様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月　 ６ 日現在

機関番号：１４３０１
研究種目：若手研究
研究期間：2020～2022
課題番号：２０Ｋ１５７５６

研究課題名（和文）NF-κB経路における直鎖状ユビキチン鎖の競合的結合

研究課題名（英文）Competitive linear ubiquitin binding in NFkB-signaling

研究代表者
Ｗａｌｉｎｄａ　Ｅｒｉｋ（WALINDA, Erik）

京都大学・医学研究科・助教

研究者番号：８０７８２３９１

交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：タンパク質へのポリユビキチン鎖の付着は、真核生物における重要な翻訳後修飾です。HOIP、HOIL-1L、SHARPINから成る直鎖ユビキチン鎖組み立て複合体（LUBAC）は、特定の種類のポリユビキチン鎖である直鎖ユビキチン鎖を合成します。HOIL-1Lは、そのNZFドメインを介して直鎖ポリユビキチンを選択的に結合することが知られているタンパク質の一つです。HOIL-1L NZFドメインと直鎖ユビキチンの結合形態の構造は既知ですが、認識特異性のいくつかの側面は未解明です。

研究成果の学術的意義や社会的意義
Scientific significance: understanding non-proteolytic functions of ubiquitin chains, particularly in immune signaling, survival, inflammation, immune disorders, cancer, and infectious diseases.

Social significance: potential to contribute to the development of treatments for immune disorders.

研究成果の概要（英文）：In these two yearsl our research has proceeded rather smoothly. We were able to publish several aspects of this project in various international peer-reviewed journals.

The project focuses on protein ubiquitylation, attaching ubiquitin to target proteins. Initially, it studied K48-linked polyubiquitin chains for degradation. The discovery of linear ubiquitin chains and LUBAC's role in immune signaling necessitated understanding them further. The study aims to explore HOIL-1L's binding specificity to linear ubiquitin chains, conformational changes, preference for M1-linked polyubiquitin, and its role in HOIL-IL and LUBAC. Key findings include optimized purification of LUBAC fragments for cancer drug screening, investigating cyclization's impact on ubiquitin chain recognition by OTUB1, and examining linear ubiquitin recognition by HOIL-1L NZF domain. These results enhance understanding of linear ubiquitin binding and the ubiquitin code.

研究分野：生物物理学

キーワード：ubiquitin　ubiquitin binding　NZF domain　M1 ubiquitin chains　linear ubiquitin chains　NMR spectroscopy　ITC　MD simulations

1 Research background at the time of the start of this project [研究開始投資の背景]

Protein ubiquitylation, a prominent post-translational modification in eukaryotic cells, distinguishes itself from other modifications like phosphorylation and acetylation. Unlike those modifiers, ubiquitin can attach to target proteins in two forms: as a single unit (monoubiquitylation) or as polymeric chains (polyubiquitylation). The formation of polyubiquitin chains involves a coordinated action between a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a substrate protein-specific ubiquitin ligase (E3). These chains connect subsequent ubiquitin units through the carboxyterminal glycine (G76) carboxylic acid group and the ε-amino group of seven internal lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, and K63). Initially, focus was primarily on K48-linked polyubiquitin chains, which play a role in transporting target proteins to the proteasome for degradation. However, as time progressed, the non-proteolytic functions of ubiquitin chains gained significance. In an intriguing discovery in 2006, an additional modification involving the α-amino group of the N-terminal methionine residue (M1) was identified. This "linear" (M1-linked) chain formation is exclusive to a distinct ubiquitin ligase enzyme complex known as the linear ubiquitin chain assembly complex (LUBAC). LUBAC comprises the catalytic RBR-type E3 ubiquitin ligase HOIP, the elusive RBR E3 ligase HOIL-1L (recently found to have a regulatory influence on HOIP's catalytic activity), and the accessory protein SHARPIN. The molecular interactions between specific domains of HOIP, HOIL-1L, SHARPIN, and other intracellular proteins, as well as the trimming of linear ubiquitin chains by deubiquitinating enzymes OTULIN and CYLD, are currently active areas of research.

LUBAC, the assembly complex responsible for generating linear ubiquitin chains, plays a significant role in various physiological functions, particularly in orchestrating inflammatory responses. One well-documented example is its involvement in the activation of nuclear factor κB (NF-κB), a pivotal transcription factor known as the "master-switch" of immunological processes that govern cell survival, proliferation, and inflammation. The intricate signaling mechanisms underlying LUBAC-mediated NF-κB activation have been extensively examined in scientific literature. Considering the critical role of LUBAC in immune signaling and preventing cell death, its components have been implicated in a range of conditions, including immune-related disorders (such as autoinflammatory and autoimmune diseases), cancer, and infectious diseases. Remarkably, one of the LUBAC components, HOIL-1L, has also emerged as a significant factor in certain neuromuscular disorders, such as cardiomyopathy with polyglucosan bodies. However, the exact connection between HOIL-1L, immunological pathways, and the precise functioning of LUBAC still remains elusive.

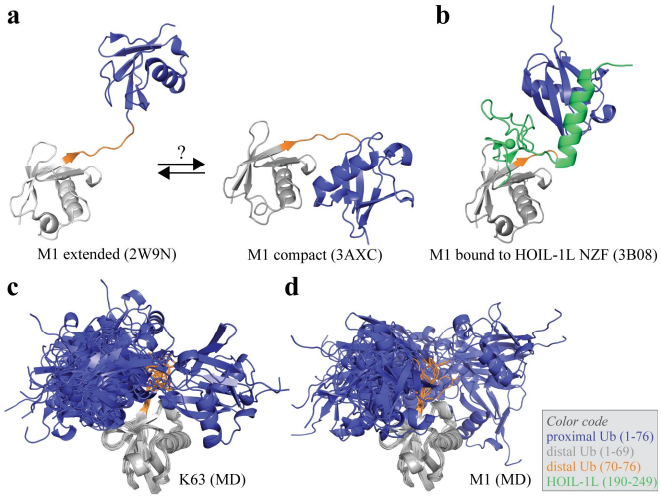

Fig. 1. Dynamic aspects of polyubiquitin chains and their implications on "polyubiquitin reader" domains

2 Research objectives [研究の目的]

The research objective of this study was to address several unanswered questions regarding the binding specificity of HOIL-1L, a component of LUBAC, to linear ubiquitin chains – and from this specific case, to extract a *general* understanding of linear ubiquitin binding, i.e., the *asymmetric* interaction of one binding protein with two distinct ubiquitin moieties. While previous research has identified other proteins with

high affinity for linear ubiquitin chains, such as NEMO and ABIN/Optineurin, the selective binding of HOIL-1L to linear ubiquitin chains through its NZF domain remains intriguing. Although the crystal structure of the bound NZF domain provides insights into the interaction with linear polyubiquitin, certain aspects still require clarification. Firstly, the structure of the free form of the NZF domain and any conformational changes occurring during binding are unknown. Secondly, the specific preference of HOIL-1L for M1-linked polyubiquitin, compared to K63-linked chains and monoubiquitin, needs further explanation (Fig. 1). Lastly, the role of M1-linked polyubiquitin binding within the context of full-length HOIL-1L and LUBAC requires a deeper understanding. To address these knowledge gaps and gain broader insights into polyubiquitin recognition specificity, this research combined solution-state NMR spectroscopy, molecular dynamics simulations, isothermal titration calorimetry, and structural modeling. The findings make progress towards a comprehensive understanding of the linear ubiquitin binding mechanism of HOIL-1L, thereby potentially shedding light on the dynamics of ubiquitin code recognition in general.

3 Research methods [研究の方法]

Protein purification methods for all samples employed in this study have been previously published (see *Biomol. NMR Assign.* 13). NMR spectroscopy experiments were conducted using a Bruker Avance II spectrometer with a 5 mm $^{15}N/^{13}C/^{1}H$ *z*-gradient triple resonance cryoprobe from Bruker BioSpin. Prior to NMR measurements, the purified HOIL-1L NZF domain was exchanged into an NMR buffer containing 20 mM HEPES at pH 7.0, 50 mM sodium chloride, 1 mM TCEP, and 5% D_2O. The sample was then placed in Shigemi tubes with a final volume of 300 μL. For triple resonance experiments, the concentration of the NZF domain was set at 1 mM, while for titration experiments, the starting concentration was 0.1 mM. Additional NOESY experiments with a mixing time of 150 ms were performed to guide structure calculation, including ^{15}N-edited NOESY-HSQC and ^{13}C-edited NOESY-HSQC. Calibration of the ^{1}H chemical shift was done using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), while the heteronuclear ^{13}C and ^{15}N shifts were calibrated indirectly with respect to the proton chemical shift. Data acquisition was carried out using Bruker TopSpin, NMR data processing was performed using NMRPipe, and spectrum analysis was conducted using CcpNmr Analysis version 2.4.1. In the heteronuclear NOE experiments (using the Bruker pulse program hsqcnoef3gpsi3d), the recycle delay was set to 5 seconds (~ 10 T_1 (^{15}N)) to ensure sufficient spin-lattice relaxation and restoration of equilibrium magnetization between acquisitions. Saturation of ^{1}H was achieved by a series of 120° 1H pulses during the 5-second recycle delay, while saturation was turned off in the control experiment. The free NZF domain was measured at a concentration of 1.0 mM. For measuring heteronuclear NOE values in the M1-linked diubiquitin-bound form of the NZF, the sample concentrations were 0.5 mM NZF and 2 mM diubiquitin. Two independent measurements were performed to obtain average values and standard error of the mean. ^{15}N relaxation dispersion measurements utilized the Bruker pulse sequence hsqcNr2rex3d with a CPMG relaxation time of 50 ms and τ_{CP} values ranging from 0 to 25 ms. Multiple data points were measured at specific τ_{CP} values to calculate experimental uncertainties. Transverse ^{15}N relaxation measurements were conducted using the CPMG pulse train as described previously, with R_2 experiment delays ranging from 0 to 0.144 s. Multiple data points were measured at specific delays to estimate experimental uncertainties using the GLOVE package through least-squares estimation of R_{ex} and R_2, obtained through Monte-Carlo simulations with 100 iterations.

For NMR structure calculation, CYANA version 3.98.13 was employed. The input peak lists were obtained by peak-picking the ^{13}C- and ^{15}N-edited 3D NOESY-HSQC spectral strips in CcpNmr Analysis using root resonances from the respective HSQC spectra. An initial structure was generated in

CYANA based on NOE and dihedral angle restraints obtained from TALOS+. This structure was then refined in CYANA against a set of residual dipolar couplings by using a PEG bicelle alignment medium. To prepare the PEG bicelle alignment medium, 50 μL of pentaethylene glycol monododecyl ether ($C_{12}E_5$ PEG, Sigma Aldrich) was mixed with 200 μL of NMR buffer (20 mM HEPES pH 7.0, 50 mM sodium chloride, 1 mM TCEP) and 50 μL of deuterium oxide. Small aliquots (1 μL) of hexanol (Sigma Aldrich) were stepwise added with vigorous vortexing after each addition until a clear solution was obtained, with a final addition of approximately 16 μL of hexanol. The PEG bicelle solution was mixed 1:1 with the protein solution and allowed to equilibrate in the NMR magnet for at least one hour before measurements. Two independent IPAP-HSQC experiments were conducted in the presence and absence of the nematic phase to estimate the residual dipolar coupling (D_{NH}) and scalar coupling (J_{NH}) values. The average D_{NH} values obtained from the measurements were used for subsequent analysis, and the uncertainties in D_{NH} were estimated as the standard error of the mean (S.E.M.) based on the two measurements. The alignment tensor was determined using the initial CYANA structure independently in the program REDCAT and in CYANA using the program-provided macro *FindTensor.cya*. A small number of RDCs were selected for this determination, and the correlation coefficient for the initial tensor estimate was 0.99. The number of RDC restraints used for structure determination was gradually increased, and the final structure calculation protocol was executed 200 times independently with different random number seeds in the CYANA protocol to ensure the reliability of the obtained result.

4 Research results [研究の結果]

As outlined in the publications section, various aspects of this project have been submitted to peer-reviewed international journals. Section 4.3. is unpublished and under revision at the time of reporting.

4.1. Purification optimization for LUBAC fragments

The LUBAC-LTM (linear ubiquitin chain assembly complex tethering motif) domain comprises two distinct accessory components of LUBAC, namely HOIL-1L and SHARPIN, but exhibits a unified globular structure. Disrupting the intricate interaction between LTM motifs destabilizes LUBAC in lymphoma cells, compromising its stability and suggesting that targeting this interaction holds promise for developing new agents against LUBAC-dependent cancers. To facilitate the screening of small-molecule inhibitors that selectively disrupt the LTM-LTM interaction, obtaining highly pure samples of the LTM domain is essential. Ideally, such samples should solely consist of the LTM itself, enabling the elimination of false positives (compounds binding to other regions of LUBAC) during the screening process. In the published study, we presented a straightforward strategy that successfully enabled the efficient bacterial production of isolated LUBAC LTM domain with high yield and purity. This strategy incorporates three key elements: (1) structural analysis suggesting tandem expression from $SHARPIN^{LTM}$ to $HOIL\text{-}1L^{LTM}$ direction; (2) bacterial expression downstream of EGFP for convenient monitoring of expression and solubility; (3) gentle low-temperature folding using autoinduction. The formation of properly folded LTM was confirmed through size-exclusion chromatography and heteronuclear NMR spectroscopy. Starting from 200-ml cultures, significant quantities (~7 mg) of highly pure protein suitable for structural studies could be obtained. This strategy will greatly benefit drug-screening endeavors targeting LUBAC LTM and may serve as a valuable reference for similar cases, wherein smaller folded fragments need to be isolated from larger protein complexes for specific downstream applications.

4.2. Recognition of ubiquitin chains by deubiquitinating enzyme OTUB1

The conjugation of K48-linked ubiquitin chains to intracellular proteins primarily serves as a signal for proteasomal degradation. The enzyme E2-25K is responsible for synthesizing both canonical (noncyclic) and cyclic K48-linked ubiquitin chains. While the cyclic conformation is expected to limit the flexibility of ubiquitin subunits within the chain, potentially hindering molecular recognition by ubiquitin binding proteins, several proteins have been found to associate with cyclic ubiquitin chains similar to noncyclic chains. However, the molecular mechanism underlying the recognition of cyclic ubiquitin chains remains unclear. In the published study, we investigated the impact of cyclization on the cleavage of ubiquitin chains and the molecular recognition of cyclic diubiquitin by the K48-linkage specific deubiquitinating enzyme OTUB1, using NMR spectroscopic analyses. Compared to noncyclic diubiquitin, cyclic diubiquitin undergoes slow but detectable cleavage to monoubiquitin by OTUB1. Interestingly, upon cleavage of the ubiquitin chain, cyclic diubiquitin exhibited a transition from an "autoinhibited" conformation to a partially accessible conformation, facilitated by its interaction with OTUB1 through specific recognition patches and adjacent surfaces on the ubiquitin subunits. These observations suggest that cyclic ubiquitin chains may persist in cells despite the presence of deubiquitinating enzymes, and that intracellular proteins can recognize these chains in a distinct manner from noncyclic ubiquitin chains.

4.3. Recognition of linear ubiquitin in solution by the HOIL-1L NZF domain

HOIL-1L is a protein that selectively binds linear polyubiquitin through its NZF domain. While the structure of the HOIL-1L NZF domain bound to linear diubiquitin is known, there are still some unanswered questions regarding its recognition specificity. In this study, we utilized NMR and other biophysical methods to demonstrate the transition of the NZF domain from its free form to the specific state when bound to linear ubiquitin, while excluding other potential ubiquitin species after weak initial binding. Analysis of the free NZF domain's solution structure revealed changes in conformational stability upon binding to linear ubiquitin, suggesting that the flexibility of the NZF tail might facilitate association through a fly-casting mechanism (Fig. 2). Conserved electrostatic contacts were observed between the NZF core and tail, which were significantly influenced by charge modification at a phosphorylation site called threonine-207. Phosphomimetic mutations decreased the affinity of the NZF domain for linear ubiquitin, and molecular dynamics simulations illustrated how charge repulsion disrupted the NZF core-tail interactions, weakening the stability of the linear ubiquitin-bound conformation. These findings provide insights into the molecular factors that determine the binding of linear ubiquitin and shed light on the dynamic nature of the ubiquitin code, emphasizing the essential role of the NZF domain in the full-length HOIL-1L protein.

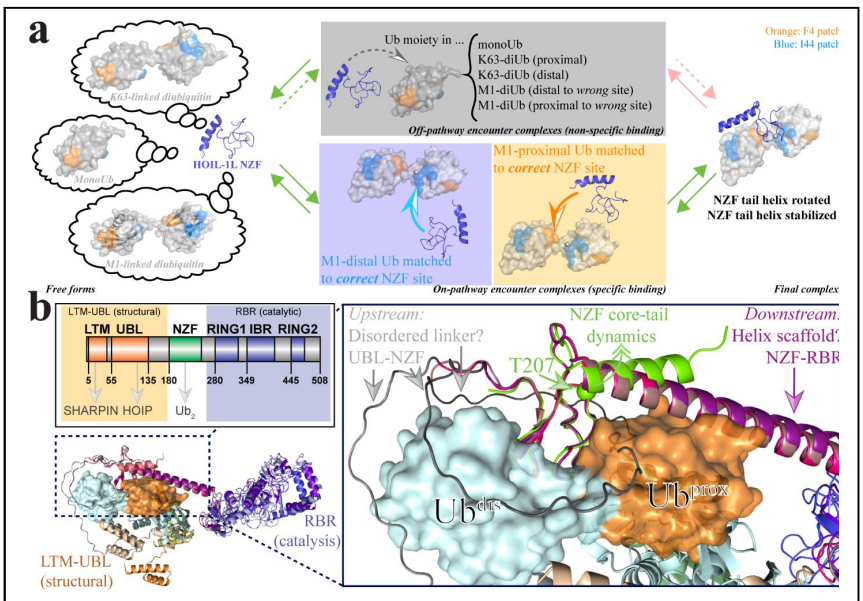

Fig. 2. Current model for dynamic recognition of linear ubiquitin chains by HOIL-1L NZF

５．主な発表論文等

〔雑誌論文〕　計6件（うち査読付論文　0件／うち国際共著　0件／うちオープンアクセス　0件）

１．著者名 Iwakawa Naoto、Morimoto Daichi、Walinda Erik、Shirakawa Masahiro、Sugase Kenji	４．巻 143
２．論文標題 Multiple-State Monitoring of SOD1 Amyloid Formation at Single-Residue Resolution by Rheo-NMR Spectroscopy	５．発行年 2021年
３．雑誌名 Journal of the American Chemical Society	６．最初と最後の頁 10604～10613
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/jacs.1c02974	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Morimoto Daichi、Osugi Masanori、Mahana Yutaka、Walinda Erik、Shirakawa Masahiro、Sugase Kenji	４．巻 15
２．論文標題 Backbone resonance assignments of the A2 domain of mouse von Willebrand factor	５．発行年 2021年
３．雑誌名 Biomolecular NMR Assignments	６．最初と最後の頁 427～431
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/s12104-021-10041-8	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Nishizawa Mayu、Walinda Erik、Morimoto Daichi、Kohn Benjamin、Scheler Ulrich、Shirakawa Masahiro、Sugase Kenji	４．巻 143
２．論文標題 Effects of Weak Nonspecific Interactions with ATP on Proteins	５．発行年 2021年
３．雑誌名 Journal of the American Chemical Society	６．最初と最後の頁 11982～11993
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/jacs.0c13118	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Walinda Erik、Morimoto Daichi、Sorada Tomoki、Iwai Kazuhiro、Sugase Kenji	４．巻 187
２．論文標題 Expression, solubility monitoring, and purification of the co-folded LUBAC LTM domain by structure-guided tandem folding in autoinducing cultures	５．発行年 2021年
３．雑誌名 Protein Expression and Purification	６．最初と最後の頁 105953～105953
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.pep.2021.105953	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

1．著者名 Hirakawa Takuya、Walinda Erik、Morimoto Daichi、Sugase Kenji	4．巻 23
2．論文標題 Rigorous analysis of the interaction between proteins and low water-solubility drugs by qNMR-aided NMR titration experiments	5．発行年 2021年
3．雑誌名 Physical Chemistry Chemical Physics	6．最初と最後の頁 21484～21488
掲載論文のDOI（デジタルオブジェクト識別子） 10.1039/D1CP03175A	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

1．著者名 Mahana Yutaka、Ohki Izuru、Walinda Erik、Morimoto Daichi、Sugase Kenji、Shirakawa Masahiro	4．巻 7
2．論文標題 Structural Insights into Methylated DNA Recognition by the Methyl-CpG Binding Domain of MBD6 from Arabidopsis thaliana	5．発行年 2022年
3．雑誌名 ACS Omega	6．最初と最後の頁 3212～3221
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acsomega.1c04917	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （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