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ダイジェスト

JB REVIEW

LUBAC-mediated linear ubiquitination in tissue homeostasis and disease

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In addition to its role in the ubiquitin–proteasome system of protein degradation, polyubiquitination is involved in the regulation of intracellular events. Depending on the type of ubiquitin–ubiquitin linkage used, polyubiquitin can assume several types of structures. The spatiotemporal dynamics of polyubiquitin involve multiple adaptor proteins and induce different downstream outputs. Linear ubiquitination, in which the N-terminal methionine on the acceptor ubiquitin serves as the site for ubiquitin–ubiquitin conjugation, is a rare and atypical type of polyubiquitin modification. The production of linear ubiquitin chains is dependent on various external inflammatory stimuli and leads to the transient activation of the downstream NF-κB signalling pathway. This in turn suppresses extrinsic programmed cell death signals and protects cells from activation-induced cell death under inflammatory conditions. Recent evidence has revealed the role of linear ubiquitination in various biological processes under both physiological and pathological conditions. This led us to propose that linear ubiquitination may be pivotal in the 'inflammatory adaptation' of cells, and consequently in tissue homeostasis and inflammatory disease. In this review, we focused on the physiological and pathophysiological roles of linear ubiquitination *in vivo* in response to a changing inflammatory microenvironment.

Keywords: cancer, inflammatory adaptation, LUBAC, TNF signalling, ubiquitin

RAPID COMMUNICATION

A lipid scramblase TMEM41B is involved in the processing and transport of GPI-anchored proteins

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Protein modification by glycosylphosphatidylinositol (GPI) takes place in the endoplasmic reticulum (ER). GPI-anchored proteins (GPI-APs) formed in the ER are transported to the cell surface through the Golgi apparatus. During transport, the GPI-anchor structure is processed. In most cells, an acyl chain modified to the inositol of GPI is removed by a GPI-inositol deacylase, PGAP1, in the ER. Inositol-deacylated GPI-APs become sensitive to bacterial phosphatidylinositol-specific phospholipase C (PI-PLC). We previously reported that GPI-APs are partially resistant to PI-PLC when PGAP1 activity is weakened by the deletion of selenoprotein T (SELT) or cleft lip and palate transmembrane protein 1 (CLPTM1). In this study, we found that the loss of TMEM41B, an ER-localized lipid scramblase, restored PI-PLC sensitivity of GPI-APs in SELT-knockout (KO) and CLPTM1-KO cells. In TMEM41B-KO cells, the transport of GPI-APs as well as transmembrane proteins from the ER to the Golgi was delayed. Furthermore, the turnover of PGAP1, which is mediated by ER-associated degradation, was slowed in TMEM41B-KO cells. Taken together, these findings indicate that inhibition of TMEM41B-dependent lipid scrambling promotes GPI-AP processing in the ER through PGAP1 stabilization and slowed protein trafficking.

Keywords: TMEM41B, scramblase, protein trafficking, lipid remodeling, glycosylphosphatidylinositol.

Abbreviations: Baf A1, bafilomycin A1; BFP, blue fluorescent protein; CHX, cycloheximide; CLPTM1, cleft lip and palate transmembrane protein 1; COP-II, coat protein complex II; DKO, double knockout; EtNP, ethanolamine-phosphate; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPI, glycosylphosphatidylinositol; GPI-AP, GPI-anchored protein; KO, knockout; Man, mannose; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; SELT, selenoprotein T; sgRNA, single-guide RNA

REGULAR PAPER
BIOCHEMISTRY
Protein Interaction and Recognition

Interaction of FlhF, SRP-like GTPase with FliF, MS ring component assembling the initial structure of flagella in marine Vibrio

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Vibrio alginolyticus forms a single flagellum at its cell pole. FlhF and FlhG are known to be the main proteins responsible for the

polar formation of single flagellum. MS-ring formation in the flagellar basal body appears to be an initiation step for flagellar assembly. The MS-ring is formed by a single protein, FliF, which has two transmembrane (TM) segments and a large periplasmic region. We had shown that FlhF was required for the polar localization of *Vibrio* FliF, and FlhF facilitated MS-ring formation when FliF was overexpressed in *Escherichia coli* cells. These results suggest that FlhF interacts with FliF to facilitate MS-ring formation. Here, we attempted to detect this interaction using *Vibrio* FliF fragments fused to a tag of Glutathione S-transferase in *E. coli*. We found that the N-terminal 108 residues of FliF, including the first TM segment and the periplasmic region, could pull FlhF down. In the first step, signal recognition particle (SRP) and its receptor are involved in the transport of membrane proteins to target them, which delivers them to the translocon. FlhF may have a similar or enhanced function as SRP, which binds to a region rich in hydrophobic residues.

Keywords: Translocon, GTPase, FlhG, FlhF, Basalbody

Acetylation of MLH1 by CBP increases cellular DNA mismatch repair activity

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The DNA mismatch repair (MMR) proteins recognize and repair DNA base pair mismatches and insertions/deletions of DNA that have occurred during DNA replication. Additionally, they are involved in regulation of the DNA damage response, including cell cycle checkpoints and apoptosis. Therefore, regulation of these proteins is essential for maintaining genomic integrity. It has been recognized that post-translational modifications, such as phosphorylation, ubiquitination, and acetylation, are being used as an important means to regulate the functions and stability of MMR proteins. Here, we report that a histone acetyltransferase CREB binding protein (CBP) interacts with and acetylates MLH1, a component of the MutLα complex (MLH1–PMS2). Moreover, CBP stabilizes MLH1 by preventing it from degradation via the ubiquitin–proteasome degradation pathway. Consistently, acetylation induced by a pan-histone deacetylase inhibitor, Trichostatin A, promotes the assembly between the MutSα (MSH2–MSH6) and MutLα complexes. Furthermore, overexpression of CBP enhances MMR activities in cells. Overall, our results suggest a novel role of CBP in prolonging MLH1 stability and enhancing MutSα–MutLα complex formation, leading to increased cellular MMR activity.

Keywords: histone acetyltransferases, DNA mismatch repair, acetylation

Structural insights into recognition of SL4, the UUCG stem-loop, of human U1 snRNA by the ubiquitin-like domain, including the C-terminal tail in the SF3A1 subunit of U2 snRNP

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The pre-spliceosomal complex involves interactions between U1 and U2 snRNPs, where a ubiquitin-like domain (ULD) of SF3A1, a component of U2 snRNP, binds to the stem-loop 4 (SL4; the UUCG tetraloop) of U1 snRNA in U1 snRNP. Here, we reported the 1.80 Å crystal structure of human SF3A1 ULD (ULD^{SF3A1}) complexed with SL4. The structural part of ULD^{SF3A1} (res. 704–785) adopts a typical β-grasp fold with a topology of $\beta1$-$\beta2$-$\alpha1$-3_{10a}-$\beta3$-$\beta4$-3_{10b}-$\beta5$, closely resembling that of ubiquitin, except for the length and structure of the $\beta1/\beta2$ loop. A patch on the surface formed by three ULD^{SF3A1}-specific residues, Lys756 ($\beta3$), Phe763 ($\beta4$) and Lys765 (following $\beta4$), contacts the canonical UUCG tetraloop structure. In contrast, the directly following C-terminal tail composed of ^{786}KERGGRKK^{793} was essentially stretched. The main or side chains of all the residues interacted with the major groove of the stem helix; the RGG residues adopted a peculiar conformation for RNA recognition. These findings were confirmed by mutational studies using bio-layer interferometry. Collectively, a unique combination of the β-grasp fold and the C-terminal tail constituting ULD^{SF3A1} is required for the SL4-specific binding. This interaction mode also suggests that putative post-translational modifications, including ubiquitination in ULD^{SF3A1}, directly inhibit SL4 binding.

Keywords: UNCG tetraloop, ubiquitin-like domain, splicing, RNA binding, crystal structure

Abbreviations: BLI, bio-layer interferometry; SL4, Stem-loop 4 of U1 snRNA; ULD, ubiquitin-like domain; RMSD, root-mean-square deviation; W–C, Watson–Crick; ZKD, zinc knuckle domain

Biochemistry in Cell Membranes

The scaffold protein PDZK1 governs diurnal localization of CNT2 on the plasma membrane in mouse intestinal epithelial cells

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Diurnal oscillations in the expression of several types of cell surface transporters have been demonstrated in the intestinal epithelial cells, which are mainly generated at transcriptional or degradation processes. Concentrative nucleoside transporter-2 (CNT2) is expressed at the apical site of intestinal epithelial cells and contributes to the uptake of nucleosides and their analogs from the intestinal lumen into the epithelial cells. In this study, we demonstrated that the localization of CNT2 protein in the plasma membrane of mouse intestinal epithelial cells exhibited a diurnal oscillation without changing its protein level in the whole cell. The scaffold protein PDZK1 interacted with CNT2 and stabilized its plasmalemmal localization. The expression of PDZK1 was under the control of molecular components of the circadian clock. Temporal accumulation of PDZK1 protein in intestinal epithelial cells enhanced the plasmalemmal localization of CNT2 at certain times of the day. The temporal increase in CNT2 protein levels at the plasma membrane also facilitated the uptake of adenosine into the intestinal epithelial cells. These results suggest a novel molecular mechanism for the diurnal localization of cell surface transporters and extend our understanding of the biological clock system that generates apparent physiological rhythms.
Keywords: scaffold protein, PDZK1, CNT2, circadian clock

Biochemistry in Diseases and Aging

Full-length prion protein incorporated into prion aggregates is a marker for prion strain-specific destabilization of aggregate structure following cellular uptake

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Accumulation of insoluble aggregates of infectious, partially protease-resistant prion protein (PrP^D) generated via the misfolding of protease sensitive prion protein (PrP^C) into the same infectious conformer, is a hallmark of prion diseases. Aggregated PrP^D is taken up and degraded by cells, a process likely involving changes in aggregate structure that can be monitored by accessibility of the N-terminus of full-length PrP^D to cellular proteases. We therefore tracked the protease sensitivity of full-length PrP^D before and after cellular uptake for two murine prion strains, 22L and 87V. For both strains, PrP^D aggregates were less stable following cellular uptake with increased accessibility of the N-terminus to cellular proteases across most aggregate sizes. However, a limited size range of aggregates was able to better protect the N-termini of full-length PrP^D, with the N-terminus of 22L-derived PrP^D more protected than that of 87V. Interestingly, changes in aggregate structure were associated with minimal changes to the protease-resistant core of PrP^D. Our data show that cells destabilize the aggregate quaternary structure protecting PrP^D from proteases in a strain-dependent manner, with structural changes exposing protease sensitive PrP^D having little effect on the protease-resistant core, and thus conformation, of aggregated PrP^D.
Keywords: aggregate structure, degradation, protein, prion, PrPD, PrPSc

Biochemical Pharmacology

The anti-inflammatory and anti-oxidative effect of a classical hypnotic bromovalerylurea mediated by the activation of NRF2

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The Kelch-like ECH-associated protein 1–nuclear factor erythroid 2-related factor 2 (KEAP1–NRF2) system plays a central role in redox homeostasis and inflammation control. Oxidative stress or electrophilic compounds promote NRF2 stabilization and transcriptional activity by negatively regulating its inhibitor, KEAP1. We have previously reported that bromovalerylurea (BU), originally developed as a hypnotic, exerts anti-inflammatory effects in various inflammatory disease models. However, the molecular mechanism underlying its effect remains uncertain. Herein, we found that by real-time multicolor luciferase assay using stable luciferase red3 (SLR3) and green-emitting emerald luciferase (ELuc), BU potentiates NRF2-dependent transcription in the human hepatoblastoma cell line HepG2 cells, which lasted for more than 60 h. Further analysis revealed that BU promotes NRF2 accumulation and the transcription of its downstream

cytoprotective genes in the HepG2 and the murine microglial cell line BV2. *Keap1* knockdown did not further enhance NRF2 activity, suggesting that BU upregulates NRF2 by targeting KEAP1. Knockdown of *Nfe2l2* in BV2 cells diminished the suppressive effects of BU on the production of pro-inflammatory mediators, like nitric oxide (NO) and its synthase NOS2, indicating the involvement of NRF2 in the anti-inflammatory effects of BU. These data collectively suggest that BU could be repurposed as a novel NRF2 activator to control inflammation and oxidative stress.

Keywords: KEAP1–NRF2, drugaction toxins/drugs/xenobiotics, bromovalerylurea, anti-oxidant oxygen, anti-inflammation

CELL

Cell Cycle

3,5-bis(styryl)pyrazole inhibits mitosis and induces cell death independent of BubR1 and p53 levels by depolymerizing microtubules

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Here, we show that 3,5-bis[(1E)-2-(2,6-dichlorophenyl)ethenyl]-1H-pyrazole 2l depolymerizes microtubules and reduces the number of growing tips of microtubules. The fluorescence recovery after photobleaching experiment in live MCF-7 cells showed that pyrazole 2l suppresses spindle microtubule dynamics. Further, the compound inhibits chromosome movements, activates the spindle assembly check point and blocks mitosis in MCF-7 cells. Pyrazole 2l treatment induced cell death in a variety of pathways. Pyrazole 2l induces cell death independent of BubR1 and p53 levels of MCF-7 cells upon microtubule depolymerization. Further, pyrazole 2l increases the interaction between NF-κB and microtubules and enhances the nuclear localization of NF-κB at its half-maximal proliferation inhibitory concentration while a high concentration of the compound reduced the nuclear localization of NF-κB. Interestingly, the compound exerted significantly stronger antiproliferative effects in cancerous cells than in non-cancerous cells. The results indicated that pyrazole 2l inhibits mitosis by targeting microtubules, induces several types of cell death stimuli and suggests its potential as a lead in developing anticancer agent.

Keywords: mitotic index, microtubule dynamics, congression index, chromosome dynamics, cell death

Abbreviations: BSA, bovine serum albumin, CI, congression index, FBS, fetal bovine serum, FRAP, fluorescence recovery after photobleaching, pHH3, phospho-Histone H3 (Ser10), PI, propidium iodide, SAC, spindle assembly checkpoint

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ダイジェスト

JB REVIEW

Trans-scale thermal signaling in biological systems

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Biochemical reactions in cells serve as the endogenous source of heat, maintaining a constant body temperature. This process requires proper control; otherwise, serious consequences can arise due to the unwanted but unavoidable responses of biological systems to heat. This review aims to present a range of responses to heat in biological systems across various spatial scales. We begin by examining the impaired thermogenesis of malignant hyperthermia in model mice and skeletal muscle cells, demonstrating that the progression of this disease is caused by a positive feedback loop between thermally driven Ca^{2+} signaling and thermogenesis at the subcellular scale. After we explore thermally driven force generation in both muscle and non-muscle cells, we illustrate how *in vitro* assays using purified proteins can reveal the heat-responsive properties of proteins and protein assemblies. Building on these experimental findings, we propose the concept of 'trans-scale thermal signaling'.

Keywords: ATPase, fluorescence microscopy, heat-induced calcium release, microheating, type 1 ryanodine receptor

Abbreviations: $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; FDB, flexor digitorum brevis; HEK293 cell, human embryonic kidney 293 cell; HICR, heat-induced Ca^{2+} release; IP3R, inositol 1,4,5-trisphosphate receptor; MH, malignant hyperthermia; RCC, rapid cooling contracture; RyR1, type 1 ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TRP, transient receptor potential; WT, wild type

REGULAR PAPER
BIOCHEMISTRY
Biochemistry General

Clomipramine inhibits dynamin GTPase activity by L-α-phosphatidyl-L serine stimulation

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Three dynamin isoforms play critical roles in clathrin-dependent endocytosis. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters host cells *via* clathrin-dependent endocytosis. We previously reported that 3-(3-chloro-10,11-dihydro-5*H*-dibenzo [*b*, *f*] azepin-5-yl)-*N*, *N*-dimethylpropan-1-amine (clomipramine) inhibits the GTPase activity of dynamin 1, which is in mainly neuron. Therefore, we investigated whether clomipramine inhibits the activity of other dynamin isoforms in this study. We found that, similar to its inhibitory effect on dynamin 1, clomipramine inhibited the L-α-phosphatidyl-L-serine-stimulated GTPase activity of dynamin 2, which is expressed ubiquitously, and dynamin 3, which is expressed in the lung. Inhibition of GTPase activity raises the possibility that clomipramine can suppress SARS-CoV-2 entry into host cells.

Keywords: clomipramine, dynamin, endocytosis, inhibition, SARS-CoV-2

Abbreviations: ACE2, angiotensin-converting enzyme 2; Dyn, dynamin; His, histidine; MiTMAB, myristyl trimethyl ammonium bromide; Pi, orthophosphate; PS, L-α-phosphatidyl-L-serine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

Protein Interaction and Recognition

Mechanism of tRNA recognition by heterotetrameric glycyl-tRNA synthetase from lactic acid bacteria

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Glycyl-tRNA synthetases (GlyRSs) have different oligomeric structures depending on the organisms. While a dimeric α_2 GlyRS species is present in archaea, eukaryotes and some eubacteria, a heterotetrameric $\alpha_2\beta_2$ GlyRS species is found in most eubacteria. Here, we present the crystal structure of heterotetrameric $\alpha_2\beta_2$ GlyRS, consisting of the full-length α and β subunits, from *Lactobacillus plantarum* (*Lp*GlyRS), gram-positive lactic bacteria. The $\alpha_2\beta_{2Lp}$GlyRS adopts the same X-shaped structure as the recently reported *Escherichia coli* $\alpha_2\beta_2$ GlyRS. A tRNA docking model onto *Lp*GlyRS suggests that the α and β subunits of *Lp*GlyRS together recognize the L-shaped tRNA structure. The α and β subunits of *Lp*GlyRS together interact with the 3′-end and the acceptor region of $tRNA^{Gly}$, and the C-terminal domain of the β subunit interacts with the anticodon region of $tRNA^{Gly}$. The biochemical analysis using tRNA variants showed that in addition to the previously defined determinants G1C72 and C2G71 base pairs, C35, C36 and U73 in eubacterial $tRNA^{Gly}$, the identification of bases at positions 4 and 69 in $tRNA^{Gly}$ is required for efficient glycylation by *Lp*GlyRS. In this case, the combination of a purine base at Position 4 and a pyrimidine base at Position 69 in $tRNA^{Gly}$ is preferred.

Keywords: aminoacyl tRNA synthetase, enzyme mechanism, enzyme structure, gram-positive bacteria, transfer RNA (tRNA), X-ray crystallography

Abbreviations: aaRS, aminoacyl-tRNA synthetase; GlyRS, glycyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; AlaRS, alanyl-tRNA synthetase; SD sequence, Shine-Dalgarno sequence; HD, hitherto-undescribed superfamily of predicted phosphohydrolases; SEM, standard error of the mean; GlySA, 2-chloro-5′-O-[N-(glycyl) sulfamoyl] adenosine

Lipid Biochemistry

Apolipoprotein M supports S1P production and conservation and mediates prolonged Akt activation via S1PR1 and S1PR3

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Sphingosine 1-phosphate (S1P) is one of the lipid mediators involved in diverse physiological functions. S1P circulates in blood and lymph bound to carrier proteins. Three S1P carrier proteins have been reported, albumin, apolipoprotein M (ApoM) and apolipoprotein A4 (ApoA4). The carrier-bound S1P exerts its functions via specific S1P receptors (S1PR1-5) on target cells. Previous studies showed several differences in physiological functions between albumin-bound S1P and ApoM-bound S1P. However, molecular mechanisms underlying the carrier-dependent differences have not been clarified. In addition, ApoA4 is a recently identified S1P carrier protein, and its functional differences from albumin and ApoM have not been addressed. Here, we compared the three carrier proteins in the processes of S1P

degradation, release from S1P-producing cells and receptor activation. ApoM retained S1P more stable than albumin and ApoA4 in the cell culture medium when compared in the equimolar amounts. ApoM facilitated theS1P release from endothelial cells most efficiently. Furthermore, ApoM-bound S1P showed a tendency to induce prolonged activation of Akt via S1PR1 and S1PR3. These results suggest that the carrier-dependent functional differences of S1P are partly ascribed to the differences in the S1P stability, S1P-releasing efficiency and signaling duration.

Keywords: Apolipoprotein A4, Apolipoprotein M, LC-MS/MS, Sphingosine 1-phosphate

Abbreviations: ApoA4, Apolipoprotein A4; ApoM, Apolipoprotein M; CHO, Chinese hamster ovary; ERK, Extracellular signal-regulated kinase; LC-MS/MS, Liquid chromatography–tandem mass spectrometry; LPP, Lipid phosphate phosphatase; Mfsd2b, Multiple facilitator superfamily domain containing 2B; PBS, Phosphate-buffered saline; S1P, Sphingosine 1-phosphate; S1PR1, Sphingosine 1-phosphate receptor 1; S1PR3, Sphingosine 1-phosphate receptor 3; SphK, Sphingosine kinase; Spns2, Spinster homolog 2; TBS-T, Tris-buffed saline containing 0.1% Tween20

Analytical Biochemistry

γ-enolase (ENO2) is methylated at the N τ position of His-190 among enolase isozymes

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Protein methylation is mainly observed in lysine, arginine and histidine residues. Histidine methylation occurs at one of two different nitrogen atoms of the imidazole ring, producing *N* τ-methy l histidine and *N*π-methy l histidine, and it has recently attracted attention with the identification of SETD3, METTL18 and METTL9 as catalytic enzymes in mammals. Although accumulating evidence had suggested the presence of more than 100 proteins containing methylated histidine residues in cells, much less information has been known regarding histidine-methylated proteins than lysine- and arginine-methylated ones, because no method has been developed to identify substrates for histidine methylation. Here, we established a method to screen novel target proteins for histidine methylation, using biochemical protein fractionation combined with the quantification of methy l histidine by LC-MS/MS. Interestingly, the differential distribution pattern of *N*τ-methylated proteins was found between the brain and skeletal muscle, and identified γ-enolase where the His-190 at the *N*τ position is methylated in mouse brain. Finally, *in silico* structural prediction and biochemical analysis showed that the His-190 in γ-enolase is involved in the intermolecular homodimeric formation and enzymatic activity. In the present study, we provide a new methodology to find histidine-methylated proteins *in vivo* and suggest an insight into the importance of histidine methylation.

Keywords: enolase, histidine methylation, Mus musculus, γ-enolase, *N*τ-methylhistidine

Abbreviations: ADMA, asymmetric dimethylarginine; DML, dimethyllysine; HEK293T, human embryonic kidney 293T; HIC, hydrophobic interaction chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MMA, monomethylarginine; MRM, multiple reaction monitoring; *N*-PLA, *N*-propyl-L-arginine; SAM, *S*-adenosylmethionine; SDMA, symmetric dimethylarginine; TML, trimethyllysine

MOLECULAR BIOLOGY

DNA-Protein Interaction

Characterization of Staphylococcus aureus Rec X protein: Molecular insights into negative regulation of RecA protein and implications in HR processes

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Homologous recombination (HR) is essential for genome stability and for maintaining genetic diversity. In eubacteria, RecA protein plays a key role during DNA repair, transcription, and HR. RecA is regulated at multiple levels, but majorly by RecX protein. Moreover, studies have shown RecX is a potent inhibitor of RecA and thus acts as an antirecombinase. *Staphylococcus aureus* is a major food-borne pathogen that causes skin, bone joint, and bloodstream infections. To date, RecX's role in *S. aureus* has remained enigmatic. Here, we show that *S. aureus* RecX (SaRecX) is expressed during exposure to DNA-damaging agents, and purified RecX protein directly interacts

physically with RecA protein. The SaRecX is competent to bind with single-stranded DNA preferentially and double-stranded DNA feebly. Significantly, SaRecX impedes the RecA-driven displacement loop and inhibits formation of the strand exchange. Notably, SaRecX also abrogates adenosine triphosphate hydrolysis and abolishes the LexA coprotease activity. These findings highlight the role of the RecX protein as an antirecombinase during HR and play a pivotal role in regulation of RecA during the DNA transactions.

Keywords: *Staphylococcus aureus*, RecX, recombination, RecA, antirecombinase

CELL

Cell Death

Ferroptosis model system by the re-expression of BACH1

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Ferroptosis is a regulated cell death induced by iron-dependent lipid peroxidation. The heme-responsive transcription factor BTB and CNC homology 1 (BACH1) promotes ferroptosis by repressing the transcription of genes involved in glutathione (GSH) synthesis and intracellular labile iron metabolism, which are key regulatory pathways in ferroptosis. We found that BACH1 re-expression in *Bach1*$^{-/-}$ immortalized mouse embryonic fibroblasts (iMEFs) can induce ferroptosis upon 2-mercaptoethanol removal, without any ferroptosis inducers. In these iMEFs, GSH synthesis was reduced, and intracellular labile iron levels were increased upon BACH1 re-expression. We used this system to investigate whether the major ferroptosis regulators glutathione peroxidase 4 (*Gpx4*) and apoptosis-inducing factor mitochondria-associated 2 (*Aifm2*), the gene for ferroptosis suppressor protein 1, are target genes of BACH1. Neither *Gpx4* nor *Aifm2* was regulated by BACH1 in the iMEFs. However, we found that BACH1 represses *AIFM2* transcription in human pancreatic cancer cells. These results suggest that the ferroptosis regulators targeted by BACH1 may vary across different cell types and animal species. Furthermore, we confirmed that the ferroptosis induced by BACH1 re-expression exhibited a propagating effect. BACH1 re-expression represents a new strategy for inducing ferroptosis after GPX4 or system Xc^- suppression and is expected to contribute to future ferroptosis research.

Keywords: transcription, fibroblasts, ferroptosis, extracellular signal, BACH1

Abbreviations: *AIFM2*, apoptosis-inducing factor mitochondria-associated 2; ANOVA, analysis of variance; BACH1, BTB and CNC homology 1; *Bach1*$^{-/-}$ mice, Bach1 knockout mice; BTB, Broad complex, Tramtrack, Bric-a-brac domain; bZIP, basic leucine zipper; ChIP-seq, chromatin immunoprecipitation sequencing; CNC, Cap'n'Collar region; DAPI, 4′,6-diamidino-2-phenylindole; DFX, deferasirox; DMSO, dimethyl sulfoxide; EMT, epithelial–mesenchymal transition; Ferr-1, ferrostatin-1; FINs, ferroptosis inducers; FSP1, Ferroptosis suppressor protein 1; Fth1, ferritin heavy chain 1; Ftl, ferritin light chain; GCL, glutamate-cysteine ligase; Gclc, GCL catalytic subunit; Gclm, GCL modifier subunit; GEO, Gene Expression Omnibus; GPX4, glutathione peroxidase 4; GSH, glutathione; HO-1 (Hmox1), heme oxygenase 1; iMEFs, immortalized MEFs; KuO, Kusabira Orange; MAFK, musculoaponeurotic fibrosarcoma oncogene homolog bZIP transcription factor K; mBACH1, Bach1 gene of Mus musculus; 2-ME, 2-mercaptoethanol; MEFs, mouse embryonic fibroblasts; NRF2, nuclear factor-erythroid 2-related factor 2; NSA, necrosulfonamide; PDAC, pancreatic ductal adenocarcinoma; PI, Propidium iodide; Ptgs2, prostaglandin-endoperoxide synthase 2; RSL3, (1S,3R)-RSL3; Slc40a1, solute carrier family 40 member 1; Slc7a11, solute carrier family 7 member 11; TFRC, transferrin receptor 1; Z-VAD.FMK, Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

BIOTECHNOLOGY

Cell and Tissue Engineering

Development of supercooling preservation method of adherent cultured human cells

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Cryopreservation of mammalian cells is an important technology; however, freezing damage due to osmotic pressure differences and ice crystal formation is inevitable. In addition, cryopreserved cells cannot be used immediately after thawing in many cases. Therefore, in this study, we developed a method for supercooling and preserving adherent cells using a precision temperature-controlled CO_2 incubator. The effects of the cooling rate from 37 to −4°C, the warming rate from −4 to 37°C and a preservation solution on cell viability after storage were examined. Human hepatocarcinoma-derived cell line HepG2 cells,

preserved with Hypo Thermosol FRS at −4℃ with a cooling rate of −0.028℃/min (24 h from 37℃ to −4℃) and warming to 37℃ at a rate of +1.0℃/min (40 min from −4 to 37℃), displayed high cell viability after 14 days of preservation. The superiority of supercooling preservation at −4℃ was demonstrated by comparing the obtained results with that of refrigerated preservation at +4℃. Cells preserved for 14 days under optimal conditions showed no cell shape abnormalities and may be used for experiments immediately after thawing. The optimized supercooling preservation method determined in this study is suitable for the temporary preservation of adherent cultured cells.

Keywords: adherent cells, cell preservation, cell viability, HepG2, supercooling preservation

Abbreviations: CS, Cryo Scaeless DMSO-Free; DMSO, dimethyl sulfoxide; FRS, Hypo Thermosol FRS; PI, propidium iodide; ST, Stem-Cellbanker DMSO Free; TK, Thelio Keep; UW, University of Wisconsin