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# Alternative Splicing of Ryanodine Receptors Modulates Cardiomyocyte $\text{Ca}^{2+}$ Signaling and Susceptibility to Apoptosis

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**Abstract**— $\text{Ca}^{2+}$  release via type 2 ryanodine receptors (RyR2) regulates cardiac function. Molecular cloning of human RyR2 identified 2 alternatively spliced variants, comprising 30- and 24-bp sequence insertions; yet their role in shaping cardiomyocyte  $\text{Ca}^{2+}$  signaling and cell phenotype is unknown. We profiled the developmental regulation and the tissue and species specificity of these variants and showed that their recombinant expression in HL-1 cardiomyocytes profoundly modulated nuclear and cytoplasmic  $\text{Ca}^{2+}$  release. All splice variants localized to the sarcoplasmic reticulum, perinuclear Golgi apparatus, and to finger-like invaginations of the nuclear envelope (nucleoplasmic reticulum). Strikingly, the 24-bp splice insertion that was present at low levels in embryonic and adult hearts was essential for targeting RyR2 to an intranuclear Golgi apparatus and promoted the intracellular segregation of this variant. The amplitude variability of nuclear and cytoplasmic  $\text{Ca}^{2+}$  fluxes were reduced in nonstimulated cardiomyocytes expressing both 30- and 24-bp splice variants and were associated with lower basal levels of apoptosis. Expression of RyR2 containing the 24-bp insertion also suppressed intracellular  $\text{Ca}^{2+}$  fluxes following prolonged caffeine exposure (1 mmol/L, 16 hours) that protected cells from apoptosis. The antiapoptotic effects of this variant were linked to increased levels of Bcl-2 phosphorylation. In contrast, RyR2 containing the 30-bp insertion, which was abundant in human embryonic heart but was decreased during cardiac development, did not protect cardiomyocytes from caffeine-evoked apoptosis. Thus, we provide the first evidence that RyR2 splice variants exquisitely modulate intracellular  $\text{Ca}^{2+}$  signaling and are key determinants of cardiomyocyte apoptotic susceptibility. (*Circ Res*. 2007;100:874-883.)

**Key Words:** ryanodine receptor ■  $\text{Ca}^{2+}$  ■ alternative splicing ■ cardiomyocyte ■ apoptosis

Alternative splicing of pre-mRNA transcripts is a powerful mechanism to generate remarkable protein diversity from single genes and is a prevalent feature of human gene processing. The plasticity of multiprotein signaling networks is greatly augmented by the regulated expression of distinct protein variants that expand the cellular signaling repertoire. The spatiotemporal encoding of intracellular  $\text{Ca}^{2+}$  signaling is exquisitely modulated by alternative splicing of phosphodiesterases, plasma membrane  $\text{Ca}^{2+}$ -ATPases, Trp channels, L-type  $\text{Ca}^{2+}$  channels, inositol 1,4,5-trisphosphate receptors, sodium calcium exchangers, sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER)  $\text{Ca}^{2+}$ -ATPases (SERCAs), and  $\text{Ca}^{2+}$ /CaM-dependent protein kinase II, according to cell type and developmental stage, and in response to neurohumoral stimuli, mechanical forces, and cellular stress.

Ryanodine receptors (RyRs) are enormous tetrameric channels that transduce an array of cellular inputs to elicit synchronous  $\text{Ca}^{2+}$  release from the SR to initiate muscle contraction and regulate numerous processes.<sup>1,2</sup> The functional diversity of RyRs is reflected in the structural organization of the polypeptide as a series of contiguous functional domains.<sup>3</sup> The human genes encoding RyR isoforms are some of the largest and most complex in the genome, comprising >100 exons. To date, 13 distinct splice variants have been identified in RyR isoforms cloned from mammalian and insect species (see Figure 1A and the online data supplement [Table I] at <http://circres.ahajournals.org>), reflecting the evolutionary importance of alternative splicing in RyR2 modulation.  $\text{Ca}^{2+}$  release channels formed from RyR1 subunits containing a devel-

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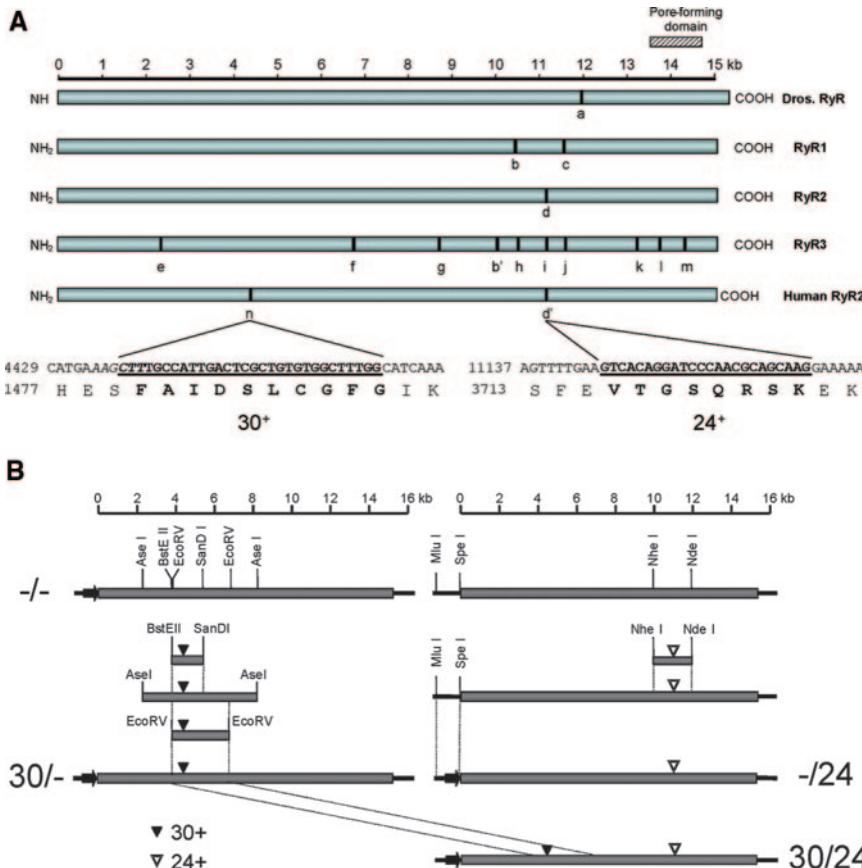
From the Department of Cardiology (C.H.G., S.A.R., B.M.A.B., N.L.T., E.V.C., C.J.H., F.A.L.), Wales Heart Research Institute, Cardiff University, UK; Department of Physiology (R.E.A.T.), University College London, UK; Institute of Membrane and Systems Biology (D.S.S.), University of Leeds, UK; Department of Haematology (C.P.), Cardiff University School of Medicine, UK; and Department of Biochemistry (W.C.C.), Louisiana State University, New Orleans.

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**Figure 1.** Construction of human RyR2 containing 30-bp and 24-bp splice insertions. A, Thirteen RyR splice variants have been identified in human, rabbit, mouse, mink, dog, and *Drosophila* (see supplemental Table I). RyR1 and RyR3 variants b and b', respectively, are equivalent. The 24-bp insertion in human RyR2 (d') is identical to that in the rabbit and canine RyR2 sequences. B, Construction of eGFP-tagged RyR2 containing 30-bp (RyR2<sup>30/-</sup>), 24-bp (RyR2<sup>-/24</sup>), or both (RyR2<sup>30/24</sup>) splice insertions. 224 indicates eGFP.

developmentally regulated, disease-linked splice insertion exhibited distinct  $\text{Ca}^{2+}$ -releasing profiles in skeletal myotubes,<sup>4</sup> and a smooth-muscle-specific deletion in RyR3 that did not form functional homotetrameric channels, inhibited  $\text{Ca}^{2+}$  release when expressed in heteromeric combination with RyR isoforms that lacked insertions<sup>5,6</sup> (Figure 1A and supplemental Table I, variants b and k, respectively).

Two alternatively spliced variants of the human cardiac RyR channel (RyR2) corresponding to a 30-bp insertion (encoding FAIDSLCGFG) and a 24-bp insertion (encoding VTGSQRSK) (variants n and d', respectively; Figure 1A and supplemental Table I) have been identified.<sup>7</sup> However, their roles in cardiac development and cell signaling have remained a mystery. Here, we present the first functional characterization of human RyR2 splice variants and show that cellular architecture and  $\text{Ca}^{2+}$  signaling are profoundly modulated following their expression in HL-1 cardiomyocytes. Our data highlight an important role for the 24-bp insertion in protecting cardiomyocytes from stimulation-induced apoptosis and thus identify RyR2 alternative splicing as a key determinant of cardiomyocyte susceptibility to apoptosis. Furthermore, we show that the expression profiles of RyR2 splice variants exhibits distinct species-, tissue-, and developmentally specific regulation, and we evaluate the implications of our findings in the context of cardiac development and future therapeutic approaches to cardiac disease.

## Materials and Methods

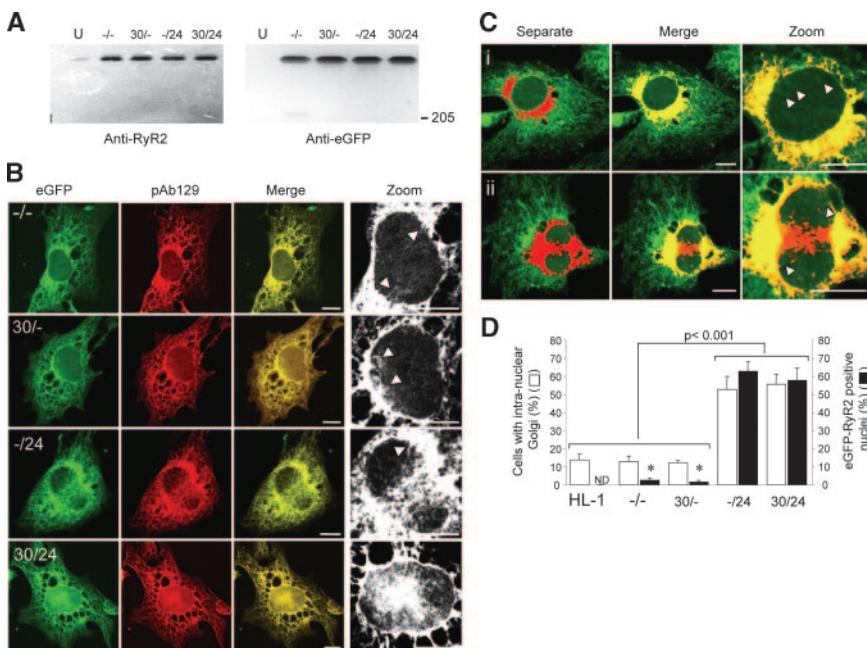
### Construction, Expression, and Detection of Human RyR2 Splice Variants

The recombinant human RyR2 routinely expressed in our laboratory lacks the 30-bp and 24-bp splice insertions and is subsequently referred to as RyR2<sup>-/-</sup>. The construction of enhanced green fluorescence protein (eGFP)-tagged RyR2 containing the 30-bp exon (RyR2<sup>30/-</sup>), 24-bp exon (RyR2<sup>-/24</sup>), or both insertions (RyR2<sup>30/24</sup>) is shown in Figure 1B and the online data supplement. N-terminal tagging of RyR2<sup>-/-</sup> with *Discosoma* sp red fluorescent protein (DsRed) was performed as described.<sup>8</sup>

HL-1 cardiomyocytes were cultured and transfected with recombinant RyR2 as described.<sup>8</sup> Cells expressing eGFP-tagged RyR2 were selected by G418-sulfate resistance (500  $\mu\text{g}/\text{mL}$ ) and were isolated using fluorescent-activated cell sorting (FACS) based on eGFP intensity.<sup>9</sup> FACS-enriched cells were used as the background for DsRed-tagged RyR2<sup>-/-</sup> expression. The intracellular localization of recombinant RyR2 was visualized using eGFP or DsRed fluorescence,<sup>10</sup> and pAb129 was used for immunodetection of endogenous and recombinant RyR2.<sup>8</sup> Antibodies to eGFP, DsRed, calreticulin (an ER marker), and a Golgi matrix protein (GM130) are described in the online data supplement. Profiling the species, tissue, and developmental aspects of RyR2 splice variant expression are described in the online data supplement.

### $\text{Ca}^{2+}$ Imaging and Signal/Noise Analysis

$\text{Ca}^{2+}$  release triggered by the acute addition of caffeine (10 mmol/L) or following tonic RyR2 activation by prolonged exposure to caffeine (1 mmol/L, 16 hours) was determined in single fluo3-loaded cells using confocal microscopy (RS2, Leica Microsystems).<sup>11</sup> Analysis of the amplitude and temporal variability in resting and caffeine-activated  $\text{Ca}^{2+}$  fluxes in nuclear and cytoplasmic regions



**Figure 2.** Intracellular segregation of RyR2 splice variants in HL-1 cardiomyocytes. A, Immunoblot analysis of total cell lysates (200 µg) obtained from FACS-enriched HL-1 cardiomyocytes expressing RyR2 splice variants or from nontransfected control cells (U). We determined equivalent relative expression of RyR2<sup>-/-</sup>, 3.84±0.28; RyR2<sup>30/-</sup>, 4.31±0.47; RyR2<sup>-/24</sup>, 3.61±0.52; RyR2<sup>30/24</sup>, 4.07±0.61 when compared with endogenous RyR2 expression assigned 1.0, based on densitometric analysis of 3 separate FACS enrichments. B, The intracellular localization of RyR2 splice variants was visualized using endogenous eGFP fluorescence (green) or pAb129 immunodetection (red). Coincident signals appear yellow (Merge). Bar represents 10 µm. C, HL-1 cardiomyocytes stained with antibodies against ER (calreticulin, green) and GA (GM130, red) demonstrated 2 distinct patterns of GA architecture (i and ii), comprising 88.7% and 11.3% of the total cell population, respectively. Coincident pixels appear yellow (Merge). Arrowheads indicate NR that is distinct

from the transnuclear distribution characteristic of INGA (ii). Bar represents 10 µm. D, The presence of INGA was determined by GM130 staining (white bars), and the localization of RyR2 splice variants was visualized by eGFP fluorescence (black bars). Data are given as means±SEM (n=4, >300 cells per experiment). \*P<0.005 within grouping. ND indicates not determined.

was performed as described (see the expanded Materials and Methods section in the online data supplement).<sup>9,11</sup>

### Determination of Cell Viability and Mode of Cell Death

Cell viability, and the extent of apoptosis versus necrosis (where "necrosis" is used to describe a mode of cell death that is distinct from apoptosis<sup>12</sup>), was determined.<sup>11</sup>

### Transcriptional and Protein Expression Analysis of Bcl-2

The levels of Bcl-2 mRNA transcripts in untreated cells or following their prolonged exposure to caffeine (1 mmol/L, 16 hours) were determined using quantitative PCR. The expression levels and phosphorylation status of the endogenous Bcl-2 protein was determined by immunoblotting. Detailed methods are included in the online data supplement.

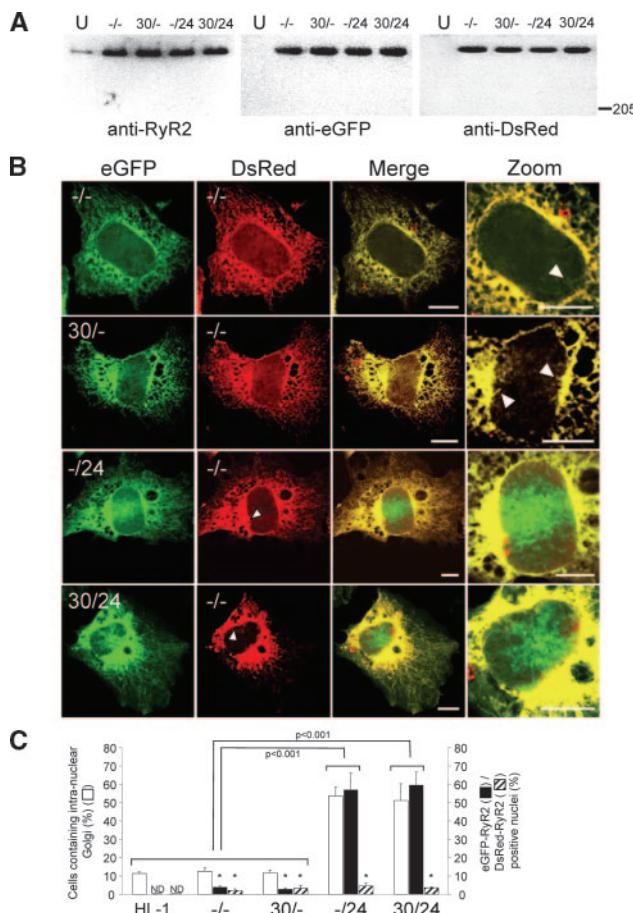
## Results

### Alternative Splicing Determines the Cellular Localization of RyR2

Recombinant eGFP-tagged RyR2 splice variants were overexpressed to equivalent levels in FACS-enriched HL-1 cardiomyocytes (Figure 2A). Coincident fluorescent signals from N-terminal eGFP and C-terminal pAb129 immunodetection confirmed the expression of full-length recombinant RyR2. All RyR2 splice variants were localized to an extensive lattice-like network consistent with ER, the nucleoplasmic reticulum (NR) (finger-like invaginations of the nuclear envelope [NE]),<sup>13,14</sup> and a perinuclear accumulation characteristic of the Golgi apparatus (GA) (Figure 2B). The localization of recombinant RyR2 to these compartments was indistinguishable for the cellular distribution of endogenous RyR2 (supplemental Figure I). The localization of RyR2 splice variants to these compartments was confirmed by antibody mapping of ER and GA (using compartment-specific markers calreticulin and GM130, respectively), although the extensive colocalization of GA and ER in the perinuclear region of HL-1 cardiomyocytes precluded the unambiguous identification of all RyR2 splice variants to perinuclear GA (Figure 2Ci). However, we identified a subpopulation of HL-1 cells (comprising ≈11% of the total cell population) that exhibited a remarkably distinct intranuclear GA organization (INGA) that accounted for 12.1±4.3% of the total cellular GA content (Figure 2Cii). The different GA architecture in HL-1 populations did not affect cellular morphology (Figure 3), size (760±92 µm<sup>2</sup> versus 830±118 µm<sup>2</sup>), viability (89±10% versus 86±15%), or nuclear size (132±24 µm<sup>2</sup> versus 153±27 µm<sup>2</sup>), where data refer to INGA-negative versus INGA-positive HL-1 populations, respectively.

Recombinant RyR2 containing the 24-bp insertion (RyR2<sup>-/24</sup> and RyR2<sup>30/24</sup>) exhibited striking intranuclear distribution, localizing to fibrillar-like structures that were distinct from the NR, yet were indistinguishable from the INGA morphology identified using anti-GA antibodies (Figure 2B and 2C). Analysis of fluorescent signal intensity revealed that INGA-localized RyR2<sup>-/+</sup> and RyR2<sup>+/+</sup> accounted for 9.3±3.6% and 11.4±4.6% of the total cellular content of recombinant RyR2, respectively, entirely consistent with the proportion of cellular GA that comprises INGA (11.3%). Costaining of GA and RyR2 splice variants revealed that INGA localization was restricted to RyR2<sup>-/24</sup> and RyR2<sup>30/24</sup> and that the number INGA-positive cells was increased by more than 4-fold following RyR2<sup>-/24</sup> and RyR2<sup>30/24</sup> expression (Figure 2D). Analysis of discrete FACS-isolated cell populations ex-

hibited a significant increase in the percentage of INGA-positive cells (Figure 2D). Recombinant RyR2<sup>-/24</sup> and RyR2<sup>30/24</sup> expressed at similar levels (Figure 2A) yet exhibited distinct cellular distributions (Figure 2B). The difference in cellular distribution was evident in the absence of endogenous RyR2 (Figure 2B, -/-) and was independent of the presence of endogenous RyR2 (Figure 2B, 30/24). The difference in cellular distribution was evident in the absence of endogenous RyR2 (Figure 2B, -/-) and was independent of the presence of endogenous RyR2 (Figure 2B, 30/24).



**Figure 3.** Intranuclear localization of RyR2 requires a 24-bp insertion. **A**, Cell lysates (200  $\mu\text{g}$ ) from HL-1 cardiomyocytes coexpressing eGFP-tagged splice variants and DsRed-tagged RyR2 $^{-/-}$  were analyzed by immunoblotting as indicated. **B**, The intracellular localization of eGFP-tagged variants (green) and DsRed-tagged RyR2 variants (red) were determined. Coincident signals appear yellow (Merge). Arrowheads depict intranuclear structures consistent with NR. Bar represents 10  $\mu\text{m}$ . **C**, The occurrence of INGA (white bars) and the localization of eGFP-tagged RyR2 splice variants (black bars) and DsRed-tagged RyR2 $^{-/-}$  (hatched bars) were quantified. Data are derived from analysis of  $>120$  cells ( $n=6$ ). \* $P<0.005$  within grouping. ND indicates not determined.

pressing different levels of eGFP-tagged RyR2 splice variants showed that the INGA localization of RyR2 $^{-/-}$  and RyR2 $^{30/-}$  was not an artifact of cellular overexpression (supplemental Figure II). Although RyR2 $^{-/-}$  and RyR2 $^{30/-}$  were found in the NR invaginations, we did not detect these variants in INGA-like structures, suggesting that the 24-bp insertion was a key determinant of RyR2 localization to this intranuclear compartment.

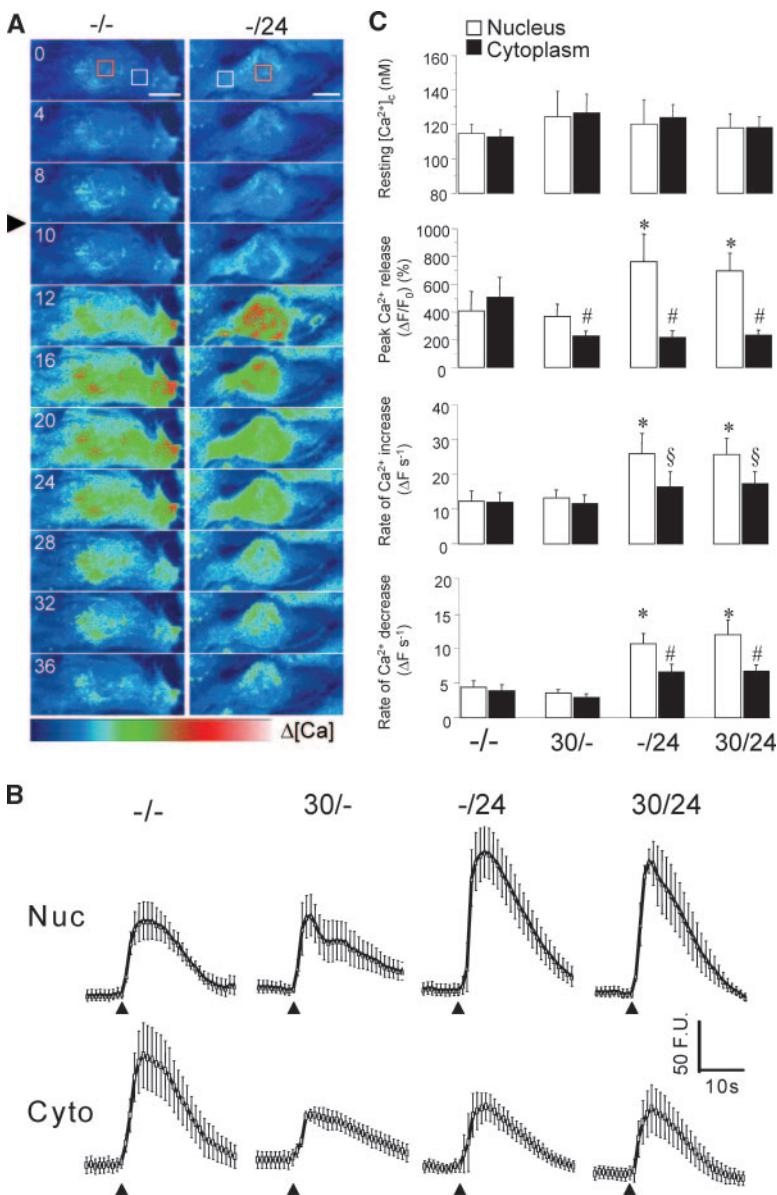
To further investigate these observations, we used the obligate tetramerization required for DsRed fluorescence.<sup>15</sup> eGFP-tagged RyR2 splice variants and DsRed-tagged RyR2 $^{-/-}$  were coexpressed to equivalent levels (Figure 3A). eGFP-tagged RyR2 $^{-/-}$  and RyR2 $^{30/-}$  extensively colocalized with DsRed-tagged RyR2 $^{-/-}$  in the ER network, NR projections, and perinuclear regions (Figure 3B, arrowheads), but we did not detect these variants in INGA. Coexpression of eGFP-tagged RyR2 $^{-/-}$  or RyR2 $^{30/24}$

with DsRed-tagged RyR2 $^{-/-}$  also resulted in significant colocalization in extranuclear and NR compartments (Figure 3B). Importantly, we determined a striking intracellular segregation of RyR2 splice variants, because INGA localization was exclusively restricted to eGFP-tagged RyR2 $^{-/-}$  and RyR2 $^{30/24}$  and there was a marked absence of homotetrameric DsRed-tagged RyR2 $^{-/-}$  in this compartment (Figure 3B and 3C, “green only” intranuclear fluorescence). Moreover, the proportion of INGA-localized eGFP-tagged RyR2 $^{-/-}$  and RyR2 $^{30/24}$  in these coexpression experiments was similar to those in which these were the only variants expressed (RyR2 $^{-/-}$ , 11.9 $\pm$ 2.8% and RyR2 $^{30/24}$ , 9.6 $\pm$ 3.7% of the total cellular recombinant protein), indicating that coexpression of DsRed-tagged RyR2 did not inhibit the INGA localization of RyR2 containing the 24-bp splice insertion (Figure 3C). These data strongly suggested that although all RyR2 splice variants exhibited limited nuclear localization by virtue of NR invaginations (Figures 2B and 3B), the 24-bp insertion was necessary for RyR2 localization in INGA, thereby conferring subcellular segregation of RyR2 variants.

### RyR2 Splice Variants Modulate Cardiomyocyte $\text{Ca}^{2+}$ Signaling and Susceptibility to Apoptosis

Using caffeine as a quasiphysiological activator of  $\text{Ca}^{2+}$ -dependent RyR2  $\text{Ca}^{2+}$  release, we investigated the role of differential cellular localization of RyR2 splice variants on cardiomyocyte  $\text{Ca}^{2+}$  signaling. Cytoplasmic and nuclear  $\text{Ca}^{2+}$  levels were comparable in nonstimulated cells expressing all RyR2 variants (Figure 4C), but only cells expressing RyR2 containing the 30-bp or 24-bp exons exhibited suppressed cytoplasmic  $\text{Ca}^{2+}$  transients in response to acute caffeine stimulation (Figure 4B and 4C), possibly reflecting reduced  $\text{Ca}^{2+}$  release from the ER and the perinuclear GA. In contrast, nuclear  $\text{Ca}^{2+}$  mobilization (comprising  $\text{Ca}^{2+}$  release from the NE, NR, and inward diffusion of  $\text{Ca}^{2+}$  from the cytosol) was markedly augmented only in RyR2 $^{-/-}$  and RyR2 $^{30/24}$  cells (Figure 4B and 4C). The amplitude of caffeine-evoked cytoplasmic and nuclear  $\text{Ca}^{2+}$  mobilization was not an index of the cellular  $\text{Ca}^{2+}$  content because the total cellular  $\text{Ca}^{2+}$  mobilization in response to thapsigargin-mediated (5  $\mu\text{mol/L}$ ) ER depletion was equivalent in each cell type (RyR2 $^{30/-}$ , 104 $\pm$ 18%; RyR2 $^{-/-}$ , 97 $\pm$ 11%; and RyR2 $^{30/24}$ , 93 $\pm$ 16%; normalized to RyR2 $^{-/-}$ , 100%). We did not determine the potential effects of RyR2 splice variant expression on discrete cytoplasmic and nuclear  $\text{Ca}^{2+}$  storage and/or availability. Consequently, the altered caffeine-induced nuclear and cytoplasmic  $\text{Ca}^{2+}$  mobilization in cells expressing RyR2 containing 30-bp or 24-bp insertions were probably attributable to factors other than cellular  $\text{Ca}^{2+}$  content (see Discussion).

Analysis of the temporal aspects of caffeine-evoked  $\text{Ca}^{2+}$  mobilization revealed that the rate of cytoplasmic  $\text{Ca}^{2+}$  increase following caffeine addition was comparable in cells expressing all RyR2 variants, although RyR2 $^{-/-}$  and RyR2 $^{30/24}$  exhibited a significantly faster recovery of resting cytoplasmic  $\text{Ca}^{2+}$  levels (Figure 4C). In contrast, the augmented nuclear  $\text{Ca}^{2+}$  transients mediated by RyR2 $^{-/-}$  and RyR2 $^{30/24}$  (Figure 4B and 4C) exhibited more rapid rise times and recovery to basal levels than cytoplasmic  $\text{Ca}^{2+}$  transients (Figure 4C).

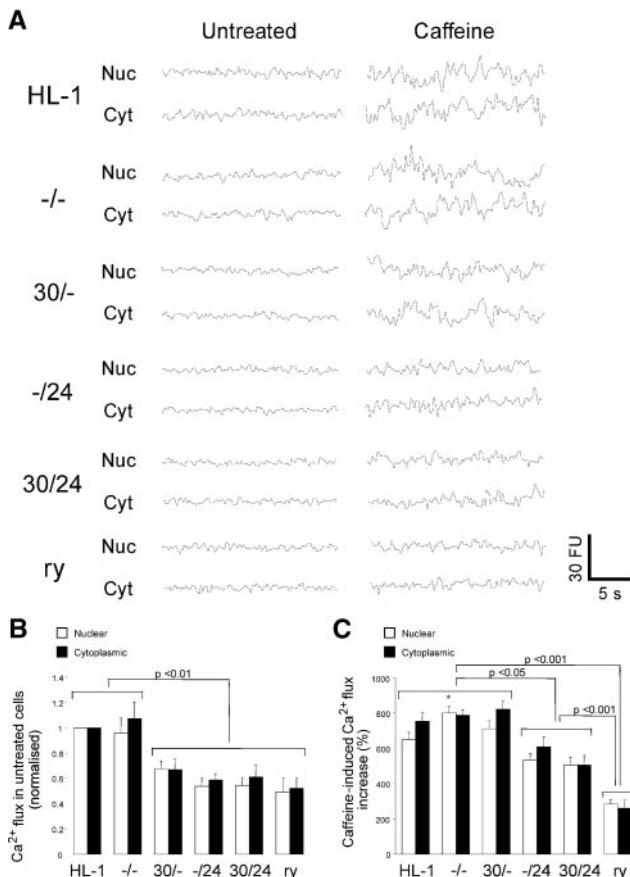


**Figure 4.** RyR2 splice variants differentially modulate cellular  $Ca^{2+}$  release following acute channel activation. A, Pseudocolored images of fluo3-loaded cells expressing RyR2 splice variants before and after the application of a bolus of caffeine (10 mmol/L) (black arrow) at time intervals (seconds) shown. Typical cytoplasmic and nuclear regions analyzed are given in white and red, respectively. Bar represents 10  $\mu$ m. B, The average profiles of  $Ca^{2+}$  mobilization in nuclear and cytoplasmic regions triggered by caffeine addition (10 mmol/L) (arrowhead). Data are given as mean  $\pm$  SEM ( $n > 12$  cells in at least 4 experiments). C and D, The amplitude (C) and temporal (D) aspects of nuclear and cytoplasmic  $Ca^{2+}$  release were quantified. \* $P < 0.05$  when compared with RyR2<sup>-/-</sup>; # $P < 0.05$  when compared within grouping and with RyR2<sup>-/-</sup>; § $P < 0.05$  when compared within grouping.

Normal cardiomyocyte function occurs against a background of large cytoplasmic  $Ca^{2+}$  fluctuations, and normal cell phenotype is maintained following acute caffeine-induced  $Ca^{2+}$  mobilization.<sup>8</sup> However, small but persistent perturbations in  $Ca^{2+}$  homeostasis arising from chronic exposure to caffeine (1 mmol/L) promoted cardiomyocyte dysfunction.<sup>16</sup> Thus, we investigated the functional impact of RyR2 splice variants on cellular  $Ca^{2+}$  signaling and phenotype in response to prolonged exposure to caffeine (1 mmol/L). Under nonstimulated conditions, nuclear and cytoplasmic  $Ca^{2+}$  amplitude variation ( $Ca^{2+}$ “flux”) was suppressed in all cells expressing RyR2 containing 30<sup>+</sup> and 24<sup>+</sup> insertions when compared with that occurring in cells expressing RyR2<sup>-/-</sup> (Figure 5A and 5B), indicating that reduced basal  $Ca^{2+}$  fluxes were independent of the distinct subcellular localization of RyR2<sup>30/-</sup>, RyR2<sup>-/24</sup>, and RyR2<sup>30/24</sup> (Figure 2). The reduced  $Ca^{2+}$  fluxes were prob-

ably caused by altered basal activity of RyR2 splice variants, because a comparable reduction in the magnitude of  $Ca^{2+}$  flux through RyR2<sup>-/-</sup> channels was achieved following ryanodine inhibition (Figure 5A and 5B). However, tonic activation of RyR2 following prolonged caffeine exposure (1 mmol/L, 16 hours), manifested as persistently increased nuclear and cytoplasmic  $Ca^{2+}$  fluxes in RyR2<sup>-/-</sup> and RyR2<sup>30/-</sup> cells (Figure 5A and 5C). In contrast, caffeine-triggered  $Ca^{2+}$  fluxes were significantly reduced in RyR2<sup>-/24</sup> and RyR2<sup>30/24</sup> cells (Figure 5A and 5C).

We next investigated the phenotypic consequences of differential suppression of  $Ca^{2+}$  fluxes by distinct RyR2 splice variants. The extent of cell death (occurring via necrosis and apoptosis) within HL-1 populations is typically 8% to 10%, and this is not affected by the expression of RyR2<sup>-/-</sup> or other splice variants (Figure 6A). However, the reduced “resting” nuclear and cytoplasmic  $Ca^{2+}$  flux in nonstimulated cardiomyocytes expressing RyR2 contain-



**Figure 5.** The 24-bp insertion suppresses intracellular  $\text{Ca}^{2+}$  fluxes during prolonged caffeine exposure. **A**,  $\text{Ca}^{2+}$  traces from nuclear and cytoplasmic regions in untreated cells or those exposed to caffeine (1 mmol/L, 16 hours). The effect of ryanodine (1 mmol/L) on  $\text{RyR2}^{-/-}$  is shown (ry). **B**, The amplitude and temporal variability ("noise") in  $\text{Ca}^{2+}$  signal fluxes obtained from nuclear and cytoplasmic regions (white and black bars, respectively) in nonstimulated cells was normalized to the signal variability in nontransfected HL-1 cells (assigned 1).<sup>8,9</sup> **C**, The increased nuclear and cytoplasmic  $\text{Ca}^{2+}$  signal variability following prolonged exposure to caffeine (1 mmol/L, 16 hours) was calculated. Data are given as the mean  $\pm$  SEM ( $n=4$  experiments,  $>20$  cells per experiment). \* $P<0.05$  when compared with nuclear  $\text{Ca}^{2+}$  signal variability in HL-1 cells and those expressing  $\text{RyR2}^{30/-}$ .

ing 30-bp or 24-bp insertions was associated with a significant reduction in the basal levels of apoptosis (Figure 6A and 6B). Apoptosis was markedly increased in caffeine-activated cells expressing  $\text{RyR2}^{-/-}$  (Figure 6A). Notably, although  $\text{RyR2}^{30/-}$  reduced nuclear and cytoplasmic  $\text{Ca}^{2+}$  fluxes in nonstimulated cells, and reduced the basal levels of apoptosis (Figure 6A),  $\text{RyR2}^{30/-}$  did not protect cells from apoptosis following prolonged caffeine exposure (Figure 6A). In contrast, apoptosis was dramatically reduced in cells expressing  $\text{RyR2}^{-/24}$  and  $\text{RyR2}^{30/24}$  following caffeine stimulation (Figure 6A). Thus our data reveal a clear link between the magnitude of  $\text{RyR2}$ -dependent nuclear and cytoplasmic  $\text{Ca}^{2+}$  fluxes and the extent of apoptosis (Figure 6B), a finding that was reinforced by the pronounced anti-apoptotic effects of channel inhibition using ryanodine (Figures 5C and 6).

These data established a clear functional consequence of 24-bp exon insertion, namely a markedly reduced caffeine-

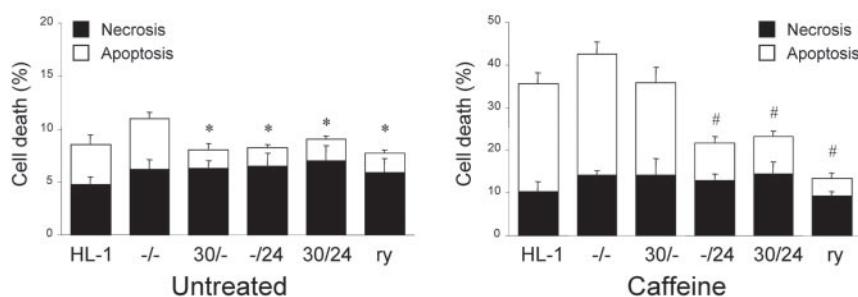
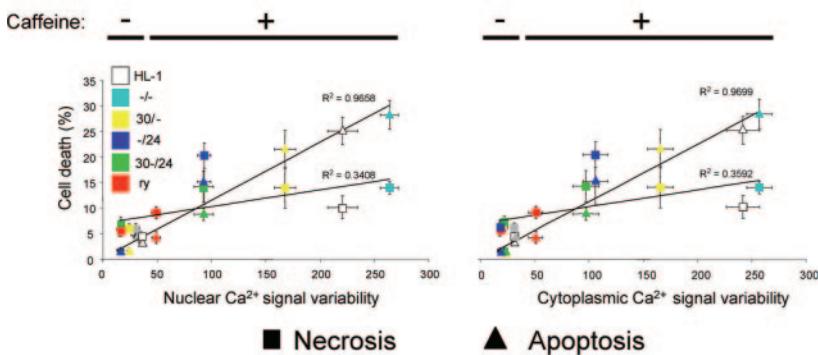
induced  $\text{Ca}^{2+}$  flux that was innately linked to a reduced apoptotic propensity. Because the Bcl-2 family of proteins have been shown to be involved in the signaling of apoptosis following agonist-triggered  $\text{RyR2}$ -dependent  $\text{Ca}^{2+}$  release,<sup>17</sup> we investigated whether the antiapoptotic effects of 24-bp splice insertion following prolonged caffeine exposure was linked to alterations in the expression or posttranslational modification of Bcl-2. We did not detect significant changes in Bcl-2 transcription or in the cellular levels of Bcl-2 protein in cells expressing  $\text{RyR2}$  splice variants (Figure 7A and 7B). However, we determined significantly increased levels of Bcl-2 phosphorylation in  $\text{RyR2}^{-/24}$  and  $\text{RyR2}^{30/24}$  cells. These data suggest that the protection from apoptosis conferred by expression of  $\text{RyR2}$  containing the 24-bp splice insertion may be linked to an alteration in the phosphorylation status of Bcl-2.

### RyR2 Alternative Splicing Exhibits Developmental Regulation and Tissue and Species Specificity

We determined the species, tissue, and developmental profile of  $\text{RyR2}$  alternative splicing. In human cardiac tissue, we identified pronounced developmental regulation of 30-bp-containing mRNA transcripts, and this insertion was far more abundant in embryonic heart (>90%) than in adult heart tissue, where it accounted for <20% of the total  $\text{RyR2}$  (Figure 7A). The disparate relative abundance of the 30-bp insertion in human and mouse embryos strongly indicated a species-dependent regulation of this variant in developing mammalian heart (Figure 7C and 7D). We did not detect the 30-bp insertion in mRNA transcripts derived from adult rat, rabbit, and mouse hearts, and thus the exclusive detection of this variant in adult human heart may represent an intriguing feature of human cardiac regulation (Figure 7D).  $\text{RyR2}$  is also the predominant isoform in brain, and, in stark contrast to its profile in adult heart, the 30-bp insertion was abundant in adult human hippocampus, suggesting that, in humans, alternative splicing of the 30-bp exon exhibits tissue-specific regulation (Figure 7B). In contrast, mRNA transcripts containing the 24-bp splice variant were detected at comparable levels in all species investigated (≈15% to 25% of total  $\text{RyR2}$ ) (Figure 7D), and we found no evidence of its developmental regulation (Figure 7C). Our screening strategy did not resolve the abundance of  $\text{RyR2}$  containing both 30-bp and 24-bp insertions ( $\text{RyR2}^{30/24}$ ) in mammalian tissues, although our functional characterization suggests that channels formed from  $\text{RyR2}^{30/24}$  are indistinguishable from those of  $\text{RyR2}^{-/24}$ .

### Discussion

We investigated the role of  $\text{RyR2}$  splice variants in cardiomyocytes and present the first evidence that developmentally regulated, tissue-specific splice variants of  $\text{RyR2}$  have profound effects on organelle structure,  $\text{Ca}^{2+}$  signaling, and susceptibility to apoptosis. In skeletal myotubes, nuclear  $\text{Ca}^{2+}$  signaling is shaped via  $\text{RyR1}$  localized to NR projections deep into the nucleus,<sup>14</sup> and nuclear and cytoplasmic  $\text{Ca}^{2+}$  release events in cardiomyocytes are markedly different.<sup>18</sup> We now demonstrate that in HL-1 cardiomyocytes, all  $\text{RyR2}$  variants exhibited NR localization, although the 24-bp inser-

**A****B**

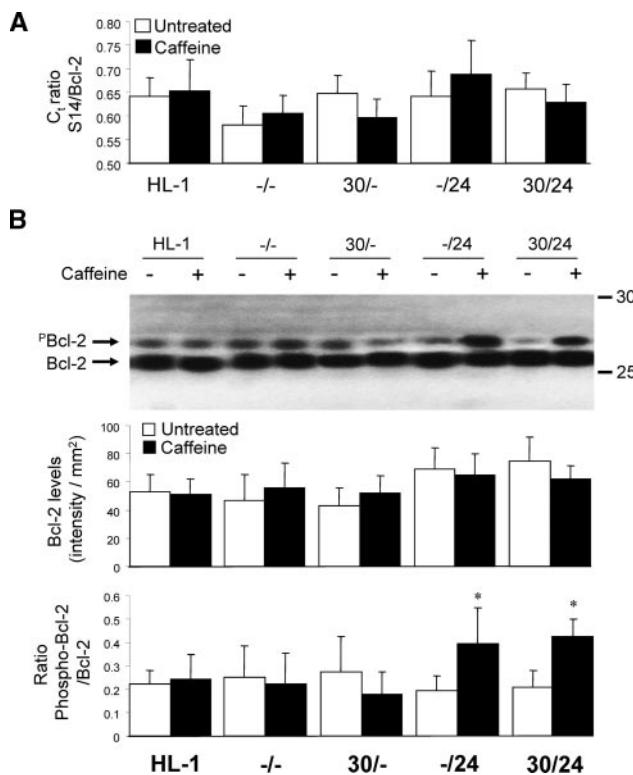
**Figure 6.** Expression of RyR2 containing the 24-bp splice insertion protects cells from caffeine-induced apoptosis. A, The extent of apoptosis vs necrosis in resting and caffeine-activated cells is shown. \* $P<0.05$  and # $P<0.001$ , respectively, when compared with nontransfected HL-1 cardiomyocytes. B, The mode of cell death was plotted against the  $\text{Ca}^{2+}$  signal flux (see Figure 5) in nuclear and cytoplasmic compartments in nonstimulated (−) and caffeine-activated (1 mmol/L, 16 hours) (+) cells expressing RyR2 splice variants (color coded as shown). Data are plotted as the mean  $\pm$  SEM ( $n=4$ , >20 cells per experiment), and  $R^2$  values are given. In A and B, the effects of ryanodine inhibition of RyR2<sup>−/−</sup> is given (ry).

tion was essential for the localization of this RyR2 splice variant to a distinct intranuclear structure that was morphologically identical to an intranuclear GA, presently termed INGA (Figures 2 and 3). Nuclear Golgi “tendrils” have been previously identified in atrial cardiomyocytes<sup>19</sup>, and tubular transnuclear structures that modulate intracellular  $\text{Ca}^{2+}$  signaling have been reported,<sup>20</sup> suggesting that INGA is not an idiosyncratic feature of HL-1 cells (supplemental Figure III). However, our data suggest that currently unknown molecular mechanisms may act as “gate-keepers” to selectively incorporate RyR2 channels containing the 24-bp insertion into this compartment. Consequently, an important conclusion of the present study is that alternative splicing contributes to the intracellular segregation of RyR2 variants. The role of alternative splicing in determining RyR targeting to other cellular compartments including mitochondria,<sup>21</sup> cytoplasmic GA,<sup>22</sup> and plasma membrane<sup>23</sup> is unknown. The protein sequences encoded by the 30- and 24-bp insertions do not represent known organelle targeting motifs, but they do exhibit limited sequence homology with domains present within  $K^+$  channels and ankyrin (Figure IV). The 30-bp insertion deletes a putative protein kinase C phosphorylation site found at 1479Ser-Ile-Lys1481, although the functional significance of this remains to be determined.

The present study provides evidence that RyR2 splice variants fundamentally contribute to shaping nuclear and cytoplasmic events, a significant finding given that spatially distinct  $\text{Ca}^{2+}$  release events underpin altered gene transcription.<sup>24</sup> Consequently, our data suggest that the precise control of cardiomyocyte  $\text{Ca}^{2+}$  handling via differential RyR2 splice variant expression may play an important role in modulating diverse aspects of cardiac cell

signaling, rather than in simply controlling the mechanics of heart muscle contraction. We do not yet know the precise basis for the differential  $\text{Ca}^{2+}$  release mediated by the distinct RyR2 splice variants, although we interpret our results to suggest that cell-compartment-specific regulation by currently unknown nuclear and/or cytoplasmic factors (including the relative contribution of ER-, NR-, GA-, and INGA-derived  $\text{Ca}^{2+}$  sources, the differences in subcellular architecture, and differential nuclear and cytoplasmic protein–protein interactions) may contribute to the distinct  $\text{Ca}^{2+}$  modulation.

We reveal a strong link between reduced RyR2-dependent  $\text{Ca}^{2+}$  fluxes following 24-bp insertion and the protection from caffeine-induced apoptosis (Figures 5 and 6). This important finding is entirely consistent with the reduced channel activity determined in some 1,4,5-trisphosphate receptor and RyR3 splice variants,<sup>5,25</sup> although further work is needed to conclusively determine whether the INGA localization of the 24-bp-containing splice variant is a critical determinant of the observed antiapoptotic effects. Importantly, our data show that the suppression of caffeine-evoked  $\text{Ca}^{2+}$  fluxes mediated by RyR2 containing the 24-bp splice insertion was associated with altered Bcl-2 phosphorylation. This new evidence indicates that the interplay between  $\text{Ca}^{2+}$  flux, Bcl-2 phosphorylation, and apoptosis can be modulated by the specific expression of distinct RyR2 splice variants. The precise mechanisms through which Bcl-2 family proteins transduce RyR2-dependent  $\text{Ca}^{2+}$  fluxes in apoptotic signaling remain to be determined, and one should consider that caffeine-evoked apoptosis may be attributable to other mechanisms, in addition to perturbations in  $\text{Ca}^{2+}$  fluxes,



**Figure 7.** Expression of RyR2 containing the 24-bp splice insert is associated with increased phosphorylation of Bcl-2 following caffeine stimulation. A, The levels of Bcl-2 transcription in untreated cells or those exposed to caffeine (1 mmol/L, 16 hours) was determined using quantitative PCR as described in the online data supplement. The ratios of the threshold crossing point ( $C_t$ ) of a control transcript, S14 ribosomal protein, to that of Bcl-2 in each instance were calculated (mean  $\pm$  SEM,  $n=3$  cell populations in each instance). B, Immunoblot analysis of Bcl-2 expression in untreated cells or following prolonged exposure to caffeine (1 mmol/L, 16 hours). Phosphorylated Bcl-2 ( $p$ Bcl-2) appears as the upper band of the doublet. Data are plotted as means  $\pm$  SEM and are derived from 4 separate blots. \* $P<0.05$  when compared within group and when compared with caffeine-treated HL-1 cells or cells expressing RyR2<sup>-/-</sup> and RyR2<sup>30/-</sup>. Reduced Bcl-2 phosphorylation was consistently observed in RyR2<sup>30/-</sup> cells following caffeine exposure but did not reach significance when compared with caffeine-treated HL-1 cells ( $P=0.14$ ) and untreated RyR2<sup>30/-</sup> cells ( $P=0.27$ ).

including the activation of a number of cAMP-dependent pathways.

Although we show that developmentally regulated RyR2 splicing occurs in human heart (Figure 7), the relative abundances of 30-bp and 24-bp insertions identified in embryonic and adult transcripts do not provide an accurate insight into the channel subunit stoichiometry occurring *in vivo*. The adult human heart is a highly heterogeneous tissue composed of numerous cell types tailored for specialized function. It is possible that the 2 RyR2 splice variants can be coexpressed in the same cells, or differentially distributed in distinct cell types within the myocardium.<sup>26</sup> Furthermore, we do not know the precise stoichiometric arrangement of the RyR2 tetramers generated in our experiments, although the approximate 4-fold overexpression of recombinant RyR2 would predict that most recombinant RyR2 channels exist in homotetrameric com-

bination. Notably, the 24-bp insertion appeared to be “dominant,” because the functional characteristics of RyR2 containing both 24- and 30-bp splice insertions were indistinguishable from RyR2 containing only the 24-bp insertion.

### The Therapeutic Potential of Manipulating Cardiac Phenotype by Alternative Splicing

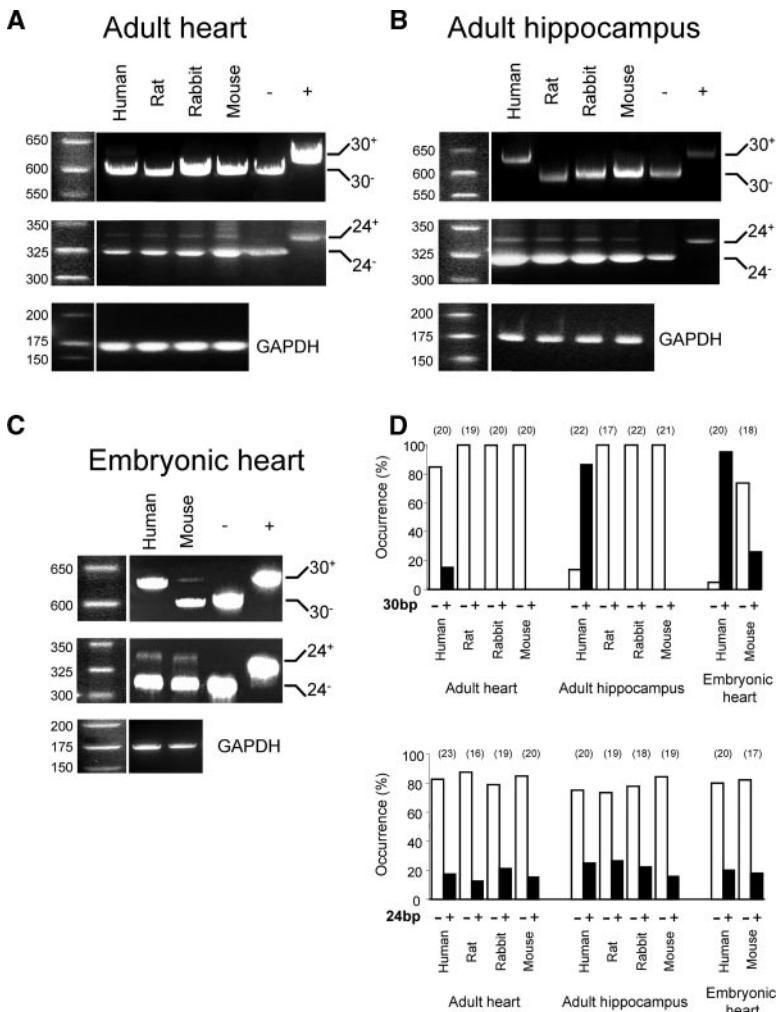
During development, cell-specific regulatory pathways dictate the intrinsic susceptibility of the cells to die. Embryonic human hearts were characterized by very high levels of 30-bp-containing transcripts, splice variants that do not protect cells from increased  $\text{Ca}^{2+}$  fluxes and apoptosis. The abundance of this variant in embryonic heart (Figure 8) may limit the incorporation of antiapoptotic RyR2 containing the 24-bp insertion into functional tetramers, and we speculate that the high levels of RyR2 containing the 30-bp splice insertion may indirectly confer apoptotic susceptibility during cardiac development. Apoptosis is a critical determinant of embryonic myocardial architecture, but it may also be important in normal adult heart remodeling.<sup>27</sup> The long-term adaptive value of apoptosis in the adult myocardium, and its place as a target for therapeutic intervention, remains to be determined. The adult heart can be repaired by gene-based reprogramming of embryonic signaling patterns,<sup>28</sup> and thus our demonstration that cardiomyocyte  $\text{Ca}^{2+}$  signaling and phenotype can be fine-tuned by the expression of functionally distinct RyR2 splice variants potentially represents a novel approach to future cardiac therapies.

### Limitations of Study

We have not succeeded in generating antibodies that specifically recognize the distinct RyR2 splice variants, and it is not currently possible to investigate the developmental profiles and intracellular localization of these variants in native cardiac tissue, or primary cell cultures at the protein level. Accordingly, at the present time, a functional characterization of RyR2 splice variants is restricted to heterologous expression systems. Despite this, HL-1 cardiomyocytes retain the contractile and phenotypic properties of adult cardiomyocytes<sup>29</sup> and express recombinant RyR2 that, in our hands, is indistinguishable from the endogenous protein.<sup>8,9</sup> Thus HL-1 cells represent the best heterologous system currently available for evaluating the role of RyR2 splice variants in cardiomyocyte  $\text{Ca}^{2+}$  signaling. Analysis of HL-1-derived mRNA transcripts (at passage nos. 37, 55, and 87) showed that endogenous RyR2 lacked the 30- and 24-bp insertions (0/18 and 0/25 transcripts, respectively), and although this is entirely consistent with the progressive loss of RyR1 splice insertions during skeletal myotube culture,<sup>4</sup> HL-1 cells may not reflect the RyR2 splicing events that occur in the myocardium *in vivo*.

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**Figure 8.** Developmental regulation and tissue specificity of RyR2 alternative splicing. RyR2 alternative splicing in adult heart (A), adult hippocampus (B), and embryonic heart (C) from various species was determined. Recombinant RyR2 fragments with or without splice insertions (+ and -, respectively) were used as controls. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts was used to verify equivalent amounts of cDNA templates. D, The presence (+, black bars) or absence (-, white bars) of 30- and 24-bp insertions in PCR-amplified fragments. The number of fragments analyzed by DNA sequencing is given in parentheses.

## Disclosures

None.

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