HHMI
HOWARD HUGHES MEDICAL INSTITUTE

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Dr. Yves Brun
Department of Biology
Indiana University
Jordan Hall 142, 1001 E 3rd Street
Bloomington, IN 47405-7005

Dear Dr. Yves Brun,

I would like to be considered for a faculty position in your department (advertised in the 2 September issue of *Science*). I am currently a postdoctoral fellow in the Howard Hughes Medical Center, Department of Molecular Genetics and Microbiology at Duke University Medical Center. My research interest is to understand the molecular mechanisms of cell cycle regulation and its interaction with other cellular functions in multi cellular organisms. I am currently studying the APC (anaphase-promoting complex) in the model organism *Drosophila melanogaster.*

As part of my graduate work with Dr. Fumio Hanaoka (Osaka University, Japan), I characterized the molecular mechanisms of human nucleotide excision repair (NER) reaction, which is deficient in cancer prone Xeroderma Pigmentosum (XP) patients. Reconstituting the NER reaction on chromatin structured-DNA with purified proteins, I found that a chromatin-remodeling factor is important for NER on nucleosome linker DNA. In addition, I identified Centrin 2 (Cen2) as a third subunit of XPC (XP group C responsible gene) complex, and showed that Cen2 enhances NER activity by stabilizing XPC. I also contributed to the purification and ultimate identification of DNA polymerase η (eta), which is mutant in XP group V patients.

In order to learn genetic approaches with a model organism, I joined Robin Wharton's laboratory (Duke University, North Carolina) as a post-doctoral fellow. Using *Drosophila* genetics and *Xenopus* biochemical assays, I showed that *Drosophila* ORC1 (origin recognition complex protein 1) degradation is mediated by the APC *via* a novel APC-targeting sequence, the O-box (ORC1-destruction box). This study reveals a direct involvement of the APC, known primarily for its role as a mitosis regulator, in regulation of DNA replication.

Since finding this new APC-targeting sequence, I have searched for potential APC substrates possessing the O-box, and found two novel substrates in *Drosophila*. I am currently trying to uncover the roles of degradation of these new APC substrates *in vivo*. In addition, I am investigating the molecular mechanism of O-box-dependent ubiquitylation by the APC using biochemical approaches. I believe that my current research will reveal new insights into APC-dependent protein degradation in multi-cellular organisms. This will be of benefit to us to understand biological events where the APC substrates play important roles.

I am confident that not only my research project but also my unique carrier in biochemistry and genetics will strengthen your department. Also, my experience in studying a human disease and a model organism may be valuable for translational research in your department.

Enclosed please find my curriculum vitae, a brief statement of research plan, a summary of past accomplishments, a statement of teaching interest, and representative publications. Please let me know if I can provide additional information in support of my application.

Sincerely,

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**Research accomplishment**

Faithful inheritance and maintenance of genomic information is essential for life to sustain its integrity. I have been interested in studying the molecular mechanisms underlying this fundamental task for life in terms of DNA repair for PhD thesis and DNA replication for post-doctoral training.

**PhD thesis research**

During my thesis work with Dr. Fumio Hanaoka (Osaka University, Japan), I elucidated the molecular mechanism of nucleotide excision repair (NER) which is deficient in cancer-prone Xeroderma Pigmentosum (XP) patients. Primarily using biochemical approaches, I pursued two projects independently and contributed to two other projects.

**1) Characterization of NER on chromatin DNA**

Eukaryotic DNA is wrapped around the histones, so access to damaged DNA is limited. In order to understand the molecular mechanism of NER reaction on chromatin, I reconstituted a cell-free reaction with purified proteins. Fractionating crude cell extracts and replacing fractions by purified XP gene products, I reconstituted the reaction with six components. However, I found that chromatin DNA is poorly repaired compared to naked DNA, suggesting the involvement of chromatin specific factors for NER. Examining chromatin-remodeling complexes, I found that ACF (ATP-dependent chromatin-remodeling complex) enhances NER activity. This work shows that nucleosome-remodeling activity is involved in NER.

**2) Idetification and Charaterization of Centrin 2 as a XPC subunit**

During the reconstitution experiments, I identified Centrin 2 (Cen2) as a third subunit of XPC complex. Despite the fact that Cen2 is reported to be a component of the centrosome, I found that Cen2 is predominantly localized the nucleus, with localization primarily depending on XPC. Examining the effect of Cen2 in a reconstituted NER assay, I showed that Cen2 enhances NER by stabilizing the XPC protein. These findings reveal new insights of XPC complex formation and Cen2 function in NER.

**3) Characterization of hHR23B function in NER**

I was involved in characterization of a second subunit of the XPC complex, hHR23B. We had previously shown that hHR23B stimulates XPC activity in NER by an unknown mechanism. In order to understand the molecular mechanism of this stimulation, we examined XPC-binding and NER-stimulatory activity of hHR23B with a number of deletion mutants. We identified a 56-amino acid peptide that is necessary and sufficient for both activities, strongly suggesting that binding of hHR23B alters the conformation of XPC and stimulates its activity.

**4) Identification and Characterization of DNA polymerase η (eta)**

XP group V (variant) patients have a normal level of NER, rather show deficiency in translesion DNA replication. However, the gene mutant in XP-V was not known. Purifying proteins which

complement a lack of translesion replication in XP-V cell extracts, I isolated a 54kDa protein. Further characterization showed this protein is a new class of DNA polymerase, which can incorporate a "correct" base opposite a damaged base. We identified the gene encoding this DNA polymerase, pol η(eta), and found mutation in the gene in all XP-V patients. These findings reveal that a lack of error-free translesion DNA polymerase results in an accumulation of mis-incorporated bases which later causes cancer in XP-V patients.

**Post-doctoral research**

In order to learn genetic approaches with a model organism system, I joined Dr. Robin Wharton's laboratory (Duke University, North Carolina) as a post-doctoral fellow. Combining fly genetics approaches and biochemical assays, I found three important aspects of cell cycle regulation in *Drosophila*.

**1) Identification of the APC as an ORC1 regulator**

In the beginning of the DNA replication initiation, the origin recognition complex (ORC) consisting of ORC1 to 6 plays a key role. ORC binds directly to the DNA replication origin, and recruits other DNA replication initiation factors. In *Drosophila*, we had found that the level of ORC1 protein fluctuates during the cell cycle. Mis-expression of ORC1 results in an ectopic DNA replication, suggesting that ORC1 serves as a limiting factor to ensure DNA replication once per cell cycle.

In order to understand the molecular mechanism of ORC1 degradation, I first determined when ORC1 disappears. Double staining with cell cycle markers reveals that ORC1 protein diminishes at the end of M phase. Since the anaphase-promoting complex (APC) mediates degradation of several cell cycle proteins during the M and G1 phase, I tested whether the APC mediates ORC1 degradation. By genetic gain- and loss-of function analyses, I showed that the APC mediates ORC1 degradation at the end of M phase and down-regulates ORC1 accumulation during much of G1 phase. Using an *in vitro* APC-dependent ubiquitylation assay with purified proteins, I demonstrated that the APC directly ubiquitylates ORC1.

**2) Identification of a novel APC-targeting sequence, the O-box**

The APC recognizes discrete short sequences known as the destruction box (D-box) and KEN-box. Mutation of those motifs abolishes APC-dependent ubiquitylation and results in stabilization of the APC substrates *in vivo*. Although we mutated four of these sequences in ORC1, the mutant protein was still ubiquitylated and degraded *via* the APC. Examining the APC-dependent ubiquitylation with a variety of ORC1 mutant proteins *in vitro*, I identified a novel APC-targeting sequence named the O-box (ORC1-destruction box). Point mutations of the O-box completely stabilize ORC1 *in vivo*, showing that the O-box is targeted by the APC for degradation.

**3) Identification of other APC substrates possessing the O-box.**

Since I found a novel APC-targeting sequence, I searched for proteins possessing sequences similar to the O-box. Narrowing down the candidates by looking at their annotated gene functions, I obtained nine genes involved in cell cycle regulation. First, I examined *S. Pombe* Cut2 which is already shown to be targeted by the APC *via* its N-terminus D-box sequences. Deletion of the D-box reduces, but doesn't eliminate, APC-dependent ubiquitylation. Remaining activity is diminished by mutation of the O-box. Examining other candidates using an *in vitro* APC-dependent ubiquitylation assay, I found that *Drosophila* Asp is a novel APC substrate. Mutation of the O-box significantly reduces ubiquitylation of Asp, demonstrating that the O-box serves as a degradation signal in other cell cycle proteins.

*Marito Araki, PhD*

# Understanding APC-dependent protein degradation *via* a novel motif, the O-box, in *Drosophila melanogaster*

## Research interest

My long term research goal is to elucidate the molecular mechanisms of cell cycle regulation in metazoans, and to define the roles of cell cycle regulators in other cellular functions. In particular, I am interested in studying the APC (anaphase-promoting complex), one of the key ubiquitin ligase complexes (E3) in cell cycle regulation. The APC regulates progression through and exit from mitosis by mediating the sequential degradation of the A- and B-type Cyclins and securin/Pds1. In addition, the APC mediates degradation of other proteins that function in processes other than mitosis such as DNA replication initiation, asymmetric cell division, TGF-β signaling, and meiosis. These APC substrates have been studied primarily in yeast or *in vitro* using *Xenopus* extracts, and so the role regulated degradation of these proteins is not well-understood in multi-cellular organisms. Defining the roles of APC-dependent protein degradation *in vivo* will benefit our understanding of carcinogenesis, development, and other processes where APC substrates play crucial roles.

## Research plan

I propose to pursue three major projects to define the APC function in *Drosophila*. 1) Identification of novel APC substrates, 2) Investigation of the role for regulated degradation of APC substrates, and 3) Characterization of the molecular mechanisms of APC-dependent ubiquitylation *via* the O-box, a new APC-targeting sequence.

I have chosen *Drosophila melanogaster* as a model system to study the APC for four reasons: **1) High quality genomic information.** The genome sequence is well-annotated, and other resources such as cDNA and Bac clones are readily available. **2) Excellent genetics tools.** A large number of tissue-specific promoter lines are available for gain-of function analysis. The FRT/FLP system in combination with mutant fly lines can be used for tissue-specific loss-of function experiments. **3) Mutant availability.** Deficiency, P-element insertion, and mutant fly lines that cover more than 85% of the euchromatic genome are available for loss-of function analysis *in vivo*. Gene targeting is also feasible for the generation of mutant animals. **4) Highly conserved genes.** Most fly genes, including those encoding the APC components, are conserved in higher eukaryotes.

### 1) Identification of novel APC substrates.

The APC recognizes discrete short sequences on its substrate proteins. I have recently identified a novel APC-targeting sequence, the O-box (ORC1-destruction box), which is responsible for ORC1(origin recognition complex protein 1)-degradation in *Drosophila*. By searching for proteins possessing sequences similar to the O-box in the database, I have found 54 potential APC substrates. So far, I have examined 21 of these using an *in vitro* APC-dependent ubiquitylation assay, and identified Asp (abnormal spindle protein) and Bicoid as novel APC substrates.

**2) Investigation of the roles for regulated degradation of APC substrates in *Drosophila* tissues.**

Since ubiquitylation by APC is a rate-limiting step for the proteasome-dependent degradation of APC substrates, mutation in the APC-targeting motif stabilizes the APC substrate *in vivo*. When the APC-targeting sequence is mutated, Cyclin B, the best-characterized APC substrate, is stabilized and ectopically activates Cdk1 (Cdc2). This causes cells to arrest in G2/M, showing that the APC-dependent degradation of Cyclin B is required for progression through the mitosis.

As described above, I have recently shown that Asp as a novel APC substrate. I have already shown that mutation of the O-box on Asp perturbs the APC-dependent ubiquitylation *in vitro*, demonstrating that Asp is targeted by the APC *via* the O-box. In order to define roles for the degradation of Asp, I will examine the phenotype resulting from expression of a non-degradable Asp protein *in vivo*. This experiment could shed a light on a poorly characterized role of Asp protein in spindle organization during cell division.

I will also investigate the role of Bicoid ubiquitylation by the APC. Interestingly, ubiquitylation of Bicoid by the APC remains at the oligo- or mono-ubiquitylation level, which has completely different physiological roles (e.g. protein trafficking). However, I favor the idea that Bicoid needs to be properly modified upon being targeted by the APC, which may be lacking in an *in vitro* system. During the early development in *Drosophila*, Bicoid plays a crucial role in establishing the anterior-posterior axis formation and then rapidly disappears by an unknown mechanism. So, I will examine phenotypes caused by ectopically stabilizing Bicoid proteins with a mutated O-box.

**3) Characterization of the molecular mechanism(s) of the APC dependent-ubiquitylation.**

Despite the fact that APC-dependent ubiquitylation has been reconstituted with purified proteins, the mechanism by which the APC recognizes its substrates is poorly understood. To date, two major APC-targeting sequences, the D- and KEN-boxes, have been well studied. In recent reports, these targeting motifs seem to play distinct roles during the degradation of yeast Hsl1: the KEN-box mediates interaction between the APC-activator and substrate, and the D-box enhances the interaction between the APC activator and APC core subunits in a ternary complex.

I will elucidate the role of the O-box in the APC-dependent ubiquitylation. Since ORC1 ubiquitylation is solely depending on the O-box, I assume that the O-box possesses both of the function that are carried out separately by KEN- and D-boxes of yeast Hsl1. The O-box might directly bind to the APC activator, causing a conformational change that stabilizes the interaction between the activator and the APC core. Alternatively, the O-box might be recognized differently from the way other motifs are recognized by the APC activator and/or APC core.

Currently, I am testing whether the O-box interacts directly with the APC activator and enhances the interaction between the activator and APC core subunits using *in vitro* binding assays. To further define the interaction during the ubiquitylation reaction, I will perform cross-linking

experiments with chemically modified O-box containing proteins. In future, structural analysis might be necessary to elucidate the molecular mechanism of substrate recognition by APC activator and the APC core *via* the O-box.

I believe this research will provide a solid foundation on which to build a productive, independent research program focused on the role of the APC in multi-cellular organisms.

**Teaching Interests**

My teaching philosophy is to develop learners' scientific thinking skills, since I believe that analytical thinking is the foundation of science. I also emphasize learning facts and figures, since thinking is based on knowledge. However, basic knowledge doesn't yield a good product unless it is handled in appropriate and effective ways. So, I place a significant emphasis on thinking skills.

In order to achieve this goal, I would like to get students to consider problems in the discipline and how people have solved them in the past. In practice, I will give a problem to students and let them discuss in the class the problem and its solution. The problem could be solved in a different way, or maybe in several different methods due to technical advances. This gives students further opportunities to gain an ability to adjust to varied circumstances. In planning and leading a course, I will tailor the problems to the level of the students, encourage them to think about the problem, and enhance discussions by providing critical cues.

From my experience in teaching, I believe that motivation is the most important element in learning. In order to motivate learners, I will make my teaching style learner-oriented. I will provide a mix of activities to involve the students as much as possible because I believe that involvements stimulate motivation. I will facilitate problem-based learning, group study, small group discussion, and guided discussion. I would like to minimize lectures, rather, I will provide readings or group projects before the class to foster group discussion and interactive participation in the class. Students sometimes may resist this style in the beginning, but with encouragement, they will rise to the challenge and begin to initiate interactions themselves. I believe I am enthusiastic and have an ability to find an effective way to do this.

Other than teaching in the classroom, I like teaching students in the laboratory to train prospective scientists. During my thesis study, I worked in a big laboratory consisting of twenty graduate students and several senior scientists. Probably because I am patient, I was often consulted by lab members, and learned a great deal about mentorship in the process. I dealt with a wide variety of problems arose in and out of the laboratory, so I am confident that I will be a good teacher not only in the classroom but also in the laboratory.