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The enigmatic role of the *ankyrin repeat domain 1* gene in heart development and disease

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ABSTRACT It has been proposed that the ankyrin repeat domain 1 (ANKRD1) factor (also known as CARP) plays a critical role in transcriptional regulation, myofibrillar assembly and stretch sensing during heart development and cardiac insults. ANKRD1/CARP has also been reported to negatively regulate cardiac gene expression in cell-based promoter-reporter assays. Consequently, rapid up-regulation of the *ankrd1* gene in myocardium in response to developmental stimuli or pathological insults has tended to be interpreted in the context of the inhibitory effects of ANKRD1 on cardiomyocyte gene expression. Surprisingly, a total *ankrd1* knockout resulted in a complete lack of phenotype, suggesting that ANKRD1/CARP is not crucial for regulation of cardiac gene expression *in vivo*. In this essay, we summarize (1) the accumulated evidence for the apparent multifunctional properties of this enigmatic protein, (2) the distinct chamber-dependent regulation of *ankrd1* expression patterns in the heart, both during development and cardiac injury, and (3) ANKRD1 involvement in networks regulating adaptation of the myocardium to stress. Whenever feasible, we present the results obtained in patients together with those obtained in the relevant animal and cellular models. A close examination of the findings still fails to define ANKRD1 as a negative regulator of cardiac gene expression *in vivo*, but rather indicates that its augmented expression can represent an adaptive response of the myocardium to stress both during development and various heart insults.

KEY WORDS: *CARP, heart development, heart failure, stress-responsive regulator, expression patterns*

Introduction

Throughout development, morphogenetic processes drive the formation of the highly specialized four-chambered mammalian heart. Once cardiac development is completed, postnatal functional maturation of the heart takes over extending the use of developmental signaling factors and pathways to adulthood. As a result, many of the cardiac-restricted factors originally characterized within a purely developmental context are now becoming significant in clinically relevant cardiac research (Hoshijima and Chien, 2002; Akazawa and Komuro, 2003b; Franco and Campione, 2003; Olson and Schneider, 2003; Olson, 2004; Olson, 2006).

The gene for cardiac ankyrin repeat domain 1 (ANKRD1) transcriptional co-factor (also known as CARP), through linking the process of heart development to heart disease, stimulated much interest in studying its potential functions in cardiac muscle tissue. In the fetal heart, ANKRD1/CARP has been implicated in negative transcriptional regulation of cardiac gene expression (Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997). Later, ANKRD1/CARP expression has been found to be rapidly induced in cardiomyocytes in response to hypertrophy stimuli, as well as, at heart failure (HF), suggesting an involvement of *ankrd1* activities in physiological and pathological remodeling of ventricular myocardium (Aihara *et al.*, 2000; Zolk *et al.*, 2002; Nagueh *et al.*, 2004). Recently, *ankrd1* has been identified as a candidate gene that can play a role in congenital heart disease (Cinquetti *et al.*, 2008).

The available information on the molecular functions of *ankrd1* suggests that the gene plays a critical role in transcriptional

Abbreviations used in this paper: ANKRD1, ankyrin repeat domain 1; CARP, cardiac ankyrin repeat protein; AF, atrial fibrillation; HF, heart failure; MARPs, muscle ankyrin repeat proteins; CASQ2, cardiac calsequestrin; MuRF1/MuRF2, muscle-specific RING finger 1 and 2 ubiquitin ligases; SRF, serum response factor; LV/RV, left/right ventricular; MLC2v, myosin light chain 2 ventricular; ANF, atrial natriuretic factor; DCM, dilated cardiomyopathy; Dox, Doxorubicin; YB-1, Y box binding protein 1; TNNC, cardiac troponin C; MLP, muscle LIM-only protein; FHF/SHF, first/second heart field; MVECs, microvascular endothelial cells.

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regulation, myofibrillar assembly, stretch sensing, and in communication between the sarcoplasm and the nucleus (Granzier and Labeit, 2004; Miller *et al.*, 2004). However, one decade after the discovery of ANKRD1/CARP as a fetal cardiac-restricted transcriptional co-factor (Zou *et al.*, 1997), the *in vivo* functions of ANKRD1/CARP remain elusive, largely because neither heart-specific deletion/down-regulation of the gene nor cardio-restricted *ankrd1* transgenesis have been reported yet. Of note, a total *ankrd1* knockout resulted in a complete lack of phenotype (Barash *et al.*, 2007). Nevertheless, rapid *in vivo* up-regulation of *ankrd1* in fetal, neonatal and adult heart in response to stress underscores its importance as a regulator of physiological and pathological processes that is only beginning to be appreciated (Baudet, 2003; Granzier and Labeit, 2004; Miller *et al.*, 2004; Torrado *et al.*, 2005b; Samaras *et al.*, 2006; Samaras *et al.*, 2007; Cinquetti *et al.*, 2008).

In this paper, we revise the properties and regulation of ANKRD1/CARP with particular reference to cardiac muscle development and disease by discussing several important issues related to: (1) the molecular properties of ANKRD1/CARP as a versatile factor that may exert pleiotropic functions in the cardiomyocyte at multiple levels, (2) the unique expression features of *ankrd1* that may be linked to regional specification of the four-chambered heart, (3) the modulations of *ankrd1* regional expression at various heart insults, and (4) the ANKRD1/CARP function as a negative regulator of cardiac gene expression. A close examination of the findings fails to define ANKRD1/CARP as a negative regulator of cardiac gene expression *in vivo*, but rather indicates its involvement in an adaptive response of the heart to biomechanical alterations relevant to both heart development and heart disease.[1]

ANKRD1 is a putatively multifunctional factor: the integration of sequence-based predictions and experimental data

ANKRD1/CARP is a founding member of a family of conserved muscle ankyrin repeat proteins (MARPs; Ikeda *et al.*, 2003; Miller *et al.*, 2003). Sequence analysis and *in silico* sequence-based predictions revealed the following features in mammalian ANKRD1s (Fig. 1): four tandem ankyrin repeats, the coiled-coil domain, nuclear localization signal motif, potential PEST protein degradation sequence, and putative phosphorylation and glycosylation sites (Chu *et al.*, 1995; Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997; Aihara *et al.*, 1999; Miller *et al.*, 2003; Torrado *et al.*, 2004; Witt *et al.*, 2005).

Ankyrin repeats have been found in many proteins spanning a wide range of interesting functions such as transcriptional regulation, cell-cycle control, developmental cell-fate decisions, cytoskeleton integrity, cellular mechanosensory, and endocytosis (Mosavi *et al.*, 2004). This can be attributed, at least in part, to the capacity of ankyrin repeat-containing proteins to interact with other polypeptides. As detected by yeast two-hybrid screening, the N2A region of the sarcomeric no-contractile protein, titin, interacts with ANKRD1/CARP. The N2A titin fragment bound to two distinct ANKRD1/CARP sites, one of which encompass the sequence containing ankyrin repeat 2 (Miller *et al.*, 2003) (see Fig. 1). Using pull-downs, co-immunoprecititation and *in vitro* binding assays, we identified a strong and selective interaction of ANKRD1/CARP with cardiac calsequestrin-2 (CASQ2) and 37- and 42-kDa fragments of titin. Mapping of the peptides involved in the ANKRD1-CASQ2 interaction revealed that three of the five binding sequences for CASQ2 are located in ankyrin repeat 2, 3, and 4 of ANKRD1/CARP (Torrado *et al.*, 2005a)[2].

We detected the coiled-coil domain in the N-terminal part of ANKRD1/CARP and demonstrated that the domain can contribute to ANKRD1/CARP self-dimerization (Torrado *et al.*, 2004). Later this data was confirmed and extended by other approaches (Witt *et al.*, 2005; Torrado *et al.*, 2008). The ANKRD1-ANKRD1 self-interaction represents a weak interaction (Witt *et al.*, 2005; Mikhailov and Torrado, 2006) thus reducing the probability that in cardiomyocytes ANKRD1/CARP oligomerization via the coiled-coil domain could be sufficient to promote a stable multivalent interaction of ANKRD1/CARP dimers with two or more molecules, permitting those molecules to function in a coordinated way.

In addition to ANKRD1/CARP interactions with titin and CASQ2 which are mediated, at least partially, by its binding sites localized within the ankyrin repeats and coiled-coil domain (see Fig. 1), several other partners of ANKRD1/CARP have been identified. In particular, using various combinations of two-hybrid, pull-down and co-immunoprecipitation assays, it has been demonstrated that ANKRD1/CARP can interact with Y-box transcription factor 1 (YB-1; Zou *et al.*, 1997), as well as, sarcomeric protein, myopalladin (Bang *et al.*, 2001), desmin (Witt *et al.*, 2005), and muscle-specific RING finger proteins (MuRF1/MuRF2; Witt *et al.*, 2008). It is

TABLE 1

PREDICTION SCORES FOR CYTOPLASMIC AND NUCLEAR LOCALIZATIONS OF MAMMALIAN ANKRD1/CARP

Acc. No.	Species	Subcellular localization	Reliability	
			PSORT II	CELLO
NP_055206	human	Cytoplasmic	47.8 %	69.80 %
		Nuclear	26.1 %	19.50 %
NP_999087	pig	Cytoplasmic	65.2 %	68.70 %
		Nuclear	17.4 %	20.28 %
NP_001029550	bovine	Cytoplasmic	56.5 %	69.18 %
		Nuclear	26.1 %	20.58 %
NP_001075523	rabbit	Cytoplasmic	47.8 %	62.80 %
		Nuclear	26.1 %	23.98 %
NP_037352	rat	Cytoplasmic	52.2 %	59.96 %
		Nuclear	21.7 %	23.14 %
NP_038496	mouse	Cytoplasmic	52.2 %	64.44 %
		Nuclear	21.7 %	20.62 %

CELLO scores have been converted to the percentage

Note 1: Ankyrin repeat domain 1 (cardiac muscle) – symbol approved by the HUGO Gene Nomenclature Committee (NGNC database). Aliases: C-193, CARP, MARP, MCARP. Discussion on ANKRD1 as a surrogate marker of ventricular hypertrophy (Kuo *et al.*, 1999; Aihara *et al.*, 2000), as well as, a cardiac-restricted target for Doxorubicin (Jeyaseelan *et al.*, 1997; Chen *et al.*, 2007) is outside the purposes and scope of the present article.

Note 2: ANKRD1/CARP and CASQ2 are highly detected and co-distributed in ventricular Purkinje cells, and ANKRD1/CARP selectively and specifically interacts with endogenous CASQ2 in LV-derived extracts (Torrado *et al.*, 2005a).

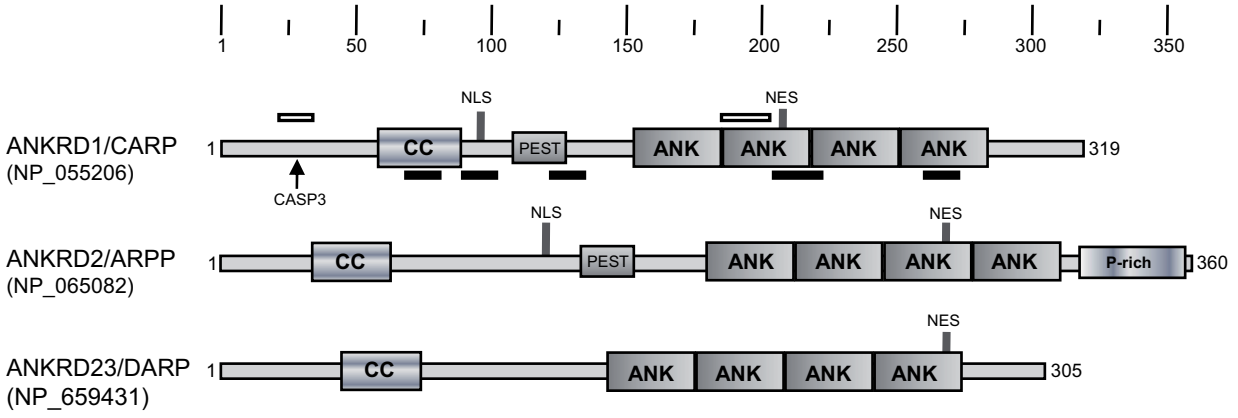

Fig. 1. Muscle ankyrin repeat protein family. *Human cardiac ankyrin repeat protein (ANKRD1/CARP), ankyrin repeat protein with a proline-rich (P-rich) region (ANKRD2/ARPP) and diabetes related ankyrin repeat protein (ANKRD23/DARP) sequences are shown. Coiled-coil (CC) and ankyrin repeat (ANK) domains, nuclear localization (NLS) and nuclear export (NES) signals, and PEST-like motifs were mapped using the COILS, ScanProsite, PredictNLS, NetNES and PESTfind algorithm, respectively. CASP3, caspase-3 cleavage site. On human ANKRD1/CARP, two titin-binding (horizontal white rectangles) and five CASQ2-binding (horizontal black rectangles) sequences are shown.*

interesting to note that only full-length ANKRD1/CARP (but not mutated versions of the protein) effectively binds with myopalladin (Bang *et al.*, 2001). The list of potential ANKRD1/CARP physical partners is not limited to the molecular interactions discussed, because several as of yet uncharacterized myocardial proteins were found to be capable of interacting with ANKRD1/CARP (Torrado *et al.*, 2005a; Witt *et al.*, 2005). The diverse range of ANKRD1/CARP binding partners, including transcription factors (YB-1), myofibrillar (titin, myopalladin), intermediate filament (desmin), calcium-handling (CASQ2), and ubiquitin ligase (MuRF1/MuRF2) proteins suggests both the pleiotropic and integrative functions of ANKRD1/CARP in cardiomyocytes. Consequently, it is crucial to our further understanding of the functions of ANKRD1/CARP to elucidate these interactions *in vivo*.

The primary structure of ANKRD1/CARP also contains several features that indicate the possible dual nuclear and cytoplasmic functions of the protein (i.e., the nuclear localization and nuclear export signal; see Fig. 1). Moreover, the results of our computer analyses of ANKRD1/CARP sequences by PSORT II (Nakai and Horton, 1999) and CELLOv2.5 (Yu *et al.*, 2006) algorithms yielded highly confidential predictions for cytoplasmic localization of the proteins, with much lower prediction scores for their nuclear localization (Table 1).

These sequence-based predictions are well in line with both cytoplasmic and nuclear localization of immuno-decorated ANKRD1/CARP on human (Ishiguro *et al.*, 2002; Nagueh *et al.*, 2004) and porcine (Torrado *et al.*, 2005a; Torrado *et al.*, 2006) heart sections. At the immunoelectron-microscopy level, ANKRD1/CARP epitopes were detected in the central I-band, as well as, within the nucleolus of mouse fetal (Miller *et al.*, 2003) and postnatal (Bang *et al.*, 2001) cardiomyocytes. Consistent with these findings, FLAG- (Jeyaseelan *et al.*, 1997) or GFP-tagged (Miller *et al.*, 2003; Zolk *et al.*, 2003) ANKRD1/CARP was localized in both the nucleus and cytoplasm of transfected neonatal rat cardiomyocytes. ANKRD1/CARP is enriched in the insoluble myofibrillar bound fraction (Torrado *et al.*, 2005a; Hayashi *et al.*, 2008), consistent with its strong binding to titin and myopalladin. Although additional studies are required to determine the significance of ANKRD1/CARP subcellular localization, it has been proposed (Granzier and Labeit, 2004; Miller *et al.*, 2004) that ANKRD1/CARP can function: (1) as a component of a titin-associated stretch-sensing complex in the myofibril and (2) as a co-factor of YB-1-regulated transcription in the nucleus.

Summarizing, the integration of sequence-based predictions and experimental data points to potentially pleiotropic functions of the cardiac ANKRD1/CARP brought about via protein-protein interactions and dual nuclear-cytoplasmic localization.

ANKRD1 expression patterns in fetal, neonatal and mature heart: atrial *versus* ventricular and left *versus* right asymmetry

The developmental patterns and developmental modulation of cardiac *ankrd1* expression in mammals are not yet completely understood. In mice, at relatively early stages of cardiac embryogenesis (stage E8 – E10) *ankrd1* expression assessed by section or whole-mount *in situ* hybridization was found to be uniform throughout all cardiac tissue compartments (Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997). Shortly after, intensity of *ankrd1* hybridization signals started to decline in ventricular myocardium remaining strong in the atrium and outflow tract (Zou *et al.*, 1997). In late fetal and neonatal mice, *ankrd1* hybridization signal was found to be stronger in the atrial as compared to ventricular myocardium (Jeyaseelan *et al.*, 1997). In 11-14-week-old human fetuses ANKRD1/CARP-positive myocytes (as revealed by immunocytochemistry) were uniformly distributed throughout the heart (Ishiguro *et al.*, 2002).

Ankrd1 expression was found to be significantly and selectively reduced in the fetal heart of *Nkx2.5* knockouts, indicating that *Nkx2.5* regulates transcription of *ankrd1* (Zou *et al.*, 1997). On the other hand, the *ankrd1* gene was up-regulated in the postnatal heart of transgenic mice overexpressing *Nkx2.5* (Takimoto *et al.*, 2000). It has been demonstrated that *Nkx2.5* can indirectly control *ankrd1* promoter activity through a transcription factor, GATA-4, that activates the mouse *ankrd1* promoter through a proximal GATA-4 *cis*-element (Kuo *et al.*, 1999). Recently, *Nxk2.5* was found to bind *in vivo* to the 2.5-kb upstream regulating region of the *ankrd1* gene, a region essential for *ankrd1* expres-

sion (van Loo *et al.*, 2007). Other factors may also be involved in activating the *ankrd1* promoter in cardiomyocytes (Aihara *et al.*, 2000; Maeda *et al.*, 2002). In this sense, serum response factor(SRF)-null neonatal cardiomyocytes exhibit an attenuation of *Nkx2.5*, *gata-4* and *ankrd1* expression (Balza and Misra, 2006), and *ankrd1* is presently a hypothetical SRF target (Miano *et al.*, 2007). Deficiency in the *Sp3* transcription factor also results in downregulation of *ankrd1* expression in ventricular (but not atrial) myocardium although *Nkx2.5* and *gata-4* expression was not disturbed in the $Sp3^{-/-}$ heart. The 2.5-kb upstream region of the *ankrd1* gene contains *Sp3* binding sites suggesting that *ankrd1* can be a direct target of the *Sp3* gene (van Loo *et al.*, 2007). The authors of this work suggest that *Nkx2.5* and *gata-4* are required for initial induction of *ankrd1* expression in developing mouse heart whereas *Sp3* is essential to maintain the appropriate expression pattern of *ankrd1* selectively in the ventricles from stage E12.5 onwards.

Concerning the developmental dynamics of *ankrd1* expression in the left ventricular (LV) myocardium, Northern blot hybridization analysis in mice (Kuo *et al.*, 1999) and cDNA microarray profiling in rats (Sehl *et al.*, 2000) revealed higher levels of the *ankrd1* transcript in fetal as compared to those in adult ventricular myocardium. In other work, however, total cardiac levels of the *ankrd1* mRNA were found to be significantly higher in adults than those in early postnatal mouse heart (Johnatty *et al.*, 2000). A gradual increase in ventricular *ankrd1* expression from late-fetus, neonate, to adult stages was demonstrated in rats (Aihara *et al.*, 2000) and pigs (Torrado *et al.*, 2004; Torrado *et al.*, 2005b), suggesting an association of *ankrd1* expression with physiological hypertrophic remodeling of LV myocardium. In this sense, maternal undernutrition in sheep in early- to mid-gestation led to fetal ventricular hypertrophy which in turn resulted in significant *ankrd1* up-regulation in the LV (Han *et al.*, 2004).

Collectively, these results indicated that: (1) *ankrd1* expression is not only exclusively associated with cardiac fetal development, but also represents an expression feature constitutive of the postnatal and adult ventricular myocardium, (2) embryonic expression of *ankrd1* seems to become repressed in the late fetal phase, and (3) ventricular *ankrd1* expression is reactivated to some extent from early neonatal stage towards the adulthood.

One of the attractive features derived from cardiac *ankrd1* profiling studies is that in the fetal (Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997) and neonatal (Gessler *et al.*, 2002) mouse as well as, in perinatal and early postnatal piglet heart (Torrado *et al.*, 2004), the gene is expressed at higher levels in atria as compared to ventricles (i.e., atrial *versus* ventricular expression). Another, but not less intriguing and provocative, observation is that in newborn and early postnatal piglets pan-cardiac *ankrd1* expression is characterized by distinct left- and right-side patterns resulting in an overall left *versus* right asymmetry of *ankrd1* transcript and protein in both atrial and ventricular myocardium (Torrado *et al.*, 2004; Torrado *et al.*, 2005b; Torrado *et al.*, 2006).

How are these specific patterns of *ankrd1* transcriptional activity established? Differential expression of the gene can partly be explained by distinct 5' *cis* regulatory elements of the *ankrd1* gene that can direct chamber-dependent gene expression, such as atrial *versus* ventricular and left *versus* right (Kuo *et al.*, 1999). Whereas the regulatory *cis*-sequences responsible for chamber-dependent expression of *ankrd1* have been identified, the trans-acting factors interacting with these sites are not yet precisely delineated.

As mentioned above, Nkx2.5 can interact with GATA-4 and activates, in turn, the mouse *ankrd1* promoter that contains the GATA-4 binding site in its upstream regulatory region (Kuo *et al.*, 1999). The *Nkx2.5* transcript is distributed homogeneously throughout the fetal and adult mouse myocardium (Komuro and Izumo, 1993; Schwartz and Olson, 1999). *Gata-4* is also broadly expressed across atrial and ventricular regions of the developing mouse heart (Heikinheimo *et al.*, 1994; Brewer and Pizzey,

TABLE 2

ANKRD1/CARP EXPRESSION LEVELS IN DILATED CARDIOMYOPATHY AND HEART FAILURE

Model	Cardiac phenotype	Clinical phase	*Ankrd1* expression	Reference
MLP-deficient mice	early - VH	end-stage HF	transcript (up)	Arber *et al.*, 1997
	late - DCM	end-stage HF	transcript (up)	Baumeister *et al.*, 1997
CASQ2-overexpressing mice	early - VH	depressed contractility	transcript (up)	Ihara *et al.*, 2002
	late - DCM	end-stage HF	transcript (up)	
Gs-alpha-overexpressing mice	late - DCM	advanced HF	transcript (up)	Gaussin *et al.*, 2003
IR and IGF-1R double-knockout mice	DCM	early-stage HF	transcript (no change)	Laustsen *et al.*, 2007
		end-stage HF	transcript (up)	
Ventricular pacing in dogs	DCM-like	end-stage HF	transcript (up)	Zolk *et al.*, 2002
F vs NF human hearts	IDCM	end-stage HF	protein (up)	Nagueh *et al.*, 2004
F vs NF human hearts	IDMC	end-stage HF	transcript (up)	Zolk *et al.*, 2002
			protein (up)	

MLP - muscle LIM-only protein; CASQ2 - cardiac calsequestrin; Gs-alpha - guanidin nucleotide-binding alpha; IR - insulin receptor; IGF-1R - insulin growth factor-1 receptor; VH - ventricular hypertrophy; DCM - dilated cardiomyopathy; IDCM - idiopatic DCM; F - failing heart; NF - non-failing heart.

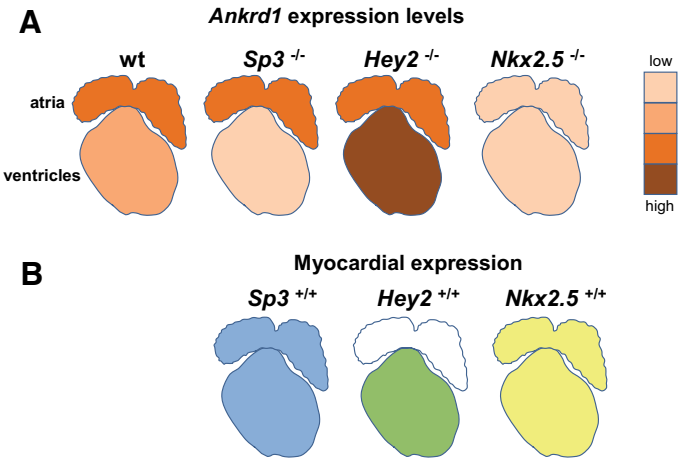

Fig. 2. Regulatory signals that may influence atrial *versus* ventricular expression of the *ankrd1* gene in the fetal myocardium. (A) *Simplified schematics of* ankrd1 *expression in the myocardium of* Sp3*-,* Hey2*- and* Nkx2.5*-defficient, and wild-type (wt) mouse embryos. Color gradient scale represents low-to-high* ankrd1 *expression levels.* **(B)** *Simplified schematics of expression levels of* Sp3 *(blue),* Hey2 *(green) and* Nkx2.5 *(yellow) in the myocardium of wide-type mouse embryos are shown. Correlative expression data (A,B) provides a context for a minimal model of regulation of* ankrd1 *expression in atrial* versus *ventricular myocardium. See text for further details.*

2006). Thus, the results on *Nkx2.5* and *gata-4* expression patterns in the mouse heart do not suggest an obvious mechanism by which distinct, atrial *versus* ventricular, patterns of *ankrd1* expression may be achieved.

During heart development basic helix-loop-helix transcription factors Hey1 and Hey2 show a clear regionally restricted expression, the first being active exclusively in the atria and the second in the ventricles (Leimeister *et al.*, 1999)[3]. Hey2 can suppress cardiac gene expression through a direct inhibitory interaction with GATA-4 that interferes with DNA binding (Kathiriya *et al.*, 2004; Xiang *et al.*, 2006). It has been demonstrated that *in vivo Hey2* limits the level of GATA-4/6-driven genes, such as *ankrd1* and atrial natriuretic factor (*anf*), to a certain level, while loss of *Hey2* activity leads to up-regulation of these genes in ventricular myocardium (Fig. 2). These effects were not associated with inhibition of *gata-4/6* expression in the ventricles of *Hey-2*-defficient embryos. In the atria of *Hey2* knockouts, *ankrd1*, *anf* and *gata-4* levels were unchanged, consistent with the ventricular restriction of *Hey2* expression (Fischer *et al.*, 2005). These results suggest that the *ankrd1* atrial *versus* ventricular pattern in the fetal and neonatal heart is not exclusively driven by the distribution of positive factors, but likely by the imposition of region-specific restraints on expression. Of note, *Hey-2*-mediated inhibition of *ankrd1* expression in the ventricular myocardium can be counterbalanced by positive *ankrd1* regulators, others than *Nkx2.5* and *gata-4* (see Fig. 2). One of such positive regulators, the transcriptional factor *Sp3* is expressed ubiquitously in the developing mouse heart[4]. Nevertheless, *Sp3*-deficient mice display a highly reduced expression of *ankrd1* only in the ventricles: *ankrd1* expression is almost completely lost from the ventricular myocardium, excepting a region in the septum that coincides with the position of the future ventricular conductive system (van Loo *et al.*, 2007).

Regulatory mechanisms resulting in left *versus* right asymmetric ANKRD1/CARP patterns in the newborn and early postnatal heart (Torrado *et al.*, 2004; Torrado *et al.*, 2005b; Torrado *et al.*, 2006) remain to be elucidated. HAND1/eHAND and HAND2/dHAND transcription factors that have distinct, left *versus* right, roles in the development of ventricular myocardium (Akazawa and Komuro, 2003a) are equally expressed in both the left (LV) and right ventricle (RV) of neonatal piglets, suggesting that these factors are not involved in *ankrd1* expression sidedness (Torrado *et al.*, 2004).

Ankrd1 expression sidedness seen in the late fetal – early neonatal pig heart is actually at the end of a process of L-R axis determination and establishment. While transcription factors like *Nkx2-5* function in both the first (FHF) and second (SHF) heart field, others appear to be restricted to the FHF or the SHF. *Tbx5*, the T-box family transcription factor, represents one such factor that is expressed in the FHF and, later in development, in atria and LV, but not in the RV and outflow tract. Ablation of *Tbx5* in mice results in a severe hypoplasia of the LV and atrial compartment, while the RV and outflow tract are not affected (Bruneau *et al.*, 2001). Interestingly, *Tbx5* interacts directly with the *Nkx2-5* factor and, *in vitro*, cooperatively activates expression of the *ankrd1* gene (Hiroi *et al.*, 2001). Thus, it seems probable that the association and positive functional coupling of *Tbx5* and *Nkx2-5* might lead to left predominant *ankrd1* expression in the heart.

The cardiac wall contains not only working myocytes, but also impulse-conducting cells, myofibroblasts, and endothelial cells. Expression of *ankrd1* in cardiac fibroblasts and endothelial cells has not been reported. Using immunohistochemical detection, we found significant ANKRD1/CARP enrichment in ventricular Purkinje cells of early postnatal piglets (Torrado *et al.*, 2005a). As revealed by Western blot, the ANKRD1/CARP protein content in the Purkinje-cell enriched samples was about 2-fold higher as compared to that in working myocytes (Torrado *et al.*, 2006). The results indicate that ANKRD1/CARP may therefore play a previously unknown role in postnatal cardiac development of the specialized conduction system in ventricular myocardium.

Note 3: Like other Hairy-related transcriptional factors, Hey1 and Hey2 are controlled by a Notch signaling pathways and can act as transcriptional repressors. In the mouse, there is strong expression of *Hey2* in the fetal ventricular myocardium that is gradually lost during postnatal development. Null mutants of *Hey2*, depending on genetic background, show either a marked cardiac hypertrophy or display various cardiac malformations (reviewed in: Brand, 2003).

Note 4: In the mouse, ablation of *Sp3* results in a severe hypoplasia of the ventricular myocardium, which failed to form a compact layer. Among the genes studied, only *ankrd1* showed a deregulated expression in *Sp3-/-* ventricular myocardium; the expression of *Nkx2-5* and *gata-4* was not different between wild type and *Sp3-/-* hearts (van Loo *et al.*, 2007).

It is well accepted that loading conditions differ drastically between different chambers of fetal and postnatal heart. Therefore, chamber-dependent expression of ANKRD1/CARP from late fetal stages through adulthood can be influenced by different types of biomechanical stress in atrial *versus* ventricular and left *versus* right myocardium.

Modulations of ANKRD1 expression in diseased myocardium: the result of myocardial damage and dysfunction regardless of the cause

Initial evidence for the possible involvement of the *ankrd1* gene in cardiac pathology was obtained using muscle LIM-only protein (MLP)-deficient mice which had developed a severe dilative enlargement of the heart (resembling human dilated cardiomyopathy – DCM) followed by ventricular hypertrophy and HF after birth. Significant up-regulation of the *ankrd1* gene was observed in both dilated and hypertrophied ventricles (Arber *et al.*, 1997; Baumeister *et al.*, 1997). Later, it has been demonstrated that DCM-like phenotypes resulted from very different initial insults are also associated with a strong *ankrd1* up-regulation in the LV myocardium (Table 2 and references wherein). Further, *ankrd1* mRNA and protein levels were significantly increased in LV myocardium samples obtained from patients with end-stage HF due to idiopathic non-ischemic DCM (Zolk *et al.*, 2003; Nagueh *et al.*, 2004; Torrado *et al.*, 2005b). Cardiac-tissue fractionation experiments followed by Western blot identification suggested that ANKRD1/CARP is mainly localized in nuclei and weakly expressed in the cytoplasm of human failing and non-failing myocardium (Zolk *et al.*, 2002). However, these findings were not confirmed by immunohistochemical analysis of normal and diseased human myocardium by which ANKRD1/CARP-specific immunoreactivity was localized in the cytoplasm, being barely detectable in the nucleus (Ishiguro *et al.*, 2002; Nagueh *et al.*, 2004).

At first glance, these results point to *ankrd1* up-regulation as the end-stage DCM-specific feature which might be responsible for dysregulation of gene expression in failing DCM-myocardium (Zolk *et al.*, 2002; Baudet, 2003; Zolk *et al.*, 2003). However, *ankrd1* expression has been demonstrated to be up-regulated in the LV at various different pathophysiological states, namely, in animals models of cardiac hypertrophy (Kuo *et al.*, 1999; Aihara *et al.*, 2000; Johnatty *et al.*, 2000), non-lethal cardiac ischemia (Depre *et al.*, 2001), genetically induced (Gotthardt *et al.*, 2003) or cardiotoxic CM (Torrado *et al.*, 2004; Torrado *et al.*, 2006), and in the heart of animals with streptozotocin-induced diabetes (Lehti *et al.*, 2007) as well as, in patients with ischemic CM (Zolk *et al.*, 2002) or ventricular septal defects (Zhang *et al.*, 2006). Certainly, induction of *ankrd1* overexpression in LV myocardium is a heart disease-relevant phenomenon, but this gene augmented expression is associated with a wide range of different cardiac disease states.

Although this field of study is extensive, several gaps in our knowledge on *ankrd1* expression patterns at HF still exist. Given known limitations for myocardial biopsies in rodent models, as well as in patients, these studies almost uniformly used LV-derived specimens, although the gene is expressed in all cardiac chambers. To overcome such limitations, we examined the fingerprint of *ankrd1* expression throughout the heart in a large-animal model that is most relevant to human heart, i.e., a pig model of HF. Neonatal piglets exposed to different doses of cardiotoxic antibiotic, Doxorubicin (Dox), develop either systolic or diastolic HF (Torrado *et al.*, 2004; Torrado *et al.*, 2005b; Torrado *et al.*, 2006). At diastolic HF, normal atrial *versus* ventricular *ankrd1* expression pattern (Torrado *et al.*, 2004) is oppositely regulated (at mRNA and protein levels), and is augmented about 2-fold in each ventricle, but equally reduced in both atria as compared to controls. *Ankrd1* down-regulation in atria is a differential sign of diastolic HF, since systolic HF results in *ankrd1* up-regulation in both atria and ventricles (Torrado *et al.*, 2006). A marked *ankrd1* up-regulation (at mRNA and protein levels) was also observed in atria in a porcine model of pacing-induced atrial fibrillation, with slightly increased nuclear localization of ANKRD1/CARP protein in the fibrillating atria as compared to sham controls (Chen *et al.*, 2007). In human HF due to DCM, no difference in ANKRD1/CARP proteins levels was observed between right atria from failing and non-failing donor hearts (Zolk *et al.*, 2002).

Taken together, the data suggest that: (1) distinct *ankrd1* regulatory programs may be operative in diseased ventricular *versus* atrial myocardium, (2) the vast majority of HF phenotypes is associated with *ankrd1* up-regulation in diseased ventricular myocardium, and (2) up-regulation of *ankrd1* expression at various different forms of HF is unlikely to be a common molecular key that unlocks the pathological process, but rather appears to be a case of non disease-specific transcriptional adaptation of the heart to myocardial stress and injury.

Correlation between elevated levels of ANKRD1/CARP in the LV myocardium and various end-stage HF-phenotypes has tended to be interpreted as an indicator of an unfavorable clinical outcome (Zolk *et al.*, 2002; Baudet, 2003; Zolk *et al.*, 2003). However, it was unclear whether ventricular *ankrd1* overexpression is causally related to the development of HF malignant phenotypes or if it is one of the adaptive reactions that antagonize the progressive loss of cardiac performance efficiency.

ANKRD1 as a negative regulator of cardiac gene expression: *in vitro versus in vivo* paradigm

Few studies have addressed this question explicitly at the cellular level *in vitro*. As mentioned above, ANKRD1 has been identified as a nuclear cardiac ankyrin repeat protein (abbreviated as CARP) by two-hybrid screening of a neonatal rat ventricular cDNA library, using the transcriptional YB-1 factor as bait. Co-immunoprecipitation and GST-ANKRD1/CARP pulldown studies further confirmed that ANKRD1/CARP does form a physical complex with YB-1 in cardiomyocytes. Results of *in vitro* cell co-transfection assays revealed that ANKRD1/CARP prevents the activation of the promoter of the myosin light chain 2 ventricular (*mlc2v*) gene probably by binding with and inhibiting activity of YB-1 (Zou *et al.*, 1997).

Evidence consistent with the definition of ANKRD1/CARP as a possible negative regulator of cardiac gene expression (Zou *et al.*, 1997) is also provided by Jeyaseelan (Jeyaseelan *et al.*, 1997), who show that forced *ankrd1* expression in cultured neonatal cardiomyocytes inhibits activity of atrial natriuretic factor (*anf*) and troponin C (*tnnc*) gene promoters. Later, however, these results have ironically been cited by other authors who listed cardiac α-actin, skeletal α-actin (Aihara *et al.*, 2000) and β-myosin

heavy chain (Zolk *et al.*, 2003), along with *mlc2v* and *tnnc*, as target genes which expression could be negatively regulated by ANKRD1/CARP. Using *in vitro* engineered heart tissue, it has been demonstrated that adenoviral-mediated *ankrd1* overexpression in neonatal rat cardiomyocytes inhibits their contractile responsiveness to elevated concentrations of exogenously added catecholamines (Zolk *et al.*, 2003). The relevance of these findings to a proposed ANKRD1/CARP inhibition of cardiac gene expression remains uncertain, because the transcriptional changes of cardiomyocytes in response to *ankrd1* forced expression have not been reported.

The ANKRD1/CARP factor has often been referred to as a negative regulator of *mlc2v* expression during cardiac development. It has even been proposed that one role of ANKRD1/CARP is to downregulate *mlc2v* in atrium domains, thereby limiting *mlc2v* expression to the ventricular myocardium (Krieg, 2001). Such interpretations of the results obtained from *in vitro* manipulated neonatal cardiomyocytes have not been yet experimentally confirmed *in vivo*. In this sense, the reduced expression of both *ankrd1* (Zou *et al.*, 1997) and *mlc2v* (Lyons *et al.*, 1995) in a *Nkx2.5*-deficient background does not correlate well with the demonstration that ANKRD1/CARP inhibits the *mlc2v* reporter gene activity *in vitro*. Moreover, *mlc2v* was not identified as the gene downregulated in the LV myocardium of MLP-deficient mice (Gao *et al.*, 2006), and no decreased levels of MLC2v and TNNC were detected in $MLP^{-/-}$ hearts characterized by very high levels of *ankrd1* expression as compared to controls (Arber *et al.*, 1997). In patients, end-stage DCM is associated with a marked activation of the *ankrd1* (see Table 2) and selective upregulation (not inhibition) of the *mlc2v* gene in failing as compared to non-failing LV myocardium (Haase *et al.*, 2002). In addition, our comparative analysis of left and right ventricular biopsies from newborn piglets characterized by a 10-fold difference in the *ankrd1* transcript and protein presentation did not reveal any differences in either MLC2v transcript and protein content between the two ventricular chambers (Torrado *et al.*, 2004).

ANKRD1/CARP negative regulation of the *anf*-promoter observed in *ankrd1*-transfected neonatal cardiomyocytes *in vitro* (Jeyaseelan *et al.*, 1997) is not in line with results on temporal and regional-specific expression of the *ankrd1* and *anf* gene *in vivo*. *Anf is* initially expressed in both the atrial and ventricular domains with higher levels of expression in the LV than in the RV. Expression of the *anf* gene becomes atrial-restricted later during cardiac development, at about the time of birth in mammals (Habets *et al.*, 2003; Small and Krieg, 2004). As mentioned above, in the early postnatal mammalian heart *ankrd1* is expressed in atria at much higher levels than in ventricles. Therefore, cardiac *ankrd1* hyper-production *in vivo* is not necessarily associated with inhibition of *anf* expression.

Although no evidence has yet been provided, induction of *ankrd1* expression has been claimed to inhibit cardiac protein synthesis and cell enlargement, suggesting therefore that *in vivo* ANKRD1/CARP might have inhibitory effects on ventricular hypertrophy (Aihara *et al.*, 2000; Han *et al.*, 2004). These works, however, offer no direct evidence for such an ANKRD1/CARP anti-hypertrophic mechanism.

How is it that ANKRD1/CARP inhibits *mlc2v*- and *anf*-gene activities in *in vitro* cultured cardiomyocytes, but not in the myocardium itself *in vivo*? This can be attributable to differences between the results obtained in *in vitro versus in vivo* models. *In vitro* binding assays and cell-culture transfections with plasmid constructs not always correspond to *in vivo* assays. Discordance between *in vitro* cell-culture studies and animal models *in vivo* may also be explained by the presence of trans-acting factors in the hearts absent in the cardiomyocyte cultures used for transfection analyses. Formally, *in vitro* established associations should be mechanistically confirmed in animal models to demonstrate their biological relevance. In this regard, the proposed role for ANKRD1/CARP as a negative regulator of *mlc2v, tnnc and anf* gene expression has not been validated in animal models of cardiac hypertrophy (van den Bosch *et al.*, 2006) associated with significant *ankrd1* up-regulation in the LV myocardium of experimental animals as compared to controls. In other experimental settings (Arber *et al.*, 1997; Torrado *et al.*, 2004; Torrado *et al.*, 2006), no appreciable differences in the level of expression and content of contractile proteins were observed between the cardiac samples characterized by quite different levels of *ankrd1* expression. More broadly, it becomes particularly difficult to interpret, in this light, the significance of *in vivo* ANKRD1/CARP over-production in failing myocardium (see section above) from the standpoint of ANKRD1/CARP negative regulation of cardiac gene expression (Zolk *et al.*, 2002), simply because in many HF phenotypes a remarkably significant *ankrd1* up-regulation is associated with re-activation (not inhibition) of the *anf* gene expression (Nanni *et al.*, 2006; Nishikimi *et al.*, 2006) in the ventricular myocardium.

In conclusion, the analysis of available data still fails to define ANKRD1/CARP as a negative regulator of cardiac gene expression *in vivo*, but rather indicates that its augmented expression may represent a universal adaptive response of the myocardium to numerous different hemodynamic, biomechanical, genetics or cardio-toxic stimuli.

ANKRD1 as a stress-inducible cardiac factor: from expression patterns to functional interpretation

As discussed above, many studies have implicated *ankrd1* over-production in LV hypertrophy at heart development, growth and disease, suggesting that its up-regulation is a common feature of the adaptive response of ventricular myocardium to pressure overloads. It should be noted, in this sense, that cardiac hypertrophy represents a general response to cardiac stress overloads, and that hypertrophic growth *per se* does not trigger cardiac dysfunction (Perrino *et al.*, 2006). In fact, *ankrd1* was found to be up-regulated at both physiological (Aihara *et al.*, 2000; Torrado *et al.*, 2004) and experimental pressure-induced LV hypertrophy not accompanied by altered systolic function (Weinberg *et al.*, 2003).

It was reported that an adenoviral *ankrd1*-expressing vector implanted subcutaneously in rats promotes neovascularization and increased blood perfusion (Shi *et al.*, 2005), which raises the intriguing question of whether or not ANKRD1/CARP might promote angiogenesis (Boengler *et al.*, 2003) in response to myocardial ischemia. Of note, transient ischemia/perfusion without irreversible cardiac damage is associated with a rapid *ankrd1* up-regulation even when neither hypertrophy nor apoptosis are detected in stunned LV myocardium (Depre *et al.*, 2001).

In mice, lacking *ankrd1*, no structural, molecular and functional

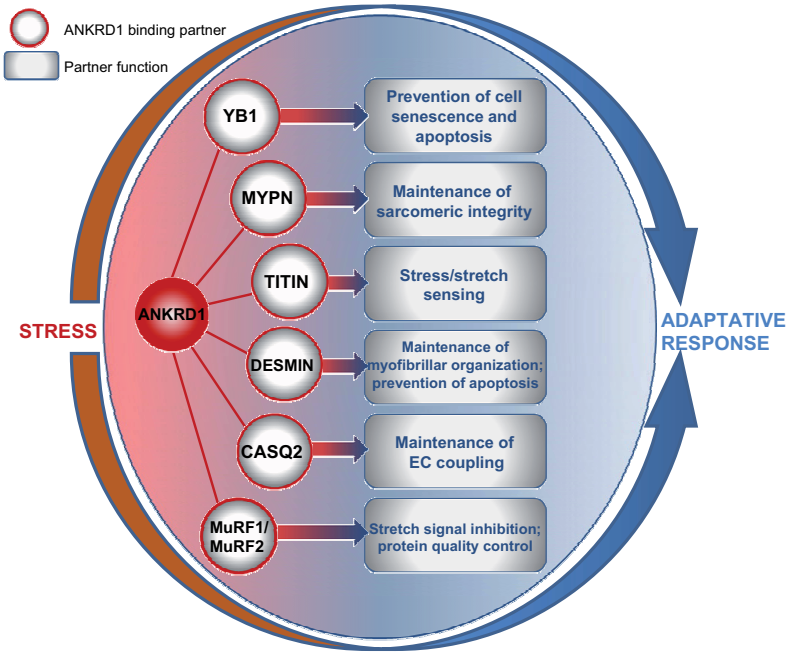

Fig. 3. ANKRD1/CARP as an integral part of cardiomyocyte stress-response circuits. *The direct interactions of ANKRD1/CARP with its binding partners suggest how this factor may be involved in stress response pathways. YB-1, Y-box transcriptional factor 1; MYPN, myopalladin; CASQ2, cardiac calsequestrin. MuRF1/MuRF2, muscle-specific RING finger 1 and 2 ubiquitin ligases; EC, excitation-contraction.*

alterations were detected in skeletal muscles. This outcome seems not to be ascribed to a redundancy within this protein family, because *ankrd1* removal is not associated with compensatory upregulation of the two remaining MARPs in muscle tissues (Barash *et al.*, 2007). Given that *ankrd1* is involved in muscle cell response to oxidative stress (Avivi *et al.*, 2006), we suggest that the phenotype of the *ankrd1* knockout might become apparent when the mice are exposed to hypoxic conditions.

Forced *ankrd1* expression in rat embryonic H9c2 cardiomyoblasts increased their resistance to hypoxia-induced apoptosis (Han *et al.*, 2005). These observations are consistent with the hypothesis that ANKRD1/CARP plays a role in the protection of human microvascular endothelial cells (MVECs) from stress-induced apoptosis. In fact, *ankrd1* transduction of Dox-treated MVECs increased cell survival that correlated with a decrease in caspase activity (Samaras *et al.*, 2007)[5]. In cardiac muscle, the inhibition of gene expression by Dox has been linked to reduced activity of *Nkx2.5*, which protects heart from Dox through controlling transcriptional homeostasis of its cardio-restricted target genes, *ankrd1* and *anf* (Toko *et al.*, 2002). In support of this model is the observation that suppression of *ankrd1* expression in adult rat ventricular cardiomyocytes results in myofibrillar disarray similar to that of Dox-treated cells (Chen *et al.*, 2006). In addition, rapid and significant *ankrd1* induction was observed after the passive stretch of beating fetal rat cardiomyocytes *in vitro*, indicating that the gene might be involved in the adaptive response of the myocardium to sarcomeric stretch-induced stress (Miller *et al.*, 2003).

As mentioned above, ANKRD1/CARP can interacts with other proteins with a variety of functions related to prevention of premature cell senescence and apoptosis (YB-1), management of sarcomeric integrity (myopalladin) and myofibril orientation (desmin), biomechanical stress sensing (titin), calcium handling (CASQ-2), and control of protein quality (MuRF1/MuRF2) (Fig. 3). Consequently, it is crucial for our further understanding of the functions of ANKRD1/CARP to elucidate these interactions and the effects that ANKRD1/CARP has on each of these molecules. The recent report found that ANKRD1/CARP is a natural caspase-3 substrate, carrying at least one putative caspase-3 cleavage site at its N-terminus (Ju *et al.*, 2007). This cleavage site is located to one of the two titin-binding sequences identified in ANKRD1/CARP (see Fig. 1). Although this question has not been addressed *in vivo*, this finding suggests that: (1) ANKRD1/CARP can be one of apoptotic caspase-3 targets in cardiac muscle and (2) caspase-3-mediated cleavage of ANKRD1/CARP may affect its interaction with titin and, probably, with myopalladin.

The recent crop of results from *ankrd1* expression association studies might seem like a sudden development. However, this blooming follows a relatively long period during which the primary concepts were developed and assembled. Insights into the function of ANKRD1/CARP originated from early studies on its inhibitory activity in functional promoter-reporter assays *in vitro*. Later, the interest has been focused on *ankrd1* regulation at various stress conditions *in vivo*, including hemodynamic overload, cardiac hypertrophy and ischemia. A close examination of the findings strongly suggests that stress-induced activation of the *ankrd1* gene is not only associated with a significant myocardial remodeling, but also involved in different signaling pathways that optimize response of cardiomyocytes to stress.

Prospects

Overexpression of ANKRD1/CARP in myocardium *in vivo* suggests that this factor may significantly contribute to physiological and pathological heart remodeling. In the last few years, there has been certain progress in understanding the physiological and pathological roles of ANKRD1/CARP in the heart. It is surprising, however, that most investigations have focused almost exclusively on the induction of *ankrd1* expression in *in vitro* cultured primary cardiomyocytes or non-relevant cell lines. Thus, ANKRD1/CARP-mediated pathways in cardiomyocytes in *in vivo* settings remain elusive. After a relatively long stagnation, we now need to probe *in vivo*, using target downregulation assays, if *ankrd1* overexpression reflects this intrinsic role as a cardiac adaptive stress-response reaction. It is also hoped that the biological importance of ANKRD1/CARP will be revealed by the generation of transgenic animals exhibiting ANKRD1/CARP overexpression in the heart. Although overexpression models produce non-

Note 5: Ventricular myocytes are surrounded by MVECs. Besides promoting blood flow, MVECs can modulate cardiomyocyte structure and function.

physiological levels of transcript and protein in the target tissue, they can still provide valuable insights into normal gene function *in vivo*. A modification of the transgenic approach to block the action of a target ANKRD1/CARP protein through the use of transgenes encoding dominant negative ANKRD1/CARP protein variants or using transgenic RNA interference approaches can provide useful information in cases where a total *ankrd1* gene knockout results in subtle or no alterations.

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