Molecular cloning in yeast by in vivo homologous recombination of the yeast putative α1 subunit of the voltage-gated calcium channel

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Abstract ***Saccharomyces cerevisiae*** **has only one gene encoding a putative voltage-gated Ca^{2+} channel pore-forming subunit, *CCH1*, which is not possible to be cloned by conventional molecular cloning techniques using *Escherichia coli*. Here, we report the successful cloning of *CCH1* in yeast by in vivo homologous recombination without using *E. coli*. Overexpression of the cloned *CCH1* or *MID1* alone, which encodes a putative stretch-activated Ca^{2+} channel component, does not increase Ca^{2+} uptake activity, but co-overexpression results in a 2- to 3-fold increase. Overexpression of *CCH1* does not substantially complement the lethality and low Ca^{2+} uptake activity of a *mid1* mutant and vice versa. These results indicate that co-overproduction of Cch1 and Mid1 is sufficient to increase Ca^{2+} uptake activity.**

Keywords: Voltage-gated Ca^{2+} channel; Stretch-activated channel; Mechanosensitive channel; Homologous recombination; Yeast; *Saccharomyces cerevisiae*

1. Introduction

The *Saccharomyces cerevisiae CCH1* gene product, Cch1, has sequence similarity to the α1 pore-forming subunit of the dihydropyridine-sensitive (L-type) family of mammalian voltage-gated Ca^{2+} channels [1,2]. In contrast, the *MID1* gene product, Mid1, has no overall sequence similarity to known mammalian ion channels except for potential transmembrane segments [3], although it has stretch-activated Ca^{2+}-permeable channel activity when expressed in mammalian cells [4,5]. Genetic analyses showed that yeast cells lacking *CCH1* or *MID1* and those lacking both genes have the same phenotype, including low Ca^{2+} uptake activity and loss of viability after exposure to the mating pheromone α-factor [1,2]. It is hypothesized that the Cch1 and Mid1 proteins are necessary constituents of a common Ca^{2+} influx system [1,2,6–9], and this is supported by the observation that Mid1 co-immunoprecipitates with Cch1 [6]. However, it cannot be proven by gene disruption analysis that these two proteins are sufficient for the formation of the Ca^{2+} influx system. To overcome this obstacle, molecular cloning of the encoding genes and consequent in vivo or in vitro reconstitution experiments are essential. Unfortunately, the molecular cloning of *CCH1* is not possible using conventional techniques with *Escherichia coli*, because the *CCH1* sequence is harmful to this organism [2].

In *S. cerevisiae*, the ends of DNA molecules are extremely recombigenic [10], allowing for targeted recombination between two DNA molecules which have homologous sequences at their ends. Based upon this property, in vivo cloning techniques have been developed that co-transform a linearized vector with a gene of interest [11–13]. In the present study, we employed this technique to clone the *CCH1* gene in a *cch1* mutant and found that the cloned gene complements the mutation, thereby indicating that it is functional. A further study showed that overexpression of *CCH1* or *MID1* from a multicopy plasmid does not increase Ca^{2+} uptake activity, but co-overexpression induces a 2- to 3-fold increase in growing and mating pheromone-treated cells, suggesting that overproduction of Cch1 and Mid1 is sufficient to increase Ca^{2+} uptake activity. The co-overexpression system is useful for functional analysis of animal, plant and fungal Ca^{2+}-permeable channel candidates and their regulators.

2. Materials and methods

2.1. Yeast strains and media

The yeast genetic manipulation and recombinant DNA techniques were previously described [14,15]. *E. coli* strain DH5α was used (Toyobo Co., Osaka). The isogenic wild-type yeast strains used were H207 (*MAT***a** *his3*-Δ1 *leu2-3,112 trp1-289 ura3-52 sst1-2*) and H208 (*MAT*α *his3*-Δ1 *leu2-3,112 trp1-289 ura3-52 sst1-2*) [3]. The following mutant strains are derivatives of H207: H311 (*MAT***a** *mid1*-Δ5::*HIS3*) [16], H313 (*MAT***a** *cch1*Δ::*TRP1*) and H315 (*MAT***a** *cch1*Δ::*TRP1 mid1*-Δ5::*HIS3*). To construct H312, H313 and H315, the *CCH1*-knockout plasmid pKC289 (a gift from K.W. Cunningham) [6] was cut with *Eco*RI and introduced into H208. Successful disruption of the *CCH1* gene was confirmed by PCR and tetrad analysis. The resulting strain, H314 (*MAT*α *cch1*Δ::*TRP1*), was crossed with H311 (*MAT***a** *mid1*-Δ5::*HIS3*), and the diploids formed were sporulated and dissected. A Trp^- His^+ segregant (MATα *mid1*-Δ5::*HIS3*) was designated

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H312, a Trp^+ His^- segregant (*MAT***a** *cch1*Δ::*TRP1*) was designated H313, and a Trp^+ His^+ segregant (*MAT***a** *cch1*Δ::*TRP1 mid1*Δ5::*HIS3*) was designated H315.

Rich medium YPD and synthetic media SD and SD.Ca100 were prepared as previously described [3]. The SD.Ca100 medium contained 100 μM $CaCl_2$, while SD medium contained 681 μM $CaCl_2$.

2.2. Plasmids

Yeast shuttle vectors YCplac33 [*CEN4 URA3*], YCplac111 [*CEN4 LEU2*], YEplac195 [2 μm *URA3*] and YEplac181 [2 μm *LEU2*] were gifts from Akio Sugino [17]. YCpMID1 [*CEN4 URA3 MID1*], YEpMID1 [2 μm *URA3 MID1*] and YEpMID1 [2 μm *LEU2 MID1*] were constructed by inserting the *MID1*-containing *Bam*HI–*Xho*I fragment of YCpMID1-23 [3] between the *Bam*HI and *Sal*I sites of YCplac33, YEplac195 and YEplac181, respectively. To construct YCp33Rec [*CEN4 URA3*] and YEp195Rec [2 μm *URA3*], the vectors used for in vivo homologous recombination, the Rec sequence, a 105 bp *Eco*RI-*Bam*HI fragment of pGADT7-Rec (BD Biosciences Clontech, Palo Alto, CA) was inserted between the *Eco*RI and *Bam*HI sites located in the multi-cloning site (MCS) of YCplac33 and YEplac195, respectively. The Rec sequence has a *Sma*I site 40 bp downstream of the *Eco*RI site.

2.3. Cloning of CCH1

The PCR primers used are listed in Table 1. To clone the *CCH1* gene by in vivo homologous recombination, it was amplified by PCR with LA Taq DNA polymerase (Takara Bio Inc., Otsu, Shiga) using primers No. 1 and No. 2 (Table 1), which possess a part of the *CCH1* non-coding region and vector sequence, and chromosomal DNA of the wild-type strain H207 as a template (see Fig. 1). The PCR products were mixed with linearized YCp33Rec or YEp195Rec, which had been cut with *Eco*RI and *Sma*I, and co-introduced into strain H313 (*cch1*Δ::*TRP1*). The resulting transformants were examined for the presence of the *CCH1* gene and flanking primer sequences by PCR analysis with Ex Taq DNA polymerase (Takara Bio Inc.), and sequence-positive transformants were further examined for viability 8 h after the addition of α-factor. If the construction of *CCH1*-carrying plasmids was successful, the transformants were viable like wild-type strains because of the presence of functional *CCH1*. Viable strains were selected and single colony isolation was repeated twice. The confirmed strains were designated H313/YCpCCH1Rec and H313/YEpCCH1Rec.

Table 1
PCR primers used in this study

No.	Primer	Sequence
1	CCH1-5'-500	5′-GGGTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTG
		CTGCAGTAGAATTGCATAAATTTCAGCTTGGGT-3′
2	CCH1-3'-142	5′-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA
		GCGCATTTTTTTCTTTTCTTTTTTCCC-3′
3	CCH1-5'-500F-Kpn	5′-GGGGTACCTAGAATTGCATAAATTTCAGCTTGGGT-3′
4	CCH1-5'R-Bam	5′-GGGGATCCAGTACTAACAATATATTCTATTTAGTAATG-3′
5	CCH1-3'F-Pst	5′-CCCTGCAG**TAA**ATTATATAAGGGCATATTTAAGAGG-3′
6	CCH1-3'-142R-Sph	5′-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3′
7	CCH1-5'F-Bam	5′-GGGATCC**ATG**CAGGGGAGAAAAAGGACGCT-3′
8	CCH1-5'-72R-Xho	5′-CCCTCGAGTGCTGCATTGTCCCCAAATGGA-3′
9	CCH1-3'-67F-Xho	5′-CCCTCGAGGATGGAGCCCCCGGAAGAAC-3′
10	CCH1-3'R-PstSal	5′-CCCTGCAGGTCGACTCTATCAATTAGATCATTTGCGCTATC-3′
11	GFP-5'F-Sal	5′-GGGTCGACAGTAAAGGAGAAGAACTT-3′
12	GFP-3'R-Pst	5′-CCCTGCAGGTCGATCGAGTTATTTGTATAGTTCATCCAT-3′

Underlines represent the sequences corresponding to each end of the linearized vector.
Double underlines represent 27-nucleotide sequences corresponding to 500–474 nucleotides upstream of the initiation codon of *CCH1* and to 116–142 nucleotides downstream of the stop codon, respectively.
Boxes indicate restriction sites used to construct the plasmids.
Bold triplets indicate the stop and initiation codons.

To construct wild-type strains carrying YEpCCH1Rec, H313/YEpCCH1Rec cells were mated with H208 (*MAT*α *CCH1*$^+$ *trp1-289 ura3-52*) cells, and the resulting diploids were sporulated and dissected. Trp^- Ura^+ segregants were assessed for the presence of the *CCH1* gene and flanking primer sequences by PCR analysis, and sequence-positive segregants were designated H207/YEpCCH1Rec. Likewise, to construct *mid1* mutants carrying YEpCCH1Rec, H313/YEpCCH1Rec cells were mated with H312 (*MAT*α *mid1*-Δ5::*HIS3 trp1-289 ura3-52*) cells, and Trp^- Ura^+ His^+ segregants were selected as described above and designated H311/YEpCCH1Rec.

2.4. Construction of Cch1-GFP and Cch1-HAx4

To construct plasmids carrying genes encoding Cch1 tagged with green fluorescence protein (GFP) or with four times-repeated hemagglutinin (HAx4) at the C-terminus of Cch1 by in vivo homologous recombination, we first constructed plasmids (named GFP-plus plasmids or HAx4-plus plasmids) that carried the 5′-non-coding (500 bp), 5′-coding (72 bp) and 3′-coding (67 bp) regions of *CCH1*, the tag (GFP or HAx4), and the 3′-non-coding region (142 bp) of *CCH1*, in which each tag was located just before the stop codon of *CCH1*. These regions were amplified by PCR with LA Taq DNA polymerase and the following sets of primers: for the amplification of the 5′-non-coding region, the primers No. 3 and No. 4 were used; for the 3′-non-coding region, No. 5 and No. 6; for the 5′-coding region, No. 7 and No. 8; and for the 3′-coding region, No. 9 and No. 10. As a template, DNA prepared from H313/YEpCCH1Rec was used. The resulting PCR products were cut with the appropriate restriction enzymes and inserted stepwise into YCplac111 and YEplac181. The GFP sequence amplified with primers No. 11 and No. 12 and the template plasmid pS65T-C1 (BD Biosciences Clontech) followed by digestion with *Sal*I and *Pst*I were inserted into the resulting plasmids cut with *Sal*I and *Pst*I to give the GFP-plus plasmids. Likewise, the HAx4 sequence obtained from pUSR-cof-HA1 [18] was inserted into the resulting plasmids to give the HAx4-plus plasmids. The GFP-plus and HAx4-plus plasmids were linearized with *Xho*I and introduced into *cch1*Δ cells with the *CCH1* sequence amplified by PCR using primers No. 7 and No. 10 and chromosomal DNA of the wild-type strain as a template. The resulting transformants were then examined by PCR and cell viability was assessed to determine whether it contained plasmids carrying the fusion genes encoding functional Cch1-GFP or Cch1-HAx4. Successful plasmids were designated YCpCCH1-GFP, YCpCCH1-HAx4, YEpCCH1-GFP and YEpCCH1-HAx4.

2.5. Fluorescence microscopy and immunoblotting

Fluorescence microscopy on cells expressing Cch1-GFP or Mid1-GFP was performed as described previously [16]. Immunoblot analysis was carried out according to the method described previously [3] except that SDS–PAGE samples were heated for 3 min at 70 °C. A mouse monoclonal antibody against the HA-tag (12CA5, Babco, Berkeley, CA) and affinity-purified rabbit antibodies against the C-terminal peptide of Mid1 [19] were used to detect Cch1-HAx4 and Mid1, respectively.

2.6. Determination of Ca^{2+} accumulation

Exponentially growing cells or those treated with 6 μM α-factor in SD.Ca100 medium were incubated with 185 kBq of $^{45}CaCl_2$ per ml (1.8 kBq/nmol). At appropriate intervals, samples were taken, filtered through Millipore filters (type HA; 0.45 μm) that had been presoaked in 5 mM $CaCl_2$ and washed five times with the same solution. The radioactivity retained on the filters was counted with a scintillation mixture, Ready Protein (Beckman Japan, Tokyo), in a liquid scintillation counter.

2.7. Determination of shmoos and viability

The methods used were previously described [3].

2.8. Statistical analysis

Results are shown as means ± S.D. from at least three independent experiments. Statistical significance was determined for Fig. 2A using a one-way ANOVA followed by the Sheffe's method of a multiple comparison test, with a maximum P value of <0.05 required for significance. We did not perform statistical analysis for other figures, such as Fig. 2B, Figs. 3 and 4, because the generations of each sample differ from strain to strain in terms of the horizontal axis of the figures.

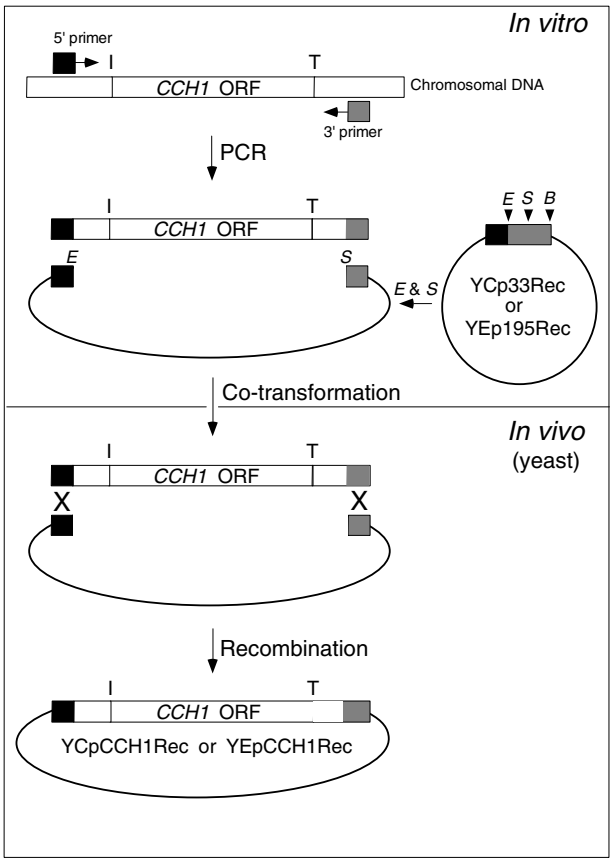

Fig. 1. Scheme of the cloning of the *CCH1* gene by in vivo homologous recombination in yeast. The *CCH1* gene was amplified by PCR using primers containing sequences homologous to a linearized plasmid at both ends. The PCR product was mixed with the linearized plasmid and co-transformed into yeast cells, in which homologous recombination occurred to produce a plasmid containing the *CCH1* gene. Abbreviations used: I, initiation codon; T, termination codon; ORF, open reading frame; *E*, *Eco*RI site; *S*, *Sma*I site; *B*, *Bam*HI site.

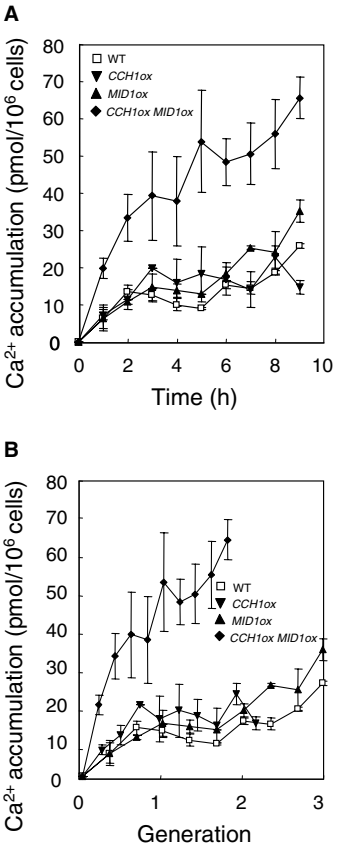

Fig. 2. Ca^{2+} accumulation of cells overexpressing *CCH1*, *MID1* or both in the exponentially growing phase. Exponentially growing cells of WT, *CCH1*ox, *MID1*ox, and *CCH1*ox *MID1*ox strains in SD.Ca100 medium received 185 kBq/ml (1.8 kBq/nmol) $^{45}CaCl_2$ at time 0 and were then examined for Ca^{2+} accumulation at the indicated times. Ca^{2+} accumulation is shown as a function of time (A) and generation (B) as the abscissa axis. Data are means ± S.D. from at least three independent experiments. In (A), statistical analysis showed that Ca^{2+} accumulation in *CCH1*ox *MID1*ox cells was significantly greater than that in WT, *CCH1*ox, and *MID1*ox cells ($P < 0.05$).

3. Results and discussion

3.1. Cloning of CCH1 by in vivo homologous recombination

Since several research groups including ours could not clone the *CCH1* gene by conventional cloning methods using *E. coli* as a host [2], we employed another method using in vivo homologous recombination in *S. cerevisiae*. The *CCH1*-open reading frame (ORF) with 5′-500 bp and 3′-142 bp flanking regions fused with sequences homologous to the ends of linearized vector YCp33Rec [*URA3*] (low-copy) and YEp195Rec [*URA3*] (multi-copy) was amplified by PCR using primers No. 1 and No. 2 listed in Table 1. The 5′-primer (No. 1) contained the distal 27 nucleotides of the 5′-flanking region and a part of the vector sequence, and the 3′-primer (No. 2) contained the distal 27 nucleotides of the 3′-flanking region and a part of the Rec sequence in the vectors. The PCR products and the linearized YCp33Rec or YEp195Rec were co-introduced into the *cch1* null mutant H313 (Fig. 1), and the resulting Ura^+ transformants were examined for the presence of the *CCH1* sequence by PCR analysis. Sequence-positive transformants were then examined to determine whether the cloned *CCH1* gene was able to complement the α-factor-induced death phenotype of the *cch1* mutant. To carry this out, six transformants containing YEp195Rec with *CCH1* were randomly selected and incubated for 8 h with α-factor, after which cell viability was determined. Their viability was 53–82%, whereas the viability of *cch1* mutant containing the empty vector YEp195Rec (H313/YEp195Rec) was 6.6 ± 3.9% ($n = 3$). This indicates that the *CCH1* gene cloned in this way was functional. Similar results were obtained for transformants containing YCp33Rec with *CCH1* (data not shown). We named the complementing plasmid YCpCCH1Rec or YEpCCH1Rec and H313 strains bearing this plasmid H313/YCpCCH1Rec or H313/YEpCCH1Rec. The viability of H313/YEpCCH1Rec (53–82%) was slightly lower than that of wild-type strain H207 bearing the empty vector (88%; $n = 3$). However, we believe that it was not due to the partial activity of the cloned *CCH1* gene carrying a putative loss-of-function mutation, because the viability of the *mid1* mutant bearing YEpMID1 or YCpMID1, which possess the *MID1* gene with no mutation, was also slightly lower than that of the wild-type strains. However, we cannot exclude this possibility, since DNA sequencing was not applicable for *CCH1* gene cloned in yeast cells. Therefore, we examined the YEpCCH1Rec plasmid that possessed the highest complementing activity hereafter.

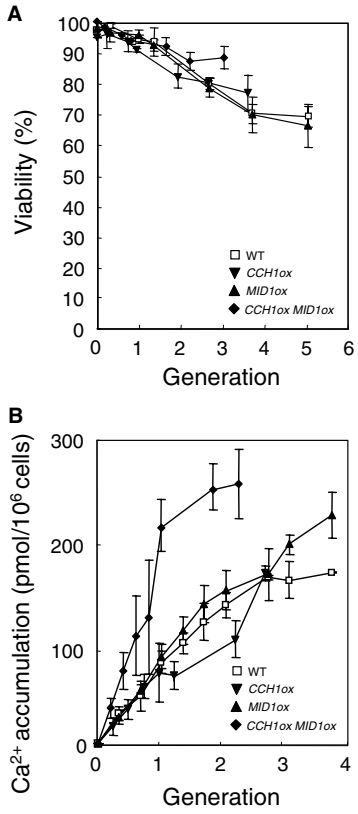

Fig. 3. Viability and Ca^{2+} accumulation of cells overexpressing *CCH1*, *MID1* or both after exposure to α-factor. (A) The viability of WT, *MID1*ox, *CCH1*ox *and CCH1*ox *MID1*ox cells was determined after the addition of 6 μM α-factor by the methylene blue liquid method [3]. Data are means ± S.D. from at least three independent experiments. (B) Ca^{2+} accumulation of each strain. Exponentially growing cells of each strain in SD.Ca100 medium received 6 μM α-factor and 185 kBq/ml (1.8 kBq/nmol) $^{45}CaCl_2$ at time 0. Data are means ± S.D. from at least three independent experiments.

It appeared that YEpCCH1Rec and YCpCCH1Rec did not replicate in *E. coli*. The plasmids were isolated from H313/YEpCCH1Rec and H313/YCpCCH1Rec strains and introduced into *E. coli* by conventional transformation techniques. However, no transformant was obtained.

To transfer YEpCCH1Rec to the wild-type strains, H313/YEpCCH1Rec was crossed with a *MAT*α wild-type strain, H208, and the resulting diploids were sporulated and dissected. From the segregants, *MAT***a** wild-type strains carrying YEpCCH1Rec, designated H207/YEpCCH1Rec, were selected and used in further experiments. Hereafter, we call cells of the H207/YEpCCH1Rec strain *CCH1*ox cells. Likewise, we call H207/YEpMID1 cells *MID1*ox cells, H207 cells bearing both YEpCCH1Rec and YEpMID1 *CCH1*ox *MID1*ox cells, and H207 cells bearing the corresponding empty vectors WT cells unless otherwise mentioned.

3.2. *CCH1-overexpression results in cell cycle retardation*

*CCH1*ox cells grew more slowly in liquid and solid media than WT cells. Therefore, we determined the mean doubling time (T_D) of cells grown in SD.Ca100 medium at 30 °C. The T_D of *CCH1*ox cells was 4.2 ± 0.2 h, whereas that of *MID1*ox and WT cells was 3.0 ± 0.2 h. The T_D of *CCH1*ox *MID1*ox cells was 5.0 ± 0.3 h. Therefore, overexpression of *CCH1*, but not *MID1*, retards cell cycle progression and co-overexpression of *CCH1* and *MID1* has an additional effect on cell cycle progression.

3.3. *Co-overexpression of CCH1 and MID1 increases Ca^{2+} uptake activity in growing cells*

We examined the effect of overexpression of *CCH1* on Ca^{2+} uptake activity. Cells of various strains were grown in SD.Ca100 medium to the exponentially growing phase and incubated further with $^{45}CaCl_2$, after which Ca^{2+} uptake activity was measured. Since T_D varies from strain to strain, the data obtained were plotted against generation time as well as incubation time (Fig. 2A and B). In all the strains examined, only *CCH1*ox *MID1*ox cells showed elevated activity for the initial Ca^{2+} uptake rate and longer term Ca^{2+} accumulation. The Ca^{2+} uptake activity of *CCH1*ox cells was unchanged, although cell cycle progression was retarded in this strain. These results suggest that a Ca^{2+} channel in growing cells requires the Cch1 and Mid1 proteins for their functionality. In addition, cell cycle retardation caused by overexpression of *CCH1* is not due to increased Ca^{2+} influx.

3.4. *Co-overexpression of CCH1 and MID1 increases Ca^{2+} uptake activity in α-factor-treated cells*

Previous studies with mutants lacking *CCH1*, *MID1* or both showed that Cch1 and Mid1 co-operate in common Ca^{2+} influx systems including an α-factor-induced Ca^{2+} influx system [1,2,7]. Therefore, we examined if overexpression of *CCH1* and *MID1* affects cell viability and Ca^{2+} uptake activity after cells were exposed to α-factor. Fig. 3A shows that the viability of cells exposed to α-factor was the same among *CCH1*ox, *MID1*ox, *CCH1*ox *MID1*ox, and WT cells, except that *CCH1*ox *MID1*ox cells maintained a slightly higher viability after three generations had passed. Fig. 3B shows that among the four strains examined, only *CCH1*ox *MID1*ox cells possessed enhanced activity in initial Ca^{2+} uptake rate and longer term Ca^{2+} accumulation. In contrast, *CCH1*ox cells did not exhibit increased activity. *MID1*ox cells possess no elevated activity [3]. It should be noted that overexpression of *CCH1*, *MID1* or both did not affect dose–response curves to α-factor and kinetics for differentiation into cells having a mating projection (called shmoos) in response to α-factor, respectively (data not shown). Therefore, the increase in the Ca^{2+} uptake activity of *CCH1*ox *MID1*ox cells is not due to changes in their sensitivity and responsiveness to α-factor.

3.5. *Overexpression of CCH1 does not complement the mid1Δ mutation*

Molecular cloning of *CCH1* on a multicopy plasmid in yeast allowed us to test whether *CCH1* can complement phenotypes of the *mid1*Δ mutant when overexpressed. Exponentially growing cells of H311/YEpCCH1Rec (*mid1*Δ*CCH1*ox) were incubated with α-factor, and cell viability and Ca^{2+} uptake activity were determined at appropriate intervals. Fig. 4A shows that *mid1*Δ*CCH1*ox cells did not have a significantly higher viability than *mid1*Δ cells. In addition, Ca^{2+} uptake activity was the same between *mid1*Δ*CCH1*ox and *mid1*Δ cells (Fig. 4B). These results indicate that overexpression of *CCH1* does not complement the *mid1*Δ mutation.

Fig. 4C shows that there was no significant difference in viability and Ca^{2+} uptake activity between *cch1*Δ*MID1*ox cells

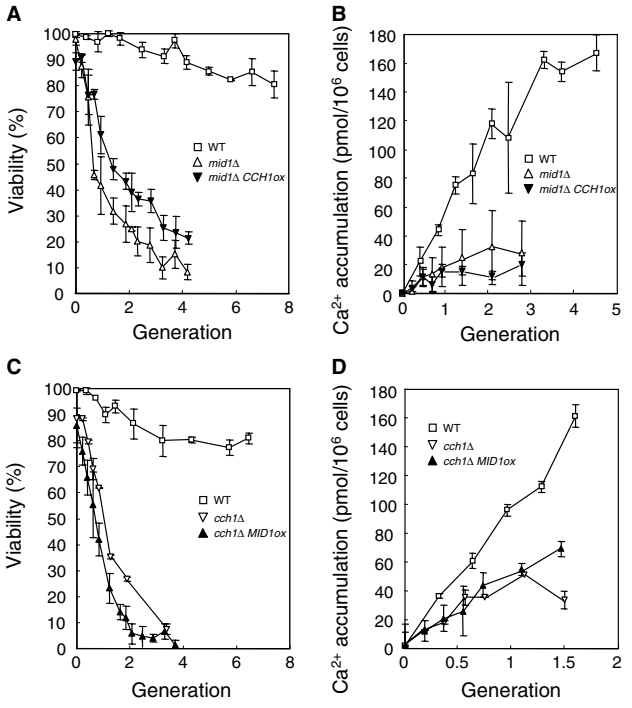

Fig. 4. Viability and Ca^{2+} accumulation of *mid1*Δ cells overexpressing *CCH1 and cch1*Δ cells overexpressing *MID1*. (A and B) Viability and Ca^{2+} accumulation of WT, *mid1*Δ and *mid1*Δ *CCH1*ox cells. Each strain was the *mid1*Δ mutant H311 harboring YCpMID1 (low-copy), empty vector YEplac195Rec or YEpCCH1Rec (multi-copy), respectively. The generation time of each strain is as follows, wild-type, 2.4 0.1 h; *mid1*Δ, 4.4 ± 0.1 h; *mid1*Δ *CCH1*ox, 4.3 ± 0.3 h. (C and D) Viability and Ca^{2+} accumulation of WT, *cch1*Δ and *cch1*Δ *MID1*ox cells. Each strain was the *cch1*Δ mutant H313 harboring YCpCCH1Rec (low-copy), empty vector YEplac195 or YEpMID1 (multi-copy), respectively. The generation time of each strain is as follows, wild-type, 2.8 ± 0.1 h; *cch1*Δ, 4.8 ± 0.5 h; *cch1*Δ *MID1*ox, 4.9 ± 0.5 h. Experimental conditions were the same as those in Fig. 3. All data are means ± S.D. from at least three independent experiments.

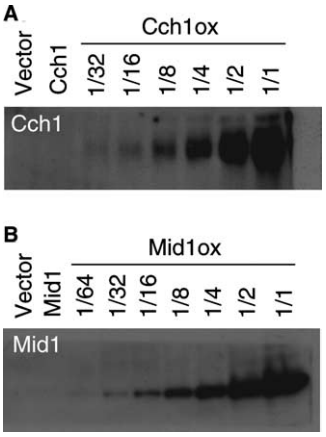

Fig. 5. Semi-quantitative estimation of the amount of overexpressed Cch1 and Mid1. (A) H313 (*cch1*Δ) cells harboring YEplac181 (vector), YCpCCH1-HAx4 (Cch1) or YEpCCH1-HAx4 (Cch1ox); (B) H311 (*mid1*Δ) cells harboring YEplac195 (vector), wild-type H207 cells harboring YEplac195 (Mid1), or H311 cells harboring YEpMID1 (Mid1ox). Each cell type was cultured to the exponentially growing phase and total cell extracts were prepared by homogenization with glass-beads. The extracts for Cch1ox or Mid1ox cells were serially diluted with the extracts of the corresponding vector-harboring cells. Samples (20 μg protein each) were subjected to 4–20% SDS–PAGE followed by immunoblotting with antibodies against the HA-tag or C-terminal peptide of Mid1.

and *cch1*Δ cells (Fig. 4C and D), indicating that *MID1* overexpression does not complement the *cch1*Δ mutation.

3.6. Semi-quantitative analysis of overexpressed Cch1 and Mid1 proteins

The copy number of multicopy plasmids is usually 10–40 per host cell [20]. Ca^{2+} uptake activity elevated by the co-overexpression of *CCH1* and *MID1* was only 2- to 3-fold, however (Figs. 2 and 3). To investigate the cause of this discrepancy, we performed an immunoblot analysis. The *CCH1-HAx4* gene encoding the Cch1 protein tagged with four hemagglutinin (HA) antigens was cloned into a low copy and multicopy plasmid in the *cch1*Δ mutant strain H313. The fusion gene complemented the lethality of the *cch1*Δ mutant upon exposure to α-factor (data not shown), indicating that the Cch1-HAx4 protein was functional. Protein samples were prepared, sequentially diluted, and subjected to immunoblotting. Fig. 5A shows that under conditions where non-diluted Cch1-HAx4 from the low copy plasmid was barely detectable, a 32-fold-diluted sample from the multicopy plasmid was still detectable. This indicates that overexpression of *CCH1* resulted in more than a 32-fold increase in protein content. As for Mid1, overexpression of *MID1* also resulted in an at least 32-fold increase compared to the endogenous content (Fig. 5B).

It is possible that excess Cch1 produced from the multicopy plasmid was mostly mislocalized. To investigate this possibility, the Cch1-GFP protein was produced from low copy and multicopy plasmids and subjected to fluorescence microscopy. Cch1-GFP produced from the low copy plasmid was barely observed (Fig. 6A), while that from the multicopy plasmid was easily seen in the plasma membrane (Fig. 6B). Similar fluorescent images were obtained with Mid1-GFP (Fig. 6E and F). It is noted that both Cch1-GFP and Mid1-GFP were also localized in an intracellular compartment. As for Mid1 and Mid1-GFP, this compartment is shown to be the endoplasmic reticulum (ER), and localization to the ER is not due to overexpression from multicopy plasmids [16,19].

These data suggest that the 2- to 3-fold-increase in Ca^{2+} uptake activity is not attributable to a small increase in Cch1 or Mid1 content or the mislocalization of Cch1 or Mid1. Why is the increase in Ca^{2+} accumulation only 2–3-fold in *CCH1ox MID1ox* cells irrespective of these facts? We suppose that it is due to the presence of a cellular factor responsible for the assembly or functional regulation of Cch1 and Mid1, and that this factor is limiting in *CCH1*ox *MID1*ox cells. Further studies are needed to identify such a factor. In the mating process, an α-factor-inducible, cycloheximide-sensitive factor has been suggested as a candidate [21].

It should be noted that measurements of Ca^{2+} accumulation, the method we employed, do not necessarily point to an increase in Ca^{2+} channel activity in *CCH1ox MID1ox* cells and may detect changes in Ca^{2+} transport through other means. However, considering that Cch1 is structurally homologous to mammalian voltage-gated Ca^{2+} channels [1,2] and Mid1 has stretch-activated Ca^{2+}-permeable channel

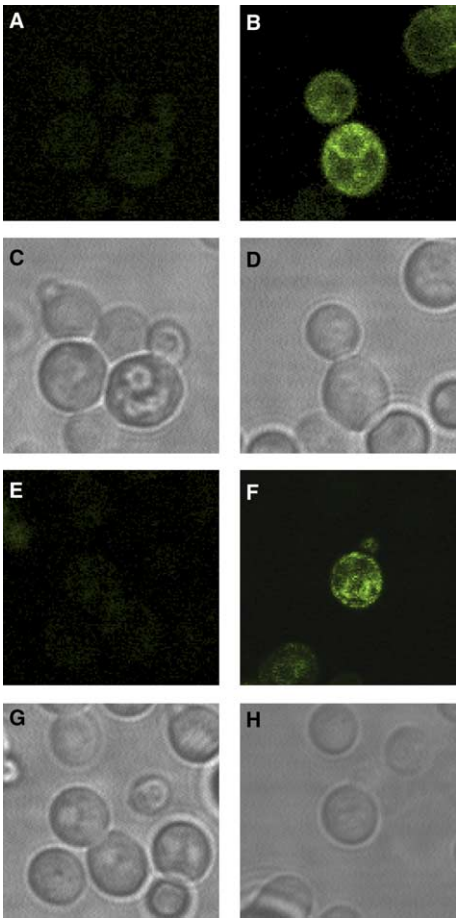

Fig. 6. Subcellular localization of Mid1 and Cch1 in exponentially growing cells. Exponentially growing cells of the *cch1*Δ mutant H313 harboring YCpCCH1-GFP (A, C) or YEpCCH1-GFP (B, D) and the *mid1*Δ mutant H311 harboring YCpMID1-GFP (E, G) or YEpMID1-GFP (F, H) in SD.Ca100 medium were observed by confocal fluorescence microscopy (A, B, E and F) or differential interference contrast microscopy (C, D, G and H). Similar results were obtained for two additional experiments.

activity [4,5], we believe that at least the increase in the initial Ca^{2+} accumulation rate reflects an increase in Ca^{2+} channel activity. Electrophysiological analysis is needed to clarify this matter.

If Cch1 acts as a voltage-gated Ca^{2+} channel, how is it activated in exponentially growing cells and those exposed to α-factor? We have no data to answer this question at present, but speculate that expansion of the plasma membrane accompanied with budding and mating projection formation might activate Mid1. The activated Mid1 might depolarize the plasma membrane to activate Cch1.

In summary, while previous studies with the *cch1*, *mid1* and *cch1 mid1* mutants have shown that Cch1 and Mid1 are necessary components for Ca^{2+} influx [1,2,7], our overexpression experiments with the cloned *CCH1* and *MID1* genes clearly indicate that they are sufficient components. The *CCH1*ox *MID1*ox strain may serve as a new tool to explore factors or subunits that upregulate or downregulate the Cch1/Mid1 channel.

Finally, we would like to highlight the usefulness of the present study. Many eukaryotic putative channels have been cloned but not functionally proven to be channels (for example, see [22]). The expression of such channels with and without Mid1 in the yeast strains described here may solve this problem.

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References

[1] Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G. and Sanders, D. (1997) FEBS Lett. 419, 259–262.
[2] Paidhungat, M. and Garrett, S. (1997) Mol. Cell. Biol. 17, 6339–6347.
[3] Iida, H., Nakamura, H., Ono, T., Okumura, M.S. and Anraku, Y. (1994) Mol. Cell. Biol. 14, 8259–8271.
[4] Kanzaki, M., Nagasawa, M., Kojima, I., Sato, C., Naruse, K., Sokabe, M. and Iida, H. (1999) Science 285, 882–886.
[5] Kanzaki, M., Nagasawa, M., Kojima, I., Sato, C., Naruse, K., Sokabe, M. and Iida, H. (2000) Science 288, 1347.
[6] Locke, E.G., Bonilla, M., Liang, L., Takita, Y. and Cunningham, K.W. (2000) Mol. Cell. Biol. 20, 6686–6694.
[7] Muller, E.M., Locke, E.G. and Cunningham, K.W. (2001) Genetics 159, 1527–1538.
[8] Bonilla, M., Nastase, K.K. and Cunningham, K.W. (2002) EMBO J. 21, 2343–2353.
[9] Matsumoto, T.K., Ellsmore, A.J., Cessna, S.G., Low, P.S., Pardo, J.M., Bressan, R.A. and Hasegawa, P.M. (2002) J. Biol. Chem. 277, 33075–33080.
[10] Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J. (1981) Proc. Natl. Acad. Sci. USA 78, 6354–6358.
[11] Ma, H., Kunes, S., Schatz, P.J. and Botstein, D. (1987) Gene 58, 201–216.
[12] Oldenburg, K.R., Vo, K.T., Michelis, S. and Paddon, C. (1997) Nucleic Acid Res. 25, 451–452.
[13] Takita, Y., Takahara, M., Nogami, S., Anraku, Y. and Ohya, Y. (1997) Yeast 13, 763–768.
[14] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY.
[15] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Short Protocols in Molecular Biology, 3rd edn. John Wiley & Sons, New York, NY.
[16] Tada, T., Ohmori, M. and Iida, H. (2003) J. Biol. Chem. 278, 9647–9654.
[17] Gietz, R.D. and Sugino, A. (1988) Gene 74, 527–534.
[18] Moriyama, K. and Yahara, I. (2002) J. Cell Sci. 115, 1591–1601.
[19] Yoshimura, H., tada, T. and Iida, H. (2004) Exp. Cell Res. 293, 185–195.
[20] Rose, M.D. and Broach, J.R. (1991) Methods Enzymol. 194, 195–230.
[21] Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 10482–10486.
[22] Lee, J.-H., Cribbs, L.L. and Perez-Reyes, E. (1999) FEBS Lett. 445, 231–236.