Auxin Proteo Controller (AID System Kit)

BioROIS Co., Ltd.

Munehiro Tomikawa Ph.D.

What is BioROIS Co., Ltd. ?

BioROIS Co., Ltd. is a venture business company having functions of research development, manufacturing, marketing and sales of new products supported by bio-related patents belonging to National institute of Genetics (NIG) in the Research Organization of Information and Systems (ROIS).

Main purpose of the establishment of BioROIS Co., Ltd. is to participate In supports to scientist engaged in the field of life science through the research development and supply of new products necessary for their new technology and research.

BioROIS Co., Ltd. has tried to develop not only domestic business of NIG Card but also international business with American partner.

This company was established at December 13 in 2006.
A motto of the company:

"More Convenient to Scientists"

Company Overview

- ◆Founded : December 13, 2006
- ◆Head Office : 5F Mishima Suruga-Building 15-26, Ichibanchou, Mishima-shi Shizuoka-ken 411-0036, JAPAN
- ◆TEL/FAX : TEL/ +81-55-994-9855 FAX/ +81-55-973-7575
- ◆E-mail : info@biorois.com
- ◆URL : http://www.biorois.com/eng/
- ◆Capital : 20million
- ◆Business :
  - ・ Analysis method of gene function and it's application.
  - ・ Research Development of technology and contract Business of cells with knock-out gene.
  - ・ Devices and apparatus for transport and storage of bio-resources.
  - ・ Production and sales of bio-resources.

“Why now ?
We need quick and accurate understanding of gene function”

Strategies in the life science industry have changed significantly since complete nucleotides sequence of human genes which is the source of all human life phenomena has been determined at 2003.

It has become critical for the life science industry to refine quickly and accurately the target genes of disease and to identify the molecular target of drug discovery, diagnosis and treatment.

Approach to understanding gene function

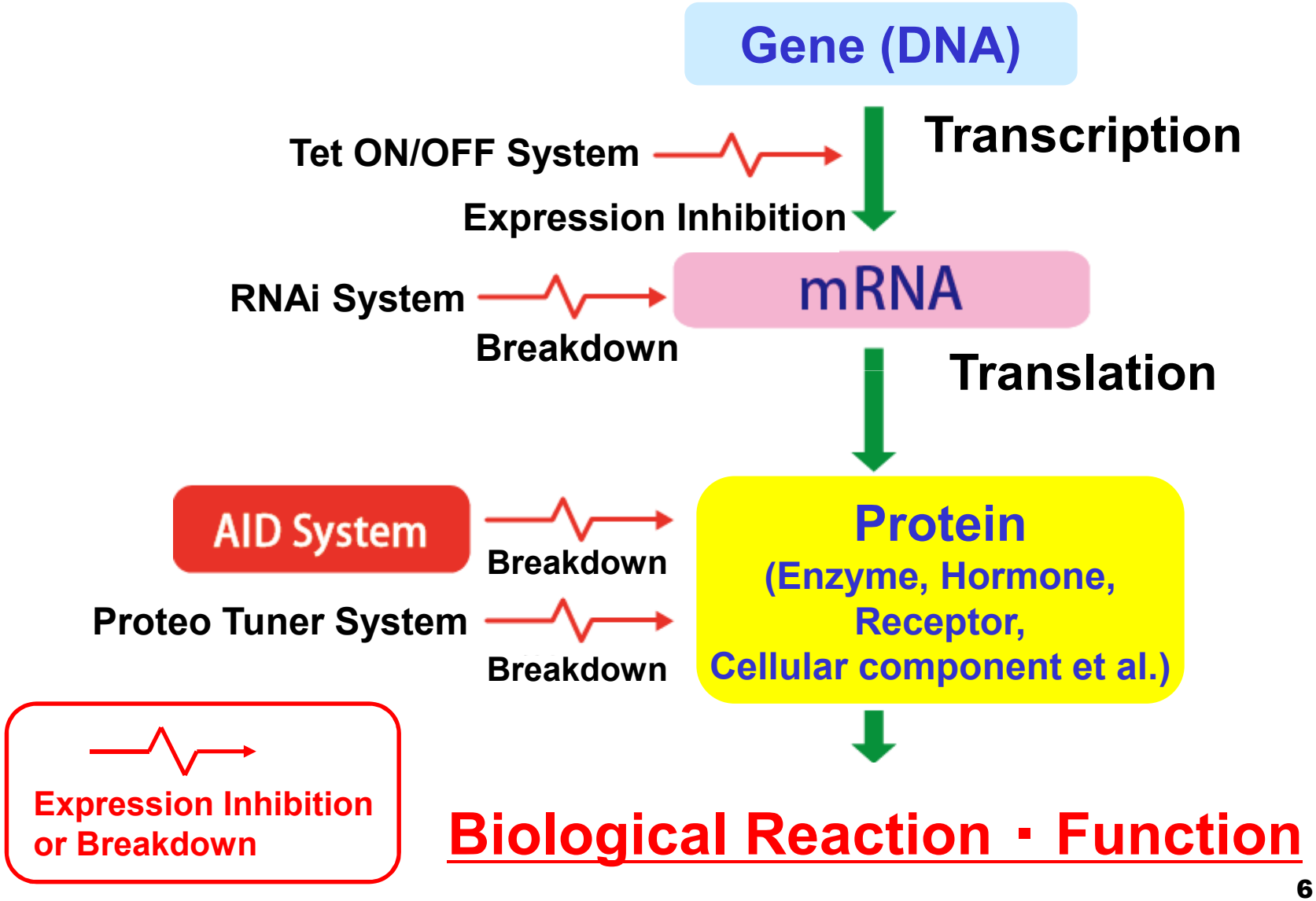
Analysis of the effects in cell after breakdown of specific protein (gene) function.

As an example, in the case of breakdown of enzyme function which is involved in the reaction A → B,
The accumulation of A, not B happens in cells.

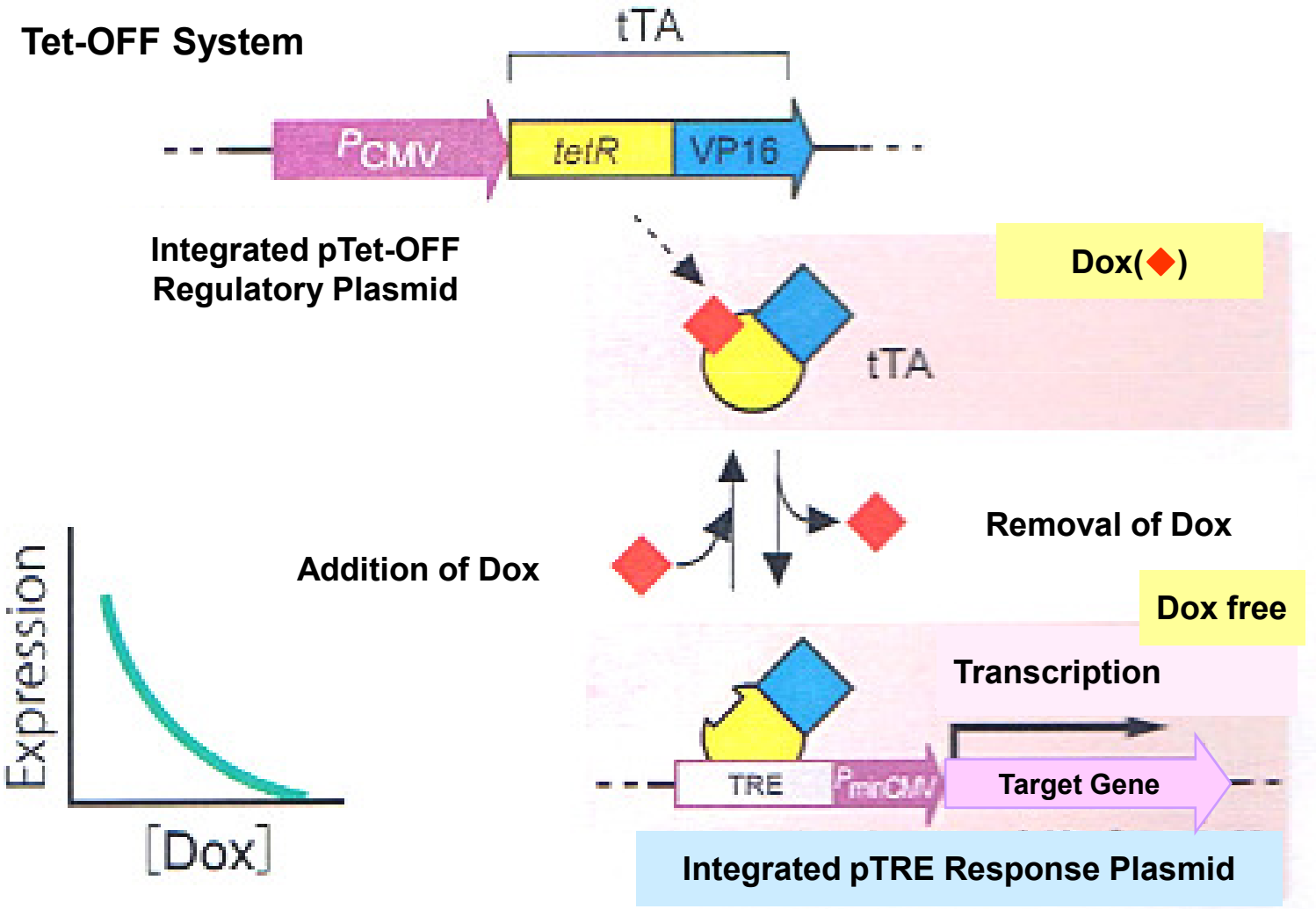
We can prove that the target enzyme is involved in the reaction from A to B.

Development of efficient and rapid degradation method of protein (gene) has been required to understand protein (gene) function.

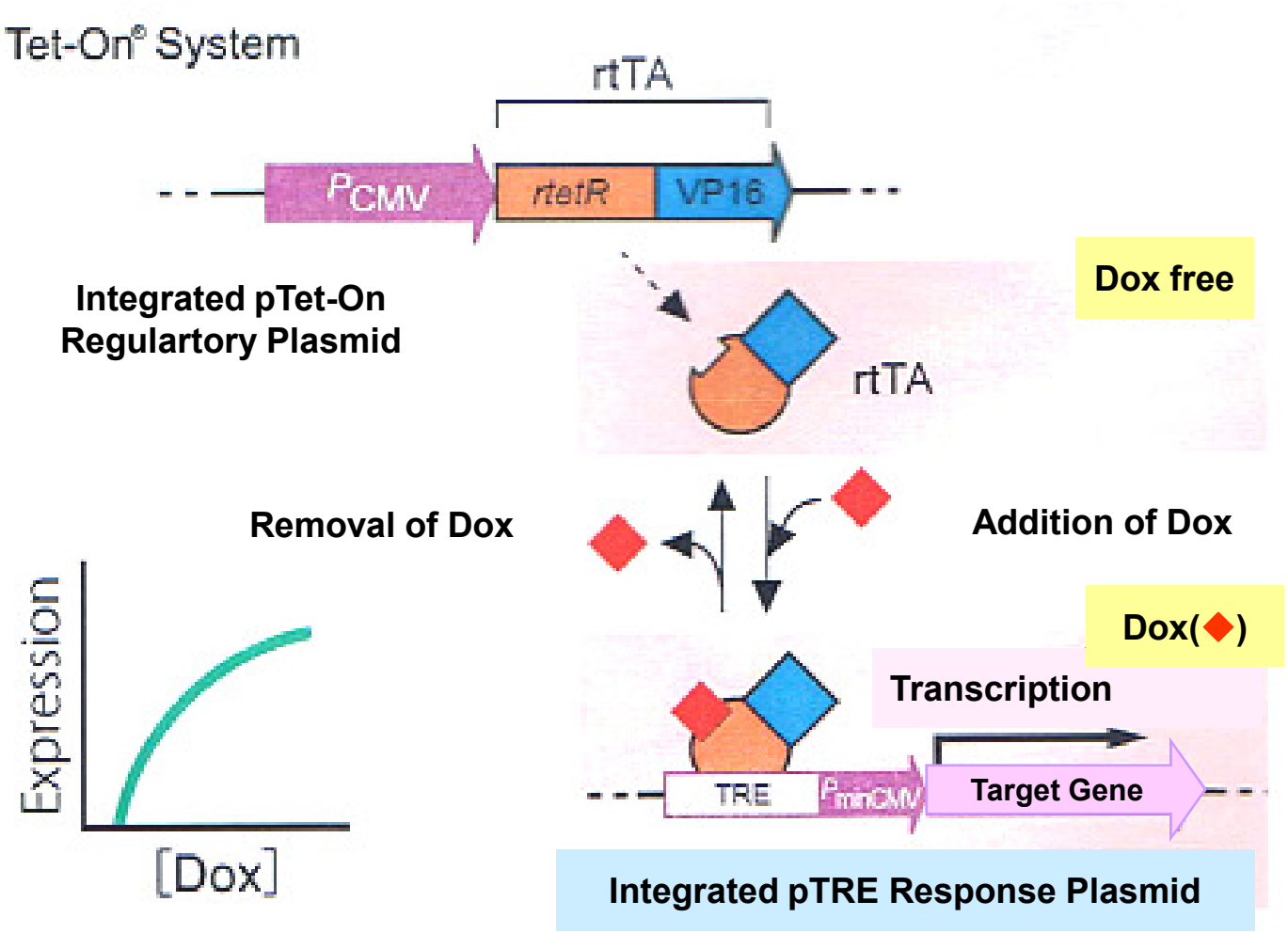
Principle of approach to understanding gene function



Tet-Off System



Tet-On System



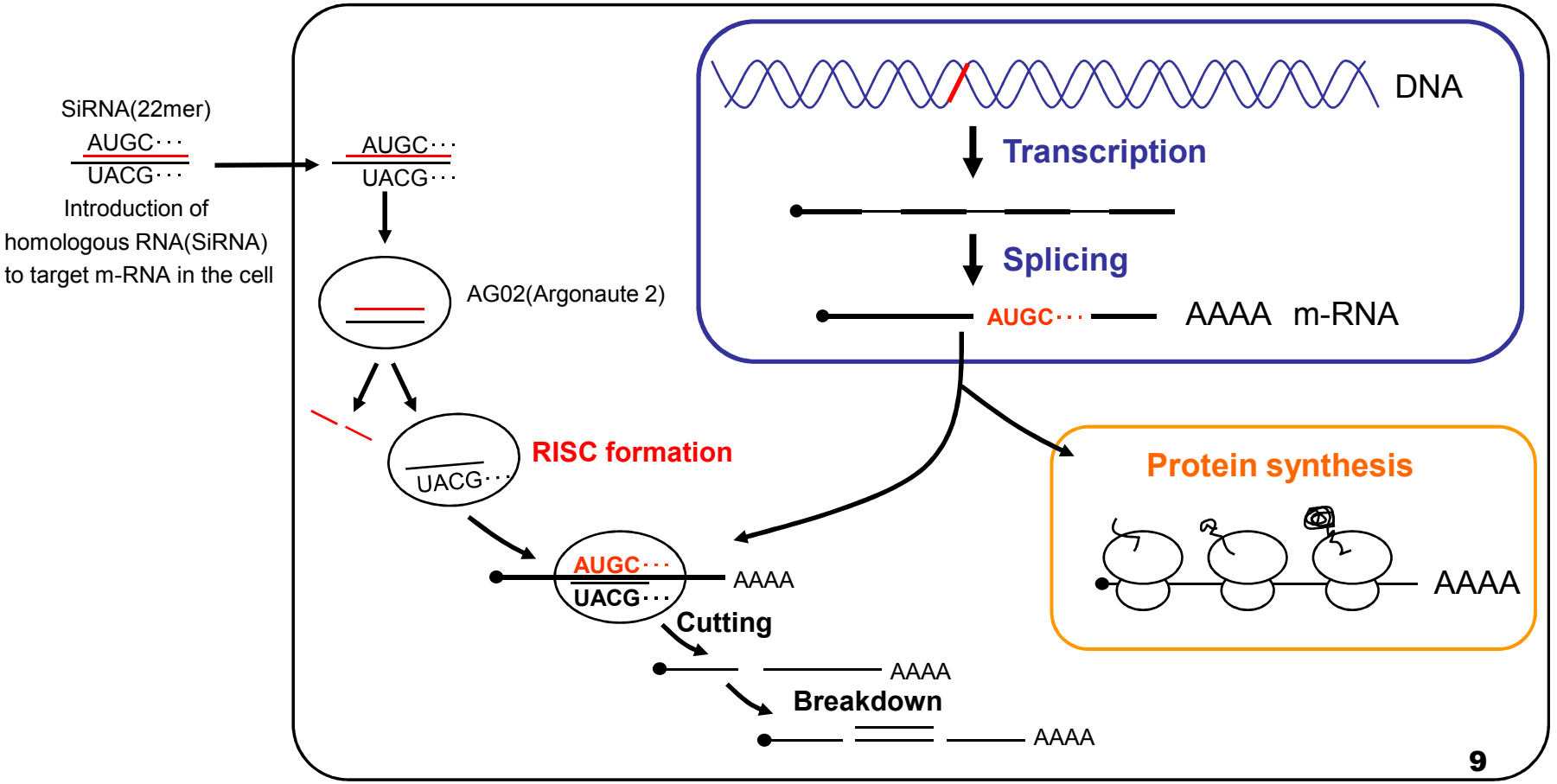
m-RNA Breakdown by RNAi Method

Strength

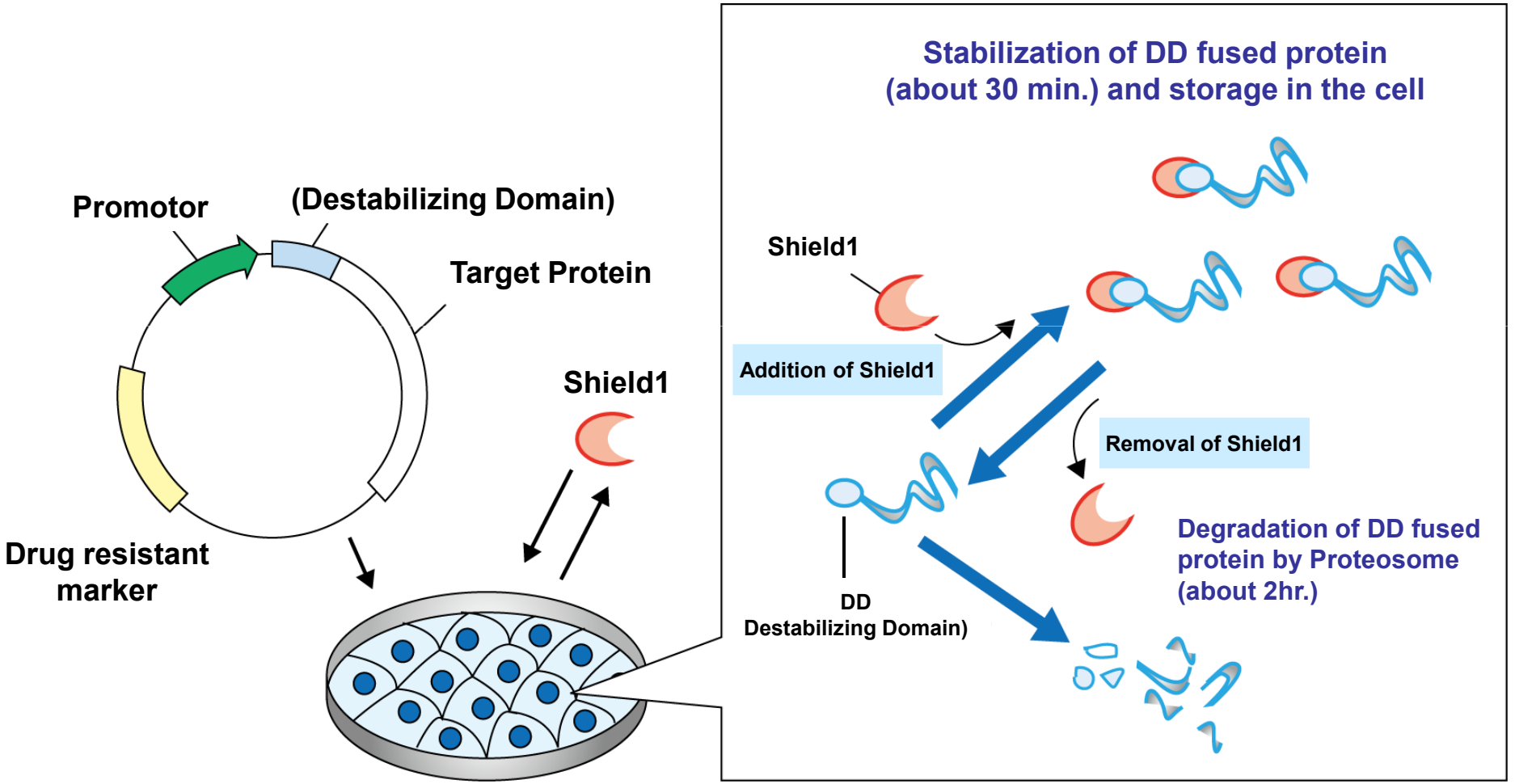
Anyone can easily do

Weak Point

- **m-RNA breakdown of off-target gene m-RNA**
- **Breakdown less than 70%**



Proteo Tuner System



Development of gene function analysis by rapid proteolysis

Prof. Fukagawa (National Institute of Genetics) has a long history of development of gene knockout method using animal cells (Nature Cell Biol. 2004, 2006, Cell 2008)

Dr. Kanemaki (Osaka University) has studied proteolysis methodology using the yeast.

They have jointly developed AID system which we can rapidly and effectively break down protein in animal cells. (Tokugan 2009-110449, Nature Methods 2009)

Nature Methods 6, p917-923, 2009

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]

Plants have evolved a unique system in which the plant hormone auxin directly induces rapid degradation of the AUX/IAA family of transcription repressors by a specific form of the SCF E3 ubiquitin ligase. Other eukaryotes lack the auxin response but share the SCF degradation pathway, allowing us to transplant the auxin-inducible degron (AID) system into nonplant cells and use a small molecule to conditionally control protein stability. The AID system allowed rapid and reversible degradation of target proteins in response to auxin and enabled us to generate efficient conditional mutants of essential proteins in yeast as well as cell lines derived from chicken, mouse, hamster, monkey and human cells, thus offering a powerful tool to control protein expression and study protein function.

Studies of protein function *in vivo* are greatly helped by systems that allow conditional inactivation or depletion of the protein of interest. Ideally, inactivation of the target protein would be rapid and efficient so that the immediate consequences can be assessed before the phenotype is complicated by the accumulation of secondary effects. Reversibility of inactivation would be an additional advantage. In practice, these features are hard to achieve in most eukaryotic species, unless a small-molecule inhibitor has already been developed for the target protein.

Other systems aim to deplete a target protein directly by exploiting specific protein degradation pathways[7,8]. Some of these have been achieved by modulating ubiquitin ligases such as SCF (Skp1, Cullin and F-box) complexes[9–11]. An advantage of these systems is that endogenous proteins can be rapidly controlled as long as a specific domain or small molecule that interacts with the target protein is known[12,13]. As a more general approach to protein knockdown, a domain to induce degradation (known as a 'degron') can be fused to the protein of interest. The temperature-sensitive degron system, using a degradation pathway based on the N-end rule[14,15], has been used to characterize many essential proteins in budding yeast[16,17]. This approach is, however, of limited use in mammalian cells that are less resistant to changes in temperature, although a recent study described its application to chicken DT40 cells[18]. Another method involves fusion with the destabilizing domain derived from FKBP12 (ddFKBP)[19]. This method has been used to control protein expression in mammalian cells, their parasites and transgenic organisms[20–22]. We compare current degradation-based methods in **Supplementary Table 1**.

Here we describe an alternative system for rapid protein depletion, based on transplanting the auxin-dependent degradation pathway from plants into other eukaryotic species, which we call the auxin-inducible degron (AID) system. This system allowed us to induce rapid depletion of a protein of interest (within

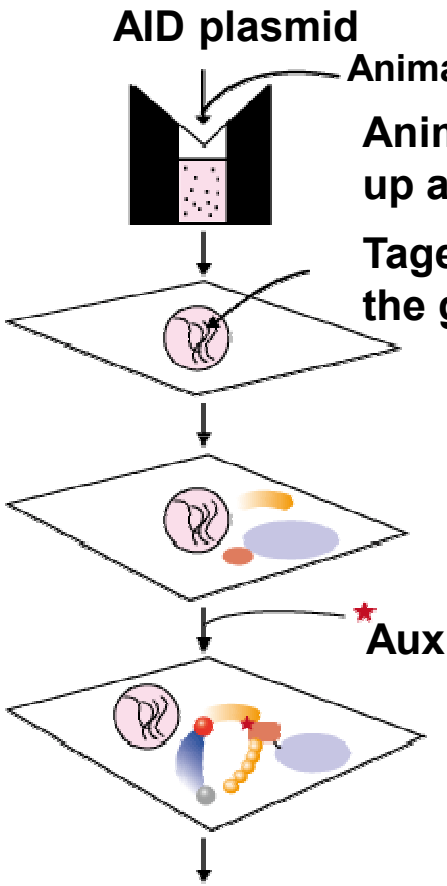
What is the AID System ?

（1）Principle

- Plant-specific protein degradation mechanism was applied to animal cells.
- In plants, target protein of TIR which is not present in animal cells is broken down by addition of auxin.

TIR derived from plants is expressed in animal cells and a part of protein IAA sequence (AID tag) that becomes the target of TIR is added to an arbitrary protein. The aid tag fused protein is broken down by the addition of auxin (plants hormone)(A New concept).

Introduction of AID plasmid into animal cells



Animal cells and AID plasmid are mixed up and the electrical pulse is given.

Taget Protein DNA is taken into the genome in the cells.

Expression of exogenous genes (Expression of TIR1 and aid tag protein)

Ubiquitin proteasome system is activated by the addition of auxin to cultured cells

A rapid degradation of the target protein happens.

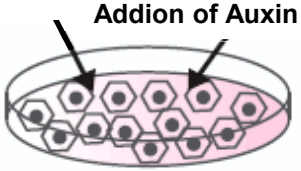
AID (Auxin inducible Degron) System

（2）System Design

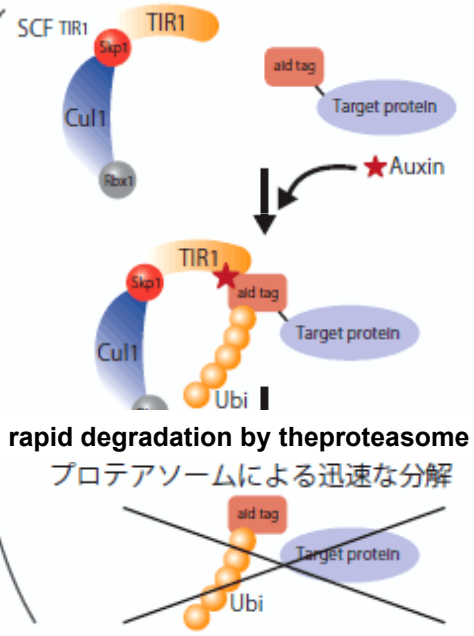
1. Introduction of AID Vector (Introduction of TIR and target protein genes)

Introduction of TIR and Target protein genes

Addion of Auxin



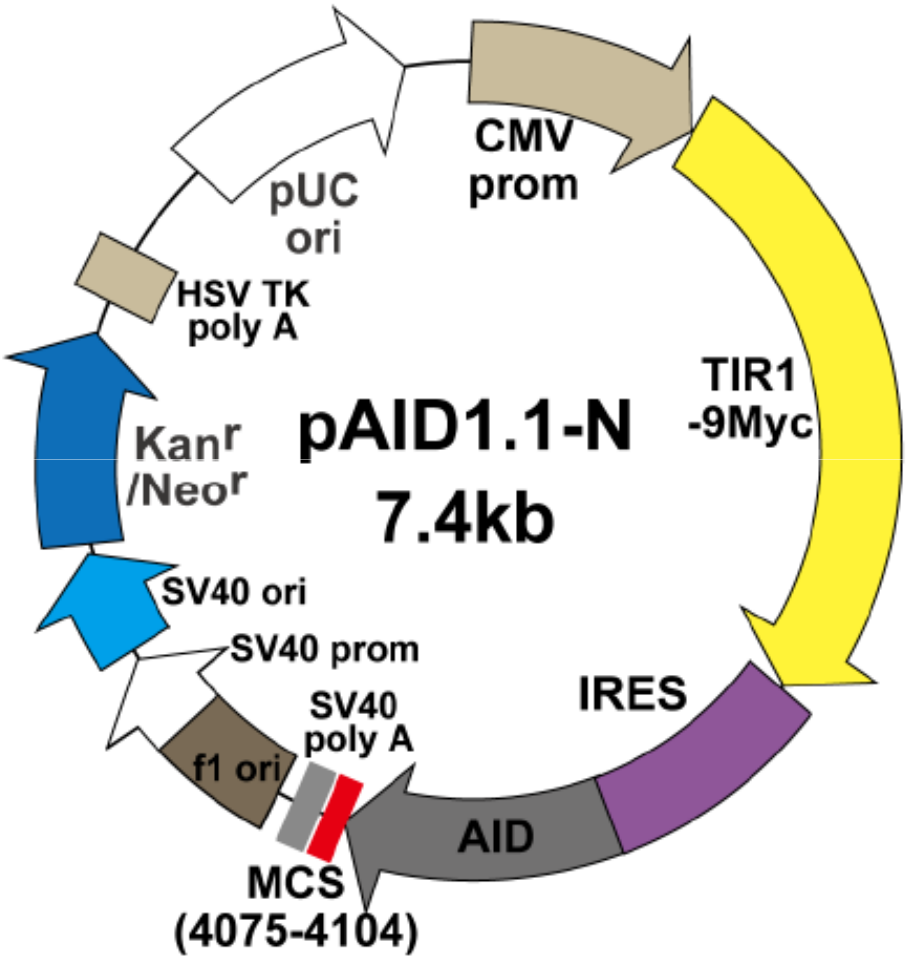
2. Addion of Auxin



AID vector plasmid consists of TIR derived from plant and the protein of interest fused with aid-tag (25-kD degron),
This vector plasmid is transplanted into the animal cells and two proteins are expressed.

Target protein is degraded by the addition of auxin.

pAID1.1-N Vector



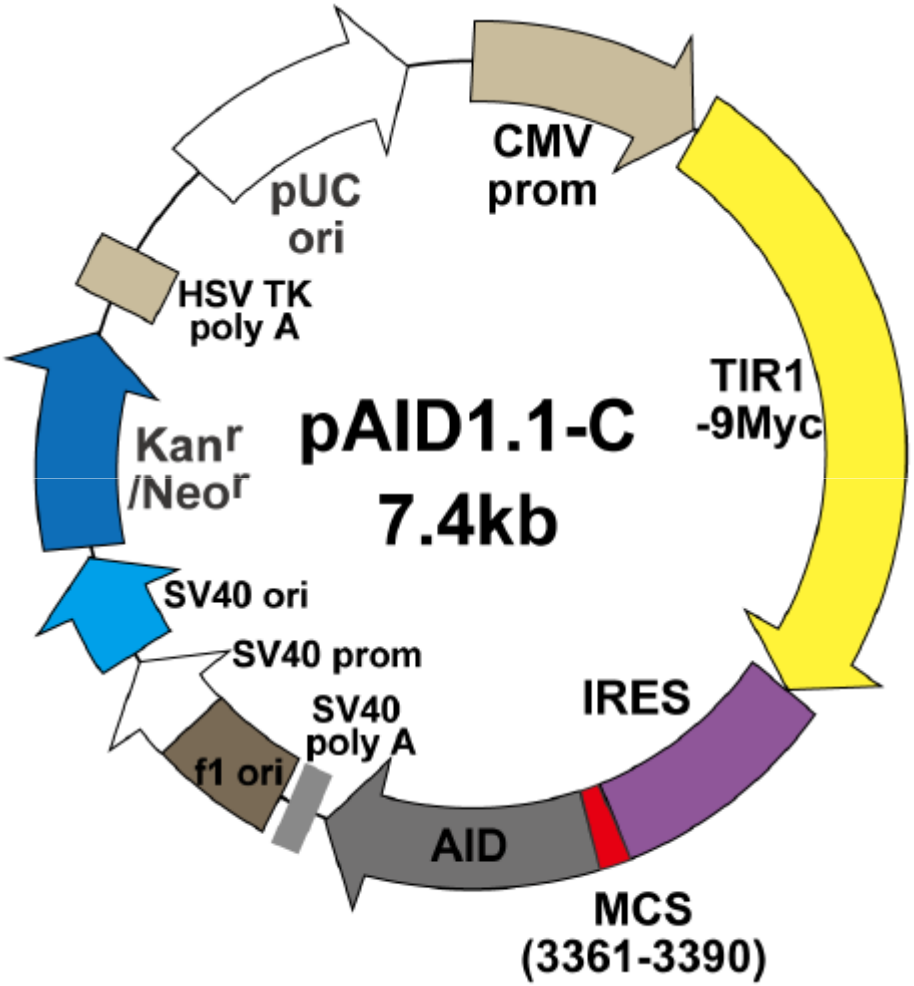
AID degron

G A G A G A G A G A

5' GGA GCT GGT GCA GGC GCT GGA GCG GGT GCC GAT ATC GAA TTC CGA TCG ACG CGT AGT ACT 3'

Linker EcoRV EcoRI PvuI MluI ScaI

pAID1.1-C Vector



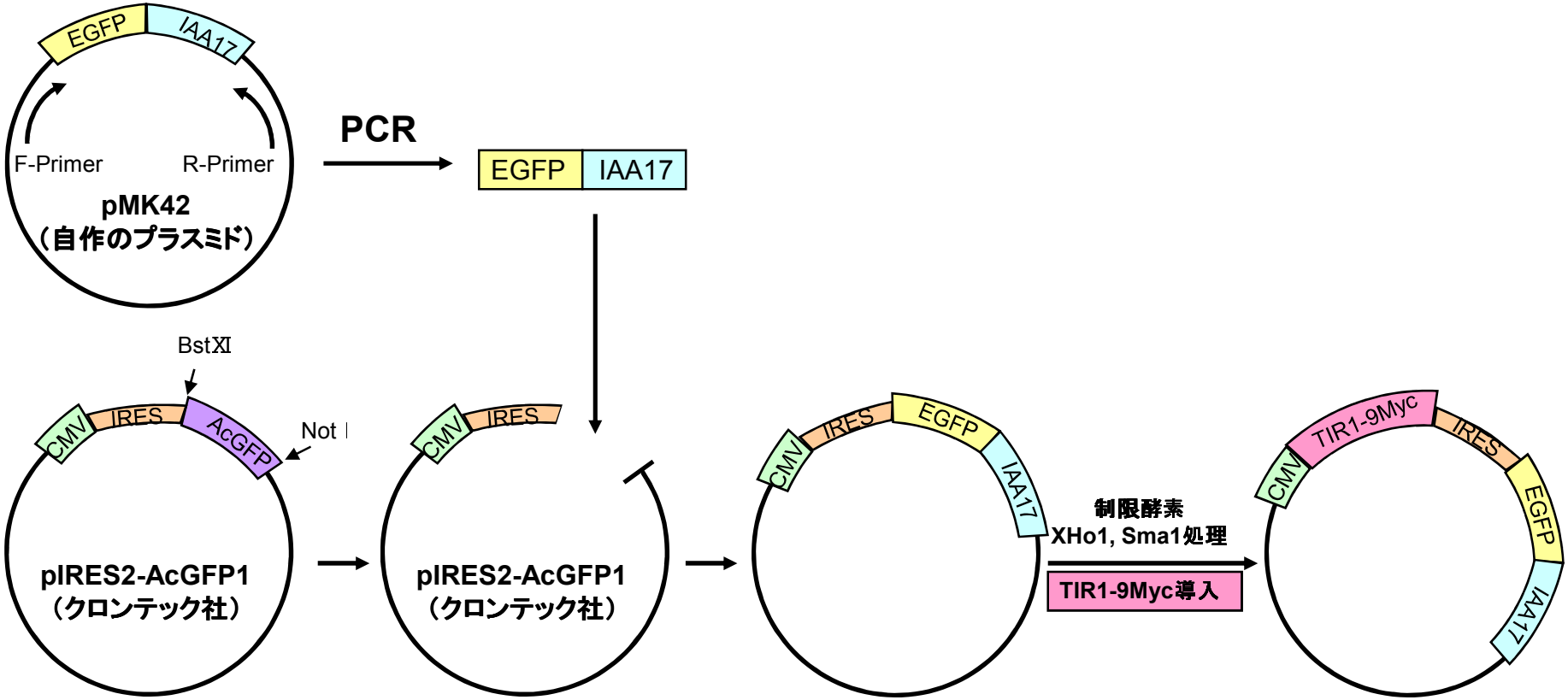
AID degron

M G A G A G A G A G A

5' ATG TGC GAT ATC GAA TTC CGA TCG ACG CGT AGT ACT GGA GCT GGT GCA GGC GCT GGA GCG GGT GCC 3'

EcoRV *EcoRI* *PvuI* *MluI* *ScaI* Linker

Preparation of GFP expression Vector incorporated in AID System

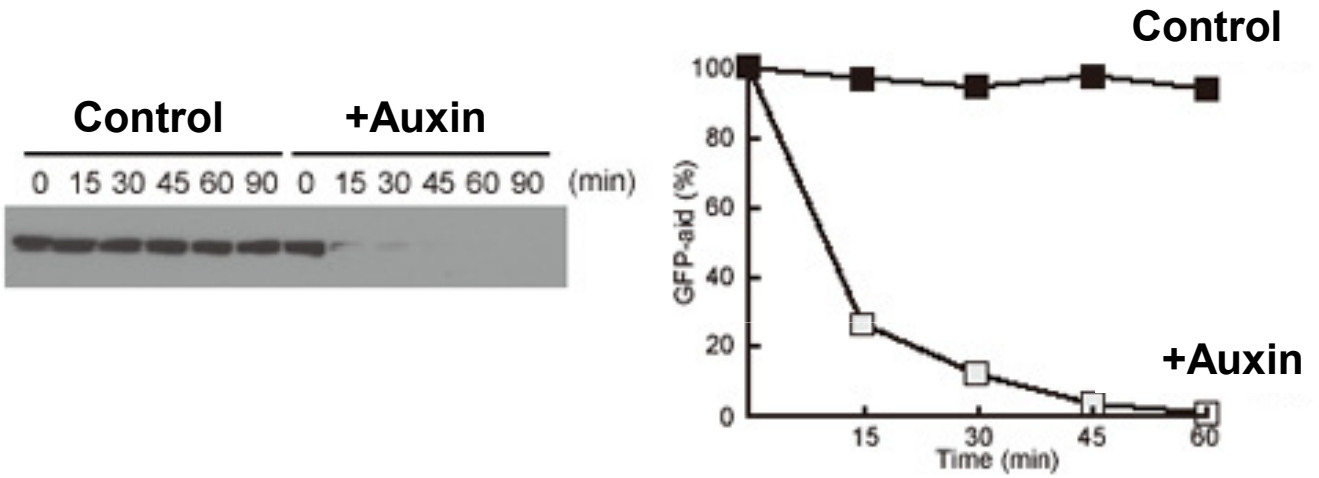


F-Primer：
5‘－CAGTGAATTCCCACAACCATGGTGAGCAAGGGCGAGGA－3’

R-Primer：
5‘－GGTCAGCGGCCGCTGGGTACCTTAAACCTTACG－3’

Rapid Degradation of GFP-aid in HEK 293 cells

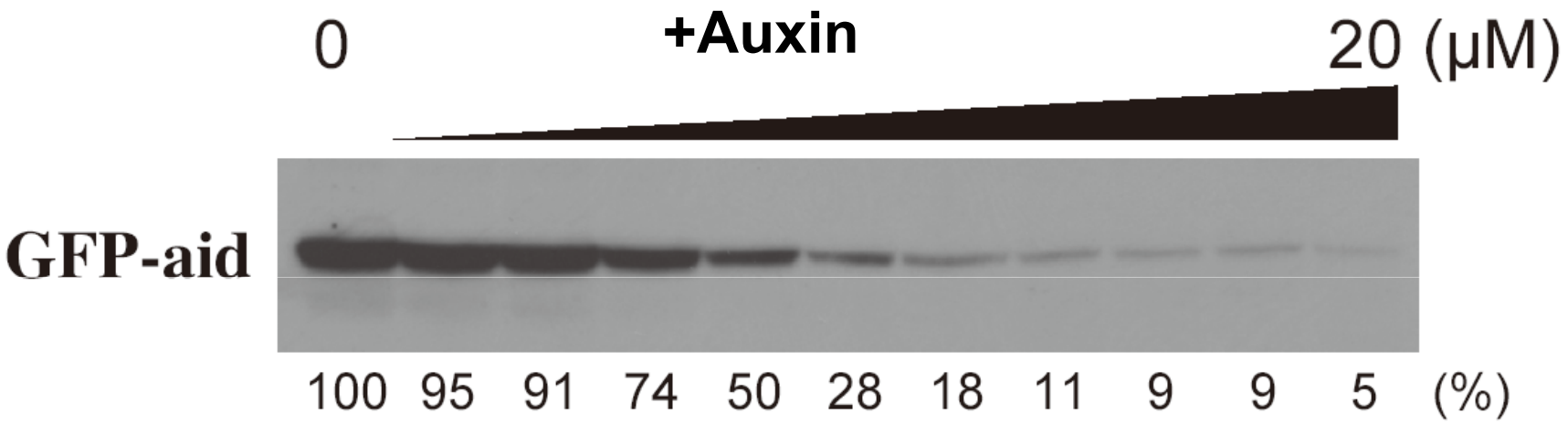
(3) Rapid Degradation of GFP (Green Fluorescent Protein)



A 233 stable cell line expressing GFP-aid was established using pAID1.1-C, cells were taken at indicated time points for immunoblotting after addition of 500μM IAA (a natural auxin) to cultune medium.

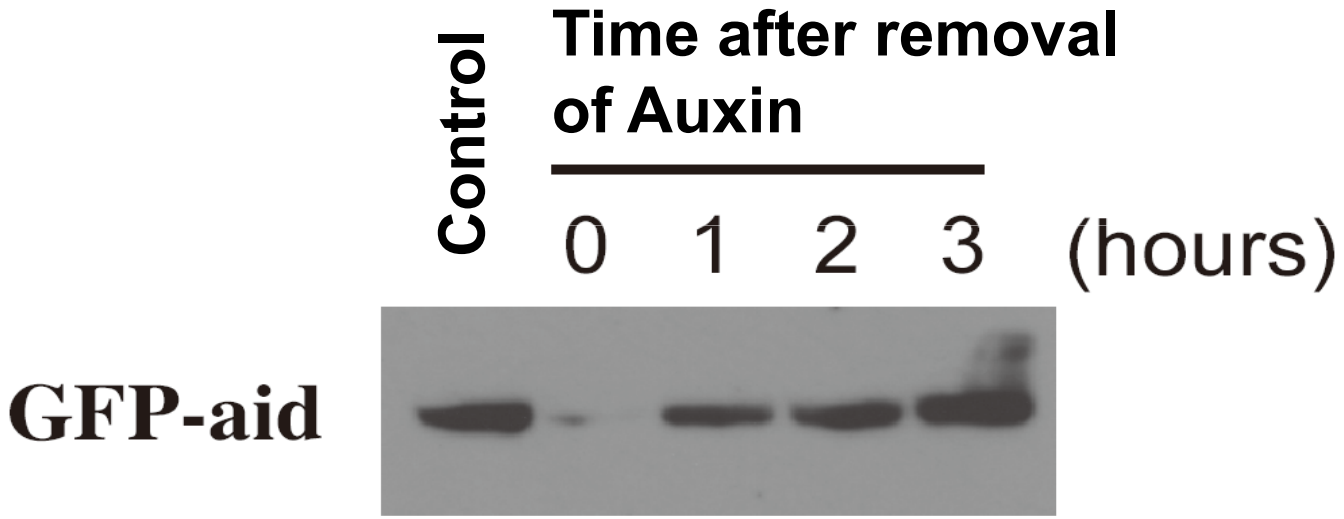
The majority of GFP-aid protein was depleted within 30minutes by the addition of Auxin.

Degradation of GFP-aid depending on Auxin Concentration



A stable 233 cell line as shown above was treated with different concentrations of auxin. Expression levels of GFP-aid were inversely correlated with auxin concentrations.

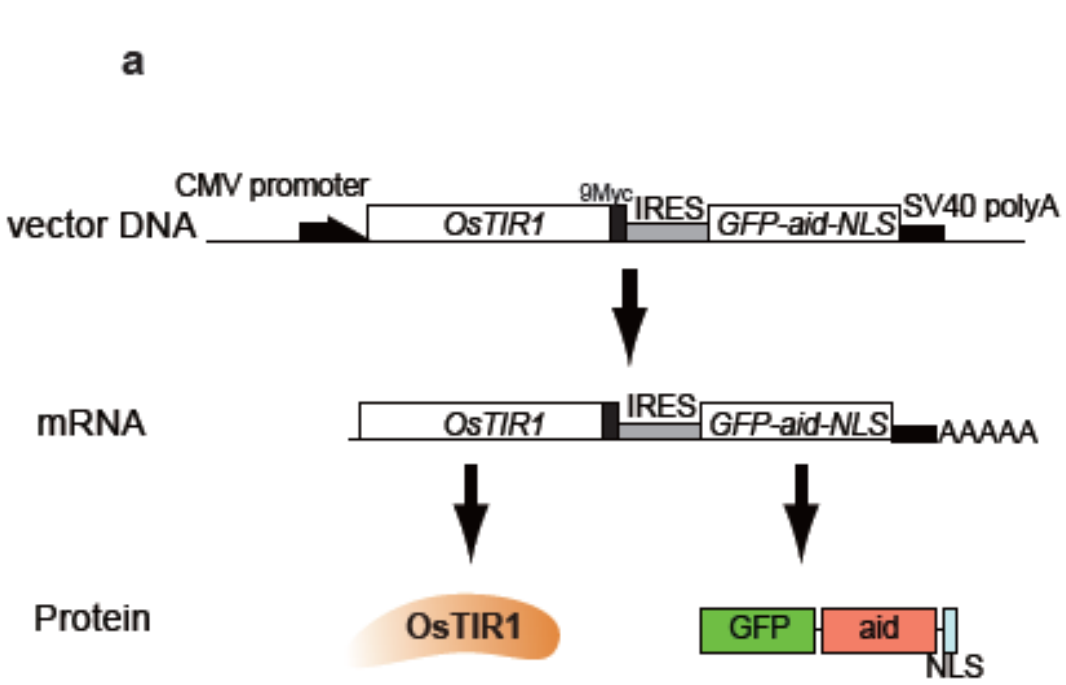
Reversibility of Degradation or Expression of GFP-aid in HEK 293 cells

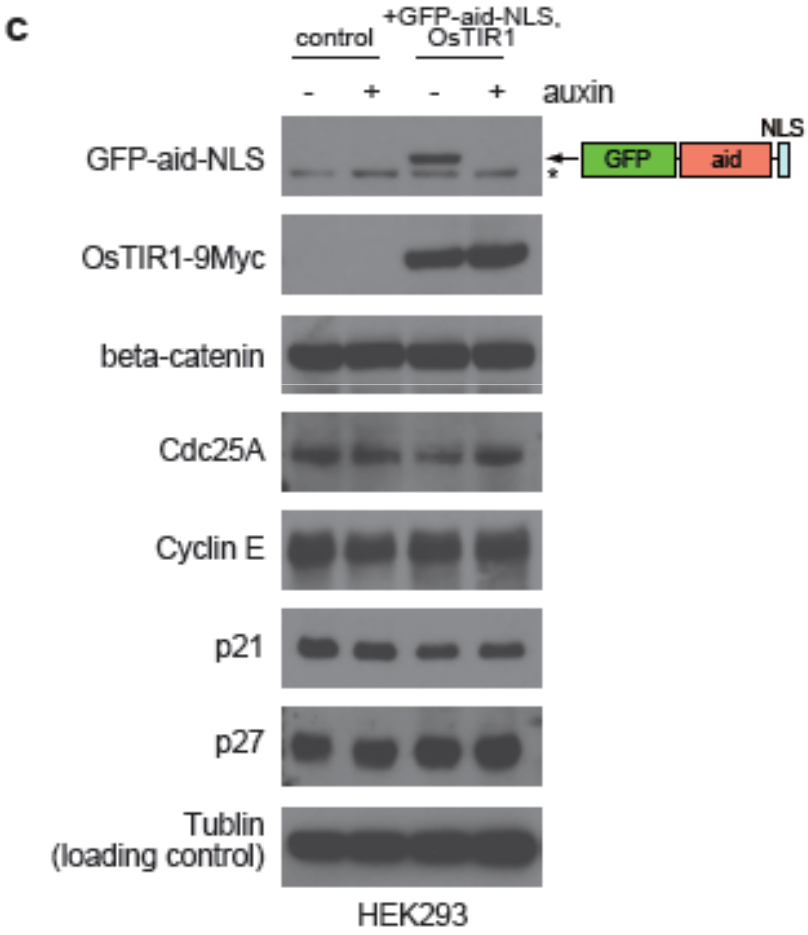


After degradation of GFP-aid in cells, the cells were transfered to culture medium without auxin in order to observe re-expression of GFP-aid.

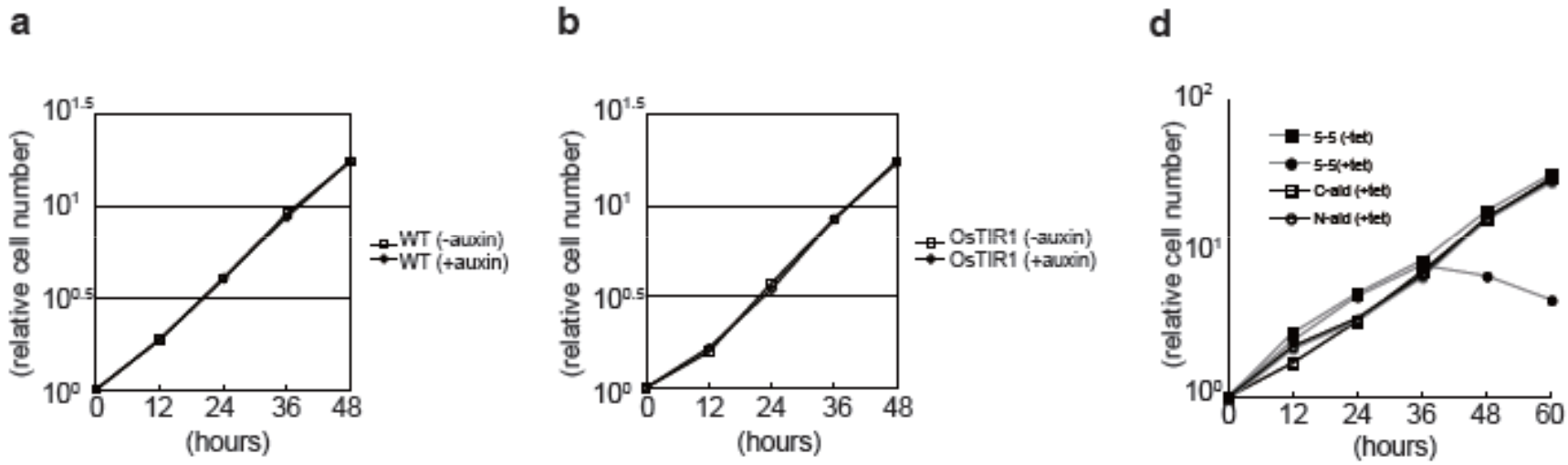
As shown here, GFP-aid was re-expressed within 1 hour after removal of auxin.

Selective degradation of GFP-aid by Auxin in HEK 293 Cells

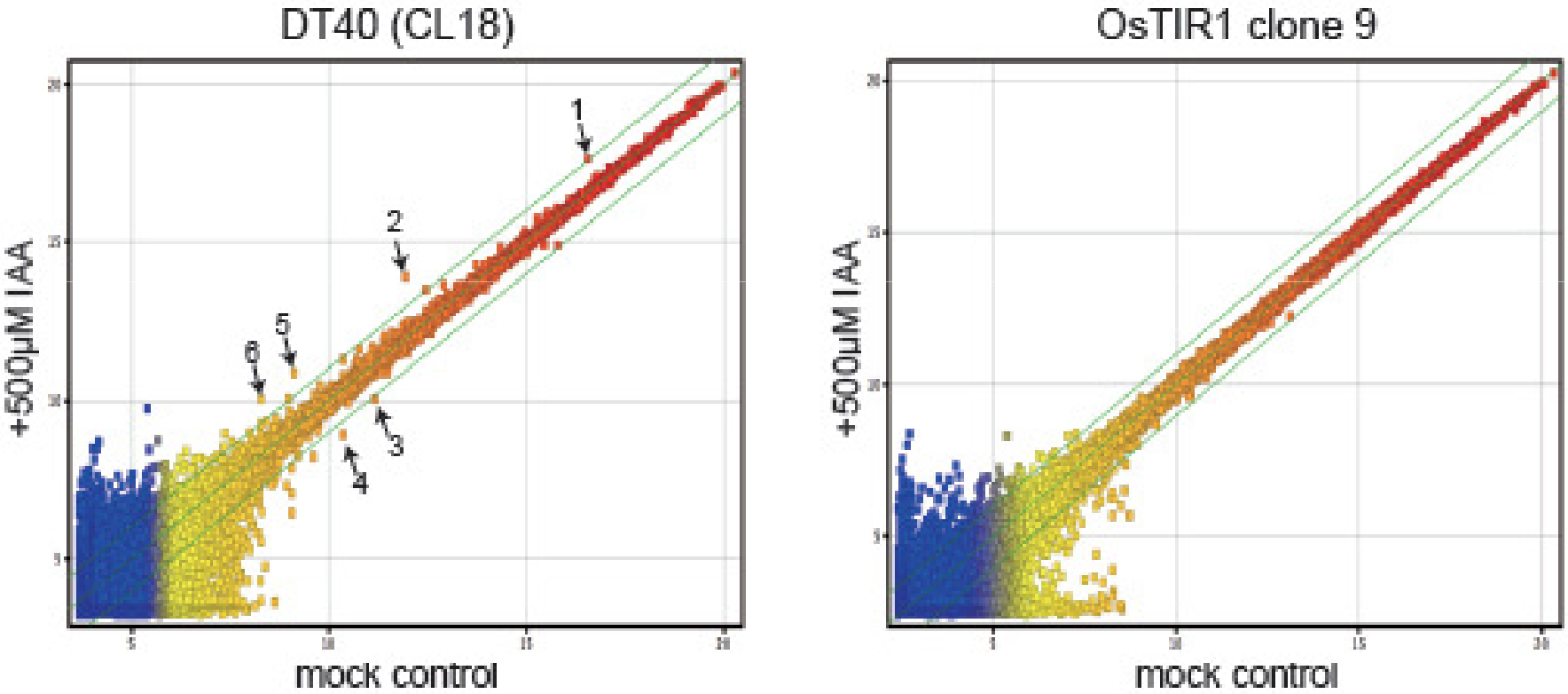




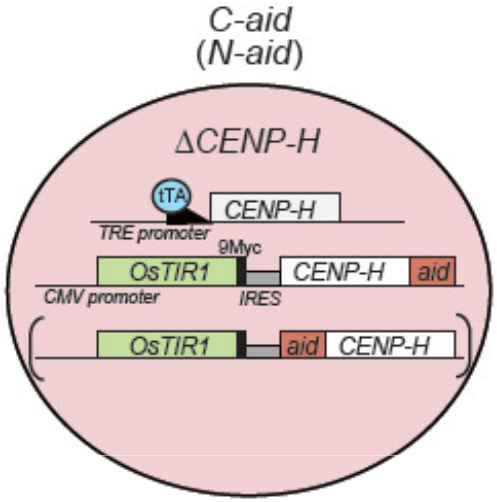
Effect of Auxin on Growth of DT40 cells

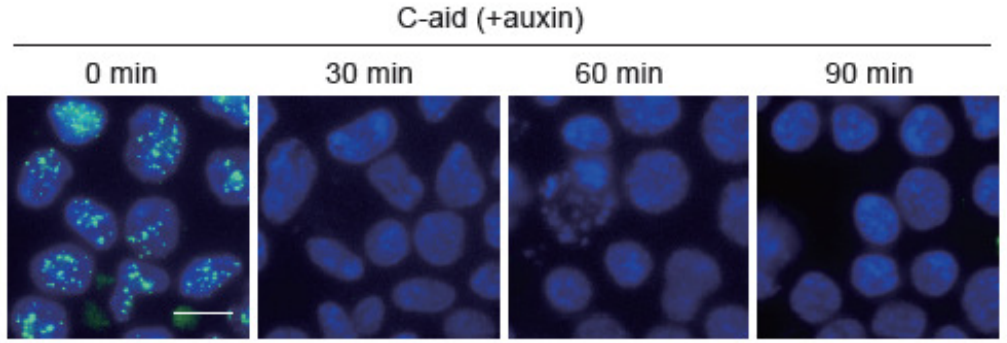


Effect of Auxin on Gene Expression in DT40 cells



Comparison of CENP-H Protein Degradation between AID System and Tet OFF System in DT40 Cells





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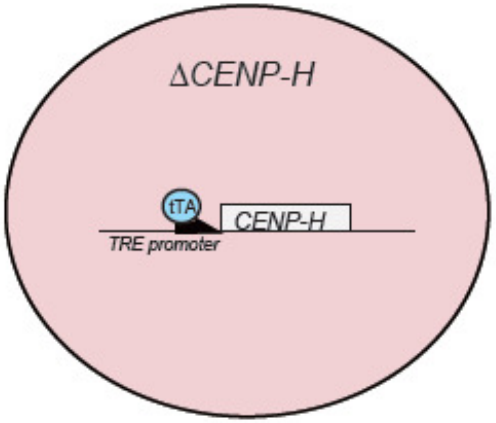
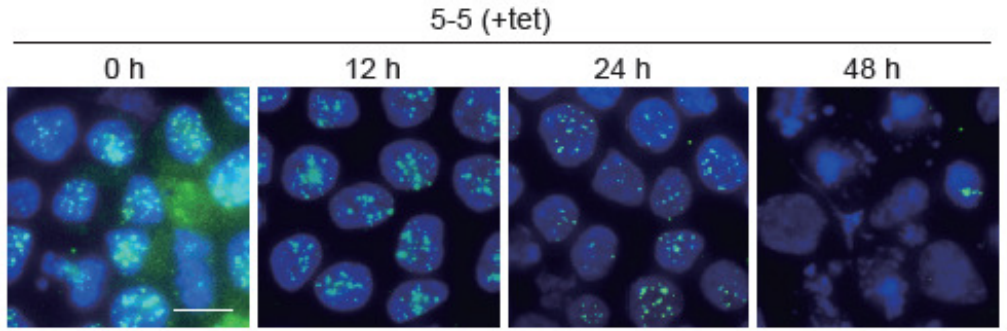




The degradation speed of a green target protein is extremely faster (30min.) in the AID system than the Tet ON/OFF system (48hr.)

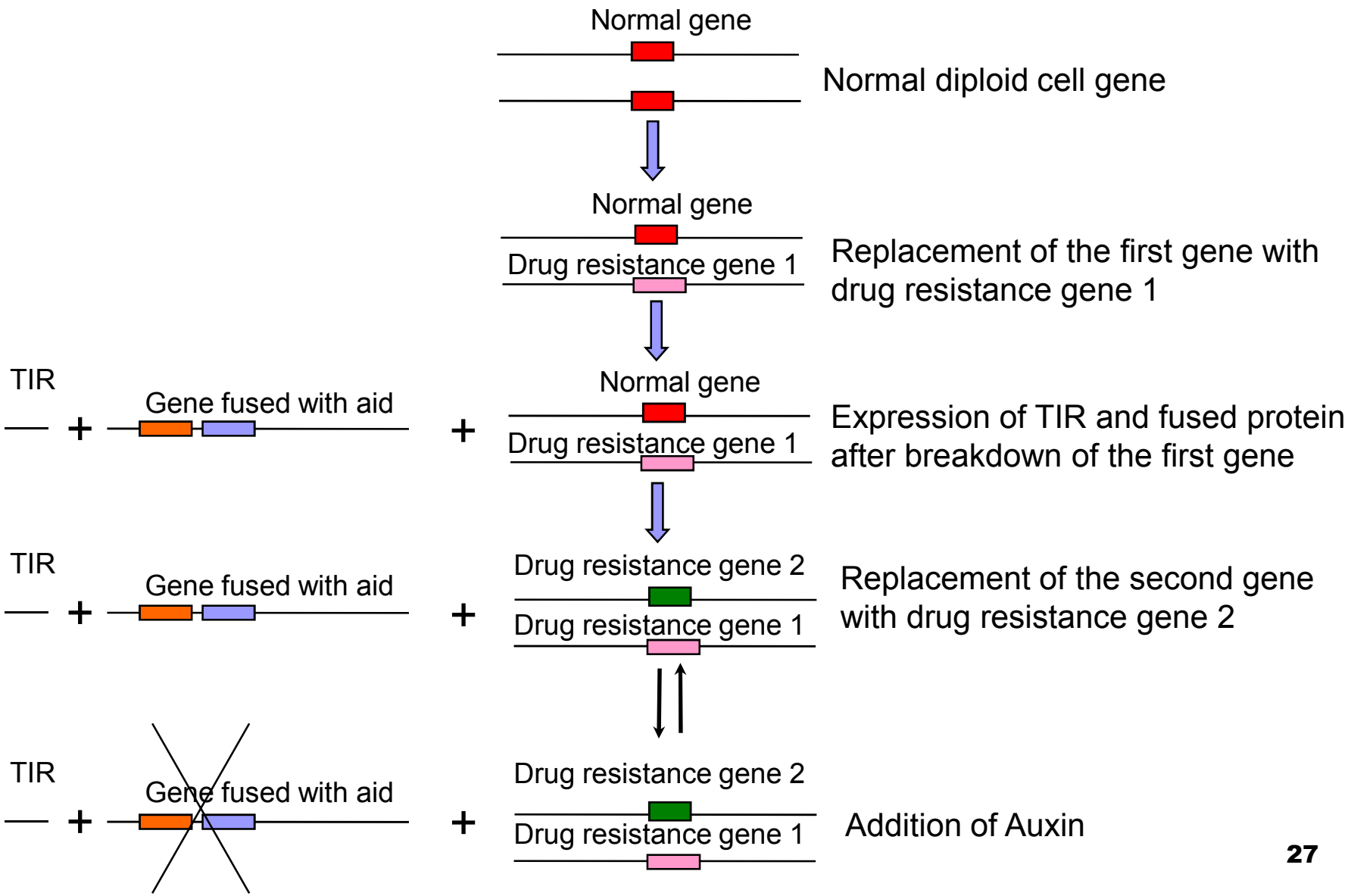
Comparison between AID system and Already-known Systems

	Mechanism	Degradation Speed	Preparation Method
AID System	Proteolysis	30 min.	DNA reombination
Tet ON/OFF	mRNA Expression in hibition	24 ~ 48 hr	Si RNA or Sh RNA
RNAi法	mRNA Degradation	24 ~ 48 hr	DNA recombination
Proteo Tuner	Proteolysis	4 hr	DNA recombination

Characteristics of AID System

1.Protein degradation is extremely rapid in comparison with other systems.
2.Auxin, inducer doesn't have toxicity for the animal cells.

Breakdown method of protein essential for cell growth by combination of AID system with gene knockout technique



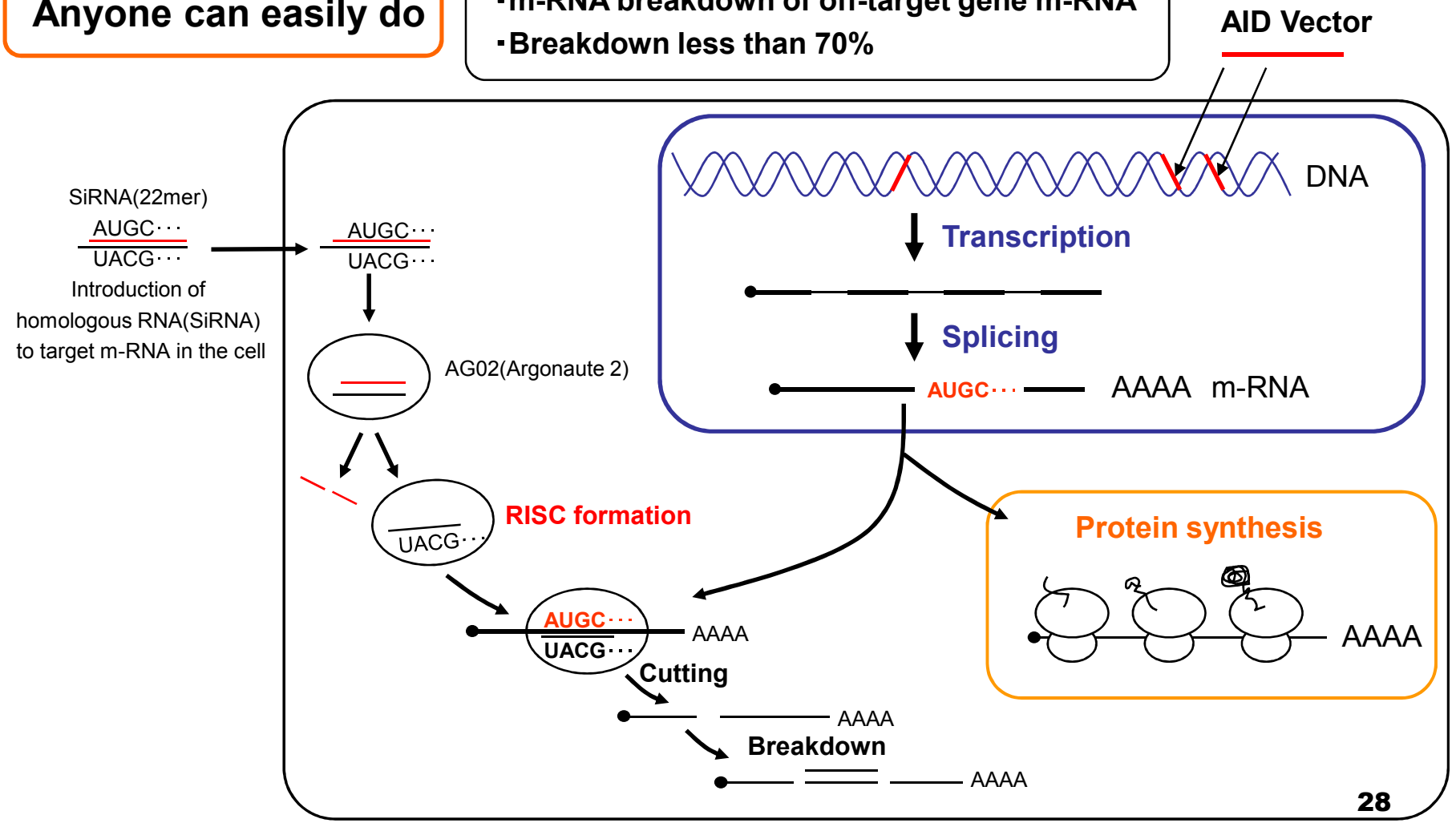
Combination of RNAi Method and AID System for Depletion of Endogenous and Exogenous Taget Proteins

Strength

Anyone can easily do

Weak Point

- **m-RNA breakdown of off-target gene m-RNA**
- **Breakdown less than 70%**



Business Plans of AID System Technology

「Title of Invention」	Proteolysis inducing method in the mammalian cells
「Application Number」	Tokugan 2009-110449
「Application Date」	April 30, 2009
「Inventor」	Masato Kanemaki, Tatsuo Fukagawa et. al.
「Application」	Osaka University, National Institute of Genetics

BioROIS has gotten exclusive license with sublicensing right of these patents from Osaka University and NIG.

1) Sale of improved AID system Kit

(Competitive products: Clontech Tet ON/OFF System, Proteo-tuner et. al.)

2) Contract business of Target protein proteolysis inducing cells.

(Competitor: Contract business of knockout mouse in the venture company in the USA or Europe.

AID System Kit Consists of four Products

AID System Applications
Precise functional analysis of human genes (proteins)

1. Discovery of Disease-related Genes or Diagnostic Marker
2. Specification of Drug Discovery-related Genes
3. Drug Screening (Target Screening) and Pharmacological Evaluation
4. System Clarification of Vital Phenomena

Different Points compare AID System with other products

	AID System	A	B
Action Mechanism	Proteolysis **(Protein degradation time 30 min)**	mRNA Expression Inhibition **(Protein degradation time 24 – 48 hr)**	mRNA Breakdown **(Protein degradation time 24 – 48 hr)**
Preparation Method	DNA recombination (Introduction of one plasmid)	DNA recombination (Introduction of two plasmids – Regulatory plasmid, Response plasmid)	Introduction of SiRNA or ShRNA (homologous SiRNA 22 mer)
Induction of Reaction	Plant Hormone (Auxin)	Tetracycline (Tet.) (Doxycycline) (Dox.)	Nuclease Complex (RISC:RNA Induced Silencing Complex)
Characteristics (Strength)	①Protein expression is controlled by the presence or absence of auxin and dependently on the amount of auxin in one plasmid-transplanted cells ②Applied to mammalian cells and yeast	①m RNA expression is controlled by the presence or absence of Tet. (Dox.) and dependently on the amount of Tet. (Dox.) in two plasmids-transplanted cells ②Applied to mammalian cells	①Anyone can easily do experiment ②Degradation of targeted mRNA is induced by introduction of homologous RNAi ③Applied to mammalian cells
Week Point	①Not so much data	①Necessary to use Tet.(Dox.) fee FBS	①OFF target (possible degradation of mRNA of non-targeted gene) ②not complete degradation of mRNA (about 70%)