HDAC6 Modulates Cell Motility by Altering the Acetylation Level of Cortactin

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Supplementary Experimental Procedures

Plasmids, Recombinant Proteins, and Antibodies

Several plasmids used in this study have previously been described. They include the expression plasmids for Flag-tagged HDAC5, Flag-tagged HDAC6 (pBJ-HDAC6F), Flag-HDAC6 (H216/611A) (Grozinger et al., 1999), the expression plasmid for GST-tagged full-length cortactin (pXZ122) (Uruno et al., 2001), the expression plasmid for Flag-tagged PCAF (Yang et al., 1996), the expression plasmid for HA-tagged p300 (Aizawa et al., 2004), the expression plasmids PXY68 and PXY69 for PCAF mutants Δ579-608 and Δ609-624 (Yang et al., 1996; Blanco et al., 1998), and the expression plasmids for Myc-tagged full-length cortactin (aa 1-546) and various Myc-tagged and Flag-tagged cortactin fragments (Weed et al., 2000; Wu and Parsons, 1993). Additional cortactin deletion mutants were generated by PCR and subcloned into the pRK5-Myc and pGEX vectors to create expression vectors for other Myc-tagged and GST-tagged cortactin fragments, respectively. The plasmid expressing Rac1G12V was purchased from the Guthrie cDNA Resource Center. More extensive details of all plasmid constructions are available upon request.

The rabbit polyclonal anti-acetyl-cortactin antibody was raised against a mixture of two lysine-acetylated peptides corresponding to the cortactin repeat region (FGGacKFGVGKC and

FGGacKYGVGKC, where acK denotes acetyl-lysine). These antibodies were first tested by ELISA against the non-acetylated and acetylated peptides to determine the extent of antibody cross reactivity. Once an animal with a specific anti-acetyl-cortactin antibody response was identified, antibody from the selected animal was purified over non-acetylated and acetylated peptide sepharose. The polyclonal anti-HDAC6 antibody has been described previously (Bertos et al., 2004). The anti-acetyl-lysine antibody, the anti-acetyl-H4 antibody, the anti-cortactin antibody (4F11), the anti-SIRT2 antibody, recombinant PCAF, and recombinant p300 were purchased from Upstate (Millipore). Anti-Flag, anti-HA, anti-tubulin, anti-acetyl-tubulin, and anti-β-actin antibodies were purchased from Sigma. Anti-Myc antibody was purchased from Santa Cruz. Anti-AcK382-p53, anti-p53, and anti-His6 antibodies were purchased from Calbiochem. Alexa-594- and Alexa-488-conjugated secondary antibodies were purchased from Molecular Probes. Anti-phospho-ERK and anti-ERK antibodies were purchased from Cell Signaling.

GST Pull-Down Assay

DH5α cells harboring either the pGST or the pGST-cortactin expression plasmid were grown to log phase and induced with isopropyl-thio-β-D-galactoside (IPTG) for 4 hr. After sonication in STE buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol) containing 1% sarcosyl (w/v, final concentration), solubilized proteins were recovered by centrifugation and incubated with glutathione-agarose beads in the presence of 3% Triton X-100 (final concentration) for 30 min at 4°C and washed several times with ice-cold phosphate buffer saline (PBS).

For binding reactions, beads were mixed with histidine-tagged HDAC6 proteins (expressed in bacteria and purified using the QIAexpress Ni-NTA Protein Purification System) for 1-h at room temperature. Unbounded proteins were washed extensively with STE buffer containing 0.1% NP-40, and bound proteins were eluted from the beads by boiling in SDS loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Final products were analyzed on a SDS-polyacrylamide gel and detected by Western blotting with anti-His6 antibodies.

Two-Dimensional Gel Electrophoresis

The first-dimensional isoelectric focusing (IEF) was carried out on an IPGphor system (Amersham Biosciences). The immunoprecipitates were dissolved in 50 μl 2D specific lysis buffer (9 M urea, 4% CHAPS, 40 mM Tris-base, 40 mM DTT) and loaded onto an immobilized pH gradient (IPG) strip (pH 4-7, 24 cm, GE Healthcare) in a total volume of 450 μl rehydration solution (8 M urea, 2% [wt/vol] CHAPS, 10 mM DTT, 0.5% [vol/vol] IPG buffer [pH 4-7], trace amount of bromophenol blue) for 10 h. After rehydration, IEF was initially carried out at 200 V. The voltage was gradually increased to 10,000 V and then kept constant for 5-6 h at 20ºC (approximately 60–80 kVh in total). After IEF, the IPG strip was immediately equilibrated in 5 ml SDS equilibration buffer (6 M urea, 50 mM Tris-HCl [pH 8.8], 30% glycerol, 2% SDS, 1% [wt/vol] DTT, trace amount of bromophenol blue) for 15 min with gentle shaking. The second dimensional separation was carried out on 8% SDS-PAGE gels. Western blotting analyses were performed using anti-acetyl-lysine antibodies, and the blot was stripped and re-probed with anti-cortactin antibodies. Spots corresponding to acetylated cortactin and cortactin were quantified using ScnImage Software.

Tandem Mass Spectrometry Acetylation Site Analysis

Gel slices containing acetylated cortactin were washed once with water and twice with 50 mM ammonium bicarbonate in 50% acetonitrile. The slices were then divided in half and proteolyzed overnight with modified sequencing grade trypsin (Promega) or with chymotrypsin. Extracted peptides were sequenced using a nanoflow liquid chromatograph coupled to an electrospray LTQ ion trap mass spectrometer. The peptides were separated on a custom packed C18 reverse phase column using a 1% per minute gradient at 250 nl/min from 1% B to 51% B (A: 0.03% acetic acid; B: 100% acetonitrile/0.03% trifluoroacetic acid). Four tandem mass spectra were acquired for each MS survey scan (spray voltage 3 kV, 30% normalized collision energy, scanning m/z 395-1,600, isolation width = 2.5 amu, dynamic exclusion on). Preliminary sequencing of peptides was facilitated by NCBI nr database correlation with the algorithm SEQUEST (Eng et al., 1994), an in-house spectrum review workbench, FuzzyIons, and GraphMod, a program for analyzing the position(s) of a modification within a peptide sequence for a given MS/MS spectrum. All spectra were manually inspected for completeness of ion assignments and intensity-based signatures (*e.g.*, neutral loss(es), proline ions). For database searches, oxidized methionine and acetylated lysine were selected as variable modifications, and two missed cleavages were allowed.

Three-Dimensional Modeling of Cortactin

The template search using the Modeller program (Jones, 1999) yielded an SH3 motif (carboxy-terminal residues 489-546) from the structure of a homologue in the Protein Data Bank (PDB code 1JO8). The GenThreader method (McGuffin et al., 2000) was used for protein fold

recognition to obtain a template for the N-terminal acidic domain (residues 2-112, PDB code 1YOZ). Finally, the cortactin domain was built from a prediction of secondary structure using the server PSIPRED (Jayasinghe et al., 2001) and from the orientation of the helices based on their hydrophobic moments calculated with MExP (Chen et al., 2003). The three domains were docked with ZDOCK (Xiang and Honig, 2001), and the first template was constructed with Jackal (Xiang and Honig, 2001).

The initial template was subjected to molecular dynamics simulation under Langevin ensemble at 1,000 K for 2 ns and simulated annealing was performed by slow cooling to 300 K for a 3 ns total simulation. All molecular dynamics simulations were performed in a vacuum with the NAMD program using CHARMM27 force field. A step descent minimization protocol was applied to the converged structure for 5,000 steps. The final structure was refined using the Modeller 8 software package, tested for secondary restrictions, and deposited in the Protein Data Bank (PDB code 2F9X).

Cell Migration Assay

One day before a migration assay, cells were serum-starved. Next, a cell suspension containing 0.5 - 1.0 x 10^6 cells/ml was prepared in serum free media and 300 μl of the cell suspension solution was added to the inside of each insert. After adding 500 μl of media containing 10% fetal bovine serum to the lower well of the migration plate, the migration plate was incubated in a cell culture incubator for 8-9 h. The media in the inside of the insert was carefully aspirated and cotton-tipped swabs were used to gently remove non-migratory cells from the interior of the inserts. Subsequently, the inserts were stained in 400 μl Cell Stain Solution provided by the kit for 10 min at room temperature. After washing the stained inserts several

times in a beaker of water, the inserts were allowed to air dry. The stained cells were either captured by camera or quantitated by applying 200 μl of extraction solution from the kit to each well and then incubating for 10 min and measuring the OD 560 nm on a plate reader.

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Supplemental Data

Table S1. Quantification of the percentage of cells co-expressing active Rac1 and the wildtype, 9KQ, or 9KR cortactin that show a leading edge

	Flag-cortactin (WT) HA-Rac1G12V	Flag-9KQ HA-Rac1G12V	Flag-9KR HA-Rac1G12V
Total cell numbers	95	59	100
Cells showing a leading edge	68	26	76
Percentage of cells showing a leading edge	71.6%	44%	76%

Supplemental Figure 1

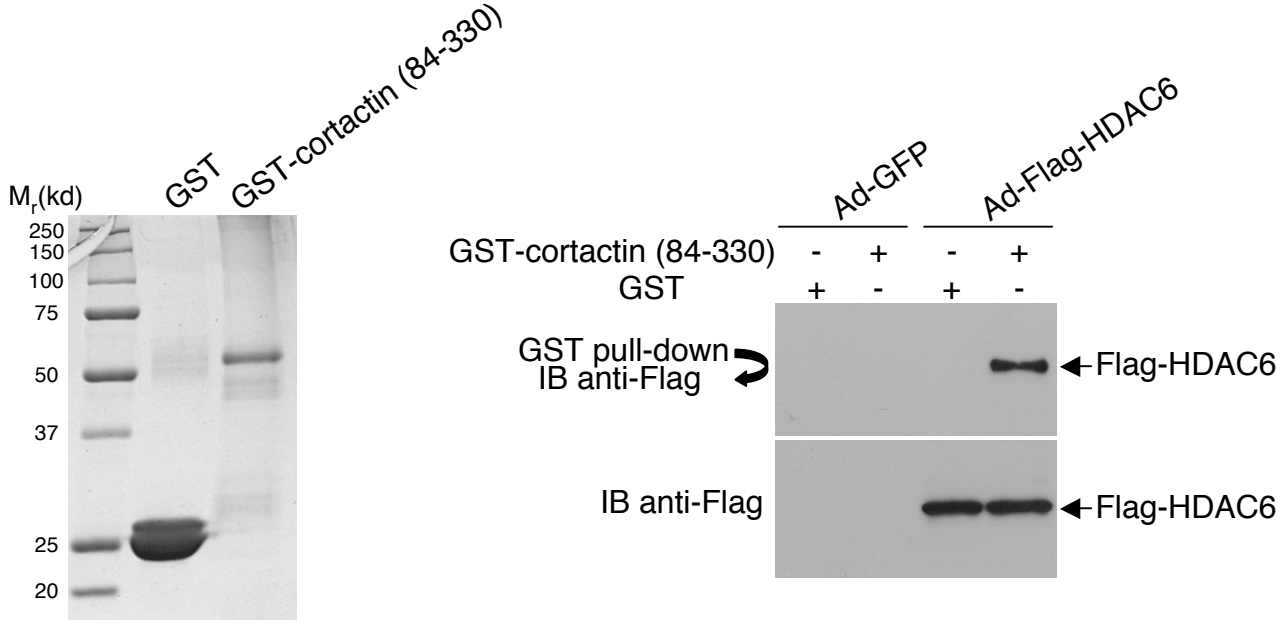

Figure S1. The cortactin repeat region alone is sufficient for interaction with HDAC6. GST and GST fused to the repeat region of cortactin (84-330) were expressed and purified from bacteria (left panel). 293T cells were transduced with adenoviruses expressing either GFP or Flag-HDAC6. Thirty hours post-infection, cells were lysed and lysates were incubated overnight at 4°C with GST or GST-cortactin (84-330) conjugated to glutathione-Sepharose beads. After extensive washing, bound proteins were eluted and analyzed by Western blotting with anti-Flag antibodies (right top panel). Expression of Flag-HDAC6 was determined by a direct Western blot with anti-Flag antibodies (right bottom panel).

Supplemental Figure 2

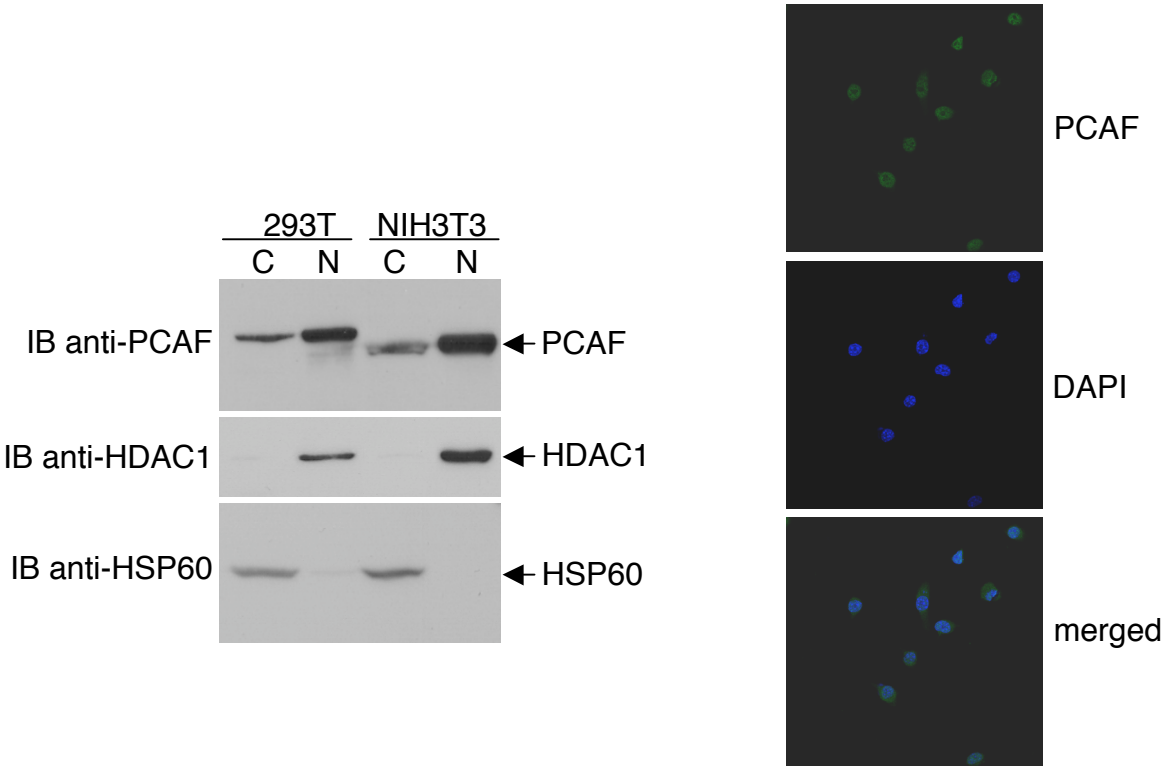

Figure S2. Subcellular localization of endogenous PCAF. Left panels, 293T or NIH3T3 cells grown in 100 mm tissue culture plates were collected by scraping into PBS, centrifuged, and resuspended in 1 ml of ice cold RSB buffer (10 mM Hepes [pH 6.2], 10 mM NaCl, 1.5 mM $MgCl_2$, and 0.5 mM phenylmethylsulfonyl fluoride) with a Kontes Dounce homogenizer (10 strokes with a type A pestle). The resulting suspension was centrifuged for 10 min at 700 *g* in 4°C and the pellet was resuspended again in 1 ml of RSB buffer. After homogenization as before, the suspension was combined with the supernatant obtained from the previous step and centrifuged further for 10 min at 700 *g*. The final pellet (N, nuclear fraction) was washed with RSB buffer and resuspended in 2 ml of SDS sample buffer and the supernatant (C, cytoplasmic fraction) was combined with 2 ml of of 2X SDS sample buffer. A small aliquot of each fraction was subjected to electrophoresis and Western blotted with anti-PCAF (top panel). The blot was sequentially stripped and re-blotted with anti-HDAC1 and anti-HSP60 antibodies to assess purity of fractionation. Right panels, NIH3T3 cells were immunostained with anti-PCAF and 4',6-diamidino-2-phenyl-indole-stained. The merged image represents the overlay of the two panels.

Supplemental Figure 3

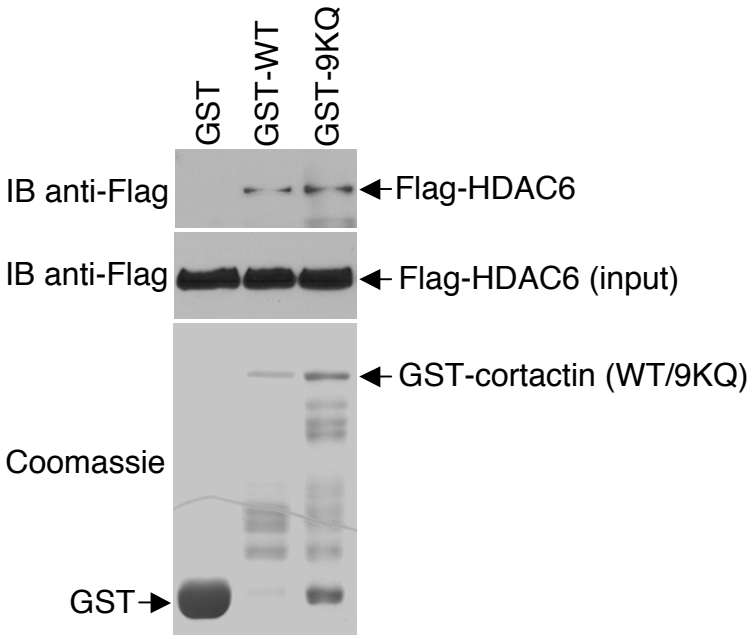

Figure S3. Lysine to glutamine mutations in cortactin does not affect its binding to HDAC6. 293T cells were infected with adenoviruses that express Flag-HDAC6. Thirty hours post-infection, cells were treated with lysis buffer and cell extracts were prepared and incubated overnight with GST, GST-WT or GST-9KQ coupled to agarose beads. Protein-bound beads were washed extensively with lysis buffer, eluted, and subjected to SDS-PAGE, followed by western blot analysis with anti-Flag antibodies (top panel). Expression of Flag-HDAC6 and GST fusion proteins were monitored by a direct Western blot with anti-Flag and Coomassie blue staining, respectively.

Supplemental Figure 4

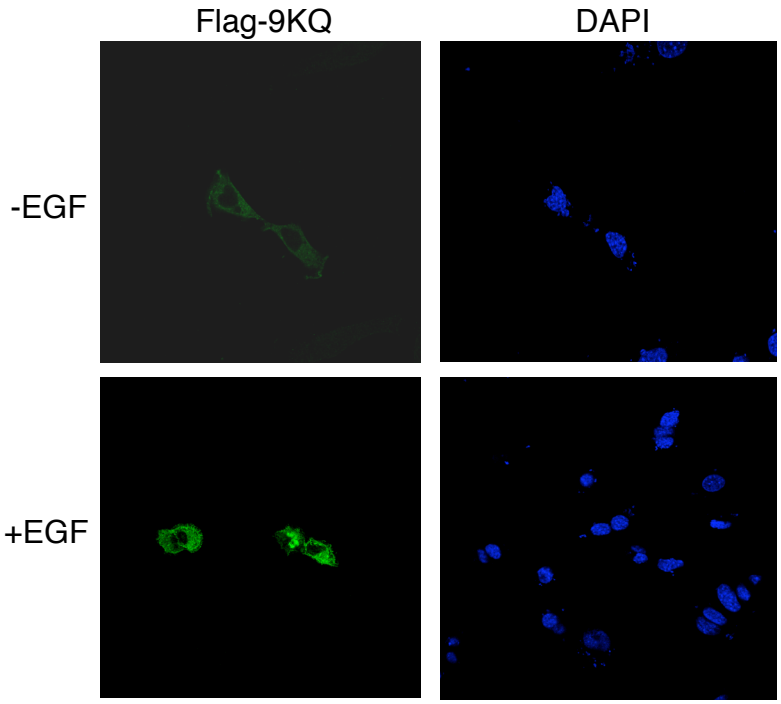

Figure S4. Subcellular localization of 9KQ mutant. NIH3T3 cells were transfected with Flag-9KQ and serum starved overnight. Cells were then either left untreated (-EGF) or treated with 20 ng/ml EGF for 10 min (+EGF). Cells were immunostained with anti-Flag antibodies and Alexa-488 conjugated anti-mouse secondary antibodies and analyzed by confocal microscopy.

Supplemental Figure 5

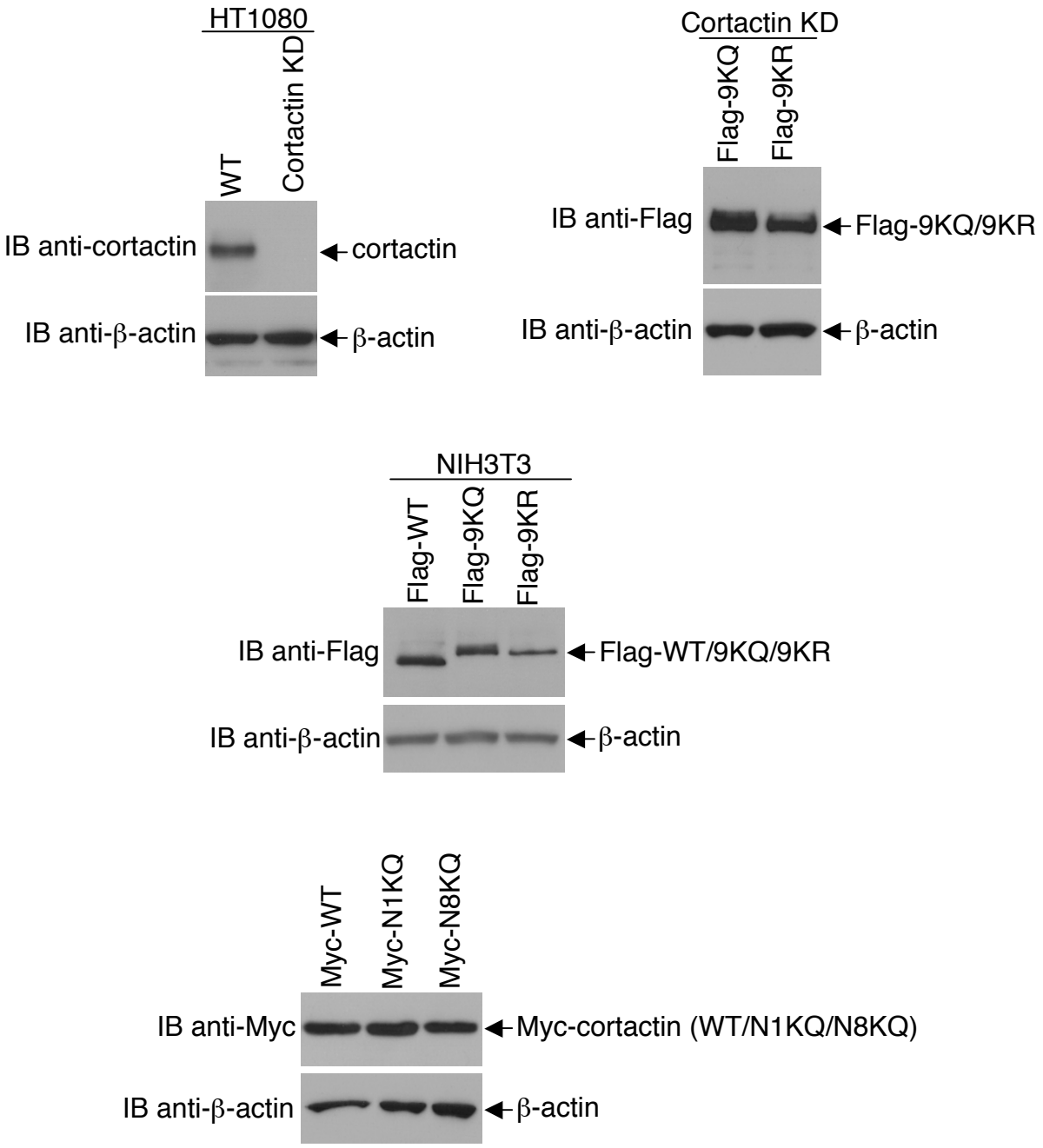

Figure S5. Expression of endogenous cortactin and overexpressed wildtype and mutant cortactins. Cell lysates prepared from transfected or untransfected cells were separated on SDS-PAGE and Western blotted with the indicated antibodies.