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Heart, Brain, and Body Wall Defects in Mice Lacking Calreticulin

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Calreticulin is a ubiquitously expressed protein, which has been implicated in a large number of cellular functions, including calcium storage and signaling, protein folding, and cell attachment. To examine the role of calreticulin during *in vivo* development, mice deficient in calreticulin were generated by targeted inactivation of the calreticulin gene. Calreticulin-deficient mutants die *in utero,* mostly in late gestation. Half of these embryos had decreased cardiac cell mass, associated with increased apoptosis of cardiac myocytes. *In vitro* differentiation cultures of calreticulin-deficient embryonic stem cells resulted in fewer embryoid bodies with contractile activity than cultures derived from calreticulin +/− stem cells ($P < 0.001$). Sixteen percent of the mutants exhibited exencephaly secondary to a defect in neural tube closure. Embryos surviving until Embryonic Day 16.5 had omphalocele. Lack of calreticulin did not influence survival of embryonic fibroblasts under various endoplasmic reticulum stress conditions. However, calreticulin did influence cell migration in a calcium- and substrate-dependent manner. We conclude that calreticulin is not essential during the early stages of embryonic development, but is important for the development of heart and brain and for ventral body wall closure. The observed abnormalities are compatible with a role of calreticulin in the modulation of cellular calcium signaling. © 2000 Academic Press

Key Words: **brain development; calcium signaling; cardiac development; endoplasmic reticulum; migration.**

INTRODUCTION

Calreticulin (CRT) is a 46-kDa protein, which is expressed in every higher organism [1]. It is highly conserved, with over 90% amino acid identity among the human, rabbit, rat, and mouse forms [2]. The protein is localized primarily to the endoplasmic reticulum (ER), but also has been reported to occur in many other locations including the nucleus, the nuclear envelope, the cytosol, and the outer surface of the plasma membrane [3].

CRT was first identified as a high-affinity calcium-binding protein [4], and early research centered on its role as an ER calcium storage molecule [2]. Later CRT became recognized as a multifunctional protein which can interact with a large number of proteins and is involved in a wide variety of divergent processes [1]. These include regulation of steroid-dependent gene expression via direct interaction with steroid receptors [5, 6], regulation of integrin activity [7], and interaction with extracellular matrix glycoproteins [8].

In the past few years the focus of CRT research has again shifted to two additional functions. A rapidly increasing number of publications have described its role as a molecular ER chaperone which assists in the folding of glycoproteins [3, 9]. Consistent with this proposed function, CRT expression is increased under conditions which impair protein folding [10–13]. Furthermore, CRT was identified as an important modulator of various calcium signaling processes [14–16]. CRT can regulate calcium release from the ER [14, 17] and influences the store-operated calcium current via the plasma membrane [18]. Additionally, we have demonstrated that CRT is essential for the integrin-mediated influx of extracellular calcium [19]. Although these studies linked differing calcium signaling pathways to CRT, the findings are not mutually exclusive, given the complexity and interdependence of calcium currents in the cell [20].

The idea that one protein can perform so many apparently unrelated functions is intriguing and so far no consensus has emerged as to how the multitude of proposed CRT actions can be reconciled with each other. A part of the seemingly contradictory findings may be due to differences in cell types and experimental conditions used. For example, while CRT unquestionably can bind Ca^{2+}, the calcium storage function of CRT may not be equally relevant in all circumstances. Thus, overexpression of CRT has been found to increase the calcium stores in HeLa cells [21], but not in Xenopus oocytes [14]. Likewise, downregulation of

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CRT expression by antisense treatment diminished calcium stores in a neuroblastoma/glioma hybrid cell line [22], whereas ER calcium stores were normal in CRT-deficient embryonic stem (ES) cells [19]. It appears that the response which can be elicited from cultured cells by manipulating their CRT levels depends on the other components of cellular equipment and the extracellular environment.

Which of the proposed functions of CRT are relevant in a more physiological context remains unknown at present. Clearly, more *in vivo* data are required to put the results of *in vitro* experiments into perspective and to provide a rational framework for further studies on CRT function. To examine the role of CRT during *in vivo* development, we generated CRT-deficient mice by targeted inactivation of the CRT gene. CRT-deficient mouse embryos die *in utero* and have cardiomyopathy, exencephaly, and omphalocele. Our results confirm the phenotype described for a CRT-deficient strain carrying a different mutation [23]. Our studies also identified an additional phenotype of CRT-deficient mice, namely exencephaly. Since we have established the mutation on an inbred background and observed the same penetrance of each phenotype, we conclude that the expression of the phenotypes is not dependent on genetic background.

MATERIALS AND METHODS

Generation of CRT-deficient mice. The targeted inactivation of the CRT gene in ES cells was described previously [19]. Mutated ES cells were microinjected into blastocysts of C57Bl6/J females. The resulting chimeras were used to generate heterozygous offspring. DNA from mouse tails or embryos were tested for the presence of the targeted allele using Southern blot analysis as described [19]. Expression of the CRT protein was measured by Western blot analysis of protein extracts prepared from Embryonic Day 14.5 (E14.5) littermates [19].

Histological analysis. Embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin. For immunohistochemistry polyclonal anti-CRT [19], anti-alpha-Bax and anti-p53 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used as primary antibodies and biotinylated anti-rabbit IgG antibody as a secondary antibody. Sections were stained with the avidin-biotin complex (Vector Laboratories, Burlingame, CA) and DAB (3,3′-diaminobenzidine)/hydrogen peroxide. Terminal UTP nucleotide end labeling (TUNEL) for detection of apoptotic cells was performed using the In Situ Cell Death Kit (Boehringer Mannheim Canada, Laval, Quebec).

Differentiation of ES cells. ES cells were differentiated into embryoid bodies by a two-stage hanging drop method, as described [24]. After 5 days, embryoid bodies were transferred to a gelatin-coated 24-well tissue culture dish. Differentiation was monitored by observation of contractile activity as assessed visually under the microscope. Each well was screened daily for contractile activity until Day 14 after the start of differentiation. Differentiation along neuroectodermal lineages was induced by incubating embryoid bodies in medium containing 0.5 μM retinoic acid, as described [25]. This protocol has been shown to promote neural and repress mesodermal differentiation [25]. After the 4-day induction period, aggregates were dispersed with trypsin and plated in tissue-culture wells coated with 0.1% gelatin.

FIG. 1. The targeted inactivation of CRT in mice creates a null allele. (a) Southern blot analysis of a representative Embryonic Day 14.5 (E14.5) litter showing the disrupted CRT allele. The *Bam*H1 diagnostic fragments are 7.8 and 6.5 kb for the targeted and wild-type alleles, respectively. Genotypes are indicated at the top of each lane. (b) Western blot of wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) E14.5 embryos. Note the absence of CRT protein in the sample from a −/− embryo, confirming the null mutation.

Cell migration assays. Mouse embryo fibroblasts were prepared from E14.5 embryos as described [19]. Migration of these cells was assessed in a modified "random" Boyden chamber transwell assay (6-well cell culture inserts containing polyethylene terephthalate membranes, 8.0 μm pore size and 1.0×10^5 pore density, Becton Dickinson, Franklin Lakes, NJ). Membranes were coated with the indicated substrates on both sides and blocked with 2% BSA. Cells (3×10^5) were then plated in the upper well. After a 4-h run in serum-free DMEM, cells on the lower membrane were fixed and stained, as described [26]. The number of stained cells per field (magnification: 100×) was counted in 8 fields for each membrane. Locomotion of ES-derived neural cells was tested by a wound healing motility assay [27]. The "wound" was created in a confluent monolayer of cells by scraping with a plastic pipette tip. The progress of cells moving into the wound area was photographed after 6 h.

Survival under cell stress. Experimental conditions for inducing cells stress in mouse embryonic fibroblasts were chosen following Zinszner *et al.* [28]. Ionomycin-induced cell death was measured by LDH release using the cytotoxicity detection kit (Boehringer Mannheim Canada), as described [29]. The assay is based on the measurement of lactate dehydrogenase (LDH) activity released by damaged cells. The LDH activity is determined by a coupled enzymatic reaction using a colorimetric reagent. Viability of cells incubated with thapsigargin and tunicamycin was assessed by trypan blue exclusion.

RESULTS

Heterozygous mice were viable and fertile and were intercrossed to obtain CRT-deficient (CRT −/−) mice. Genotype analysis of 665 F2 C57Bl/129Sv hybrids and 68 F2 129Sv inbred mice at weaning demonstrated no viable CRT-deficient mice (not shown). Genotyping of live embryos (Fig. 1A) revealed that the mutated allele was transmitted to the progeny with the expected Mendelian ratios until E13.5 (Table 1). Embryonic lethality was detected at E14.5 (Table 1). The distribution of

genotypes in two F2 C57Bl/129Sv outbred strains generated by independent targeting events suggested that one-third of the CRT −/− mice died at E14.5, and two-thirds between Day 14.5 and birth (Table 1). Western blot analysis of live E14.5 embryos revealed a complete absence of CRT protein, confirming that the engineered mutation created a true null allele (Fig. 1B) [19].

Heart

To determine the cause of the embryonic lethality, embryos delivered by cesarean section were examined morphologically and histologically. Fourteen out of the 28 examined CRT-deficient mutant hearts (50%) displayed signs of cardiomyopathy at E14.5 (Fig. 2). These hearts had thinner muscular walls of ventricles and ventricular septum, as well as fewer trabeculae (Fig. 2b). Expression of CRT in embryonic heart at this stage of development has been reported previously [23]. The cardiomyopathy of CRT −/− embryos was associated with increased apoptosis of cardiomyocytes, as evidenced by TUNEL analysis (Figs. 2c and 2d). Furthermore, *in vitro* differentiation of CRT −/− ES cells resulted in fewer embryoid bodies with contractile activity than cultures derived from CRT +/− stem cells (Fig. 2e).

Brain

In addition to the cardiac defects, CRT-deficient embryos had exencephaly (Fig. 3a) secondary to the failure to close the cranial neural tube (Figs. 3b and 3c). Exencephaly was present in 16% of CRT-deficient embryos of the two lines of C57Bl/129Sv hybrids and was also noted in the 129Sv inbred strain, demonstrating that this phenotype is not a peculiarity of the outbred genetic background (Table 2). No closure defects were observed at the level of the spine (not shown). Neural tube closure is a complex process involving various cellular functions which might be influenced by CRT [30]. Cell adhesion and migration are of obvious importance for neural tube closure. We therefore examined the influence of CRT deficiency on cell migration. A wound healing assay of ES cells differentiated into neuroectodermal lineages showed slower migration of the CRT-deficient cells compared to heterozygous cells (Figs. 4a to 4d). The influence of CRT on cell migratory behavior was further examined in a random mode transwell assay [26]. Mouse embryonic fibroblasts derived from E14.5 CRT-deficient embryos migrated slower on high concentrations of fibronectin and laminin, but migrated faster on low substrate concentrations (Fig. 4e). Wild-type and CRT −/− fibroblasts migrated similarly when these experiments were repeated in a cell culture medium where Ca^{2+} was complexed to EGTA (Fig. 4e). Migration on collagen type I was similar for both genotypes (data not shown).

Neural tube closure can also be disturbed by conditions causing ER stress [26] and CRT has been proposed to be an ER stress protein [10–13]. ER stress can be induced by inhibition of glycosylation following tunicamycin treatment or by depletion of ER Ca^{2+} stores by thapsigargin or ionomycin [28]. Overexpression of CRT increases cell survival under these conditions, while downregulation by antisense treatment decreases viability [12, 22]. We therefore tested whether CRT is important for mouse embryonic fibroblast survival when treated with stress-inducing agents, choosing conditions similar to previous reports [12, 28]. Survival of CRT-deficient fibroblasts was not different from wild-type cells when incubated with ionomycin or thapsigargin (Fig. 5). In tunicamycin-treated cells viability after 48 hours was even slightly increased in CRT −/− cells (Fig. 5).

Body Wall

CRT-deficient embryos surviving until E16.5 failed to withdraw the physiological umbilical hernia (Fig. 6a). Levels of the proapoptotic proteins p53 and Bax were increased in this membrane (Fig. 6, compare panels c and e to panels b and d). These results suggest that signal transduction cascades leading to apoptosis may be perturbed in CRT-deficient cells.

DISCUSSION

The intriguing multitude of functions attributed to CRT and the high degree of conservation in the CRT sequence between species have led to the suggestion that CRT is essential for cell survival [18]. In fact, downregulation of CRT expression by antisense treatment quickly induces apoptosis in a neuronal cell line [31]. However, CRT obviously is not equally important for all types of cells and at all times. We have previously demonstrated that CRT-deficient ES cells are

TABLE 1

Genotypes of Progeny from Intercrosses between Heterozygous CRT-Targeted Parents

Age	No. litters	+/+	+/−	−/−
E9.5	4	5 (17%)	15 (50%)	10 (33%)
E13.5	3	4 (28%)	7 (50%)	3 (21%)
E14.5	43	88 (28%)	171 (54%)	56 (18%)
E15.5	4	7 (23%)	22 (71%)	2 (7%)
E16.5	2	6 (38%)	8 (50%)	2 (13%)
E17.5	7	10 (29%)	20 (59%)	4 (12%)
Birth	78	144 (32%)	288 (65%)	14† (3%)

Note. E, embryonic day; †, deceased.

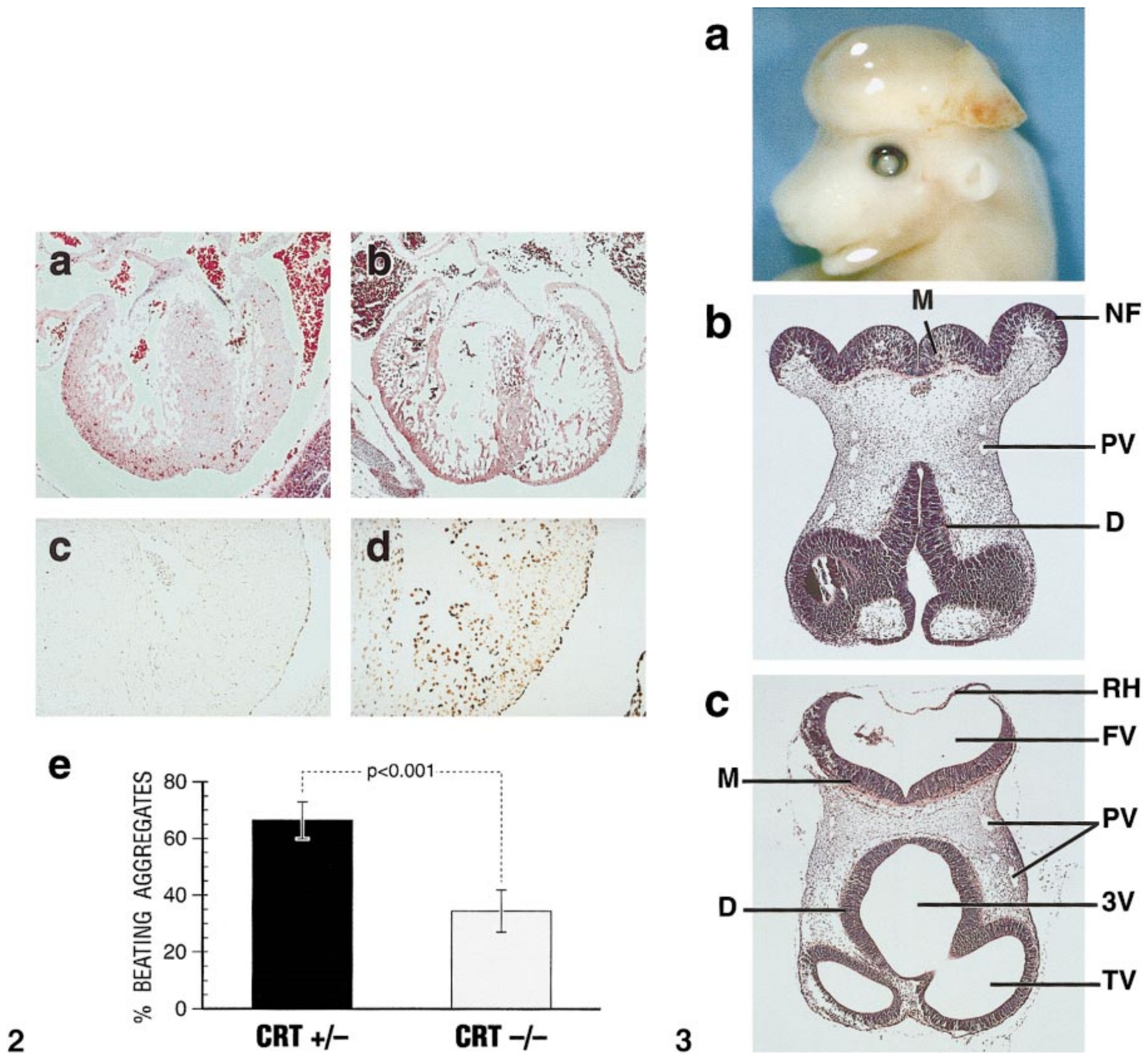

FIG. 2. Cardiac histology of wild-type and CRT-deficient embryos. (a, b) Histological analysis of E14.5 CRT −/− (b) and littermate control (a) hearts. Ventricular walls and muscular septum are thinned in the CRT-deficient heart, and there is less trabeculation. (c, d) Representative view of TUNEL-stained sections of wild-type (c) and CRT-deficient (d) hearts. Apoptotic cells are present in the mutant but not the wild-type heart. (e) Embryoid bodies derived from CRT-deficient ES cells were impaired in their capacity to differentiate into contracting cardiomyocytes when compared to embryoid bodies derived from CRT +/− ES cells. Means ± SD of three independent experiments are shown. A total of 92 embryoid bodies was examined for each genotype. Significance calculated by χ^2 test.

FIG. 3. Analysis of CRT exencephaly mutants. (a) 16% of CRT-deficient embryos had exencephaly, irrespective of genetic background. (b, c) Histological analysis of CRT-deficient (b) and wild-type (c) brains at E10.5. The cranial neural tube is completely closed in the wild-type, but remains open in the mutant embryo. Failure to close the neural tube results in hypercellularity. 3V, third ventricle; D, diencephalon; FV, fourth ventricle; M, metencephalon; NF, neural folds; PV, primary head veins; RH, roof of hindbrain; TV, telencephalic vesicle.

viable and exhibit normal growth rates [19]. We show here that complete lack of CRT is compatible with survival during early embryonic development, as the majority of CRT-deficient embryos were still alive at E14.5. This observation confirms the results obtained with a strain of CRT-deficient animals containing a different mutation [23]. No live-born CRT-deficient animals were detected in the offspring of heterozygous matings, however, proving that CRT is indeed essential for survival of the organism as a whole. Our results further show that CRT is essential for the development of the heart and ventral body wall closure, as was also reported by Mesaeli *et al.* [23]. Additionally, we show that CRT is essential for brain development.

The defect most likely responsible for death *in utero* is cardiac failure. Among the three affected organ systems only the heart is essential for survival in the late stages of gestation. Histology revealed cardiomyopathic changes and increased apoptosis in cardiomyocytes of CRT −/− embryos. These abnormalities either

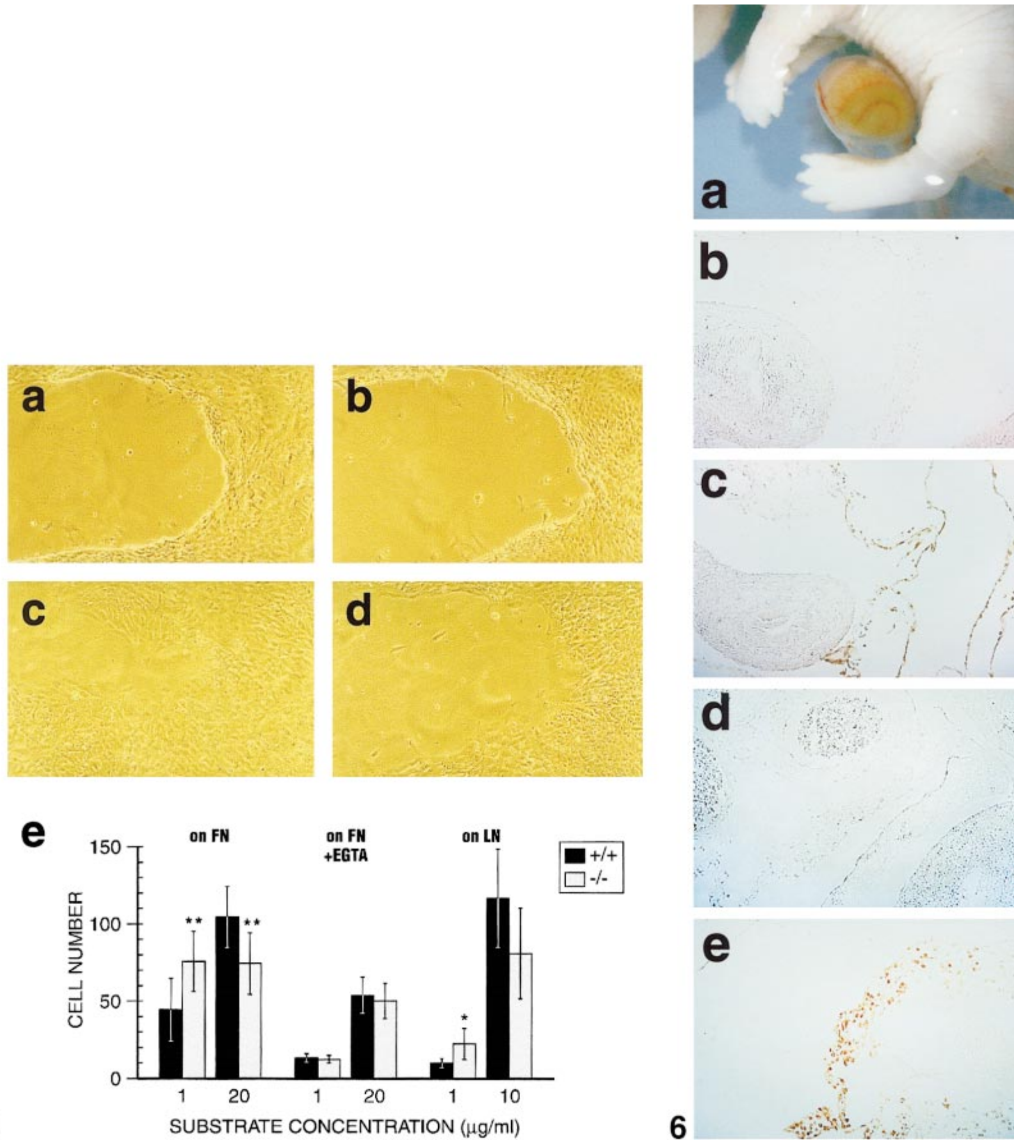

FIG. 4. Abnormal migration of CRT-deficient cells. (a–d) Wound healing assay of ES cell-derived neural cells. Homozygous CRT mutant (−/−, b and d) and heterozygous control (+/−, a and c) ES cells were treated with retinoic acid to induce differentiation along neuroectodermal lineages. Monolayers of differentiated cells were "wounded" with a pipette tip and migration into the wound area was monitored by microphotography. (a and b) Time 0, (c and d) 6 h. The CRT-deficient cells migrate slower to fill the wound than heterozygous control cells. (e) Transwell migration assay of wild-type (+/+) and CRT-deficient (−/−) mouse embryonic fibroblasts. Migration of the cells was assessed in a Boyden chamber where membranes were coated with the indicated substrates. Cells on the lower membrane were fixed, stained, and counted after 4 h. CRT-deficient fibroblasts display increased motility on membranes coated with low concentrations of fibronectin (FN) and laminin (LN), but decreased motility on high substrate concentrations. This difference was abolished when EGTA was added to the tissue culture medium to chelate Ca^{2+}. Results show the means ± SD of at least two experiments performed in triplicate. $*P < 0.05$ vs wild-type cells; $**P < 0.01$ vs wild-type cells (*t* test for unpaired samples).

FIG. 6. CRT-deficient embryos fail to retract the umbilical hernia. (a) Omphalocele in CRT-deficient embryos surviving past E16.5. (b–e) Immunohistochemical staining of umbilical hernia at E14.5. The expression of the proapoptotic proteins p53 (b, c) and Bax (d, e) was measured in heterozygous control and homozygous mutant littermates. Note that expression of the proapoptotic proteins is dramatically increased in the membrane covering the umbilical hernia of CRT −/− embryos (c, e) when compared to control embryos (b, d).

might be a direct effect of CRT deficiency in the cells or could be secondary to decreased cardiac function. The latter explanation appears more plausible, as all embryos died *in utero,* even though cardiac cell mass was decreased in only 50% of CRT mutants. Decreased function of cardiomyocytes is also suggested by our *in vitro* differentiation studies which revealed a lower number of embryoid bodies exhibiting contractile activity in the CRT-deficient strain.

Which of the proposed CRT functions is essential for

heart function remains to be determined. The observed phenotype fits well with a role of CRT in calcium signaling, as one of the basic processes of heart function—excitation-contraction coupling—is regulated by calcium shifts from the ER to the cytosol [32]. CRT has been shown to modulate ER calcium release and/or re-uptake by calcium pumps [14, 17, 23], although it is not yet known whether this also occurs in cardiomyocytes.

In this respect the striking phenotypic similarities between CRT-deficient embryos and those lacking FKBP12 must be acknowledged [33]. Similar to CRT −/− embryos, FKBP12-deficient embryos die *in utero* during the last days of gestation, have cardiomyopathy and exencephaly. The functions proposed for FKBP12 also overlap with those of CRT. FKBP12 can act as a chaperone for protein folding and is a modulator of intracellular calcium signaling [34]. FKBP12 has been shown to stabilize ryanodine receptors, which serve as ER calcium release channels mainly in excitable cells, such as cardiomyocytes and neurons [33]. Whether CRT also interacts with ryanodine receptors or other components of the excitation-contraction machinery in the cardiomyocyte is not known at present.

In addition to heart function, CRT is important for cranial neural tube closure. CRT is highly expressed in the developing brain at about the time neurulation occurs [23]. Neurulation is a complex process, which necessitates a finely tuned interplay between neuroepithelial cells and the underlying mesenchyme [30]. Neural tube closure is disrupted in several knockout models, where proteins involved in cell migration and calcium signaling were inactivated [reviewed in 35]. Exencephaly can also be provoked by conditions inducing cellular stress [30]. We therefore tested functional deficits in cells lacking CRT which might be responsible for causing exencephaly in CRT-deficient embryos.

We found no evidence for decreased survival of CRT-deficient fibroblasts under various conditions of cellular stress. However, we detected migratory abnormalities in fibroblasts and in neuronal cells derived from CRT −/− ES cells. Interestingly, migration behavior of CRT-deficient fibroblasts depended on the concentration of extracellular matrix substrate. Regulation of cytoplasmic calcium during migration might be abnormal in cells lacking CRT, as the integrin-mediated calcium influx after cell attachment is abolished [19]. In accordance with this hypothesis, wild-type and CRT-deficient fibroblasts exhibited similar migration speed in calcium-free media.

CRT-deficient embryos failed to close the ventral body wall, resulting in omphalocele. The mechanisms involved in body wall closure have not yet been elucidated in detail, but obviously cell migration and attachment do play a role [36]. The pathogenetic role of disturbed apoptotic signaling, as observed in CRT-deficient embryos, is unclear at present.

In conclusion, this study shows that CRT is not essential during the early stages of embryonic development, but is important for the development of heart and brain and for ventral body wall closure. The observed abnormalities are compatible with a role of CRT in the modulation of cellular calcium signaling.

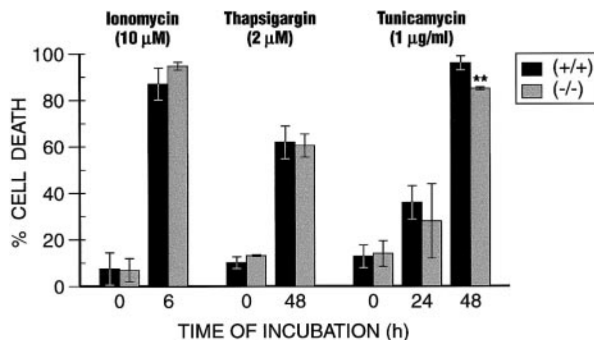

FIG. 5. Survival of wild-type (+/+) and CRT-deficient (−/−) mouse embryonic fibroblasts following ER stress. Cell stress was induced by treatment with the drugs indicated above the bars. Means ± SD of three experiments performed in triplicate are plotted. $^{**}P < 0.01$ vs wild-type cells (t test for unpaired samples).

TABLE 2

Penetrance of the Phenotypes Observed in CRT-Deficient Embryos

Phenotype	Penetrance
Cardiomyopathy	50%
Omphalocele	50%
Exencephaly	16%

Note. All of the described phenotypes were observed at the same frequency in all strains (two outbred strains derived from independent targeting events and one inbred strain), suggesting that the expression of the phenotypes is not dependent on genetic background.

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