how to knock-down *in vivo* the expression of endogenous proteins?
Tools for negative regulation of protein level
Tools for negative regulation of protein level

- at genomic level:
  - DNA
  - ZFN
  - TALEN
  - CRISPR/Cas9

- use a nuclease as catalytic domain
- the cleavage of target gene induced DSB/DNA repair mechanism: ORF disruption
ZFN
Zinc Finger Nucleases

- structure and mechanism

A guide to genome engineering with programmable nucleases

Hyongbum Kim and Jin-Soo Kim

NATURE REVIEWS | GENETICS
VOLUME 15 | MAY 2014 | 321
**TALE**
Transcription Activator-Like Effectors

- **Origin**: secreted by *Xanthomonas*
  modulate gene expression in host plants and to facilitate bacterial colonization

- **Structure**

  ![Diagram of TALE structure]

  - Hyper-variability of the amino acids at the 12th and 13th positions of each repeat directed
    the recognized sequence by a simple one-to-one code between these two critical aa and each
    DNA base in the target sequence
  - Optimal size of DNA target sequence: 19-pb (about 17.5 repeat domains)

  ➢ **TALE have been utilized to create site-specific gene-editing tools**
  by fusing target sequence-specific TAL effectors
TALE mechanism (1)

- **TALE Nucleases**: Disruption of gene: Nucleases + DNA repair

Modularly assembled designer TALE nucleases for targeted gene knock-out or gene replacement like knock-in (reporter gene) or specific mutation by homologous recombinaison with donor plasmid

• TALE-type transcription factors: induce or repress gene expression by interaction of the functional domain with endogenous transcription complex

• Bind to target gene promoter sequence-specifically
• Recruit or block transcription complex via a trans-activator or trans-repressor domain as functional domain in TALE-TF
CRISPR/Cas9 system II
Clustered Regularly Interspaced Short Palindromic Repeats

• **Origin:**
  - mechanism of bacterial immune system against viruses infections
  - Cas 9 from *Streptococcus pyogenes/Neisseria meningitidis/Staphylococcus aureus*

• **mechanism:**
  to create deletion,  
  insertion,  
  or replacement by homologous recombinaison with donor plasmid
CRISPR/Cas9 structure and mechanism

- On-target sqc(20nt)
- scaffold domain
- Cas9 fixation domain

Protospacer Adjacent Motif

AGCTGGGGATCAACTATAGCG CGG
DNA repair mechanisms after the nuclease action

(a) Non-homologous end joining

- Gene disruption (via small insertions or deletions)
- Insertion (up to 14 kb by synchronized donor cleavage in vivo)
- NHEJ-mediated ligation of broken DNA ends
- Simultaneous cleavage by two nuclease(s)
- Deletion
- Inversion

(b) Homology-directed repair

- Gene addition
  - Provide donor template containing transgene(s)
- Gene correction
  - Provide donor template containing modified gene sequence
## Comparison of genome engineering technologies

<table>
<thead>
<tr>
<th></th>
<th>ZFN</th>
<th>TALEN</th>
<th>CRISPR-Cas9</th>
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Tools for negative regulation of protein level

- at genomic level:
  - DNA:
    - Zinc Finger Nuclease
    - TALEN
    - CRISPR/Cas9
  - mRNA:
    - shRNA
    - miRNA
Post Transcriptional Gene Silencing

- **shRNA**: short hairpin RNA is a siRNA-like transcripts with stem-loop structure

- **miRNA**: gene-specific sequence of shRNA into a miR-30 context
shRNA/miRNA maturation
sh/miRNA mechanism

- **RISC**
  - RNA Induced Silencing Complex

**ssRNA**

- **Cleavage**
  - Degradation

- **Translational inhibition**

- **Deadenylation**
  - Decapping
  - Degradation

**perfect complementarity between ssRNA/mRNA**

**imperfect complementarity between ssRNA/mRNA 3’UTR**

Höck & Meister (2008)
miRNA Avantage

Add many Sh On-target sequence to increase efficiency of interference
Tools for negative regulation of protein level

- **at genomic level:**
  - DNA
    - Zinc Finger Nuclease
    - TALEN
    - CRISPR/Cas9
  - mRNA
    - shRNA
    - miRNA

- **at protein level:**
  - specific neutralizing antibodies > membranar protein
  - peptide-directed degradation > cytosolic protein
Neutralizing Antibodies

Application for:

- soluble protein
- extracellular protein (receptor)
Neutralizing Antibodies (NA)

**In Vivo AAV1 Transduction With hRheb(S16H) Protects Hippocampal Neurons by BDNF Production**

Min-Tae Jeon¹,², Jin Han Nam³, Won-Ho Shin⁴, Eunju Leem¹,², Kyoung Hoon Jeong¹,², Un Ju Jung⁵, Young-Seuk Bae¹,², Young-Ho Jin⁶, Nikolai Kholodilov⁷, Robert E Burke⁸, Seok-Geun Lee⁹,¹¹, Byung Kwan Jin³,⁴ and Sang Ryong Kim¹,²,¹²,¹³

*Molecular Therapy* vol. 23 no. 3, 445–455 mar. 2015

- **Thrombin** - a serine protease of the trypsin family, a key enzyme of the blood coagulation system - can act as a **neurotoxin**, leading to the death of hippocampal neurons and AD-like cognitive impairment. Its expression is increased in the brains of patients with AD. It accumulates in senile plaques, reactive microglial cells, and neurofibrillary tangles in AD brains and microvessels.

- **BDNF expression** - a neurotrophin that mediates neuronal survival and Differentiation - is decreased in AD brains. BDNF delivery has **neuroprotective effects** in animal models of AD.

- **Rheb** - a member of the Ras family of small GTP-binding proteins - mediates the activation of mTORC1, which enhances the cell survival by producing BDNF. Rheb(S16H) is an active form.

- hRheb(S16H)-induced BDNF on the activation of mTORC1 may contribute to neuroprotection in the thrombin-treated hippocampus.
• rat model in CA1 region
• thrombin-induced neurotoxicity in vivo
• hRheb(S16H) protects hippocampal neurons from thrombin-induced neurotoxicity

• Stereotaxic injection of BDNF neutralizing antibodies (100, 200 or 400 ng, 4 μl at 0.5 μl/minute) and thrombin (20 U) at 3 weeks after AAV-hRheb(S16H) injection.
Chaperon-Mediated Autophagie (CMA) by lysosomal degradation pathway

- CMA is a type of autophagy specific for proteins containing a pentapeptide motif biochemically related to KFERQ
- Could be easily generalized to degrade any native cytosolic endogenous protein
  - Use of a chimeric peptide which directed endogenous protein degradation by lysosome
  - Induce a conditional knock-down
  - A simple, fast and easily reversible technique
CMA-peptide design

| Cell membrane penetrating domain (CMPD) | Protein binding domain (PBD) | CMA targeting motif (CTM) |

TAT 47-57: derived from HIV-1 arginine-rich cell-penetrating peptide

peptide-protein of interest interaction domain
Based on a well-known protein/protein interaction motif

directed protein degradation by peptide-lysosomal proteins interaction
from CMA substrate proteins like RNaseA (KFERQ), hsc70 (QKILD), or hemoglobin (QRFFE)
CMA-peptide mechanism

Systemic or local injection

In situ production?
• **DAPK1**, Death-associated protein kinase 1, a calcium-calmodulin–regulated protein kinase normally inactive in the brain. Activation and recruitment of DAPK1 to NMDARs under excitotoxic stimulation or cerebral ischemia. *DAPK1 activation induce apoptosis.*

PBD : DAPK1 interact with C-terminal residues 1292–1304 of GluN2B
GluN2B can bind **only the active** and not the inactive form of DAPK1

---

**TAT 47-67_GluN2B 1292-1304_CTM**

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<th>Component</th>
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<td>CMPB</td>
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<tr>
<td>PBD</td>
<td>KKNRNKLRRQHYS</td>
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<tr>
<td>CTM</td>
<td>KFERQKILDQRFEE</td>
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</tbody>
</table>

**CMA peptide : TAT-GluN2B-CTM**

```
YGRKKRRQRRR
KKNRNKLRRQHYS
KFERQKILDQRFEE
```

**Control peptide : TAT-GluN2B**

```
YGRKKRRQRRR
KKNRNKLRRQHYS
```

**No cell-permeable peptide : GluN2B-CTM**

```
KKNRNKLRRQHYS
KFERQKILDQRFEE
```
In vitro CMA-peptide efficiency on cultured cortical neurons (1)

- **Bath application**:
  - TAT-GluN2B or TAT-GluN2BCTM (200 μM)
  - mixed GluN2B-CTM with the intracellular delivering carrier peptide Pep-1 at a 1:4 ratio (50/200 μM) for 30 min to form a plasma membrane-permeant peptide complex.
  - bath-applied: 60 min before and during NMDA treatments (50 μM; 30 min)

- ➢ in a dose-dependent manner
- ➢ in a time-dependent manner

➢ TAT- or Pep-1-mediated synthetic peptides to **rapidly** and **reversibly** degrade its endogenous binding partner DAPK1 following NMDA treatment
• **α-synuclein**, a protein implicated in neuro-degenerative synucleinopathies such as Parkinson’s disease.
  PBD : α-syn have a strongly interact with β-synuclein (βsyn 36-45)

• **PSD-95**, a membrane-associated guanylate kinase concentrated at glutamatergic synapses and involved in synapse stabilization.
  PBD : PSD-95 interact with 9 aa Cterminal of GluN2B

*In vitro* CMA-peptide efficiency on cultured cortical neurons (2)
**In vivo CMA-peptide efficiency : protocol**

- Rat model of focal ischemia: middle carotid artery occlusion (MCAo)

- Timecourse

- Injections: saline, TAT-GluN2B-CTM or TAT-GluN2B

![Diagram showing the protocol](image_url)
In vivo CMA-peptide efficiency: results

1- Control of ischemia insult:
2,3,5-Triphenyltetrazolium chloride staining of transverse brain sections

- saline-treated rats revealed that unilateral MCAo reliably induced insult mostly in the ipsilateral

2 - DAPK1 expression

- The knock-down of DAPK1 produce neuroprotection against ischemic insult in vivo

3- Neuroprotection:
Fluorojade staining
## Comparison of tools

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- **ZFN**: Zygote-activated nuclear cassette
- **TALEN**: Transcription activator-like effector nucleases
- **CRISPR-Cas9**: Clustered regularly interspaced short palindromic repeats with CRISPR associated protein 9
- **shRNA**: Short hairpin RNA
- **miRNA**: MicroRNA
- **CMA**: Cas9-mediated adeno-associated virus
- **pb**: Pico base
- **AAV**: Adeno-associated virus
- **LV**: Lentiviral vector
## miRNA maturation

![Diagram of miRNA maturation](image)

### Natural microRNA in nervous system

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Function</th>
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<tr>
<td>miR-302; miR-124</td>
<td>différentiation neuronale</td>
<td>(Hohjoh and Fukushima, 2007)</td>
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<tr>
<td>miR-1</td>
<td>facteur de croissance; développement neuronal</td>
<td>(Lewis et al., 2003)</td>
</tr>
<tr>
<td>miR-29</td>
<td>régulation de l’apoptose</td>
<td>(Mott et al., 2007)</td>
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