De novo dNTP production is essential for normal postnatal murine heart development

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Running title: De novo versus salvage dNTP production

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ABSTRACT

The building blocks of DNA, dNTPs, can be produced de novo or can be salvaged from deoxyribonucleosides. However, to what extent the absence of de novo dNTP production can be compensated for by the salvage pathway is unknown. Here, we eliminated de novo dNTP synthesis in the mouse heart and skeletal muscle by inactivating ribonucleotide reductase (RNR), a key enzyme for the de novo production of dNTPs, at embryonic day 13. All other tissues had normal de novo dNTP synthesis and theoretically could supply heart and skeletal muscle with deoxyribonucleosides needed for dNTP production by salvage. We observed that the dNTP and NTP pools in wild-type postnatal hearts are unexpectedly asymmetric, with unusually high dGTP and GTP levels compared with those in whole mouse embryos or murine cell cultures. We found that RNR inactivation in heart led to strongly decreased dGTP and increased dC, dT, dATP, and dATP pools; aberrant GTP replication; defective expression of muscle-specific proteins; progressive heart abnormalities; disturbance of the cardiac conduction system; and lethality between the second and fourth weeks after birth. We conclude that dNTP salvage cannot substitute for de novo dNTP synthesis in the heart and that cardiomyocytes and myocytes initiate DNA replication despite an inadequate dNTP supply. We discuss the possible reasons for the observed asymmetry in dNTP and NTP pools in wildtype hearts.

Mammalian cells can obtain deoxyribonucleoside triphosphates (dNTPs) via two pathways – de novo synthesis and salvage (Figure 1A) (1). A key player in de novo dNTP synthesis is ribonucleotides reductase (RNR), which converts ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs) that are subsequently phosphorylated to dNTPs by nucleoside diphosphate kinases (1). Mouse RNR contains two subunits, the large RRM1 and the small RRM2 subunits, both of which are essential for enzyme activity (2). In addition, the alternative small subunit RRM2B is required for the production of the dNTPs used for mitochondrial DNA (mtDNA) synthesis and nuclear DNA repair in non-dividing cells (3,4). The RRM1 subunit contains the catalytic site and two allosteric sites, whereas the RRM2 and RRM2B subunits harbour a tyrosyl radical needed for catalysis (1).

The salvage pathway is coupled to the degradation of unutilised dNTPs to deoxynucleosides (dNs) by 5'--nucleotidases (5), and the triphosphohydrolase SAMHD1 (SAM domain and HD domain-containing protein 1) (Figure 1A) (6). dNs diffuse into the extracellular environment following their concentration gradient and are taken up by neighbouring cells through specialised nucleoside transporters. Once inside the cells,
dNs are phosphorylated stepwise by several enzymes, first to dNMPs by the dN kinases thymidine kinase 1, thymidine kinase 2, deoxyguanosine kinase, and deoxycytidine kinase, and later by other kinases to dNDPs and dNTPs (1,5).

To what extent the dNTP salvage pathways can substitute for de novo production is not known. Here, we investigated whether inactivation of de novo dNTP synthesis in developing mouse cardiomyocytes and myocytes could be rescued by salvage of dNs from other tissues that have normal de novo dNTP synthesis. In mice, cardiomyocyte proliferation progressively slows down after birth and stops at about postnatal day 11 (P11) (7). However, dNTPs continue to be in demand because the decline in proliferation coincides with cardiomyocyte binucleation that takes place between P5 and P10, peaking at around P7. Finally, mouse cardiomyocytes undergo polyploidisation during postnatal weeks two and three. Cardiomyocyte proliferation, binucleation, and polyploidisation account for about 30%, 57%, and 13% of postnatal DNA synthesis in mouse hearts, respectively (7). In this study, we created a tissue-specific deletion of Rrm1, thus blocking de novo dNTP synthesis in late prenatal and postnatal cardiomyocytes and skeletal muscle. Rrm1 knockout (KO) pups were born viable and had functional hearts without gross abnormalities, but they started to die after the first postnatal week due to heart failure. Our results indicate that cardiomyocytes initiate DNA synthesis despite the absence of de novo dNTP production and that the salvage pathway is unable to support DNA synthesis in the cells lacking RRM1.

Results

De novo dNTP synthesis in cardiac and skeletal muscle is essential for postnatal survival

To inactivate de novo dNTP synthesis in cardiac and skeletal muscle, we obtained mice with a homozygous deletion of Rrm1 by setting up an Rrm1<sup>fllox/+</sup> Ckn<sup>Cre/+</sup> × Rrm1<sup>fllox/+/ Cre/+</sup> Ckn<sup>Cre/+</sup> cross. Because mouse embryos start to express creatine kinase type M from embryonic day (E)13 (8), we analysed embryos from this cross at E16.5 and E18.5 and in pups at P1. At these stages, Rrm1<sup>fllox/+/ Cre/+</sup> knockout embryos and pups followed Mendelian distributions (Table S1) and showed no obvious size or morphological differences compared to their wild-type (WT) littermates (Figure 1B).

To increase the probability of obtaining Rrm1 KO pups in a litter, we set up Rrm1<sup>fllox/+</sup> Ckn<sup>Cre/+</sup> × Rrm1<sup>fllox/+/ Cre/+</sup> Ckn<sup>Cre/+</sup> × Rrm1<sup>fllox/+/ Cre/+</sup> crosses and monitored the offspring every morning from P1 to P30. When a pup was found dead, its tail was taken for genotyping, while its heart, hind leg muscles, and diaphragm were isolated and stored at −80°C until further analysis. The Rrm1 KO pups appeared progressively smaller and they had lower body weight compared to their WT littermates (Figure 1C and 1D). In contrast to their littermates, the Rrm1 KO pups started to die soon after birth, and most of them died before weaning. The median survival age of Rrm1 KO pups was 11.5 days (Figure 1E). These results suggest that de novo dNTP synthesis is essential for the survival and growth of Rrm1 KO pups.

To assess how inactivation of de novo dNTP synthesis affects dNTP levels, we measured dNTP pools in the hearts isolated from P15 – P17 pups. The dNTP pool in WT hearts was unexpectedly asymmetric with an unusually high dGTP level compared to what is observed in whole mouse embryos and in cell culture (Figure 1G and 1H) and Refs (9,10). Possible explanations for this asymmetry are provided in the Discussion. The Rrm1 KO hearts had a strongly decreased pool of dGTP (~3-fold), but surprisingly had increased pools of dCTP (~2-fold) and dTTP (~1.7-fold), and no change in the pool of dATP compared to WT (Figure 1F). This result can be interpreted to mean that in the absence of de novo synthesis, salvage pathways might be able to compensate for the production of dCTP, dTTP, and dATP, but not dGTP.

Progressive anatomical alterations in the hearts of Rrm1 KO pups

During the first week after birth, the morphology of Rrm1 WT and KO hearts was very similar, but at later stages Rrm1 KO hearts showed enlargement of both the left and the right atria (Figure 2A). A more detailed anatomical examination using optical projection tomography (OPT) scanning revealed interventricular septal (IVS) thinning in P7 Rrm1 KO hearts (by ~27% compared to the septa of WT hearts), but no significant changes in the thickness of the left ventricular (LV) or right ventricular (RV) walls or any other abnormalities (Figure 2B and 2C). In contrast, P15 Rrm1 KO hearts had dramatic thinning of the IVS as well as the LV and RV walls (by ~69%, ~46%, and ~45%, respectively, compared to WT hearts) (Figure 2D and 2E).
Furthermore, while the thickness of the LV wall and IVS increased during the period between P7 and P15 in Rrm1 WT hearts, it decreased during the same period in Rrm1 KO hearts, which suggests that the proliferation and/or growth of cardiomyocytes is abnormal in the absence of de novo dNTP synthesis.

**Inactivation of de novo dNTP synthesis disrupts nuclear organisation and muscle fibre structure in postnatal mice**

Murine cardiomyocytes proliferate during the first week, multinucleate during the first and second weeks, and become polyploid during the second and third weeks after birth (7). Because dNTPs are required for these processes, we examined whether deletion of Rrm1 would block these processes in postnatal mice. The LVs of P7 and P10 hearts were stained with phalloidin and DAPI to visualise F-actin and nuclei, respectively (Figure 3A and 3B). While no significant differences were observed at P7, at P10 the Rrm1 KO cardiomyocytes had a ~20% lower nuclear density compared to WT (Figure 3C). P10 Rrm1 KO hearts also exhibited unstructured organisation of cardiac fibres and abnormal nuclear shape (Figure 3B). Therefore, we analysed cardiomyocyte nuclei at an even later stage (P14) and observed that most Rrm1 KO nuclei were fragmented or interconnected like a mesh with some surrounding micronuclei, in contrast to the Rrm1 KO nuclei at P7 (Figure 3D). Furthermore, increased DNA breakage and a greater number of apoptotic cells were detected in P14–P16 Rrm1 KO hearts (Figure S2). To investigate whether the fragmentation of nuclei in Rrm1 KO heart is associated with a failure of DNA synthesis, we quantified the relative amounts of DNA and RNA in P7, P9, and P15 hearts. We found that the proportion of DNA was significantly lower in Rrm1 KO hearts compared to WT, which suggests that DNA synthesis is defective in Rrm1 KO cardiomyocytes (Figure 3E).

The effect of Rrm1 deletion on skeletal muscle development was less pronounced than in the heart. P15 Rrm1 KO gastrocnemius (Gas) muscles had thinner fibres but ~20% more nuclei per fibre (Figure S3A and S3C). A small number of Gas muscle nuclei were situated close to one another and were slightly deformed, but the majority of the nuclei had a normal shape and size (Figure S3B). Furthermore, we observed no significant change in the DNA/RNA proportion in the tibialis anterior (TA) muscle of P15 Rrm1 KO mice (Figure S3D). Neither DNA breakage nor apoptotic cells were detected in Rrm1 KO skeletal muscle by TUNEL assay and active Caspase 3 staining, respectively. To confirm that Rrm1 was deleted in skeletal muscles, we performed immunostaining of the RRM1 protein in P16 pups and found a dramatic reduction of the RRM1 signal in Rrm1 KO muscles compared to WT (Figure S3E). Taken together, these findings suggest that homozygous deletion of Rrm1 driven by the CKMM-Cre recombinase affects nuclear organisation and muscle fibre structure in cardiac muscle more so than in skeletal muscle.

Mitochondria replicate their DNA even in non-dividing, terminally differentiated cells that have very limited de novo dNTP synthesis (11). Therefore, mitochondria rely more on dNTP salvage pathways than do actively dividing cells, and they have two dedicated deoxynucleoside kinases, thymidine kinase 2 and deoxyguanosine kinase (5). Despite this, the mtDNA copy number was decreased in P9 and P15 Rrm1 KO hearts, indicating that salvage pathways alone are not sufficient to sustain normal mtDNA replication (Figure S4A). In contrast, the mtDNA copy number did not decrease significantly in P15 Rrm1 KO TA muscle (Figure S4B), again indicating that the deletion of Rrm1 has a milder effect on DNA synthesis in skeletal muscle than in heart muscle.

**Aberrant expression of muscle-specific proteins in Rrm1 KO mice**

Inactivation of de novo dNTP synthesis in cardiomyocytes strongly affected the expression levels of the muscle-specific proteins dystrophin and desmin (Figure 4A). The expression levels of laminin were not significantly affected, but the pattern of laminin staining was different in the P16 Rrm1 KO heart due to pronounced fibrosis and altered heart morphology (Figure 4A). In addition, quantification of laminin-stained heart sections showed a ~34% decrease in cardiomyocyte number per mm² and a ~26% increase of connective tissue in Rrm1 KO hearts in comparison to WT hearts (Figure 4B). At the same time, Rrm1 KO cardiomyocytes showed a ~50% increase in size (Figure 4C). Similar to cardiac fibres, Rrm1 KO skeletal fibres from the hind legs (TA and Gas muscle) and diaphragm membranes showed reduced levels of dystrophin and desmin, but normal levels of laminin (Figure S5A, S5B, S5F, and S5G). In addition, Rrm1 KO Gas muscle had ~2.5-fold fewer nuclei per muscle fibre (Figure S5E).
However, in contrast to the heart, the changes in skeletal fibres were more subtle, and the Rrm1 KO mice displayed only a ~7% decrease in myocyte number and a ~14% increase in connective tissue (Figure S5C), and the size of the skeletal fibres was ~2.5-fold smaller compared to WT (Figure S5D).

**Disturbance of the cardiac conduction system**

The obvious defects observed in Rrm1 KO cardiomyocytes by the end of the second week prompted us to investigate the conduction system of the hearts using ECG. Rrm1 KO hearts of P15 pups showed a ~26% prolonged P-wave (from 10.61 to 13.38 ms), and the P-wave was biphasic (Figure 5A and 5B), indicating enlargement of the atria as observed in Figure 2A, right panel. The absence of a Q-wave indicated dramatically weakened conduction of the septum, which could be explained by the thinning of the septum observed in Figure 2D. The PQ interval in the Rrm1 KO hearts was increased by ~46% due to the increase of the P-wave and the PQ segment, which indicated a severe blockage of the conduction at or from the atrioventricular node. Similarly, an increase in PR interval duration by ~43% and a ~31% increase in duration of the QRS complex suggested that there was a defect in the conduction system of the LV (Figure 5B). As a result of the prolonged P-wave, PQ and PR intervals, and QRS complex, the heart rate of Rrm1 KO pups was decreased ~30% relative to WT (Figure 5C).

**Discussion**

The ability of the dNTP salvage pathway to compensate for the absence of de novo dNTP synthesis is not known. We addressed this question by inactivating de novo dNTP synthesis in cardiomyocytes and myocytes at the late prenatal and postnatal stages of development. We reasoned that the deletion of Rrm1 in cardiomyocytes and myocytes starting at E13 would progressively decrease the levels of the large RNR subunit, leading to inactivation of de novo dNTP synthesis. We found that inactivation of RNR in heart and skeletal muscle resulted in lethality between the second and fourth week after birth, associated with progressive anatomical and histological alterations in the hearts, defective expression of muscle-specific proteins, and disturbance of the cardiac conduction system. Are these defects in Rrm1 KO hearts caused by the shortage of dNTPs?

To assess the effect of Rrm1 deletion on dNTP metabolism, we analysed dNTP pools in WT and Rrm1 KO mouse hearts. To our surprise, we observed a strong asymmetry among the individual dNTPs not only in Rrm1 KO hearts, but also in WT hearts. In contrast to the dNTP pools found in proliferating mouse fibroblasts and in whole mouse embryos where dGTP is the least abundant dNTP (12,13), the amount of dGTP in the heart of P15-P17 pups was more than double the sum of dATP, dTTP, and dCTP (Figure 1F). The unusually high dGTP pool was also found in the hearts but not in the spleens of adult mice (Figure S6). Interestingly, exceptionally high dGTP levels were previously reported in heart mitochondria of adult rats (14-16). In the mitochondria of several other rat tissues, dGTP was also reported to be the most abundant DNA precursor, with intermediate values found in skeletal muscle, kidney, and brain (14,16). Whether or not dGTP levels are unusually high in the mitochondria of mouse liver is debated (17). In contrast, the dGTP pool in the cytosol of different rat tissues, including heart, was reported to be much lower than in mitochondria and to be similar in size to the other three dNTPs (14). We could not measure mitochondrial vs. cytosolic dNTP pools in postnatal mouse hearts because the amount of material was too low, but if (i) the cytosolic dGTP pool in the mouse heart is also low and close in size to the other three dNTP pools as it was observed in rat hearts and (ii) the dGTP pool in the Rrm1 KO mouse heart is equally reduced in the cytosol and the mitochondria (~3-fold), then the cytosolic dGTP pool in the Rrm1 KO heart will be very low and will be limiting for normal DNA replication. This would cause replication fork stalling and collapse, in turn leading to cell death. Slower DNA replication caused by the shortage of dGTP would lead to slower utilisation of the other three dNTPs, which would result in their accumulation as observed in Figure 1F, perhaps in part due to their salvage from the dNs produced by the other cell types in the heart in which Rrm1 is not deleted.

dNTP pool imbalances are mutagenic (18-20), and it is therefore difficult to envision how high DNA replication fidelity could be maintained under the extremely imbalanced dNTP pools we measured in the heart. For example, in the presence of the biased dNTP concentrations found in the heart, the fidelity of DNA synthesis in vitro by the normally highly accurate mtDNA polymerase gamma is
Reduced, with error frequencies increased by as much as 3-fold (16). We therefore speculate that most of the dGTP found in the heart might be bound to proteins and thus not be available to nuclear or mitochondrial DNA polymerases. GTP-binding and GTP-metabolising proteins are especially enriched in the mitochondria. For example, adenylate kinase 3 is located in the mitochondrial matrix and is unique in that it uses GTP instead of ATP as a phosphate donor to phosphorylate AMP (21). Furthermore, fission and fusion of mitochondria are regulated by dynamin family proteins that act as GTPases and that require a large amount of GTP as an energy source (22). A recently described nucleoside diphosphate kinase-like protein, dynamin-based ring motive-force organizer I (DYNAMO1), locally generates a high concentration of GTP for mitochondrial dynamin GTPases (23). Interestingly, we found that GTP was the most abundant NTP in the heart after ATP (Table S2), with GTP levels ~5-fold higher than CTP and ~2-fold higher than UTP. In contrast, in actively dividing mouse fibroblasts and whole E13.5 embryos GTP levels were higher than CTP but lower than UTP (9) and Table S2. We propose that GTP-binding proteins found in mitochondria also bind dGTP because it is very similar to GTP, and this would explain the high dGTP levels previously reported in heart mitochondria. At the same time, protein-bound dGTP would be sequestered and be unavailable to participate in DNA replication and would therefore not increase the mutation frequencies in nuclear DNA or mtDNA.

Our findings demonstrate that the salvage pathway of dGTP is not able to compensate for the lack of de novo dNTP synthesis in mouse heart and skeletal muscle, despite normal de novo dNTP synthesis in all other tissues. Inefficient salvage might be explained by relatively low expression of dN kinases in heart and skeletal muscle (24). Perhaps inactivation of dNTP-catabolising enzymes such as 5' nucleotidases or SAMHD1 might rescue Rrm1 KO cardiomyocytes from dying by increasing dNTPs such that salvage would be sufficient to maintain cell viability. Inactivation of SAMHD1 might be especially effective because this results in a greater increase in dGTP than the other three dNTPs in whole mouse embryos (~2-fold for dCTP and dTTP, ~3-fold for dATP, and ~5.5-fold for dGTP) (13).

Our results also show that limiting the dNTP supply in cardiomyocytes does not lead to their exit from the cell cycle. Rather, cardiomyocytes appear to lack control mechanisms that prevent them from initiating DNA replication when dNTPs are limiting. The observed nuclear fragmentation, increased DNA breakage, decreased proportion of DNA compared to RNA, and increased number of apoptotic cells in the Rrm1 KO heart are indicative of DNA replication failure caused by dNTP shortage, although we cannot exclude that other RRM1 functions (25) might contribute to the observed phenotypes. Therefore, just like in vitro cultured cells treated with hydroxyurea or other drugs that deplete dNTPs (26,27), cardiomyocytes in vivo attempt to replicate their DNA even when dNTPs are in short supply.

Experimental procedures

Generation of transgenic mice

The Rrm1<sup>fllox</sup> mice were generated by homologous recombination of a targeting vector in C57BL/6 embryonic stem cells. The NotI-linearised vector, carrying the sequence from exon 7 to exon 12 of the Rrm1 gene and a Neomycin (Neo) selection cassette, was transfected into the embryonic stem cells by electroporation. Neo-resistant clones were injected into the blastocysts of surrogate mice to obtain chimeric transgenic mice. At the Rrm1 locus of the transgenic mice, a distal loxP site was inserted 159 bp upstream of exon 9 and the Neo cassette was inserted 149 bp downstream of exon 11. Two clusters of FRT sites-loxP sites were flanked on two ends of the Neo cassette. Mice carrying the Neo cassette in the germ line were mated to Flp recombinase-expressing mice to remove a large part of the Neo cassette, leaving a remnant of the Neo cassette that carries one FRT and one loxP site. The Neo cassette remnant was confirmed using PCR with NDE1 and NDE2 primers. In the presence of a Cre recombinase protein, the region between the distal loxP site and the loxP site in the Neo cassette remnant is excised, which can be detected by PCR using ORRM3 and NDEL2 primers (Figure S1A).

Ckmn<sup>-Cre</sup> mice [Tg(Ckmn-cre)1Lrsn], constructed as described in (28), express Cre recombinase under the control of the creatine kinase type M (Ckmn) promoter exclusively in skeletal and cardiac muscles from embryonic day E13 in mice (8). The truncated Rrm1 gene is translated into a 285 amino acid protein that does not possess the active site (Figure S1C). Skeletal muscle-specific and cardiac muscle-specific knockout of the Rrm1 gene was achieved using the Rrm1<sup>fllox</sup> Ckmn<sup>+/+</sup> ×...
Rrm1<sup>lox/lox</sup> Ckm<sup>Cre/+</sup> cross. Such a cross also provided WT, hemizygous, and homozygous KO phenotypes at a ratio of 5:2:1 for the survival analysis.

Maintenance of transgenic mice

All mice were maintained at the animal facility at Umeå University under pathogen-free conditions. Mice were housed in a 12-hour dark/light cycle environment with ad libitum access to food and water. Both Rrm1<sup>lox</sup> and Ckm<sup>Cre</sup>-<sup>Cre</sup> strains were maintained in the C57BL/6 background. Genotyping at the Rrm1 locus was performed using PCR with NDE11 (5'-CAG GAC TGG TCA TTG AGT TGT CCC-3'), NDEL2 (5'-GCC TTC TCC TGA TGT GTC TGA AGA CAG-3'), and ORRM3 (5'-GTA GGG TTG TTG AGG TAG CTC AAC-3') primers, resulting respectively in a 518 bp (WT band), 671 bp (floxed band), and 457 bp (knockout band) product (Figure S1B). The presence of Ckm<sup>Cre</sup> was confirmed with PCR using Cre1F and Cre1R primers as described in (28).

All animals and experiments carried out in this study were approved by the ethical committee at Umeå University and complied with the rules and regulations of the Swedish Animal Welfare Agency and with the European Communities’ Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to minimise the number of animals used and to minimise their suffering.

dNTP pool measurement

Pups at the age of P15–P17 were euthanised by cervical dislocation, followed by rapid isolation of hearts; the tails were taken for genotyping. To extract E13.5 embryos, mothers were euthanised by cervical dislocation and the embryos were dissected in ice-cold PBS solution; the tails were taken for genotyping. The samples were placed in 700 µl ice-cold 12% (wt/v) TCA and 15 mM MgCl₂, frozen in liquid nitrogen and stored at −80°C. SV40 large T antigen-immortalised MEF cells were grown in DMEM medium supplemented with 10% FBS, 2 mM glutamate, 1% non-essential amino acids and 1% penicillin and streptomycin, and washed with ice-cold PBS twice. Approximately 3x10⁶ cells were scraped in 500 µl ice-cold 12% (wt/v) TCA and 15 mM MgCl₂. Nucleotide extraction was performed as described in (13). Sample cleaning using SPE (solid-phase extraction) columns and HPLC (high pressure liquid chromatography) analysis were performed as described in (9,29). The p-value was computed with Mann–Whitney U-tests.

Survival analysis of postnatal pups

Postnatal pups from six pairs of the cross Rrm1<sup>lox/lox</sup> Ckm<sup>Cre/+</sup> × Rrm1<sup>lox/lox</sup> Ckm<sup>+/+</sup> were checked every morning from P1 to P30. When a pup was found dead, its tail was saved for genotyping, and its heart, hind leg muscles, and diaphragm were isolated, mounted on thin cardboard in OCT embedding medium (Tissue Tek, Miles, Elkhart IN, USA), and frozen in propane chilled with liquid nitrogen. The samples were stored at −80°C until further processing. The survival curves were plotted with GraphPad Prism 7.04. Median survival calculations and comparisons of survival curves were performed with the Mantel–Cox test.

Quantification of DNA and total RNA

Pups at the age of P7 and P9 were euthanised in a CO₂ box and pups at the age of P15 were euthanised by cervical dislocation. The hearts and TA muscles were isolated and processed as described in (30) to obtain genomic DNA and RNA. DNA and RNA concentrations were measured using the Qubit dsDNA HS and RNA HS assay kits, respectively, and a Qubit 2.0 Fluorometer (Molecular Probes). The difference in DNA/RNA ratios was calculated with an unpaired t-test with Welch’s correction.

OPT imaging and analyses

Pups at the age of P7 were euthanised in a CO₂ box and pups at the age of P15 were euthanised by cervical dislocation. The hearts were fixed in 2% freshly made paraformaldehyde (PFA) in PBS for 2 hours at room temperature. The fixed tissues were subsequently processed for OPT imaging as described in (31), omitting the antibody labelling procedure. All steps of the OPT scanning and sample reconstruction are described in detail in (32). Briefly, the samples were aligned around their centre of mass (33) and the autofluorescence of the samples was detected using a Bioptics 3001 OPT scanner (SkyScan, Belgium). The filters used to detect the autofluorescence from the hearts were excitation 565/30 (Chroma) and emission 620/60 (Chroma). Tomographic reconstructions were performed using the NRecon v1.6.9.18 software (Bruker microCT, Belgium). Sliced images and videos were created in Fiji software (34). The thickness of the LV wall, RV wall, and IVS was measured...
using Fiji, and the difference was computed with the Mann–Whitney U-test.

**Quantification of the nuclear density**

To quantify the nuclear density in cardiac muscles, pups at P10 were euthanised in a CO₂ box for at least 20 minutes. Their hearts were isolated and immediately placed in Eppendorf tubes containing ice-cold PBS, and their tails were taken for genotyping. The hearts were then fixed in 2% PFA in PBS for 2 hours at room temperature. The PFA solution was removed and the hearts were incubated in 0.1 M glycine in PBS for 5 min to quench the excess PFA. The hearts were permeabilised in 0.1% Triton X-100 in PBS for 1 min, followed by three washes with PBS for 5 min each. The hearts were incubated in PBS containing 0.33 µM rhodamine-phalloidin (Thermo Fisher Scientific) and 2 µg/ml DAPI (Thermo Fisher Scientific) overnight at 4°C. The hearts were then washed in 0.1% Tween-20 in PBS for 5 hours in a dark, cold room. Thin slices of LVs were scanned with a Nikon A1 confocal microscope to create z-stacks of images within a cuboid volume of 211.41 µm × 211.41 µm × 2.7 µm. Fiji software was used for z-stack processing and nuclei quantification. Statistical significance was computed with an unpaired t-test.

To isolate Gas muscles, P15 pups were euthanised by cervical dislocation and their muscles were dissected out and kept at −80°C. These muscles were processed as described above for the hearts. Thin slices of Gas muscles were scanned with a Nikon A1 confocal microscope to create z-stacks of images within a cuboid volume of 318.18 µm × 318.18 µm × 2.7 µm. Fiji software was used for z-stack processing and nuclei quantification. The thickness of the muscle fibres was measured at the maximal width using the z-stack images. Statistical significance was computed with an unpaired t-test.

**Imaging of nuclei**

Hind leg muscles and hearts of P14 or P16 KO pups from the survival study or of the wild type control pups at the same age were cryosectioned to obtain slices 7–10 µm thick. These slides were processed similarly to what was described above under “Quantification of nuclear density in cardiac muscle” except for a 15-min fixation in PFA solution and a 2-hour wash in 0.1% Tween-20 in PBS in a dark, cold room. The sections were mounted in fluorescence mounting medium containing 500 µg/l DAPI (Dako Omnis, GM304) and scanned with a Nikon A1 confocal microscope as single images or as z-stacked images, which were processed with Fiji software.

**Immunohistochemistry**

Hearts, hind leg muscles, and diaphragms from Rrm1 KO pups in the survival study or from age-matched WT pups were used for immunohistochemical staining. Five to seven-µm thick serial tissue sections were made with a cryostat (Leica CM3050) at −20°C and mounted on glass slides. The sections were stained using a rabbit polyclonal antibody directed against the basement membrane protein laminin (Z0097, Dako Sweden, dilution 1:5000), the C-terminal of the membrane-associated protein dystrophin (GTX 1527, GeneTex Inc., Taiwan, dilution 1:3000), and the intermediate filament protein desmin (ab 1520, Abcam, UK, dilution 1:2000). To stain laminin in a double staining of laminin-desmin and laminin-dystrophin, a sheep polyclonal antibody against laminin (pc128, The Binding Site, UK) was used instead of the rabbit polyclonal antibody. Bound primary antibodies were visualised by indirect immunofluorescence. A mounting medium with DAPI was used to visualise the nuclei (H-1500, Vector Lab, Burlingame, CA, USA). The detailed laboratory techniques are described in (35).

**Morphometric analysis**

All cardiac and skeletal muscle samples were scanned at 40× magnification with a fluorescence microscope (Leica DM6000B, Leica Microsystems CMS GmbH, Wetzlar, Germany) equipped with a digital high-speed fluorescence charged couple device camera (Leica DFC360 FX). For measurements of cardiomyocyte and muscle fibre area and the amount of extracellular matrix, two or three photos were randomly selected from each cross-section, and the sizes of the cells were measured using a customised morphometric software (Leica QWin Standard V3.5.1 software, Leica Microsystems Ltd. Heerbrugg, Switzerland). The cross-sectional cell area was measured by tracing the circumference of the muscle cell membrane stained for the primary antibody Z0097 against laminin. The amount of extracellular tissue, i.e., connective tissue, including vessels and nerves, was calculated by subtracting the total muscle cross-sectional area from the total tissue area of each scanned photo. The investigator was blinded to the origin of the
samples. The difference in size of cardiomyocytes or myofibres between \textit{Rrm1} KO and WT was tested with an unpaired t-test.

\textbf{Electrocardiogram (ECG) recording and analysis}

P15 pups were anesthetised by intraperitoneal injection of a mixture of 10 mg/ml ketamine and 1 mg/ml xylazine, and the injected volume was based on the body weight. The body temperature was monitored throughout the ECG recording by a rectum-inserted thermocouple probe. Breathing was monitored through a homemade sensor at the beginning of each experiment to confirm the stable health conditions of the animal. ECG electrodes were placed in a lead I configuration on the Einthoven surface by gently inserting them subcutaneously. The analogue signals were amplified through a DAM50 Extracellular Amplifier (WPI), acquired via an LIH 8+8 data acquisition board (HEKA), digitized at 10 kHz, and recorded through the Patchmaster acquisition software (HEKA). Data were analysed offline using the Patchmaster software. The ECG parameters (P-wave, PQ, PR, and RR intervals) were measured semi-automatically and blind to the experimental conditions. Statistical analysis was performed with an unpaired t-test.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
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Figure 1. De novo dNTP synthesis in cardiac and skeletal muscle is required for postnatal survival. (A) A simplified scheme of deoxynucleoside triphosphate (dNTP) production in mammals. In the de novo synthesis pathway, ribonucleoside diphosphates (NDPs) are reduced to deoxynucleoside diphosphates (dNDPs) by ribonucleotide reductase (RNR) (in green) and these dNDPs are...
phosphorylated to dNTPs by several kinases. Excess dNTPs are degraded to 2’-deoxynucleosides (dNs) by SAM domain and HD domain-containing protein 1 (SAMHD1) (in yellow) or 5’-nucleotidases (5’NUC) (in orange). dNs can penetrate cellular membranes and exit into the extracellular space. In the salvage pathway, dNs are phosphorylated to dNMPs by specialised kinases, including thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), deoxyguanosine kinase (DGUOK), and deoxycytidine kinase (DCK) (in blue). (B) Embryos at E16.5. (C) Size comparison of P16 pups. (D) Weight of postnatal pups at P9. Each value is represented as a circle or a triangle, and horizontal bars represent the median values of 10 WT pups and 5 Rrm1 KO pups. The p-value was computed with Mann–Whitney U-tests. (E) Kaplan–Meier survival curve of postnatal pups from P1 to P30. Data are from 26 WT pups and 20 Rrm1 KO pups. The p-value was calculated with the log-rank (Mantel–Cox) test, **** p < 0.0001. (F) dNTPs of P15–P17 hearts normalised to total NTPs. Data are shown as mean ± SEM with circles and triangles representing single values from 7 WT pups and 6 Rrm1 KO pups. The p-values were calculated with Mann–Whitney U-tests, ns: non-significant. (G) dNTPs of E13.5 embryos normalised to total NTPs. Data are shown as mean ± SEM with n = 4. (H) dNTPs of immortalised mouse embryonic fibroblasts normalised to total NTPs. Data are shown as mean ± SEM with n = 2.
Figure 2. Anatomical aberrations in Rrm1 KO hearts. (A) Representative images of hearts from P7 and P15 pups. Atrial enlargement was clearly seen in the Rrm1 KO heart at P15. (B) Coronal (left) and transverse (right) sections of OPT-reconstructed P7 hearts. (C) Quantification of the thickness of the left ventricle (LV) wall, right ventricle (RV) wall, or interventricular septum (IVS) from the samples in (B). Data are represented as median values (horizontal bars) from three hearts of each genotype. The p-values were calculated with Mann–Whitney U-tests. (D) Coronal (left) and transverse (right) sections of OPT-reconstructed P15 hearts. (E) Quantification of the thickness of the LV wall, RV wall, and IVS from the samples in (D). Data are represented as median values (horizontal bars) from 2 WT hearts and 4 Rrm1 KO hearts. The p-values were calculated with Mann–Whitney U-tests.
Figure 3. Disorganisation of cardiac muscles and abnormalities of cardiomyocyte nuclei in Rrm1 KO hearts. (A) Images from the LVs of P7 hearts stained with phalloidin and DAPI, scale bar: 20 µm. (B) Images from the LVs of P10 hearts stained with phalloidin and DAPI, scale bar: 20 µm. (C) Quantification of the cardiomyocyte nuclear density in the samples in (A) and in (B). Data are shown as median values (horizontal bars) from 2 WT pups and 2 Rrm1 KO pups at P7 and from 4 WT pups and 6 Rrm1 KO pups at P10. The p-values were computed with Mann–Whitney U-tests, ns: non-significant. (D) LVs of P7 or P14 hearts were stained with DAPI, scale bar: 10 µm. (E) Proportions of DNA and RNA per total extracted nucleic acid from the hearts of P7, P9, and P15 pups. Data are shown as means ± SEM with n = 6–9. The p-values were computed with an unpaired t-test with Welch’s correction, **** p < 0.0001.
**Figure 4. Aberrant expression of muscle proteins in Rrm1 KO hearts.** (A) Cardiac muscle sections from P16 pups immunostained for desmin, dystrophin C-terminal, or laminin (green) together with DAPI staining of the nuclei (blue). Positively stained fibres are marked (+), and negatively stained fibres are marked (*). Scale bar: 25 µm. (B) The proportion of cardiomyocytes to connective tissue and the cardiomyocyte size were quantified from two or three images of laminin-stained sections of the sample shown in (A). Data for Rrm1 KO cardiomyocytes are shown as means ± SEM with n = 541 for WT and n = 363 for Rrm1 KO. (C) Cardiomyocyte size was computed from the sections in (A). Data are shown as means ± SEM with n = 541 for WT cardiomyocytes and n = 363 for Rrm1 KO cardiomyocytes. The p-value was computed with an unpaired t-test with Welch’s correction, **** p < 0.0001.
Figure 5. Disturbance of ECG in Rrm1 KO hearts. (A) Representative lead I ECG traces of P15 WT pups and Rrm1 KO pups with a scale bar of 100 ms. Traces in the grey squares are magnified as shown on the right side with a scale bar of 25 ms. (B) The durations of the ECG parameters were quantified from the data in (A). (C) Heart rates of the WT and Rrm1 KO pups used in (A). Data in (B) and (C) are shown as means ± SEM, n = 158 for WT and n = 21 for Rrm1 KO. These data were compiled from 11 WT pups and 2 Rrm1 KO P15 pups. The p-values were calculated with an unpaired t-test with Welch’s correction, ****p < 0.0001.
De novo dNTP production is essential for normal postnatal murine heart development
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