Identification of the YEATS domain of GAS41 as a pH-dependent reader of histone succinylation

Yi Wang*,1, Jing Jin*,1, Matthew Wai Heng Chung*,1, Ling Fengb, Hongyan Sunb,c, and Quan Haoa,2

*School of Biomedical Sciences, University of Hong Kong, Hong Kong, China; bDepartment of Chemistry, City University of Hong Kong, Hong Kong, China; and cKey Laboratory of Biochip Technology, Biotech and Health Centre, Shenzhen Research Institute of City University of Hong Kong, Shenzhen 518057, China

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Lysine succinylation is a newly discovered posttranslational modification with distinctive physical properties. However, to date rarely have studies reported effectors capable of interpreting this modification on histones. Following our previous study of SIRT5 as an eraser of succinyl-lysine (Ksuc), here we identified the GAS41 YEATS domain as a reader of Ksuc on histones. biochemical studies showed that the GAS41 YEATS domain presents significant binding affinity toward H3K122suc upon a protonated histidine residue. Furthermore, cellular studies showed that GAS41 had prominent interaction with H3K122suc on histones and also demonstrated the coenrichment of GAS41 and H3K122suc on the p27 promoter. To investigate the binding mechanism, we solved the crystal structure of the YEATS domain of Ya9, the GAS41 homolog, in complex with an H3K122suc peptide that demonstrated the presence of a salt bridge formed when a protonated histidine residue (His39) recognizes the carboxyl terminal of the succinyl group. We also solved the apo structure of GAS41 YEATS domain, in which the conserved His43 residue superimposes well with His39 in the Ya9 structure. Our findings identified a reader of succinyl-lysine, and the binding mechanism will provide insight into the development of specific regulators targeting GAS41.

posttranslational modifications (PTMs) discovered on histones provide another layer of gene transcription regulation which complement the cis-regulatory elements in our genome. Emerging evidence shows that these PTMs, also collectively termed “histone codes,” function individually or in combinations to regulate nuclear processes such as transcription, DNA damage repair, replication, and chromatin condensation (1). Lysine succinylation (Ksuc) was initially identified as a novel protein PTM on Escherichia coli isocitrate dehydrogenase, serine hydroxymethyltransferase, and glyceraldehyde-3-phosphate dehydrogenase A (GAPDH) (2). Subsequently, Xie et al. (3) confirmed lysine succinylation as the recognition mechanism underlying crotonyl-lysine readout (4, 5). The dynamic system of histone codes falls under the control of histone lysines contributed by succinylation may imply its unique interactions (2). The dynamic system of histone codes falls under the control of “writer” and “eraser” enzymes dedicated to specific modification sites and types (1, 4). Meanwhile, the “readers” translate the histone code and disseminate the message across myriad cell phenotypes and behaviors (4, 5). Succinyl-CoA is believed to be a cofactor for succinyltransferase activity on various substrate proteins, analogous to the addition of acetyl groups, or alternatively, it may directly react with freely exposed lysines for succinylation to take place (2, 6, 7). Recently, acetate transferase KAT2A was found to couple with nuclear-translocated α-Ketoglutarate Dehydrogenase complex to write succinylation from the coenzyme onto H3K79 (8). Our previous study identified SIRT5, one of the NAD-dependent deacylases, as a potent desuccinylase and demalonylase on lysines (9, 10). However, little is known about the readers for the distinct Ksuc, which motivates the studies that discover potential candidates and advance our knowledge of its biological functions.

YEATS domain was initially characterized in Ya9 as a crucial subunit of yeast histone acetyltransferase NuA4 (11). The YEATS domain-containing proteins, including Ya9, Tafl4, ENL, AF9, and Glisoma-Amplified Sequence-41 (GAS41), are involved in acetyltransferase or methyltransferase complexes, which participate in the processes of chromatin remodeling and gene transcription (12). Shi and coworkers (13, 14) illustrated that human AF9 and ENL YEATS domains could recognize acetyl-lysine (Kac) to mediate normal physiological processes, but the readout could also underlie severe pathological conditions. Subsequently, studies have reported human AF9, YEATS2, and ENL and yeast Tafl4 YEATS domains as readers of crotonyl-lysine (Kcr), whereby AF9 YEATS domain senses an enrichment of H3K18cr deposited by p300 to coordinate a mechanism for transcriptional activation of inflammation genes (15–18). YEATS domain adopts a classical immunoglobulin fold structure of eight antiparallel β-strands and two α-helices (19). Currently, all solved YEATS domain structures reveal the consistent positioning of the aliphatic modified-lysine ligand sandwiched between two aromatic residues, establishing a robust π-π-π stacking as the recognition mechanism underlying crotonyl-lysine readout capability of Tafl4 and AF9 (15–18).

Results and Discussion

Prediction of the Readout of GAS41 or Ya9 Toward Ksuc. In favor of hydrophobic interactions, the extremely low binding affinity

Significance

Posttranslational modifications (PTMs) on histone lysines regulate gene expression and physiological functions. Succinylation is a newly discovered PTM with distinctive features. However, rarely studies have shown the function of succinylation on histone lysines. Our biochemical and structural studies demonstrate that GAS41, an oncogene-coded protein, can act as the reader of succinylation on histone H3K122. The functional significance of the pH-dependent histidine of GAS41 recognizing succinyl lysine (Ksuc) could have implications in local pH-dysregulated circumstances. The mechanism illustrated by the structures also provides an important insight into the development of specific regulators targeting the GAS41 YEATS domain in the future.


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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes 5Y8V and 5PBV).

1Y.W., J.J., and M.W.H.C. contributed equally to this work.
2To whom correspondence should be addressed. Email: qhao@hku.hk.

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Fig. 1. The YEATS domains of Yaf9 and GAS41 are pH-dependent succinyl readers on histones. (A) Different chemical structures of acetyl-lysine (Kac), crotonyl-lysine (Kcr), and succinyl-lysine (Ksuc). The succinyl acyl group is colored in red. (B) Microarray screening assay of Yaf9 and GAS41 YEATS domains against H3 and H4 peptides succinylated on previously identified succinyl-lysine sites. H3K122suc binds both Yaf9 and GAS41 (highlighted in dashed box). (C) Peptide pull-down assay of succinyl-lysine peptides against Yaf9 or GAS41 YEATS domain. (D) Peptide pull-down assays of GAS41 WT against different acyl groups on H3K122 peptides under pH 6.0, pH 7.0, and pH 7.4 conditions. GAS41 mutant (H43F) shows decreased binding affinity toward H3K122suc peptide. Images are representative of three independent experiments for each condition. (E) Statistical analysis of the efficiency of peptide pull-down assays in D. (F) Confirmation of GAS41 knockdown (KD) by Western blot. GAPDH was used as loading control. (G) Western blot for the identification of H3K122suc in anti-FLAG immunoprecipitates. FLAG-tagged GAS41 proteins were used as loading control. (H) The coenrichment of GAS41 and H3K122suc on p21 promoter. ChIP-qPCR analysis using anti-FLAG antibody and IgG control to probe the enrichment of FLAG-tagged WT or mutant (H43F) GAS41 on p21 promoter in cells (Left). ChIP-qPCR analysis using anti-H3K122suc antibody and IgG control to probe the enrichment of H3K122suc on p21 promoter in cells (Right). A Western blot with anti-FLAG and anti-GAPDH antibodies shows that both constructs are expressed at appropriate levels.
between AF9 and Ksuc was attributed to a key hydrophobic aromatic residue (Phe28) on loop 1 located at the end of the acyl-binding pocket of its YEATS domain (17). The residue at this position may have a crucial role in the recognition of different acyl groups, which has displayed a major discrepancy across human YEATS domains with a phenylalanine (Phe) in AF9 and ENL, a serine (Ser) in YEATS2, and a histidine (His) in GAS41 (SI Appendix, Fig. S1D) (15). As a comparative case, YEATS2 can favorably bind 2-hydroxysobutyryl-lysin (Khib) while AF9 cannot, probably due to the contribution of a hydrogen bond by the Ser254 hydroxyl group in YEATS2 (15, 17). Intriguingly, GAS41 has a histidine residue at this position. GAS41, otherwise known as YEATS4 (YEATS Domain-containing Protein 4), is a newly discovered oncogene regulating the p53-p21 pathway (20). However, despite the availability of crystal structures of its homolog Ya9f, the role of GAS41 YEATS domain has been left unexplored (19). In concordance with GAS41, Ya9f has a histidine residue that surrogates the key Phe residue recognizing Kcr in human AF9 when superimposed (SI Appendix, Fig. S2A and B). As histidine could gain a positive charge when protonated (21), therefore, we proposed that human GAS41, as well as yeast Ya9f, may have the preference for an acyl group with a negative charge, such as the succinyl group.

**Screening of the Potential Ksuc Sites Binding to GAS41 or Ya9f.** To test our hypothesis, we first applied peptide microarray assay to profile the binding affinities of yeast Ya9f or human GAS41 YEATS domain against N-terminal biotinylated peptides bearing a subset of succinyl-lysine sites on histone H3 or H4 (Ksuc-H3/H4) (19). Different experimental methods have been used to measure pH of resting yeast at 5.8 and 6.35, and that of fermenting yeast at 6.1 and 7.1 (22, 23); the intracellular pH of yeast yeast at 5.8 and 6.35, and that of fermenting yeast at 6.1 and 7.1 (22, 23); the intracellular pH of yeast

$$pH = \frac{(\text{H}^+)}{[\text{H}_2\text{O}]}$$

measurement of GAS41 or Ya9f Binding Toward Acetyl-, Crotonyl-, Succinyl H3K122 Peptides.** Next, we compared the binding capabilities of different acyl groups on H3K122 against GAS41 YEATS domain under different pH conditions. After testing the intracellular pH value varies in different cellular organelles (from ~pH 4.7 to ~pH 7.4), owing to the activities of various metabolic pathways such as ATP production in the cytoplasm (25). The regulation of nuclear pH is hypothesized to have a mechanism similar to that in the cytoplasm, but this requires experimental validation (25). Therefore, we chose pH 6.0, pH 7.0, or pH 7.4 for the following experiments. The results of peptide pull-down revealed that the H3K122suc peptide, rather than the others, showed prominent binding ability toward GAS41 under pH 6.0 or pH 7.0 (Fig. 1D). Mutation of the proposed key residue from His to Phe (H343F) significantly decreased the binding affinity. When the pH reached 7.4, H3K122suc demonstrated dramatically decreased binding toward GAS41, while H3K122cr and H3K122ac showed increased affinity to GAS41 (Fig. 1D). Quantification by statistical analysis supported the scenario of the above peptide pull-down experiment (Fig. 1E). Together, the GAS41 YEATS domain can interact with H3K122suc presumably upon a protonated histidine. Furthermore, we measured the binding affinities of both GAS41 and Ya9f YEATS domains against different acylations on H3K122 peptide with surface plasmon resonance spectroscopy (SPR). In line with the peptide pull-down assay, the SPR experiment was also performed at pH 6.0, 7.0, and 7.4 (Table 1). Each measurement was repeated three times. At pH 7.4, affinity of H3K122suc binding was not determined, but at pH 7.0, it was calculated to be 48.34 ± 3.8 μM. This suggests that the proportion of protonated His43 increases from pH 7.4 to 7.0. Affirmatively, a stronger binding affinity between GAS41 and the H3K122suc peptide was determined to be 2.937 ± 0.21 μM at pH 6.0. The mutation H43F in GAS41 sharply decreased its affinity toward the H3K122suc peptide (Table 1). Together, upon pH variation from pH 7.4 to pH 6.0, we observed an increase in binding affinity of GAS41 toward H3K122suc, in contrast to the corresponding decrease in affinity toward H3K122cr (Table 1). The affinities did not vary as much toward H3K122ac from pH 7.4 to 7.0. Additionally, Ya9f was able to bind H3K122suc with an affinity of 27.44 ± 3.4 μM at pH 6.0 while it has no detectable binding toward H3K122cr and H3K122ac. Altogether, the results suggest that GAS41 and Ya9f YEATS domains can interact with H3K122suc peptide in a pH-dependent manner, probably through the protonation of His43, which facilitates recognition of the Ksuc carboxyl terminal.

**Cellular Studies of GAS41 Binding H3K122suc on Histones.** Subsequently, we investigated whether the interaction between GAS41 YEATS domain and H3K122suc is physiologically relevant in cells. It is previously described that GAS41 could act as a transcription factor involved in cancer development, while H3K122suc was detected in HeLa cells (3, 20). Peptide dot-blots assays showed that the anti-H3K122suc antibody specifically recognizes H3K122suc, but not other modification types or sites including unmodified histone H3K122, H3K122ac, H3K122cr, and other identified Ksuc sites on H3 (SI Appendix, Fig. S3). We then generated GAS41 knockdown (KD) HeLa cells (Fig. 1F), in which FLAG-tagged constructs of full-length GAS41 WT or mutant (H343F) were overexpressed. FLAG immunoprecipitation (FLAG-IP) was then carried out to analyze the content of H3K122suc in FLAG immunoprecipitates. Western blot analysis showed that GAS41 WT, rather than the mutant (H343F), had prominent interaction with H3K122suc in cells.

### Table 1. The binding affinities of GAS41 and Ya9f YEATS domains against different acylations on H3K122 peptides measured by surface plasmon resonance spectroscopy

<table>
<thead>
<tr>
<th>Peptides</th>
<th>pH</th>
<th>GAS41</th>
<th>Ya9f</th>
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<tr>
<td>H3K122suc</td>
<td>6.0</td>
<td>2.937 ± 0.21 μM (WT)</td>
<td>27.44 ± 3.4 μM</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>48.34 ± 3.8 μM (WT)</td>
<td>ND</td>
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<tr>
<td></td>
<td>7.4</td>
<td>ND (WT)</td>
<td>ND</td>
</tr>
<tr>
<td>H3K122cr</td>
<td>6.0</td>
<td>79.82 ± 1.0 μM</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>31.27 ± 2.6 μM</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>18.23 ± 4.3 μM</td>
<td>ND</td>
</tr>
<tr>
<td>H3K122ac</td>
<td>6.0</td>
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<td>ND</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>51.94 ± 6.1 μM</td>
<td>ND</td>
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<tr>
<td></td>
<td>7.4</td>
<td>47.68 ± 6.7 μM</td>
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*Kₐ values are calculated with an equilibrium model, except when marked with an *, the values are calculated with kinetic rate constants. ND, not detectable. Choice of analysis and the original rate constants are available in the SI Appendix.
suggesting the essential role of this key histidine residue during the interaction. Furthermore, we also performed ChIP-qPCR analysis to investigate cellular coenrichment of GAS41 and H3K122suc. As reported, GAS41 was located on p14ARF and the p21 promoter in proliferating cells (26). Our data underscored the fact that GAS41 WT enriches on the p21 promoter and showed the low occupancy of the mutant (H43F) accordingly (Fig. 1H, Left histogram). H3K122ac was also detected on the p21 promoter (26–29). Thus, H3K122suc is proposed to competitively occupy the p21 promoter region. ChiP-qPCR results confirmed the enrichment of H3K122suc on the p21 promoter in cells (Fig. 1H, Right histogram). Therefore, GAS41, but not the mutant, could be coenriched with H3K122suc on the p21 promoter, implicating a relevant physiological interaction in cells. Collectively, the cellular data supported that GAS41 could act as a reader of histone succinylation in cells.

**Molecular Mechanism of H3K122suc Readout.** To gain insight into the molecular basis underlying Ksuc readout by GAS41, we performed crystallographic studies of GAS41 and Yaf9 YEATS domains in complex with the H3K122suc peptide. Crystals of the Yaf9/H3K122suc complex could diffract to 2.0-Å resolution after optimization. Statistics of the crystallographic data collection and refinement are summarized in SI Appendix, Table S1. The overall structure of Yaf9 shows a typical YEATS domain with eight antiparallel β-strands capped by two short α-helices (Fig. 2A). Despite our efforts to cocrystallize GAS41 with H3K122suc peptide, only the apo structure of GAS41 was obtained at 2.6-Å resolution (SI Appendix, Table S1). The structure of GAS41

![Image of molecular basis of H3K122suc readout by Yaf9 and GAS41 YEATS domains]

**Fig. 2.** Molecular basis of H3K122suc readout by Yaf9 and GAS41 YEATS domains. (A) The overall structure of Yaf9 YEATS domain (yellow) in complex with H3K122suc (green). (B) The apo structure of GAS41 YEATS domain (red). (C) The Ksuc readout by Yaf9 via the hydrophilic interactions between H39, E38, and T69 residues and carboxyl terminal of succinyl group. 2Fo-Fc omit map (1σ) shows that the succinyl-lysine is well-defined in the pocket. (D) Superimposition of the pockets of Yaf9/H3K122suc (yellow) and GAS41 (Red) YEATS domains. (E) The electrostatic potential map for the interactions between residues and succinyl group. The negative potential is colored in red while the positive potential is colored in blue. (F) Interactions between H3 peptide (green) and Yaf9 YEATS domain (yellow).
again demonstrates a classical YEATS domain that superimposes well with Ya9 (Fig. 2B and SI Appendix, Fig. S4). In the complex, the succinyl-lysine is anchored in the groove of Ya9 formed between loops 1 and 4 (Fig. 2A). 2Fo–Fc omit map (1σ) shows the complete density of H3K122suc anchored inside the pocket (Fig. 2C). Remarkably, the carboxyl terminal of H3K122suc could be recognized by His39 via a salt bridge as well as by Glu38 and Thr69 through hydrogen bonds (Fig. 2C). The pattern of H3K122suc bound to Ya9 YEATS domain is similar to that of H3K9cr2 being recognized by human AF9 (PDDB ID code 5HUB) (SI Appendix, Fig. S5). The succinyl-lysine superimposes well with crotonyl-lysine in the pockets of both yeast Ya9 and human AF9 YEATS domains (SI Appendix, Fig. S5). The His39 of Ya9 aligns best with Phe28 of human AF9, which distinguishes Ya9 as a reader of Ksuc (SI Appendix, Fig. S5). Superimposition of the residues Trp89 and Tyr70 of Ya9 to Tyr78 and Phe59 of AF9, respectively, demonstrates the typical binding pocket constituents adopted by the YEATS domains for the readouts of different acyl-lysines (SI Appendix, Fig. S5). The distance between the imidazole ring of His39 and the carboxyl group is 2.6 Å, which is comparable to that between Arg105 and H3K9cr2 (2.7 Å) in the SIRT1 complex structure (10), indicating a strong electrostatic interaction.

Notably, His43 of GAS41 is conserved and superimposes well with His39 in Ya9 in the pocket, demonstrating a similar layout of the H3K122suc-binding pocket residues (Fig. 2D). As aforementioned, mutation of this His to Phe (H3F3) significantly decreased the binding affinity of GAS41 to the H3K122suc peptide in both pull-down and SPR assays (Fig. 1 D and E and Table 1), which again emphasizes the key role of this histidine for acyl recognition. Additionally, the electrostatic potential was calculated to illustrate charge distribution in the interaction environment within the binding pocket (Fig. 2E). Meanwhile, the structure reveals an aromatic sandwiching cage, which specifically accommodates the flat carbonyl group (C=O) on the ligand by snugly clamping it via π stacking into a well-oriented position and permits other associated intermolecular interactions (SI Appendix, Fig. S6). Moreover, 2Fo–Fc omit map (1σ) shows that the flanking residues of the H3K122suc peptide could be well-defined in the structure (SI Appendix, Fig. S7). They form intimate contacts with the surface residues of Ya9 YEATS domain (Fig. 2F). The hydrophobic interaction between the residues of H3K122suc-containing M1, P2, I4, Q6, and L7, and the residues H67, W89, F92, and H118 of Ya9 stabilizes the binding (Fig. 2F). It may explain why H3K122suc could stand out to bind both Ya9 and GAS41, probably due to the additional interactions available for the specific peptide sequence. Moreover, hydrogen bonds formed between the residues H67, E91, R116, and H118 of Ya9 and the main chain of H3K122suc peptide also contribute to the interaction (Fig. 2F).

Therefore, GAS41 is able to recognize Ksuc with a mechanism similar to Ya9, utilizing the characteristic salt bridge established with a protonated histidine residue.

Conclusion

With a rapidly expanding library of PTM marks on histones, it is exciting to understand their interplay and the emerging important roles that lysine acylations play in epigenetic regulation on nucleosomes. Succinylation is a newly discovered PTM with distinct structural and chemical properties. YEATS domain-containing proteins have been reported as readers of Kac or Kcr on histones. Our cellular and structural studies revealed that GAS41 is a lysine succinylation reader owing to a protonated histidine residue located inside the pocket. As reported, intracellular pH could be regulated by global histone acetylation at a genomic level by the relevant physiological functions in normal or cancer cells (30). The wax and wane of the binding affinities of GAS41 toward H3K122suc and H3K122cr indicate the switching role of GAS41 upon pH variation. The functional significance of the pH-dependent histidine switch of GAS41 for Ksuc recognition could have implications in local pH-dysregulated circumstances (31). This cross-talk for GAS41 recognizing Kac, Kcr, or Ksuc upon intracellular pH variation would be an interesting area for further investigation. Currently, there are more than 20 Ksuc sites identified on various histones in cancer cells (3), which urges further investigation on modifications on profiling the roles of histone Ksuc sites and other types of acyl-lysines with a terminal carbonyl group including malonyl- and glutaryl-lysine, to comprehensively elucidate GAS41-mediated epigenetic regulation.

Materials and Methods

Full details are provided in SI Materials and Methods. The in vitro screening of the potential Ksuc sites binding to GAS41 or Ya9 was achieved by microarray assay using proteins against N-terminal biotinylated peptides that bear a subset of succinyl-lysine sites on histone H3 or H4. The measurement of GAS41 or Ya9 binding toward acetyl-, crotonyl-, succinyl H3K122 was performed by peptide pull-down and SPR assay. For the cellular study, the coenrichment of GAS41 and H3K122suc on the p27 promoter was detected by ChIP-qPCR. Then, the crystallizations of GAS41 and Ya9 with H3K122suc were carried out and diffraction data were collected at the Shanghai and Taiwan synchrotron facilities.

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