Supplementary Information

Materials and Methods

Cell lines and cell culture - Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % fetal calf serum, 1 % chicken serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humidified 5% CO_2 atmosphere at 40 °C. A Coulter Counter (Z1 Coulter Particle Counter, Beckman) was used for cell counting.

Plasmid constructions for establishment of CaM knock out cell lines - For CaMI targeting and ratCaM knock-in (pCaMI ko) the following elements were cloned into pBluescript KS (+) in 5´ -> 3´direction: a chicken CaMI upstream flanking sequence (containing exon 3 and part of exon 4, 2.2 kb); the hygromycin resistance gene under the control of the ß-actin promoter and thymidine kinase polyadenylation site (3.5 kb, kindly provided by Dr. T. Kurosaki (Kansai Medical University, Osaka, Japan); a rat CaMIII transgene (Nojima, 1989) under the control of the tetracycline (tet) responsive sequence and the SV40 polyadenylation signal (1), and a 1.5 kb downstream CaMI targeting sequence (containing part of exon 3 and exon 5, 1.8 kb). Flanking chicken CaM sequences were generated by PCR using genomic chicken DNA as template. The total insert size was 9 kb. The cell line 8-5 (2) with the CaMII gene eliminated was transfected with the linearized pCaMI ko plasmid and the tet transactivator (3) in a molar ratio of 1:10 using standard electroporation methods (4). The second CaMI allele was knocked out with a construct made of the same CaMI flanking sequences and containing the zeocyin resistance gene cassette (kindly provided by T. Kurosaki) for selection. Selection of the clones expressing the selection markers was performed according to standard procedures (4).

Generation of the HA-CaM gpt constructs and stably HA-CaM expressing clones - The Eco.gpt cassette kindly provided by Dr T. Kurosaki was cloned into the pCMV vector (Clontech), carrying a rat CaMII wt gene with an N-terminal HA tag. The latter construct was generated by PCR-assisted subcloning of the rat CaMI sequence from the pET-CM vector (5) into the BglII-NotI sites of the pCMV-HA vector (Clontech). Site-directed mutagenesis was performed by PCR on the HA-CaMwt eco.gpt plasmid to generate a mutant HA-CaM using the Quick Change Lightning site-directed mutagenesis kit (Stratagene) and two complementary primers bearing the particular substitution. The CaM1,2; CaM3,4 and CaM(Y99F/Y138F) double mutants were obtained by performing PCR with the plasmid carrying one mutation and the primers with the other mutation. CaM1,2,3,4 was created by mutating CaM1,2 to CaM1,2,3 followed by mutating this construct with the CaM4 primers. The resulting plasmids are listed in Table 1. Selection of stably transfected clones was done using medium containing 125 µg/ml xanthine, 10 µg/ml hypoxanthine and 7.5 µg/ml mycophenolic acid.

Analysis of cell survival - Cells ($1.5x10^5$) were harvested and resuspended in 250 µl phosphate buffered saline (PBS). Cells were analyzed by flow cytometry using a FACScan analytical cytometer (Becton Dickinson). The fraction of living cells was gated in the forward (FSC) / sideward (SSC) scatter dot-plot, quantified and expressed as percentage of the fraction of living DT40 wt cells (relative survival). In the preliminary experiments, propidium iodide (PI) staining was used for gaiting and quantification of the PI-negative live cells population. This technique produced the same results as gaiting of live cells using FSC/SSC dot-plots described above. Therefore, the latter approach was used in all experiments described in this work. The data are representative of the experiments repeated at least two times.

Preparation of cell extracts - Cells ($1-2x10^6$) were harvested, washed twice with PBS, and lyzed on ice in 20 µl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 % (v/v) NP-40, supplemented with 1 mM dithiothreitol (DTT) and the complete EDTA-free protease inhibitors cocktail (Roche). Cell extracts to be used for the detection of phospho-Tyr99CaM were supplemented with phosphatase inhibitors (Cocktail 1 and 2, Sigma). After 10 min incubation on ice, the samples were clarified by centrifugation at 15,000 x g for 15 min. The supernatants were stored at -20 °C until used.

Western blot analysis - Proteins were separated by 15 % SDS-PAGE and transferred to a polyvinyl difluoride membrane (Hybond-P, Amersham Biosciences) using a semi-dry blotter (EBU 4000, CBS Scientific) and a discontinuous buffer system according to (6). After protein transfer, the membranes were incubated in Tris buffered saline-Tween (TBS-T: 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.05 % Tween-20) supplemented with 0.2 % (v/v) glutaraldehyde for 10 min in order to immobilize CaM to the membranes (reviewed in (7)). The membranes were thoroughly washed in TBS-T and blocked for 1 hour in TBS-T containing 5 % skim milk powder. The membranes were probed under standard conditions with primary antibody (1:1000) followed by the secondary antibody (1:2000) unless specified otherwise. The signal was visualized using a chemiluminescent reagent (ECL Plus Western Blotting Detection System, GE Healthcare). Coomassie blue staining of gels after blotting was used to confirm equal loading.

CaM preparation for mass spectrometry (MS) analysis - DT40 cells were lyzed as described above (see Preparation of cell extracts) in a lysis buffer supplemented with 5 mM EGTA. The lysate was heated at 100 °C for 90 s followed by cooling on ice and removal of denatured protein by centrifugation (15.000 g for 5 min). The supernatant was diluted in 50 mM Tris-HCl pH 7.5 supplemented with 10 mM $CaCl_2$. Elution by EGTA was done according to (8). Fractions containing CaM were identified by Western blot analysis, dialyzed against 1 mM Tris-HCl pH 7.5, lyophilized and dissolved in water. The purified product was run on SDS-PAGE along with vertebrate recombinant CaM from *E. coli* (Jena Bioscience) as a control. The bands of recombinant CaM and native CaM were excised for analysis by mass spectrometry (MS). Protein samples were reduced and alkylated with iodoacetamide, i.e. carbamidomethylated and digested in-gel with trypsin. The extracted peptides were concentrated using ZipTip micropurification columns and eluted onto an anchor chip target for analysis on a Bruker autoflex II MALDI TOF/TOF instrument. The peptide mixtures were analyzed in positive reflector mode for accurate peptide mass determination. The MS spectra were combined and used for database searching using the Mascot database as well as in-house databases (Alphalyse, Odense, Denmark).

Pulse-chase labeling - After four days of tetracycline treatment HA-CaM wt and HA-CaM (K115R) cells were labeled according to the procedure described in (9). The cells were starved for 1 hour in methionine-free RPMI 1640 medium supplemented with 2 mM glutamine and dialyzed 10 % fetal calf serum and 1 % chicken serum. The cells were pulse-labeled for 10 minutes with 80 µCi/ml ^{35}S methionine (Premium stabilized translation, PerkinElmer) and chased in complete RPMI 1640 medium. Equal volumes of cell suspension were withdrawn at 1, 2 and 4 h time points. Cells were lysed with lysis buffer and CaM was purified according to the procedure described above. Data were analyzed using a Storm 840 PhosphorImager (Molecular Dynamics) and ImageJ software.

Cell cycle analysis – Cells ($1.5x10^6$) were harvested, washed and resuspended in 420 μl PBS. Then 1000 μl ice-cold ethanol was added to the cells, which were then incubated for 1 hour at -20 ºC. The cells were pelleted and resuspended in 500 μl PBS containing 10 μg/ml propidium iodide and 50 μg/ml RNAse A and incubated for 45 minutes in the dark at 37 ºC followed by flow cytometry analysis. The non apoptotic cells were gated in the FSC/SSC plot and analyzed for their FL2 signal, distinguishing G_0/G_1, S and G_2/M phases. The percentage of cells in each cell cycle phase is shown as a percentage of the combined population. The experiments were done in triplicates.

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1. Genomic status of the CaM gene loci in wt and generated CaM knock out cell lines used in this study Cell lines 1G3, ET1-50 and ET1-55 additionally contain the stably and randomly integrated tet transactivator pUHD 152 (tet-off system). Black boxes indicate exons (labeled with roman numbers) and dashed lines indicate plasmid sequences.

Figure S2. Cell cycle analysis Distribution of the cell cycle phases (G_0/G_1, S and G_2/M phases) in (A) untreated ET1-50 cells (CTR) as well as tetracycline treated (B) ET1-50 2G9, (C) CaM1,2 A, (D) CaM3,4 B, (E) CaM1,2,3,4 B and (F) ET1-50 cells at the indicated times after start of treatment. The error bars represent SEM.

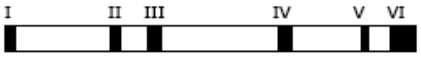
Figure S3. Identification of posttranslational modification of CaM (A) Western blot analysis of proteins isolated from ET1-50 cells untreated or treated with tet for 96 h. Top panel: the blot was probed with anti CaM phosphoTyr99 antibody; middle panel: with anti CaM antibody; bottom panel: Coomassie stained post blot (loading control). **(B)** Identification of trimethylated CaMLys115 vertebrate CaM (vCaM) isolated from DT40 cells and recombinant CaM (rCaM) prurified from in *E. coli* were subjected to trypsin digestion following mass analysis. The marked peaks in the selected part of the spectrum correspond to the peptide HVMTNLGEKLTDEEVEDEMIR without K115 methylation (peak group 1), the same peptide with K115 trimethylation (peak group 2) and the same peptide containing monooxydized Met (peak group 3) and dioxydized Met peak group 4). The latter three peptides were only found in CaM isolated from DT40 cells and not in recombinant CaM.

Figure S4. Stability of HA-CaM (K115R) Cell lines expressing HA-CaM wt and HA-CaM (K115R) were pulse-labeling and chased for indicated periods. The radioactivity of each band was determined and normalized to the value at the 0 h time point. The means of two experiments are plotted against the chase period in the lower panel.

Figure S1

A. DT40 [CaMI$^{+/+}$ CaMII$^{+/+}$]

genomic organisation of CaMI alleles

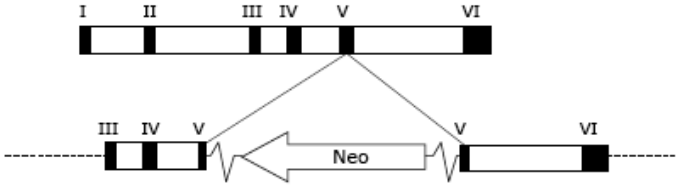


genomic organisation of CaMII alleles

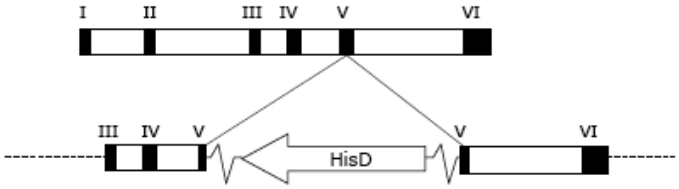
B. 8-5 [CaMI$^{+/+}$ CaMII$^{-/-}$]

(Schmalzigaug et al. 2001)

Targeting of 1st CaMII allele
with pCaMII-Neo KO cassette



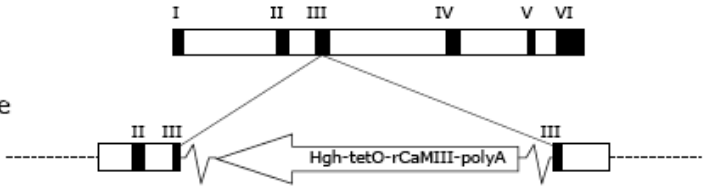
Targeting of 2nd CaMII allele
with pCaMII-HisD KO cassette



C. 1G3 [CaMI$^{+/rCaMIII}$ CaMII$^{-/-}$]

(this study)

Targeting of 1st CaMI allele
with pTetO-rCaMIII-Hgh cassette



D. ET1-50 [CaMI$^{-/rCaMIII}$ CaMII$^{-/-}$]

(this study)

Targeting of 2nd CaMI allele
with pCaMI-Zeo KO cassette

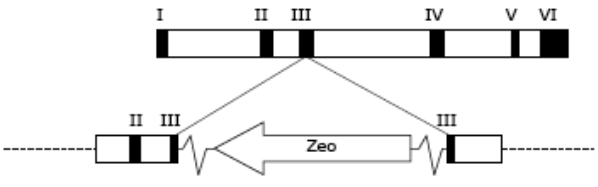


Figure S2

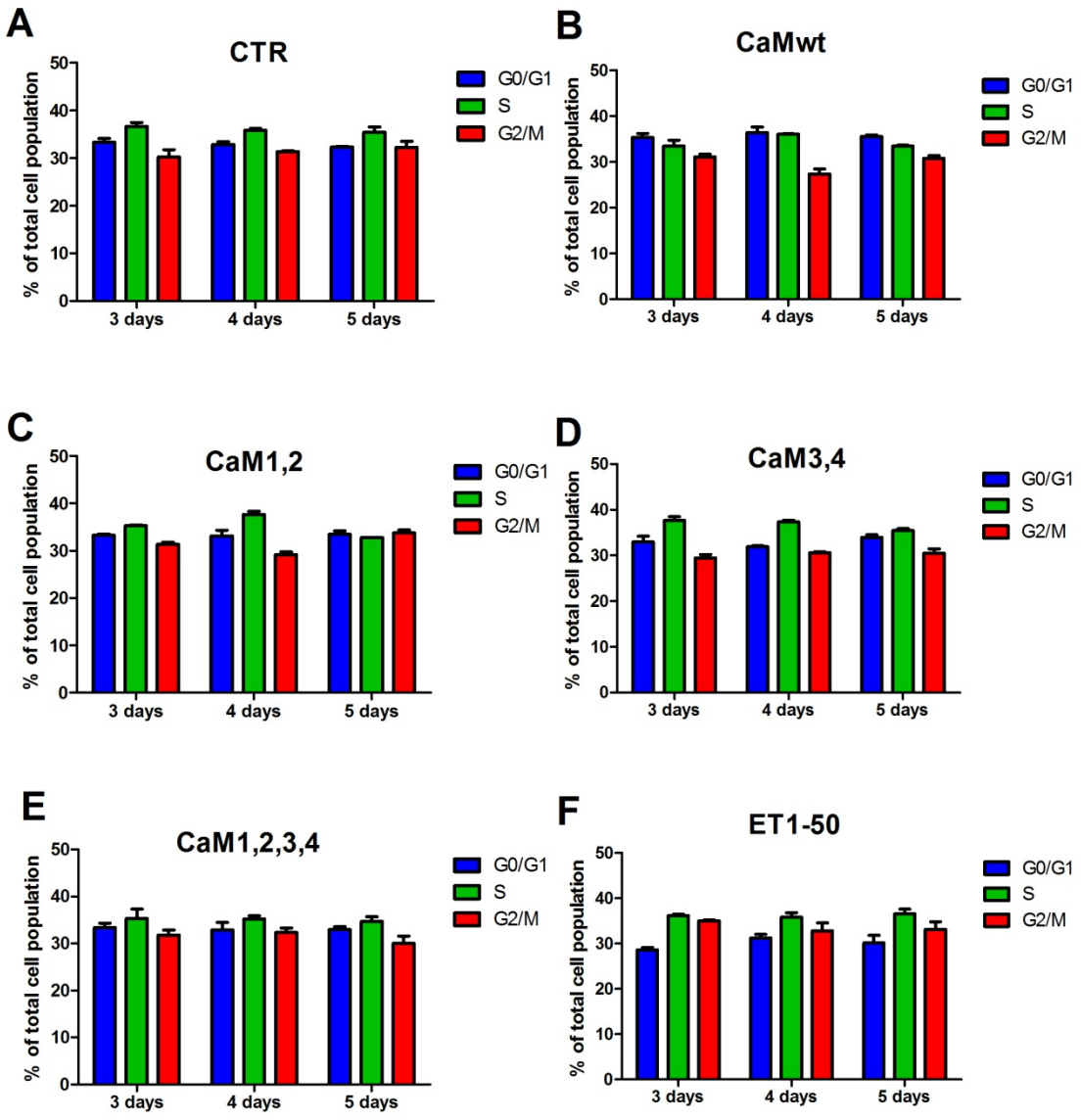
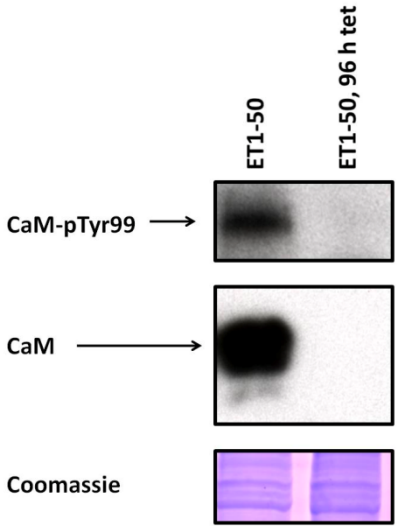

A
CTR
B
CaMwt
C
CaM1,2
D
CaM3,4
E
CaM1,2,3,4
F
ET1-50
% of total cell population
0
10
20
30
40
50
3 days
4 days
5 days
G0/G1
S
G2/M

Figure S3

A


ET1-50
ET1-50, 96 h tet
CaM-pTyr99
CaM
Coomassie

B

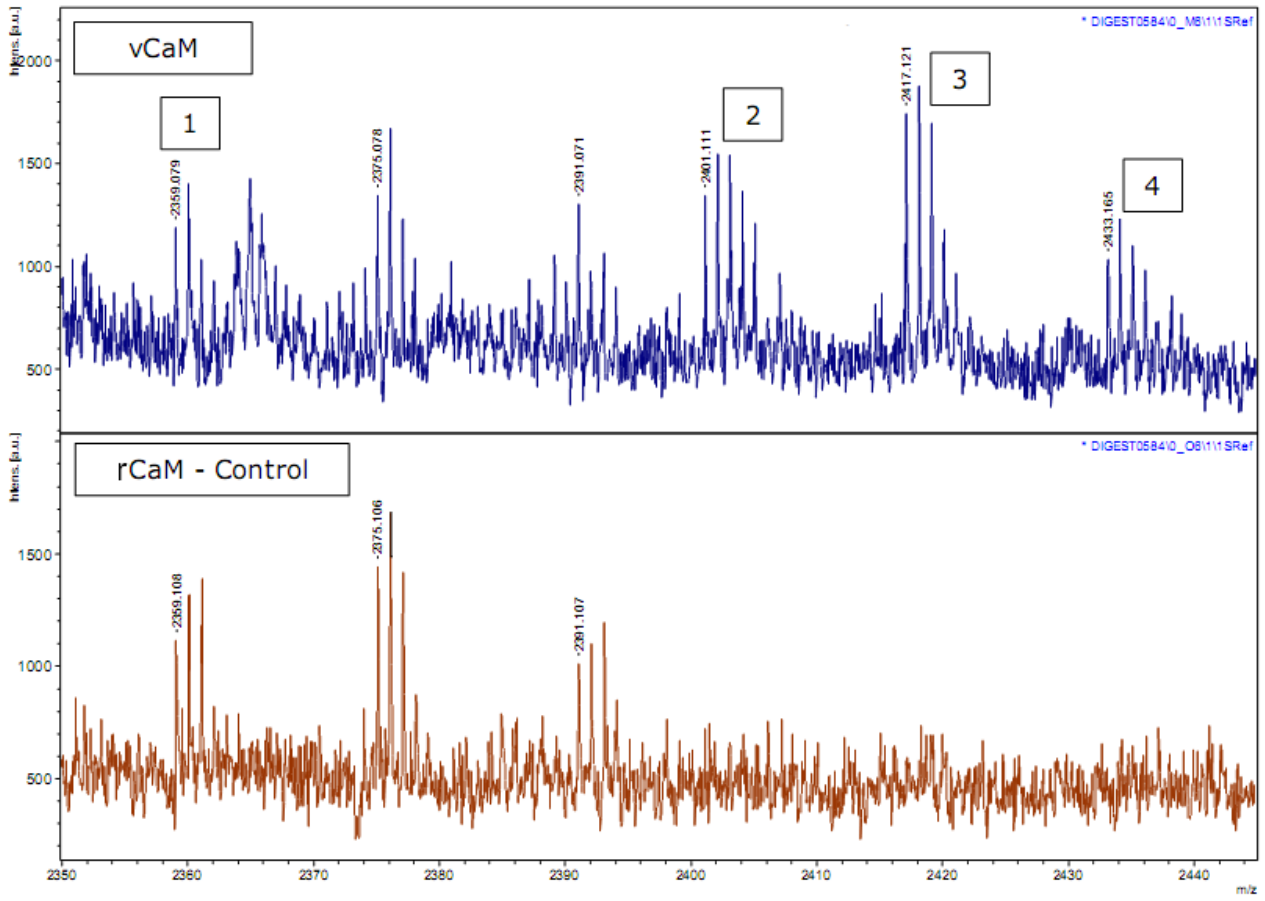
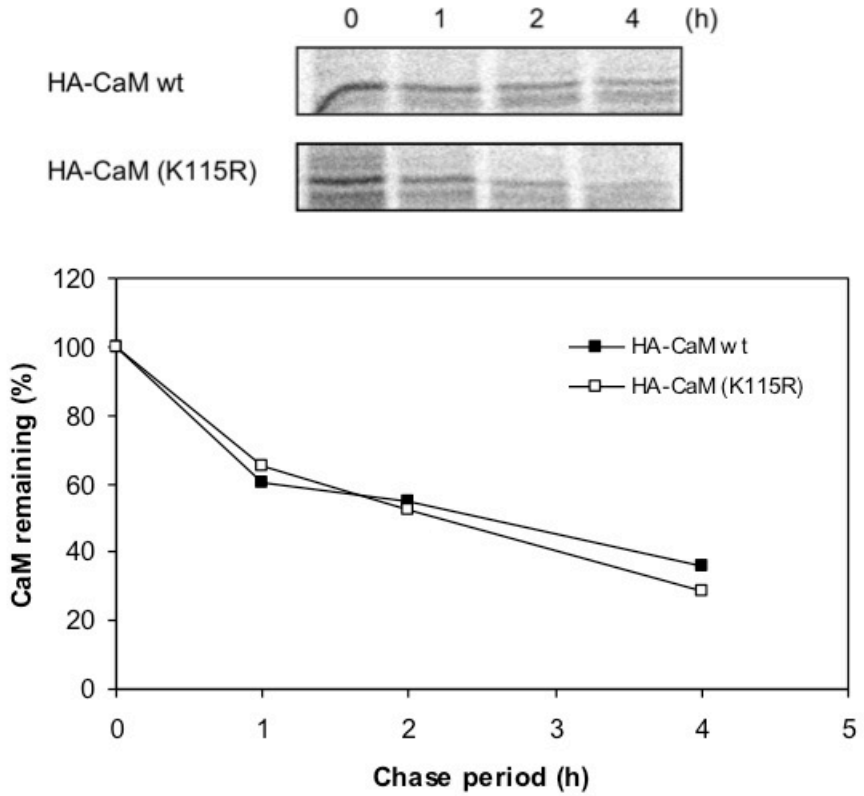

vCaM
1
2
3
4
2359.079
2375.078
2391.071
2401.111
2417.121
2433.165
rCaM - Control
2359.108
2375.106
2391.107
2000
1500
1000
500
2350
2360
2370
2380
2390
2400
2410
2420
2430
2440
m/z

Figure S4



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