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Research Article

Post-natal heart adaptation in a knock-in mouse model of calsequestrin 2-linked recessive catecholaminergic polymorphic ventricular tachycardia

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ABSTRACT

Cardiac calsequestrin (CASQ2) contributes to intracellular Ca^{2+} homeostasis by virtue of its low-affinity/high-capacity Ca^{2+} binding properties, maintains sarcoplasmic reticulum (SR) architecture and regulates excitation–contraction coupling, especially or exclusively upon β-adrenergic stimulation. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease associated with cardiac arrest in children or young adults. Recessive CPVT variants are due to mutations in the CASQ2 gene. Molecular and ultra-structural properties were studied in hearts of $CASQ2^{R33Q/R33Q}$ and of $CASQ2^{-/-}$ mice from post-natal day 2 to week 8. The drastic reduction of CASQ2-R33Q is an early developmental event and is accompanied by down-regulation of triadin and junctin, and morphological changes of jSR and of SR-transverse-tubule junctions. Although endoplasmic reticulum stress is activated, no signs of either apoptosis or autophagy are detected. The other model of recessive CPVT, the $CASQ2^{-/-}$ mouse, does not display the same adaptive pattern. Expression of CASQ2-R33Q influences molecular and ultra-structural heart development; post-natal, adaptive changes appear capable of ensuring until adulthood a new pathophysiological equilibrium.

Abbreviations: ANF, atrial natriuretic factor; Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma-2; CASQ2, cardiac calsequestrin; CASQ2-R33Q, mutant cardiac calsequestrin; CRT, calreticulin; CRU, calcium release unit; CT1, cardiac triadin; EC coupling, excitation–contraction coupling; Egr-1, early growth response-1; Elk-1, E-twenty-six (ETS)-like transcription factor 1; EM, electron microscopy; ERK1/2, extracellular-signal-regulated kinases 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRP78, glucose-related protein 78; GRP94, glucose-related protein 94; JC, junctin; jSR, junctional SR; KI, knock-in; KO, knock-out; NCX-1, Na^+/Ca^{2+} exchanger; PCs, peripheral couplings; PI3K, hosphatidylinositol 3-kinases; RYR2, ryanodine receptor 2; SE/SD, standard error/standard deviation; SERCA, SR Ca^{2+}-ATPase; SOCE, Store-Operated Calcium Entry; SR/ER, sarco/endoplasmic reticulum; STIM1, stromal interaction molecule 1; TA, triggered activity; TRPC, transient receptor potential channel; T-tubule, transverse-tubule; UPR, unfolded protein response; VT, ventricular tachycardia; WT, wild type

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Introduction

The sarcoplasmic reticulum (SR) is the major organelle responsible for intracellular Ca^{2+} cycling which, in heart, is tightly controlled on a beat-to-beat basis. Ca^{2+} release units (CRUs), intracellular junctions between SR and transverse (T)-tubules, are made up of a quaternary molecular complex containing the ryanodine receptor (RYR2), the SR Ca^{2+} release channel, the anchoring proteins triadin (CT1) and junctin (JC), and calsequestrin (CASQ2). CASQ2 is localized in the lumen of junctional SR (jSR) and plays an important role in regulating myoplasmic Ca^{2+} concentration [1]. CASQ2 not only determines intra-SR Ca^{2+} by virtue of its low-affinity, high-capacity Ca^{2+} binding properties but also seems to maintain SR architecture [2] and regulates excitation–contraction (EC)-coupling, especially or exclusively upon β-adrenergic stimulation [3].

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease associated with cardiac arrest in children or young adults [4]. The diagnosis of CPVT is most commonly established in subjects suddenly and unexpectedly manifesting stress- or emotion-induced ventricular tachycardia (VT) and syncope. The dominant CPVT variant results from inherited abnormalities of intracellular Ca^{2+} regulation caused by mutations in the RYR2 gene [5], whereas the recessive CPVT variant is due to mutations in the CASQ2 gene [6]. The clinical phenotype of the two CPVT variants is virtually identical and both appears to share the same cellular pathogenetic mechanism entailing SR Ca^{2+} overload and spontaneous diastolic SR Ca^{2+} release evoking VT [7–9].

Our laboratories have been studying the functional consequences of the point mutation R33Q located in the first domain of CASQ2. Since the content of CASQ2-R33Q was drastically reduced in myocytes of the adult knock-in (KI) $CASQ2^{R33Q/R33Q}$ mouse, our experimental model of recessive CPVT [9], the post-natal development of KI hearts (from post-natal day 2 to post-natal week 8) was studied in order to assess the ultra-structural and molecular adaptation. The key issues at stake are when CASQ2-R33Q was reduced and when and what type of adaptive responses occurred in heart right after birth. The main findings are (a) morphological changes of jSR and of SR–T tubule junction are evident at post-natal week 2, (b) reduction of CASQ2-R33Q by about 80% is an early post-natal event and is accompanied by a drastic and concurrent reduction of CT1, whereas JC down-regulation is observed only at post-natal week 8, (c) up-regulation of glucose-related protein 78 (GRP78) from post-natal day 2 onward is evidence of endoplasmic reticulum (ER) stress, (d) pro-survival signals are detected from post-natal week 2 onward, as inferred by increase of Bcl-2-associated X protein (Bcl-2), (e) the transcription factor early growth response-1 (Egr-1) and stromal interaction molecule 1 (STIM1), an Egr-1-dependent Ca^{2+}-regulating protein, are up-regulated, (f) only at post-natal week 8, changes are detected in channels associated to SOCE: Orai1 is drastically reduced whereas transient receptor potential channel (TRPC)3 and TRPC6 are up-regulated, and (g) another model of recessive CPVT, the $CASQ2^{-/-}$ mouse, does not display qualitatively and temporally the same molecular adaptive pattern.

Expression of the mutant CASQ2-R33Q and its precocious and persistent removal influence the molecular and ultra-structural heart development; ensuing, multiple, post-natal adaptive responses, including ER stress, are capable of ensuring until adulthood a new, long-lasting pathophysiologic equilibrium to be hampered only under emotional stress or β-adrenergic stimulations.

Material and methods

Antibodies

Source of specific antibodies: CASQ2 from Thermo Scientific; ERK1/2, Orai1, TRPC3 and TRPC6 from Sigma; GRP78, glucose-related protein 94 (GRP94), calreticulin (CRT) from Abcam; Bcl-2, Bcl-2-associated X protein (Bax), Egr-1, total E-twenty-six (ETS)-like transcription factor 1 (Elk-1) and phosphorylated Elk-1, Na^+/Ca^{2+} exchanger (NCX-1) from SantaCruz; caspases 3, 9, 12, Akt and phosphorylated (T308) Akt, phosphorylated (Thr202/Tyr204) ERK1/2, STIM1 from Cell Signaling; antibodies for CT1 and JC, generous gifts from Dr. Isabelle Marty and Dr. Steven Cala, respectively.

Animal models

Transgenic homozygous KI $CASQ2^{R33Q/R33Q}$, transgenic homozygous knock-out (KO) $CASQ2^{-/-}$ and control C57BL6 wild-type (WT) male mice were previously described [9,10]. All animal experimental protocols were approved by the Animal Care and Use Committee of University of Padova.

Electron microscopy (EM)

Fixed hearts were embedded in an epoxy resin and ultrathin sections were cut, stained, and analyzed as previously described [9].

Protein profile of heart homogenates and quantitative densitometry

Hearts were snap frozen in liquid nitrogen and homogenized in 3% SDS, 1 mM EGTA with protease inhibitors. Quantitative western blotting was carried out on whole heart homogenates from WT, KI and KO mice ($n=4$ for each group). Equal amounts of heart homogenates (30–200 μg) were analyzed by SDS-PAGE [9]. Following transfer to membranes, immunoblots were revealed with the corresponding primary antibodies and secondary antibodies conjugated with either alkaline phosphatase or horseradish peroxidase. In the latter case, visualization was achieved using ECL Western Blotting substrate (Pierce). For quantitative densitometry, intensity of each protein band in blots developed only with alkaline phosphatase was determined by Scion Image software. Protein-signal densities were normalized to the corresponding actin-signal densities within a linear relationship of antigen concentration versus signal density. Data were expressed as mean ± standard error (SE). Comparisons between means of two groups were performed by unpaired two-tales Student's *t*-test. Differences were considered significant at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$.

Real-time (RT)-PCR

Total RNA from WT and KI hearts of 2-week-old and 8-week-old mice was isolated using TRIzol reagent (Invitrogen) and quantified spectrophotometrically. Two micrograms of total RNA from each sample were subjected to random hexamer primed first-strand cDNA synthesis, using Superscript III reverse transcriptase (Invitrogen). RT-PCR quantification of target genes, CASQ2 and atrial natriuretic factor (ANF), and reference genes, β_2-microglobulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was performed by the SYBR Green method with an Applied Biosystems 7500 Fast Real Time PCR System [11]. Calculations were made using the Applied Biosystems software based upon threshold (Ct) values. RT-PCR reaction conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, 1 min at 60 °C for CASQ2 and 1 min at 56 °C for ANF, followed by the melting curve protocol. All samples were run in triplicate. Oligonucleotide primers were CASQ2 5′CTGAAGGGTGACCGCACGAT3′ (forward) 5′TTCACGATCTCCACTGGGTCTT3′ (reverse); ANF 5′AACCTGCTAGACCACCTGGA3′ (forward) 5′CTGCTTCCTCAGTCTGCTA3′ (reverse); β_2-microglobulin 5′TTTCTGGTGCTTGTCTCACTGA3′ (forward) 5′ATGTTCGGCTTCCCATTCTC3′ (reverse); GAPDH 5′GTATGACTCCACTCACGGCAAA3′ (forward) 5′TTCCCATTCTCGGCCTTG3′ (reverse).

Results

Morphology of jSR and SR–T-tubule junction in homozygous CASQ2$^{R33Q/R33Q}$ mice from birth to adulthood and comparison with CASQ2$^{-/-}$ mice

In mammalian cardiomyocytes, jSR is closely juxtaposed to surface membranes, either sarcolemma or T-tubules: CRUs are peripheral couplings (PCs), where jSR comes in close contact with the sarcolemma, and dyads, where jSR connects to T-tubule in the fiber interior at the Z-lines (internal junctions). In mouse, up to post-natal days 3–4, CRUs are present only as PCs, whereas dyads are predominant in mature cardiomyocytes. Imbalanced expression of SR proteins alters the jSR architecture, both in cardiac and skeletal muscles [3,12,13]. Hearts from adult CASQ2$^{R33Q/R33Q}$ mice display ultra-structural changes such as disarray of junctional electron-dense material, referable to CASQ2 polymers, dilatation of jSR, yet normal total SR volume [9].

Cardiomyocytes of both WT and KI mice were analyzed at post-natal day 2 and post-natal week 2 using EM. At post-natal day 2, CRUs were exclusively found as PCs (Fig. 1A–F), whereas at post-natal week 2, both PCs and dyads were present (Fig. 1G–L). Frequency of both PCs and dyads did not vary between WT and KI mice at both the ages (data not shown). In WT myocytes, the jSR was usually narrow and flat (Fig. 1A, B, G, and H) and CASQ2 was usually visible as a chain-like electron-dense polymer (Fig. 1A, G, single arrow) running parallel to the junctional membrane. On the other hand, jSR from KI myocytes displayed altered features: electron-dense chain-like polymers were either absent or dispersed in dyads (Fig. 1I–L), similarly to that seen in adult KI [9]; changes were only marginally affecting PCs, which were not significantly different in morphology from WT; PCs displayed a slightly more variable/dilated profile and an apparently less condensed electron-dense material referable to CASQ2-R33Q. However, some electron-dense material, even forming a chain-like polymer, was still visible (Fig. 1, white arrows).

The jSR of cardiomyocytes from adult KI mice is wider than that of WT cardiomyocytes [9]. Thus, measurements of the jSR size of both PCs and internal dyads were performed at post-natal day 2 and post-natal week 2: width of the jSR cisternae was significantly increased and more variable, as indicated by the high standard deviation (SD), in dyads of KI myocytes at post-natal week 2: 45 ± 18 nm versus 25 ± 3 nm (Table 1). On the other hand, there was no significant difference in PCs between WT and KI myocytes, at both the ages (Table 1). Thus, morphological changes in developing KI hearts occur quite early during post-natal development and affect in particular dyad junctions.

It has been reported that the absence of CASQ2 in KO adult myocytes induces disappearance of the chain-like polymers and widening of the jSR lumen [3,10]. Here, ultra-structural measurements were also carried out on developing KO hearts (Table 1, third column): data show that not only dyads but also PCs were significantly wider in KO myocytes as compared to WT, both at post-natal day 2 and at post-natal week 2. By and large, ultra-structural remodeling of jSR seems to be related to CASQ2 content.

Time course of expression of CASQ2R33Q and junctional proteins in homozygous CASQ2$^{R33Q/R33Q}$ mice from birth to adulthood

The content of mutant CASQ2-R33Q was found to be reduced in hearts obtained from 8 week-old CASQ2$^{R33Q/R33Q}$ mice, as compared to that of age-matched WT mice, thus explaining the absence of electron-dense materials, referable to CASQ2, observed in adult CRUs in association with reduction of CT1 and JC [9]. Here, we monitored the expression of CASQ2-R33Q, CT1 and JC in KI mice from birth to adulthood, i.e., from post-natal day 2 to post-natal week 8, in comparison to that of WT mice. In WT hearts, the relative abundance of CASQ2 (solid line, square symbol), of glycosylated and un-glycosylated forms of CT1 (solid line, circle and triangle symbols, respectively) and of JC (dashed line) changed during post-natal development (Fig. 2A), in agreement with previous data [14,15]. Fig. 2B shows that CASQ2-R33Q was down-regulated at post-natal day 2 (38.6 ± 8.1% of CASQ2 content in WT mice; $P<0.01$) and its amount was further reduced in the following stages (at post-natal day 9, 23.1 ± 2.4%, $P<0.001$; at 2 weeks, 24.1 ± 3.7%, $P<0.001$; at 8 weeks, 18.9 ± 1.8%, $P<0.001$). In confirmation of Rizzi et al. [9], who examined only adult specimens, no variation in CASQ2-R33Q mRNA content was observed at all ages investigated (data not shown). Thus, disposal of CASQ2-R33Q was a persistent process throughout adulthood. Fig. 2C shows the expression pattern of the glycosylated and un-glycosylated forms of CT1 and of JC. Right after birth, both forms of CT1 were down-regulated in KI mice as compared to WT mice; in details, at post-natal day 2, the un-glycosylated CT1 was 33.6% (±0.9%; $P<0.001$), at post-natal day 9, 47.2% (±0.8%; $P<0.05$), at 2 weeks, 58.6% (±1.4%; $P<0.01$) and at 8 weeks, 41.8% (±1.9%; $P<0.001$) of the amount measured in age-matched WT mice. The glycosylated CT1 was hardly detectable in KI hearts at all ages, i.e., it was very much decreased as compared to WT values. As we observed in WT mice (Fig. 2A), JC was hardly detectable until post-natal week 2; in KI mice, it was not significantly up-regulated at week 2 (162.8 ± 27.1%), whereas at week 8 was drastically reduced (34.9 ± 3.3%; $P<0.01$; [9]).

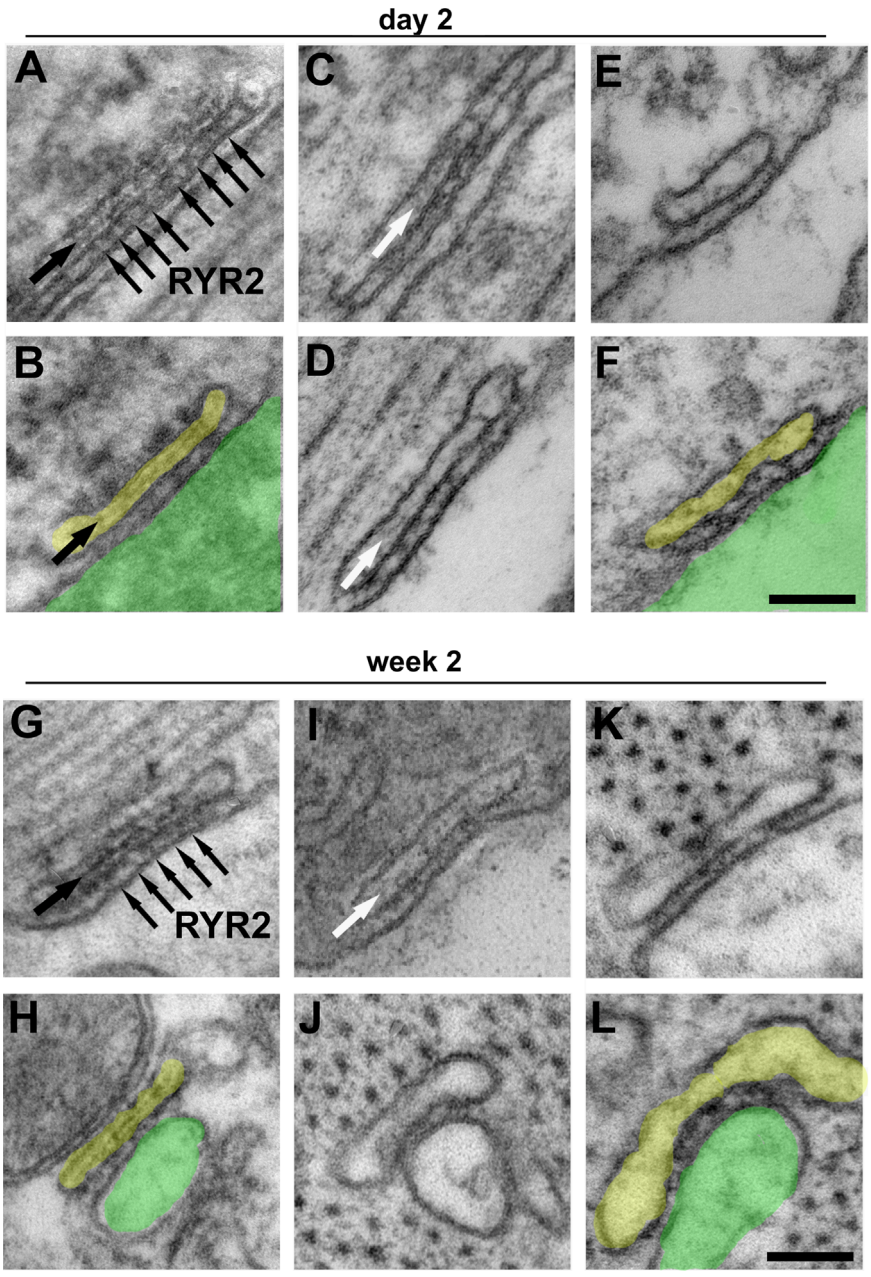

Fig. 1 – Electron microscopy of PCs and dyads in cardiomyocytes from WT and KI mice. (A and B) In 2 day-old WT myocytes, jSR associates to plasmalemma to form PCs and displays a narrow lumen profile. An intraluminal, chain-like electron-dense polymer representing CASQ2 is already visible (single arrow in A). Smaller arrows in (A) point to the cytoplasmic domain of RYR2. (C–F) jSR lumen of KI myocytes appears only minimally different from WT counterpart; there is a slightly more variable/dilated jSR and an apparently less condensed electron-dense material (D and E, white arrow). However, some electron dense material, sometimes even forming a chain-like polymer, is still visible (C, white arrows). (G and H) In 2 week-old WT myocytes, jSR associates to either plasmalemma (G) or invaginating T-tubules (H). Single arrows in (G) and (H) point to the chain-like electron-dense polymer, whereas smaller arrows (G) point to the cytoplasmic domain of RYR2. (I–L) In KI myocytes, the jSR profile appears more variable in shape. CASQ2 polymers are missing and electron-dense material, if any, is quite disperse. However, such changes affect dyads rather than PCs (I, white arrow). Green refers to extracellular space, yellow to jSR lumen. Bar: 0.1 µm.

Thus CASQ2-R33Q is reduced but not totally absent in KI mice right after the birth, reaching very low levels in comparison to CASQ2 of WT mice at post-natal day 9 and remaining thereafter constant and very low. Decrease of CASQ2-R33Q was accompanied by a marked down-regulation of CT1. Interestingly, the un-glycosylated CT1/CASQ2-R33Q relative ratio in KI hearts is changing from 0.6 ± 0.1 to 2.8 ± 0.3, from post-natal day 2 to post-natal week 8, whereas the un-glycosylated CT1/CASQ2 ratio in WT mice changed from 0.7 ± 0.3 to 1.5 ± 0.3. Thus, during post-natal differentiation, there is a progressive imbalance of CT1/CASQ2-R33Q ratio compared to CT1/CASQ2.

Table 1 – The width of jSR cisternae in dyads of KI myocytes at post-natal week 2 is increased (*$P<0.001$) and more variable in shape, similarly to what already observed in the adult stage [9] and in KO hearts at both post-natal week 2 and adult stage [10]. In KO mice, PCs are wider as early as post-natal day 2. Data are given as mean ± SD. n = Number of either dyads or PCs examined. Each data set was obtained from 3 hearts for each group.

Age	$CS2^{+/+}$	$CS2^{R33Q/R33Q}$	$CS2^{-/-}$
2 Days: only PCs	26 ± 4 nm (n=153)	26 ± 4 nm (n=255)	34 ± 10 nm* (n=124)
2 Weeks: PCs	25 ± 3 nm (n=62)	29 ± 7 nm (n=95)	54 ± 23 nm* (n=65)
2 Weeks: dyads	25 ± 3 nm (n=165)	45 ± 18 nm* (n=154)	45 ± 23 nm* (n=75)
Adult (week 8 for KI mice [9], week 20 for KO mice [10]): dyads	26 ± 4 nm (n=139)	41 ± 10 nm* (n=108)	37 ± 12 nm* (n=78)

CASQ2-R33Q-induced activation of ER stress and involved signaling pathways

ER stress is associated to various intrinsic and extrinsic factors such as mutated proteins and ER Ca^{2+} depletion. The ubiquitous unfolded protein response (UPR) counteracts disruption of ER homeostasis and entails transient attenuation of new protein synthesis, degradation of misfolded proteins and eventually but not necessarily apoptosis in order to mitigate or eliminate ER stress [16].

CASQ2-R33Q is a missense mutation of CASQ2 and is likely post-translationally down-regulated since it might be recognized as misfolded protein [17] within the ER/SR, activating the ER stress. Time course of ER stress activation was, thus, studied in KI hearts by monitoring two established markers, i.e., GRP78 and GRP94 [16], whose expression in WT hearts was down-regulated during the post-natal period (Fig. 3A, solid and dashed lines for GRP78 and GRP94, respectively). GRP78 was found to be up-regulated (Fig. 3B) since post-natal day 2 (150 ± 11.2%; $P<0.05$) and attained a stable, plateau value thereafter (202.26 ± 18.2%, 9-day-old $P<0.01$; 181.7 ± 15.8%, $P<0.05$, 2-week-old and 214.7 ± 37.1%, $P<0.01$, 8-week-old), i.e., from post-natal day 9 until adulthood there was a two-fold increase of GRP78, as compared to WT mice. On the contrary, as shown in Fig. 3B, GRP94 in hearts of KI mice was not up-regulated until post-natal week 2. GRP94 attained higher values (about 150%) in adulthood without any significant difference from WT mice.

The next question was whether the reduced content of CASQ2-R33Q in KI mice would trigger the compensatory expression of intra-luminal Ca^{2+}-binding proteins such as CRT, which is also known as an ER molecular chaperone and a marker of ER stress [18], and whose expression in WT mice (Fig. 3A, dotted line) is finely regulated during heart development, i.e., it is up-regulated during embryogenesis and down-regulated in adulthood where it is expressed at low levels [19]. As shown in Fig. 3B, CRT was slightly but significantly up-regulated in hearts from KI mice at post-natal day 2 (118.62 ± 4.9%; $P<0.05$), undistinguishable from WT until post-natal week 2, and up-regulated at post-natal week 8 (186.1 ± 22.4%; $P<0.05$).

Thus, GRP78 appears to be always up-regulated from the earliest post-natal time point onward into adulthood, whereas CRT only after post-natal week 2.

ER stress can elicit both pro-survival and pro-apoptotic signals. To this aim, expression of pro-survival protein Bcl-2 and of its counterpart, Bax, was monitored. Bcl-2 was hardly detectable in hearts of both WT and KI mice until post-natal day 9 (Fig. 3C); afterwards, Bcl-2 was abruptly up-regulated by about two-fold in hearts of 2 week-old KI mice and over-expression was also detected at post-natal week 8 (Fig. 3C). On the contrary, Bax was expressed at very low levels at all ages in both WT and KI mice (Fig. 3C). Since the Bcl-2/Bax ratio determines the susceptibility of cells to a death signal [20], it can be concluded that pro-survival signals prevail in KI hearts. This result was confirmed by studying the activation of apoptosis, which is often induced by persistence of ER stress. Expression of the cleaved forms of caspases 3, 9 and 12 was studied and no activation of the apoptotic pathway was detected (data not shown).

One of the signaling cascades ruled by pro-survival signals is the phosphatidylinositol 3-kinases (PI3K)–Akt pathway. Akt improves cell survival by phosphorylating several negative modulators of cell death and suppresses apoptosis by directly controlling the Bcl-2/Bax ratio [21]. In 2-week-old KI hearts, increased phosphorylation of Akt at residue Thr308 was detected (Fig. 3D).

In $CASQ2^{R33Q/R33Q}$ mice, evidence of ER stress accompanies jSR development and CRUs deranged maturation from post-natal day 2 onward; CASQ2-R33Q-induced ER stress activation in turn promotes pro-survival pathways and downgrades pro-apoptotic pathways throughout the entire period of investigation.

Cell signaling pathways activated in hearts of $CASQ2^{R33Q/R33Q}$ mice

It is known that several fetal genes contain promoter elements that can be transcriptionally activated upon ER stress [22,23]. Egr-1 gene is an immediate early gene activated by various ER-stress stimuli [24] and is involved in regulation of cardiac genes controlling Ca^{2+} homeostasis, such as STIM1 [25], CASQ2 [26], NCX [27] and SR Ca^{2+}-ATPase (SERCA2) [28]. On these premises, time course of Egr-1 expression was studied. No differences were detected in Egr-1 expression between KI and WT mice until post-natal day 9 (data not shown). On the contrary, Egr-1 was sharply up-regulated at post-natal week 2 in KI hearts (Fig. 4A) and returned to basal levels at post-natal week 8 (Fig. 4B). The expression of relevant Egr-1-dependent transducer proteins, such as STIM1 and NCX-1, was also investigated. At post-natal week 2, STIM1 was drastically up-regulated in KI hearts whereas no differences were observed in expression of NCX-1, which is present at this age as a doublet (Fig. 4A). In 8 week-old KI hearts, STIM1 was still increased even if Egr-1 was no longer up-regulated.

Egr-1 expression is regulated by ERK1/2-dependent pathways [29]. Increased levels of the phosphorylated form of ERK2 (p-ERK2) and of its downstream target, Elk-1 [30], were detected in hearts from 2 week-old KI mice (Fig. 4A).

STIM1 is a Ca^{2+} binding/sensor protein that activates Store-Operated Calcium Entry (SOCE) by interacting with cytoplasmic

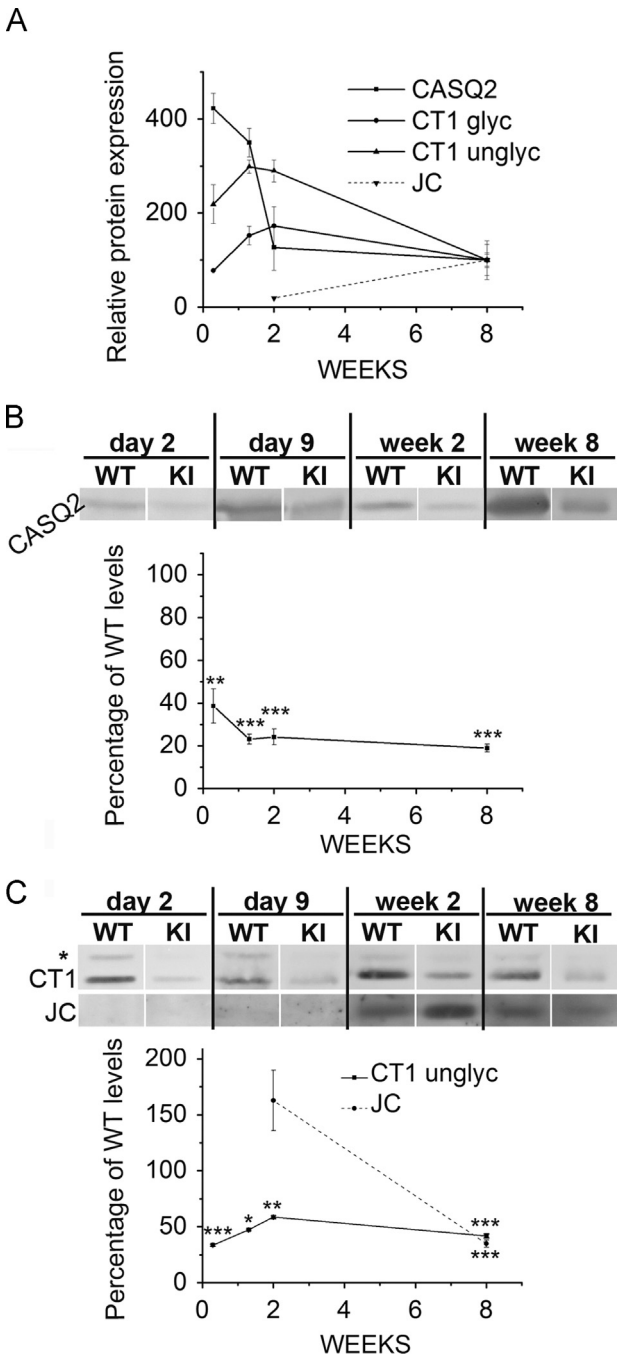

Fig. 2 – Expression of CASQ2, CT1 and JC in WT and KI hearts at different ages. Average percentages in WT hearts (A) of CASQ2 (solid line, square symbol), glycosylated (solid line, circle symbol) and un-glycosylated (solid line, triangle symbol) CT1 and JC (dashed line) are plotted versus age and are given as mean ± SE for $n=4$. In (B) and (C), average percentages in KI hearts of CASQ2-R33Q (B), un-glycosylated CT1 (solid) and JC (dashed) (C) are plotted versus age and are given as mean ± SE for $n=4$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$. Blots are representative images of each group and each experiment was repeated at least thrice.

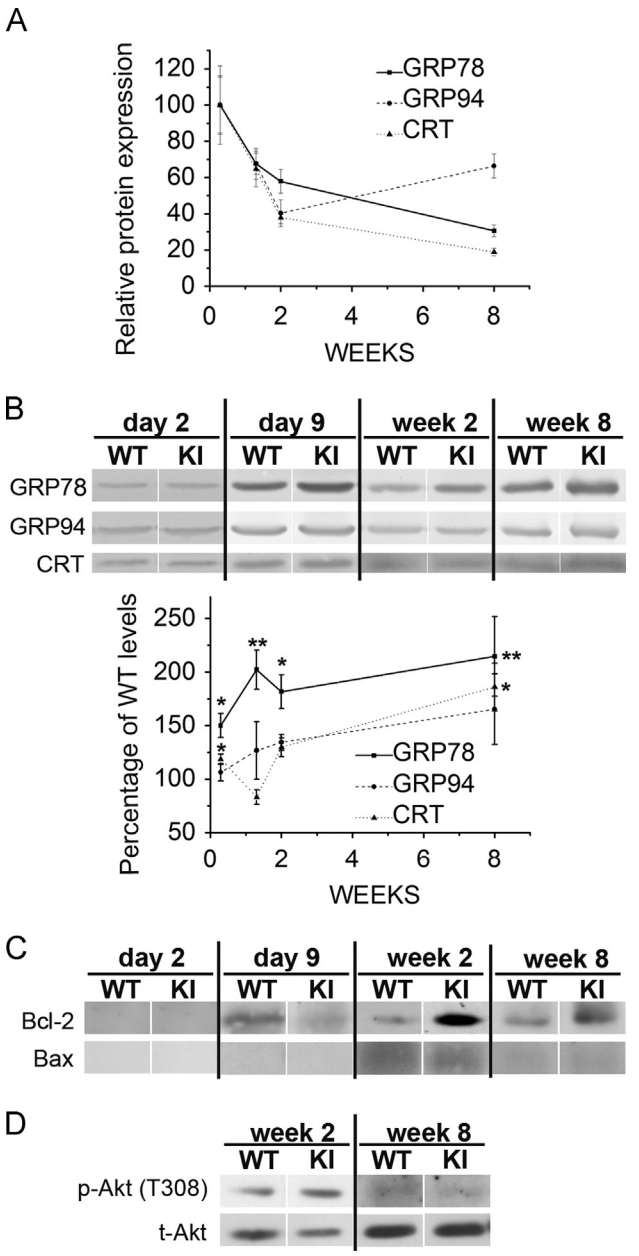

Fig. 3 – ER stress markers were monitored at different ages in hearts from WT and KI mice. Average percentages in WT (A) and in KI (B) of GRP78 (solid line), GRP94 (dashed line) and CRT (dotted line) are given as mean ± SE for $n=4$. $^{*}P<0.05$ and $^{**}P<0.01$. Activation of pro-survival factors (C) was studied by comparing the Bcl-2/Bax ratio at different ages in WT and KI mice. Dependence of Bcl-2 activation from Akt pathway (D) was inferred from phosphorylation of Akt at residue Thr308 (T308) versus total Akt (t-Akt). Blots are representative images of each group, and each experiment was repeated at least thrice.

components of plasma membrane Ca^{2+} channels such as Orai1 and/or TRPCs [31,32]. In this context, we investigated Orai1 and two members of the TRPC family, TRPC3 and TRPC6, whose expression was previously ascertained in mammalian hearts [33,34]. At post-natal week 2, no differences were observed in Orai1 and TRPC6 expression between WT and KI, whereas TRPC3 was hardly detectable in both WT and KI hearts. At post-natal week 8, instead, Orai1 was drastically reduced whereas TRPC3

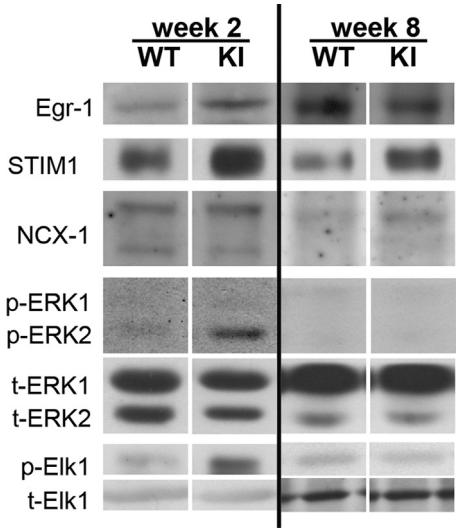

Fig. 4 – Activation of Egr-1, of its dependent transducer proteins, STIM1 and NCX-1, and of ERK1/2 pathways were studied in hearts from WT and KI mice at post-natal week 2 (left) and post-natal week 8 (right). Blots are representative images of each group, and each experiment was repeated at least thrice.

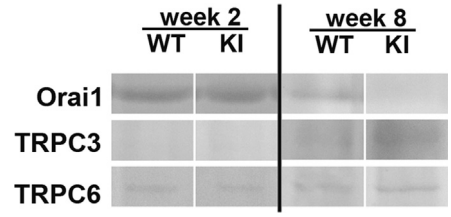

Fig. 5 – Expression of SOCE related Ca^{2+} channels, Orai1, TRPC3 and TRPC6 were studied in hearts from WT and KI mice at post-natal week 2 (left) and post-natal week 8 (right). Blots are representative images of each group, and each experiment was repeated at least thrice.

and TRPC6 were up-regulated in KI hearts (Fig. 5).Thus, in relation to very low levels of CASQ2-R33Q, a complex network of intracellular pathways is activated at the critical turning point of post-natal week 2 allowing adaptation and change of important Ca^{2+} handling proteins.

The mere lack of CASQ2 does not appear to cause adaptive responses which are instead due to expression and removal of the mutant CASQ2-R33Q

Biochemical changes and signs of ultra-structural modification in hearts of adult CASQ2$^{-/-}$ mice, i.e., up-regulation of CRT, disappearance of the chain-like polymers and widening of the jSR lumen, have been previously reported [3,10]. As shown in Table 1, widening of both PCs and dyads was detected in 2-day and 2-week old KO mice. Thus, the next question was whether CASQ2 ablation evoked adaptive, post-natal changes resembling those described in KI mice. In hearts of KO mice, CT1s and JC were hardly detectable until post-natal week 2; at week 2, un-glycosylated CT1 was very low, whereas JC was slightly up-regulated; both CT1 and JC were subsequently drastically reduced (Fig. 6A). As to ER stress, no up-regulation of GRP78 and GRP94 was observed in 2 week- and 8 week-old KO mice (Fig. 6B). On the contrary, up-regulation of CRT from post-natal week 2 onward was detected and attained high levels at post-natal week 8 (Fig. 6B). Finally, up-regulation of Bcl-2 and of STIM1 was observed only at post-natal week 8, for the latter without up-regulation of Egr-1 (Fig. 6C). As to Orai1 channels, a drastic reduction was observed at post-natal weeks 2 and 8 (Fig. 6C). As to TRPC channels, only at post-natal week 8 there was up-regulation of both TRPC3 and TRPC6 in KO hearts (Fig. 6C).

Thus, the bulk of adaptive changes outlined for KI mice **until** post-natal week 2 appears to be due exclusively to expression and removal of CASQ2-R33Q (*mechanism 1*). In particular, up-regulation of GRP78 **before** and up-regulation of GRP78 and Bcl-2 **after** post-natal week 2 indicates that specific pathways of ER stress are continuously activated by the presence of CASQ2-R33Q. On the other hand, in KO mice, delayed and sustained CRT increment, **after** post-natal week 2, and delayed up-regulation of Bcl-2, at post-natal week 8 (Fig. 6), indicate that different etiological factors are involved (*mechanism 2*): as depicted in Fig. 7, disruption of ER Ca^{2+} homeostasis, particularly intraluminal SR Ca^{2+}depletion, caused by either ablation or drastic reduction of CASQ2, is sufficient to evoke and sustain a sizable ER stress response, whose marker might be the CRT increase [18]. Thus, in CASQ2$^{R33Q/R33Q}$ mice, mechanisms 1 and 2 are activated in series and from post-natal week 2 appear to be simultaneously operational in order to maintain ER homeostasis.

Discussion

Development, transition and establishment of the adapting and adapted phenotypes in the CASQ2$^{R33Q/R33Q}$ mouse [9], one of the recessive CPVT experimental models, are monitored from post-natal day 2 onward: a detailed ultra-structural and molecular analysis of the recessive CPVT cardiac phenotype is reported here for the first time in order to determine the onset of adaptive changes that might either lead or accompany the key pathogenetic event of CPVT, i.e., diastolic Ca^{2+} release from SR [3,9].

Mutant CASQ2-R33Q protein is drastically reduced right after birth and is kept at very low levels, i.e., ~20% of WT amounts, throughout adulthood. Recent studies have confirmed that loss of one constituents of the quaternary Ca^{2+} release channel complex may not only affect the expression of other constituents but also derange the functional and structural integrity of the quaternary complex [35]. In CASQ2$^{R33Q/R33Q}$ mice, such a paradigm is observed as early as post-natal week 2. At post-natal day 2, CRUs-only in the form of PCs- are not significantly different in WT and KI hearts: jSR width is similar and still contains electron-dense material as a chain-like polymer, whereas PCs are dilated as early as post-natal day 2 in KO mice. At week 2, on the other hand, changes are evident in the newly formed internal dyads of KI hearts: jSR is wider in size and mostly empty, as shown in adult specimens [9]. Moreover, at the protein level, reduced expression of CASQ2-R33Q is accompanied by a drastic reduction of un-glycosylated CT1 and virtual absence of glycosylated CT1, from post-natal day 2 onward. It has been shown that synthesis and/or stability of CT1 is CASQ2-dependent [2], that post-translational glycosylation of CT1 regulates its expression and trafficking and

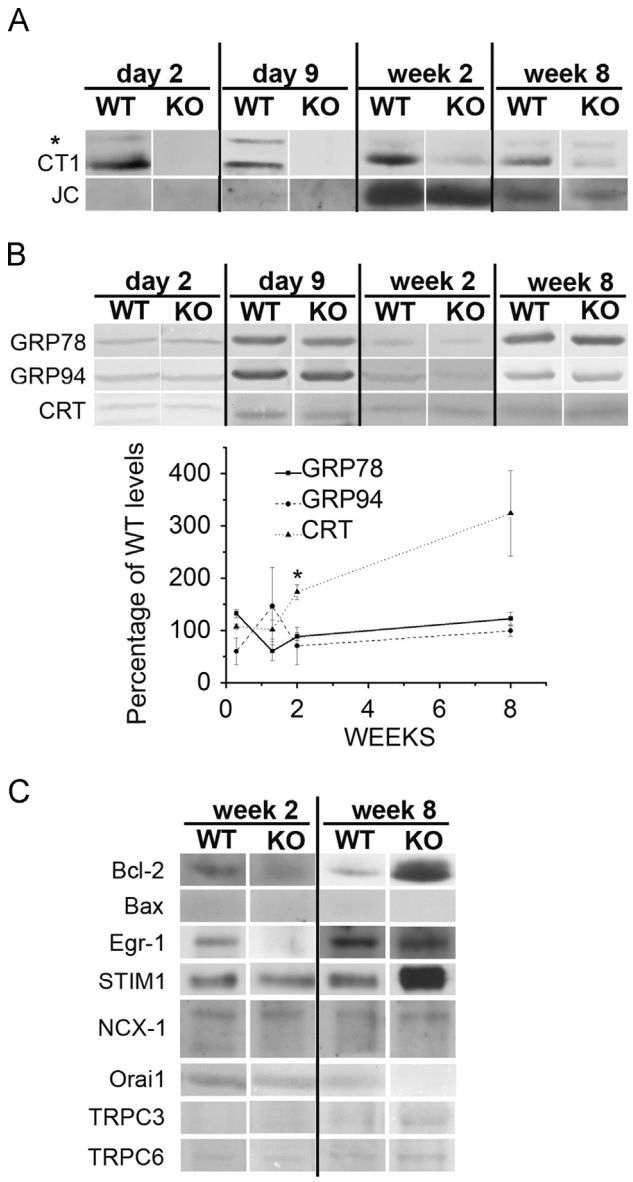

Fig. 6 – Characterization of CASQ2$^{-/-}$ hearts at different ages entailed analysis of expression of (A) glycosylated (asterisk) and un-glycosylated CT1 and JC, (B) ER stress-related proteins, GRP78 (solid), GRP94 (dashed) and CRT (dotted). Average percentages are given as mean ± SE for $n=4$. *$P<0.05$. In (C), expression of Bcl-2, Bax, Egr-1, STIM1, NCX-1, Orai1, TRPC3 and TRPC6 was studied in WT and KO mice at post-natal week 2 (left) and post-natal week 8 (right). Blots are representative images of each group, and each experiment was repeated at least thrice.

that un-glycosylated CT1 is more prone to active degradation relative to the more stable glycosylated form [36]. Thus, expression of CASQ2-R33Q prevents CT1 from attaining suitable levels and stable conformation. The importance of the CT1/CASQ2 ratio for SR Ca^{2+} handling in ventricular myocytes has been recently addressed [37]; during post-natal differentiation, the CT1/CASQ2-R33Q ratio changes with an excess of the un-glycosylated CT1 in adulthood, although both the proteins are reduced. Since the un-glycosylated CT1/CASQ2 interaction is Ca^{2+}-dependent and the Ca^{2+}-sensitivity of the un-glycosylated CT1/CASQ2-R33Q interaction is abnormal determining altered control of RYR2 open probability [38], the present data further explain the altered SR Ca^{2+} release in adult KI hearts [9,39]. As to JC, another component of the quaternary complex, we observed its reduction in CASQ2$^{R33Q/R33Q}$ hearts only in adulthood, in agreement with previous results [9], whereas at post-natal week 2 it was found to be up-regulated. In JC$^{-/-}$ mice, JC ablation did not affect the expression of other key SR proteins, such as CASQ2 and CT1 [40], whereas its expression was down-regulated in the absence of either CT1 or CASQ2 [9,35]. These data indicate that JC expression is not directly regulated by either reduction or absence of CASQ2, as it happens for CT1, but it is controlled by distinct and secondary mechanisms.

Since mRNA for CASQ2-R33Q was unchanged in CASQ2$^{R33Q/R33Q}$ mice, protein disposal occurs via post-translation mechanisms whose molecular details are yet to be unveiled. The R33Q mutation on CASQ2 alters both the conformational flexibility [17,41] and the functionality of the protein [42], so mutant CASQ2-R33Q might affect not only intra-luminal SR Ca^{2+} concentration but also protein folding equilibrium. It is well known that alterations in intra-luminal Ca^{2+} concentration and ER accumulation of unfolded proteins elicit cellular stress responses, particularly ER stress signals, in order to restore ER homeostasis. The ubiquitous cellular response is the up-regulation of a class of ER stress proteins among which GRP78, GRP94 and CRT, whose high levels are indicative of ER stress [16]. In CASQ2$^{R33Q/R33Q}$ mice, GRP78 was strongly up-regulated as of post-natal day 2, in temporal coincidence with reduction of CASQ2-R33Q, and remained increased thereafter. CRT increase was milder, delayed but detectable at post-natal week 8. From birth into adulthood, thus, ER stress is activated in KI hearts.

ER stress is well understood in non-muscle ER but has not been fully elucidated in heart [18]. It is always an adaptive response but, if unresolved, can lead to apoptosis; the ER is a critical organelle in controlling cell life and death through a wide array of either adaptive/pro-survival or pro-apoptotic pathways. In CASQ2$^{R33Q/R33Q}$ mice, the well-known anti-apoptotic factor Bcl-2 was up-regulated through activation of the Akt pathway only at post-natal week 2 whereas at post-natal week 8 it was not, thus suggesting a different regulation. Notwithstanding persistent ER stress, the present data indicate that KI cardiomyocytes are effectively protected by Bcl-2 up-regulation and pro-apoptotic pathways are not activated throughout adulthood. It is also intriguing that Bcl-2 has been reported to repress apoptosis by inhibiting ER Ca^{2+} release [43]; moreover, ER-localized Bcl-2 lowers the steady-state level of intra-ER Ca^{2+}, possibly by modulating a passive Ca^{2+} leak, and thereby reduces stimulus-induced ER Ca^{2+} fluxes [44]. Thus, Bcl-2 might also contribute to counteract RYR2-mediated diastolic Ca^{2+} release propaedeutic to triggered activity (TA) and VT, at least between post-natal week 2 and week 8. The latter is a mere speculation amenable, however, of future, direct experimentation.

In some pathological conditions [18], ER stress triggers the fetal gene program which favors cardiac remodeling and, in some cases, leads to hypertrophy. At the molecular level, in fact, many fetal genes contain promoter elements that can be transcriptionally activated by transducers of ER stress [18]. This appears to be the case of the CASQ2$^{R33Q/R33Q}$ phenotype, at least with respect to Akt and ERK1/2, established pro-hypertrophic transducers, and

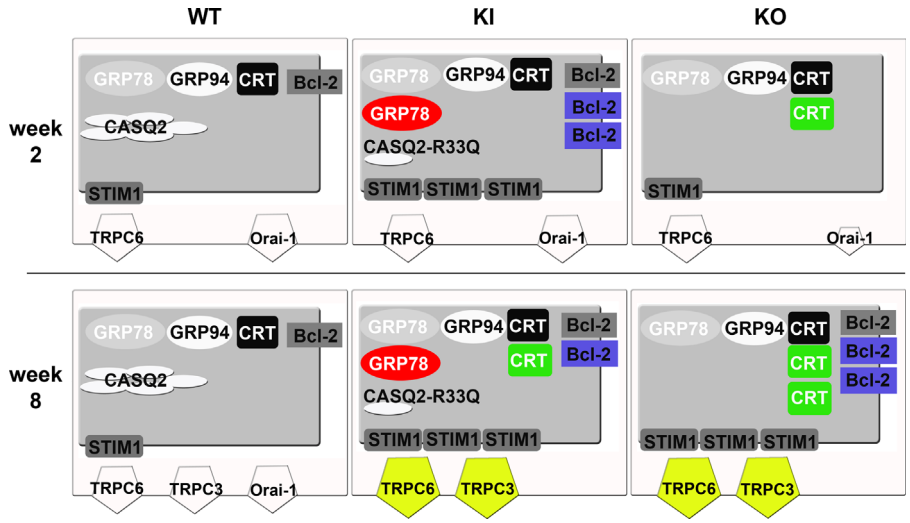

Fig. 7 – Schematic representation of SR/ER at post-natal week 2 and post-natal week 8. ER stress is highlighted by incremental content of GRP78 (red), CRT (green) and Bcl-2 (blue). Adaptation is highlighted by the increased content of STIM1 and of TRPC3 and TRPC6 in plasma membrane (yellow). Note temporal, qualitative and quantitative differences in the adapting phenotypes of KI and KO mice.

ANF (data not shown) that were up-regulated at post-natal week 2. Since pro-hypertrophic transducers were no longer activated at post-natal week 8 (Figs. 3 and 4 for Akt and ERK1/2, respectively; data not shown for ANF) and no signs of hypertrophy were detected during post-natal development and adulthood [9], activation of fetal genes appears to be transient and unrelated to hypertrophy. Interestingly, re-expression of CRT has been shown to attenuate hypertrophic responses [45]: in both CPVT models, thus, increased levels of CRT might exert an additional, long-term, cardio-protective effect.

Egr-1, one of the immediate early genes up-regulated by ER stress [24], has been reported to regulate genes controlling cardiac Ca^{2+} homeostasis, with a positive feedback on STIM1 [25] and a negative feedback on CASQ2 [26] and NCX-1 [27]. An increase of the intracellular Ca^{2+} concentration is often the prerequisite for enhanced Egr-1 gene transcription [46]. In $CASQ2^{R33Q/R33Q}$ mice, Egr-1 was up-regulated at post-natal week 2, NCX-1 was unchanged and no effect on mRNA for CASQ2-R33Q could be ascribed to Egr-1 up-regulation. On the contrary, activation of Egr-1 was paralleled by activation of STIM1 at post-natal week 2, whereas STIM1 appeared to be up-regulated in the absence of Egr-1 activation at post-natal week 8. Once again, we observed that expression of the same Ca^{2+}-related protein displayed a different age-dependent upstream regulation.

SOCE is an ubiquitous mechanism linking ER/SR Ca^{2+} depletion to activation of plasma membrane Ca^{2+} channels. Ca^{2+} influx via SOCE promotes refilling of depleted ER/SR Ca^{2+} stores and generation of long lasting increases of cytoplasmic Ca^{2+}. SOCE has been described both in neonatal [47] and in adult cardiomyocytes [48] and correlates with STIM1 [47], which acts as sensor for Ca^{2+} level in ER/SR and coupler between Ca^{2+} store depletion and activation of SOCE channels. SOCE channels might be formed solely by Orai1 [49,50] or by both Orai1 and TRPCs. Current evidence indicates that STIM1, Orai1 and TRPCs dynamically interact forming a ternary complex that mediates SOCE in a number of different cells [51]. The SR is functional in neonatal cardiomyocytes but cytoplasmic Ca^{2+} transients are independent of SR Ca^{2+} release, i.e., Ca^{2+} transients largely rely upon influx of extracellular Ca^{2+} [52] until T-tubules–SR junctions achieve full development. Interestingly, there is an evidence that ER stress plays a role in SOCE regulation: stress-triggered STIM1-re-expression and consequent SOCE activation seem to be critical elements in the upstream, Ca^{2+} dependent control of cardiac hypertrophy [47]. Egr-1-dependent up-regulation of STIM1, around post-natal week 2, appears to be the part of the complex adapting phenotype of $CASQ2^{R33Q/R33Q}$ mice and might have effects both on intracellular Ca^{2+} homeostasis and on SR development via SOCE. The SR Ca^{2+} content of adult $CASQ2^{R33Q/R33Q}$ cardiomyocytes was reported to be decreased by 35% as compared to that of WT cardiomyocytes [9], i.e., the intraluminal SR milieu should favor a compensatory SOCE activation. Thus, persistent Egr-1-independent up-regulation of STIM1 in adult cardiomyocytes, i.e., when L-type Ca^{2+} channels take control over EC coupling and SR Ca^{2+} release becomes essential for EC coupling [53], might not only determine a prolonged increase of cytoplasmic Ca^{2+} concentration, but also step up intra-luminal SR Ca^{2+}.

Both *in vitro* studies and experiments in transgenic and KO mice have suggested that TRPC3 and TPRC6 may assemble to form receptor-activated cation channels. Such TRPC-dependent pathways may have a central role in the development of cardiac hypertrophy and/or arrhythmias [54,55]. At post-natal week 8, i.e., in the adult stage of KI hearts, Orai1 was down-regulated whereas both TRPC3 and TRPC6 were up-regulated (Fig. 5). Thus, STIM1-TRPC-dependent SOCE might predispose to diastolic Ca^{2+} release and be pathogenetically relevant in CPVT. The latter is a mere speculation amenable, however, of future, direct experimentation.

The study of the $CASQ2^{-/-}$ mouse [2,3,10] as well as of several KI mice carrying different CASQ2 mutations [9,56], highlighted that both absence and reduction of CASQ2 evoke a similar phenotype in CPVT, relying upon the same cellular pathogenesis for TA. On the other hand, the $CASQ2^{-/-}$ mouse is adapting quite differently up to post-natal week 2 (Table 1 and Figs. 6 and 7): widening of PCs as early as post-natal day 2, no up-regulation of GRP78, no Egr-1-dependent up-regulation of STIM1, down-regulation of Orai1 and initial up-regulation of CRT. In the

adapted, adult phenotype, ER stress entails medium levels of CRT and high levels of GRP78 in KI mice, but only high levels of CRT in KO mice. Bcl-2 up-regulation, Egr-1-independent up-regulation of STIM1 and TRPC3/6 up-regulation at post-natal week 8 appear to be, instead, features shared by both models of recessive CPVT

Persistent disposal of CASQ2-R33Q and reduction of CT1 and JC are the primary pathogenetic events determining derangements of SR Ca^{2+} release, on the one hand, and evoking multiple, post-natal adaptive changes, on the other: such changes appear to ensure in the adult, adapted mouse a new, long-lasting patho-physiologic equilibrium. Up to post-natal week 2, $CASQ2^{R33Q/R33Q}$ mice, because of the specific pattern of ER stress response, accommodate to mutant CASQ2 differently than adult mice. In the case of $CASQ2^{-/-}$ mice, ER stress might be mainly due to alteration of intra-SR Ca^{2+}, either Ca^{2+} depletion or reduced luminal Ca^{2+} buffer capacity [57]. By comparing the adapted phenotypes of $CASQ2^{R33Q/R33Q}$ and $CASQ2^{-/-}$ mice (Fig. 7), it is tempting to suggest that two ER stress mechanisms are operational: mechanism 1 dependent upon disposal of CASQ2-R33Q from post-natal day 2 onward, mechanism 2 dependent upon SR Ca^{2+} depletion from post-natal week 2 onward. By and large, increased cytosolic Ca^{2+} concentration, reduced Ca^{2+} SR content and CASQ2-R33Q disposal appear to be concomitant and co-operative mechanisms for adaptation in $CASQ2^{R33Q/R33Q}$ mice.

Both $CASQ2^{-/-}$ mice, despite a different and delayed pattern of biochemical adaptation, and $CASQ2^{R33Q/R33Q}$ mice, with a biphasic pattern of adaptation, end up to display the same deranged mechanism of SR Ca^{2+} release in adulthood, i.e., reduced refractoriness of RYR2 leading to VT [8,9,39]. It is not known when VT first manifest and if there is an asymptomatic window in the experimental models of recessive CPVT. Additional work is required to further dissect molecular pathways of the adapting phenotype in order to (a) assess whether a completely compensated phenotype occurs in early ages—in temporal coincidence with SR Ca^{2+}release incompetence to support Ca^{2+} transients, (b) identify factors determining the transition, if any, from the compensated phenotype to the decompensated phenotype displaying VT episodes, and (c) determine the involvement, if any, of ER stress responses and SOCE in such a transition. Moreover, it is not known whether asymptomatic young human patients carrying one of the homozygous CASQ2 mutations undergo a transition from complete compensation without VT to relative compensation with VT episodes.

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