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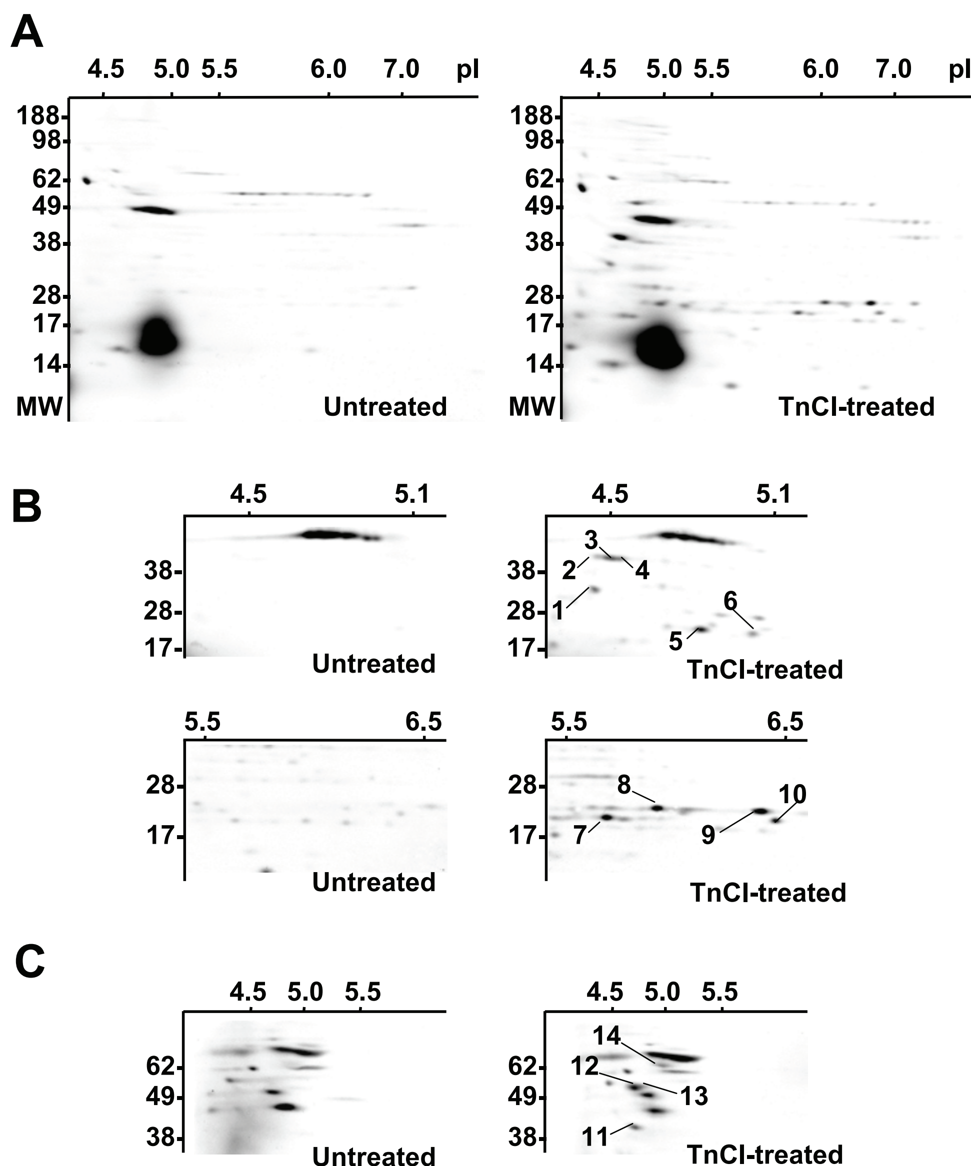

Figure S1 Identification of proteins oxidized in JLP-119 lymphoma cells treated with TnCl using 2D gel electrophoresis and IAF-labeling of Cys residues. To identify oxidant-sensitive proteins that are responsible for mediating the induction of apoptosis in response to TnCl, cysteinyl-containing proteins were tagged with fluorescently-labeled sulfhydryl-reactive reagents as described by others[15,16,32]. Using this procedure, only cellular proteins containing oxidized Cys residues at the time of cell lysate collection are labeled. (**A**) Protein disulfides from untreated cells and cells treated with 0.5 mM of TnCl for 1 h in PBS were labeled with IAF and resolved by 2D electrophoresis (IEF with pH 3–10 strips and 4-12% SDS-PAGE) as described in "Methods". All of the new spots seen in lysates from TnCl-treated cells (compared to untreated cells) had isoelectric points between pH 4 and 8. The figure shows the areas of the gels containing spots. (**B**) To increase protein resolution and highlight major changes in treated samples, the same proteins shown in (**A**) were separated using non-linear IEF strips with pH gradients from 3–5.5 and 5–6.5 followed by SDS-PAGE. (**C**) 5'IAF-labeled proteins derived from the crude mitochondrial fraction of treated and untreated cells were resolved by 2D electrophoresis (IEF with pH 3–10 strips and 4-12% SDS-PAGE). In all cases, the left-hand panels are extracts from control (untreated) cells and the right-hand panels are from TnCl-treated cells. The numbering of the spots is arbitrary. Gel images are representative of at least three independent experiments. Proteins were selected for further analysis only if they occurred consistently in three separate experiments and showed more than a 2-fold increase in oxidation in response to TnCl.

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Protein	spot #	accession #[2]	MW/pI (approx)	cys #	# peptides / spot
Molecular Chaperones					
Calreticulin	12	P27797	50/4.6	3	9
DJ-1 (PARK7)	8	Q99497	20/6.0	3	22
HSP60	14	P10809	62/4.9	3	167
Nucleophosmin (B23)	11	P06748	42/4.7	3	12
	3		42/4.5		17
14-3-3 ε	1	P62258	32/4.3	3	11
14-3-3 τ	1	P27348	32/4.3	5	7
Cytoskeletal/structural					
Cofilin-1	7	P23528	18/5.8	4	12
	10		18/6.4		15
Similar to Laminin receptor 1	3	Q6IPD1	42/4.5	2	31
	4		42/4.6		13
Tropomyosin 3	1	Q5VU58	32/4.3	2	20
Tropomyosin α-4 chain	1	P67936	32/4.3	2	16
Tropomyosin α-1 chain	1	P09493	32/4.3	13	12
Tubulin β-2 chain	13	P07437	50/4.8	8	16
Antioxidant					
Glutathione S-transferase	6	P09211	21/5.0	4	12
Peroxiredoxin-1	9	Q06830	20/6.3	4	16
Peroxiredoxin-2	5	P32119	22/4.8	3	19
Protein disulfide-isomerase	13	P07237	50/4.8	7	4
Other					
Chromatin assembly factor 1	12	Q09028	50/4.7	5	4
GAPDH	4	Q5JR58	42/4.6	7	4
Hepatoma-derived growth factor	2	P51858	42/4.4	2	12
T-complex protein 1 ε	14	P48643	62/4.9	8	13
dUTP pyrophosphatase	6	P33316	21/5.0	5	10

[1]Liquid chromatography electrospray ionization tandem mass spectrometry.

[2]Accession number on NCBI/Uniprot website.

Figure S2 Summary of cellular protein targets of TnCl oxidation in human B lymphoma cells as identified by 2-D gel electrophoresis and LC-ESI-MS/MS. Protein spots excised from gels shown in Supplementary Information, Fig. 1 were destained and digested with trypsin (Promega) overnight at 37 °C. The peptides were extracted from the gel and analyzed by reversed-phase nanoflow LC coupled on-line with a linear ion trap mass spectrometer (LTQ, Thermo Electron) for tandem MS (nanoLC-MS/MS)[1]. The raw MS/MS data were searched using SEQUEST (ThermoFinnigan, San Jose, CA) against the human protein database downloaded from the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/proteome/index.html). Peptide mass tolerance of 2.0 Da and fragment ion tolerance of 0.5 Da were allowed with tryptic specificity allowing two missed cleavages. During the SEQUEST analysis, dynamic modification of cysteine with 5-iodoacetamidofluorescein (mass addition of 387.1 Da) was also set. The SEQUEST filtering criteria were as described previously[1].

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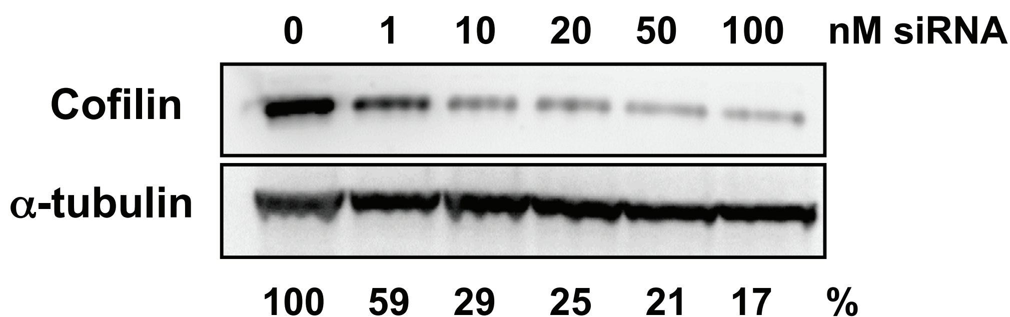
A



B

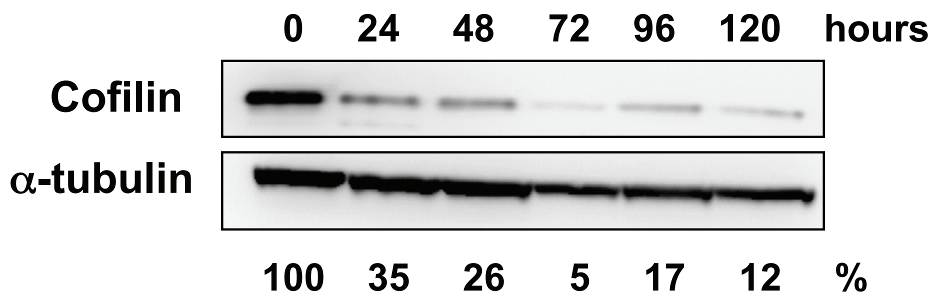


Figure S3 Targeted silencing of cofilin protein expression using siRNA. The conditions for optimizing the knockdown of cofilin were established using different concentrations of siRNA and different time points following siRNA transfection. The siRNA sequences used for the targeted silencing of cofilin protein expression are described in Methods. Cells were transfected using the Dharmafect siRNA transfection reagent into cells plated the previous day at 1.5 x10^5 cells per well in 6-well plates. (A) The siRNA pool was used at 1-100 nM and cofilin levels were evaluated three days after transfection by Western blot immunoassay. (B) Using 100 nM siRNA, the time course of cofilin silencing was determined over the course of 1-5 days (24-120 hours) by Western blot immunoassay. Densitometric quantification of the cofilin levels is shown below each figure and is presented as a percent of control levels normalized to the α-tubulin level in each lane.

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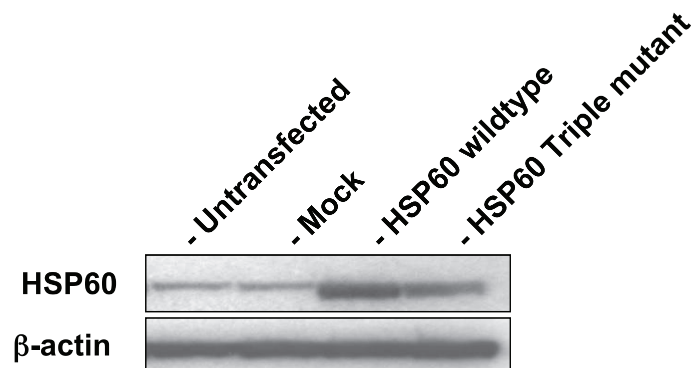

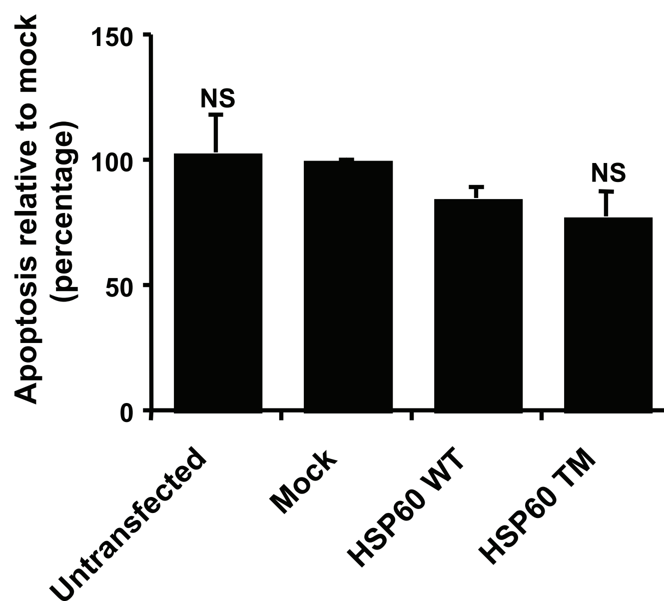

Figure S4 TnCl-induced apoptosis is not dependent upon oxidation of Cys residues in HSP60. (**A**) COS-7 cells were transfected for 6 h with expression plasmids containing different constructs of wildtype or mutant HSP60 in which all three Cys residues were mutated to Ala as described in "Methods". Total cell lysates were extracted 48 h after transfection and the expression of wild-type and mutant HSP60 proteins was followed by Western-blot immunoassay using an anti-HSP60 antibody. Alpha-tubulin protein was used as an internal control for protein loads. The blot shown is representative of 3 separate experiments. (**B**) The transfected cells described in (A) were treated with 1.5 mM TnCl for 1 h in complete media, washed, and then incubated at 37 °C in fresh media. Apoptosis was assessed by flow cytometry 24 h after TnCl treatment and is calculated relative to the levels in mock transfected cells. The data represent the mean ± SD (n=3) from at least 3 separate experiments. NS, not significantly different from wildtype transfected, TnCl-treated cells.

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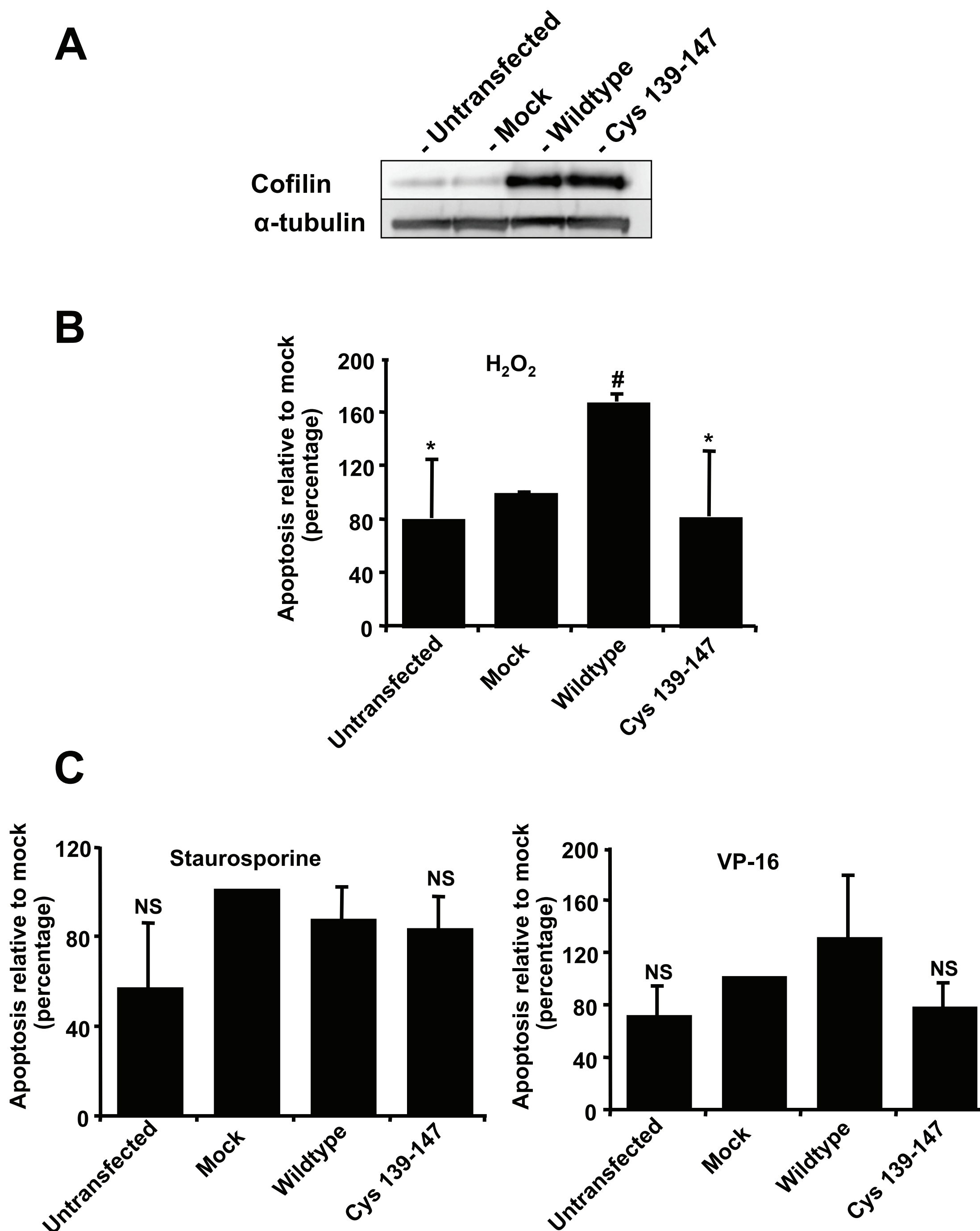

Figure S5 Oxidation of cofilin Cys residues is required for H_2O_2-induced apoptosis but not staurosporine- or VP-16-induced apoptosis. (**A**) COS-7 cells were transfected for 6 h with expression plasmids containing different constructs of wildtype or mutated cofilin. Total cell lysates were extracted 48 h after transfection and the expression of wildtype and mutant cofilin proteins was followed by Western-blot immunoassay using an anti-cofilin antibody. Alpha-tubulin protein was used as an internal control for protein loads. The blot shown is representative of 3 separate experiments. Transfected COS-7 fibroblasts at 80% confluence were exposed (1 day after transfection) for 1 h to (B) 1 mM H_2O_2 in complete DMEM medium; 100 μM VP-16 (C, left panel), or 1 μM staurosporine (C, right panel). The poly (ADP-ribose) polymerase inhibitor 3-aminobenzamide (500 μM) was added to cells 30 min prior to H_2O_2 to promote apoptosis over necrosis [26]. Apoptosis was assessed by flow cytometry 24 h after each treatment and is calculated relative to the levels in mock transfected cells. The data were analyzed using the Student's t-test and show the mean ± SD from 3 separate experiments (n=3). *Different from treated wildtype cells with a $p<0.05$. NS: not significantly different from treated wildtype cells. # Different from mock transfected cells with a $p<0.05$.

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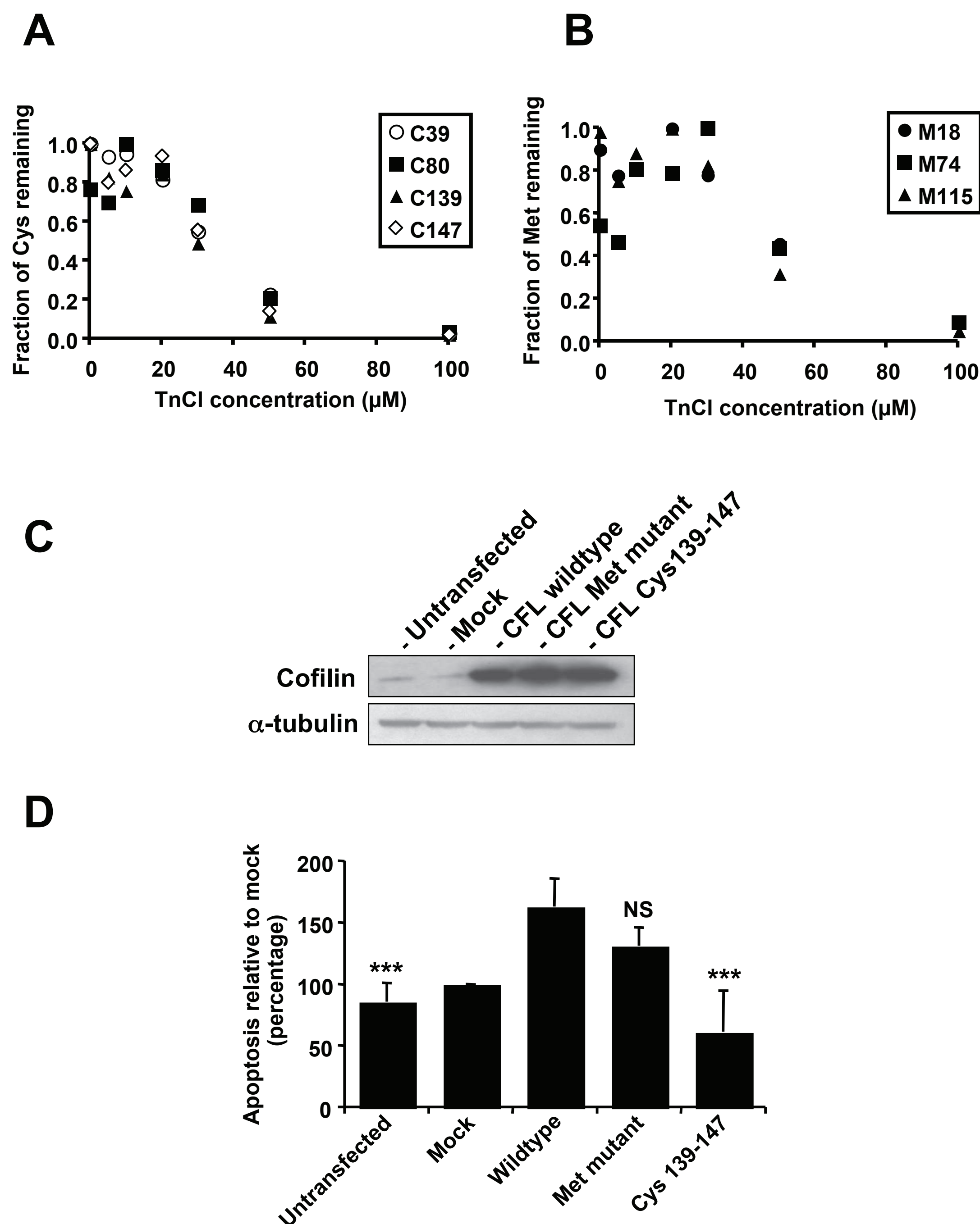

Figure S6 TnCl induces oxidation of Met residues in cofilin but this oxidation is not required for apoptosis. (A and B) Human recombinant cofilin was exposed for 30 min to the concentrations of TnCl shown in the figure (5-100 μM). Each Cys and Met residue in the protein was identified and quantified following LysC digestion and LC/MS peptide analysis as described in "Methods". The data show the fraction of each specific amino acid residue remaining relative to the initial amounts found in native, fully reduced cofilin and are from a single experiment. The lag in oxidation of the Cys and Met residues in cofilin (*i.e.*, the absence of protein oxidation at TnCl concentrations at ≤ 20 μM) is due to the presence of low molecular weight scavengers in the cofilin samples, as determined by MS, so the concentrations of TnCl required to oxidize cofilin are actually lower than those depicted in the figure. TnCl-induced apoptosis is not dependent upon oxidation of Met residues in cofilin (**C**) COS-7 cells were transfected for 6 h with expression plasmids containing different constructs of wild type or mutated cofilin (CFL) in which all three Met residues were mutated to Leu residues as described in Methods. Total cell lysates were extracted 48 h after transfection and the expression of wildtype and mutant cofilin proteins was followed by Western-blot immunoassay using an anti-cofilin antibody. Alpha-tubulin protein was used as an internal control for protein loads. The blot shown is representative of 3 separate experiments. (**D**) The transfected cells described in (**C**) were treated with 1.5 mM TnCl for 1 h in complete media, washed, and then incubated at 37 °C in fresh media. Apoptosis was assessed by flow cytometry 24 h after TnCl treatment and is calculated relative to the levels in mock transfected cells. The data represent the mean ± SD from at least 3 separate experiments (n=3). *** indicates a significant difference from wildtype transfected TnCl-treated cells with a p<0.001. NS, not significantly different from wildtype transfected, TnCl-treated cells.

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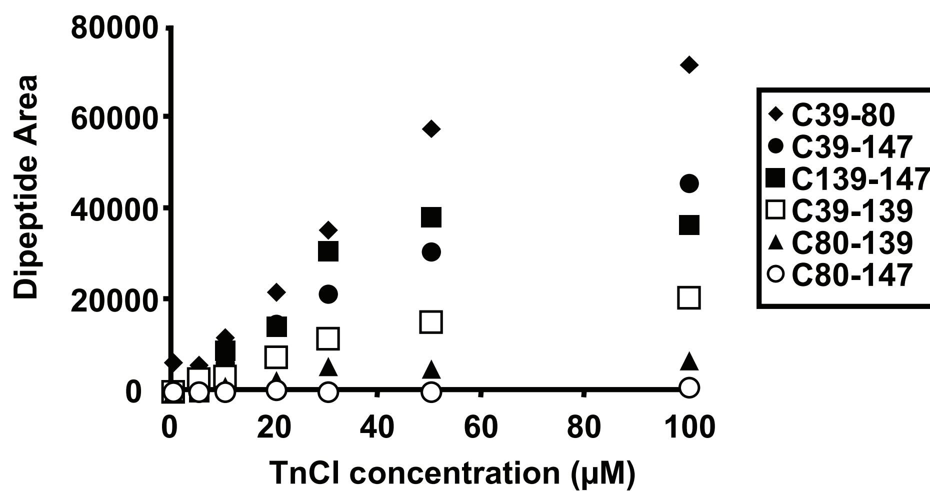

Figure S7 Mapping and quantification of disulfide bonds formed in cofilin exposed to different concentrations of TnCl. Human recombinant cofilin was exposed for 30 min to the concentrations of TnCl shown in the figure (5-100 μM). Each Cys-containing dipeptide in the protein was identified and quantified following LysC digestion and LC/MS peptide analysis as described in "Methods". The data show the integrated areas of each dipeptide peak based on the UV chromatograms (210 nm).

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Figure 1A

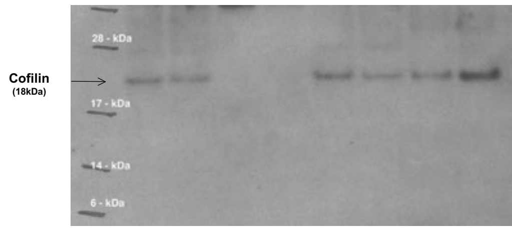


Figure 1D

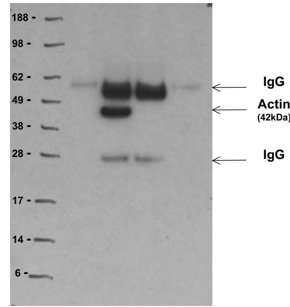
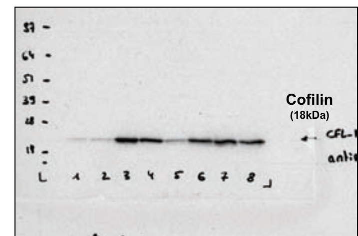


Figure 3C



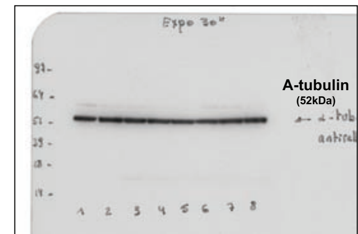
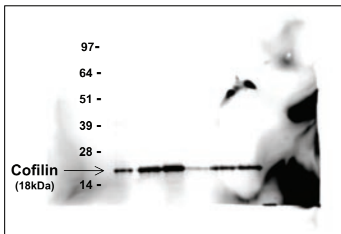


Figure 3E



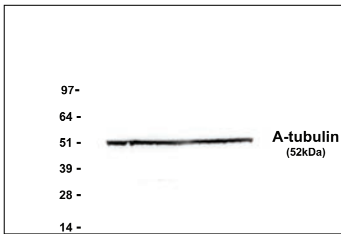
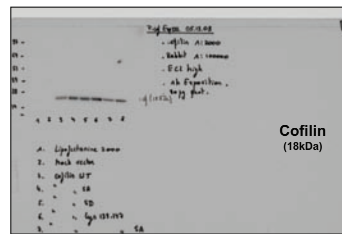
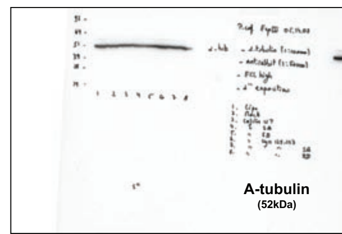


Figure 5A





Supplementary Figure 3A

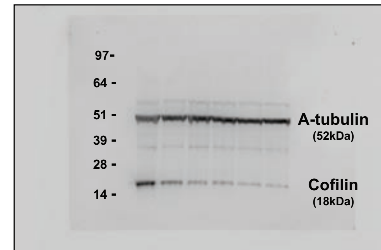
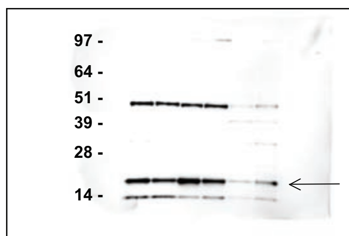
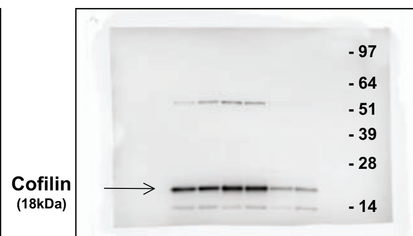
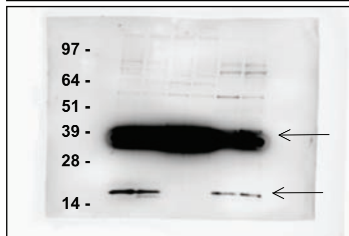


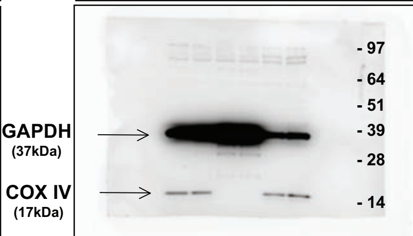
Figure 4A





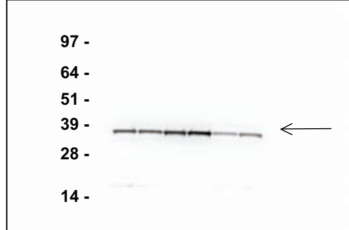
Cofilin
(18kDa)

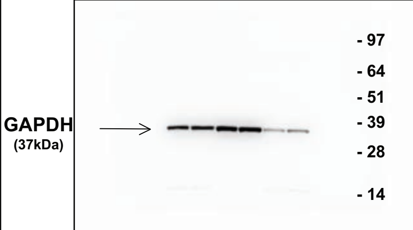




GAPDH
(37kDa)

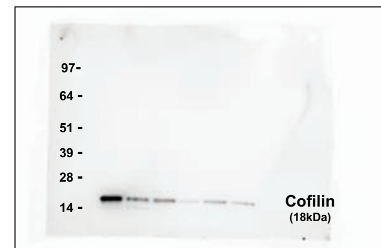
COX IV
(17kDa)





GAPDH
(37kDa)

Supplementary Figure 3B



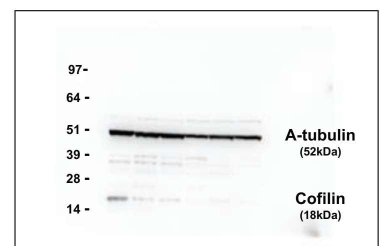


Figure S8 Full scans of blots

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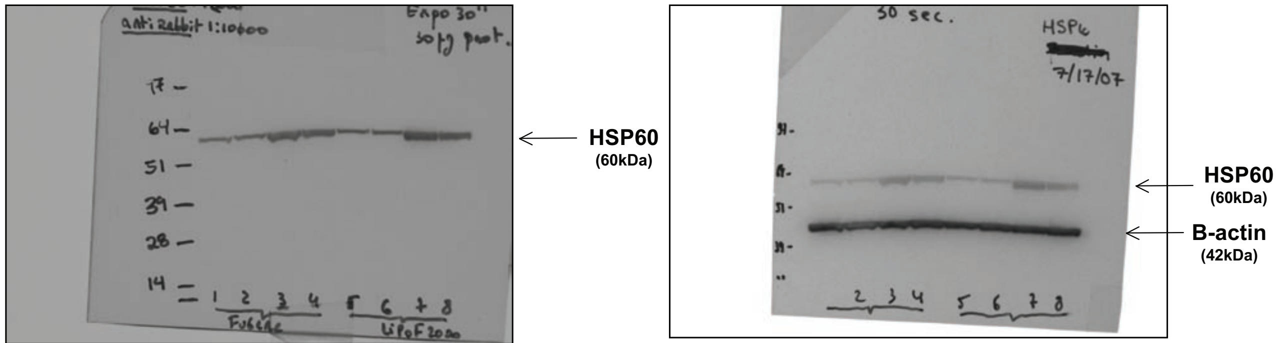
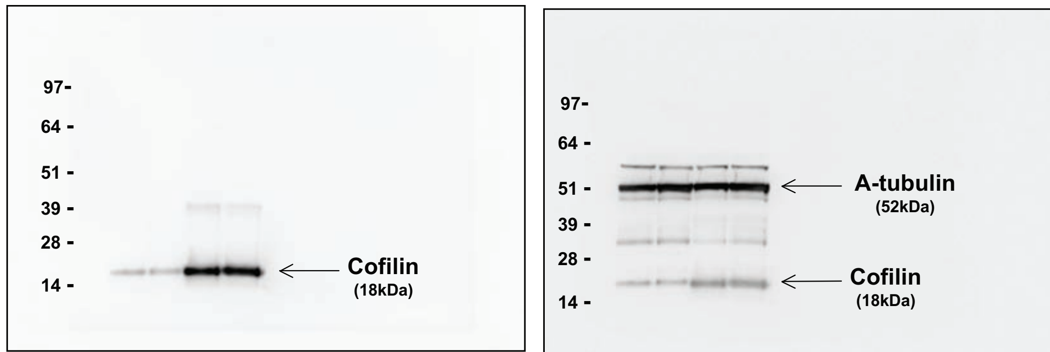
Supplementary Figure 4A



Supplementary Figure 5A



Supplementary Figure 6C

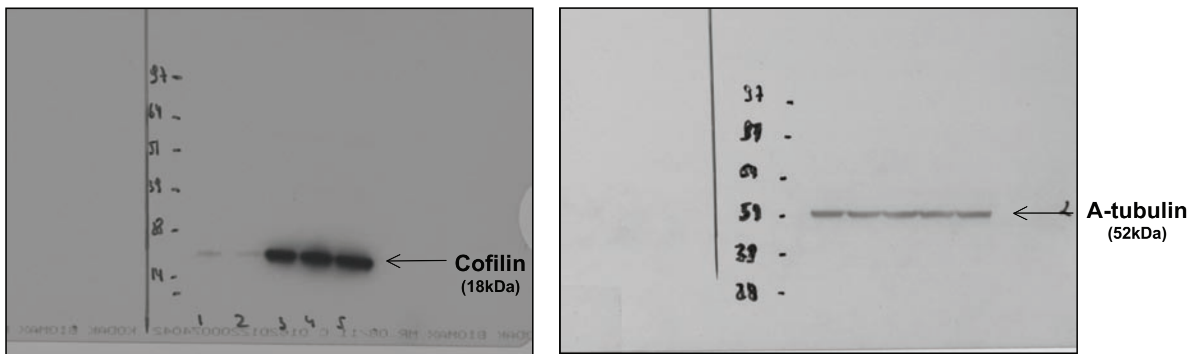


Figure S8 continued

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Supplementary Information, References

1. Yu, L.R., *et al.* Global analysis of the cortical neuron proteome. *Mol Cell Proteomics* **3**, 896-907 (2004).

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