Supplemental information

Human CIA2A (FAM96A) and CIA2B (FAM96B) integrate maturation of different subsets of cytosolic-nuclear iron-sulfur proteins and iron homeostasis

Oliver Stehling[1,#], Judita Mascarenhas[1,#], Ajay A. Vashisht[2], Alex D. Sheftel[1,3], Brigitte Niggemeyer[1], Ralf Rösser[1], Antonio J. Pierik[1], James A. Wohlschlegel[2], and Roland Lill[1,4,5,*]

[1] Institut für Zytobiologie, Philipps-Universität Marburg, Robert-Koch-Str. 6, 35033 Marburg, Germany

[2] Department of Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

[3] Current address: University of Ottawa Heart Institute, 40 Ruskin St., Ottawa, ON K1Y 4W7, Canada

[4] Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

[5] LOEWE Zentrum für Synthetische Mikrobiologie SynMikro, Hans-Meerwein-Str., 35043 Marburg, Germany

[#] These authors contributed equally to this work

[*] Correspondence: Dr. Roland Lill, E-mail: lill@staff.uni-marburg.de
Phone: +49-6421-2866449, Fax: +49-6421-2866414

Running title:
CIA1, CIA2A and CIA2B mature diverse Fe/S proteins

Supplemental Data

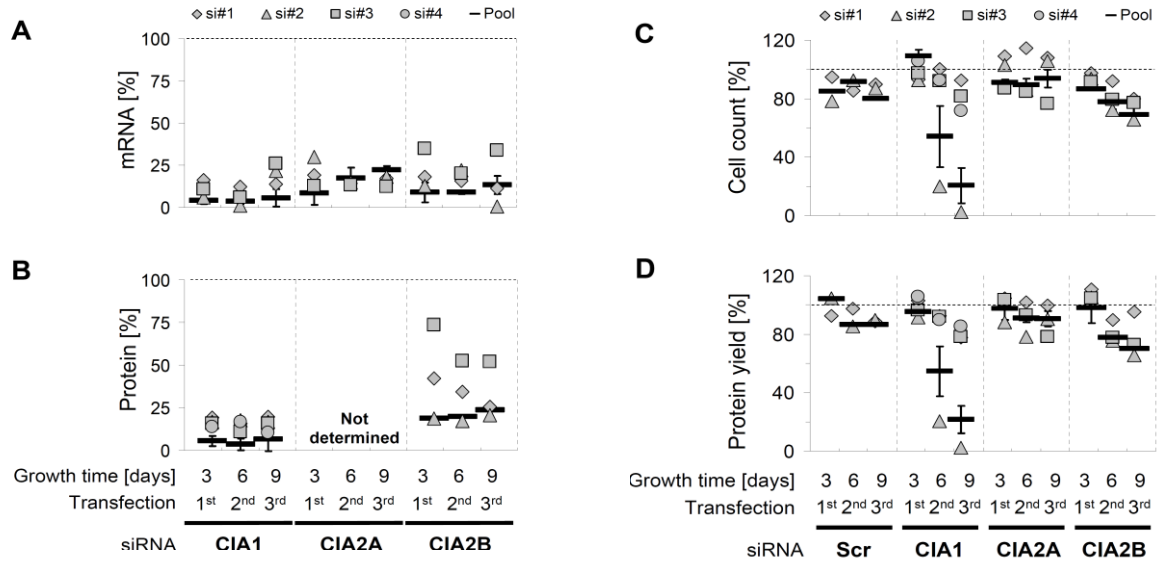

Figure S1, related to Figure 1. Efficiency of RNAi-mediated CIA2A, CIA1, and CIA2B depletion. HeLa cells were transfected three times (1st, 2nd, 3rd) at a 3-day interval with individual (grey symbols) or pooled (black bars; ± SD) siRNAs directed against CIA1, CIA2A, CIA2B, or with scrambled (Scr) siRNAs as indicated, or were subjected to mock-transfection. After a growth time of 3, 6, and 9 days, cells were harvested and analyzed. Residual mRNA **(A)** and protein **(B)** levels of depleted proteins were determined by qRT-PCR and immunoblotting and presented relative to β-actin mRNA and protein levels, respectively. At each harvest cumulative cell numbers **(C)** and cumulative protein yields **(D)** were determined. All values are normalized to mock-transfected cells (set to 100%; dashed line).

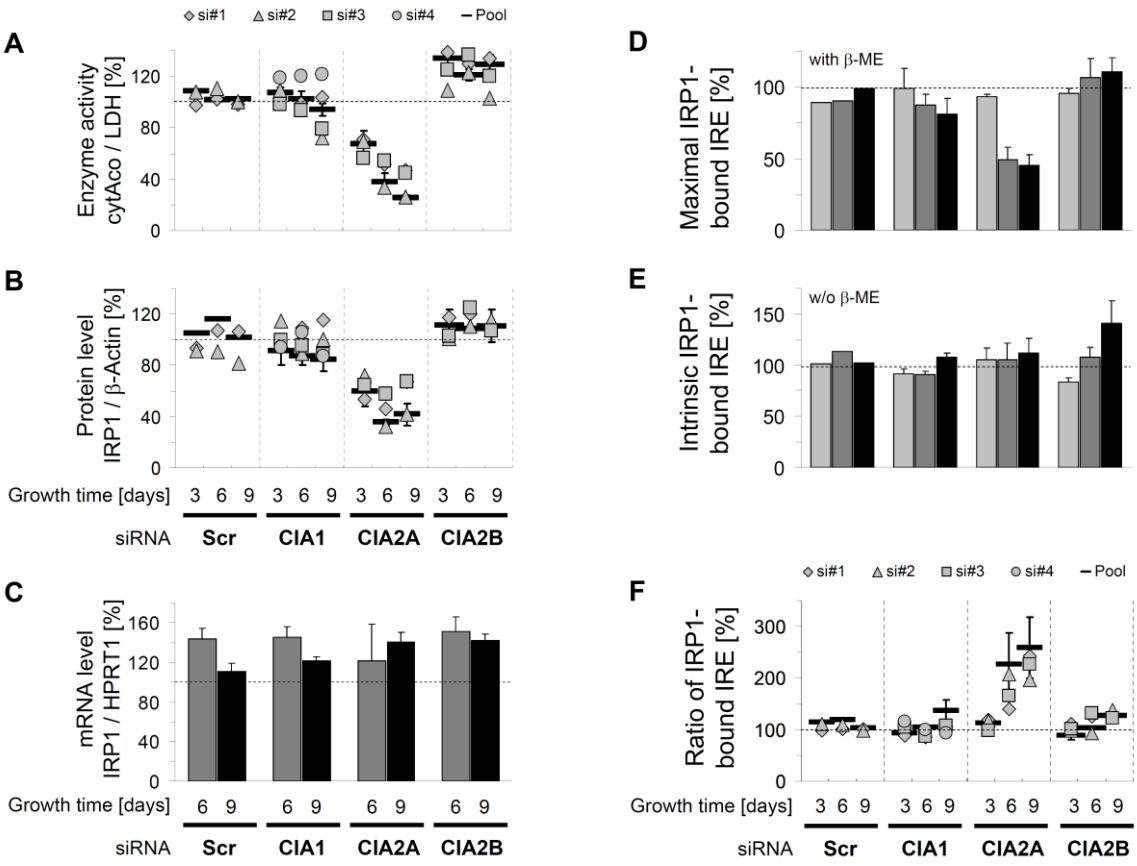

Figure S2, related to Figure 2. Depletion of CIA2A, but not of CIA2B or CIA1, impairs cytAco activity, steady-state levels, and IRE-binding capacity of the cytosolic Fe/S protein IRP1. HeLa cells were transfected with individual (grey symbols) or pooled (black bars; ± SD) siRNAs according to Fig. S1. Cells were harvested 3, 6, and 9 days after the first transfection (cf. Fig. 2). **A)** Cells were fractionated as in Fig. 1 and cytosolic aconitase activity of IRP1was determined relative to the activity of the cytosolic non-Fe/S cluster-containing enzyme lactate dehydrogenase (LDH). Ratios were normalized to mock-transfected cells. **B)** Samples of mock- and siRNA-transfected cells were subjected to immunoblotting. IRP1- and β-actin-associated chemiluminescence was quantified and the ratio normalized to mock-transfected cells. **C)** IRP1 and hypoxanthine phosphoribosyl transferase 1 (HPRT1) mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. **D-F)** IRP1 binding activity to ^{32}P-labeled IRE of human ferritin mRNA was analyzed by native gel electrophoresis and subsequent phosphorimaging according to Fig. 2C. IRE-binding capacity in the presence **(D)** or absence **(E)** of β-mercaptoethanol (β-ME) was calculated as the ratio of IRP1 binding activity **(F)** and normalized to mock-transfected cells. In parts A and B the IRP1 binding activities upon transfection with pooled siRNAs are shown. Values of mock-transfected cells were set to 100% (dashed line).

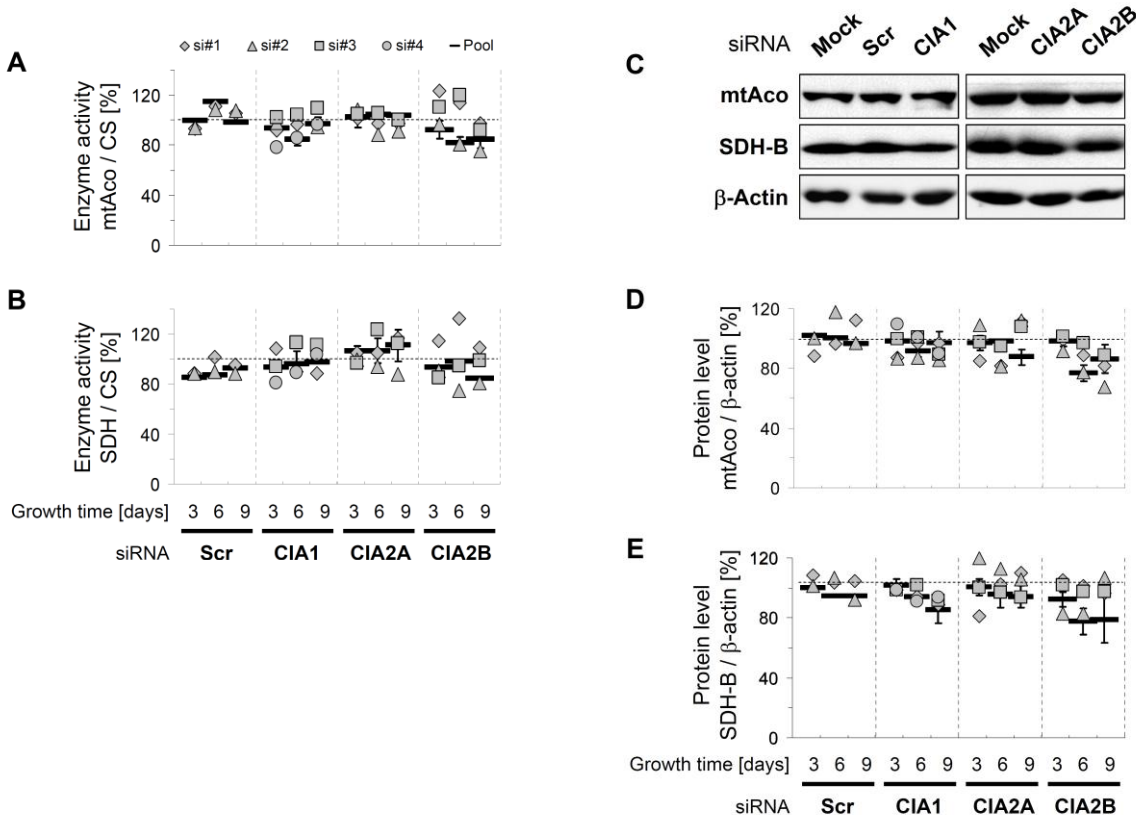

Figure S3, related to Figures 2 and 3. Depletion of CIA1, CIA2A, and CIA2B hardly affects specific activities and cellular steady-state levels of mitochondrial Fe/S proteins aconitase and succinate dehydrogenase. HeLa cells were transfected with individual (grey symbols) or pooled (black bars; ± SD) siRNAs and treated according to Fig. S2. **A-B)** Cells were fractionated as in Fig. 1, and the activities of the mitochondrial Fe/S enzymes aconitase (mtAco) (**A**) and succinate dehydrogenase (SDH) (**B**) were determined relative to the activity of the mitochondrial non-Fe/S cluster-containing enzyme citrate synthase (CS). Ratios were normalized to mock-transfected cells. **C-E)** Samples of mock- and siRNA-transfected cells were subjected to immunoblotting and stained for mtAco, the Fe/S subunit of respiratory complex II (SDH-B), and for β-actin. Representative immunoblots of samples prepared 9 days after the first transfection are shown in (**C**). mtAco- (**D**), SDH-B- (**E**) and β-actin-associated chemiluminescence was quantified and the ratio normalized to mock-transfected cells. Values of mock-transfected cells were set to 100% (dashed line).

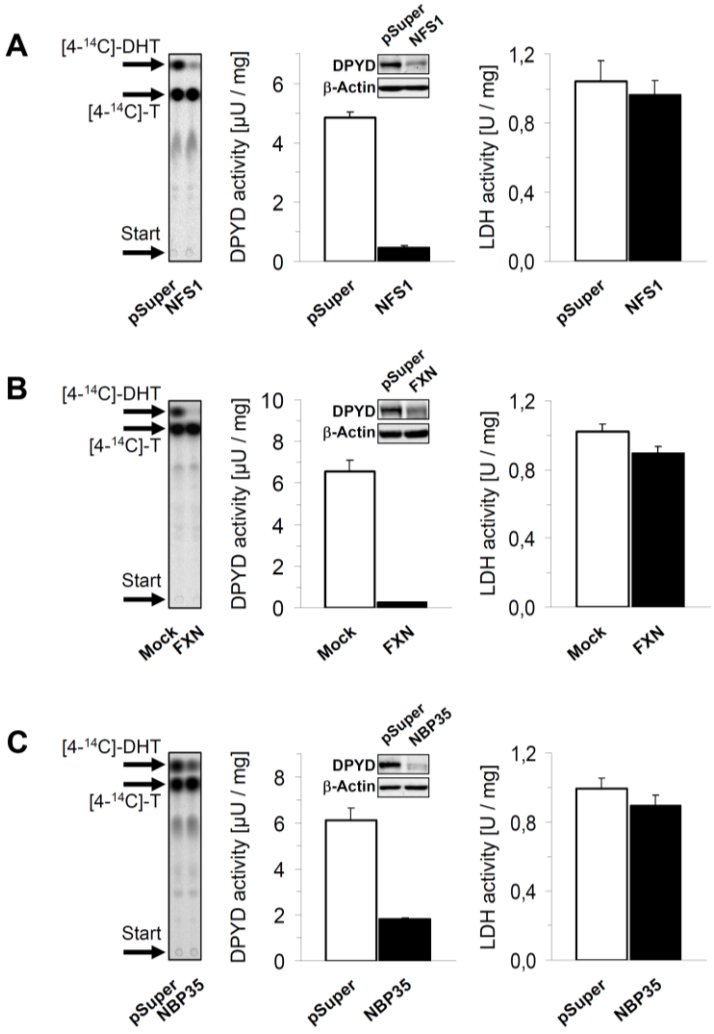

Figure S4, related to Figure 3A-C. Depletion of NFS1, FXN, or NBP35 impairs enzyme activity and levels of the cytosolic Fe/S protein DPYD. HeLa cells were depleted for the mitochondrial ISC assembly components NFS1 **(A)** and FXN **(B)** for 6 days, as well as for the CIA factor NBP35 **(C)** for 9 days by RNAi (Biederbick et al., 2006; Stehling et al., 2004; Stehling et al., 2008). Cells were analyzed for DPYD-dependent conversion of [4-^{14}C]-thymine ([4-^{14}C]-T) to [4-^{14}C]-dihydrothymine ([4-^{14}C]-DHT) by thin layer chromatography and autoradiography. Autoradiographs (left panels) show that the product of DPYD function, [4-^{14}C]-DHT, is less abundant (C) or virtually absent (A-B) when Fe/S protein assembly is impaired. Specific DPYD enzyme activity was calculated from the proportion of [4-^{14}C]-DHT formed from [4-^{14}C]-T (middle panels) and compared to specific LDH activity (right panels). Immunoblotting revealed that loss of DPYD activity is accompanied by a less severe decrease in DPYD protein (inserts in middle panel) which thus may not be causative but a consequence of the impaired enzyme activity. Representative experiments are shown. All values are given as mean ± SD.

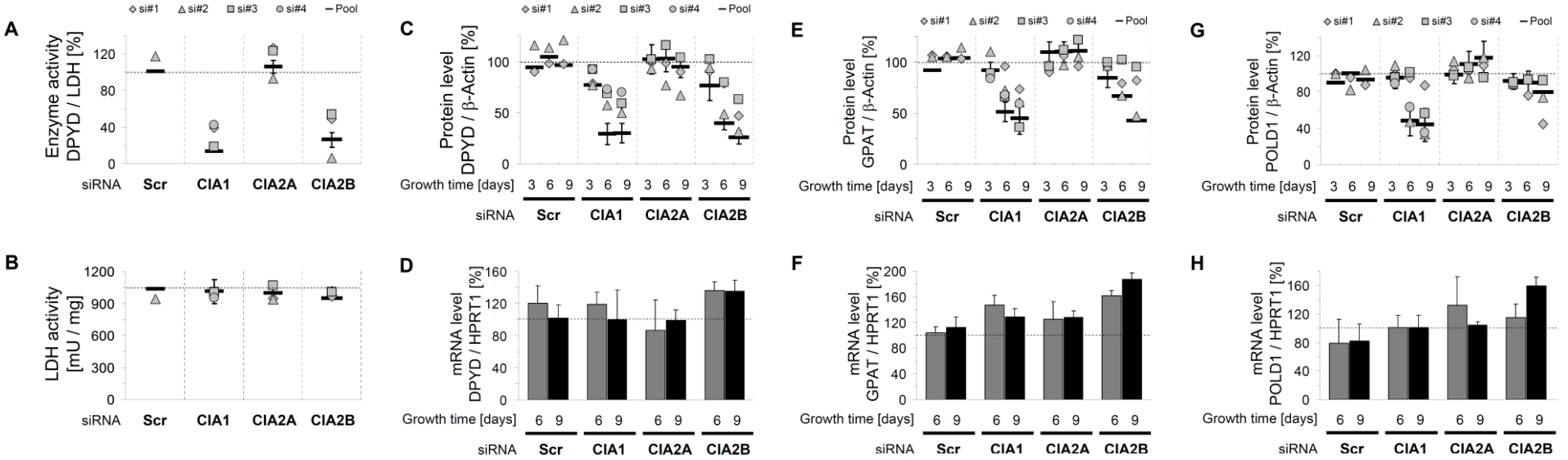

Figure S5, related to Figure 3. Depletion of CIA1 and CIA2B, but not of CIA2A, impairs maturation of the cytosolic Fe/S proteins DPYD, GPAT, and POLD1. HeLa cells were transfected with individual (grey symbols) or pooled (black bars; ± SD) siRNAs, treated according to Fig. S2, and analyzed for enzyme activity, steady-state protein levels, and mRNA levels of DPYD, GPAT, and POLD1. **A)** DPYD-dependent formation of [4-^{14}C]-dihydrothymine ([4-^{14}C]-DHT) from [4-^{14}C]-thymine ([4-^{14}C]-T) was determined 9 days after the first transfection by thin layer chromatography and subsequent autoradiography. DPYD enzyme activity was calculated from the proportion of [4-^{14}C]-DHT formed from [4-^{14}C]-T, expressed relative to LDH activity, and normalized to mock-transfected cells. **B)** Specific LDH activity in HeLa cells depleted for CIA1, CIA2A, and CIA2B. **C)** DPYD- and β-actin protein levels were determined by immunoblotting and the ratios were normalized to mock-transfected cells. **D)** DPYD and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. **E)** GPAT- and β-actin protein levels were determined by immunoblotting and the ratios were normalized to mock-transfected cells. **F)** GPAT and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. **G)** POLD1- and β-actin protein levels were determined by immunoblotting and the ratios were normalized to mock-transfected cells. **H)** POLD1 and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. Values of mock-transfected cells were set to 100% (dashed line).

Figure S6, related to Figure 4.

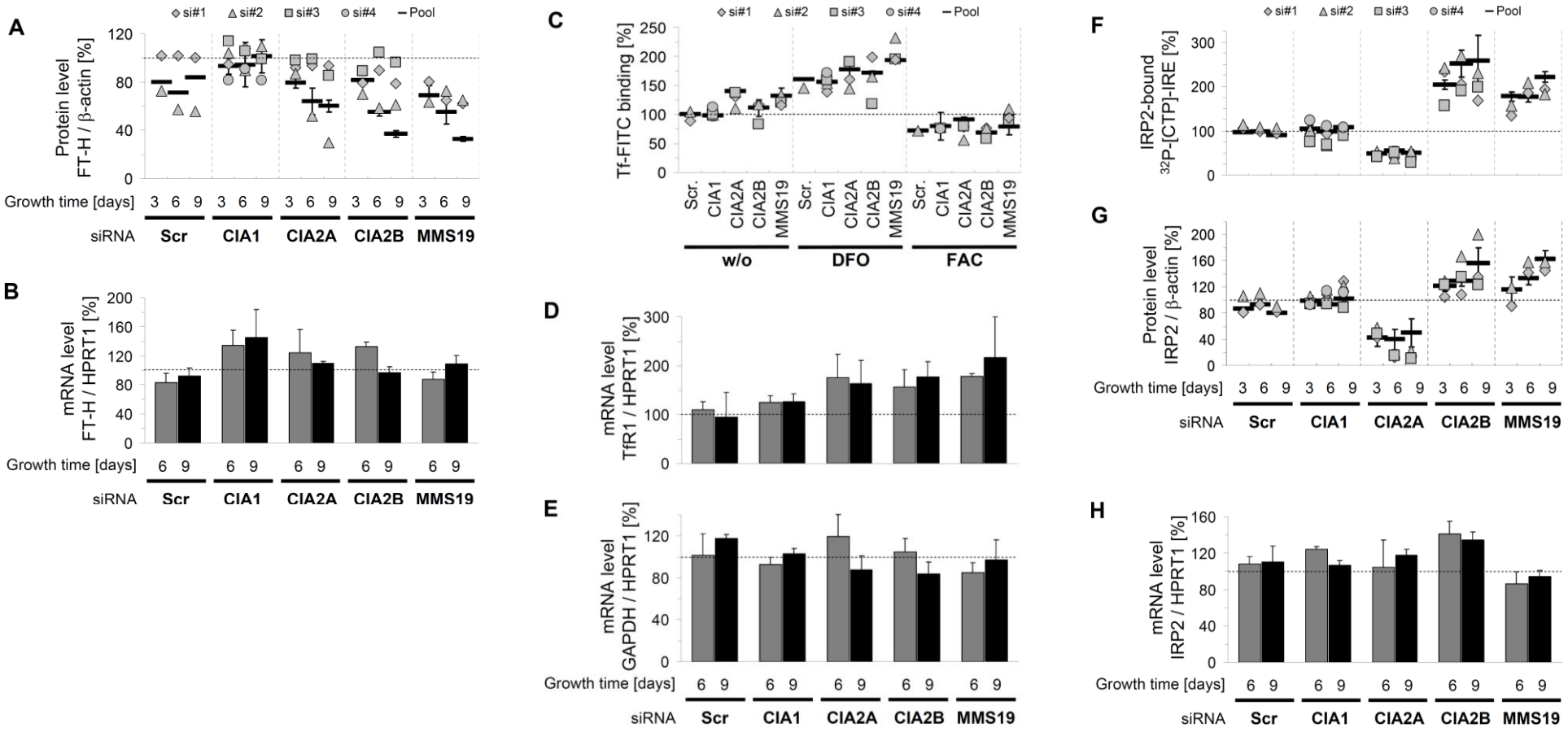

Figure S6, related to Figure 4. Depletion of CIA2A and of CIA2B-MMS19 interferes with cellular iron metabolism by conversely affecting IRE-binding activity of IRP2. HeLa cells were transfected with individual (grey symbols) or pooled (black bars; ± SD) siRNAs directed against CIA1, CIA2A, CIA2B, and MMS19, and treated according to Fig. S2. **A)** Steady-state protein levels of ferritin heavy chain (FT-H) and β-actin were determined by immunoblotting (cf. Fig. 4A) and the ratios were normalized to mock-transfected cells. **B)** FT-H and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. **C)** HeLa cells were analyzed for FITC-conjugated transferrin (Tf-FITC) binding as a measure of transferrin receptor (TfR) expression (cf. Fig. 4B). Directly after the third transfection varying numbers of HeLa cells were seeded into 48-well-plates. 24 h prior to analysis, cells were supplemented either with 125 µM deferoxamine (DFO), 125 µM ferric ammonium citrate (FAC), or remained without supplementation (w/o). On the next day cells were labeled with Tf-FITC, and analyzed for cell-associated fluorescence. Fluorescence intensity was corrected for cell density dependency and normalized to mock-transfected cells. **D-E)** TFR1, GAPDH, and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. **F)** IRE binding (IRP2 supershift method) was probed by a ^{32}P-labeled IRE of human ferritin mRNA according to Fig. 4C, and analyzed by native gel electrophoresis and subsequent phosphorimaging. ^{32}P-IRE-binding was normalized to mock-transfected cells. **G)** Steady-state protein levels of IRP2 were determined by immunoblotting. IRP2- and β-actin-associated chemiluminescence was quantified and the ratio normalized to mock-transfected cells. **H)** IRP2 and HPRT1 mRNA levels were determined by qRT-PCR and ratios were normalized to mock-transfected cells. Values of mock-transfected cells were set to 100% (dashed line).

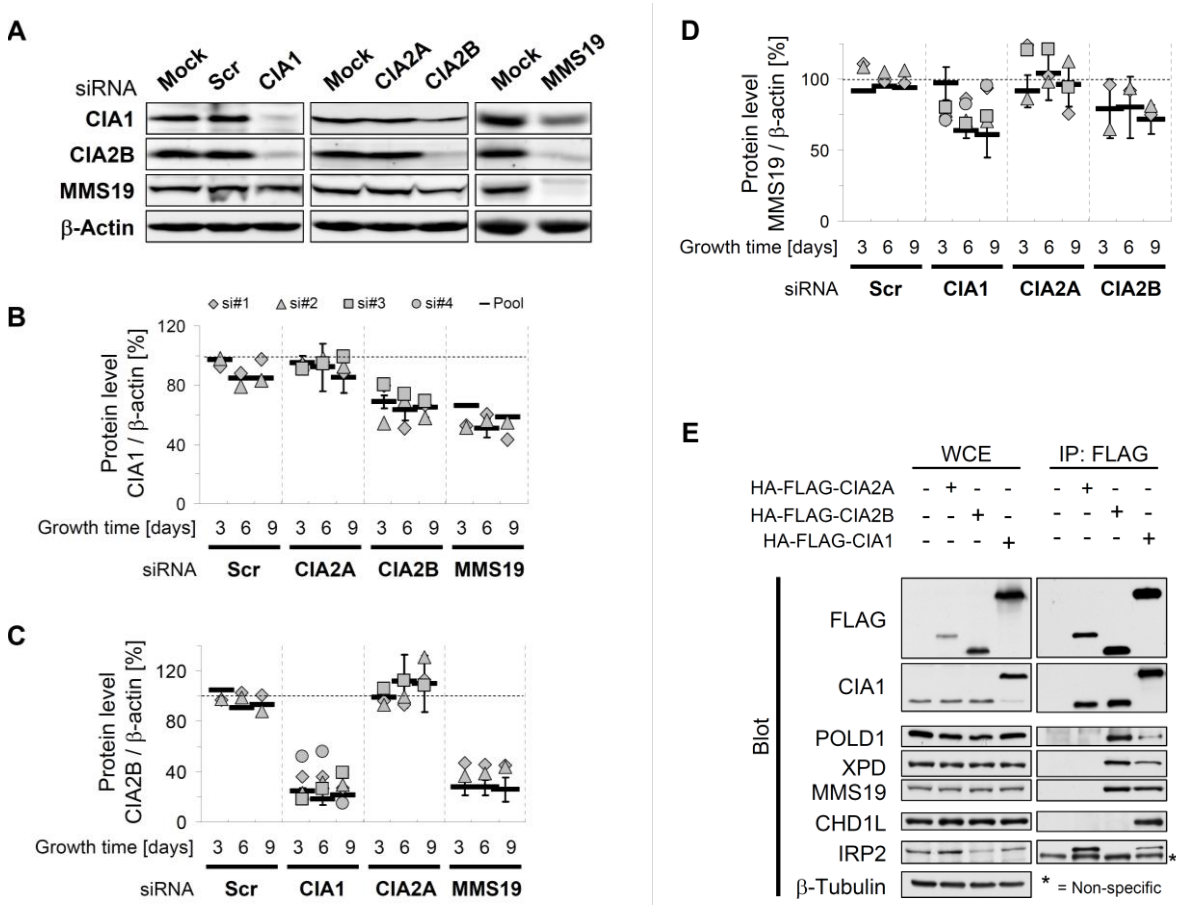

Figure S7, related to Figure 5 and Table S4. CIA1, CIA2B, and MMS19 are dependent on each other and form distinct CIA targeting complexes to allow for the interaction with different Fe/S target proteins. A-D) HeLa cells were transfected with individual (grey symbols) or pooled (black bars; ± SD) siRNAs and grown according to Fig. S1 and S6. Cells were analyzed for CIA1, CIA2B, MMS19, and β-actin steady-state protein levels by immunoblotting. Representative immunoblots of samples prepared 9 days after the first transfection are shown **(A)**. CIA1- **(B)**, CIA2B- **(C)**, and MMS19- **(D)** associated chemiluminescence was quantified, calculated as ratio to the β-actin signal, and normalized to mock-transfected cells (set to 100%; dashed line). All values are given as mean ± SD. **(E)** Flp-In™ TREx™-293 cells and cells stably expressing inducible 3xHA-3xFLAG-CIA2A, -CIA2B, and -CIA1 were induced with doxycycline (500 ng/mL) overnight. Cell lysates were prepared and used for immunoprecipitation with anti-FLAG antibodies. Whole cell extracts (WCE) and immunoprecipitates (IP: FLAG) were immunostained with specified antibodies (IB).

Supplemental Experimental Procedures

Vectors and siRNAs. In order to add a C-terminal myc-tag to murine CIA2A (for nomenclature see Table S1) the open reading frame (ORF) was cloned into the mammalian expression vector pEF1/myc-His A (Invitrogen) via *Eco*RI and *Xho*I / *Psp*XI restriction sites. An N-terminal EGFP-tag was added by cloning the *Eco*RI / *Xho*I fragment into pEGFP-N3 (Clontech). CIA2B was fused to a C-terminal myc-tag by amplifying its ORF using the primer pair 5'-GGTACCGCCATGGTAGGCGGCGGC-3' / 5'-ATCTCGAGGGAGCGGGCTGAC AGGCACT-3' and cloning the PCR product into the pEF1/myc-His A vector via *Kpn*I and *Xho*I / *Psp*XI restriction sites. An N-terminal EGFP-tag was added by cloning an *Eco*RI / *Xho*I fragment into pEGFP-C1 (Clontech) via the *Eco*RI / *Sal*I restriction sites.

For mass spectrometric studies human CIA2A, CIA2B, and CIA1 ORFs were amplified using Phusion Taq polymerase (NEB) with primers containing *Att*B sites, and subcloned into pDONR221 vector using the Gateway cloning system (Invitrogen). The Gateway cloning system was also used to transfer genes into Gateway-adapted expression plasmids that encode 3xHA-3xFLAG N-terminal tags (pcDNA5-FRT-TO-3xHA-3xFLAG).

Vectors encoding shRNAs directed against mRNAs of human *NFS1* and *NBP35* were described previously (Biederbick et al., 2006; Stehling et al., 2008). Small interfering RNAs (siRNAs) against *FXN* (frataxin) mRNA were purchased from Ambion. Sequences are listed in Table S2. SiRNAs against MMS19 have been described previously (Stehling et al., 2012).

Antibodies. MMS19 antiserum was obtained from ProteinTech Group (Manchester, UK) or raised in the Lill laboratory (Stehling et al., 2012); rabbit anti-mitochondrial aconitase was a kind gift from L. Szweda, USA; affinity-purified rabbit anti-murine GPAT was graciously donated by H. Puccio, Illkirch Cedex, France; rabbit anti-IRP1 serum was a generous gift of B. Galy, Heidelberg, Germany; rabbit anti-MIA40 was kindly provided by J. Herrmann, Kaiserslautern, Germany; monoclonal mouse anti-IRP1 (clone 295B) and rabbit anti-IRP2 were kindly provided by R. Eisenstein, Wisconsin, USA; mouse anti-IRP2, mouse anti-beta actin, rabbit anti-DPYD, mouse anti-XPD, mouse anti-CHD1L, rabbit anti-myc, and rabbit anti-FLAG came from Santa Cruz biotechnology; rabbit anti-human ferritin was purchased from ICN. Mouse anti-POLD1 was from Santa Cruz Biotechnology and rabbit anti-POLD1 from Protein Tech Group. Mouse anti-transferrin receptor (clone H68.4) came from Zymed, and mouse anti-Complex II (30 kDa Fe/S protein subunit) from MitoSciences. FLAG-M2

(F1804) and β-tubulin (T5293) antibodies were from Sigma. Peroxidase- (Biorad, Germany or Jackson Immunoresearch Laboratories) or biotin- (Vecta Laboratories, Burlingame, USA) conjugated goat anti-rabbit and anti-mouse antibodies as well as the ABC system (Vecta Laboratories, Burlingame, USA) were used as secondary reagents.

Quantitative real-time (qRT) PCR. HeLa cells were transfected thrice at a three day interval, harvested, washed, pelleted, snap-frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was prepared using the peqGOLD RNApure reagent (Peqlab). Complementary DNA (cDNA) was amplified using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). For quantification of cDNA the Maxima SYBR Green/ROX qPCR Master Mixes of Fermentas and Thermo Fisher Scientific (MA, USA) were used. QRT-PCR primer sequences are listed in Table S3.

RNA Mobility Shift Assay (REMSA). IRP binding activity of ^{32}P-labeled IRE of human ferritin mRNA was analyzed by a REMSA-related RNAse digestion protection assay according to Leibold and Munro (1988) and Müllner et al. (1989) with minor modifications. HeLa cells were harvested by trypsination, washed twice with PBS, and snap-frozen in liquid nitrogen as cell pellets. Upon use, pellets were lysed in 'Munro buffer' (10 mM Hepes, pH 7.6; 3 mM $MgCl_2$; 40 mM KCl; 5% glycerol; 1 mM DTT; 1 mM PMSF; 0.2% NP40) (Leibold and Munro, 1988) and lysates were cleared by centrifugation. For the IRE-IRP binding reaction cleared lysate samples corresponding to 1.8 µg protein were incubated with 125,000 cpm of a α-^{32}P[CTP]-labeled ferritin IRE-probe (Stehling et al., 2007). In order to induce maximal IRE-binding activity of IRP1, aliquots were pre-treated with 1.7% β-ME. IRE-binding of IRP2 was optimized by addition of 0.3% β-ME according to a modification which improved the signal-to-noise ratio (Zumbrennen et al., 2009). Subsequently, RNAse T1 was added to degrade free IRE probes and probe stretches not covered by the IRP proteins. Finally, reaction mixtures were mixed with sample buffer (30 mM Tris/HCl pH 7.5; 40% sucrose; 0.2% bromophenol blue; 10 µg/µl heparin) in a 2:1 ratio. In some cases the sample buffer contained polyclonal rabbit anti-IRP1 or anti-IRP2 antiserum to allow separation of IRP1- and IRP2-bound IREs during native gel run. Samples were loaded onto 8% polyacrylamide gels and separated at 10 V/cm for 2.5 hours using low concentrated TBE buffer (3.3 mM Tris, 3.3 mM borate, 0.66 mM EDTA). Gels were dried and subjected to autoradiography for three to four days.

Table S1, related to Experimental Procedures.

Nomenclature used for the human CIA proteins

Name used	Other name(s)	Yeast homolog
NBP35	NUBP1	Nbp35
CFD1	NUBP2	Cfd1
IOP1	NARFL, PRN, HPRN, Let1L	Nar1
CIA1	CIAO1, WDR39	Cia1
CIA2A	FAM96A	Cia2
CIA2B	FAM96B, MIP18	Cia2
MMS19	-	Met18, Mms19

To simplify the nomenclature of the human CIA proteins, we used names that, with the exception for IOP1, were derived from the names of the corresponding *S. cerevisiae* CIA homologs.

Table S2 related to Experimental Procedures.

siRNA sequences

Target	siRNA ID	siRNA#	Sense	Antisense
CIA1	S17970	1	GGAAGAGGAUGACUGGGUAtt	UACCCAGUCAUCCUCUUCCtc
	S17971	2	GGAUGUCAAGCAUGUGGUUtt	AACCACAUGCUUGACAUCCtg
	S228500	3	AUUGCUUGGUGUCAGCUGAtt	UCAGCUGACACCAAGCAAUgt
	S17972	4	CAGUGAAGCUGUACCGGGAtt	UCCCGGUACAGCUUCACUGtg
CIA2A	S38636	1	GUUUAUGAUUUGAUUAGAAtt	UUCUAAUCAAAUCAUAAACtt
	S38637	2	AGUUCAGGAGAUAAAUGAAtt	UUCAUUUAUCUCCUGAACUtc
	S38638	3	AUCUGGUUAUUAUCAGGUUtt	AACCUGAUAAUAACCAGAUat
CIA2B	S28461	1	GUUUCAAGAUGGACGUGCAtt	UGCACGUCCAUCUUGAAACgc
	S28462	2	AGUUGAACGUAGUAGAGCAtt	UGCUCUACUACGUUCAACUcc
	S28463	3	CGCGAGAUCUUCGAUCUGAtt	UCAGAUCGAAGAUCUCGCGtg
MMS19	s34552	1	AGGCCCUAGUGCUCAGAUAtt	UAUCUGAGCACUAGGGCCUtt
	s34553	2	GACUCUGAAUGCUUGCUGUtt	ACAGCAAGCAUUCAGAGUCtg
	s34551	3	CGGGAG**U**UUUUGGAACUGAtt	UCAGUUCCAAAAACUCCCGca
FXN	S5360	1	GAACCUAUGUGAUCAACAAtt	UUGUUGAUCACAUAGGUUCct
	S5361	2	AGAGCUCACUAAAGCCUUAtt	UAAGGCUUUAGUGAGCUCUgc
	S5362	3	GGAGUGGUGUCUUAACUGUtt	ACAGUUAAGACACCACUCCca

The sense and antisense siRNA sequences used in this study to deplete the indicated CIA proteins or frataxin (FXN) are listed. The bold underlined nucleotide represents a mismatch present in the commercial siRNA (see Stehling et al., 2012).

Table S3, related to Experimental Procedures.

qRT primer sequences

Target	forward	reverse
CIA1	GCTTCCACTCAAGGACCATT	GTTGGGATCCTCCTGAAACA
CIA2A	CGGATCATGGAAGAGAAAGC	CTTTCCGAGACCACTTCCAG
CIA2B	GCTTTCACACCAACCATTCC	AATGTGCACGTCCATCTTGA
IRP1	CTGCTTGGGTCAGGTTCG	GTACAGGATCCAATGGCTCAG
IRP2	ACGCCTTTGAGTACCTTATTGAA	CCCGTATTGAGTAAGGCAGAA
DPYD	GCTGTCCCTGAGGAGATGGA	GTCCGAACAAACTGCATAGCAA
GPAT	TGATCACTCTGGGACTCGTG	AAGACCCATTCCCTTGTGTG
POLD1	AGGTAGTACTGCGTGTCAATGG	CCCTACGTGATCATCAGTGC
FT-H	GGTGCGCCAGAACTACCA	CCACATCATCGCGGTCAAAG
TFR1	GGTTGCAAATGCTGAAAGC	AAGGAAGGGAATCCAGGTGT
β-Actin	CTCTTCCAGCCTTCCTTCCT	AGCACTGTGTTGGCGTACAG
HPRT1	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

The nucleotide primer sequences for qRT PCR analysis of the amounts of the mRNAs of the indicated proteins are depicted.

Table S4 (Excel file), related to Figures 5D and S7E.
Proteins interacting with CIA1, CIA2A, or CIA2B as identified by MudPIT

Immunoprecipitation and immunoblotting was performed as described (Vashisht et al., 2009). Equal amounts of protein lysate were added to the immunoprecipitation reactions using affinity matrices for anti-FLAG M2 (A2220; Sigma) and incubated for 2 h at 4°C. Beads were washed three times with lysis buffer, and bound proteins were analyzed by immunoblotting with relevant antibodies. For proteomic mass spectrometry experiments, the protein complexes were eluted using FLAG peptide, digested by Lys-C and trypsin proteases, and analyzed by MudPIT on a LTQ-Orbitrap XL mass spectrometer as previously described (Stehling et al., 2012; Vashisht et al., 2009). One important caveat related to the groupings described here are the difficulties in reproducibly sampling low abundance protein interactors using the semi-quantitative proteomic methods employed here. This could result in interactors being mistakenly classified as exclusively associated with a particular CIA factor while in reality they are associated with multiple CIA factors at levels that are below our detection limit.

Supplemental Reference

Leibold, E.A., and Munro, H.N. (1988). Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. Proceedings of the National Academy of Sciences of the United States of America 85, 2171-2175.