Supporting Information

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SI Materials and Methods

Generation of a Conditional Cytoglobin-Knockout Mouse Line. A 10.5-kb region used to construct the targeting vector was first subcloned from a C57BL/6 BAC clone (RPCI23 330N7). The region was designed so that the short homology arm (SA) extended 1.5 kb 3′ to exon 3 of the murine cytoglobin (*Cygb*) gene, and the long homology arm (LA) extended 5′ to exon 2 and was 7.7 kb in length. Subsequently, a pGK-gb2 loxP/FRT-flanked Neo cassette was inserted 3′ to exon 3, and a single loxP site was inserted at the 5′ side of exon 2. The targeting region was 1.3 kb and included exons 2 and 3. The targeting vector was linearized with NotI and was electroporated into hybrid (129svev/C57BL/6) ES cells. G418-resistant colonies were screened for the targeted integration and retention of the loxP cassette by PCR and injected into C57BL/6 blastocysts (Fig. S2*B*). Chimeras with high percentage of coat color contribution from the injected ES cells were bred to WT C57BL/6 mice to generate F1 heterozygous mice. Germ-line integration was confirmed by both PCR and Southern blot analyses (Fig. S2 *C* and *D*). The mouse line was backcrossed 15 generations into a pure C57BL/6J background strain before in vivo experiments were initiated.

Mice with skeletal muscle-specific deletion of *Cygb* [$Cygb^{flox/flox}$: *Myo-Cre* (+) or $Cygb^{-/-}$] were generated by crossing homozygous loxP mice ($Cygb^{flox/flox}$) with *Myo-Cre* transgenic mice (1). Mice lacking the *Myo-Cre* transgene [$Cygb^{flox/flox}$: *Myo-Cre* (−) or $Cygb^{-/+}$] were used as control mice. To verify the deletion of *Cygb* from myogenic progenitor cells (MPCs), primary MPCs were isolated from $Cygb^{-/-}$ skeletal muscle, and quantitative RT-PCR (qRT-PCR) and Western blot analysis demonstrated complete absence of *Cygb* from the $Cygb^{-/-}$ MPCs (Fig. 3 *A–D*).

Southern Blot Analysis. Southern Blot analysis was undertaken to verify germ-line integration in the F1 heterozygous mice ($Cygb^{flox/+}$) and was performed using a standard protocol (2). In brief, DNA from the tails of WT and F1 heterozygous mice was digested with a restriction endonuclease recognizing the Bglll restriction site and was electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a reactive probe targeted against the SA (Fig. S2*D*). The expected WT fragment size was 6.0 kb, and the expected loxP fragment size was 4.4 kb. The primers used for the SA Southern probe were

Probe primer 1: CCA AGT CAC CTC TCC CGG TAC
Probe primer 2: CGT GAT GCT CTA GAG ATG GCG AGG

Genotyping of the Conditional Cygb-Knockout Mouse Line. Genotyping of $Cygb^{flox/flox}$ mice was undertaken using two separate semiquantitative PCR reactions (Table S1). The primer set for reaction 1 resulted in no amplicon for a WT allele, whereas a loxP allele demonstrated a 250-bp amplicon. With the primer set for reaction 2, a WT allele was identified with the appearance of a 342-bp amplicon, whereas a loxP allele demonstrated a 2.1-kb amplicon.

Histological and Immunohistochemical Studies. Tissues for histological and immunohistochemical (IHC) studies were prepared as previously described (3). Paraffin processing, embedding, sectioning, and H&E staining were performed using standard procedures. IHC staining of fixed muscle tissue sections was performed using standard protocols using polyclonal rabbit anti-*Cygb* serum [1:100; University of Texas Southwestern (UTSW) Antibody Core Facility], polyclonal rabbit anti-MyoD serum (1:200; Santa Cruz) and DAPI nuclear stain (Molecular Probes). Finally, TUNEL staining for apoptotic cells was performed on tissue sections as previously described (4). Apoptotic cells were labeled with fluorescein, and the sections were counterstained with propidium iodide.

Morphometric Analyses. Myocytes with central or peripheral nuclei were determined by visual inspection of H&E-stained sections. For each sample, 200–250 myocytes were counted, and the myocyte cross-sectional area was measured using the National Institutes of Health software ImageJ (Image J 1.37V).

C2C12 Myoblast Differentiation and Immunocytochemical Studies. On day 0, C2C12 myoblasts were plated in equal numbers under normoxic conditions (21% O_2, 5% CO_2, 74% N_2) in DMEM supplemented with 20% FBS and antibiotics (100 U penicillin/mL and 100 μg streptomycin/mL) as previously described (5). Immunocytochemistry (ICC) studies were done using standard ICC protocols using the polyclonal rabbit anti-*Cygb* serum (1:100; UTSW Antibody Core Facility), polyclonal rabbit anti-MyoD serum (1:200; Santa Cruz), polyclonal mouse anti-Desmin (1:200; DakoCytomation), and either DAPI or Hoechst nuclear stain (5). Induction of differentiation of C2C12 myoblasts into mature myotubes was initiated by growing the cells in DMEM supplemented with 2% heat-inactivated horse serum, 10 μg/mL insulin (Gibco-BRL), and 10 μg/mL transferrin (Gibco-BRL) for 5 d.

Isolation of Primary MPCs. MPCs were isolated and cultured from the hindlimbs of 8-wk-old male mice (6). The hindlimb muscle was enzymatically digested using collagenase and collagenase/dispase solution, followed by sequential filtration to obtain a heterogeneous population consisting of MPCs, fibroblasts, and endothelial cells. The cell preparation was preplated on uncoated tissue-culture plates to remove fibroblasts, endothelial cells, and other contaminating cell types. The floating MPCs then were plated on collagen-coated plates and grown in growth medium for 48 h before the initiation of experimental protocols. Induction of differentiation was initiated by growing the MPCs for 5 d in differentiating medium containing 5% horse serum.

Isolated MPCs were cultured in growth or differentiation medium at 37 °C in a humidified tissue culture incubator with 5% CO_2. The MPCs were cultured in the following media:
Plating medium. DMEM was supplemented with 10% horse serum (Sigma), 0.5% chick extract (MP Biomedicals), 4 mM L-glutamine (Sigma), and 1% penicillin and streptomycin solution (Sigma).
Growth medium. DMEM was supplemented with 20% horse serum or 20% FBS (Gibco), penicillin/streptomycin (50 U/mL/50 mg/mL), 4 mM L-glutamine, 10 mM Hepes, and 3% chicken embryo extract.
Differentiation medium. DMEM was supplemented with 5% horse serum, penicillin/streptomycin (each 50 U/mL), 4 mM L-glutamine, and 10 mM Hepes.

Western Blot Analysis, RNA Isolation, and qRT-PCR. Protein from cell lysates, cellular fractions (i.e., nuclear and cytosolic), or whole-muscle tissue were isolated from control and experimental groups. Western blot analysis was performed as previously described using various antibodies [α-tubulin (1:800; Sigma); caspase 3 (1:1,000; Cell Signaling); cytochrome *c* (1:1,000; Santa Cruz); Cygb (1:100; UTSW Antibody Core Facility); myogenic differentiation (MyoD) (1:500; Santa Cruz); poly-ADP ribose polymerase (PARP) (1:500; Santa Cruz;); specificity protein 1 (1:500; Santa Cruz); voltage-dependent anion channel (VDAC) (1:1,000; Cell Signaling); myoglobin (1:1,000; Dakocytomation); myogenin (1:10; Developmental Studies Hybridoma Bank,

University of Iowa); GFP (1:1,000; Invitrogen); and GAPDH (1:800; Santa Cruz;)] (3. 5, 7).

Total RNA was isolated from the control or the experimental cells or tissues using the TriPure Isolation Kit (Roche Diagnostics Corporation). Reverse transcription was performed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen) to obtain cDNA, and qRT-PCR was performed as previously described (5). The qRT-PCR data were analyzed using the ΔΔCt method, and individual gene expression was normalized to 18s-ribosome (*18s-Rib*) expression.

Primers Sets. qRT-PCR was undertaken to assess the transcript levels for a variety of genes in this study. The primer sets used in this study are noted in Table S2.

Myoglobin-Overexpressing Studies. Primary MPCs isolated from the hindlimbs of either $Cygb^{+/+}$ or $Cygb^{-/-}$ mice were infected for 60 min with either an adenovirus overexpressing myoglobin (AdV-Mb) or an empty, control adenovirus (AdV-Ctrl) at a multiplicity of infection of 100. After 60 min, the infected MPCs were washed twice with the culture medium and incubated for 36 h. Subsequently, the MPCs were incubated in differentiating medium for 5 d. At the end of this period the cells were used for ICC analyses, or the protein was harvested for Western blot analyses. All the adenoviruses used in this study were generated and propagated according to previously published protocols (8).

1. Li S, et al. (2005) Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. *Proc Natl Acad Sci USA* 102(4):1082–1087.
2. Garry DJ, et al. (1998) Mice without myoglobin. *Nature* 395(6705):905–908.
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4. Lu JR, et al. (2002) Control of facial muscle development by MyoR and capsulin. *Science* 298(5602):2378–2381.
5. Singh S, et al. (2009) Calcineurin activates cytoglobin transcription in hypoxic myocytes. *J Biol Chem* 284(16):10409–10421.
6. Musarò A, Barberi L (2010) Isolation and culture of mouse satellite cells. *Methods Mol Biol* 633:101–111.
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8. Gerard RD, Meidell RS (1995) *Adenovirus Vectors in DNA Cloning - A Practical Approach: Mammalian Systems*, eds Glover DM, Hames BD (Oxford Univ Press, Oxford, UK), pp 285–307.

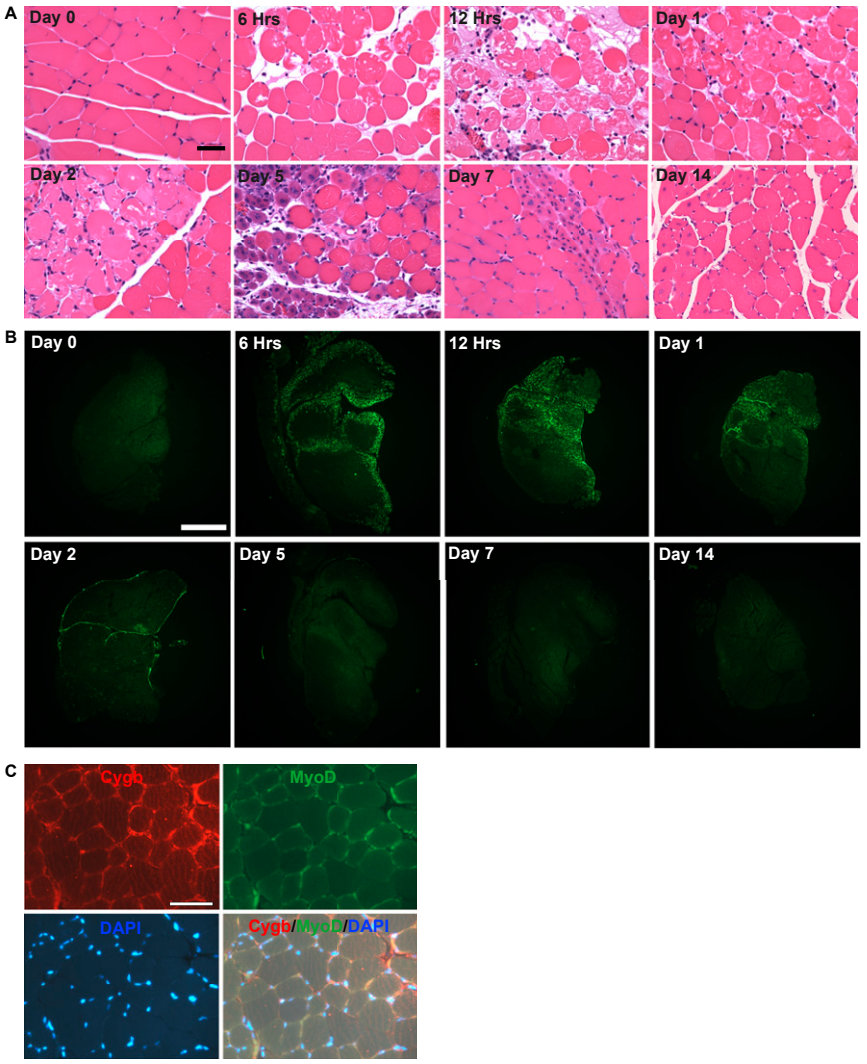

Fig. S1. Time course of muscle regeneration in CTX-injured WT mice. (*A*) H&E staining indicated marked inflammation and myocyte cell death during the first 24–48 h after CTX injury to the hindlimb muscles of WT mice. Between 2–7 d post-CTX injury there was evidence of muscle regeneration as evident by the appearance of centrally nucleated myocytes. By 14 d post-CTX injury the muscle architecture was completely restored in CTX-injured WT mice. (Scale bar: 40 μm.) (*B*) TUNEL assays supported enhanced apoptotic cell death within 24–48 h after CTX injury to the hindlimb muscles of WT mice. (Scale bar: 1 mm.) (*C*) Immunohistochemistry performed at day 5 post-CTX injury showed colocalization of Cygb and MyoD in cells surrounding myofibers. (Scale bar: 40 μm.)

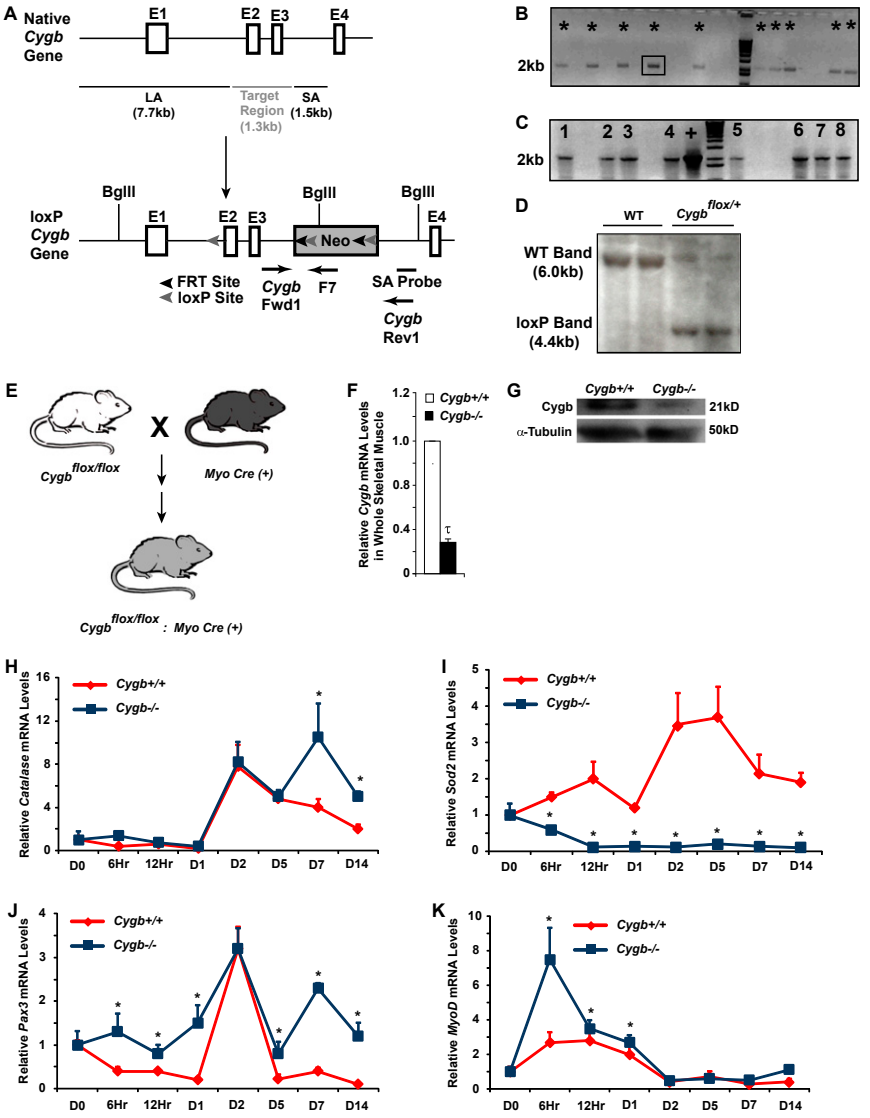

Fig. S2. Altered transcriptome expression in injured mice with muscle-specific deletion of Cygb. (*A*) Schematic of the targeting strategy for generating a conditional *Cygb*-knockout mouse model. A genotyping strategy was designed using the primers *Cygb* Fwd1, *Cygb* Rev1, and F7. (*B*) Multiple targeted ES clones (*) were identified by RT-PCR as having incorporated the conditional *Cygb*-knockout targeting construct. The ES clone identified by the black box was the clone that successfully generated a chimeric mouse with the conditional *Cygb*-knockout targeting construct. (*C*) Genotyping of mice from the F_1 generation revealed several mice heterozygous for the *Cygb-loxP* construct. The positive ES clone identified by the black box in *B* was used as a positive control for the genotyping run. (*D*) The genotyping was confirmed by Southern blot analysis using genomic tail DNA from F_1 generation mice. (*E*) Schematic demonstrating the cross required to generate skeletal muscle-specific *Cygb*-knockout mice. (*F* and *G*) qRT-PCR and Western blot performed on whole hindlimb muscle confirmed an ~ 70% knockdown of *Cygb* expression in $Cygb^{-/-}$ mice. The residual *Cygb* expression observed in the $Cygb^{-/-}$ mice was presumed to result from the expression of *Cygb* in other cell types that exist within the muscle (e.g., fibroblasts and endothelial cells). (*H* and *I*) qRT-PCR data supported increased oxidative stress in the hindlimb muscles of CTX-injured $Cygb^{-/-}$ mice [i.e., increased *catalase* transcript levels (*H*) but decreased superoxide dismutase 2 (*Sod2*) mRNA levels (*I*)]. (*J* and *K*) Dysregulation of some of the myogenic regulatory factors was noted in CTX-injured $Cygb^{-/-}$ mice. $^{\tau}P < 0.05$ $Cygb^{-/-}$ vs. $Cygb^{+/+}$, $n = 3$ in each group; $^{*}P < 0.05$ $Cygb^{-/-}$ vs. $Cygb^{+/+}$, $n = 3$ at each time point. Pax3, paired box 3.

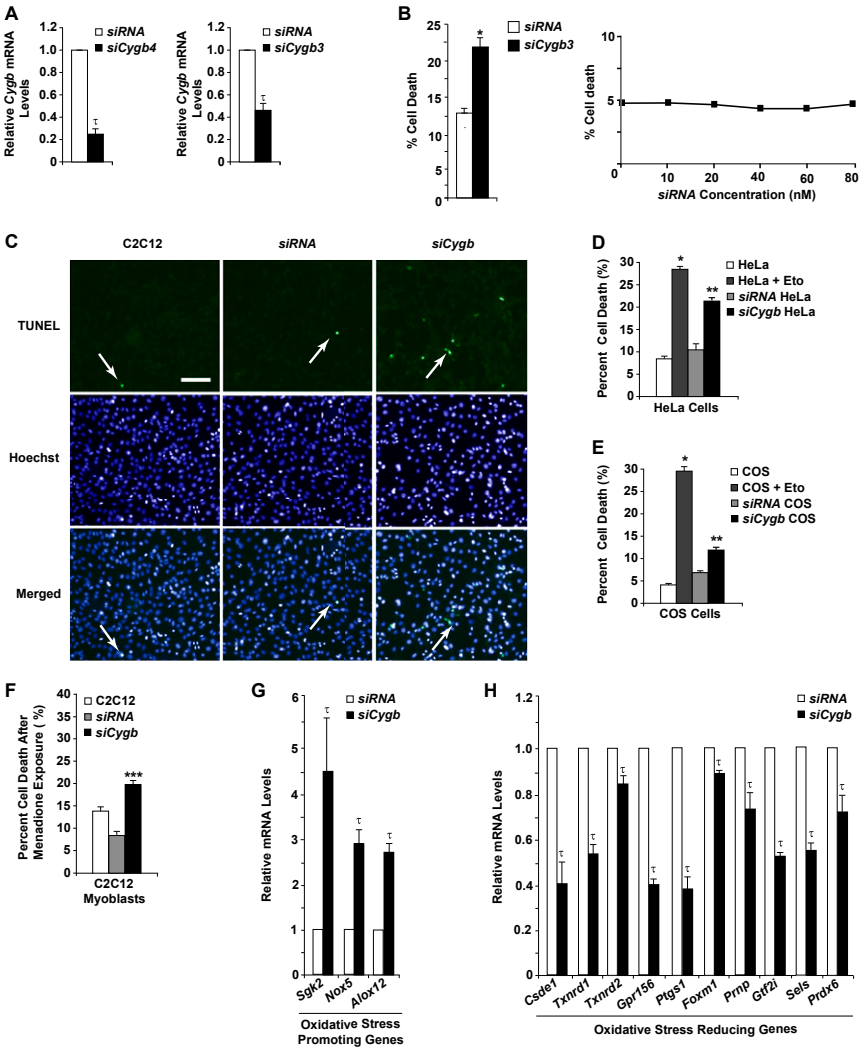

Fig. S3. Loss of Cygb induces cell death and oxidative stress in a variety of cell lines. (*A*) qRT-PCR data demonstrated significant knockdown of Cygb within C2C12 myoblasts using two different Cygb-targeting oligonucleotides, siCygb4 and siCygb3. Because siCygb4 silenced Cygb gene expression to a greater degree than siCygb3, the majority of the small, interfering RNA (siRNA) studies undertaken in this work used siCygb4, which is referred to in this paper as "siCygb." (*B*) Knockdown of Cygb induced cell death in siCygb3 C2C12 myoblasts as compared with control C2C12 myoblasts transfected with the scrambled siRNA oligonucleotide (control). The control siRNA did not have any effect on cell death over a wide range of concentrations (0–80 nM). (*C*) Representative images from TUNEL assays demonstrated an increase in TUNEL-positive nuclei in siCygb myoblasts as compared with the control cells (siRNA and C2C12 myoblasts). The white arrows identify TUNEL-positive cells. Hoechst staining was used to identify the nuclei of the myoblasts. (Scale bar: 100 μm.) (*D* and *E*) FACS analysis revealed increased cell death in HeLa (siCygb-HeLa) (*D*) and COS (siCygb-COS) (*E*) cells, which are deficient in Cygb under basal conditions, as compared with the respective control cells. Nontransfected HeLa and COS cells were exposed to etoposide as a positive control to demonstrate the robustness of the assay to induce cell death. (*F*) Increased cell death, as measured by FACS analysis, was noted in C2C12 myoblasts deficient in Cygb (siCygb) exposed to menadione as compared with control cells (C2C12 and siRNA cells) exposed to this free radical-generating agent. (*G*) qRT-PCR data demonstrated that the mRNA transcript levels of various genes involved in promoting oxidative stress were up-regulated in *siCygb* as compared with control siRNA C2C12 myoblasts. (*H*) qRT-PCR data demonstrated that the mRNA transcript levels of various genes involved in reducing oxidative stress were down-regulated in *siCygb* as compared with control siRNA C2C12 myoblasts. $^{\tau}P < 0.005$ *siCygb* vs. siRNA; $*P < 0.005$ stressed cells vs. nonstressed cells; $**P < 0.05$ *Cygb*-knockdown cells vs. siRNA-transfected cells; $***P < 0.05$ menadione-treated *siCygb* cells vs. menadione-treated siRNA or C2C12 cells; $n = 6$ in each group.

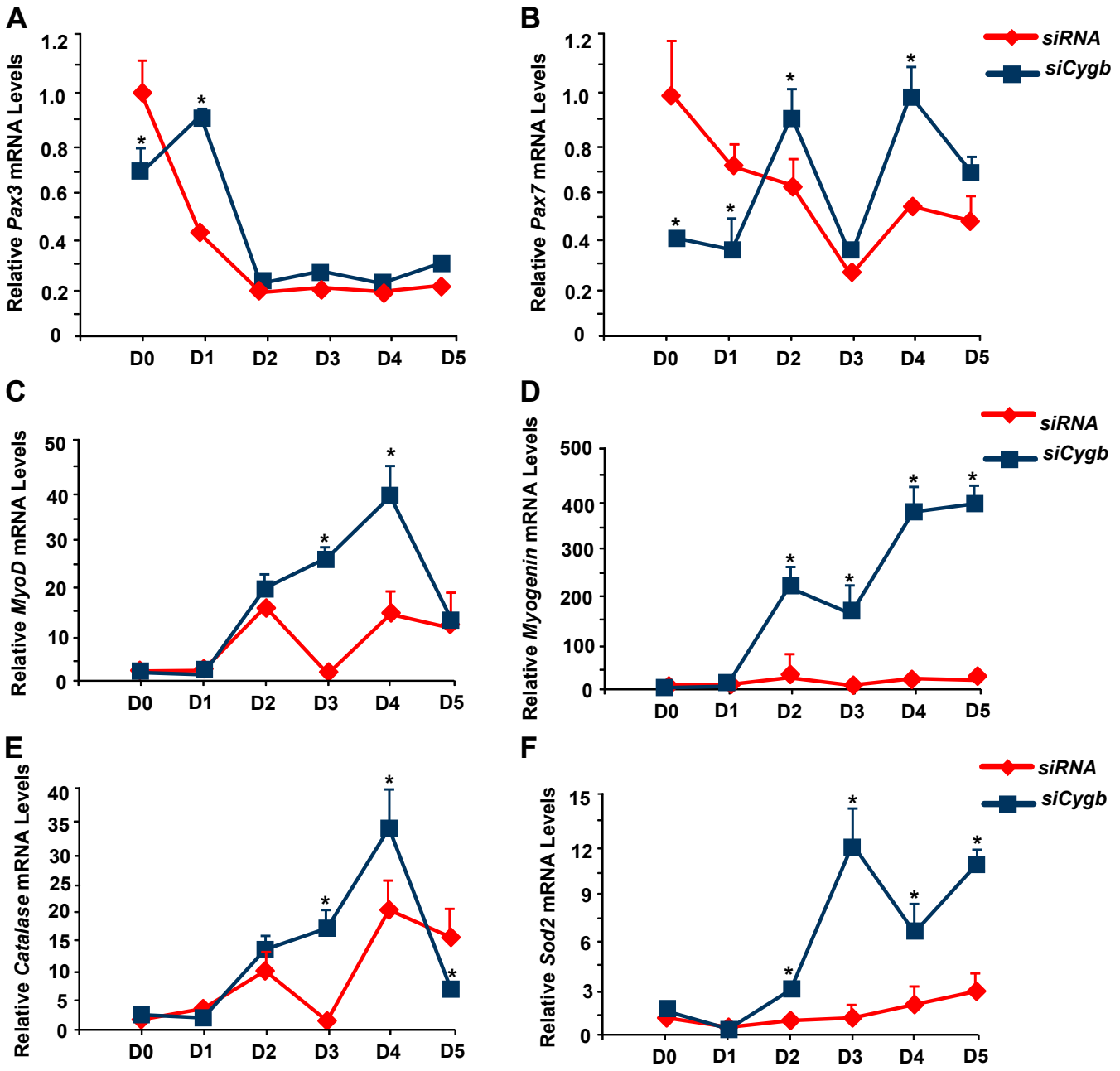

Fig. S4. Deficiency in Cygb results in the dysregulation of key myogenic transcription factors and oxidative stress genes during the differentiation of C2C12 myoblasts. (*A–D*) qRT-PCR analysis of mRNA transcript levels of *Pax3, Pax7, MyoD*, and *myogenin* demonstrated altered expression patterns during differentiation in *siCygb* cells as compared with control siRNA cells (days 0–5). These altered expression patterns of the key myogenic transcription factors supported the concept that differentiation is impaired in C2C12 myoblasts that are deficient in Cygb. (*E* and *F*) mRNA expression of *catalase* and *Sod2* remained consistently high during the course of C2C12 differentiation in *siCygb* as compared with control siRNA cells, indicating elevated oxidative stress. *$P < 0.05$ *siCygb* vs. siRNA; $n = 3$ in each group at each time point.

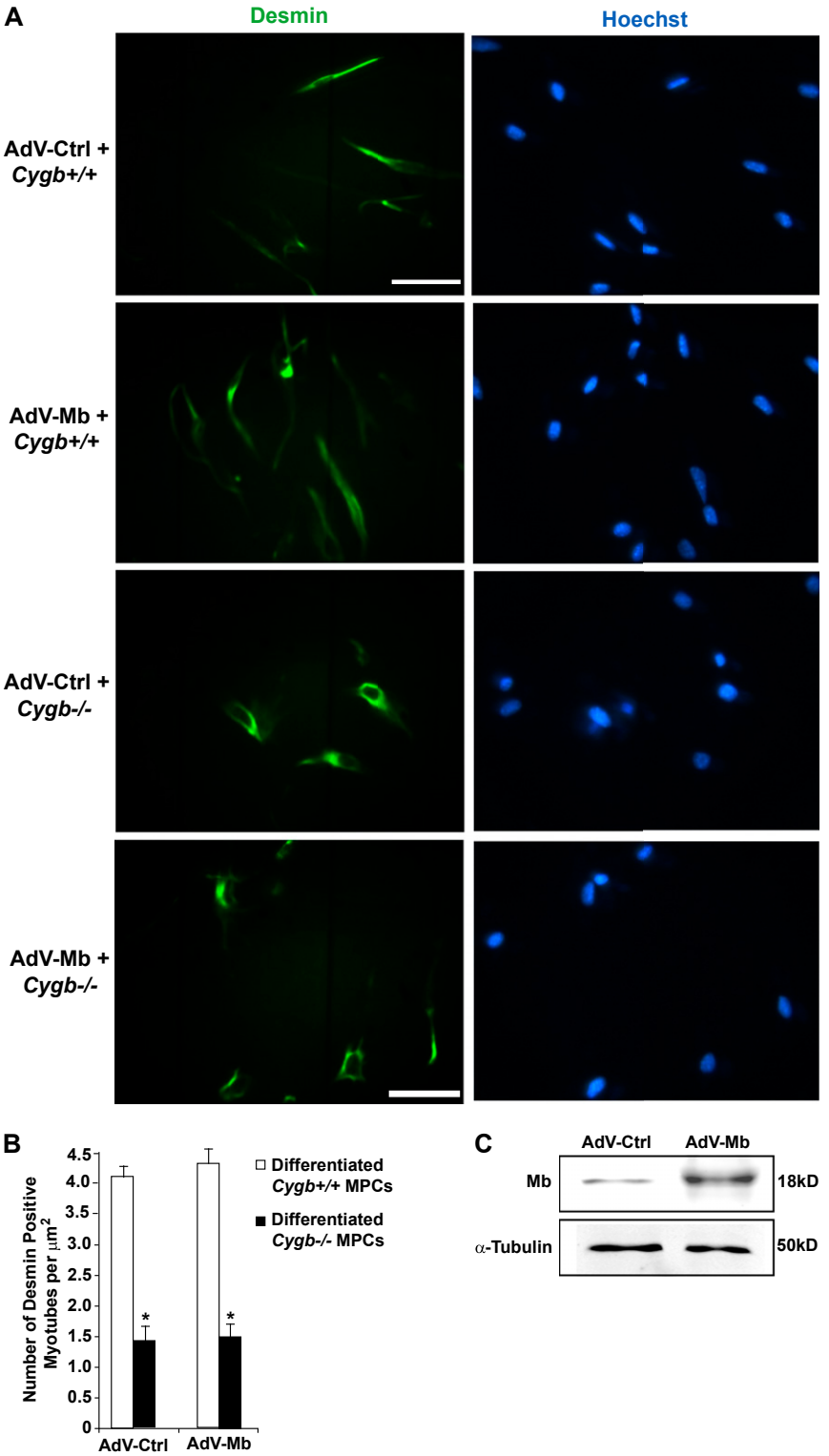

Fig. S5. Overexpression of myoglobin fails to rescue the impaired differentiative capacity observed in primary MPCs isolated from the hindlimbs of either $Cygb^{+/+}$ or $Cygb^{-/-}$ mice. (*A*) Representative ICC images revealed decreased desmin staining in differentiated $Cygb^{-/-}$ MPCs transduced with adenovirus overexpressing myoglobin [Adv-Mb as compared with differentiated $Cygb^{+/+}$ MPCs transduced with control adenovirus (AdV-Ctrl)]. (*B*) Quantification of the MPCs that differentiated into elongated myotubes demonstrated that myoglobin failed to rescue the impaired ability of the primary $Cygb^{-/-}$ MPCs to differentiate fully. (*C*) Western blot analysis confirmed the ability of AdV-Mb to overexpress myoglobin (Mb) in control MPCs. α-Tubulin was used as the loading control. (Scale bar: 40 μm.) *$P < 0.005$ $Cygb^{-/-}$ vs. $Cygb^{+/+}$.

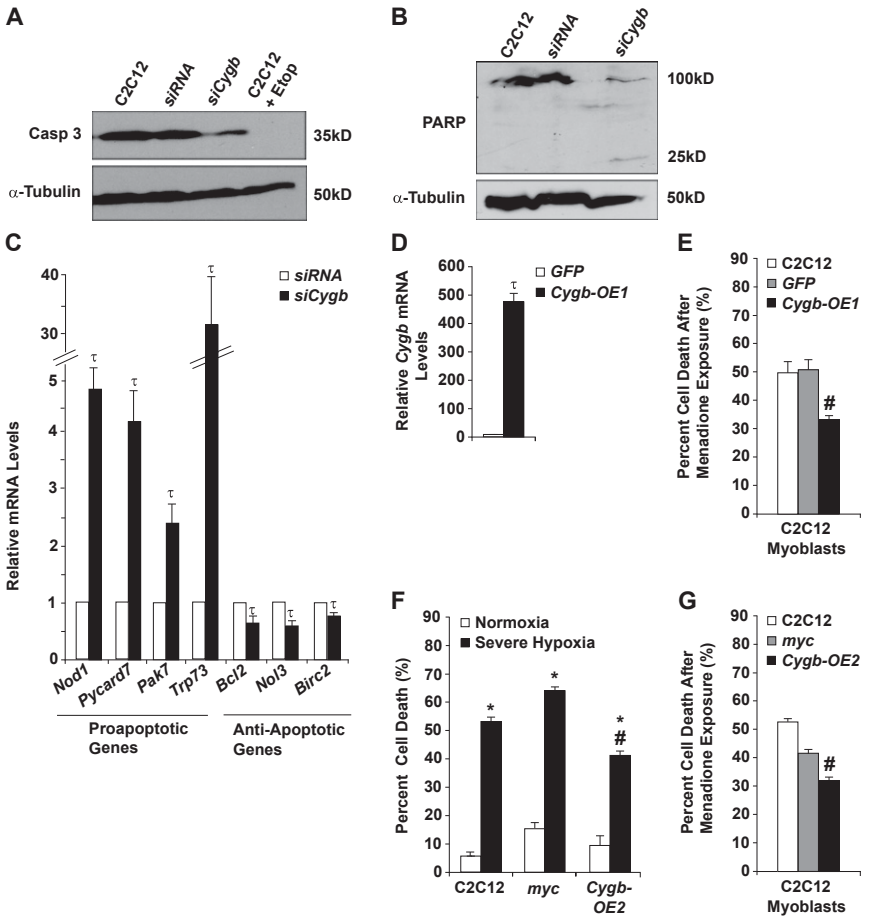

Fig. S6. Cygb has a cytoprotective role under various oxidative stress conditions. (*A*) Western blot analysis demonstrated the cleavage of caspase 3 (i.e., diminished expression of the total caspase 3 protein at 35 kD) in *siCygb* myoblasts as compared with control cells (nontransfected C2C12 myoblasts and siRNA myoblasts). C2C12 myoblasts exposed to etoposide (Etop) were used as a positive control. Note the complete absence of the total caspase 3 protein in the C2C12+Etop lane. (*B*) Western blot analysis demonstrated the cleavage of PARP (i.e., diminished expression of the total PARP protein at 100 kD) in *siCygb* myoblasts as compared with control cells (nontransfected C2C12 myoblasts and siRNA myoblasts). Note that the cleaved product of PARP is seen at 25 kD in the *siCygb* lane. α-Tubulin was used as the loading control. (*C*) The mRNA transcript levels of established anti- and proapoptotic genes were assessed in *siCygb* and siRNA control C2C12 myoblasts using qRT-PCR. The proapoptotic genes were up-regulated, and antiapoptotic genes were down-regulated in C2C12 myoblasts deficient in Cygb. (*D*) qRT-PCR data demonstrated significant overexpression of *Cygb* within C2C12 myoblasts (*Cygb-OE1*) compared with control cells overexpressing GFP only. (*E*) Decreased cell death as measured by FACS was noted in menadione-treated C2C12 myoblasts overexpressing *Cygb-OE1* as compared with the respective menadione-treated control myoblasts. (*F* and *G*) FACS analysis revealed decreased death in hypoxic or menadione-treated *Cygb-OE2* C2C12 myoblasts as compared with hypoxic or menadione-treated control myoblasts (C2C12 myoblasts and *myc*-transfected C2C12 myoblasts). $^{\tau}P < 0.05$ *siCygb* or *Cygb-OE1* vs. siRNA or *GFP*, respectively; $n = 6$ in each group. $^{*}P < 0.05$ stressed cells vs. nonstressed cells; $^{\#}P < 0.05$ stressed *Cygb-OE1* or *Cygb-OE2* cells vs. stressed control cells; $n = 6$ in each group. Casp 3, caspase 3; *Cygb-OE1*, C2C12 myoblasts overexpressing a *Cygb-GFP* fusion protein; *Cygb-OE2*, C2C12 myoblasts overexpressing a Cygb-myc fusion protein; *GFP*, C2C12 myoblasts overexpressing a GFP protein and used as a control for *Cygb-OE1* cells; *myc*, C2C12 myoblasts overexpressing a myc fusion protein and used as a control for *Cygb-OE2* cells; PARP, poly-ADP ribose polymerase; *siCygb*, C2C12 myoblasts deficient in *Cygb* via siRNA; siRNA, C2C12 myoblasts transiently transfected with a scrambled siRNA oligonucleotide and used as a control for *siCygb* cells.

Table S1. Genotyping primers for the conditional cygb-knockout mouse line

	Forward primer	Reverse primer
Reaction 1	*Cygb CKO* fwd1 primer: GGCTTGCAGACACATTGAAA	F7 primer: GGAACTTCGCTAGACTAGTACGCGTG
Reaction 2	*Cygb CKO* fwd1 primer: GGCTTGCAGACACATTGAAA	*Cygb CKO* rev1 primer: TGGCTCCAGCTCTGTTAAGG

Table S2. qRT-PCR primer sets for genes involved in oxidative stress or apoptosis

Gene	Forward primer	Reverse primer
Alox12	GTTCCACACATCCGTTACACT	CCGAGTAAGCAACTGAACATGG
Bcl2	GTCGCTACCGTCGTGACTTC	CAGACATGCACCTACCCAGC
Birc2	TGTGGCCTGATGTTGGATAAC	GGTGACGAATGTGCAAATCTACT
Catalase	CTCACGTTCCGCAGCTCT	TAGACTGCCTCTCCATCTGC
Csde1	TGAACGGACAAGTTGTGTGC	CCTGTTGGACTCTGACCCG
Cygb	CCAACTGCGAGGACGTGG	AGTTCACAAAGAACCTCACCAGG
Desmin	GTGGATGCAGCCACTCTAGC	TTAGCCGCGATGGTCTCATAC
Foxm1	CTGATTCTCAAAAGACGGAGGC	TTGATAATCTTGATTCCGGCTGG
Gpr156	TGGATCGGAGACCCCTTCAT	AGAAAGGTCCACATAATCCCCA
Gpx	TGGACTGCTACAAAGACGTGAAA	GAACATACTTGAGCCCAGGAAGAA
Gtf2i	AAAGCCCTCCAATTTTCCTTTCA	TCCACACCTGATGCAGTAGTA
Mb	TCTAGCCCAATCACACGCC	AACTCCAGGTACTTGACCGG
MyoD	CCACTCCGGGACATAGACTTG	AAAAGCGCAGGTCTGGTGAG
Myogenin	GGCAATGCACTGGAGTTCG	AGCCGCGAGCAAATGATC
Nod1	GAAGGCACCCCATTGGGTT	AATCTCTGCATCTTCGGCTGA
Nol3	TTGACCGGGAACGGAAACG	CCAAGGCTTCGTACTCGGG
Nox5	CGGTCTTTCGAGTGGTTTGT	GAAGAAGACCTGCACCTTGC
Pak7	AAGATCGAAATATCTGGCCCATC	TGTGTCTGCTAACAGGCTGTG
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT
Pax7	TCTCCAAGATTCTGTGCCGAT	CGGGGTTCTCTCTCTTATACTCC
Prdx6	CGCCAGAGTTTGCCAAGAG	TCCGTGGGTGTTTCACCATTG
Prnp	ATGGCGAACCTTGGCTACTG	CCTGAGGTGGGTAACGGTTG
Ptgs1	ATGAGTCGAAGGAGTCTCTCG	GCACGGATAGTAACAACAGGGA
Pycard	CTTGTCAGGGGATGAACTCAAAA	GCCATACGACTCCAGATAGTAGC
Sels	GACCGAGAGCCTGCGATTC	AGCCCTCAGTCGAAGGGAG
Sgk2	TCCAGCCCAGTTGGAGTTC	CCGTAGTTCCCTTTGCCAATG
Sod2	ACGTTGCCTTCCCAGGAT	GGCTGAAGAGCGACCTGAGT
Titin	CCAGGCCCTCCAAACAACC	CCATTCACCAACACTCACATCAC
Trp73	GCACCTACTTTGACCTCCCC	GCACTGCTGAGCAAATTGAAC
Txnrd1	CCCACTTGCCCCAACTGTT	GGGAGTGTCTTGGAGGGAC
Txnrd2	GATCCGGTGGCCTAGCTTG	TCGGGGAGAAGGTTCCACAT