Supplemental Materials

Molecular Biology of the Cell

Peters et al.

Supplemental Figures

Supplemental Figure 1: Ric-8 is depleted by dsRNAs directed against the 5'/3' UTR of the gene. Protein levels were determined by immunoblot with anti-Ric-8 and an antibody to α-tubulin was used as a loading control.

Supplemental Figure 2: Myc-tagged Cta functions as a proxy for wild-type and constitutively inactive Cta. (A) Expression of Myc-Cta rescues the ability of cells depleted of endogenous Cta to respond to Fog. Transfected cells were identified using an anti-Myc antibody. Scale bar= 20 μm. (B) Myc-Cta can rescue constriction in response to Fog in the absence of endogenous Cta, while constitutively inactive Myc-Cta_{GA} cannot. Quantification shows percentage of S2R+ cells within a population transfected with Myc-Cta or Myc-Cta_{GA} depleted of endogenous Cta able to contract in response to Fog application (±SEM).

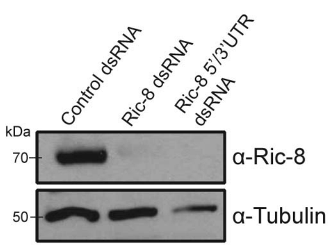
Supplemental Figure 3: Membrane localization of Cta is reduced in the absence of Ric-8. (A) Myc-Cta localizes to the membrane in cells treated with control dsRNA. (B) Myc-Cta localization is decreased at the membrane in cells treated with Ric-8 dsRNA. S2 cells were treated with either control or Ric-8 dsRNA and stained for Myc (green); Membrane=Gap43 cherry expression (magenta). Note the enrichment of Cta in the membrane ruffles, shown in insets, which is absent in cells treated with Ric-8 RNAi. Enlarged images of boxed areas are shown in insets. Scale bar = 20 μm.

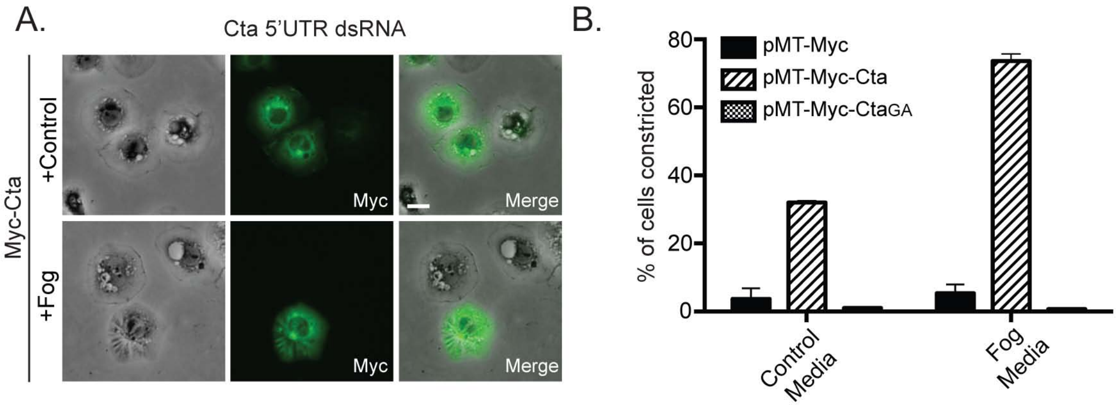
Supplemental Figure 4: Mito-Ric-8-GFP co-localizes with a mitochondrial specific antibody. (A) S2 cells transfected with Mito-Ric-8-GFP co-localize with an antibody specific to mitochondria while (B) cells transfected with Ric-8GFP do not. Enlarged images of boxed areas are shown in insets. Scale bar = 20 μm.

Supplemental Figure 5: Sequence alignment of Ric-8 across taxa reveals evolutionarily conserved residues. Residue clusters used for Ric-8 mutational analysis are highlighted in yellow. The number of the mutant cluster is indicated below its residues.

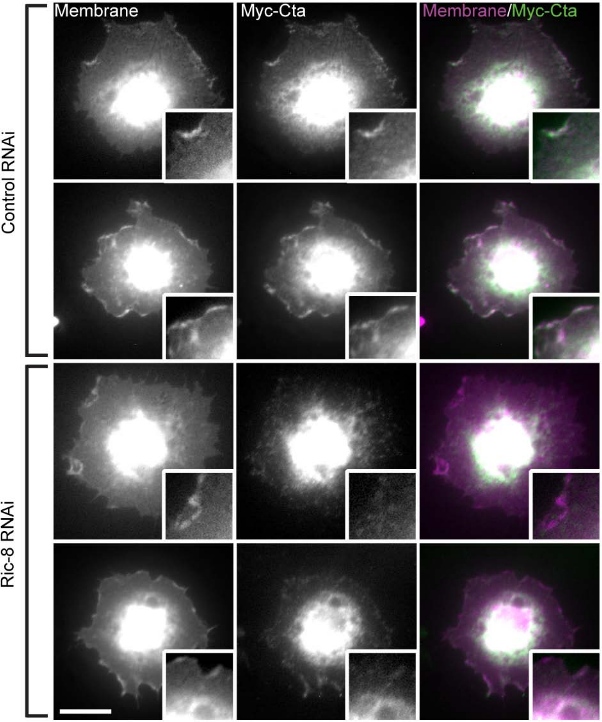
Supplemental Figure 6: Location of individual point mutants comprising mutant clusters that strongly inhibit Cta_{GA} binding are mapped onto a structural model of Ric-8. Note that glutamic acid-487 is buried within the predicted molecule.

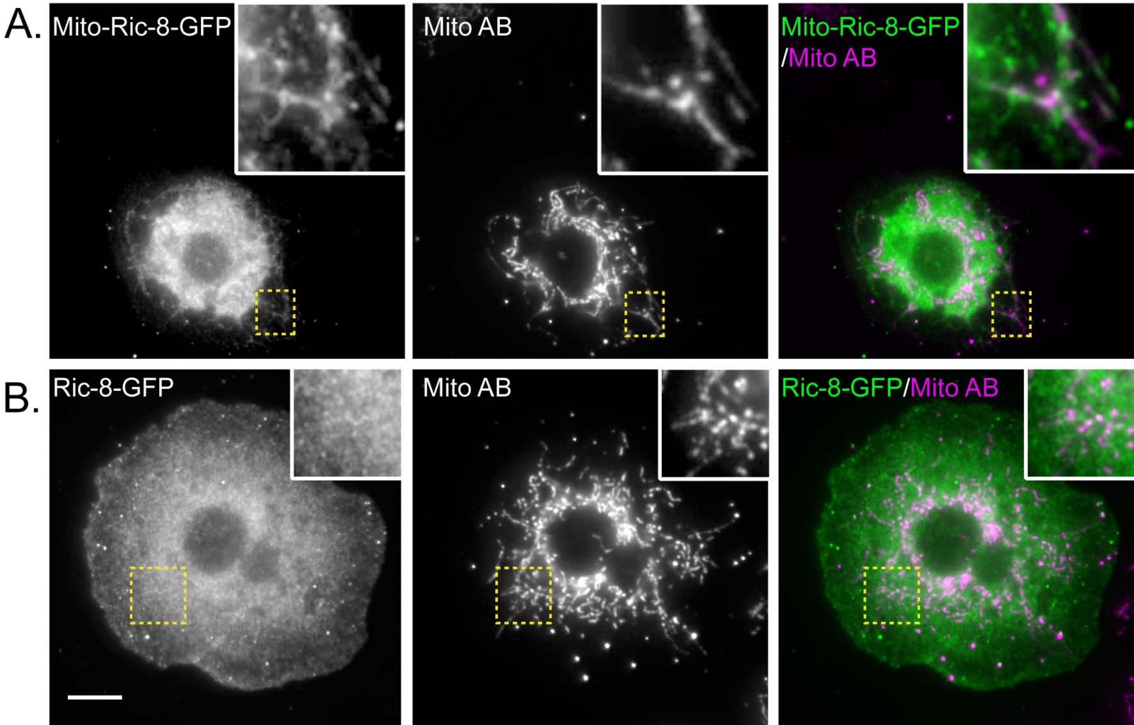
Supplemental Figure 7: Wild-type and constitutively active Cta exhibit differential binding to Ric-8 cluster mutants as compared to inactive Cta. (A-D) Similarly to Myc-Cta_{GA}, both Myc-Cta and Myc-Cta_{QL} are deficient in binding Ric-8-GFP cluster mutant 1. However, unlike Myc-Cta_{GA}, Myc-Cta and Myc-Cta_{QL} are capable of binding mutants 9, 10 and 13. (A) S2 Cells were transfected with GFP, Ric-8-GFP or cluster Ric-8-GFP mutants and wild-type Myc-Cta. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (B) Quantification of IPs presented in S7A. The pulldown:input ratios were determined using quantitative densitometry, and normalized to Ric-8-GFP (±SEM; error bars, $p<0.05$). (C) S2 Cells were transfected with GFP, Ric-8-GFP or cluster Ric-8-GFP mutants and Myc-Cta_{QL}. IPs were performed with GFP-binding protein and probed with anti-GFP and anti-Myc. (D) Quantification of IPs presented in S7C. The pulldown:input ratios were determined using quantitative densitometry, and normalized to Ric-8-GFP (±SEM). Dashed lines indicate where two separate gels have been combined.


Control dsRNA
Ric-8 dsRNA
Ric-8 5'/3'UTR dsRNA
kDa
70
50
α-Ric-8
α-Tubulin



A.
Cta 5'UTR dsRNA
Myc-Cta
+Control
+Fog
Myc
Merge
Myc
Merge
B.
pMT-Myc
pMT-Myc-Cta
pMT-Myc-CtaGA
% of cells constricted
80
60
40
20
0
Control Media
Fog Media


Membrane
Myc-Cta
Membrane/Myc-Cta
Control RNAi
Ric-8 RNAi


A.
Mito-Ric-8-GFP
Mito AB
Mito-Ric-8-GFP
/Mito AB
B.
Ric-8-GFP
Mito AB
Ric-8-GFP/Mito AB

```
Xenopus         MPAMDLGALLDELESGDQELVQKSLAEYNQENSQCFFFNAEQRE-ERKKLGELVISFLNR 59
Zebrafish       ---MDLNAIIEKMETGDQDAALTALQTYNKEKSQCFSFTSGEEE-DRERLGELVLSFLER 56
Mouse           ---MEPRAVADALETGEEDAVTEALRSFNREHSQSFTFDDAQQE-DRKRLAKLLVSVLEQ 56
Chicken         ---MELRTVVATVESGEQDAVLKVLQIYNQEKSQCFTFDDEERE-ERKKMAQLLIKFLER 56
Human           -------------------------RDYSDKHRATFKFESTDED-KRKKLCEGIFKVLIK 34
Drosophila      ----METEHLKRLEAKEADHIPAILDEFNTKNADLLVFDSFRTDNLWHELWLAIFGILDD 56
                                           :. ::   : *      :   ..:   :. .*

Xenopus         DLQPSCQIACLETIRILSRDKYALSPFTGRSAIQTLAQYAGLDYS--------------- 104
Zebrafish       DLQPSCQLACLETIRILSRDKKSLSPFATRHAMQILIRHAGLGQ---------------- 100
Mouse           GLSPKHRVTWLQTIRILSRDRSCLDSFASRQSLHALACYADITV---------------- 100
Chicken         ELQPSCQVTCLESIRILSRDKYCLEPFTTEEGLKTLSRHAGIDY---------------- 100
Human           DIPTTCQVSCLEVLRILSRDKKVLVPVTTKENMQILLRLAKLNE---------------- 78
Drosophila      QRLSHLHTQCLNTVRILTRDEFSLQTNYIEQEVNTLLKLARIEAGSLKLPATPDELKQEE 116
                   .  :   *: :***:**.  * .   .  :: *   *  :
                             Mutant 1
Xenopus         --EEMEMPCIPDGESAVEALKGLCNIIYNSVEAQEVAKDLRLVCGLARRLKLYNETRSSH 162
Zebrafish       --GEGVTPEIPDLEVIVEALKCLCNIVFNSEAAQEAAADLQLMVGLAERLKQCREPQWNH 158
Mouse           --SEEPIPQSPDMDVLLESLKCLCNLVLSSPTAQMLAAEARLVVRLAERVGLYRKRSYPH 158
Chicken         --SEELIREVPDLEVILESLKCLCNIVFSSPRAQELTAEARLVVGLTKRIKLYNERSLPH 158
Human           --LDDSLEKVSEFPVIVESLKCLCNIVFNSQMAQQLSLELNLAAKLCNLLRKCKDRKFIN 136
Drosophila      REEPQLEPSQAQSEVIAEALKCLCNLVYQSSDCRRQCLRQHCLDAILKRVASS--MRHPC 174
                          .:     *:** ***:: .*  .:      .    :  .  :
                               Mutant 2
Xenopus         ESKFFDLRLLFLLTALSVDMRRQLAQELRGVSLLTDALESTLALKWSDIYEVVTDHLA-- 220
Zebrafish       DVRFFDLRLTFLITALRVDVRAQLAHELRGVSLLSEALDATFGLCWPDMYEVARAGFDGC 218
Mouse           EVQFFDLRLLFLLTALRTDVRQQLFQELHGVRLLTDALELTLG--------VAPKENP-- 208
Chicken         EVKFFDLRLLFLLTALRVDIRQQLAQELRGISLMTDTLELTLGVKWMDPYEVATEEGL-- 216
Human           DIKCFDLRLLFLLSLLHTDIRSQLRYELQGLPLLTQILESAFTIKWTDEYESAIDHNG-- 194
Drosophila      ALEYYDMKLLFLLTALEPAARSRLQIDLNGLTYMTKWLDDKLGE---------------- 218
                  . :*::* **:: *    * :*  :*.*:  ::. *:  :
                  Mutant 3
Xenopus         ---PPLGKEETERVMEILKALFNITFDISRREVDEEEAALYRHLAAILRHCLLRQSDGED 277
Zebrafish       SELPPLGRQETERVMEILKILFNVTFDSNRRHVDEEEAATYRHLGAILRHCIMSSAEGEE 278
Mouse           --PVMLPAQETERAMEILKVLFNITFDSVKREVDEEDAALYRYLGTLLRHCVMVEAAG-D 265
Chicken         --LPPLPRQETERAMEILKVLFNITFDSSKREVDEEDAALYRHLGALLRHCLMISADGED 274
Human           ---PPLSPQETDCAIEALKALFNVTVDSWKVHK-ESDSHQFRVMAAVLRHCLLIVGPTED 250
Drosophila      ---DSVGEEQLNIICELLKVMFNVTSAP-DKSPNEYEIQSLHLTGVLRELLLRFGDLATE 274
                     :  :: :   * ** :**:*         * :    :  ..: .  :       :
                             Mutant 4
Xenopus         RTEEFHGHTVNLLVNLPLMCLDVLLTPKVEQG---------------------------- 309
Zebrafish       RTEEMHSHTVNLLGNLPLPCLDVLLMPKVQQG---------------------------- 310
Mouse           RTEEFHGHTVNLLGNLPLKCLDVLLALELHEG---------------------------- 297
Chicken         RTEEFHSHTVNLLGNLPLKCLDVLLTPKVRPG---------------------------- 306
Human           KTEELHSNAVNLLSNVPVSCLDVLICPLTHEETAQEATTLDELPSNKTAEK---ETVLKN 307
Drosophila      KDRAVVTHAINLLTNISGSCLTELTLRCSNAELESHKEREQDNEKEKDTEAGAGAKPREC 334
                : . .  :::*** *:.  **  *     .
                        Mutant 5
Xenopus         -SVEYMGMNMDTVEVLLQFLHRRLDR---GHKLREMLTPVLNLLTESSRVHRETRKFLRA 365
Zebrafish       -SIEYMGVNMDAVKVLVEFMEKRLDR---GNKLKETLLPSLNLLTESARIHRETRKFLRN 366
Mouse           -SLEFMGVNMDVISALLAFLEKRLHQ---THRLKECVAPVLNVLTECARMHRPARKFLKA 353
Chicken         -SLEYMGVNMDAVNILLDFLERRLDR---GHKLKESLTPVLNLLTESARVHRQTRKFLKA 362
Human           NTMVYNGMNMEAIHVLLNFMEKRIDK---GSSYREGLTPVLSLLTECSRAHRNIRKFLKD 364
Drosophila      CSQCFEKRNVRSLDVLLRYLRQSLAQQEAEASSHELLSPVLTVLVKCARSDRVMRHYLRQ 394
                 :  :   *:  :  *: ::.: : :       :* : * *.:*.:.:* .*  *::*:
                                                               Mutant 6
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Xenopus         KVLPPLRDVKNRPEVGNTLRNKLVRLMTHVDTDVKHCAAEFLFVLCKENVSRFVKYTGYG 425
Zebrafish       KVLPPLRDVKNRPEVGNALRNKLVRLMTHIDTDVKHCAAEFLFVLCKESVSRFIKYTGYG 426
Mouse           QVLPPLRDVRTRPEVGDLLRNKLVRLMTHLDTDVKRVAAEFLFVLCSESVPRFIKYTGYG 413
Chicken         KVLPPLRDVKNRPEVGNSLRNKLVRLMTHIDTDVKHCAAEFLFVLCKESVSRFVKYTGYG 422
Human           QVLPPLRDVTNRPEVGSTVRNKLVRLMTHVDLGVKQIAAEFLFVLCKERVDSLLKYTGYG 424
Drosophila      EILPPLRDVSQRPEVGQELRNHLCRFLTLPAMILRDLSAELLFVLCKENVGRMIKYTGYG 454
                ::*******  *****. :**:* *::*     ::  :**:*****.* *  ::******
               Mutant 7,    8,     9,                 10,     11

Xenopus         NAAGLLAARGLLAGGRGEG--CYSEDDDTDTEEYREAKANINPVTGRVEEKQPNPMDGMT 483
Zebrafish       NAAGLLAARGLMRGGRDPG--HYSEDEDSDTEEYREAKPHINPVTGRVEEEQPNPMEGMT 484
Mouse           NAAGLLAARGLMAGGRPEG--QYSEDEDTDTEEYREAKASINPVTGRVEEKPPNPMEGMT 471
Chicken         NAAGLLAARGLMAGGREEG--EYSEDEDTDTEEYKEAKPNINPVTGRVEEKLPNPMEGMT 480
Human           NAAGLLAARGLLAGGRGDN--WYSEDEDTDTEEYKNAKPNINLITGHLEEPMPNPIDEMT 482
Drosophila      NAAGLFAKRGILDCRRVEGTDYSSDSEDSDTEEYKQQQQGINPVLGCVEPRSKSHLDDIS 514
                *****:* **::   *  .    *:.:*:*****:: :  ** : * :*    . :: ::
                    Mutant 12             Mutant 13
Xenopus         EEQKEYEAMKLVNMFDKLSREQIIQPMGVTSDGRLGPLDEAAQKMLQRQ---ESSDLDSD 540
Zebrafish       DEQKEYEAMKLVNMFDKLSREQVIQPMKIGADGKMTSMEPHELHHLASQQFGESNNSDSD 544
Mouse           EEQKEHEAMKLVNMFDKLSRHRVIQPMGMSPRGHLTSLQDAMCETMEGQ---LSSDPDSD 528
Chicken         EEQKEYEAMKLVNMFDKLSREQVIQPMGITPSGNLAPMENAIRDMADER---SSSDSDLG 537
Human           EEQKEYEAMKLVNMLDKLSRYCIPIHFLLGF----------------------------- 513
Drosophila      EEQKEYEAMQLVNLIEQLRQGGIVKPAMIDKDGRPQPLEHILQLQEELPQQQLDQKRKT- 573
                :****:***:***::::* :  :     :
              Mutant 14
Xenopus         SD-- 542
Zebrafish       SDTN 548
Mouse           PD-- 530
Chicken         LD-- 539
Human           ----
Drosophila      ----
```
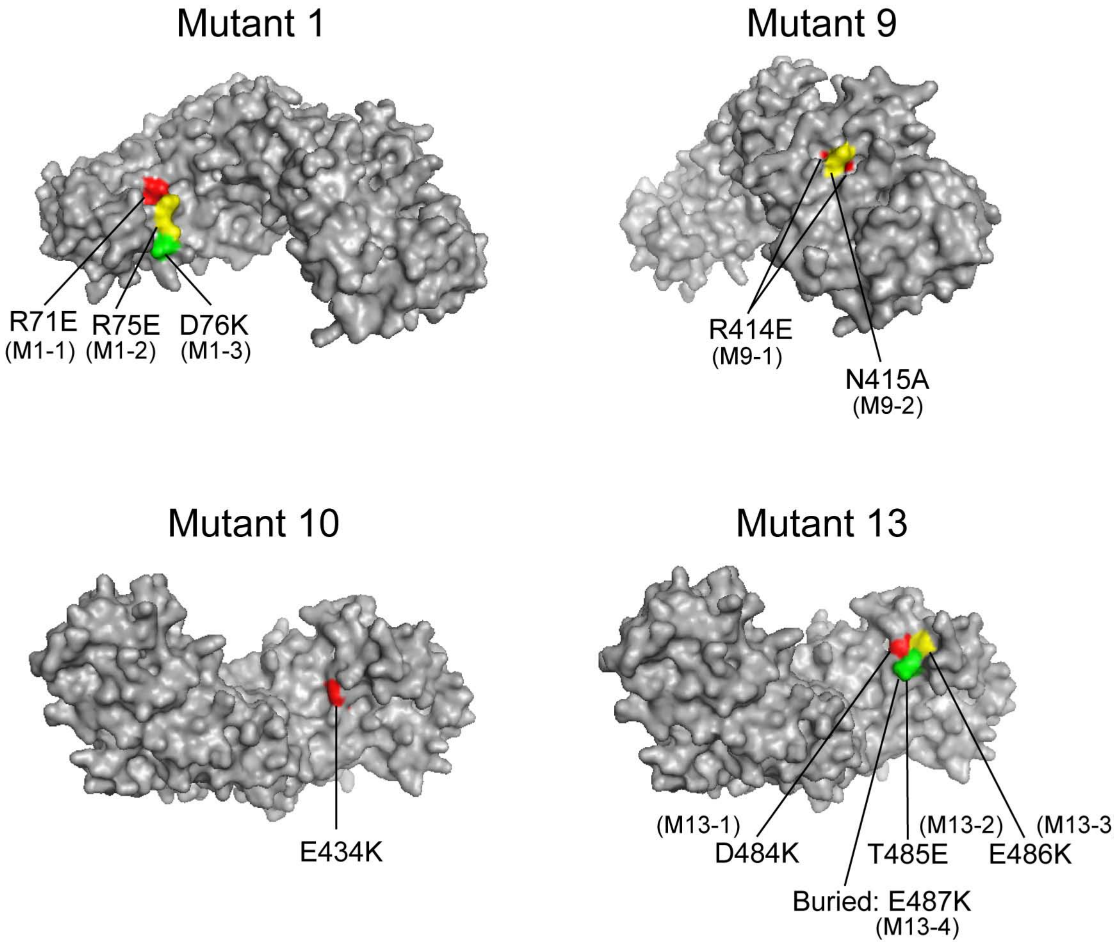
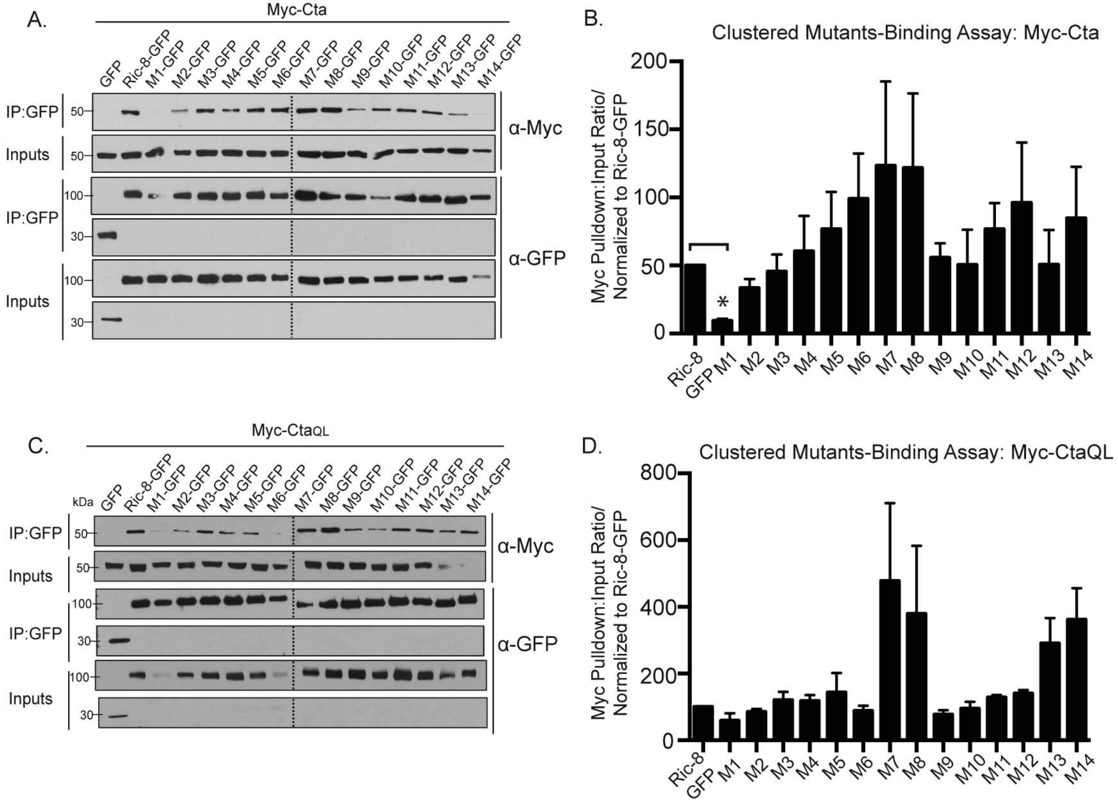


Mutant 1
R71E
(M1-1)
R75E
(M1-2)
D76K
(M1-3)
Mutant 9
R414E
(M9-1)
N415A
(M9-2)
Mutant 10
E434K
Mutant 13
(M13-1)
D484K
(M13-2)
T485E
(M13-3)
E486K
Buried: E487K
(M13-4)


A.
Myc-Cta
GFP
Ric-8-GFP
M1-GFP
M2-GFP
M3-GFP
M4-GFP
M5-GFP
M6-GFP
M7-GFP
M8-GFP
M9-GFP
M10-GFP
M11-GFP
M12-GFP
M13-GFP
M14-GFP
IP:GFP
Inputs
IP:GFP
Inputs
50
50
100
30
100
30
α-Myc
α-GFP
B.
Clustered Mutants-Binding Assay: Myc-Cta
Myc Pulldown:Input Ratio/
Normalized to Ric-8-GFP
200
150
100
50
0
*
Ric-8
GFP
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14
C.
Myc-CtaQL
kDa
GFP
Ric-8-GFP
M1-GFP
M2-GFP
M3-GFP
M4-GFP
M5-GFP
M6-GFP
M7-GFP
M8-GFP
M9-GFP
M10-GFP
M11-GFP
M12-GFP
M13-GFP
M14-GFP
IP:GFP
Inputs
IP:GFP
Inputs
50
50
100
30
100
30
α-Myc
α-GFP
D.
Clustered Mutants-Binding Assay: Myc-CtaQL
Myc Pulldown:Input Ratio/
Normalized to Ric-8-GFP
800
600
400
200
0
Ric-8
GFP
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14