Japan-Mexico Workshop on “Pharmacobiology” & “Nanobiology”
@ UNAM, Mexico City

Molecular components supporting ryanodine receptor-mediated Ca^{2+} release: roles of junctophilin and TRIC channel in cardiac Ca^{2+} release

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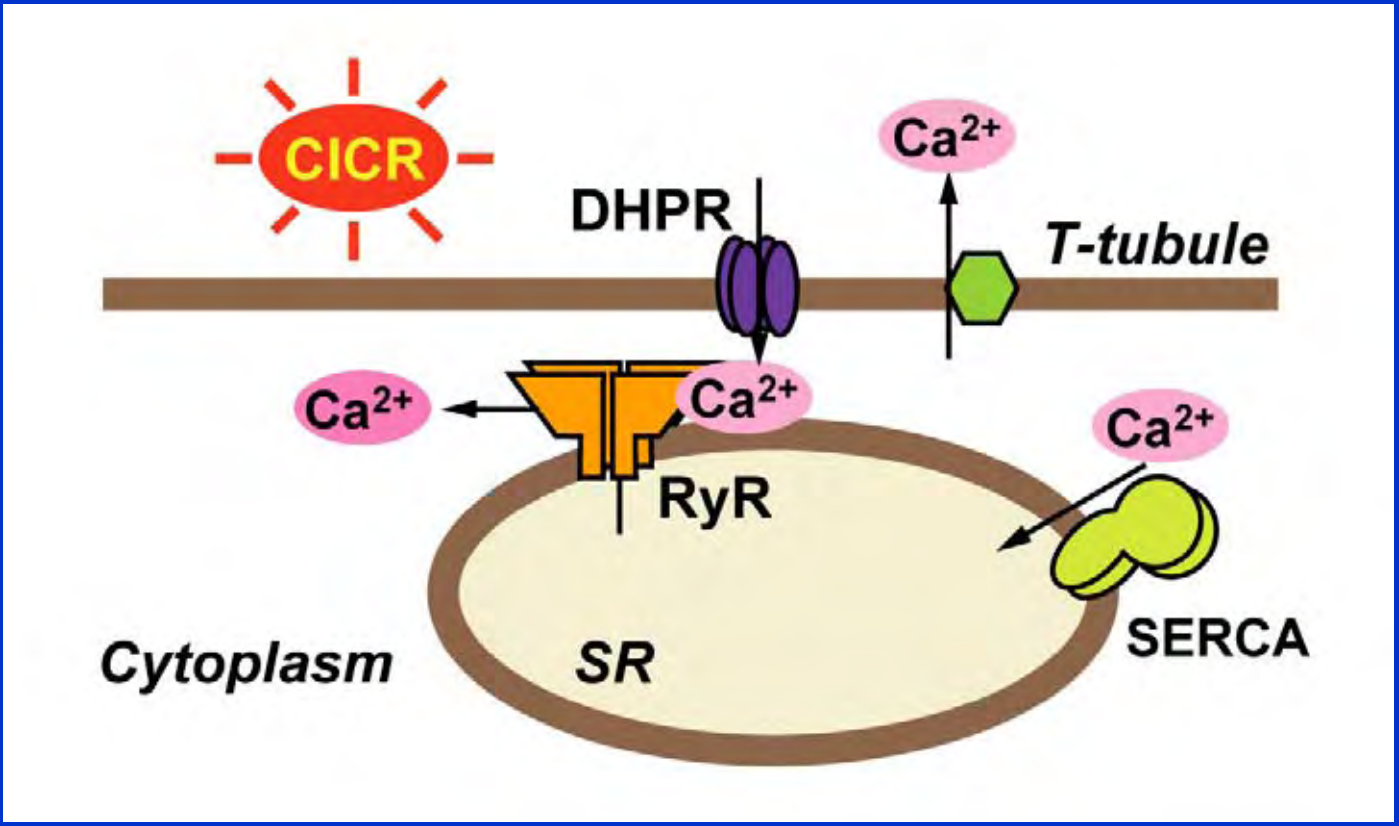
Cardiac Ca^{2+}-induced Ca^{2+} release (CICR)



DHPR: dihydro-pyridin receptor / L-type voltage-gated Ca^{2+} channel

RyR: ryanodine receptor / Ca^{2+} release channel

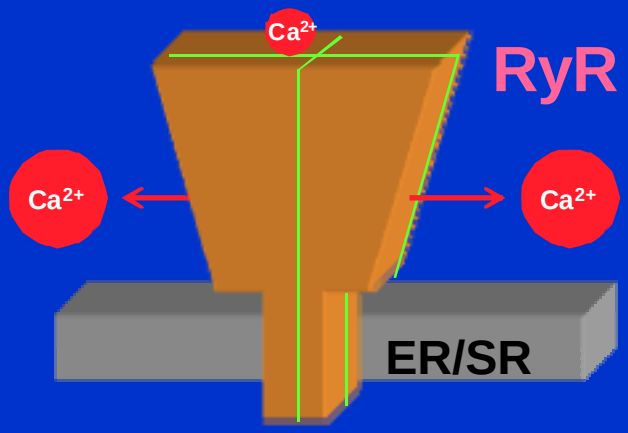
SERCA: SR/ER Ca^{2+}-pump

1) Cell-surface depolarization opens DHPR to generate Ca^{2+} influx.

2) Inflowing Ca^{2+} binds to RyR, opens its channel and triggers Ca^{2+} release.

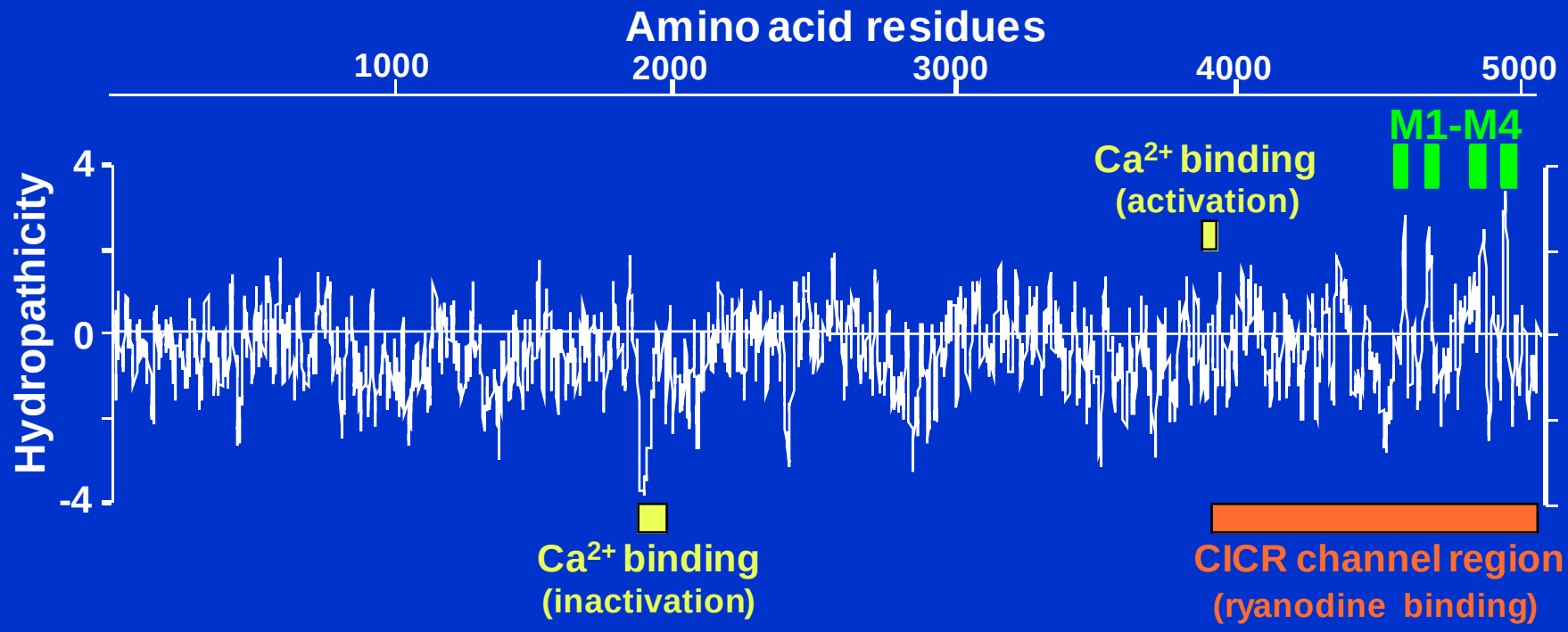
3) Cytoplasmic Ca^{2+} binds to troponin and generates muscle force.

Cardiac excitation-contraction (E-C) coupling requires synchronized channel activation of DHPR and RyR.



Ryanodine receptor (RyR) functioning as Ca^{2+} release channel on SR/ER

Hydropathicity plot of RyR-1



Ryanodine receptor subtypes

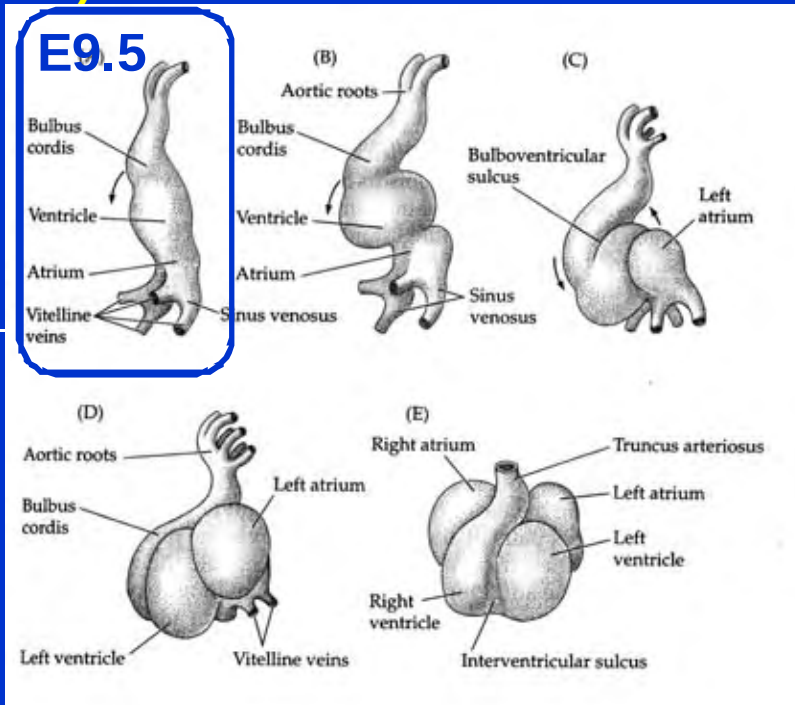
subtype	locus	tissue distribution	knockout mouse	human disease
RyR1	mouse 7A2-B3 human 19q13.1	skeletal muscle brain	neonatal lethality respiratory failure	malignant hyperthermia*
RyR2	mouse 13 human 1q42-43	cardiac & smooth muscles, brain	embryonic lethality heart failure	polymorphic tachycardia**
RyR3	mouse 2E5-F3 human 15q14-15	skeletal & smooth muscles, brain	impaired memory hyperlocomotion	

*MacLennan et al. Nature 343, 559, 1990.
**Priori et al. Circulation 103, 196, 2001.

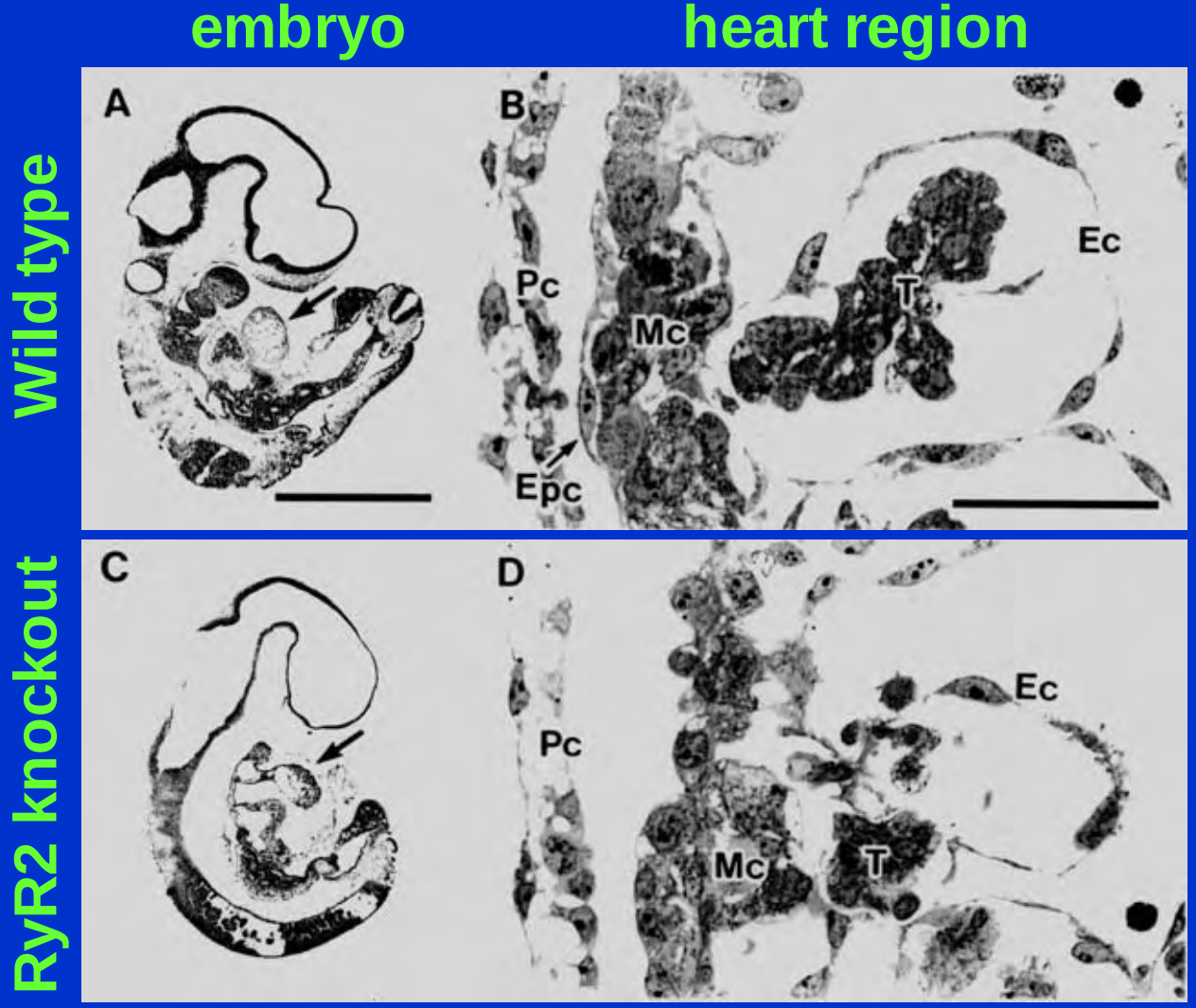
RyR2-knockout mice exhibit cardiac failure at early embryonic stage

Pups obtained by mating between RyR2(+/-) mice

embryonic day	+/+	+/-	-/-
E8.5	8	12	3
E9.5	32	38	24 (heartbeats)
E10.5	30	31	18 (cardiac arrest)
E11.5	9	15	12 (autolysis)
E12.5	3	3	1 (autolysis)
E18.5/P0	22	32	0

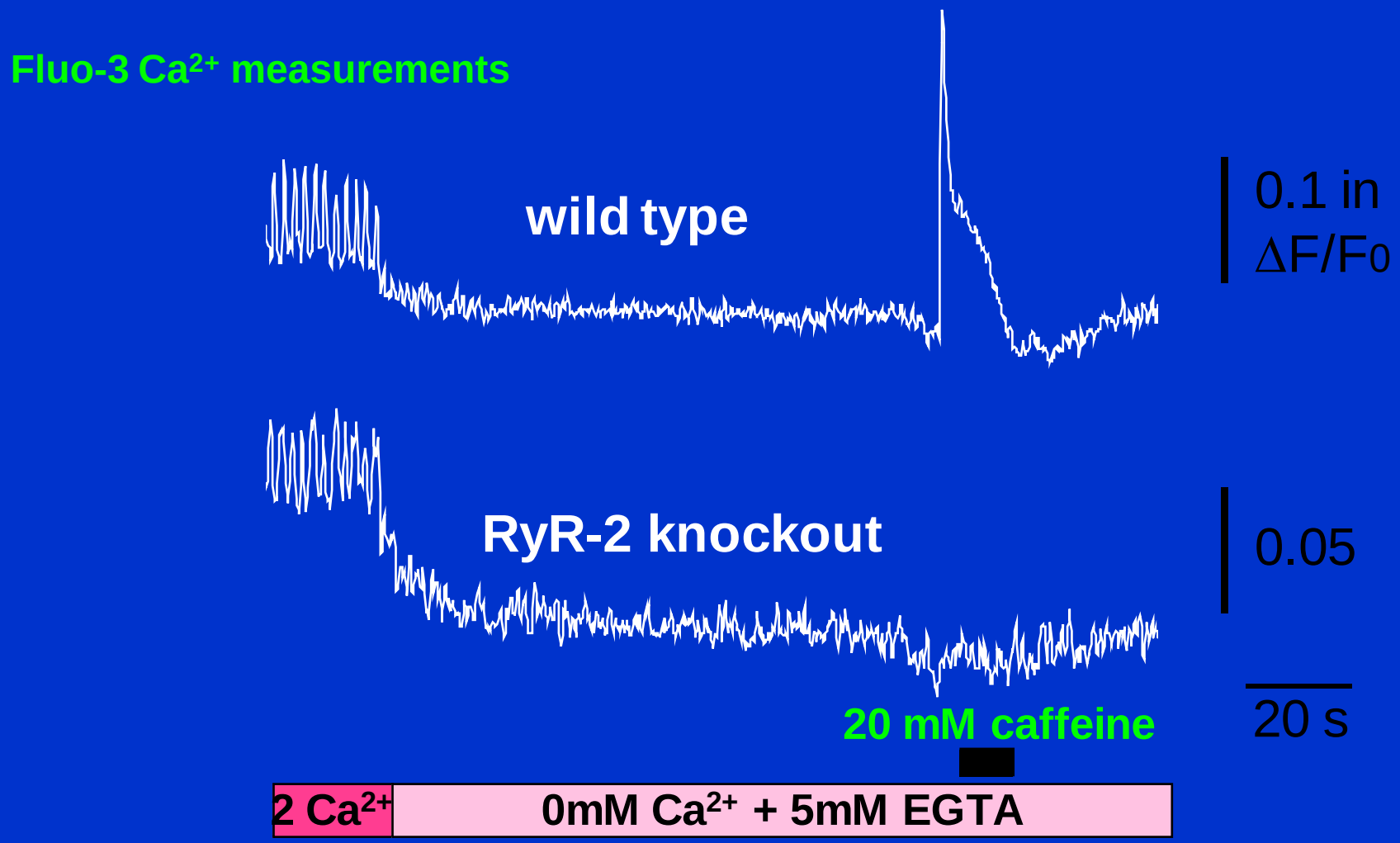


Histology of E9.5 RyR2-knockout embryo



RyR2-KO embryos show delayed development at this stage, but the mutant cardiac tubes show beating and retain normal cardiomyocytes.

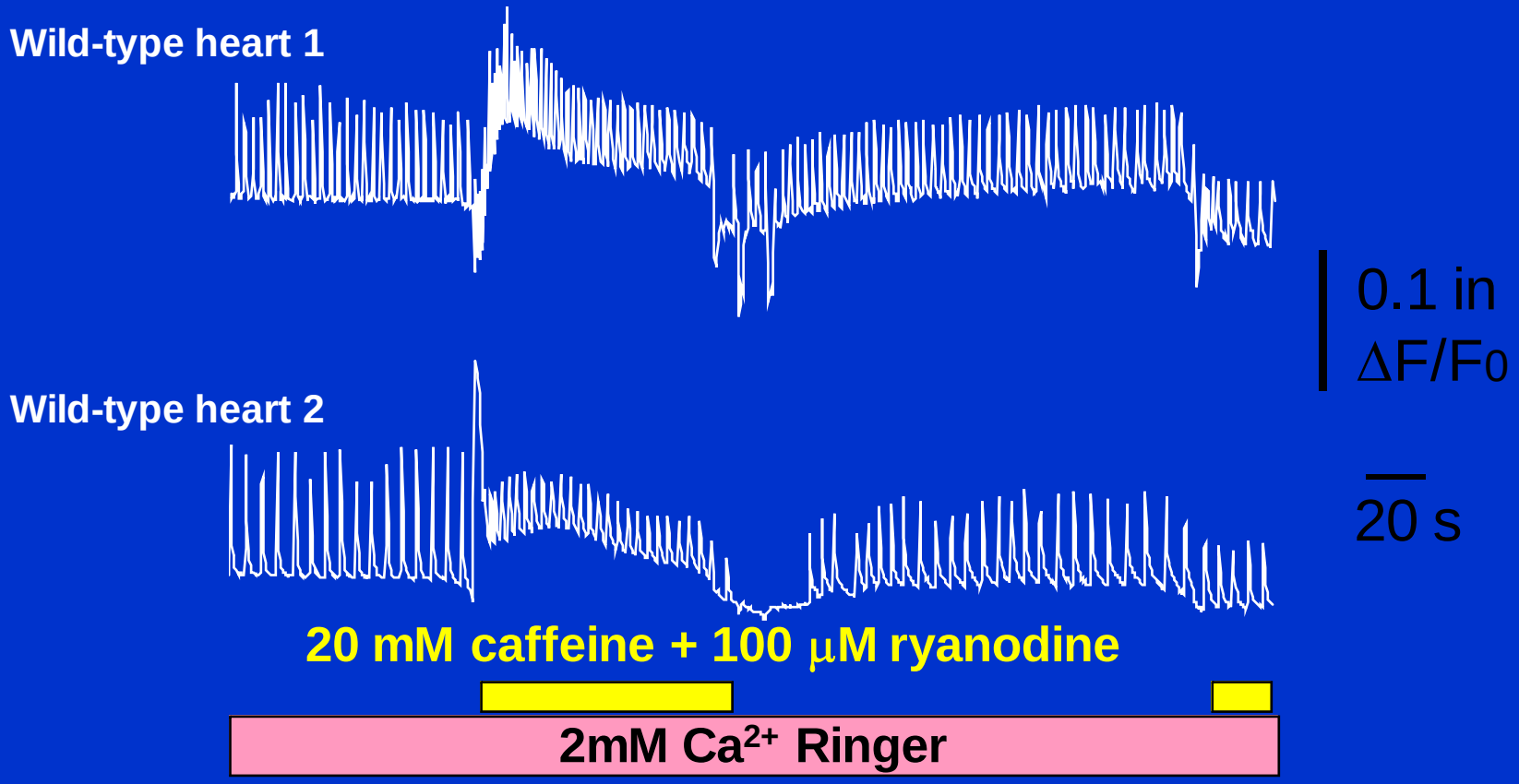
E9.5 RyR2-knockout cardiomyocytes lose caffeine-induced Ca^{2+} release



Of RyR subtypes, only RyR2 is expressed in embryonic cardiomyocytes.

E9.5 and E10.5 cardiomyocytes retain spontaneous Ca^{2+} oscillations under store-depleted conditions

Fluo-3 Ca^{2+} measurements using wild-type embryonic hearts



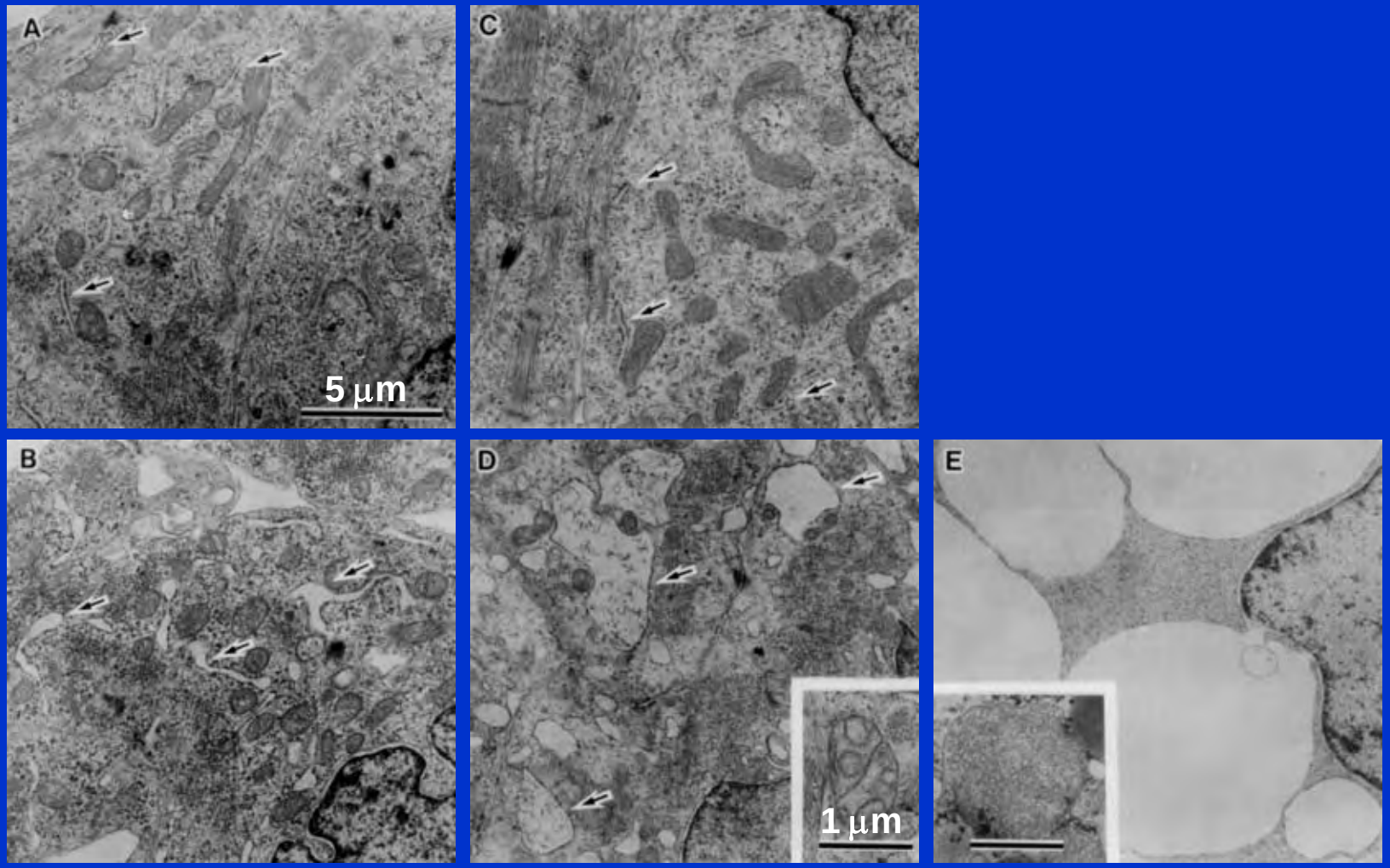
The loss of RyR2-mediated Ca^{2+} release dose not abolish Ca^{2+} oscillations in embryonic cardiomyocytes. Why does the RyR2-KO heart stop beating?

EM detects swollen SR elements and degraded mitochondria in RyR2-knockout cardiomyocytes

E8.5 E9.5 E10.5

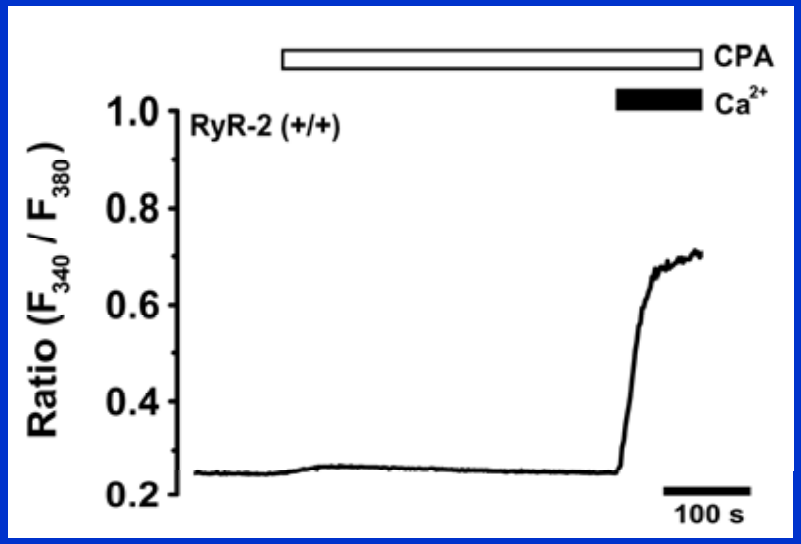
Wild type

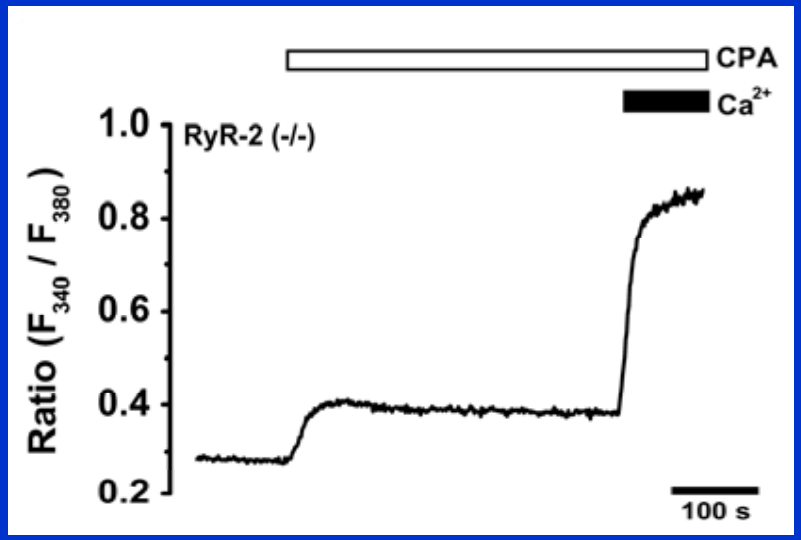
RyR2 knockout



	E8.5	E9.5	E10.5
Morphological abnormalities	SR: x Mt: -	SR: xx Mt: xx	SR: xxx Mt: xxx

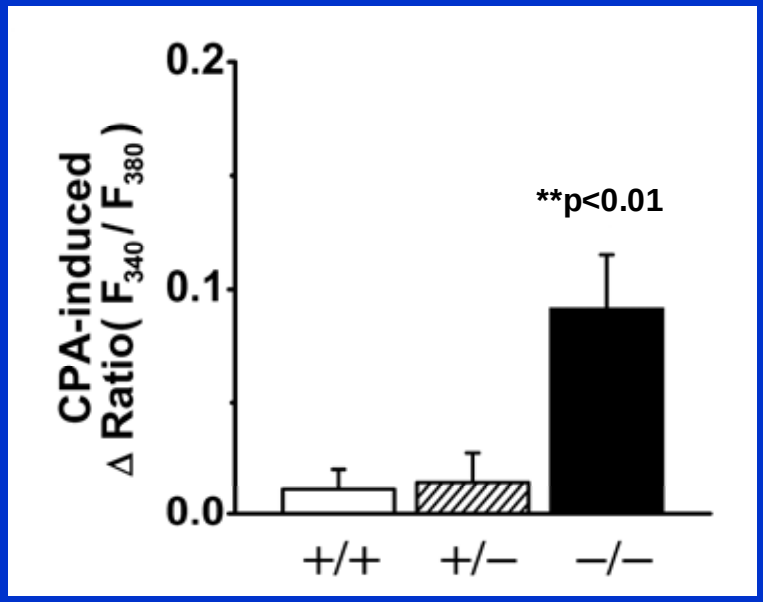
Ca^{2+} overloading of the swollen SR in RyR2-knockout cardiomyocytes





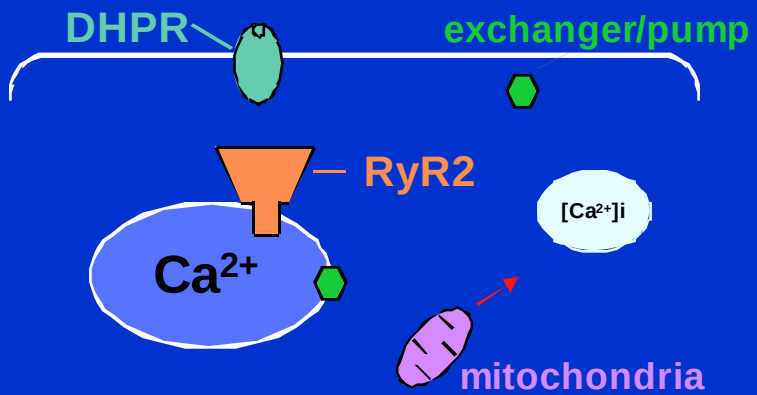
Fura-2 Ca^{2+} measurement in single cell preparations

CPA: cyclopiazonic acid
SR Ca^{2+}-ATPase inhibitor

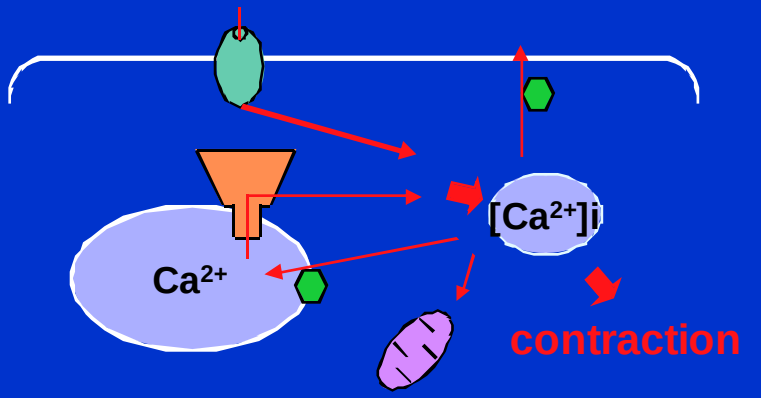


As well as contributing to CICR (Ca^{2+} signal amplification), RyR2 prevents SR Ca^{2+} overloading in embryonic cardiomyocytes

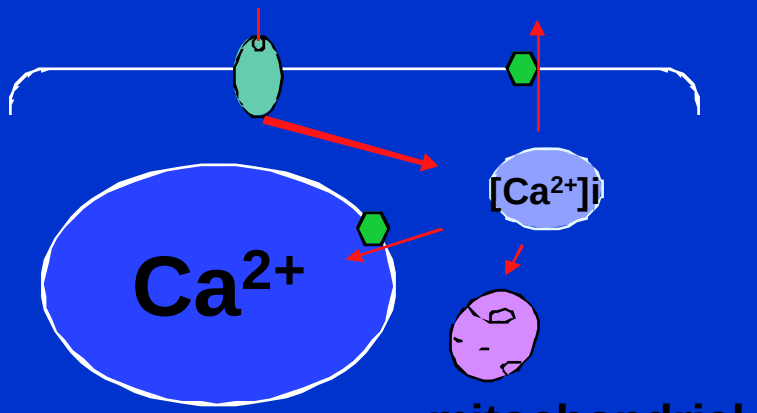
Resting state



Excitation state



RyR2-knockout myocytes



SR overloading abolishes its Ca^{2+} buffering in the cytoplasm, likely induces excess Ca^{2+} entry to other organelle and finally damages mitochondria.

Damaged mitochondria produce cell-death signals including *Cyt c* release.

➡ **cell death signals**

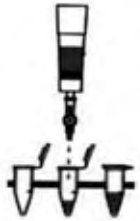
Our immuno-proteomic survey is useful for the identification of muscle membrane proteins

1. Triad-rich membrane preparation from rabbit skeletal muscle

2. Injection of membrane proteins into mice

3. mAb library production from immunized mouse spleen

4. Screening mAbs by immuno-staining muscle sections



5. Immuno-affinity purification of antigen proteins

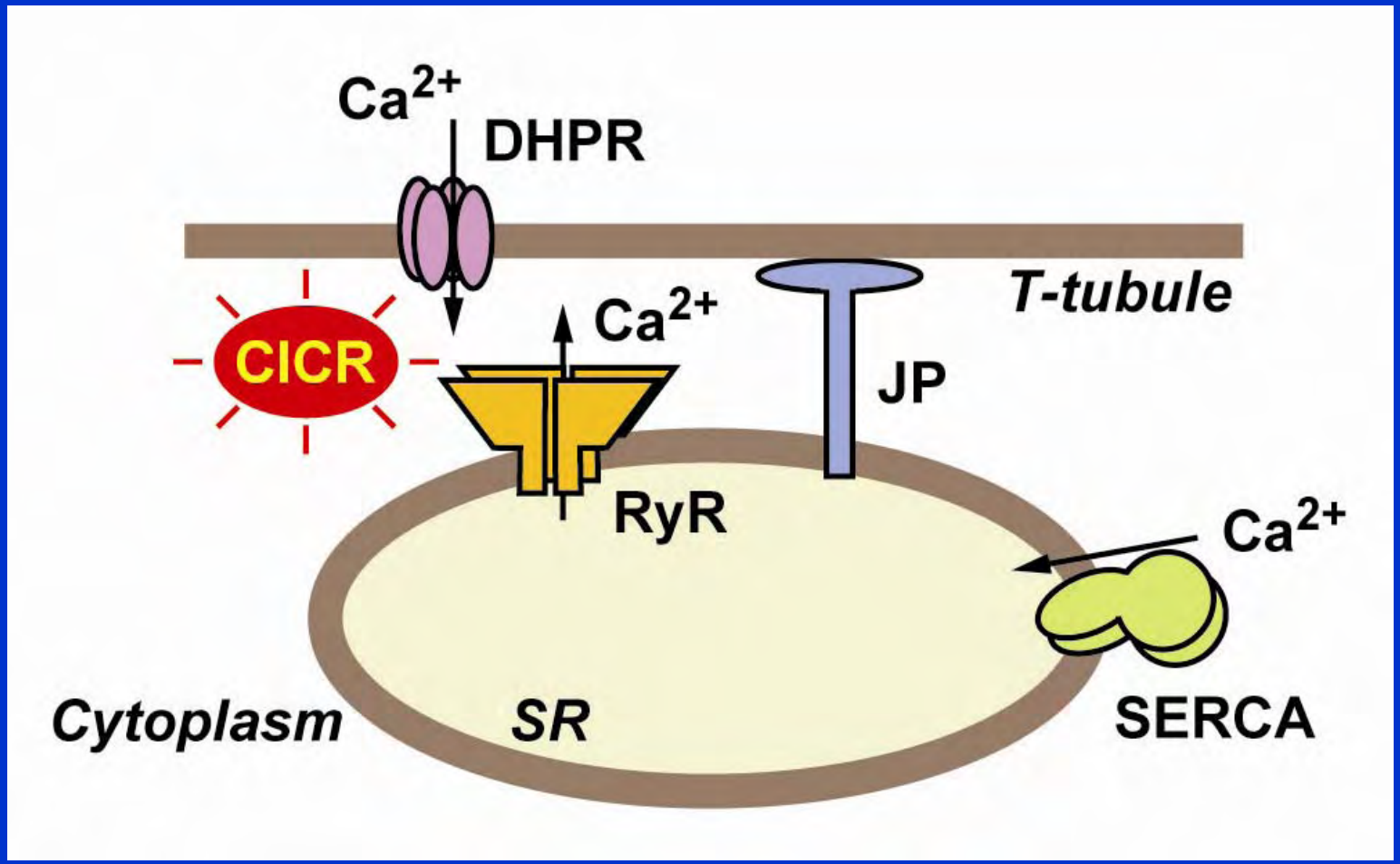
6. DNA cloning, DNA expression and protein-protein interaction analyses of target proteins

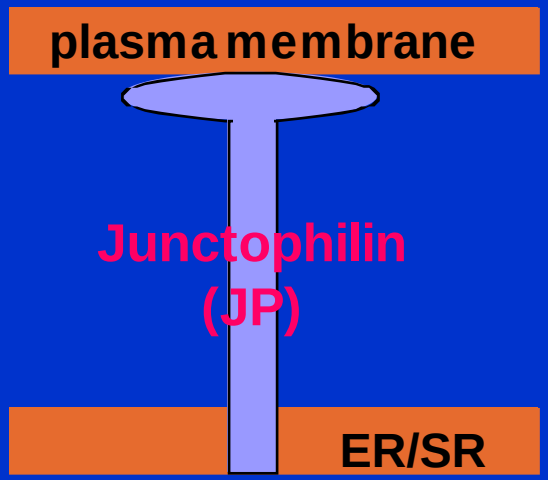
7. Mutant mice generation to know roles of target proteins

Mitsugumins identified in our screening

mitsugumins	structure	function
MG29	synaptophysin family	T-SR structure
MG23	multi-TMs	?
MG72 (junctophilin)	MORN motif protein	JMC formation
MG53	RBCC family	membrane repair
MG33 (TRIC channel)	trimer of multi-TMs	cation channel
MG56 (calumin)	single TM	SR/ER Ca^{2+} binding

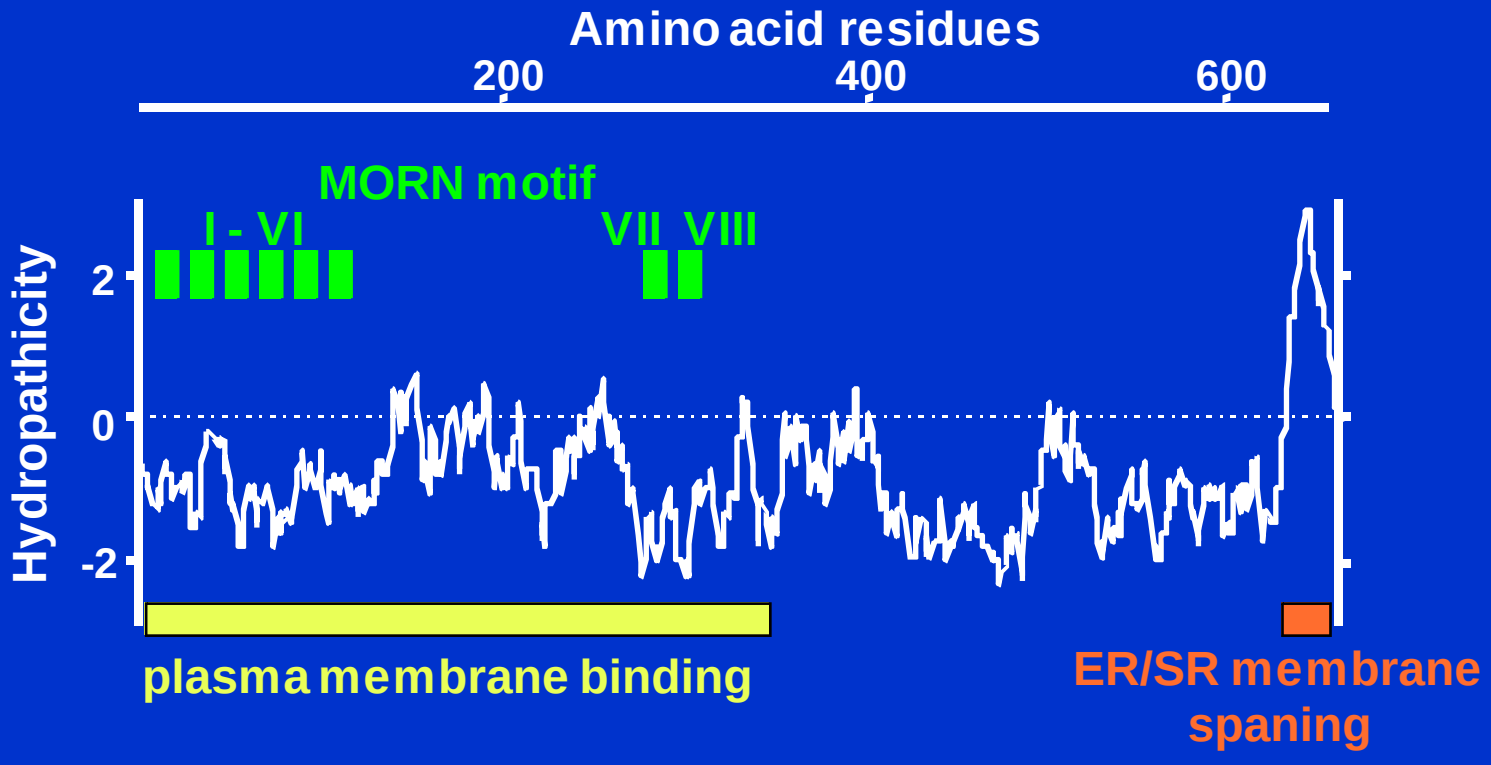
Because the cytoplasm has Ca^{2+}-buffering property, efficient CICR probably requires co-localization of DHPR and RyR in junctional membrane complexes





Junctophilin contains MORN motifs in the cytoplasmic region and an ER/SR membrane-spanning segment in the C-terminal end

Hydropathicity plot of JP type 1



MORN motifs shared by different proteins

I	YCGGWEEGKAHGHG
II	YSGSWSHGFEVVGG
III	YQGYWAQGKRHGLG
IV	YRGEWSHGFKGRYG
V	YEGTWSNGLQDGYG
VI	YQGQWAGGMRHGYG
VII	YMGEWKNDKRNGFG
VIII	YEGEWANNKRHGYG

Junctophilin

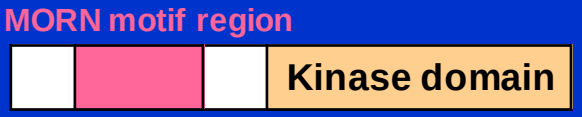
100 aa

I	YDGRWLSGKPHGRG
II	YSGMFRNGLEDGYG
III	YVGHEKEGKMCGQG
IV	FEGCFQDNMRHGHG
V	FIGQWVMDKKAGYG
VI	YMGMWQDDVCQGNG
VII	YEGNFHLNKMMGNG
VIII	YEGEFSDDWTSGKG

Alsin (amyotrophic lateral sclerosis 2 gene)

I	YTGQWYDSFPHGHG
II	YIGDWYNGKTMGNG
III	YEGEFKSGYMDGIG
IV	YKGQWVMNLKHGHG
V	YDGEWRRGLQEGQG
VI	YIGEWKNGTICGKG
VII	YDGFWDEGFPRGNG
VIII	YVGHWSKDPEEMNG

A. thaliana PIP 5-kinase



I	YEGQFVEGEKKGQG
II	YEGEFVDGQPHGQG
III	YEGEFVDGQPTGKG
IV	YEGTLKNGQPDGEG
V	YEGEFQSGEFSGQG
VI	FQGQFKQGLPSGQG
VII	YQGEIRDGQPAGEG
VIII	YQGQFVAGKFAGEG

Cyanobacterium putative adaptor

MORN motif region interacts with various phospholipids

Overlay assay:

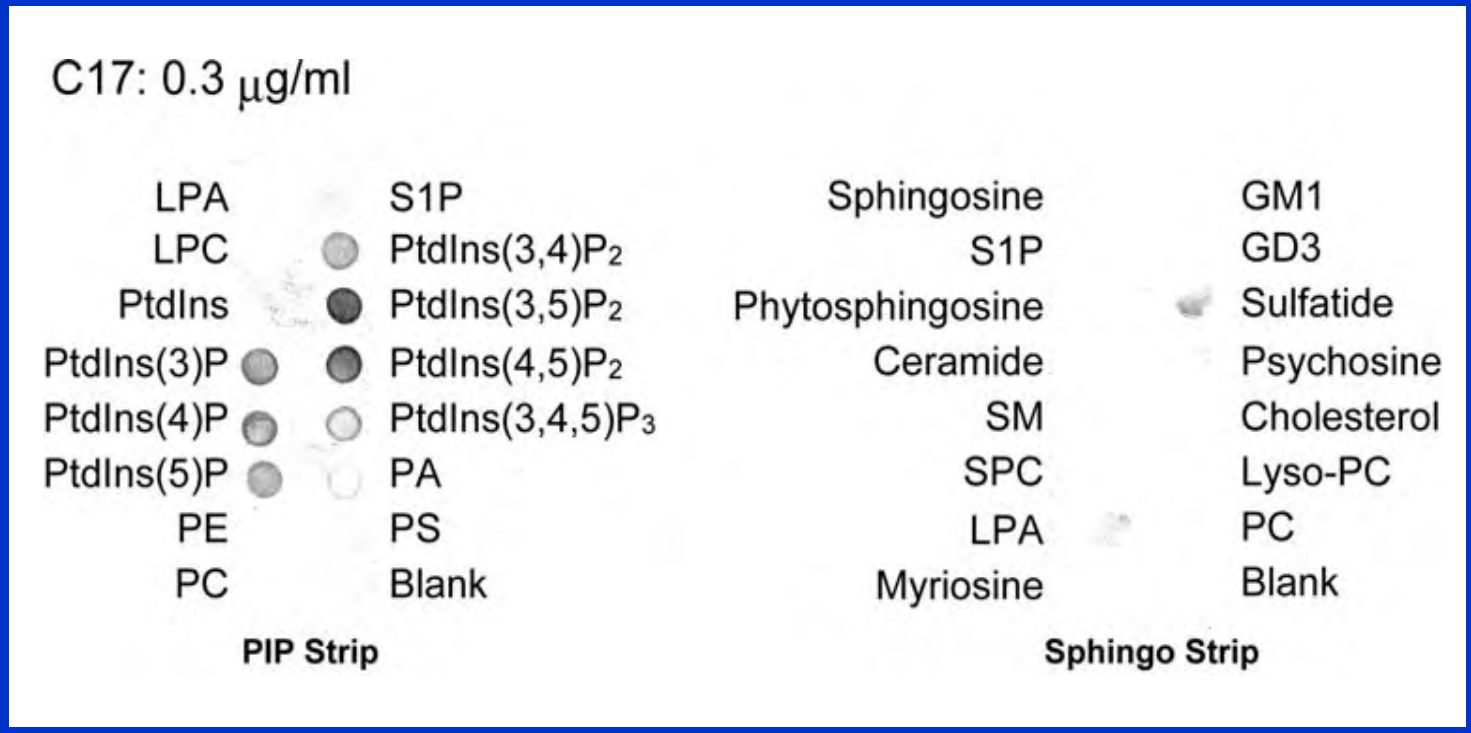
Production of recombinant JP protein

↓

react with PIP & Sphingo-Strip™

↓

Detection of protein bound using mAb



PIP_2 is enriched in PM, and PIP is enriched in endosome.

The data suggest that MORN motifs are responsible for phospholipid-binding to interact with membrane systems.

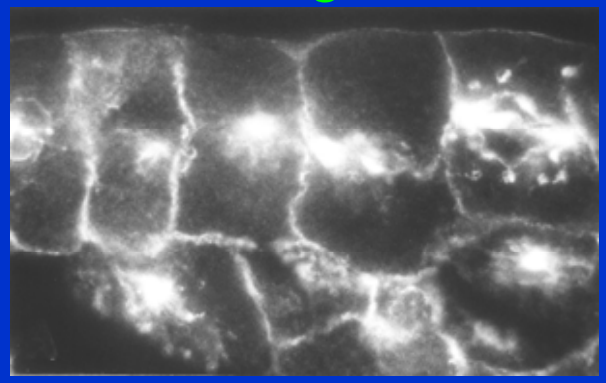
Junctophilin forms JMC

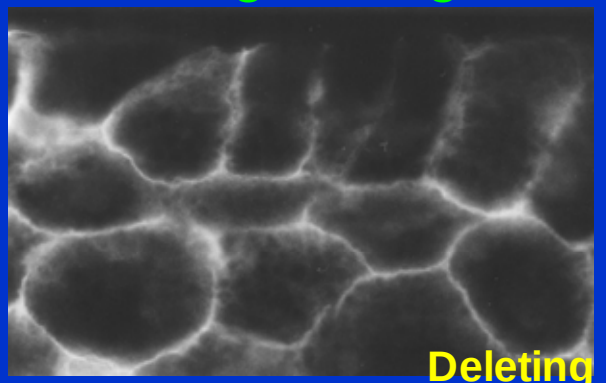
JP-cRNA expression in amphibian embryonic cells

Full-length JP

JP lacking TM segment

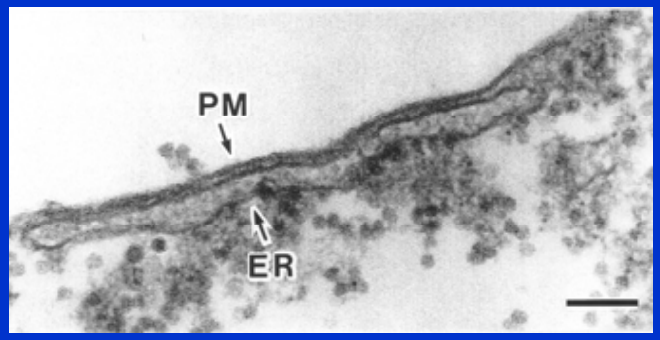
Immunodetection of expressed JP





Deleting MORN motifs inhibits PM association.

EM observation of cell periphery



Formation of junctional membrane complex

No junctional membrane structure

Junctophilin forms junctional membrane complex by interacting with the plasma membrane and spanning the ER/SR membrane.

Junctophilin subtypes

subtype	locus	tissue distribution	knockout mouse	human disease
JP1	mouse 1A2-5 human 8q21	skeletal muscle	neonatal lethality contraction deficiency	
JP2	mouse 2H1-3 human 20q12	skeletal, cardiac & smooth muscles	embryonic lethality heart failure	Hypertrophic cardiomyopath
JP3	mouse 8E human 16q23-24	brain (neurons)	no obvious phenotype	Huntington's disease type 2*
JP4	mouse 14C1-2 human 14q11.1	brain (neurons)	no obvious phenotype	

Double knockout mice lacking both JP-3 & 4:
weaning lethality, abolished memory and motor learning

*Holmes et al. Nature Genetics 29, 377, 2001.

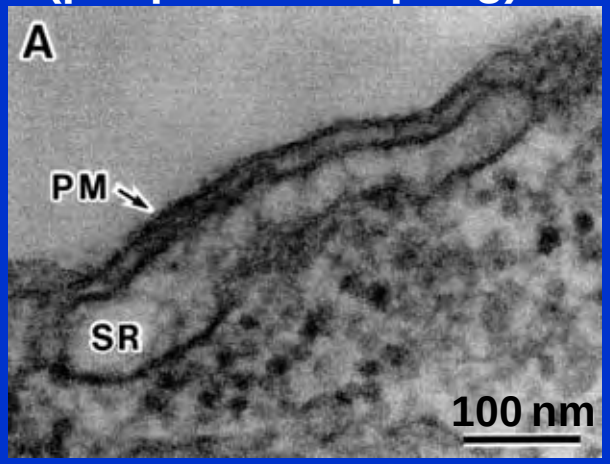
JP2-knockout mice exhibit cardiac failure at early embryonic stage

Pups obtained by crosses between heterozygous mutants

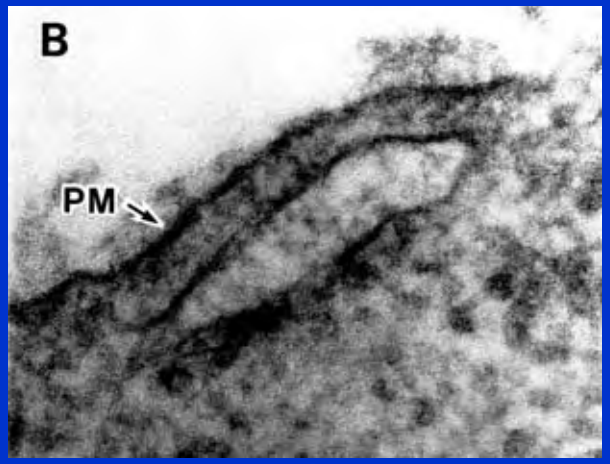
embryonic day	+/+	+/-	-/-
E9.5	44	72	40 (weak heartbeats)
E10.5	10	27	11 (cardiac arrest in ~60% embryos)
E11.5	5	18	5 (autolysis)
E18.5/P0	18	35	0

Junctional membrane structures in E9.5 embryonic cardiomyocytes

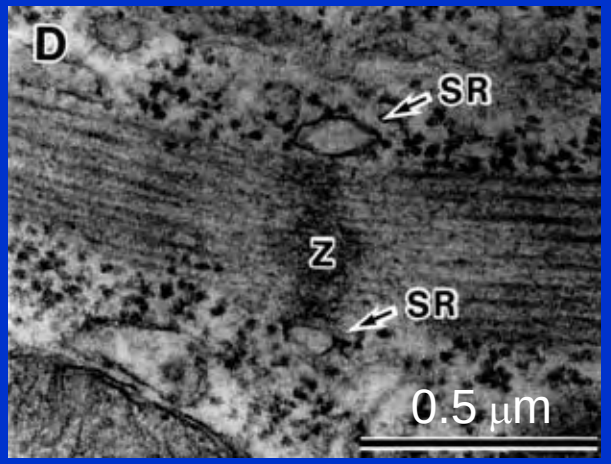
12-nm junction (peripheral coupling)



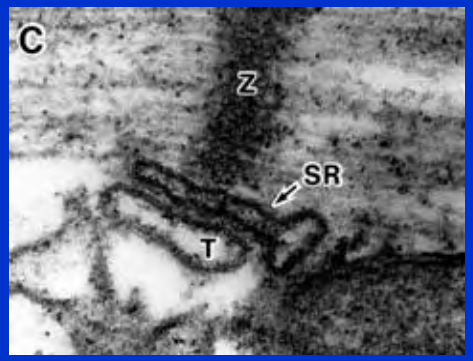
30-nm junction



Z line-SR junction



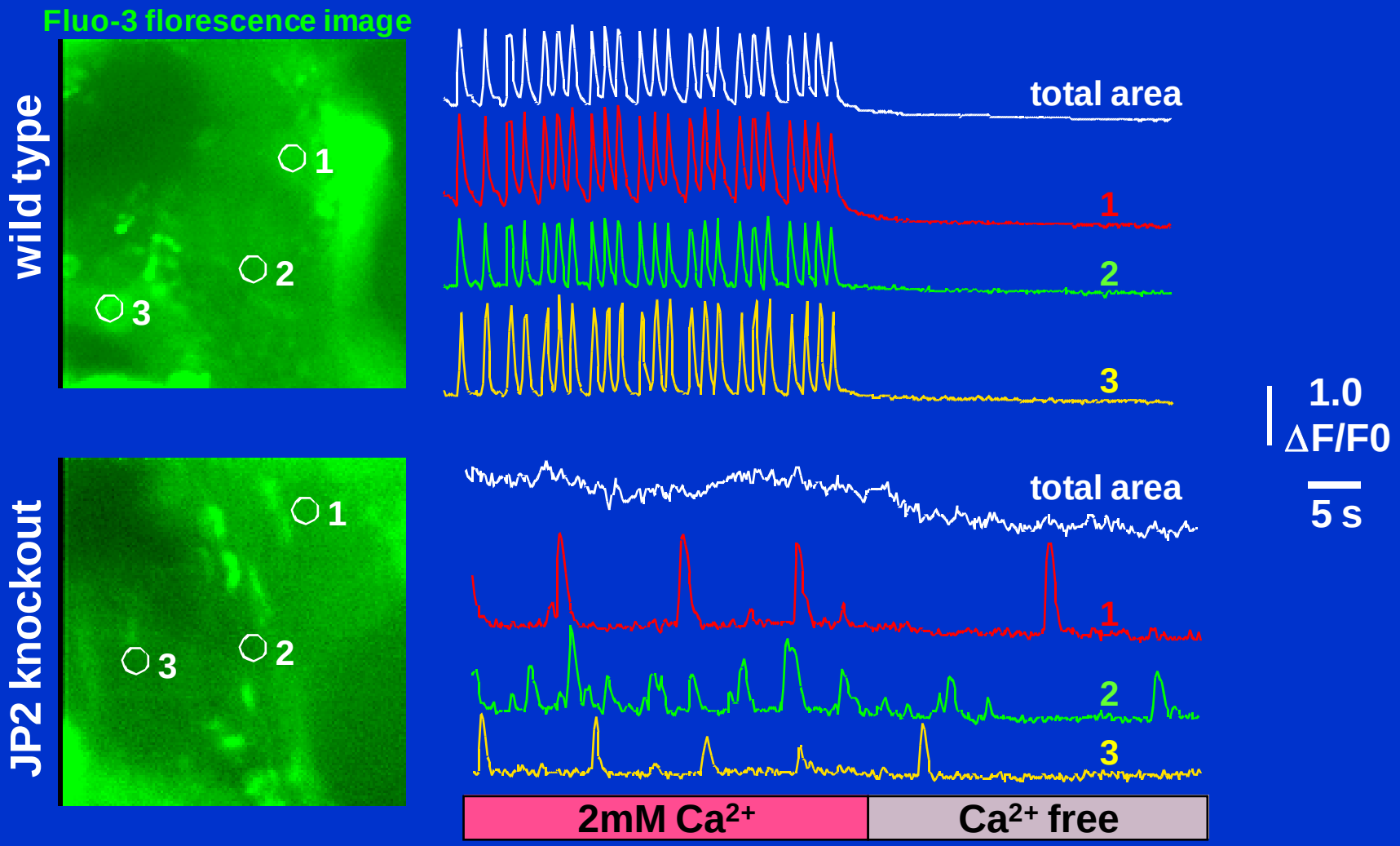
	12-nm junction (peripheral coupling)	30-nm junction	Z line-SR junction
wild-type	12.4 ± 0.2	2.2 ± 0.3	91 ± 2.2
JP2-KO	1.5 ± 0.7 * (*p<0.01)	2.2 ± 0.9	91 ± 2.0
	(junctions / 100 μm plasma membrane)		(% of SR-bearing Z line)



diad with 12 nm gap in adult myocytes

In embryonic cardiomyocytes, JP2 likely generates peripheral couplings.

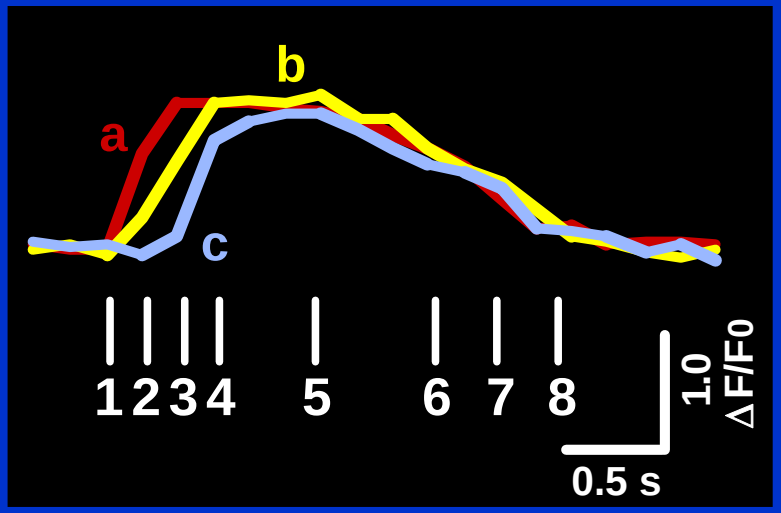
Cardiomyocytes show random Ca^{2+} transients in hearts from E9.5 JP2-knockout embryos



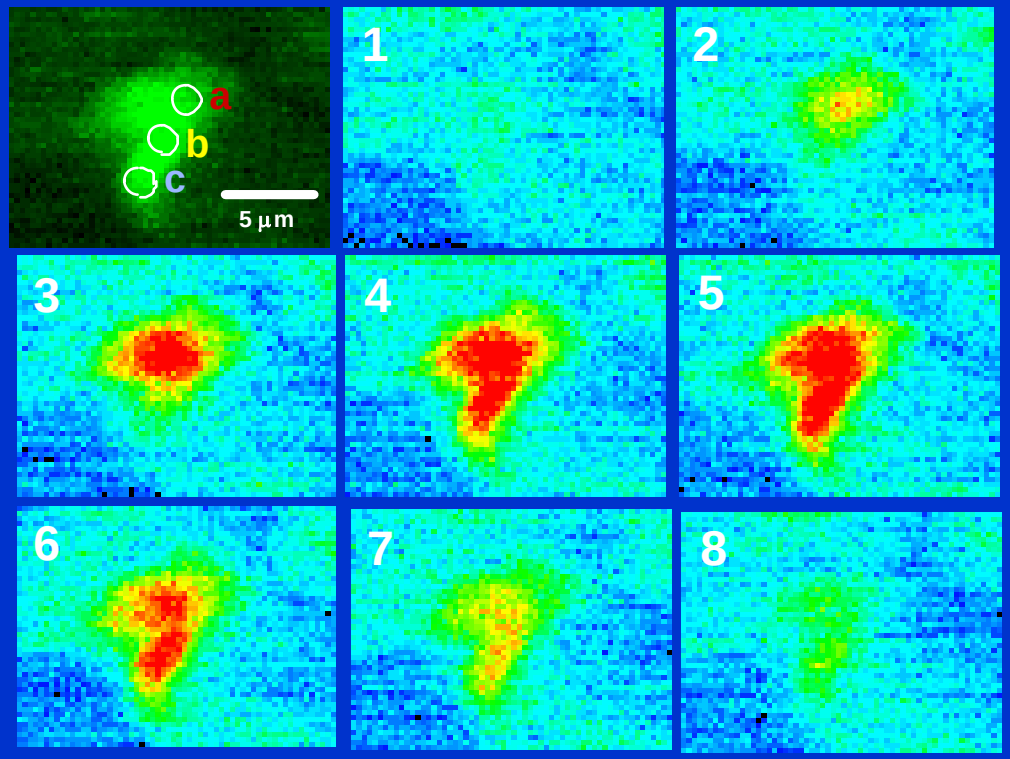
Since the application of caffeine and ryanodine abolish the random transients in JP2-knockout hearts, the random transients are generated by Ca^{2+} release.

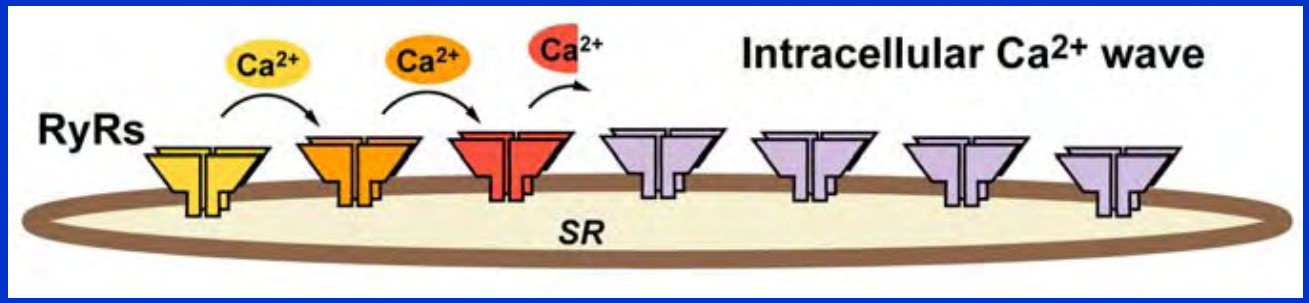
Ca^{2+} waves compose random transients in JP2-knockout cardiomyocytes

Analysis of a single event of random Ca^{2+} transient



Pseudocolor images at indicated frames

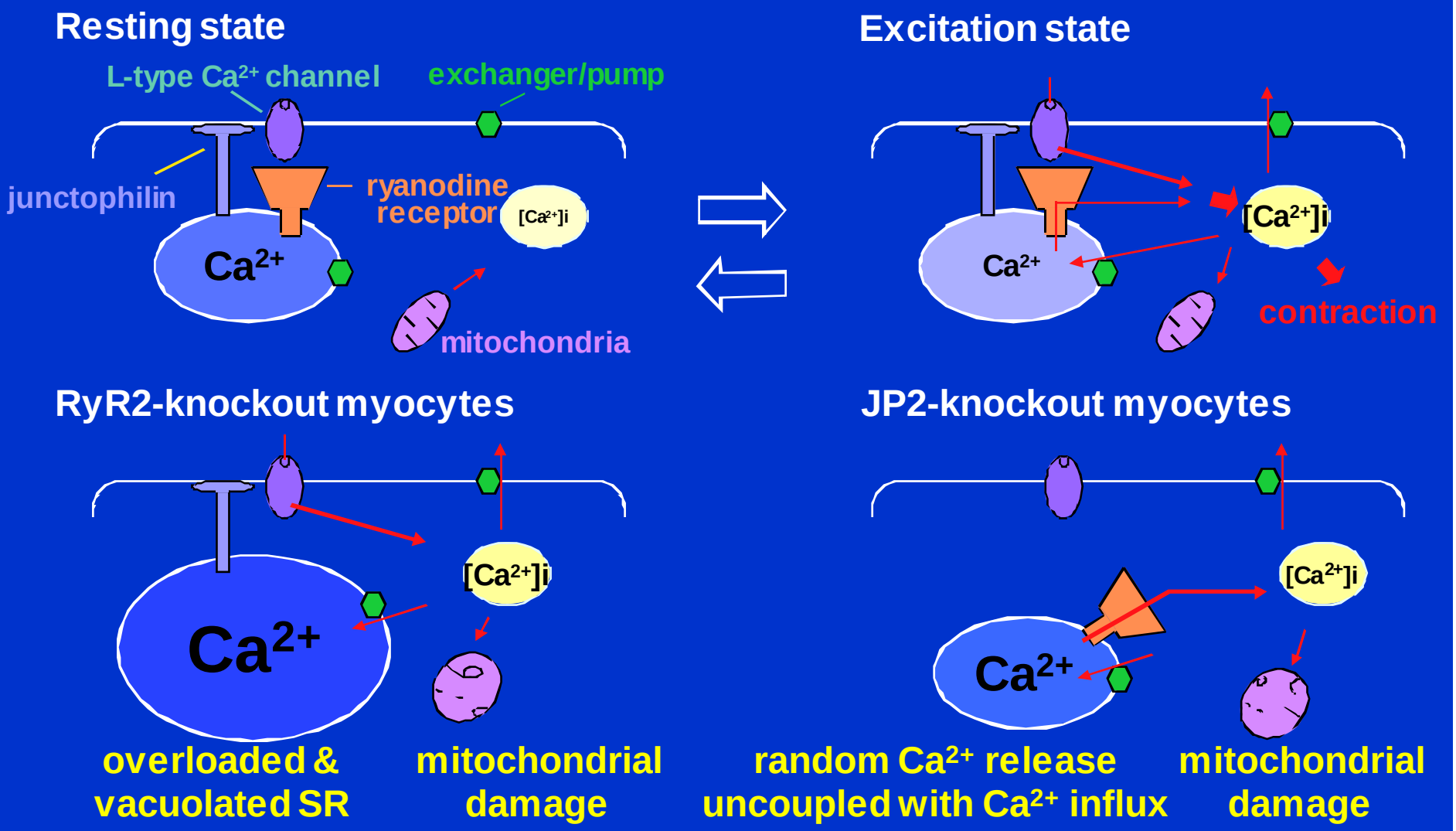




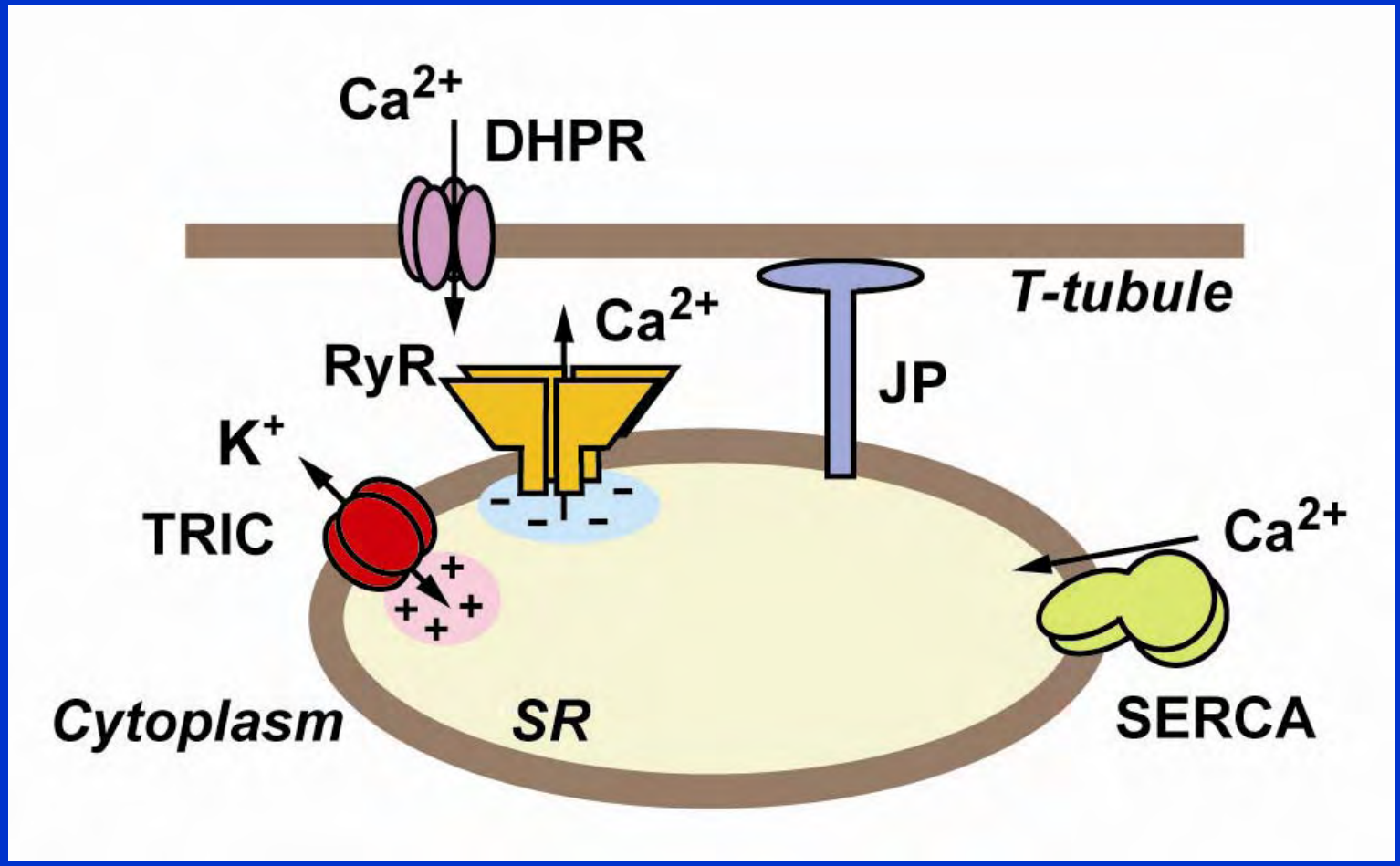
Ca^{2+} concentration

low high

Loss of JP2-mediated JMC formation inhibits DHPR-RyR2 functional coupling, and thus likely generates SR overloading and RyR2-mediated Ca^{2+} waves

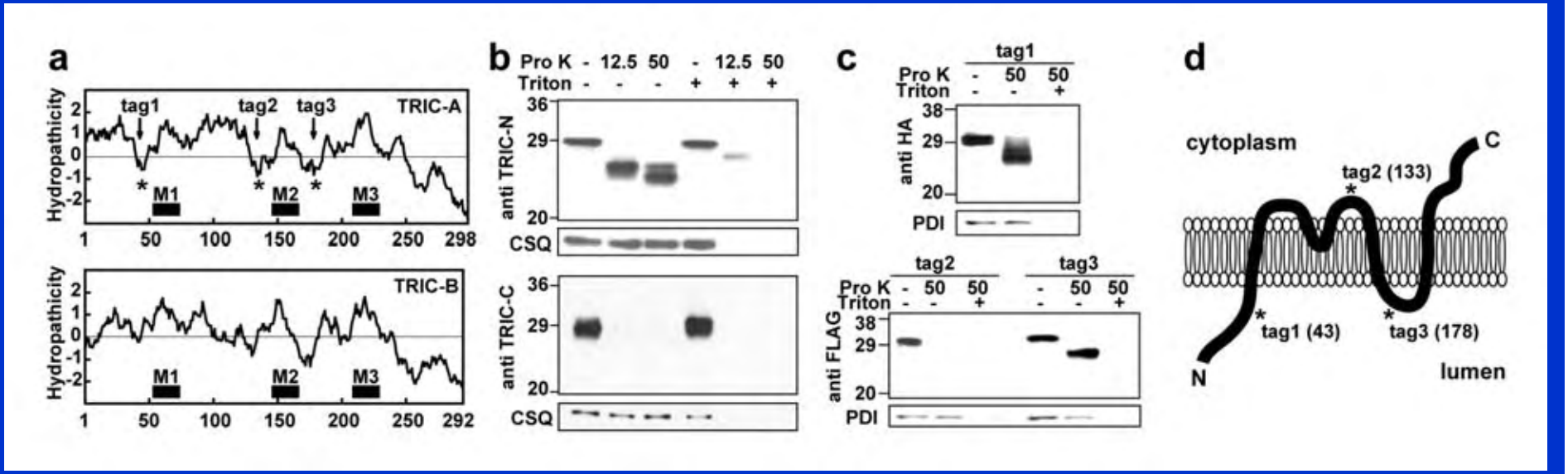


Efficient Ca^{2+} release is likely supported by counter-ion movement across ER/SR membrane



Without counter-ion channels, negative potential would be generated by initial Ca^{2+} release and inhibit following Ca^{2+} release.

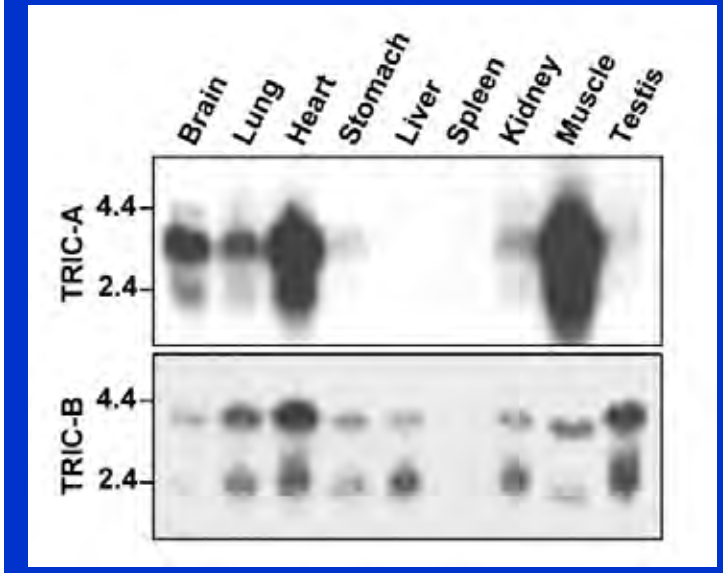
TRIC (trimeric intracellular cation) channels contain three transmembrane segments



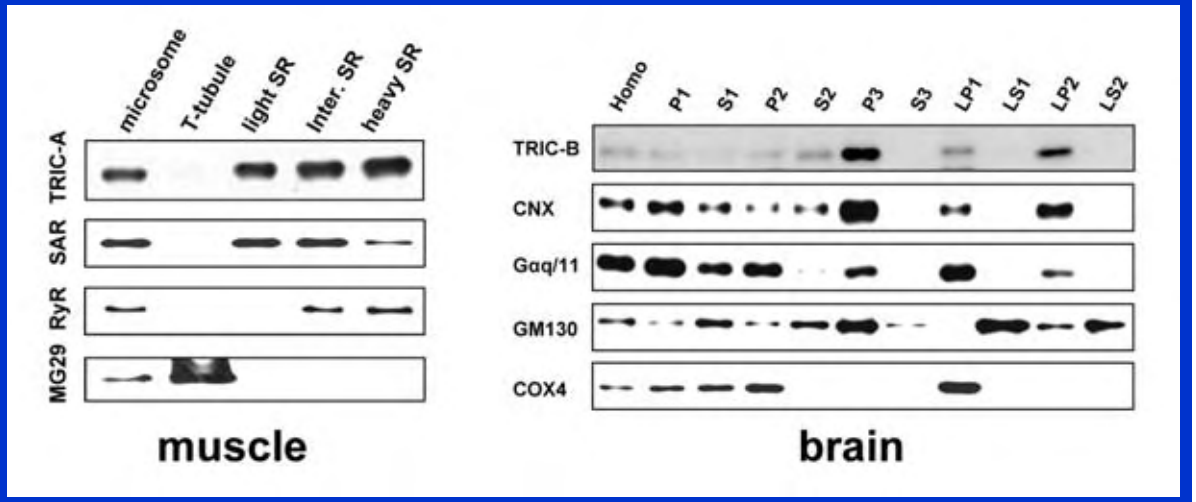
The C-terminus was proteinase-sensitive, and thus is assigned to the cytoplasmic side. Moreover, TRIC became hyper-sensitive when the FLAG tag was inserted between M1 and M2, suggesting that this putative cytoplasmic loop is likely associated with membrane lipids.

TRIC channels are ubiquitously expressed and localized on intracellular membranes

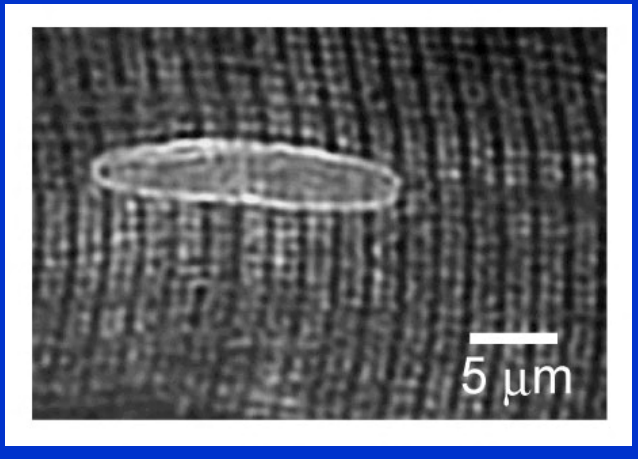
Northern blotting (mouse)



Cell fractionation and Western blots



TRIC-A immunostaining (muscle)

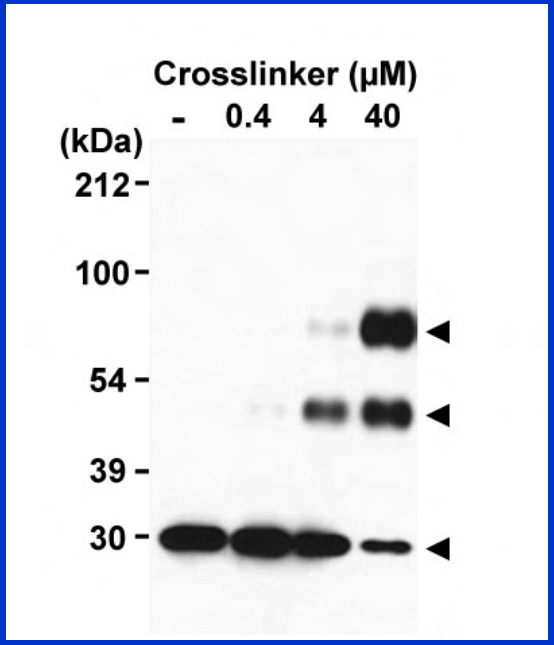


TRIC-A: excitable cell-specific subtype
TRIC-B: common subtype

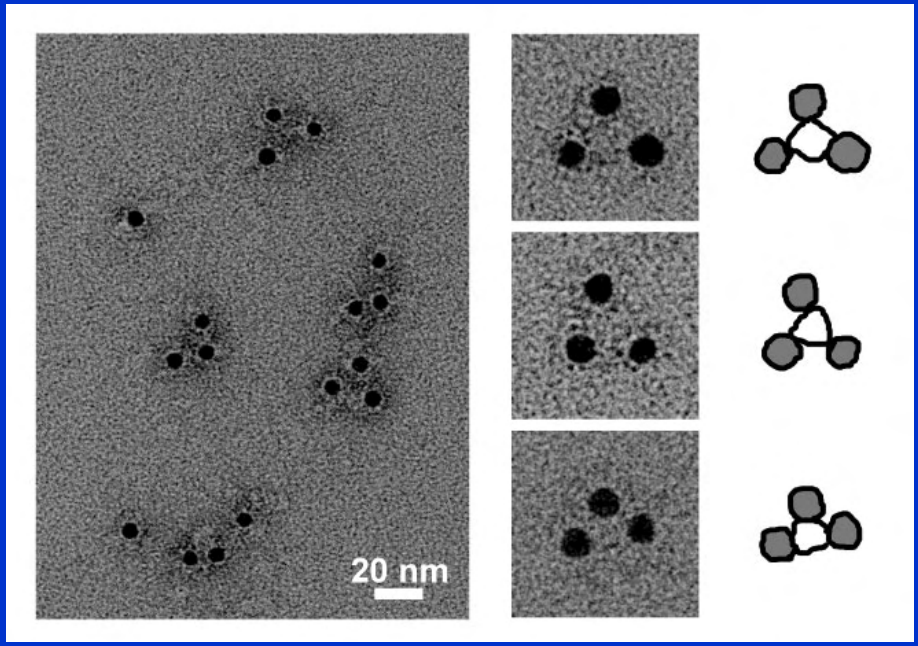
TRIC subtypes are localized on the ER/SR and nuclear membranes.

Homo-trimeric structure of TRIC channel

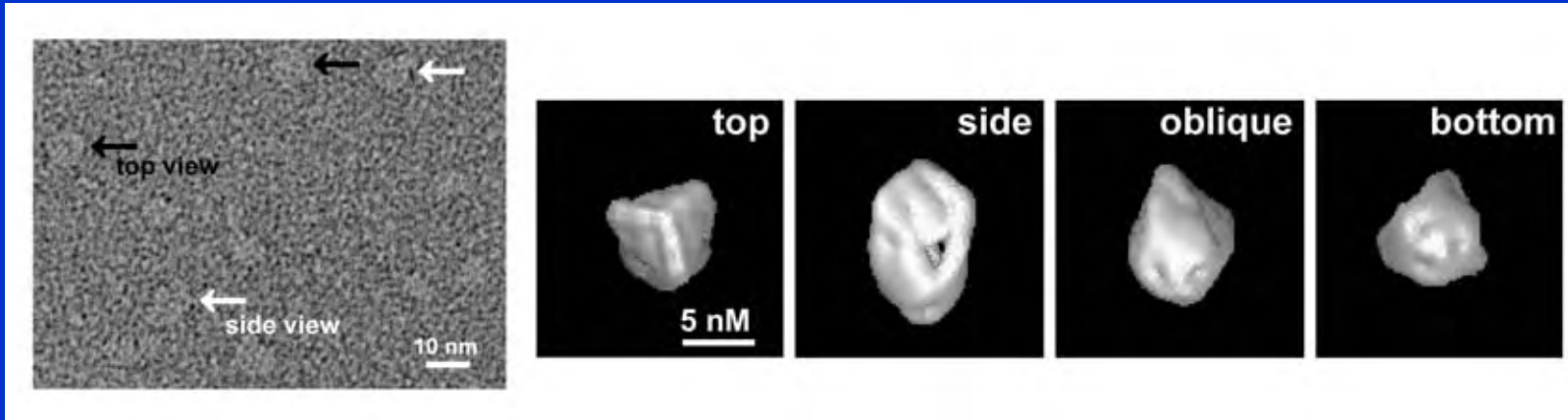
Chemical crosslinking



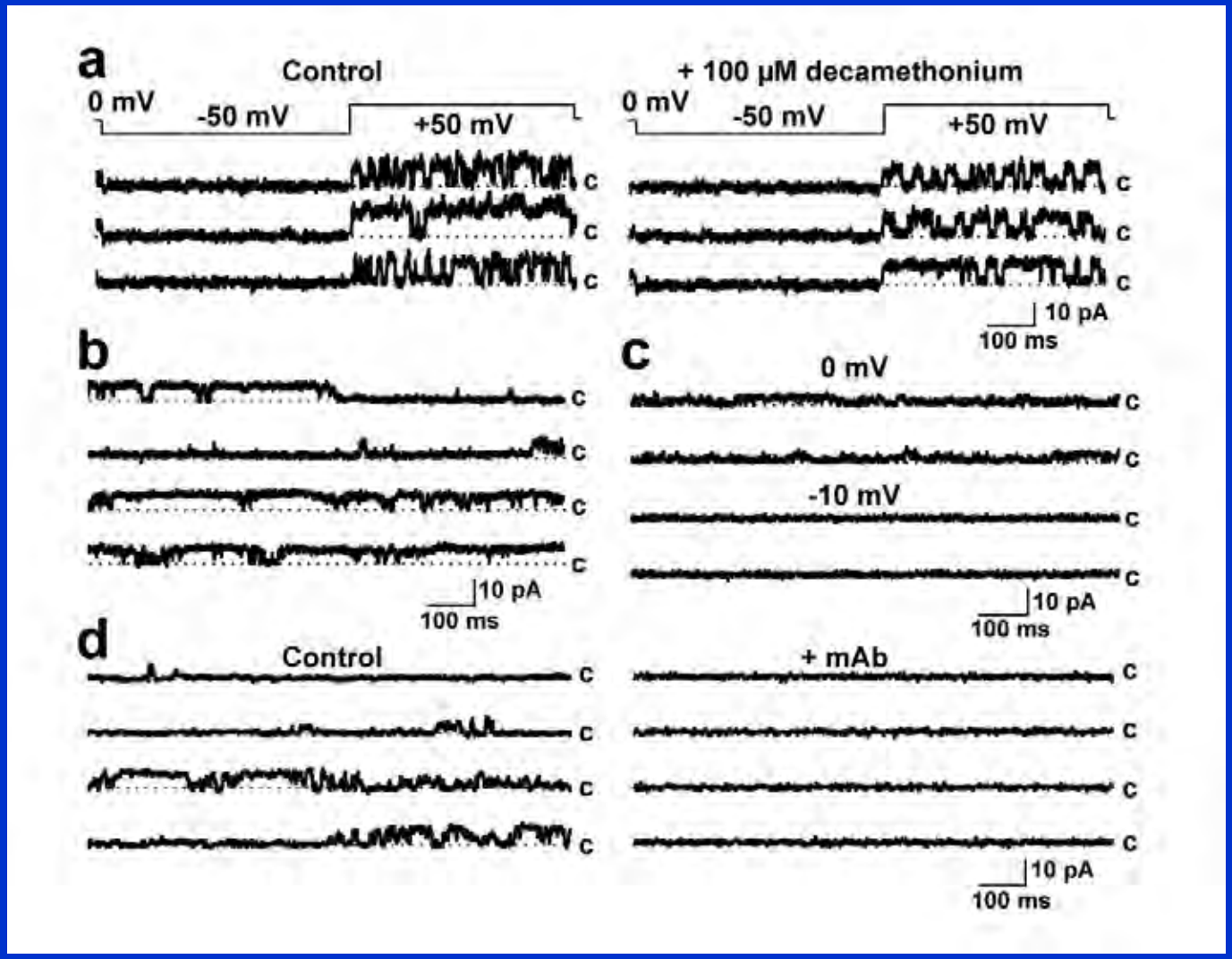
Immunogold staining



3D reconstruction of purified TRIC particles



Purified native and recombinant TRIC-A preparations forms a monovalent cation-selective channel



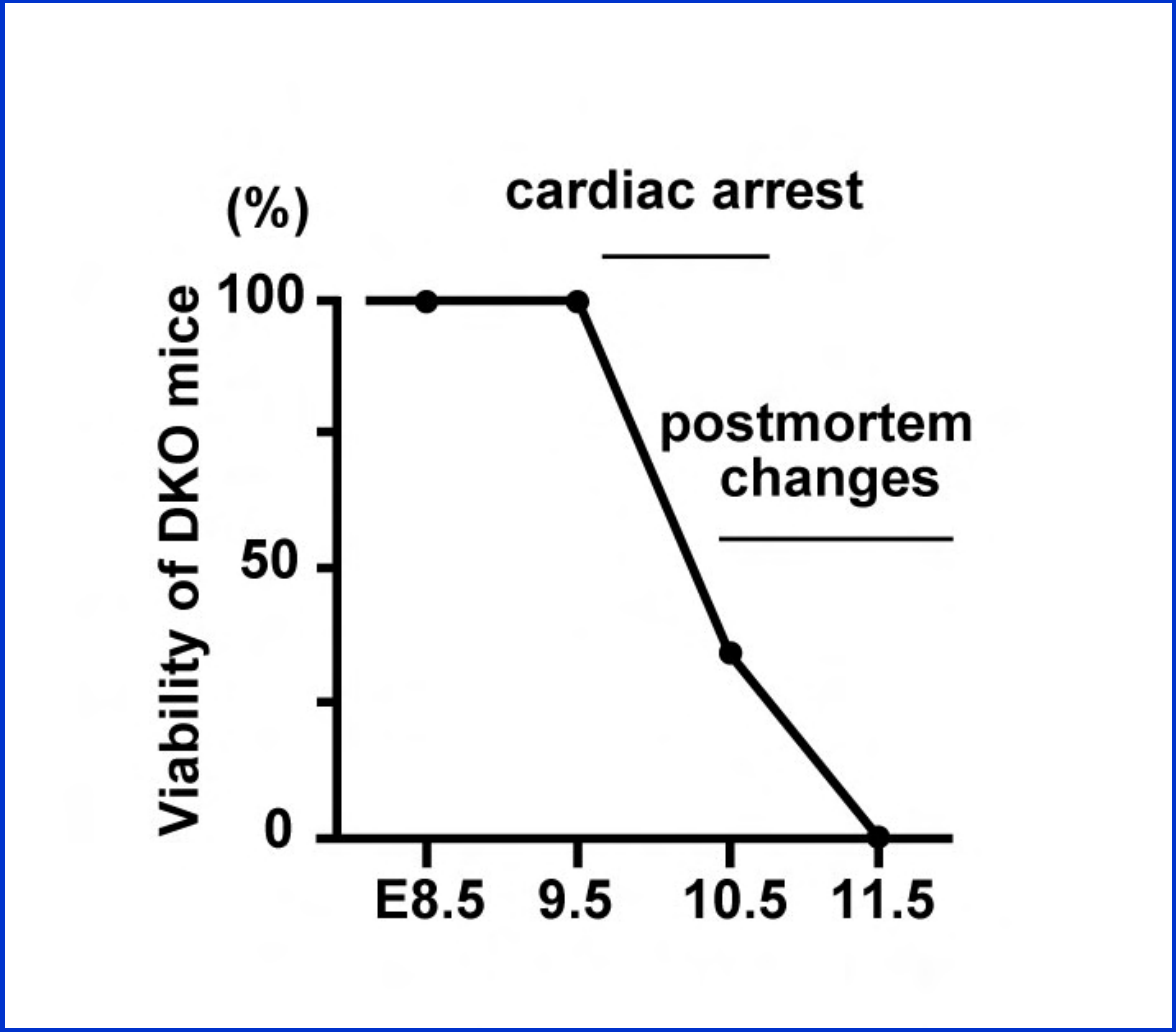
TRIC-A shows moderate selectivity for K^+ over Na^+ (P_{K^+}/P_{Na^+} = 1.5).

TRIC channel subtypes

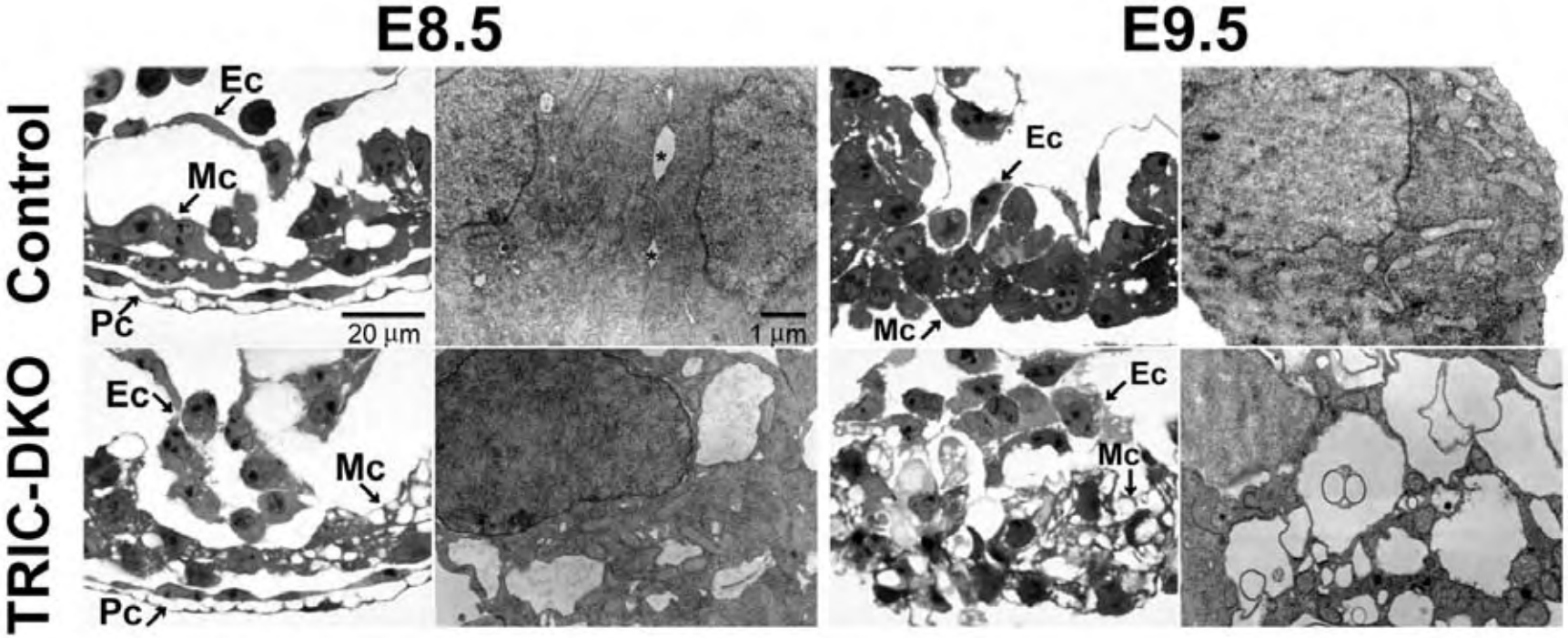
subtype	locus	tissue distribution	knockout mouse	human disease
TRIC-A	Mouse 8B3.3 Human 19p13.1	predominant in excitable cells	no obvious phenotype?	
TRIC-B	mouse 4B2 Human 9q3.1	ubiquitous	neonatal lethality ?	

TRIC-A & B double-knockout mouse:
embryonic lethality (heart failure)

Double-knockout mice lacking TRIC-A and B exhibit embryonic heart failure

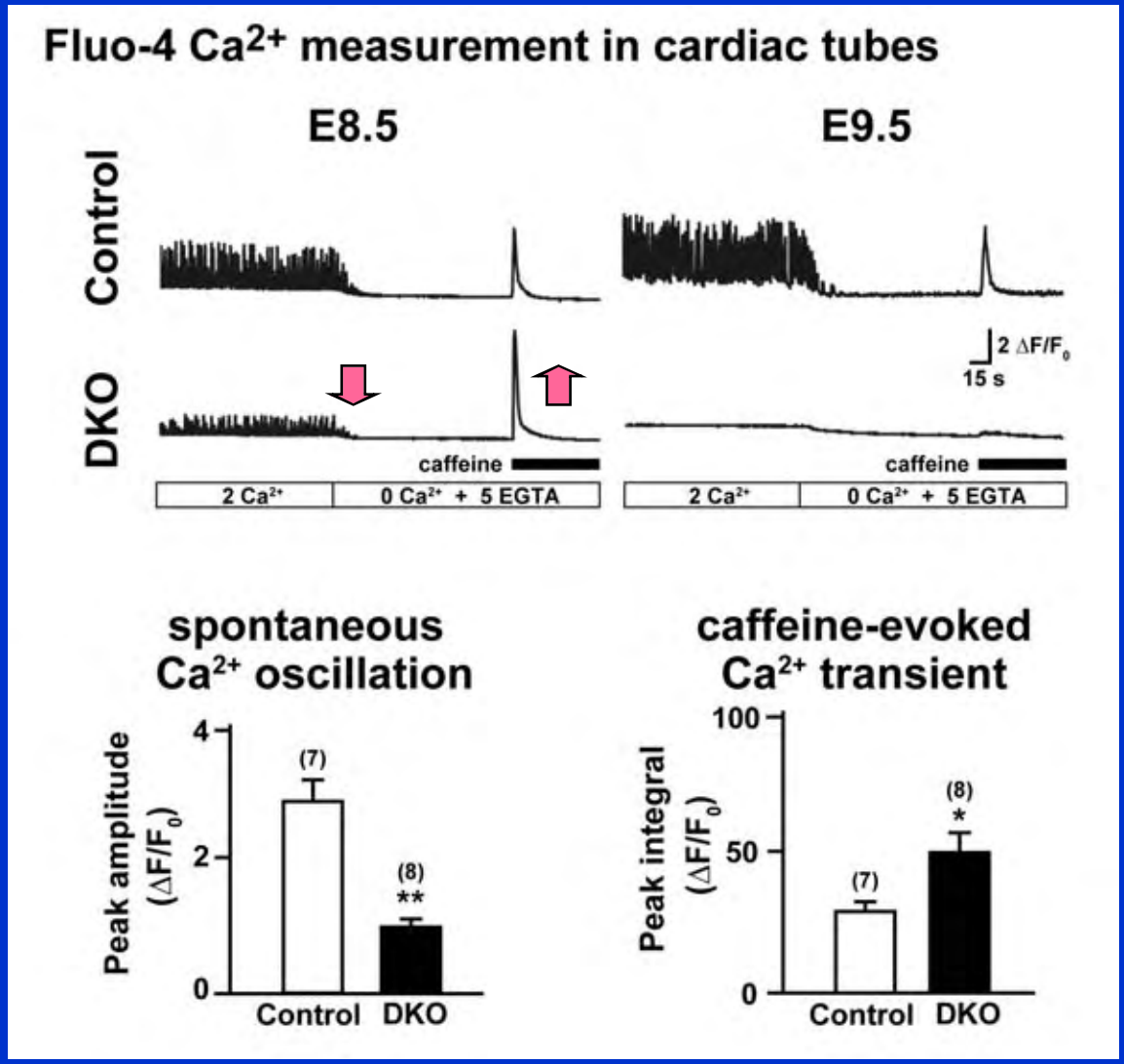


Toward cardiac arrest, the ER/SR becomes swollen in mutant cardiomyocytes from TRIC-DKO embryos



Because this abnormality is identical to that of RyR2-knocout myocytes, severe SR Ca^{2+} overloading is predicted in TRIC-DKO myocytes.

E8.5 TRIC-DKO cardiomyocytes exhibit weak spontaneous Ca^{2+} oscillations, but facilitated caffeine-induced transients



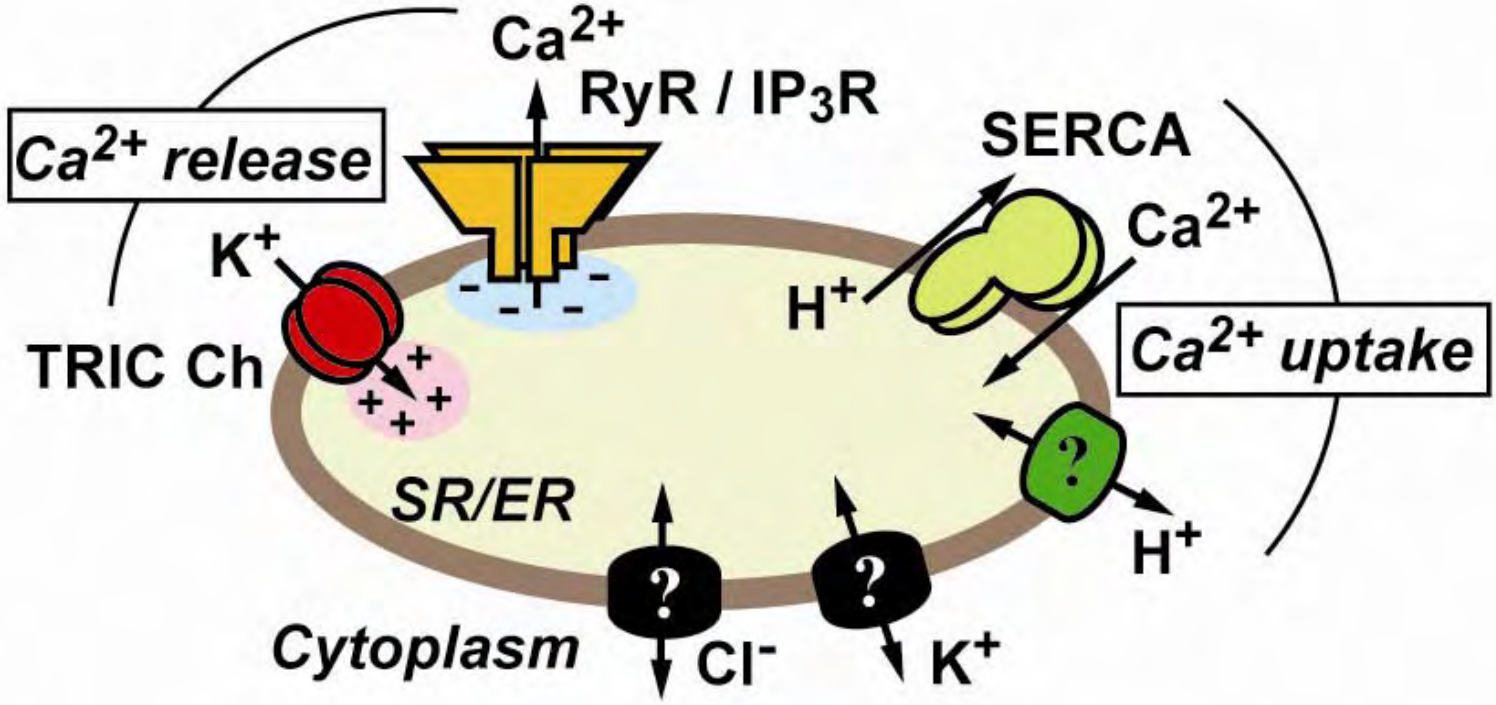
1)Despite Ca^{2+} overloading in the SR, CICR is not well functioning in E8.5 TRIC-DKO cardiomyocytes.

2)The expression levels of major Ca^{2+}-handling proteins including DHPR and RyR are normal in E8.5 DKO cardiomyocytes.

RyR2-mediated Ca^{2+} release is probably inhibited under TRIC-null conditions.

TRIC channels support SR Ca^{2+} release by neutralizing excessive membrane potentials

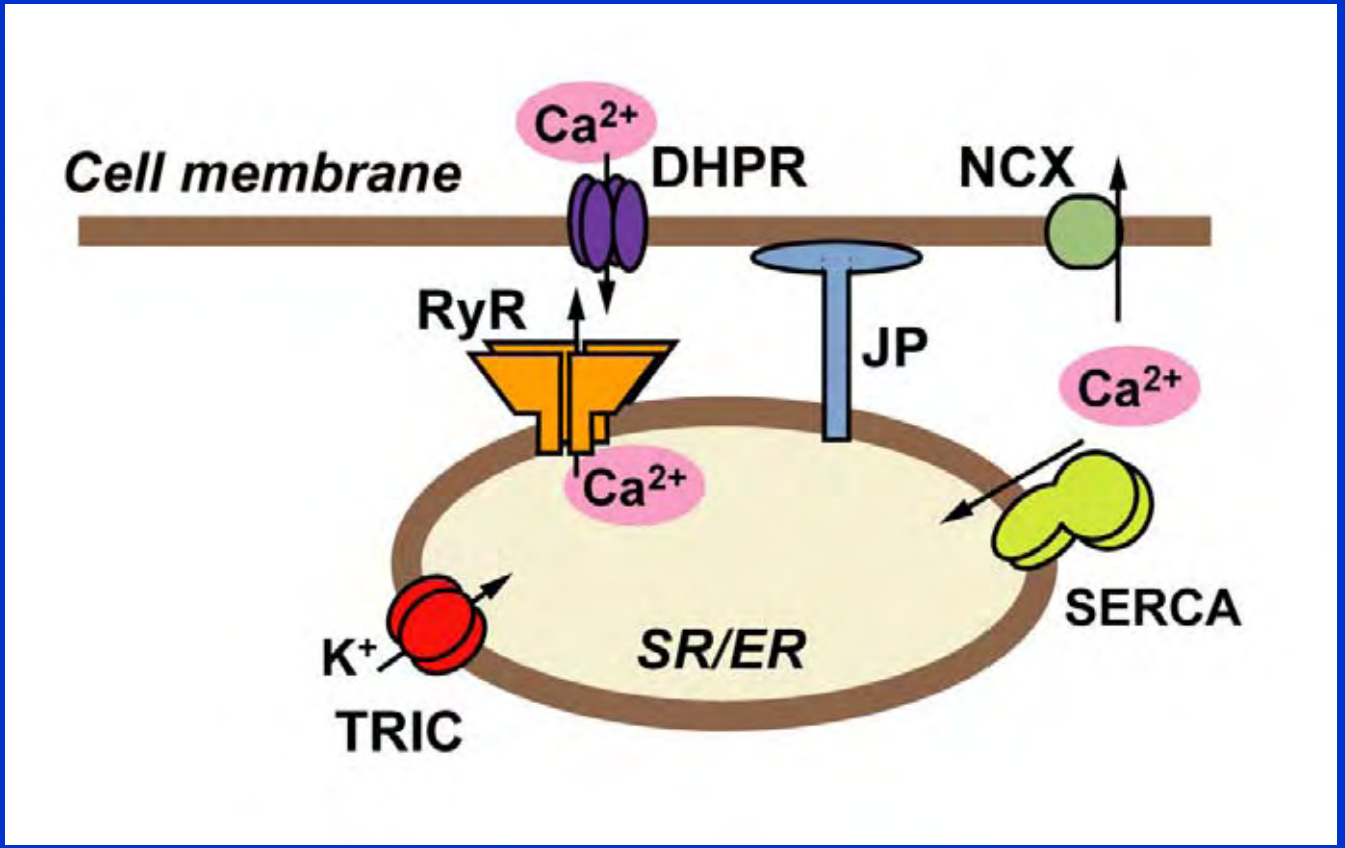
TRIC channel is counter-ion channel coupled with RyR



There are still several unknown SR/ER channels detected by previous electrophysiological studies.

Embryonic cardiomyocytes is a model system in assessing Ca^{2+}-handling proteins from excitable cells

Ca^{2+}-handling proteins crucial in embryonic heart beating



We are still looking for new molecules that essentially contribute to SR/ER Ca^{2+} handling in excitable cells.

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