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研究課題名（和文）GANP may regulate the c-Myc expression in germinal centers (GCs), that is essential for the affinity maturation of B cells

研究課題名（英文）GANP may reulates the c-Myc expression in germinal center (GCs), that is essential for the affinity maturation of B cells)

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研究成果の概要（和文）：GANPは、B細胞のc-Myc　mRNAの5'UTR領域と相互作用することでc-Mycの翻訳を調節します。GANPは、GCでセレクションされるB細胞におけるc-Mycのアップレギュレーションに必要です。 GANPによる増強されたc-Myc発現は、B細胞のポジティブセレクションの調節シグナルである可能性があります。

研究成果の学術的意義や社会的意義
Our studies suggest that GANP regulates the c-Myc mRNA in cooperation with translation initiation factor eIF4E, for the positive selection of high affinity GC B cells during immune responses. Our research direction holds great promise for the discovery of new targets for therapeutic applications.

研究成果の概要（英文）：Germinal Center (GC)-associated nuclear protein (GANP) was discovered as up-regulated molecule in GC B cells upon T cell dependent immune responses, GANP-deficient mice failed to generate affinity-maturation of Ag-specific antibodies in B cells thus; GANP is critical molecule for the regulation of adaptive immune system.
We investigated mRNAs and proteins associated with GANP in B cells. GANP interacts with many kinds of RNAs for various functions of cancer development, cell cycling, DNA repair, and protein translation. GANP interacts with the translation initiation factor eIF4E and efficiently targets the transcription factor c-Myc mRNA. Furthermore, GANP regulates c-Myc translation via interacting with the 5’ untranslated region of c-Myc mRNA in B cells. The results indicated that GANP is necessary for the upregulation of c-Myc in B cells undergoing the selection process in the GCs. The augmented c-Myc expression by GANP is likely a regulatory signal for B cell positive selection.

研究分野：Immune Regulations

キーワード：GANP　c-Myc　eIF4E

## １．研究開始当初の背景

Acquired immunity is dependent on affinity maturation and class switching of antibodies (Abs) against exogenous antigens (Ags). Ag-reactive B-cell clone proliferate and differentiate in germinal centers (GCs) of lymphoid organs. The B-cells undergo somatic hypermutation (SHM) of the rearranged immunoglobulin (Ig) variable (V) region gene by the activation induced cytidine deaminase (AID). As an AID-associated protein, GANP is a 210-kDa protein upregulated in GCs upon T-cell dependent (TD)-Ag responses (Kuwahara K et al, Blood, 2000). GANP has a unique structure, the middle portion is homologous to the Saccharomyces Sac3 that is involved in mRNA export (Wickramasinghe et al, Curr Biol, 2010). The N-terminal contains the GC-rich regions that potentially associate with the NPCs. GANP interacts with the TREX-2 components, ENY2, PCID2, Centrin2/3, and DSS1, and facilitates the NXF1-dependent mRNP export in mammalian cells. GANP-deficient mice failed to generate high levels somatic hypermutations (SHM) at the rearranged IgV-locus and could not generate affinity-maturation of Ag-specific antibodies in B cells thus; GANP is critical molecule for the regulation of adaptive immune system.

## ２．研究の目的

GC-associated nuclear protein (GANP) was discovered as up-regulated molecule in GC B cells upon T cell dependent immune responses, contains multiple domains having different functions. GANP expression is ubiquitous in mammalian cells but is increased in Ag-driven GC B cells. GANP expressed at high levels in various hematological disorders including Hodgkin's disease, acute myelogenous leukemia, chronic lymphocyte leukemia, and myelodyscrasia syndrome in the clinical specimens. The overexpression of GANP in B-cell targeted transgenic mice developed the B-lineage lymphomas with Hodgkin-like characteristic. Similar high-level expression of GANP was also demonstrated in cases of other malignancies such as malignant melanomas and cholangiocarcinomas. It has been shown previously, mammalian cells require GANP for efficient mRNP nuclear export to nuclear pore complex (NPCs) via NXF1 dependent pathway. Depletion of GANP reduces the amount of NXF1 at NPCs. However, detail molecular function of mammalian GANP regarding its RNA binding targets in immune cells has not been determined. This study aim to investigate the novel role of GANP and its binding RNA targets during immune responses in GC B cells.

## ３．研究の方法

(1) To investigate GANP-interacting target molecules in B cells, we carried out mass spectrometry (MS) analysis from ramos B cells using anti-GANP Ab mediated immunoprecipitation.
(2) GANP interact with mRNA cap complex in B cells (from MS data), next we performed the cap-binding assay using 7-Methyl-GTP-Agarose (m7-GTP-agarose) affinity resins, for the detection of cap binding complex with GANP.
(3) Investigation of GANP-binding mRNA targets using high-throughput sequencing of crosslinked immunoprecipitated RNA (HITS-CLIP) using human Ramos B cells and mouse splenic B cells (Illumina platform).
(4) HITS-CLIP data indicate that GANP markedly interacts with *c-Myc* mRNA both in human and mouse B cells, we confirmed the binding of GANP with the *c-Myc* mRNAs using RNA IP (RIP) assay, followed by the gene-specific q-PCR.
(5) Next, we examined the effect of GANP on *c-Myc* expression in Ramos B cell using *Ganp*-KD (Si oligos) and GANP over-expression (mammalian expression vectors).
(6) To confirm the interaction of GANP with the *c-Myc* mRNA affecting the *c-Myc* translation, we carried out luciferase reporter assay, using the *5'UTR sequence* of the *human c-Myc* mRNA at the 5' side of the *luciferase* gene (pGL3).
(7) To address whether GANP regulates *c-Myc* expression in GC B cells, we used *Aicda-cre* mice to generate GANP depletion, specifically in GC B cells. GC B cells were isolated from 6-8-week-old *Aicda-cre* $Ganp^{Flox/Flox}$ mice after 8 days of immunization with sheep red blood cells (SRBC) followed by FACS and western blot.
(8) To explore the role played by GANP during GC B cell development, we performed single-cell (sc) transcriptomic analysis (10X genomics) mouse GC B cells, in response to T cell–dependent antigens after SRBC immunizations.

４．研究成果

**(1) GANP interacts with the translation initiation complex in B cells**

To investigate GANP-interacting target molecules in B cells, we carried out mass spectrometry (MS) analysis of IP with the endogenous GANP from three independent biological replicates of Ramos B cells. I found that GANP directly interacts with the translation initiation components (eIF4E, eIF4G, and eIF4A). I further confirmed the interaction between GANP and translation initiation complex using co-precipitation experiments; I found that GANP interact with eIF4E with its binding partner eIF4G and eIF4A in ramos B cells lysates (Figure 1. a, b, c, d).

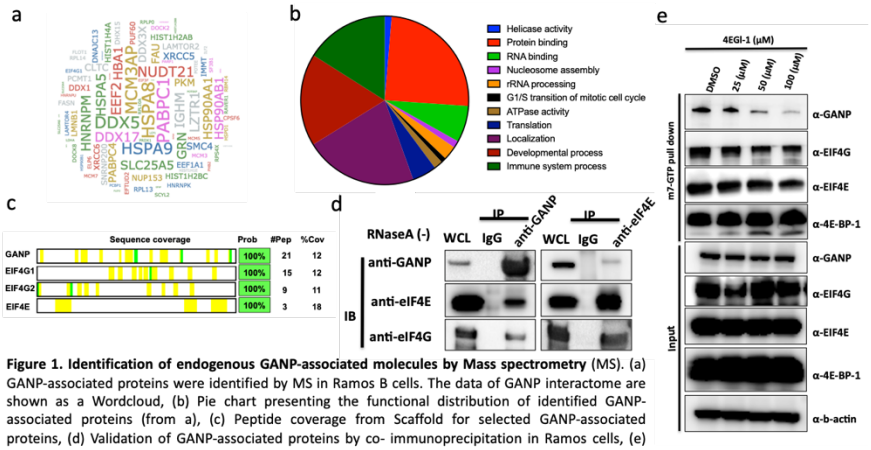


**Figure 1. Identification of endogenous GANP-associated molecules by Mass spectrometry** (MS). (a) GANP-associated proteins were identified by MS in Ramos B cells. The data of GANP interactome are shown as a Wordcloud, (b) Pie chart presenting the functional distribution of identified GANP-associated proteins (from a), (c) Peptide coverage from Scaffold for selected GANP-associated proteins, (d) Validation of GANP-associated proteins by co- immunoprecipitation in Ramos cells, (e) Cap-binding assay using inhibitor reagent 4EGI-1 that can dissociate the interaction between eIF4E and eIF4G from cap-containing mRNA.

**(2) GANP interact with translation complex in a cap-dependent manner**

We examined whether GANP interacts with eIF4E in a cap-dependent manner by using 7-Methyl-GTP-Agarose (m7-GTP-agarose) affinity resins, which traps eIF4E and its two binding partners. GANP interacts with eIF4E and eIF4G as the cap-binding complex from the B cell lysate. To confirm this result, we used an inhibitor reagent 4EGI-1 that can dissociate the interaction between eIF4E and eIF4G from cap-containing mRNA in cells as well as in extracts. I found that GANP interacts with translation initiation components in a cap dependent manner (Figure 1. e).

**(3) Investigation of GANP-binding mRNA targets in B cells**

To investigate the GANP-binding mRNA targets, we employed high-throughput sequencing of immunoprecipitated (IP) and crosslinked RNA (HITS-CLIP) using human Ramos B cells and mouse splenic B cells (Figure 2. a, b). To identify the most consistent RNA targets of GANP, we classified the genes that exert the higher number of the CLIP tags in each cluster. Initial screening identified 3161 human genes and 3351 mouse genes as the target RNAs associated strongly with GANP (Figure 2. c). Notably, 1278 RNA transcripts are common in both humans and mice. Gene ontology (GO) term analysis revealed enrichment of genes involved in cellular process, cellular component organization, signaling, biological regulation, translation initiation and immune regulation.

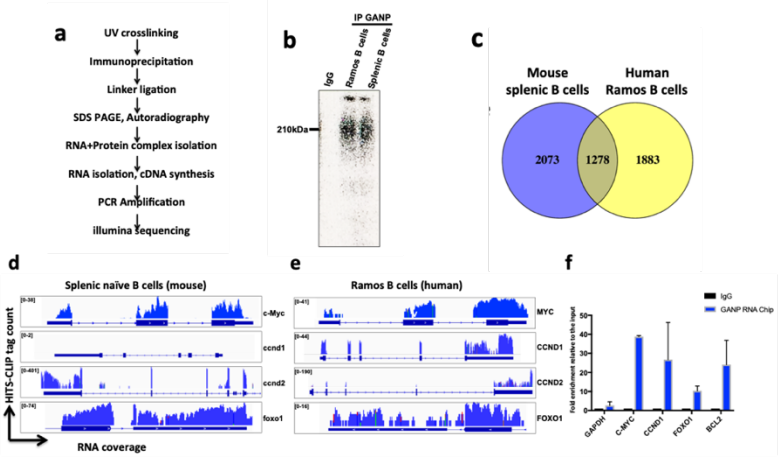


**Figure 2. GANP HITS-CLIP of Human ramos b cells and Mouse spleenic b cells using anti-GANP antibody.**
(a) Schematic representation of HITS-CLIP. (b) GANP-RNA complexes were excised from the PAGE-gel for library preparation after immunoprecipitation and radiolabeling. IgG(negative control), (c) Overlap between human and mouse GANP-RNA targets. (d,e) GANP associated with the transcription factors *c-Myc*, CCND1 and CCND2 mRNA from human and mice (CLIP tag count and RNA coverage).(f) RNA immunoprecipitation assay using anti-GANP antibody in human ramos B cells and HITS-CLIP target, cDNA were were generated and amplified with gene specific primers.

**(4) GANP strongly interacts with the immune regulatory transcripts in B cells**

We found that GANP markedly interacts with the major transcription factors (*c-Myc*, *Ccnd1* and *Foxo1* mRNA) associated with GC B cell function from HITS-CLIP and GO term analysis. Amongst these RNAs, GANP markedly interacts with *c-Myc* mRNA both in human and mouse B cells (Figure2. d, e). We confirmed the binding of GANP with the mRNAs using RNA IP (RIP), followed by the gene-specific q-PCR. GANP indeed binds with *c-Myc* mRNA at a very high level in Ramos B cells (Figure2. f). The HITS-CLIP and RIP seq analysis showed the significant binding of GANP with *c-Myc* mRNA, suggesting that GANP is one of the *c-Myc*-binding cofactors working at the post-transcriptional process in B cells.

**(5) Downregulation of GANP decreases *c-Myc* translation**

Next, we examined the effect of GANP on *c-Myc* expression in Ramos B cells. *Ganp*-KD significantly decreased the protein level of *c-Myc* and Bcl2 but did not cause any change in the expression of eIF4E, eIF4A and GAPDH protein. The results suggest that GANP significantly targets the restricted and specified mRNAs such as *c-Myc* and *Bcl-2* in B cells. Conversely, overexpression of GANP enhances the *c-Myc* translation (Figure3. a-b).

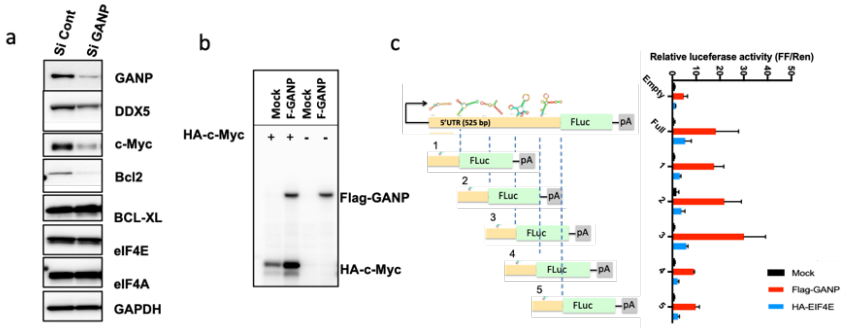


**Figure 3.** (a) Ramos B cells transfected with Si cont or Si GANP followed by western blot. (b) 293T cells transfected with GANP or c-Myc, c-Myc and GANP expression was checked by western blot. (c) luciferase reporter plasmid was generated containing 5'UTR of c-Myc, luciferase activity was measured by dual luc assay.

**(6) GANP enhances translation activity by interacting with 5'UTR of *c-Myc* mRNA**

Both human and mouse *c-Myc* mRNAs have a long and highly structured 5′ UTR region (located in the exon 1) that serves for both cap-dependent and -independent *c-Myc* protein expression. To confirm the interaction of GANP with the mRNA, we prepared reporter constructs carrying the *5'UTR sequence* of the *human c-Myc* mRNA at the 5' side of the *luciferase* gene (pGL3). GANP introduction to the reporter transfectants significantly augmented the luciferase activity, in contrast siRNA-mediated *Ganp*-knockdown (*Ganp*-KD) reduced the expression of luciferase activity, which suggested that GANP targets the *c-Myc* 5'UTR for efficient mRNA translation (Figure 3. c).

**(7) Downregulation of GANP decreases *c-Myc* translation in GC B cells**

To address whether GANP regulates *c-Myc* expression in GC B cells, we used *Aicda-cre* mice to generate GANP depletion, specifically in GC B cells. GC B cells were isolated from 6-8-week-old *Aicda-cre Ganp*$^{Flox/Flox}$ mice after 8 days of immunization with sheep red blood cells (SRBC). Absence of GANP significantly reduced B cell number in GCs with the markers of B220, GL7 and Fas after SRBC-immunization in comparison with the control (Figure 4). The GC specific *Ganp*-KO decreased the expression of *c-Myc* and Bcl2 proteins, but did not affect their expressions in the non-GC B cells. Data suggesting that GANP is a regulator for selective *c-Myc* and Bcl2 expression in GC B cells.

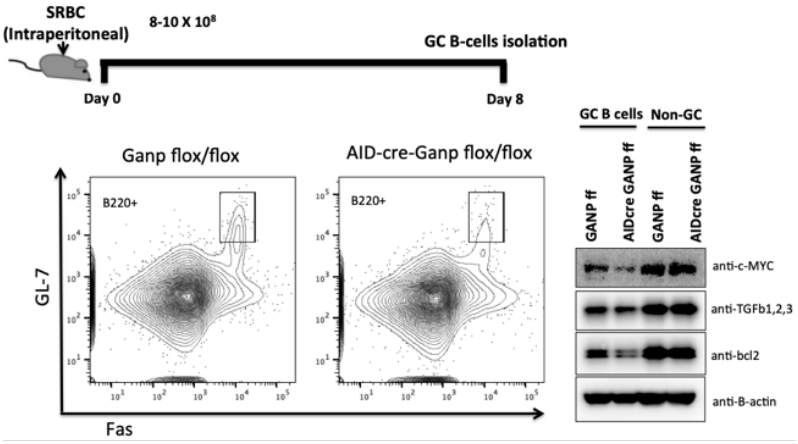


**Figure 4.** GC b cells isolation from 6-8 week old mouse after SRBC immunization, c-Myc protein level was checked by immunoblot.

**(8) Single cell RNA-seq analysis from GC B cells**

GC is organized into two major compartment dark zone (DZ) and light zones (LZ), *c-Myc* expression mainly in the LZ is required for B cell survival. It has been shown recently, B cell receptor (BCR) and CD40 ligand; both signals were required to induce *c-Myc* and cyclic reentry of GC B cells. Positive selection of high-affinity GC B cells is driven by BCR and follicular T helper cells. However, how different affinity-related signaling events control the *c-Myc* in a manner that links to the positive selection is poorly understood. GANP may control the *c-Myc* mRNA or protein expression together with some binding co factors in GC B cells for affinity maturation and B cell selection.

To explore the role played by GANP during GC B cell development, we performed single-cell (sc) transcriptomic analysis mouse GC B cells, in response to T cell–dependent antigens after SRBC immunizations.

The GC B cells were sorted from GANP-WT, *Aicda-cre Ganp*$^{Flox/Flox}$ & GANP-overexpressing-Tg mice, after immunization using GL7, Fas, B220, CXCR4 and CD86 markers followed by Sc-RNA sequencing. We identified 11 clusters, which were annotated based on their gene expression signatures. About 50% of the analyzed GC B cells display high gene expression associated with the S-G2-M stages of the cell cycle, expressing high CXCR4 representing DZ or intermediate phenotypes (Figure 5. a, b). live or dead cells distinguished by mitochondrial percentage (Figure 5. c). Gene expression profile showed that CXCR4 high and CD86 expression is low in dark zone and CD86 high, CXCR4 low in light zone populations (Figure 5. d, e), we also confirmed various GC B cells associated molecules for example, Foxo1, *c-Myc*, Aicda, Akt, Bcl2, Bach2, Irf4 and Ccnd2.

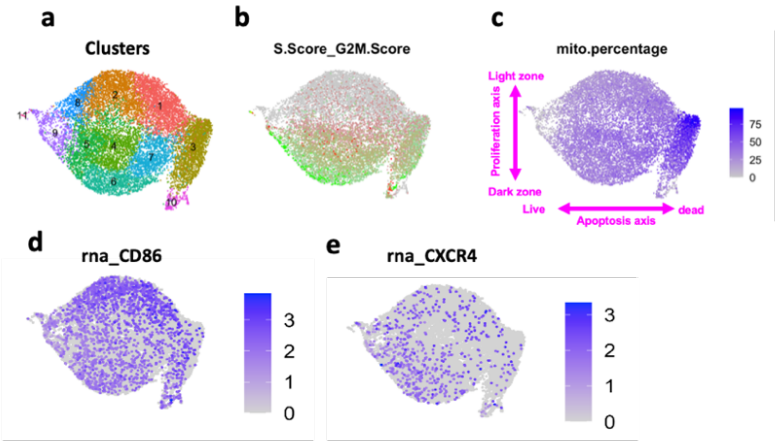


**Figure 5. Single cell RNA-seq analysis of GC B cells after immunizations using GANP WT, GC specific GANP KO and GANP overexpressing Tg mice (10x genomics). (a) GC b cells clusters according to differentially expressed genes (DEG). (b) GC b cells clusters showing cell cycle stage. (c) Mitochondrial percentage showing live and dead cells. (d, e) CXCR4 and CD86 expression showing the light zone and dark zone GC b cells.**

**(9) B cell receptor (BCR) analysis**

We found that GC B cells from GANP overexpressing-Tg mice are much higher in numbers as compared to *Aicda-cre Ganp*$^{Flox/Flox}$ and GANP-WT mice (Figure 6. a). After single-cell transcriptomic analysis, we identified multiple functionally linked subpopulations, including centroblasts, centrocytes and the distinct precursors of memory B cells, plasma cells and intermediate B cells among the different clusters (Figure 6. b). Finally, we examined the B cell receptor (BCR) analysis in different GC B cells clusters, interestingly we found that GANP overexpressing GC B cells enhanced the complementarity-determining regions (CDRs) which are the part of variable chains in immunoglobulins (antibodies), data suggest that GANP is an important molecule for the GC b cell affinity maturation and selection.

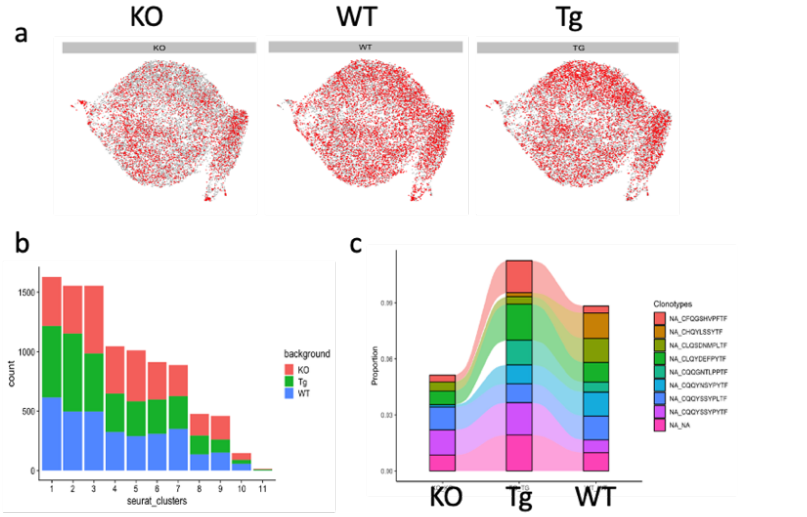


**Figure 6. Single cell RNA-seq analysis of GC B cells after immunizations using GANP WT, GC specific GANP KO and GANP overexpressing Tg mice (10x genomics). (a) GC b cells clusters among the different mice. (b) GC b cells clusters comparisons among the different mice . (c) B cell receptor (BCR) analysis among the different mice.**

Taken together, we studied the molecules associated with a transcription exportation complex (TREX-2) component GANP interacting with AID in B cells. GANP interacts with eIF4E and translation initiation complex for cap-containing mRNAs and enhances the translation activity in the reporter assay. GANP strongly associates with *c-Myc* mRNA through the 5'-untranslated region and enhances the *c-Myc* translation by cap-dependent and *IRES*-dependent pathways. GANP overexpression causes an increase of *c-Myc* expression, and reciprocally GANP ablation showed the reverse effect in GC B cells in mice. Sc-transcriptomics analysis showed that, GANP overexpression enhanced the LZ and intermediate population in GC B cells in comparison with GANP-KD and GANP-WT. Overall GANP regulates post-transcriptional *c-Myc* expression in GC B cells undergoing the B cell selection.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

| | |
|---|---|
| １．著者名<br>Chalise Jaya Prakash、Hashimoto Shigeru、Parajuli Gyanu、Kang Sujin、Singh Shailendra Kumar、Gemechu Yohannes、Metwally Hozaifa、Nyati Kishan Kumar、Dubey Praveen Kumar、Zaman Mohammad Mahabub-Uz、Nagahama Yasuharu、Hamza Hanieh、Masuda Kazuya、Kishimoto Tadamitsu | ４．巻<br>116 |
| ２．論文標題<br>Feedback regulation of Arid5a and Ppar-γ2 maintains adipose tissue homeostasis | ５．発行年<br>2019年 |
| ３．雑誌名<br>Proceedings of the National Academy of Sciences | ６．最初と最後の頁<br>15128～15133 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1073/pnas.1906712116 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスとしている（また、その予定である） | 国際共著<br>該当する |

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| １．著者名<br>Sakaguchi Masaji、Cai Weikang、Wang Chih-Hao、Cederquist Carly T.、Damasio Marcos、Homan Erica P.、Batista Thiago、Ramirez Alfred K.、Gupta Manoj K.、Steger Martin、Wewer Albrechtsen Nicolai J.、Singh Shailendra Kumar、Araki Eiichi、Mann Matthias、Enerback Sven、Kahn C. Ronald | ４．巻<br>10 |
| ２．論文標題<br>FoxK1 and FoxK2 in insulin regulation of cellular and mitochondrial metabolism | ５．発行年<br>2019年 |
| ３．雑誌名<br>Nature Communications | ６．最初と最後の頁<br>1582 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1038/s41467-019-09418-0 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスとしている（また、その予定である） | 国際共著<br>該当する |

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

| | 氏名<br>（ローマ字氏名）<br>（研究者番号） | 所属研究機関・部局・職<br>（機関番号） | 備考 |
|---|---|---|---|

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

| 共同研究相手国 | 相手方研究機関 |
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