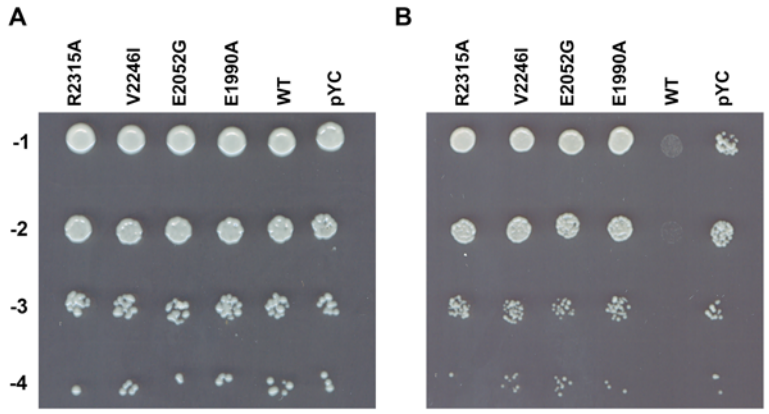


**Fig. S1**. Mutations in $ACD_{Vc}$ that have decreased levels of actin cross-linking activity in HeLa cells, do not have differences in viability when expressed in yeast. Dilutions of *Sc* containing either pYC, pYC-ACD (WT), or pYC-ACD with the indicated mutants, were spotted onto plates containing either Glu (A) or gal and raf (B) and grown for 48 hrs at 30°C.

**Table S1.** Oligonucleotide primers used in this study. Restriction enyzme recognition sequences are underlined.

| Name | Sequence 5'-3' |
|---|---|
| *ACD LSM* | |
| ACDentr UP | CACCATGAGATCTCAACCAACGGGTCAACTGG |
| ACDentr DOWN | GCTAGCTCGATACTCCTGATACCAAG |
| *Ala Substitution* | |
| S1986A sense | GCCATTTCGCATCGACAGCAATTGGTATAGAAAATGAGTTATC |
| S1986A anti | GATAACTCATTTTCTATACCAATTGCTGTCGATGCGAAATGGC |
| E1990A sense | CATCCATTGGTATAGCAAATGAGCTCTCCGGTCTGTCCGG |
| E1990A anti | CCGGACAGACCGGAGAGCTCATTTGCTATACCAATGGATG |
| N1991A sense | CATCCATTGGTATAGAAGCTGAGTTATCCGGTCTG |
| N1991A anti | CAGACCGGATAACTCAGCTTCTATACCAATGGATG |
| N2003A sense | TGGTTTTACCGAAAGCTTCAGCGCAGACTTTTGG |
| N2003A anti | CCAAAAGTCTGCGCTGAAGCTTTCGGTAAAACCA |
| S2004A sense | GGTTTTACCGAAAAACGCAGCGCAGACTTTTGGC |
| S2004A anti | GCCAAAAGTCTGCGCTGCGTTTTTCGGTAAAACC |
| T2023A sense | ACCCATTGTTCATGCTAGCCAAGGATATGAATCAAGG |
| T2023A anti | CCTTGATTCATATCCTTGGCTAGCATGAACAATGGGT |
| K2024A sense | CCCATTGTTCATGCTAACCGCGGATATGAATCAAGGTGG |
| K2024A anti | CCACCTTGATTCATATCCGCGGTTAGCATGAACAATGGG |
| D2025A sense | GTTGTTCATGCTAACCAAGGCTATGAATCAAGGTGGTT |
| D2025A anti | AACCACCTTGATTCATAGCCTTGGTTAGCATGAACAAC |
| *ACD Yeast* | |
| pYC-ACD UP | GGTACCATGGGAAGTCAACCAACGGG |
| pYC-ACD DOWN | TCTAGATGTGAGCGTCTCATGGTTATC |
| pYC-ACD recomb UP | GGAAGTCAACCAACGGGTCAACTGGC |
| pYC-ACD recomb DOWN | TGTGAGCGTCTCATGGTTATCAGTATAAGGAGCGGTAATTTTC |
| Glu1992Ala | TTCGCATCGACATCCATTGGTATAGAAAATGCGTTATCC |
| Glu2052Ala | CAATGATATTCAAGGGGTGAACAACTGGCAGACGCATACGATTGCACTGGTTAC |
| Ser2058Ala | GTGAACAACTGGCAGACGCATACGATTGAACTGGTTACATATCCTGCTGAAATC |
| His2083Ala | GAGGCAATGCTATGGCTTGCGAAAGAGTTTACCGATGCTATCAATCAGT |
| Asn2085Ala | CTATGGCTTGCGAAAGAGTTTACCGATCATATCGCTCAGTCTAAC |
| Ser2087Ala | GCGAAAGAGTTTACCGATCATATCAATCAGGCTAACCACCAAAGC |
| His2089Ala | GCGAAAGAGTTTACCGATCATATCAATCAGTCTAACGCCCAAAGC |
| His2111Ala | CGTTTCACTCTGGTTATATCGAACTCTAAGGCTCTTATTG |
| Ser2133Ala | GATGCACAAGGCAAGACCATAGGAATGACCCCTGCTGGCC |
| Arg2155Ala | GCGAAAGAATTTGGTACAAGCTCGTCGCCGGAAGTCGCACTGCTTGAATC |
| Ser2159Ala | GGTACAAGCTCGTCGCCGGAAGTCAGACTGCTTGAAGCTGCGCC |
| Ser2195Ala | GCACAAAATGTGTATGCCTATCTCACGGCTG |
| Lys2205Ala | TCTGTCTATTCAAAAACAGCAGATTTGGCCGCAGAGTAT |
| Glu2313Ala R | CTGCGAAACGCAAATAAGATCGCTGGTTCAGGCTGTTGCACACTTCCTGTC |
| Arg2315Ala R | TACAAAGTCACTTAATGCACTCGGTACACTGGCAAACTC |
| Lys2327Ala R | ACTTTCACCGTTGACGCTGTTGCGGGTA |
| Lys2337Ala R | TTGCCGAGTCAAAATGATCGAGTGCCGCAACATC |

**Table S2.** The location of each linker-scanning insertion and its ability to crosslink actin upon transient transfection and expression from the indicated plasmid in COS-7 cells. +, crosslinking indistinguishable from WT; -, no detectable crosslinking; nd, not determined.

| RtxA AA# | pDEST-ACD | pEGFP-ACD | RtxA AA# | pDEST-ACD | pEGFP-ACD |
|---|---|---|---|---|---|
| P1996 | + | *nd* | L2092 | - | - |
| K1979 | - | + | L2095 | - | - |
| S1984 | - | + | K2110 | + | *nd* |
| S1986 | - | + | I2121 | - | + |
| G1988 | - | - | P2132 | + | *nd* |
| L1993 | - | - | A2137 | - | - |
| G1995 | - | + | S2149 | + | *nd* |
| V1997 | - | - | S2151 | + | *nd* |
| N2003 | - | - | E2153 | - | + |
| A2005 | - | + | L2167 | + | *nd* |
| V2011 | + | *nd* | L2180 | + | *nd* |
| H2012 | + | *nd* | D2181 | + | *nd* |
| S2014 | - | + | N2188 | - | + |
| P2018 | + | *nd* | Y2197 | - | + |
| L2022 | - | - | E2206 | - | + |
| G2029 | - | + | Y2210 | + | *nd* |
| Y2031 | + | *nd* | N2212 | - | + |
| N2033 | + | *nd* | D2213 | - | + |
| Q2041 | + | *nd* | F2221 | - | + |
| G2042 | + | *nd* | W2237 | - | - |
| N2045 | + | *nd* | L2249 | - | + |
| Q2047 | - | + | L2268 | + | *nd* |
| T2048 | - | + | S2275 | - | - |
| H2049 | - | + | G2288 | - | *nd* |
| T2050 | + | + | I2295 | + | *nd* |
| Y2056 | - | + | V2303 | + | *nd* |
| S2058 | - | + | Q2304 | + | *nd* |
| E2067 | - | + | P2318 | - | - |
| L2074 | - | - | L2321 | + | *nd* |
| W2075 | - | + | S2322 | + | *nd* |
| L2076 | - | - | K2327 | + | *nd* |
| A2077 | - | + | S2330 | + | *nd* |
| K2078 | - | + | T2331 | + | *nd* |
| E2079 | - | + | D2343 | + | *nd* |
| S2087 | - | + | K2348 | + | *nd* |

**Table S3.** Ala-substitutions in ACD constructed in either pEGFP-ACD or pYC-ACD based on LSM and error-prone PCR approaches. Each plasmid was screened for actin cross-linking in transiently transfected HeLa cells (pEGFP-ACD plasmids) or the ability to allow *S. cerevisiae* growth when plated on media containing galactose (pYC-ACD plasmids). The symbols in the pEGFP-ACD columns indicate; +, mutant displayed similar actin crosslinking as wild-type; -, mutant completely abolished actin crosslinking activity; +/-, mutant reduced actin crosslinking activity compared to wild-type. The symbols in the pYC-ACD column indicate functional ACD (no growth, (+)) or defective ACD (growth, (-)) on gal+raf plates. Not all mutations were constructed on both plasmids (*nd*).

| **RtxA AA#** | **pEGFP-ACD** | **pYC-ACD** | **RtxA AA#** | **pEGFP-ACD** | pYC-ACD |
|---|---|---|---|---|---|
| Wild-type | **+** | **+** | D2122 | **+** | *nd* |
| S1986 | **+** | *nd* | K2126 | **+** | **+** |
| E1990 | **-** | **-** | T2127 | **+** | **+** |
| N1991 | **+** | *nd* | S2133 | *nd* | **+** |
| E1992 | **+/-** | **-** | Q2135 | **+** | *nd* |
| N2003 | **+** | *nd* | T2138 | **+** | *nd* |
| S2004 | **+** | *nd* | S2159 | *nd* | **+** |
| T2023 | **+** | *nd* | S2195 | *nd* | **+** |
| K2024 | **+** | **+** | K2205 | *nd* | **+** |
| D2025 | **+/-** | **-** | N2224 | **+** | *nd* |
| N2027 | **+** | *nd* | T2229 | **+** | *nd* |
| Q2028 | **+** | *nd* | K2232 | **+** | *nd* |
| S2058 | *nd* | **+** | K2234 | **+** | *nd* |
| R2069 | **+** | *nd* | N2235 | **+** | *nd* |
| K2070 | **+** | *nd* | T2243 | **+** | *nd* |
| L2074 | **+** | *nd* | K2244 | **+** | *nd* |
| L2076 | **+** | *nd* | D2256 | **+** | *nd* |
| H2089 | *nd* | **+** | S2273 | **+** | *nd* |
| L2092 | **+** | *nd* | E2289 | **+** | *nd* |
| L2095 | **+** | *nd* | H2293 | **+** | **+** |
| S2097 | **+** | *nd* | R2315 | **+/-** | **-** |
| D2099 | **+** | *nd* | K2327 | *nd* | **+** |
| R2101 | **+** | *nd* | K2337 | *nd* | **+** |
| H2111 | *nd* | **+** | | | |

**Table S4.** Ala-substitutions in ACD constructed in either pEGFP-ACD or pYC-ACD based on a structural alignment of the glutamine synthetase and γ-glutamylcysteine synthetase active site (**Fig. 5A**). The degree of actin cross-linking in HeLa cells transfected with each pEGFP-ACD plasmid was tested; +, indistinguishable from wild-type level of actin crosslinking; -, no actin crosslinking activity; +/-, reduced actin crosslinking activity compared to wild-type. The symbols in the pYC-ACD column indicate functional ACD (no growth, (+)) or defective ACD (growth, (-)) on gal+raf plates. Not all mutations were constructed on both plasmids (*nd*).

| **RtxA AA#** | **pEGFP-ACD** | **pYC-ACD** |
|---|---|---|
| Wild-type | **+** | **+** |
| E2052 | **+/-** | **-** |
| H2083 | *nd* | **-** |
| N2085 | *nd* | **+** |
| S2087 | *nd* | **+** |
| R2155 | *nd* | **+** |
| R2242 | **+** | *nd* |
| R2313 | **+/-** | **-** |