DISSERTATION ABSTRACT

The role of Nucleoporin Rae1 in cell fate determination

細胞の運命制御における Rae1 の機能解析

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CHAPTER I.

Rae1 in Cancer: The preliminary study of the role of Rae1 in breast cancer

ABSTRACT

Nuclear pore complexes (NPCs), alongside as having the main function as nucleocytoplasmic trafficking, have also implicated in numerous basic cellular processes including mitosis, differentiation, chromatin organization, epigenetic regulation and so forth, which correlated with many diseases. Previously, we and others showed that nucleoporin Rae1 is a binding platform for NuMA, cohesin subunits SMC1 to prevent aneuploidy during mitosis. Bioinformatic data analysis from The Cancer Genome Atlas (TCGA) showing that Rae1 is extensively amplified and overexpressed in several solid cancers including colon and breast cancer respectively. Here, we first performed transcriptomic and proteomic study of Rae1 in MCF7 breast cancer cells to provide in depth-understanding about the global change of differentially expressed genes. We performed RNA sequence from Rae1 depleted cell, and mass spectrometry (MS) of Rae1 immunoprecipitates from MCF7 cell lysates. From the Phenotypic assay, our work suggested that Rae1 might be important to overcome susceptibility of apoptosis in MCF7 as depletion of Rae1 lead to the increasement of apoptotic marker under the anoikis condition.

Keywords: Rae1, MCF7, mass spectrometry, apoptosis

CHAPTER II.

Rae1 in viral disease: Overexpression of SARS-CoV-2 protein ORF6 dislocates Rae1 and Nup98 from the nuclear pore complex

ABSTRACT

The novel human betacoronavirus SARS-CoV-2 has caused an unprecedented pandemic in the 21st century. Several studies have revealed interactions between SARS-CoV-2 viral proteins and host nucleoporins, yet their functions are largely unknown. Here, we demonstrate that the open-reading frame 6 (ORF6) of SARS-CoV-2 can directly manipulate localization and functions of nucleoporins. We found that ORF6 protein disrupted nuclear rim staining of nucleoporins Rae1 and Nup98. Consequently, this disruption caused aberrant nucleocytoplasmic trafficking and led to nuclear accumulation of mRNAtransporters such as hnRNPA1. Ultimately, host cell nucleus size was reduced and cell growth was halted.

Keywords: SARS-CoV-2, Rae1, ORF6, hnRNPA

CHAPTER III.

Label-free tomographic imaging of nanodiamonds in living cells (A new promising method to visualize tagged Rae1 in near future)

ABSTRACT

Given the huge impact of Rae1 in our laboratory's long work history, it is reasonable to consider the new, fast and reliable system dedicating for better labeling and imaging of this protein inside living cells. Nanodiamonds (NDs) have acknowledged growing attention due to their decent biocompatibility and photostability with its application as a molecular label is actually not a new phenomenon. However, despite its extensive utilization, major drawback such as phototoxicity from laser irradiation to detect the fluorescence signal still limits this long-time tracking of nanodiamonds in living cells. Other researcher of our laboratory in collaboration with Kyoto University has succesfully detect Rae1 in a conjugation form with Nanodiamonds (DNDs-HPG-Rae1) using high speed AFM (unpublished data). Here, we performed novel quantitative morphological and biophysical celullar analysis by optical diffraction tomography (ODT) using various sizes and introduction methods of NDs. ODT is an inexpensive and noninvasive microscopy technique, which images cells and subcellular structures as a function of their refractive index (RI); hence, can reduce phototoxicity. In this sudy, the very high RI of NDs in HeLa cells can be clearly discriminated from cellular structures. Also, as our data indicated no significant changes of viability of cells receiving NDs treatment, we hopefully can continue to bioconjugate Rae1 into NDs and successfully tracked it inside the living cells by ODT in the near future.

Keywords:
Rae1, fluorescence, nanodiamonds, refractive-index tomography, optical diffraction tomography (ODT)

CHAPTER I.

Nulear Pore Complexes (NPCs) were the largest protein complexes in eukaryotic cells (~60 MDa in yeast and 90–120 MDa in humans) and are composed of ~30 types of nucleoporins (Nups). The structure of NPCs demonstrate an eightfold rotational symmetry, along with a membrane-embedded scaffold that built around a central transport channel, a cytoplasmic ring, a nuclear ring and eight filaments attached to each ring. The nuclear filaments are connected to a distal nuclear ring forming the 'nuclear basket' of the NPC [1,2,]. One of nucleoporins called Rae1 is a conserved protein previously identified as an mRNA export protein of 41 kDa which was cross-linked to poly(A)-containing mRNA, therefore known also as Mrnp41. It contains four tryptophan-aspartic acid (WD) motifs and has been shown to localize in the nucleoplasm, nuclear membrane, and giving the meshwork-like structures in the cytoplasm. The binding of Rae1 spesifically to the GLEBS motif of Nup98 enable them to interact and work together in mRNA export or spindle assembly to prevent chromosome mis-segregation [3-8]. Both depletion and overexpression of Rae1 lead to increased formation of multipolar spindles, an effect that can be rescued by NUMA depletion or co-overexpression, respectively. Imbalances in NUMA, SMC1 or Rae1 interactions cause formation of multipolar spindles and aneuploidy, increasing genomic instability and promoting tumorigenesis [3-4]. Rae1 and Nup98 are kinetically partitioned among other

components in both cytoplasmic and nuclear compartments. Between the cytoplasm and nucleus, the complex acts as regulators for BMAL1 shuttling, and as maintenance of the correct pace of the circadian clock [9]. In addition, Rae1 was found to mediate ZEB1 expression by promoting epithelial-mesenchymal transition in breast cancer [10].

As finding the bonafide theraphy in breast cancer continued to encounter adversity, attention to the emerging field of study called epigenetics are growing more nowadays. This has also drawn interest in the development of antitumor agents altering the abnormal chromatin structure of the cancer cell and modifying the gene-expression profiles (epitherapeutics). Epigenetics can also be affected by cellular signaling pathways and extracellular stimuli. Given the importance of epigenetics in influencing cell functions, a better understanding of both normal and abnormal epigenetic processes are necessary [11].

Using data from METABRIC & TCGA from cbioportal (http://www.cbioportal.org), we found that amplification and gain was the first and second common abnormalities suggesting breast cancers prone to show over-expression of Rae1. Further, we compared the protein levels of Rae1 between breast cancer cell line MCF7 and normal breast cell line MCF10A by western blotting analysis and the localization by immunofluorescence. (**Figure 1A and B**). Consistent with in-silico data, the expression levels of Rae1 in MCF7 was elavated in MCF7 comparing to that in MCF10. Notably, Rae1 in MCF7 cells localized in both nuclear membrane and nucleoplasm while the localization of Rae1 was mainly in the nuclear membrane in MCF10A, suggesting the cancer specific of Rae1 inside nucloplasm.

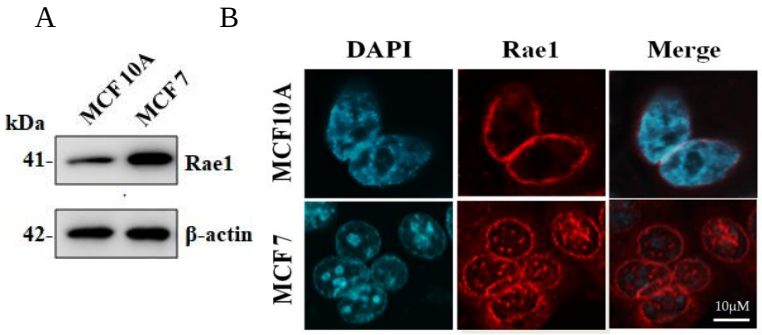

Figure 1. Endogenous Rae1 protein of MCF10A and MCF7 by western blotting (A) and Immuno-fluorescence (B).

We next asked whether over-expressed Rae1 is functionally involved in resistance to anoikis, which plays a pivotal role during progression of cancers [48]. After introducing siRNA, MCF7 cells were seeded on the anchorage resistant plate for 96 and 120 hours, and detected cleaved PARP, a hallmark of apoptosis by WB (**Figure 2**). Rae1-depleted MCF7 cells showed marked increasement of cleaved PARP1, suggesting the role of Rae1 in preventing anoikis.

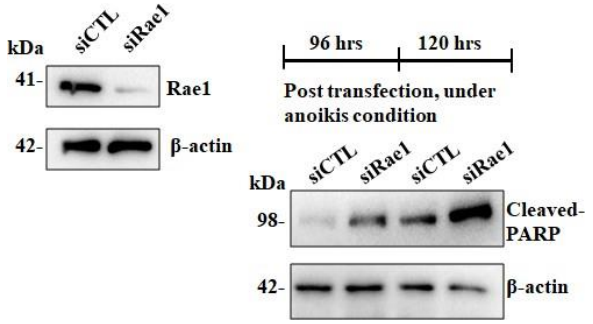

Figure 2. Cleaved PARP protein level by western blotting under anoikis conditions following Rae1 knockdown in MCF7

In order to understand the biological processes and signaling pathways upon Rae1 knock-down in MCF7 cells, RNA-seq analysis was performed in MCF7 cells silenced for Rae1. Gene ontology (GO) analysis revealed that loss of Rae1 increased a subset of genes highly enriched in processes regulating type I interferon related events, positive regulation of actin filament bundle assembly and cell death, and gland development.

Since Rae1 is localized in nucleoplasm, we hypothesized that Rae1 might be involved in transcriptional or epigenetic function, and are inspired to see Rae1 binding partners in breast cancer cells. Therefore, we utilized mass spectrometer (MS) to obtain peptide mass profiles from silver-stained protein in MCF, where several protein interactors of Rae1 were selected based on the peptides that give high peptide spectrum match (PSM) score [13]. **Figure 3** shows the candidates for the query peptides. Unsurprisingly, we identified several established binding protein partner of Rae1 including Nup98 [12]. Importantly, we identified several epigenetic regulator such as HDAC2 and Rad21. These results imply that Rae1 might be involved in epigenetic regulation by collaborating newly identifed epigenetic regulators.

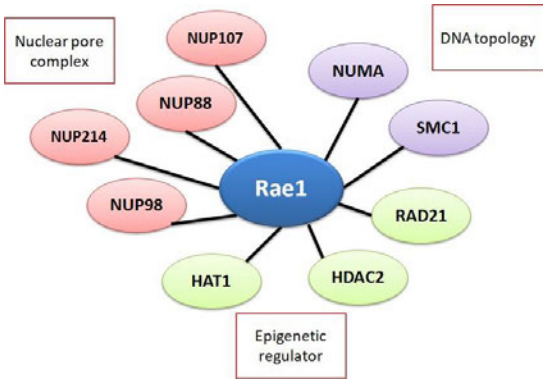

Figure 3. Protein partners of Rae1 selected from Mass Spectrometry

In conclusion, we found that upregulated Rae1 is functionally involved in preventing anoikis, which is caused by novel role of Rae1 in nucleoplasm related to epigenetic regulation.

CHAPTER II

Severe acute coronavirus respiratory syndrome coronavirus-2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus that caused a life-threatening disease known as COVID-19. An overriding exponential rate of new cases and deaths has urged the scientists across the world to develop vaccines and antiviral drugs that inhibit infection with or replication of SARS-CoV-2. The genomic size SARS-CoV-2 is 29.9 kb and encodes 28 confirmed proteins. The genome comprises of the 5'- untranslated region (5'-UTR) with non-structural proteins (Nsp1-16) and the 3′ end untranslated region (3'-UTR) comprises genes encoding the four structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N), as well as eight accessory proteins (Orf3a, Orf3b, Orf6, Orf7a, Orf7b, Orf8, Orf9b and Orf10) [14].

Viruses with RNA proliferate in the cytoplasm of their host cells and they do not need to enter the nucleus. However, they tend to hijack the host nuclear transport for suppressing the interferon (IFN)-inducing antiviral responses. Moreover, cells infected by these RNA viruses undergo NPC structural and functional nucleocytoplasmic disorder. This will leave the possibility for the various host proteins to become jammed in the

cytoplasm. Most likely, the overall activity of the NPC continues after infection because certain imports and exports keep occuring even when the cell is infected [15-18].

Recently, more than 1000 putative virus-host protein-protein interactions (PPIs) in HEK293T cells has been identified by utilizing immunoprecipitation coupled with mass spectrometry (IP-MS) of epitope-tagged viral proteins to map SARS-CoV-2-host PPIs [19,20]. Reportedly, Nup98 exhibited to bind with Ribonucleic acid export 1 (Rae1) as Nup98/Rae1 complex. The ORF6 interacts with the complex (an interferon-inducible mRNA nuclear export complex that is hijacked or degraded by multiple viruses) as depicted in **Figure 4** [19].

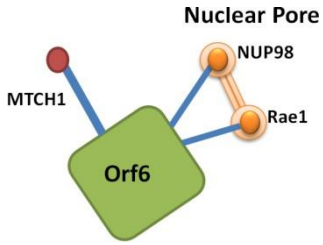

Figure 4. Illustration of Interaction of Orf6 of SARS-CoV-2 with protein complex (Nup98-Rae1).

It remains largely unknown why SARS-CoV2 ORF6 needs to interact with nucleoporins after infection because SARS-CoV2 hijacks the protein-making machinery of the host cell to translate its RNA directly into new copies ofthe virus.

In this study, first we aim to look the Rae1 localization in ORF6-overexpressing cells. ORF6 overexpression caused Rae1 to be displaced from the nuclear membrane to the cytoplasm in HEK293T cells (Figure 5) and the human lung cancer cell line PC9 (Figure not shown here).

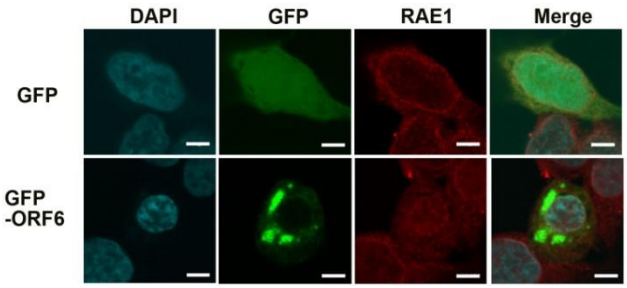

Figure 5 Confocal imaging of GFP and Rae1 localization in HEK293T cells transfected with pEGFP-N1 or pEGFP-ORF6

In Figure 6, the top row for each protein shows mock-transfected GFP vector control samples, while the bottom row shows GFP-ORF6-transfected cells. The signal for hnRNPA1 coincided with DNA staining in control cells, suggesting that these proteins were in the nucleus. Here, overexpression of SARS-CoV-2 ORF6 alone did not shift hnRNPA1 to the cytoplasm, according to our findings. In GFP-ORF6-transfected cells, the density of hnRNPA1 was dramatically enhanced and consistently remained inside the nuclear area. This finding also suggests that ORF6 overexpression caused hnRNPA1 to accumulate in the nucleus.

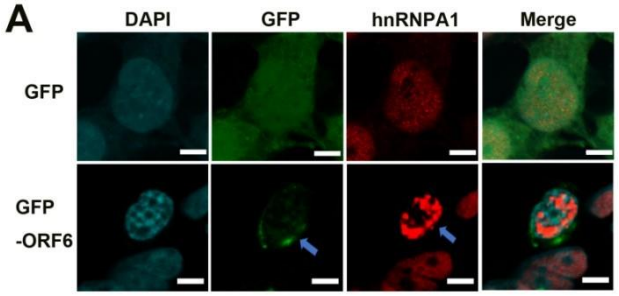

Figure 6. ORF6 overexpression resulted in nuclear accumulation of mRNA binding protein hnRNPA1 by hijacking Rae1 function

If our hypothesis is correct, overexpression of FLAG-Rae1 and GFP-ORF6 would partially relieve hnRNPA1 stacking through sequestration of endogenous nuclear hnRNPA1. Consistent with our prediction, we found that hnRNPA1 nuclear accumulation was partially relieved by hnRNPA1 nuclear stacking (**Figure 7**).

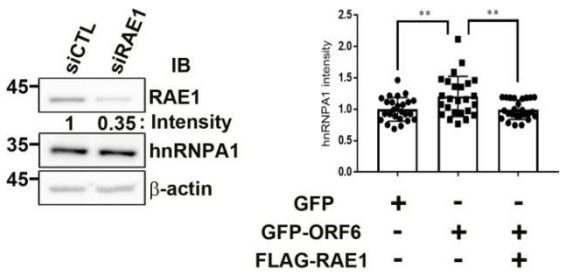

Figure 7 Immunoblot analysis of Rae1 and hnRNPA1 in HEK293T cells transfected with control or Rae1 siRNA and quantification of relative nuclear hnRNPA1 intensity in HEK293T cells transfected with GFP or GFP-ORF6, or co-transfected with GFPORF6 and FLAG-Rae1

In conclusion, the Orf6 of SARS-CoV-2 has the ability to displace the Rae1 AND Nup98 complex from its main origin, resulting the disturbance of nucleocytoplasmic trafficking and evntually the accumulation of mRNAtransporters like hnRNPA1 (**Figure 8).**

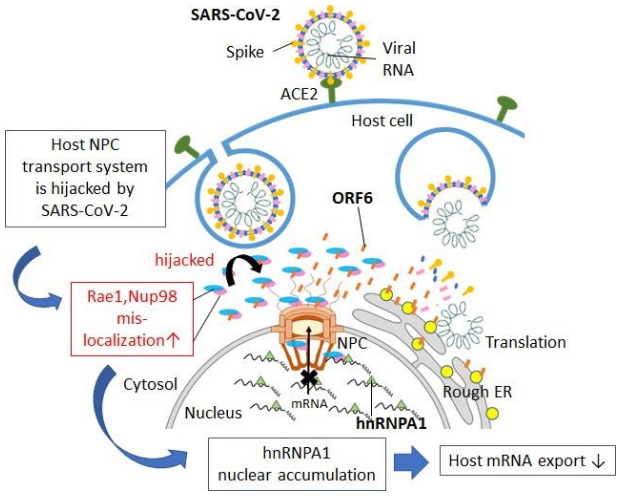

Figure 8. The pathogenesis of SARS-CoV-2 in association with Nucleocytoplasmic trafficking.

CHAPTER III

The study of the role of nucleoporin Rae1 in living cells is strongly correlated to the recombinant protein expression and purification experiments performed multiple times in our laboratory. Additionally, several fusion tags with nups have been developed to support identification and characterization of Rae1 in cancer cell. Our previous studies of Rae1 and other nucleoporins have utilize Hemaglutinin (HA) tag, Histidine tag, Flag tag, and GFP tag [3,4,7,8,28,29]. Despite the superior results yielded from those studies, it is undeniable that such techniques have special preparation that might be labouring. In other hand, these tags system usually followed by laser-based imaging that can raise the possibility of gaining phototoxicity and photobleacing side effect to the living cells. Therefore, this issues encourage us to study the other alternative of faster and less toxic protein labeling-imaging system.

In recent years, nanodiamonds (NDs) have emerged as a highly promising material for various biomedical applications namely fluorescent imaging and drug

delivery [21-23]. They are generally considered to be physically and chemically stable, bioinert and biocompatible due to their highly stable chemical structure, which makes them attractive to be employed as multifunctional probes [24-26].

All our experiments showed that nanodiamonds, thanks to their high RI values, can be successfully imaged inside cells and can be clearly discriminated from cellular structures using ODT. For the first series of ND cell uptake by electroporation, we observed a decreasing RI value with decreasing ND size. This is expected, since a single 100 nm nanodiamond in a voxel will take a larger volumetric fraction than 35 nm or 5 nm NDs. On top of this, small particles have a larger surface-to-volume ratio, where effects like coverage by a protein corona will more strongly reduce the RI. In the second series, ND cell uptake by endocytosis was studied. A remarkable reduction of RI values for 100 nm NDs were observed when the incubation time was doubled from 3 h to 6 h. This speaks for a disaggregation of ND particles with longer incubation time, which could be caused by a protein corona formation and is in agreement with Ref. [27], where a prior coating of the NDs with proteins from cellular medium (FBS) is an effective option to prevent inter-particle aggregation and achieve size reduction. In the third series of experiments using electroporation, we see a similar reduction of RI values for 100 NDs, when coated with a HPG polymer layer, which protects from aggregation. This result is visible from the RI tomogram in the case of 100 nm NDs showing smaller spot sizes. This demonstrates the effectiveness of HPG coating to prevent NDs of different sizes from aggregation after cellular uptake.

In conclusion, nanodiamonds (NDs) with their high RI are ideal particles to be visualized by ODT. Internalized NDs can be detected inside a living cell within less than a second and can be clearly discriminated from cellular structure. The very weak usage of laser irradiation in ODT was able to ensure the prolonged cells' life span while performing experiment (**Figure 9)**. This fundamental new method of NDs imaging can give many benefits in the future study; specifically NPC in our field, as the bioconjugation of Rae1 in the NDs (DNDs-HPG-Rae1) has also been successfully detected using high speed AFM.

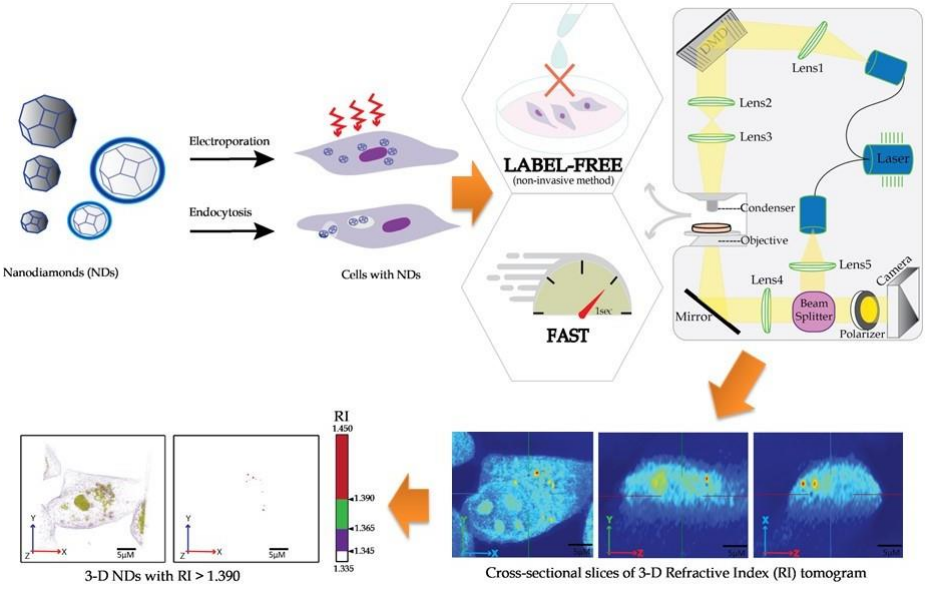

Figure 9. The principle and advantage of ODT to visualize NDs in a living cell.

REFERENCES

[1] M. Raices, M. A. D'Angelo, Nuclear pore complex composition: a new regulator of tissue-specific and developmental functions, Nat Rev Mol Cell Biol. 13 (2012) 687-99.

[2] F. Alber, S. Dokudovskaya, L. M. Veenhoff, et al., The molecular architecture of the nuclear pore complex, Nature 450 2007) 695-701.

[3] R. W. Wong, Interaction between Rae1 and cohesin subunit SMC1 is required for proper spindle formation, Cell Cycle 9 (2010) 198e200.

[4] R.W. Wong, G. Blobel, Cohesin subunit SMC1 associates with mitotic microtubules at the spindle pole, Proc. Natl. Acad. Sci. U. S. A 105 (2008) 15441e15445.

[5] K.B. Jeganathan, D.J. Baker, J.M. van Deursen, Securin associates with APCCdh1 in prometaphase but its destruction is delayed by Rae1 and Nup98 until the metaphase/anaphase transition, Cell Cycle 5 (2006) 366e370.

[6] T. Funasaka, V. Balan, A. Raz, R.W. Wong, Nucleoporin Nup98 mediates galectin-3 nuclear-cytoplasmic trafficking, Biochem. Biophys. Res. Commun. 434 (2013) 155e161.

[7] T. Funasaka, H. Nakano, Y. Wu, C. Hashizume, L. Gu, T. Nakamura, W. Wang, P. Zhou, M.A. Moore, H. Sato, R.W. Wong, RNA export factor Rae1 contributes to Nup98-HOXA9-mediated leukemogenesis, Cell Cycle 10 (2011) 1456e1467.

[8] Y. Ren, H. S. Seo, G. Blobel, A. Hoelz, Structural and functional analysis of the interaction between the nucleoporin Nup98 and the mRNA export factor Rae1, Proc. Natl. Acad. Sci. U.S.A. 107(2010) 10406-11.

[9] J.H. Oh, J.Y. Lee, S. Yu, et al., Rae1 mediated ZEB1 expression promotes epithelial-mesenchymal transition in breast cancer, Sci. Rep. 9 (2019) 2977.

[10] Y. Cheng, C. He, M. Wang, et al., Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. Sig Transduct Target Ther**.** 4 (2019) 62.

[11] O. Witt, H. E. Deubzer, T. Milde, I, Oehme: HDAC family: what are the cancer relevant targets? Cancer Lett. 277(2009) 8-21.

[12] C. Chen, J. hou, J. J Tanner, J. Cheng, Bioinformatics Methods for Mass Spectrometry-Based Proteomics Data Analysis, Int. J. Mol. Sci. 21 (2020**)** 2873.

[13] A. Fabregat, K. Sidiropoulos , G. Viteri, et al., Reactome pathway analysis: a highperformance in-memory approach, BMC bioinformatics 18 (2017) 142.

[14] H. Ben, G. Hua, Z. Peng, L.S. Zheng, Characteristics of SARS-CoV-2 and COVID-19, Nat Rev Microbiol. 19 (2021) 141-154.

[15] J. A. Hiscox, The interaction of animal cytoplasmic RNA viruses with the nucleus to facilitate replication. Virus Res. 95 (2003) 13-22.

[16] M.K. Weidman, R. Sharma, S. Raychaudhuri, et al., The interaction of cytoplasmic RNA viruses with the nucleus. Science 288 (2000) 1374-1377.

[17] J.Y. Lin, T.C. Chen, K.F. Weng, et al., Viral and host proteins involved in picornavirus life cycle. J. Biomed. Sci. 16 (2009) 103.

[18] E.S. Sajidah, K.S. Lim, R.W. Wong, How SARS-CoV-2 and Other Viruses Build an Invasion Route to Hijack the Host Nucleocytoplasmic Trafficking System, Cells 10 (2021**)** 1424.

[19] D.E. Gordon, G.M. Jang, M. Bouhaddou, et al., A SARS-CoV-2 protein interaction map reveals targets for drug repurposing, Nature 583 (2020) 459e468.

[20] M. Bouhaddou, D. Memon, B. Meyer, et al., The global phosphorylation landscape of SARS-CoV-2 infection, Cell 182 (2020) 685e712.

[21] V. N. Mochalin, O. Shenderova, D. Ho, Y. Gogotsi, The properties and applications of nanodiamonds, Nat Nanotechnol. 7 (2011) 11–23.
[22] V. Vaijayanthimala, D. K. Lee, S. V. Kim, et al., Nanodiamond-mediated drug delivery and imaging: challenges and opportunities. Expert Opin Drug Deliv 12(2015)735–49.
[23] K. Turcheniuk, V.N. Mochalin, Biomedical applications of nanodiamond (Review). Nanotechnology 28 (2017) 252001.
[24] S. J. Yu, M. W. Kang, H. C. Chang, et al., Bright Fluorescent Nanodiamonds: No Photobleaching and Low Cytotoxicity, J Am Chem Soc. 127(2005)17604–5.
[25] C. C. Fu, H. Y. Lee, K. Chen, et al., Characterization and application of single fluorescent nanodiamonds as cellular biomarkers, Proc Natl Acad Sci U S A. 104(2007)727–32.
[26] A. M. Schrand, H. Huang, C. Carlson, et al., Are Diamond Nanoparticles Cytotoxic ? J Phys Chem B. 111(2007)1–7.
[27] S. R. Hemelaar, A. Nagl, F. Bigot, et al., The interaction of fluorescent nanodiamond probes with cellular media, Microchim Acta. 184(2017)1001–9.
[28] K. Kato, D.K. Ikliptikawati, A. Kobayashi., et al, Overexpression of SARS-CoV-2 protein ORF6 dislocates Rae1 and Nup98 from the nuclear pore complex, Biochem biophys Res Com. 536 (2021) 59-66.
[29] R.W.Wong, G. Blobel, E. Coutavas, Rae1 interaction with NuMA is required for bipolar spindle formation, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 19783e19787.

令和　3年　7月　26日

学位論文審査報告書（甲）

1．学位論文題目（外国語の場合は和訳を付けること。）

The role of Nucleoporin Rae1 in cell fate determination（細胞の運命制御における Rae1 の機能解析）

2．論文提出者　(1) 所　　属　自然システム学　　専攻

(2) 氏（ふり）名（がな）　DINI KURNIA IKLIPTIKAWATI（でぃに くるにあ いくりぷてぃかわてぃ）

3．審査結果の要旨（600～650字）

核膜孔複合体（NPC）は、核膜における分子輸送孔を形成する。NPC 構成タンパク質の一つ Rae1 の機能は RNA 輸送に限定されず、細胞分裂やウイルス感染応答、がん病態制御など、基本細胞生理から病態進行など幅広く関与する。本学位論文では、①乳癌における Rae1 の機能、②ウイルス感染応答における Rae1 の動態・機能、③Rae1 の動態イメージング法の確立にむけた研究成果が審査された。まず、Rae1 が乳癌の発症や浸潤過程に重要なアノイキス（接着不全による細胞死）抵抗性を制御することを報告している。さらに、MCF7 細胞における Rae1 の局在は核質にも認められ、エピジェネティクス制御因子と協力し、アノイキス抵抗性に関与する FYN 遺伝子の発現を制御する可能性を見出している。次いで、SARS-CoV-2 由来タンパク質 ORF6 が Rae1 を標的とし、宿主細胞の RNA 核外搬出過程を阻害することを発見した。最後に、ナノダイヤモンド（ND）をタグとした新規分子イメージング法の開発に向けた研究成果について報告した。高い屈折率（RI）を持つ ND の特徴を生かし、ND のサイズや化学修飾に伴う RI を指標にしたホログラフィックイメージング法の情報基盤を確立した。近い将来、Rae1 を含めた生体分子を ND でラベルすることで、RI を指標とした革新的分子イメージング法の創出が期待される。これらの成果は、ウイルス感染や病態進行に伴う細胞運命の決定メカニズムにおける Rae1 の重要な機能を明らかにした成果であること、新たな生命科学技術開発に資する成果であると判断し、本研究が博士（学術）に値するものと評価した。

4．審査結果　(1) 判　　定（いずれかに○印）　（合　格）・　不合格

(2) 授与学位　博　士（学術）