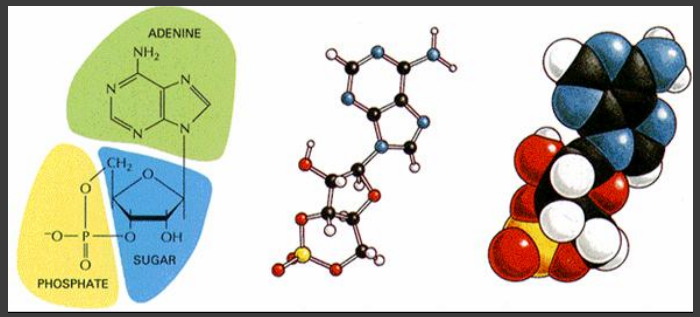
# SPATIO-TEMPORAL DYNAMICS OF INTRACELLULAR DIFFUSIBLE MESSENGERS:CAMP, CA2+, NO

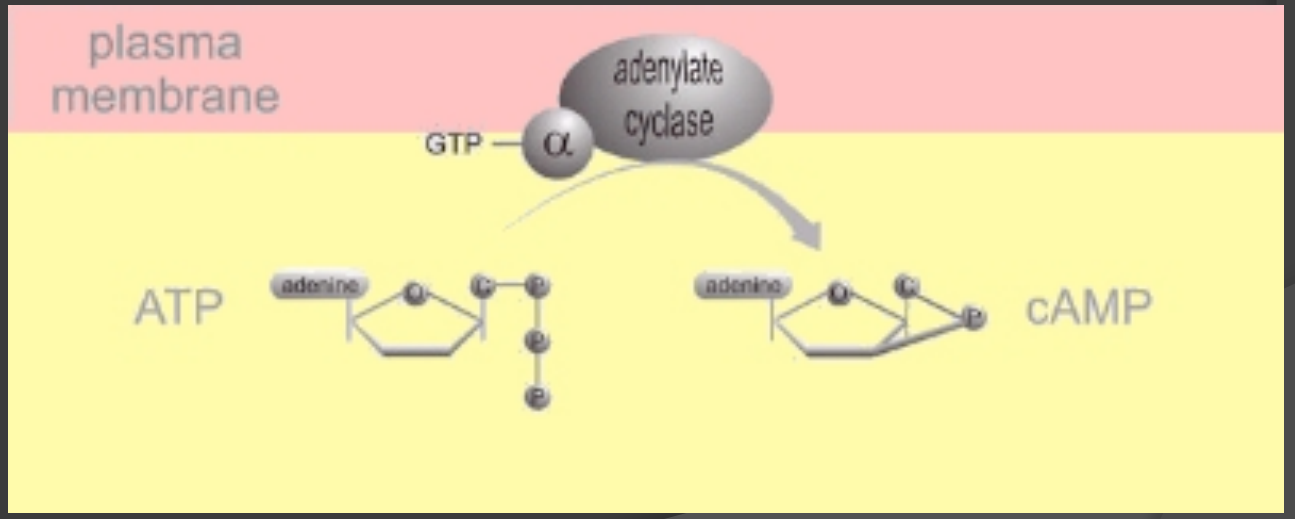
# Second messengers

Molecular mediators of signal transduction. Cells carefully, and rapidly, regulate the intracellular concentrations. Second messengers can be used by multiple signaling networks (at the same time).

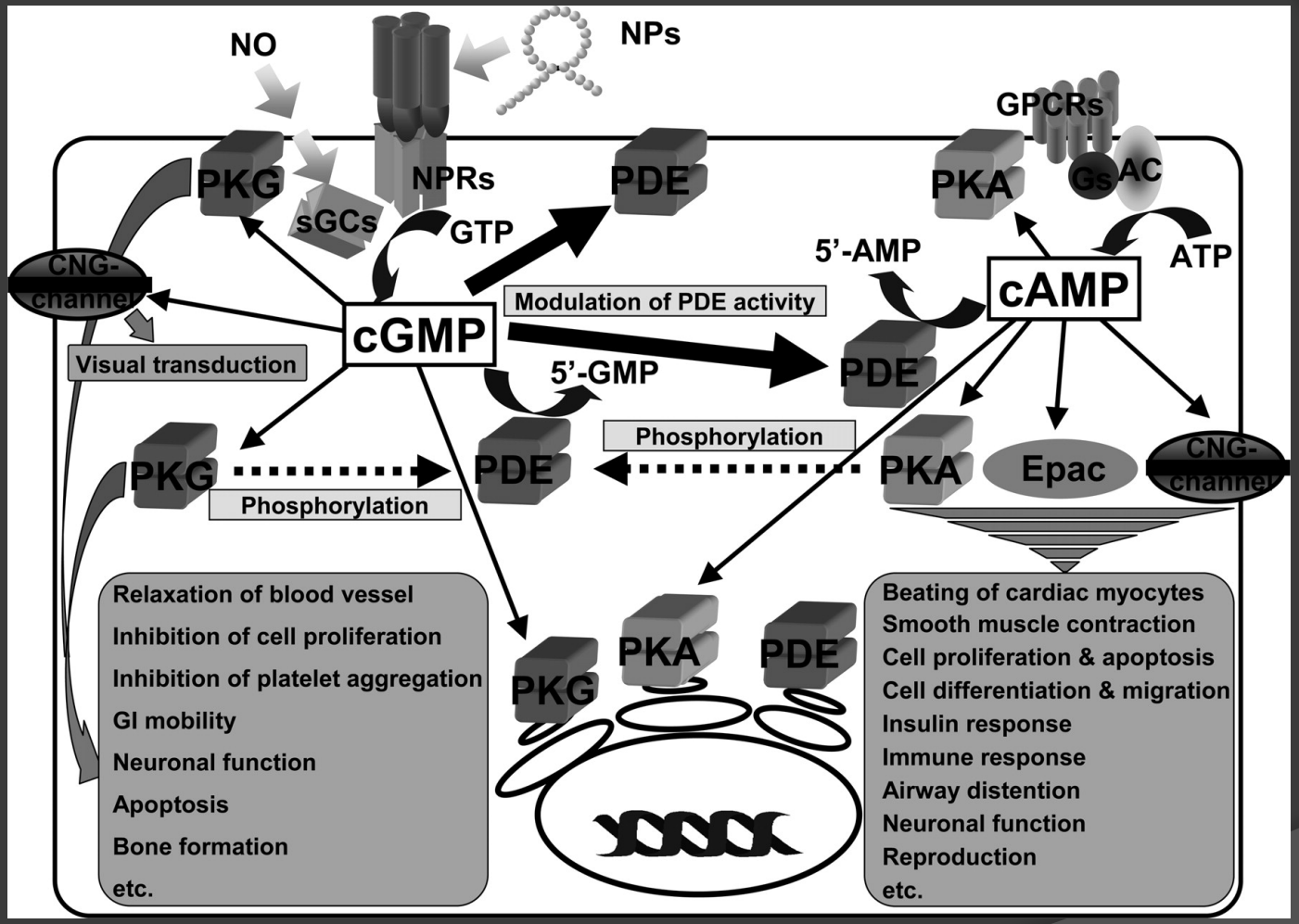
- Cyclic nucleotides: cAMP, cGMP
- Inositol phosphate (IP)
- Diacylglycerol (DAG)
- Calcium
- Nitric oxide (NO)
- Reactive oxygen species (ROS)

# cAMP is a very well studied second messenger



# Cyclic nucleotide signaling and regulation




Omori, K. et al. Circ Res 2007;100:309-327

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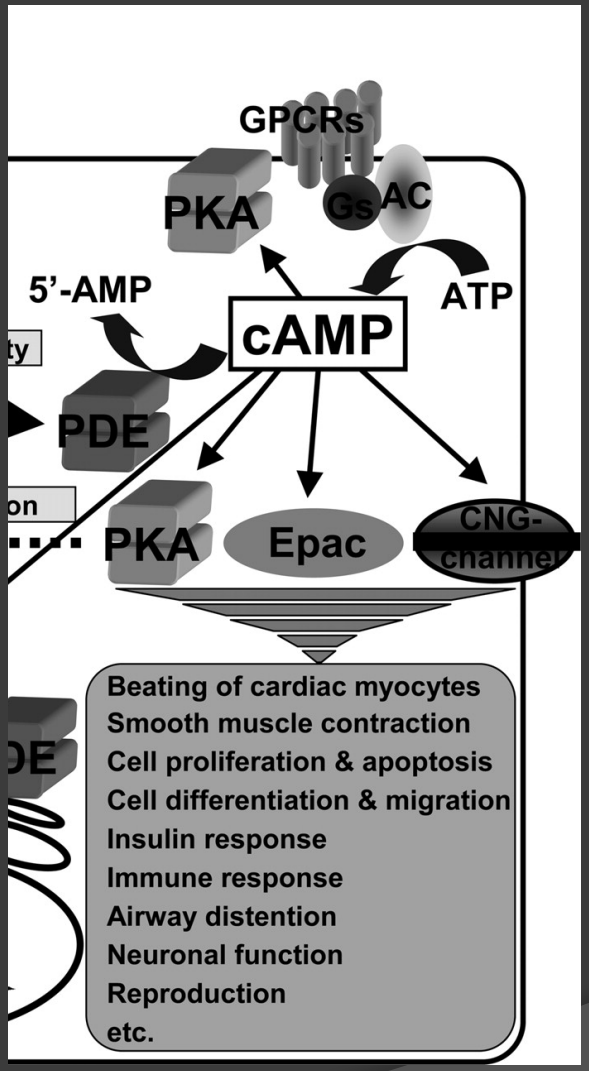
## Cyclic nucleotide signaling and regulation

cAMP mediates a huge amount of intracellular events acting on few effectors: PKA, EPAC, CNG-channels

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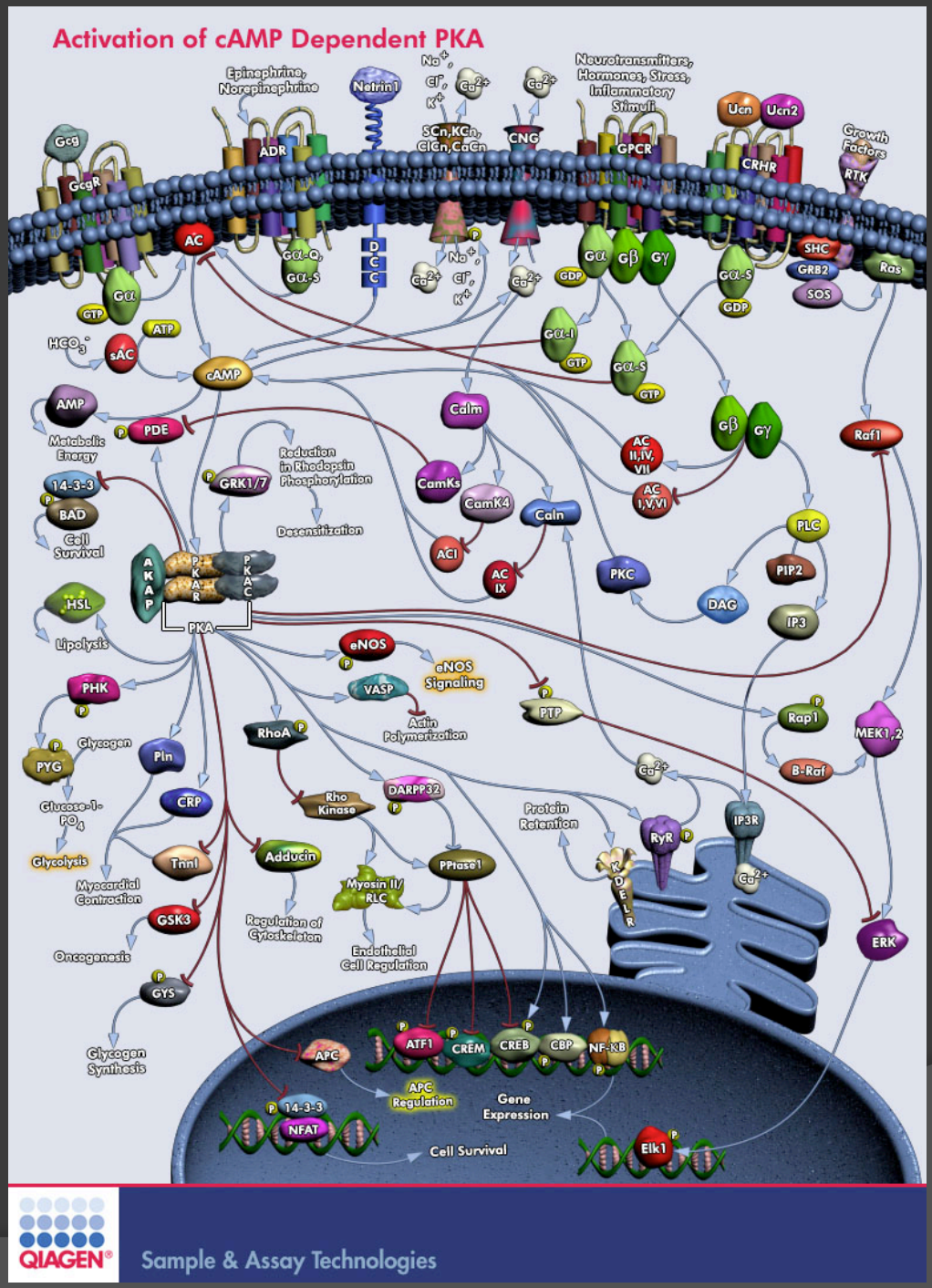

Activation of cAMP Dependent PKA
Epinephrine, Norepinephrine
Netrin 1
Neurotransmitters, Hormones, Stress, Inflammatory Stimuli
Ucn
Ucn2
Growth Factors
Gcg
GcgR
ADR
SCn,KCn, ClCn,CaCn
CNG
GPCR
CRHR
RTK
AC
GTP
ATP
sAC
cAMP
AMP
Metabolic Energy
PDE
Reduction in Rhodopsin Phosphorylation
GRK1/7
Desensitization
14-3-3
BAD
Cell Survival
Calm
CamKs
CamK4
Caln
ACI
AC IX
AC II,IV, VII
AC I,V,VI
PKA
HSL
Lipolysis
eNOS
eNOS Signaling
VASP
Actin Polymerization
PHK
Glycogen
PYG
Glucose-1-PO4
Glycolysis
Pln
CRP
Tnnl
Myocardial Contraction
GSK3
Oncogenesis
GYS
Glycogen Synthesis
RhoA
Rho Kinase
DARPP32
Adducin
Myosin II/ RLC
PPtase1
Regulation of Cytoskeleton
Endothelial Cell Regulation
PTP
PKC
PLC
PIP2
DAG
IP3
Raf1
Rap1
B-Raf
MEK1,2
ERK
Protein Retention
RyR
IP3R
KDELR
APC
ATF1
CREM
CREB
CBP
NF-KB
APC Regulation
Gene Expression
14-3-3
NFAT
Cell Survival
Elk1
SHC
GRB2
SOS
Ras
QIAGEN
Sample & Assay Technologies

# Localizzazione spaziale dei segnali cAMP/PKA

- Il fatto che cAMP agisca attraverso un limitato numero di effettori originando così tanti effetti intracellualri suggerisce che esista una compartimentalizzazione spaziale delle dverse vie attivate da cAMP
- Nella cellula coesistono vie di segnale per cAMP che sono spazialmente segregate:
- Ruolo chiave svolto da AKAPs (A-kinase anchoring proteins), proteine scaffold che ancorano PKA in uno specifico microdominio cellulare in prossimità di mdulatori e target specifici
- PKA nei diversi pool verranno attivate dal rilascio localizzato di cAMP (localizzazione spaziale sia delle proteine G regolatorie che delle adenilato ciclasi nonché delle fosfodiesterasi)

# Tools per studio dinamiche cAMP

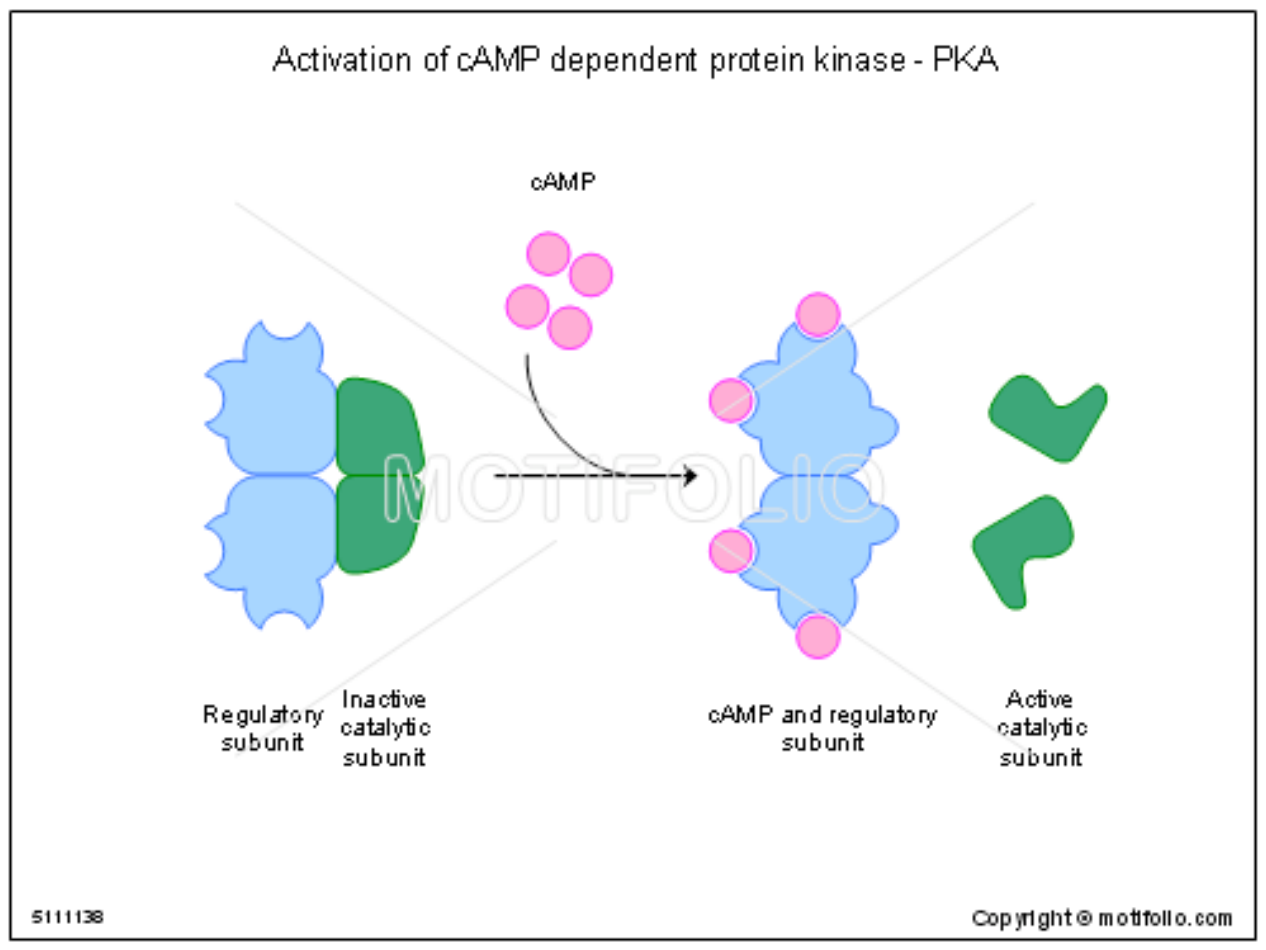
- **Saggi RIAs** sono utilizzati comunemente per valutare rilascio cAMP totale.
  - Svantaggio: bassa risoluzione temporale e assenza di risoluzione spaziale: inadeguati per lo studio dei dettagli molecolari del signalling cAMP.

- FRET basata su PKA:
  - Microiniezione

  Subunità catalitica (C) PKA – fluoresceina / Subunità regolatoria (R) PKA – rhodamine

  Svantaggi: il complesso proteico da microiniettare è troppo voluminoso (non appropriato per tutti i modelli cellulari); biotossicità della sonda; tendenza della sonda ad aggregare ed immunoprecipitare

# PKA


Activation of cAMP dependent protein kinase - PKA
cAMP
Regulatory subunit
Inactive catalytic subunit
cAMP and regulatory subunit
Active catalytic subunit
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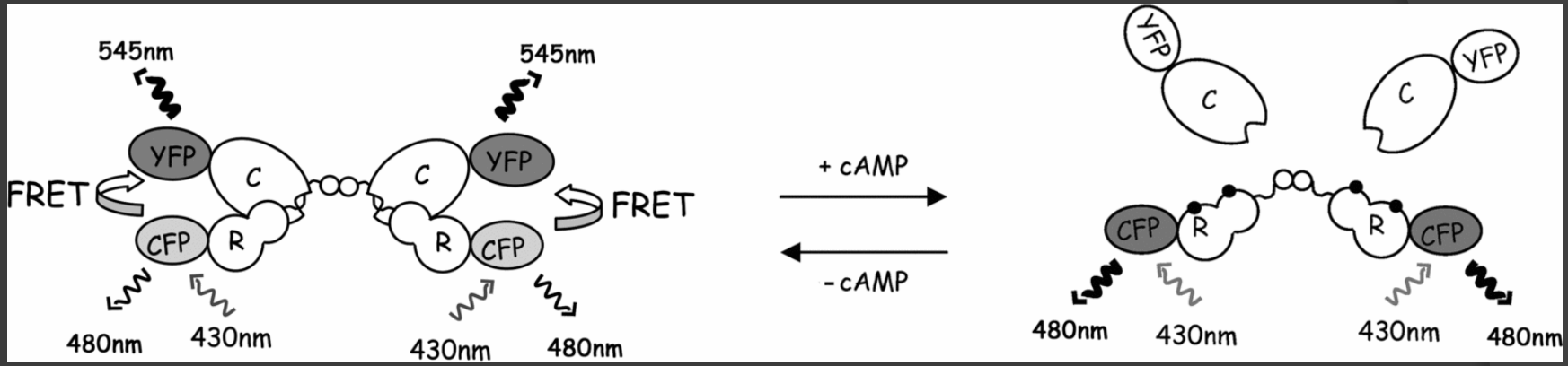
# FRET:

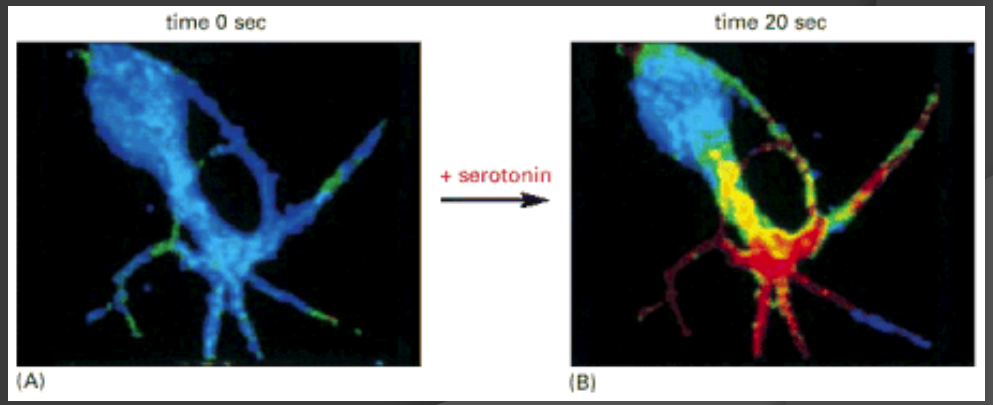
- Utilizzo di sonde “proteiche”:

PKA (R) – CFP / PKA (C) - YFP

Biochem. Soc. Trans. (2005) 33, 1323-1326




Vantaggi: compartimentalizza in compartimenti specifici attorno alla proteina multiscaffolding AKAP

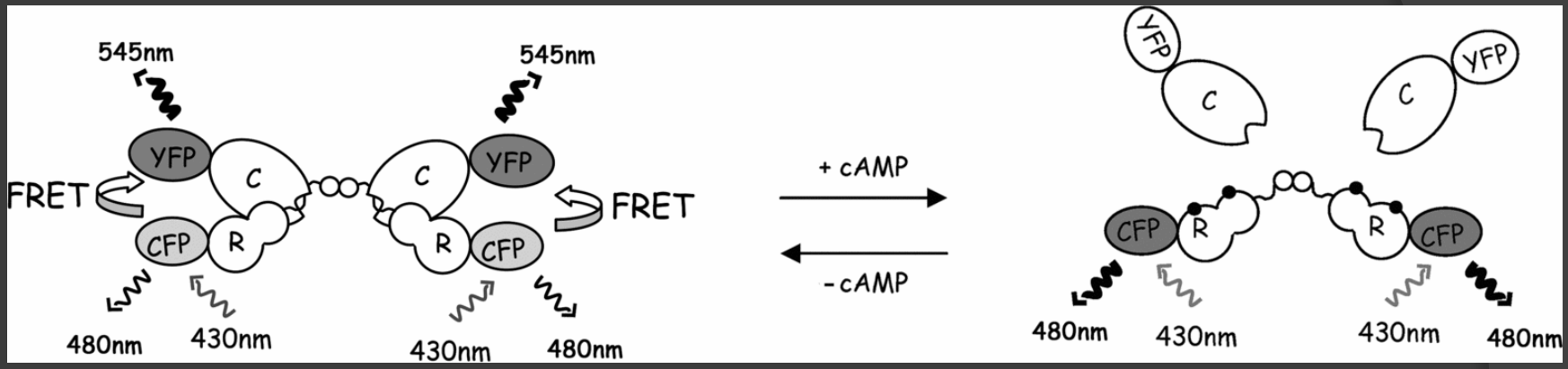



Misurare cAMP da specifici compartimenti subplasmatici

# FRET:

- Utilizzo di sonde "proteiche": **PKA**

PKA (R) – CFP / PKA (C) - YFP

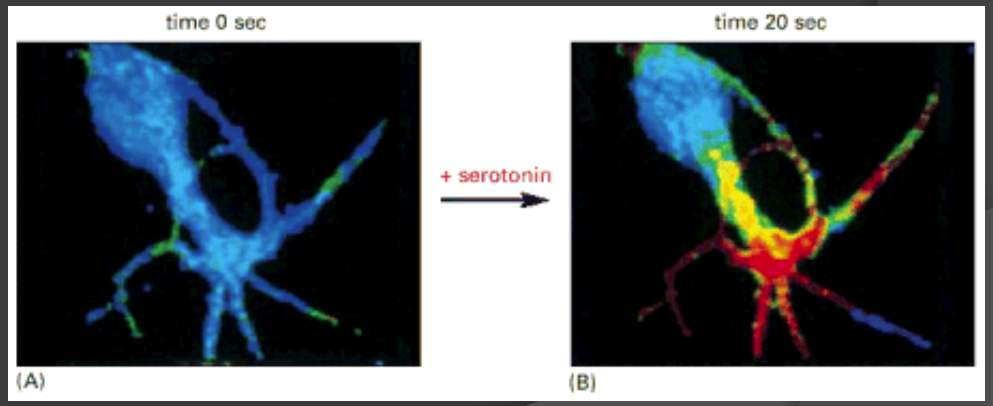



Biochem. Soc. Trans. (2005) 33, 1323-1326

Svantaggi:

•Il sensore consiste di due proteine di fusione indipendenti e quindi bisogna calibrare i livelli di espressione

• **PKA (C) - YFP** è catalicamente attiva quindi la sua overespressoine potrebbe avere degli effetti cellualri



- FRET:
  - **Utilizzo di EPAC**

# Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator

*Bas Ponsioen[1,2*], Jun Zhao[3*], Jurgen Riedl[3], Fried Zwartkruis[3], Gerard van der Krogt[1], Manuela Zaccolo[4], Wouter H. Moolenaar[2], Johannes L. Bos[3+] & Kees Jalink[1++]*

[1]Division of Cell Biology, and [2]Division of Cellular Biochemistry and Centre of Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands, [3]Department of Physiological Chemistry and Centre of Biomedical Genetics, UMCU, Utrecht, The Netherlands, and [4]Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, Padova, Italy



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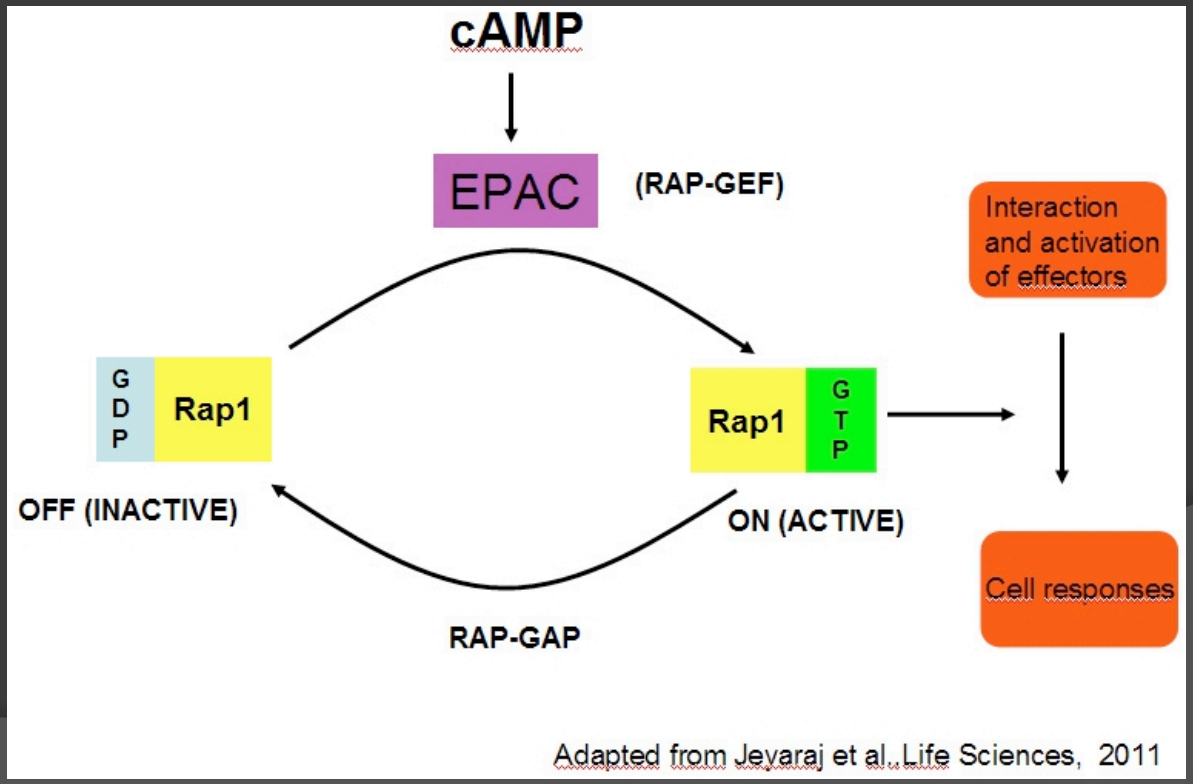
Costrutto di fusione CFP – EPAC – YFP, che funziona come sensore di cAMP, cambiando conformazione in seguito al legame con cAMP

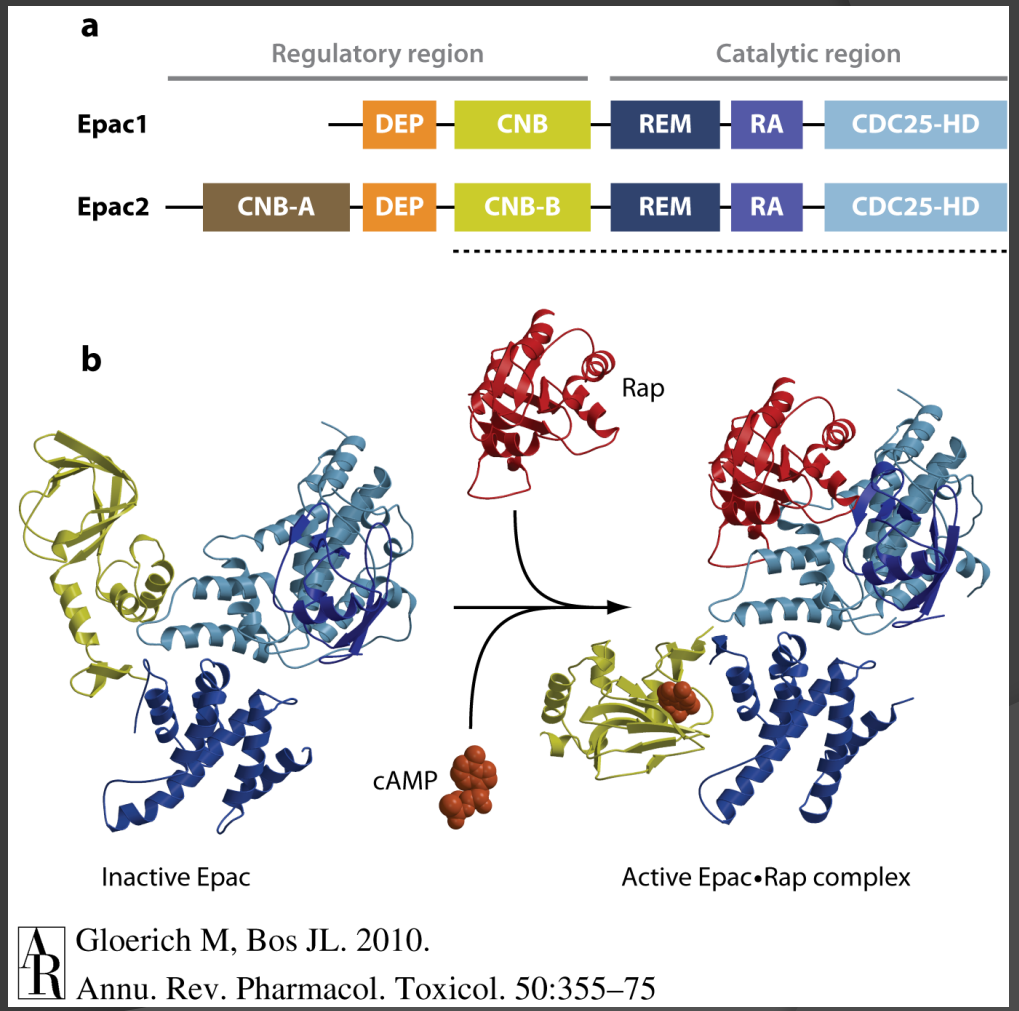
# EPACS

EPACs are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2. Rap GTPases cycle between an inactive GDP-bound and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. Rap proteins are involved in many biological processes, most notably the regulation of cell adhesion through integrins and cadherins.



# EPACS

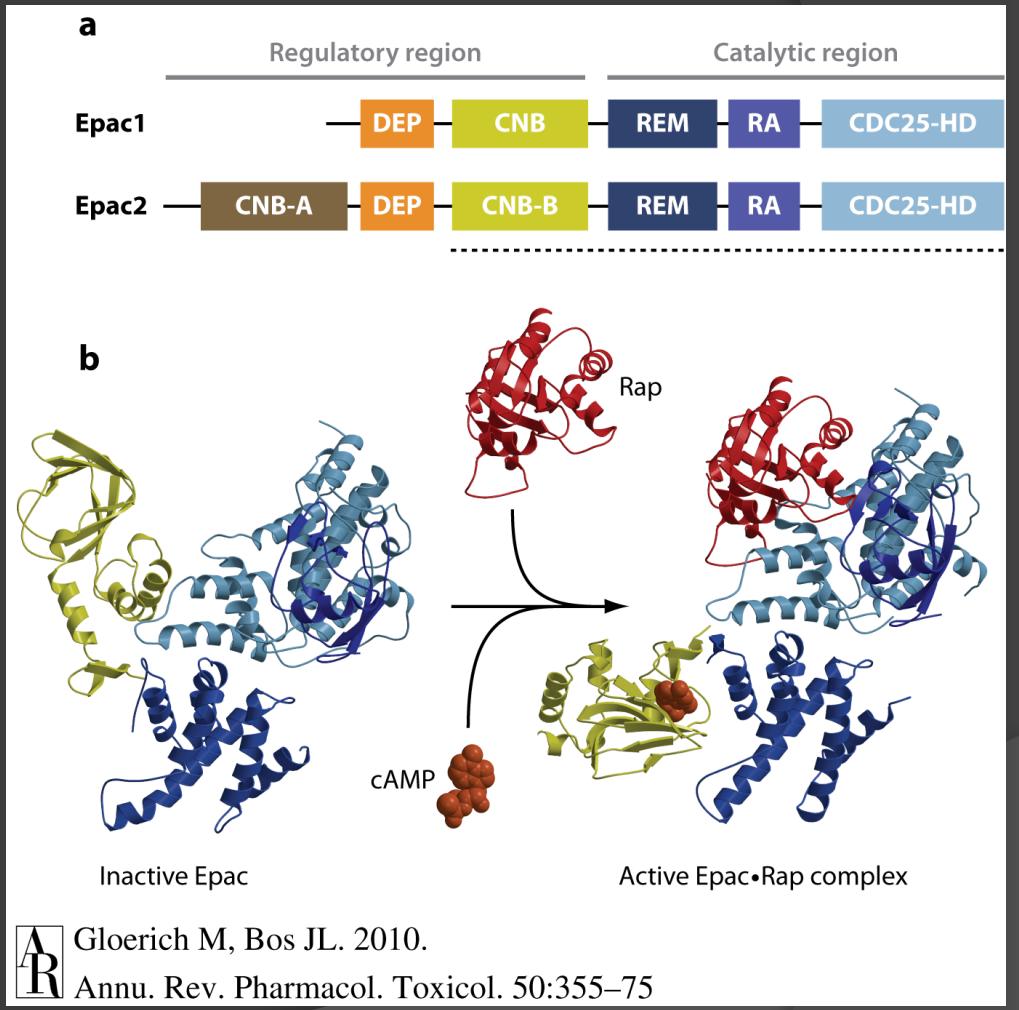
The GEF EPAC1 consists of a C-terminal catalytic domain characteristic of exchange factors for Ras family GTPases and an N-terminal regulatory domain. The latter domain contains a cAMP-binding site similar to those of protein kinase A (PKA) and, in addition, a DEP domain that mediates membrane attachment




Gloerich M, Bos JL. 2010.
Annu. Rev. Pharmacol. Toxicol. 50:355–75

# EPACS

It has been hypothesized that the regulatory domain of EPAC functions as an auto-inhibitory domain, which is relieved from inhibition by cAMP, but direct proof for this notion is lacking. In this model, EPAC is folded in an inactive conformation at low cAMP levels, thereby preventing Rap binding due to steric hindrance. cAMP binding unfolds the protein, allowing Rap to bind.

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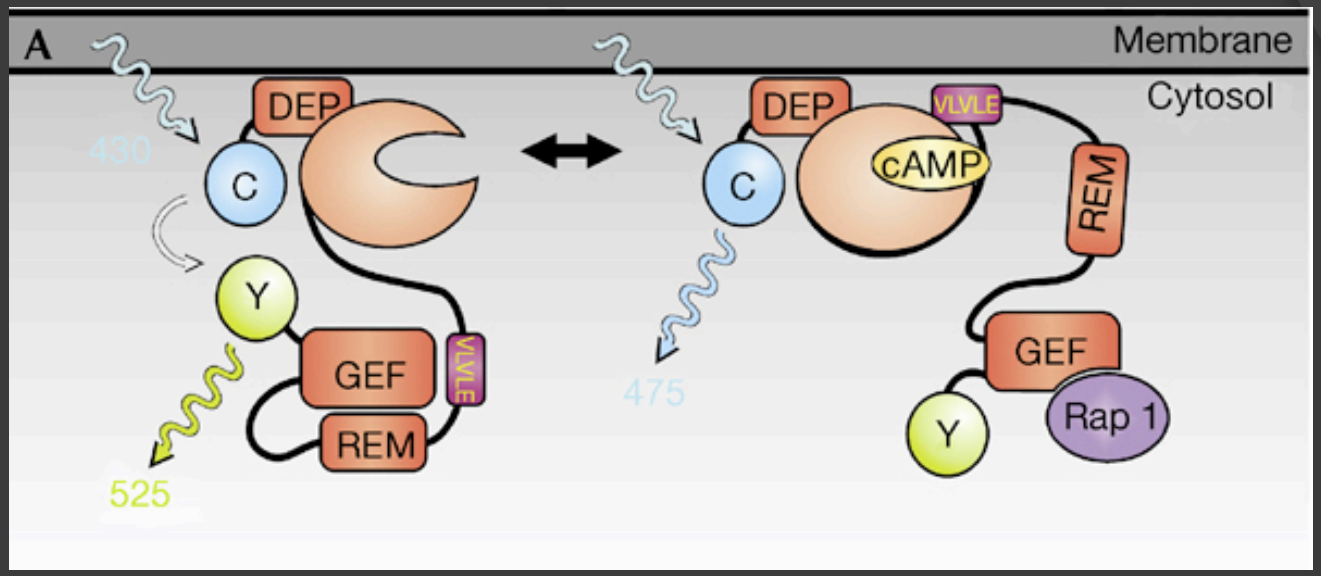
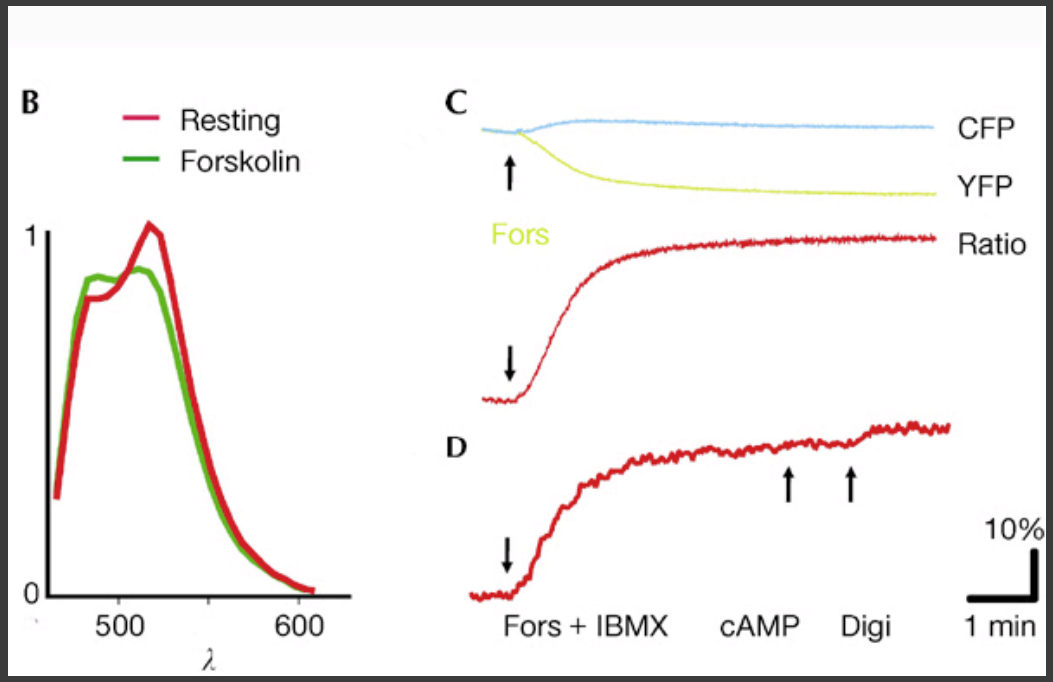


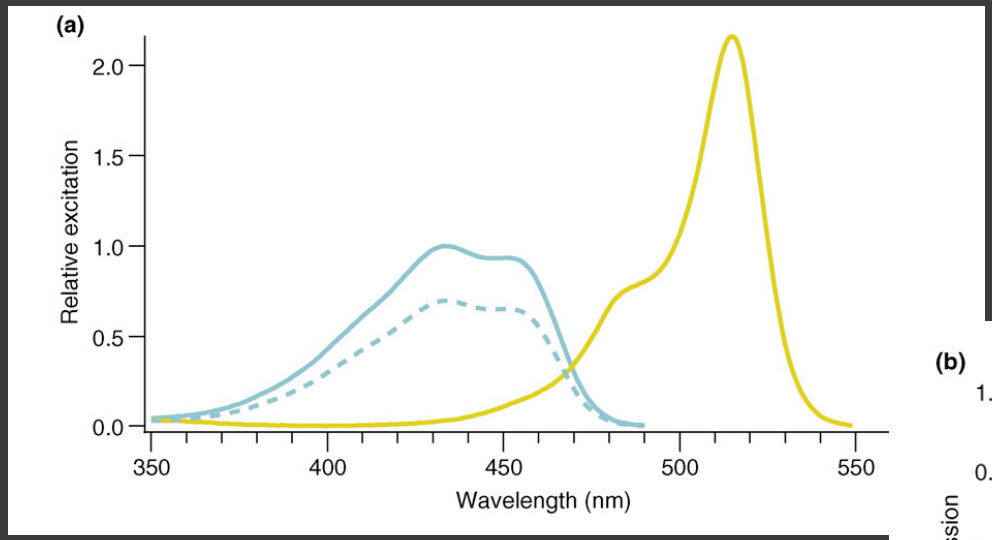

FRET EPAC-based sensors aims to measure EPAC activation in vivo by sandwiching EPAC between cyan fluorescent protein (CFP) at the N-term and yellow fluorescent protein (YFP) at the C-term and then measure fluorescence resonance energy transfer (FRET) between the two fluorescent moieties.

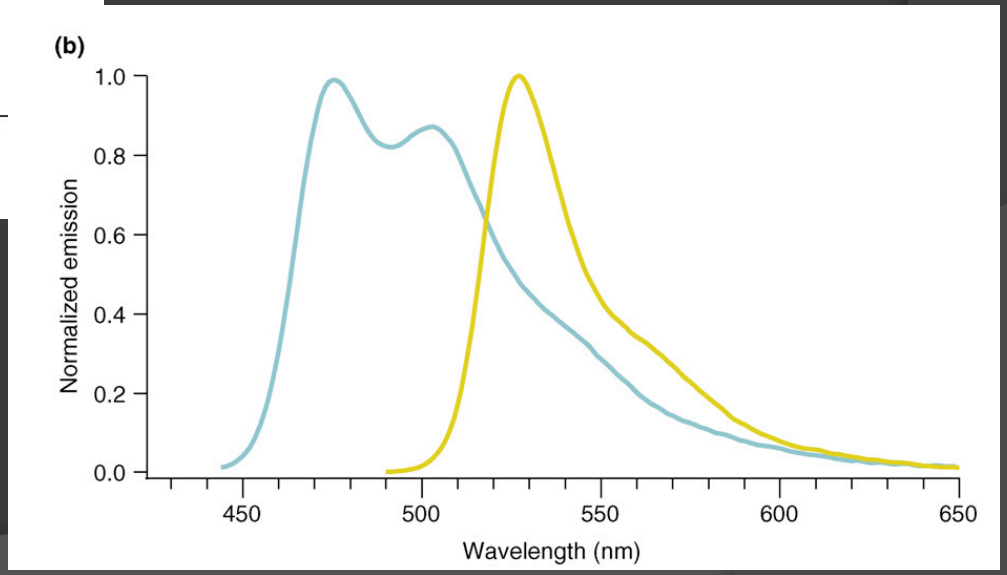


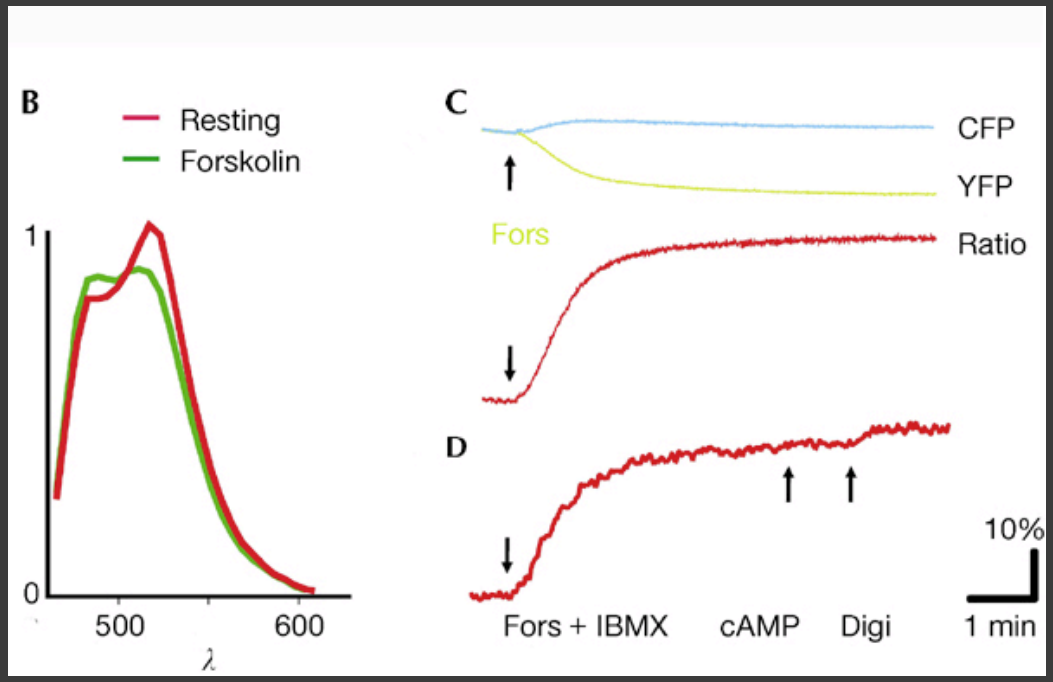

- Fluorescence spectra of these cells revealed significant FRET (Fig 1B, red line), indicating that CFP and YFP are in close proximity (3–4 nm).
- Stimulation with forskolin, a direct activator of adenylyl cyclase, significantly decreased FRET (green line).

# Fluorescent Proteins and FRET: CFP-YFP

CFP and YFP remain the best couple although the significant cross-talk between both the excitation and emission spectra

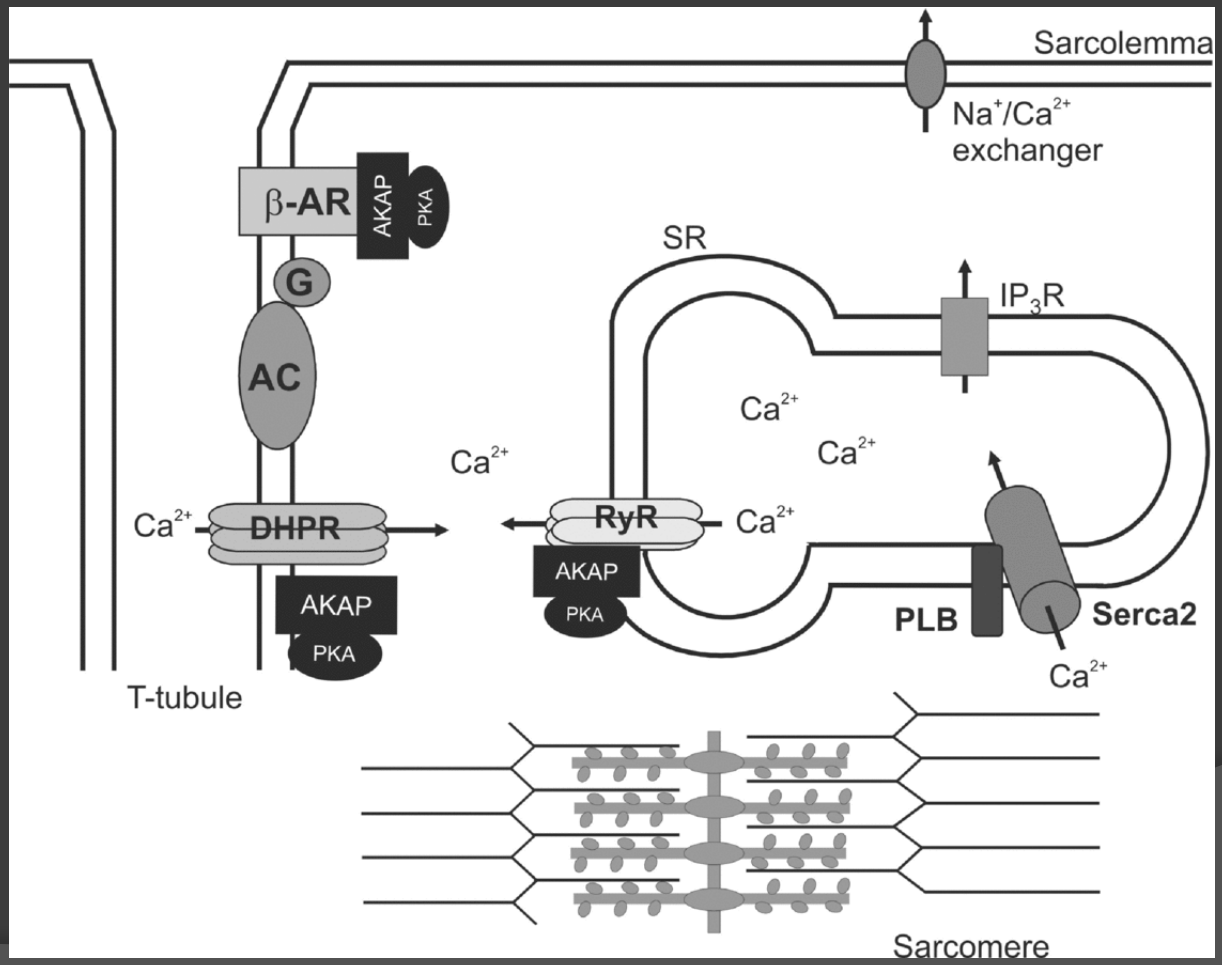


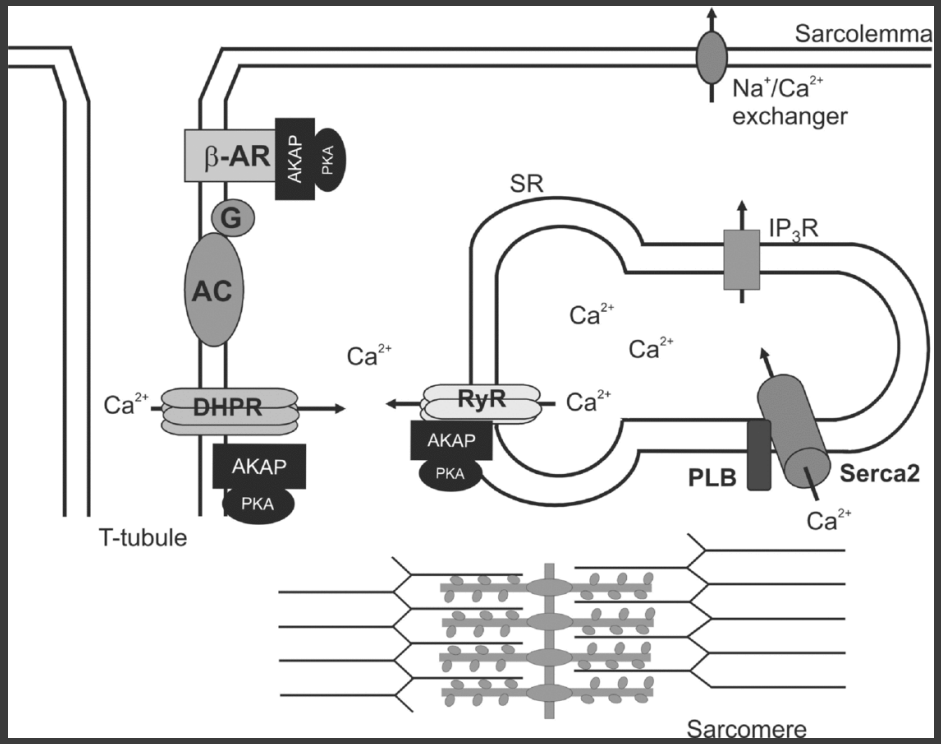



- In the presence of the phosphodiesterase inhibitor IBMX (100 mM), forskolin evoked an average decrease of 73% in CFP/YFP emission ratio. This reflects near-complete saturation of cAMP binding to EPAC, as deduced from experiments where cells were subsequently permeabilized with digitonin (10 mg/ml) in the presence of 2mM extracellular cAMP (Fig 1D). This caused at most a moderate further drop in FRET.

# La segregazione del signalling cAMP è fondamentale in alcuni modelli cellulari quali i cardiomiociti

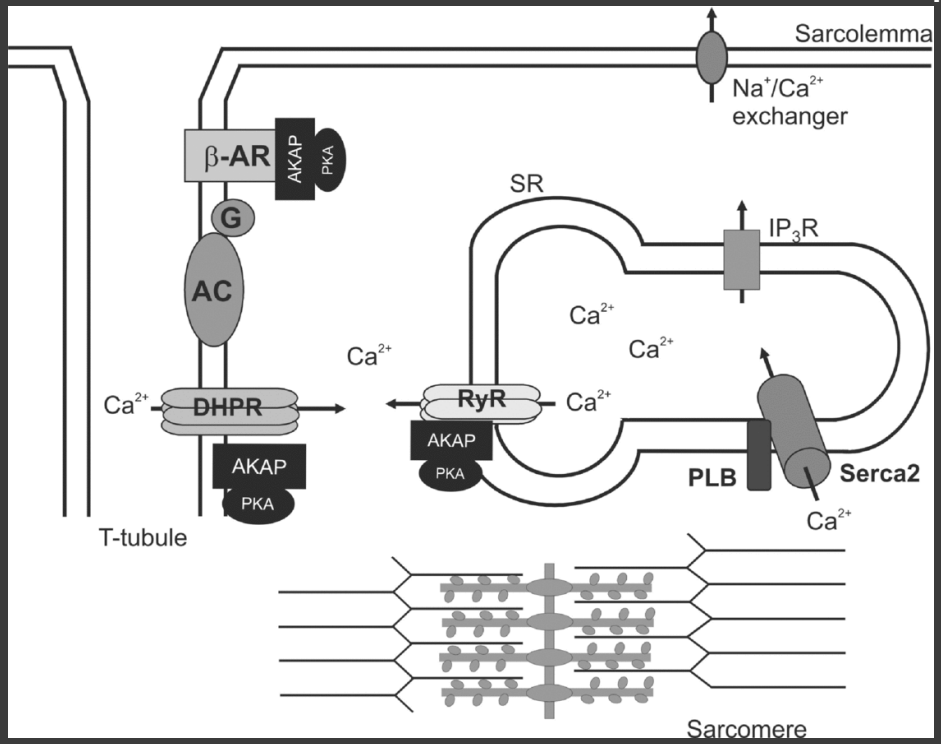


# cAMP nei cardiomiociti



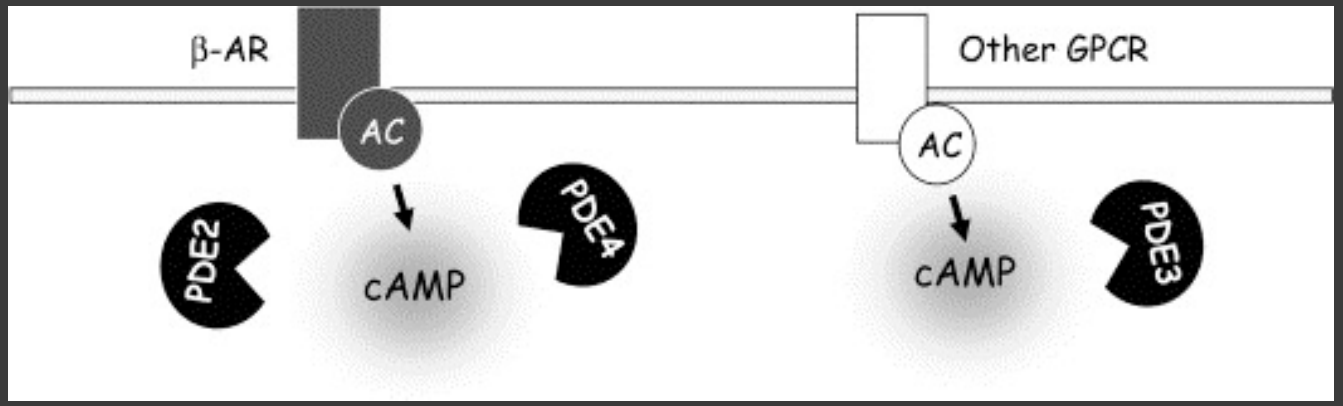

cAMP is a key regulator of excitation–contraction coupling. Sympathetic control of the strength of cardiac myocytes' contraction is exerted by PKA mediated phosphorylation of L-type Ca2+ channels DHPR (dihydropyridine receptor) and the ryanodine receptor, with the consequent increase in the amount of Ca2+ ions available for the sarcomere at the systole.

# cAMP nei cardiomiociti

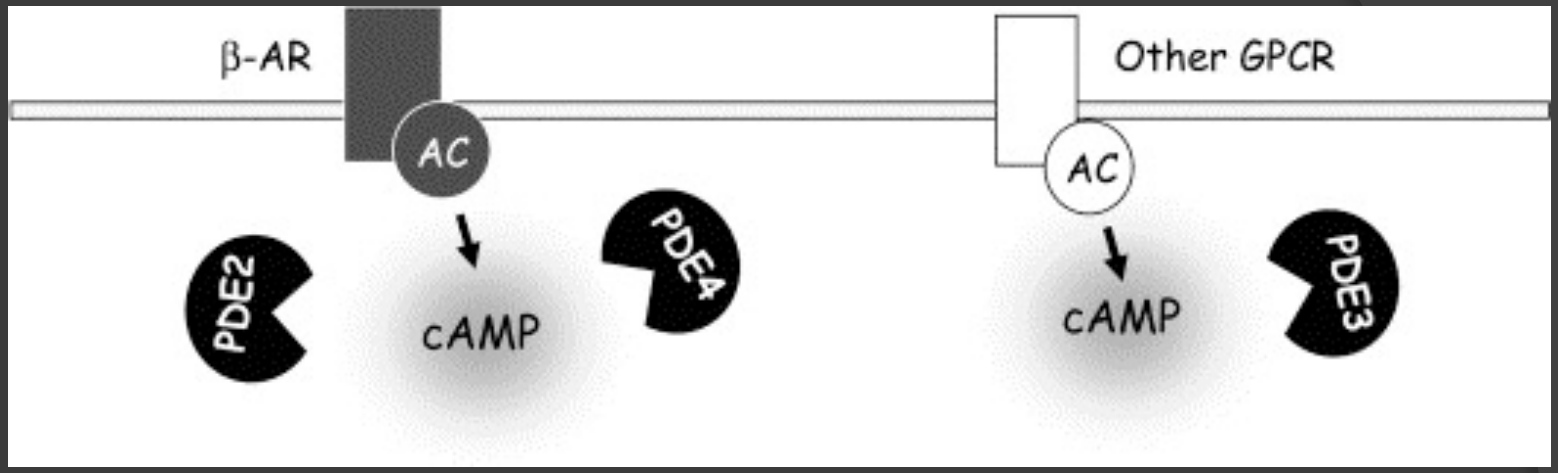



PKA also controls the reuptake of Ca2+ in the sarcoplasmic reticulum at the diastole by phosphorylating phospholamban and thus increasing the activity of the sarcoplasmic reticulum Ca2+ pump. Moreover PKA can phosphorylates sarcomeric proteins, such as the myosinbinding Protein C and troponin I, thus exerting control also on the sensitivity of the sarcomere to Ca2+.

Nei cardiomiociti si trovano microdomini con elevata concentrazione do cAMP in seguito a stimolazione con catecolamnine i quali sono dipendenti dall'attività delle PDEs




Individual PDEs are functionally coupled to a selected pool of adenylyl cyclases (AC) activated upon stimulation of specific G protein-coupled receptors (GPCR). The activity of individual phosphodiesterases (PDE) is selectively engaged in the presence of specific stimuli and is responsible for the generation of distinct microdomains in which the concentration of cAMP is locally modulated.




PDE4 was found to be the main regulator of the amplitude and duration of the cAMP transient generated by catecholamines. In particular, PDE4 was suggested to be functionally coupled with the adenylate cyclase activated by *β-adrenoceptor, whereas PDE3 seemed* to act in a different compartment.

Research Article 107

# Direct demonstration of discrete $Ca^{2+}$ microdomains associated with different isoforms of adenylyl cyclase

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*Accepted 3 November 2009*
*Journal of Cell Science 123, 107-117 Published by The Company of Biologists 2010*
*doi:10.1242/jcs.062067*

- Direct interplay between Ca2+ and cAMP signalling is fundamental to some of the most elaborate aspects of cellular homeostasis, including hormone and neurotransmitter release, cardiac contraction, cell migration and synaptic development. To a considerable extent, the co-ordinated interaction between these two ubiquitous messengers is controlled by the Ca2+-sensitive adenylyl cyclases (AC1, AC5, AC6 and AC8).
- AC1 and AC8 are stimulated by Ca2+ in a calmodulin (CaM)-dependent manner

Research Article 107

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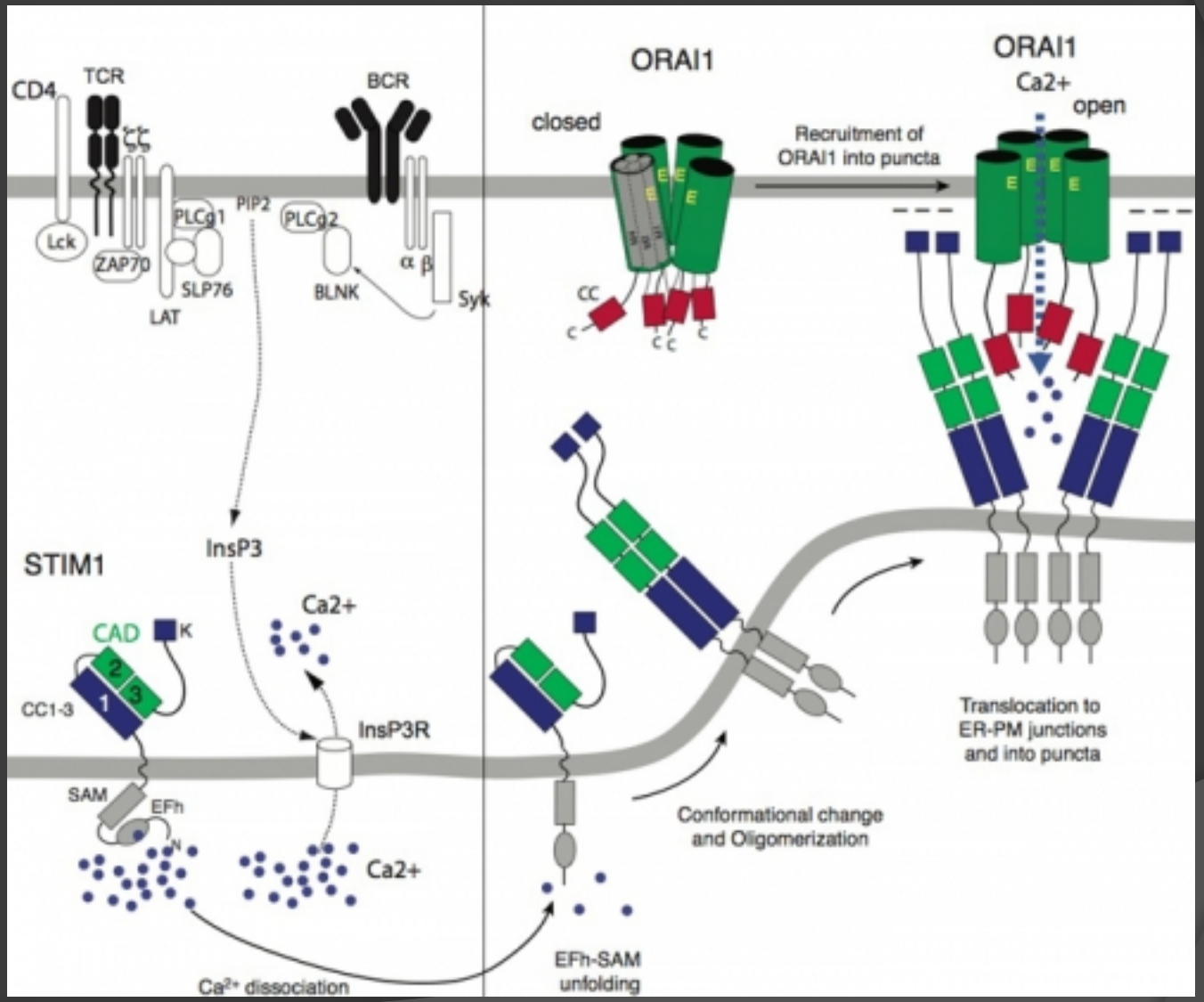
Previous studies have demonstrated a robust selectivity of the Ca2+-sensitive ACs for distinct modes of Ca2+ increase. In particular, the ACs are uniquely sensitive to Ca2+ rises mediated by store-operated Ca2+ entry (SOCE), which occurs as a consequence of endoplasmic reticulum (ER) store depletion.

- Ca2+-sensitive ACs display remarkably limited sensitivity to Ins(1,4,5)*P3-mediated Ca2+ release from the ER in* the absence of external Ca2+ and to other forms of Ca2+ entry mediated by ionophore, arachidonic acid or 1-oleoyl-2-acetyl-snglycerol (OAG). Thus, despite the enzymes displaying a simple dose-dependent Ca2+ sensitivity in vitro, in the intact cell, equivalent (or higher) Ca2+ rises originating from non- CCE sources are far less effective at regulating the ACs. The reason for this selectivity is currently unknown.

# Store-Operated Ca2+ Entry (SOCE or CCE)



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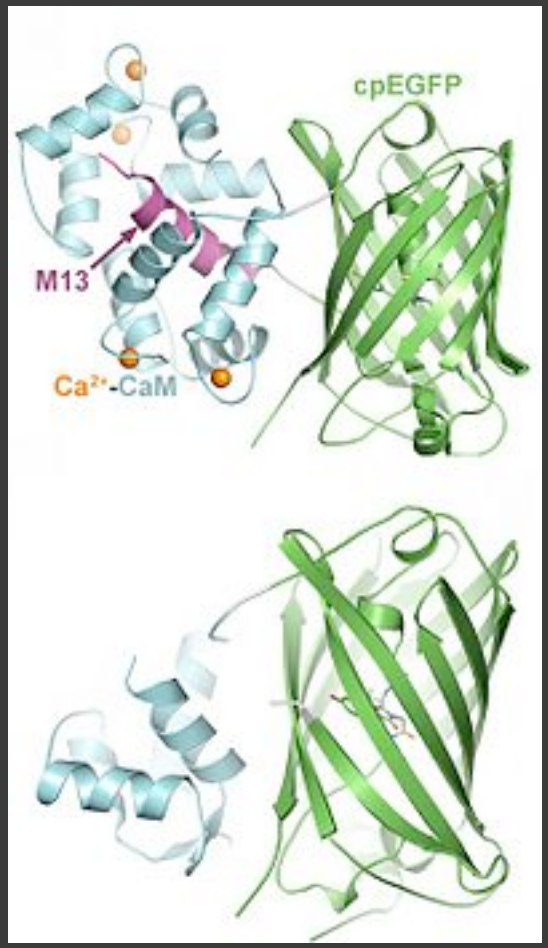
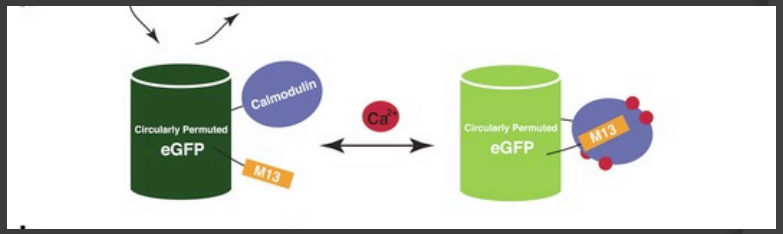
By comparing the Ca2+ signals seen within the immediate vicinity of the Ca2+- stimulated AC8 and the Ca2+-insensitive AC2 with more widespread Ca2+ changes monitored within the bulk cell cytosol or subplasmalemmal regions, we would be able to directly establish whether the selectivity of AC8 for CCE is dictated by its residence in a unique Ca2+ microdomain.




- GCaMP2 is a genetically encoded, high-affinity Ca2+ sensor that exhibits large fluorescent shifts in response to physiological Ca2+ changes. The sensor comprises a circularly permuted EGFP flanked by CaM and a CaM-binding peptide (M13) from myosin light chain kinase. Increases in Ca2+ promote Ca2+-CaM-M13 interaction and a conformational change within the sensor, resulting in an increase in EGFP fluorescence





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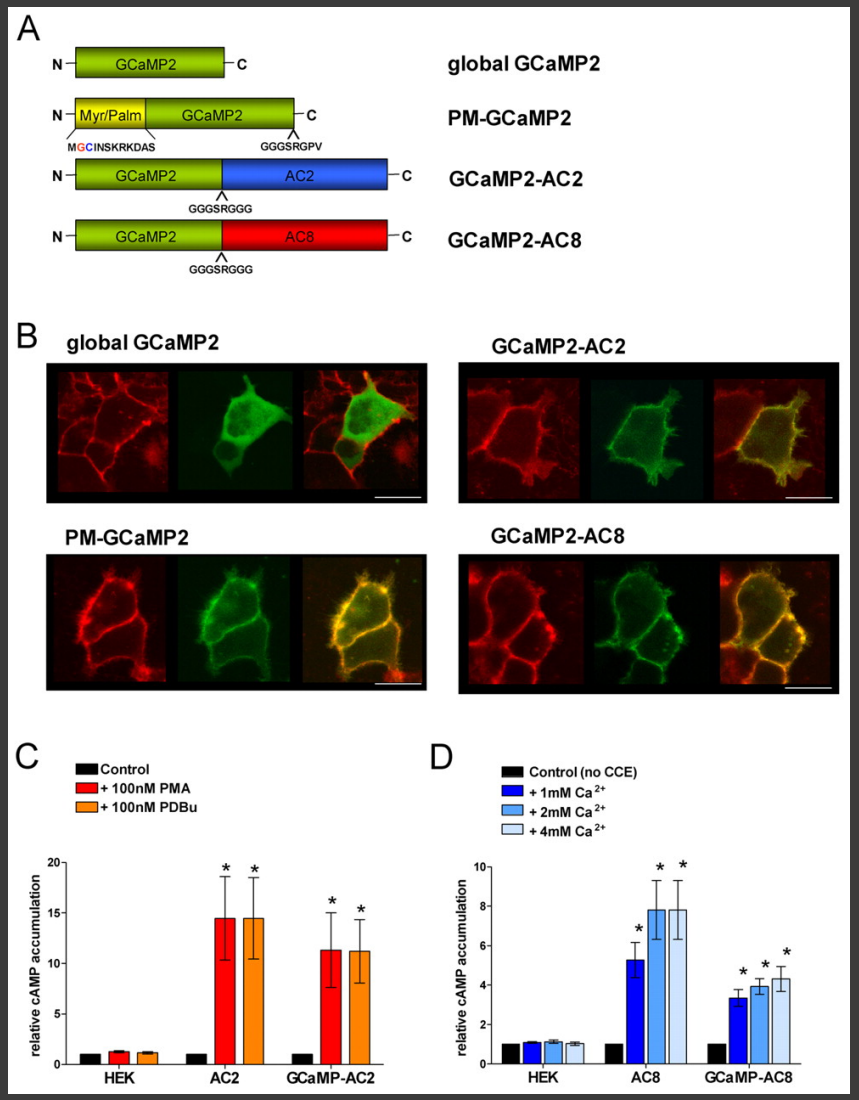


- a plasma-membrane-targeted version of GCaMP2 was generated by the addition of an ‘SH4’ sequence motif derived from Lyn kinase. This sequence is modified to generate a palmitoylation and myristoylation group at the N-terminus of GCaMP2, which effectively targets the sensor to the PM (this new construct is referred to as PM-GCaMP2).

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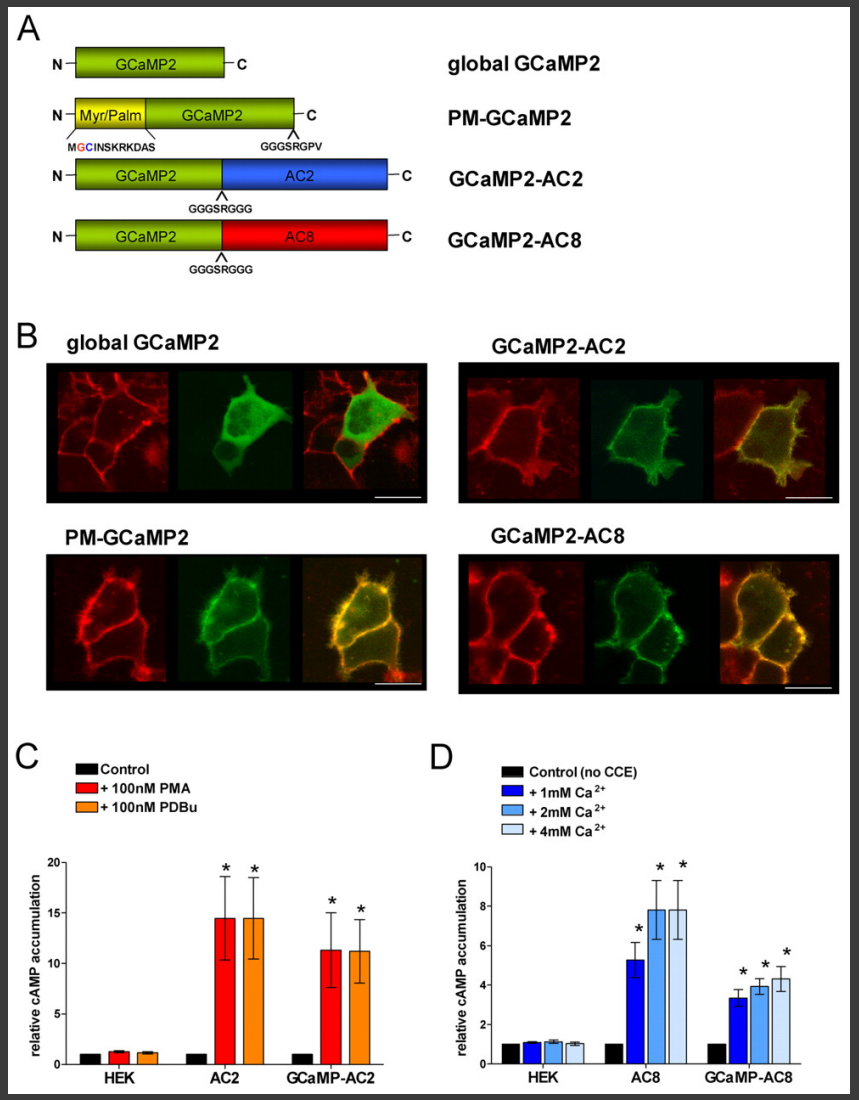


- Ca2+-stimulated AC8 is thought to reside in lipid rafts
- and Ca2+-insensitive AC2 to reside in non-raft regions of the plasma membrane

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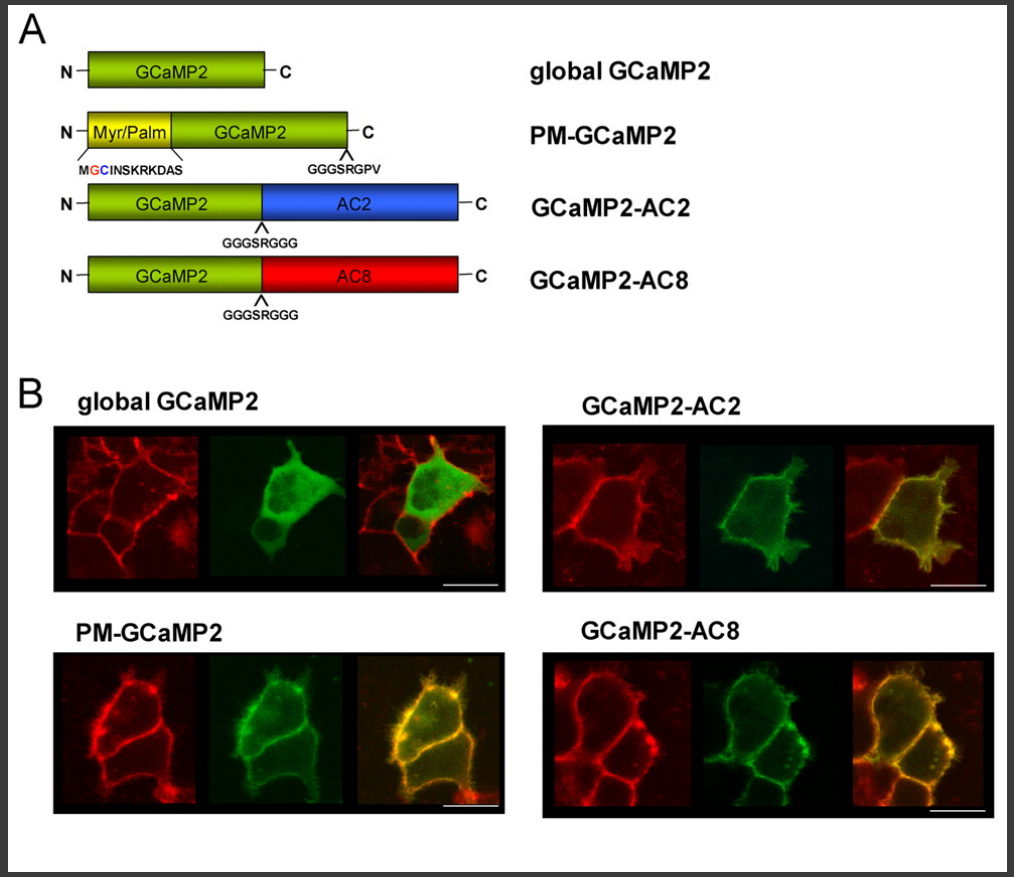
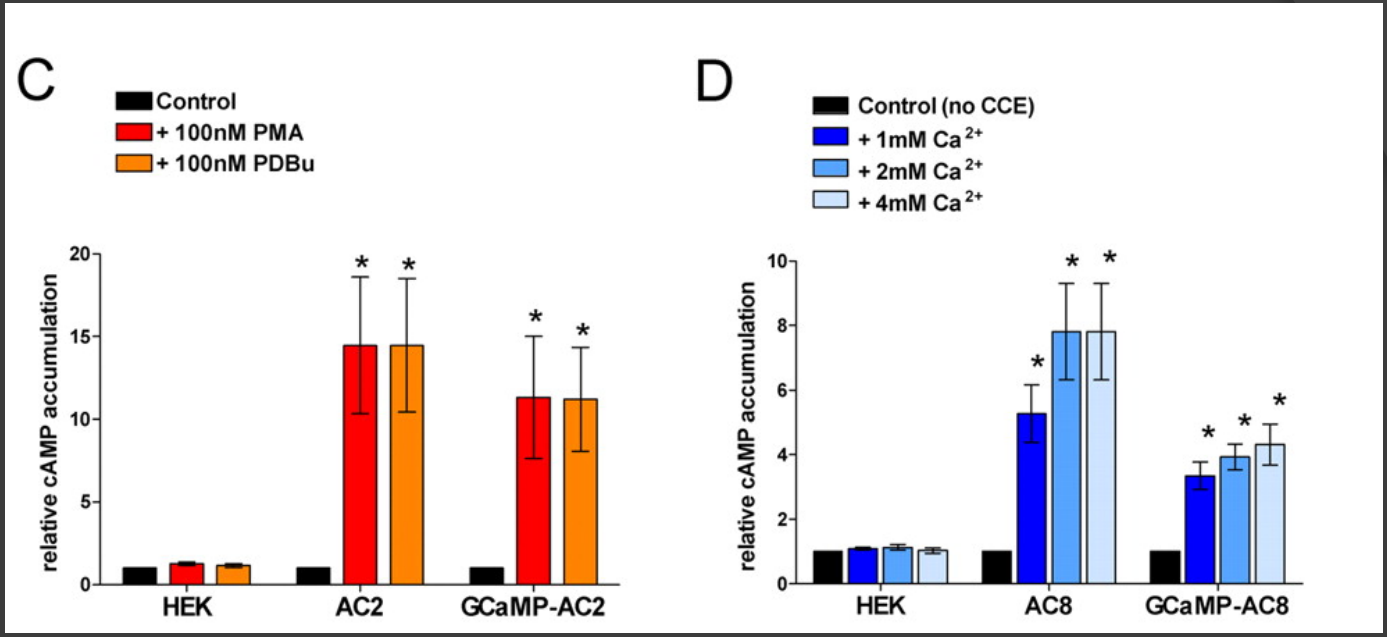



- Good colocalization of PM-GCaMP2, GCaMP2-AC2 and GCaMP2-AC8 with the CellMask PM marker. PM-GCaMP2 and GCaMP2-AC8 consistently displayed good colocalization with the PM marker,

Willoughby, D. et al. J Cell Sci 2010;123:107-117




- Cell population cAMP assay data presented in Fig. 1C revealed significant enhancement of GCaMP2- tagged AC2 activity during treatment with the phorbol esters, phorbo myristate acetate (PMA, 100 nM) or phorbol dibutyrate PDBu, 100 nM).
- The data confirmed that the tagged AC2 was localized to the same regulatory domains of the PM as the wild-type homologue.

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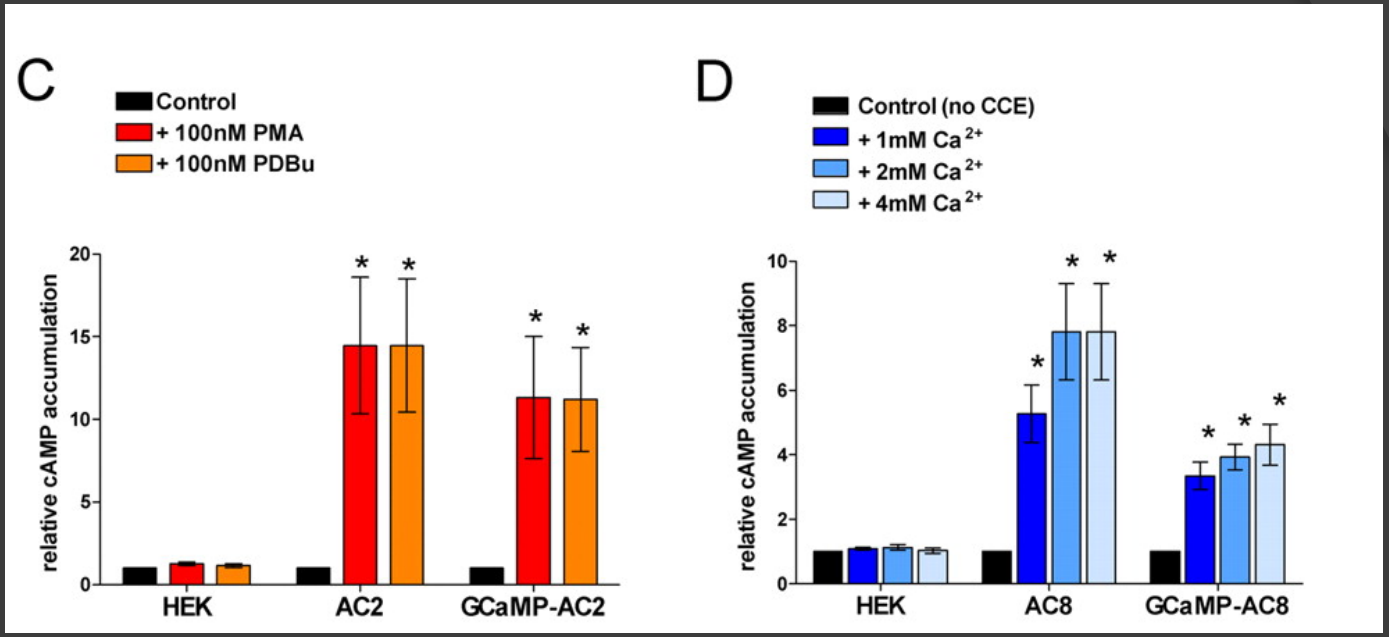


- A comparison of GCaMP2-AC8 and wild-type AC8 activity during a 1-minute period of SOCE, following prior store depletion using 100 nM Tg in Ca2+-free conditions, revealed that both versions of AC8 responded well to increasing degrees of CCE (Fig. 1D).
- These data confirmed that GCaMP2-AC8 was correctly targeted to regions of the PM associated with CCE.

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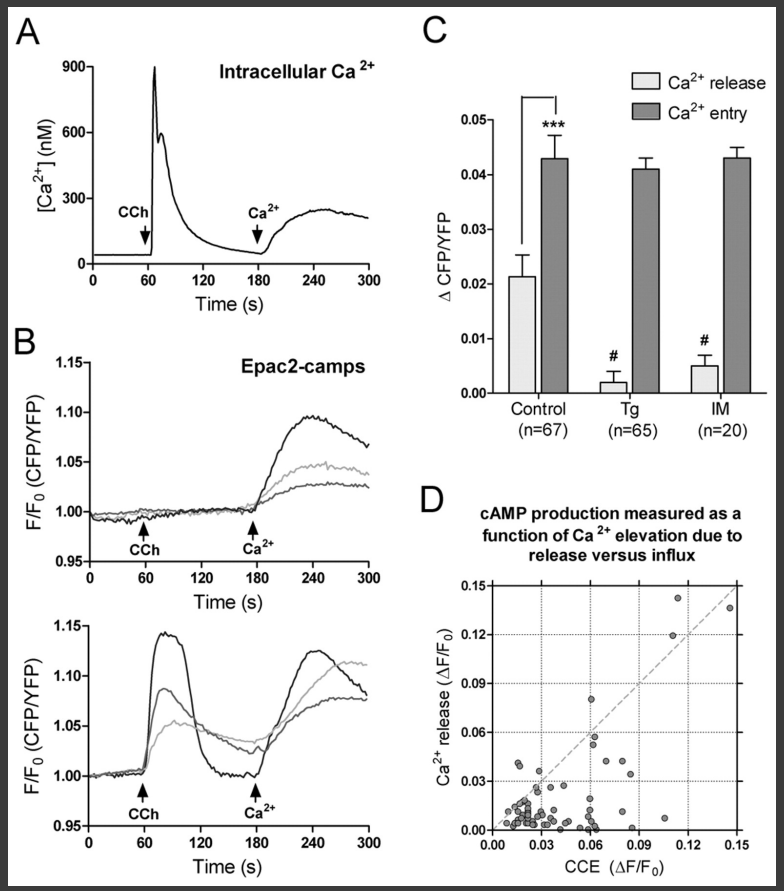
# Stimulation of AC8 by CCh-induced Ca2+ release versus CCE at the single-cell level




- Prior to examining the subcellular [Ca2+] changes detected by our newly designed GCaMP2-based sensors, we first analysed the effects of Ins(1,4,5)*P*3-induced Ca2+ release and SOCE on cAMP production in individual HEK293 cells stably expressing AC8 (HEK-AC8).
- To monitor changes in cAMP production, the cytosolic FRET-based cAMP sensor Epac2-camps was transiently expressed in the HEK-AC8 cells

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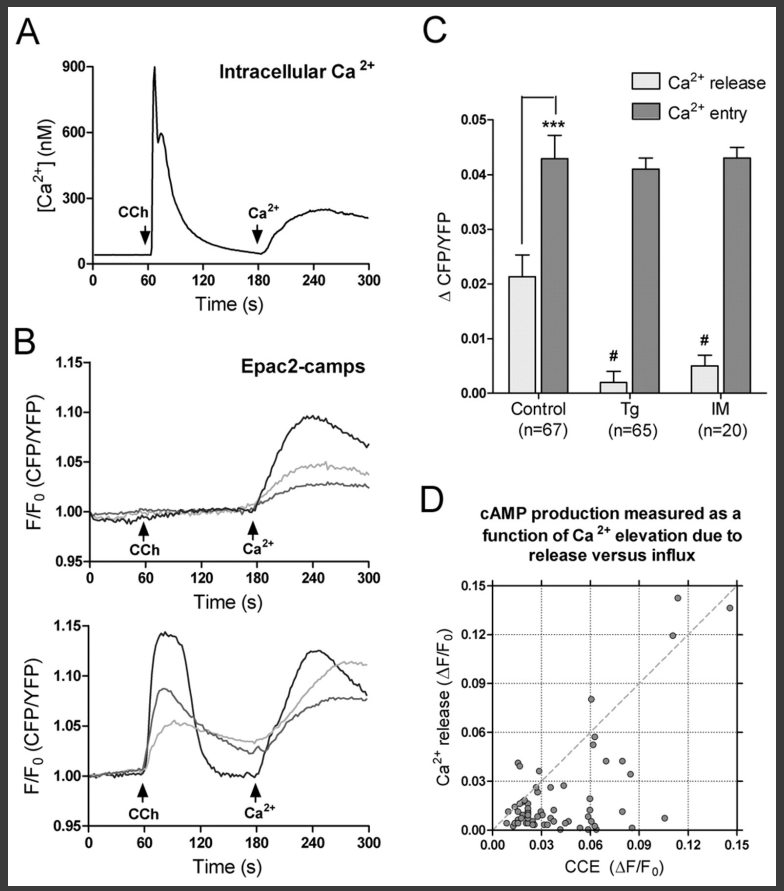
# Stimulation of AC8 by CCh-induced Ca2+ release versus CCE at the single-cell level




- Addition of a muscarinic agonist, carbachol (CCh; 500 uM), in Ca2+-free conditions mobilized ER stores to generate a large rise in cytosolic [Ca2+], as detected with the ratiometric Ca2+ indicator Fura-2 (Fig. 2A). In parallel real-time cAMP measurements, the majority of cells (39 out of 67 cells tested) exhibited no increase in cAMP production during Ca2+ mobilization from the ER (see Fig. 2B, top panel for example traces). The remaining 28 cells displayed varying degrees of cAMP production in response to the Ins(1,4,5)*P3-mediated Ca2+* signal

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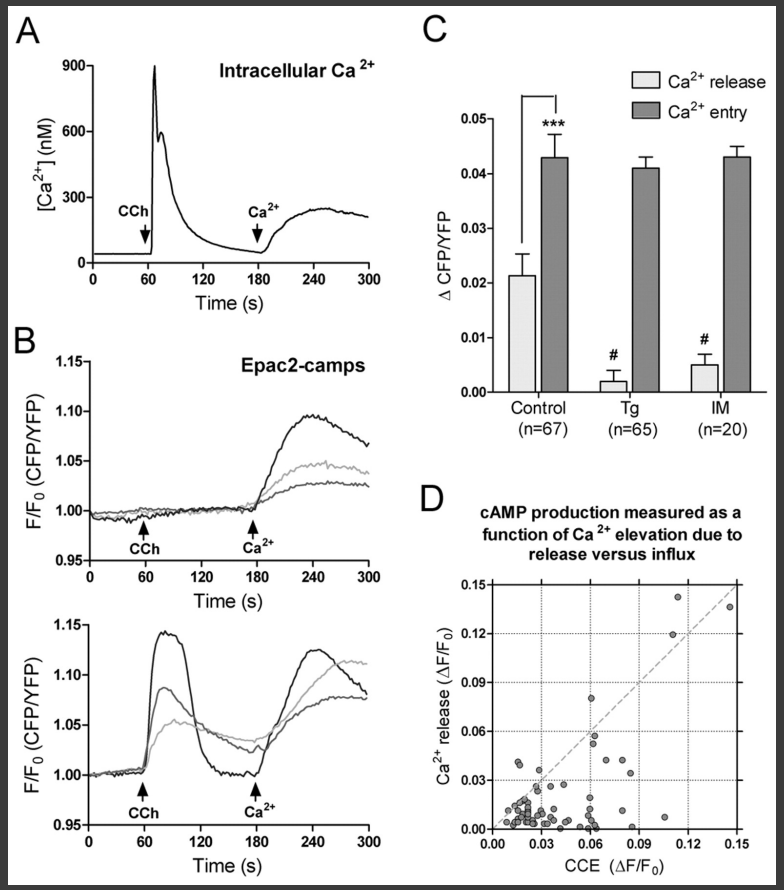
# Stimulation of AC8 by CCh-induced Ca2+ release versus CCE at the single-cell level




- The subsequent addition of 2 mM external Ca2+ to trigger SOCE (Fig. 2A, top panel) was accompanied by enhanced cAMP production in all cells tested (Fig. 2B). Data analyses revealed that, on average, SOCE produced a twofold greater increase in cAMP production in HEK-AC8 cells than CCh-induced Ca2+ mobilization (Fig. 2C).

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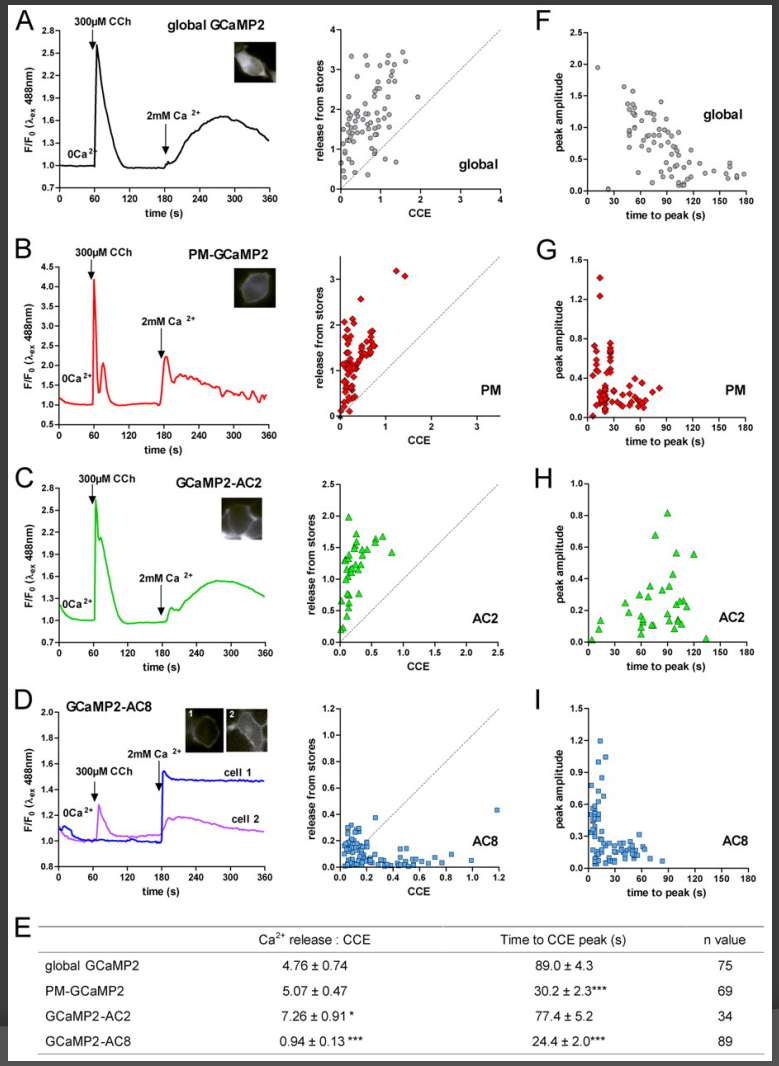
# Selectivity of GCaMP2-AC8 for detection of Ca2+ changes associated with CCE rather than Ins(1,4,5)P3-mediated Ca2+ release from the ER.




| | $Ca^{2+}$ release : CCE | Time to CCE peak (s) | n value |
|---|---|---|---|
| global GCaMP2 | 4.76 ± 0.74 | 89.0 ± 4.3 | 75 |
| PM-GCaMP2 | 5.07 ± 0.47 | 30.2 ± 2.3*** | 69 |
| GCaMP2-AC2 | 7.26 ± 0.91 * | 77.4 ± 5.2 | 34 |
| GCaMP2-AC8 | 0.94 ± 0.13 *** | 24.4 ± 2.0*** | 89 |

- Using the same protocol to induce Ca2+ mobilization and SOCE as described above, we examined local changes in Ca2+ in the immediate vicinities of AC8 and the Ca2+-insensitive AC2. Changes in Ca2+ were also assessed using global or PM-targeted GCaMP2 sensors.
- Representative traces of fluorescent intensity shifts for each GCaMP2 construct are presented in Fig. 3A-D alongside scatter plot analyses of the peak responses of each sensor to Ca2+ release and entry in all cells tested

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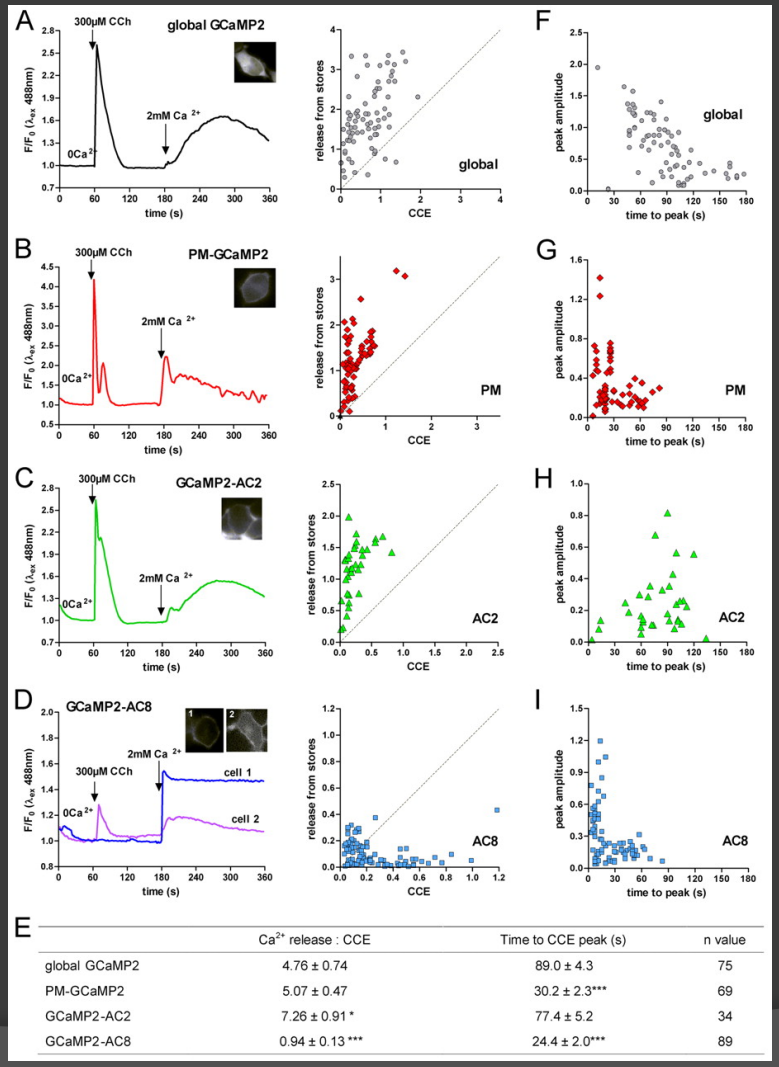


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| GCaMP2-AC2 | 7.26 ± 0.91 * | 77.4 ± 5.2 | 34 |
| GCaMP2-AC8 | 0.94 ± 0.13 *** | 24.4 ± 2.0*** | 89 |

**67% of GCaMP2-AC8-expressing cells** (60 out of 89 cells tested) detected **little or no change in local Ca2+ levels during CCh-evoked Ca2+ release** (e.g. Fig. 3D, cell 1), indicating that AC8 might reside at sites within the PM that are 'distant' or 'shielded' from Ins(1,4,5)*P3-receptor-mediated Ca2+* signals. The remaining GCaMP2-AC8 cells reported a modest transient rise in fluorescent signal during CCh-evoked Ca2+ release (Fig. 3D, cell 2).

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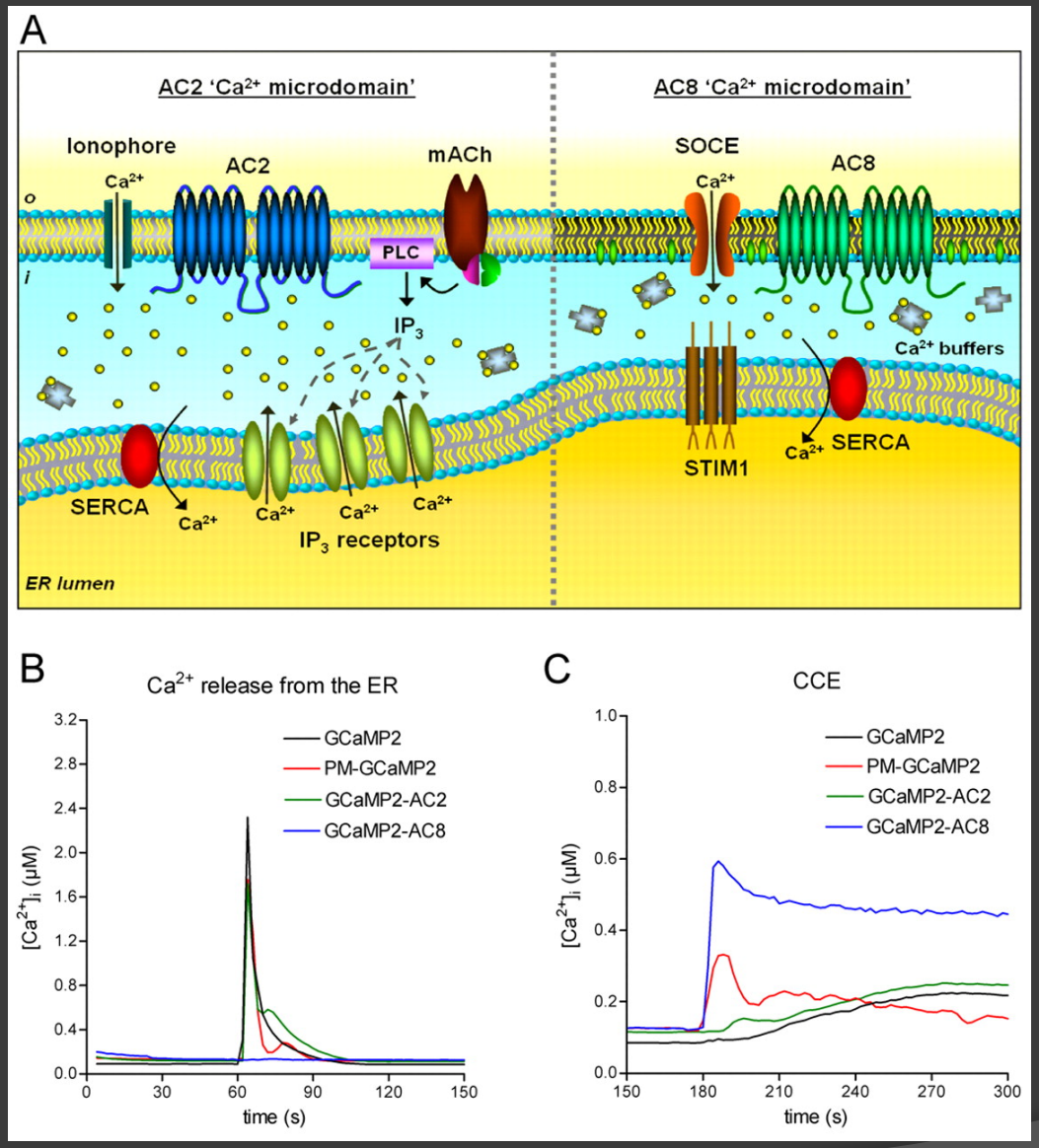


- The data point towards the targeting of AC8 and AC2 to distinct microdomains of the PM where they appear to colocalize with a different assortment of Ca2+ regulatory proteins (Fig. 6A).
- (**B) Calibrated Ca2+** signals from single cells expressing each of the four GCaMP2 sensors in response to CCh-induced Ca2+ mobilization.
- (**C) Calibration of GCaMP2** sensor signals in the same cells during SOCE.

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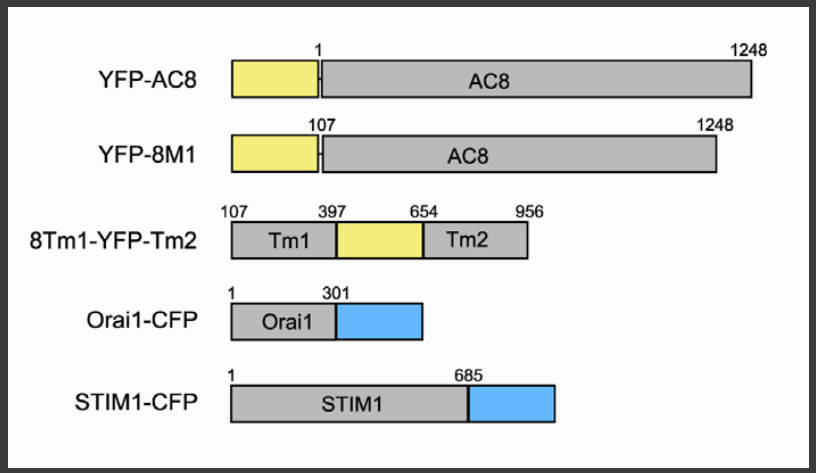
` **Direct Binding Between Orai1 and AC8 Mediates Dynamic Interplay Between Ca 2+ and cAMP Signaling**

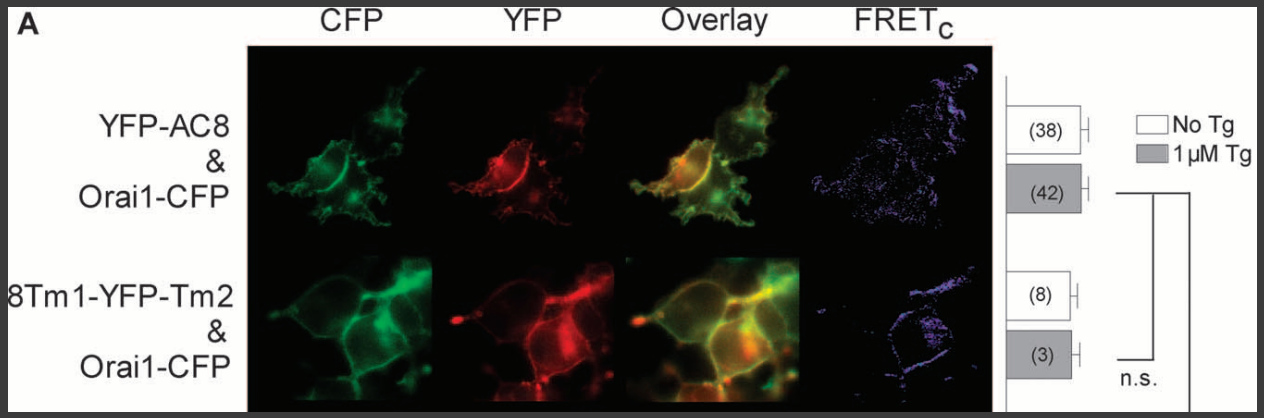
Debbie Willoughby, Katy L. Everett, Michelle L. Halls, Jonathan Pacheco, Philipp Skroblin, Luis Vaca, Enno Klussmann and Dermot M. F. Cooper (10 April 2012) *Science Signaling* **5** (219), ra29. [DOI: 10.1126/scisignal.2002299]

We used multidisciplinary approach involving fluorescence resonance energy transfer (FRET), glutathione S-transferase (GST) pulldown, coimmunoprecipitation, and peptide array analyses to identify a direct protein-protein interaction between AC8 and Orai1.

We used live-cell imaging with high-resolution AC-targeted Ca2+ and cAMP biosensors, together with small interfering RNA (siRNA) knockdown of Orai1, to demonstrate a role for Orai1 in generating dynamic changes in Ca2+ concentration within the AC8 microdomain to stimulate cAMP production.




Analysis of FRET images, produced a normalized corrected FRET (NFRETc) value of 6.5 ± 0.6 (x 105) in cells expressing YFP-tagged AC8 (YFP-AC8) and CFP–tagged Orai1 (Orai1-CFP), consistent with a protein-protein interaction between AC8 and Orai1.




We observed a similar degree of FRET when Orai1-CFP was coexpressed with 8Tm1-YFP-Tm2 [a construct containing the full-length N terminus and transmembrane domains of AC8 but lacking much of its catalytic C1 and C2 domains, with YFP sandwiched between AC8 residues 397 and 654]. The observation of FRET between Orai1-CFP and the catalytically inactive 8Tm1-YFP-Tm2 construct suggests that **the interaction of Orai1 with AC8 is unaffected by cAMP signaling.**

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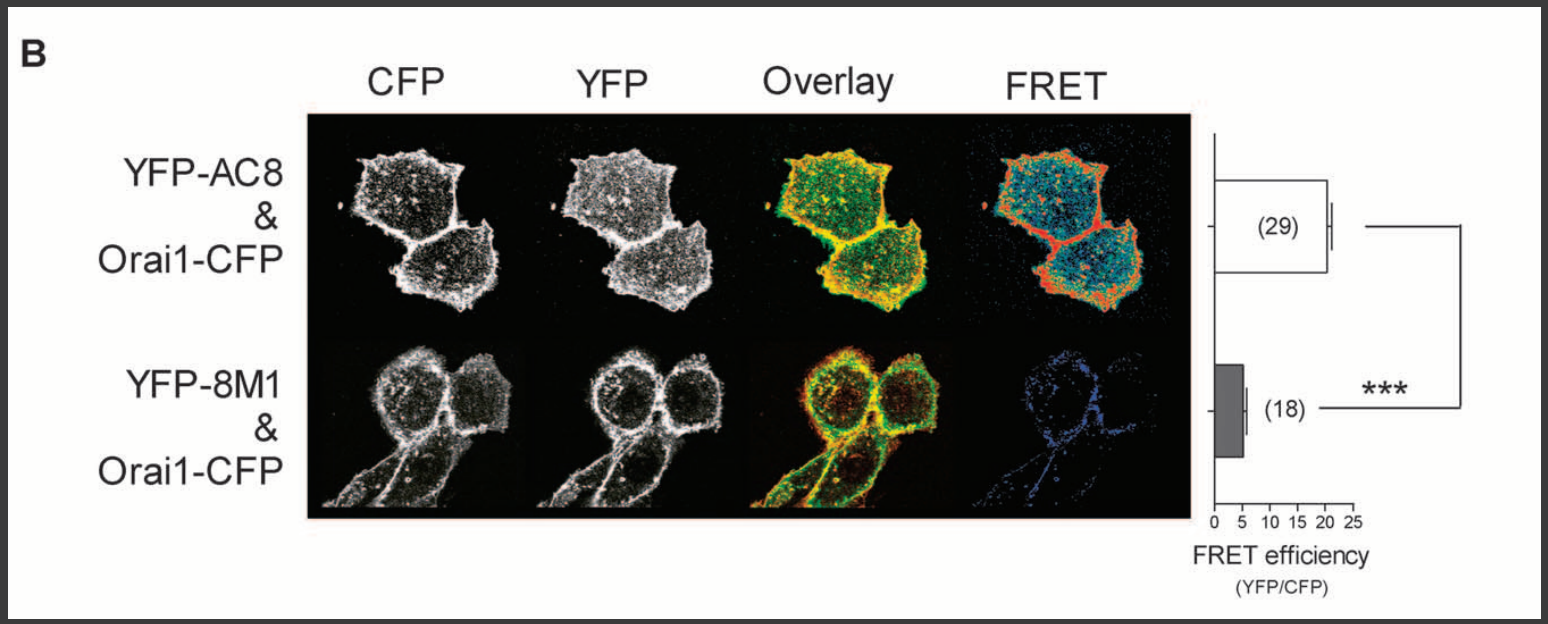


In contrast, FRET between Orai1-CFP and YFP-8M1 [a fluorescently tagged N-terminally truncated form of AC8 that lacks the first 106 residues] was markedly reduced compared to FRET between Orai1-CFP and YFP-AC8. This loss of FRET signal suggests that the **N terminus of AC8,** which is critical for AC8 regulation by SOCE, was also **required for AC8 and Orai1 interaction**.

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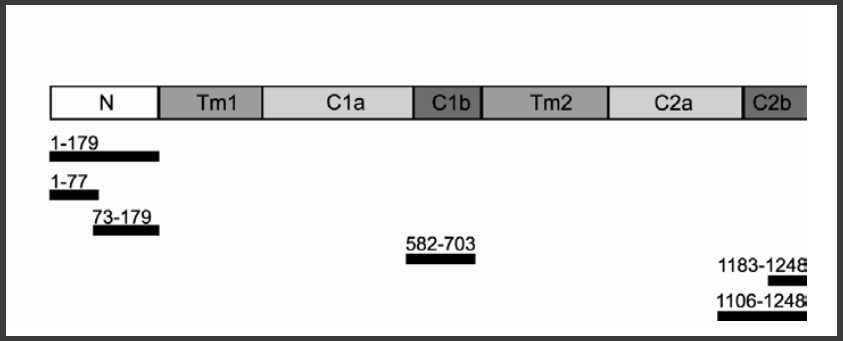
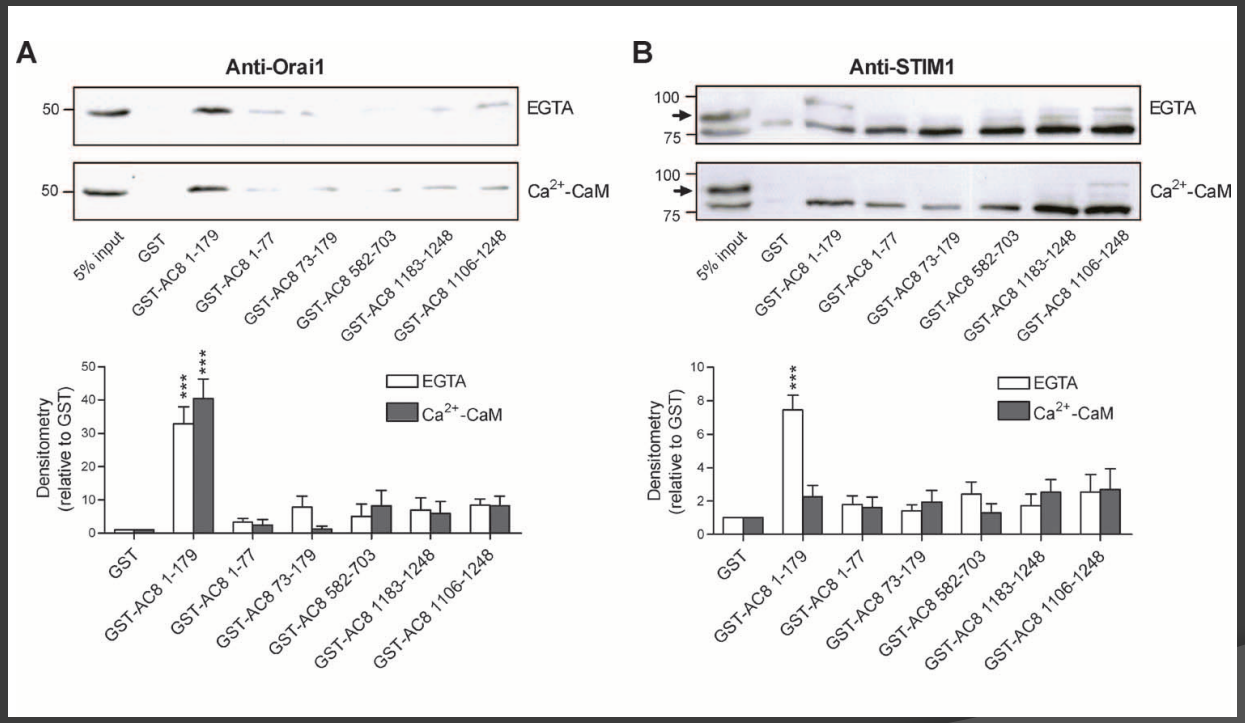
# AC8 and Orai1 interact via their N termini




we tagged cytosolic fragments of AC8 with GST and used them to pull down endogenous Orai1 from HEK293 cell lysates

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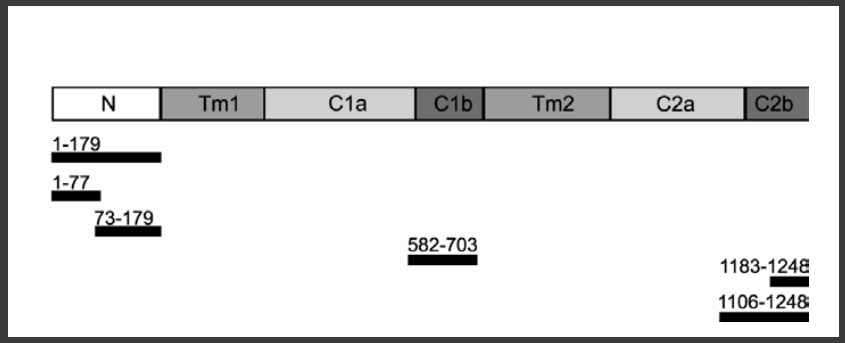


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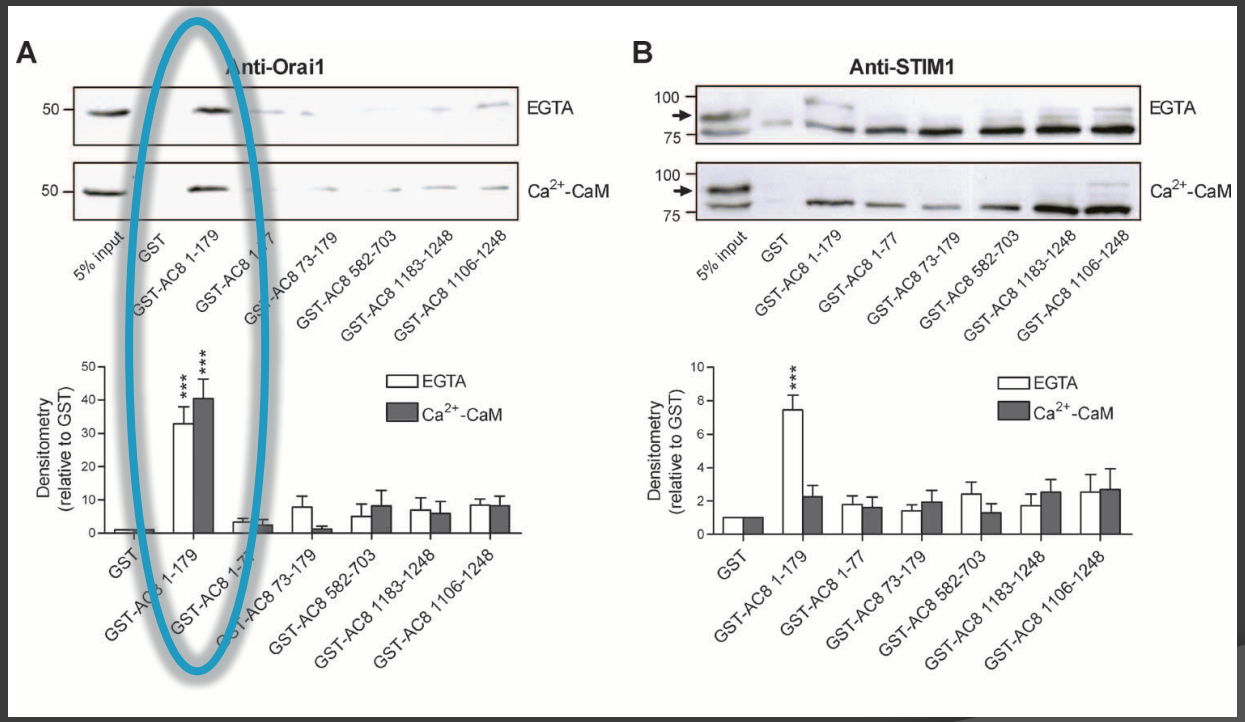


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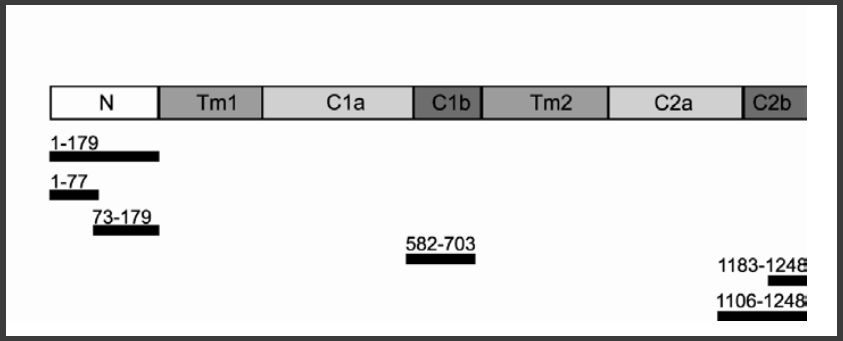


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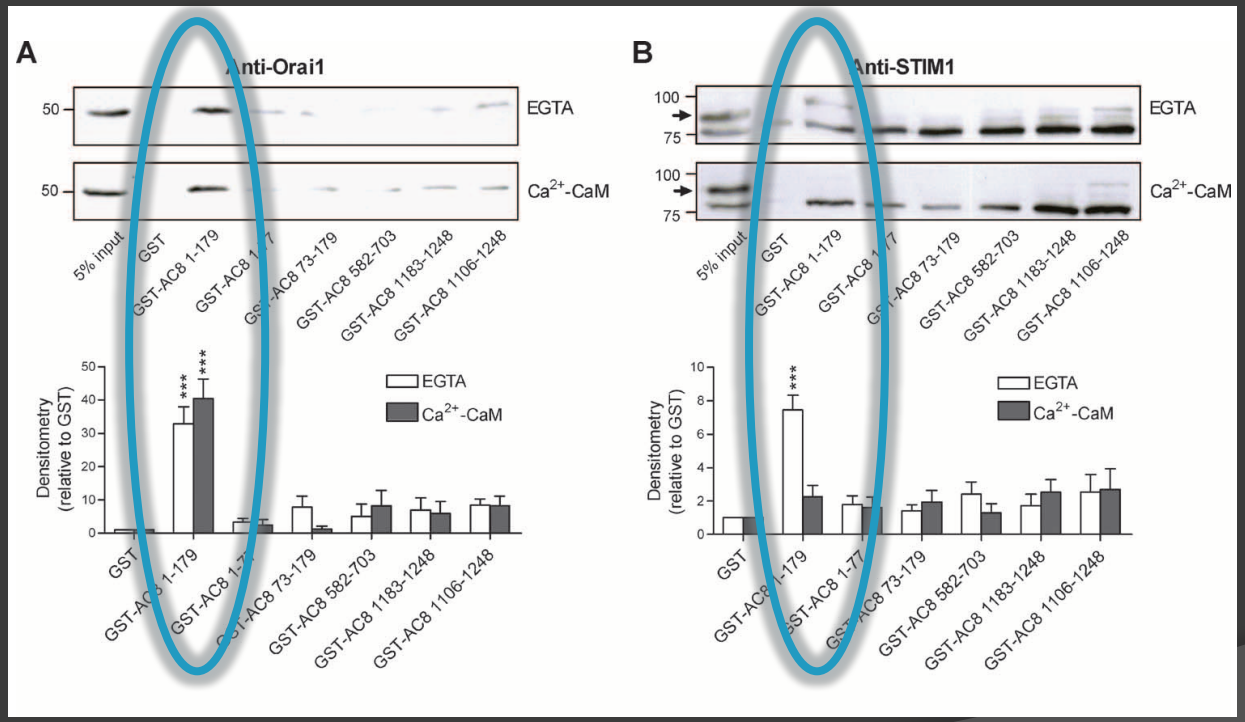


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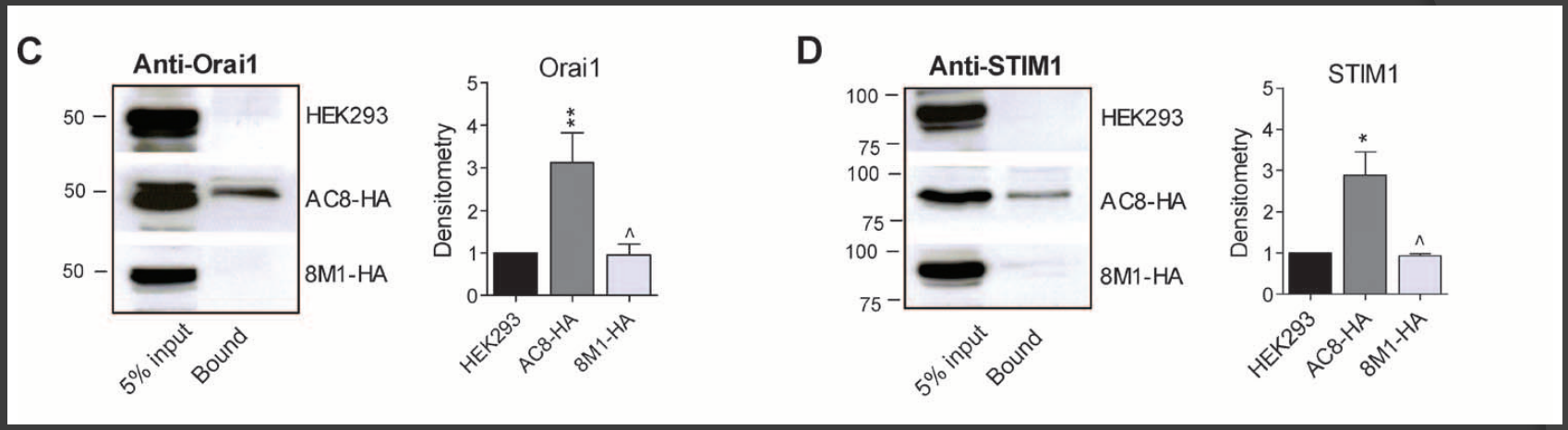
# AC8 and Orai1 interact via their N termini




Affinity coimmunoprecipitation with hemagglutinin (HA)–tagged AC8 confirmed that both Orai1 and STIM1 interacted with full-length AC8 (AC8-HA) (Fig. 3, C and D) but not with the N-terminally truncated form (8M1-HA)

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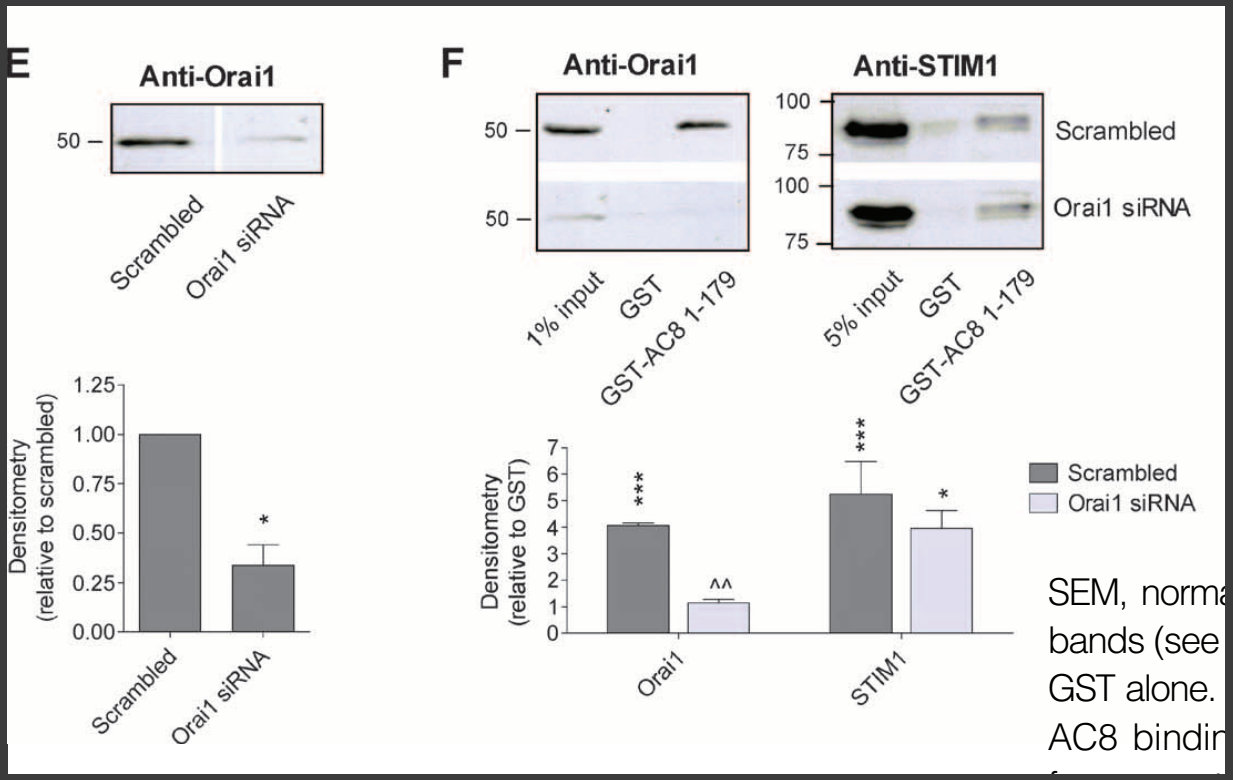
# AC8 and Orai1 interact via their N termini




Orai1-selective siRNA reduced Orai1 abundance in HEK293 cells (Fig. 3E). This was accompanied by a significant decrease in the pulldown of Orai1 by GST-AC8 1–179 (Fig. 3F), confirming the selectivity of the interaction between Orai1 and the N terminus of AC8.

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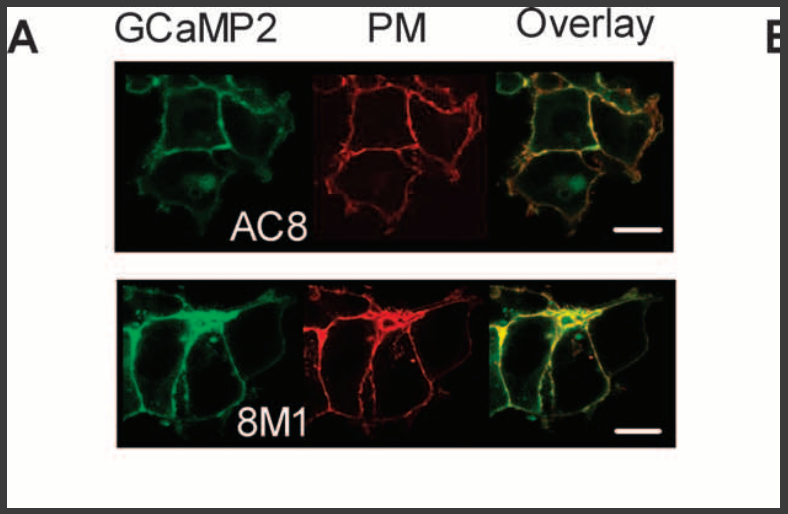
# Orai1 dictates Ca2+ changes within the “AC8 microdomain”




We compared local Ca2+ changes within the immediate vicinities of AC8 and its N-terminally truncated form, 8M1 (which did not bind Orai1)

Both targeted sensors, GCaMP2-AC8 and GCaMP2-8M1, localized to the plasma membrane of HEK293 cells

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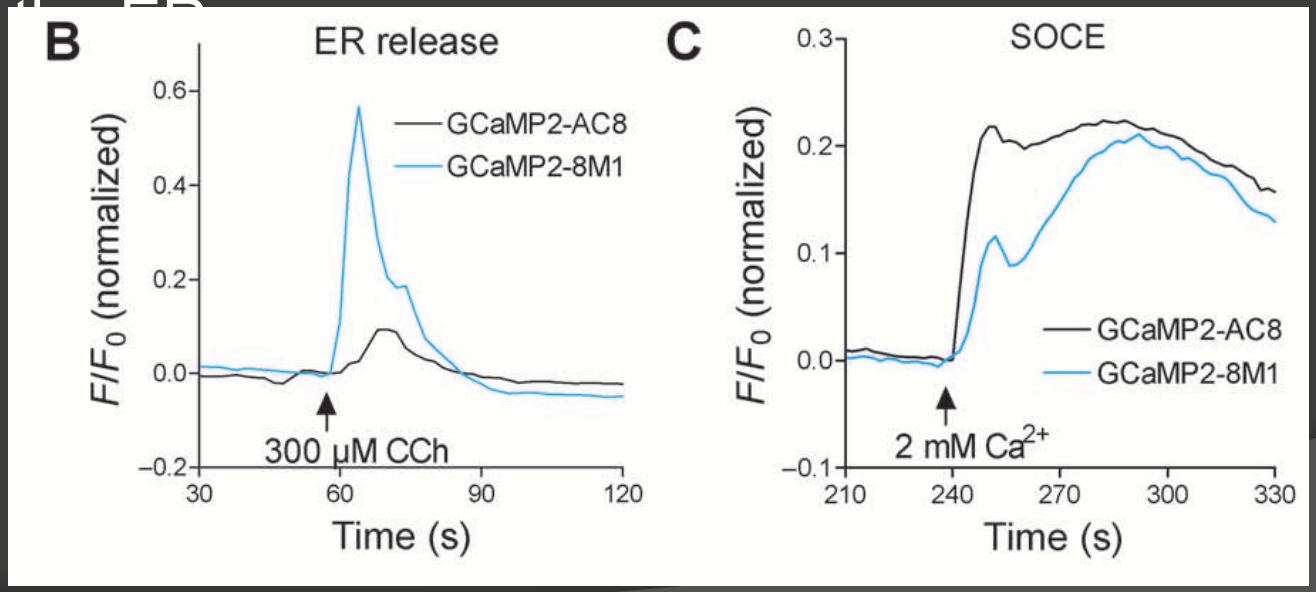
# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

they reported different Ca2+ profiles within their respective microenvironments. GCaMP2-AC8 reported a large rapid Ca2+ change during SOCE but showed poor sensitivity to Ca2+ signals arising from IP3-induced Ca2+ release from

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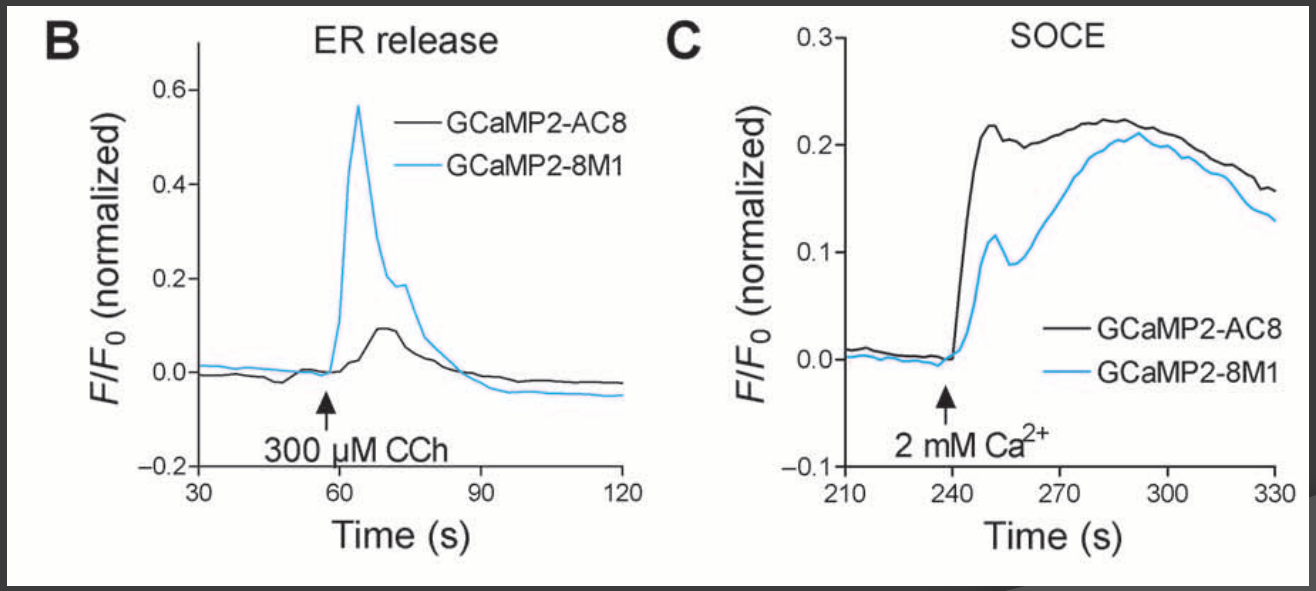
# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

In contrast, GCaMP2-8M1 reported a robust increase in Ca2+ during Ca2+ mobilization from the ER and a smaller, slower response to SOCE.

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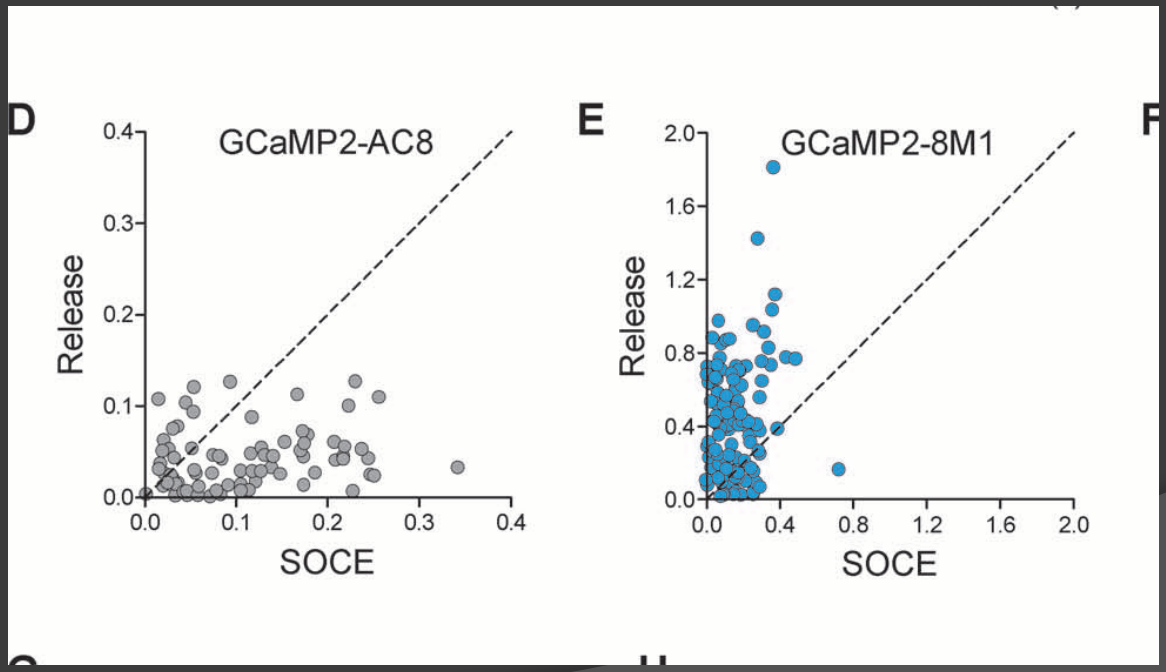
# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

This preferential response to SOCE is apparent in a scatter plot showing the peak response of each cell tested to carbachol (CCh)–induced Ca2+ release from the ER in the absence of external Ca2+ and the subsequent SOCE after readdition of Ca2+ to the bath.

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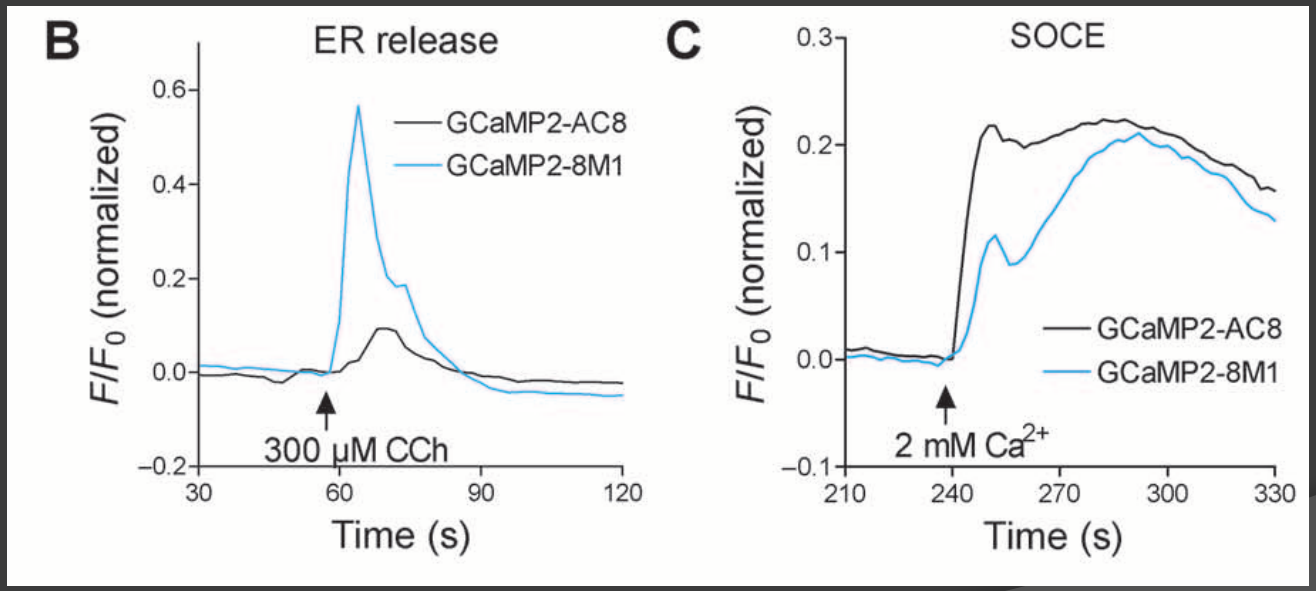
# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

These data suggest that interaction with Orai1 places AC8 in a specific cellular microdomain that experiences rapid changes in Ca2+ concentration during SOCE. Removal of the N terminus to produce 8M1, which largely precludes binding to Orai1, places the AC in a functionally distinct region of the plasma membrane.

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# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

To investigate the role of Orai1 in controlling the AC8 microdomain, we used siRNA to knock down endogenous Orai1. Orai1 knockdown was confirmed by ~75% decrease in peak SOCE detected by GCaMP2- AC8 (Fig. 6, G and I; P < 0.0001 compared to scrambled controls). Scatter plot analysis confirmed the dependence on Orai1 of Ca2+ entry within the AC8 microdomain (Fig. 6J). Orai1 knockdown decreased the slower Ca2+ entry reported by GCaMP2-8M1 by 45% (Fig. 6, H, I, and K; P < 0.001 compared to scrambled siRNA).

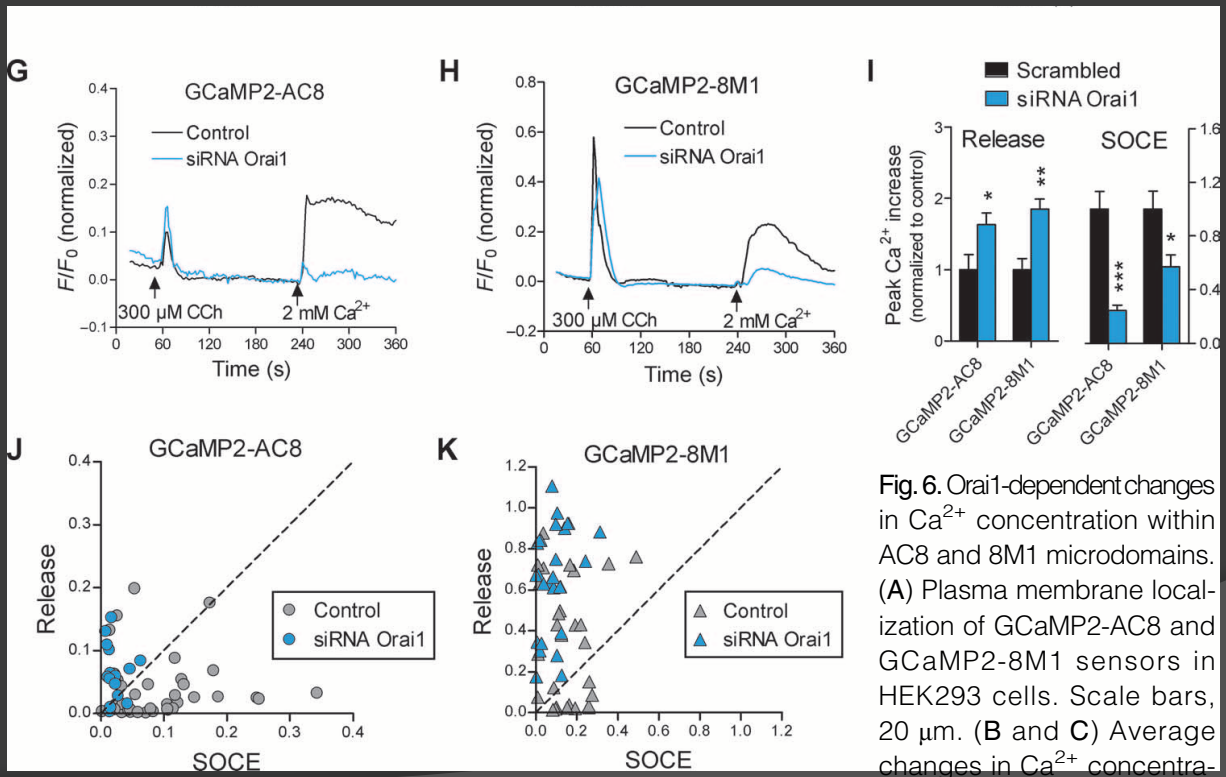


Fig. 6. Orai1-dependent changes in $Ca^{2+}$ concentration within AC8 and 8M1 microdomains. (A) Plasma membrane localization of GCaMP2-AC8 and GCaMP2-8M1 sensors in HEK293 cells. Scale bars, 20 µm. (B and C) Average changes in $Ca^{2+}$ concentra-

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# Orai1 dictates Ca2+ changes within the “AC8 microdomain”

These findings support the hypothesis that the Orai1-AC8 interaction establishes robust Ca2+ events within the immediate vicinity of AC8 to promote Ca2+- stimulated cAMP production.

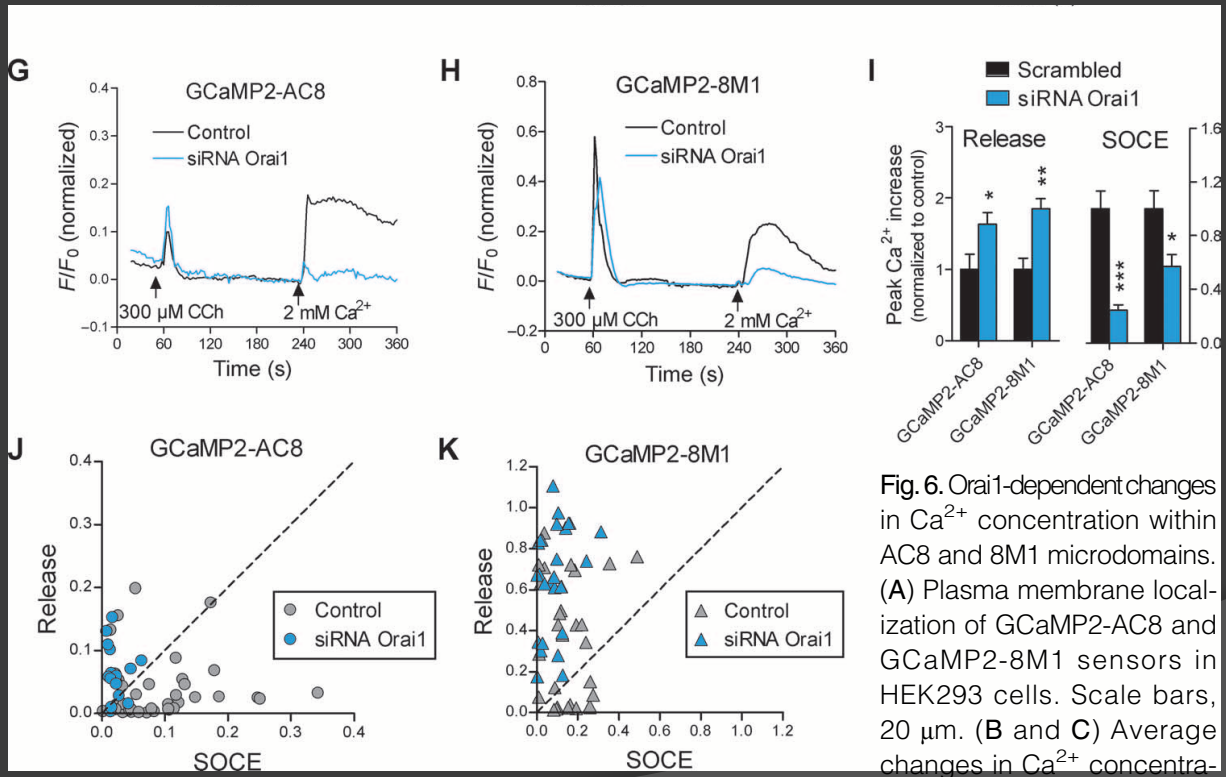


Fig. 6. Orai1-dependent changes in $Ca^{2+}$ concentration within AC8 and 8M1 microdomains. (A) Plasma membrane localization of GCaMP2-AC8 and GCaMP2-8M1 sensors in HEK293 cells. Scale bars, 20 µm. (B and C) Average changes in $Ca^{2+}$ concentra-

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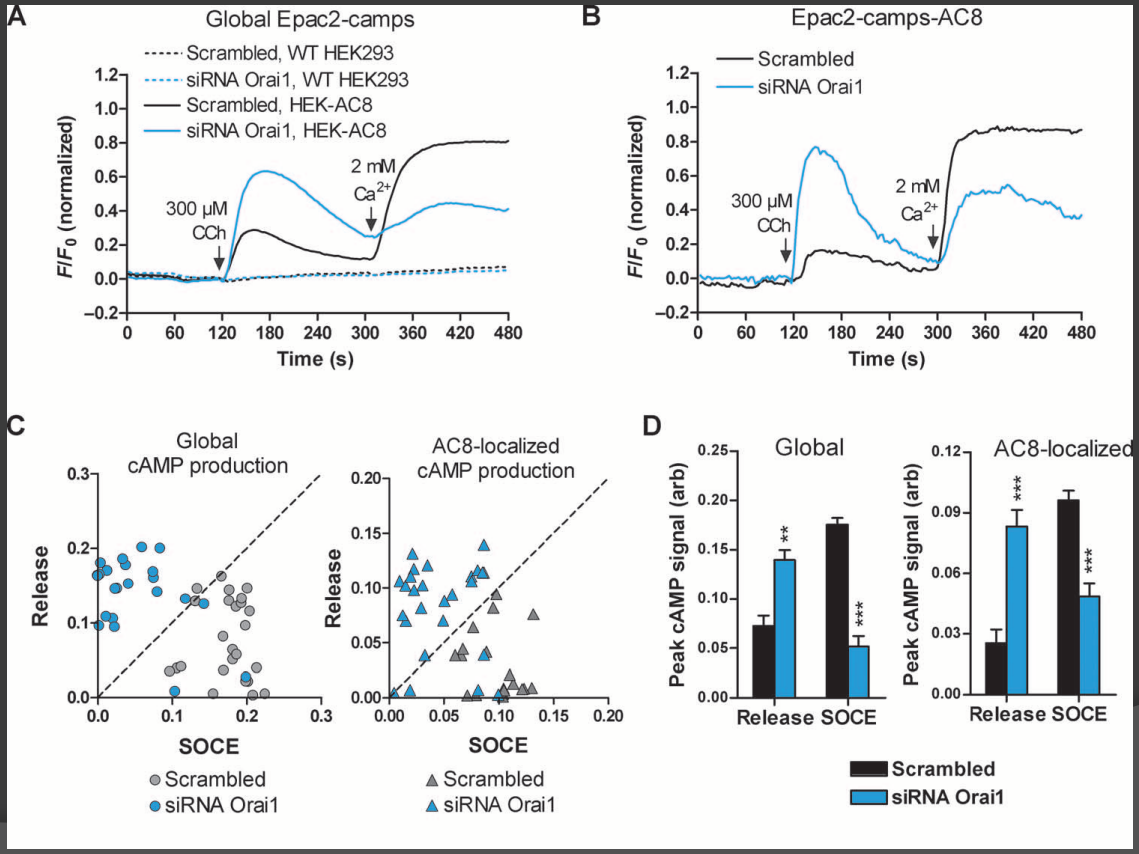
# Orai1 affects the activity and subcellular targeting of AC8

Next, we used the FRET-based cAMP sensor Epac2-camps to determine the effects of Orai1-dependent Ca2+ signaling on AC8 activity. Orai1 knockdown in HEK-AC8 cells was accompanied by ~70% decrease in SOCE-dependent cAMP accumulation. In contrast, Orai1 knockdown was accompanied by increased cAMP accumulation in response to CCh-induced ER Ca2+ release in the absence of external Ca2+

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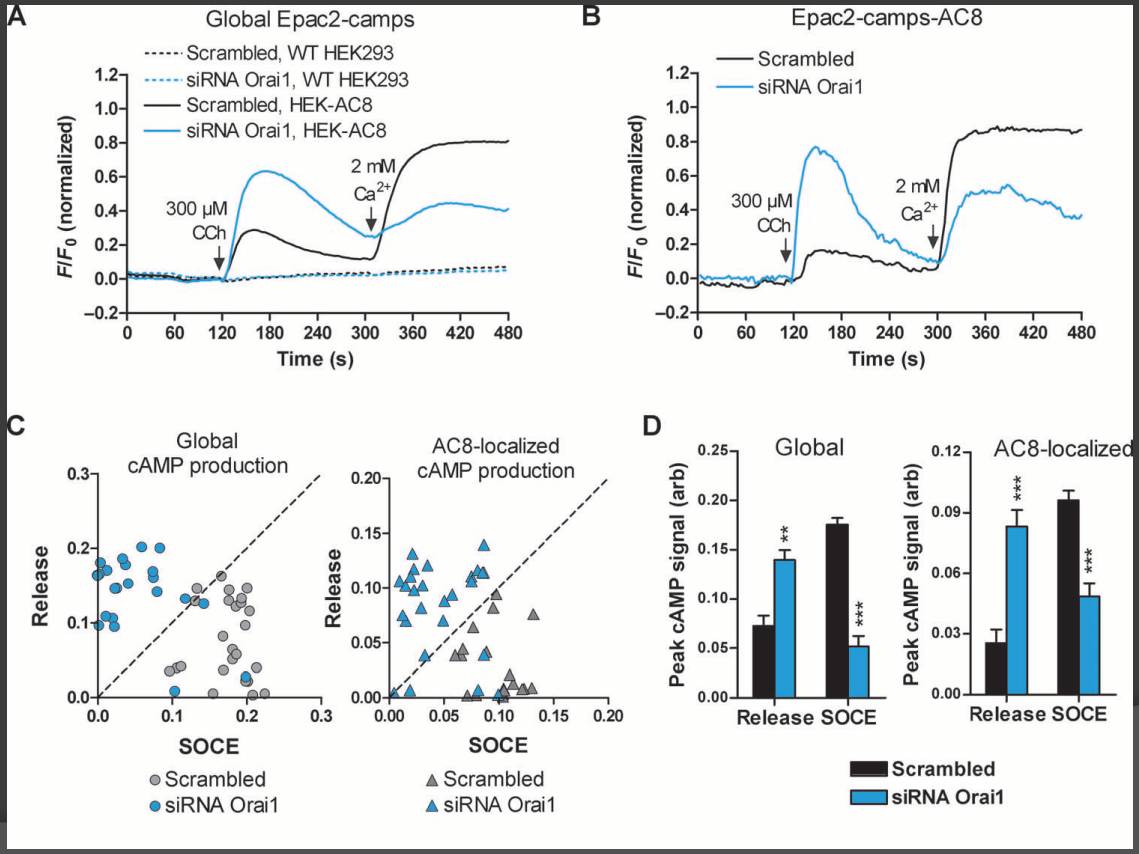
# Orai1 affects the activity and subcellular targeting of AC8

We hypothesized that when AC8 is unable to bind Orai1 it may delocalize to a different region of the cell. In this scenario, AC8 could function like 8M1, which does not bind Orai1 and shows a larger response to Ca2+ release from the ER.

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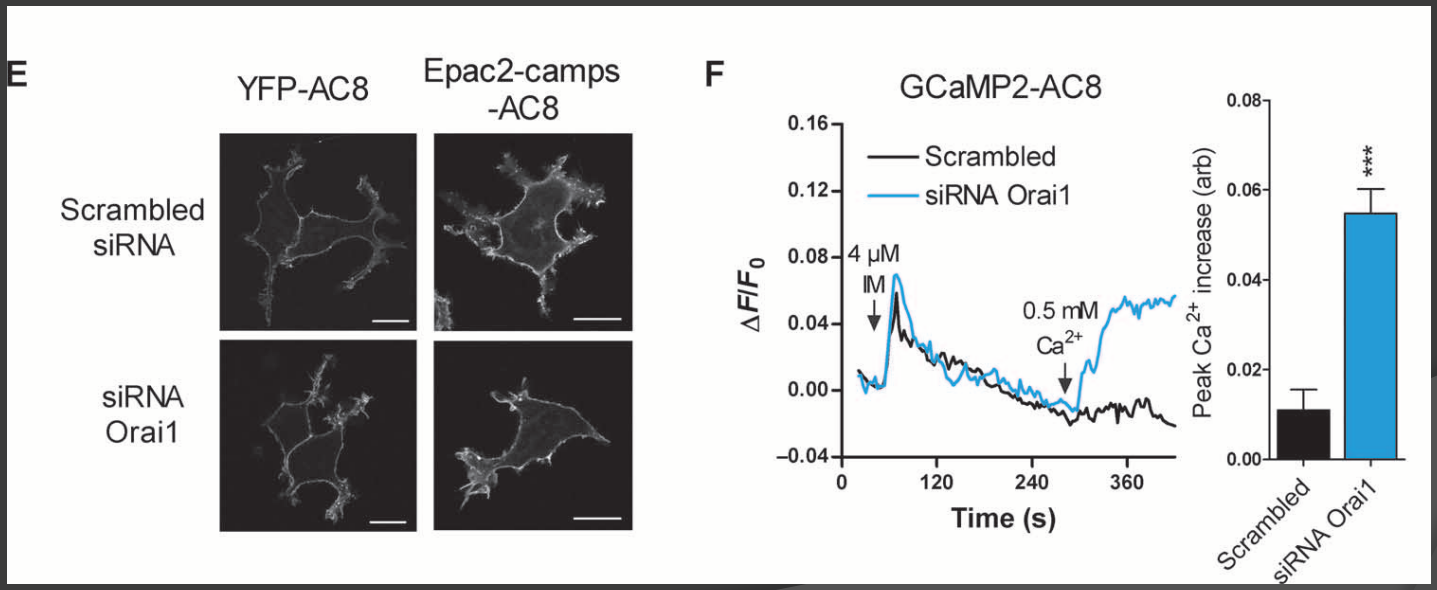
# Orai1 affects the activity and subcellular targeting of AC8

To address the possible delocalization of AC8 to a different plasma membrane Ca2+ microdomain when it cannot bind Orai1, we examined the responsiveness of GCaMP2-AC8 to ionophore-mediated Ca2+ entry. Typically, AC8 is shielded from ionophore-induced Ca2+ entry, as assessed with GCaMP2-AC8. However, Orai1 knockdown enabled the detection of substantial Ca2+ entry byGCaMP2-AC8 in response to ionomycin treatment

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