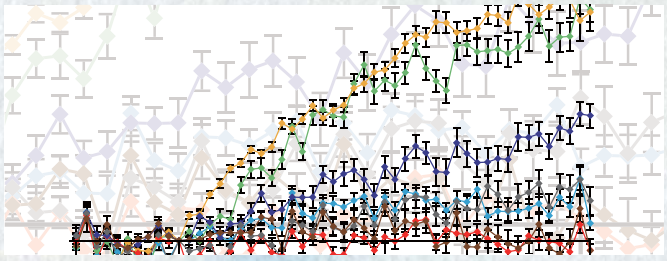
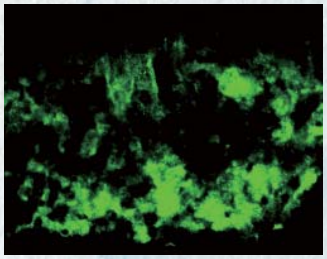
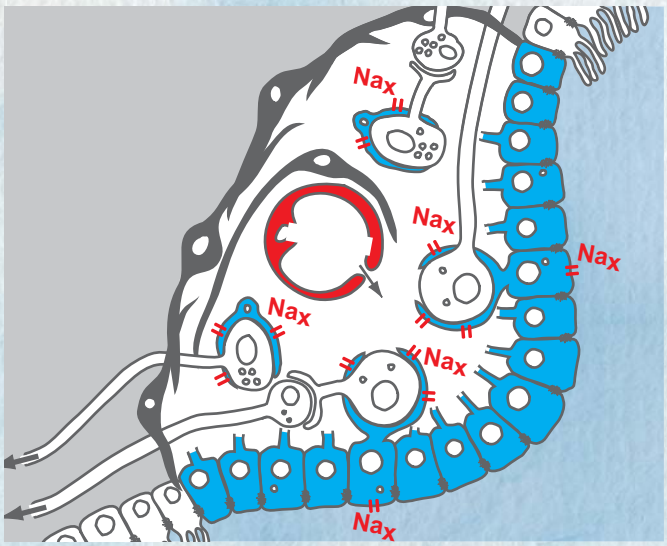
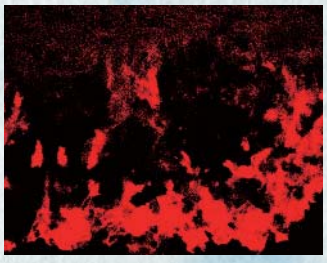
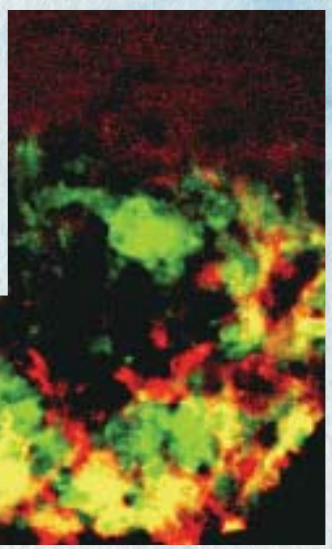
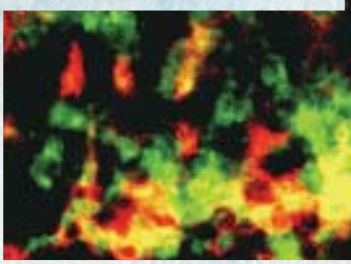










National Institute for Basic Biology

# 2013 ANNUAL REPORT

# CONTENTS



The photographs on the cover show a series of studies on neural mechanisms for sodium (Na) homeostasis in animals. Na homeostasis is essential to life, and $Na^+$ concentrations ($[Na^+]$) in plasma and cerebrospinal fluid (CSF) are continuously monitored in the brain by $Na_x$, an extracellular $[Na^+]$ sensitive Na channel, to control salt-intake behaviors. $Na_x$ is expressed in glial cells in the circumventricular organs (e.g. subfornical organ [SFO]), which lack a blood-brain barrier. Previous *in vitro* studies indicated that an apparent threshold value of $Na_x$ for $[Na^+]$ is ~150 mM, though $[Na^+]$ in plasma and CSF is strictly controlled at 135–145 mM in mammals. This discrepancy has now been resolved by our study which showed that the $[Na^+]$ dependency of $Na_x$ is shifted to the lower-concentration side by endothelin-3 produced in the SFO in response to dehydration (Hiyama *et al*., Cell Metab., 2013). See page 35 of this report for details.

This is my first address in the Annual Report of the National Institute for Basic Biology (NIBB) as a Director General of the Institute, which I undertook last October. Since my appointment, I have been enjoying the high level research activities of the Institute and its effective function as a center for collaborative research in Japan. At the same time I feel my strong responsibility to maintain the liberal atmosphere for research and discussion that NIBB has kept for many years, which I believe is a basis for these remarkable activities.

Because of the relative decline of Japan's status in the worldwide economy, the government is now keen to pull out actual profits from science. In addition, it has become apparent recently that certain researchers did not have sufficient ethics as a scientist and committed excessive misconduct in a number of institutions in Japan. These circumstances have set a strong headwind against basic sciences, through which NIBB must stride properly. In my view, two directions will be very important. One is for all NIBB members. Every person in NIBB should do his/her best in accomplishing good research in basic biology. Good science, even in a basic research field, will eventually benefit human beings. The history of science tells us this is true. The other is for the administrative board of NIBB. The methods of science are changing significantly in the era of computer technology and data sharing through the internet. Inspection of recent scientific misconduct cases indicates that research institutions must have high ethical standards to prevent them. NIBB should work hard to enhance the realization of each individual's ethical obligations as a leading institution in this respect.

Please find in this booklet a summary of the research, collaborative, educational, and international activities of NIBB in 2013. I would like to note that we welcomed several new colleagues in 2013, including one adjunct professor, two assistant professors, six specially appointed assistant professors, and five NIBB research fellows, while two colleagues transferred to other institutes.

Finally I would like to congratulate Prof. Mitsuyasu Hasebe for winning the Janette Siron Pelton Award from the Botanical Society of America. I would also like to congratulate Prof. Mikio Nishimura, Prof. Taisen Iguchi, Assoc. Prof. Takashi Murata, and Assoc. Prof. Shuji Shigenobu for winning awards from academic societies and governments, as detailed on page 7.

To establish NIBB as an international leading institute in the field of basic biology, we always welcome your suggestions, comments and queries concerning our activities, in addition to your warm support.

Masayuki Yamamoto
Director General of NIBB
July 22, 2014

# ORGANIZATION OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) was founded in 1977 on a hill overlooking the old town of Okazaki in Aichi prefecture as one of the Inter-University Research Institutes to promote and stimulate studies of biology. NIBB conducts first-rate research on site as well as in cooperation with national, public and private universities and research organizations. NIBB attempts to elucidate the general and fundamental mechanisms underlying various biological phenomena throughout a wide variety of biological fields, such as cell biology, developmental biology, neurobiology, evolutionary biology, environmental biology, and theoretical biology.

NIBB and four other national institutes, the National Astronomical Observatory (NAO), the National Institute for Fusion Science (NIFS), the National Institute for Physiological Sciences (NIPS), and the Institute for Molecular Science (IMS), constitute the National Institutes of Natural Sciences (NINS), which is one of the four Inter-University Research Institute Corporations. NIBB's activities are supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. The Okazaki Institute for Integrative Bioscience was established as a common facility for the three institutes (NIBB, NIPS, and IMS) of the Okazaki campus.

Buildings in the Myodaiji area (left) and Yamate area (right).

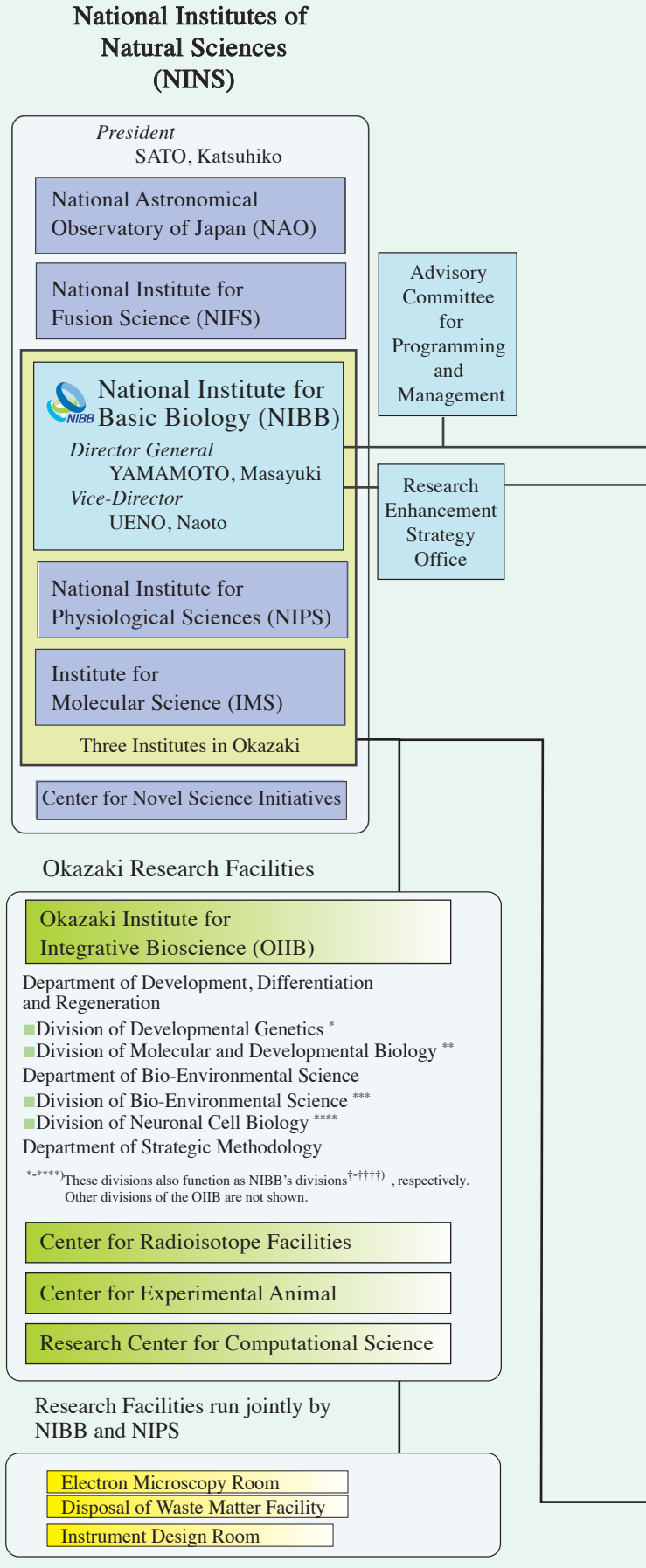
## Policy, Decision Making, and Administration

The Director General oversees the operation of NIBB, assisted by the Advisory Committee for Programming and Management. The Advisory Committee, comprised of eleven professors within NIBB and ten leading biologists outside NIBB, advises the Director General on important matters such as planning joint research programs as well as on the scientific activities of NIBB. The Advisory Committee makes recommendations on the appointments of new Directors General, faculty appointments, NIBB's annual budget and future prospects.

The Research Enhancement Strategy Office, aimed at supporting researchers in order to improve NIBB's abilities as a collaborative research institution, was restructured in 2013 from the former Strategic Planning Department, Office of Public Relations, and Office of International Cooperation. The Office is made up of five groups (p. 83) and its activities are mainly carried out by URAs (University Research Administrators) in close collaboration with the NINS's Research Enhancement Promotion Headquarters.

Administration of NIBB is undertaken by the Okazaki Administration Office under the direct auspices of the Administration Office of NINS.

## Organization



# National Institute for Basic Biology (NIBB)

As of April 1, 2014

## Research Units

- **Cell Biology**
  - Laboratory of Cell Response
  - Laboratory of Neuronal Cell Biology††††
  - Laboratory of Cell Sociology
- **Developmental Biology**
  - Division of Morphogenesis
  - Division of Developmental Genetics †
  - Division of Molecular and Developmental Biology ††
  - Division of Embryology
  - Division of Germ Cell Biology
  - Laboratory of Molecular Genetics for Reproduction
- **Neurobiology**
  - Division of Molecular Neurobiology
  - Division of Brain Biology
  - Division of Brain Circuits
  - Laboratory of Neurophysiology
- **Evolutionary Biology and Biodiversity**
  - Division of Evolutionary Biology
  - Division of Symbiotic Systems
  - Laboratory of Morphodiversity
  - Laboratory of Bioresources
  - Laboratory of Biological Diversity
- **Environmental Biology**
  - Division of Molecular Environmental Endocrinology †††
  - Division of Environmental Photobiology
  - Division of Seasonal Biology (Adjunct)
- **Theoretical Biology**
  - Laboratory of Genome Informatics
- **Imaging Science**
  - Laboratory for Spatiotemporal Regulations

## Research Support Facilities

- **NIBB Core Research Facilities**
  - Functional Genomics Facility
  - Spectrography and Bioimaging Facility
  - Data Integration and Analysis Facility
- **NIBB BioResource Center**
  - Model Animal Research Facility
  - Model Plant Research Facility
  - Cell Biology Research Facility
- **NIBB Center of the Interuniversity Bio-Backup Project (IBBP Center)**
- **NIBB Center for Model Organism Development**

## Research Support Section

- **Technical Division**

## Administration Offices

- Evaluation and Information Group
- Public Relations Group
- International Cooperation Group
- Collaborative Research Group
- Gender Equality Promotion Group

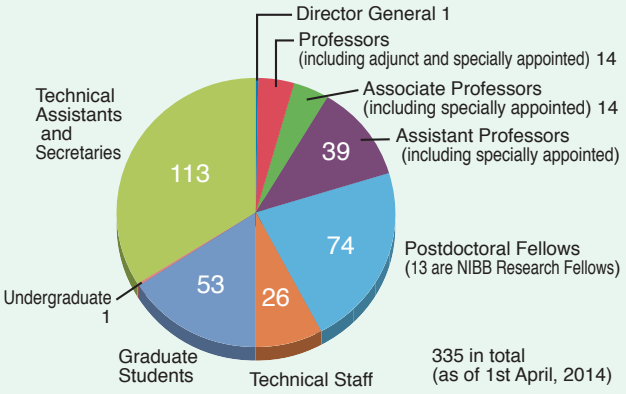
- **Okazaki Administration Office**

## Research and Research Support

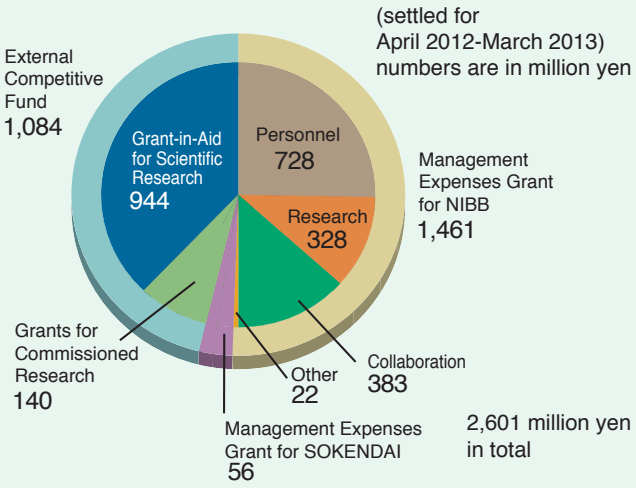
NIBB's research programs are conducted in research units and research support units. Each research unit is called either a division, led by a professor, or a laboratory, led by an associate professor or an assistant professor. Each research unit forms an independent project team. The NIBB Core Research Facilities and NIBB BioResource Center were launched in 2010 to support research in NIBB and also to act as an intellectual hub to promote collaboration among the researchers of NIBB and other institutions. The NIBB Center of the Inter-University Bio-Backup Project (IBBP Center) was founded in 2012 to prevent loss of invaluable biological resources. Projects for the development of bioresource preservation technology are solicited by the IBBP center from 2013. The NIBB Center for Model Organism Development was founded in 2013 to promote development of new model organisms and research using them. The Technical Division manages the activities of the technical staff and helps to promote the activities of each research unit and to maintain the common resources of both the research support units of NIBB and the research facilities of the Okazaki campus. Center for Radioisotope Facilities are one of the latter and ran by the technical staff of NIBB.

The Okazaki Institute for Integrative Bioscience was founded to conduct interdisciplinary molecular research on various biological phenomena and is shared by all three institutes in Okazaki. Some of the divisions also function as NIBB divisions.

### Members in NIBB




### Financial Configuration of NIBB




### Members of the Advisory Committee for Programming and Management (as of June, 2014)

| | | |
|---|---|---|
| **Non-NIBB members** | HAKOSHIMA, Toshio | Professor, Nara Institute of Science and Technology |
| | HIGASHIYAMA, Tetsuya | Professor, Nagoya University |
| | KONDO, Takao | Designated Professor, Nagoya University |
| | KURUMIZAKA, Hitoshi | Professor, Waseda University |
| | MIZUSHIMA, Noboru | Professor, The University of Tokyo |
| | MORI, Ikue | Professor, Nagoya University |
| | OHTA, Kunihiro | Professor, The University of Tokyo |
| | TAKABAYASHI, Junji ## | Professor, Kyoto University |
| | TANAKA, Ayumi | Professor, Hokkaido University |
| | TSUKITA, Sachiko | Professor, Osaka University |
| **NIBB members** | FUJIMORI, Toshihiko | Professor, National Institute for Basic Biology |
| | HASEBE, Mitsuyasu | Professor, National Institute for Basic Biology |
| | IGUCHI, Taisen | Professor, Okazaki Institute for Integrative Bioscience |
| | KAWAGUCHI, Masayoshi | Professor, National Institute for Basic Biology |
| | KOBAYASHI, Satoru | Professor, Okazaki Institute for Integrative Bioscience |
| | MATSUZAKI, Masanori* | Professor, National Institute for Basic Biology |
| | NODA, Masaharu | Professor, National Institute for Basic Biology |
| | TAKADA, Shinji # | Professor, Okazaki Institute for Integrative Bioscience |
| | UENO, Naoto | Professor, National Institute for Basic Biology |
| | YAMAMORI, Tetsuo | Professor, National Institute for Basic Biology |
| | YOSHIDA, Shosei | Professor, National Institute for Basic Biology |

# Chairperson
## Vice-Chair
* new member from April 2014

# GOALS OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) sets five goals for its activities in pursuing the progress of biology. We contribute to the world-wide community of biologists through our efforts to accomplish these goals. This chapter briefly explains four of these goals. The last goal, the promotion of academic research, is accomplished through our research activities, which are introduced throughout this brochure.


## Promotion of Collaborative Research

### Collaborative Research Support

Research activities in collaboration with NIBB's divisions/laboratories using NIBB's facilities are solicited from external researchers. "Individual collaborative research projects" are the basic form of collaboration support which provide external researchers with travel and lodging expenses to visit NIBB's laboratories for collaborative research. For the use of NIBB's unique and excellent research instruments, such as the large spectrograph, the DSLM, and next generation DNA sequencers experimental projects are solicited and reviewed to provide machine time and travel expenses. "Priority collaborative research projects" are carried out as group research by internal and external researchers to develop pioneering research fields. "Collaborative research projects for model organism/technology development" and "Collaborative research projects for bioresource preservation technology development" are for developing and establishing new model organisms and new research technology. Projects for the development of bioresource preservation technology are solicited by the IBBP center. For these projects, research expenses in addition to travel expenses are provided

| year | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|
| Priority collaborative research projects | 4 | 6 | 5 | 2 |
| Collaborative research projects for model organisms/ technology development | 2 | 2 | 3 | 4 |
| Individual collaborative research projects | 68 | 88 | 89 | 89 |
| NIBB workshops | 3 | 6 | 6 | 4 |
| Collaborative experiments using the large spectrograph | 8 | 9 | 14 | 15 |
| Collaborative experiments using the DSLM | 7 | 8 | 5 | 9 |
| Collaborative experiments using the next generation DNA sequencer | 11 | 45 | 47 | 41 |
| Facility Use (Training Course Facility) | 1 | 0 | 2 | 1 |
| Collaborative research projects for bioresource preservation technology development | | | | 9 |
| total | 94 | 164 | 171 | 174 |

Collaborative Research Projects by Year

### NIBB Core Research Facilities

The NIBB Core Research Facilities were launched in 2010 to support research in NIBB and also to act as an intellectual hub to promote collaboration between NIBB and other academic institutions. They consist of three facilities that are developing and providing state-of-the-art technologies through functional genomics, bioimaging and bioinformatics (p. 70).

The Functional Genomics Facility maintains a wide array of core research equipment, including next generation DNA sequencers. The facility is dedicated to fostering NIBB's collaborative research by providing these tools as well as expertise. The current focus is supporting functional genomics works that utilize mass spectrometers and DNA sequencers, holding training courses as one of these undertakings (p. 89). The Spectrography and Bioimaging Facility manages research tools, such as confocal microscopes, DSLM and the large spectrograph, and provides technical support and scientific advice to researchers. These two facilities hold specially appointed associate professors, an expert in each field, with a mission to manage each facility as well as conducting his own academic research. The Data Integration and Analysis Facility supports analysis of large-scale biological data, such as genomic sequence data, gene expression data, and imaging data. For this purpose, the facility maintains high-performance computers with large-capacity storage systems.

### NIBB Center of the Inter-University Bio-Backup Project (IBBP Center)

To prevent damage to important biological resources by natural disasters, NIBB established the IBBP Center in 2012 in collaboration with seven national universities for multiplicate preservation of genetic libraries and other invaluable bioresources under cutting-edge research (p.79).

## International Cooperation and Outreach

### Collaborative Programs with Overseas Institutes

NIBB takes a leading role in the collaborative research programs between the European Molecular Biology Laboratory (EMBL) and the National Institutes of Natural Sciences (NINS) and promotes personal and technological exchange through joint meetings, exchange between researchers and graduate students, and the introduction of experimental equipment. The 10th NIBB-EMBL symposium "Quantitative Bioimaging" was held in March, 2013 (p. 87).

NIBB formed an agreement with the Max Planck Institute for Plant Breeding Research (MPIPZ) and the Temasek Life Sciences Laboratory (TLL), of Singapore to promote joint research projects, collaborative symposia, training courses and student exchange programs. The 4th NIBB-MPIPZ-TLL Joint Symposium "Arabidopsis and Emerging Model Systems" was held in November, 2012 at NIBB.

### NIBB Conference

The NIBB Conferences are international conferences on hot topics in biology organized by NIBB's professors. Since the first conference in 1977 (the year of NIBB's foundation), NIBB Conferences have provided researchers in basic biology with valuable opportunities for international exchange. The 61st conference "Cellular Community in Mammalian Embryogenesis" was held in July, 2013 (p. 86).

### International Practical Course

With the cooperation of researchers from Japan and abroad the NIBB international practical course is given at a laboratory specifically prepared for its use. The 7th course "Genetics, Genomics, and Imaging in Medaka and

Zebrafish" was held jointly with TLL and the National University of Singapore (NUS) in July, 2012 at NUS and TLL. Graduate students and young researchers from various areas including Germany, China, India, and Italy, were provided with training in state-of-the-art research techniques. International conferences and courses are managed by the International Cooperation Group of the Research Enhancement Strategy Office (p. 84).

### Bio-Resources

The National BioResource Project (NBRP) is a national project for the systematic accumulation, storage, and supply of nationally recognized bio-resources (experimental animals and plants, cells, DNA, and other genetic resources), which are widely used as materials in life science research. To promote this national project, NIBB has been appointed as a center for research on Medaka (*Oryzia latipes*) whose usefulness as a vertebrate model was first shown by Japanese researchers. The usability of Medaka as a research material in biology has drawn increasing attention since its full genome sequence recently became available. NIBB is also a sub-center for the NBRP's work with Japanese morning glory. The NIBB BioResource Center has equipment, facilities, and staff to maintain Medaka and Japanese morning glory safely, efficiently, and appropriately. The center also maintains other model organisms, such as mice, zebrafish, *Arabidopsis*, *Lotus japonicus*, and *Physcomitrella patens*, and provides technical support and advice for the appropriate use of these organisms (p. 76).

Strains of Japanese morning glory maintained in the center

### Outreach

NIBB's outreach activities aim to present cutting edge research results to the public via mass media through press releases or directly through the internet, such as web pages, a Facebook page, and a Twitter account. Our triannual open campus event was held in 2013 at which we welcomed more than 1,300 local citizens. NIBB also cooperates in the education of undergraduate and younger students through lectures and workshops. Outreach activities are mostly managed by the Public Relations Group of the Research Enhancement Strategy Office (p. 83).

## Development of New Fields of Biology

### Bioimaging

NIBB aims to maximize the application of modern light microscopes and biophotonic probes for real time visualization of biological phenomena and to develop new imaging techniques. As part of our collaborative work with EMBL, NIBB introduced a DSLM, which is effective for the three-dimensional observation of living samples, and has developed an improved model using two-photon optics (p. 69). The application of the adaptive optics to microscopy is under way in collaboration with the National Astronomical Observatory. The Advisory Committee on Bioimaging, comprised of leading researchers in the bioimaging field in Japan, is organized to formulate advice on NIBB's imaging research. The Bioimaging Forum provides an opportunity for researchers and company engineers to frankly discuss practical difficulties and needs regarding imaging. A training course in bioimage analysis was also held in 2013 (p. 89).

### Okazaki Biology Conferences

NIBB holds Okazaki Biology Conferences (OBC) that, with the endorsement of the Union of Japanese Societies for Biological Science, aim to explore new research fields in biology and support the formation of international communities in these fields. Dozens of top-level researchers from Japan and abroad spend nearly one week together for intensive discussions seeking strategies for addressing future critical issues in biology. Past Conferences have promoted the formation of international researcher communities. The OBC8 "Speciation and Adaptation II: Environment and Epigenetics" was held in March, 2012, and the OBC9 "Marine Biology II" was held in October, 2012.

## Cultivation of Future Researchers

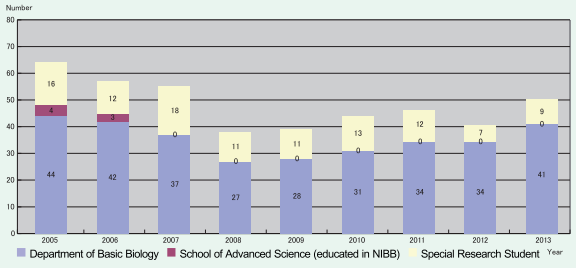
NIBB constitutes the Department of Basic Biology in the School of Life Science of the Graduate University for Advanced Studies (SOKENDAI). The department provides a five-year course for university graduates and a three-year doctoral course for graduate students with a master's degree.

Graduate students enrolled in other universities and institutions can apply to be special research students eligible to conduct research for fixed periods of time under the supervision of NIBB professors.

In both cases above, graduate students can receive financial support from NIBB based on the research assistant (RA) system from the beginning of the five-year course.

Due to the international collaboration with EMBL, graduate students are encouraged to attend PhD students symposia held at EMBL and provided an opportunity to give oral and poster presentations, at least once during their doctoral program (p. 88).

Students from Japan and abroad can also come to NIBB through our Internship Program. Internships give students an excellent way to build international connections while experiencing hands on research in a world class research institute (p. 90).




Graduate students educated by NIBB

## Personnel changes in 2013*

### Newly assigned in NIBB

| Name | Position | Research Unit | Date |
|---|---|---|---|
| TOKUTSU, Ryutaro | Assistant Professor | Division of Environmental Photobiology | January 1 |
| KIMURA, Tetsuaki | Speccially Appointed Assistant Professor | IBBP Center | March 1 |
| TANAKA, Daisuke | Speccially Appointed Assistant Professor | IBBP Center | March 16 |
| HIRA, Riichiro | Assistant Professor | Division of Brain Circuits | March 29 |
| YOSHIMURA, Takashi | Adjunct Professor | Division of Seasonal Biology | April 1 |
| TABATA, Ryo | NIBB Research Fellow | Division of Intercellular Signaling Biology | April 1 |
| YAMAGUCHI, Takeshi | NIBB Research Fellow | Division of Morphogenesis | April 1 |
| SHINOMIYA, Ai | Speccially Appointed Assistant Professor | Division of Seasonal Biology | June 1 |
| SHIMMURA, Tsuyoshi | Speccially Appointed Assistant Professor | Division of Seasonal Biology | June 1 |
| IMAI, Akihiro | NIBB Research Fellow | Division of Evolutionary Biology | July 1 |
| HATTORI, Masayuki | NIBB Research Fellow | Spectrography and Bioimaging Facility | July 1 |
| YAMAMOTO, Masayuki | Director General | | October 1 |
| TANIGUCHI, Atsushi | NIBB Research Fellow | Laboratory for Spatiotemporal Regulations | October 1 |
| KATO, Kagayaki | Speccially Appointed Assistant Professor | Laboratory of Biological Diversity | November 1 |
| KIMORI, Yoshitaka | Speccially Appointed Assistant Professor | Laboratory of Biological Diversity | November 1 |

### Newly affiliated in other universities and institutes

| Name | New Affiliation | Position | Date |
|---|---|---|---|
| HAYASHI, Makoto | Nagahama Institute of Bio-Science and Technology | Professor | April 1 |
| KATSU, Yoshinao | Hokkaido University | Associate Professor | April 1 |

* Changes in professors, associate and assistant professors, and NIBB research fellows are shown.

## Awardees in 2013

| Name | Position | Award |
|---|---|---|
| NISHIMURA, Mikio | Professor | The Japanese Society of Plant Physiologists Award |
| IGUCHI, Taisen | Professor | The Minister of the Environment Award |
| SHIGENOBU, Shuji | Speccially Appointed Associate Professor | NINS Young Researcher Award |
| HASEBE, Mitsuyasu | Professor | The Jeanette Siron Pelton Award (Botanical Society of America) |
| MURATA, Takashi | Associate Professor | The Japanese Society of Plant Morphology Paper Award (Hirase Award) |

## DIVISION OF CELL MECHANISMS †

*Professor*
NISHIMURA, Mikio

*Associate Professor*
HAYASHI, Makoto

| | |
|---|---|
| *Assistant Professor:* | *MANO, Shoji* |
| | *YAMADA, Kenji* |
| *Technical Staff:* | *KONDO, Maki* |
| *NIBB Research Fellow:* | *GOTO-YAMADA, Shino* |
| *Postdoctoral Fellow:* | *OIKAWA, Kazusato* |
| | *KANAI, Masatake* |
| | *WATANABE, Etsuko* |
| | *TANAKA, Mina* |
| | *KAMIGAKI, Akane* |
| | *NITO, Kazumasa* |
| | *CUI, Songkui* |
| *SOKENDAI Graduate Student:* | *SHIBATA, Michitaro* |
| *Technical Assistant:* | *SAITO, Miyuki* |
| | *HIKINO, Kazumi* |
| | *YOSHINORI, Yumi* |
| | *YAMAGUCHI, Chinami* |
| | *NAKAI, Atsushi* |
| | *CUI, Songkui** |
| *Secretary:* | *UEDA, Chizuru* |

Since plants spread their roots in the ground, they must survive in a given environment. To adapt to the environment, they positively utilize environmental changes in their life cycle as important signals that are necessary for their survival. Plant cells can induce, degenerate and differentiate their organelles to adapt to environmental changes. The flexibility of plant organelles is the basis of the strategy for environmental adaptation in plants.

The aim of this division is to clarify the molecular mechanisms underlying the induction, differentiation, and interaction of organelles, and to understand the integrated function of individual plants through organelle dynamics. The Scientific Research on Innovative Areas "Environmental sensing of plants: Signal perception, processing and cellular responses" was started to clarify the molecular mechanisms underlying organelle differentiation and interaction.

### I. Reversible transformation of plant peroxisomes

Dramatic metabolic changes that underlie the shift from heterotrophic to autotrophic growth occur in the greening of seed germination. Accompanying these metabolic changes are the functional transformations of many constitutive organelles. Etioplasts differentiate into chloroplasts while mitochondria acquire the ability to oxidize glycine. Glyoxysomes, which are peroxisomes engaged in the degradation of reserve oil stored in the oil body via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence. Gene expression, alternative splicing, protein translocation and protein degradation control the functional transformation between glyoxysomes and leaf peroxisomes.

### II. Transcriptomics, proteomics and phenomics of plant peroxisomes

Enzymes localized in plant peroxisomes are synthesized in the cytosol and function after their post-translational transport into peroxisomes. Almost all of the peroxisomal matrix proteins contain one of two targeting signals (PTS1 and PTS2) within the molecules. PTS1 is a unique tripeptide sequence found in the carboxyl terminus of the mature proteins. In contrast, PTS2 is involved in a cleavable amino terminal presequence of peroxisomal proteins that are synthesized as a precursor protein with larger molecular mass.

We identified 256 gene candidates of PTS1- and PTS2-containing proteins and another 30 genes of non-PTS-containing proteins from the *Arabidopsis* genome. Custom-made DNA microarrays covering all these genes were used to investigate expression profiles of the peroxisomal genes in various organs. They revealed that peroxisomes in root cells play a pivotal role in polyamine catabolism. We also made a two-dimensional protein map of glyoxysomes and leaf peroxisomes isolated from *Arabidopsis* and soybean. Peptide MS fingerprinting analyses allowed us to identify novel peroxisomal membrane proteins, i.e. voltage-dependent anion-selective channel and adenine nucleotide carrier 1 (PNC1). We also found that peroxisomal membrane ATP-binding cassette transporter promotes seed germination by inducing pectin degradation under the control of abscisic acid signaling. The overall results provide us with new insights into plant peroxisomal functions.

Bioinformatic analysis of the *Arabidopsis* genome predicted the presence of 15 kinds of genes, called *PEX* genes, for peroxisomal biogenesis factors. We demonstrated that PEX5 and PEX7 form a cytosolic receptor complex and recognize PTS1- and PTS2-containing proteins, respectively. PEX14 is a peroxisomal membrane docking protein that captures the receptor-cargo complex. We also comprehensively investigated whether or not these predicted *PEX* genes function in peroxisome biogenesis by generating knock-down mutants that suppress *PEX* gene expression by RNA-interference. Phenotypes of these mutants allowed us to identify the functional *PEX* genes, which can be classified into two groups: *PEX* genes regulating for peroxisomal protein import and *PEX* genes regulating for peroxisomal morphology. We continue to investigate the detailed molecular functions of other *PEX* genes. Of these, we recently proposed that function of PEX7 is maintained by a quality control mechanism involving RabE1c.

### III. Identification of novel components essential for peroxisome biogenesis

To better understand peroxisome biogenesis and functions, we isolated a number of *Arabidopsis* mutants having aberrant peroxisome morphology (*apem* mutants) based on them having a different pattern of GFP fluorescence from the parent plant, GFP-PTS1, in which peroxisomes with normal sizes and numbers can be visualized with GFP.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

†: This laboratory was closed on 31 March, 2014.

Of these *apem* mutants, the *APEM1* gene encodes dynamin-related protein 3A, which is involved in division of both peroxisomes and mitochondria. *APEM2* and *APEM4* were revealed to encode proteins homologous to PEROXIN 13 (PEX13) and PEX12, respectively, and both proteins are responsible for protein translocation on peroxisomal membranes. In addition, we revealed that APEM3 and APEM9 are the peroxisomal membrane proteins. APEM3 is a transporter, whose defect causes enlargement of peroxisomes, and APEM9 is the plant-specific PEX to tether the PEX1-PEX6 complex on peroxisomal membranes. Recently, we found that *APEM10* encodes the Lon protease 2, which modulates peroxisome degradation processed by autophagy.

From an analysis using *peroxisome unusual poisoning* (*peup*) *1*, *peup2* and *peup4* mutants, which were isolated as showing abnormal position and an increase in number of peroxisomes, we revealed that autophagy selectively degrades oxidized peroxisomes (Figure 1).

We are currently characterizing other *apem* and *peup* mutants. From these analyses, we will be able to identify the components responsible for peroxisome biogenesis, functions and maintenance, and to address the mechanism at the molecular level.

## IV. ER derived organelles for protein storing and defense strategy

Plant cells develop various types of endoplasmic reticulum (ER)-derived structures with specific functions. ER bodies are ER-derived compartments observed in *Arabidopsis*. They are rod-shaped structures surrounded by ribosomes, and are widely distributed in the epidermal cells of whole seedlings. Undamaged rosette leaves have no ER bodies, but accumulate ER bodies after wounding or jasmonic acid treatment. This suggests that ER bodies function in the defense against herbivores. ER bodies in seedlings include β-glucosidase PYK10. When plant cells are damaged, PYK10 forms large protein aggregates. The aggregate formation increases glucosidase activity, possibly producing toxic products. *Arabidopsis nai1* mutants have no ER bodies in the entire plant and do not accumulate PYK10. *NAI1* encodes a transcription factor and regulates the expression of *PYK10* and *NAI2*. The *Arabidopsis nai2* mutant has no ER bodies and reduced accumulation of PYK10. *NAI2* encodes a unique protein that localizes to the ER body. Membrane protein of ER body 1 (MEB1) and MEB2 are integral membrane proteins of the ER body and have iron/manganese transport activity. These results suggest that the ER body has specific membrane proteins that are involved in defense against metal stress as well as pathogens and herbivores. NAI2 interacts with MEB1 and MEB2. NAI2 deficiency relocates MEB1 and MEB2 to the ER network. The results suggest that NAI2 facilitates the accumulation of MEB1 and MEB2 on the ER body membrane (Figure 2). These findings indicate that NAI2 is a key factor that enables ER body formation. We are now investigating the function of NAI2 on ER body formation by heterologously expressing it in onion and tobacco cells.

## V. Roles of molecular chaperones on cell differentiation

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures. We have found that HSP90 inhibitor induces genes with heat shock response element (HSE) motifs in their promoters, suggesting that heat shock transcription factor (HSF) is involved in the response. *Arabidopsis* HSFs interacted with HSP90.2. Thus, it appears that in the absence of heat shock, HSP90 actively suppresses

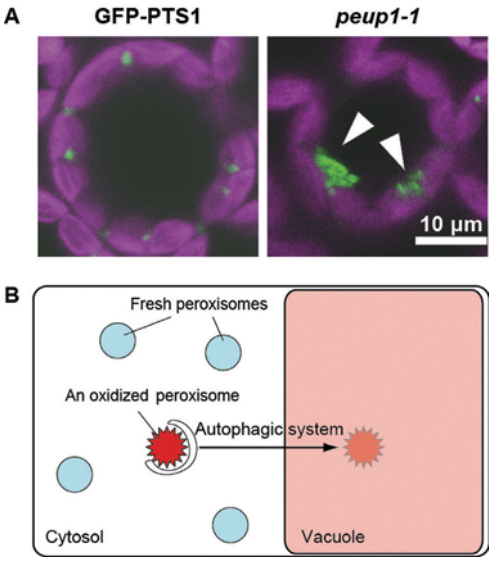



Figure 1. PEUP1/ATG2 is involved in autophagy to degrade oxidized peroxisomes. (A) Compared to the parent plant, GFP-PTS1, the *peup1-1* mutant contains peroxisome aggregates (arrowheads). Chloroplasts and peroxisomes are shown in magenta and green, respectively. (B) The scheme of the quality control of peroxisomes by autophagic system. Oxidized peroxisomes are selectively removed from the cytosol via autophagy.

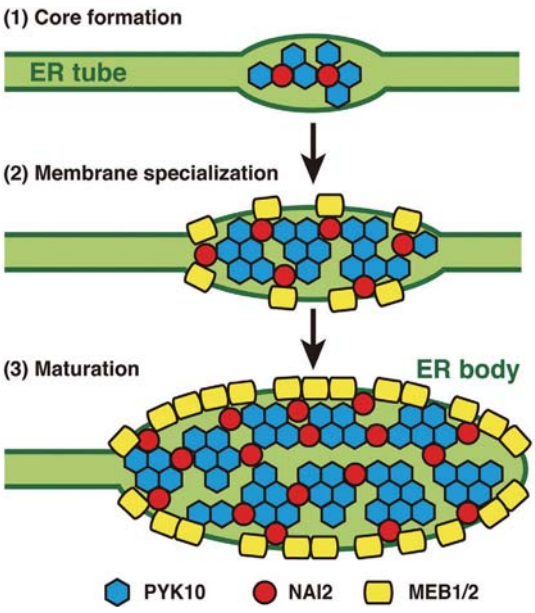



Figure 2. Models of ER body formation. PYK10 and NAI2 may physically interact to form ER body structures from the ER. NAI2 forms a complex with MEB1 and MEB2 to generate the ER body-specific membrane.

HSF function. During heat shock, HSP90 is transiently inactivated, which leads to HSF activation. These data indicate that HSP90 regulates correct gene expression for heat acclimatization in plants. We also observed that HSP90 is involved in hormone responses in *Arabidopsis*. The evolutional and functional characterizations are now being investigated.

## VI. Update of The Plant Organelles Database 3 (PODB3) and Plant Organelles World

The Plant Organelles Database 3 (PODB3) was built to promote a comprehensive understanding of organelle dynamics. This public database is open to all researchers. PODB3 consists of six individual units: the electron micrograph database, the perceptive organelles database, the organelles movie database, the organellome database, the functional analysis database, and external links. The electron micrograph database, which was added as new content, provides information on the ultrastructures in plant cells (Figure 3). The perceptive organelles database shows organelles' dynamics in response to environmental stimuli. The organelles movie database contains time-lapse images and 3D structure rotations. The organellome database is a compilation of static image data of various tissues of several plant species at different developmental stages. The functional analysis database is a collection of protocols for plant organelle research. The amount of included data is increasing day by day. In addition, we updated the website, Plant Organelles World, which is based on PODB3 as an educational tool to engage members of the non-scientific community. We expect that PODB3 and Plant Organelles World will enhance the understanding of plant organelles among researchers and the general public who want to explore plant biology.


Figure 3. The graphical user interface of the electron micrograph database in PODB3 (http://podb.nibb.ac.jp/Orgenellome).

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- Cui, S., Fukao, Y., Mano, S., Yamada, K., Hayashi, H., and Nishimura, M. (2013). Proteomic analysis reveals that the Rab GTPase RabE1C is involved in the degradation of the peroxisomal protein receptor PEX7 (peroxin 7). J. Biol. Chem. *288*, 6014-6023.
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- Tameshige, T., Fujita, H., Watanabe, K., Toyokura, K. Kondo, M., Tatematsu, K., Matsumoto, N., Tsugeki, R., Kawaguchi, M., Nishimura, M., and Okada, K. (2013). Pattern dynamics is adaxial-abaxial specific gene expression are modulated by a plastid retrograde signal during Arabidopsis thaliana leaf development. PLoS Genetics *9*, e1003655.
- Tamura, K., Iwabuchi, K., Fukao, Y., Kondo, M., Okamoto, K., Ueda, H., Nishimura, M., and Hara-Nishimura, I. (2013). MyosinXI-i links the nuclear membrane to the cytoskeleton to control nuclear movement and shape in Arabidopsis. Curr. Biol. *23*, 1776-1781.
- Yamada, K., Nagano, A.J., Nishina, M., Hara-Nishimura, I., and Nishimura, M. (2013). Identification of two novel endoplasmic reticulum body-specific integral membrane proteins. Plant Physiol. *161*, 108-120.

〔Original paper (E-publication ahead of print)〕

- Mano, S., Nakamura, T., Kondo, M., Miwa, T., Nishikawa, S., Mimura, T., Nagatani, A., and Nishimura, M. The Plant Organelles Database 3 (PODB3) update 2014: integrating electron micrographs and new options for plant organelle research. Plant Cell Physiol. 2013 Sep. 18.

〔Review articles〕

- Cui, S., Mano, S., Yamada, K., Hayashi, M., and Nishimura, M. (2013). Novel proteins interacting with peroxisomal protein receptor PEX7 in *Arabidopsis thaliana*. Plant Signal. Behav. *8*, e26829.
- Mano, S., Kimori, Y., Takeda, T., Miwa, T., Nishikawa, S., Mimura, T., Nagatani, A., and Nishimura, M. (2013). Using image-based resources: databases for plant organelle dynamics and applications based on image information. In Introduction to Sequence and Genome Analysis. Edited by Mishra, A., Nawaz, Z. and Shahid, Z. pp.83-109. iConcept Press.

## DIVISION OF INTERCELLULAR SIGNALING BIOLOGY †


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Cell-to-cell signaling mediated by secreted signals and membrane-localized receptors is one of the critical mechanisms by which growth and development of multicellular organisms are cooperatively regulated. Signal molecules that specifically bind receptors are generally referred to as ligands. Because membrane-localized receptors act as master switches of complex intracellular signaling, identification of the ligand-receptor pair is one of the central issues of post-genome research. We are working to clarify the mechanisms by which plant development is regulated through identification of novel ligands such as small peptides and their specific receptors using *Arabidopsis* genome information, biochemical analysis and phenotypic observation.

## I. Secreted peptide signals

Following complete sequencing of the *Arabidopsis* genome, a number of genes encoding small secreted peptides have been identified by *in silico* database analysis. Based on our own analysis, we identified 979 putative secreted peptide genes with an open reading frame (ORF) size between 50 and 150 amino acids in the *Arabidopsis* genome. These 979 ORFs include many functionally uncharacterized peptides. Although estimation of the total number of biologically relevant secreted peptide signals is difficult at present, the presence of many "orphan receptors" among receptor-like kinases in *Arabidopsis* suggest that a substantial number of intercellular signals remain to be identified.

One structurally characteristic group of peptide signals is "post-translationally modified small peptides". These peptides are characterized by the small size of mature peptides (less than 20 amino acids) and the presence of post-translational modifications. In these peptide signals, peptide chain length and post-translational modifications are generally very important for their receptor binding activity and physiological functions.

### 1-1 Root meristem growth factor (RGF)

Root meristem growth factor (RGF) is a 13-amino-acid tyrosine-sulfated peptide involved in maintenance of the root stem cell niche in *Arabidopsis* identified by our group in 2010. RGF was identified in a search for sulfated peptides that recover root meristem defects of the loss-of-function mutant of tyrosylprotein sulfotransferase (*tpst-1*). TPST is a post-translational modification enzyme that catalyzes tyrosine sulfation of secreted peptides and proteins. This approach is based on the assumption that the severe short root phenotype of the *tpst-1* mutant reflects deficiencies in the biosynthesis of all the functional tyrosine-sulfated peptides, including undiscovered peptide signals. RGFs are produced from ≈100-amino-acid precursor peptides via post-translational sulfation and proteolytic processing. RGF family peptides are expressed mainly in the stem cell area and the innermost layer of central columella cells, and diffuse into the meristematic region. RGF peptides regulate root development by stabilizing PLETHORA transcription factor proteins which are specifically expressed in root meristem and mediate patterning of the root stem cell niche (Figure 1).

To gain more insight into RGF signaling, we have developed a positive screening system to identify *Arabidopsis* mutants with altered response to RGF peptides. Several mutants that are less sensitive to RGF have been identified and are currently being further analyzed.

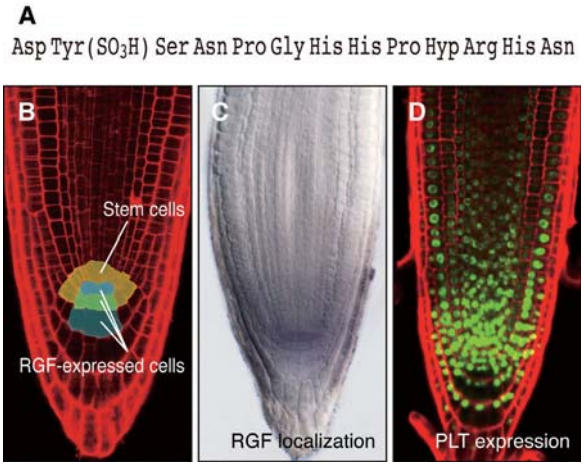


Figure 1. Mode of action of RGF peptide. (A) Structure of RGF1. (B) Expression domain of *RGF* genes. (C) Localization of RGF peptides visualized by anti-RGF1 antibody. (D) Expression pattern of PLETHORA (PLT2-GFP) transcription factor, a downstream target of RGF signaling.

### 1-2 Other novel peptide signal candidates

The common feature of known small post-translationally modified peptide signals is that they are encoded by multiple paralogous genes whose primary products are approximately 70- to 110-amino-acid cysteine-poor secreted polypeptides that share short conserved domains near the C-terminus. We have identified several novel polypeptide families that fulfill the above criteria by *in silico* screening and determined their mature structures by analyzing apoplastic peptide fractions by nano LC-MS/MS. Functional analysis of these peptides is now going on.

## II. Post-translational modification mechanisms

Post-translational modifications are known to affect peptide conformation through steric interactions with the peptide backbone, thereby modulating the binding ability and specificity of peptides for target receptor proteins. To date, the following types of post-translational modification have

†: This laboratory was closed on 31 March, 2014.

been identified in secreted peptide signals in plants: tyrosine sulfation and hydroxyproline arabinosylation (Figure 2).

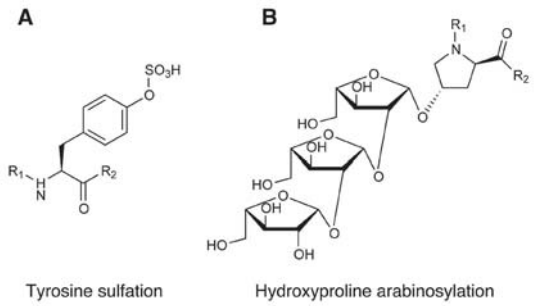


Figure 2. Post-translational modifications in secreted peptide signals in plants.

### 2-1 Tyrosine sulfation

*Arabidopsis* tyrosylprotein sulfotransferase (AtTPST) is a Golgi-localized 62-kDa transmembrane protein identified by our group in 2009. AtTPST is expressed throughout the plant body, and the highest levels of expression are observed in the root apical meristem. A loss-of-function mutant of *AtTPST* (*tpst-1*) displayed a marked dwarf phenotype accompanied by stunted roots (Figure 3), loss of root stem cells, pale green leaves and early senescence, indicating the important roles of sulfated peptides in plant growth and development. Three known sulfated peptide signals, PSK, PSY and RGF, can almost fully restore root defects of *tpst-1* when added to the culture medium, but can not fully restore phenotypes in the above-ground parts of the plant. This observation suggests that as yet undiscovered sulfated peptides may regulate plant development. A search for novel sulfated peptide signals is now in progress.

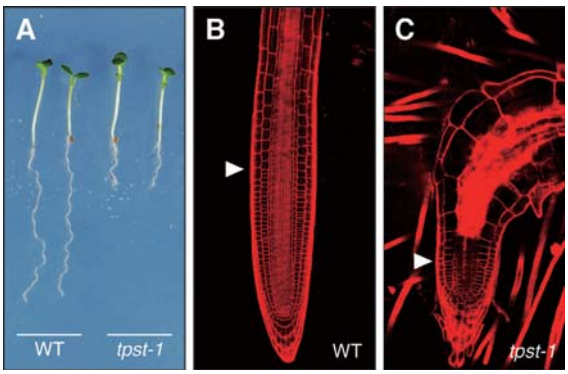


Figure 3. Phenotypes of *Arabidopsis tpst-1* mutant. White arrowheads indicate the root meristem boundary.

### 2-2 Hydroxyproline arabinosylation

Hyp residues in several secreted peptide signals, such as *Arabidopsis* CLV3 and *Lotus japonicus* CLE-RS2 are further modified with an *O*-linked L-arabinose chain. This modification is physiologically important for these peptide signals. CLE-RS peptides are hydroxyproline O-arabinosylated 13-amino-acid glycopeptides that are responsible for autoregulation of nodulation in leguminous plants.

Biosynthesis of Hyp-bound β-1,2-linked triarabinoside involves two distinct arabinosyltransferases. The first is responsible for the formation of a β-linkage with the hydroxyproline (hydroxyproline arabinosyltransferase), and the second forms a β-1,2-linkage between arabinofuranose residues (arabinosyltransferase). Arabinosyltransferase has already been reported, but there have been no reports on hydroxyproline arabinosyltransferase (HPAT).

*Arabidopsis* HPAT was recently purified and identified as a Golgi-localized transmembrane protein. Loss-of-function mutations in *HPAT* genes in *Arabidopsis* cause pleiotropic phenotypes that include enhanced hypocotyl elongation, defects in cell wall thickening, early flowering, and early senescence. In addition, a double mutation in *HPAT1* and *HPAT3* significantly impairs the growth of pollen tubes, thereby causing a transmission defect through the male gametophyte (Figure 4). Detailed phenotypic analyses of loss-of-function mutants of *HPAT* genes will provide a more complete picture of how hydroxyproline O-arabinosylated glycoproteins and glycopeptides contribute to plant growth and development.

### 2-3 Chemical synthesis of arabinosylated peptides

Arabinosylation of hydroxyproline (Hyp) is a posttranslational modification often found in secreted peptide signals in plants. We have succeeded in the stereoselective total synthesis of β-1,2-linked tri-arabinosylated CLV3 peptide ([$Ara_3$]CLV3). Comparison of mono-, di- and tri-arabinosylated CLV3 glycopeptides revealed that the biological activity increased progressively as arabinose chain length increased. Thus, arabinose chain length of CLV3 is important for its biological activity.

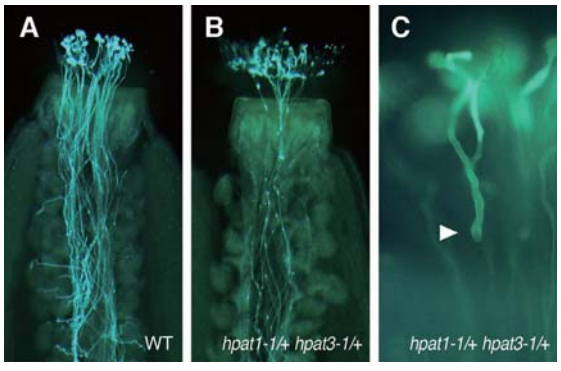


Figure 4. A double mutation in *HPAT1* and *HPAT3* significantly impairs the growth of pollen tubes. Arrowhead indicates aberrant pollen tube.

### 2-4 Conformation of arabinosylated peptides

NMR spectroscopy and NOE-based structure calculations revealed the structural impact of the arabinose chain on peptide conformation. The arabinose chain of [$Ara_3$]CLV3 extends toward the C-terminal end of the peptide, and its non-reducing end is positioned proximal to the peptide backbone. Consequently, the arabinose chain causes distinct distortion in the C-terminal half of the peptide in a highly

directional manner. The established synthetic route of [$Ara_3$]CLV3 will greatly contribute to our understanding of the biology and biochemistry of arabinosylated peptide signals in plants.

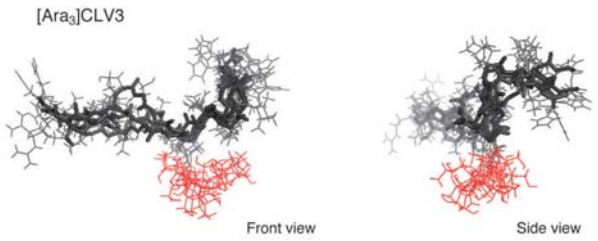


Figure 5. Energy-minimized structure of [$Ara_3$]CLV3 resulting from a simulated annealing protocol that incorporated NOE-derived distance restraints.

## III. Receptors for secreted peptide signals

The receptors or putative receptors for peptide signals identified to date belong to the receptor kinase (RK) or receptor-like protein (RLP) families. Among RKs, the largest subfamily is the leucine-rich repeat RK (LRR-RK) family, which consists of 216 members in *Arabidopsis*. The majority of receptors for small post-translationally modified peptide signals belong to this family. Especially, an increasing number of LRR X and LRR XI members are now being confirmed as receptors for several endogenous small peptide ligands, suggesting that these subgroups are an attractive target for binding analysis with novel peptide signals.

### 3-1 Receptor expression library

Although both genetic and biochemical methods have been used to identify ligand-receptor pairs in plants, genetic redundancy often interferes with the former approach, and the low levels at which ligand and receptor molecules are often present in tissues can make the latter approach very difficult. As described in the above section, an increasing number of ligand candidates are being identified. If individual receptor kinases could be functionally overexpressed in certain cells at sufficiently high levels and sufficiently high quality for biochemical binding analysis, such a receptor library would facilitate identification of ligand-receptor pairs in plants. To this end, we established a functional and efficient expression system of plant receptor kinases in tobacco BY-2 cells and prepared an expression library of all the potential receptor candidates.

### 3-2 Structural basis for ligand recognition

Leucine-rich repeat receptor kinases (LRR-RKs) comprise the largest subfamily of the transmembrane receptor kinases in plants. In several LRR-RKs, a loop-out region called an "island domain" that intercepts the extracellular tandem LRRs at a position near the transmembrane domain constitutes the ligand-binding pocket, but the absence of the island domain in numerous LRR-RKs raises questions about which domain specifically recognizes the corresponding ligands in non-island domain-carrying LRR-RKs. We determined, by photoaffinity labeling followed by chemical and enzymatic digestion, that BAM1, a CLV1/BAM family LRR-RK whose extracellular domain is comprised of 22 consecutive LRRs, directly interacts with the small peptide ligand CLE9 at the LRR6-8 region that is relatively distal from the transmembrane domain (Figure 5). Multiple sequence alignment and homology modeling revealed that the inner concave side of LRR6-8 of the CLV1/BAM family LRR-RKs is slightly deviatory from the LRR consensus. Our results indicate that ligand recognition mechanisms of plant LRR-RKs are more complex and diversified than anticipated.

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- Shinohara, H., and Matsubayashi, Y. (2013). Chemical synthesis of *Arabidopsis* CLV3 glycopeptide reveals the impact of hydroxyproline arabinosylation on peptide conformation and activity. Plant Cell Physiol. *54*, 369-374.

## LABORATORY OF NEURONAL CELL BIOLOGY

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The transport of specific mRNAs and local control of translation in neuronal dendrites represent an important gene expression system that provides localized protein synthesis in dendrites at just the right time and place. It is believed that this system controls the location at which neurites will connect to each other, thereby forming neural networks. Our main interest is to understand the mechanisms and roles of mRNA transport and local translation in neuronal dendrites.

It is known that specific mRNAs are recruited into "RNA granules" in neuronal dendrites. RNA granules are macromolecular complexes composed mainly of mRNAs, ribosomes and RNA-binding proteins, and mediate the transport and local translation of their mRNA cargoes in response to synaptic stimulation (Figure 1). We are researching RNA granule factors regulating mRNA transport and local translation, their target mRNAs, and the mechanisms of localized protein synthesis using mice in order to better understand its relation to the formation of synapses and neural networks, memory, learning, and behavior.

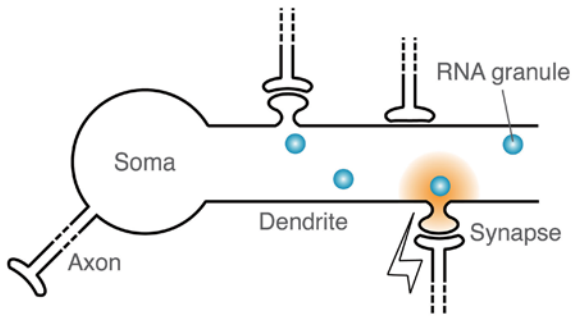


Figure 1. A model for local translation in neuronal dendrites. Specific mRNAs are recruited into RNA granules and transported to dendrites. Translation of the mRNAs is induced locally upon synaptic stimulation, which modifies local postsynapses to regulate synaptic connection and network formation.

### I. Analyses of RNG105 conditional knockout mice

We previously identified RNA granule protein 105 (RNG105), an RNA-binding protein, as a component of RNA granules. RNG105 is responsible for mRNA transport to dendrites, which is required for the encoded proteins to be translated and function in dendrites for proper networking of neurons (Shiina *et al*., J. Neurosci. *30*, 12816-12830, 2010). RNG105 knockout mice exhibit reduced dendritic synapse formation and reduced dendritic arborization, which results in poor development of neuronal networks. The knockout neonates die soon after birth due to respiratory failure, which is associated with defects in fetal brainstem development (Shiina *et al*., J. Neurosci. *30,* 12816-12830, 2010).

To investigate the role of RNG105 in higher brain functions, we have generated RNG105 conditional knockout (cKO) mice using the alpha-CaMKII-Cre/loxP system. Expression of RNG105 was markedly reduced in the cerebrum, especially in the hippocampal pyramidal neurons of adult cKO mice (Figure 2A). To investigate comprehensively the changes in the dendritic localization of mRNAs in RNG105 cKO neurons, we dissected brain slices to isolate dendritic areas of hippocampal CA1 pyramidal neurons (Figure 2B). Quantitative RT-PCR experiments confirmed that dendritic mRNAs, but not somatic mRNAs, were concentrated in the isolated dendrite fragments, which indicated that dendrites of hippocampal neurons were successfully isolated. We are going to compare the mRNAs contained in the dendrite fragments between wild-type and RNG105 cKO mice using next-generation sequencing to understand the effect of RNG105 on mRNA transport to neuronal dendrites in adult mouse brains.

We further analyzed behavior of RNG105 cKO mice. Open field behavior tests revealed that exploratory activity of RNG105 cKO mice in a novel environment was not changed between the first and later trials, although exploratory activity of wild-type mice was reduced with increasing number of trials, suggesting that the cKO mice had some problems in being acclimated to a new environment. Passive avoidance test is one of the learning and memory tests, in which mice receive an electric foot shock in a room and thereafter they remember the situation and do not enter the room for more than several days. In this test, RNG105 cKO mice did not enter the room at 5 minutes after they received a foot shock, but entered the room after 24 hours. These results suggested that RNG105 cKO mice acquired short-term, but not long-term memory, which is consistent with a model that dendritic mRNA transport and local translation are key bases for the conversion of short-tem memory to long-term memory.

RNG105 has one paralog, RNG140, which has RNA-binding domains highly conserved with RNG105. RNG105 and RNG140 are localized to different kinds of RNA granules and their timing of expression is also different: RNG105 is highly expressed in embryos, but RNG140 is highly expressed in adults (Shiina and Tokunaga, J. Biol. Chem. *285,* 24260-24269, 2010). We have obtained RNG140 knockout mice and are going to investigate the role of RNG140 in higher brain functions in adult mice.

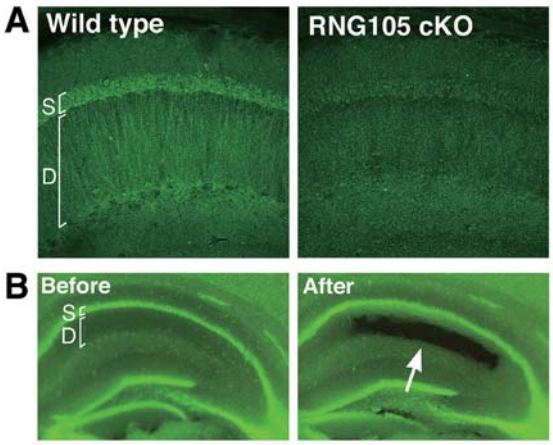


Figure 2. (A) Brain CA1 region of hippocampal slices from wild-type and RNG105 cKO mice were immunostained with an anti-RNG105 antibody. S, somatic layer; D, dendritic layer of hippocampal pyramidal neurons. RNG105 was reduced in pyramidal neurons of RNG105 cKO mice. (B) A hippocampal slice from a wild-type mouse stained with YO-PRO1 to visualize nuclei. Left and right panels are before and after the isolation of the dendritic area of CA1 pyramidal neurons. An arrow indicates the dendritic area removed by dissection.

## II. Molecular mechanism of RNA granule assembly and disassembly

We used proteomic analyses to identify proteins associated with RNG105. RNG105 fused to green fluorescence protein (GFP) was expressed in cultured A6 cells and immunoprecipitated with an anti-GFP antibody, and the immunoprecipitates were analyzed by mass spectrometry. Among the identified proteins, we focused on nuclear factor 45 (NF45) and its binding partner, nuclear factor associated with dsRNA 2 (NFAR2). Expression of NFAR2 in cells enhanced the assembly of RNG105-containing RNA granules, whereas expression of NF45 disassembled the RNA granules (Figure 3).

NFAR1 and NFAR2 are splice variants and their different properties from one another have been unclear. We have found that NFAR2, but not NFAR1, has the ability to localize with and enhance the assembly of RNG105-containing RNA granules through NFAR2-specific GQSY domain (Figure 3). The GQSY domain interacted with RNG105-containing messenger ribonucleoprotein (mRNP) complexes and was structurally and functionally similar to the low complexity (LC) sequence domain of FUS/TLS, which is known to drive RNA granule assembly.

Another domain of NFAR2, DZF domain, was dispensable for the interaction with the RNG105 mRNP complexes, but involved in positive and negative regulation of RNA granule assembly by being phosphorylated by PKR, a master kinase inducing RNA granule assembly, and by association with NF45, respectively (Figure 3).

Our results suggest a model that NFAR2 functions as a connector of RNG105 mRNP complexes through its multivalent domains, i.e., the GQSY domain and the DZF domain, in the assembly of RNA granules. The connector function may be enhanced by phosphorylation by PKR and blocked by NF45 binding. We are going to elucidate the roles of NFAR2 and NF45 in neurons, including their relation to neurodegeneration, because defective regulation of RNA granule assembly by LC sequence domain-containing proteins such as FUS/TLS and TDP-43 is recently suggested to be associated with neurodegenerative disease such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD).

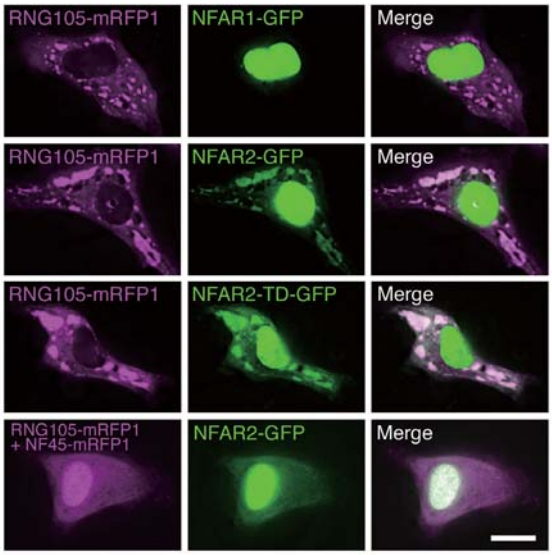


Figure 3. Effects of NFAR2, its phosphorylation by PKR, and NF45 on the assembly of RNG105-containing RNA granules. A6 cells were co-transfected with RNG105-monomeric red fluorescent protein 1 (mRFP1) and NFAR1-GFP, NFAR2-GFP, phosphomimetic NFAR2 at PKR phosphorylation sites (NFAR2-TD-GFP), or NFAR2-GFP plus NF45-mRFP1. NFAR2 was predominantly localized to the nucleus, and also co-localized to and enlarged RNG105-containg RNA granules. RNA granules were much more increased in size by phosphomimetic NFAR2, but disassembled by co-expression of NF45. Scale bar, 10 μm.

## LABORATORY OF CELL SOCIOLOGY

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Mammalian eggs implant onto the maternal uterine wall after hatching at the blastula stage, due to a lack of enough nutrients to support development until birth. In order to transfer nutrients and oxygen into the fetus, and metabolic wastes into the mother, the placenta has evolved to establish close connections between the maternal and fetal vasculature. There is amazing diversity from one animal to another in the features, such as gross structures, microscopic arrangements and developmental processes, but the function of this organ is invariable.

Primates and rodents have the ancestral type of placenta, classified as heomochorial, in which maternal blood is not contained within endothelial cell lined vessels but rather is in direct contact with epithelial cells of the fetal placenta that are derived from the trophoblast cell lineage. In mice, maternal blood coming out from the spiral arteries invaded by trophoblast cells that replace the endothelium passes across the fetal layers of the placenta and then enters into the labyrinth where fetal blood vessels form and feto-maternal exchange occurs. Maternal and fetal vascular circuits in the established mouse placenta are shown schematically in Figure 1.

Notch2 null mutation induces lethality around embryonic day 11 because of impaired maternal vasculature formation. However, both cellular and molecular events in the process of vasculature formation are little known, especially as to how the maternal blood bed expands among the tightly packed trophoblast mass. Our histological studies showed that a stream of maternal blood along the interface between labyrinthine and spongiotrophoblast was slightly opened in the mutant placenta. Expression of the Notch2 gene is observed in spongiotrophoblast and Giant cells, but in neither the labyrinthine trophoblast nor in the multi-nuclear Syncytiotrophoblast that is derived by cell fusion of the mono-nuclear labyrinthine trophoblast (Figure 2). Thus, we conclude that cellular and molecular events to form the maternal blood bed occur in the spongiotrophoblast and Giant cell layers.

We have assumed that the cellular event that expands the maternal blood bed is the deletion of spongiotrophoblast cells in a significant number from the developing placenta. Thus, we are trying to detect trophoblast cell death or detachment with various methods.

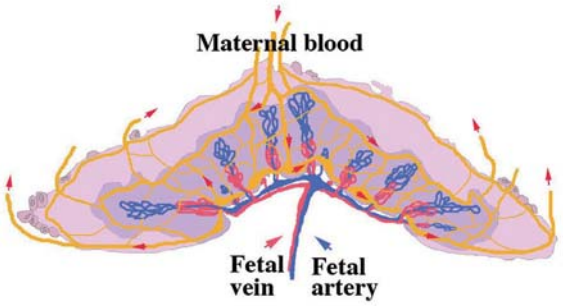


Figure 1. Schematic maternal and fetal vascular circuits in mouse placenta. Maternal blood flows in streams drawn in yellow in the direction shown by small red arrows. Fetal blood flowing in the artery (blue line) reaches the border of the spongio- and labyrinth layers and returns to the fetal vein (red line).

### Publication List

〔Original paper〕

● Gasperowicz, M., Surmann-Schmitt, C., Hamada, Y., Otto, F., and Cross, J.C. (2013). The transcriptional co-repressor TLE3 regulates development of trophoblast giant cells lining maternal blood spaces in the mouse placenta. Dev. Biol. *382*, 1-14.

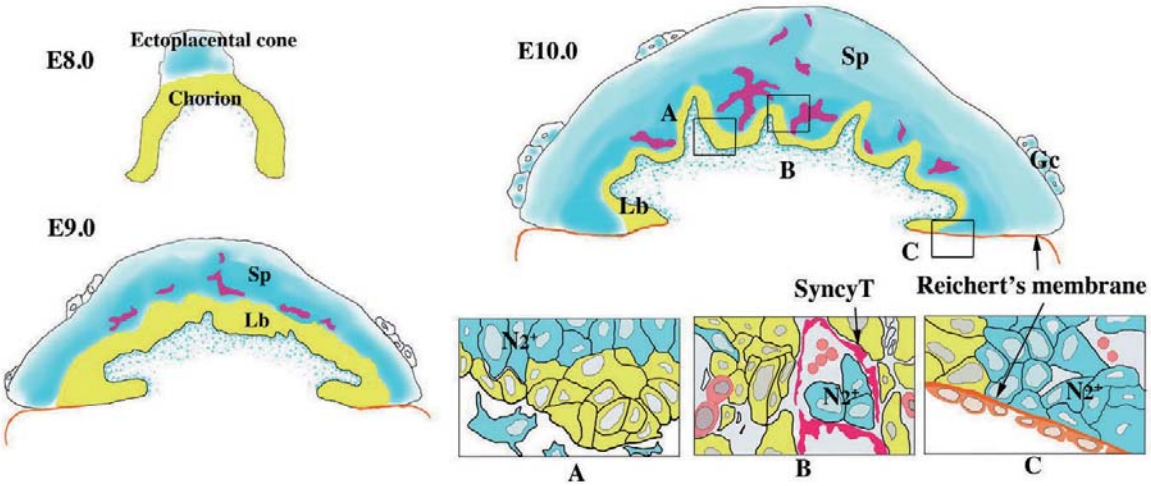


Figure 2. Expression of Notch2 gene in developing mouse placenta. The Notch2 gene is expressed in the ectoplacental cone at E8.0, but not in the chorion. At E9.0, the ectoplacental cone and chorion differentiate into spongiotrophoblast (Sp) and labyrinthine trophoblast (Lb), respectively. The expression is detected in Sp. At E10.0, differentiation of syncytiotrophoblast (SynT) and vasculogenesis of fetal blood are obvious in the placenta. Notch2 expression occurs in Sp, Giant cell layer (Gc), and mesenchymal cells derived from allantois. However, it is not detected in SynT and Lb far away from maternal blood or separated by SynT.

## DIVISION OF MORPHOGENESIS

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The complex morphogenesis of organisms is achieved by dynamic rearrangements of tissues during embryogenesis, in which change in cellular morphology as well as orchestrated cell movements are involved. For cells to know how they should change their shape and where they should move, information called “cell polarity” is essential. How then is the cell polarity established within cells? Is it intrinsically formed within the cells or triggered by extracellular cues? Furthermore, little is known as to how coordinated and complex cell movements are controlled in time and space. We attempt to understand the mechanisms underlying these events using several model animals, including frogs, fish, mice and ascidians, taking physical parameters such as force in consideration, in addition to conventional molecular and cellular biology.

### I. Biological significance of force for morphogenesis

Physical forces are a non-negligible environmental factor that can guide the morphogenesis of organisms. Such forces are generated by tissue-tissue interactions during early development where drastic tissue remodeling occurs. One good example is neural tube formation. In vertebrates, the neural tube is the primordial organ of the central nervous system and is formed by the bending of the neural plate that is a flat sheet of neuroepithelial cells. The tissue remodeling is driven by cellular morphogenesis in which selected cells in the neural plate change their shapes from cuboidal to an elongated wedge-like shape, generating a force for the tissue-bending. Recent studies have revealed that this cell shape change is controlled by cytoskeletal dynamics, namely the remodeling of F-actin and microtubules (Suzuki, M. *et al*. Dev. Growth Differ., 2012). We are currently investigating how the cellular morphogenesis is spatiotemporally controlled.

Another example is the axial mesoderm, which elongates along the anterior-posterior axis during gastrulation cell movements by which rearrangements of the three germ layers is driven. The axial mesoderm is led by the anteriorly precedent tissue leading edge mesoderm (LEM). When surgically isolated the LEM migrates fairly rapidly toward the predetermined anterior side, while the following axial mesoderm shows little directed tissue migration. We hypothesized that the LEM generates a pulling force on the following axial mesoderm (AM). We measured the force generated by LEM and estimated it to be approximately 40 nN and confirmed by laser ablation experiments that LEM generates tension on AM (Figure 1). We concluded that the anterior movement associated with the force generation is essential for proper notochord formation (Hara ,Y. *et al*. Dev. Biol., 2013).

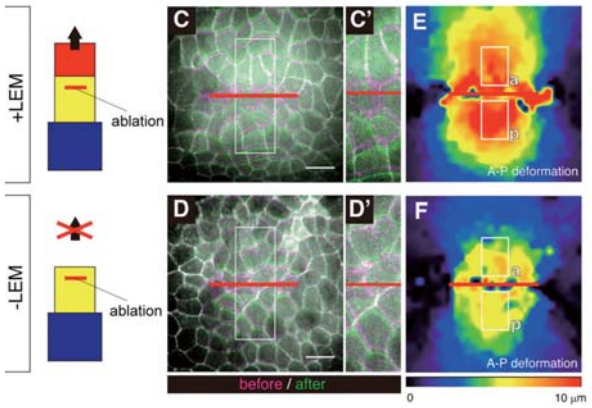


Figure 1. Laser ablation of dorsal mesoderm with and without LEM. Deformation map indicates that more significant displacement in AM after laser cutting of the tissue with LEM (upper panels, E) than without LEM (lower panels, F). From Hara, Y. *et al*., 2013.

### II. The roles of PCP core components in mouse development

In epithelia, the roles of planar cell polarity (PCP) have been extensively studied, whereas in non-epithelia, they have yet to be fully understood. We are exploring the roles of PCP in the mesenchymal and neural tissues by using mouse genetics and *RNAi* knock-down technology

Recently, we generated a hypomorphic allele of mouse *Prickle1*, one of the core PCP factors. As we have reported (Tao, H. *et al*. Proc. Natl. Acad. Sci., USA, 2009) *Prickle1* null/null mice die around E6.0 in gestation due to the failure of gastrulation. In contrast, *Prickle1* hypomorphic mutant mice survive to P0. Interestingly, the mutant mice had shortened noses. Detailed analyses at the cell level suggested that PCP signaling involving core components such as Prickle governs convergent extension of nasal cartilage cells which is required for the lengthening of the nose. Our finding further suggests that PCP signaling employed multiple times at different places during development may be one of the universal mechanisms of organ morphogenesis. We hope that in combination with comparative genomic analysis, these

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

PCP mutant mice will serve as good models that can explain morphological variations of mammals.

## III. Regulation of FGF-2 secretion and localization

Protein secretion is a fundamental process for every organism. Most of the secreted proteins have a signal peptide at the amino-terminal region. Proteins with the signal peptide are secreted through a conventional secretory pathway including ER and Golgi apparatus. Recently, however, a growing number of proteins have been found to be secreted not through the conventional ER-Golgi pathway. Those include some of the FGF (fibroblast growth factor) family members such as FGF-2. It is known that despite the lack of signal sequences, they are still secreted and play important biological roles as extracellular ligands. It is not fully understood how FGFs lacking signal peptides are secreted. In order to clarify the secretory mechanism of such proteins, we are taking a biochemical approach and performing microscopic analysis. We searched for proteins that interact with FGF-2 in the cytoplasm by mass spectrometry analysis. So far, several proteins that interact with FGF-2 have been identified. Interestingly, some of them are probably secreted through an unconventional secretory pathway as well as FGF-2. These proteins, thus, may be involved in the regulation of FGF-2 secretion. In addition, we observed the localization of secreted FGF-2 in the *Xenopus* embryonic cells (Figure 2). FGF-2 forms dots on the cell surface, suggesting that it associates with certain cell surface structures. This localization may be related to its secretion mechanism, or FGF signaling through binding with the FGF receptor. This study will contribute to clarifying a novel regulatory system for the FGF signaling pathway.

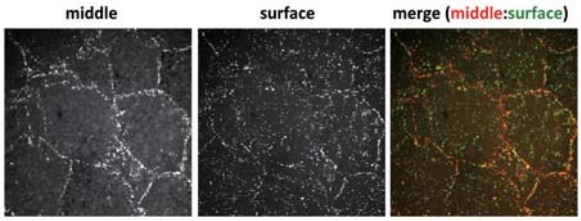


Figure 2. The localization of FGF-2 on the cell surface of *Xenopus* embryonic cells. mRNA encoding Flag-tagged FGF-2 is injected into embryos. The explant was excised from the animal pole region and FGF-2 was detected by immunofluorescense using laser scanning confocal microscopy. "middle" and "surface" indicate the focal planes of each image. In the merged image, the middle image was colored in red, and the surface image was colored in green. FGF-2 forms dot-like structures on the cell surface.

## IV. Cellular morphogenesis during neural tube closure

For the morphogenesis of organs, cellular morphogenesis as well as cellular behaviors plays critical roles. During early development of the central nervous system (CNS) in vertebrates, the neuroepithelial cells undergo a typical shape change, called apical constriction (AC), the cumulative action of which causes the neural plate to bend to form the neural tube. In AC, cell apices are contracted and stabilized, causing cells to adopt wedge-like shapes from columnar ones. Recent studies have revealed that AC is controlled by cytoskeletal dynamics, namely the remodeling of F-actin, and non-muscle myosin II activity, yet how AC is dynamically controlled in time and space is not fully understood.

We found that during *Xenopus* neural tube closure intracellular calcium ion ($Ca^{2+}$) dynamically fluctuates throughout the neural plate at single-cell to whole-tissue levels. Spatio-temporal patterns of the $Ca^{2+}$ fluctuations appeared to be differentially regulated by the membrane-bound $Ca^{2+}$ channel, inositol triphosphate receptor, and the extracellular signals. $Ca^{2+}$ fluctuations temporally preceded the repeated acceleration of the closing movements. $Ca^{2+}$ fluctuations correlated with apical constriction at the single cell level, and manipulation of cytoplasmic $Ca^{2+}$ caused cell shape change similar to apical constriction thorough actomyosin contractility. These data suggest that intracellular $Ca^{2+}$ is a positive regulator of apical constriction. We propose a dynamic $Ca^{2+}$-dependent mechanism and a Rho/ROCK-dependent mechanism coordinately control apical constriction in order to enable embryos to ensure the primitive central nervous system formation.

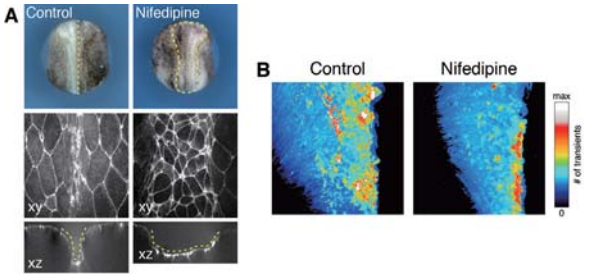


Figure 3. $Ca^{2+}$ fluctuation is required for *Xenopus* neural tube closure. (A) Inhibition of $Ca^{2+}$ signaling delayed neural tube closure (right). F-actin staining (middle, bottom) showed the failure of apical constriction and formation of the neural folds. (B) Intracellular $Ca^{2+}$ patterns visualized by $Ca^{2+}$ probe. The inhibitor treatment reduced total number of $Ca^{2+}$ transients in the neural plate.

## V. Notochord and evolution of chordates

Amphioxus occupy a key phylogenic position for deciphering the origin and evolution of chordates. While they develop well-organized somites, their neural tubes lack a brain-like structure; hence, they are considered acraniate. Their notochords contain myofibrils. In order to characterize these three tissues of adult amphioxus, we examined their differential gene expression profiles by RNA-seq analysis. There were 1,018, 1,079, and 737 genes that were highly expressed in the notochord, somite muscle, and neural tube, respectively. Of the genes expressed in the notochord and muscle, 564 (55% of notochord genes and 52% of muscle genes) were shared, suggesting a molecular affinity between the two. On the other hand, only 119 and 104 neural tube genes were shared with notochord and somite muscle, respectively, indicating independence of genes expressed in

the neural tube. GO annotation analyses showed that genes associated with extracellular components are preferentially found in the notochord. Those associated with organelles are found in somite muscle, and those involved in synapsis and those that encode molecular transducers appear in the neural tube. The present repertoire of genes provide a molecular basis for future evo-devo studies of early chordate evolution.

## VI. Epigenetic diversification associated with subfunctionalization of duplicated genes

Polyploid organisms offer a model for understanding the evolutionary effects of gene duplication. The African clawed frog *Xenopus laevis* has experienced recent allopolyploidization, which gave rise to the pseudotetraploid genome. We found that the duplicated MyoD genes (MyoDa and MyoDb) are subfunctionalized; complementarily decreasing in the expression levels of each gene in different developmental stages, and this set of genes play roles corresponding to those of the unduplicated *Xenopus tropicalis* MyoD. To reveal the mechanisms that make MyoDa and MyoDb genes subfunctionalized, genetic and epigenetic differences between MyoDa and MyoDb were investigated. A nucleotide sequence comparison showed that MyoDa and MyoDb are 87% identical in amino acid coding regions, 5' UTR and 3' UTR. In contrast, the first intron is relatively diverged (73% identical), especially with a CpG depletion in MyoDa. We then measured DNA methylation levels in the first intron, as DNA methylation is a well-known cause of CpG depletion by deamination mutations. By means of bisulfite sequencing, we showed epigenetic differences between the duplicated MyoDa and MyoDb. The first intron of MyoDa is highly methylated, instead that of MyoDb is completely unmethylated. The constant DNA methylation levels in early developmental stages irrespective of gene activity indicated that DNA methylation is not a direct regulator. Alternatively, DNA methylation in the first intron may primarily regulate the accessibility of polycomb proteins and addition of silencing epigenetic mark trimethylated histone H3 Lysine 27 (H3K27me3), which exclusively appears on DNA methylation free regions.

### Publication List

〔Original papers〕


- Hara, Y., Nagayama, K., Yamamoto, T.S., Matsumoto, T., Suzuki, M., and Ueno, N. (2013). Directional migration of leading-edge mesoderm generates physical forces: Implication in *Xenopus* notochord formation during gastrulation. Dev. Biol. *382*, 482-495.
- Paemka, L., Mahajan, V.B, Skeie, J.M., Sowers, L.P., Ehaideb, S.N., Gonzalez-Alegre, P., Sasaoka, T., Tao, H., Miyagi, A., Ueno, N., Takao, K., Miyakawa, T., Wu, S., Darbro, B.W., Ferguson, P.J., Pieper, A.A., Britt, J.K., Wemmie, J.A., Rudd, D.S., Wassink, T., El-Shanti, H., Mefford, H.C., Carvill, G.L., Manak, J.R., and Bassuk, A.G. (2013). PRICKLE1 interaction with SYNAPSIN I reveals a role in autism spectrum disorders. PLoS One *8*, e80737.
- Suzuki, M.M., Yoshinari, A., Obara, M., Takuno, S., Shigenobu, S., Sasakura, Y., Kerr, A.R., Webb, S., Bird, A., and Nakayama, A. (2013). Identical sets of methylated and nonmethylated genes in *Ciona intestinalis* sperm and muscle cells. Epigenetics Chromatin *6*, 38.
- Uno, Y., Nishida, C., Takagi, C., Ueno, N., and Matsuda, Y. (2013). Homoeologous chromosomes of *Xenopus laevis* are highly conserved after whole-genome duplication. Heredity *111*, 430-436.


〔Review articles〕


- Hashimoto, M., Morita, H., and Ueno, N. (2013). Molecular and cellular mechanisms of development underlying congenital diseases. Congenital Anomalies *54*, 1-7.
- Takagi, C., Sakamaki, K., Morita, H., Hara, Y., Suzuki, M., Kinoshita, N., and Ueno, N. (2013). Transgenic *Xenopus laevis* for live imaging in cell and developmental biology. Dev. Growth Differ. *55*, 422-433.

## DIVISION OF DEVELOPMENTAL GENETICS

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Germ cells are specialized cells that can transmit genetic materials from one generation to the next in sexual reproduction. All of the other cells of the body are somatic cells. This separation of germ and somatic cells is one of the oldest problems in developmental biology. In many animal groups, a specialized portion of egg cytoplasm, or germ plasm, is inherited by the cell lineage which gives rise to germ cells. This cell lineage is called germline. The germline progenitors eventually migrate into the gonads, where they differentiate as germline stem cells (GSC) to form eggs and sperm when the organisms are physically matured. Our laboratory aims to find the molecular mechanisms regulating germline segregation and GSC niche function in *Drosophila*.

### I. Role of maternal Ovo protein in the germline of *Drosophila* embryos

It has been proposed that germline-specific gene expression is initiated by the function of maternal factors that are enriched in the germ plasm. However, such factors have remained elusive. We have done a genome-wide survey of maternal transcripts that are enriched in the germ plasm and encode transcription factors for germline-specific gene expression of *vasa* and/or *nanos*. We finally identified 6 transcripts required for germline-specific gene expression by knockdown experiments using RNA interference (RNAi). Among the 6 transcripts, we focused on *ovo*. The *ovo* gene encodes a DNA-binding, C2H2 Zn-finger protein that is involved in oogenesis and in epidermal development. The *ovo* gene produces at least three alternate isoforms. Ovo-A and Ovo-B function as a negative and a positive transcriptional regulator in the germline, respectively. Ovo-Svb is expressed in the epidermal cells and is required for their differentiation. We found that Ovo-B is the major isoform expressed in PGCs during embryogenesis. To understand its function, we over-expressed the Ovo-A repressor only in the primordial germ cells (PGCs), and examined their developmental fate. Our data shows that the reduction in maternal Ovo-B activity results in a decrease in the number of primordial germ cells during post-embryonic stages. Thus, maternal Ovo-B has an essential role in germline development in both sexes. Experiments for identifying the downstream genes regulated by Ovo-B in germline are now on-going.

### II. Germline Functional Genomics and Systems Biology using a *Bombyx mori* cell line

Conserved mechanisms regulating germline properties have remained largely unknown. Despite extensive use of *vasa* expression as a marker for germline in various animal species, systematic analyses on regulators for *vasa* expression have not been accomplished. With availability of genomic information and development of various *omics* technologies, functional genomics and/or systems biology approaches have been becoming more powerful to elucidate complex regulatory systems. However, such approaches require parallel data collection for many samples on many parameters to extract information on the systems, which is prohibitively difficult to perform using germline cells isolated from animals.

BmN4-SID1, a cell line derived form a *Bombyx mori* ovary, is known to express a germline gene, *piwi* (Kawaoka *et al*. 2009) and carries a nuage-like structure with *Vasa* protein (Tatsuke *et al*. 2010). Without any feeder cells, BmN4-SID1 can easily be propagated for large-scale experiments. Furthermore, a knockdown system by RNAi in the cell line has been established by introducing *C. elegans* SID1 (Mon *et al*. 2012). These properties are suitable to obtain parallel and scalable data collection from genetically manipulated germline cells. Therefore, we aimed to establish resources using BmN4-SID1 for functional genomics and systems approaches on germline properties. First, approximately 375 million reads of 50bp paired-end sequences were obtained by RNA-seq. Since the genetic background of BmN4 is unclear and gaps exist in the reference *B. mori* genome sequence, the obtained reads were used to build transcript contigs by the *de novo* assembler Trinity. Fifty thousand eight hundred eight contigs fully or partially matching the current gene models of *B. mori* were obtained. These contigs include *piwi* and *vasa* as well as the genes involved in sex differentiation and meiosis. With the structural information of the BmN4-SID1 transcripts, we carried out *in silico* dsRNA designed to efficiently knockdown each of the transcripts. These resources provide a platform enabling genome-wide perturbations and system-level measurements.

We have initiated RNAi screening for regulators of *vasa* and/or *piwi* expression. Among 300 regulatory genes which are conserved between *D. melanogaster* and *B. mori* and are involved in fertility of *D. melanogaster*, 7 genes including *ovo* were identified to regulate *vasa* and/or *piwi* expression. In addition, we have initiated elucidation of topology of regulatory network for germline gene expression (Figure 1). In this analysis, we also aimed to identify regulatory relationships between the genes known to be involved in germline development and the ones in other developmental processes. Since our transcriptome analyses on *D. melanoster* and *M. musculus* germline indicated that most of genes encoded by the genomes were dynamically regulated

during their development, it is intriguing to examine functional association between the genes involved in germline development and in biological processes that were not known to be involved in germline development. We are now performing RNAi-based knockdown of the genes including the 7 genes which are required for vasa expression in the cell line. The RNA was isolated from the dsRNA-treated BmN4-SID1, and then was analyzed by RNA-seq. The transcriptome data was used to infer regulatory relationships among the genes perturbed.

Although BmN4-SID1 does not fully maintain germline properties such as the ability to produce gametes, it has advantages on performing high-throughput and/or massive phenotyping on germline gene expression signatures to build testable hypotheses. Further studies using comparative functional analyses with model organisms such as *D. melanogaster* that are feasible for *in vivo* studies would identify developmental roles of genes and/or regulatory networks identified using BmN4-SID1.

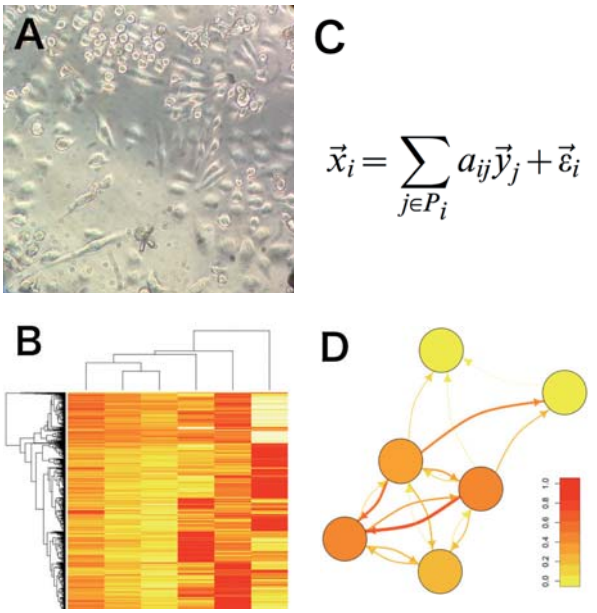


Figure 1. The workflow of modeling the gene regulatory network in BmN4-SID1. (A) BmN4-SID1 soaked in the culture media containing dsRNA. (B) Collection of network states obtained by transcriptome analyses. RNA-seq analyses were performed to collect mRNA expression differences between control and RNAi-treated cells, as inputs for network modeling. (C) EdLEGG for network modeling. The EdLEGG algorithm was applied to the mRNA expression difference data. $\bar{x}_i$ and $\bar{y}_j$ are gene expression differences caused by knockdown of gene *I* and *J*, respectively. In the linear combination, regulatory relationships between gene *I* and *J* are estimated by coefficients $a_{ij}$. (D) The resultant network model obtained so far. Nodes and links represent the genes targeted by RNAi and regulatory relationships between these genes, respectively.

## III. The role of HSPGs in germline stem cell niche of *Drosophila*

Stem cells posses the remarkable capacity to generate daughter cells that retain a stem-cell identity and others that differentiate. Stem cells reside in dedicated cellular microenvironments termed stem-cell niches. These niches dictate stem-cell identity, maintain the stem cell population, and coordinate proper homeostatic production of differentiated cells. The GSC niche in *Drosophila* gonads is a useful model system for studying the stem-cell niche, because the cellular components of this niche have been characterized and the signaling pathways, such as BMPs and JAK/STAT which are essential for GSC maintenance, are known. Ligands for these signaling pathways (niche signals) are secreted from the niche cells, and are received by GSCs to activate the pathway responsible for GSC maintenance. Thus, the GSC niche is defined as the specialized region retaining a sufficient amount of niche signals for GSC maintenance. However, it is not well understood how the distribution of the niche signals is precisely controlled in the GSC niche.

To address this question, we have been investigating the function of Heparan Sulfate Proteoglycans (HSPGs) in the GSC niche. HSPGs are an evolutionally conserved family of sugar modified protein, which is an essential component of the extracellular matrix. One of the important functions of HSPGs during animal development is to regulate distribution of growth factors in extracellular space by binding to them. Thus, we speculated that HSPGs could retain a sufficient amount of niche signals for GSC maintenance. We found that Glypcan, a membrane-associating type of HSPG, is an essential component of the GSC niche both in female and male gonads. Glypican was highly expressed in niche cells both in ovary and testis, and its mutations caused a significant reduction in GSC number. In the GSC of the mutant ovary, the signaling pathway activated by Dpp (a BMP homologue acting as a niche signal) was impaired. Conversely, ectopic expression of Glypican in female gonads caused an increase in the number of GSCs with Dpp signaling. These results strongly suggest that Glypican defines the female GSC niche by regulating distribution of Dpp.

The question of whether the other HSPGs have functions in the GSC niche remains unclear. Since disrupting biosynthesis of all HSPGs by knockdown of NDST gene caused more severe GSC-loss phenotype than Glypican mutant, the other types of HSPGs could have functions in the GSC niche. We found that Syndecan and Perlecan, two evolutionally conserved groups of HSPGs, are essential in the GSC niche. These HSPGs were highly expressed in female GSC niche cells, and reduction of their function in niche cells caused a decrease in GSC number. Surprisingly, in these ovaries, ectopic GSC-like cells were also observed. This phenotype has not been observed in Glypican mutants. Thus, we speculated that Syndecan and Perlecan could regulate Dpp distribution in the GSC niche, in a way distinct from Glypican. We have succeeded to visualize Dpp protein distribution in the female GSC niche (Figure 2A). When Glypican was ectopically expressed in female gonads, Dpp distribution was ectopically observed (Figure 2B). We are now trying to visualize Dpp distribution in Syndecan and Perlecan mutant ovaries. Furthermore, we have also succeeded to visualize the GSC niche signal in male gonads (Figure 2, C, D). This enables us to study HSPGs function in the male GSC niche.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

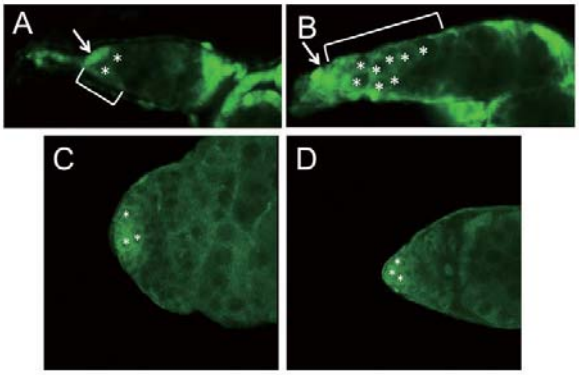


Figure 2. Distribution of GSC niche signals.
(A,B) Dpp distribution in distal tip region (germarium) of normal ovary (A) and of ovary expressing Glypican in the somatic cells throughout germarium (B). Green signal indicates Dpp protein (Brackets). Dpp distribution is expanded in *dally*-expressing ovary, compared to that observed in normal ovary. Arrows show niche cells, which are the source of Dpp. Asterisks indicate GSCs. (C, D) Distribution of male GSC niche signals, Upd (JAK/ STAT ligand, Green, C) and Gbb (BMP ligand, Green, D). Distribution of both niche signals were limited within the male GSC niche. Asterisks indicate GSCs.

## Publication List

### 〔Original papers〕


- Dejima, K., Takemura, M., Nakato, E., Peterson, J., Hayashi, Y., Kinoshita-Toyoda, A., Toyoda, H., and Nakato, H. (2013). Analysis of Drosophila glucuronyl C5-epimerase: implications for developmental roles of heparan sulfate sulfation compensation and 2-O-sulfated glucuronic acid. J. Biol. Chem. 288, 34384-34393.
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### 〔Original paper (E-publication ahead of print)〕


- Lim, R., Anand, A., Nishimiya-Fujisawa, C., Kobayashi, S., and Kai, T. Analysis of Hydra PIWI proteins and piRNAs uncover early evolutionary origins of the piRNA pathway. Dev. Biol. 2013 Dec. 16.

## DIVISION OF MOLECULAR AND DEVELOPMENTAL BIOLOGY

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The body and tissues of the developing embryo are repeatedly divided into sub-regions specified by characteristic gene expression and morphology. The process that gives rise to these sub-regions, according to a defined pattern, is called “pattern formation” or “patterning.” Our laboratories aim to understand the molecular mechanism underlying pattern formation by several different approaches.

The most popular model to explain the patterning process is the “morphogen gradient and threshold” theory. Many genetic results indicate that secreted signal proteins such as Wnt, BMP, and Hedgehog function as morphogens in many aspects of the patterning process. In spite of the accumulation of genetic evidence, however, the biochemical characteristics of morphogens, including modification and higher order structure, remain to be elucidated. Thus, one of our major goals is to reveal *the real image* of morphogens and the molecular mechanism underlying the formation of morphogen gradients, including the secretion and extracellular transport of these morphogens.

The segmental sub-regions of several specific tissues, including the paraxial mesoderm (or somites) and the pharyngeal pouches, by contrast, appear not to be simply directed by the morphogen gradient and threshold, but by a unique mechanism proceeding periodically. For instance, somites are sequentially generated in an anterior-to-posterior order by converting oscillatory gene expression into periodical structures. The molecular mechanism underlying this conversion and morphological segmentation, however, is not yet fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of *this other and unique mode of patterning* that underlie the periodical and sequential sub-division in the development of somites and pharyngeal arches.

## I. Structural features of Wnt proteins secreted from polarized epithelial cells

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. In a classical view, it has been proposed that secreted Wnt proteins spread and function over a distance of several cells in many aspects of morphogenesis. In contrast, accumulating evidence also implies that Wnt proteins appear to transmit their signals locally in some particular cases, presumably since their secretion and transport are under tight control. To understand the molecular mechanism underlying morphogenesis mediated by extracellular transmission of Wnt signals, it will be required to reveal manners of diffusion and/or accumulation of Wnt proteins in the extracellular space. Of note, Wnt proteins are specifically modified with fatty acids during their secretion, as shown by several groups including us, suggesting that some specific structural feature caused by this modification may affect movement of Wnt proteins in the extracellular space.

Epithelial cells, as well as other type of cells, secret Wnt proteins during morphogenesis. Wnt proteins are secreted both from the apical and baso-lateral side of epithelial cells, but evidence suggested that the action range of Wnt proteins may be different according to side of secretion. On the other hand, structural features of secreted Wnt proteins appear to be heterogeneous. For instance, in Drosophila imaginal disc epithelium, Wg, the ortholog of vertebrate Wnt1, could be loaded onto lipoprotein particles and transported over large distances. Recent reports showed that Wnts were also secreted on exosome-like vesicles from cultured mouse L cells or Drosophila S2 cells. However, what is the possible contribution of lipoprotein particles or exosome in polarized secretion of Wnts is still unknown.

To better understand the molecular basis of generating a variety of signaling ranges, we examined structural features of Wnt proteins secreted from either the apical or the baso-lateral side of epithelial cells by using the MDCK (Madin-Darby Canine Kidney) cell as a model system. MDCK cells that stably express mouse Wnt3a were grown on a polarized monolayer on tissue culture inserts, which separate an upper apical compartment from the lower compartment. We found that polarized MDCK cells preferentially secreted Wnt3a from the basolateral side. To determine if the protein is present in the culture medium as freely soluble protein or associated with exosomes or with lipoprotein partiles, we fractionated conditioned medium by centrifugation. We found most Wnt3a proteins were present in the medium, indicating that they were secreted as a freely soluble form. Wnt3a proteins were also found to be packaged onto exosome-like vesicles and were exclusively secreted basolaterally. In addition, we found that the conserved serine residue, S209, which is required for lipid modification, was essential for proper secretion of Wnt3a from MDCK cells. These results indicated that Wnt3a secretion via exosomes specifically occurred on the basolateral side and required lipid modification (Figure 1).

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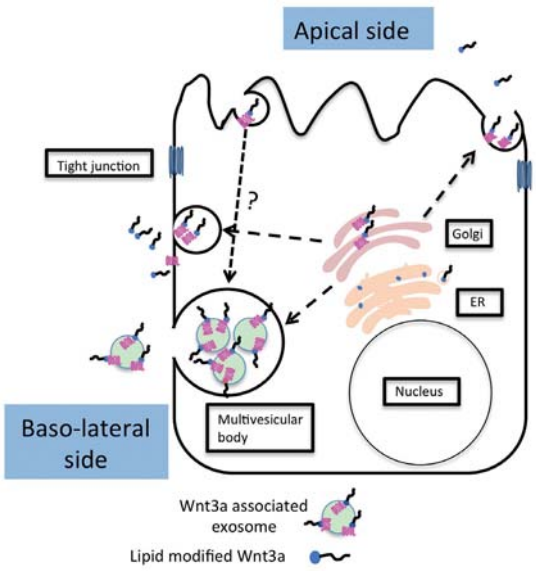


Figure 1. Model of polarized secretion of Wnt proteins form epithelial cells. Most Wnt3a proteins are secreted into both apical and basolateral sides. In addition, some Wnt3a proteins were also found to be packaged onto exosome-like vesicles and were exclusively secreted basolaterally. It is unclear how these exosome-like vesicles are formed during their secretion process..

## II. Molecular mechanism of somite development

Somites are segmental epithelial blocks located symmetrically on either side of the neural tube. Somites are periodically generated in an anterior to posterior manner from their precursor, the presomitic mesoderm (PSM), which is located posterior to newly formed somites. This periodic generation is achieved by a complex and dynamic mechanism in the PSM. First, the molecular clock, the so-called segmentation clock, creates oscillatory expression of particular genes, *hairy* and some *notch*-related genes, in the posterior PSM. Because the phase of oscillation is gradually shifted along the posterior-to-anterior axis, a wave of the oscillation appears to move in a posterior-to-anterior fashion. This oscillatory gene expression subsequently resulted in periodical generation of morphologically segmented somites.

The spatial pattern of somites is defined by positioning of intersomitic boundaries and rostro-caudal patterning within a somite. This spatial pattern is established in the anterior PSM by converting the oscillatory gene expression into spatially restricted patterns of gene expression. Several intra-cellular molecules, as well as FGF and Wnt signaling, are involved in this conversion. In the mouse, the prospective segmentation boundary is periodically established in the anterior PSM at the rostral border of Mesp2 expression domain. Mesp2, one of the key regulators in this conversion, is initially expressed at the most anterior region of the Tbx6 protein domain. This expression is not anteriorly extended beyond the anterior border of Tbx6 protein domain because Mesp2 expression requires Tbx6 proteins. Thus, the anterior border formation of Tbx6 protein domain is a more fundamental process in the positioning of the segmentation boundary (Figure 2).

Importantly, this border is not consistent with the anterior border of Tbx6 mRNA, rather it is regulated by a proteasome-mediated mechanism. Although the molecules directly executing this proteolysis are still unclear *Mesp2*, as well as *Ripply1* and *2*, have shown to be required for the down-regulation of Tbx6 proteins by analysis of embryos defective in these genes. Since expressions of *Ripply1* and *2* are eliminated in Mesp2 deficient mouse embryos, we previously proposed the following model; *Mesp2,* whose expression is activated in the most anterior part of the Tbx6 domain, causes retreat of the Tbx6 protein domain through activation of *Ripply1* and *2* expression, and the retreated Tbx6 subsequently defines the next segmentation border and *Mesp2* expression. However, it is still to be elucidated whether Mesp2 causes the retreat of the Tbx6 protein border through the activation of Ripplys' expression or that Mesp2 and Ripplys regulate this retreat in parallel. In addition, contrasting to the analysis with mouse mutants, Ripplys have also been implicated in acting in transcriptional repression in association with co-repressor Groucho/TLE and some T-box proteins, including zebrafish and Xenopus Tbx6. Therefore, it is also unclear whether Ripplys regulate Tbx6 proteins at the protein level in other species besides mouse.

For better understanding of somite patterning, we have examined zebrafish embryos, in which the gene functions involved in this process seem to be relatively precisely understood. In this study, by generating an antibody specific for zebrafish Tbx6/Fss, previously referred to as Tbx24, we examined the relationship between spatial distribution of

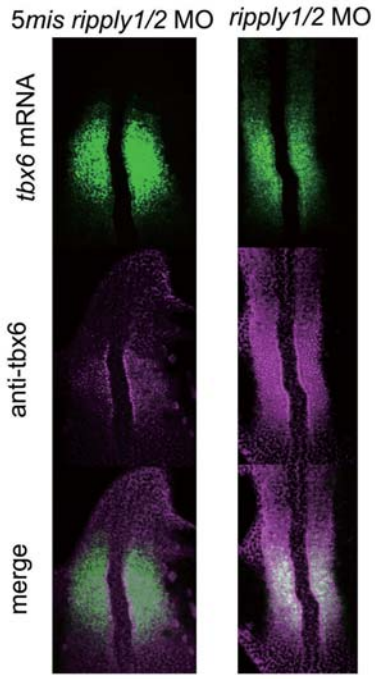


Figure 2. The zebrafish tbx6 mRNA expression expands from the PSM anteriorly to several newly formed somites. In contrast, the anterior border of the protein is restricted in the PSM to define the segmentation boundary. This restriction is regulated by ripply1/2 as observed in ripply1/2 defective embryos where the tbx6 protein domain is expanded anteriorly.

Tbx6 proteins and positioning of intersomitic boundaries in zebrafish embryos. We found that the anterior border of Tbx6 defines the segmentation boundary in zebrafish as previously shown in mouse. However, the dynamics of Tbx6 proteins are partly different from that in the mouse, suggesting that the spatial dynamics of the Tbx6 protein domain is also involved in the rostro-caudal patterning. Furthermore, we directly examined the ability of Ripply or Mesp to reduce the level of Tbx6 proteins in zebrafish eggs. Ripply, but not Mesp, down-regulated Tbx6 protein level when these mRNAs were co-injected into zebrafish eggs. Importantly, this down-regulation is dependent on physical interaction between Ripply and Tbx6. Consistent with these results, *ripply1* and *2* morphants showed expansion of Tbx6 protein domain in zebrafish embryos (Figure 2). These results strongly suggest that Ripply, but not Mesp, is a direct regulator of Tbx6 protein level in the establishment of intersomitic boundaries.

## III. Establishment of gene knock out methodology based on TALEN-mediated gene editing in the zebrafish

Zebrafish are one of the most suitable model animals for genetic analysis. Until now, chemical mutagenesis-mediated screening provided us a great number of mutant strains, which contribute to studies of morphogenesis of the vertebrates. In addition to this well-established genetic approach, recent advances in genome editing technologies have made it possible to create any mutation of a gene of interest.

One of these technologies is based on Transcription Activator-Like Effector Nuclease (TALEN). To establish efficient conditions for TALEN-mediated gene disruption in zebrafish, we designed 48 TALENs for targeting 14 genes and confirmed that 31 of the 48 TALENs could induce mutations in zebrafish. As a result, we succeeded in efficiently generating gene disrupted lines for all of the genes tested, indicating that TALEN-mediated gene disruption is a convenient technology for generating mutation of a gene of interest in a laboratory setting. By using this technology, we generated mutants for many genes specifically expressed during early embryogenesis (Figure 3).

### Publication List

〔Original papers〕


- Hisano, Y., Ota, S., Takada, S., and Kawahara, A. (2013). Functional cooperation of spns2 and fibronectin in cardiac and lower jaw development. Biol. Open. *2*, 789-794.
- Takahashi, Y., Yasuhiko, Y., Takahashi, J., Takada, S., Johnson, R.L., Saga, Y., and Kanno, J. (2013). Metameric pattern of intervertebral disc/vertebral body is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somites in the mouse embryo. Dev. Biol. *380*, 172-184.


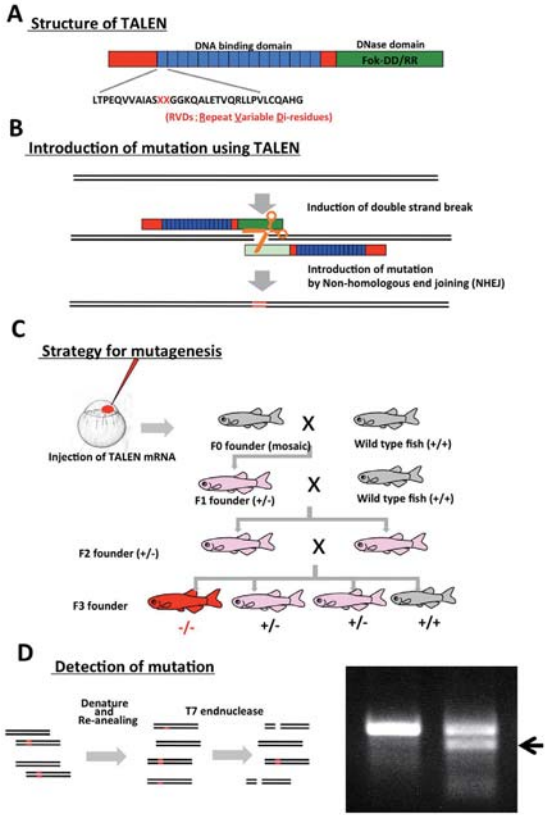


Figure 3. Example of TALEN-mediated gene disruption. A. Structure of TALEN construct. TALEN is an artificial nuclease containing Transcritional Activator Like Effector (TALE) of Xenthomonas and DNase domain. TALE domain is constituted from repeat units, each of which specifically recognize one particular nucleotide. The specificity of each unit is defined RDV sequence. B. Mutagenesis by TALEN. Two TALENs designed to recognize a particular site in the genome cause double strand break down, resulting in deletion and/or small insertion. C. Generation of homozygous mutant fish by crossing. D. Detection of mutation generated by TALEN. Genome DNA containing targeted site by TALEN was amplified by PCR. By heat denaturing and subsequent renaturation of the PCR products, DNA from embryos carrying mutations generate heteroduplex containing a mismatched sequence. Because this mismatched sequence can be digested by T7 endonuclease, heterozygous mutations can by detected. PCR products shown in the photo are example of T7 assay; Right lane: control embryo, Left lane: TALEN injected embryo

## DIVISION OF EMBRYOLOGY

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The aim of our research is to understand the events underlying early mammalian development during the period from pre-implantation to establishment of the body axes.

Early events during embryogenesis in mammals are relatively less understood, as compared to in other animals. This is mainly due to difficulties in approaching the developing embryo in the uterus of the mother. Another characteristic of mammalian embryos is their highly regulative potential. The pattern of cell division and allocation of cells within an embryo during the early stages vary between embryos. The timing of the earliest specification events that control the future body axes is still under investigation. Functional proteins or other cellular components have not been found that localize asymmetrically in the fertilized egg. We want to provide basic and fundamental information about the specification of embryonic axes, differentiation of cell lineages, behaviors of cells, and the regulation of body shape in early mammalian development. We are also interested in the relationship between embryos and their environment, the oviducts and the uterus.

## I. Establishment of live imaging system for observation of early mouse development

To understand the mechanisms underlying embryonic development and morphogenesis, it has become common to observe the behavior of cells and gene expression in living embryos in a variety of animal species. Progress in GFP technologies, genetic engineering and embryonic manipulation concomitantly with the progress in optics and microscope technologies, including development of highly sensitive photo-detectors, has allowed us to observe developing embryos live, even in mammals. It is useful to visualize nuclei, cell shapes, cytoskeleton or other organelles to observe cells and cell behaviors in living mouse embryos. We have established a series of transgenic mouse lines for live imaging, which is a part of a collaborative project taking place in the Laboratory for Animal Resources and Genetic Engineering, Riken CDB. In each mouse, cDNA encoding fusion protein with fluorescent protein and a localization sequence were inserted into Rosa26 locus. The sequence for the fusion protein was following stop sequences that are surrounded by loxP sites on both sides. These loxP sites can be recognized by an enzyme, Cre recombinase, that catalyzes recombination between two loxP sites to remove the stop sequences. Thus, when Cre recombinase is activated in a spatial-temporally specific manner, the following reporter fluorescent proteins are expressed in a specific way. And once this irreversible reaction is induced in the germ line, the derived offspring possess the transgene without the stop sequence and express the reporter fusion protein ubiquitously.

We are also establishing several reporter mouse lines in the lab to study gene expression patterns during the peri-implantation stage of mouse development. In these mice, cDNA encoding fluorescent proteins are connected to the mouse genomic DNA sequences of enhancer/promoter region of important gene encoding factors regulating cell differentiation in these stages. Combining these two types of reporter transgenic mouse lines, we have started analysis of behaviors of cells comparing gene expression properties at the single cell level. This year, we have reported establishment of mouse lines to monitor the cell cycle.

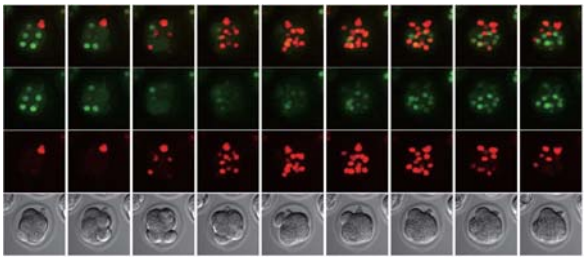

Figure 1. Examples of a morula expressing fucci2, cell cycle markers. The G(1) and S/G(2)/M phases are distinguished by mCherry(red) and mVenus (green) signals respectively.

For the live imaging of early mammalian embryos, a combined microscope and incubation system is an important tool. Conventional CO2 incubators provide better conditions compared with microscope top incubation chambers, including stability of temperature and humidity, to support embryonic development in vitro. Incubation microscopes have also recently become commercially available, however, these are expensive for personal use. We have modified an incubation microscope with wide field fluorescent illumination, which is relatively inexpensive. We added a spinning disc confocal system and sensitive EM-CCD camera for observation of developing mouse embryos with less photo-toxicity and higher spatial resolution.

We are also trying to observe and reveal aspects of cell shape, morphogenesis, cell lineage, gene expression and cell differentiation by combining these techniques.

## II. Histological observation of mouse embryos developing in the uterus

One of the characteristics of mammalian development is that embryos develop in the uterus after implantation. Direct interaction between embryos and the cells of the uterus is important. In studies on developmental biology of mammalian embryos, embryos are usually removed from the uterus, and isolated embryos are analyzed. We have been analyzing early embryonic development of mice comparing

the changes in the uterine epithelium. To observe the morphogenesis of embryos within the uterus, serial sections of implanted uteruses were made, stained with hematoxylin and eosin, and the images of the embryos within the uteruses are captured to make high resolution three-dimensional re-constructions. Figure 2 shows an example of a section. We are preparing samples obtained from various developmental stages. Precise positions of embryonic implantation and changes in the uterine epithelium correlating with embryonic development will be examined using these images.

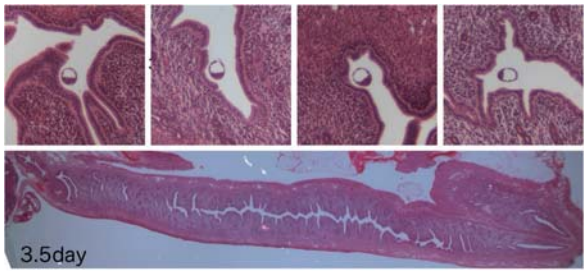


Figure 2. A pregnant uterus 3.5 days after fertilization. Developing embryos and the shape of uterus epithelium can be observed.

## III. Studying early development of rabbits as a new model of mammalian embryogenesis

Mice have been the main experimental animal used for the study of mammalian developmental biology because genetics and genetic engineering including transgenesis and targeted mutagenesis are applicable for mice. We also have been mainly studying mouse embryogenesis. However, rodent embryos do not necessarily provide ideas common in mammalian embryogenesis because the styles of early embryonic development differ between species, and rodent embryos do not provide a typical style when compared with other animals. We searched for animals that are suitable for the study of early embryogenesis comparing possible candidates, and decided to use rabbits. We are studying morphological changes and gene expression during early stages of development until the peri-implantation stage. We are focusing especially on the formation of germ layers and body axes, and compare with corresponding stages in mice.

## IV. Formation, maintenance of cell polarity and tissue morphogenesis in the mouse oviduct

The oviducts (fallopian tubes) are tubes connecting the periovarian spaces and the uterine horns. The ova released from the ovary are transported through the oviduct, where fertilization occurs with the spermatozoon moving from the uterus. We are focusing on the region of the oviduct close to the opening to the ovary. In this region many multi-ciliated cells exist. These cilia move in one direction along the ovary-uterus axis. This directional movement of multi-cilia might play a major role in the transportation of ovum from periovarian space, although muscle contractions also play roles in the region close to the uterus. This suggested that the ovum was transported by the directionally beating cilia. This directional beating of cilia was based on the polarity of the microtubule assembly in the cilia. Skeletal microtubules in the cilia are arranged in a “9 +2”array, and the central two bundles are aligned facing the same direction in each cell. This suggests that oviduct epithelial cells possess polarity along the cell surface parallel to the longitudinal axis of the oviduct; this type of cellular polarity is called “Planer Cell Polarity (PCP)”. We have been studying how PCP is established during development, and how this polarity is maintained over a long period in later stages.

We have been focusing on several genes involved in PCP formation. The molecular basis of PCP formation has been genetically studied, particularly using fruit fly, and several genes have been shown to be involved in the regulation of PCP formation. Homologues of these genes are also known in mammalian species, and some of them have been shown to play similar roles. Disruption of functions of some of these genes resulted in abnormal PCP formation in skin, inner ear, and early embryos of mice. We are also focusing on some of the PCP regulators, and are trying to understand how oviduct epithelial cells establish and maintain polarity. We have been trying to reveal mechanisms including the molecular functions, cellar shape, tissue morphology and involvement of mechanical forces in this system.

## V. Analysis of mechanical properties of cells during embryonic development

Mechanics is one of the essential components for biological processes including cell shape transformation, and tissue morphogenesis etc. However, how mechanical properties such as force and material stiffness regulate these processes is poorly understood. To approach this problem, measuring cellular geometric information and mechanical properties are necessary. We developed an image processing based technique to measure cellular geometric information from fluorescent microscopic images and a framework to theoretically estimate the mechanical properties. The image processing technique enabled us to robustly detect cell contour from images with high noise and non-uniform illumination, although the algorithm of the technique is quite simple. By employing the image processing technique, we successfully extracted geometric information of early embryonic cells during cytokinesis in *C. elegans* and of mouse cells in cell sheets. In the framework for estimating mechanical properties, geometric information was combined with a mechanical simulation, which was technically based on the data assimilation (Figure 3). We spatio-temporally

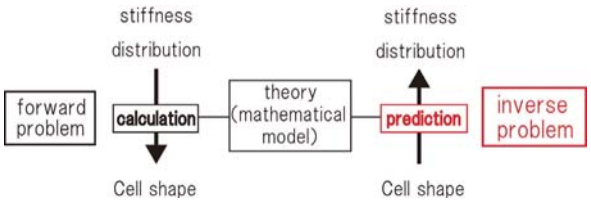


Figure 3. Theoretical estimation of cell surface stiffness. Schematic illustration of estimation.

estimated cell surface stiffness during cytokinesis by systematically fitting the *in vivo* cell shape to the mechanical simulation. We found that cell polar and equatorial regions were stiffer and softer, respectively. Further theoretical modeling showed that the relative difference of stiffness between the two regions could be a primary determinant for cleavage furrow ingression during cytokinesis. We speculated that the relative contributions of cell surface stiffness and the contractile ring could explain the contractile ring dependent and independent cytokinesis.

## Publication List

### 〔Original papers〕


- Abe, T., Sakaue-Sawano, A., Kiyonari, H., Shioi, G., Inoue, K., Horiuchi, T., Nakao, K., Miyawaki, A., Aizawa, S., and Fujimori, T. (2013). Visualization of cell cycle in mouse embryos with Fucci2 reporter directed by Rosa26 promoter. Development *140*, 237-246.
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- Xu, G., Shen, J., Ishii, Y., Fukuchi, M., Dang, T.C., Zheng, Y., Hamashima, T., Fujimori, T., Tsuda, M., Funa, K., *et al*. (2013). Functional analysis of platelet-derived growth factor receptor-beta in neural stem/progenitor cells. Neuroscience *238*, 195-208.


### 〔Review articles〕


- Abe, T., Aizawa, S., and Fujimori, T. (2013). Live imaging of early mouse embryos using fluorescently labeled transgenic mice. Methods Mol. Biol. *1052*, 101-108.
- Abe, T., and Fujimori, T. (2013). Reporter mouse lines for fluorescence imaging. Develop. Growth Differ. *55*, 390-405.

## DIVISION OF GERM CELL BIOLOGY

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Production of numerous sperm for a long period in testis is fundamental for continuity of life across generations. This process of spermatogenesis is supported by the robust stem cell system.

The Division of Germ Cell Biology aims to fully understand the mouse sperm stem cell system under the context of *in vivo* tissue architecture. We have revealed some characteristics of this potent and interesting stem cell system. First, differentiating germ cells that had been believed to be irreversibly committed for differentiation still retain the self-renewing potential and can contribute to stem cell pool maintenance ("potential stem cells"). Secondly, "reversion" from potential stem cells occurs at a higher frequency when testicular tissue is damaged and regeneration is induced. Thirdly, the undifferentiated spermatogonia population including both "actual" and "potential" stem cells are preferentially localized near vasculature (vasculature-associated niche). We also discovered that stem cells turn over at an unexpected frequent and stochastic manner under a steady-state situation that continuously produces sperm (Nakagawa *et al*., Dev. Cell 2007; Yoshida *et al*., Science 2007; Nakagawa *et al*., Science 2010; Klein *et al*., Cell Stem Cell 2010). These observations have thrown doubt on the prevailing stem cell model, the "$A_s$ model", postulating that the stem cell function is restricted to the singly isolated spermatogonia ($A_s$ cells).

In 2013, we performed single-cell-level analyses of a tiny spermatogonial population (viz. GFRα1+) taking advantage of pulse-labeling and live-imaging studies, combined with biophysical modeling. The results indicate that the entirety of GFRα1+ spermatogonia (including both $A_s$ and syncytia) comprise a single equipotent stem cell pool and cells continually interconvert between $A_s$ and syncytial states. This novel paradigm would replace the "$A_s$ model" that was proposed over 40 years ago (Hara *et al*., Cell Stem Cell *in press*).

## I. Background: Testicular cells and stem cell models

In mammalian testis, a huge number of sperm are continuously produced over the reproduction period (1-2 years in mice), which relies on stem cell activity. For decades, the cellular identity and behavior of spermatogenic stem cells has been argued as a fundamental question in the field of spermatogenesis. However, cellular identity and behaviors of stem cells remain poorly understood in mouse spermatogenesis.

The process of spermatogenesis takes place in seminiferous tubules (Figure 1). In mice, mitotic stages of spermatogenic cells, called "spermatogonia", are divided into "undifferentiated" and "differentiating" populations. "Undifferentiated spermatogonia" are found as singly isolated cells ($A_s$), or syncytia consisting mainly of 2 ($A_{pr}$), 4 ($A_{al\text{-}4}$), 8 ($A_{al\text{-}8}$), or 16 ($A_{al\text{-}16}$) cells. The formation of syncytia is due to incomplete cell division, a germline-specific cell division process where cytokinesis does not complete and the cytoplasmic connection between daughter cells persists via intercellular bridges.

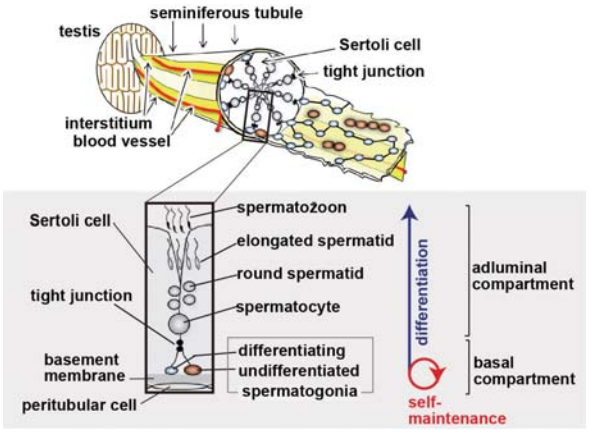


Figure 1. Spermatogenesis in seminiferous tubules. Spermatogenesis progresses from the basal- to adluminal compartment. Stem cells are thought to reside primarily within undifferentiated spermatogonia (brown) which are distributed sparsely on the basement membrane. Figures are modified from Cell Stem Cell (DOI: 10.1016/j.stem.2014.01.019) with permission.

Based on the detailed morphological analyses of fixed specimens, it was proposed in 1971 that stem cell activity may be restricted to the population of $A_s$ spermatogonia, while interconnected $A_{pr}$ and $A_{al}$ syncytia are irreversibly committed to differentiation and no longer contribute to the stem cell pool (Huckins, 1971; Oakberg, 1971). This hypothesis is known as the "$A_s$ model". On the other hand, over the last decade, studies from some groups, including ours, have shown that the population of undifferentiated spermatogonia can be divided on the basis of their heterogeneous gene expression. To summarize, In undisturbed steady-state spermatogenesis, the GFRα1+ subpopulation (mainly $A_s$, $A_{pr}$ and fewer $A_{al}$, shown later in Figure 3) is thought to reside on the top of the hierarchy: As well as maintaining their own population, GFRα1+ cells also give rise to the second, Ngn3+, subpopulation of undifferentiated spermatogonia (comprised of more $A_{al}$ and fewer $A_s$ and $A_{pr}$). Ngn3+ cells, although retaining an ability to revert into GFRα1+ cells that becomes apparent during regeneration after tissue insult, mostly differentiate into differentiating spermatogonia and then meiotic

spermatocytes and haploid spermatids.

These studies indicate that the dynamics of GFRα1+ spermatogonia is the essential question to fully understand identity and behavior of the mouse sperm stem cells. In our previous study, inspired by the $A_s$ model, GFRα1+ $A_s$ spermatogonia were hypothesized to be the primary population of stem cells (Figure 2; Nakagawa *et al*., Science 2010). However, this idea warrants experimental evaluation.

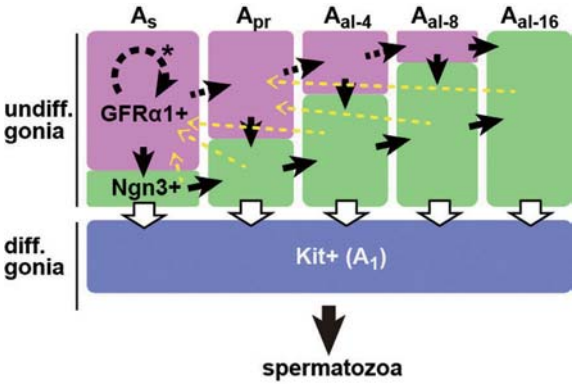


Figure 2. Extended $A_s$ model. A previously proposed hierarchy of GFRα1+ and Ngn3+ subpopulations of undifferentiated spermatogonia, as well as Kit+ differentiating spermatogonia (modified from Nakagawa *et al*., 2010). Black and white solid arrows indicate processes that had been directly observed, whereas the black broken arrows represent a presumptive dynamics of GFRα1+ cells, in which only GFRα1+ $A_s$ self-renew (asterisk). Yellow broken arrows indicate the processes of "reversion", which occur infrequently in steady state.

Following the aforementioned background, we have been challenging to understand the identity and dynamics of the stem cells by analyzing the fate behaviors of GFRα1+ spermatogonia at a single-cell-resolution in undisturbed mouse testis. To achieve this, we took advantage of originally established approaches of *in vivo* live-imaging and pulse-labeling studies, combined with biophysical modeling analyses that were performed in collaboration with Dr. Ben Simons of the University of Cambridge, UK.

## II. Population asymmetric stem cell maintenance

The population of GFRα1+ spermatogonia lies scattered unevenly on the basement membrane of seminiferous tubules (Figure 3), but their local density over a prolonged tubule length was found to be remarkably constant in adult mice. To trace the fate behavior of GFRα1+ spermatogonia, we established *GFRα1-CreER$^{T2}$; CAG-CAT-GFP* mice that can irreversibly label GFRα1+ spermatogonia with GFP by administrating 4OH-tamoxifen into the abdomen, in collaboration with Dr. Hideki Enomoto of Kobe University, Japan. Using this model, we obtained the clonal fate data of GFRα1+ spermatogonia from several days to over a year, and showed that, although the entirety of GFRα1+ spermatogonia maintained their own population while giving rise to differentiating progeny, the individual GFRα1+ spermatogonia followed highly variable and intricate fate behaviors. These findings suggest that the maintenance of GFRα1+ spermatogonia is achieved by "population asymmetry", in which balanced stem cell maintenance and production of differentiating progeny are achieved at a population-level. This paradigm is a recently emerged concept in the stem cell biology field, marking a stark contrast to the classical "division asymmetry", in which every cell division provides asymmetrically fated daughter cells (one self-renewing and one differentiating).

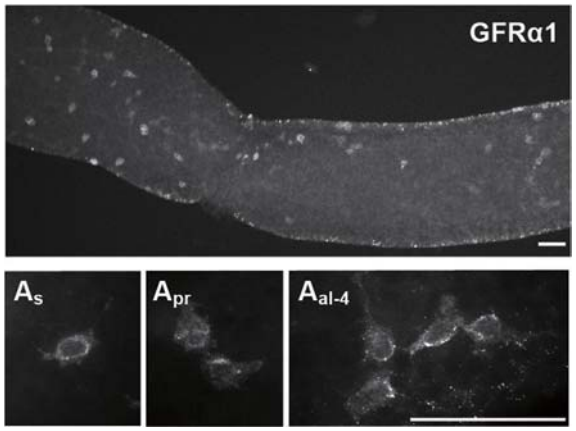


Figure 3. Morphology of GFRα1+ spermatogonia. Immunofluorescence for GFRα1 in a whole-mount seminiferous tubule specimen. Lower, higher magnification of GFRα1+ $A_s$, $A_{pr}$, and $A_{al-4}$. Bars, 50$\mu$m.

## III. Interconversion of stem cells between different morphological states

We next investigated the behavior of GFRα1+ spermatogonia, by means of *in vivo* live-imaging of *GFRα1-GFP* mouse testis up to 3 days, again collaborating with Dr. Hideki Enomoto. From the numerous 3-day-long observations that altogether effectively cover one year of spermatogonial behavior, it is indicated that GFRα1+ cells continually change their morphological states between $A_s$, $A_{pr}$ and $A_{al}$ spermatogonia through a combination of "incomplete cell division (leading to syncytial extension)" and "syncytial fragmentation (through breakage of intercellular bridges)", while giving rise to differentiating progeny from all of these morphological states. The live-imaging study further provides quantitative insight into the dynamics of GFRα1+ cells: incomplete cell division and syncytial fragmentation of GFRα1+ spermatogonia appear to occur at constant rates, independent of the unit length.

From the live-imaging study, it was also apparent that GFRα1+ spermatogonia were in constant movement in the basal compartment without showing any systematic pattern. This contrasts with the behavior of Ngn3+ spermatogonia, which are less motile in the vasculature-associated region, before actively spreading over the basal compartment on transition into $A_1$ spermatogonia (Yoshida *et al*., Science 2007).

## IV. Stem cell dynamics governed by the rates of incomplete cell division and syncytial fragmentation

The live-imaging measurements indicate that the rates of the fundamental processes of "incomplete division" and "syncytial fragmentation" may be constant, independent of unit length. We were then motivated, to try to capture the dynamics of GFRα1+ spermatogonia using a biophysical modeling scheme, which essentially depends only on the aforementioned two measured rates.

Intriguingly, the model could accurately predict the wide range of intricate clonal fate behaviors experimentally observed including that of pulse-labeled GFRα1+ cells in steady-state over wide time scales from several days to over a year. Moreover, the model also nicely predicted the fate behaviors of GFRα1+ spermatogonia during regeneration following tissue insult. Given the ability of such a simple model to predict the complex *in vivo* behavior from steady-state to regeneration, we believe that the principles that define the dynamics of the GFRα1+ compartment have been successfully resolved.

## V. Conclusion: A proposed stem cell dynamics

Figure 4 illustrates the proposed stem cell dynamics, in which GFRα1+ units continuously extend via incomplete division and fragment via intercellular bridge breakdown, while giving rise to Ngn3+ progeny. Interestingly, no GFRα1+ cell in this scheme meets the generic strict definition of "a stem cell", a cell that repeats self-renewal (viz. production of one or more cell(s) identical to the parental state following proliferation). Rather, individual cells constantly change their morphological states reversibly between single cells and variable lengths of syncytia. Through this process, at the population level, the population of GFRα1+ cells is maintained, while producing differentiating progeny.

To conclude, in contrary to the long-held "$A_s$ model", we propose that the entirety of GFRα1+ spermatogonia comprises a single "stem cell pool". In particular, the stem cells can be defined, not as a particular cell type, but as a heterogeneous population in which cells continually interconvert between different states. We believe such a concept of "*dynamic heterogeneity*" emerging in mouse spermatogenesis may provide a novel paradigm for other tissue stem cell systems.

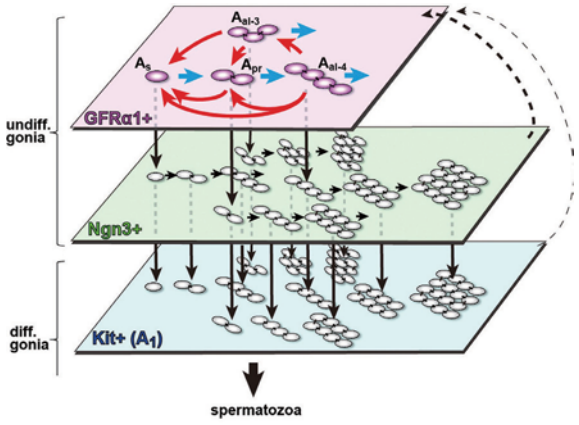


Figure 4. A proposed stem cell dynamics. On the top of the differentiation hierarchy, GFRα1+ spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between states of $A_s$, $A_{pr}$ and $A_{al}$ spermatogonia through incomplete cell division (blue arrows) and syncytial fragmentation (red arrows), while giving rise to Ngn3+ cells. After leaving the GFRα1+ compartment, differentiation-destined cells follow a series of transitions (GFRα1+ Ngn3+ Kit+; downward black arrows) that accompany the extension of syncytial length (rightward black arrows). Ngn3+ and, to a lesser extent, Kit+ cells retain the capacity to revert back into the GFRα1+ compartment in a context-dependent fashion (broken arrows).

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- Nakamura, Y., Tasai, M., Takeda, K., Nirasawa, K., and Tagami, T. (2013). Production of functional gametes from cryopreserved primordial germ cells of the Japanese quail. J. Reprod. Dev. *59*, 580-587.
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〔Review article〕

- Nakamura, Y., Kagami, H., and Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. Dev. Growth Differ. *55*, 20-40.

## Laboratory of Molecular Genetics for Reproduction

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*TAKEDA, Manami*

### Laboratory scope

Reproduction is a universal and fundamental system for organisms to produce generations. To accomplish this purpose efficiently, organisms develop sexual reproduction. Vertebrates, however, exhibit a variety of reproductive systems. The many modes of sex differentiation is one of the main components that contribute to variety. Our laboratory aims to reveal the fundamental mechanisms underlying reproduction, especially focusing on the mechanism of sex differentiation, and to understand how the mechanisms are related to a variety of reproductive systems.

We use medaka fish (*Oryzias latipes*) and have been generating transgenic medaka enabling us to analyze how different cell lineages are involved in the process of gonad formation and sex differentiation in vivo. Additionally, in order to identify the genes essential for reproduction, we carried out a mutational screening of medaka with defective phenotypes and disrupted several candidate genes. With these two unique analytical methods (visualizing cells and mutants), we are attempting to unveil both the fundamental mechanisms and the specific mechanism that produce a variety of reproductive systems.

### I. Critical contribution of germ cells to direct sex differentiation

In gonochoristic vertebrates such as medaka and humans, a gene on the sex chromosome is responsible for the determination of sex. Once the process of sex determination is triggered by the gene, the animal begins to develop into either female or male and does not change the direction during its life cycle. The sex differentiation is unidirectional. On the other hand, it has been described that sex is a consequence of balancing between female and male process (biphasic process) because sex reversal is often reported even in gonochoristic vertebrates.

As the results of our previous studies, we have revealed that germ cells are critical for the biphasic process. In the absence of germ cells, we found that medaka exhibit complete male secondary characteristics at both endocrine and gene levels (Kurokawa *et al*., 2007 PNAS). This indicates that germ cells are essential for formation of ovaries. In addition, in the absence of germ cells, somatic cells are predisposed to male development even if the individuals do not have a Y chromosome. This view indicates that, other than sex determination genes, germ cells critically contribute to establishment of biphasic status.

Supporting this view, we have previously identified the gene responsible for this regulation, *amhrII* (anti-Müllerian hormone receptor). The ligand for this receptor is AMH (anti-Müllerian hormone), which is known to be secreted from male supporting cells (Sertoli cells) during mammalian sex differentiation, and is critical for Müllerian duct (female reproductive organ) regression in mammalian males. But in teleosts, there are no organs equivalent to the Müllerian duct. In addition, AMH belongs to a phylogenetically old and conserved type of TGFβ superfamily. These collectively suggest some conserved function other than Müllerian duct regression.

The mutant, called *hotei*, has a mutation in *amhrII* and exhibits a hypertrophic phenotype of germ cells and male to female sex reversal. This suggested that an AMH system regulates germ cell number and sex (Morinaga *et al*., 2007 PNAS).

Our analysis using the mutant indicates that an AMH system regulates germline stem cells. In the absence of the AMH signal, proliferation of a mitotically active type of germline stem cell is promoted in the gonads. The enhanced proliferation of germline stem cells, but not a direct effect of AMH impairment, causes sex reversal from male to female even if the medaka possesses a Y chromosome (Nakamura *et al*., 2010 Science, Nakamura *et al*., 2012 Development).

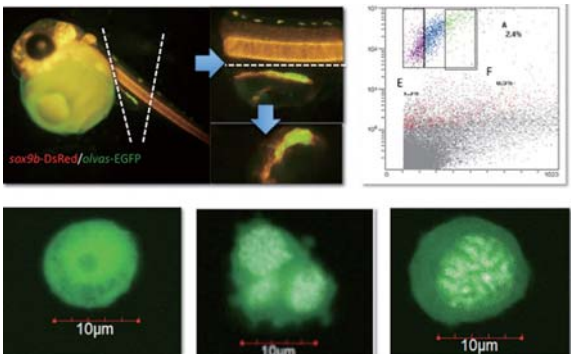


Figure 1. Isolation of different types of germ cells. Upper left; transgenic medaka with fluorescent germ cells. Upper right: Cell-sorting conditions that allow isolation of different germ cells. Bottom images (from left to right); stem-type germ cell, cystic germ cells (type II) and early diplotene germ cell.

### II. The plasticity of germ cell sex

These results raise a still unaddressed fundamental issue of reproduction, the sex of germ cells. Before critical contribution of germ cells to sex differentiation, the sex of germ cells has to be determined.

The reciprocal transplant experiments between female and male by other groups show that the germline stem cells are sexually plastic or indifferent. This implies that the sex of germ cells may be determined at an early phase of gametogenesis. Our previous study indicates that germline

stem cells commit gametogenesis through synchronous and successive division before they enter meiosis (Saito *et al*., 2007 Dev. Biol.). This division is called typeII division. It is therefore possible that sex is determined in the process between the mitotically active type of germline stem cells and cystic germ cells undergoing typeII division.

With employment of transgenic medaka that visualize germ cells (Tanaka *et al*., 2001 PNAS), we have established cell-sorting conditions to isolate stem-like germ cells, cystic germ cells and germ cells at an early diplotene stage (Figure 1). Parallel with this establishment, we prepared medaka microarray input by compiling all the public medaka databases available. These enabled us to search the transcripts that feature in each stage of germ cells.

Interestingly, the analysis reveals the presence of sexually different transcripts in stem type germ cells. The sexually dimorphic expression can be also recognized in primordial germ cells at earlier stages (Figure 2).

First we addressed if the different expression occurred as a germ-cell autonomous event or is regulated by somatic cells. For this purpose, we generated chimeric medaka with different sex of germ cells (XX somatic cells vs XY germ cells or XY somatic cells vs XX germ cells). The expression of the transcripts is consistently enriched in XY germ cells when compared with XX germ cells, and this difference did not depend on the somatic sex at all. The results clearly demonstrate that the sexually different expression is regulated in a germ cell-autonomous manner.

It is generally accepted that sex determination gene is expressed in the somatic cells surrounding germ cells at the onset of gonadal formation. Our finding, therefore, made us suspect that sex determination gene is also expressed in primordial germ cells at stages earlier than gonadal formation and might cause the sexually different expression of the transcripts. We performed in situ hybridization and, as expected, detected the expression of the sex determination gene in the primordial germ cells of males. Then, the expression of the sex determination gene was knocked down by injection of grip-RNA. Unexpectedly, however, downregulation of sex determination gene does not affect any sexually different expression of the transcripts. This demonstrates that the Y chromosome-, but not the sex determination gene, dependent mechanism is involved in the sexually different expression of primordial germ cells.

The sexually dimorphic event is also manifested in the primordial germ cell behavior. We found that isolated primordial germ cells, before onset of sex determination genes in the somatic cells, exhibit the sexually different rate of proliferation in culture. Very interestingly, the sexually different expressing gene in the primordial germ cells affect the proliferation by the overexpression and knockdown experiments.

All the results mentioned above demonstrate the sexual plasticity of germ cells and several mechanisms, other than sex determination genes, that confer sexually different characters at cellular levels: importance of a Y chromosome. Actually we found the sexually different gene is mapped near the sex determination locus on the Y chromosome and that sex-specific SNPs are present in the promoter region of the sexually dimorphic gene. These results collectively suggest that the difference of the two sex chromosomes, but not sex determination gene, can contribute to manifestation of sexually different character at the cellular levels.

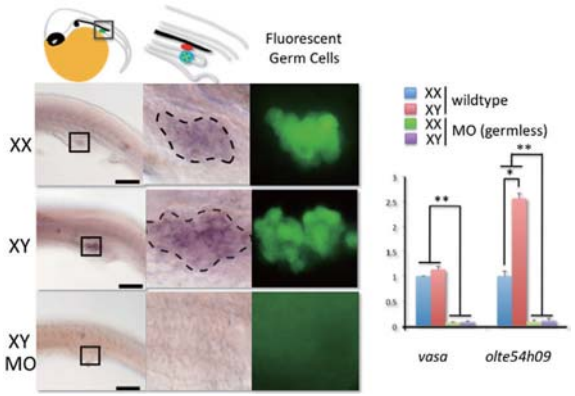


Figure 2. Presence of the transcripts that exhibit sexually different expression in the primordial germ cells.

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### 〔Original papers〕


- Herpin, A., Adolfi, M.C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., Engel, M., Tanaka, M., Guiguen, Y., and Schartl, M. (2013). Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. Mol. Biol. Evol. *30*, 2328-2346.
- Ishikawa, T., Okada, T., Ishikawa-Fujisawa, T., Todo, T., Kamei, Y., Shigenobu, S., Tanaka, M., Saito, T.L., Yoshimura, J., Morishita, S., Toyoda, A., Sakaki, Y., Taniguchi, Y., Takeda, S., and Mori, K. (2013). ATF6a/b-mediated adjustment of ER chaperone levels is essential for development of the notochord in medaka fish Mol. Biol. Cell *24*, 1387-1395.
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- Morohashi, K., Baba, T., and Tanaka, M. (2013). Steroid hormones and the development of reproductive organs. Sex. Dev. *7*, 61-79.
- Nishimura, T., and Tanaka, M. (2013). Function of germ cells in sex differentiation. In Sexual Plastility and Gametogenesis in Fishes, S. Subramanian, ed. (Nova Biomedical, New York), pp.291-304.
- Tanaka, M., and Capel, B. (2013). Forward to the special issue on sex determination. Dev. Dyn. *242*. (Editors of this Special Issue)

## LABORATORY OF PLANT ORGAN DEVELOPMENT †

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*TSUZUKI, Yumiko*
*Secretary:* *SAKAGAMI, Mari*

Plant organs, leaves, flowers, and roots show impressive, symmetrical shapes, based on an ordered arrangement of differentiated cells. The organs are formed from a group of undifferentiated cells located at the tip of the stem or the root. In the case of leaves, the process of organogenesis starts with the formation of a leaf primordium in the peripheral zone of the shoot apical meristem (SAM) at a fixed position, following an order called phyllotaxis. Cells in the primordium then proliferate and differentiate according to three spatially fixed axes: the apical-basal axis, the lateral (central-marginal) axis, and the adaxial-abaxial (foreside-backside) axis. In the course of proliferation and differentiation, plant cells are believed to exchange information with neighboring or separated cells in order to regulate organ architecture. We are trying to understand the mechanisms of information exchange between plant cells during the development of lateral organs by using genetic, and biochemical approaches.

### I. Genetic approach

Several factors regulating leaf development along the adaxia-abaxial axis are identified in *Arabidopsis thaliana* and other plants. The adaxial- and abaxial-specific tissue differentiation and the lamina expansion are determined by the precise expression of the adaxial marker genes, such as *HD-Zip III* including *PHABULOSA* (*PHB*), and the abaxial marker genes, such as *FILAMENTOUS FLOWER* (*FIL*) and *YABBY*. Using the reporter gene-system, we visualized the activity pattern of microRNA165 (miR165), which targeted the *HD-Zip III* messenger RNA, and showed that miR165 from *MIR165A* locus, which was expressed in the abaxial epidermis, act non-cell-autonomously in a present-or-absent manner on the abaxial-side cells, indicating that miR165 is likely to move toward the adaxial side. When replacing either the miRNA precursor or mature miRNA sequence of miR165 with the corresponding parts of other miRNA, it was revealed that the formation of the miR165 activity pattern depends both on the precursor backbone and the mature miRNA sequence of miR165. Thus we proposed that the rigid patterning of miR165 activity is confined by the *MIR165A* precursor sequence (Tatematsu *et al*., submitted).

Detailed analysis of temporal and spatial expression pattern of *FIL* in a developing leaf primordium using reporter gene combined with the Cre-LoxP recombinant system showed dynamic shift of the *FIL*-expression domain. This result indicates that the boundary between the expression domain of *FIL* and that of *PHB* gradually shifted from the adaxial side to the abaxial side during leaf development. We also found that several mutants defective in the adaxial-abaxial cell differentiation and the lamina expansion showed altered speed of the boundary shifting, indicating that the boundary shifting correlates with the precise leaf development. Furthermore, genetic analysis and chemical-treatment experiments revealed that the state of expression of chloroplast genome-encoding genes might be a factor determining the speed of the boundary shifting between the *PHB*- and the *FIL*-expression domains (Tameshige *et al*., 2013).

### II. Biochemical approach

We are taking a biochemical approach to isolate small peptides, which have a role in the intercellular signaling system of the SAM, from the apoplastic region of the curds of cauliflower (*Brassica oleracea* L. var. *botrytis*). We obtained a putative lipid transfer protein (LTP), which increased the number of SAMs in Arabidopsis seedlings when applied exogenously. The cauliflower *LTP* gene was highly expressed in the curd, and the expression of the Arabidopsis ortholog was observed in the L1 cell layers of the SAM. When expressed under constitutive active promoter in Arabidopsis, the transgenic plants showed increased number of SAMs near the shoot apex. Moreover, we analyzed RNAi knockdown lines of Arabidopsis *LTP*, and found that some seedlings of the knockdown lines had filamentous leaves and lost the SAM. Thus, we concluded that LTP proteins in apoplasts are responsible for SAM formation and maintenance (Yabe, Tatematsu, Tsuchida *et al*., manuscript in preparation).

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〔Original papers〕


- Ikeuchi, M., Tatematsu, K., Yamaguchi, T., Okada, K., and Tsukaya, H. (2013). Precocious progression of tissue maturation instructs basipetal initiation of leaflets in *Chelidonium majus* subsp. *asiaticum* (*Papaveraceae*). Am. J. Bot. *100*, 1116-1126.
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〔Review article〕


- Nakata, T., and Okada, K. (2013). The leaf adaxial-abaxial boundary and lamina growth. Plants *2*, 174-202.


†: This laboratory was closed on 31 March, 2013.

## DIVISION OF MOLECULAR NEUROBIOLOGY

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We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system (CNS), mainly using mice. This research covers many developmental events including the patterning of the retina, neuronal terminal differentiation, axonal navigation, branching and targeting, synapse formation, refinement and plasticity. The scope of our interests also encompasses the mechanisms for various functions of the mature brain, including body-fluid regulation, behavior control, learning, and memory.

### I. Mechanisms for neural circuit formation

Topographic maps are fundamental features of neural networks in the nervous system. We have long studied the molecular mechanisms for regional specification in the developing retina as the basis of the topographic retinotectal projection. We are now focusing our attention on the molecular mechanisms underlying axonal navigation, branching, and arborization for synapse formation, along with elimination of mistargeted axons and branches. Among the region-specific molecules in the developing retina, we have already found several molecules that induce abnormal branching or arborization when their expression was experimentally manipulated *in vivo*.

One is adenomatous polyposis coli 2 (Apc2), which is preferentially expressed in the nervous system from early developmental stages through to adulthood. The knockdown of *Apc2* in chick retinas reduced the stability of microtubules in retinal axons, and yielded abnormal behaviors of growth cones including a reduced response to ephrin-A2 and misprojection in the tectum without making clear target zones. In *Apc2*-deficient mice, robust defects in neuronal lamination were observed in the cortex, hippocampus, cerebellum, and olfactory bulb. These laminary abnormalities are a result of dysregulated neuronal migration by a cell-autonomous mechanism. Apc2 is distributed along actin fibers as well as microtubules in neurons. Our investigation suggests that Apc2 is involved in the signaling pathway from membrane receptors for extracellular guidance factors to the intracellular migration machinery. The phenotypes observed in *Apc2*-deficient mice suggest that mutations in *APC2* in humans may cause a neurodevelopmental disorder.

Another molecule is SPARC-related protein containing immunoglobulin domains 1 (SPIG1), which is a secretory protein expressed in a region-specific manner in the developing retina. The knockdown of *SPIG1* in chick retinas induces abnormal branching and arborization of retinal axons on the tectum. We are now investigating the molecular mechanism underlying the regulation of axon branching by SPIG1.

### II. Development of direction-selective retinal ganglion cell subtypes

Visual information is transmitted to the brain by roughly a dozen distinct types of retinal ganglion cells (RGCs) defined by characteristic morphology, physiology, and central projections. However, because few molecular markers corresponding to individual RGC types are available, our understanding of how these parallel pathways develop is still in its infancy.

The direction of image motion is computed in four cardinal directions and coded by direction selective (DS) ganglion cells in the retina. Particularly, the ON DS ganglion cells are critical for mediating the optokinetic reflex. We generated a knock-in mouse in which SPIG1-expressing cells are labeled with GFP. We successfully visualized both upward-motion-preferring and downward-motion-preferring ON DS ganglion cells (SPIG1$^{+}$ and SPIG1$^{-}$ ganglion cells, respectively) by a combination of genetic labeling and conventional retrograde labeling in the medial terminal nucleus.

It is not known at which circuit location along the flow of visual information the cardinal direction selectivity first appears. In collaboration with Dr. Roska's Group, we

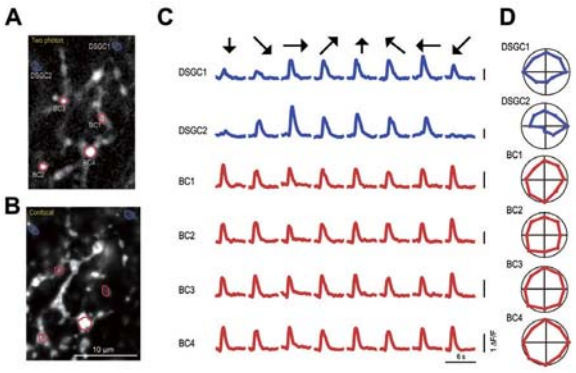



Figure 1. Concerted activity at subcellular resolution within the circuit of a single ON DS ganglion cell. **A**, Top view of two-photon image of bipolar cell axon terminal and ON DS ganglion cell dendrite labeled with GCaMP3 expressed from transsynaptic rabies virus initiated from an ON DS ganglion cell. Regions of interest are marked by colored lines. BC, bipolar cell; DSGC, ON DS ganglion cell. **B**, Confocal picture of the same region as in (A). **C**, Calcium transients in ON DS ganglion cell dendrites (blue) and bipolar cell axon terminals (red) recorded by two-photon imaging in response to stimuli moving in eight different directions. Labels to the left of traces indicate locations of the recorded compartments in (A). **D**, Polar plots of peak responses to each direction of motion.

recorded the concerted activity of the neuronal circuit elements of single ON DS ganglion cells at subcellular resolution by combining GCaMP3-functionalized transsynaptic viral tracing and two-photon imaging. While the visually evoked activity of the dendritic segments of the DS ganglion cells were direction selective, direction-selective activity was absent in the axon terminals of bipolar cells (Figure 1). Furthermore, the glutamate input to DS ganglion cells, recorded using a genetically encoded glutamate sensor, also lacked direction selectivity. Therefore, these results suggest that cardinal direction selectivity appears first at the dendrites of DS ganglion cells.

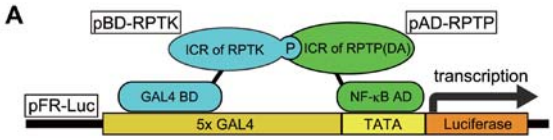
## III. Physiological roles of receptor-like protein tyrosine phosphatases

Protein tyrosine phosphorylation plays crucial roles in various biological events such as cellular proliferation, differentiation, survival, migration, and metabolism. Cellular tyrosine phosphorylation levels are governed by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The physiological functions and regulatory mechanisms of receptor-like PTPs (RPTPs) are not fully elucidated. We have been making efforts to reveal the functional roles of the R3 and R5 subfamilies of RPTPs.

### 3-1 R3 RPTP subfamily

The human genome contains 58 and 20 genes for RPTKs and RPTPs, respectively. In some studies including ours, RPTPs have been shown to be involved in the regulation of RPTKs through dephosphorylation as substrates. However, our understanding about the roles of individual RPTPs in the regulation of RPTKs is still limited.

The R3 RPTP subfamily, which is comprised of Ptprb, Ptprh, Ptprj, and Ptpro, reportedly plays pivotal roles in the development of several tissues including the vascular and nervous systems. We examined enzyme-substrate relationships between the four R3 RPTP subfamily members and 21 RPTK members selected from 14 RPTK subfamilies by using a mammalian two-hybrid system with substrate-trapping RPTP (RPTP(DA)) mutants (Figure 2A). Among the 84 RPTP-RPTK combinations conceivable, we detected 30 positive interactions: 25 of the enzyme-substrate relationships were novel (Figure 2B). We randomly chose several RPTKs assumed to be substrates for R3 RPTPs, and validated the results of this screen by *in vitro* dephosphorylation assays, and by cell-based assays involving overexpression and knock-down experiments. Because their functional relationships were verified without exception, it is probable that the RPTKs identified as potential substrates are actually physiological substrates for the R3 RPTPs. Interestingly, some RPTKs were recognized as substrates by all R3 members, but others were recognized by only one or a few members: This study is the first to demonstrate the similarities and differences in substrate-specificities among a RPTP subfamily. The enzyme-substrate relationships identified in the present study will shed light on physiological roles of the R3 RPTP subfamily.




B

RPTPs

| RPTKs | Ptpro | Ptprb | Ptprj | Ptprh |
|---|---|---|---|---|
| EphA4 | +++ | +++ | ++ | ++ |
| EphB2 | +++ | +++ | ++ | ++ |
| TYRO3 | +++ | +++ | ++ | ++ |
| RET | +++ | +++ | +++ | – |
| MET | ++ | +++ | +++ | – |
| RON | – | – | – | – |
| LTK | + | +++ | ++ | – |
| FGFR1 | + | ++ | – | – |
| ERBB2 | ++ | +++ | – | – |
| EGFR1 | – | – | – | ++ |
| ROS | – | +++ | – | – |
| PDGFRa | – | + | – | – |
| VEGFR1 | – | – | – | ++ |
| VEGFR2 | – | – | – | ++ |
| VEGFR3 | – | – | – | – |
| DDR1 | – | – | – | – |
| DDR2 | – | – | – | – |
| ROR1 | – | – | – | – |
| ROR2 | – | – | – | – |
| TIE1 | – | – | – | – |
| TRKB | – | – | – | – |

Figure 2. Identification of RPTK-RPTP interactions by a mammalian two-hybrid system using substrate-trapping mutants of RPTP. **A**, The strategy to detect RPTK-RPTP interactions. COS7 cells were transfected with a pBD-RPTK (encoding a fusion protein GAL4 BD domain and intracellular region (ICR) of RPTK) and a pAD-RPTP (encoding a fusion protein NFkB AD and ICR of RPTP(DA)) expression plasmid, together with the reporter plasmid pFR-Luc. Thus, the protein interactions can be assessed with a standard luciferase assay. Luciferase activities induced are expected to correlate with the strength of interaction. **B**, Summary of specific interactions between DA mutants of RPTP and RPTKs. The experiments were carried out in triplicate. Signal intensities were classified into four groups: -, none; +, weak; ++, medium; +++, strong.

### 3-2 R5 RPTP subfamily

Protein-tyrosine phosphatase receptor type Z (Ptprz) is predominantly expressed in glial and neuronal cells in the central nervous system (CNS). We are now focusing our efforts on determining the roles of Ptprz signaling in the regulation of hippocampal synaptic plasticity, dopamine transporter internalization, oligodendrocyte differentiation, etc.

## IV. Brain systems for body-fluid homeostasis

Sodium (Na) is a major electrolyte of extracellular fluids and the main determinant of osmolality. Na homeostasis is essential to life and $Na^+$ concentrations in plasma and cerebrospinal fluid (CSF) are continuously monitored to maintain a physiological level of $Na^+$ in body fluids. We have previously shown that $Na_x$, which structurally resembles voltage-gated sodium channels ($Na_v1.1$–$1.9$), is a concentration-sensitive Na channel.

In the brain, $Na_x$ channels are preferentially expressed in astrocytes and ependymal cells in the sensory circumventricular organs, such as the subfornical organ (SFO) and organum vasculosum of the lamina terminalis

(OVLT), where $Na_x$-positive glial cells are involved in sensing an increase in $[Na^+]$ in body fluids. *$Na_x$*-deficient mice do not stop ingesting salt even when dehydrated, while wild-type mice avoid salt. This behavioral defect of *$Na_x$*-deficient mice is recovered by a site-directed transfer of the *$Na_x$* gene with an adenoviral vector into the SFO. $Na_x$ thus functions as the brain's $Na^+$-level sensor for the homeostatic control of $[Na^+]$ in body fluids.

In our previous *in vitro* studies, $Na_x$ showed a threshold value of ~150 mM for $[Na^+]_o$. However, $[Na^+]$ in serum and CSF is strictly controlled at 135–145 mM in mammals including humans, suggesting that the brain sensor(s) can detect an increase in $[Na^+]$ in this range to strictly maintain the physiological level. We therefore presumed that the threshold value of $Na_x$ for $[Na^+]_o$ must be modulated *in vivo* by some mechanism.

Recently, we found that endothelin-3 (ET-3) shifts the $[Na^+]_o$ dependency of $Na_x$ activation to the lower-concentration side in a dose-dependent manner (Figure 3A and B). This shift enables $Na_x$ to gate even when $[Na^+]_o$ is in the physiological range. $ET_BR$ signaling promoted activation of $Na_x$ through protein kinase C (PKC) pathways leading to extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Importantly, expression of ET-3 in the SFO was time-dependently enhanced during dehydration (Figure 3C). Moreover, BQ788, a specific blocker of $ET_BR$ attenuated the salt-aversive behavior in wild-type mice induced by dehydration (Figure 3D). These results strongly indicate that regulated expression of ET-3 in the SFO is involved in the control of salt-intake behavior through improvement of the $[Na^+]_o$ sensitivity of $Na_x$ *in vivo*.

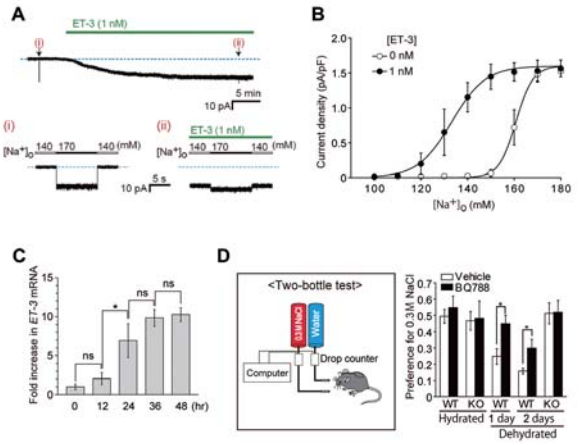



Figure 3. ET-3 expression in the subfornical organ enhances the sensitivity of $Na_x$ to suppress salt intake. **A**, A representative whole-cell current response of a SFO cell obtained from WT mice on application of hypertonic $Na^+$ during ET-3 application. The transient application of hypertonic $Na^+$ (170 mM) was performed at the time points indicated by arrows, 5 min before (i) and 30 min after (ii) the start of the ET-3 application (1 nM). The dotted lines represent the basal level under the 140 mM $[Na^+]_o$ condition without ET-3. The currents induced by an increase in $[Na^+]_o$ (+30 mM) at the time points (i) and (ii) are magnified below. **B**, Relationships between the current density and $[Na^+]_o$ in the presence or absence of 1 nM ET-3. The current amplitudes from the basal level were measured and plotted. For comparisons, the current changes due to the shift of the $E_{Na}$ were compensated. Data are mean ± SEM (n = 6 for each point). The $C_{1/2}$ values were determined after curves fitting (solid lines). **C**, Changes in the signal intensity of the *in situ* hybridization for ET-3 mRNA in the SFO during dehydration. Signal intensities are shown as relative values against that at 0 hr. *$p < 0.05$, two-tailed Student's t test; ns, not significant; data are mean ± SEM (n = 8 for each). **D**, Preference ratios for 0.3 M NaCl in the two-bottle test during 12 hr. Wild-type mice (WT) and *$Na_x$*-deficient mice (KO) received an i.c.v. infusion of BQ788 or vehicle were compared. The preference ratio was defined as the ratio of the volume of 0.3 M saline ingested to total fluid intake. *$p < 0.05$, two-tailed Student's t test; data are mean ± SEM (n = 10 for each).

Very recently, we demonstrated that $Na_x$ was involved in the regeneration process of injured peripheral nerves by enhancing lactate release from non-myelinating Schwann cells, where $Na_x$ was activated by ET-1 through $ET_BR$ signaling. This finding may bring new strategies to promote peripheral nerve regeneration.

## Publication List

### 〔Original papers〕


- Ayoub, E., Hall, A., Scott, A.M., Chagnon, M.J., Miquel, G., Hallé, M., Noda, M., Bikfalvi, A., and Tremblay, M.L. (2013). Regulation of the Src kinase-associated phosphoprotein 55 homologue by the protein tyrosine phosphatase PTP-PEST in the control of cell motility. J. Biol. Chem. *288*, 25739-25748.
- Hiyama, T.Y., Yoshida, M., Matsumoto, M., Suzuki, R., Matsuda, T., Watanabe, E., and Noda, M. (2013). Endothelin-3 expression in the subfornical organ enhances the sensitivity of $Na_x$, the brain sodium-level sensor, to suppress salt intake. Cell Metabolism *17*, 507-519.
- Sakuraba, J., Shintani, T., Tani, S., and Noda, M. (2013). Substrate specificity of R3 receptor-like protein-tyrosine phosphatase subfamily towards receptor protein-tyrosine kinases. J. Biol. Chem. *288*, 23421-23431.
- Yonehara, K., Farrow, K., Ghanem, A., Hillier, D., Balint, K., Teixeira, M., Jüttner, J., Noda, M., Neve, R.L., Conzelmann, K.-K., and Roska, B. (2013). The first stage of cardinal direction selectivity is localized to the dendrites of retinal ganglion cells. Neuron *79*, 1078-1085.


### 〔Original paper (E-publication ahead of print)〕


- Unezaki, S., Katano, T., Hiyama, T.Y., Tu, N.H., Yoshii, S., Noda, M., and Ito, S. Involvement of $Na_x$ sodium channel in peripheral nerve regeneration via lactate signaling. Eur. J. Neurosci. 2013 Nov. 29.


### 〔Review article〕


- Noda, M., and Sakuta, H. (2013). Central regulation of body-fluid homeostasis. Trends Neurosci. *36*, 661-673.

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*#: SRPBS (Strategic Research Program for Brain Sciences), NIPS*

This year we have reported three findings. One is methylation of genes and their controls selectively expressed in the association area by methyl-binding proteins in macaque monkeys. The other is that we confirmed the existence of ocular dominance columns in the new world monkey marmosets. The third is the primate visual area selective gene expression, which pattern was different from that previously reported.

## I. DNA methylation and methyl-binding proteins control differential gene expression in distinct cortical areas of macaque monkey

The neocortex, which is present only in mammals and is enlarged in primates, consists of anatomically and functionally distinct areas that form different sensory modalities and functions. Studies have been done to address the underlying mechanisms that control the formation of the cortical layers and primary sensory areas. However, the molecular mechanisms that form and maintain these areas remain to be elucidated. The different regional specializations relate to the different gene expression profiles and resultant distinctive histochemical phenotypes seen in different cortical regions. Recent analyses of gene expression patterns in rodents reveal four patterning centers that control graded transcription in the neocortex. It can be expected that primates, with their much greater arealization, will have other molecular specifications besides that found in rodents.

We have isolated genes that are specifically expressed in the neocortical areas in primates, and reported two groups of genes that are differentially expressed either in the macaque primary visual cortex or in the occipital lobe (Tochitani *et al*., 2001; Takahata *et al*., 2009; Watakabe *et al*., 2009), or in the association areas of cortex including the frontal cortex (Komatsu *et al*., 2005; Takaji *et al*., 2009; Sasaki *et al*., 2010). Pronounced area-selectivity and activity-dependency of these genes occur in primates, but not in the rodents, lagomorphs or carnivores we examined. We thus suggest that the mechanisms underlying this gene expression may be an important clue to the evolution of the primate cerebral cortex (Takahata *et al*., 2011; Yamamori, 2011). We previously clarified the functions of two of the V1-selective genes, HTR1B and HTR2A, in the macaque primary visual cortex (Watakabe *et al*., 2009), and functional studies are currently underway for other molecules. Identifying mechanisms underlying these unique area-selective genes is important for understanding the development, evolution and function of the primate neocortex.

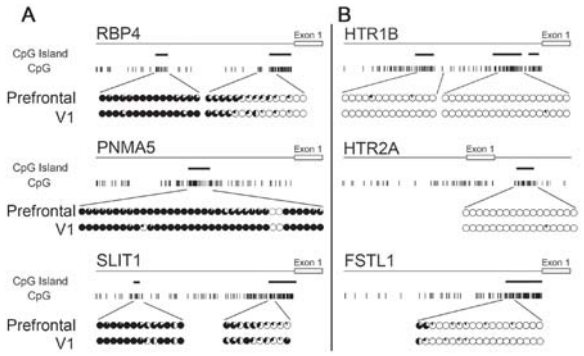


Figure 1. Methylation of association area- and V1-selective gene promoters.
Map of CpG base motifs in each AA- (A) and V1-selective gene (B) and their methylation status in the vicinity of the promoter region for tissues from the prefrontal cortex of area 46 (upper rows) and V1 (lower rows). The sites of CpG islands (CpGI) are represented by thick bars. The fraction of methylated DNA at each CpG site is shown by the proportion of the black area in each circle, with a full black circle indicating complete methylation. The left and right panels show the AA- and V1-selective gene promoters, respectively. The CGI in the promoter region of the AA-selective genes was hypermethylated, whereas that of the V1-selective genes was almost completely unmethylated. The same pattern was observed for genomic DNA obtained from both adult macaque PFC and V1 tissues (Cited from Hata *et al*., J. Neurosci. 2013 33(50):19704-19714).

This year, we demonstrated striking differences of DNA methylation between the promoter regions of the genes selectively expressed in the association areas (AAs) and the primary visual cortex (V1) in macaque monkeys (figure 1). Although the methylation levels of promoters of each area-selective gene showed no regional difference, MBD4, among five known methyl-binding proteins, was enriched in the AAs (Figure 2). MBD4 was mainly observed in NeuN-positive cells, specifically bound to and activating the AA-selective genes both in culture and *in vivo* in the macaque monkey neocortex (Figure 3). Thus, our results provide evidence for the critical role of DNA methylation and methyl-binding proteins in the differential gene expression profiles in the primate neocortex (Published in Hata *et al*., J. Neurosci. 33(50):19704-19714, 2013)

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

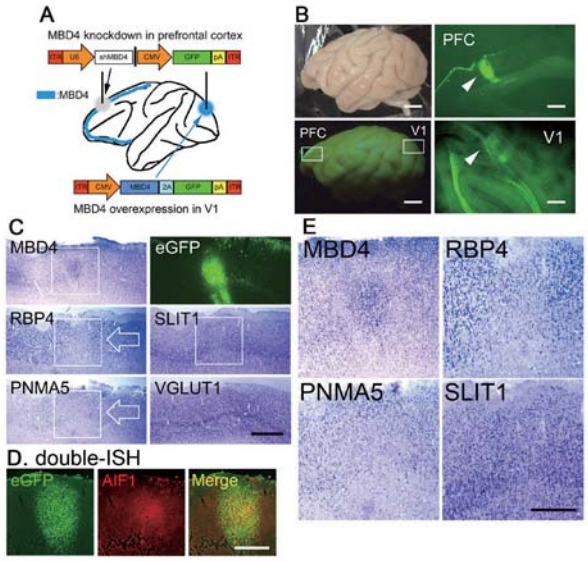


Figure 2. Expressions of the AA-selective RBP4, PNMA5 and SLIT1 in sections including PFC in AAV-shMBD4-injected monkey
A. An illustration of the AAV1-mediated gene transfer procedures in the macaque brain. AAV1-U6-shMBD4 was injected into PFC to examine the effects of loss of function of MBD4, whereas AAV1-CMV-MBD4 was injected into V1 to examine the gain of function of MBD4. B. Four weeks later, eGFP signals were observed in the injected sites in PFC and V1. Scale bar = 10 mm. C. The ISH signals of MBD4, RBP4, PNMA5, SLIT1 and VGLUT1 (an excitatory neuron specific marker gene) are shown. The white boxes of MBD4, RBP4 and PNMA5 indicate areas around the injection site, demonstrating decreased expression, but note that MBD4 ISH signals were enhanced at the injected site, possibly due to the damage-induced proliferation of glial cells or the migration of glial cells from uninjected regions. RBP4 and PNMA5 expression were restricted to neurons, and these signals were decreased around the injected site (white arrows). The MBD4 signals were only enhanced in the center region, with decrease observed in the signals just outside this focus, forming a donut-like appearance of MBD4 expression. Scale bar = 2 mm. D. Double-ISH of eGFP (green, left) and AIF1/Iba1 (red, middle), an activated microglial marker. A merged image (right) is also shown. Scale bar = 2 mm. E. Higher magnification photomicrographs of the white boxes shown for MBD4, RBP4, PNMA5 and SLIT1 in the upper panels, delineating clear boundaries of the decreased areas of expressions (except for SLIT1). Scale bar = 1 mm. Cited from Hata *et al*., J. Neurosci. 2013 33(50):19704-19714).

## II. Dynamic changes of gene expression in the primary visual cortex revealed by monocular activation in adult marmosets

The primary visual cortex (V1) of primates is estimated to occupy more than 30% of the cerebral cortex. It has many characteristic features that enable highly complex information processing, e.g., formation of distinct functional columnar structures (ocular dominance and orientation columns, or color domains etc.), and parallel processing. We previously showed that there are a group of genes that are conserved in several species of primate but not in ferrets or mice (Takahata *et al*., 2008, 2012), suggesting that there are primate-specific mechanisms for expression of these genes. An important common feature of these genes was the activity-dependent expression in V1, which we showed by monocular inactivation of retinal activity using tetrodotoxin (TTX) (Tochitani *et al*., 2001; Takahata *et al*., 2009; Watakabe *et al*., 2009; Yamamori, 2011). Whereas this experiment revealed the requirement for retinal activity in gene expression in V1, it has not been clear how the incoming visual inputs induce the expression of these genes.

Synaptic transmission triggers the expression of a group of genes, which play roles in neural plasticity, differentiation, proliferation etc. Immediate early genes (IEGs) are classic activity-dependent genes, which are expressed within minutes of stimulation without the requirement for de novo protein synthesis. IEGs, such as c-Fos and Zif268 (also known as early growth response 1, Egr-1), are often used as the marker of neural activities. Previous studies in rodents demonstrated that visual stimulation induces the expression of Zif268 and c-Fos proteins at the peak level within one hour from stimulus onset, suggesting that input-driven gene activation in V1 reaches the maximum level within a short period of time. Compared with rodents, primates have far more developed visual systems. To our knowledge, however, there has been no information about visually evoked transcription in primate V1 within one hour. We designed a series of monocular visual stimulation experiments using adult marmosets, in order to understand the transcriptional regulation of the activity-dependent genes in primates.

We selected common marmosets (*Callithrix jacchus*), a New World monkey, because of its size, ease of handling, and transgenic (Sasaki *et al*., 2009) and gene manipulation potentials (e.g., Watakabe *et al*., 2012). One debated issue in marmoset vision research is whether ocular dominance columns (ODCs) exist or not. With particular relevance to our study, Markstahler *et al*. (1998) reported columnar ZIF268 immunostaining in layer IVCβ two hours after

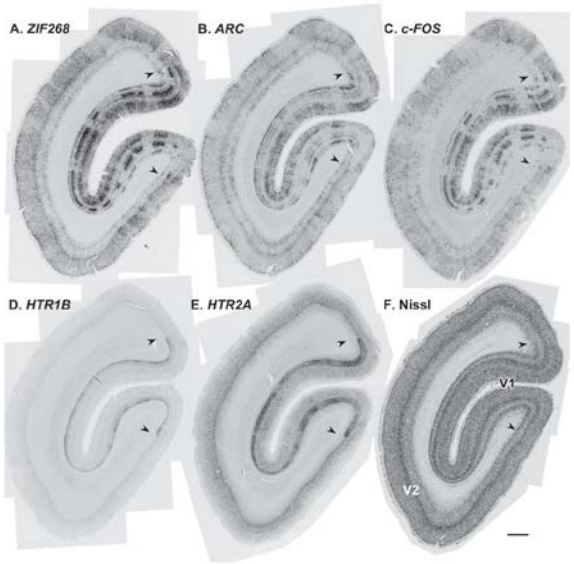


Figure 3. mRNA expression patterns of five stimulation events in visual cortices of adult marmosets. (A-E) mRNA expression patterns for activity-dependent genes we examined in the visual cortex 24 min after light stimulation: (A) ZIF268, (B) ARC, (C) c-FOS, (D) HTR1B, (E) HTR2A. (F) Cortical section stained for Nissl substance. Adjacent sections were analyzed. Arrowheads indicate V1/V2 boundaries. The columnar signal patterns within V1 indicate ocular dominance columns. Scale bar: 1 mm. visual cortex (Cited from Nakagami *et al*., Front. Neural Circuits. 7:43, 2013)

monocular visual stimulation following transient (24 h) monocular TTX injection, which they called "physiological ODCs". To investigate the visually evoked gene expression in primates, marmoset V1 is potentially a very good model.

We modified Markstahler's method to examine the mRNA expression of a set of activity-dependent genes in adult marmoset V1. We showed that these genes were expressed in a columnar fashion in V1 of all the monocularly visual stimulated marmosets, which shows strong evidence for the segregation of right and left eye inputs in the marmoset activity-dependent genes induced by monocular light.

Using this experimental system, we investigated the detailed time course of expression for (1) HTR1B and HTR2A mRNAs that represent the primate-specific V1-enriched and activity-dependent genes that we have previously reported (Watakabe *et al*., 2009; Takahata *et al*., 2012), and (2) IEGs of c-FOS, ARC (also known as Arg3.1), and ZIF268. Each of these activity-dependent genes revealed complex and characteristic time courses upon visual stimulation, demonstrating the inputs-evoked dynamic regulation of gene expression profile in marmoset V1 (Published in Nakagami *et al*., Front. Neural Circuits. 7:43, 2013).

## III. Genes selectively expressed in the visual cortex of the OLD World monkey

Among the genes selectively expressed in the primary visual cortex, this year we have reported a new class of gene, SEMA 7A, whose expression is different from previously reported V1-selective gene expressions such as those of OCC1/FSTL1, HTR1B and HTR2A in that it is already expressed in the mid-embryonic stage (embryonic day 83 in macaque monkey) at the time when thalamocortical projections start. In addition, in contrast to the V1 selective gene we previously reported, SEMA 7A shows only week activity-dependent gene expression when examined by monocular inhibition by TTX injection into one eye (Komatsu *et al*., Published as a book Chapter, *In* Cortical Development, Eds. Kageyama R and Yamamori T Springer, Tokyo, 263-276). These features of SEMA 7A suggest a different role in primate cortical development from those suggested in mice, such as roles in axon branching and/or presynaptic punctate formation in the thalamocortical projections. We therefore are currently working to explore possible functions that explain the result in monkeys.

### Publication List

〔Original papers〕


- Hata, K., Mizukami, H., Sadakane, O., Watakabe, A., Ohtsuka, M., Takaji, M., Kinoshita, M., Isa, T., Ozawa. K., and Yamamori, T. (2013). DNA methylation and methyl-binding proteins control differential gene expression in distinct cortical areas of macaque monkey. J. Neurosci. *33*, 19704-19714.
- Moritoh, S., Komatsu, Y., Yamamori, T., and Koizumi, A. (2013). Diversity of retinal ganglion cells identified by transient GFP transfection in organotypic tissue culture of adult marmoset monkey retina. PLoS ONE *8*, e54667.
- Nakagami, Y., Watakabe, A., and Yamamori, T. (2013). Monocular inhibition reveals temporal and spatial changes in gene expression in the primary visual cortex of marmoset. Front. Neural Circuits. *7*, 43.


〔Review article〕


- Komatsu, K., Toita, S., Ohtsuka, M., Takahata, T., Tochitani, S., and Yamamori, T. (2013). Genes selectively expressed in the visual cortex of the Old World monkey. *In* Cortical Development (Eds. Kageyama, R., and Yamamori, T.) Springer, Tokyo, 263-276.

## DIVISION OF BRAIN CIRCUITS

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MATSUZAKI, Masanori

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| *Postdoctoral Fellow:* | *MASAMIZU, Yoshito* |
| | *TANAKA, Yasuyo* |
| *SOKENDAI Graduate Student:* | *OHKUBO, Fuki* |
| | *HASEGAWA, Ryota* |
| *Visiting Scientist:* | *KATO, Daisuke* |
| *Visiting Graduate Student:* | *TERADA, Shinichiro* |
| | *HIRA, Riichiro** |
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| | *SAITO, Junko* |
| | *KOBAYASHI, Tomoko* |
| *Secretary:* | *SUGIYAMA, Tomomi* |

Various firing patterns of many cortical neurons represent information processing in the brain. The microarchitecture of synaptic connections control information processing in cortical circuits. The structure and location of synapses determine and modify the strength of this information processing. The aim of our laboratory is to reveal how information is formed, maintained, selected, and decoded in the brain at the levels of single cells and of single synapses. To do so, we mainly use two-photon microscopy that allows us to see fluorescence signals from deep within living tissue, developing novel photostimulation methods and animal behavioral tasks. The goals of our recent studies are to reveal how voluntary movement is represented in cortical circuits. One of the most important problems in neuroscience is how a variety of spatio-temporally heterogeneous neural activity in the cortex emerges moment-by-moment at multiple stages of a movement.

### I. Fine-scale spatio-temporal dynamics of the neuronal activity in mouse motor areas during a voluntary movement

Functional clustering of neurons is frequently observed in the motor cortex. However, it is unknown if, when, and how fine-scale (<100 μm) functional clusters form relative to voluntary forelimb movements. In addition, the implications of clustering remain unclear. To address these issues, we conducted two-photon calcium imaging of mouse layer 2/3 motor cortex, the rostral forelimb area (RFA) and the caudal forelimb area (CFA), during a self-initiated lever-pull task. In the imaging session, after 8-9 days of training, head-restrained mice had to pull a lever for ~600ms to receive a water drop, and then had to wait for > 3s to pull it again. We found two types of task-related cells in the mice: cells whose peak activities occurred during lever pulls (pull cells) and cells whose peak activities occurred after the end of lever pulls (post-pull cells). The activity of pull cells was strongly associated with lever-pull duration. In approximately 40% of imaged fields, functional clusterings were temporally detected during the lever pulls. Spatially, there were ~70 μm-scale clusters that consisted of more than four pull cells in approximately 50% of the fields (primary cluster in Figure 1). Ensemble and individual activities of pull cells within the cluster more accurately predicted lever movement trajectories than activities of pull cells outside the cluster. This was likely because clustered pull cells were more often active in the individual trials than pull cells outside the cluster. This higher fidelity of activity was related to higher trial-to-trial correlations of activities of pairs within the cluster (Figure 2). We propose that strong recurrent network clusters may represent the execution of voluntary movements.

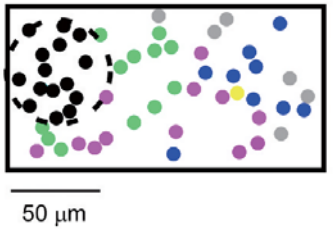


Figure 1. An example of imaged fields in the mouse motor areas. Black, green, blue, magenta, yellow, and grey circles indicate primary-clustered pull cells, other clustered pull cells, non-clustered pull cells, post-pull cells, other task-related cells, and non-task-related cells, respectively. Dotted circles surround the primary clusters.

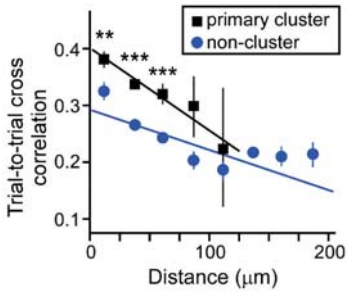


Figure 2. Trial-to-trial cross correlations relative to cellular distances for 1075 pairs of primary-clustered cells (black squares) and 1680 pairs of non-clustered cells (blue circles) across 30 fields that included the primary cluster and ≥3 non-clustered cells (16 mice). Regression lines are shown in the same colors as the cluster types. ** $p < 0.001$ and *** $p < 0.0001$.

### II. *In vivo* optogenetic tracing of functional corticocortical connections between motor forelimb areas

Interactions between distinct motor cortical areas are essential for coordinated motor behaviors. In rodents, the motor cortical forelimb areas are divided into at least two distinct areas, the RFA and the CFA. The RFA is thought to be an equivalent to the premotor cortex in primates, whereas the CFA is believed to be an equivalent to the primary motor cortex. Although reciprocal connections between the RFA and the CFA have been anatomically identified in rats, it is unknown whether there are functional connections between these areas that can induce postsynaptic spikes. We used an *in vivo* Channelrhodopsin-2 (ChR2) photostimulation method to trace the functional connections between the mouse RFA and CFA. This was done in either ChR2 transgenic mice, where the L5b neurons express ChR2-EYFP, or in mice where both the upper layers and L5b were transfected with an adeno-associated virus that encoded ChR2-EYFP. Simultaneous electrical recordings were utilized to detect spiking activities induced by synaptic inputs originating from

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

photostimulated areas (Figure 3). This method demonstrated that neurons in the upper layers, but not L5b, of the CFA induce strong postsynaptic responses in L5 of the RFA, and that RFA neurons in L5b induce strong postsynaptic responses in L5 of the CFA. The onset latency of electrical responses evoked in remote areas upon photostimulation of the other areas was approximately 10 ms, which is consistent with the synaptic connectivity between these areas.

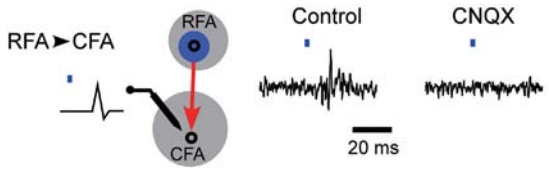


Figure 3. Left, a schematic of the experimental arrangement for photostimulation (blue) of the RFA (gray) with simultaneous electrical recording in the CFA. RFA photostimulation evoked postsynaptic firing activities in the CFA via the synaptic projection (red). Right, representative data showing evoked activities in the CFA when the RFA was photostimulated. Control conditions and application of CNQX to the CFA are shown.

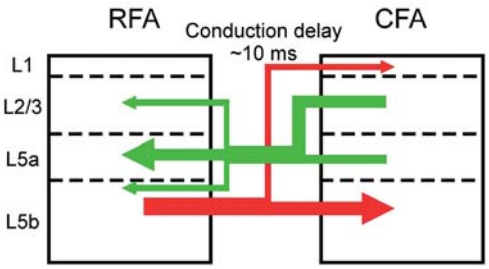


Figure 4. Asymmetric synaptic connectivity between the RFA and the CFA.

We also determined the anatomical connections between the RFA and the CFA. The anatomical results supported the idea that the RFA receives strong functional projections from layer 2/3 and/or layer 5a, but not from layer 5b, of the CFA. Further, the CFA receives strong projections from layer 5b neurons of the RFA. Our results suggest that neuronal activities in the RFA and the CFA during movements are formed through asymmetric reciprocal connections (Figure 4).

## III. *In vivo* photoinhibition mapping method using halorhodopsin

We are developing *in vivo* photoinhibition methods as well as the *in vivo* photostimulation method using ChR2. We examined whether the Thy1-eNpHR2.0 (enhanced halorhodopsin) transgenic mice are suitable for *in vivo* photoinhibition. For this purpose, we illuminated the motor cortex of these mice with green light (559 nm wavelength) while measuring neuronal activity and forelimb movement. Spontaneous multiunit activity (MUA) was recorded from a single electrode that was inserted into the motor cortex. Illumination near the tip of the electrode clearly inhibited spontaneous MUA (Figure 5), with a rapid recovery of activity afterwards. In contrast, illumination 1 mm away from the recording site had no effect on MUA. To examine the spatial range of photoinhibition mediated by eNpHR2.0 *in vivo*, we measured upward local field potentials (LFPs) that presumably reflect chloride ion influx into eNpHR 2.0-expressing neurons (Figure 5). Illumination near the tip of the recording electrode (location 1) induced large LFPs, while illumination at more distant sites produced smaller LFPs (locations 2 and 3). By scanning the position of the light spot, while measuring LFPs, we could make a two-dimensional map of the spatial range of photoinhibition (Figure 5). The width of the area exhibiting light-induced LFPs was 0.65 mm in the experiment shown in Figure 5.

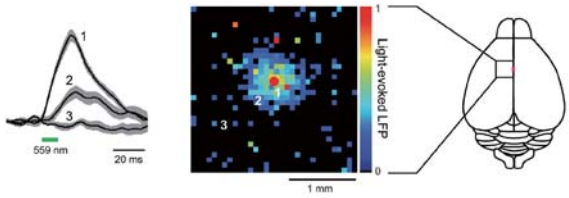


Figure 5. Left, LFPs caused by activation of eNpHR2.0, recorded at the location indicated by the red circle in the middle, in response to light spots positioned at the numbered locations in the middle. Middle, map of amplitudes of LFPs evoked when eNpHR2.0 was activated. Each of the 32 x 32 pixels in the map was illuminated (559 nm) and the amplitude of the LFP evoked at each pixel was encoded into the pseudocolor scale shown at right. Right, schematic dorsal view of the cortical surface; boxed region is the photostimulation mapping area and the magenta square denotes the bregma.

We also examined *in vivo* photoinhibition of limb movements induced by intracortical microstimulation in Thy1-eNpHR2.0 mice. Stimulation of the right motor forelimb area in the motor cortex produced movements of the left forelimb. Whole-field illumination of the right cortical surface with orange light (594 nm) clearly inhibited left forelimb movement and movements were restored rapidly once the light was turned off. These results show that cortical activity and limb movement can be photoinhibited *in vivo* using the Thy1-eNpHR2.0 mouse, indicating that this mouse is an excellent tool for disruption of neural circuit activity *in vivo*.

## Publication List

〔Original papers〕


- Asrican, B., Augustine, G.J., Berglund, K., Chen, S., Chow, N., Deisseroth, K., Feng, G., Gloss, B., Hira, R., Hoffmann, C., Kasai, H., Katarya, M., Kim, J., Kudolo, J., Lee, L., Lo, S., Mancuso, J., Matsuzaki, M., Nakajima, R., Qui, L., Tan, G., Tang, Y., Ting, J.T., Tsuda, S., Wen, L., Zhang, X., and Zhao, S. (2013). Next-generation transgenic mice for optogenetic analysis of neural circuits. Front. Neural Circuits *7*, 160.
- Hayama, T., Noguchi, J., Watanabe, S., Takahashi, N., Hayashi-Takagi, A., Ellis-Davies, G.C.R., Matsuzaki, M., and Kasai, H. (2013). GABA promotes the competitive selection of dendritic spines by controlling local $Ca^{2+}$ signaling. Nature Neurosci. *16*, 1409-1416.
- Hira, R., Ohkubo, F., Ozawa, K., Isomura, Y., Kitamura, K., Kano, M., Kasai, H., and Matsuzaki, M. (2013). Spatiotemporal dynamics of functional clusters of neurons in the mouse motor cortex during a voluntary movement. J. Neurosci. *33*, 1377-1390.
- Hira, R., Ohkubo, F., Tanaka, Y.R., Masamizu, Y., Augustine, G.J., Kasai, H., and Matsuzaki, M. (2013). In vivo optogenetic tracing of functional corticocortical connections between motor forelimb areas. Front. Neural Circuits *7*, 55.

## LABORATORY OF NEUROPHYSIOLOGY

*Associate Professor*
WATANABE, Eiji

*NIBB Research Fellow:* *NAKAYASU, Tomohiro*
*Visiting Scientist:* *AONO, Sachiko*

In order to interact successfully with the environment, animals must deduce their surroundings based on sensory information. The visual system plays a particularly critical role in such interactions with the environment.

"Why can we see?" This question is fundamental for a thorough understanding of vision-dependent animals, including human beings. In order to better understand the visual system of animals, we are researching animal behaviors through psychophysical and computational methods.

### I. Psychophysical study of Medaka fish

One of our major subjects is the psychophysical and computational study of medaka (*Oryzias latipes*). We have made progress in studies of the prey-predator interaction using medaka and zooplankton. Visual motion cues are one of the most important factors for eliciting animal behavior, including predator-prey interactions in aquatic environments. To understand the elements of motion that cause such selective predation behavior, we used a virtual plankton system where the predation behavior in response to computer-generated prey was analyzed. First, we performed motion analysis of zooplankton (*Daphnia magna*) to extract mathematical functions for biologically relevant motions of prey. Next, virtual prey models were programmed on a computer and presented to medaka, which served as predatory fish. Medaka exhibited predation behavior against several characteristic virtual plankton movements, particularly against a swimming pattern that could be characterized as pink noise motion. Analyzing prey-predator interactions via pink noise motion will be an interesting research field in the future (Matsunaga & Watanabe, 2012).

This year, we have made progress in studies of the schooling behaviors of medaka (Figure 1). Many fish species are known to live in groups. Visual cues have been shown to play a crucial role in the formation of shoals (a shoal is defined as social group of fish). Using biological motion stimuli, depicting a moving creature by means of just a few isolated points, we examined for the first time whether physical motion information is involved in the induction of shoaling behavior. To generate biological motion stimuli, medaka were videotaped and then six points were placed along the body trunk using computer software. We found that the presentation of biological motion could prominently induce shoaling behavior. We have shown what aspects of motion (such as movement speed and temporal order) are critical in the induction of shoaling behavior (Nakayasu & Watanabe, 2013). Motion or behavioral characteristics can be valuable in recognizing animal species, sex, and group members. Studies using biological motion stimuli will enhance our understanding of how non-human animals extract and process the information which is vital for their survival.

Figure 1. Shoal of Medaka fish (*Oryzias latipes*).

### II. Psychophysical study of Human vision

Another of our major subjects is the psychophysical and theoretical studies of the visual illusions of human beings (*Homo sapiens*). One recent focus of this debate is the flash-lag effect, in which a moving object is perceived to lead a flashed object when both objects are aligned in actual physical space. This effect has been utilized for understanding human motion perception. We developed a simple conceptual model explaining the flash-lag effect (Delta model, Watanabe *et al*., 2010). This year, we have made progress in studies of novel visual illusion, shelf-shadow illusion (Figure 2).

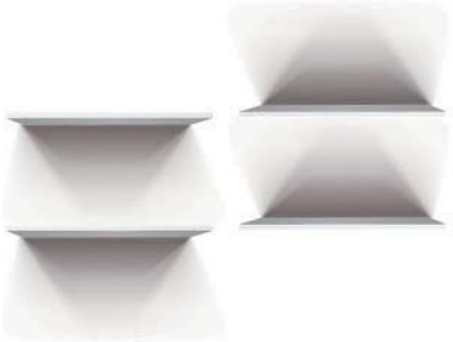

Figure 2. Shelf-Shadow Illusion. Upward shadows look darker than downward shadows. Third award of The 5th Illusion Contest in Japan.

### Publication List

〔Original paper〕

● Hiyama, T.Y., Yoshida, M., Matsumoto, M., Suzuki, R., Matsuda, T., Watanabe, E., and Noda, M. (2013). Endothelin-3 expression in the subfornical organ enhances the sensitivity of $Na_x$, the brain sodium-level sensor, to suppress salt intake. Cell Metabolism *17*, 507-519.

〔Original paper (E-publication ahead of print)〕

● Nakayasu, T., and Watanabe, E. Biological motion stimuli are attractive to medaka fish. Animal Cognition 2013 Oct. 20.

## DIVISION OF EVOLUTIONARY BIOLOGY

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| | *KAJIKAWA, Ikumi* |
| | *MASUOKA, Tomoko* |
| | *MATSUZAKI, Yoko* |
| | *NISHI, Tayo* |
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## I. Evolution of Complex Adaptive Characters

The theory of natural selection and the neutral theory of molecular evolution are powerful concepts in evolutionary biology. However, even with such theories, there still remain unexplained phenomena, one of which is the evolution of complexity. It is difficult to explain the mechanisms needed to evolve complex adaptive traits at cellular and organismal levels, such as cell division machinery, regeneration, novel organ development, host race change, and mimicry. Such traits comprise many components and become adaptive only when all components are gathered together. However, based on evolutionary theory, each component should evolve one by one according to the accumulation of mutations. We aim to reveal the genetic networks regulating the complex traits and to infer the mechanisms needed to evolve complex characters.

## II. Evolution of Regeneration: Reprogramming of Differentiated Cells to Pluripotent Stem Cells

Different species have different morphology and also cellular characters vary between species. Stem cells self-renew and repeatedly produce differentiated cells during development. Conversely, differentiated cells can be converted into stem cells in some organisms. In plants, regeneration of a stem cell leads to a generation of a new individual, which is an effective strategy for propagation. The ability of reprogramming is different from species to species but the reason is unknown. The moss *Physcomitrella patens* has a rapid reprogramming ability (see http://www.nibb.ac.jp/evodevo/ERATO/movie/MacMovie.mp4) and is feasible for use in experiments. Cells in a dissected leaf are reprogrammed to become chloronema apical stem cells within 24 hours. However, current systems for controlled transgene expression remain limited and we developed an estrogen receptor mediated inducible gene expression system, based on the system used in flowering plants (Kubo *et al*. 2013). After identifying the appropriate promoters to drive the chimeric transducer, we succeeded in inducing transcription over 1,000-fold after 24 h incubation with β-estradiol. The *P. patens* system was also effective for high-level long-term induction of gene expression; transcript levels of the activated gene were maintained for at least seven days on medium containing β-estradiol. We also established two potentially neutral targeting sites and a set of vectors for reproducible expression of two transgenes. This β-estradiol-dependent system will be useful to test genes individually or in combination, allowing stable, inducible transgenic expression in *P. patens*.

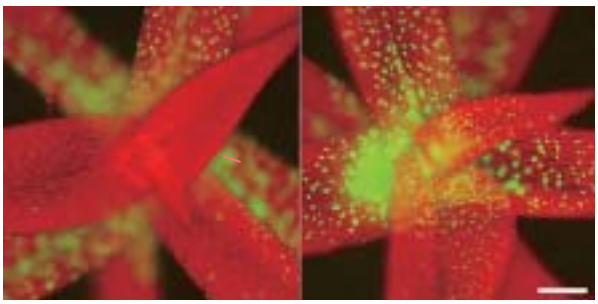

Figure 1. Spatial expression patterns of GFP induced by two different β-estradiol-dependent systems.

Genome-wide changes of chromatin modifications are required for the cell-fate transition including reprogramming to ensure a transcription profile that fits the new cell fate. However, the mechanisms and the timing of the changes during the cell-fate transition are still largely unclear. We analyzed genome-wide levels of active and repressive histone modifications, trimethylation of histone H3 at lysine 4 (H3K4me3) and H3K27me3, respectively, in the reprogramming process of *P. patens* with chromatin immunoprecipitation-sequencing (ChIP-seq). We revealed that the genome-wide changes occur at the last moment of the reprogramming process. We are currently attempting to specify the actual moment of the changes by performing 4D (3D + time) live imaging of chromatin modifications in a single *P. patens* nucleus. To detect H3K27me3, we introduced a gene encoding a fluorescent protein fused to a *Drosophila melanogaster* Polycomb protein, which binds to



Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

H3K27me3, into *P. patens*. We are now performing live imaging for H3K27me3 during the reprogramming, and producing novel detectors for other chromatin modifications including H3K4me3. This study was mainly conducted by Yosuke Tamada.

## III. Evolution of Regeneration: Master Regulator for Reprogramming *STEMIN*

Animal somatic cells can be reprogrammed to induce pluripotent stem (iPS) cells by introducing four transcription factors, while such factors have not been identified in plants. On the basis of the transcriptional profile during moss reprogramming (Nishiyama *et al*., 2012), we selected genes, of which transcript levels increase during reprogramming, and induced each candidate gene in gametophores using the estrogen inducible system. As a result, we identified a gene encoding a member of a plant-specific transcription factor, STEM CELL-INDUCING FACTOR (STEMIN), that was able to induce direct reprogramming of differentiated leaf cells into chloronema apical stem cells without wounding signals. In addition, *STEMIN* promoter was activated at leaf cells that underwent reprogramming. Deletion of the *STEMIN* and its two paralogous genes delayed reprograming after leaf excision. Together, we suggest that STEMIN is a single master regulatory transcription factor governing *de novo* stem cell formation. Masaki Ishikawa was this study's main researcher.

## IV. Evolution of Elaborated Cell Division Machinery: Phragmoplast

The cells of land plants and their sister group, charophycean green algae, divide by the insertion of cell plates at cytokinesis. This is in contrast to other green algae, in which the invagination of the plasma membrane separates daughter cells at cytokinesis. The cell plate appears in the middle of daughter nuclei, expands centrifugally towards the cell periphery, and finally fuses to the parental cell wall. Cell wall materials are transported to the expanding cell plate with a phragmoplast, which is mainly composed of microtubules. Centrifugal expansion of the phragmoplast is a driving force for that of the cell plate, although elucidating the molecular mechanism for the expansion was a challenge. We have found that γ-tubulin complexes on existing phragmoplast microtubules nucleate new microtubules as branches. Although elongation of the branched microtubules is likely a driving force of the phragmoplast expansion, the mechanism by which phragmoplast microtubules redistribute towards the cell periphery is unclear. We found that the phragmoplast array comprises stable microtubule bundles and dynamic microtubules. We found that the dynamic microtubules are nucleated by γ-tubulin on stable bundles. The dynamic microtubules elongate at the plus ends and form new bundles preferentially at the leading edge of the phragmoplast. At the same time, they are moved away from the cell plate, maintaining a restricted distribution of minus ends. We propose that cycles of attachment of γ-tubulin complexes onto the microtubule bundles, microtubule nucleation and bundling, accompanied by minus-end-directed motility, drive the centrifugal development of the phragmoplast. Takashi Murata was this study's main researcher.

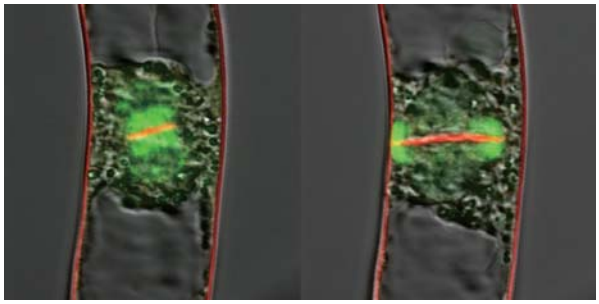

Figure 2. Centrifugal expansion of the phragmoplast. Green, phragmoplast; Red, cell plate and membrane.

## V. Evolution of Life cycles

Land plants undergo an alternation of generations, in which multicellular bodies are produced in both haploid and diploid generations. Distinct developmental programs initiate after meiosis and fertilization in the haploid and diploid generations, respectively. Green alga, the sister to land plants, is inferred to have had a haplontic life cycle and the developmental program for the sporophyte generation was elaborated during land plant evolution. To understand the evolution of alteration of generations, we first analyzed molecular switches to start each generation. Previously we found that a polycomb repression complex 2 gene *CURLY LEAF* (*CLF*) represses initiation of sporophyte pluripotent stem cells in the gametophyte generation and the decrease of the protein was correlated to the start of the sporophyte generation (Okano *et al*. 2010 PNAS). We also found that *CLF* represses sporophyte stem cell activity in the sporophyte generation, and the timing of *CLF* expression regulates the length of the sporophyte generation and resultant body growth. This year, we found the other switch to start the haploid generation as a collaborative work with Drs. Keiko Sakakibara and John Bowman's groups in Hiroshima University and Monash University, respectively. Deletion of the class 2 KNOTTED1-LIKE HOMEOBOX (KNOX2) transcription factors in the moss *Physcomitrella patens* resulted in the development of gametophyte bodies from diploid embryos without meiosis. This indicates that KNOX2 acts to prevent the haploid-specific body plan from developing in the diploid plant body. Our findings indicate critical roles for the evolution of CLF and KNOX2 in establishing an alternation of generations in land plants.

## VI. Molecular mechanisms of mimicry

An excellent example of mimicry is the flower-mimicry of the orchid mantis *Hymenopus coronatus* with pink and white coloration and petal-like legs. Biochemical analyses indicated that the reduced form of xanthommatin, a common red pigment of the ommochrome family, almost solely contributes to the pink color. On the other hand, the oxidized form of xanthommatin and a mixture of ommochrome pigments were found in mantises with brown and yellowish-red color, respectively. These results suggest that the unique pink coloration of the orchid mantis is formed by the

predominance of the reduced form of xanthommatin. This work was mainly done by Hiroaki Mano.

## VII. Molecular mechanisms of host shifting

In phytophagous insects a precise combination of performance and preference traits for particular host plants is crucial for host shifting because a new host plant can be incorporated into an insect's diet if adults accept it for oviposition and if the larvae are able to complete their development on it. However, very little is known about the genetic bases of the performance and preference. A QTL analysis of a tiny moth, *Acrocercops transecta* revealed that only a restricted region of a single autosome was responsible for the larval performance, suggesting that a small number of genetic changes to larval performance allowed the successful host shifting. Identification of the responsive genes is in progress with Dr. Issei Ohshima in Kyoto Prefecture University.

## VIII. Molecular mechanisms of Plant Movement using *Mimosa pudica*

The molecular mechanisms and evolutionary significance of plant movement, including seismonastic and nyctinastic movements, are enigmatic. To introduce the sensitive plant *Mimosa pudica* as a model, we established a method for transformation. We used a cotyledonary node as a target of *Agrobacterium*-mediated gene transfer because of its ability of shoot regeneration. We obtained a large number of transformed calluses (55-60%) and succeeded in regenerating transgenic plants with a transformation efficiency of >5%. This study was conducted mainly by Hiroaki Mano.

## IX. Evolution of pitcher leaves in carnivorous plants

Carnivorous plants form specialized leaves that are capable of attracting, trapping, and digesting prey and absorbing nutrients. The unusual plants evolved from non-carnivorous plants but their evolutionary process is mostly unknown. To understand the genomic changes associated with the evolution of carnivory, we sequenced 2-Gbp genome of the Australian pitcher plant *Cephalotus follicularis* in collaboration with Beijing Genomics Institute. Whole-genome shotgun data corresponding to 100-fold depth were produced by Illumina and PacBio sequencers. A *de novo* assembly yielded a total of 1.6 Gbp in 16,307 scaffolds with 99.5 kb of contig N50 and 287 kb of scaffold N50. Transcript-based and homology-based gene prediction with RNA-seq reads found 32,973 gene models. Genomic data enable us to deduce the origin and evolution of carnivory-related genes, such as digestive enzyme genes. This study was conducted mainly by Kenji Fukushima and Tomoko Shibata.

## Publication List

### 〔Original papers〕


- Kim, S.Y., Colpitts, C.C., Wiedemann, G., Jepson, C., Rahimi, M., Rothwell, J.R., McInnes, A.D., Hasebe, M., Reski, R., Sterenberg, B.T., *et al*. (2013). Physcomitrella PpORS, basal to plant type III polyketide synthases in phylogenetic trees, is a very long chain 2'-oxoalkylresorcinol synthase. J. Biol. Chem. *288*, 2767-2777.
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## DIVISION OF SYMBIOTIC SYSTEMS

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| | *FUKUHARA, Mai* |
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*Rhizobium*–legume symbiosis is one of the most successful mutually beneficial interactions on earth. In this symbiosis, soil bacteria collectively called rhizobia supply the host legumes with ammonia produced through bacterial nitrogen fixation, in contrast to host plants providing the rhizobia with their photosynthetic products. To accomplish this biotic interaction, leguminous plants develop root nodules in which to confine rhizobia. This novel lateral organ differentiation (i.e., nodulation) is triggered by Nod factors secreted by rhizobia. On the other hand, more than 80% of land plant families have symbiotic relationships with arbuscular mycorrhizal (AM) fungi. Despite marked differences between the fungal and bacterial symbioses, common genes are required for both interactions. Using a model legume *Lotus japonicus*, we are trying to unveil the mechanisms of both symbiotic systems.

## I. Nodulation

### 1-1 Endoreduplication is essential for initiation of root nodule organogenesis and rhizobial infection

During early nodule development, rhizobia-derived Nod factors induce the dedifferentiation of root cortical cells. The activated cortical cells then proliferate to form the primordium of the symbiotic nitrogen-fixing root nodule. Recent identification and functional analyses of the putative cytokinin receptors *Lotus japonicus* LOTUS HISTIDINE KINASE 1 (LHK1) and *Medicago truncatula* CYTOKININ RESPONSE 1, have led to a greater understanding of how the activation of cytokinin signaling is crucial to the initiation of nodule organogenesis. In particular, it has been shown that in the *L. japonicus spontaneous nodule formation 2* (*snf2*) mutant, a gain-of-function form of LHK1 confers the constitutive activation of cytokinin signaling, resulting in the formation of spontaneous nodule-like structures in the absence of rhizobia.

We identified a novel nodulation-deficient mutant named *vagrant infection thread 1* (*vag1*) after suppressor mutant screening of *snf2* mutants in *L. japonicus*. The *VAG1* gene encodes a protein that is putatively orthologous to *Arabidopsis* ROOT HAIRLESS 1/HYPOCOTYL 7, a component of the plant DNA topoisomerase VI that is involved in the control of endoreduplication. Nodule phenotype of the *vag1* mutant shows that VAG1 is required for the ploidy-dependent cell growth of rhizobial infected cells. Furthermore, the *VAG1* gene mediates a local emergence of endoreduplicated cortical cells during early nodule development, which is essential for the initiation of cortical cell proliferation leading to nodule primordium formation (Figure 1). In addition, a misguided infection thread formation in the *vag1* mutant indicates that the endoreduplication of cortical cells is required for the guidance of symbiotic bacteria to host meristematic cells. Overall, our results provide new insights into the role of endoreduplication in plant development and plant–microbe symbiosis.

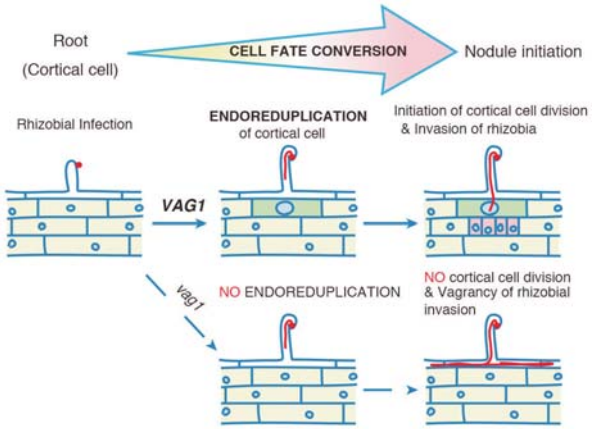


Figure 1. Model for the conversion of cortical cell fate toward root nodule initiation.

### 1-2 Analysis of a non-nodulating *L. japonicus* mutant *daphne* reveals a negative regulation of rhizobial infection

The main events for creation of nodules are divided into rhizobial infection and nodule organogenesis. For proper establishment of symbiosis, it is essential that the two phenomena proceed synchronously in different root tissues, the epidermis and cortex. Owing to their complexity most of those have defects in both infection and organogenesis pathways, the molecular interaction between these symbiotic signalings has still remained elusive.

Our study began with the isolation of a unique mutant from $C^{6+}$ beam mutagenized seeds of *L. japonicus*, named *daphne*. *daphne* showed a novel symbiotic phenotype, non-nodulation and increased number of rhizobial infections. This mutant allowed us to focus on the interrelationship of the two different symbiotic pathways. Characterization of the locus responsible for these phenotypes revealed a chromosomal translocation upstream of *NIN* gene in the *daphne* genome. Genetic analysis using a known *nin* mutant revealed that *daphne* is a novel *nin* mutant allele. Although the *daphne*

mutant showed reduced expression of *NIN* after rhizobial infection, the spatial expression pattern of *NIN* in epidermal cells was broader than that in the wild type. Over-expression of *NIN* strongly suppressed hyper-infection in *daphne*, and *daphne* phenotypes were partially rescued by cortex specific expression of *NIN*. These observations suggested that *daphne* mutation enhanced the role of NIN in the infection pathway due to a specific loss of the role of NIN in nodule organogenesis. Based on the results, we provide evidence that a bifunctional transcription factor NIN negatively regulates infection but positively regulates nodule organogenesis during the course of the symbiosis.

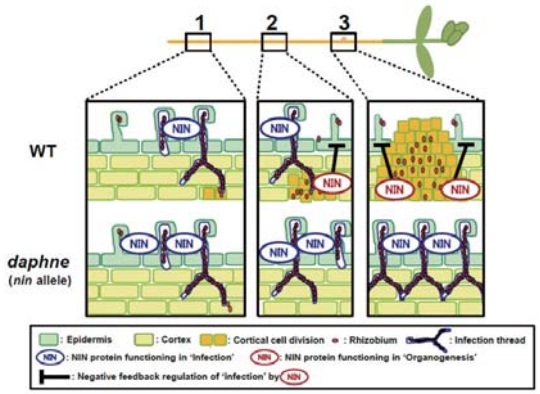


Figure 2. A model of inhibition of rhizobial infection processes mediated by NIN. In the wild type, NIN functions in both rhizobial infection (blue) and organogenesis (red). In the earlier stage, NIN (blue) is predominant but in the later stage, the proportion of NIN (red) has increased with nodule development. It is assumed that a potential negative correlation between the organogenesis and infection pathway (black bars) regulates the amount of infection and restricts the region of rhizobial susceptibility.

### 1-3 Nodulation by global control

In the legume-rhizobia symbiosis, the nodule number is tightly restricted by the host through a systemic suppression called Autoregulation Of Nodulation (AON). AON provides a long-distance control of nodulation via the root-to-shoot-to-root negative feedback. In *L. japonicus*, autoregulation is mediated by CLE-RS genes that are specifically expressed in the root, and the receptor kinase HAR1 that functions in the shoot. However, the mature functional structures of CLE-RS gene products and the molecular nature of CLE-RS/HAR1 signaling governed by these spatially distant components remain elusive. We showed that CLE-RS2 is a post-translationally arabinosylated glycopeptide derived from the CLE domain. Chemically synthesized CLE-RS glycopeptides cause significant suppression of nodulation and directly bind to HAR1 in an arabinose-chain and sequence-dependent manner. In addition, CLE-RS2 glycopeptide specifically produced in the root is found in xylem sap collected from the shoot. We propose that CLE-RS glycopeptides are the long sought mobile signals responsible for the initial step of autoregulation of nodulation.

## II. Arbuscular mycorrhiza symbiosis

In order to obtain insights about molecular mechanisms in AM symbiosis, we performed transcriptome analysis in *L. japonicus* by RNAseq using next generation sequencing technology. This analysis revealed that phytohormone gibberellin (GA) biosynthesis genes were up-regulated during AM symbiosis (Figure 3A,B). GA has been reported to inhibit AM fungal infection by genetic and pharmacological analyses. However, our transcriptome analysis and phytohormone quantification analysis revealed GA accumulation in the roots infected with AM fungi, suggesting that *de novo* GA synthesis plays a role in AM symbiosis development.

We found pleiotropic effects of GAs on the AM fungal infection. In particular, the morphology of AM fungal colonization was altered by the status of GA signaling in the host root. Exogenous GA treatment inhibited hyphal entry into the host root and arbuscule formation, however, it enhanced hyphal branching in the cortex (Figure 3C,D). On the other hand, inhibition of GA biosynthesis inhibited hyphal branching, resulting in low hyphal density in the host root (Figure 3E). Alterations in GA conditions also affected expression of the AM symbiosis-induced genes that are required for AM fungal infection and hyphal elongation in the host root. These studies demonstrated that GA signaling interacts with symbiotic responses and regulates AM colonization of the host root.

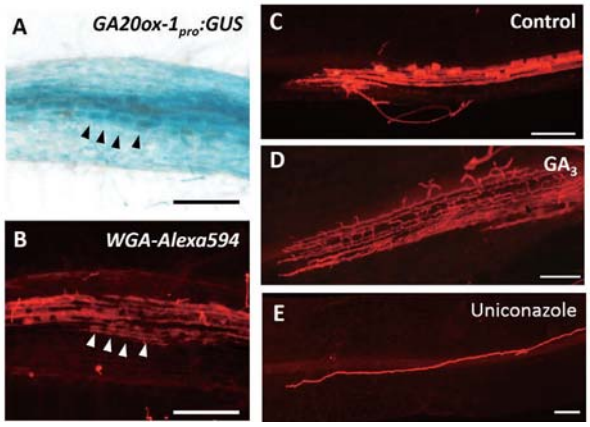


Figure 3. A GA biosynthesis gene *GA20 oxidase-1* was induced around AM inner hyphae. (A) Transgenic roots carrying the *GA20ox-1*$_{pro}$*:GUS* were stained with X-gluc. (B) The inner fungal structures were stained with WGA-Alexa594 at 3 wai with *R. irregularis*. Arrowheads indicate a symbiotic structure 'arbuscule'. (C-E) GA treatment increased hyphal branching (D) compared with mock treated roots (C). On the other hand, GA biosynthesis inhibitor 'uniconazole' treatment inhibited hyphal branching in the host roots. Scale bars = 200 μm.

## III. Evolutionary dynamics of nitrogen fixation in the legume-rhizobia symbiosis

The stabilization of host-symbiont mutualism against the emergence of parasitic individuals is pivotal to the evolution of cooperation. One of the most famous symbioses occurs between legumes and their colonizing rhizobia, in which rhizobia extract nutrients (or benefits) from legume plants while supplying them with nitrogen resources (or costs) produced by nitrogen fixation. Natural environments, however, are widely populated by ineffective rhizobia that extract benefits without paying costs and thus proliferate

more efficiently than nitrogen-fixing cooperators. How and why this mutualism becomes stabilized and evolutionarily persists has been extensively discussed. To better understand the evolutionary dynamics of this symbiosis system, we constructed a simple model based on the continuous snowdrift game with multiple interacting players (Figure 4A). We investigated the model using adaptive dynamics and numerical simulations. We found that symbiotic evolution depends on the relative strength between the benefit and cost, and that cheaters can emerge when these quantities are balanced (Figure 4B). This result suggests that the symbiotic relationship is robust to the emergence of cheaters, and may explain the prevalence of cheating rhizobia in nature. Our model also predicts that, although cheaters possess a short-term advantage over cooperators, their evolutionary persistence is not guaranteed. These findings provide a theoretical basis of the evolutionary dynamics of legume-rhizobia symbioses, which is extendable to other single-host, multiple-colonizer systems.

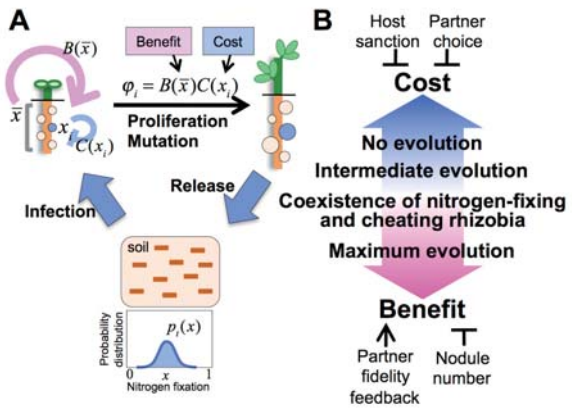


Figure 4. Schematic representation of the model. (A) The probability distribution of rhizobia strategy (i.e. nitrogen fixation) is iteratively changed in the cycle of infection, proliferation/mutation, and release steps. (B) The evolution of the legume-rhizobia symbiosis depends on the cost-benefit balance.

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- Miyata, K., Kawaguchi, M., and Nakagawa, T. (2013). Two distinct EIN2 genes cooperatively regulate ethylene signaling in *Lotus japonicus*. Plant Cell Physiol. *54*, 1469-1477.
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### 〔Review article〕


- Suzaki, T., Ito, M., and Kawaguchi, M. (2013). Genetic basis of cytokinin and auxin functions during root nodule development. Front. Plant Sci. *4*, 42.

## Laboratory of Morphodiversity

*Associate Professor*
KODAMA, Ryuji

The aim of this laboratory is to observe the variety of morphogenetic processes that occur in the course of the ontogenesis of multicellular organisms and to analyze the mechanisms of such processes, mainly by morphological methods. The accumulation of such analyses of the embryogenetic processes of related species is expected to provide an insight into the evolution of morphogenetic processes. This laboratory uses the wings of lepidopteran insects as the main target of morphological studies.

The laboratory was moved from Myodaiji-area to Yamate-area in July 2012.

### I. Wing morphogenesis

The wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. Due to its simple construction, this tissue is a good material for studying cellular interactions in the course of morphogenesis.

The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing. The marginal dying area is called “the degeneration region” and the internal area, which develops into the adult wing, is called “the differentiation region”.

Cell death in the degeneration region proceeds very rapidly and finishes in a half-day to one-day period in *Pieris rapae* and in several other examined species. It was shown that the dying cells in the degeneration region have characteristics in common with the apoptotic cell death in mammalian cells, such as fragmented and condensed nuclei containing short DNA fragments detected by TUNEL staining. The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. The macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between the basal surfaces of the dorsal and ventral epithelium in the differentiation region, which occurs at the time of prominent cell death and excludes macrophages out of the differentiation region. This realized concentration of macrophages seems to accelerate the shrinkage of the degeneration region.

A possible physiological role of the cell degeneration at the wing margin is to make space for the growth of the marginal scales. Marginal scales are extremely elongated scales that grow densely on the edge of the wing. These scales are considered to be important in stabilizing the turbulence occurring posterior to the wing. The movements of the marginal scales are closely monitored by sensory scales and bristles growing among them (Yoshida and Emoto, Zool. Sci. *28*, 430-437, 2011).

Trachea and tracheoles are both important in delivering air into the wing and their patterns coincide with that of the boundary of degeneration and differentiation zones at the distal end of the wing. According to previous observations, the pattern formation of wing epithelium is often dependent on tracheal and tracheole patterns. Basic research on the development of tracheal pattern formation is being done by scanning and transmission electron microscopy (Figure 1) to describe the exact pathway and time course of the formation of the elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of the tracheal pattern and the epithelial cell pattern.

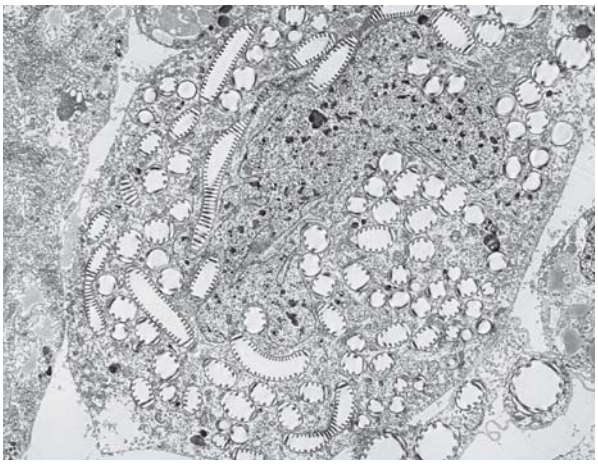

Figure 1. Transmission electron micrograph of the tracheole cell. Many cross-sections of the tracheoles can be observed within the cytoplasm of the tracheole cell. As the cell migrates, the tracheole is laid behind.

### II. Other research activities

This laboratory also conducts morphological observation of various animal tissues by scanning and transmission electron microscopy and immuno-electron microscopic analyses in collaboration with other laboratories of NIBB. Training in specimen preparation and instrument operation for such observations is also given.

## LABORATORY OF BIORESOURCES

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NARUSE, Kiyoshi

| | |
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| | *HARA, Ikuyo* |
| | *ISHIKAWA, Hiroe* |
| | *SHIBATA, Emiko* |
| | *TAKAKI, Chikako* |
| | *KANIE, Yuta* |
| *Secretary:* | *SUZUKI, Tokiko* |

Medaka is a small egg-laying "secondary" fresh water fish found in brooks and rice paddies in Eastern Asia. This species has a long history as an experimental animal, especially in Japan. Our laboratory has conducted studies on evolution of the sex determination system using medaka and relatives, identification of the causal gene of mutants for PGC migration and pigment cell development, and the gonadal development of medaka. In addition to these activities, our laboratory is stepping forward to lead the National BioResource Project Medaka (NBRP Medaka).

### I. Evolution of the sex chromosome and sex determination genes in *Oryzias* fish

Recent studies have demonstrated that *Oryzias* species have different genetic sex-determination systems (XX/XY and ZZ/ZW) (Figure 1). Furthermore, the sex chromosomes differ in their origin and degree of differentiation. These findings suggest the repeated creation of new sex chromosomes from autosomes during evolution of *Oryzias* fishes, possibly in association with the formation of new sex-determining genes. We are now trying to positionally clone the novel sex-determining genes in these species. Identification of these genes would provide a clue to understand the evolutionary process underlying frequent turnover of sex determination mechanisms.

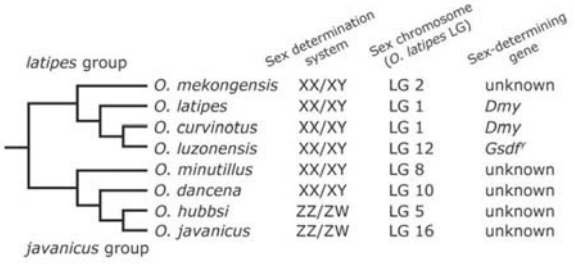


Figure 1. Phylogenetic relationships and sex determination mechanisms in *Oryzias* fishes.

### II. Genetic dissection of migration of primordial germ cells in medaka

Germ cells are responsible for the sustainability of life over generations in many multicellular animal species. To elucidate the mechanisms underlying the development of primordial germ cells, we identified multiple mutations affecting the migration and development of the primordial germ cells in medaka in a prior large-scale mutagenesis screening project, and have analyzed a set of them to date. We focused on three mutants that have defects in primordial germ cell migration, *kamigamo*, *shimogamo*, and *naruto* that were isolated in the screening project. Positional cloning and analysis of the genes carrying the mutations are now in progress. In addition, two mutations, *kamigamo* and *shimogamo*, cause cystic pronephric ducts simultaneously with abnormal positioning of the primordial germ cells. Therefore, the analysis of these mutations will be important in giving basal knowledge underlying the mechanisms of human cystic kidney diseases.

### III. The function of estrogen in the medaka ovary

Estrogens have been generally considered to play a critical role in the ovarian differentiation of teleost fish by Yamamoto's model. In medaka, estrogen treatment has induced functional male-to-female sex reversal. To clarify the function of estrogen during ovarian development, we examined the role of ovarian aromatase, which is responsible for catalyzing the conversion of testosterone to estrogen. We isolated two tilling mutant strains of ovarian aromatase. In these tilling mutants, one amino acid in ovarian aromatase ORF altered the STOP codon. Mutant females seemed to develop normal ovaries but yolk accumulation was not observed in the ovarian follicles and most of the ovarian follicles underwent atresia in the adult ovary. Even more surprisingly, spermatogenesis was observed within the mutant ovary. These results suggest that the hypothesis that endogenous estrogens drive ovarian differentiation needs to be modified to the hypothesis that estrogens are essential for the maintenance of ovarian differentiation.

### IV. Positional cloning of pigment cell mutants in medaka

Animal body colors are generated primarily by neural crest-derived pigment cells in the skin. Whereas mammals and birds have only melanocyte on the body surface, fish have a variety of pigment cell types, also referred to as chromatophores: melanophores, xanthophores, iridophores and so on. Compared with other fish species, medaka has a unique chromatophore type, leucophores. However, the genetic basis of chromatophore diversity has so far remained barely understood. Here, we found that three loci, leucophore free (*lf*), *lf-2* and white leucophore (*wl*), in medaka, which affect leucophore and xanthophore formation, encode slc2a15b, pax7a and slc2a11b, respectively. As lf-2, a loss-of-function mutant for pax7a, exhibits defects in formation of xanthophore and leucophore precursor cells, pax7a is critical

for fate specification of the xanthophore/leucophore lineage. This genetic evidence implies that leucophores develop in the same cell lineage as xanthophores, although it has been believed that leucophores are related to iridophores because they have the property of purine-dependent light reflection in common. Our identification of slc2a15b and slc2a11b as crucial for pigmentation of leucophores and xanthophores in medaka revealed that the existence of these two genes in the genome coincides with the trait of having xanthophores or their characteristic intracellular organelle in non-mammalian vertebrates. Considering that birds have yellow-pigmented irises with xanthophore-like intracellular organelles, our findings provide clues for uncovering evolutionary diverse mechanisms of pigment cell formation in animals.

## V. National BioResource Project Medaka (NBRP Medaka) (http://www.shigen.nig.ac.jp/medaka/)

In 2007, NIBB was selected as the core facility of NBRP Medaka. Our laboratory is taking an active part in this project. With the goal of facilitating and enhancing the use of medaka as a model organism, we provide, maintain and collect living resources such as standard strains, inbred strains, and mutants in addition to frozen resources such as EST/cDNA, BAC/ Fosmid clones and hatching enzymes, as well as integrated information on medaka (Figure 2). We have been providing BAC clones of medaka related species, a library screening system employing a 3D PCR strategy for evolutionary studies, and the TILLING screening system for promoting the reverse genetic approach. NBRP Medaka aims to establish a first rate biological resource with the highest possible levels of accessibility and ease of use.

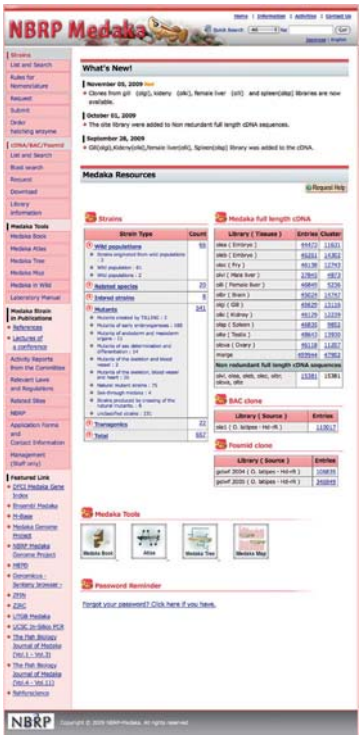


Figure 2. NBRP Medaka website.

## Publication List

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### 〔Original paper (E-publication ahead of print)〕


- Zhang, X., Guan, G., Chen, J., Naruse, K., and Hong, Y. Parameters and efficiency of direct gene disruption by zinc finger nucleases in medaka embryos. Marine Biotech. 2013 Oct. 23.

## LABORATORY OF BIOLOGICAL DIVERSITY

### KAMADA Group

*Assistant Professor:* *KAMADA,Yoshiaki*

Nutrients are indispensable for life. Thus, perception of the nutrient environment is also essential for cells. To recognize cellular nutrient conditions, eukaryotic cells employ Tor (target of rapamycin) protein kinase. Tor forms two distinct protein complexes, TORC (Tor complex) 1 and TORC2. TORC1 regulates rapamycin-sensitive branches of the TOR pathway, such as protein synthesis, cell cycle and autophagy. TORC1 is thought to act as a nutrient sensor, because rapamycin, a TORC1 inhibitor, mimics a starved condition. On the other hand, TORC2, whose function is insensitive to rapamycin, is responsible for actin organization and cell integrity. So far, it is not clear whether TORC2 also perceives nutrient signals.

The aim of our research group is to reveal the molecular mechanisms of how Tor receives nutrient signals and how the TORC1/2 pathways regulate each phenomenon. We have been studying Tor signaling in the budding yeast *Saccharomyces cerevisiae*, and have found three novel branches of the TOR signaling pathway (Figure 1).

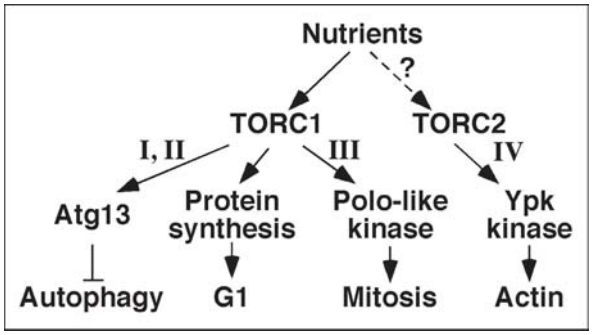


Figure 1. Tor signaling pathway of the budding yeast. Our group has found three branches of the Tor pathway.

## I. TORC1 phosphorylates Atg13, the molecular switch of autophagy

Autophagy is mainly a response to nutrient starvation, and TORC1 negatively regulates autophagy. The Atg1 kinase and its regulators, i.e. Atg13, Atg17, Atg29, and Atg31 collaboratively function in the initial step of autophagy induction, downstream of TORC1. Atg1 is a Ser/Thr protein kinase, the activity of which is essential for autophagy and is largely enhanced following nutrient starvation or the addition of rapamycin. This regulation involves phosphorylation of Atg13.

We found that Atg13 is directly phosphorylated by TORC1. Phosphorylated Atg13 (during nutrient-rich conditions) loses its affinity to Atg1, resulting in repression of autophagy. On the other hand, under starvation conditions Atg13 is immediately dephosphorylated and binds to Atg1 to form Atg1 complex. Atg1 complex formation confers Atg1 activation and consequently induces autophagy. We determined phosphorylation sites of Atg13 and generated an unphosphorylatable Atg13 mutant (Atg13-8SA). Expression of Atg13-8SA induces autophagy bypassing inactivation of TORC1, the same as starvation treatment or rapamycin. These results demonstrate that Atg13 acts as a molecular switch for autophagy induction.

## II. Monitoring in vivo activity of TORC1 by phosphorylation state of Atg13

Since Atg13 has turned out to be a substrate of TORC1, *in vivo* activity of TORC1 can be monitored by the phosphorylation state of Atg13. Various conditions and mutants have been examined to determine what kind of nutrients TORC1 recognizes and how nutrient signals are transmitted to TORC1.

## III. How and why TORC1 regulates mitotic entry?

TORC1 regulates protein synthesis, which is important for promotion of the cell cycle at G1 (G0). Little is known, however, about whether or not TORC1 is involved in other stages of the cell cycle.

We generated a temperature-sensitive allele of *KOG1 (kog1-105)*, which encodes an essential component of TORC1, and found that TORC1 plays an important role in mitotic entry (G2/M transition). Since Cdc5, the yeast polo-kinase is mislocalized and inactivated in *kog1-105* mutant cells, TORC1 mediates G2/M transition via regulating polo-kinase. Recently we discovered a physiological role of TORC1 in mitosis; autophagy negatively controlled by TORC1 plays an important part in maintenance of genome stability under starvation conditions.

## IV. Ypk2 kinase acts at the downstream of TORC2 to control actin organization

Genetic studies have shown that TORC2 controls polarity of the actin cytoskeleton via the Rho1/Pkc1/MAPK cell integrity cascade. However, the target (substrate) of TORC1 was not yet identified. We found that Ypk2, an AGC-type of protein kinase whose overexpression can rescue lethality of TORC2 dysfunction, is directly phosphorylated by TORC2.

### Publication List

〔Original paper〕

● Matsui, A., Kamada, Y., and Matsuura, A. (2013). The role of autophagy in genome stability through suppression of abnormal mitosis under starvation. PLoS Genetics *9*, e1003245.

## LABORATORY OF BIOLOGICAL DIVERSITY

### OHNO Group

*Assistant Professor:* *OHNO, Kaoru*

The aim of this laboratory is to research reproductive hormones in invertebrates and to analyze the mechanisms by which they work. The comparisons of such molecules and mechanisms in various species are expected to provide insights into the evolution of reproductive hormone systems.

## I. Gonadotropins in the starfish, *Asterina pectinifera*

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, LH and FSH are structurally and functionally conserved across various species, whereas no such molecule has been identified in invertebrates. The insect parsin hormones are assumed to be the physiological counterpart of LH and FSH in mammals. Some gonadotropic hormones, such as the egg development neurosecretory hormone of the mosquito, the egg-laying hormone of the sea hare, and the androgenic gland hormone of the terrestrial isopod, have been found in invertebrate species. More recently, an insulin-like peptide was reported to be responsible for the regulation of egg maturation in the mosquito, *Aedes aegypti*, demonstrating the involvement of insulin signaling in egg maturation among invertebrates.

The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates. GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine, which in turn induces the maturation of the oocytes. In this sense, GSS is functionally identical to vertebrate LH, especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte. Considering the functional similarity that GSS shares with vertebrate LH, it is very important from an evolutionary point of view to know the chemical and molecular structure of GSS. We cloned the gene encoding GSS referred to amino acid sequence of purified GSS from radial nerves of the starfish, *Asterina pectinifera*. Interestingly, phylogenetic analyses revealed that it belonged to the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, to the subclass of relaxin/insulin-like peptides (Figure 1).

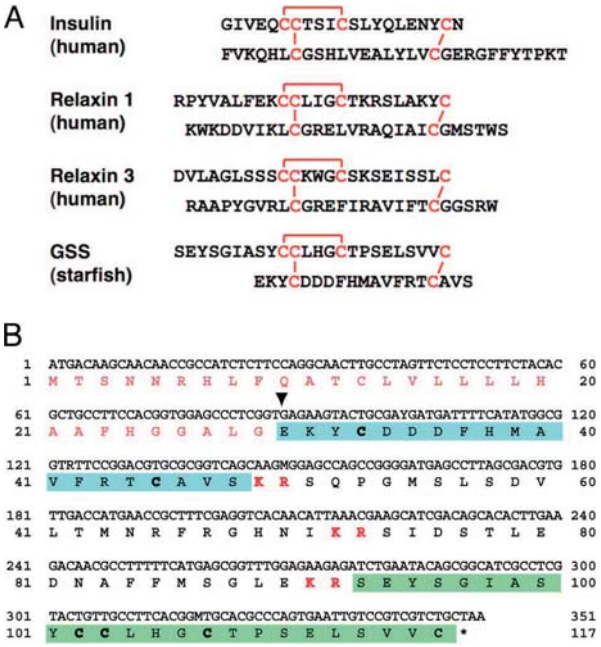


Figure 1. Amino acid sequence of starfish GSS. (A) Comparison of the heterodimeric structure of starfish GSS with those of various representative members of the insulin superfamily. The cysteine bridges are shown in red. (B) Coding DNA sequence and predicted amino acid sequences of GSS. Sequences of A and B chains are shown in green and blue boxes, respectively. Characters shown in red boldface indicate basic dipeptides that are the sites of proteolytic cleavage. Inverted triangle shows the deduced cleavage site of the signal peptide.

## II. Search for reproductive hormones in invertebrates

In a collaborative effort with Prof. Yoshikuni's Laboratory of the Kyushu Univ. and Dr. Yamano and Dr. Awaji of the National Research Institute of Aquaculture, Fisheries Research Agency (NRIA), we are searching for reproductive hormones in invertebrates; sea urchin, sea cucumber, oyster, and shrimp. While the collaborators are partially purifying physiological materials which induce egg maturation from nerve extracts and analyzing them with a tandem mass spectrometer, we are creating and analyzing EST libraries from nerve tissues and developing a database of the mass analysis performed in this laboratory.

## LABORATORY OF BIOLOGICAL DIVERSITY

### HOSHINO Group


*Assistant Professor:* *HOSHINO, Atsushi*
*Technical Assistant:* *NAKAMURA, Ryoko*
*TAKEUCHI, Tomoyo*


While genomic structures as well as their genetic information appear to stably transmit into daughter cells during cell division, and also into the next generation, they can actually vary genetically and/or epigenetically. Such variability has had a large impact on gene expression and evolution. To understand such genome dynamisms in eukaryotes, especially in plants, we are characterizing the flower pigmentation of morning glories.

## I. Flower pigmentation patterns of the morning glories

*Ipomoea nil* (Japanese morning glory), *I. purpurea* (the common morning glory), and *I. tricolor* have been domesticated well as floricultural plants, and their various spontaneous mutations have been isolated. The wild type morning glories produce flowers with uniformly pigmented corolla, whereas a number of mutants displaying particular pigmentation patterns have been collected (Figure 1). Because flower pigmentation patterns are easily observed, the molecular mechanisms underlying these phenomena provide fine model systems for investigating genome variability.

*Margined*, *Rayed* and *Blizzard* of *I. nil* are dominant mutations. While these mutants show distinct flower pigmentation patterns, the same pigmentation gene is repressed by non-coding small RNA in the whitish parts of the corolla. It is suggested that distinct regulation of small RNA cause the difference in pigmentation patterns. The recessive mutations, *duskish* of *I. nil* and *pearly-v* of *I. tricolor*, confer variegated flowers, and epigenetic mechanisms are thought to regulate flower pigmentation. We are currently characterizing detailed molecular mechanisms of these mutations.

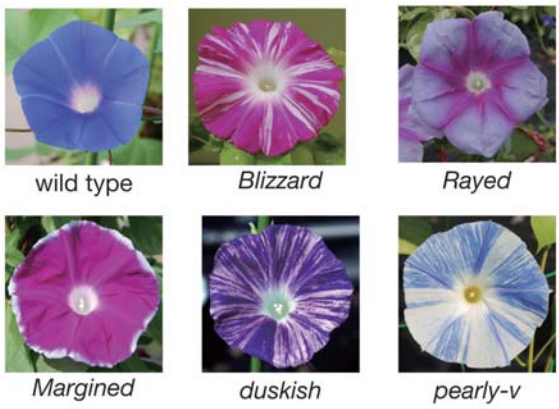


Figure 1. Flower phenotypes of the morning glories.

## II. *de novo* sequencing of Japanese morning glory genome

Although morning glories are studied worldwide, especially in plant physiology and genetics, no whole nuclear genome sequences of any *Ipomoea* species are available. To facilitate the studies of our group as well as all morning glory researchers, we are conducting *de novo* genome sequencing of *I. nil*, having a genome of about 800 Mbp. We chose the Tokyo-kokei standard line for genome sequencing, and employed not only shotgun sequencing using high-throughput DNA sequencers but also BAC end sequencing. We are collaborating with several laboratories in Japan.

## III. BioResource of morning glories

NIBB is the sub-center for National BioResource Project (NBRP) for morning glory. In this project, we are collecting, maintaining and distributing standard lines, mutant lines for flower pigmentation, and DNA clones from EST and BAC libraries of *I. nil* and its related species. *I. nil* has been one of the most popular floricultural plants since the late Edo era in Japan. It has extensive history of genetic studies and also has many advantages as a model plant; simple genome, large number of mutant lines, and efficient self-pollination. Our collection includes 220 lines and 147,000 DNA clones.

## IV. Flower color and vacuolar pH

Flower color is not only determined by pigments. It is dependent on several factors, such as colorless pigments, metal ions, and pH in the vacuole where flower pigments are accumulated. Petunia blooms red or violet flowers, and mutations in any one of the seven loci, named *PH1-PH7*, result in a bluish flower color (Figure 2). We successfully isolated *PH1* that encodes $P3_B$-ATPase, hitherto known as $Mg^{2+}$ transporters in bacteria. Although PH1 itself is not a proton transporter, it can boost PH5 ($P3_A$-ATPase) proton transport activity that has been known to be essential for vacuolar hyperacidification. PH1 and PH5 physically interact with each other, and co-localize in the vacuolar membrane. The heteromeric P-ATPase pump of PH1 and PH5 is sufficient to hyperacidify vacuoles creating red pigmentation of petunia flowers.

Figure 2. The petunia unstable *ph1* mutant. The bluish pigmentation is due to a failure to hyperacidify vacuoles.

## LABORATORY OF BIOLOGICAL DIVERSITY

### TSUGANE Group

*Assistant Professor:* *TSUGANE, Kazuo*
*Visiting Scientist:* *HAYASHI-TSUGANE, Mika*

Active DNA transposons are important tools for gene functional analysis. The endogenous non-autonomous transposon, *nDart1-0*, in rice (*Oryza sativa* L.) is expected to generate various transposon-insertion mutants because *nDart1-0* elements tend to insert into genic regions under natural growth conditions. The transpositions of *nDart1-0* were promoted by an active autonomous element, *aDart1-27*, on chromosome 6. By using the endogenous *nDart1*/*aDart1-27* system in rice, a large-scale *nDart*-inserted mutant population could be easily generated under normal field conditions, and the resulting tagging lines were free of somaclonal variation.

## I. A mutable albino allele in rice reveals that formation of thylakoid membranes requires *SNOW-WHITE LEAF1* gene Activation and Epigenetic Regulation of DNA Transposon *nDart1* in Rice

To understand chloroplast biogenesis and development, various chloroplast-defective mutants have been analyzed, yet they remain to be discovered. Although analyses of albino plants provide important information about mechanisms of plastid development, albino mutants are seedling lethal under natural growth conditions, owing to the complete loss of photosynthetic apparatus. Variegated mutants are excellent models for exploring the mechanism of chloroplast biogenesis because green and white sectors in the leaves allow an increased chance of survival. The variegation caused by somatic excision of DNA transposon is known as a mutable allele. The endogenous *nDart1*/*aDart1* tagging system is a powerful tool for investigating various unidentified and/or uncharacterized albino alleles. We report a novel variegated albino mutant, *snow-white leaf1-variegated* (*swl1-v*), caused by insertion and excision of *nDart1-0* in the *SNOW-WHITE LEAF1* (*SWL1*) gene (Figure 1). We have developed a specific method (nDart1-0-iPCR) for efficient detection of *nDart1-0* insertions and successfully identified the *snow-white leaf1* (*swl1*) gene in a variegated albino (*swl1-v*) mutant obtained from the *nDart1*-promoted rice tagging line. The variegated albino phenotype was caused by insertion and excision of *nDart1-0* in the 5'-untranslated region of the *SWL1* gene predicted to encode an unknown protein with the N-terminal chloroplast transit peptide. *SWL1* expression was detected in various rice tissues at different developmental stages. However, immunoblot analysis indicated that SWL1 protein accumulation was strictly regulated in a tissue-specific manner. In the *swl1* mutant, formations of grana and stroma thylakoids and prolamellar bodies were inhibited (Figure 2). This study revealed that SWL1 is essential for the beginning of thylakoid membrane organization during chloroplast development. Furthermore, we provide a developmental perspective on the *nDart1*-promoted tagging line to characterize unidentified gene functions in rice.

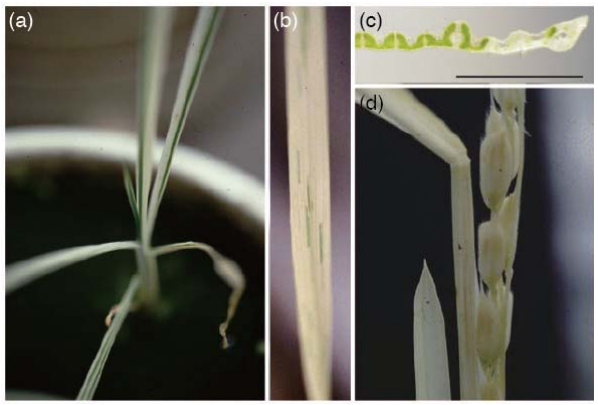


Figure 1. Phenotypes of swl1-v plants. Mutable albino plants with large green sectors (a) and with fine sectors (b). (c) Transverse section of the leaf blade. Scale bar = 1.0 mm. (d) Panicles.

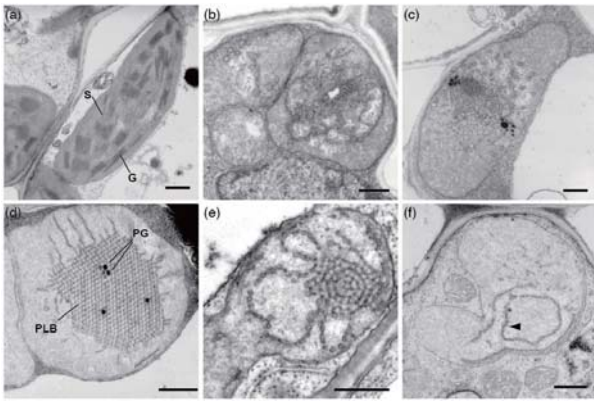


Figure 2. Plastid ultrastructures of WT and *swl1-stb* plants.
Chloroplasts from tertiary leaf blades of WT (a) and swl1-stb (b and c) seedlings grown under light. Etioplasts from the tertiary leaf blades of WT (d) and swl1-stb (e and f) seedlings germinated in the dark. G, grana thylakoid; S, stroma thylakoid; PG, plastoglobule; PLB, prolamellar body. The arrowhead indicates a ring-shaped structure formed from vesicles. Scale bars = 500 nm.

### Publication List

〔Original paper (E-publication ahead of print)〕

● Hayashi-Tsugane, M., Takahara, H., Ahmed, N., Himi, E., Takagi, K., Iida, S., Tsugane, K., and Maekawa, M. A mutable albino allele in rice reveals that formation of thylakoid membranes requires SNOW-WHITE LEAF1 gene. Plant Cell Physiol. 2013 Oct. 21.

## LABORATORY OF BIOLOGICAL DIVERSITY

### JOHZUKA Group

*Assistant Professor:* *JOHZUKA, Katsuki*
*Technical Staff:* *ISHINE, Naomi*

Chromosome condensation is a basic cellular process that ensures the faithful segregation of chromosomes in both mitosis and meiosis. This process is required not only for shrinking the length of chromosome arms, but also for resolving entanglements between sister-chromatids that are created during DNA replication. Any abnormality in this process leads to segregation errors or aneuploidy, resulting in cell lethality. Studies in the past decade have demonstrated that chromosome condensation is mainly achieved by condensin, a hetero-pentameric protein complex, widely conserved from yeast to humans. Despite its conservation and importance for chromosome dynamics, how condensin works is not well understood. Recent studies reveal that condensin functions are not restricted to chromosome condensation and segregation during cell divisions. They are required for diverse DNA metabolism such as genome stability, transcriptional regulation, and cell differentiation.

Our research interest is to understand the mechanism and regulation of chromosome condensation. We have been studying the role of condensin in the budding yeast *Saccharomyces cerevisiae*. Microscopic observation indicated the nucleolar localization of condensin. Consistent with this, the ribosomal RNA gene (rDNA) repeat is the most condensed region in the genome during mitosis. We have found that condensin specifically binds to the RFB site located within the rDNA repeat. To date, the best characterized condensin binding region is the rDNA repeat on the right arm of chromosome XII in budding yeast. We further discovered the multiple protein network required to recruit condensin to the RFB site.

## I. Dynamic relocalization of condensin during meiosis

Our genetic screening indicated that two proteins, Csm1 and Lrs4, were required for condensin recruitment to the RFB site. Physical interactions between Csm1/Lrs4 and subunits of condensin are important for recruitment of condensin to the RFB site. These proteins are known as components of monopolin complex that are required for faithful segregation of homologous chromosomes during meiotic division I. During meiosis I, monopolin complex re-localizes from rDNA repeat to the centromere and acts for ensuring sister-chromatid co-orientation. Re-localization of Csm1/Lrs4 proteins suggested the re-localization of condensin from rDNA repeat to centromere. As expected, chromatin-IP experiments indicated that condensin re-localizes to the centromere during meiosis I. Condensin might clamp sister-chromatids together during meiosis I.

## II. Condensin-dependent chromatin folding

The RFB site, which consists of a ~150bp DNA sequence, functions as a cis-element for recruitment of condensin to chromatin in the yeast genome. If the RFB site is inserted into an ectopic chromosomal locus, condensin can associate with the ectopic RFB site. To explore the role of condensin in chromosome organization, we have constructed a strain in which two RFB sites are inserted on an ectopic chromosome arm with an interval of 15kb in the cell with complete deletion of chromosomal rDNA repeat. Using this strain, condensin-dependent chromatin interaction between two RFBs was examined by chromosome conformation capture (3C) assay. We found the condensin-dependent chromatin interaction between the two RFB sites on the chromosome arm. This result indicates that condensin plays a role in chromatin interaction between condensin binding sites, and this interaction leads to creation of a chromatin loop between those sites (Figure 1). It is thought that condensin-dependent chromatin folding is one of the basic molecular processes of chromosome condensation. During the cell cycle stages, the RFB - RFB interaction signal increases in metaphase and reaches maximum level in anaphase. In addition to the RFB – RFB interaction, the chromatin interactions between internal regions of two RFBs increases in anaphase. Thus, the configuration of chromatin fiber changes from a simple loop into a complicated twisted shape as the cell cycle progresses from metaphase to anaphase.

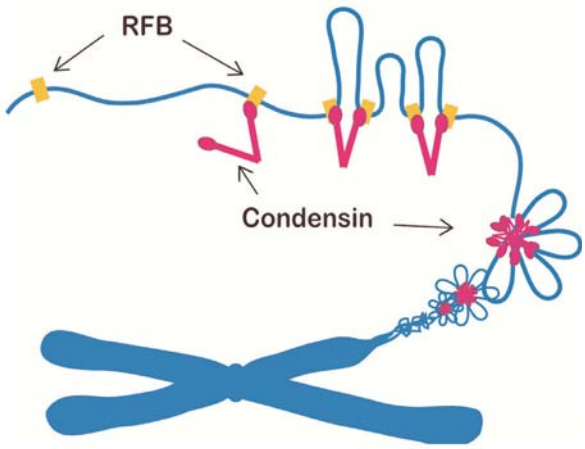


Figure 1. A schematic model of chromosome condensation. Condensin makes chromatin interactions between adjacent binding sites (RFB, for example). This lead to a folding of chromatin fibers between the sites, as a basic process of chromosome condensation.

## LABORATORY OF BIOLOGICAL DIVERSITY

### KATO Group

*Specially Appointed Assistant Professor:*
*KATO, Kagayaki*

Organogenesis is accomplished by a series of deformations of the planar cell sheet into a three-dimensional shape during embryogenesis. This drastic structural change is an integrated result of individual cell behaviors in response to spatio-temporally controlled mechanisms.

To better understand the programs underlying organ formation, it is required to analyze individual cells' morphology and dynamics quantitatively. However, due to the massive images generated by 4D microscopy and their ambiguity, this made it difficult to perform these analyses.

To unveil organogenesis from the point of view of distinct cell behaviors, we developed software applications that are capable of describing cell dynamics out of 4D time-lapse imaging data sets by employing several approaches.

## I. Automated cell tracking system

Epithelial morphogenesis in the fruit fly *Drosophila melanogaster* embryo is considered to be an excellent model for collective cell migrations. Drastic cell rearrangements lead drastic structural changes to build elaborate tubular organs such as the tracheal network. We developed a software pipeline, which automatically recognizes individual cell shapes out of 3D space and tracks them through time. This system extracts cell boundaries and reconstitutes cell shapes as a skeletonized chain of voxels spanning 3D space. This abstract form of cell visualization makes it possible to describe morphometric quantities and kinetics of cells in single-cell resolution (Figure 1). Obtained cell dynamics are subjected to comparative analyses among wild type and several mutants to unveil genetic programs underlying organogenesis.

## II. Particle tracking for tissue deformation analysis

Besides cell boundary extraction, we also developed a derived algorithm for particle image velocimetry (PIV). This system is designed to track local features identified in individual images along a time-series to measure cell dynamics in organogenesis out of hazy images obtained by experimental constraints. This implementation detects local characteristics, such as uneven fluorescence, over the specimen and tracks these patterns along a time-series. By applying this tracking algorithm, we can successfully measure tissue deformation without recognizing individual cells such as in *Xenopus* neuroectoderm (Figure 2).

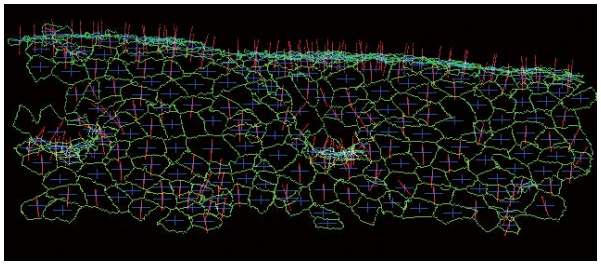

Figure 1. Visualized apical cell surface of *Drosophila* embryonic epidermal cells. A time-lapse confocal microscopic data set of fly embryo expressing E-cadherin-GFP was subjected to our automatic cell shape recognition system. Cell boundaries (green), center of gravity (blue) and normal vector (magenta) are indicated for each cell.

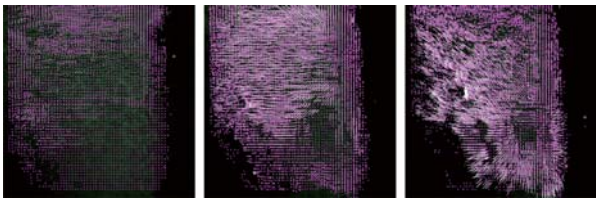

Figure 2. Collective cell migration of *Xenopus* neuroectodermal cells visualized as optical flow along a time-series. A modified PIV method successfully tracks uneven subcellular distribution of GFP signal over time. Dr. M. Suzuki (Prof. Ueno's laboratory at NIBB) performed microscopy.

## III. A GUI application for manual image quantification

Biologically significant image features are not always significant to computational algorithms due to their structural instability. This kind of difficulty requires human eye inspection for feature extractions. A GUI (Graphical User Interface) application we developed can easily visualize 4D imaging data and has made manual feature annotations easy (Figure 3). This application is freely available at our website (https://is.cnsi.jp/).

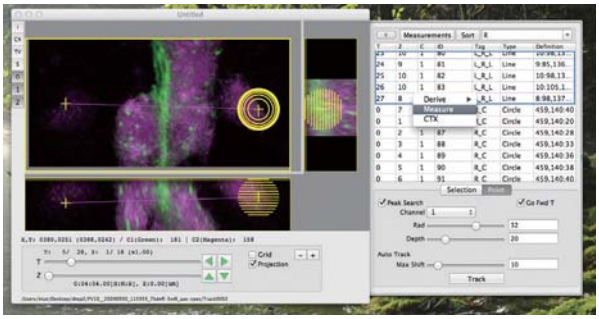

Figure 3. A lightweight/native 4D stack viewer equipped with functions for manual feature extraction.

## LABORATORY OF BIOLOGICAL DIVERSITY

### KIMORI Group

*Specially Appointed Assistant Professor:*
*KIMORI, Yoshitaka*

Image processing methods significantly contribute to visualization of biomedical targets acquired from a variety of imaging techniques, including: wide-field optical and electron microscopy, X-ray computed tomography, magnetic resonance imaging, and mammography. Quantitative interpretation of the deluge of complicated biomedical images, however, poses many research challenges. We have developed new computational methods based on mathematical morphology for quantitative image analysis. One of the most important purposes of image processing is to derive meaningful information, which is expressed as image structural properties. Mathematical morphology is a nonlinear image processing method based on set theory and is useful for the extraction of the structural properties from an image. It can be used as a fundamental tool to analyze biomedical images.

## I. Novel image processing method based on mathematical morphology

Image processing is a crucial step in the quantification of biomedical structures from images. As such, it is fundamental to a wide range of biomedical imaging fields. Image processing derives structural features, which are then numerically quantified by image analysis. Contrast enhancement plays an important role in image processing; it enhances structural features that are barely detectable to the human eye and allows automatic extraction of those features. To effectively recognize a region of interest, specific target structures must be enhanced while surrounding objects remain unmodified. A contrast enhancement technique which used mathematical morphology enables selective enhancement of target structures. Based on set theory, mathematical morphology applies shape information to image processing.

Mathematical morphology operates by a series of morphological operations, which use small images called structuring elements (typically, a single structuring element is used). The structuring element acts as a moving probe that samples each pixel of the image. Since the structuring element moves in a fixed direction across the image, some intricate images (in particular, those whose structural details contain a variety of directional characters) may not be properly processed. Consequently, an artifact in the shape of structuring elements may be generated at the object periphery. Since objects in biomedical images consist of delicate structural features, this drawback is an especially serious problem.

To overcome this problem, we have proposed an extension of conventional mathematical morphology called rotational morphological processing (RMP). The RMP based morphological filters have been applied to a wide variety of biomedical images, including electron micrographs, light micrographs and medical images such as mammographic images and chest X-ray images.

In this study, we have developed a novel RMP-based contrast enhancement method. The method uses a top-hat contrast operator, a well-known and commonly used morphological operation for extracting local features from a low-contrast image. Two types of top-hat operations exist; white top-hat (*WTH*) and black top-hat (*BTH*). *WTH* and *BTH* extract structures brighter and darker than the surrounding areas, respectively. In the proposed method, these RMP-based top-hat operators are computed in parallel. We applied the proposed method to enhancement of structural features in medical images: a mammographic image and a chest radiographic image. The performance of the method was subjectively and quantitatively evaluated by the contrast improvement ratio (*CIR*). The efficiency of the method was clearly demonstrated. Figure 1 shows chest radiographic images enhanced by the proposed method.

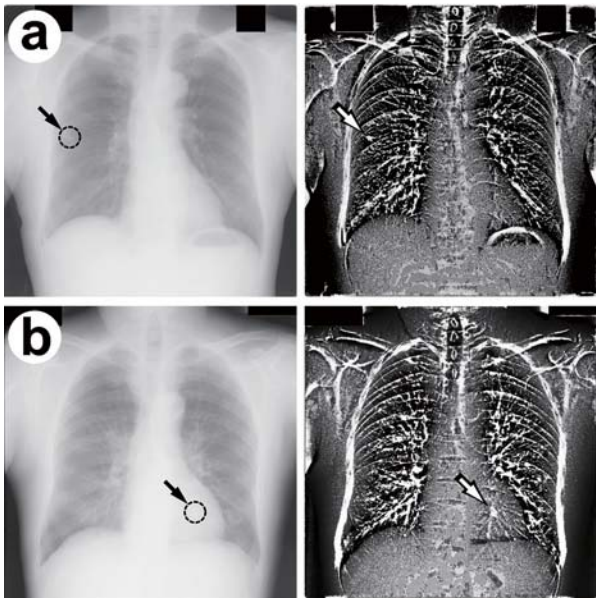


Figure 1. Enhancement of chest radiographic images. Left panel: Original chest radiographic images ((a): JPCLN80, (b): JPCLN152). Arrow in each image indicates nodule. Right panel: contrast enhanced images obtained by the proposed method. Chest radiographic images were obtained from the standard digital image database (Japanese Society of Radiological Technology).

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〔Original papers〕


- Kimori, Y. (2013). Morphological image processing for quantitative shape analysis of biomedical structures: effective contrast enhancement. J. Synchrotron Rad. *20,* 848-853.
- Kimori, Y., Baba, N., and Katayama, E. (2013). Novel configuration of a myosin II transient intermediate analogue revealed by quick-freeze deep-etch replica electron microscopy. Biochemical J. *450,* 23-35.

## Division of Molecular Environmental Endocrinology

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Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ERs) and evoke estrogenic effects. Early evidence that exposure to estrogenic chemicals during development could pose a threat to human health came from studies of a synthetic hormone, diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments showed that exposure of animals to sex hormones during critical windows of perinatal life caused irreversible alterations to the endocrine and reproductive systems of both sexes. The immune and nervous systems, bone, muscle, and the liver were also affected. Although many of these chemicals can bind to ERs in wildlife and humans, the molecular basis for the action of environmental estrogens remains poorly understood. Thus, understanding the molecular mechanisms through which environmental estrogens and sex hormones act during critical developmental windows is essential.

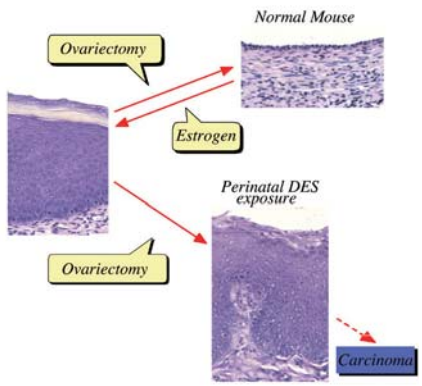


Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

## I. Developmental origin of adult disease: Perinatal estrogen exposure induces persistent changes in reproductive tracts

The emerging paradigm of the "embryonic/fetal origins of adult disease" provides a powerful new framework for considering the effects of endocrine disrupters on human and animal health. In 1971, prenatal DES exposure was found to result in various abnormalities of the reproductive tract in women. This syndrome was named the DES syndrome. Similar abnormalities have been demonstrated in experimental animals exposed perinatally to estrogens. Developmental estrogen exposure in mice, for example, induces persistent proliferation of vaginal epithelial cells (Figure 1). We found that the persistent changes in the vagina in mice exposed neonatally to estrogens result from the persistent activation of erbBs and ERα, and sustained expression of EGF-like growth factors (Figure 2). Currently, we are analyzing the methylation status in the mouse vagina using MeDIP (methylated DNA immunoprecipitation) coupled with a microarray (MeDIP-chip). We found several differentially methylated or demethylated DNA profiles in neonatally DES-exposed mouse vaginae and controls. We thus consider that neonatal DES exposure affects DNA methylation profiles, resulting in persistent abnormalities in mouse reproductive organs.

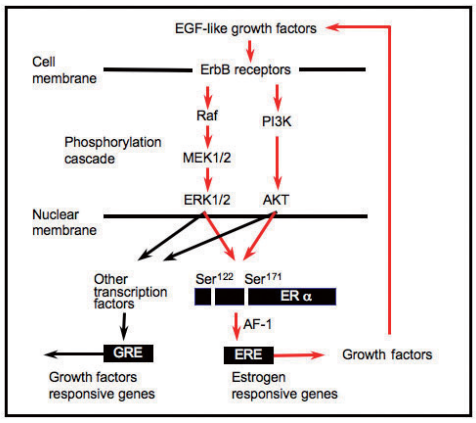


Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vaginae.

## II. Estrogen receptors of birds, reptiles, amphibians and fishes

Steroid and xenobiotic receptors (SXR) have been cloned from various animal species (fish, amphibians, reptiles, birds, and mammals) by our group and we have demonstrated species-specific differences in their responses to various environmental and endogenous chemicals (receptor gene zoo). Thus, simple predictions of chemical effects based on data from a few established model species are not sufficient to develop real world risk assessments. ER and ER-like genes have been cloned from various animal species including rockshell, *Amphioxus*, lamprey, lungfish, sturgeon, gar, roach, stickleback, mosquitofish, mangrove *Rivulus*, catshark, whale shark, Japanese giant salamander, Tokyo

salamander, newt, axolotl, toad, *Silurana tropicalis*, American alligator, Nile crocodile, freshwater turtle, Japanese rat snake, Okinawa habu, and vultures. Functional studies showed that the *Amphioxus* ER sequence does not bind estrogen but *Amphioxus* steroid receptor and lamprey ER exhibited ligand-dependent transactivation, proving that invertebrate and primitive vertebrates, such as the Agnatha, have a functional ER. We found that medaka ER subtypes have their specific functions, and medaka, zebrafish and stickleback ERs are more sensitive to estrogen/estrogen-like chemical exposures than other fishes by reporter gene assay. Thus, these approaches are efficient to evaluate the relationship between species and their sensitivities to chemicals.

## III. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates

Vertebrates show diverse sexual characteristics which are regulated by androgens. To elucidate the evolutionary history and functional diversification of androgen receptor (AR) genes in vertebrates, we cloned the AR cDNAs from a shark, basal ray-finned fishes (Actinopterygii), namely bichir and sturgeon (Acipenseriformes), and teleosts including a basal teleost, arowana (Osteoglossiformes). Molecular phylogenetic analysis revealed that a gene duplication event gave rise to two different teleost ARs (α and β) and likely occurred in the actinopterygian lineage leading to teleosts after the divergence of Acipenseriformes but before the split of Osteoglossiformes. Functional analysis revealed that the shark AR activates the target gene via androgen response element by classical androgens. The teleost ARα showed unique intracellular localization with a significantly higher transactivation capacity than that of teleost ARβ. These results indicate that the most ancient type of AR, as activated by the classic androgens as ligands, emerged before the Chondrichthyes-Osteichthyes split and the *AR* gene was duplicated during a teleost-specific gene duplication event (Figure 3).

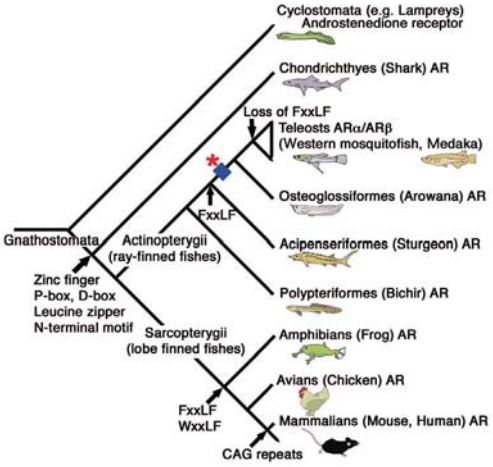


Figure 3. Evolutionary relationships of androgen receptor sequences.

## IV. Papillary process formation in medaka

Androgens play key roles in the morphological specification of male type sex characteristics and reproductive organs, whereas little is known about the developmental mechanisms. Medaka show a prominent masculine sexual character, papillary processes in the anal fin, which has been induced in females by exogenous androgen exposure. We have identified androgen dependent expressions of *Bmp7* and *Lef1* are required for the bone nodule outgrowth leading to the formation of the papillary process in the postal region of the anal fin. We have also developed a testing method for screening of chemicals having androgen and anti-androgenic activity using the anal fin in juvenile medaka.

## V. Sex differentiation mechanism in Daphnids

*Daphnia magna* has been used extensively to evaluate the organism- and population-based responses of toxicity or reproductive toxicity tests. These tests, however, provide no information about the mode of action of the tested compounds. Therefore, we applied an ecotoxicogenomic assessment of *D. magna*. We established a *Daphnia* EST database and developed an oligonucleotide-based DNA microarray with high reproducibility and demonstrated the usefulness of the array for the classification of toxic chemicals as well as for the molecular understanding of chemical toxicity in a common freshwater organism. *D. magna* reproduce asexually (parthenogenesis) when they are in an optimal environment for food, photoperiod and population density. Once environmental conditions become sub-optimal, they alter their reproductive strategy from asexual to sexual reproduction (Figure 4). Chemicals are able to affect the sex determination of *D. magna* and we found that juvenile hormone (JH) agonists (insect growth regulators), for example, induce the production of male offspring. The molecular basis of environmental sex determination is largely unknown in *D. magna*. To understand the molecular mechanisms of this phenomenon we isolated sex determination-related genes. Also, we have developed a method to inject genes into *D. magna* and *D. pulex* embryos which will allow us to study gain- and loss-of function analyses in more detail in this species. Using these techniques, we demonstrated that DSX1 (double sex 1), one

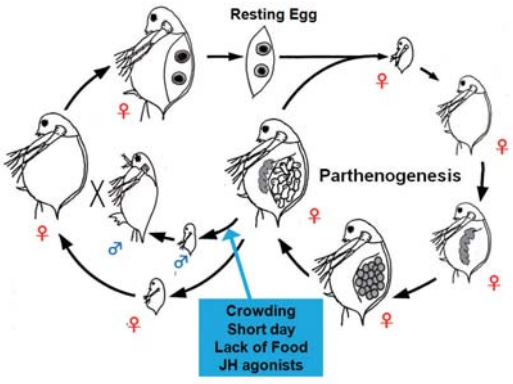


Figure 4. Life cycle of *Daphnia*.

of the DM-domain genes, is essential for male differentiation in *D. magna*. To further explore the signaling cascade of sexual differentiation in *D. magna*, a gene expression profile of JH-responsive genes is essential. We are identifying JH-responsive genes in the ovary of *D. magna* exposed to JH agonist and methyl farnesoate (JH identified in decapods) at the critical timing of JH-induced sex determination in *D. magna*. We have identified JH receptor (heterodimer of methoprene-tolerant and steroid receptor co-activator) in daphnids.

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- Hirakawa, I., Miyagawa, S., Mitsui, N., Miyahara, M., Onishi, Y., Kagami, Y., Kusano, T., Takeuchi, T., Ohta, Y., and Iguchi, T. (2013). Developmental disorders and altered gene expression in the tropical clawed frog (*Silurana tropicalis*) exposed to 17α-ethinylestradiol. J. Appl. Toxicol. *33*, 1001-1010.
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- Katsu, Y., Lange, A., Miyagawa, S., Urushitani, H., Tatarazako, N., Kawashima, Y., Tyler, C.R., and Iguchi, T. (2013). Cloning, expression and functional characterization of carp, *Cyprinus carpio* estrogen receptors and their differential activations by estrogens. J. Appl. Toxicol. *33*, 41-49.
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- Oka, T., Mitsui-Watanabe, N., Tatarazako, N., Onishi, Y., Katsu, Y., Miyagawa, S., Ogino, Y., Yatsu, R., Kohno, S., Takase, M., Kawashima, Y., Aoki, Y., Guillette, L.J.Jr., and Iguchi, T. (2013). Establishment of transactivation assay systems using fish, amphibian, reptilian and human thyroid hormone receptors. J. Appl. Toxicol. *33*, 991-1000.
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- Nakamura, A., Takanobu, H., Tamura, I., Yamamuro, M., Iguchi, T., and Tatarazako, N. Verification of responses of Japanese medaka (*Oryzias latipes*) to antiandrogens, vinclozolin and flutamide, in short-term assays. J. Appl. Toxicol. 2013 Sep. 24.
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- Toyota, K., Kato, Y., Miyakawa, H., Yatsu, R., Mizutani, T., Ogino, Y., Miyagawa, S., Watanabe, H., Nishide, H., Uchiyama, I., Tatarazako, N., and Iguchi, T. Molecular impact of juvenile hormone agonists on neonatal *Daphnia magna*. J. Appl. Toxicol. 2013 Sep. 5.


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- Bergman, Å, Heindel, J.J., Kidd, K.A., Jobling, S., Zoeller, R.T., Becher, G., Bjerregaard, P., Bornman, R., Brandt, I., Brian, J.V., Kortenkamp, A., Muir, D., Ochieng, R., Skakkebaek, N.E., Iguchi, T., Toppari, J., and Woodruff, T.J. (2013). State of the Science of Endocrine Disrupting Chemicals 2012. WHO and UNEP, pp. 260.
- Bergman, Å, Heindel, J.J., Kidd, K.A., Jobling, S., Zoeller, R.T., Becher, G., Bjerregaard, P., Bornman, R., Brandt, I., Brian, J.V., Kortenkamp, A., Muir, D., Ochieng, R., Skakkebaek, N.E., Iguchi, T., Toppari, J., and Woodruff, T.J. (2013). The impact of endocrine disruption: A consensus statement on the state of the science. Environ. Health Perspect. *121*, A104-106.
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## DIVISION OF ENVIRONMENTAL PHOTOBIOLOGY

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Plants and algae have a large capacity to acclimate themselves to changing environments. We are interested in these acclimation processes, in particular, how efficiently yet safely they harness sunlight for photosynthesis under the fluctuating light conditions. Using a model green alga, we are studying the molecular mechanisms underlying the photoacclimation of the photosynthetic machinery. We are also applying the knowledge obtained in the studies of a model green alga to various phytoplankton including diatoms in the subarctic North Pacific, prasinophytes in the subtropical Mediterranean Sea, and *Symbiodinium* in corals and sea anemones in tropical oceans, to explore how these environmentally important photosynthetic organisms thrive in their ecological niche.

## I. Macroorganization of photosynthetic machinery

Photosystem (PS) II is a multiprotein complex that splits water and initiates electron transfer in photosynthesis. The central part of PSII, the PSII core, is surrounded by light-harvesting complex II proteins (LHCIIs). In higher plants, two or three LHCII trimers are seen on each side of the PSII core whereas only one is seen in the corresponding positions in a unicellular green alga *Chlamydomonas reinhardtii*. We re-examined the supramolecular organization of this PSII-LHCII supercomplex in *C. reinhardtii* by solubilizing the thylakoid membranes with n-dodecyl-α-D-maltoside and subjecting them to gel-filtration. This newly-prepared PSII-LHCII supercomplex bound twice as much LHCII than the previously reported supercomplex and retained higher oxygen-evolving activity. Single-particle image analysis of the electron micrographs revealed that the PSII-LHCII supercomplex had a novel supramolecular organization, with three LHCII trimers attached to each side of the core (Figure 1).

## II. Acclimation of photosynthesis

Using *C. reinhardtii*, we investigate the molecular mechanisms underlying the acclimation processes of the photosynthetic complexes by means of biochemistry, molecular genetics, absorption and fluorescence spectroscopy, and bio-imaging.

### 2-1 Non-photochemical quenching

Absorption of light in excess of the capacity for photosynthetic electron transport is damaging to photosynthetic organisms. Several mechanisms exist to avoid photodamage, which are collectively referred to as non-photochemical quenching (NPQ). This term comprises at least two major processes: state transitions (qT), the change in the relative antenna sizes of photosystem II and I, and energy-dependent quenching (qE), the increased thermal dissipation triggered by lumen acidification. Recently, *npq4*, a mutant strain of *C. reinhardtii* that is qE-deficient and lacks the ancient light-harvesting protein LHCSR3 was reported. Applying the newly-established procedure described above, we isolated the PSII-LHCII supercomplex from both WT *C.*

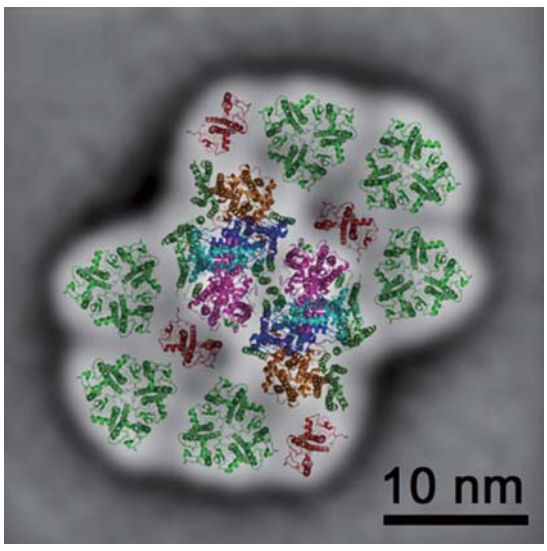


Figure 1. A top view of the PSII-LHCII supercomplex in *C. reinhardtii* as revealed by single particle analysis of electron micrographs.

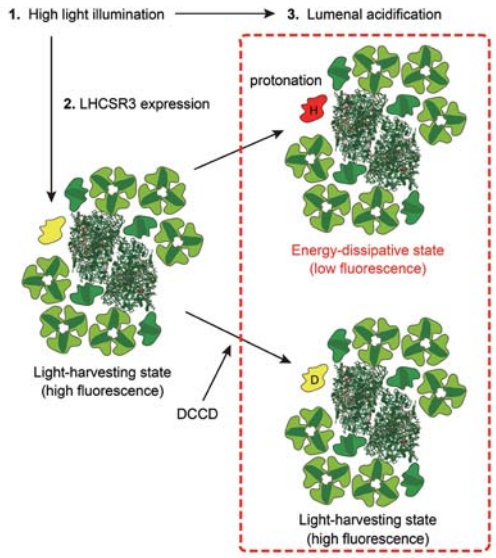


Figure 2. A model for the induction of qE in *C. reinhardtii*.

*reinhardtii* and the *npq4* mutant grown in either low light or high light. LHCSR3 was present in the PSII-LHCII supercomplex from the high light-grown WT but not in the supercomplex from the low light-grown WT or the *npq4* mutant. The purified PSII-LHCII supercomplex containing LHCSR3 showed a normal fluorescence lifetime at a neutral pH (7.5) by single-photon counting analysis but exhibited a significantly shorter lifetime (energy-quenching) at pH 5.5, which mimics the acidified lumen of the thylakoid membranes in high light-exposed chloroplasts. The switching from light-harvesting mode to energy-dissipating mode observed in the LHCSR3-containing PSII-LHCII supercomplex was sensitive to DCCD (dicyclohexylcarbodiimide), a protein-modifying agent specific to protonatable amino acid residues. We conclude that the PSII-LHCII-LHCSR3 supercomplex formed in high light-grown *C. reinhardtii* cells is capable of energy dissipation upon protonation of LHCSR3 (Figure 2).

Furthermore, to investigate the respective roles of qE and qT in photoprotection, a mutant (*npq4 stt7*-9) was generated in *C. reinhardtii* by crossing the state transition-deficient mutant (*stt7-9*) with the *npq4* mutant. The comparative phenotypic analysis of the WT, the single mutants and the double mutant reveals that both state transitions and qE are induced in high light. Moreover, the double mutant presents an increased photosensitivity with respect to the single mutants and WT. We suggest therefore that besides qE, state transitions also play a photoprotective role during high light acclimation of the cells.

### 2-2 Cyclic electron flow

Photosynthetic light reactions establish electron flow in the chloroplast's thylakoid membranes, leading to the production of the ATP and NADPH that participate in carbon fixation. Two modes of electron flow exist—linear electron flow (LEF) from water to $NADP^+$ via photosystem (PS) II and PSI in series and cyclic electron flow (CEF) around PSI. Although CEF is essential for satisfying the varying demand for ATP, the exact molecule(s) and operational site are as yet unclear. In the green alga *C. reinhardtii*, the electron flow shifts from LEF to CEF upon preferential excitation of PSII, which is brought about by an energy balancing mechanism between PSII and PSI (state transitions). We isolated a protein supercomplex composed of PSI with its own light-harvesting complex (LHCI), the PSII light-harvesting complex (LHCII), the cytochrome $b_6f$ complex (Cyt *bf*), ferredoxin (Fd)-NADPH oxidoreductase (FNR), and the integral membrane protein PGRL1 from *C. reinhardtii* cells under PSII-favoring conditions. Spectroscopic analyses indicated that upon illumination, reducing equivalents from downstream of PSI were transferred to Cyt *bf*, while oxidized PSI was re-reduced by reducing equivalents from Cyt *bf*, indicating that this supercomplex is engaged in CEF (Figure 3). Thus, formation and dissociation of the PSI-LHCI-LHCII-FNR-Cyt *bf*-PGRL1 supercomplex not only controlled the energy balance of the two photosystems, but also switched the mode of photosynthetic electron flow.

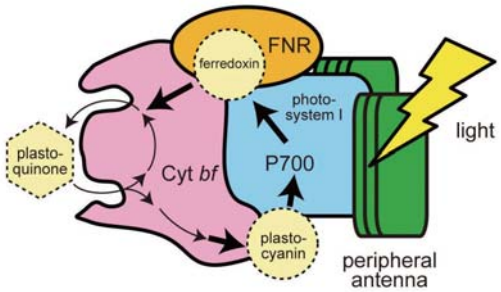


Figure 3. Cyclic electron flow by way of the CEF supercomplex.

## III. Ecophysiology of marine phytoplankton

Prasinophyceae are a broad class of early-branching eukaryotic green algae. These picophytoplankton are found ubiquitously throughout the ocean and contribute considerably to global carbon-fixation. *Ostreococcus tauri*, as the first sequenced prasinophyte, is a model species for studying the functional evolution of light-harvesting systems in photosynthetic eukaryotes.

We isolated and characterized *O. tauri* pigment-protein complexes to understand the diversity and the evolutional traits of the light-harvesting systems in a primitive green alga. Two PSI fractions were obtained by sucrose density gradient centrifugation in addition to free LHC fraction and PSII core fractions. The smaller PSI fraction contains the PSI core proteins, LHCI, which are conserved in all green plants, Lhcp1, a prasinophyte-specific LHC protein, and the minor, monomeric LHCII proteins CP26 and CP29. The larger PSI fraction contained the same antenna proteins as the smaller, with the addition of Lhca6 and Lhcp2, and a 30% larger absorption cross-section. When *O. tauri* was grown under high-light conditions, only the smaller PSI fraction was present. The two PSI preparations were also found to be devoid of far-red chlorophyll fluorescence (715-730 nm), a signature of PSI in oxygenic phototrophs. These unique features of *O. tauri* PSI may reflect primitive light-harvesting systems in green plants and their adaptation to marine ecosystems.

Our newest project is the study of photoacclimation of *Symbiodinium*, which live in a symbiotic relationship with corals, and other Cnidarians. We are particularly interested in those living with corals and sea anemones and are trying to elucidate how their photosynthetic machinery is acclimated to the variable light and temperature environments in the tropical ocean.

### Publication List

〔Original papers〕


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## DIVISION OF SEASONAL BIOLOGY (ADJUNCT)

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Animals living outside the tropics adapt various physiology and behavior to seasonal changes in the environment. As animals use changes in day length and temperature as seasonal cues, these phenomena are referred to as photoperiodism and thermoperiodism, respectively. Medaka provides an excellent model to study these mechanisms because of their rapid and robust seasonal responses. In addition, genomic sequences and transgenic approaches are available in this species. In this division, we are trying to uncover the underlying mechanisms of seasonal adaptation using medaka fish.

## I. Identification of seasonal sensor in fish

In our previous study, we uncovered the signal transduction pathway regulating photoperiodism in birds and mammals. The pars tuberalis of the pituitary gland and the mediobasal hypothalamus are considered to play a critical role in the regulation of photoperiodism. Long-day induced thyrotropin (thyroid-stimulating hormone: TSH) in the pars tuberalis, a master factor regulating photoperiodism, acts on the TSH receptor in the mediobasal hypothalamus to induce *DIO2* expression. DIO2 (type 2 iodothyronine deiodinase) is a thyroid hormone activating enzyme, which converts prohormone thyroxine ($T_4$) to bioactive triiodothyronine ($T_3$).

Most fish living outside the tropics also exhibit a seasonal response and involvement of thyroid hormones in seasonality has been described. However, fish do not possess an anatomically distinct pars tuberalis, which is the regulatory hub of photoperiodism in birds and mammals. Therefore, we tried to identify the photoperiodic center in fish using masu salmon.

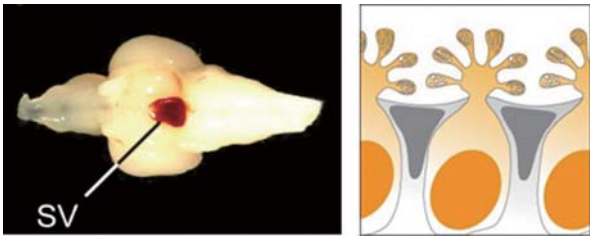


Figure 1. Left, Ventral view of the masu salmon brain. SV: saccus vasculosus. Right, Schematic drawing of the coronet cells.

### 1-1 Expression analysis of key genes regulating photoperiodism in the masu salmon

Photoperiodic regulation of TSH and DIO2 were observed in the saccus vasculosus of masu salmon (Figure 1). In addition, localization of rhodopsin family genes were observed in the saccus vasculosus. Immunohistochemical studies demonstrated that TSH, DIO2 and photoreceptor proteins are expressed in the coronet cells of the saccus vasculosus, suggesting the existence of a photoperiodic signaling pathway from light input to neuroendocrine output in this organ.

### 1-2 Functional analysis of saccus vasculosus

We have cultured saccus vasculosus *in vitro* and exposed them to short day or long day conditions. Isolated saccus vasculosus had the capacity to respond to photoperiodic signals. In addition, removal of the saccus vasculosus abolished the photoperiodic response of the gonad *in vivo*. Although the physiological role of the saccus vasculosus has been a mystery for several centuries, these findings indicated that the saccus vasculosus acts as a seasonal sensor in fish.

## II. Genome-wide association study of seasonal time measurement

It is well established that the circadian clock is somehow involved in seasonal time measurement. However, it remains unknown how the circadian clock measures day length. Additionally, it is not known how animals adapt to seasonal changes in temperature. It has been reported that medaka populations that were caught at higher latitudes have more sophisticated responses to day length and temperature. For example, medaka fish caught in Hokkaido have a critical day length (i.e., duration of light period required to cause a response) of 13 h, while those caught in Okinawa have an 11.5 h critical day length. To uncover the underlying mechanism of seasonal time measurement, we are planning to perform a genome-wide association study in medaka populations collected from various latitudes all over Japan.

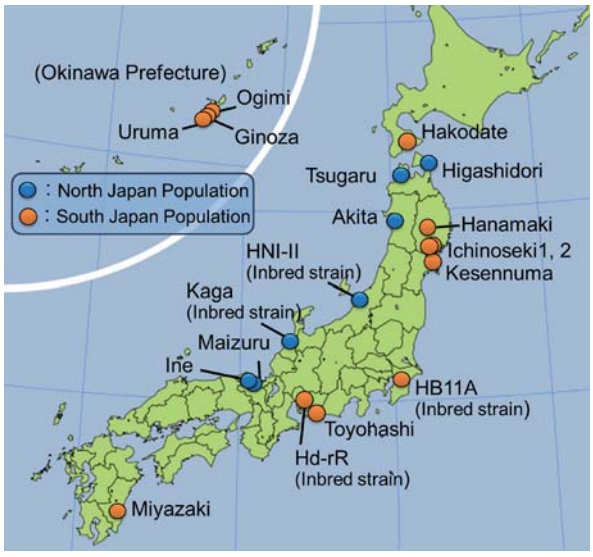


Figure 2. Medaka populations collected and used in our study.

### 2-1 Variation in seasonal responses with latitude in medaka fish

To perform a genome-wide association study, we have collected thousands of medaka fish from all over Japan (Figure 2). This initial year, we have examined the effects of changing day length and temperature to determine the critical day lengths and critical temperatures that will cause seasonal responses in the gonad.

## III. Transcriptome analysis of seasonality in medaka fish

Homeotherms such as birds and mammals do not show clear seasonal responses to changing temperature. In contrast, poikilothermal animals also use changing temeperature as a calendar. Medaka provides an excellent model to uncover this mechanism. To elucidate the signal transduction pathway regulating seasonal reproduction in medaka fish, we have examined transcriptome analysis.

### Publication List

〔Original paper〕

- Nakane, Y., Ikegami, K., Iigo, M., Ono, H., Takeda, K., Takahashi, D., Uesaka, M., Kimijima, M., Hashimoto, R., Arai, N., Suga, T., Kosuge, K., Abe, T., Maeda, R., Senga, T., Amiya, N., Azuma, T., Amano, M., Abe, H., Yamamoto, N., and Yoshimura, T. (2013). The saccus vasculosus of fish is a sensor of seasonal changes in day length. Nature Commun. *4*, 2108.

〔Review articles〕

- Ikegami, K., and Yoshimura, T. (2013) Seasonal time measurement during reproduction. J. Reprod. Develop. *59*, 327-333.
- Yoshimura, T. (2013). Thyroid hormone and seasonal regulation of reproduction. Front. Neuroendocrinol. *34*, 157-166.

## LABORATORY OF GENOME INFORMATICS

*Assistant Professor*
UCHIYAMA, Ikuo

*Postdoctoral Fellow:* *CHIBA, Hirokazu*

The accumulation of biological data has recently been accelerated by various high-throughput "omics" technologies including genomics, transcriptomics, and proteomics. The field of genome informatics is aimed at utilizing this data, or finding the principles behind the data, for understanding complex living systems by integrating the data with current biological knowledge using various computational techniques. In this laboratory we focus on developing computational methods and tools for comparative genome analysis, which is a useful approach for finding functional or evolutionary clues to interpreting the genomic information of various species. The current focus of our research is on the comparative analysis of microbial genomes, the number of which has been increasing rapidly. Since different types of information about biological functions and evolutionary processes can be extracted from comparisons of genomes at different evolutionary distances, we are developing methods to conduct comparative analyses not only of distantly related but also of closely related genomes.

### I. Microbial genome database for comparative analysis

The microbial genome database for comparative analysis (MBGD; URL http://mbgd.genome.ad.jp/) is a comprehensive platform for microbial comparative genomics. The central function of MBGD is to create orthologous groups among multiple genomes from precomputed all-against-all similarity relationships using the DomClust algorithm (see Section II below). By means of this algorithm, MBGD not only provides comprehensive orthologous groups among the latest genomic data available, but also allows users to create their own ortholog groups on the fly using a specified set of organisms. To efficiently explore the diversity of the microbial genomic data, MBGD pre-calculates ortholog tables for each major taxonomic group in the Taxonomy database, and provides several pages to display the entire picture of each pre-calculated ortholog table. For some closely related taxa, MBGD also provides the conserved synteny information (core genome alignment) pre-calculated using the CoreAligner program (see Section III below). In addition, MBGD provides MyMBGD mode, which allows users to add their own genomes to MBGD.

The database continues to grow and now contains more than 2500 published genomes including 41 eukaryotic microbes and 4 multicellular organisms. To further enhance the database, we are now preparing to incorporate genomic data released as draft sequence data, which are now growing as rapid as complete sequences. Data will be incorporated in some pre-computed ortholog tables, and also will be provided for users to incorporate in their analysis through the MyMBGD function.

### II. Improvement of the methods for constructing orthologous groups among multiple genomes at the domain level

As a core technology of our comparative genomics tools, we have developed a rapid automated method of ortholog grouping, named DomClust, which is effective enough to allow the comparison of many genomes simultaneously. The method classifies genes based on a hierarchical clustering algorithm using all-against-all similarity data as an input, but it also detects domain fusion or fission events and splits clusters into domains if required. As a result, the procedure can split genes into the domains minimally required for ortholog grouping.

Although DomClust can rapidly construct orthologous groups at the domain level, its classification quality has room for improvement since it is based on pairwise sequence alignment. We developed a procedure to refine the DomClust classification based on multiple sequence alignments. The method is based on a scoring scheme, the domain-specific sum-of-pairs (DSP) score, which evaluates ortholog clustering results at the domain level as the sum total of domain-level alignment scores. We developed a refinement pipeline to improve domain-level clustering, DomRefine, by optimizing the DSP score. We applied DomRefine to domain-based ortholog groups created by DomClust using a dataset obtained from the MBGD database, and evaluated the results using COG and TIGRFAMs as the reference data. Thus, we observed that the agreement between the resulting classification and the classifications in the reference databases is improved in the refinement pipeline. Moreover, the refined classification showed better agreement than the classifications in the eggNOG databases when TIGRFAMs was used as the reference database (Figure 1).

We are also developing a method to update the clustering result incrementally, by which we can add new genomes to a reference set of ortholog groups. This approach allows us to conduct further large-scale ortholog analysis including draft genome sequences. We are also extending the algorithm for handling metagenomic data. To infer the taxonomic position of the source organism of each metagenomic sequence, we have developed a method to map each tree node of the hierarchical clustering tree generated by the DomClust algorithm onto a taxonomic tree node.

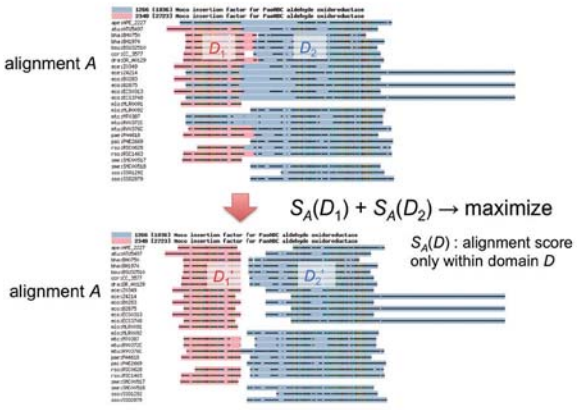


Figure 1. Definition of DSP score to evaluate domain-based classification in DomRefine.

## III. Identification of the core structure conserved among moderately related microbial genomes

Horizontal gene transfers (HGT) have played a significant role in prokaryotic genome evolution, and the genes constituting a prokaryotic genome appear to be divided into two classes: core and accessory. The core gene set comprises intrinsic genes encoding the proteins of basic cellular functions, whereas the accessory gene set comprises HGT-acquired genes encoding proteins which function under particular conditions. We consider the core structure of related genomes as a set of sufficiently long segments in which gene orders are conserved among multiple genomes so that they are likely to have been inherited mainly through vertical transfer (called "syntenic core"). To find such core structures, we developed a method named CoreAligner.

We systematically applied the method to bacterial taxa (family, genus and species) that contain a sufficient number of completed genomes stored in MBGD, and the resulting syntenic core gene sets were compared with corresponding universal core gene sets based on the conventional definition. As a result, syntenic core is generally larger than universal core, and typically the number of syntenic core genes is more stable than universal core when the number of genomes in the given taxa increases.

## IV. Development of a workbench for comparative genomics

We are developing a comparative genomics workbench named RECOG (Research Environment for COmparative Genomics), which aims to extend the current MBGD system by incorporating more advanced analysis functionalities including phylogenetic pattern analysis, the ingroup/outgroup distinction in ortholog grouping and the core structure extraction among related genomes. The central function of RECOG is to display and manipulate a large-scale ortholog table. The ortholog table viewer is a spreadsheet like viewer that can display the entire ortholog table, containing more than a thousand genomes. In RECOG, several basic operations on the ortholog table are defined, which are classified into categories such as filtering, sorting, and coloring, and various comparative analyses can be done by combining these basic operations, such as "Neighborhood gene clustering" and "Phylogenetic pattern clustering." In addition, RECOG allows the user to input arbitrary gene properties such as sequence length, nucleotide/amino acid contents and functional classes, and compare these properties among orthologs in various genomes. We continue to develop the system and apply it to various genome comparison studies under collaborative research projects.

## V. Utilizing ortholog data to integrate microbial database using Semantic Web technologies

Orthology is a key to integrate knowledge about various organisms through comparative analysis. Moreover, presence/absence of orthologs in each genome can be an important clue to understand the relationship between gene functions and species phenotype/habitat. Toward this goal, we are trying to integrate various types of microbial data with genome/metagenome data and orthology relation using Semantic Web technologies. For this purpose, we described orthology relation and related data stored in MBGD and other databases using Resource Description Framework (RDF) and launched a SPARQL endpoint to query the database via the SPARQL language. This is part of the MicrobeDB project, a collaborative project under the National Bioscience Database Center.

## VI. Identification of mobile genes and its application to characterizing *H. pylori* pan-genome repertoire

Gene contents of the same bacterial species can have great variation and thus the whole repertoire of genes in a bacterial species, termed the pan-genome, can be very large. We analyzed the pan-genome identified among 30 strains of the human gastric pathogen *Helicobacter pylori* isolated from various phylogeographical groups. We identified co-occurring gene clusters using phylogenetic pattern clustering combined with neighboring gene clustering implemented in the RECOG system. In addition, we developed a method to define mobility of genes against the reference coordinate determined by the syntenic core alignment created by CoreAligner, and classified each accessory gene into mobility classes. On the basis of these analyses, we characterized the repertoire of accessory genes in *H. pylori* strains in terms of co-occurring gene clusters and mobility. This work is in collaboration with Prof. Kobayashi, Univ. Tokyo.

### Publication List

〔Original papers〕

- Uchiyama, I., Mihara, M., Nishide, H., and Chiba, H. (2013). MBGD update 2013: the microbial genome database for exploring the diversity of microbial world. Nucleic Acids Res. *41*, D631-D635.
- Yahara, K., Furuta, Y., Oshima, K., Yoshida, T., Azuma, T., Hattori, M., Uchiyama, I., and Kobayashi, I. (2013). Chromosome painting in silico in a bacterial species reveals fine population structure. Mol. Biol. Evol. *30*, 1454-1464.

〔Original paper (E-publication ahead of print)〕

- Toyota, K., Kato, Y., Miyakawa, H., Yatsu, R., Mizutani, T., Ogino, Y., Miyagawa, S., Watanabe, H., Nishide, H., Uchiyama, I., Tatarazako, N., and Iguchi, T. Molecular impact of juvenile hormone agonists on neonatal *Daphnia magna*. J. Appl. Toxicol. 2013 Sep. 5.

## LABORATORY FOR SPATIOTEMPORAL REGULATIONS

*Associate Professor*
NONAKA, Shigenori

*Technical Staff:* *KAJIURA-KOBAYASHI, Hiroko*
*Postdoctoral Fellow:* *TAKAO, Daisuke*
*MARUYAMA, Atsushi*
*NIBB Research Fellow:* *TANIGUCHI, Atsushi*
*Technical Assistant:* *ISHIBASHI, Tomoko*

Our laboratory is currently pursuing two paths of scientific inquiry of interest to developmental biology. One goal is to clarify the mechanism of left-right (L-R) determination. The other is to develop imaging technologies for biological analyses.

### I. Initial step for left-right asymmetry

In mammalian development, the initial left-right asymmetry first appears as cilia-driven leftward fluid flow on the ventral surface of the node, and the flow provides the initial cue for asymmetric gene expressions that actually determine asymmetric morphogenesis. The mechanism that converts the flow to the asymmetric gene expression, i.e. the flow sensing mechanism, remains controversial, with several models being proposed, and involvement of $Ca^{2+}$ being suggested.

We pursued this question by measuring $Ca^{2+}$ dynamics in the node and found that the node cells cause apparently stochastic elevation of $Ca^{2+}$, and its spatiotemporal distribution is equal at the left and right sides but becomes more frequent at the left after late headfold stage, when the flow-induced commitment occurs. This asymmetry was disrupted in dynein mutant *iv*/*iv* and *pkd2*$^{-/-}$ mutants, in accordance to their left-right phenotypes.

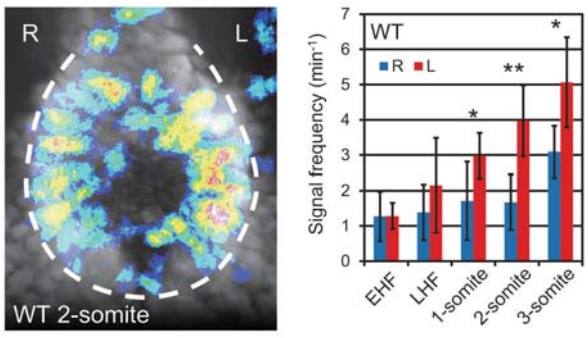


Figure 1. Left: Distribution of $Ca^{2+}$ elevation in a 2-somite wild-type node. Right: Time course of $Ca^{2+}$ elevation frequency at the left and the right sides.

### II. Development of light-sheet microscopy

Light-sheet microscopy has many advantages for live imaging including low photobleaching and phototoxicity, high penetration of depth, and fast imaging acquisition. This method has also peculiar disadvantages, however.

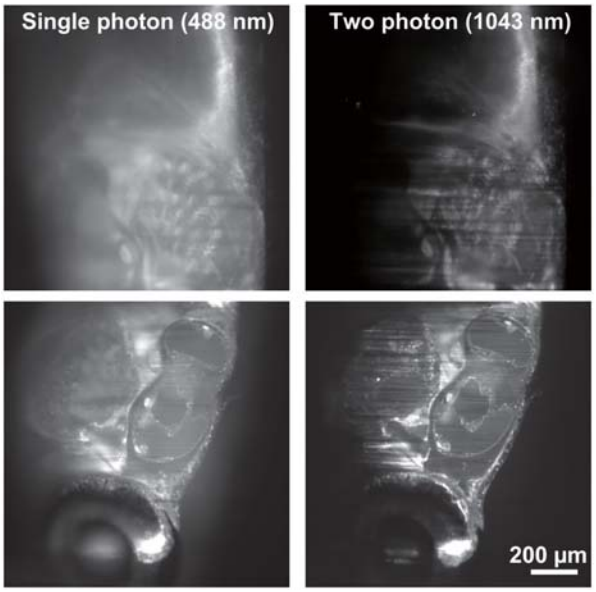


Figure 2. Two-photon light-sheet microscopy. Left: Fluorescent images of a medaka taken by a conventional (one-photon) light-sheet microscope. Right: Images of the same area taken by our two-photon light-sheet microscope.

Specifically scattering of excitation light within the specimen and illumination of areas besides the focal plane, and deterioration of contrast. A solution to these problems is combining light-sheet microscopy with two-photon excitation (TPE), but this results in a narrow field of view, because generation of TPE images requires very high photon density, i.e. focusing with a high numerical aperture (NA) lens.

We utilized a new fiber laser with high peak power to overcome this problem, and enabled observation of larger specimens using a hybrid TPE light-sheet microscope.

### Publication List

〔Original papers〕

- Ichikawa, T., Nakazato, K., Keller, P.J., Kajiura-Kobayashi, H., Stelzer, E.H., Mochizuki, A., and Nonaka, S. (2013). Live imaging of whole mouse embryos during gastrulation: migration analyses of epiblast and mesodermal cells. PLoS ONE *8*, e64506.
- Murata, T., Sano, T., Sasabe, M., Nonaka, S., Higashiyama, T., Hasezawa, S., Machida, Y., and Hasebe, M. (2013). Mechanism of microtubule array expansion in the cytokinetic phragmoplast. Nature Commun. *4*, 1967.
- Takao, D., Nemoto, T., Abe, T., Kiyonari, H., Kajiura-Kobayashi, H., Shiratori, H., and Nonaka, S. (2013). Asymmetric distribution of dynamic calcium signals in the node of mouse embryo during left-right axis formation. Develop. Biol. *376*, 23-30.

〔Review article〕

- Nonaka, S. (2013). Visualization of mouse nodal cilia and nodal flow. Methods Enzymol. *525*, 149-157.

## NIBB Core Research Facilities

*Head*
KOBAYASHI, Satoru

The NIBB Core Research Facilities were launched in 2010 to support basic biology research in NIBB. They consist of three facilities that are developing and providing state-of-the-art technologies to understand biological functions through functional genomics, bioimaging and bioinformatics.

The NIBB Core Research Facilities also act as an intellectual hub to promote collaboration among the researchers of NIBB and other academic institutions.

### Functional Genomics Facility

*Specially Appointed Associate Professor*
SHIGENOBU, Shuji

| | |
|---|---|
| *Technical Staff:* | *MORI, Tomoko* |
| | *MAKINO, Yumiko* |
| | *YAMAGUCHI, Katsushi* |
| *Technical Assistant:* | *ASAO, Hisayo* |
| | *WAKAZUKI, Sachiko* |
| | *MATSUMOTO, Miwako* |
| | *FUJITA, Miyako* |
| *Secretary:* | *ICHIKAWA, Mariko* |

The Functional Genomics Facility is a division of the NIBB Core Research Facilities and organized jointly by NIBB and NIPS for promoting DNA and protein studies. The facility maintains a wide array of core research equipment, from standard machinery like ultracentrifuges to cutting edge tools such as next generation DNA sequencers, which amount to 60 different kinds of instrument. The facility is dedicated to fostering collaborations with researchers both of NIBB and other academic institutions worldwide by providing these tools as well as expertise. Our current focus is supporting functional genomics works that utilize mass spectrometers and DNA sequencers. We also act as a bridge between experimental biology and bioinformatics.

### Representative Instruments

#### *Genomics*

The advent of next-generation sequencing (NGS) technologies is transforming today's biology by ultra-high-throughput DNA sequencing. Utilizing the SOLiD5500xl (Applied Biosystems), HiSeq2500, HiSeq1500 (Illumina), and MiSeq (Illumina) the Functional Genomics Facility is committed to joint research aiming to explore otherwise inaccessible new fields in basic biology.

During 2013 we carried out 41 NGS projects in collaboration with NIBB laboratories as well as the researchers of other academic institutions. These projects cover a wide range of species (bacteria, animals, plants, and humans) including both model and non-model organisms, and various applications such as genomic re-sequencing, RNA-seq and ChIP-seq.

Figure 1. Next-generation sequencer

#### *Proteomics*

Three different types of mass spectrometer and two protein sequencers, as listed below, are used for proteome studies in our facility. In 2013, we analyzed approximately 300 samples with mass spectrometers and 50 samples with protein sequencers.

- GC-Mass Spectrometer (JEOL DX-300)
- MALDI-TOF-MS (Bruker Daltonics REFLEX III)
- LC-Q-TOF MS (Waters Q-TOF Premier)
- Protein sequencer (ABI Procise 494 HT; ABI Procise 492 cLC)

#### *Other analytical instruments*

- Cell sorter (SONY SH800)
- Bioimaging Analyzer (Fujifilm LAS 3000 mini; GE FLA9000)
- Laser Capture Microdissection System (Arcturus XT)
- DNA Sequencer (ABI PRISM 310; ABI 3130xl)
- Real Time PCR (ABI 7500)
- Ultra Centrifuge (Beckman XL-80XP etc.)

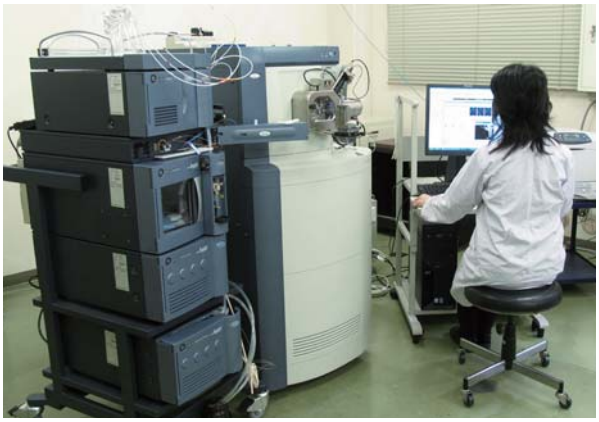

Figure 2. LC-Q-TOF mass spectrometer

## Genome Informatics Training Course

We organize NIBB Genome Informatics Training Courses every year. In 2013, we provided two two-day training courses on next-generation sequence data analyses and transcriptome analysis. These courses are designed to introduce the basic knowledge and skills of bioinformatics analysis to biologists who are not familiar with bioinformatics.

Figure 3. NIBB Genome Informatics Training Course

### Publication List on Cooperation

〔Original papers〕


- Arimura, T., Onoue, K., Takahashi-Tanaka, Y., Ishikawa, T., Kuwahara, M., Setou, M., Shigenobu, S., Yamaguchi, K., Bertrand, A.T., Machida, N., *et al*. (2013). Nuclear accumulation of androgen receptor in gender difference of dilated cardiomyopathy due to lamin A/C mutations. Cardiovasc. Res. *99*, 382–394.
- Ishikawa, T., Okada, T., Ishikawa-Fujiwara, T., Todo, T., Kamei, Y., Shigenobu, S., Tanaka, M., Saito, T.L., Yoshimura, J., Morishita, S., *et al*. (2013). ATF6α/β-mediated adjustment of ER chaperone levels is essential for development of the notochord in medaka fish. Mol. Biol. Cell *24*, 1387–1395.
- Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y., and Kawaguchi, M. (2013). Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. Nat. Commun. *4*, 2191.
- Tabata, R., Kamiya, T., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M., Fujiwara, T., and Sawa, S. (2013). Identification of an EMS-induced causal mutation in a gene required for boron-mediated root development by low-coverage genome re-sequencing in Arabidopsis. Plant Signal. Behav. *8*, e22534.
- Takahara, M., Magori, S., Soyano, T., Okamoto, S., Yoshida, C., Yano, K., Sato, S., Tabata, S., Yamaguchi, K., Shigenobu, S., *et al*. (2013). TOO MUCH LOVE, a novel kelch repeat-containing F-box protein, functions in the long-distance regulation of the Legume-Rhizobium symbiosis. Plant Cell Physiol. *54*, 433–447.
- Tokuda, G., Elbourne, L.D.H., Kinjo, Y., Saitoh, S., Sabree, Z., Hojo, M., Yamada, A., Hayashi, Y., Shigenobu, S., Bandi, C., *et al*. (2013). Maintenance of essential amino acid synthesis pathways in the *Blattabacterium cuenoti* symbiont of a wood-feeding cockroach. Biol. Lett. *9*, 20121153.
- Wang, Z., Pascual-Anaya, J., Zadissa, A., Li, W., Niimura, Y., Huang, Z., Li, C., White, S., Xiong, Z., Fang, D., *et al*. (2013). The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. Nat. Genet. *45*, 701–706.


〔Original paper (E-publication ahead of print)〕


- Uehara, M., Wang, S., Kamiya, T., Shigenobu, S., Yamaguchi, K., Fujiwara, T., Naito, S., and Takano, J. (2014). Identification and characterization of an Arabidopsis mutant with altered localization of NIP5;1, a plasma membrane boric acid channel, reveals the requirement for D-galactose in endomembrane organization. Plant Cell Physiol. 2013 Dec. 15.


### ● *Research activity by S. Shigenobu*

*Specially Appointed Associate Professor:* *SHIGENOBU, Shuji*
*NIBB Research Fellow:* *MAEDA, Taro*
*Technical Assistant:* *SUZUKI, Miyuzu*
*Visiting Graduate Student:* *OGAWA, Kota*

## Symbiosis Genomics

*"Nothing, it seems, exists except as part of a network of interactions." (Gilbert & Epel, 2008)*

Every creature on the earth exists among a network of various biological interactions. For example, many multicellular organisms, including humans, harbor symbiotic bacteria in their bodies: some of them provide their hosts with essential nutrients deficient in the host's diet and others digest foods indigestible by the host alone. In spite of numerous examples of symbioses and its intriguing outcomes, the genetic and molecular basis underlying these interactions remains elusive. The goal of our group is to establish a new interdisciplinary science "Symbiosis Genomics", where we aim to understand the network of biological interactions at the molecular and genetic level. To this end, we take advantage of state-of-the-art genomics such as next-generation sequencing technologies.

### I. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont

Aphid species bear intracellular symbiotic bacteria in the cytoplasm of bacteriocytes, specialized cells for harboring the bacteria. The mutualism is so obligate that neither can reproduce independently. The 464 Mb draft genome sequence of the pea aphid, *Acyrthosiphon pisum*, in consort with that of bacterial symbiont *Buchnera aphidicola* illustrates the remarkable interdependency between the two organisms. Genetic capacities of the pea aphid and the symbiont for amino acid biosynthesis are complementary. The genome analysis revealed that the pea aphid has undergone characteristic gene losses and duplications. The IMB antibacterial immune pathway is missing several critical genes, which might account for the evolutionary success of aphids to obtain beneficial symbionts. Lineage-specific gene duplications have occurred in genes in a broad range of functional categories, which include signaling pathways, miRNA machinery, chromatin modification and mitosis. The importance of these duplications for symbiosis remains to be determined. We found several instances of lateral gene transfer from bacteria to the pea aphid genome. Some of them are highly expressed in bacteriocytes.

Aphid research is entering the post-genome era. We analyzed the transcriptome of aphid bacteriocytes using RNA-seq technology featuring a next-generation DNA sequencer. We found thousands of genes over-represented in

the symbiotic organ in comparison with the whole body. Many genes for amino acid metabolism are found to be over-represented as expected: the plant sap-eating insect depends on the bacterial symbionts to supply essential amino acids. In addition, many kinds of novel secretion proteins that are found only in aphid species are extremely enriched in the bacteriocytes. We also found that bacteriocytes express Distal-less (Dll), a homeodomain-containing transcription factor throughout the life cycle. Future study should focus on dissecting the genetic network of these components, which should allow us to understand the genetic basis on which symbiosis generates evolutionary novelty.

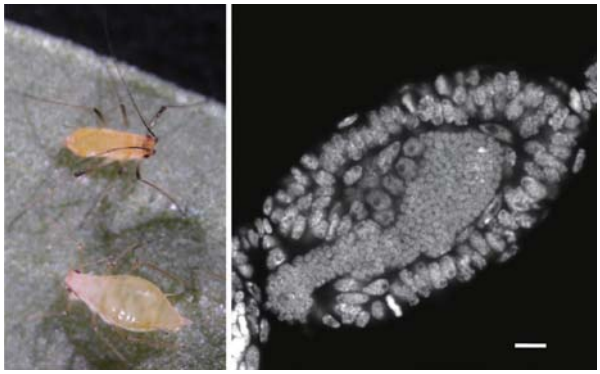

Figure 1. Pea aphids and the bacterial symbiont, *Buchnera*. Adult aphids (Left). A developing viviparous embryo which symbionts are infecting (Right). Scale bar = 20um.

## Publication List

〔Original papers〕


- Chang, C.-C., Hsiao, Y.-M., Huang, T.Y., Cook, C.E., Shigenobu, S., and Chang, T.-H. (2013). Noncanonical expression of caudal during early embryogenesis in the pea aphid *Acyrthosiphon pisum*: maternal cad-driven posterior development is not conserved. Insect Mol. Biol. *22*, 442–455.
- Hayashi, Y., Shigenobu, S., Watanabe, D., Toga, K., Saiki, R., Shimada, K., Bourguignon, T., Lo, N., Hojo, M., Maekawa, K., *et al*. (2013). Construction and characterization of normalized cDNA libraries by 454 pyrosequencing and estimation of DNA methylation levels in three distantly related termite species. PLoS ONE *8*, e76678.
- Shibata, T.F., Maeda, T., Nikoh, N., Yamaguchi, K., Oshima, K., Hattori, M., Nishiyama, T., Hasebe, M., Fukatsu, T., Kikuchi, Y., *et al*. (2013). Complete genome sequence of *Burkholderia* sp. strain RPE64, bacterial symbiont of the bean bug *Riptortus pedestris*. Genome Announc. *1*, e00441–13.
- Shigenobu, S., and Stern, D.L. (2013). Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. Proc. Royal Soc. B Biol. Sci. *280*, 20121952.
- Suzuki, M.M., Yoshinari, A., Obara, M., Takuno, S., Shigenobu, S., Sasakura, Y., Kerr, A.R., Webb, S., Bird, A., and Nakayama, A. (2013). Identical sets of methylated and nonmethylated genes in *Ciona intestinalis* sperm and muscle cells. Epigenetics Chromatin *6*, 38.


## Spectrography and Bioimaging Facility

*Specially Appointed Associate Professor*
KAMEI, Yasuhiro

*Technical Staff:* *HIGASHI, Sho-ichi*
*TANIGUCHI-SAIDA, Misako*
*UCHIKAWA, Tamaki*
*Technical Assistant:* *ICHIKAWA, Chiaki*
*Secretary:* *ISHIKAWA, Azusa*

The Spectrography and Bioimaging Facility assists both collaborative and core research by managing and maintaining research tools that use “Light”. The facility also provides technical support through management of technical staff assisting in the advancement of collaborative and core research projects, as well as academic support to researchers. Among its tools are advanced microscopes for biology and the Okazaki Large Spectrograph for photobiology. The Okazaki Large Spectrograph is the world’s largest wide spectrum exposure mechanism, capable of producing a range of wavelengths from 250 nm (ultraviolet) to 1,000 nm (infrared) along its 10 meter focal curve; allowing exposure to strong monochromatic light. The facility’s microscopes, which are cutting edge devices such as confocal and multi-photon excitation microscopes, are used by both internal and external researchers as vital equipment for core and collaborative research projects.

### Representative Instruments:

### Okazaki Large Spectrograph (OLS)

The spectrograph runs on a 30 kW Xenon arc lamp and projects a wavelength spectrum from 250 nm (ultraviolet) to 1,000 nm (infrared) onto its 10 m focal curve with an intensity of monochromatic light at each wavelength more than twice as much as that of the corresponding monochromatic component of tropical sunlight at noon (Watanabe *et al*., Photochem. Photobiol. *36*, 491-498, 1982).

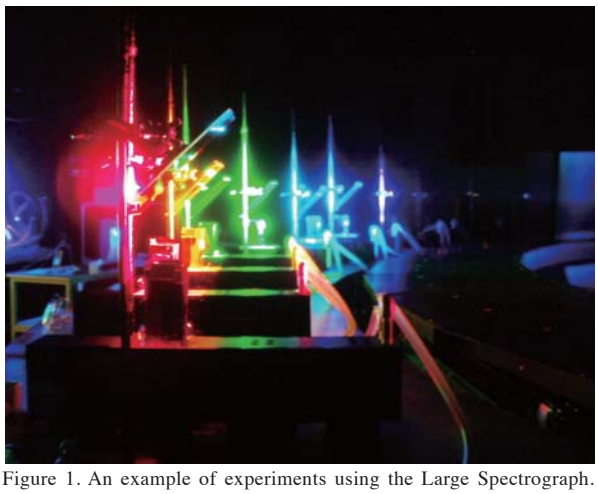

Figure 1. An example of experiments using the Large Spectrograph. Various color rays (monochromatic light from right side and reflected by mirrors) were irradiated simultaneously to samples in cooling chambers.

The spectrograph is dedicated to action spectroscopical studies of various light-controlled biological processes.

The NIBB Collaborative Research Program for the Use of the OLS supports about 10 projects every year conducted by both visiting scientists, including foreign researchers, as well as those in NIBB.

Action spectroscopical studies for various regulatory and damaging effects of light on living organisms, biological molecules, and artificial organic molecules have been conducted.

## Microscopes

This facility also has Bioimaging machines such as widefield microscopes (Olympus IX-81, BX-63 and KEYENCE BZ-8000), confocal microscopes (Olympus FV1000, Nikon A1R, Nikon A1Rsi, Carl Zeiss Duo 5 and Yokogawa CSU-X1), multi-photon microscopes (Olympus FV1000-MP, Leica SP8 MPs) and other advanced custom-made laser microscopes with special aims (Digital Scanned Light-sheet Microscope: DSLM and Infrared Laser-Evoked Gene Operator microscope: IR-LEGO) for users in NIBB and collaborative guest researchers. We began Collaborative Research Programs using these machines in 2010.

The DSLM was developed by Dr. Ernst Stelzer's group at the European Molecular Biology Laboratory (EMBL). This microscope can realize high-speed z-axis scanning in deeper tissue by illuminating a specimen from the side with a light sheet (more information is given in Dr. Nonaka's section: Lab. for Spatiotemporal Regulations). Dr. Nonaka conducted and supported 7 projects of the Collaborative Research Program for the Use of the DSLM. The IR-LEGO was developed by Drs. Shunsuke Yuba and Yasuhiro Kamei at the National Institute of Advanced Industrial Science and Technology (AIST). This microscope can induce a target gene of interest by heating a single target cell *in vivo* with a high efficiency irradiating infrared laser (Kamei *et al*. Nat. Methods, 2009). Details are described in the next section. The IR-LEGO was also used for 10 Individual Collaborative Research projects, including applications for animals and higher plants.

## Workshop and Symposium

In 2013, we held workshops (training courses) on IR-LEGO for vertebrates (frog and fish) and biological image processing. We also have been holding a "Bioimaging Forum" every year which discusses Bioimaging from various directions such as microscopy, new photo-technology, and computer science.

### Publication List on Cooperation

〔**Original papers (Selected)**〕


- Allorent, G., Tokutsu, R., Roach, T., Peers, G., Cardol, P., Girard-Bascou, J., Seigneurin-Berny, D., Petroutsos, D., Kuntz, M., Breyton, C., Franck, F., Wollman, F.-A., Niyogi, K.K., Krieger-Liszkay, A., Minagawa, J. and Finazzi, G. (2013). A dual strategy to cope with high light in *Chlamydomonas reinhardtii*. Plant Cell. *25*, 545-557.
- Ikehata, H., Higashi, S., Nakamura, S., Daigaku, Y., Furusawa, Y., Kamei, Y., Watanabe, M., Yamamoto, K., Hieda, K., Munakata, N., and Ono, T. (2013). Action spectrum analysis of UVR genotoxicity for skin: the border wavelengths between UVA and UVB can bring serious mutation loads to skin. J. Invest. Dermatol. *133*, 1850-1856.
- Shikata, T., Matsunaga, S., Iseki, M., Nishide, H., Higashi, S., Kamei, Y., Yamaguchi, M., Jenkinson, I.R., and Watanabe, M. (2013). Blue light regulates the rhythm of diurnal vertical migration in the raphidophyte red-tide alga *Chattonella antiqua*. J. Plankton Res. *35*, 542-552.
- Okuyama, T., Isoe, Y., Hoki, M., Suehiro, Y., Yamagishi, G., Naruse, K., Kinoshita, M., Kamei, Y., Shimizu, A., Kubo, T., and Takeuchi, H. (2013). Controlled cre/loxP site-specific recombination in the developing brain in medaka fish, *Oryzias latipes*. PLoS ONE. *8*, e66597.
- Murata, T., Sano, T., Sasabe, M., Nonaka, S., Higashiyama, T., Hasezawa, S., Machida, Y., and Hasebe, M. (2013). Mechanism of microtubule array expansion in the cytokinetic phragmoplast. Nat. Commun. *4*, 1967.
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- Takahara, M., Magori, S., Soyano, T., Okamoto, S., Yoshida, C., Yano, K., Sato, S., Tabata, S., Yamaguchi, K., Shigenobu, S., Takeda, N., Suzaki, T., and Kawaguchi, M. (2013). Too much love, a novel Kelch repeat-containing F-box protein, functions in the long-distance regulation of the legume-Rhizobium symbiosis. Plant Cell Physiol. *54*, 433-447.
- Yamagata, Y., Kaneko, K., Kase, D., Ishihara, H., Nairn, A.C., Obata, K., and Imoto, K. (2013). Regulation of ERK1/2 mitogen-activated protein kinase by NMDA-receptor- induced seizure activity in cortical slices. Brain Res. *24*, 1-10.
- Kimura, E., Deguchi, T., Kamei, Y., Shoji, W., Yuba, S., and Hitomi, J. (2013). Application of infrared laser to the zebrafish vascular system: gene induction, tracing, and ablation of single endothelal cells. Arterioscler. Thromb. Vasc. Biol. *33*, 1264-1270.
- Hira, R., Ohkubo, F., Tanaka, Y.R., Masamizu, Y., Augustine, J.G., Kasai, H., and Matsuzaki, M. (2013). In vivo optogenetic tracing of functional corticocortical connections between motor forelimb areas. Front. Neural Circuits. *7*, 55.
- Shimada, A., Kawanishi, T., Kaneko, T., Yoshihara, H., Yano, T., Inohaya, K., Kinoshita, M., Kamei, Y., Tamura, K., and Takeda, H. (2013). Trunk exoskeleton in teleosts is mesodermal in origin. Nat. Commun. *4*, 1639.
- Kobayashi, K., Kamei, Y., Kinoshita, M., Czerny, T., and Tanaka, M. (2013). A heat-inducible Cre/LoxP gene induction system in medaka. Genesis. *51*, 59-67.
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- Suzaki, T., Kim, C.S., Takeda, N., Szczyglowski, K., and Kawaguchi, M. (2013). TRICOT encodes an AMP1-related carboxypeptidase that regulates root nodule development and shoot apical meristem maintenance in *Lotus japonicus*. Development *140*, 353-361.


### ● *Research activity by Y. Kamei*

*Specially Appointed Associate Professor:* *KAMEI, Yasuhiro*
*NIBB Research Fellow:* *HATTORI, Masayuki*

My research group promotes two advanced microscope projects; "observation" and "manipulation" using optical and biological technologies. The aim of our "observation project" is deep-seeing in living organisms using adaptive optics (AO) which were well-developed in the field of astronomy as

a key technology of large telescopes such as the Subaru telescope in Hawaii. Although observation of telescopes on the earth may be disturbed by fluctuation in the atmosphere, AO technology can cancel this disturbance. On the other hand, living materials have particular refractive indexes, therefore, some organelles act as a disturbance of the ideal optical path for microscope observation just like the atmosphere does for telescopes. AO technology also can recover this disturbance by sensing and correcting wave fronts using a wave front sensor and deformable mirror. Hence, we developed a custom-made wide-field microscope equipped with an AO system for observation of living organisms as a collaboration with Dr. Tamada in NIBB and Dr. Hayano in the National Astronomical Observatory of Japan (NAOJ) and got high-resolution bright field and fluorescent images of living cells. Our result indicated that improvement of optical resolution was restricted in a small

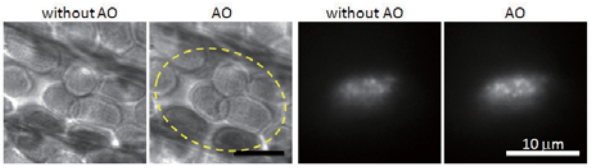


Figure 1. Effects of adaptive optics (AO) to the wide-field microscope images (bright field and fluorescence of plant cells).

area which is called the "isoplanatic patch" (Figure 1).

Second, the aim of our "manipulation project" is to control gene expression *in vivo*. Gene function analysis must be evaluated at the cell level *in vivo*. To achieve spatiotemporal-controlled gene expression we employed one of the stress responses, the heat shock response. The heat shock promoter is the transcription regulation region of heat shock proteins and all organisms have this mechanism. Positioning the target gene downstream of the promoter, we can induce the target gene expression by local heating.

Infrared (IR) beams can heat water molecules, which are the main constituent of cells, hence, we can heat a single cell by irradiating IR to a target cell using a microscope. We have developed a microscope, IR laser evoked gene operator

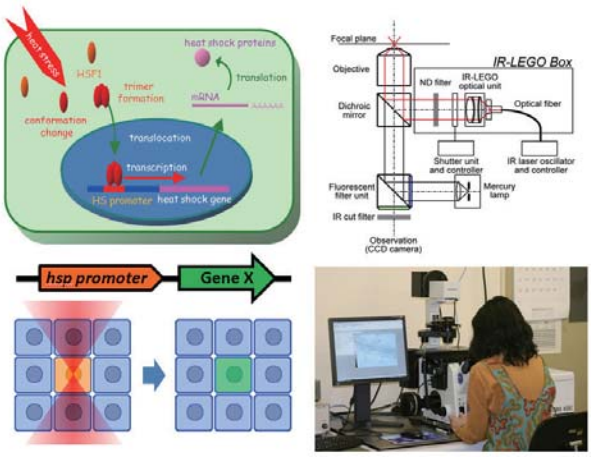


Figure 2. Schematic illustration of local gene induction system and an infrared laser-evoked gene operator (IR-LEGO) microscope system in NIBB.

(IR-LEGO), specialized for this purpose (Figure 2). The IR-LEGO microscope can irradiate an IR laser to a single cell *in vivo* such as *C. elegans*, medaka, zebrafish, *Xenopus* and *Arabidopsis*, to induce the heat shock response at a desired timing.

Optimal heating induces the heat shock response and subsequent gene expression, while an excess results in cell death. Hence, we must precisely control laser heating. We evaluated time course and spatial heating profiles, and the results presented that temperature of the target area rose rapidly and kept a constant level dependant on IR laser power, additionally, the heated area was adequately as small as a typical cell size.

With this in mind, we tried to induce gene expression in various species. At first, we reported an IR-LEGO experiment in living *C. elegans*. Target gene expression in a target cell could be induced with only 1 s-IR irradiation. Whereas the optimal power range which can induce gene induction without cell damage was limited. Excess laser power resulted in cell death or cessation of cell division. We confirmed that an optimal irradiation, e.g. 11 mW for 1 s, induced physiological gene expression in the target cell and subsequent cell division or morphogenesis underwent normal development. Next, we tried the experiment in other animals, medaka, zebrafish and *Xenopus*, and the higher plant, *Arabidopsis*, since all organisms have a heat shock response system. We succeeded in local gene induction in the species

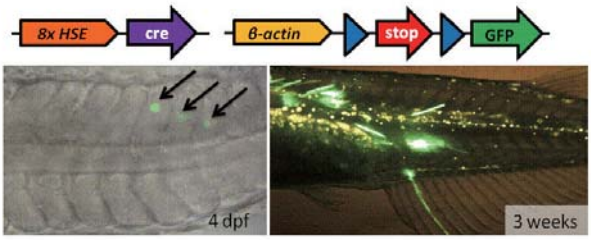


Figure 3. Cre-loxP mediated long-term GFP marking in a living medaka individual for linage tracing.

as expected.

Studies of cell fates, cell-cell interaction, or analysis of non-cell autonomous phenomena require a fine control system of gene expression in experiments. IR-LEGO will be a powerful tool for these studies in combination with molecular biological techniques, such as the cre-loxP system. By Applying IR-LEGO to a mutant and its rescue transgenic strain; using hsp-cre with a rescue gene which is sandwiched by loxP sequences, we will achieve single-cell knockout experiments in living organisms, and reveal fine interaction between the cells. We are now testing this system using medaka. We have already constructed a medaka TILLING library and a screening system for reverse genetic mutant screening, furthermore we have confirmed a system operation of a cre-loxP system in medaka using IR-LEGO (Figure 3).

## Publication List

〔Original papers〕


- Ikehata, H., Higashi, S., Nakamura, S., Daigaku, Y., Furusawa, Y., Kamei, Y., Watanabe, M., Yamamoto, K., Hieda, K., Munakata, N., and Ono, T. (2013). Action spectrum analysis of UVR genotoxicity for skin: the border wavelengths between UVA and UVB can bring serious mutation loads to skin. J. Invest. Dermatol. *133*, 1850-1856.
- Ishikawa, T., Okada, T., Ishikawa-Fujiwara, T., Todo, T., Kamei, Y., Shigenobu, S., Tanaka, M., Saito, T.L., Yoshimura, J., Morishita, S., Toyoda, A., Sakaki, Y., Taniguchi, Y., Takeda, S., and Mori, K. (2013). ATF6a/b-dediated adjustment of ER chaperone levels is essential for development of the notochord in medaka fish. Mol. Biol. Cell *24*, 1387-1395.
- Kimura, E., Deguchi, T., Kamei, Y., Shoji, W., Yuba, S., and Hitomi, J. (2013). Application of infrared laser to the zebrafish vascular system: gene induction, tracing, and ablation of single endothelal cells. Arterioscler. Thromb. Vasc. Biol. *33*, 1264-1270.
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- Shikata, T., Matsunaga, S., Iseki, M., Nishide, H., Higashi, S-I., Kamei, Y., Yamaguchi, M., Jenkinson, I.R., and Watanabe, M. (2013). Blue light regulates the rhythm of diurnal vertical migration in the raphidophyte red-tide alga *Chattonella antiqua*. J. Plankton Res. *35*, 542-552.
- Shimada, A., Kawanishi, T., Kaneko, T., Yoshihara, H., Yano, T., Inohaya, K., Kinoshita, M., Kamei, Y., Tamura, K., and Takeda, H. (2013). Trunk exoskeleton in teleosts is mesodermal in origin. Nature Commun. *4*, 1639.


## Data Integration and Analysis Facility


*Assistant Professor:* *UCHIYAMA, Ikuo*
*Technical Staff:* *MIWA, Tomoki*
*NISHIDE, Hiroyo*
*NAKAMURA, Takanori*
*Technical Assistant:* *OKA, Naomi*


The Data Integration and Analysis Facility supports research activities based on large-scale biological data analysis, such as genomic sequence analysis, expression data analysis, and imaging data analysis. For this purpose, the facility maintains high-performance computers with large-capacity storage systems. On the basis of this system, the facility supports development of data analysis pipelines, database construction and setting up websites to distribute the data worldwide. In addition to computational analysis, the Data Integration and Analysis Facility supports NIBB's information infrastructure, the maintenance of the network system in the institute and computer/network consultation for institute members.

### Representative Instruments

Our main computer system is the Biological Information Analysis System (BIAS) (Figure 1), which consists of a shared memory parallel computer (DELL PowerEdge R905; 4 nodes/16 cores, 256GB memory), a high-performance cluster system (DELL PowerEdge M1000e+M610; 32 nodes/256 cores, 768GB memory) and a large-capacity storage system (DELL Equallogic; 35TB SAS, 26TB SATA, 750GB SSD). All subsystems are connected via a high-speed InfiniBand network so that large amounts of data can be processed efficiently. Some personal computers and color/monochrome printers are also available. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for institute members. Especially, we have supported the construction and maintenance of published databases of various model organisms including XDB (*Xenopus laevis*), PHYSCObase (*Physcomitrella patens*), DaphniaBASE (*Daphnia magna*), The Plant Organelles Database, and MBGD (microbial genomes).

Figure 1. Biological Information Analysis System

The facility also provides network communication services. Most of the PCs in each laboratory, as well as all of the above-mentioned service machines, are connected by a local area network, which is linked to the high performance backbone network ORION connecting the three research institutes in Okazaki. Many local services, including sequence analysis services, file sharing services, and printer services, are provided through this network. We also maintain a public World Wide Web server that hosts the NIBB home page (http://www.nibb.ac.jp/).

### Research activity by I. Uchiyama

Assistant professor I. Uchiyama is the principal investigator of the Laboratory of Genome Informatics, which currently focuses on microbial comparative genomics studies. For details, please refer to the laboratory page (p. ##).

## NIBB BioResource Center

*Head*
IGUCHI, Taisen

*Vice head:* *FUJIMORI, Toshihiko*

To understand the mechanisms of living organisms, studies focusing on each gene in the design of life (the genome) are required. The use of model animals and plants, such as mice, Medaka (*Oryzias latipes*), zebrafish, *Arabidopsis*, *Lotus japonicus*, and *Physcomitrella patens*, makes it possible to produce genetically controlled organisms with markers placed by genetic and cell engineering technology. Such marking allows detailed studies of genes and cell functions. The model organisms mature in a short period of time; therefore, changes in cells, organs, and individuals can be totally and efficiently observed. The NIBB BioResource Center has equipment, facilities, and staff to maintain such organisms safely, efficiently, and appropriately.

### Model Animal Research Facility

| | |
|---|---|
| *Associate Professor:* | *WATANABE, Eiji* |
| | *TANAKA, Minoru* |
| | *NARUSE, Kiyoshi* |
| *Technical Staff:* | *HAYASHI, Kohji* |
| | *NOGUCHI, Yuji* |
| *Technical Assistant:* | *INADA, Yosuke* |
| | *MATSUMURA, Kunihiro* |
| | *FUJIMOTO, Daiji* |
| | *TAKAGI, Yukari* |
| | *SUGINAGA, Tomomi* |
| | *SUZUKI, Kohta* |

The worldwide genome project has almost been completed and research on basic biology has arrived at a post-genome era in which researchers are focusing on investigating the functions of individual genes. To promote the functional analysis of a gene of interest it is essential to utilize genetically altered model organisms generated using genetic engineering technology, including gene deletion, gene replacement and point mutation.

The NIBB Center for Transgenic Animals and Plants was established in April 1998 to support research using transgenic and gene targeting techniques at NIBB. The NIBB Center for Transgenic Animals and Plants was integrated into the NIBB BioResource center in April 2010, and was renamed as “The Model Animal Research Facility”.

Technical staff and supporting staff develop and promote research-supporting activities. A state-of-the-art facility for transgenic animals opened at the end of 2003 in the Yamate area.

The activities of the model animal research facility are as follows:

1. The provision of information, materials, techniques and animal housing space to researchers.
2. The use of various kinds of instruments to analyze mutant, transgenic, and gene-targeted animals.
3. The development of novel techniques related to transgenic and gene targeting technology.
4. Cryopreservation and storage of transgenic strains.

## I. Research support activities (mouse)

In 2001, the NIBB mouse facility (built under specific pathogen free (SPF) conditions) opened in the Myodaiji area and the production, breeding, analysis, cryopreservation and storage of genetically manipulated mouse strains has been conducted there since then. The new center facility building in the Yamate area strengthened research activities using genetically altered organisms. The building has five floors and a total floor space of 2,500 $m^2$ in which we can generate, breed, store and analyze transgenic, gene targeting and mutant mice under SPF conditions. The mouse housing area was constructed based on a barrier system. This building is also equipped with breeding areas for transgenic small fish, birds, and insects.

In 2013 (from January 1 to December 31), 2,439 fertilized eggs (*in vitro* fertilization; 1,192 eggs of 5 lines in which 894 eggs of 5 lines were frozen for long-term storage, frozen eggs: 1,247 of 13 lines) and 5,231 mice were brought into the facility in the Yamate area, and 58,879 mice (including pups bred in the facility) were taken out.

A number of strains of genetically altered mice from outside the facility were brought into the mouse housing area by microbiological cleaning using *in vitro* fertilization-embryo transfer techniques, and stored using cryopreservation.

Figure 1. Mouse (strain MCH(ICR))

Figure 2. Equipment for manipulating mice eggs.

A new mouse facility in the Myodaiji area opened at the beginning of 2005. The facility provides research-supporting activities to researchers in the Myodaiji area. In March 2009, we expanded the facility which includes areas for breeding, behavioral tests and transgenic studies using various kinds of recombinant viruses. In 2013 (from January 1 to December 31) 612 mice (including pups bred in the facility) were taken out.

Figure 3. Large sized autoclave in the Myodaiji area.

## II. Research support activities (small fish, birds, and insects)

The first floor of the center facility building in the Yamate area provides space and facilities to maintain small fish, chick embryos, and insects. In the laboratory room for chick embryos, a large incubation chamber is equipped and set at 42 degrees (suitable for chick embryogenesis). The researchers can manipulate chick embryos under optimal conditions, removing biohazard risks. For researchers who employ fish as an experimental model, 480 tanks (1 liter) and 450 tanks (3 liters) are available for medaka and zebrafish, respectively. Water circulates and can be maintained to suit the conditions desired for fish breeding in the aquarium systems. Currently, over three mutant lines and over fifteen transgenic lines of medaka and zebrafish are maintained in our facility. A medaka line that allows gene induction by heat treatment, in combination with a cre/loxP system, has been developed in this facility. In addition to the rooms mentioned above, a room for insects is also available. All the rooms are qualified to meet the criteria for transgenic animals, allowing researchers to generate and maintain these important biological tools.

In 2013 (from January 1 to December 31), 15,114 medaka (142 eggs, 37 embryos and 14,935 adults) were brought to the facility and 77,946 medaka (65,103 fertilized eggs, 317 embryos and 12,526 adults, including animals bred in the facility) were taken out. In the laboratory for chick embryos there were no fertilized eggs or chicken embryos brought in or taken out this year. These animals were used for research activities in neurobiology and developmental biology.

In 2007 NIBB was approved as a core facility of the National BioResource Project (NBRP) for Medaka by the Japanese Government. We have supported the activities of NBRP Medaka by providing standard strains, mutants, transgenic lines and organizing international practical courses for medaka. In 2010 we began providing the TILLING library screening system to promote the reverse genetic approach. In 2013 we shipped 417 independent medaka strains, 197 cDNA/BAC/Fosmid clones, and 165 samples of hatching enzyme to the scientific community worldwide.

Figure 4. Quarantine room of medaka and zebrafish.

## III. Research activities

The associate professors of this center - E. Watanabe, T. Naruse and M. Tanaka - are the principal investigators of the Laboratory of Neurophysiology, the Laboratory of Bioresources and the Laboratory of Molecular Genetics for Reproduction, respectively. The Laboratory of Neurophysiology is studying mechanisms of the visual system using a psychophysical approach (p. 43). The Laboratory of Bioresources has conducted a genetic and genomic analysis of quantitative traits and Mendelian phenotype variations as well as evolution of sex determination systems in medaka related species (p. 51). The Laboratory of Molecular Genetics for Reproduction is studying the molecular mechanisms of reproductive organ development and sex differentiation using mutagenized or transgenic medaka (p. 32).

## Model Plant Research Facility

● ***Plant Culture Laboratory***

| | |
|---|---|
| *Assistant Professor:* | *HOSHINO, Atsushi* |
| | *TSUGANE, Kazuo* |
| *Technical Staff:* | *MOROOKA, Naoki* |
| *Technical Assistant:* | *SUZUKI, Keiko* |

The Plant Culture Laboratory manages facilities for the cultivation of plants in general and also for the rearing of several animal species that do not fit in other facilities.

The Plant Culture Laboratory equips and manages 62 culture boxes or growth chambers, 4 phytotrons, and 12 rooms with the P1P physical containment level for

established and emerging model plants including the thale cress *Arabidopsis thaliana*, several carnivorous plants, the rice *Oryza sativa*, the moss *Physcomitrella patens*, green alga *Chlamydomonas reinhardtii* and several other flowering plants. Most culture space is fully used the whole year by more than 60 researchers from both outside and inside groups.

As well as regular culture conditions, extreme environmental conditions for light and temperature are available for various types of experiments. Three light environmental simulators (max 120,000 lux by using xenon lamp) and three chambers (3.4 $m^2$ each) that can control $CO_2$ and humidity in addition to temperature and light (max 70,000 lux) conditions are available. Autotrophic and heterotrophic culture devices are also available for researchers using cyanobacteria, algae, and cultured flowering plant cells. Aseptic experiments can be performed in an aseptic room with clean benches.

Next to the institute building of the Myodaiji area, a 386-$m^2$ experimental farm is maintained for Japanese morning glory and related *Ipomoea* species, several carnivorous plants and other flowering plants necessary to be cultivated outside. Three green houses (44, 44, and 45 $m^2$) with heating are used for the sensitive carnivorous plants, and wild-type strains of medaka fish *Oryzias* sp. Seven green houses (4, 6, 6, 6, 6, 9, and 9 $m^2$) with air-conditioning are provided for the cultivation of a rice *Oryza* sp., *Lotus japonica* and related legume species, as well as mutant lines of the Japanese morning glory. One green house (18 $m^2$) with air-conditioning meets the P1P physical containment level and is available for experiments using transgenic plants. The Plant Culture Laboratory also maintains a 46-$m^2$ building with storage and workspace. Part of the building is used for rearing of the orchid mantis.

In 2013, several analytical and imaging instruments as well as plant culture equipment were introduced. Of these, the DUAL-PAM is a chlorophyll fluorescence measuring system that can monitor both photosystem I and photosystem II activities. A tissue culture rack with dimming LEDs and pulse-width modulation controllers is used for algae culture under precise light control.

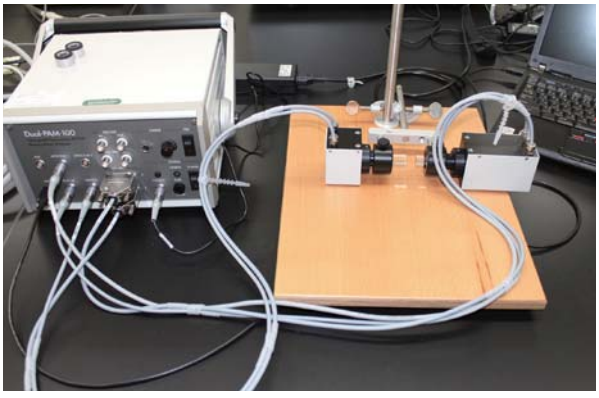


Figure 5. The chlorophyll fluorescence measuring system (DUAL-PAM).

### ● *Morning Glory BioResource Laboratory*

*Assistant Professor:* *HOSHINO, Atsushi*

The Japanese morning glory (*Ipomoea nil*) is a traditional floricultural plant in Japan, and is studied worldwide, especially in plant physiology and genetics. NIBB collects, develops and distributes DNA clones, mutant lines for flower pigmentation, and transgenic lines as a sub-center of the National BioResource Project (NBRP) Morning glory, and collaborates with the core organization center, Kyushu University. We collected several mutant lines and 30,000 BAC clones, and provided 108 DNA clones and 3 *Ipomoea* lines to both local and foreign biologists this year.

Research activities of the assistant professor A. Hoshino are shown on the laboratory page (p. 55).

## Cell Biology Research Facility

*Assistant Professor:* *HAMADA, Yoshio*
*Technical Assistant:* *SUGINAGA, Tomomi*

The Cell Biology Research Facility provides various equipment for tissue and cell culture. This laboratory is equipped with safety rooms which satisfy the P2 physical containment level, and is routinely used for DNA recombination experiments.

Research activities of assistant professor Y. Hamada, the principal investigator of the Laboratory of Cell Sociology, is shown on the laboratory page (p. 16).

Figure 6. Equipment for tissue and cell culture.

## NIBB Center of the Interuniversity Bio-Backup Project (IBBP Center)

*Head*
KOBAYASHI, Satoru

*Associate Professor*
NARUSE, Kiyoshi

*Specially Appointed Assistant Professor:*
*KIMURA, Tetsuaki*
*TANAKA, Daisuke*
*Technical Assistant:* *AKIMOTO-KATO, Ai*
*MATSUBAYASHI, Naomi*
*Secretary:* *HAMATANI, Ayako*
*SAKAMOTO, Yuki*

In order to realize a life sciences community resilient to natural disasters and calamities, the National Institutes for Natural Sciences (NINS) and Hokkaido University, Tohoku University, University of Tokyo, Nagoya University, Kyoto University, Osaka University, and Kyushu University concluded an agreement on June 1st 2012 to launch a system to 'back up' the biological resources essential to the work being done at universities and research institutions nationwide, called the 'Interuniversity Bio-Backup Project (IBBP)'.

The IBBP Center was established as a centralized backup and storage facility at NIBB, while IBBP member universities set up satellite hubs and work closely with the IBBP center to put in place reciprocal systems for backing up important biological resources that have been developed by researchers residing in the area each university satellite hub is responsible for.

Figure 1. IBBP Center

Figure 2. Cryogenic storage system. Liquid nitrogen tanks are monitored 24 hours a day and are refilled automatically.

The IBBP Center includes earthquake proof structures capable of withstanding even very large scale quakes (equipped with emergency backup power generators), cryopreservation facilities equipped with automatic liquid nitrogen feeding systems, deep freezers, and refrigerated storage (mainly for seed stocks), as well as all manner of automated laboratory equipment, cell culture tools, and the latest equipment necessary to back up the genetic resources in a collaborative manner. The specific methods of preservation are freezing of the sperm and eggs of animals, cultured plant and animal cells, and gene libraries. Plant seeds are frozen or refrigerated.

University satellite hubs receive preservation requests of biological resources from researchers and report to the Managing Project Committee of IBBP (constituted of faculty of NIBB and satellite institutes), where the relevance of the request is reviewed. When the request is sustained, biological resources to be preserved will be sent to the IBBP Center by the requesting researcher, where they will be frozen (or refrigerated) and their particular information will be registered into a database. In the event of a disaster leading to the loss of a researcher's own biological resources, preserved samples will be promptly supplied back to the researcher so they can quickly resume their work.

Through the development of this backup system biological resources that had only been stored at individual research institutes can now be preserved at the state of the art facilities of the IBBP Center, and Japan's research infrastructure has been significantly strengthened.

### I. Current status of back up for the biological resources

In 2013, IBBP Center stored 3,598 384-well plates consisting of 1,381,632 clones as cDNA/BACs clones, 1013 tubes for plant and animal samples and 618 tubes for microorganisms.

Figure 3. Cryo tube with 2D barcode. Each sample is printed with a unique barcode and is managed using a database.

## II. Collaborative Research Project for the development of new long-term storage technologies and cryo-biological study

As the IBBP Center can only accept biological resources which can be cryopreserved in liquid nitrogen, researchers cannot backup those biological resources for which cryopreservation methods are not well established, to increase the usability of IBBP, we started a collaborative research project for the development of new long-term storage technologies and cryo-biological study in 2013. This collaborative research includes two subjects 1) Establishment of new storage technology for biological resources for which long-term storage is unavailable. 2) Basic cryobiological research enabling us to improve low temperature storage for biological resources. In 2013 we had eleven applications and accepted nine proposals. We are also working to establish a research center for cryo-biological study thorough this Collaborative Research Project.

### Research activity by D. Tanaka

*Specially Appointed Assistant Professor:* *TANAKA, Daisuke*
*Technical Assistant:* *AKIMOTO-KATO, Ai*

### Cryopreservation

Cryopreservation protocols contain components which are usually developed empirically using each biological resource's specific strategy to enhance survival (Benson 2008). The theory of cryopreservation encompasses several interconnected disciplines ranging from the physiological to cryophysical. Water status and cryopreservation, in combination with physiological factors, are the most influential determinants of survival (Figure 1).

So far cryopreservation has been considered in terms of the liquid and solid (ice) phases of water. It is also possible to cryopreserve tissues by the process of "vitrification", the solidification of liquids without crystallization. This comprises a "glassy state" as the system is amorphous, lacks organized structure but possesses the mechanical and physical properties of a solid (Taylor *et al*. 2004).

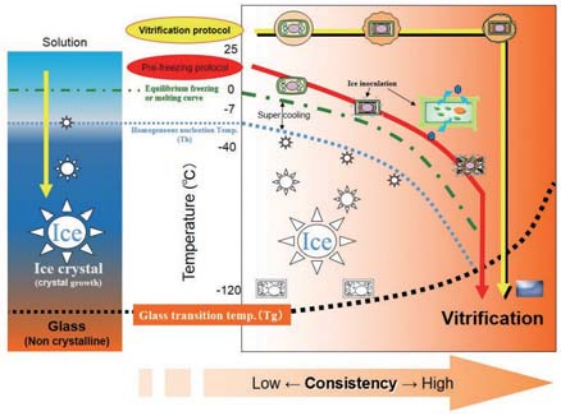



Figure 1. Phase diagram of vitrification of a solution.
Vitrification, a physical process, can be defined as the phase transition of an aqueous solution from a liquid into an amorphous glassy solid, or glass, at the glass transition temperature (Tg), while avoiding ice crystallization.

Vitrification-based protocols are known to be effective for long-term, stable preservation of plant germplasm; this protocol can reduce the cost and manpower for maintaining a large number of germplasm lines and keep many valuable genetic lines for a long term under genetically stable conditions. However, it is still not widely employed as a reliable long-term preservation protocol due to the lack of basic knowledge on the cellular and water behavior in tissues when immersed in liquid nitrogen.

In the present study, electron microscopy combined with freeze-substitution was employed to examine the ultrastructure of cells of shoot apices of a Chrysanthemum plant that were cooled to the temperature of liquid nitrogen after exposure to various steps of the Cryo-plate protocol (Figure 2-3).

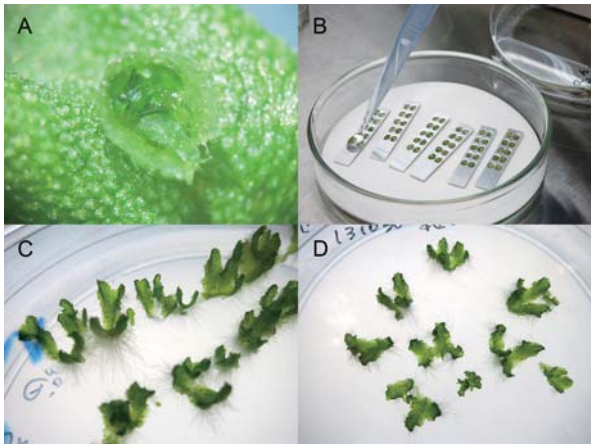



Figure 2. Cryopreservation protocol using aluminum cryo-plate.
A: Preparation of *in vitro* grown gemmae of liverwort. B: Placing precultured gemmae in a cryo-plate's wells. C: Regrowth of cryopreserved livewort line 'Takaragaike-1' 30 days after rewarming. D: Control (without cooling in liquid nitrogen).

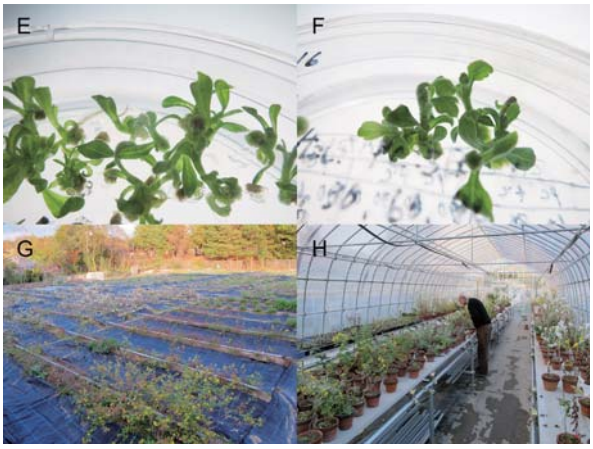



Figure 3. Plantlets regenerated from shoot apices of chrysanthemum cryopreserved by the D-Cryo-plate protocol. Shoot apices were on the post-thaw medium for 30 days. E, cryopreserved. F, control (without cooling in liquid nitrogen). G-H, Field preservation of chrysanthemum biological resources. About 2,000 races/lines are cultivated in the fields of Hiroshima University, Japan.

## Publication List

〔Original papers〕


- Matsumoto, T., Akihiro, T., Maki, S., Mochida, K., Kitagawa, M., Tanaka, D., Yamamoto, S., and Niino, T. (2013). Genetic stability assessment of wasabi plants regenerated from long-term cryopreserved shoot tips using morphological, biochemical and molecular analysis. Cryo Letters *34*, 128-136.
- Niwa, N., Akimoto-Kato, A., Sakuma, M., Kuraku, S., and Hayashi, S. (2013). Homeogenetic inductive mechanism of segmentation in polychaete tail regeneration. Dev. Biol. *381*, 460-470.


〔Original paper (E-publication ahead of print)〕


- Kondo, T., Sakuma, T., Wada, H., Akimoto-Kato, A., Yamamoto, T., Hayashi, S. TALEN-induced gene knock out in Drosophila. Dev. Growth Differ. 2013 Oct. 31.


## Research activity by T. Kimura


*Specially Appointed Assistant Professor:*
*KIMURA, Tetsuaki*


### Analysis of median fin-rays development

The vertebrate body plan has evolved by the acquisition of structures projecting from the body axis. The original structures have transformed, as an environmental adaptation, into appendages such as fins, limbs, and wings. Median fins are the oldest of such evolved structures. In order to better understand the mechanisms by which the median fins developed, we crossed two inbred lines of medaka (*Oryzias latipes*). From the results, we found that the number of anal fin-rays was determined by two genetic traits, the anteroposterior length of the anal fin and interval between the anal fin-rays. The 19-ray fish has a longer anal fin than the 17-ray fish (Figure 1A). The 19-ray fish has the same anal fin length as the 17-ray fish (Figure 1B). This indicates that the 19-ray fish has narrower intervals between fin-rays than the 17-ray fish.

Further, the pattern of rays was independent of the pattern of the somites and vertebrae. Thus, the pattern formation of fin-rays proposes a new model of bone patterning.

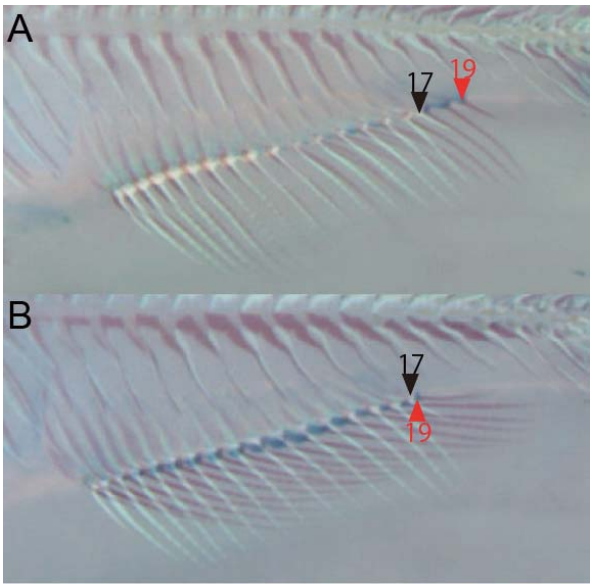



Figure 1. Superimposition of $F_2$ anal fin-rays. (A) Superimposed images of $F_2$ fish with 17 and 19 rays. (B) Superimposed images of $F_2$ fish with 17 (the same fish as in A) and 19 rays (a different fish from that in A). All three fish have 29 vertebrae. Note the pattern of the vertebrae is the same for both A and B. Arrowheads indicate the posteriormost ray. Numbers indicate total number of fin-rays.

## Publication List

〔Original paper〕


- Ohshima, A., Morimura, N., Matsumoto, C., Hiraga, A., Komine, R., Kimura, T., Naruse, K., and Fukamachi, S. (2013). Effects of body-color mutations on vitality: an attempt to establish easy-to-breed see-through medaka strains by outcrossing. G3 *3*, 1577-1585.

## CENTER FOR RADIOISOTOPE FACILITIES

*Head*
HASEBE, Mitsuyasu

*Associate Professor*
KODAMA, Ryuji

*Technical Staff:*
*MATSUDA, Yoshimi*
*(Radiation Protection Supervisor)*
*SAWADA, Kaoru*
*(Radiation Protection Supervisor)*
*IINUMA, Hideko*

*Technical Assistant:*
*ITO, Takayo*
*KAMIYA, Kiyomi*

The technical and supporting staff of the Center for Radioisotope Facilities (CRF) maintain controlled areas in compliance with the law. The CRF is responsible for monitoring the purchase of radioisotopes from JRIA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRIA.

Ms. Matsuda, Ms. Iinuma, Ms. Ito, and Ms. Kamiya maintained the Myodaiji-Area. Ms. Sawada worked in the Yamate-Area. Dr. Kodama worked in both areas.

The following are CRF's notable activities in 2013.

1. For the Common Facilities building I was repaired, the radiation controlled area of the Myodaiji-Area was closed from April 2013. (Figure1A)
2. For animal experiments and gene-recombination experiments using radioisotopes during the above repair period, the radioisotope handling rooms were set up at the NIPS building. (Figure 1B)
3. At the Yamate-area, the visual recording system's recorder and monitor expired, and were exchanged. (Figure 1C)

The number of registrants and the number of users from January 2013 to December 2013 are shown in Table 1.

Users and visitors counted by the access control system of the controlled areas numbered 2,653 during this period. The radioisotope handling rooms' users and visitors counted by record sheet numbered 292. The numbers for each area are shown in Table 2. The annual changes of registrants and the number of totals per fiscal year are shown in Figure 2. The balance of radioisotopes received and used at the CRF is shown in Table 3. The training courses on radioisotope handling were given as in Table 4.




Figure 1. The CRF's notable activities in 2013
A: The common facilities building was repaired.
B: The radioisotope handling room set up (NIPS building, room No.248)
C: The visual recording system of the controlled areas was updated.

| | Myodaiji-Area | Yamate-Area |
|---|---|---|
| registrants | 90 | 86 |
| users | 40 | 28 |

Table 1. Numbers of registrants and users at Myodaiji-Area and Yamate-Area in 2013

| | controlled area | | | radioisotope |
|---|---|---|---|---|
| | Myodaiji-Area | Yamate-Area | total | handling room |
| users | 965 | 775 | 1740 | 274 |
| visitors | 746 | 167 | 913 | 18 |
| total | 1711 | 942 | 2653 | 292 |

Table 2. Users and visitors who entered each controlled area and radioisotope handling room in 2013

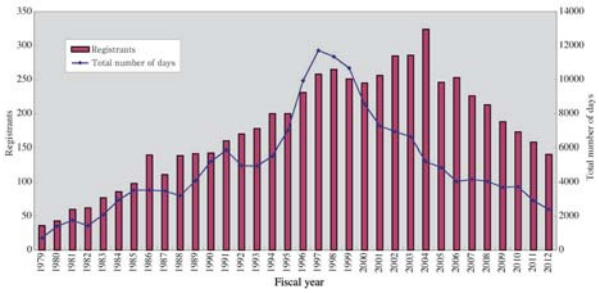



Figure 2. Annual changes of registrants and number of totals per fiscal year

| | Myodaiji-Area | Yamate-Area | | total |
|---|---|---|---|---|
| | | controlled area | radioisotope handling room | |
| $^{125}$I Received | 74000 | 148330 | — | 222330 |
| $^{125}$I Used | 55500 | 129830 | — | 185330 |
| $^{35}$S Received | 0 | 222000 | — | 222000 |
| $^{35}$S Used | 0 | 133200 | — | 133200 |
| $^{32}$P Received | 38500 | 77000 | — | 115500 |
| $^{32}$P Used | 24210 | 40800 | — | 65010 |
| $^{14}$C Received | 37000 | 220428 | — | 257428 |
| $^{14}$C Used | 4163 | 52 | 53280 | 57495 |
| $^{3}$H Received | 83250 | 8688290 | — | 8771540 |
| $^{3}$H Used | 71052 | 5550074 | 11840 | 5632966 |

Table 3. Balance of radioisotopes received and used (kBq) at each controlled area and radioisotope handling area in 2013

| training course | place | numbers of participant |
|---|---|---|
| introductory course for beginners* | Myodaiji | 1 |
| introductory course for beginners* | Yamate | 3 |
| introductory course for experts | Myodaiji | 12 |
| introductory course for experts | Yamate | 29 |
| Users training course* | Myodaiji | 67 |
| Users training course | Yamate | 49 |

*including English course

Table 4. Training courses for radiation workers in 2013

## Research Enhancement Strategy Office

*Director*
UENO, Naoto

*Vice-Director (Specially Appointed Professor (URA))*
NISHIMURA, Mikio

In order to fulfill two goals, to encourage cutting-edge academic research in the field of natural sciences through international joint research, and to contribute to the enhancement of research capabilities of universities etc. in Japan using the world's most advanced research environment for joint utilization and joint research, NINS started in 2013 a research enhancement project with the following four approaches: 1) Support for the promotion of international advanced research, 2) Support for the promotion of joint utilization and joint research in Japan, 3) Dissemination of information and enhancement of public relations in Japan and abroad, 4) Support for researchers, especially young, female or foreign researchers.

The Research Enhancement Strategy Office is aimed at supporting researchers so that NIBB improves its ability as a collaborative research institution, and was restructured in 2013 from the former Strategic Planning Department, the Office of Public Relations, and the Office of International Cooperation which existed from 2005. The Office's activities are mainly carried out by URAs (University Research Administrators) according to the advice of the group adviser chosen from NIBB's professors and in close collaboration with the NINS's Research Enhancement Promotion Headquarters.

### Evaluation and Information Group

| | |
|---|---|
| *Associate Professor:* | *KODAMA, Ryuji* |
| *Group Adviser:* | *YOSHIDA, Shosei* |

This group serves as a central office for assisting the Director General in preparing for NIBB's evaluation procedure and in planning long-range strategies for the institute.

### The main activities of the group

***1) Management of external evaluation processes***

NIBB asks non-NIBB members of the Advisory Committee for Programming and Management to answer a questionnaire on research achievements and collaborative research activities of the institute. Several of the members are further invited to participate in a meeting for the evaluation of these activities and for proposing future directions for the institute. This group manages these processes.

***2) Editing of the Annual Report (in collaboration with the Public Relations Group)***

This group edits the annual report (this print) which summarizes the annual activities of laboratories and facilities and is used in the evaluation procedures of the institute.

***3) Assistance in budget requests and long-range planning of the institute***

This group assists the Director General in preparing long-range plans for building the most advanced research facilities, and in budget requests to the government to realize and implement these plans.

***4) Assistance in making the plans and reports of the institute***

This group assists in drafting NIBB's Medium-term Goals and Plans (for a six-year-period), and in instituting Annual Plans to implement them. The department also assists in preparing Business and Performance Reports for the external evaluation on whether we are meeting the goals set both annually and for the medium-term.

### Public Relations Group

| | |
|---|---|
| *Specially Appointed Assistant Professor (URA):* | *KURATA, Tomoko* |
| *Technical Assistant:* | *OTA, Kyoko* |
| | *KAWAGUCHI, Colin* |
| | *BAN, Misato* |
| *Group Adviser:* | *FUJIMORI, Toshihiko* |

This group, in order to publicize the activities of NIBB to the widest audience, performs both routine public relations as well as communication with media and scientific organizations worldwide.

### The main activities of the group in 2013

***1) Press releases***

The office sends news on scientific achievements to newspaper and magazine reporters via leaflets, e-mails and web pages. We also arrange press conferences for some releases.

***2) Updating and maintenance of the NIBB web page***

***3) Editing of publications, production of posters and leaflets***

Publication of the pamphlet "An Introduction to the National Institute for Basic Biology". Publication of leaflets introducing the individual researchers of NIBB (in Japanese). Publication of "NIBB News" (Intra-institutional newsletter, in Japanese), "NIBB English News" (Intra-institutional newsletter, in English). Design and distribution of posters for international conferences and advertisements for the graduate school's entrance examination.

***4) Producing videos***

Creation of videos introducing NIBB and interviews with researchers that are published on the web.

***5) Organization of scientific outreach programs***

Planning the Summer Program for university students, and coordinating special classes for middle school students.

## International Cooperation Group

*Specially Appointed Assistant Professor (URA):* *TATEMATSU, Kiyoshi*
*Technical Assistant:* *TAKAHASHI, Ritsue*
*SANJO, Kazuko*
*Group Adviser:* *UENO, Naoto*

This group supports NIBB's activities related to international research collaborations, through organizing various types of international scientific meetings and technical courses, exchanging researchers and students between NIBB and oversea institutions including those with which we exchanged cooperative agreements. This group also supports NIBB's interns visiting from foreign countries.

NIBB has a mission to continually explore the leading-edge of biology and form research communities that link Japan to the world. For this purpose, NIBB holds scientific meetings including NIBB conferences and Okazaki Biology Conferences (OBC), and educational programs such as International Practical Courses. Further, NIBB is tightly interacting with the European Molecular Biology Laboratory (EMBL, European member states), the Max Planck Institute for Plant Breeding Research (MPIPZ, Germany), and Temasek Life Sciences Laboratory (TLL, Singapore) on the basis of cooperative agreements, through exchanging people and techniques and holding scientific meetings. This group supports and coordinates these international activities of NIBB.

### The main activities of the group in 2013

***1) Coordination of international conferences***

This group coordinated the following international conferences hosted by NIBB:

The 61st NIBB Conference "Cellular Community in Mammalian Embryogenesis" Okazaki, Japan, July 10-12, 2013 (p. 86)

The 10th NIBB-EMBL Joint Symposium "Quantitative Bioimaging" Okazaki, Japan, March 17-19, 2013 (p. 87)

***2) Support of dispatching researchers and students to international conferences***

This group coordinated sending NIBB researchers to the following events (related to the NIBB-EMBL exchange agreement).

The 15th EMBL PhD Symposium "Competition in Biology: The Race for Survival, from Molecules to Systems", EMBL, Heidelberg, Germany, November 21-23, 2013 (p. 90)

EMBL Workshop "Morphogen Gradients" Oxford, UK, June, 26-29, 2013

NIBB investigators' lab visits to EMBL, Heidelberg

***3) Support of education-related programs***

This group supported the following student-related activities of NIBB.

NIBB Internship Program 2013 (p. 89)

10th Life Science Retreat, Department of Life Science, SOKENDAI. October 30-31, 2013

## Collaborative Research Group

*Specially Appointed Associate Professor (URA):* *SHIGENOBU, Shuji*
*KAMEI, Yasuhiro*
*Technical Assistant:* *ICHIKAWA, Mariko*
*ICHIKAWA, Chiaki*
*Group Adviser:* *YOSHIDA, Shosei*

Specially appointed associate professors of this group also belong to NIBB core research facilities and are responsible in managing collaborative research projects and practical courses using their academic background knowledge and specialties. Based on these experiences, this group explores further promotion of information exchange and collaboration among universities and institutes, both academic and industrial, and supports the development and dissemination of new experimental equipment and methods.

In 2013, this group advised the core research facilities to enhance functionality, and new equipment such as a high-sensitivity EM-DDCP camera system, protein identification software for mass spectrometry, and a spectrophotometer were purchased.

## Gender Equality Promotion Group

*Group Adviser:* *TAKADA, Shinji*

This group supports the improvement of the research environment for female researchers in both research and livelihood that includes the research support system for the period of childbirth and childcare, etc., aiming at promoting the employment of female researchers.

In 2013, this group assisted in giving publicity to the recruitment of an associate professor from female candidates to NIBB, and contributed to the management of the research support system for the period of childbirth and childcare. This group organized an enlightenment lecture and published a brochure for gender equality promotion in collaboration with other institutes of the NINS.

## TECHNICAL DIVISION

*Head*
FURUKAWA, Kazuhiko

### Common Facility Group

*Chief:* *MIWA, Tomoki*

● ***NIBB Core Research Facilities***

| | |
|---|---|
| *Unit Chief:* | *MORI, Tomoko* |
| *Subunit Chief:* | *MAKINO, Yumiko* |
| | *YAMAGUCHI, Katsushi* |
| *Technical Staff:* | *NISHIDE, Hiroyo* |
| | *NAKAMURA, Takanori* |
| | *TANIGUCHI-SAIDA, Misako* |
| | *UCHIKAWA, Tamaki* |
| *Technical Assistant:* | *ICHIKAWA, Chiaki* |
| | *NISHIMURA, Noriko* |
| | *OKA, Naomi* |
| *Secretary:* | *ICHIKAWA, Mariko* |
| | *ISHIKAWA, Azusa* |

● ***NIBB Bioresource Center***

| | |
|---|---|
| *Subunit Chief:* | *HAYASHI, Kohji* |
| | *MOROOKA, Naoki* |
| *Technical Staff:* | *NOGUCHI, Yuji* |
| *Technical Assistant:* | *TAKAGI, Yukari* |
| | *SUZUKI, Keiko* |
| | *SUZUKI, Kohta* |

● ***Disposal of Waste Matter Facility***

*Unit Chief:* *MATSUDA, Yoshimi*

● ***Center for Radioisotope Facilities***

| | |
|---|---|
| *Unit Chief:* | *MATSUDA, Yoshimi* |
| *Subunit Chief:* | *SAWADA, Kaoru* |
| *Technical Staff:* | *IINUMA, Hideko* |
| *Technical Assistant:* | *ITO, Takayo* |

### Research Support Group

*Chief:* *KAJIURA-KOBAYASHI, Hiroko*

● ***Cell Biology***

*Unit Chief:* *KONDO, Maki*

● ***Developmental Biology***

| | |
|---|---|
| *Technical Staff:* | *TAKAGI, Chiyo* |
| | *UTSUMI, Hideko* |
| | *OKA, Sanae* |
| | *NODA, Chiyo* |
| | *MIZUGUCHI, Hiroko* |

● ***Neurobiology***

| | |
|---|---|
| *Unit Chief:* | *OHSAWA, Sonoko* |
| *Subunit Chief:* | *TAKEUCHI, Yasushi* |

● ***Evolutionary Biology and Biodiversity***

| | |
|---|---|
| *Unit Chief:* | *FUKADA-TANAKA, Sachiko* |
| *Subunit Chief:* | *KABEYA, Yukiko* |

● ***Environmental Biology***

| | |
|---|---|
| *Unit Chief:* | *MIZUTANI, Takeshi* |
| *Technical Assistant:* | *INABA, Kayo* |

### Reception

| | |
|---|---|
| *Secretary:* | *TSUZUKI, Shihoko* |
| | *KATAOKA, Yukari* |
| | *UNO, Satoko* |
| | *MIYATA, Haruko* |

The Technical Division is a support organization for researchers and research organizations within NIBB. The Technical Division develops and promotes the institute's research activities and maintains the research functions of the institute.

The Technical Division is organized into two groups: the Common Facility Group, which supports and maintains the institute's common research facilities, and the Research Support Group, which assists research activities as described in the reports of individual research divisions.

Technical staff members continually participate, through the Division, in self-improvement and educational activities to increase their capabilities and expertise in technical areas. Generally, technical staff members are attached to specific divisions so that they may contribute their special biological and biophysical knowledge and techniques to various research activities.

The Technical Division hosts an annual meeting for technical engineers who work in various fields of biology at universities and research institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems. The proceedings are published after each meeting.

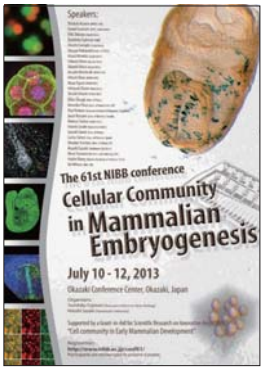
# The 61st NIBB Conference "Cellular Community in Mammalian Embryogenesis"

Organizers: Toshihiko Fujimori (NIBB), Hiroshi Sasaki (Kumamoto Univ.)

July 10(Wed)-12(Fri), 2013

July 8 heralded the end of the Japanese rainy season just in time for this year's 61st annual NIBB conference on Cellular Community in Mammalian Embryogenesis, held from July 10th through the 13th. The end of the rainy season brought the summer heat, but the heated discussions inside the halls were no less intense. Thanks to support from a Grant in Aid for Scientific Research on Innovative Areas (MEXT) "Cell Community in Early Mammalian Development," the conference hosted not just Japanese researchers, but speakers from England, Spain, Canada, China, Singapore, and Switzerland as well, with the final total of attendees and participants coming to just under 100 people, all gathered together to participate in, further, and listen to discussions of the latest research in early development in mammals.

Understanding of initial embryogenesis in mammals, particularly in mice, has advanced quite far from when the Scientific Research in Innovative Areas team first began their operations, with Japanese research groups often leading the field. Most of the first day was spent on presentations and discussions relating to research on pre-implantation embryos, with many attendees reporting astonishment at some of the findings. It was not a one-note session however, with presentations on embryology in mammals other than mice, quantitative analyses of post-implantation embryos and embryogenesis, and other related research as well. Both at home and abroad, chances for leading edge researchers to gather and discuss their findings are rare, with the tightly focused theme of this conference making this gathering even rarer, and one that will be remembered in years to come. Indeed, though throughout the world researchers sharing an interest in this topic are limited in number, it is our hope that this conference, through providing a central venue to gather, hold poster sessions, go on excursions, eat, drink, talk and discuss both findings and the future potential of that research, proved to be a fruitful event for all involved. Many attendees voiced their hope that around the world, research conferences in a similar vein to this one will continue to be held. As the hosts of the 61st NIBB conference, we are humbled and delighted, and hope to be hosts and participants to more such conferences in the future, wherever they may be.




**Speakers**

Constam, Daniel (EPFL), Plusa, Berenika (Univ. Manchester), Robson, Paul (Genome Inst. Singapore), Rodriguez, Tristan (Imp. Coll. London), Rossant, Janet (Univ. Toronto), Simon, Carlos (Univ. Valencia), Srinivas, Shankar (Univ. Oxford), Tzouanacou, Elena (Univ. Cambridge), Wilson, Val (MRC)
Aizawa, Shinichi (RIKEN CDB), Ebisuya, Miki (RIKEN CDB), Fujimori, Toshihiko (NIBB), Hamada, Hiroshi (Osaka Univ.), Kobayashi, Tetsuya (Univ. Tokyo), Kondoh, Hisato (Osaka Univ.), Meno, Chikara (Kyushu Univ.), Miura, Takashi (Kyushu Univ.), Mochizuki, Atsushi (RIKEN ASI), Niwa, Hitoshi (RIKEN CDB), Ogura, Atsuo (RIKEN BRC), Okano, Hideyuki (Keio Univ.), Onami, Shuichi (RIKEN QBiC), Osugi, Miho (Univ. Tokyo), Saito, Mitinori (Kyoto Univ.), Sasaki, Hiroshi (Kumamoto Univ.), Sawai, Satoshi (Univ. Tokyo), Suzuki, Atsushi (Yokohama City Univ.)

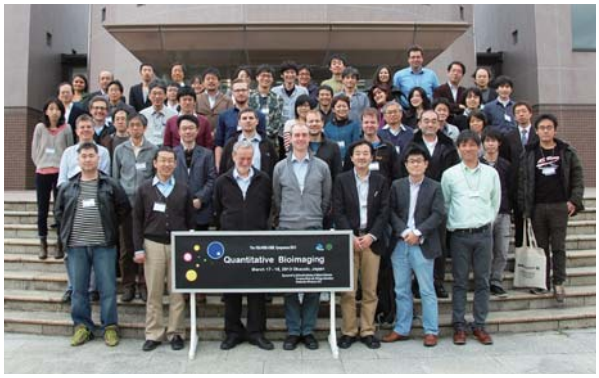
## NIBB-EMBL Collaborations

# The 10th NIBB-EMBL Symposium 2013 "Quantitative Bioimaging"

Organizers: Toshihiko Fujimori (NIBB), Naoto Ueno (NIBB), Matthias Weiss (Univ. Bayreuth), Rainer Pepperkok (EMBL)

March 17(Sun)-19(Tue), 2013

The 10th NIBB-EMBL Joint Symposium was held from March 17th to the 19th of 2013, on the topic of "Quantitative Bioimaging". Though the symposium was originally planned to be held in March 2011, it was indefinitely postponed due to the effects of the 2011 Tohoku earthquake and tsunami. It is with great pleasure then, that we saw the fruits of those preparations finally realized. Following after the 9th Joint Symposium's work, this symposium was held with the goal of facilitating deeper exploration of the state of imaging in biology, with cutting edge techniques in quantitative biological imaging, and areas showing potential for new development, being particular topics of vigorous discussion. The symposium was host to 10 speakers from abroad and 15 from within Japan, presenting on a variety of subjects including optical probes, new microscope technology, advances in super resolution microscopy, mathematical modeling, and 3D biology. After each presentation extra time was set aside for comprehensive discussions of the entire session, leading to enthusiastic debate. Inside the oral presentation venue space was set aside for poster presentations. This led to seeing many thought provoking discussions of the posters, which were not limited to the poster presentation session on the first evening, but also took place during breaks between sessions and during meal times. This was an unusual meeting with many young scientists and researchers from fields other than biology, including research fields like mathematics, physics, optics as well as engineers and technicians from industry microscope manufacturers. From the impressions of the participants from both Japan and abroad, they were stimulated to think about new possibilities in future research due to the environment being different from their normal scientific networks. With the participants coming from various academic backgrounds, ranging from students to expert researchers at the forefront of their fields, the open and natural debates and discussions were diverse and energetic; giving life to the smaller event hall and leaving an unforgettable impression on those who joined in.


**Speakers**

Dahan, Maxime (CNRS), Gratton, Enrico (UCI), Heisler, Marcus (EMBL Heidelberg), Hufnagel, Lars (EMBL Heidelberg), Kress, Holger (Univ. Bayreuth), Lakadamyali, Melike (ICFO, Spain), Pepperkok, Rainer (EMBL Heidelberg), Rippe, Karsten (DKFZ), Weiss, Matthias (Univ. Bayreuth), Wohland, Thorsten (NUS)

Aoki, Kazuhiro (Kyoto Univ.), Fujimori, Toshihiko (NIBB), Hayashi, Shigeo (RIKEN CDB), Kageyama, Ryoichiro (Kyoto Univ.), Kimura, Akatsuki (NIG), Kuroda, Shinya (Univ. Tokyo), Miyawaki, Atsushi (RIKEN BSI), Mochizuki, Atsushi (RIKEN ASI), Sasai, Yoshiki (RIKEN CDB), Sawai, Satoshi (Univ. Tokyo), Tokunaga, Makio (Tokyo Inst. Tech.), Ueno, Naoto (NIBB), Watanabe, Naoki (Tohoku Univ.), Yoshida, Shosei (NIBB)

## NIBB-EMBL Collaborations

# Sending Graduate Students to the EMBL PhD Students Symposium

Three PhD students from NIBB were funded by NIBB to participate in the 13th International EMBL PhD Students Symposium "Competition in Biology: The Race for Survival, from Molecules to Systems" (held on 21-23 November). Our students had the chance to give poster presentations at the PhD students symposium to introduce their current research. They also visit some laboratories in Europe and to exchange experimental information and discuss their research with PhD students, post-docs and PIs.

### Participating students from Japan

Rammohan Shukla
Toshiya Nishimura
Takema Sasaki

### Comments from student

• Rammohan Shukla

As a researcher it's important to present your work to both broad and domain specific audiences. While explaining your work to an audience of broad scientific backgrounds you get new perspectives toward your work. On the other hand, presenting to domain specific audiences fills the gap towards the understanding of your work. I am happy that I got the opportunity to present my work to both kinds of audience.

In Heidelberg Germany, the Ph.D symposium gave me an opportunity to interact with students of various scientific disciplines. I had a good time seeing and understanding others' work and explaining my own. It gave me an idea that while explaining to a broad audience I should focus on telling them my work as a story rather than presenting the in-depth analysis.

In Monterotondo, Italy I visited to Dr. Cornelius Gross. I had an opportunity to make a presentation of my work. That was my first scientific presentation to domain specific audience. It gave me an opportunity to skim out the most important result and organize it in a presentable form. The comments and questions asked filled up many gaps which I had towards the understanding of my work. In addition, I got many constructive suggestions from Dr. Gross to further improve my work.

Overall I had a good time deepening my understanding of science thanks to NIBB for providing me this wonderful opportunity.

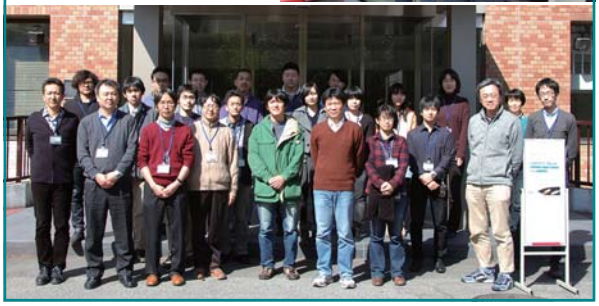
# The NIBB TALEN Training Course

Recent advancement of a reverse genetics method using an artificial nuclease (TALE nuclease) has made it possible to disrupt target genes in small fishes in an ordinary laboratory. In response to requests from domestic researchers' community using small fishes for a practical course on the gene disruption method using TALEN, we held a training course with the help of Dr. Masato Kinoshita (Kyoto Univ.) and Dr. Atsuo Kawahara (RIKEN). The same two-and-a-half-day course was given twice in tandem to accommodate the larger number of participants than expected. The course was designed so that the participants could proceed with gene disruption experiments by themselves back in their laboratories, and the practices of the key techniques of TALEN method were given, together with lectures on essential principles of gene disruption using TALEN. Participants were very active in both practices and lectures, and we could feel the high level of expectation of this method. We hope participants continue to communicate with each other and further advance their research. (Shinji Takada and Taijiro Yabe)

## "Gene disruption method using an artificial nuclease in small fishes (TALEN practical course)"

February 25 (Mon) -27 (Wed), 2013
February 27 (Wed) -March 1 (Fri), 2013

- Organizers: Dr. Shinji Takada (NIBB), Dr. Atsuo Kawahara (RIKEN), Dr. Masato Kinoshita (Kyoto Univ.), Dr. Taijiro Yabe (NIBB)
- Lecturers: Dr. Atsuo Kawahara (RIKEN), Dr. Yu Hisano (RIKEN), Dr. Masato Kinoshita (Kyoto Univ.), Mr. Satoshi Ansai (Kyoto Univ.), Dr. Taijiro Yabe (NIBB)
- Participants: 34
- Lecture: Gene disruption method using TALEN in the fish -Explanation of basic methods and introduction of practical applications-
- Practical Course: Preparation of TALEN construct and evaluation of mutation efficiency



# The NIBB Genome Informatics Training Course

NIBB organizes a series of training courses on up-to-date research techniques for researchers from mainly Japanese universities and institutions. In 2013 we held two training courses on Genome Informatics. The two-day programs offered lectures and hands-on tutorials to introduce basic knowledge and skills to deal with genomic scale large data such as those obtained by next-generation sequencing (NGS). The programs were specially designed for biologists who were not familiar with bioinformatics.

## "Introduction to Transcriptome Data Analysis"

March 14 (Thu) -15 (Fri), 2013

- Organizer: Dr. Shuji Shigenobu (NIBB Core Research Facilities)
- Lecturers: Dr. Shuji Shigenobu, Dr. Masanao Sato, Dr. Ikuo Uchiyama, Dr. Katsushi Yamaguchi
- Participants: 20 (including 2 from NIBB)
- Program:
  1. Overview: Transcriptome data analysis
  2. Introduction to statistics
  3. Introduction to "R"
  4. RNA-seq analysis pipeline
  5. Basic format of mapping data and basic tools
  6. Expression data analysis I: Normalization, differential expression analysis
  7. Expression data analysis II: Multivariate analysis
  8. Excercise

## "Introduction to Next-generation DNA Sequence Data Analysis"

September 19 (Thu) -20 (Fri), 2013

- Organizer: Dr. Shuji Shigenobu (NIBB Core Research Facilities)
- Lecturers: Dr. Shuji Shigenobu, Dr. Ikuo Uchiyama, Mr. Tomoki Miwa, Dr. Katsushi Yamaguchi, Dr. Taro Maeda
- Participants: 19 (including 3 from NIBB)
- Program:
  1. Overview: NGS data analysis
  2. UNIX for beginners
  3. NGS basic data formats
  4. NGS basic tools I: Mapping
  5. NGS basic tools II: Visualization tools
  6. NGS basic tools III: Samtools
  7. Methods for text data processing
  8. Excercise

# The NIBB Bioimage Analysis Training Course

Thanks to the enhancement of the performance of imaging devices such as microscopes and cameras, it is getting more and more important to handle large-sized and multi-dimensional image data in the biological research. Most biology researchers, however, lack the basic knowledge of image data handlings. Training courses of microscopic observations are frequently held, while coursed centered on image data handlings are seldom held. To improve such situations the spectrograpy and bioimaging facility, in collaboration with the department of imaging science, the center for novel science initiative, NINS, held a three-day practical course and lectures on the basis of image data processing and analysis. The course proceeded according to the program shown below and, at the end of the course, participants discussed over real problems some of the participants are facing. Most of the participants were satisfied with the course and we plan to hold such courses periodically in future. (Shigenori Nonaka)

October16(Wed)-18(Fri),2013

- Organizers: Dr. Takuo Yasunaga (Kyushu Inst. Tech.), Dr. Yuki Tsukada (Nagoya Univ.), Dr. Yoshitaka Kimori (NIBB), Dr. Kagayaki Kato (NIBB), Dr. Yasuhiro Kamei (NIBB), Dr. Shigenori Nonaka (NIBB), Dr. Takashi Murata (NIBB), Dr. Hiroshi Koyama (NIBB)
- Participants: 22 (including 2 from NINS)
- Program:
  - Basis of image processing and analysis (lecture and practice)
  - Usage of ImageJ software and its macros (lecture and practice)
  - Quantitative analysis of images (lecture and practice)
- Lectures:

"Dynamic description and information extraction of biological phenomena by analyzing movie data" Dr. Yuki Tsukada

"Basic knowledge of microscope for image analysis" Dr. Takashi Murata

# The NIBB Internship program

The NIBB Internship program is a hands on learning experience started in 2009 as a way to promote NIBB with overseas students and to build connections through providing education to the people who will form the core of future research networks. At the same time, this program aims to internationalize the graduate students of the Graduate University for Advanced Studies (SOKENDAI), giving them the opportunity to get to know students and interns with various cultural customs.

To participate in this program, applicants who would like to experience research at NIBB must supply the name of the lab they would like to visit as well as their reasons for choosing it and a letter of recommendation. Based on this information applicants are chosen to spend set periods of time participating in specific research activities in the lab they applied for. Round trip airfare and housing expenses are provided by the NIBB Internship Program.

In FY 2013 there were 16 applicants, of which five interns were selected. These interns were from universities located in 4 countries (India, Bangladesh, Hungary, and China) and spent periods ranging from one to twelve weeks experiencing life as a member of a research team.

## Report from a participant
## Manirui Haaque
## University of Dhaka, Bangladesh

I worked in the Laboratory of Molecular Genetics for Reproduction, under the supervision of Dr. Minoru Tanaka from 1st Octobor to 1st November, 2013. During my stay I have done several experiments but my major topic was "Detection of laminin in female gonad of medaka (*Oryzias latipes*) during development".

I successfully completed the experiments and also learned many advanced techniques. To detect laminin I learned immunohistochemistry which was the first time for me. I did this experiment using 18 dph (day post hatch) *Olvas*-transgenic medaka. GFP (Green Fluorescent Protein) was used to detect the gonad which express green color in germ cells under microscope. I have also done another experiment that was detection of laminin in matured gonad of *hotei* medka. Interestingly, we found oocyte like structure in XY male *hotei* gonad along with spermatocyte. In addition, I learned XY typing (Genotyping) of *hotei* (germcell excess medaka), DNA sequencing, microinjection, use of confocal microscopes and so on.

Every lab member was very open and ready to help. I was so overwhelmed by such a nice lab environment. This internship was very important for me to become an efficient researcher in the future.

# INDEX

# Access


## From Tokyo

Take the Tokaido Shinkansen and get off at Toyohashi, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 20 minutes from Toyohashi).

## From Osaka

Take the Tokaido Shinkansen and get off at Nagoya, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 30 minutes from Nagoya).

## From Central Japan International Airport

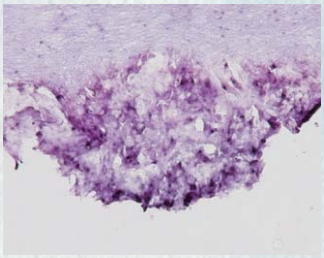
Get on the Meitetsu bus bound for JR Okazaki Station and get off at Higashi Okazaki Station (approximately one hour from the airport). Alternatively, take the Meitetsu Line for Nagoya and change at Jingu-mae Station to a Toyohashi-bound train and get off at Higashi Okazaki Station (approximately 70 minutes from the airport).

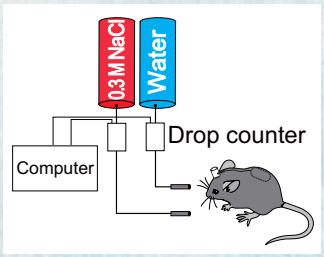

## From Higashi Okazaki Station to Each Area

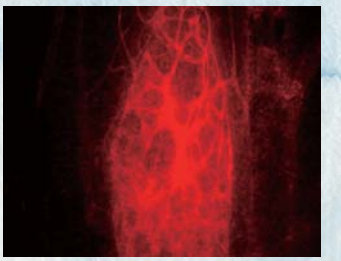
Turn left (south) at the ticket barrier and exit the station. The Institute is a 7-minute walk up the hill (Myodaiji-area) or 20-minute walk (Yamate-area).

## By car

Take the Tomei Expressway to the Okazaki Exit. Turn left at the City Office S.E. signal (approximately 10 minutes from the Exit).





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