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The polypeptide Syn67 interacts physically with human holocarboxylase synthetase, but is not a target for biotinylation

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Abstract

Holocarboxylase synthetase (HCS) catalyzes the binding of biotin to lysines in carboxylases and histones in two steps. First, HCS catalyzes the synthesis of biotinyl-5'-AMP; second, the biotinyl moiety is ligated to lysine residues. It has been proposed that step two is fairly promiscuous, and that protein biotinylation may occur in the absence of HCS as long as sufficient exogenous biotinyl-5'-AMP is provided. Here, we identified a novel polypeptide (Syn67) with a basic patch of lysines and arginines. Yeast-two-hybrid assays and limited proteolysis assays revealed that both N- and C-termini of HCS interact with Syn67. A potential target lysine in Syn67 was biotinylated by HCS only after arginine-to-glycine substitutions in Syn67 produced a histone-like peptide. We identified a Syn67 docking site near the active pocket of HCS by *in silico* modeling and site directed mutagenesis. Biotinylation of proteins by HCS is more specific than previously assumed.

Keywords

Biotin; domains; holocarboxylase synthetase; substrate; Syn67

1. Introduction

Biotin is a water-soluble vitamin that serves as a coenzyme for 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and acetyl-CoA carboxylases 1 and 2 [1]. Carboxylases play essential roles in macronutrient metabolism. Biotin is also covalently attached to at least 11 distinct lysine (K) residues in histones H2A, H3, and H4, including biotinylation of K12 in histone H4 (H4K12bio) [2–5]. Covalent modifications of histones are known to play crucial roles in gene regulation, DNA repair, and chromatin structure [2]. H4K12bio has been linked to the repression of genes [3,4] and retrotransposons [5], the cellular response to DNA damage [6], and is also enriched in telomeric repeats and pericentromeric alpha satellite repeats [3,7].

Attachment of biotin to both carboxylases and histones is mediated by holocarboxylase synthetase (HCS, EC 6.3.4.10) [4,8,9]. The following four domains have been identified and characterized in human HCS: N-terminal domain, central domain, linker domain, and C-

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terminal domain [10]. Both N- and C-termini of HCS participate in substrate recognition [10]. The central domain in HCS contains binding sites for both ATP and biotin [11–13]. The linker domain might play a role in conferring the flexibility needed for HCS to interact with a diverse group of substrates for biotinylation [10]. Mutations and knockdown of HCS decreases the abundance of biotinylated carboxylases and histones [4,8,9,14,15], leading to abnormal patterns of gene expression [4,9] and phenotypes such as altered life span and stress resistance [9,16].

HCS-mediated biotinylation of carboxylases and histones requires ATP and proceeds in two steps [17]. In the first step, HCS catalyzes the synthesis of biotinyl-5'-AMP. In the second step, the biotinyl moiety is conjugated to distinct lysine residues in target proteins. Recently it was proposed that the second step may occur in the absence of HCS, if synthetic biotinyl-5'-AMP is provided [18]. If true, this could be a mechanism to explain biotinylation of proteins as diverse as carboxylases and histones, but it would also make it somewhat difficult to explain why only some of the many lysines in histones are biotinylated [19]. Here, we used various molecular biology techniques and *in silico* modeling to test the hypothesis that not all the proteins and polypeptides that interact with HCS are targets for biotinylation, but that carboxylase- and histone-like structures are preferred HCS targets.

2. Materials and Methods

2.1 Y2H assays

Two independent Y2H assays were conducted to identify HCS-interacting proteins, following our previously published protocols for high-stringency conditions [10]. Briefly, a human liver cDNA library was fused to a Gal4 activation domain (Gal4AD) and served as prey in Y2H assays (pGADT7 vector; Clontech, Mountain View, CA). Full-length human HCS (GenBank accession #D23672) was fused to a Gal4 binding domain (BD) to produce vector pGBKT7-HCS [10], which was used as bait. In previous studies we demonstrated that pGBKT7-HCS does not auto-activate reporter genes in Y2H assays and is not toxic for *S. cerevisiae* strain AH109 [10]. Also in previous studies we demonstrated biological activity of human HCS in yeast [10]. Growth of mated yeast colonies was monitored for up to 21 d. Sterile tubes containing 20 mL of SD/-Leu broth were inoculated with recovered yeast colonies and grown for 72 h on an orbital shaker (200 rpm) at 30°C. Cells were collected by centrifugation at 2880 g at 4°C and yeast plasmids were isolated using the YEASTMAKER yeast plasmid isolation kit (Cat. # PT3049-1; Clontech) or the Zymoprep II kit (Cat. # D2004; Zymo Research, Orange, CA) following the manufacturers' protocols. A total of 425 clones that interacted with HCS were recovered in the two Y2H assays; 62 clones were chosen for further analysis, preference being given to clones from colonies that were visible within less than 14 d of incubation. Eluted plasmids (10 µL) were used to transform One Shot TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) for subsequent plasmid preparation (QIAprep Spin Miniprep kit, Qiagen, Valencia, CA) and DNA sequencing. Nucleotide sequences were converted to protein sequences, using the ExPASy DNA translation tool [20]. One polypeptide comprising 67 amino acids interacted strongly with HCS and appeared in both Y2H assays (see Results); this polypeptide was named Syn67 and became the focus of this study. Yeast was co-transformed with Syn67-Gal4AD, and either pGBKT7 vector (empty Gal4BD) or pGBKT7-HCS to confirm HCS:Syn67 interactions in a targeted Y2H assay.

Interactions of Syn67-Gal4AD with the following truncation constructs of HCS-Gal4 BD were investigated to identify HCS domains that interact with Syn67: pGBKT7-HCS₁₋₄₄₆ (HCS N-terminal domain, M1-F446), pGBKT7-HCS₄₇₁₋₅₇₅ (central domain, F471-S575), pGBKT7-HCS₆₁₀₋₆₆₈ (linker domain, T610-V668), pGBKT7-HCS₆₆₉₋₇₁₈ (C-terminal domain, H669-R718), pGBKT7-HCS_Ndel (deletion of the N-terminal domain M1-F446), and pGBKT7-HCS_Cdel (deletion of the C-terminal domain H669-R718) [10]. Plates were monitored daily

for appearance of colonies. The time of appearance was recorded accordingly as an indicator for the strength of interactions.

2.2 Recombinant HCS, Syn67, and p67

The Syn67 insert in Syn67-Gal4AD was PCR amplified using primers 5'-tggaggatccatggagtaccatacg-3' (forward) and 5'-tatatgtcgacctattggggaggggtg-3' (reverse) and cloned using vector pET-30a(+) (Novagen, Madison, WI). PCR products and pET-30a(+) were digested with *Bam*HI and *Sal*I and gel purified prior to overnight ligation, to produce plasmid Syn67-pET. The identity of Syn67-pET was confirmed by sequencing before ArcticExpress (DE3)RP *E. coli* competent cells (Cat., #230194, Stratagene, La Jolla, CA) were transformed with the plasmid. Recombinant Syn67 was purified using His Trap FF columns and an AKTA protein purification system as described for p67 [19]. Protein extracts were resolved on Novex 16% Tricine gels (Invitrogen) and stained with Coomassie blue to confirm expression of a polypeptide of the expected size (13 kDa for his-tagged Syn67). Purified Syn67 was stored at -20°C in elution buffer.

The polypeptide p67 comprises the 67 C-terminal amino acids in human propionyl-CoA carboxylase, including the biotin-binding site K669 [11]. p67 is a well-established substrate for studies of HCS [11,13]. Recombinant p67 was produced as described previously [19]. p67 is a target for biotinylation by the HCS ortholog in *E. coli*, BirA, and the biotin-containing fraction of p67 was removed using avidin beads [19].

Recombinant human HCS was prepared as described (B. Bao *et al.*, submitted) with minor modifications. Briefly, a clone coding for full-length human HCS was obtained from Yoichi Suzuki (Tohoku University, Sendai, Japan) [21]. *Sac*I and *Eco*RI restriction sites were inserted by PCR, using forward primer 5'-catatgagctcatggaagatagactccac-3' and reverse primer 5'-ctaataattctaccgccgtttggggaggatg-3'. PCR products were ligated into pSTBLUE-1 AccepTor vector (Novagen, Madison, WI), digested with *Sac*I and *Eco*RI, and subcloned into vector pRSET A (Novagen), thereby fusing a his-tag to the N-terminus of HCS. The plasmid was named "HCS- pRSET A" and codes for a fusion protein of 82 kDa; its identity was verified by sequencing. ArcticExpress™ (DE3) competent cells (Stratagene, La Jolla, CA) were transformed with HCS- pRSET A and grown overnight at 37°C in 10 mL LB broth containing 20 mg/L gentamycin and 50 mg/L ampicillin. Cultures were expanded to 1 L and grown at 30°C for about 5 h to an optical density of 0.3 to 0.6 at 600 nm. Isopropyl-beta-D-thiogalactopyranoside was added for a final concentration of 1 mM and cultures were continued at 12°C for 24 h. Cell pellets were collected by centrifugation at 2700 g for 10 min, and resuspended in 50 mM Tris buffer (pH 7.5) with protease inhibitor cocktail set III (Calbiochem, Gibbstown, NJ; Cat. # 539134). Samples were sonicated on ice and cell debris was removed by centrifugation. HCS fusion protein was purified using His Trap FF columns on an AKTA protein purification system according to manufacturer's instructions (GE Healthcare). HCS in the column fractions was identified by gel electrophoresis, Coomassie blue staining, anti-His-tag antibody (Novagen), and an antibody to the C-terminus in human HCS [22].

2.3 Limited proteolysis assays

Limited proteolysis experiments were conducted as described [23] to confirm that HCS interacts with Syn67. In proteolysis assays, interactions between two proteins slow proteolytic degradation to an extent that is detectable by Western blot analysis. Briefly, 5.85 µg recombinant Syn67 was pre-incubated in the presence or absence of 34 µg recombinant HCS in 50 µL of 75 mM Tris acetate buffer (pH 7.5), 0.3 mM dithiothreitol, and 45 mM MgCl₂ at 37°C for 2 h to allow for binding of HCS to Syn67. Ten microliters were collected as baseline value, and 200 ng trypsin (Sigma; Cat. # T6567, St. Louis, MO) was added to the remaining solution. Incubation was continued at 37°C and 10-µL aliquots were collected at timed

intervals. Proteolysis was stopped by adding NuPAGE sample buffer (Invitrogen) and heating at 95°C for 10 min. Proteins were resolved using 4–12% NuPAGE Bis-Tris gels and stained with silver stain (SilverQuest; Invitrogen).

2.4 Site-directed mutagenesis of HCS

A large number of mutations have been identified in human HCS from various ethnic backgrounds [14,21]; some of these mutation decrease HCS activity by >99% compared with wild-type HCS and require treatment of afflicted individuals with pharmacological doses of biotin [24,25]. Here we tested four of the most common HCS mutations to determine whether they affect the interactions of HCS with Syn67. The following mutants were created using the Gene Tailor Site-Directed Mutagenesis System and the manufacturer's protocol (Invitrogen); PCR reactions were conducted using high fidelity platinum taq polymerase (Invitrogen) and pGBKT7-HCS as a template: (a) The L237P mutation is caused by a C997T transition in the HCS coding region [14,26] and was created using primers 5'-accagaagttcatggcctatccctctcagggag-3' (forward) and 5'-ataggccatgaactctgtgtacaggtcttc-3' (reverse); (b) The D571N mutation is caused by a G1998A transition in the HCS coding region [24,25], and was created using primers 5'-tagcagtgagtgcccaacaacattattacag-3' (forward) and 5'-gttggccactctactcgttaagtgatgc-3' (reverse); (c) The R508W mutation is caused by a C1522T transition in the HCS coding region [21], and was created using primers 5'-ggcagaccgagggcaaaagatggggagggaatg-3' (forward) and 5'-tcctttgccctcggctcggcggcgcat-3' (reverse); and (d) the G581S mutant is caused by a G1741A transition in the HCS coding region [21,25,27] and was created using primers 5'-acagtgcacctcatgaagatcctccggagttctgg-3' (forward) and 5'-gatcttcatgaggtcactgtaataaatatc-3' (reverse). Briefly, 2 µL of mutagenesis mixture were transformed into chemically competent MAX Efficiency® DH5α-T1^R cells (F- ϕ 80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k⁻, m_k⁺) *phoA supE44 thi-1 gyrA96 relA1 tonA*) before plating on LB plates supplemented with ampicillin. The following bait plasmids were recovered and corresponded to the four mutants named above: pGBKT7-HCS_{L237P}, pGBKT7-HCS_{D571N}, pGBKT7-HCS_{R508W}, and pGBKT7-HCS_{G581S}. Interactions of mutants with Syn67-Gal4AD were investigated using Y2H assays as described above.

2.5 Molecular modeling of HCS:partner (Syn67 and p67) interactions

3D models of HCS and p67 were predicted as described in our previous studies [10,28]. The structure of Syn67 was predicted using the Phyre server and Tk-subtilisin propeptide as a template (protein database ID = 2z57) [29]. Protein-protein interaction between HCS and its two substrates p67 and Syn67 were modeled using Patchdock [30] and results were refined using FireDock [31]. Modeling of interactions was completed by visual inspection of representations generated by PyMol [32] and Deep View [33].

2.6 Biotinylation of Syn67 and Syn67-like peptides by recombinant human HCS

Syn67 contains one lysine residue embedded in a patch of arginine residues, creating a potential target site for biotinylation by HCS (see Results). Recombinant human HCS, Syn67, and p67 were prepared as described above. Biotinylation assays were conducted as described in our previous studies [19], using up to 34 µg of HCS and up to 5.85 µg of Syn67 per sample; p67 was used as positive control at an amount of 0.75 µg per sample. Proteins were resolved by gel electrophoresis and peptide-bound biotin was visualized using either IRDye® 800CW streptavidin or anti-biotin polyclonal antibodies (Abcam) and a LI-COR Odyssey infrared imaging system (LI-COR, Lincoln, NE).

In some biotinylation assays, recombinant Syn67 was replaced with the following synthetic Syn67-based peptides to identify amino acid residues that facilitate or hinder biotinylation of

Syn67 (see Results): (a) NRSRRKECFRS = truncated variant of Syn67, containing the lysine residue that is a potential target for biotinylation by HCS; (b) NRSGGKECFRS = histone-like variant of Syn67, in which glycines were substituted for arginines adjacent to the lysine residue; (c) NRSRRAECFRS = negative control, in which the lysine in Syn67 was replaced with an alanine; and (d) NRSAMKMCFRS = p67-like variant of Syn67 in which AMKM was substituted for RRKE to create the canonical biotinylation site in carboxylases [12]. All peptides were acetylated at the N-terminus to prevent biotinylation of the α -amino group. Peptides were purchased from AnaSpec (San Jose, CA) and identities and purities were confirmed by mass spectrometry and HPLC, respectively, by the manufacturer.

3. Results

3.1 Interactions between human HCS and the Syn67 polypeptide

Two Y2H assays were conducted independently, and consistently suggested that human HCS interacts with a polypeptide that was subsequently named Syn67. Syn67 comprises 67 amino acids, including a lysine near the C-terminus as a potential target site for biotinylation by HCS (Fig. 1). The lysine is embedded in a basic patch of arginines, creating an environment suspected to favor biotinylation by HCS [18].

The interaction between full-length HCS and Syn67 was among the strongest interactions seen in our Y2H screening experiments; colonies became visible within only 2–3 days of incubation of mated yeast (Fig. 2). No auto-activation of the reporter genes or non-specific binding to the Gal4 BD was observed for this peptide (data not shown). Both N- and C-termini in HCS interacted with Syn67, whereas central domain and linker domain did not interact (Fig. 2). The importance of the N- and C-termini in HCS for interactions with Syn67 were confirmed in targeted Y2H assays of truncated and mutant HCS as described below.

Physical interactions between HCS and Syn67 were confirmed by limited proteolysis assay, using recombinant full-length HCS and recombinant Syn67 (Fig. 3). If HCS was digested with trypsin in the presence of Syn67, HCS digestion was delayed by 10–15 minutes compared with Syn67-free controls. The reader should note that various modifications of the digestion protocol were tested; Syn67 always slowed but never prevented the degradation of a large fraction of rHCS in incubation mixtures. Collectively, these observations suggest that Syn67 recognizes the three-dimensional fold of full-length HCS by interacting with at least two HCS domains (N- and C-termini) known to play important roles in interactions with biotinylation targets [10].

3.2 Biotinylation of Syn67

Syn67 is not a target for biotinylation by HCS. If recombinant Syn67 was incubated with recombinant HCS and cofactors for biotinylation for up to 24 h, no Syn67-bound biotin was detectable with streptavidin (Fig. 4, **lane A**). Other incubation periods (12–18 h) were also tested, but also produced no signal (data not shown). Previous studies suggested that recombinant proteins that are targets for biotinylation by HCS, e.g. p67, undergo biotinylation by BirA in *E. coli* [19]. However, recombinant Syn67 was not biotinylated by BirA in *E. coli* (Fig. 4, **lane B**). Recombinant p67, a well-known HCS and BirA substrate [11,19,34], was deprived of the biotin-containing fraction by avidin beads before biotinylation assay (Fig. 4, **lane C**); biotin-depleted p67 was used as positive control in biotinylation assays and produced a strong signal after only 45 min of incubation with HCS (Fig. 4, **lane D**).

Syn67 became a target for biotinylation by HCS only after the amino acid sequence was altered to produce a histone-like variant of Syn67. If a truncated variant of Syn67 (peptide NRSRRKECFRS) was incubated with HCS and cofactors for enzymatic biotinylation, no

peptide-bound biotin was detectable if probed with anti-biotin and streptavidin (Fig. 5, **lane A**). If arginines adjacent to the lysine residue in Syn67 were substituted with glycines to produce a sequence reminiscent to that in histones (peptide NRSGGKECFRS), the peptide became substrate for biotinylation by HCS (Fig. 5, **lane B**). If the lysine residue in Syn67 was replaced with an alanine (peptide NRSRRAECFRS, negative control), no biotin was detectable by anti-biotin and streptavidin (Fig. 5, **lane C**). Finally, a p67-like variant of Syn67 was created by substituting AMKM for RRKE; the p67-like variant of Syn67 also was no good substrate for biotinylation by HCS (Fig. 5, **lane D**). The latter is not too surprising, given that previous studies suggested a minimum length of 67 amino acids for carboxylase-based peptides to become HCS substrates [11].

3.3 Molecular modeling of HCS interactions

HCS, p67, and Syn67 were subjected to molecular modeling. The HCS model spanned the region between E393 and D715. The root mean square deviation (RMSD) between the biotin protein ligase in *E. coli*, BirA, as a template and the HCS model was 0.52 Å, as previously reported [10]. The p67 model covered R1 to E66 with RMSD at 0.39 Å and contained six β -strands. Lysines were located at the ends of β -strands. For Syn67, the model covered M13 to E59 with the RMSD being 0.45 Å. The model contained an α -helix from A43 to V55, short two β -strands, and a major loop. The only potential biotinylation target in Syn67, K58, resides in the loop (Fig. 6). Generally, models with RMSD less than 1 Å are considered reliable for prediction [35,36].

Our previous studies revealed two potential binding sites for p67 in HCS [10]; predicted binding energies are -44 units for site A and -69 units for site B (Fig. 7). Three potential Syn67 binding sites were predicted in HCS (Fig. 7); two of these sites (denoted A and B) are shared with p67. Syn67 binding energies at sites A, B, and C were estimated to be -51, -47, and -44 units, respectively. These binding energies are similar to those calculated for antigen-antibody interactions [31]. The following residues in HCS are predicted to play important roles in the three Syn67-binding sites: (a) site A = R508-N511, Q533-Q542, P569-N570, V585-F594, G677-S678, and D710-G711; (b) site B = E444-S448, L453-R457, R501-Q502, V512-C519, and T605-E619; and (c) site C = V407-L420, N452-I455, and S607-I613.

3.4 Effects of HCS mutations on interactions with Syn67

Targeted Y2H assays with HCS mutants revealed that truncations and mutations in the predicted Syn67-binding sites substantially decreased the strength of interaction. Mated yeast transformed with Syn67 and full-length HCS produced visible colonies in as little as 2–3 d (Fig. 8A). If the C-terminus of HCS was deleted, interaction with Syn67 was abolished (Fig. 8B), consistent with the involvement of the C-terminus in substrate recognition described above. Likewise, if the N-terminus of HCS was deleted, it took about 15 d of incubation for colonies to become faintly visible (Fig. 8D). Interactions with Syn67 were also abolished in the D571N and R508W mutants (Fig. 8 E and F), even if incubation was extended to 21 d (not shown). These two mutations reside in or within immediate proximity to the Syn67 docking site A (see above), suggesting that site A, rather than sites B and C, is the substrate docking site in HCS. Two other mutations that are commonly found in human HCS (G581S and L237P) also resulted in decreased interactions with Syn67, but the effects were smaller than those seen for the D571N and R508W mutants. Colonies of the G581S mutant became visible after about 3–4 days of incubation (Fig. 8C), whereas colonies of the L237P mutant became visible after 7–8 d of incubation (Fig. 8G).

4. Discussion

To the best of our knowledge this is the first report of a protein or polypeptide interacting with HCS without being biotinylated. The polypeptide identified here, Syn67, mimics the binding characteristics of a known HCS substrate, p67. Both Syn67 and p67 bind to the central domain of HCS near the active pocket, and for both polypeptides the N- and C-termini of HCS are important for binding ([10], and this study). The importance N- and C-termini for Syn67 binding is consistent with the recently solved structure of *Aquifex aeolicus* biotin protein ligase, revealing that N- and C-terminal domains form a cavity involved in catalysis [37]. The confines of the Syn67-binding site in the central domain are being predicted as R508-N511, Q533-Q542, P569-N570, V585-F594, G677-S678, and D710-G711, based on *in silico* modeling and site-directed mutagenesis studies. This study sheds important light on a current debate in epigenetics, where it has been suggested that HCS physically interacts with histone H2A, and that the subsequent biotinylation of histone H2A depends on the intermediate biotinyl-5'-AMP but not directly on HCS [18]. This could be mis-construed as a phenomenon where biotinylation of lysines in histones might occur whenever a free ϵ -amino group is available, rather than being linked to specific biological readouts. Non-specific biotinylation of lysine residues in histones is not consistent with the existing dogma in epigenetics that distinct histone marks typically have distinct biological readouts [2]. For example, evidence has been provided that biotinylation of K12 in histone H4 participates in the repression of transposable elements, whereas other histone biotinylation marks appear not to play a role in this phenomenon [5]. We believe that a very careful analysis of HCS-mediated biotinylation events is needed, and that Syn67 might prove a useful tool in such studies.

The present study already offered some useful insights into biotinylation of lysine residues by HCS. In the Syn67 polypeptide, a lysine residue resides in a patch of basic amino acids. Despite physically interacting with HCS, the lysine residue is not a target for biotinylation. Only after the amino acid sequence in Syn67 was altered by substituting two glycines for arginines to create a histone-like peptide, became Syn67 a target for biotinylation by HCS. We interpret this observation as HCS specifically biotinylating distinct lysine residues in histones (and carboxylases). The majority of the lysine residues in histones that are known to be targets for biotinylation reside in glycine-rich patches [38,39], suggesting that glycines create an environment favorable for lysine biotinylation. It is currently unknown whether this is due to the small size of glycines or to the absence of D- and L-isomers. The former decreases steric hindrance, the latter might create additional flexibility in the substrate molecule to fit into the active site of HCS. Please note that we did not formally exclude the possibility that HCS might also biotinylate some proteins other than histones and carboxylases.

We cannot formally exclude the possibility that the His-tag in rHCS might have influenced interactions with Syn67 in limited proteolysis assays and Syn67 biotinylation studies. Importantly, this is not the case in Y2H assays where untagged HCS, HCS domains, and HCS mutants were used. Moreover, in previous studies we demonstrated that HCS (and its microbial ortholog BirA) interacts physically with histone H3 to mediate biotinylation of lysines 9, 18, and 23 ([19,39], B. Bao, et al., submitted)). In these previous studies, HCS interacted strongly with histone H3, regardless of whether experiments were conducted using His-tagged, GST-tagged, GFP-tagged, or endogenous HCS. Given that both tagged and untagged HCS revealed an affinity of HCS for Syn67, we are confident that the reported interaction is real.

The biological importance of Syn67 is currently unknown. Syn67 was identified when screening a human liver cDNA library for HCS-binding proteins. A database search revealed no known biological functions for Syn67 or related proteins. Meanwhile, Syn67 might be a useful analytical tool in the following applications. First, the histone-like Syn67 peptide might prove a useful tool in the development of HCS assays, which currently depend on the

production of recombinant p67, followed by treatment with avidin beads to remove endogenously biotinylated p67 [19]. Second, site-directed mutagenesis of individual amino acid residues in Syn67, followed by Y2H assays, can generate rapid insights into which residues are important for binding to HCS. Third, Syn67-drug conjugates might prove useful tools to deliver drugs directly to HCS, e.g., to alter the epigenome in the cell nucleus.

The lack of a good template for modeling of the 3D structure of HCS causes some uncertainty regarding our Syn67 docking predictions. In this study, the 3D structure of BirA from *E. coli* was used as a template to model HCS. Both BirA and HCS share a high degree of functional similarity [19], the size of the two proteins and the amino acid sequence are considerably different. Importantly, BirA lacks major portions of the N-terminus of HCS [40], making it impossible to reliably predict the conformation of the HCS N-terminus. Currently, no X-ray crystallography data are available for HCS. In this study, we limited HCS modeling to the sequence spanning amino acids E393-D715 in order to maintain a high level of stringency.

Abbreviations used

Ade	adenine
Arg	arginine
Gal4AD	Gal4 Activation domain
Gal4BD	Gal4 DNA binding domain
HCS	holocarboxylase synthetase
His	histidine
Kan	kanamycin
Leu	leucine
Lys	lysine
PPI	protein–protein interactions
RMSD	root mean square deviation
Y2H	yeast-two-hybrid

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MEYPYDVPDYAHMAMEASEFHPSSGINAEWPLWPGGLVNFVPATAVTRLTQVNRSRKKECFRSPPPQ

Figure 1.

The Syn67 polypeptide contains one lysine residue (gray box), embedded in a patch of basic amino acids (underlined).



Figure 2. The N- and C-termini in human HCS interact with Syn67 in yeast-two-hybrid assays. N-terminal domain, central domain, linker domain, C-terminal domain, and full-length HCS were investigated. (A) Plate layout. (B) Growth of the yeast host strain AH109 transformed with Gal4BD-HCS domains and Gal4AD-Syn67 on SD/-Leu,-Trp, +Kan medium to confirm co-transformation with both plasmids. (C) Activation of reporter genes in AH109 yeast caused by HCS:Syn67 interactions.

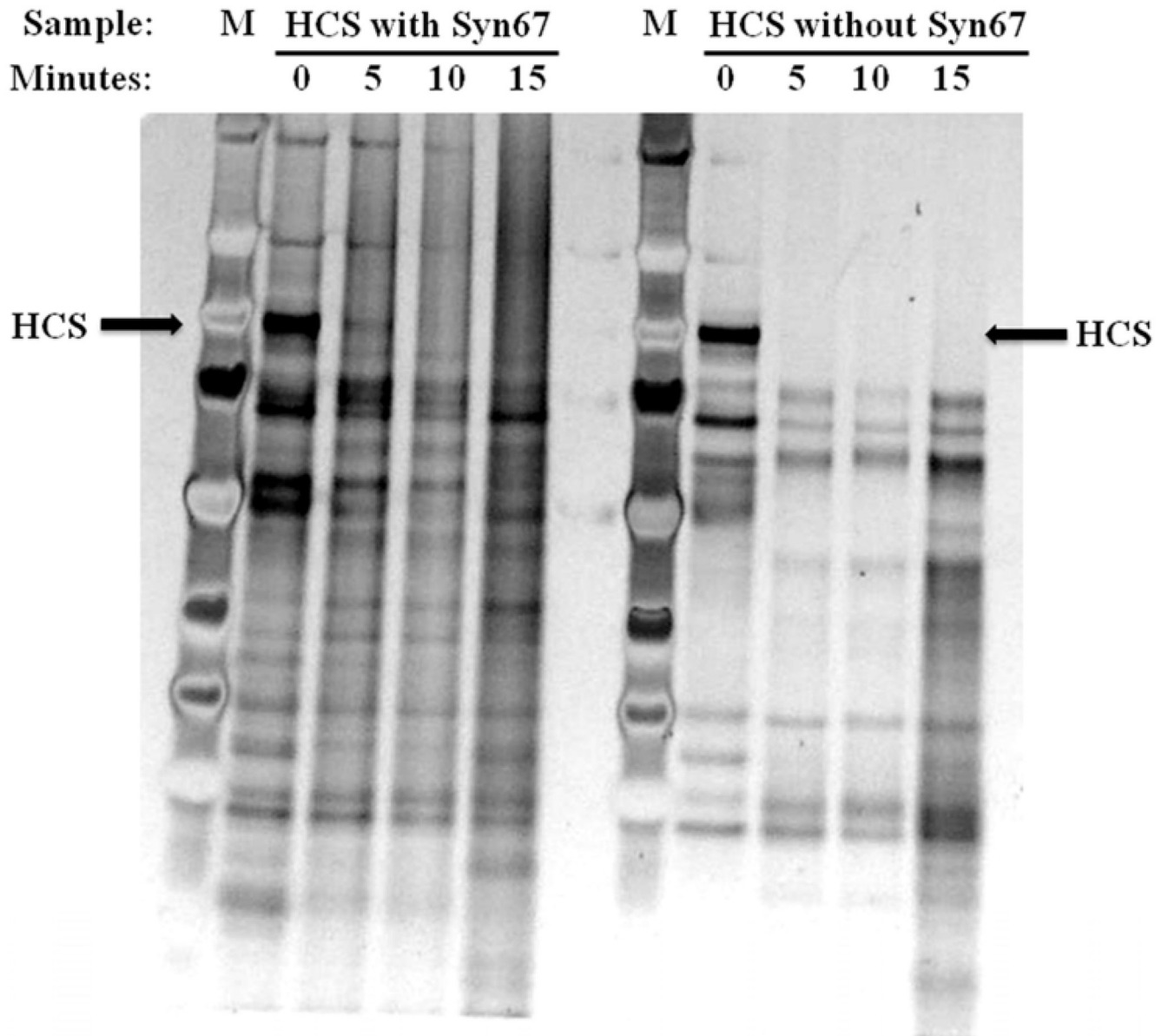


Figure 3.

Limited proteolysis assay of HCS. Recombinant HCS and Syn67 were pre-incubated to allow for interactions to occur, followed by tryptic digestion (left panel); controls were prepared by omitting Syn67 and also digested with trypsin (right panel). Aliquots were collected at timed intervals, resolved by gel electrophoresis, and stained with silver stain. M = molecular weight markers.

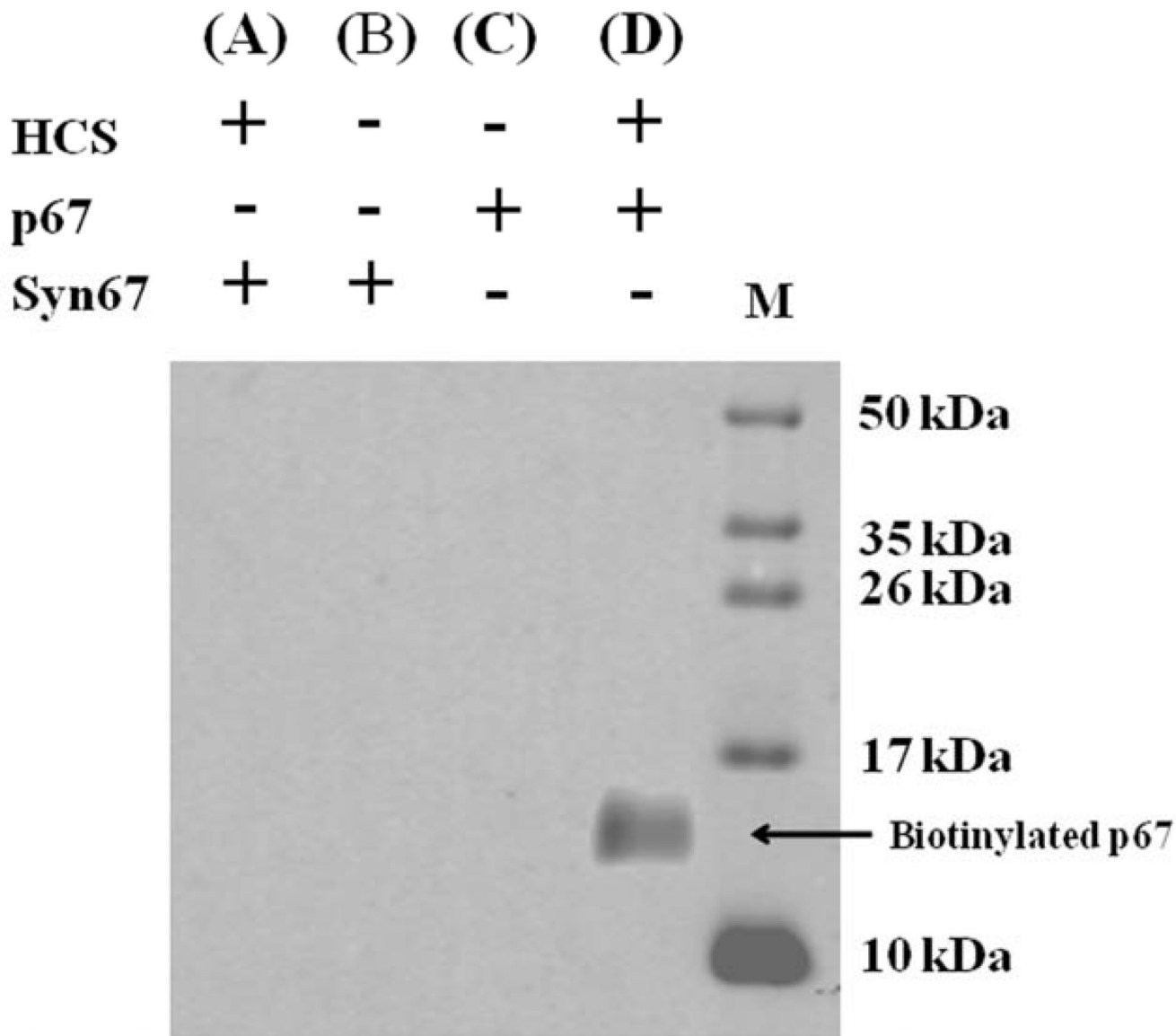


Figure 4.

Biotinylation of Syn67 and Syn67-like peptides by recombinant human HCS: (A) Syn67 is not a target for biotinylation by HCS; (B) Recombinant Syn67 does not contain endogenous biotin; (C) Recombinant p67 was biotin-depleted by using avidin beads and incubated in the absence of HCS (negative control); (D) Biotinylation of recombinant p67 by recombinant human HCS (positive control). M = molecular weight markers.

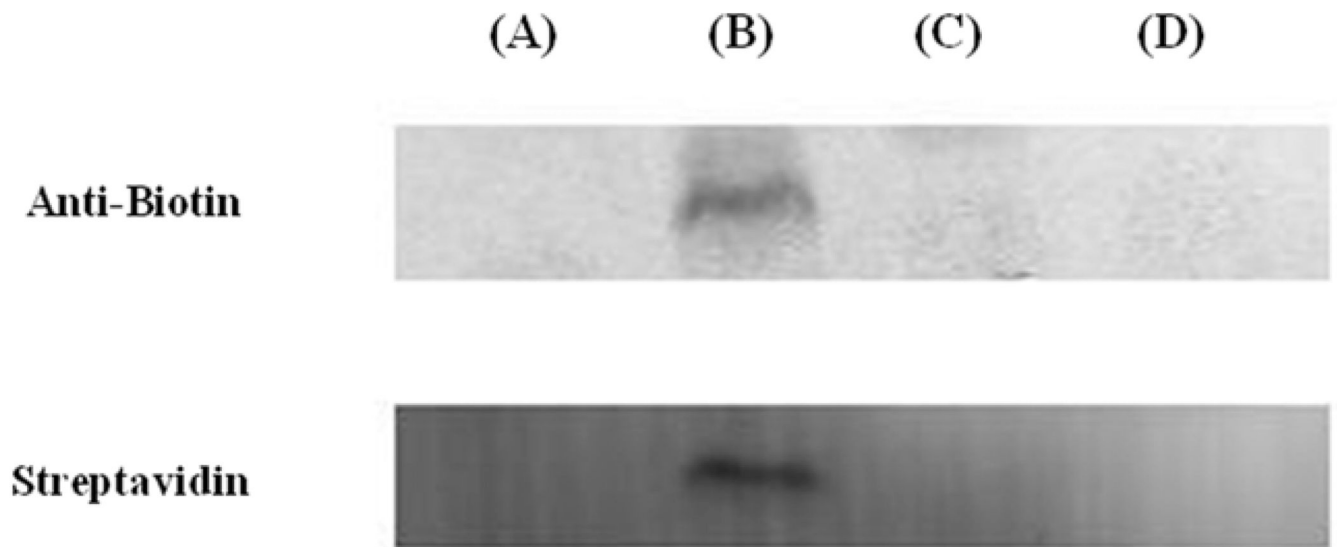


Figure 5.

A histone-like variant of Syn67 becomes a target for biotinylation by HCS. Synthetic peptides were incubated with recombinant human HCS and co-factors for biotinylation. Samples in the top panel were probed with anti-biotin; samples in the bottom panel were probed with streptavidin. (A) truncated variant of Syn67 (peptide NRSRRKECFRS); (B) histone-like variant of Syn67 (peptide NRSGGKECFRS); (C) negative control (peptide NRSRRAECFRS); and (D) p67-like variant of Syn67 (peptide NRSAMKMCFRS). The potential target site for biotinylation is shaded in gray; amino acid substitutions are underlined.

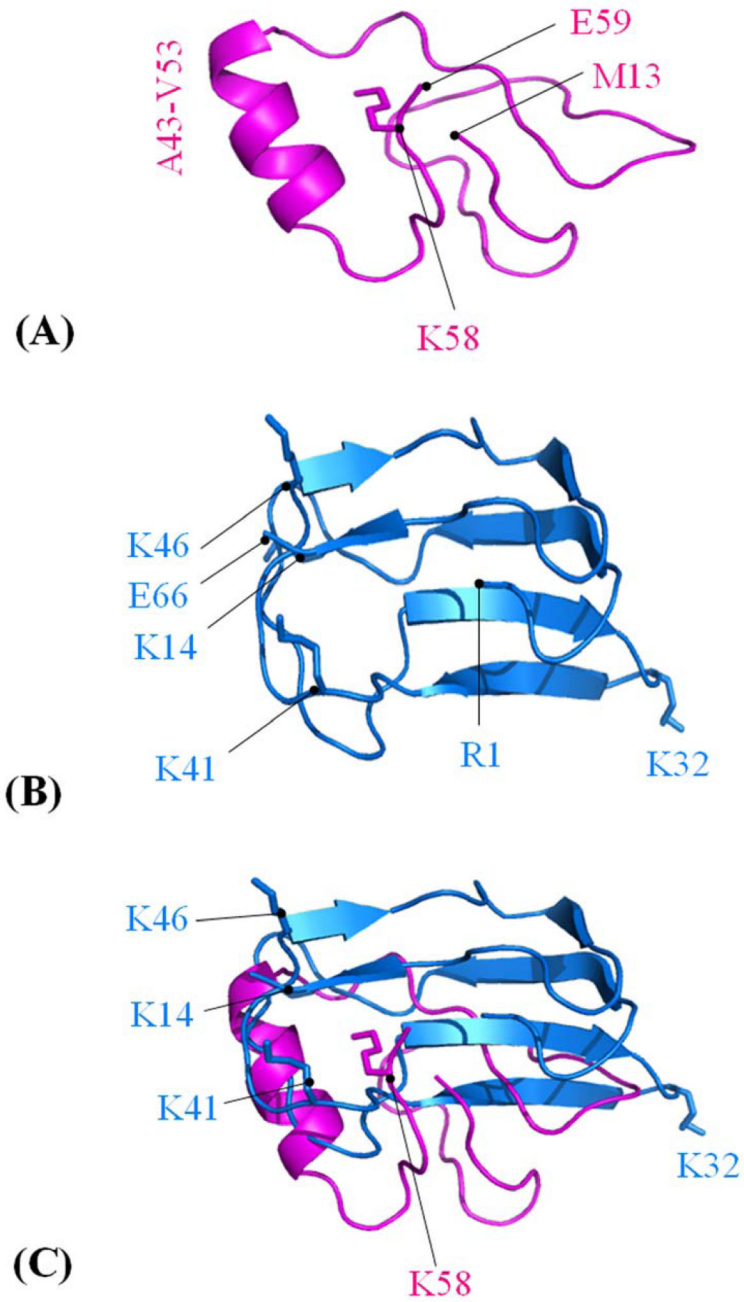


Figure 6. Prediction of three-dimensional structures of p67 and Syn67. (A) Syn67 (magenta); (B) p67 (blue); (C) superimposed Syn67 and p67.

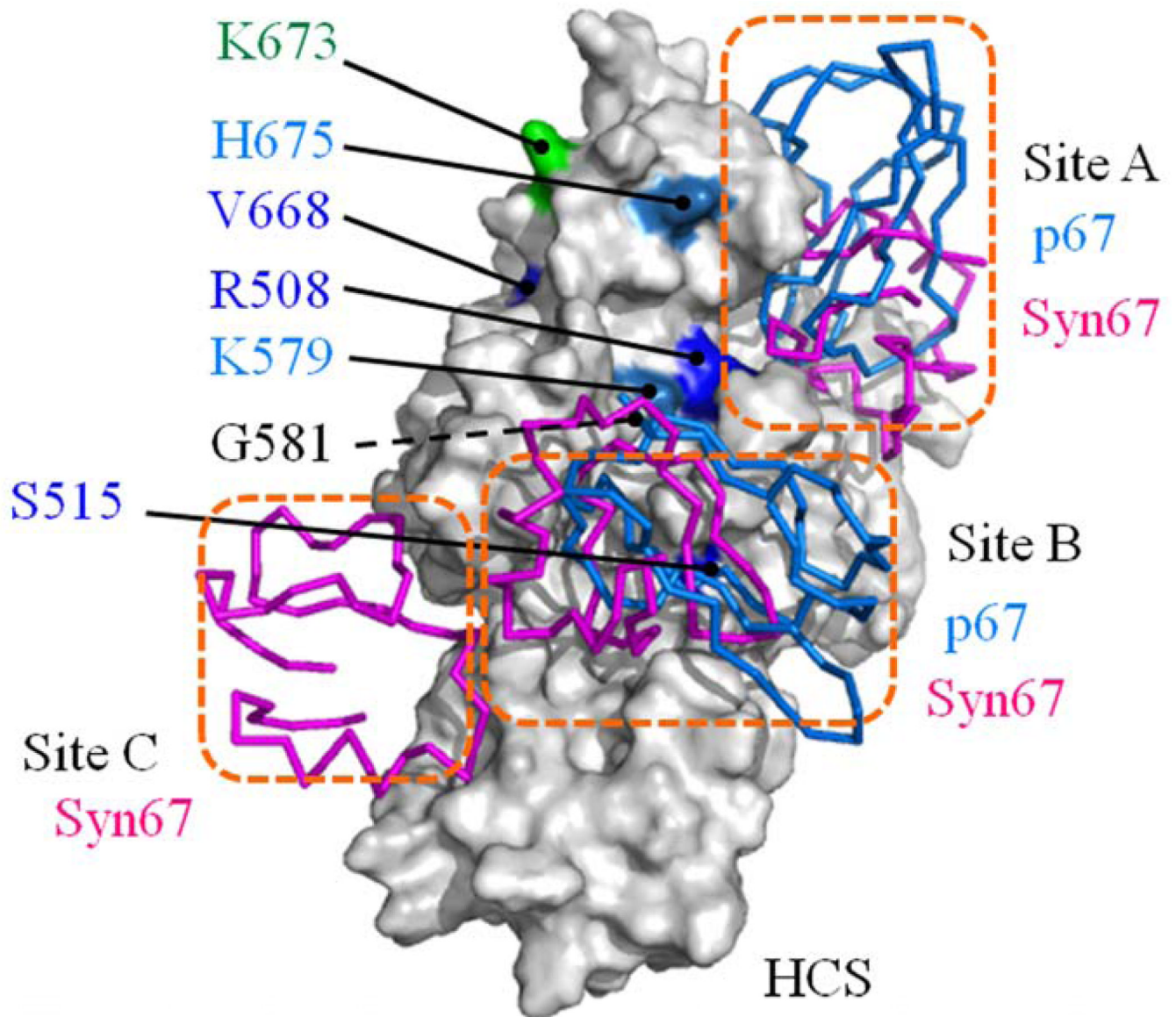


Figure 7. Prediction for p67 and Syn67 binding sites in HCS. HCS (white surface model) has two potential binding sites for p67 (blue; sites labeled A and B) and three potential binding sites for Syn67 (magenta; sites labeled A, B, and C). G581 is positioned in the back of the HCS/Syn67 complex and cannot be seen from this perspective. The approximate location of G581 in the back of the model is indicated by a dashed line. L237 is not shown because the depicted model is based on amino acid residues 373 – 715 in human HCS.

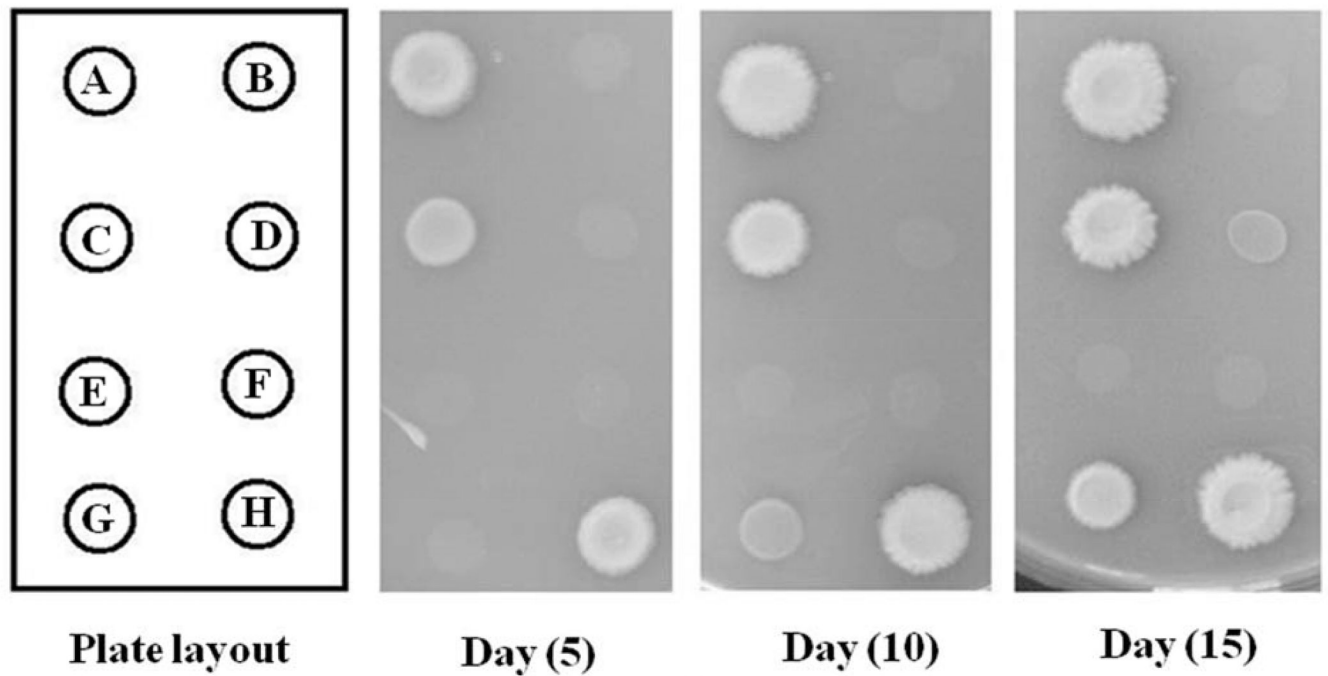


Figure 8. Effects of HCS truncations and mutations on interactions with Syn67. The yeast host strain AH109 was transformed with Gal4AD-Syn67, Gal4BD-HCS, or the following truncated or mutant variants of HCS, and grown on SD/-Leu,-Trp,-Ade,-His,+Kan medium: (A) wild-type HCS; (B) truncated HCS lacking the C-terminus; (C) HCS G581S mutant; (D) truncated HCS lacking the N-terminus; (E) HCS D571N mutant; (F) HCS R508 mutant; and (G) HCS L237P mutant. (H) Positive control (interaction of p53 with T-antigen).