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A novel mutant allele of Ncx1: a single amino acid substitution leads to cardiac dysfunction

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ABSTRACT The biological role and structure-function relationship of the Na^+Ca^{2+} exchanger NCX1 have been the subject of much investigation. Subtle mutagenesis to study the function of a protein seems only feasible in *in vitro* systems, but genetic forward screens have the potential to provide *in vivo* models to study single amino acid substitutions. In a genetic screen in mouse, we have isolated a mutant line carrying a novel mutant allele of the mouse *Ncx1* gene. In this allele, a point mutation causes the substitution of a highly conserved asparagine residue (N874) with lysine. Accepted models for NCX1 structure propose that the affected amino acid is located in one of the reentrant membrane loops and experiments *in vitro* have identified N874 as critical for the ion transport function of NCX1. We found severe circulation defects and defective placentation in homozygous *Ncx1*^{N87K4} mutant embryos, making the phenotype essentially indistinguishable from those of previously described null mutants. By *ex vivo* analysis, we demonstrated intrinsic functional abnormalities of cardiomyocytes. Western blot analysis and immunohistochemistry demonstrated normal levels and subcellular localization of the altered protein, ruling out the possibility that the abnormalities are a mere consequence of a major disturbance of protein structure. This study confirms and extends studies *in vitro* indicating the significance of amino acid N874 for the function of the NCX1 protein. It provides an *in vivo* model for this mutation and demonstrates the potential of forward genetic screens in a mammalian system.

KEY WORDS: *forward screen, mouse, heart development, Na^+Ca^{2+} exchanger*

Introduction

Ca^{2+} flux is pivotal in excitation-contraction coupling in the heart and in other processes (Lytton, 2007). In cardiomyocytes, the sodium calcium exchanger NCX1 has an important role, particularly in extrusion of Ca^{2+} during relaxation, but also in Ca^{2+} entry during contraction. NCX1, also known as SLC8A1, is a member of the superfamily of solute carriers. Three *Ncx* genes encoding structurally highly similar $Na^{+/}Ca^{2+}$ exchanger proteins are present in the mouse genome. *Ncx2* and *Ncx3* are mainly expressed in the central nervous system and skeletal muscle, respectively (Quednau *et al.,* 2004), while *Ncx1* is expressed ubiquitously in adult animals, but at much higher levels in heart, brain and kidney. Expression analysis on embryos shows predominant expression in the developing heart as of approximately the 1-somite stage (Koushik *et al.,* 1999). In four different laboratories knockout lines representing null alleles of *Ncx1* were generated (Cho *et al.*, 2000; Koushik *et al.,* 2001; Reuter *et al.,* 2002; Wakimoto *et al.,* 2000). Mouse embryos lacking a functional *Ncx1* gene were reported to die around embryonic day (E) 10.5 with striking cardiac abnormalities. Given *Ncx1*'s assumed cardiac function, these data confirmed the expected phenotype, but some controversy regarding the cause of lethality arose when (*i*) it was reported that a 'cardiac-specific' *Ncx1* knockout is viable into adulthood (Henderson *et al.,* 2004); (*ii*) two groups showed that heart-specific *Ncx1* expression in transgenic mice rescued the phenotype only to limited extent (Conway *et al.,* 2002; Cho *et al.,* 2003); (*iii*) *Ncx1* was shown to be expressed in the normal

Abbreviations used in this paper: : SNP, single-nucleotide polymorphism; PECAM, platelet endothelial cell adhesion molecule; E, embryonic day

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placenta, and placental defects were seen in *Ncx1* mutants (Cho *et al.,* 2003).

The topology and the structure-function relationship of the NCX1 protein has been subject of much research. NCX1 is a plasma membrane protein containing nine transmembrane domains, a large intracellular loop that has been directly linked to Ca^{2+} binding, and two reentrant loops, the α-1 and α-2 repeats that have been linked to ion transport (Nicoll *et al.,* 1996; Iwamoto *et al.,* 1999; Ottolia *et al.,* 2005).

Here we report a novel mutant allele of *Ncx1* that we found in a genetic screen for mutations that disturb early mouse development. We show that a point mutation leading to the substitution of one of the conserved asparagines in the α-2 repeat is sufficient to produce an essentially complete loss-of-function phenotype, as compared to published knockout studies. In addition, we demonstrate intrinsic defects of mutant cardiomyocytes, by studying Ca^{2+} homeostasis and action potential in isolated cardiomyocytes.

Results

Identification of a mutant line with early-embryonic heart defects

In the course of an ENU-mediated, phenotype-driven screen for recessive mutations leading to developmental defects (manuscript in preparation), we identified a mutant line that was designated *HI-07*. Affected mutant embryos of this line displayed extreme thoracic oedema, inflated branchial arches and abnormal folding of the heart tube (see Fig. 1). Embryos collected at E9.5 or E10.5 had similar morphology and size, but while a heart beat could usually be observed at E9.5, E10.5 embryos never had a beating heart. Yolk sacs of mutant embryos lacked a blood-filled vasculature. Mutant embryos at these stages were obtained with a frequency as expected for an autosomal recessive mutation.

Mapping

We designed a panel of 192 single nucleotide polymorphisms (SNPs), distinguishing FVB/NJ from C57Bl/6 DNA. These SNPs are dispersed evenly over the genome, allowing initial crude mapping. Analysis of DNA from 25 mutant embryos and some of their littermates indicated that the gene was located on Chromosome 17, between 75.8-86.1 Mbp. Further mapping using different SNPs in this region (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home) reduced the candidate region to 78.0-84.5 Mb. Among the approximately 55 genes located within this segment is the *Ncx1* gene. Since descriptions of homozygous mutant mouse embryos obtained through gene targeting appeared to match the *HI-07* mutant, we sequenced the coding regions of *Ncx1* in genomic DNA from mutant embryos. This demonstrated a T>A transversion of nucleotide 86 of exon 9 (Fig. 2A) that correlated completely with the phenotype. Because of the mutation, the asparagine residue at position 874 is substituted by a lysine (numbering according to the Ensembl database (www.ensembl.org), Ensembl peptide ID ENSMUSP00000083725). The occurrence of this mutation in combination with a very strong resemblance of the *HI-07* mutant phenotype to the described *Ncx1* phenotype basically excludes a noncausal relation between the two. Functional relevance of N874 emerged previously from site-directed mutagenesis studies followed by *in vitro* assays (Nicoll *et al.,* 1999). From modelling studies, it has been proposed that N874 is located in the membrane as part of the second of two re-entry loops present in the protein (Nicoll *et al.,* 1996, 1999; Iwamoto *et al.,* 1999, 2000; Ottolia *et al.,* 2005; see Fig. 2B).

Ncx1 *expression in embryos*

Reports of expression analysis of *Ncx1* at early embryonic stages are not entirely consistent, but agree on predominant expression in the heart (Koushik *et al.,* 1999; Wakimoto *et al.,* 2001). Our analyses confirmed expression in the heart at E10.5, which was essentially unchanged in mutant embryos (Fig. 1 D,E).

It is hypothetically possible that the mutation we observed in the *Ncx1N*^{874K} mutant affects the protein in a way that destabilizes it, and/or upsets its targeting to the cell membrane. To address this, we analyzed by Western blot analysis the presence of NCX1 in embryonic hearts. As shown in Fig. 3A, similar levels of 120- and 160-kD NCX1-specific bands were detected in pools of dissected wildtype, heterozygous and homozygous mutant embryos. We also compared expression and subcellular localization in wildtype *vs.* mutant embryos by immunofluorescence (Fig. 3 B-G). Fig. 3 B,C shows presence of NCX1 protein in the heart of an E9.0 wildtype embryo and similarly in an *Ncx1* mutant embryo. Fig. 3 D,F shows the plasma membrane localization of NCX1 in wildtype embryos. In mutant cells (Fig. 3 E,G) this localization is unchanged. Therefore, the N874K mutation does not bring about a general, unspecific disorder of the NCX1 protein organization. Our attempts to demonstrate expression of *Ncx1* in the embryonic vasculature of the placenta were unsuccessful, as the use of the R3F1 antibody led to artefacts in this tissue.

Placental phenotype in Ncx1 *mutant embryos*

To investigate the possibility that placentation is disturbed in

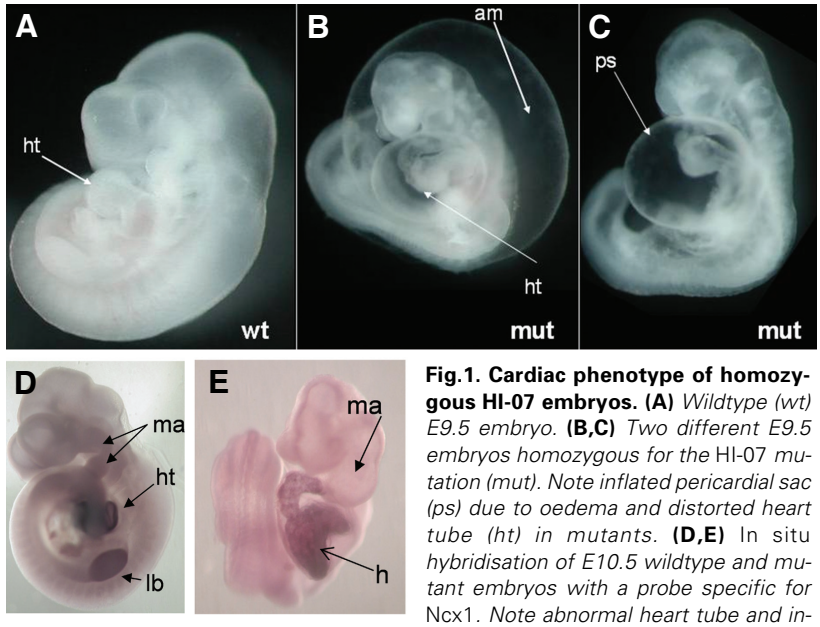

Fig.1. Cardiac phenotype of homozygous HI-07 embryos. (A) *Wildtype (wt) E9.5 embryo.* **(B,C)** *Two different E9.5 embryos homozygous for the* HI-07 *mutation (mut). Note inflated pericardial sac (ps) due to oedema and distorted heart tube (ht) in mutants.* **(D,E)** In situ *hybridisation of E10.5 wildtype and mutant embryos with a probe specific for* Ncx1*. Note abnormal heart tube and inflated mandibular arch (ma) in mutant and heart-specific expression in both embryos. Staining of the limb bud (lb) may be unspecific. am, amnion.*

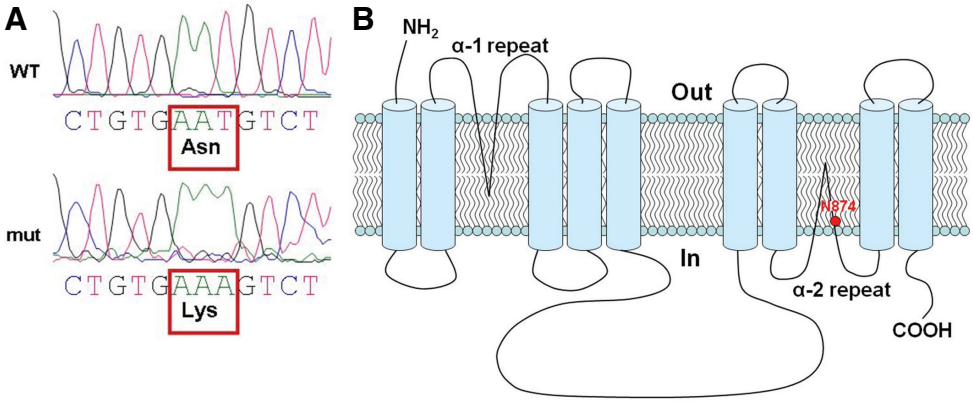

Fig. 2. Identification of *Ncx1* mutation in HI-07 mutant embryos. (A) *Sequence traces representing the mutation found in the Ncx1 allele in the HI-07 line. A T to A transversion in the coding region of Ncx1 is evident in DNA from a homozygous mutant embryo, which is predicted to lead at the protein level to replacement of an Asparagine (N874) by a Lysine.* **(B)** *Scheme depicting the topology of NCX1 protein as proposed in the literature, in particular references 12,13,15. The protein contains 9 transmembrane loops, an intracellular loop and two reentrant loops. Orientation of the membrane is indicated by 'Out' and 'In,' referring to outside and inside the cell. The red dot marked 'N874' indicates the crucial Asparagine mentioned above and discussed in the text.*

homozygous mutant *Ncx1*^{N874K} embryos, as was reported for *Ncx1* knockout embryos (Cho *et al.,* 2000), we analyzed a time series of mutants between E9.0, E9.5 and E10.5. This suggested normal development of the allantois, prior to and including attachment to the chorion. Upon attachment of the allantois, in normal embryos the labyrinth develops because of invasion of the trophectoderm layer by allantois-derived vasculature. The labyrinth layer enables exchange of nutrients and gases between the foetal and maternal circulation (Rossant and Cross, 2001). Comparison of sections of E9.5 placenta from wildtype and mutants revealed that while in the placenta of wildtype embryos the labyrinth could easily be distinguished; in the mutant placenta it had not developed (Fig. 4 A-D). Furthermore, the haemotrichorial layer that separates embryonic and maternal circulation (yellow arrow in Fig. 4B) is seen in wildtype (yellow arrow in Fig. 4C) but not in mutant placenta (Fig. 4D).

To analyze the vasculature of the placenta, we used antibodies against PECAM that specifically label the endothelial lining of the vessels. The results confirm the defective labyrinth development, but do not suggest that vascular endothelium is specifically affected (Fig. 4 E,F).

To better understand the nature of the placenta phenotype, we analyzed expression of a number of markers by *in situ* hybridization on sections of E9.5 placentas. Expression of *eHAND* as a trophoblast giant cell marker (Cserjesi *et al.,* 1995), *Cdx2* as a trophoblast lineage marker (Beck *et al.,* 1995), and *Mash2* as a spongiotrophoblast marker (Guillemot *et al.,* 1994) was essentially normal compared to the wildtype placenta, apart from differences that are evidently a direct consequence of the abnormal morphology of the mutant placenta (see Supplementary Fig. 1). These results suggest that derivatives of these lineages are present in essentially normal amounts.

Intrinsically affected properties of Ncx1 *mutant cardiomyocytes*

The occurrence of extra-embryonic defects in this *Ncx1* mutant opens the possibility that cardiac failure might not be the only or even primary cause of embryonic lethality. We therefore set out to compare intrinsic properties of cardiomyocytes isolated from mutant embryos and their wildtype litter mates. Two key components of excitation contraction coupling were studied, the action potential (AP) and the Ca^{2+} homeostasis. Hearts from E9.5 normal and mutant embryos were isolated, dissected into small pieces, dissociated enzymatically and replated. After one day of culture, cardiomyocytes from wildtype embryos were contracting regularly and synchronously in small clusters. In contrast, cardiomyocytes derived from mutant embryos were fibrillating and

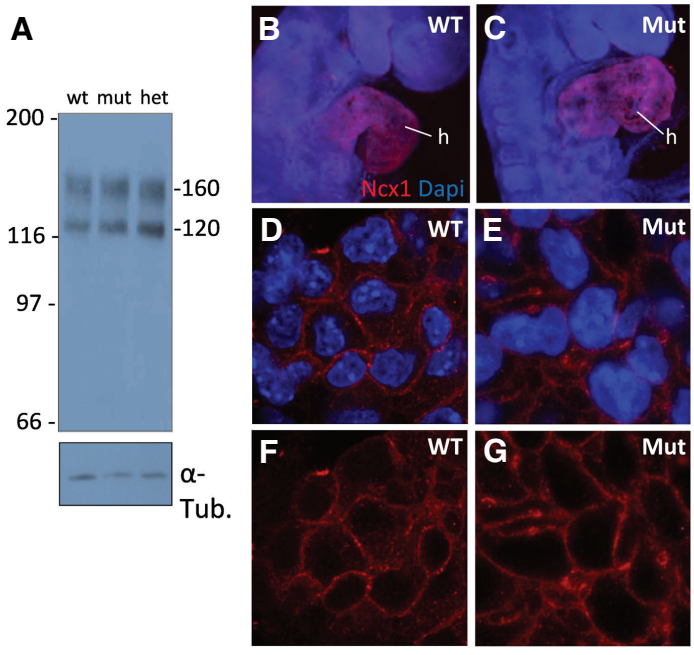

Fig. 3. Immunodetection of Ncx1 and Ncx1^{N874K}**. (A)** *Western blot analysis of pooled embryonic hearts. Hearts were dissected from E9.5 embryos and lysates of identical genotype were pooled. One slot contained 25 μg protein from pools of 11-12 hearts. Two NCX1 bands of 120 and 160 kD were detected at similar levels in wildtype (wt), heterozygous (het) and homozygous mutant (mut) material. To control for the amount of protein loaded, the membrane was incubated with an α-tubulin antibody (α-tub), as shown below.* **(B-G)** *Immunodetection of NCX1 in E9.0 embryos.* **(B,D,F)** *Wildtype embryo;* **(C,E,G)** *homozygous mutant* NCX1^{N874K} *embryo. NCX1-specific fluorescent signal appears red, DAPI staining for DNA is blue (only in B-E).* **(B,C)** *Confocal detection (z-stacks) of low power magnification demonstrates heart-specific expression.* **(D-G)** *High power magnification (optical sections) reveals plasma membrane localization in both genotypes.*

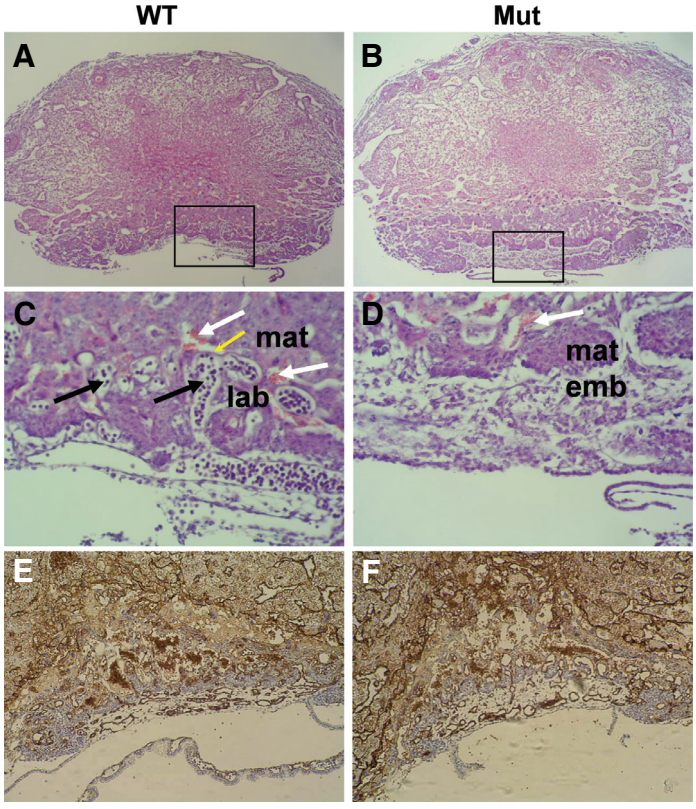

Fig. 4. (Left). Abnormal placentation in *Hl-07* mutants. (A-D) *Haematoxylin and eosin stained sections of E9.5 placentas from wildtype* **(A,C)** *or mutant* **(B,D)** *embryos. Maternal blood appears red and is indicated by white arrows. Embryonic (nucleated) erythrocytes are seen as dark round cells, and are pointed out by black arrows. Labyrinth formation manifests by the penetrance of embryonic blood into the maternal part of the placenta. This occurs in the wildtype, but not in the mutant placenta. In* **(C,D)***, 'lab' indicates area of labyrinth; 'mat' and 'emb' indicate areas of maternal and embryonic placenta, respectively. The yellow arrow indicates the thin layer known as haemotrichorial layer that separates maternal and embryonic circulation. This interface was never seen in mutant placenta.* **(E,F)** *PECAM staining of sections similar to those of panels* **(A-D)***, showing normal expression of this endothelium marker. Mutant (mut) and wildtype (wt) placenta as indicated on top of the figure.*

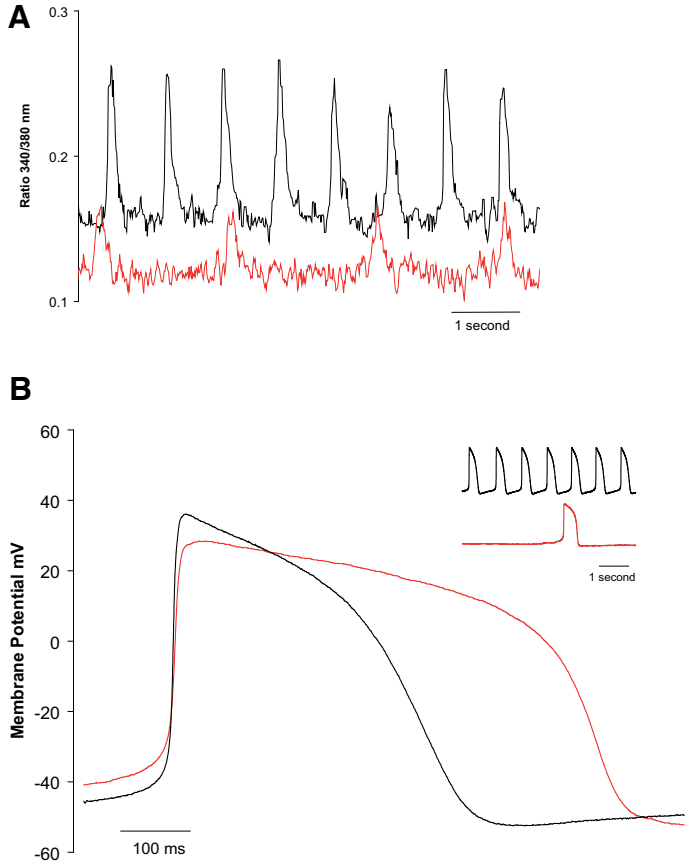

Fig. 5. Calcium transients and action potentials in wildtype vs. mutant cardiomyocytes. (A) *Representative calcium transients in fura-2 loaded, spontaneous beating wild-type black) and Ncx1^{N874K} mutant (red) cardiomyocytes.* **(B)** *Representative action potentials of spontaneous beating wild-type (black) and Ncx1874K mutant (red) cardiomyocytes.*

contracting randomly (see supplementary videos). The Ca^{2+} transients of *Ncx1^{N874K}* cardiomyocytes were smaller in amplitude and had a lower frequency (0.53 Hz) than their wild-type counterparts (1.06 Hz) (Fig. 5A). Although small Ca^{2+} transients were found, very rarely action potentials were detected, which were never repetitive. In the mutant cells we were able to measure only two prolonged action potentials out of ten recordings (Fig. 5B). These results are consistent with the notion that the *Ncx1^{N874K}* mutation causes intrinsic abnormalities in embryonic cardiomyocytes. Clearly, these malfunctions would be sufficient to explain the embryonic phenotype including early lethality.

Discussion

In addition to identifying novel roles of genes in an unbiased way, a phenotype-based approach involving generation of point mutations is also expected to yield subtly altered mutant alleles of genes. Such mutants may be of interest if they represent hypomorphic alleles, providing novel opportunities to study gene function. In addition, if a single amino acid substitution leads to a strong phenotype, as in the present study, the results may provide insight in the biochemistry and structure-function relation of the protein in question.

We have characterized a novel mouse mutant, carrying a missense mutation of the *Ncx1* gene. Our data show that homozygous *Ncx1^{N874K}* embryos exhibit a wide-ranging set of abnormalities similar to published knock-out models, including defective placentation, lack of yolk sac vascularization and abnormalities of heart development leading to impaired blood circulation. The early lethality of the *Ncx1* mutants precludes analysis of possible other functions of the gene. For instance, *Ncx1* is highly expressed in adult brain and kidney (Wakimoto *et al.,* 2001), but the study of its function in those tissues would require the use of conditional mutants. Assessing whether the *Ncx1^{N874K}* allele is hypomorph or a null mutation is complicated by certain discrepancies in the phenotypes described for the existing presumed null

mutants. Koushik *et al.* (2001) reported total absence of heartbeat in homozygous mutant embryos at all stages (see also Lux *et al.,* 2008 and Rhodes *et al.,* 2008) whereas others (Cho *et al.,* 2000 and Wakimoto *et al.,* 2000) observed at least some contractility in all or some of the mutant embryo hearts. Our observation that mouse embryos homozygous for the mutation retain a heartbeat at E9.5 therefore does not resolve the question as to remaining functionality of the NCX1^{N874K} protein.

Our *ex vivo* analyses of mutant cardiomyocytes demonstrate major functional deficits of mutant E9.5 cells that are sufficient to explain early lethality. The essential physiological role of NCX1 in the heart is further emphasized by reports of arrhythmias in zebrafish *Ncx1* mutants (Ebert *et al.,* 2005; Langenbacher *et al.,* 2005) where complications of extra-embryonic development do not apply. The doubts that have arisen as to the question whether heart failure is the primary cause of embryonic lethality around E10 were based on survival of a 'heart-specific' knock-out in which, however, only 80-90% of the cells had lost *Ncx1* activity (Henderson *et al.,* 2004). In addition, attempts to rescue the *Ncx1* phenotype by expressing *Ncx1* under control of an αMHC promoter failed to compensate more than marginally (Conway *et al.,* 2002; Cho *et al.,* 2003). Although efficacy of the expression construct at the relevant embryonic stages was not demonstrated by these authors, this suggests a potential role of *Ncx1* in placental development. On the other hand, since heart failure in the *Ncx1^{N874K}* mutant is already evident at E9.5, and our analyses of cell-specific markers in mutant placenta (Supplementary Fig. 1) showed essentially normal patterns, it is possible that the placental defect we observe is a mere secondary consequence of it, for instance as consequence of delayed development, as has been suggested for a number of mutants (Conway *et al.,* 2003). The lack of vascularization of the yolk sac may also be a consequence of impaired cardiac function. It was recently reported that haemodynamic force is necessary and sufficient to induce vessel remodeling in the mouse yolk sac. The manifestation of the *Ncx1* deficiency in our *HI-07* mutant in appearance of the yolk sac phenotype strongly resembles what these authors show for *Mlc2a* knock-out embryos, an immature appearance and failure of remodelling of the capillary plexus. Interestingly however, no labyrinth phenotype has been reported for the *Mlc2a* mutant (Lucitti *et al.,* 2007).

Iwamoto and co-workers (Iwamoto *et al.,* 1999, 2000) and others studied the structure of dog NCX1 protein. On the topological disposition of N842 (corresponding to mouse N874), these authors concluded that it is located in the membrane as part of a reentrant loop. Replacement of N842 by cysteine allowed examining its accessibility. It was demonstrated to be chemically accessible from the intracellular membrane surface and possibly also from the outside (Iwamoto *et al.,* 2000). Earlier, functional importance of this amino acid had been revealed by demonstrating that its mutation to aspartate or valine led to decreased exchange activity (Nicoll *et al.,* 1996). Mouse Ncx1 N874 is therefore likely to be a part of the mechanism responsible for ion transport. Interestingly, Iwamoto *et al.* (2000) showed that replacement of N874 with cysteine did not affect NCX1 performance, whereas subsequent treatment with methanethiosulfonate ethylammonium (MTSEA) did inhibit its activity. MTSEA is a sulfhydryl reagent that converts the neutral cysteine side-chain to a positively charged group resembling a lysine side-chain; therefore this *in vitro* experiment resembled the situation resulting from the point mutation in the *Ncx1^{N874K}* mutant.

In conclusion, our analysis of a novel loss-of-function mutant allele encoding an abnormal NCX1^{N874K} protein substantiates the importance of the conserved asparagine at position 874. By extrapolation of published work *in vitro*, it is likely that the NCX1^{N874K} protein is specifically defective in ion transport. While protein levels and subcellular location of the protein remained unaffected, a strong embryonic phenotype was observed in homozygous mutants. Our data establish for the first time *in vivo* the essential role of this residue and demonstrate the potential power of phenotype-driven genetic screen as an intermediate between gene targeting studies and studies *in vitro*.

Materials & Methods

Identification of mutant

We identified the *HI-07* mutant in the course of a genetic screen for recessive mutations affecting development. This screen will be described in more detail elsewhere. Briefly, C57BL/6 mice were injected three times with one-week intervals with 60-80 mg/ kg bodyweight of N-ethyl-N-nitrosourea and crossed with FVB/N mice. Founders were crossed with their daughters and embryos, which were potentially homozygous for ENU-induced mutations, were examined at E10.5.

Mapping procedures

We designed a SNP panel for approximate mapping of mutations from our screen. SNPs were chosen to be equally spread along all chromosomes excluding Y and to be polymorphic between C57BL/6 and FVB/N. 10 μl of PCR product was diluted with 25 μl water and 1 μl was used as template for the sequencing reactions. Sequencing reactions, containing 0.25 μl BigDYE (v1.1; Applied Biosystems), 3.75 μl 2.5x dilution buffer (Applied Biosystems), and 0.4 μM gene-specific primers in a total volume of 10 μl, were performed using cycling conditions recommended by the manufacturer. Sequencing products were purified by ethanol precipitation in the presence of 40 mM sodium acetate and analyzed on a 96-capillary 3730XL DNA analyzer (Applied Biosystems). Sequences were analyzed for the presence of polymorphisms using Polyphred (Thurneysen *et al.,* 2002). Primers for PCR amplification and sequencing were designed using the Ensembl genome database (http://www.ensembl.org).

Western blot analysis

E9.5 mouse embryos were harvested in PBS; hearts were dissected and separately lysed in 5μl RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS) including protease inhibitors. After genotyping, 11 or 12 hearts of every genotype were pooled, and protein concentrations of the pools were determined using the Bicinchoninic assay Kit (Sigma-Aldrich). 2x Laemmli sample buffer was added to 25μg protein and the mixture was heated at 70˚C before loading. Polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed according to standard procedures, including use of the ECL system for luminescence detection. Mouse-anti-NCX1 (R3F1, Swant, Bellinzona, Switzerland) was diluted 1:500 in 5% milk powder in TBS-Tween blocking solution. The NCX1-specificity of this antibody has been described (Van Nes *et al.,* 2006). After stripping, the blot was incubated with mouse-anti-α tubulin (1:2000 in TBS-Tween, t6199, clone DM1A, Sigma-Aldrich). The secondary antibody used was HRPO-coupled anti-mouse (BDbiosciences).

Immunohistochemistry

Embryos were dissected in PBS and fixed overnight in 4% paraformaldehyde (PFA) and dehydrated by increasing Methanol concentrations to 100% for storage at -20˚C. After rehydration, embryos where

blocked in PBS, 0.1% Triton X-100-1 % BSA for 1h and incubated with 1:500 mouse-anti-NCX1 (R3F1, Swant, Bellinzona, Switzerland). After washing with PBS containing 0.1% Triton X-100, embryos were incubated with the secondary antibody (Cy3-goat-anti-mouse 1:250; Jackson Immunological). Nuclei were stained with DAPI (Invitrogen). Fluorescence was analyzed by using a Leica TCS SPE confocal microscope and the Leica Application Suite software. PECAM-staining has been described (Van Nes *et al*., 2006).

Preparation of dispersed cardiomyocytes

Single cardiomyocytes were prepared essentially as described (Maltsev *et al.,* 1994). In brief, embryonic hearts were dissected, washed and then transferred to collegenase-containing buffer and incubated for 45' at 37° C. Tissue was then transferred to KB buffer (Maltsev *et al.,* 1994) and shaken at room temperature for 1 hour at 100 rpm. Small amounts of tissue were dispersed by pipetting up and down and transferred to 12-well plates containing gelatin coated cover slips and hES medium (Braam *et al.,* 2008).

Calcium measurements

The equipment used consisted of a Leica DM R upright microscope (Leica-Microsystems Wetzlar GmbH, Germany) equipped with an UV 40x water-immersion objective (0.9 NA) and a temperature-controlled specimen holder. Measurements were done in a buffer containing 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose and 0.5% bovine serum albumin, at 33 ^{0}C. The cells were labelled for 15 minutes at 37 ^{0}C with 10 μM fura2-AM (Invitrogen, Molecular Probes). The light from two excitation monochromators (SPEX fluorolog SPEX Industries EDISON, N.J, USA) was rapidly alternated between 340 nm and 380 nm and the ratio of the emission signals (wave length 505 nm) was recorded.

Expression analysis

In situ hybridization on whole mounts or sections was as described previously (Kuijper *et al.,* 2005). Probes were previously described (Van Nes *et al.,* 2006) except for *Ncx1*. In this case, two different probes of 689 and 693 bp, were made by PCR from partially overlapping parts of exon 1 using primers:
Ncx1F1, AGACTGTGTCGAACCTGACC;
Ncx1R1, TGCTGTTGACTTAGGACCTG, and
Ncx1F2, GATGGGAAAGTGGTCAACTC;
Ncx1R2, CCCAAGACAAGCAATTGAAG, respectively. Both probes gave identical results.

To ensure specificity of probes, experiments were combined with parallel hybridizationshybridisations using probes detecting known expression patterns.

Acknowledgements

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