An internal promoter drives the expression of a truncated form of *CCC1* capable of protecting yeast from iron toxicity

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Supplementary material

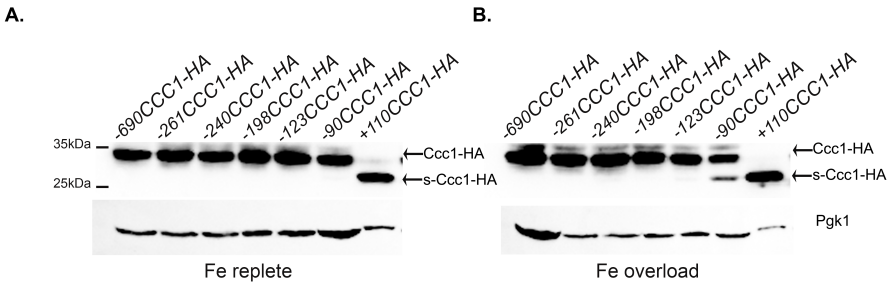

Figure S1. s-Ccc1 is repressed by elements present upstream -90 bp. *Δccc1* strain expressing the indicated constructs were grown to mid-exponential phase in the absence (A) or presence (B) of iron supplementation (2mM $FeSO_4$) and harvested. Total protein extracts were analyzed by immunoblotting using anti-HA antibody. Pgk1 was used as loading control.

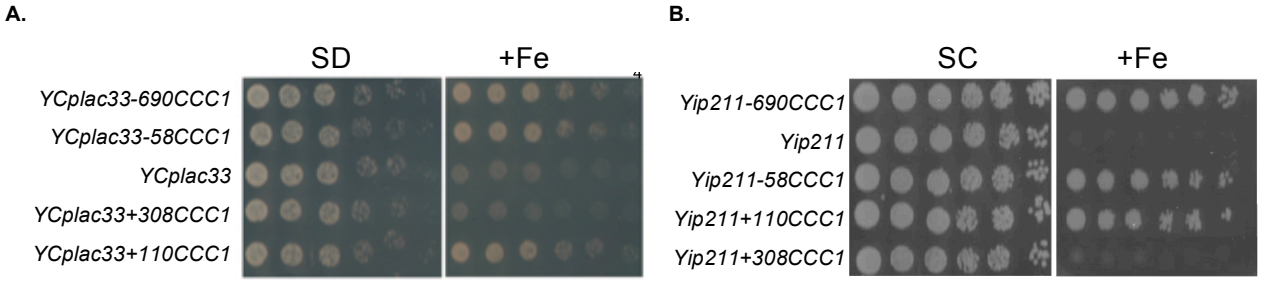

Figure S2. Removal of *CCC1* promoter and part of the gene coding sequence, still rescues Δ*ccc1* high iron–associated phenotype, independently of the vector used for cloning. The fragments *-690CCC1*, *-58CCC1*, *+110CCC1*, *+308CCC1* were cloned into (A) the centromeric vector YCplac33 or (B) the integrative vector YIplac211. The resulting constructs were used to transform a Δ*ccc1* strain. Exponentially growing cells were serially diluted and spotted onto control SC/SD plates or SC/SD plates supplemented with $FeSO_4$ (+Fe).

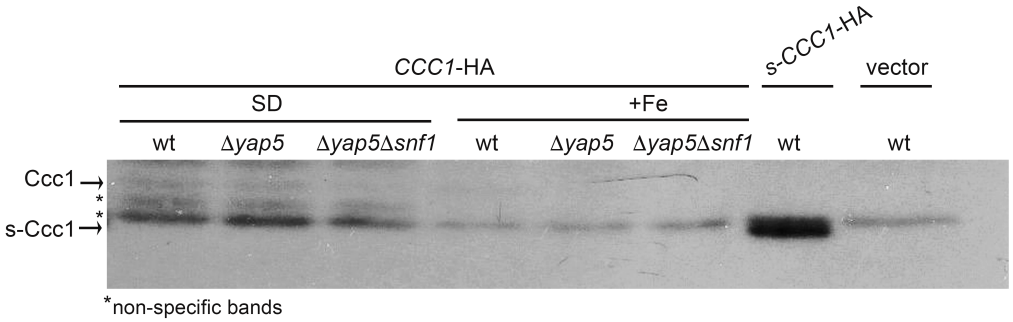

Figure S3. Deletion of *YAP5* and *SNF1* does not induce s-Ccc1 expression. Wild type, *Δyap5 and Δyap5Δsnf1* strains were transformed with either *CCC1*-HA (*-690CCC1-HA*), *s-CCC1*-HA (*+110CCC1-HA)* or the empty vector (pRS416) and left untreated (SD) or treated (+Fe) with 5mM $FeSO_4$ for 4h. Protein extracts were immunoblotted with an anti-HA antibody.

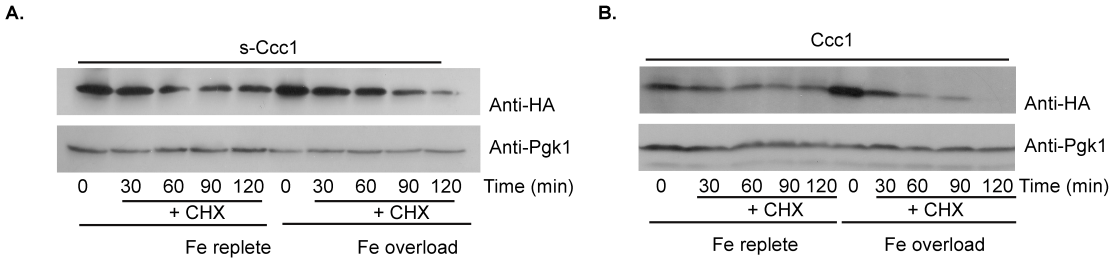

Figure S4. s-Ccc1 and Ccc1 stability. Ccc1-HA and s-Ccc1-HA protein stability was assessed by cycloheximide (CHX) chase assay. Δ*ccc1* expressing either the s-Ccc1 (A) or Ccc1 (B) (plasmids *+110CCC1-HA* and *-690CCC1-HA*, respectively) were grown to mid-exponential phase and left untreated or treated with 2 mM of $FeSO_4$ for 30 minutes. CHX was then added to a final concentration of 100 µg/mL. Cells were collected at the indicated time points and protein extracts were immunobloted using an anti-HA antibody. Pgk1 levels were used as a loading control.

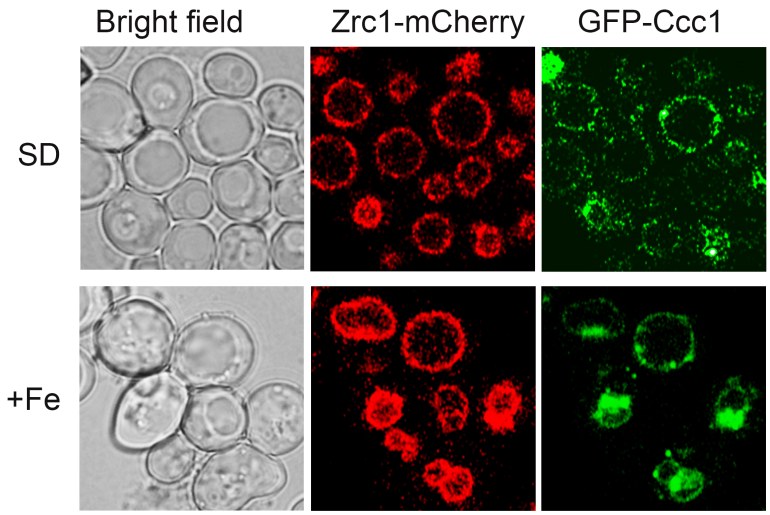

Figure S5. Ccc1 is located in the vacuole membranes. A strain with a genomic mCherry tagged copy of *ZRC1* was transformed with a plasmid encoding the fusion GFP- Ccc1. Cells were grown to mid-exponential phase and collected. Live cells were imaged by confocal microscopy under control conditions (SD) and iron overload conditions (+Fe).

Table S1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4742	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
Δ*ccc1*	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR220w::kanMX4*	EUROSCARF
ZRC1-mCherry	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR243c-mCherry his3*$^{+}$	This study
Ylp2111-690CCC1	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR220w::kanMX4 URA3::-690CCC1 ura3*$^{+}$	
Ylp2111-58CCC1	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR220w::kanMX4 URA3::-58CCC1 ura3*$^{+}$	This study
Ylp2111+110CCC1	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR220w::kanMX4 URA3::+110CCC1 ura3*$^{+}$	This study
Ylp2111+308CCC1	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR220w::kanMX4 URA3::+308CCC1 ura3*$^{+}$	This study
Δ*yap5*	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YIR018w::kanMX4*	EUROSCARF
Δ*snf1*	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDR477w::his3MX6*	This study
Δ*yap5*Δ*snf1*	MATα, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YIR018w::kanMX4 YDR477w::his3MX6*	This study

Table S2. Oligonucleotides used in the study.

Name	Sequence 5'- 3'	Use
CCC1_A1	TACAGGACCAAACCCCTCAG	*-690CCC1*
CCC1_A4	GGTTATCATGGATCACCTGA	Several *CCC1* constructs
pCCC1-240	CTTTGTTAGCCCGAATGTCTC	*-240CCC1*
pCCC1-198	GATTATATATGAAAAATTGATACAAAAC	*-198CCC1*
pCCC1-123	GTTAAAGCGACATCACTCTC	*-123CCC1*
pCCC1-114	GGTTTATAGAATAGAAATATAAC	*-114 CCC1*
pCCC1-90	GAACGAAGCCTTTAGACTCT	*-90CCC1* and *-90CCC1-HA*
pCCC1-58	TGGTATATTGAACAAAGAAA	*-58CCC1*, *-58CCC1*-HA and yEp356R-58
qCcc1_Fw	AACGCAGTGGTGACCCTTAT	qRT-PCR
qCcc1_Rev	TCCTGCGCTGAATTTCTACC	
CCC1_DM_HA_Fw	CTTTGTTAAGTTACTGGGTATGTACCCATACGATGTTCCAGATTACGCTTAAG TGTAAAGTAACAACAC	-690*CCC1-HA*
CCC1_DM_HA_Rv	GTGTTGTTACTTTACACTTAAGCGTAATCTGGAACATCGTATGGGTACATAC CCAGTAACTTAACAAAG	
EcoRI_pCCC1_Rv	ggGAATTCCCTCAACAAGGGAGTTGAT	yEp356R-58
IN_ORF_CCC1	ATGTCCATTGTAGCACTAAAGAA	yEp356R+1
IN_ORF2_CCC1	GTAGTAATAGCAATAGTTCAAG	*+110CCC1*, *+110CCC1-HA*
IN_ORF3_ CCC1	ATGTGATTATCGGGCTAAGC	*+308CCC1*
mATG_leu_2_Fw	AACTCACCGtTGTCAGTAGGG	*+110CCC1^{mut}-HA*
mATG_leu_2_Rv	CCCTACTGACAaCGGTGAGTT	
Frame_2_Fw	AATATTATGTCgCATTGTAGCACT	*-690CCC1^{frame}-HA* and *-90CCC1^{frame}-HA*
Frame_2_Rv	AGTGCTACAATGcGACATAATATT	
BamHI_mCherry_Fw2	ggCGGATCCCCGGGTTAATTAACAGTATGGTCAGCAAGGGAGAGGAAGATA A	pFA6a-mCherry-His3MX6
AscI_mCherry_2_Rv	ggcgcgccCTATTTGTATAATTCGTCCAT	
Zrc1_F2	gctgtaaactgcaatacttccaattgcctgCGGATCCCCGGGTTAATTAA	ZRC1 C-terminal mCherry tag K7
Zrc1_R1	agtatatagtttatgttctctgtagaaccatGAATTCGAGCTCGTTTAAAC	
CCC1_2ATG_Fw	ATGTCAGTAGGGAAAGATAATAGGA	GFP-s-*CCC1*
HA_ClaI_rv	ccacatcgatAGCGTAATCTGGAACATC	GFP-s-*CCC1*
T7_CCC1_Rv	TAATACGACTCACTATAGG CCAGTAACTTAACAAAGAACC	Northern blot
CCC1_F	AACGCAGTGGTGACCCTTAT	Northern blot probe
CCC1_R	ACCACCCACAACAACCATTT	Northern blot probe
Frame_2_Fw	AATATTATGTCgCATTGTAGCACT	*-690CCC1^{frame}-HA* and *-90CCC1^{frame}-HA*
Frame_2_Rv	AGTGCTACAATGcGACATAATATT	
pRS416_lacZ_Rv	GGGCTGCAGGAATTCGATATC	*-25CCC1-HA*, *+40CCC1-HA*, *+60CCC1-HA and +80CCC1-HA*
pRS416-25Fw	GAATTCCTGCAGCCC TCCCATATCTCGTGCACACAAATAT	*-25CCC1-HA*
pRS416+40Fw	GAATTCCTGCAGCCCTACAGAAAGCGAAAGGTAGTGGT	*+40CCC1-HA*
pRS416+60Fw	GAATTCCTGCAGCCCTGGTGGAACCTCAGAGTTGG	*+60CCC1-HA*
pRS416+80Fw	GAATTCCTGCAGCCCGGGGGTCTGAATCAACTCCC	*+80CCC1-HA*
SNF1_HIS_Fw	TTTGTAACAAGTTTTGCTACACTCCCTTAATAAAGTCAAC ATCGTACGCTGCAGG	Disruption of *SNF1*
SNF1_HIS_Rv	AACTTCCATATCATTCTTTTACGTTCCACCATCAATTGC TTAGGGAGACCGGCAGAT	

Table S3. Plasmids used and generated in this study.

Plasmid Name	Description	Reference
-690CCC1	PCR amplification from genomic DNA (BY4742, WT) using CCC1-A1 and CCC1-A4 primers and cloning into pRS416 (SmaI)	[1]
pRS416	*CEN, URA*	[2]
-273CCC1	*-690CCC1* digested with KpnI and cloned into pRS416 (KpnI, SmaI)	[1]
-240CCC1	PCR amplification of *-690CCC1* using CCC1-240 and -A4 primers and cloning into pRS416 (SmaI)	This study
-198CCC1	PCR amplification of *-690CCC1* using CCC1-198 and -A4 primers and cloning into pRS416 (SmaI)	This study
-114CCC1	PCR amplification of *-690CCC1* using CCC1-114 and -A4 primers and cloning into pRS416 (SmaI)	This study
-90CCC1	PCR amplification of *-690CCC1* using CCC1-90 and -A4 primers and cloning into pRS416 (SmaI)	This study
-58CCC1	PCR amplification of *-690CCC1* using CCC1-58 and -A4 primers and cloning into pRS416 (SmaI)	This study
-690CCC1-HA	*-690CCC1* with a HA epitope before the stop codon. *CCC1* was amplified using a three-step-PCR strategy as described previously [1] and cloned into pRS416 (SmaI)	This study
-90CCC1-HA	PCR amplification of *-690CCC1-HA* using CCC1-90 and CCC1-A4 primers and cloning into pRS416 (SmaI)	This study
-58CCC1-HA	PCR amplification of *-690CCC1-HA* using CCC1-58 and CCC1-A4 primers and cloning into pRS416 (SmaI)	This study
YEp356R-58	PCR amplification of *-690CCC1* using CCC1-58 and EcoRI_pCCC1_Rv and cloning in yEp356R (SmaI, EcoRI)	This study
YEp356R+1	PCR amplification of *-690CCC1* using IN_ORF_CCC1 and EcoRI_pCCC1_Rv and cloning in yEp356R (SmaI, EcoRI)	This study
YEp356R	*2μ, URA, lacZ*	[3]
Ylp211-690CCC1	PCR amplification of *-690CCC1* using CCC1-A1 and -A4 primers and cloning into Ylplac211 (SmaI)	
Ylp211-*58CCC1*	PCR amplification of *-690CCC1* using CCC1-58 and -A4 primers and cloning into Ylplac211 (SmaI)	This study
Ylp211+*110CCC1*	PCR amplification of *-690CCC1* using IN_ORF2_CCC1 and -A4 primers and cloning into Ylplac211 (SmaI)	This study
Ylp211+*308CCC1*	PCR amplification of *-690CCC1* using IN_ORF3_ CCC1and -A4 primers and cloning into Ylplac211 (SmaI)	This study
+110CCC1	PCR amplification of *-690CCC1* using IN_ORF2_CCC1 and -A4 primers and cloning into pRS416 (SmaI)	
+308CCC1	PCR amplification of *-690CCC1* using IN_ORF3_ CCC1and -A4 primers and cloning into pRS416 (SmaI)	
+110CCC1-HA	PCR amplification of *-690CCC1-HA* using IN_ORF2_CCC1 and -A4 primers and cloning into pRS416 (SmaI)	This study
+110CCC1^{mut}-HA	PCR site directed mutagenesis of *+110CCC1-HA* ATG using mATG_leu_2_Fw and mATG_leu_2_Rv	This study
-690CCC1^{frame}-HA	PCR site directed mutagenesis of *-690CCC1-HA* using Frame_2_Fw and Frame_2_Rv	This study
-90CCC1^{frame}-HA	PCR site directed mutagenesis of *-90CCC1-HA* using Frame_2_Fw and Frame_2_Rv	This study
-25CCC1-HA	PCR amplification of *-90CCC1-HA* using pRS416-25Fw and pRS416_lacZ_Rv followed by *in vivo* bacterial assembly	This study
+40CCC1-HA	PCR amplification of *-90CCC1-HA* using pRS416+40Fw and pRS416_lacZ_Rv followed by *in vivo* bacterial assembly	This study
+60CCC1-HA	PCR amplification of *-90CCC1-HA* using pRS416+60Fw and pRS416_lacZ_Rv followed by *in vivo* bacterial assembly	This study
+80CCC1-HA	PCR amplification of *-90CCC1-HA* using pRS416+80Fw and pRS416_lacZ_Rv followed by *in vivo* bacterial assembly	This study
pFA6a-mCherry-His3MX6	PCR amplified Cherry with BamHI_mCherry_Fw_2 and AscI_mCherry_2_Rv cloned in pFA6a-GFP(S65T)His3MX6 (AscI, BamHI)	This study
pFA6a-GFP(S65T)-His3MX6	*HIS3*, GFP	[4]
pUG36	*CEN, URA, pMET17, GFP-*	GenBank: AF298791.1
GFP-s-*CCC1*	PCR amplification of *CCC1-HA* using CCC1_2ATG_Fw and HA_ClaI_Rv primers and cloning into pUG36 (SmaI, ClaI), The plasmid is a fusion between GFP and +207 bp of *CCC1*.	This study
GFP-*CCC1*	PCR amplification of *CCC1-HA* using IN_ORF_1_CCC1 and The plasmid is a fusion between GFP and +1 bp of *CCC1* HA_ClaI_Rv primers and cloning into pUG36 (SmaI, ClaI)	This study
***-261CCC1*-HA**	PCR amplification of *-690CCC1*-HA using CCC1-261 and -A4 primers and cloning into pRS416 (SmaI)	This study
***-240CCC1*-HA**	PCR amplification of *-690CCC1*-HA using CCC1-240 and -A4 primers and cloning into pRS416 (SmaI)	This study
***-198CCC1*-HA**	PCR amplification of *-690CCC1*-HA using CCC1-198 and -A4 primers and cloning into pRS416 (SmaI)	This study
***-123CCC1*-HA**	PCR amplification of *-690CCC1*-HA using CCC1-123 and -A4 primers and cloning into pRS416 (SmaI)	This study

Table S4. Putative transcription factors binding sites located in the *CCC1* gene region spanning 273 bp upstream the AUG codon, according to YEASTRACT (http://www.yeastract.com).

Transcription Factor	Consensus[a]	Position[b]
Msn2, Msn4, Nrg1, Rph1	CCCCT	-270
Rtg1, Rtg3	GGTAC	-268
Gis1, Msn2, Msn4, Rph1, Com2, Usv1	AGGGG	-265
Swi4	CACGAAA	-259
Rtg1, Rtg3	GTCAC	-257
Fkh1, Fkh2	AAACA	-242
Tec1	GAATGT	-229
Stb5	CGGNS	-228
Rox1	ACAAT	-220
Pho2	WTAWTW	-199
Mot2	ATATA	-191,-189
Mot2	ATATA	-189
Pho2	WTAWTW	-155
Ash1	YTGAT	-151
Pho2	WTAWTW	-94
Fkh1,Fkh2p	RYMAAYA	-56
Mot2	ATATA	-52

[a]N- any base pair, S- C or G, W- A or T, Y- C or T

[b]bp upstream the ATG triplet encoding the first Ccc1 methionine

References

1. Pimentel, C., et al., *The role of the Yap5 transcription factor in remodeling gene expression in response to Fe bioavailability.* PLoS One, 2012. **7**(5): p. e37434.
2. Sikorski, R.S. and P. Hieter, *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* Genetics, 1989. **122**(1): p. 19-27.
3. Myers, A.M., et al., *Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions.* Gene, 1986. **45**(3): p. 299-310.
4. Longtine, M.S., et al., *Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.* Yeast, 1998. **14**(10): p. 953-61.