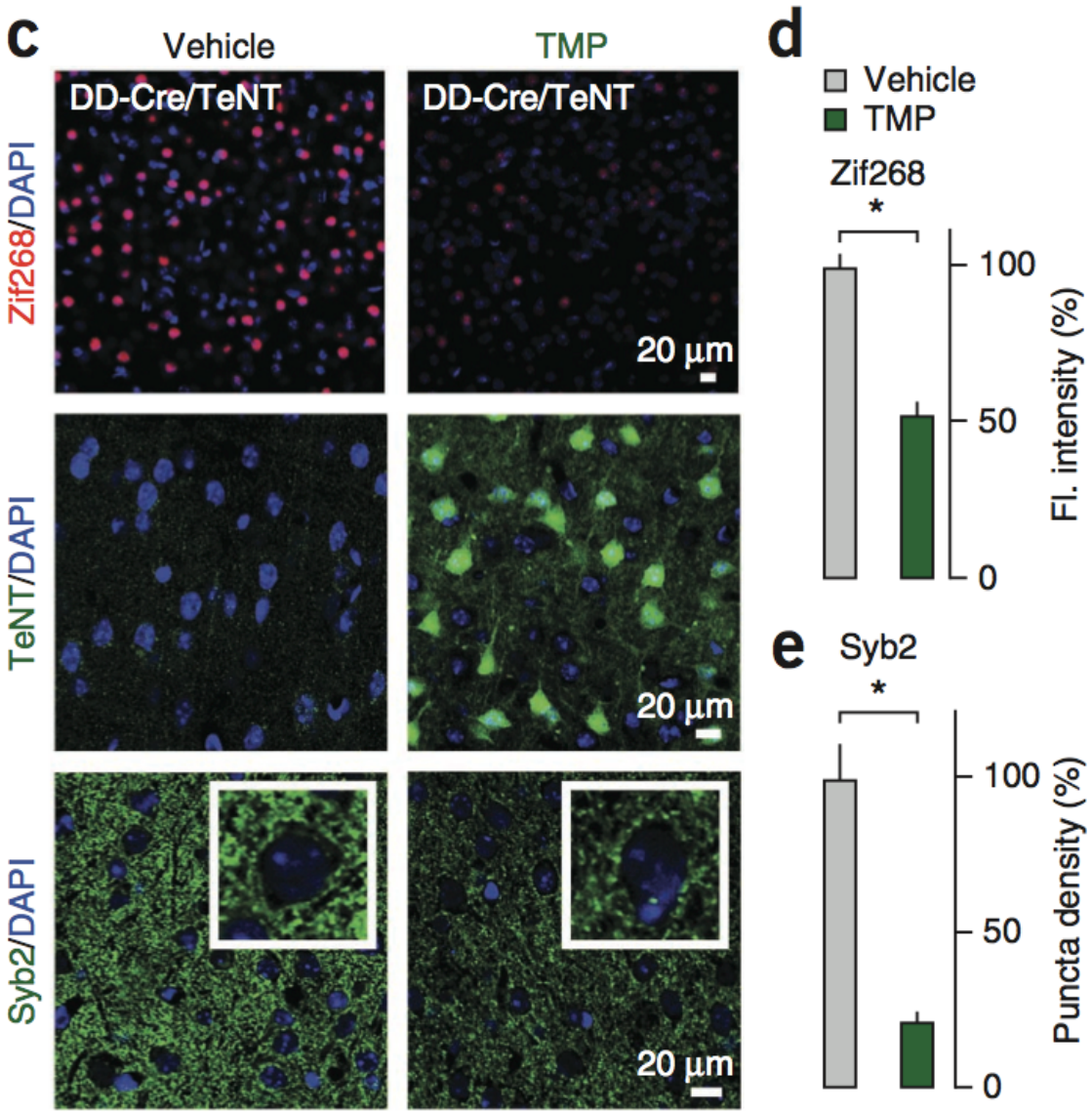

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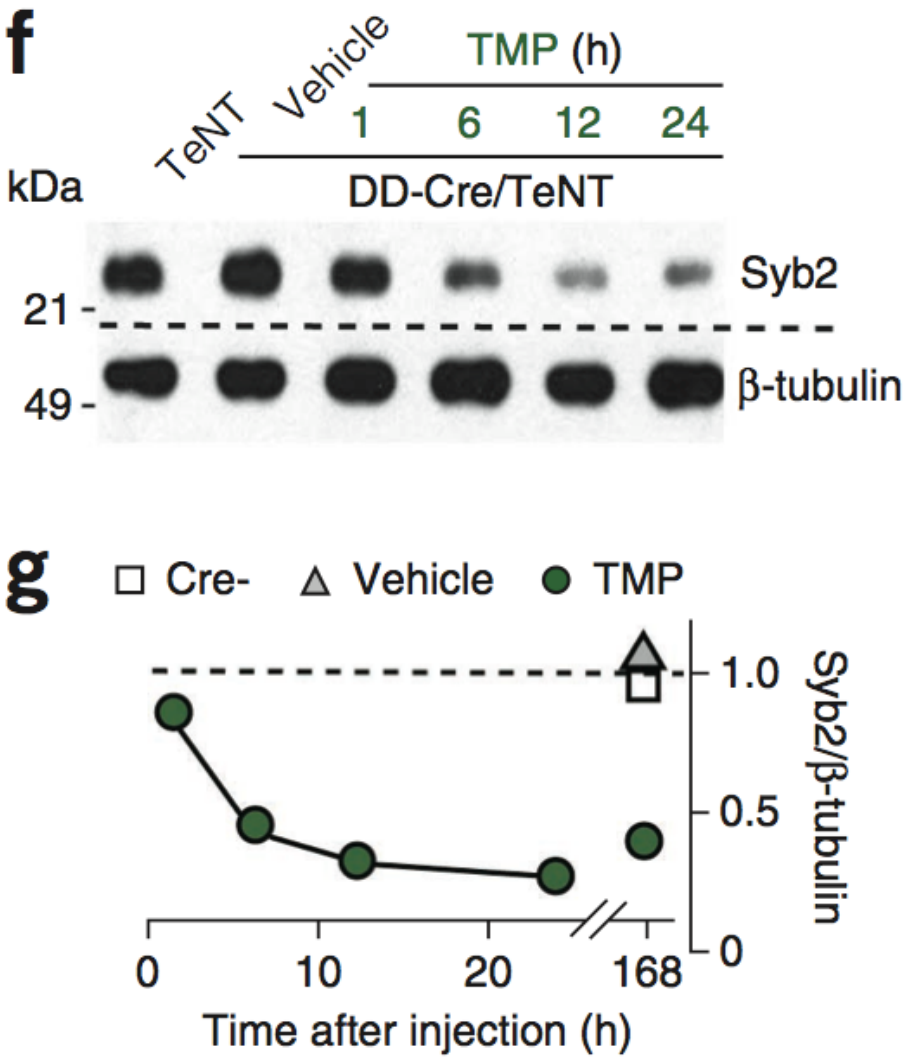
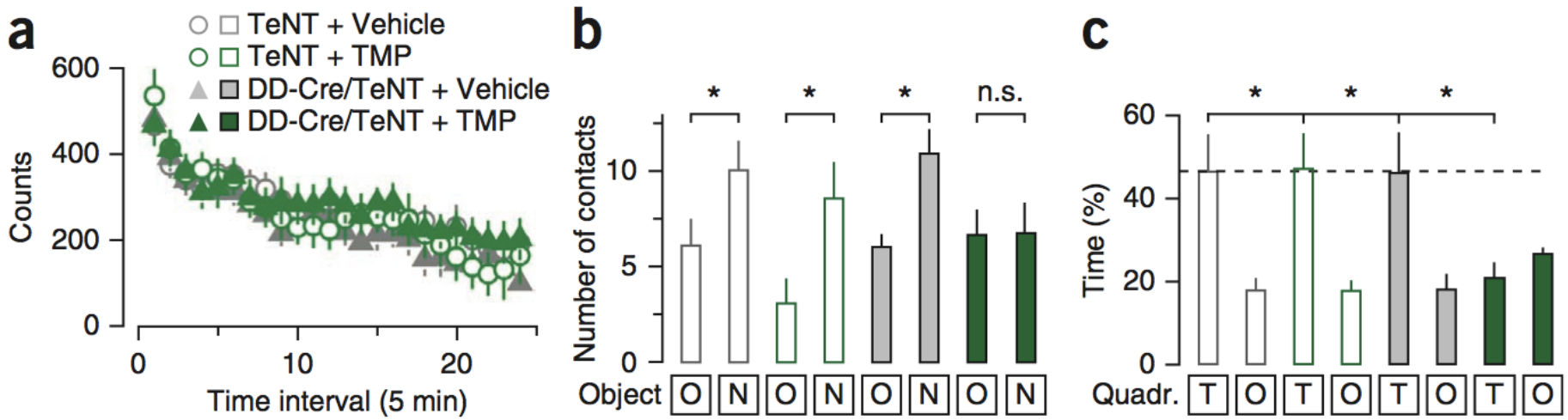


Behavioral consequences of DD-Cre-mediated synaptic silencing



Summary

! Efficient degradation of highly active proteins such as Cre in the absence of TMP induction

! After TMP induced stabilization – significant amounts of protein after 2-4h

!? DD-Cre induction possibly more efficient than Cre-ERT2 in the mouse brain

? How quickly do DD-systems react in fast developing tissues such as the embryonic mouse brain

Rapid and reversible knockdown of endogenous proteins by peptide-directed lysosomal degradation

Xuelai Fan[1,5], Wu Yang Jin[1,5], Jie Lu[1], Jin Wang[2] & Yu Tian Wang[1,3,4]

[1]Brain Research Centre and Department of Medicine, Vancouver Coastal Health Research Institute, University of British Columbia, Vancouver, British Columbia, Canada. [2]Institute of Pharmacology, Medicine College of Shandong University, Jinan, China. [3]Translational Medicine Research Center, China Medical University Hospital, Taichung, Taiwan. [4]Graduate Institute of Immunology, China Medical University, Taichung, Taiwan. [5]These authors contributed equally to this work. Correspondence should be addressed to Y.T.W. (ytwang@brain.ubc.ca).

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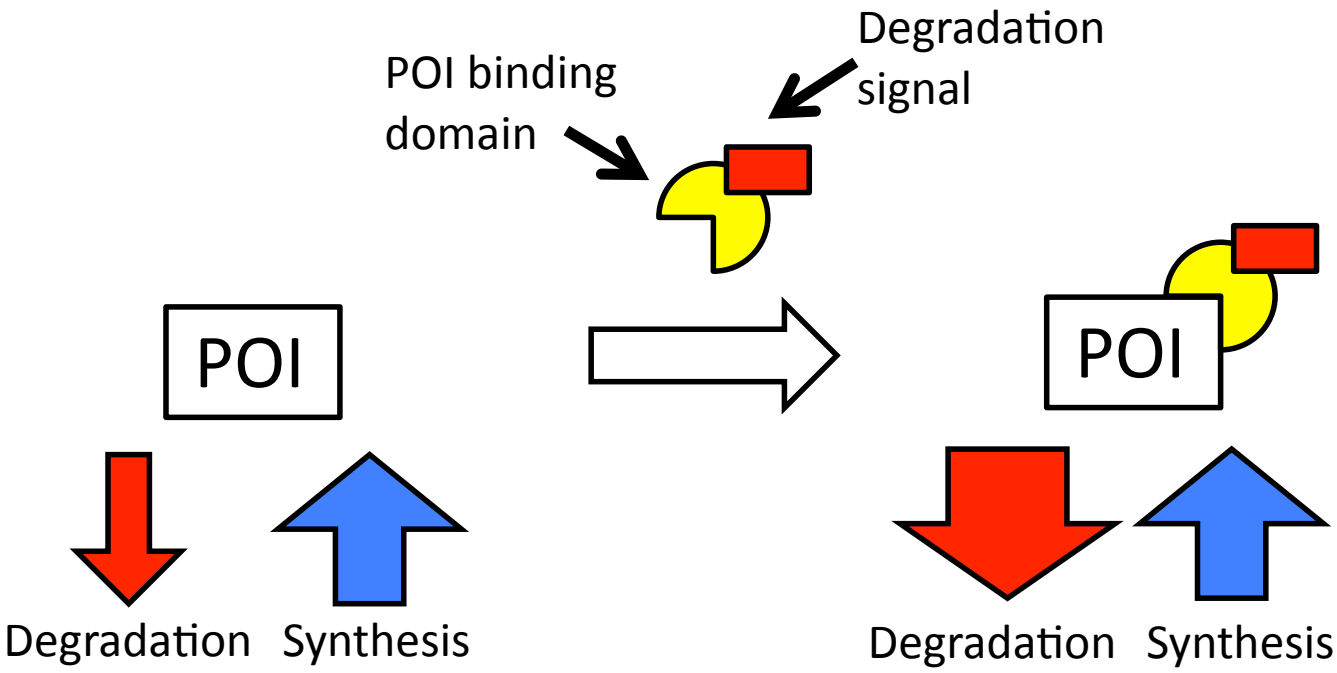


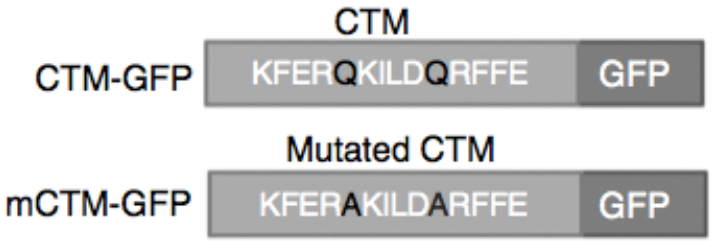
Goals of this study

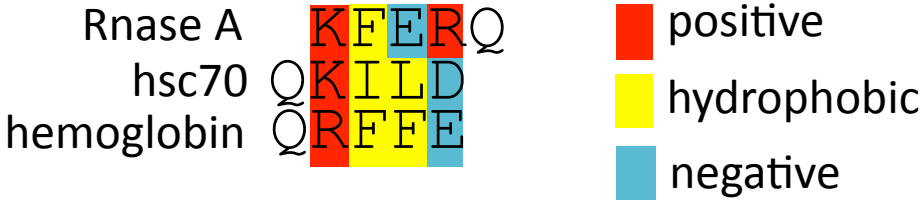
- Develop a synthetic peptide that specifically labels the activated form of DAPK1 (death-associated protein kinase 1) for lysosomal degradation

- Determine if this approach is neuroprotective *in vivo* in a rodent model of focal ischemia

Degradation signal - proof of principle

a





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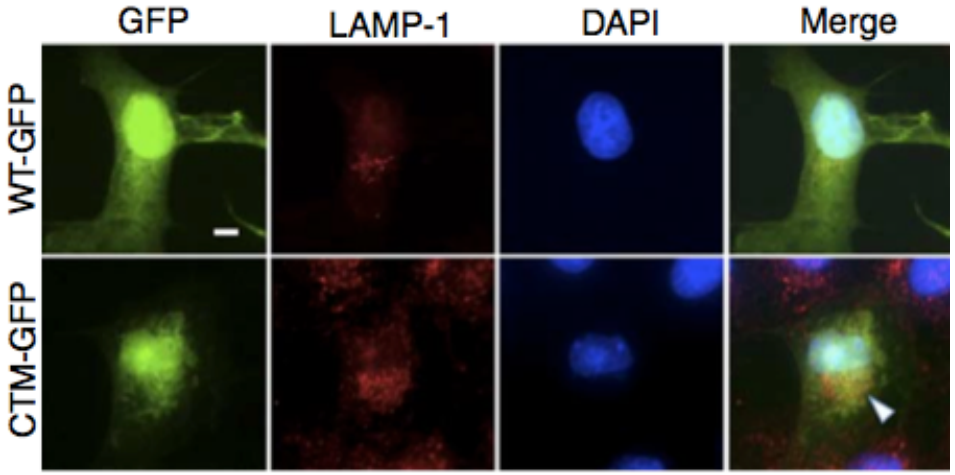
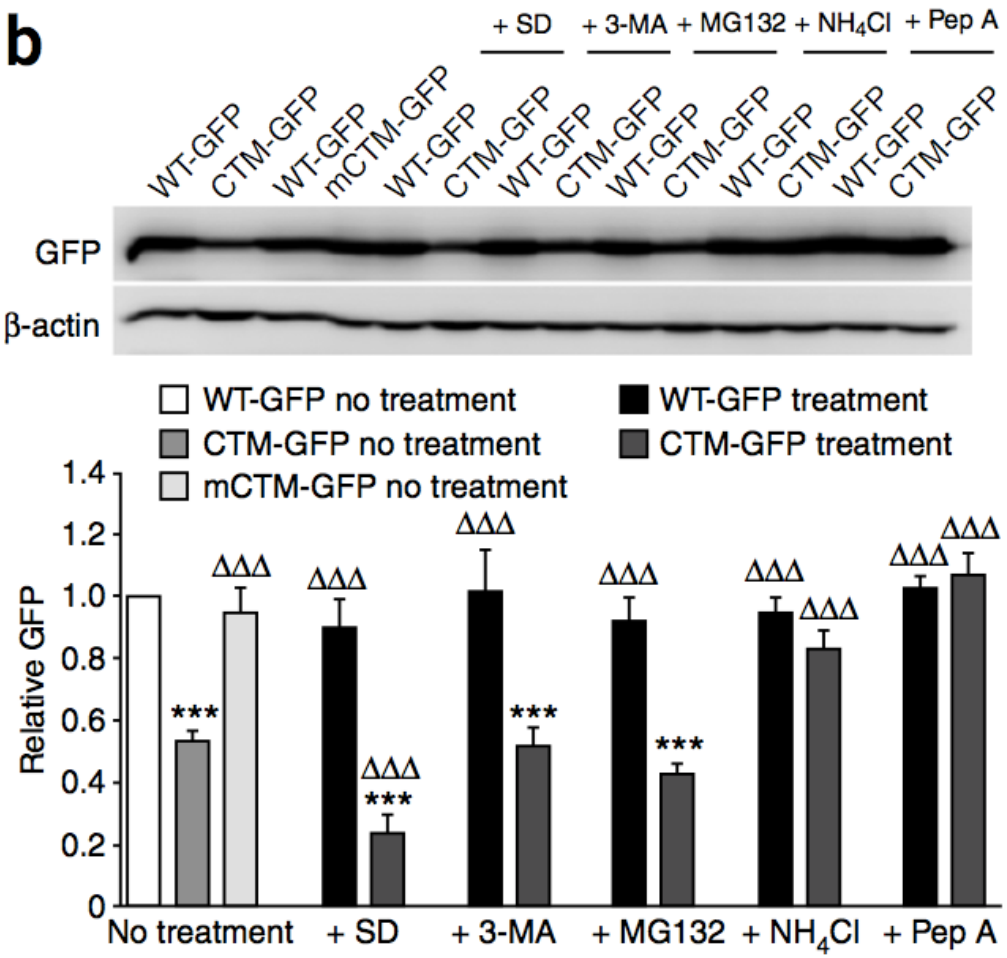


Degradation signal - proof of principle



SD (serum deprivation): enhance CMA activity

3-MA (3-methyladenine): macroautophagy inhibitor

MG132: proteasome inhibitor

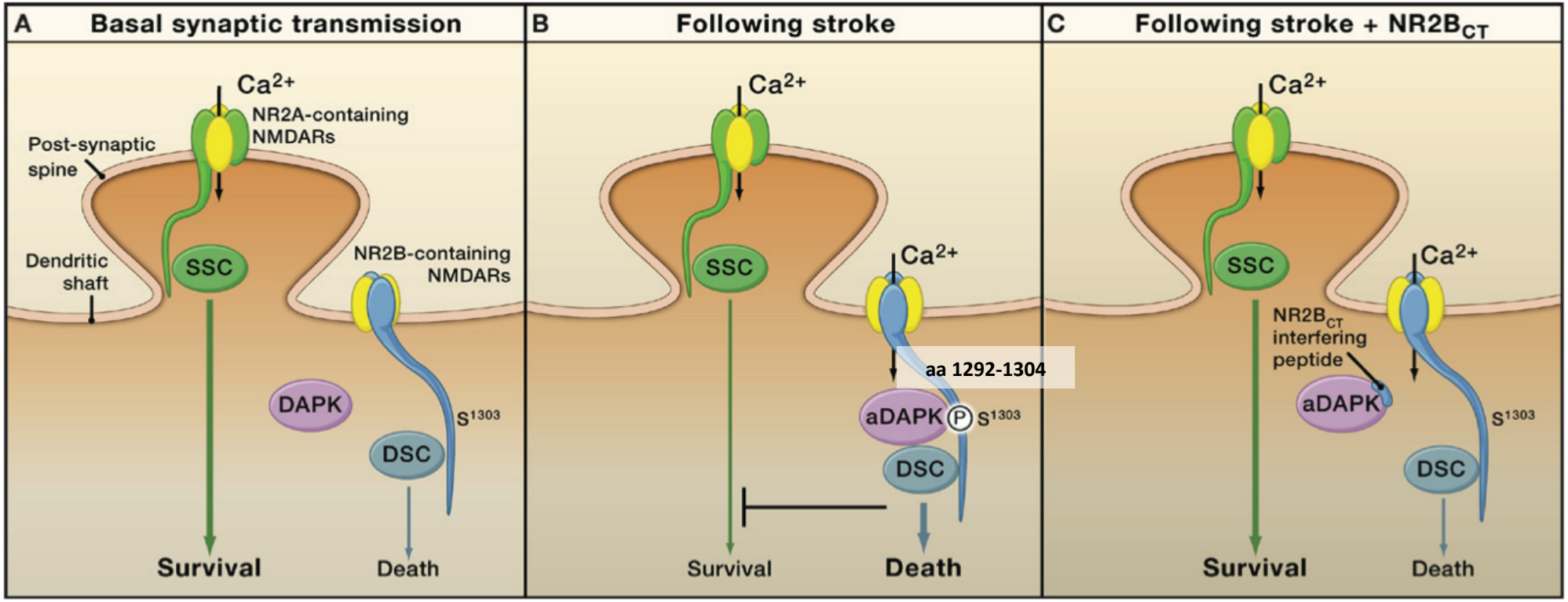
NH_4Cl: lysosome inhibitor

pepstatin A: cathepsins D and E inhibitor (lysosomal proteases)

* vs. wt-GFP
Δ vs. untreated CTM-GFP

NMDAR-DAPK1 signaling in stroke

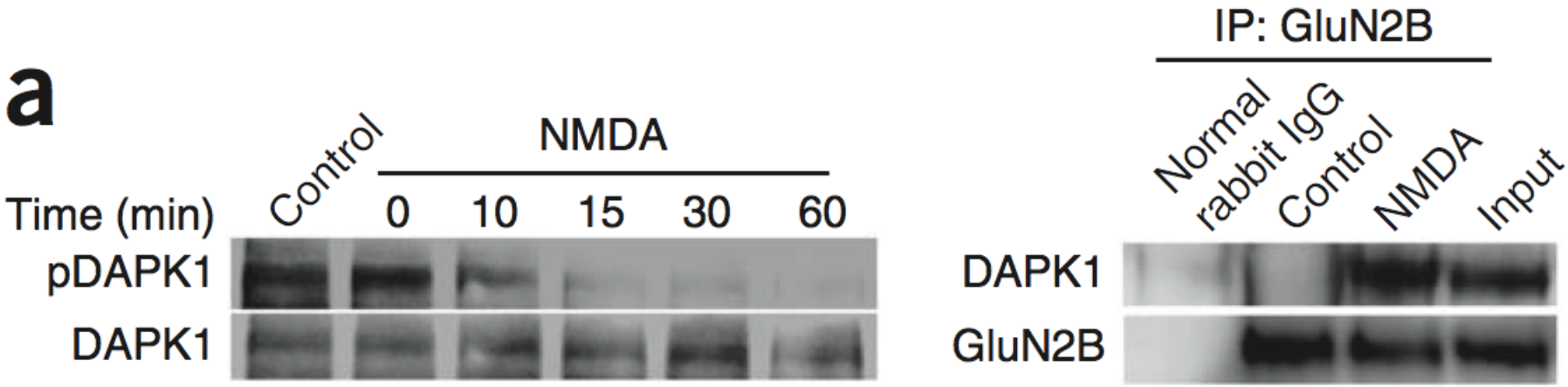
- DAPK1 - death-associated protein kinase 1
- calcium-calmodulin–regulated protein kinase
- activated by de-phosphorylation



Martin & Wang 2010, comment on Tu et al., 2010

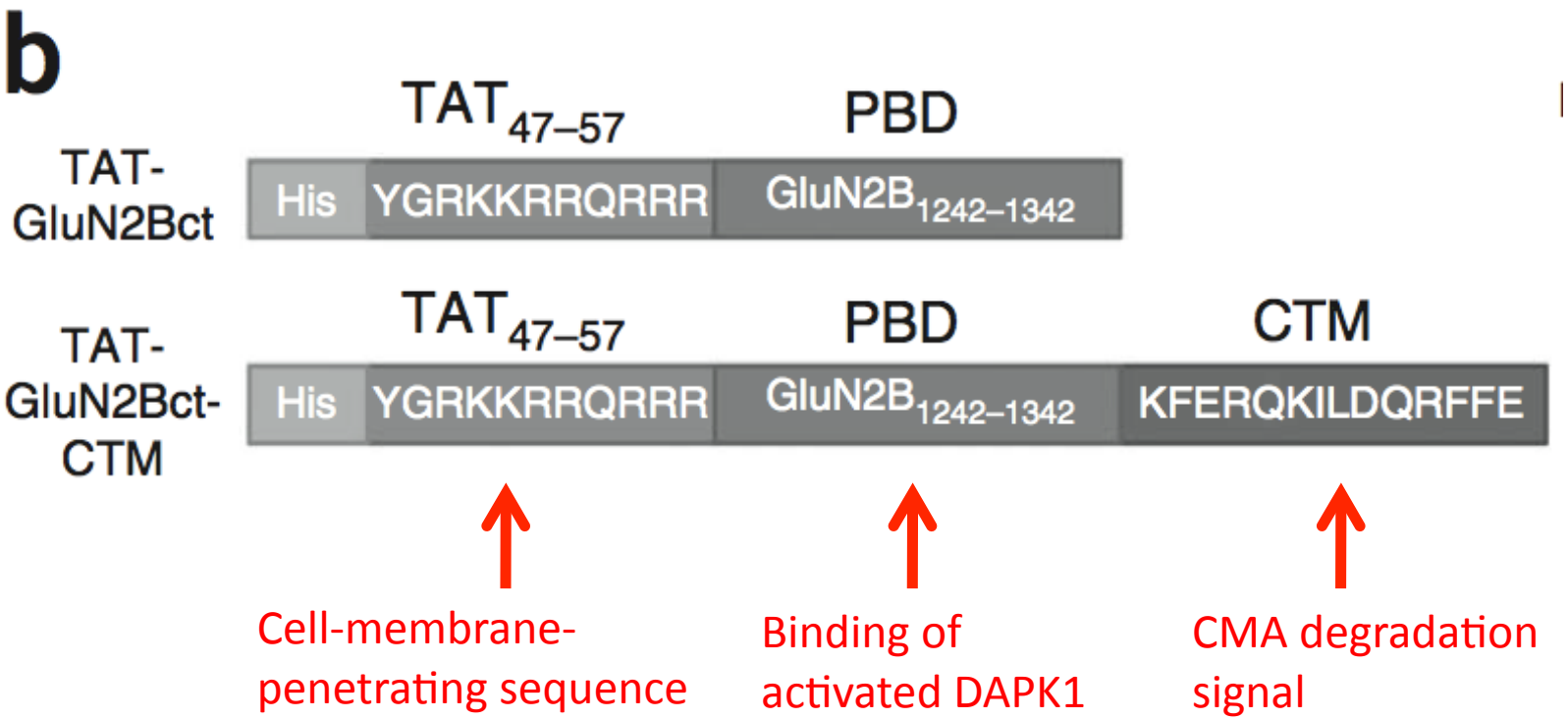
Targeted degradation of activated DAPK1

- Cultured rat cortical neurons
- NMDA-mediated excitotoxicity



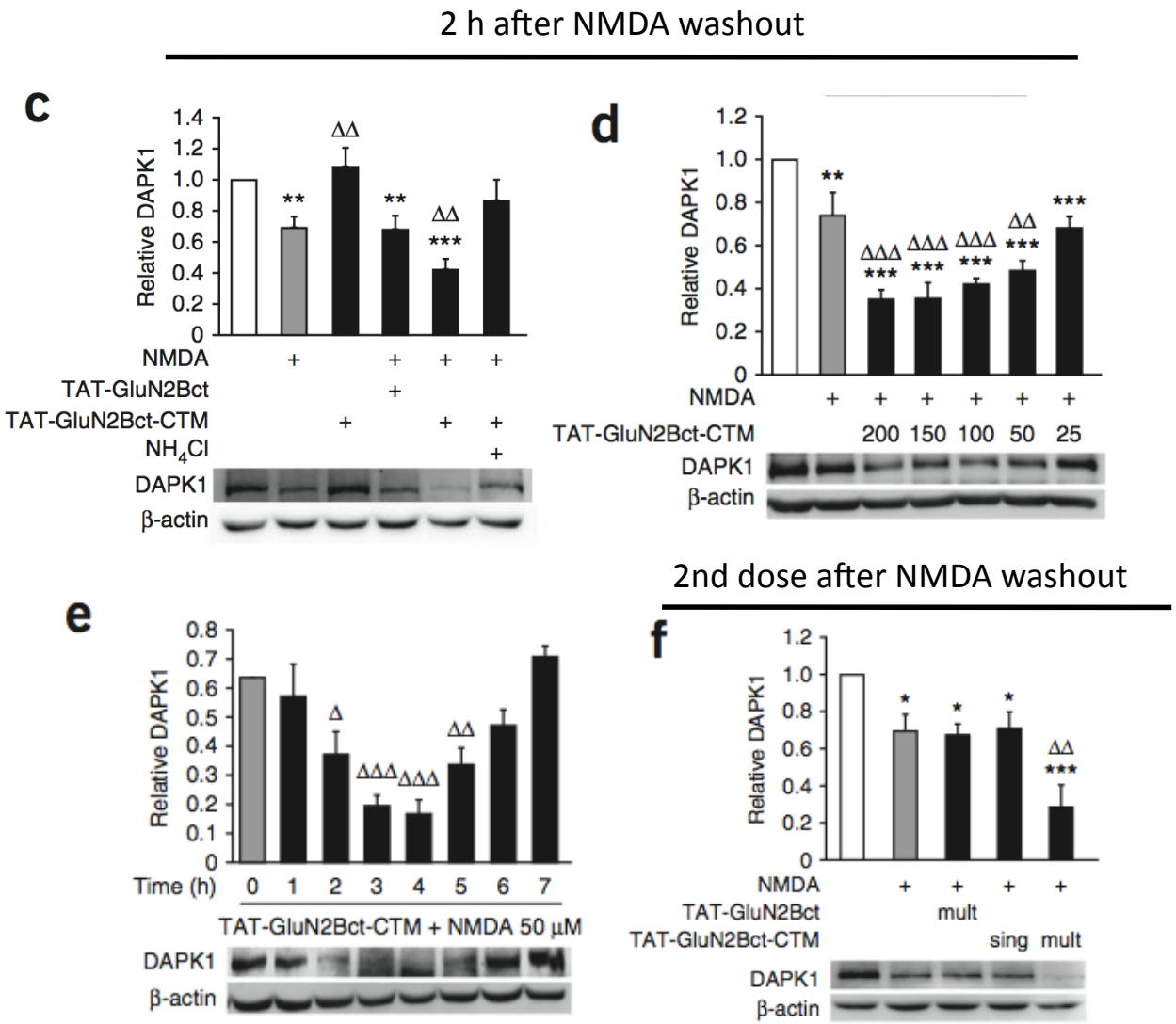
Targeted degradation of activated DAPK1

- Production of recombinant DAPK1 binding-protein in *E. coli*



Targeted degradation of activated DAPK1

- Cultured rat cortical neurons
- 30 min NMDA treatment
- Targeting peptides 60 min before and 30 min during NMDA treatment
- * vs. saline (white bar)
- Δ vs. NMDA (grey bar)



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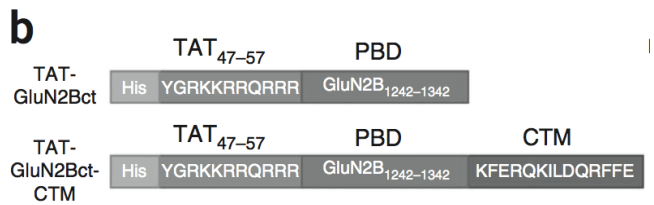
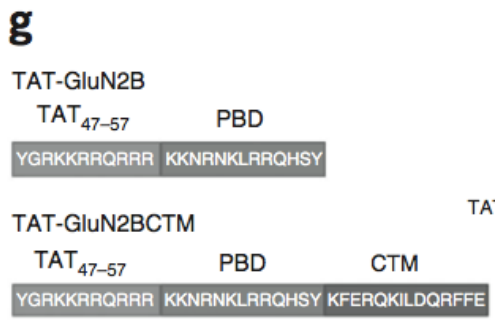
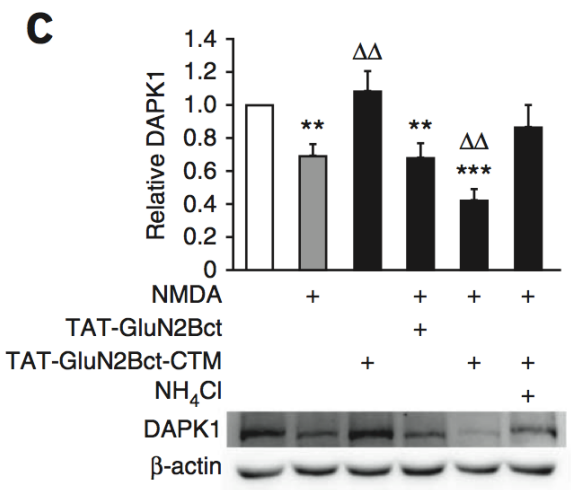
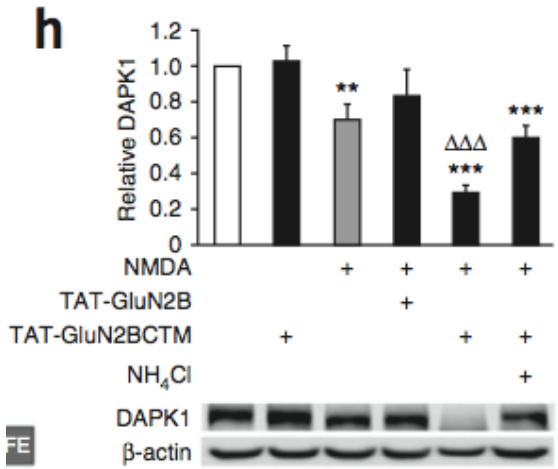




recombinant – 200 µM

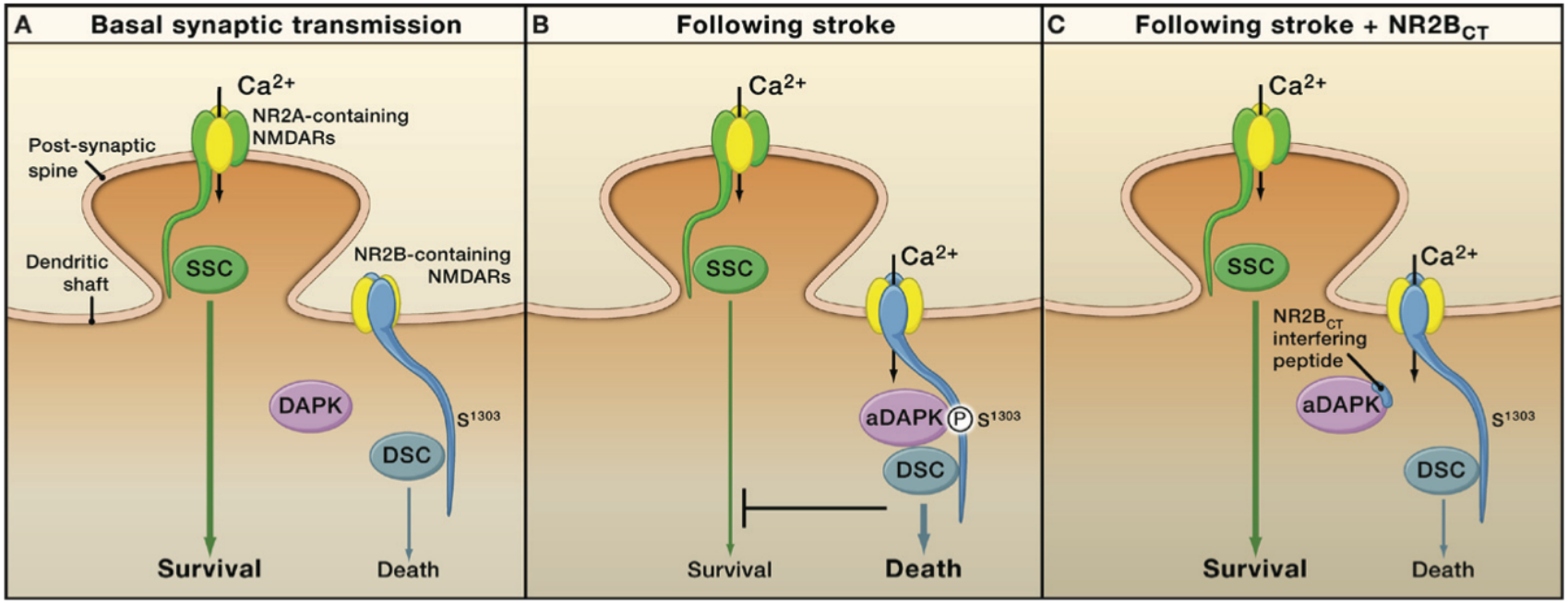


synthetic – 25 µM



Targeted degradation of activated DAPK1 successful

→ Does this protect neurons from NMDA-mediated excitotoxicity?

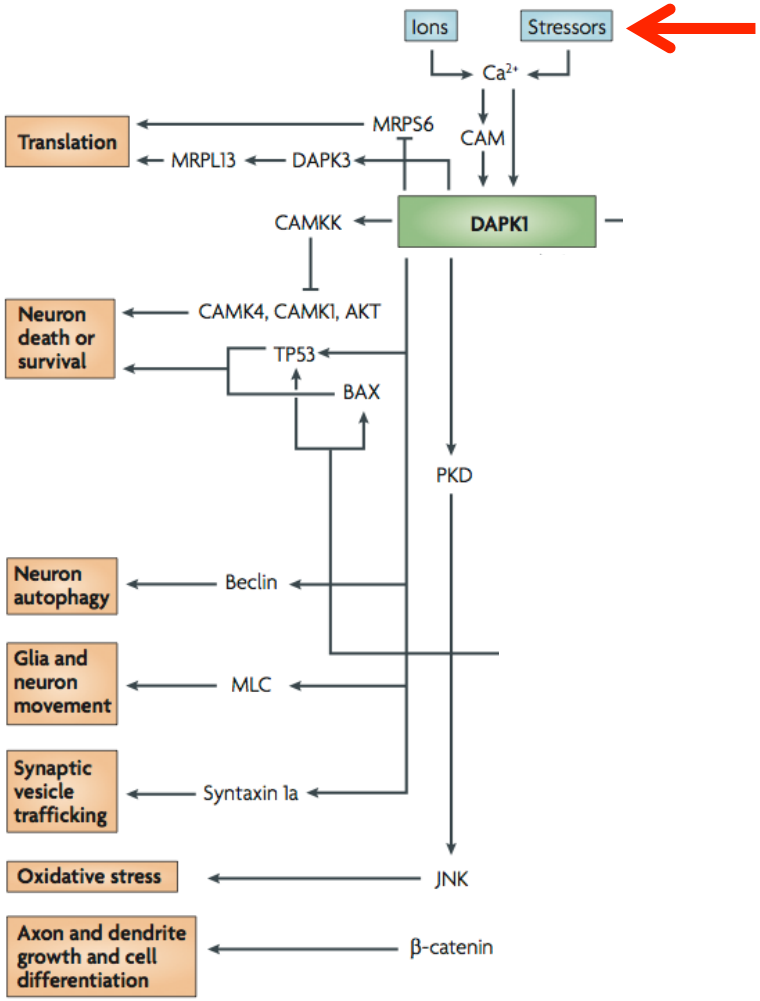


Targeted degradation of activated DAPK1 successful

→ Does this protect neurons from NMDA-mediated excitotoxicity?

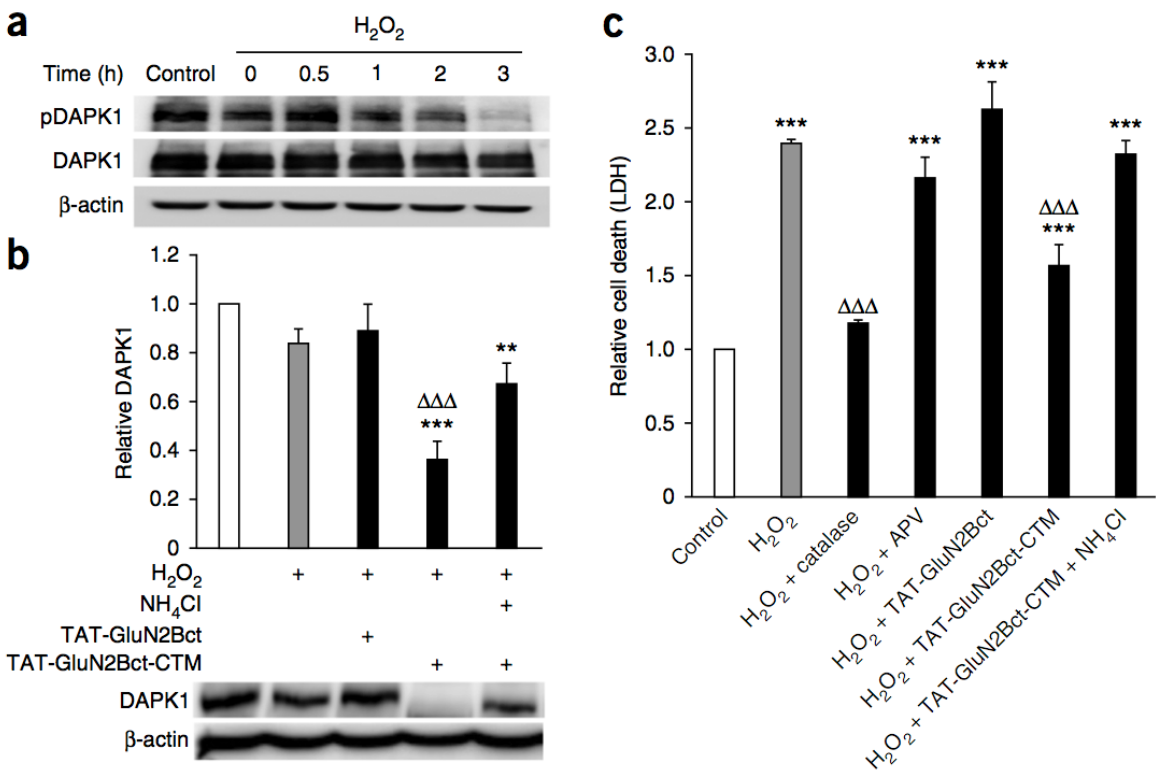
?

Blocking additional cellular stressors with degradation of activated DAPK1



Chico et al., 2009

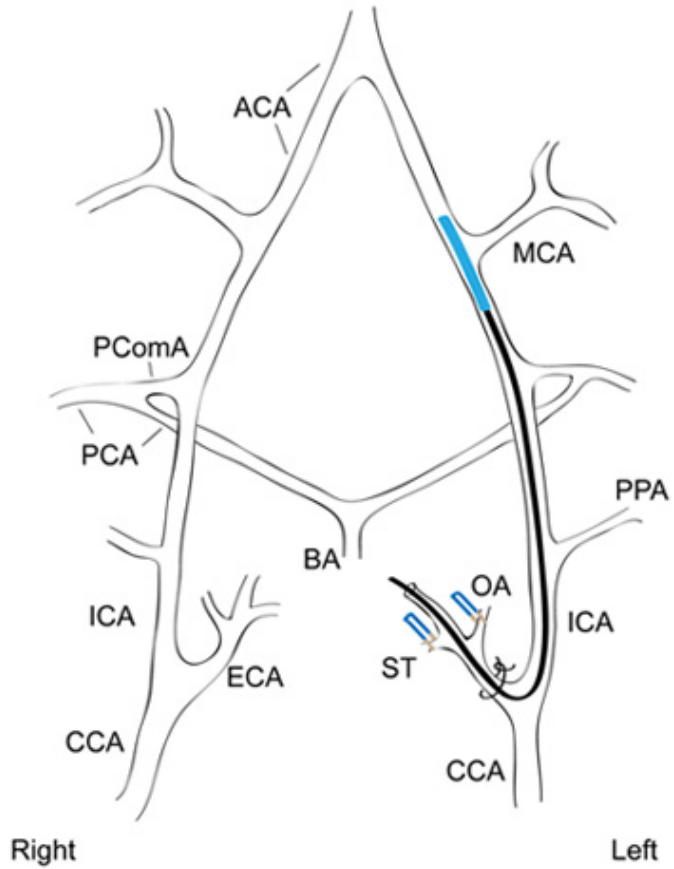
DAPK1 targeting-peptide protective against H_2O_2-induced oxidative stress



- Cultured rat cortical neurons
- 30 min H_2O_2 treatment
- Targeting peptides 60 min before and 30 min during H_2O_2 treatment
- * vs. saline (white bar)
- Δ vs. H_2O_2 (grey bar)
- APV – NMDAR inhibitor
- NH_4Cl – lysosome inhibitor

Rat model of focal ischemia

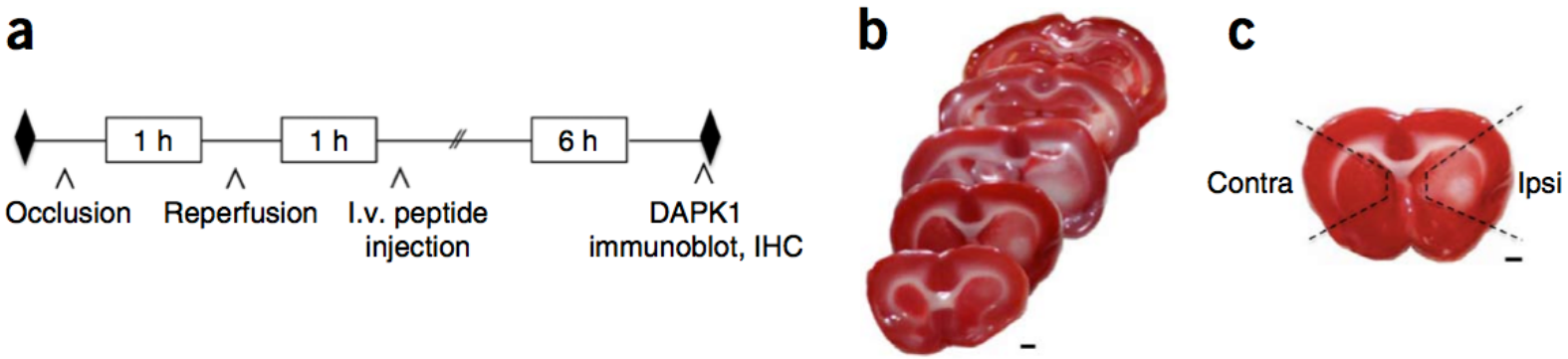
Occlusion of the middle cerebral artery
by endovascular suture



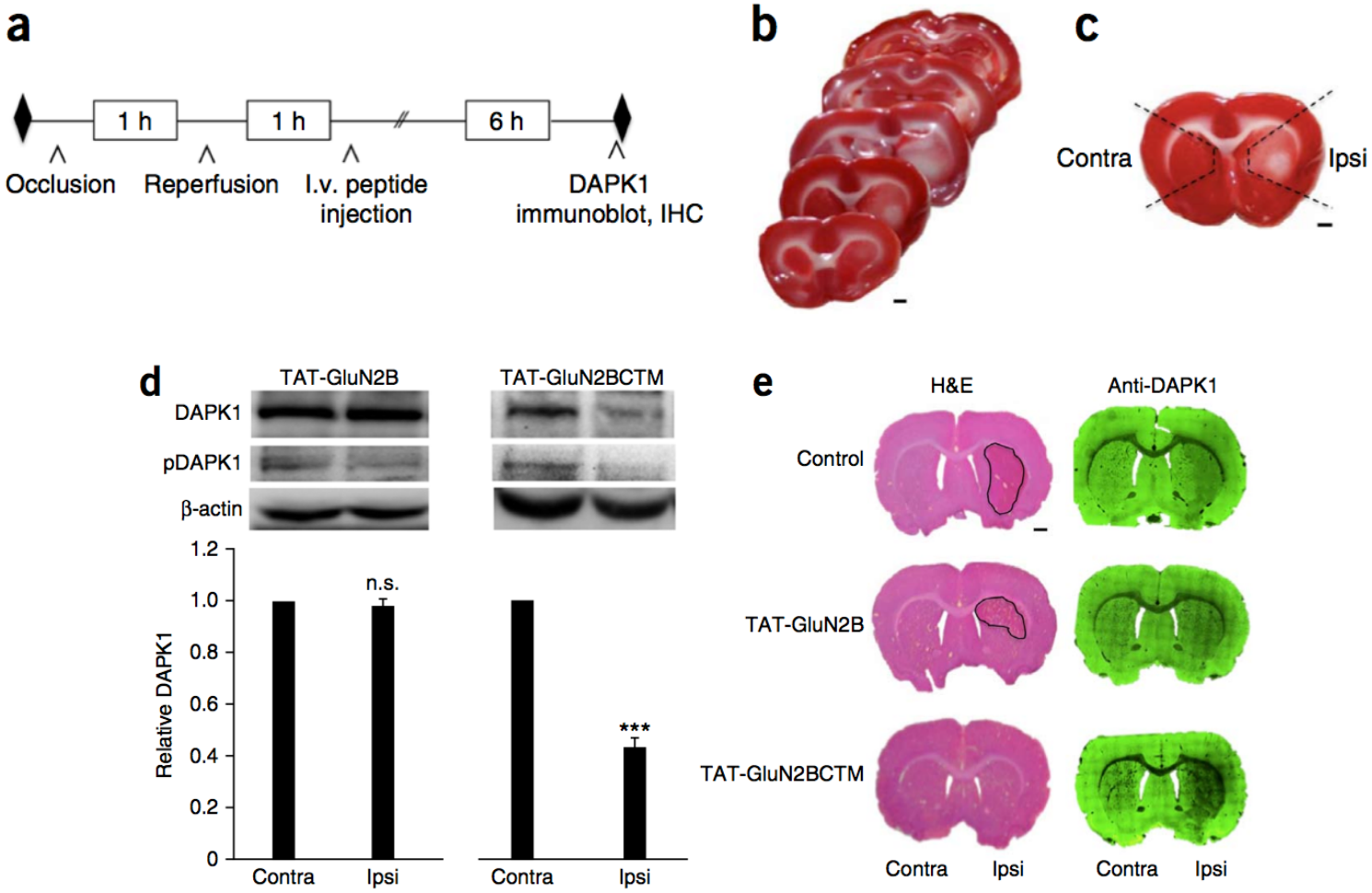
coronal sections of rat brain stained with TTC
24 hours after 1 hour transient MCAO

Uluç et al., 2011

in vivo DAKP1 protein knockdown

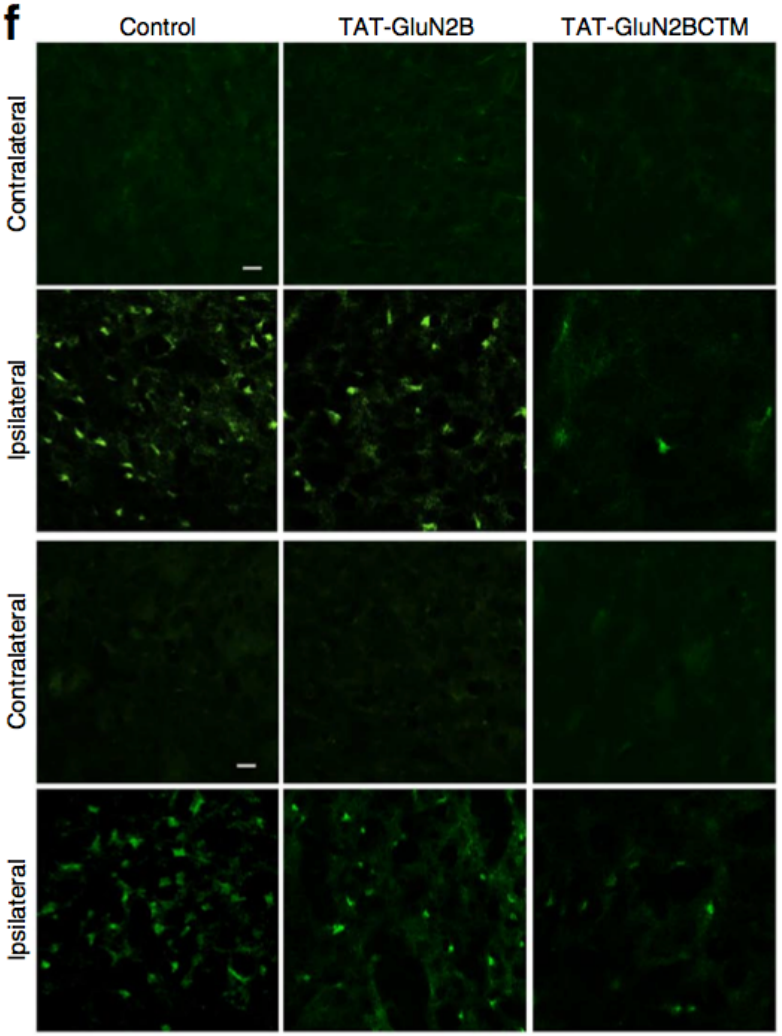


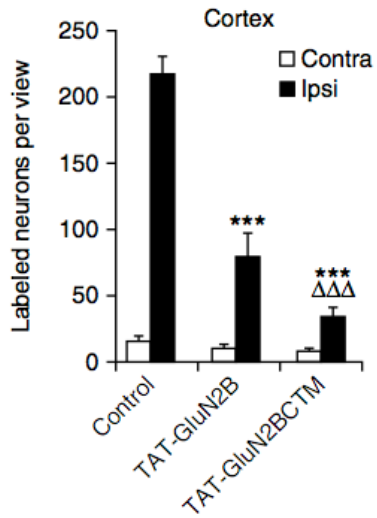
in vivo DAKP1 protein knockdown

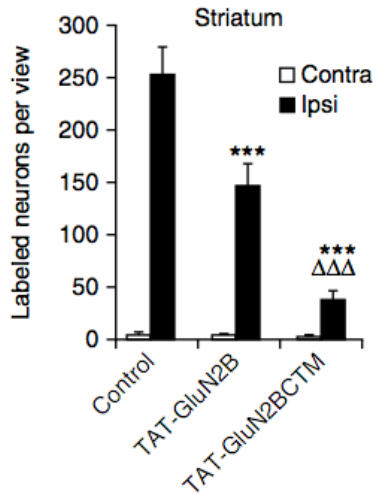


in vivo DAKP1 protein knockdown

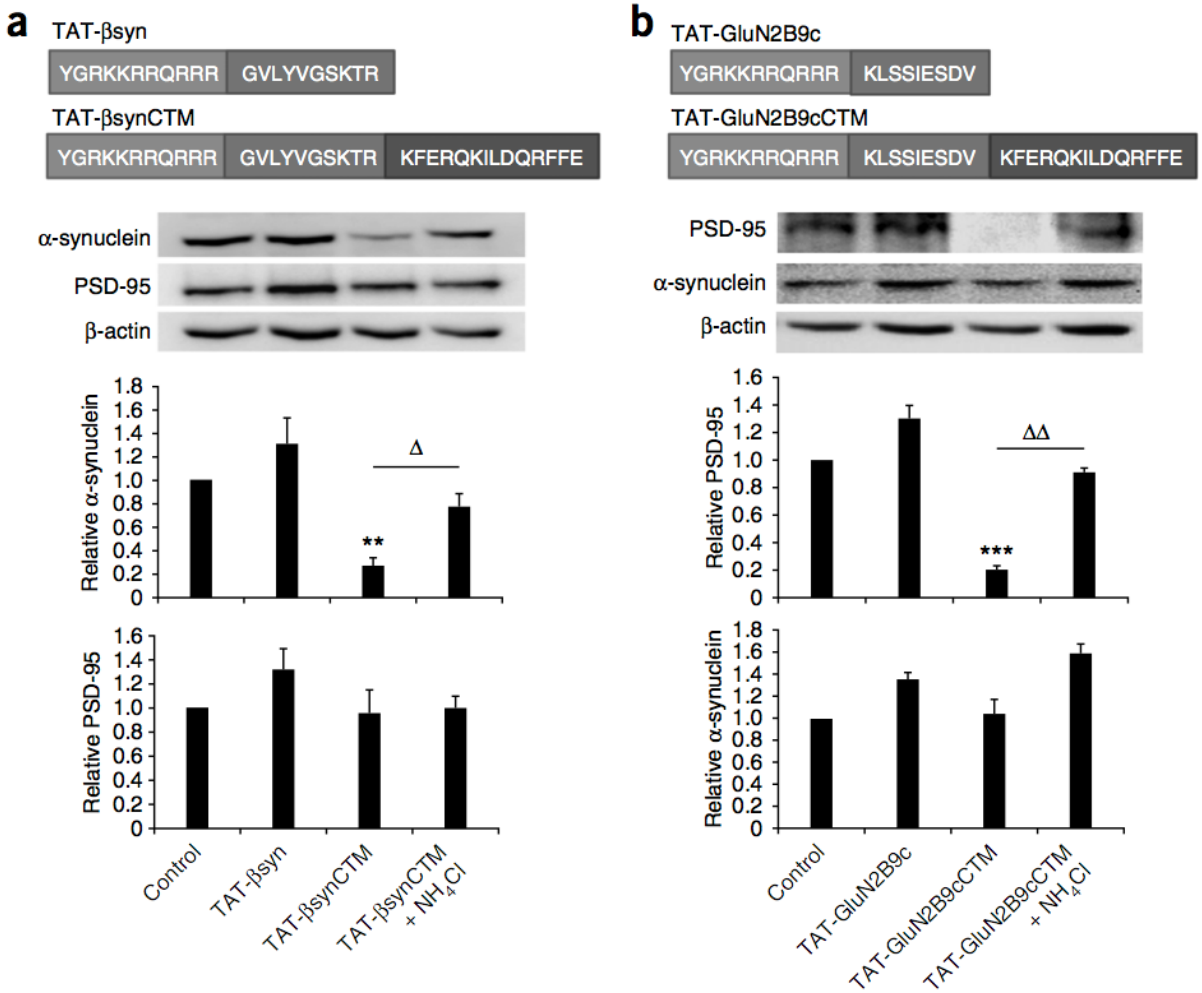
Fluoro-Jade B (degenerating neurons)



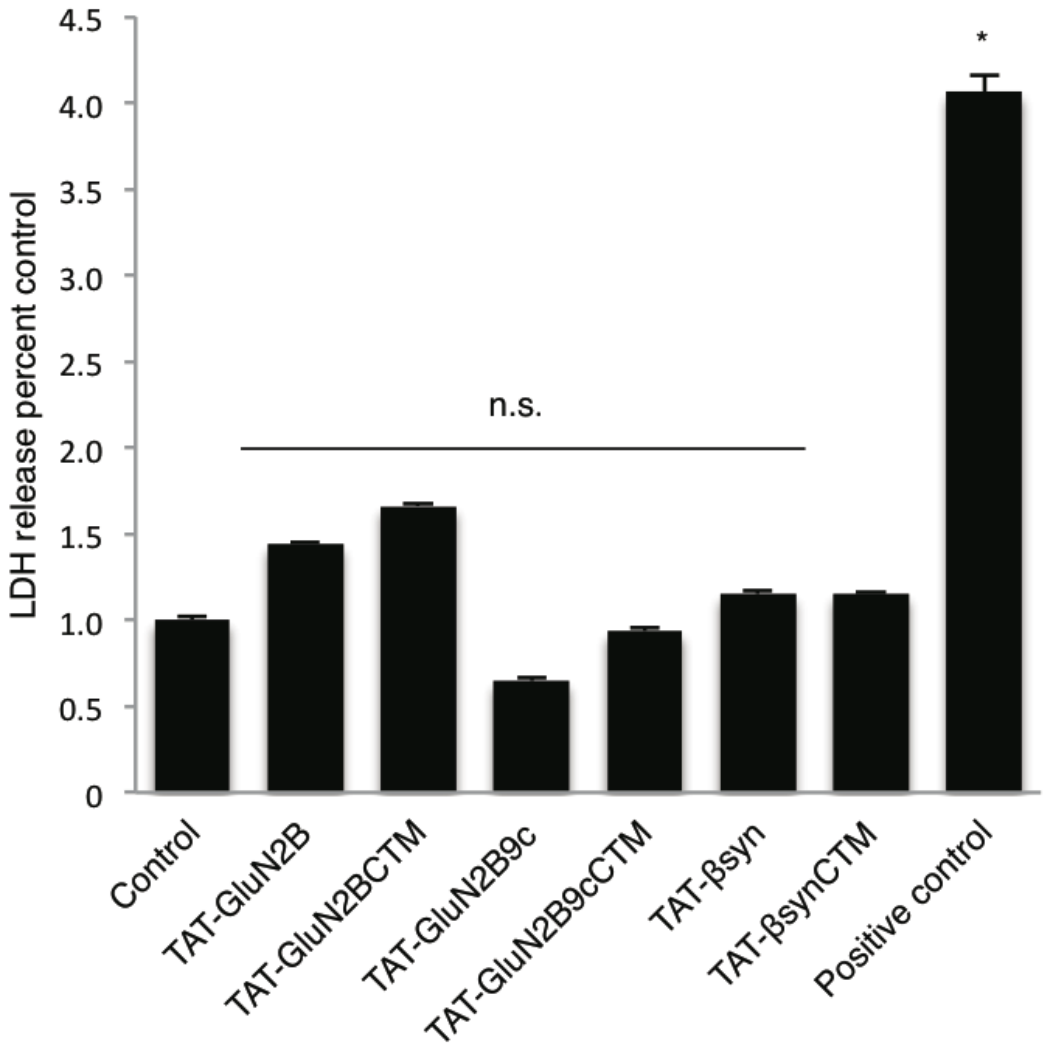




Protein knockdown of PSD-95 & α-synuclein



All targeting peptides are well-tolerated in cell culture (24 h)



Summary

! Efficient depletion of endogenous proteins as early as 4h after application of the targeting peptide

! Specific targeting of post-translationally modified versions of proteins for lysosomal degradation

! Systemic application of DAPK1 targeting peptide reduces neuronal death shortly after ischemic stroke in a rat model

? How difficult is the design of targeting peptides for other proteins of interest

? Is this approach only feasible for cyotosolic proteins

Further Reading

- Lienert, F., Lohmueller, J.J., Garg, A., Silver, P.A., 2014. Synthetic biology in mammalian cells: next generation research tools and therapeutics. Nat Rev Mol Cell Biol advance online publication.

Questions?

THANK YOU FOR YOUR ATTENTION!