Cardiac-specific deletion of the microtubule-binding protein CENP-F causes dilated cardiomyopathy

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SUMMARY
CENP-F is a large multifunctional protein with demonstrated regulatory roles in cell proliferation, vesicular transport and cell shape through its association with the microtubule (MT) network. Until now, analysis of CENP-F has been limited to in vitro analysis. Here, using a Cre-loxP system, we report the in vivo disruption of CENP-F gene function in murine cardiomyocytes, a cell type displaying high levels of CENP-F expression. Loss of CENP-F function in developing myocytes leads to decreased cell division, blunting of trabeculation and an initially smaller, thin-walled heart. Still, embryos are born at predicted mendelian ratios on an outbred background. After birth, hearts lacking CENP-F display disruption of their intercalated discs and loss of MT integrity particularly at the costamere; these two structures are essential for cell coupling/electrical conduction and force transduction in the heart. Inhibition of myocyte proliferation and cell coupling as well as loss of MT maintenance is consistent with previous reports of generalized CENP-F function in isolated cells. One hundred percent of these animals develop progressive dilated cardiomyopathy with heart block and scarring, and there is a 20% mortality rate. Importantly, although it has long been postulated that the MT cytoskeleton plays a role in the development of heart disease, this study is the first to reveal a direct genetic link between disruption of this network and cardiomyopathy. Finally, this study has broad implications for development and disease because CENP-F loss of function affects a diverse array of cell-type-specific activities in other organs.

INTRODUCTION
Cardiomyopathies are diseases of the myocardium. Classically, cardiomyopathies are divided into three categories on the basis of the phenotype of the diseased ventricles: hypertrophic, dilated or restrictive (Franz et al., 2001; Seidman and Seidman, 2001). Both hypertrophy and dilation of the ventricle can be beneficial initial adaptations to cardiac stress such as pressure or volume loading, but in cardiomyopathy such processes become excessive and maladaptive, causing ventricular dysfunction (Schafer et al., 1991). There are numerous causes of cardiomyopathy, and more recent classifications of this disease focus on primary vs secondary cardiomyopathy, depending on whether the disease is restricted to the cardiac muscle (Seidman and Seidman, 2001; Maron et al., 2006). Single gene defects in sarcomeric or other cardiac muscle proteins are important causes of primary cardiomyopathy. Most of these gene defects cause hypertrophic forms of cardiomyopathy, but others can cause either hypertrophic or dilated cardiomyopathy, depending on genetic background or on the specific function of the protein that is affected by the mutation (Franz et al., 2001; Olson et al., 2001; Seidman and Seidman, 2001; Carniel et al., 2005). For example, mutations in α-cardiac actin cause hypertrophic cardiomyopathy when they affect actin-myosin interaction (which generates the force of contraction), but cause dilated cardiomyopathy when they affect interactions between actin thin filaments and myocellular proteins outside the sarcomere (which generate transmission of force) (Olson et al., 1998). Other examples of single gene defects that can lead to dilated cardiomyopathy include mutations in α-tropomyosin (Olson et al., 2001), vinculin (Olson et al., 2002), sarcoglycan (Tsubata et al., 2000), desmin (Li et al., 1999), titin (Gerull et al., 2002) and actinin (Mohapatra et al., 2003). All lead to impaired interaction between the sarcomere and the cytoskeleton. Interestingly, although myofibrils form connections to surrounding microtubules (MTs) and MTs are implicated in sarcomere development as well as in the regulation of mitosis and vesicular transport, we find no reports associating defects in the cardiac MT network with dilated cardiomyopathy (Dellefave and McNally, 2010).

CENP-F is a large multifunctional protein associated with the MT network. In the embryonic mouse, CENP-F protein expression is ubiquitous, although its expression is highest in the heart and brain (Goodwin et al., 1999). In a serial BrdU labeling assay of cardiac morphogenesis, high-level CENP-F expression was shown to be abruptly downregulated after neonatal day 6. This coincided precisely with cessation of myocyte cell division (Soonpaa et al., 1996; Soonpaa et al., 1997; Goodwin et al., 1999; Dees et al., 2005). Low levels of this gene product are detected in the adult heart. Although a causal relationship was not established, it is of interest that a recent screen of transcriptional profiling in human end-stage dilated cardiomyopathies identified CENP-F as being downregulated 2.3-fold compared with its expression in control hearts (Colak et al., 2009). This intriguing expression pattern, and its link with cardiac disease, argues strongly for studying the effects of a cardiac-specific deletion of the CENP-F protein.

The multiple functional roles for CENP-F also support this strategy. CENP-F is an MT-interacting protein, and was first
described in cancer cell lines as a component of the outer
kinetochore and as a binding partner of the retinoblastoma (Rb)
protein (Rattner et al., 1993; Liao et al., 1995; Zhu et al., 1995).
Several studies show that CENP-F stabilizes the attachment of MTs
to the kinetochore in mitotic cells. Disruption of CENP-F in vitro
causes mitotic delay, with failure of full kinetochore assembly and
misalignment of chromosomes in a subset of cells during mitosis
(Bomont et al., 2005; Feng et al., 2006). Additionally, CENP-F binds
and inhibits ATF-4, a transcription factor linked to cellular
proliferation and differentiation (Zhou et al., 2005). Although these
and other in vitro studies link CENP-F to mitosis, disrupting CENP-
F only delays but does not completely disrupt progression of mitosis
(Ashe et al., 2004; Feng et al., 2006; Evans et al., 2007). CENP-F
interaction with Rb protein might be predicted to affect cell cycle
progression; however, studies in murine embryonic stem (ES) cells
and in avian myocyte lines suggest primary roles of this protein
complex in differentiation instead (Papadimou et al., 2005;
Robertson et al., 2008). Thus, the role of CENP-F in mitosis is
currently complex and, until now, all data implicating CENP-F in the
regulation of cell proliferation has been through biochemical and
in vitro studies.

Further studies have indicated broader functions for CENP-F
and MTs beyond the limited role in mitosis. These include
regulation of cell shape through interactions with the MT-binding
protein Nucl (Soukoulis et al., 2005) and the centrosomal-binding
protein Hook2 (Moynihan et al., 2009). Additionally, we have
demonstrated that CENP-F directs protein trafficking through its
interaction with syntaxin 4 and SNAP25, physically linking the MT
network with transport vesicles (Pooley et al., 2006; Pooley et al.,
2008). Specifically, using dye transfer studies, disruption of CENP-
F function inhibits trafficking of connexins, resulting in loss of cell-
cell coupling (Pooley et al., 2008). Connexins, in particular
connexin-43, are crucial for cell-cell transmission of the cardiac
action potential (Gollob, 2006; Shaw et al., 2007).

Here we report the first in vivo disruption of CENP-F gene
function. In these studies, we generated a mouse model utilizing the
Cre-loxP system to ablate CENP-F protein expression in a
cardiomyocyte-specific manner (termed CENP-F+/−, hereafter).
Although the overall structure of the CENP-F+/− heart was preserved,
these hearts were smaller with completely consistent changes in
morphogenesis, including decreased myocyte proliferation, thinner
chamber walls and blunted ventricular trabeculation. In the adult,
CENP-F+/− hearts had a significant reduction in the number of
intercalated discs characterized by diminished accumulation of
connexin-43 and interruption of myocyte-myocyte coupling. The
MT network of CENP-F+/− myocytes, particularly costamere-
associated tubules, was also disrupted. All of these defects are
consistent with the reported functions of CENP-F. Importantly, these
hearts invariably developed abnormal cardiac physiology, including
progressive dilated cardiomyopathy with fibrosis, heart block and a
pattern of gene expression consistent with this disease. Although it
has long been postulated that the MT cytoskeleton plays a role in
the development of heart disease, this is the first study to reveal a
direct genetic link between disruption of an MT-associated protein
and cardiomyopathy.

RESULTS
CENP-F is successfully excised by cTNT-Cre to generate a
cardiomyocyte-specific knockout
Attempts to create a globally targeted deletion of CENP-F resulted in
ES cells with significant proliferative defects such that the lines
could not be expanded (results not shown). This is consistent with
results reported by others that CENP-F knockdown in pre-
implantation bovine embryos arrest at the eight-cell stage
(Toralova et al., 2009). Therefore, we adopted a Cre-loxP approach to study
CENP-F gene function on an outbred (CD-1) mouse background.

Given the high expression of murine CENP-F in the developing
heart (Fig. 1A), mice with the first five exons of CENP-F flanked
by loxP sites (Fig. 1B) were crossed with a cardiac troponin T
(cTNT)-Cre mouse to generate a cardiomyocyte-specific targeted
deletion of CENP-F. The floxed region contains the functional
binding domains for syntaxin 4, Hook2 and SNAP25 (Pooley et al.,
2006).

Fig. 1. Cardiomyocyte-specific disruption of the
CENP-F gene. (A) In situ hybridization of an E11.5 mouse
embryo with an antisense CENP-F probe demonstrates the
distribution of CENP-F transcripts. CENP-F is
ubiquitous in the developing embryo but the highest
levels of expression are observed in the heart (arrow)
and regions of the brain. (B) A schematic presentation of
the targeted CENP-F gene with the location of loxP sites
marked by yellow arrowheads. Gray boxes indicate
CENP-F exons. The first five exons of CENP-F are removed
when crossed with the cTNT-Cre mouse line expressing
Cre recombinase in cardiomyocytes beginning at E7.5.
(C,D) Genomic PCR confirms Cre-mediated excision of
the floxed CENP-F gene segment. Lanes are as labeled. In
panel C, primers flank the 5′ loxP site and show different
sized transcripts for WT and loxP-containing genes. In
panel D, primers flank both loxP sites and amplify a
product only when recombination has occurred (only in
the heart). (E-H) CENP-F protein expression in WT (E,F)
and CENP-F−/− (G,H) heart. Anti-CENP-F antibody staining
is absent in CENP-F−/− cardiomyocytes, but retained in
the overlying epicardium, where cTNT does not
recombine (G,H; arrow).
Genotyping confirmed successful genomic incorporation of the 5’ loxP sites in CENP-FloxP mice (606 bp band) compared with wild type (WT; 572 bp band). The heterozygote exhibited both bands, as shown in Fig. 1C (first three lanes).

CENP-F ablation is specific to the heart in eTNT-Cre;CENP-FloxP mice (designated CENP-F–/– hereafter), as seen in PCR analysis with primers flanking the 5’ loxP site (Fig. 1C,D). The remainder of Fig. 1C, labeled ‘tail’ and ‘heart’, demonstrates the disappearance of the floxed band with the expression of Cre in the heart, whereas tail samples from the same mice show that the floxed allele remains intact. After Cre-mediated excision, a recombination band appears when primers flanking both loxP sites are used with the same DNA samples (Fig. 1D).

Given that our targeting strategy generated a partial deletion of the CENP-F gene, it was important to verify that expression of CENP-F protein was ablated in the hearts of CENP-F–/– mice. Fig. 1E-H demonstrates the difference in CENP-F protein expression when comparing 1-day-old (P1) WT and CENP-F–/– heart sections using an anti-CENP-F antibody. This antibody was generated against an epitope in exon 11, well downstream of the deleted sequence. In WT hearts, widely distributed CENP-F expression is observed throughout the myocardium (Fig. 1E,F), whereas absence is evident in the myocardium of CENP-F–/– mice (Fig. 1G,H). However, as an internal positive control, expression is visualized in the epicardium of the CENP-F–/– heart owing to the myocyte-specific nature of cTNT-Cre activity. Another commercially available antibody confirmed CENP-F ablation (data not shown). Taken together, these data demonstrate the cardiomyocyte-specific deletion of CENP-F.

**CENP-F–/– hearts exhibit morphogenic abnormalities**

Grossly, the heart structures of CENP-F–/– mice developed normally, including valves, endocardium and vessels. However, the ventricles of CENP-F–/– hearts were smaller and appeared distinctly more angular throughout embryonic life. This characteristic was observed in 100% of cases, and is shown in Fig. 2A. Additionally, a modest angular difference was not significantly different, although there was a trend for CENP-F–/– hearts to be 30-40% smaller (mean ± s.d.; P<0.05).

Histological examination of WT and CENP-F–/– hearts revealed that the atrial and ventricular walls on both left and right sides were invariably thinner in the CENP-F–/– heart at E12.5 and thereafter compared with controls (Fig. 2C and supplementary material Fig. S1A,B). Quantification of these data in the ventricular wall at E12.5 is given in Fig. 2D. Additionally, clear differences in trabeculation of CENP-F–/– hearts were observed. These structures were thinner and blunter when compared with WT counterparts. These differences in heart wall thickness and trabeculation persisted throughout the embryonic period. On the CD-1 background, there was no apparent in utero loss of CENP-F–/– pups. WT, heterozygotes and CENP-F–/– mice were born in expected mendelian ratios (see supplementary material Table S1).

**CENP-F–/– hearts exhibit decreased proliferation in the prenatal and neonatal periods**

Overall wall thickness and trabeculation in CENP-F–/– hearts was attenuated. A possible explanation for this observation would be impaired cardiomyocyte proliferation. Because high-level CENP-F expression parallels the period of cardiomyocyte mitosis in the heart, it is thought that CENP-F expression parallels the period of cardiomyocyte mitosis and CENP-F is thought to regulate cell proliferation in vitro, we examined the impact of CENP-F loss of function on myocyte proliferation. Prenatally, there is a threefold decrease in myocyte proliferation in CENP-F–/– hearts compared with WT (supplementary material Fig. S2). Postnatally, we examined the pattern of BrdU incorporation daily over a period of 7 days. This corresponds to the time during which high-level CENP-F expression is downregulated and mitotic rates sharply decline (Dees et al., 2005). Compared with WT hearts, CENP-F–/– hearts had significantly lower levels of BrdU incorporation on P1-P4, but equal levels on P5-P7 (Fig. 3). Phosphohistone H3 analysis of mitosis over this same timeframe confirmed the decrease in mitotic activity in CENP-F–/– hearts (Fig. 3). Analysis of apoptosis with TUNEL staining showed no significant difference between WT and knockout (KO) hearts (data not shown). These data suggest a positive effect of CENP-F on cardiomyocyte mitosis in the normal embryonic and neonatal heart, up until the time that high-level expression of CENP-F has been shown to decline. These results demonstrate that CENP-F disruption in the developing heart leads to decreased cardiomyocyte proliferation.
to decreased myocyte proliferation, reduced trabeculation, and a smaller and thin-walled heart. Thus, loss of CENP-F function consistently leads to reproducible changes in cardiac morphogenesis. With this information, we next determined whether the differentiated heart was susceptible to disease states.

Adult CENP-F<sup>-/-</sup> hearts have fewer intercalated discs

CENP-F plays a central role in intracellular protein trafficking through its interaction with SNARE proteins (Pooley et al., 2008). Specifically, movement of connexins to gap junctions is dependent on CENP-F activity (Pooley et al., 2008). Because connexins are a crucial component of the intercalated disc and are required for regulation of electrical conduction between myocytes, we examined their presence at the disc using quantitative immunostaining (see Methods for protocol on disc quantification). In comparison to WT hearts (Fig. 4A), CENP-F<sup>-/-</sup> hearts (Fig. 4B) exhibited a 3.5-fold decrease in total intercalated disc number in the mature ventricle (Fig. 4C; \( P \leq 0.001 \)). Not only were differences in overall disc number observed, but connexin-43 staining in CENP-F<sup>-/-</sup> hearts was often punctate and not organized into a ‘disc-like’ structure. Additionally, the accumulation of Bves, a protein that is also enriched at the intercalated disc (Perriard et al., 2003; Smith and Bader, 2007), was significantly diminished in CENP-F<sup>-/-</sup> hearts (supplementary material Fig. S3E,F). N-cadherin accumulation at the cell surface and disc was also severely inhibited (supplementary material Fig. S3A-D). These data indicate that loss of CENP-F function has a generalized effect on intercalated disc structure and function, and could directly relate to the conduction defects observed in electrocardiographic (ECG) analysis (see below).

CENP-F<sup>-/-</sup> myocytes have significant deficiencies in costamere structure

The costamere is essential for the transmission of mechanical force from the myofibril to the connective tissue matrix, and thus plays an important role in heart function (Pardo et al., 1983b; Pardo et al., 1983a; Anastasi et al., 2009). MTs are associated with these structures and are thought to play a role in stabilization of the myofibril and cell membrane (Prins et al., 2009). Because CENP-F is an MT-binding protein and is an important regulator of the MT network, we examined CENP-F<sup>-/-</sup> hearts for MT integrity. By confocal microscopy, we detected a qualitative decrease in the amount of tubulin accumulation in myocytes using an anti-tubulin antibody that detects all forms of the protein. These data are shown in Fig. 5A–D. At higher power, examination of WT hearts with anti-tubulin antibodies revealed the characteristic ribbed pattern of MT staining, which is associated with costameres, perpendicular to the long axis of myofibrils (three consecutive sections of a confocal z-stack focused on the myocyte cell surface are given in Fig. 5E-H). By contrast, confocal analysis through multiple sections determined that CENP-F<sup>-/-</sup> myocytes were completely devoid of this organized pattern of MT staining (Fig. 5I–L). Taken together, these data demonstrate that costamere structure is severely impaired by loss of CENP-F function, and suggest that defects in cardiac contractility might be observed.

CENP-F<sup>-/-</sup> hearts develop dilated cardiomyopathy

Dilated cardiomyopathies have not been linked to MT dysfunction, but disruption of the costamere or intercalated disc has been associated with this disease (Perriard et al., 2003; Anastasi et al., 2009; Prins et al., 2009; Geisler et al., 2010). Because both of these structures are severely compromised by loss of CENP-F function, CENP-F<sup>-/-</sup> and WT littermates were serially analyzed for cardiac function from 4 to 26 weeks after birth. No differences in body weight or heart rate were detected between experimental and control groups. Serial conscious transthoracic echocardiography was performed at 1- to 4-week intervals. Typical M-mode images are shown in Fig. 6A,B. The diastolic and systolic dimensions are as labeled with arrows (red, diastolic;
yellow systolic). These data show decreasing left ventricular systolic function of CENP-F–/– mice with age (Fig. 6D). The left ventricular posterior wall thickness was unaffected (not shown), whereas the left ventricular internal dimension in diastole was mildly increased (Fig. 6C). Thus, CENP-F–/– mice developed a progressive form of dilated cardiomyopathy. These longitudinal studies showed full or 100% penetrance of this functional phenotype in the CENP-F–/– animal model.

**CENP-F–/– hearts develop arrhythmias**

CENP-F–/– cardiomyocytes showed a marked decrease in the number of intercalated discs and a diminution in the accumulation of disc-related proteins. Thus, we assessed the conduction system of WT and CENP-F–/– hearts using continuous ECG recordings. These recordings were performed at 3 months of age, in the early stages of the cardiomyopathy detailed above. CENP-F–/– hearts displayed prolonged PR intervals, consistent with first degree heart block (not shown). Furthermore, as shown in Fig. 6E, there were rare instances of second degree heart block in CENP-F–/– animals; this was never observed in controls. More frequently, we observed sinus slowing, defined as P-P intervals greater than 200 ms, with 1:1 atrioventricular (AV) conduction, suggestive of sinus node dysfunction (Fig. 6F). This finding was observed in 4/5 CENP-F–/– animals studied, and in no controls. These data clearly demonstrate impaired cardiac conductivity, developing concurrently with dilated cardiomyopathy, with loss of CENP-F function. This is probably a consequence of the reduced integrity of the intercalated discs, either as a direct result of disrupted CENP-F-mediated trafficking of connexin proteins to the intercalated disc, or as a secondary phenomenon to intercalated disc deterioration and fibrosis.

**CENP-F–/– hearts are fibrotic and enlarged in adult mice**

Gross anatomical examination revealed a progressive dilation of CENP-F–/– hearts in comparison with WT hearts. Echo data from these mice at 1 year of age are shown in supplementary material Fig. S4 and demonstrate depressed left ventricular function. Masson’s trichrome staining demonstrated fibrosis throughout the CENP-F–/– heart (Fig. 7). This fibrosis was often continuous with the epicardium and these areas could be seen in whole-mount as nodules on the surface of the CENP-F–/– heart (Fig. 7G). Additionally, there were fewer myocytes in CENP-F–/– hearts compared with WT hearts, as determined by quantification of myosin-positive cells by immunostaining (supplementary material Fig. S5). This probably reflects lower myocyte numbers from the newborn period, with additional losses related to fibrosis. During longitudinal follow-up over a 9-month period of analysis, the experimental animal population had a 20% mortality rate, with no death of WT animals. These deaths were sudden with no sentinel symptoms of illness. Thus, disruption of CENP-F in cardiomyocytes leads to fibrosis, arrhythmia, and progressive ventricular dilation and dysfunction – a phenotype with all the hallmarks of dilated cardiomyopathy.

**Transcriptional profiling reveals both expected and novel patterns for diluted cardiomyopathy**

To obtain a broad perspective on differences between CENP-F–/– and WT hearts, we performed microarray analysis on hearts from 1-year-old mice. Principal component analyses of these datasets confirmed minimal variability between the experimental replicates, with complete segregation between CENP-F–/– and WT hearts (Fig. 8A). CENP-F–/– and WT hearts exhibited differential expression of 528 probes, of which 192 were upregulated and 336 were downregulated in CENP-F–/– hearts (Fig. 8B). The 528 probes represented a total of 251 genes, 101 of which were upregulated and 150 downregulated in CENP-F–/– hearts. Of the total number of genes, 123 matched at least one other published report on altered gene expression in dilated cardiomyopathy in humans or in animal models (Barth et al., 2006; Muchir et al., 2007; Oyama et al., 2008; Colak et al., 2009; Pretorius et al., 2009). This is shown in the heat map in Fig. 8C and in supplementary material Table S2. Importantly, CENP-F is among the genes reported to be downregulated in human end-stage dilated cardiomyopathy, corroborating our model of cardiomyopathy in CENP-F–/– hearts. These data also demonstrate that our mouse model of cardiomyopathy recapitulates many of the genetic alterations described in dilated cardiomyopathies of other causes. To look for novel patterns of gene expression, we performed analyses using the Partek, DAVID and GSEA databases. Signaling pathways that emerged from these analyses included MAPK signaling (ten genes; \( P=0.012 \)) and ubiquitin-mediated proteolysis (six genes; \( P=0.042 \)). Transcription factor pathways with significant representation among our data included C/EBP delta (seven genes; \( P=1.01 \times 10^{-2} \)) and serum response factor (SRF; seven genes \( P=1.95 \times 10^{-2} \)). Interestingly, one of the SRF-related genes was Mir133a-1, a microRNA that was downregulated in CENP-F–/– hearts and has been shown to increase arrhythmogenesis in congestive heart failure (Belevych et al., 2011). Given the arrhythmias demonstrated in mice with CENP-F–/– hearts, we looked for ion channel genes that were altered. No overt alteration of sodium channel gene...
expression was identified to directly explain the arrhythmias observed in KO mice. Several channel-related genes were altered, however. Ahnak2 is linked to L-type calcium channels and localizes to the Z band of murine cardiomyocytes (Komuro et al., 2004). Two isoforms of a potassium-channel-interacting protein were altered in CENP-F–/– hearts, one upregulated (Kcnip1) and one downregulated (Kcnip4). These related but distinct proteins have been shown to affect trafficking of the Kv4 potassium channel to the cardiomyocyte membrane, and seem to be major determinants of potassium channel stability (Shibata et al., 2003). Finally, multiple probes demonstrated downregulation of intronic sequences in the Dlg1 gene, encoding synapse-associated protein (SAP)97, which is involved in targeting and anchoring various cardiac ion channels to the plasma membrane. Channels regulated by SAP97 include Kv4 potassium channels, discussed above (El-Haou et al., 2009), and cardiac sodium channels, where it targets channels to the intercalated disc rather than to lateral membranes (Petitprez et al., 2011). Taken together, transcriptional profiling analysis in conjunction with our structural and physiological studies established that loss of CENP-F function results in dilated cardiomyopathy.

**DISCUSSION**

CENP-F is a very large multifunctional protein that has roles in divergent cell properties, including proliferation, protein trafficking and regulation of the cytoskeleton (Yang et al., 2003; Ashe et al., 2004; Bomont et al., 2005; Dees et al., 2005; Papadimou et al., 2005; Soukoulis et al., 2005; Feng et al., 2006; Evans et al., 2007; Pooley et al., 2008; Moynihan et al., 2009). Until now, CENP-F loss of function has only been studied in vitro using knockdown or dominant-negative technologies. Here, for the first time, we analyze loss of gene function in vivo and find that CENP-F, through its interaction with the MT network, plays a crucial role in heart development as well as in adult cardiac function. Importantly, this study is the first to link disruption of MT-associated function with the development of dilated cardiomyopathy, thereby identifying a novel disease mechanism. Finally, this study has broad implications for development and disease because CENP-F loss of function is likely to impact a diverse array of cell-specific activities.

**MT functions in the heart**

Thus far, the majority of cytoskeletal research in the heart has focused on the actin network. However, the MT network also plays a crucial role in heart maintenance and function. For example, MT-binding protein EB1 has recently been shown to mediate trafficking of Cx43, an essential component of the intercalated disc, to the plasma membrane (Smyth et al., 2010). Additionally, several studies in multiple animal models have observed an increase in MT network density during pressure overload induced by aortic banding (Collins et al., 1996; Ishibashi et al., 1996; Tagawa et al., 1998; Wang et al., 1999; Koide et al., 2000). A functional disturbance similar to pressure overload can be replicated by application of MT stabilizers such as Taxol (Rabkin and Sunga, 1987; Tagawa et al., 1997; Palmer et al., 1998; Takahashi et al., 1998). Conversely, MT depolymerization improves contractile function in pressure overload hypertrophy (Hein et al., 2000). These results suggest a major role for the MT network in cytoskeletal plasticity as the heart transitions from a state of hypertrophy to failure (Wang et al., 1999; White, 2011). MTs are also implicated in the pathogenesis of arrhythmias associated with cardiac hypertrophy, although the mechanisms behind this are not clear (Parker et al., 2001; White, 2011).

Recent reviews of cardiac disease have identified classes of genes, with those of the actin cytoskeleton being most prominent among
them, whose dysfunction leads to dilated cardiomyopathy (Sheikh et al., 2006; Gustafson-Wagner et al., 2007; Purevjav et al., 2010; Nikolova-Krstevski et al., 2011). Although it is clear that the MT network impacts both development and function of the heart, direct linkage of any MT-network-associated gene product to cardiac disease had not yet been established (Dellefave and McNally, 2010). Here, we show that loss of an MT-binding protein, CENP-F, in the heart results in dilated cardiomyopathy. Our physiological, morphological and molecular data are consistent with established patterns for dilated cardiomyopathy. However, CENP-F–/– hearts have a unique combination of abnormalities, including reduced mitotic activity during embryogenesis, loss of intercalated disc structure and disruption of costameres, that underline the phenotype of dilated cardiomyopathy. Our physiological, morphological and molecular data are consistent with established patterns for dilated cardiomyopathy. However, CENP-F–/– hearts have a unique combination of abnormalities, including reduced mitotic activity during embryogenesis, loss of intercalated disc structure and disruption of costameres, that underline the phenotype of dilated cardiomyopathy. All of these abnormalities can be predicted from the functions of CENP-F that are manifested in cardiomyocytes. Finally, it is of interest to note that our review and analysis of human dilated cardiomyopathy microarrays revealed a significant decrease in CENP-F expression associated with the disease (Colak et al., 2009). Although this does not establish a causal effect from loss of CENP-F expression, the current data along with the established role of the MT network in cardiomyocytes would suggest that cardiomyopathies currently described as idiopathic would arise from disruption of this crucial and multifunctional cytoskeletal structure.

**CENP-F loss of function reduces mitotic activity**

Work from our group and others has demonstrated that CENP-F is important for the regulation of DNA synthesis and cell division (Ashe et al., 2004; Bomont et al., 2005; Dees et al., 2005; Feng et al., 2006; Evans et al., 2007). All of these studies showing a reduction but not elimination of cell proliferation are consistent with our current in vivo data. CENP-F–/– mice exhibit decreased cardiomyocyte proliferation in the embryo and during the first week of life, precisely paralleling the high-level expression pattern of CENP-F (Dees et al., 2005). The thinning of the myocardial wall in CENP-F–/– hearts is presumably the result of decreased myocyte

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**Fig. 6. Mice with CENP-F–/– hearts develop dilated cardiomyopathy and dysrhythmias.** (A,B) M-mode ECG images from unanesthetized WT and CENP-F–/– mice, showing measurement of left ventricular internal dimension in diastole (LVIdd; red) and in systole (LVIDs; yellow). (C) Graph of LVIDd from WT (blue/circles) and CENP-F–/– (pink/squares) mice at ages as shown. CENP-F–/– mice show mild but progressive dilation over time, compared with WT. (D) Graph of cardiac function calculated as shortening fraction, or (LVIDd-LVIDs)/LVIDd, in WT (blue/circles) and CENP-F–/– (pink/squares) mice. CENP-F–/– mice show progressive functional impairment over time, compared with WT. (E,F) Examples of rhythm disturbances observed in CENP-F–/– mice. In E, there is a P wave (‘j’) that does not conduct, whereas the others do (‘N’), representing an episode of second degree heart block. In F, there is slowing of the sinus rate with preserved AV conduction, representing sinus node dysfunction.

**Fig. 7. Adult CENP-F–/– hearts are fibrotic and enlarged.** (A-F) Masson’s trichrome stain of 12-month-old WT (A,C,E) and CENP-F–/– (B,D,F) hearts in cross-section. Low-power magnification shows coronary enlargement in CENP-F–/– mice (B) as compared with WT (A). High-power magnification reveals significant fibrosis (blue) in CENP-F–/– hearts (D,F) as compared with WT (C,E). (G,H) Two whole-mount views of WT and CENP-F–/– hearts. CENP-F–/– hearts have nodular surfaces (G; right) and are significantly enlarged compared with WT. Scale bars: 200 μm.
proliferation and, in turn, renders the organ susceptible to dilation and disease. The decreased number of cardiomyocytes within the heart is likely to contribute to the development of dysfunction over time on the basis of a reduced ‘reserve’ of working cardiomyocytes.

CENP-F loss of function disrupts intercalated discs

Differentiated CENP-F–/– myocytes also have decreased and clearly abnormal intercalated discs. The intercalated disc is a complex, cardiac-specific structure that regulates cell-cell communication and force transduction between myocytes, and is functionally analogous to cell-cell junctions in epithelia (Perriard et al., 2003; Geisler et al., 2010). Targeted deletion of the non-receptor protein tyrosine kinase focal adhesion kinase (FAK), a principal component of the costamere, causes dilated cardiomyopathy in mice and abnormal patterns of hypertrophy in response to stress (Peng et al., 2008; Chu et al., 2011). Interestingly CENP-F, although not a component of the disc, is essential for the maintenance of its structure and function, because transmembrane proteins, including N-cadherin, Bves and connexin-43, were significantly decreased at the disc in CENP-F–/– myocytes when compared with WT myocytes. Our previous work has shown that intracellular trafficking of integral membrane proteins to the cell surface is dependent on CENP-F. CENP-F interacts with SNAPs and syntaxins of the SNARE complex and provides a physical link between this trafficking machinery and the MT network (Pooley et al., 2006; Pooley et al., 2008). In particular, we demonstrated that connexin-43 translocation to the cell surface is regulated by CENP-F (Pooley et al., 2008). The reduction of N-cadherin, Bves and connexin-43 is of particular interest because these three proteins have diverse functions at the disc in the regulation of force and electrical transduction. The loss of an MT-binding protein represents a mechanism underlying disruption of disc structure and/or function. Even though CENP-F is not a disc component, its loss clearly demonstrates a previously unknown mechanism for dilated cardiomyopathy, “a disease of the intercalated disc” (Perriard et al., 2003).

CENP-F loss of function disrupts costamere structure

Given previous work on the regulation of the MT network by CENP-F, disruption or loss of costamere structure are also consistent with loss of CENP-F function in cardiomyocytes. The costamere is a crucial component of the force transduction machinery within cardiomyocytes (Pardo et al., 1983b; Anastasi et al., 2009). Juxtaposed between the sarcolemma and the myofibril, this structure is essential for the efficient transmission of force from...
the myofibril to the sarcolemma to the connective tissue environment (Samarel, 2005). Structure of the MT network in CENP-F<sup>-/-</sup> cardiomyocytes is clearly altered, with the pattern of costamere-associated MTs being completely absent in these cells and an overall reduction in the amount of tubulin. The loss of costamere structure has been implicated in the loss of contractile function, a hallmark of CENP-F<sup>-/-</sup> hearts. Several costameric proteins are implicated in human dilated cardiomyopathy, including vinculin (Olson et al., 2002), sarcoglycan (Tsubata et al., 2000) and dystrophin (Muntoni et al., 1993; Towbin et al., 1993; Muntoni et al., 1995). Dystrophin-associated cardiomyopathy is the most common of these, in association with the X-linked disease Duchenne's muscular dystrophy. Duchenne's muscular dystrophy is characterized by progressive skeletal muscle weakness in early childhood, and with the development of slowly progressing dilated cardiomyopathy usually beginning in the teenage years (Towbin et al., 1993; Franz et al., 2001). Importantly, the Duchenne cardiomyopathy is also associated with diffuse and progressive cardiac conduction abnormalities (Sanyal and Johnson, 1982). The pathology of these hearts is characterized by thinning of the ventricular wall with fibrosis and scarring within the epicardium, a setting remarkably similar to what we observe in the CENP-F<sup>-/-</sup> end-stage heart.

**Delayed onset of cardiomyopathy**

Loss of CENP-F function in vivo results in a thin-walled heart, fewer intercalated discs and disruption of costamere-associated MTs. It is of interest to note that any one of these variations in cardiac structure could give rise to dilated cardiomyopathy. Thus, the dilated cardiomyopathy that we observe in CENP-F<sup>-/-</sup> mice is probably multifactorial in nature, even with the loss of only a single gene. We would note that the present study, although revealing the fundamental importance of CENP-F in organogenesis and adult heart function, does not determine whether adult onset of this cardiomyopathy is a disease of embryonic, neonatal or adult origin. Indeed, our data suggest that an embryonic 'hit' produces a structurally normal heart with fewer myocyte numbers and thinner walls. It is probable that ongoing stress in the mature heart, due to MT instability or dysfunction, then culminates in dilated cardiomyopathy. This is similar to the cardiomyopathy that develops in Duchenne's muscular dystrophy and many other causes of cardiomyopathies: a single gene defect present from the time of cardiogenesis results in dilated cardiomyopathy many years later. With information from this first in vivo analysis of cardiogenesis in CENP-F gene ablation, it is now possible to initiate loss of function at specific times in development and in adult life in an effort to pinpoint specific CENP-F functions that are essential for organ development and function, and whose absence leads to disease. Loss of CENP-F function leads to specific and, in retrospect, predictable changes in myocyte structure that suggest a generalized disruption of MT-network-associated activities within the cell. It will be important to determine whether other MT-associated proteins underlie developmental and disease states of the heart.

**METHODS**

**Developing the CENP-F<sup>-/-</sup> mouse**

BAC clones were used to analyze the 5′ region of the CENP-F gene and clone the gene segment into the pFRT-loxP vector (a gift from Mark Magnuson, Vanderbilt University, TN). This vector contains the loxP sites, the thymidine kinase negative-selection marker utilized for random vector integration events, and FRT sites used to delete the neomycin resistance cassette. This cloning strategy successfully isolated the first five exons of CENP-F, flanked by loxP sites. This targeting construct was electroporated into ES cells and resulting clones screened for recombination at the CENP-F locus. Two ES clones were positive for all sections of the screened locus, and were injected into blastocysts and placed in foster mothers. Founder pups were chosen by successful transmission in the germline, and progeny were backcrossed to the ICR background and maintained in this manner (Ryan D. Pooley, The role of LEK1 in recycling endosome trafficking and its function in heart development, PhD thesis, Vanderbilt University, 2006). We then crossed the CENP-F<sup>loxP/loxP</sup> mouse with cTNT-Cre mouse (a generous gift from Scott Baldwin, Vanderbilt University, TN) to target CENP-F deletion specifically in cardiomyocytes starting at embryonic day (E)7.5. All procedures involving experimental animals were performed in compliance with the Vanderbilt University Institutional Animal Care and Use Committee guidelines.

**Genotyping**

PCR genotyping was based on three features to distinguish WT CENP-F from Cre-mediated CENP-F exon 1-5 deletion: presence of the 5′ loxP site upstream of CENP-F; generation of a DNA band only being possible with recombination after Cre-mediated excision, and the presence of the cTNT-Cre gene. The primers used were: Across 5′ loxP site: 5′-AATAATGAGCTGACACC-AAAAACT-3′, 3′-GAACCTACCGTCCTGAGAACCCTG-5′; Recombination band: 5′-AATAATGAGCTGACACCAAAAACT-3′, 3′-GAGGAGCACAGGGAGGAATG-5′; cTNT-Cre: 5′-TCCGGGCTGCACGACCAA-3′, 3′-GGCGCGGGCAACCC-5′.

**Organ and tissue preparation for histochemical analysis**

Mouse hearts, both embryonic and adult, were isolated from the animal, imaged on a Zeiss Stemi 2000-C stereomicroscope with an Olympus camera (model no. 60806; Optrons) for gross anatomical analysis, fixed with methanol, and washed in PBS prior to placement in OCT compound (Sakura) and freezing. Frozen tissues were cryosectioned at a thickness of 7 μm. Successive sections were collected on glass slides and stored at ~20°C until immunohistochemical analysis. Frozen tissues slides were processed as previously described (Bader et al., 1982; Dees et al., 2005). Histological stains were visualized by fluorescence microscopy on an AX70 (Olympus) and digital images were captured and processed using Magnafire (Optrons) and Photoshop (Adobe) software. CENP-F labeling was conducted with a polyclonal antibody (Spec1) to CENP-F peptide (Soukoulis et al., 2005). Other antibodies include anti-phosphohistone-H3 (Millipore), rabbit polyclonal to N-cadherin (Abcam), DM1A (Abcam), MF20 (DSHB) and Alexa-Fluor-568–phalloidin (Invitrogen), and were used as previously described (Bader et al., 1982) or as per the manufacturer’s instructions. DAPI (Boehringer Mannheim) and TO-PRO-3 iodide (Invitrogen) were used to visualize nucleic material. Histological analysis with hematoxylin and eosin (H&E) and Masson's trichrome staining was standard.
E15.5 WT and CENP-F−/− heart sections were stained with DAPI and anti-PH3 antibodies. PH3-positive nuclei were quantified from 25 different fields of view (20× magnification) from multiple sections. For BrdU labeling, neonatal mice were subcutaneously injected with 0.1 ml BrdU labeling reagent per 100 g of body weight as per the manufacturer’s instructions (Roche). After 90 minutes the animals were sacrificed, and heart tissue processed and sectioned as above. Tissue fixation, and anti-BrdU primary and secondary antibody staining for this procedure were all performed using Roche reagents and protocols (Dees et al., 2005). In situ hybridization analyses of mouse embryos were conducted using published methods (Yutzey et al., 1994). Three prime coding and non-coding sequences (1.6 kb) of murine CENP-F were cloned into pGEM-T Easy, as described previously (Ashe et al., 2004). Antisense and sense probes were generated using T7 and SP6 polymerases, respectively. Hybridization, washing and antibody detection of probes were standard. No reaction was detected with sense controls.

**Quantification of intercalated discs**

Frozen heart sections were post-fixed in a 50:50 mixture of acetone and methanol for 10 minutes at –20°C. Sections were rehydrated and stained with monoclonal anti-connexin-43 (Sigma) and DAPI or TOPRO3 to stain the DNA. Secondary Alexa-Fluor-488 anti-mouse IgM antibodies (Invitrogen) were used to recognize connexin-43-enriched intercalated discs. Anti-Bves antiserum was also used to visualize intercalated discs (Smith and Bader, 2006). For quantification purposes, images were taken (Olympus Ax70 at 40× magnification) from 20 random fields of view on multiple sections and the number of discs present in each field was quantified. For standardization purposes, we identify a disc as a contiguous area positive for connexin-43 staining and greater than 5 μm in length. Representative confocal images were acquired on a Zeiss LSM 510 inverted microscope maintained by the Vanderbilt Cell Image Shared Resource Core. Quantification is shown as fold difference between WT and CENP-F−/− animals at 1 year of age.

**Cardiomyocyte quantification**

Frozen heart sections were stained with anti-MF20 and DAPI. MF20-positive cells were quantified from 30 different fields of view on multiple sections (20× magnification) for WT and CENP-F−/− hearts at 1 year of age.

**Echocardiography**

Imaging was performed using a Hewlett-Packard Sonos 5500 or a VisualSonics Vevo 2100 system, with a 12 MHz transducer in unanesthetized mice held in a left lateral decubitus position. Mice were imaged in a parasternal long axis view to study the septum, posterior wall, apex and left ventricular outflow tract, and in a short axis view at the chordal level to study symmetry of wall thickness and contraction (Rottman et al., 2007). From these studies, M-mode images were recorded on a strip chart at a speed of 100 mm/second. Dimensions from the M-mode tracings and the summary interpretation were entered in the database after review of the data quality, raw data and interpretation by a trained echocardiographer. This work was performed in the Vanderbilt University Murine Cardiovascular Core.

**Clinical issue**

Cardiomyopathies are diseases of the myocardium and can often be classified as hypertrophic, restrictive or dilated. Progress has been made in identifying the genetic basis of these life-threatening diseases, particularly of hypertrophic cardiomyopathy. Although genes that can cause either hypertrophic or dilated cardiomyopathy (depending on the mutation) have been identified, most cases of dilated cardiomyopathy remain idiopathic in terms of their genetic origin. Undoubtedly, some genetic defects that cause dilated cardiomyopathy involve proteins not yet clinically associated with this disease. Without a more complete understanding of the origin of this disease and how it disrupts heart structure and function, the design of new therapies will be limited.

**Results**

The authors characterized an in vivo cardiomyocyte-specific knockout of CENP-F, a large microtubule-associated protein with multiple functions, including the regulation of cell shape, mitosis and subcellular vesicles trafficking. At the onset of heart development, CENP-F−/− hearts were smaller, exhibited decreased myocyte proliferation and had thinner walls than age-matched controls. These hearts also contained fewer intercalated discs (the myocyte-specific junctional complexes responsible for electrical and force transduction), and costameres (the myocyte-specific cytoskeletal link between myofilbrils and the cell membrane) were severely disrupted. Impairment of both the intercalated disc and the costamere has been associated with cardiomyopathy; this study demonstrates that CENP-F is crucial for establishment and/or maintenance of these structures. Over 12 months, CENP-F−/− hearts became enlarged, fibrotic and developed arrhythmias, all of which are hallmarks of disease associated with progressive dilated cardiomyopathy. Furthermore, transcriptional profiling of CENP-F−/− hearts revealed findings that are in line with previous reports of dilated cardiomyopathy in model organisms and humans.

**Implications and future directions**

These findings represent an advance in the analysis of heart disease, and reveal a new disease mechanism involving disruption of a particular gene product, and more broadly of the microtubule network. This is also the first report of an in vivo knockout of CENP-F and the first reported genetic link between disruption of a microtubule-associated protein and heart disease. The complexity and diversity of microtubule components suggests that a proportion of dilated cardiomyopathies might be caused by mutations in the many genes that encode several other microtubule-associated proteins. Future investigation of this issue might provide insight into other forms of heart disease and dysfunction.

**Electrocardiography**

Small (<4 gm) telemetry devices (Data Sciences) were implanted under anesthesia in the peritoneum and the wires tunneled to the left shoulder and right leg of WT and CENP-F−/− animals. Recovery occurred within a day and, via a telemetry receiver placed under the cage, ECG data were obtained for at least 30 minutes per animal in unanesthetized, unrestrained animals. A total of 48 hours of recordings were obtained and scanned for RR intervals greater than 200 ms. An electrophysiologist examined all data (raw and analyzed) and insured quality control. This work was performed in the Vanderbilt University Murine Cardiovascular Core.

**Microarray analysis**

RNA samples were obtained from WT and KO mouse hearts, labeled and hybridized to Affymetrix Mouse Gene 1.1 ST arrays, yielding a total of 12 arrays (four groups; n=3). Microarray images were scanned with an Affymetrix high resolution GenePix 4000B scanner in the Vanderbilt Functional Shared Resource
functions and pathways were performed using DAVID (Bina, 2008). Statistical analyses (including correction for multiple hypothesis testing) for identification of overrepresented ontologies, (data not shown) demonstrated comparable hybridization signals across replicate samples. Using Partek, pairwise comparisons of average group values and one-way ANOVA for analysis of WT versus KO heart were performed. Only probes that resulted in a fold change of at least 1.5 and P-value of less than 0.05, with or without Benjamini and Hochberg multiple hypothesis correction (Hochberg and Benjamini, 1990), were considered as significantly altered. Individual pairwise comparisons were also performed to ascertain the level of consistency and to identify the top altered genes for each major comparison. Gene functions were determined using publicly available records using NCBI Entrez Gene, Stanford SOURCE, Aceview and PubMed databases. Sequences for differential probes not associated with transcripts, based on Affymetrix database annotations, were retrieved from the Affymetrix NetAffx Analysis Center website (http://www.affymetrix.com/index.affx) and searched in the UCSC Genome Browser (http://genome.ucsc.edu/) using BLAT (Bina, 2008). Statistical analyses (including correction for multiple hypothesis testing) for identification of overrepresented ontologies, functions and pathways were performed using DAVID (http://david.abcc.ncifcrf.gov) and the Partek Genomics Suite.

ACKNOWLEDGEMENTS

We thank the members of the Dees and Bader laboratories for their many constructive and insightful discussions concerning this work.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

E.D., P.M.M., K.L.M., J.N.R. designed and conducted experiments and wrote the manuscript, R.P.H. designed and conducted experiments. D.M.B. designed experiments and wrote the manuscript.

FUNDING

This work was supported by the National Institutes of Health (NIH) [grants HL087050, HL037675 and U24DK059637]; by an AHA pre-doctoral fellowship (to P.M.M.); and by 5 T32HL007751, Training Grant in Mechanisms of Vascular Disease (R.D.P., C.L.G. and J.N.R. designed and conducted experiments and wrote the manuscript.

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