



## BASIC SCIENCE ARTICLE

# The newborn *Fmr1* knockout mouse: a novel model of excess ubiquinone and closed mitochondrial permeability transition pore in the developing heart

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**BACKGROUND:** Mitochondrial permeability transition pore (mPTP) closure triggers cardiomyocyte differentiation during development while pathological opening causes cell death during myocardial ischemia-reperfusion and heart failure. Ubiquinone modulates the mPTP; however, little is known about its mechanistic role in health and disease. We previously found excessive proton leak in newborn *Fmr1* KO mouse forebrain caused by ubiquinone deficiency and increased open mPTP probability. Because of the physiological differences between the heart and brain during maturation, we hypothesized that developing *Fmr1* KO cardiomyocyte mitochondria would demonstrate dissimilar features.

**METHODS:** Newborn male *Fmr1* KO mice and controls were assessed. Respiratory chain enzyme activity, ubiquinone content, proton leak, and oxygen consumption were measured in cardiomyocyte mitochondria. Cardiac function was evaluated via echocardiography.

**RESULTS:** In contrast to controls, *Fmr1* KO cardiomyocyte mitochondria demonstrated increased ubiquinone content and decreased proton leak. Leak was cyclosporine (CsA)-sensitive in controls and CsA-insensitive in *Fmr1* KOs. There was no difference in absolute mitochondrial respiration or cardiac function between strains.

**CONCLUSION:** These findings establish the newborn *Fmr1* KO mouse as a novel model of excess ubiquinone and closed mPTP in the developing heart. Such a model may help provide insight into the biology of cardiac development and pathophysiology of neonatal heart failure.

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**IMPACT:**

- Ubiquinone is in excess and the mPTP is closed in the developing FXS heart.
- Strengthens evidence of open mPTP probability in the normally developing postnatal murine heart and provides new evidence for premature closure of the mPTP in *Fmr1* mutants.
- Establishes a novel model of excess CoQ and a closed pore in the developing heart. Such a model will be a valuable tool used to better understand the role of ubiquinone and the mPTP in the neonatal heart in health and disease.

**INTRODUCTION**

Heart failure in the newborn is a challenging clinical condition that results from a variety of congenital and acquired conditions.<sup>1,2</sup> Cardiac dysfunction can manifest from structural abnormalities or ischemia-reperfusion injury related to surgery in children with congenital heart disease and can develop as a consequence of primary cardiomyopathies in infants with a structurally normal heart.<sup>1–3</sup> Unfortunately, therapy for neonatal heart failure remains nonspecific due to a lack of mechanistic insight and incomplete understanding of normal cardiomyocyte development.<sup>1,2</sup>

Recent investigation has helped to elucidate the role of mitochondria in the developing heart.<sup>2</sup> In embryonic cardiomyocytes, the respiratory chain is immature and bioenergetic capacity is generated primarily via anaerobic glycolysis.<sup>4,5</sup> As the heart matures, the mitochondrial membrane potential is established and oxidative phosphorylation becomes the predominant source

of cellular energy.<sup>2,4</sup> This change, mediated by closure of the mitochondrial permeability transition pore (mPTP), reduces oxidative stress and triggers cardiomyocyte differentiation.<sup>6</sup> Murine cardiac mitochondria are relatively immature at birth, but undergo maturation in the first 2 weeks of life.<sup>2</sup> However, the probability of mPTP opening remains high in postnatal cardiomyocyte mitochondria, rendering the neonatal heart vulnerable to injury.<sup>2</sup>

High-conductance opening of the mPTP collapses the mitochondrial membrane potential, uncouples oxidative phosphorylation, results in mitochondrial swelling, and leads to apoptosis or necrosis.<sup>4,7,8</sup> Pathological mPTP opening is thought to play a role in myocardial ischemia-reperfusion injury and heart failure.<sup>4,9,10</sup> A number of agents, such as cyclosporine A (CsA), are known to target and inhibit the mPTP and have been shown to protect the heart in preclinical models.<sup>10,11</sup> However, such approaches have

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failed to translate to the clinical scenario and, currently, mitochondrial therapy for neonatal heart failure is limited to nonspecific agents such as carnitine and coenzyme Q (CoQ).<sup>12–14</sup>

CoQ, or ubiquinone, is a lipophilic mobile electron carrier within the respiratory chain.<sup>15</sup> In addition, CoQ<sub>10</sub> and other ubiquinone analogs have been shown to have biological activity on the mPTP, modulating channel opening by inhibition or activation.<sup>16</sup> In a handful of clinical trials, CoQ supplementation was shown to be safe and effective in adult heart failure patients, improving cardiac function and short-term survival while reducing major adverse cardiovascular events.<sup>17,18</sup> However, mechanistic data are lacking and CoQ therapy for cardiomyopathy in children has been poorly studied.<sup>19</sup> Thus, there is a need to investigate the biological effects of CoQ on pathological opening of the mPTP in the developing heart. In order to do so, as a first step, novel and relevant preclinical models need to be identified and established.

Interestingly, the physiological role of the mPTP in developing cardiomyocytes differs from that of immature neurons.<sup>4</sup> In contrast to cardiac mitochondria, inhibition and closure of the pore in neural progenitors enhances proliferation while prolonged mPTP opening promotes neuronal differentiation.<sup>4,20</sup> We previously found excessive proton leak in forebrain mitochondria in a newborn mouse model of Fragile X syndrome (*Fmr1* knockout) caused by CoQ deficiency and an open CsA-sensitive channel (the mPTP).<sup>21</sup> Fragile X syndrome (FXS) is caused by silencing of the *Fmr1* gene and loss of expression of the gene product, fragile X mental retardation protein (FMRP).<sup>22</sup> FMRP is a translational repressor that binds to the mRNA of all of the known enzymes of the ubiquinone biosynthesis pathway; however, it is unknown how loss of FMRP causes changes in CoQ levels.<sup>23</sup> Because of the physiological differences between the immature heart and brain, we hypothesized that developing *Fmr1* KO cardiomyocyte mitochondria would demonstrate dissimilar features from immature neurons. In stark contrast to the *Fmr1* KO forebrain and the immature wild-type heart, we found increased levels of ubiquinone, decreased proton leak, and closed probability of the mPTP in newborn *Fmr1* KO cardiomyocyte mitochondria. The importance of these findings is that it establishes a model of excess CoQ and a closed pore in the developing heart. Such a model will be a valuable tool in the quest to better understand the role of ubiquinone and the mPTP in neonatal heart failure in future work.

## METHODS

### Animals

The care of the animals in this study was in accordance with NIH and Columbia University Medical Center Institutional Animal Care and Use Committee guidelines and conformed to the provisions of the Animal Welfare Act (NIH/DHHS) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Six- to eight-week old hemizygous male (*Fmr1*<sup>-Y</sup>) (FVB.129P2-Pde6b<sup>+</sup> Tyr<sup>c-ch</sup> *Fmr1*<sup>tm1Cgr/J</sup>) and homozygous female (*Fmr1*<sup>-/-</sup>) mice were acquired (Jackson Laboratory, Bar Harbor, ME), paired, and mated to yield *Fmr1* KO neonatal male pups. Control (FVB.129P2-Pde6b<sup>+</sup> Tyr<sup>c-ch</sup>/AntJ) hemizygous male and homozygous female breeding pairs were also acquired (Jackson Laboratory, Bar Harbor, ME) and mated to yield pups that expressed wild-type FMRP. Experiments were performed on 10-day-old male pups given the male predominance in Fragile X syndrome. For FMRP expression, FVB male pups were evaluated on P7 and P10.

### Immunoblot analysis

Samples (10 µg) of forebrain and cardiac ventricular homogenate were subjected to SDS-acrylamide gel electrophoresis and immunoblotting. FMRP expression was assessed using a primary polyclonal rabbit anti-FMRP antibody (Abcam ab17722). Cytosolic protein loading was assessed with a primary monoclonal antibody

to mouse  $\alpha$ -tubulin (Millipore Sigma T5168). Appropriate secondary antibodies were utilized, signal was detected with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and density was measured using scanning densitometry. FMRP density was normalized to tubulin and FVB P7 heart values were arbitrarily set to equal 1.

### Mitochondrial isolation

Cardiac ventricles were harvested and homogenized in ice-cold isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM egtazic acid (EGTA), 5 mM HEPES-KOH (pH 7.2) and 1 mg mL<sup>-1</sup> of fatty-acid-free bovine serum albumin (BSA)). The homogenate was spun at 1100  $\times g$  for 5 min at 4 °C. Supernatant (0.8 mL) was removed and centrifuged at 18,500  $\times g$  for 10 min at 4 °C 15 vol% Percoll gradient. The mitochondria fraction was collected and resuspended in 1 mL of washing buffer (250 mM sucrose, 5 mM HEPES-KOH (pH 7.2), 1 mM EGTA and 1 mg mL<sup>-1</sup> of BSA). The suspension was centrifuged at 10,000  $\times g$  for 10 min at 4 °C. The mitochondrial pellet was resuspended in 0.035 mL of washing buffer and mitochondrial protein concentrations were determined using the method of Lowry.

### Steady-state electron transport chain enzyme activity

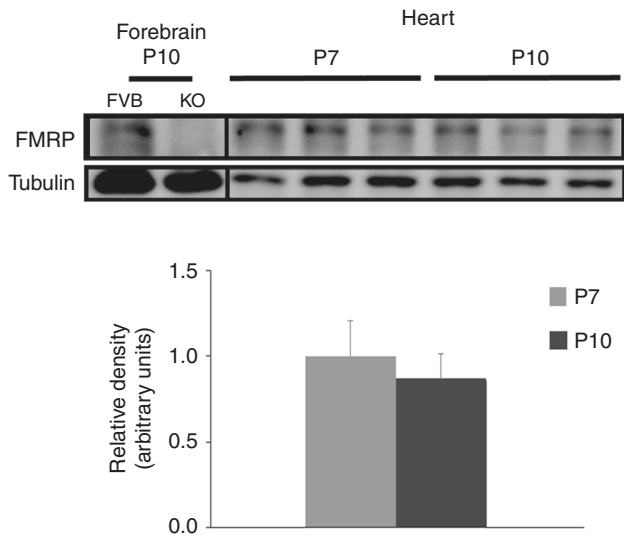
Respiratory chain enzyme complex activities were measured spectrophotometrically as previously described in 1-mL volume.<sup>24,25</sup> Rotenone-sensitive Complex I specific activity was measured in isolated mitochondria (40 µg) using 4.8 mM<sup>-1</sup> cm<sup>-1</sup> as the extinction coefficient of NADH at 340 nm with the reference wavelength of 380 nm. TFA (2-thenoyltrifluoroacetone)-sensitive Complex II activity was determined in isolated mitochondria (40 µg) using 19.1 mM<sup>-1</sup> cm<sup>-1</sup> as the extinction coefficient of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. For Complex III, antimycin A-sensitive first-order rate constants were calculated in isolated mitochondria (4 µg) using 18.5 mM<sup>-1</sup> cm<sup>-1</sup> as the extinction coefficient of cytochrome c at 550 nm. Rotenone-sensitive Complex I + III linked activity and antimycin A-sensitive Complex II + III linked activity were determined separately in isolated mitochondria (40 µg) using 18.5 mM<sup>-1</sup> cm<sup>-1</sup> as the extinction coefficient of cytochrome c at 550 nm.

### Ubiquinone quantification

Ubiquinone was extracted from isolated cardiomyocyte mitochondria (1.5–3 mg) using Tween-20 (3%), cold (-20 °C) methanol (300 µL), and light petroleum (450 µL).<sup>26,27</sup> Samples were centrifuged at 2200  $\times g$  for 15 min at room temperature. Supernatant was collected, evaporated in a glass tube, and resuspended in 100% ethanol. Total ubiquinone was calculated in 400 µL from the difference in spectra (oxidized minus sodium borohydride reduced) using an absorption coefficient of 12.25 mM<sup>-1</sup> cm<sup>-1</sup> at 275 nm.<sup>27</sup>

### Proton leak kinetics

Proton leak relative to the proton motive force was measured as previously described.<sup>28</sup> Oxygen consumption and mitochondrial membrane potential were measured simultaneously using ventricular cardiomyocyte mitochondria (0.1 mg) in 1 mL of respiration buffer (200 mM sucrose, 25 mM KCl, 2 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM HEPES-KOH (pH 7.2), 5 mM MgCl<sub>2</sub>, 0.2 mg mL<sup>-1</sup> BSA) containing 80 ng mL<sup>-1</sup> nigericin (to collapse  $\Delta$ pH) and 5 µM rotenone at 37 °C. Membrane potential was determined using an ion sensitive electrode selective for the lipophilic cation, tetraphenylphosphonium (TPP<sup>+</sup>) (World Precision Instruments, Sarasota, FL), and calculated using the Nernst equation as previously described.<sup>29</sup> Mitochondrial respiration was initiated with 5 mM succinate. State 4 was induced with oligomycin (2.5 µg mL<sup>-1</sup>) and respiration was titrated with malonate (up to 2 mM). In separate experiments, titrations of malonate (0.1 mM) were used to induce voltage-gated proton leak during state 4 respiration and



**Fig. 1 Wild-type fragile X mental retardation protein (FMRP) expression in the developing heart.** Immunoblot of FMRP in cardiac ventricles is depicted. FVB mice were assessed on postnatal days 7 (P7) and P10 ( $n=3$  mice per time point). Densities were normalized to the tubulin loading control. Graphical representation of relative densities is shown. Normalized values from P7 FVB mouse heart were arbitrarily set to equal 1. Forebrain homogenate from P10 FVB and *Fmr1* KO mice served as positive and negative controls, respectively. As expected, no FMRP was detected in forebrain of a P10 *Fmr1* KO mouse. Values are expressed as means  $\pm$  SD.  $P$  = nonsignificant.

cyclosporine A (CsA, 1  $\mu$ M), carboxyatractyloside (cAT, 1  $\mu$ M), and guanosine diphosphate (GDP, 0.75 mM) were added to specifically inhibit the mPTP, the adenine nucleotide translocase (ANT), and uncoupling proteins (UCPs), respectively to determine source of leak.<sup>30,31</sup> cAT was added after CsA given the potential to open the mPTP. The effect of the CsA solvent, ethanol, was tested independently. The ubiquinone analog, decylubiquinone (DUB, 100  $\mu$ M), was used in separate experiments to antagonize endogenous CoQ.<sup>21</sup>

#### Mitochondrial oxygen consumption

Ventricular cardiomyocyte mitochondria (50  $\mu$ g) were added to 0.5 mL of respiration buffer (200 mM sucrose, 25 mM KCl, 2 mM  $K_2HPO_4$ , 5 mM HEPES-KOH (pH 7.2), 5 mM  $MgCl_2$ , 0.2 mg mL<sup>-1</sup> BSA). Oxygen consumption was measured using a Clark-type electrode (Oxytherm, Hansatech, UK) with Complex I-dependent substrates (10 mM glutamate and 5 mM malate) or Complex II-dependent substrate (10 mM succinate in the presence of 5  $\mu$ M rotenone) at 32 °C. State 2 respiration was determined following the addition of substrates and ADP (200  $\mu$ M) was added to initiate state 3 respiration. The respiratory control ratio (RCR) was calculated as state 3 respiration divided by the state 4 rate observed following ADP consumption. The ADP-to-oxygen ratio (ADP:O) was calculated from the amount of ADP and oxygen consumed during state 3 respiration. Separately, state 4 respiration was induced with oligomycin (2.5  $\mu$ g mL<sup>-1</sup>) and uncoupled state 3 respiration was induced with dinitrophenol (DNP) (70  $\mu$ M).

#### Echocardiography

Transthoracic echocardiographic measurement of left ventricular cardiac function was performed as previously described.<sup>32</sup> Mice were lightly sedated with i.p. ketamine (12.5 mg kg<sup>-1</sup>) and xylazine (2 mg kg<sup>-1</sup>) and then subjected to two-dimensional echocardiography using a Vevo770 High-Resolution Imaging System (VisualSonics, Inc., Toronto, Canada) with a 55-MHz transducer (RMV708, VisualSonics, Inc., Toronto, Canada). M-

mode images and two-dimensional parasternal short axis images at the midpapillary level were obtained and recorded in a digital format by an investigator blinded to mouse genotype (K.M.). Images were then analyzed offline in a blinded manner (K.M.). Heart rate, end-diastolic left ventricular dimension, and end-systolic left ventricular dimension were measured. The percent fractional shortening was then calculated.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). Data were assessed for normality by examining histograms and box plots and are presented as means  $\pm$  SD. The sample number of animals studied for each experiment is indicated in each figure. Differences between the two strains were assessed using a two-tailed, unpaired Student's  $t$  test. Significance was set at  $P < 0.05$ .

## RESULTS

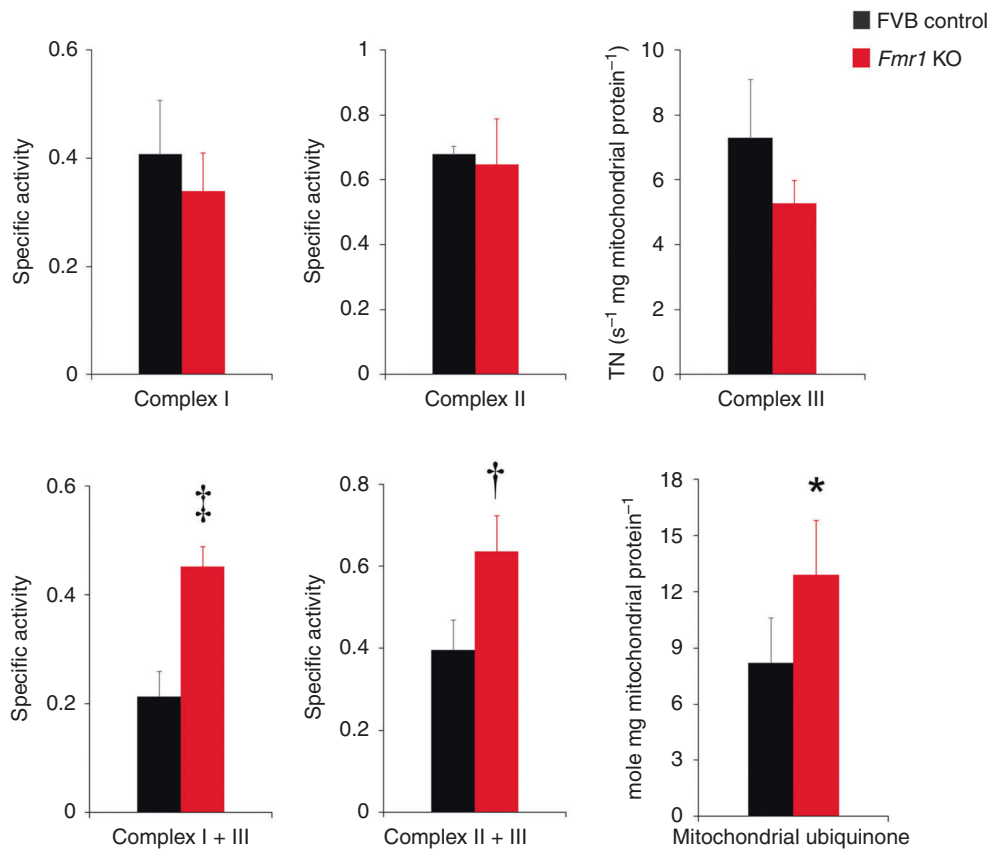
Fragile X mental retardation protein (FMRP) is expressed in the developing heart

FXS is caused by genetic silencing of the *Fmr1* gene and loss of the gene product, FMRP.<sup>22</sup> Although predominantly expressed within the brain and testis, FMRP is ubiquitous.<sup>33</sup> However, little is known about FMRP expression in the developing heart. So, we first assessed for wild-type FMRP expression within the immature ventricular myocardium of FVB controls in the first 2 weeks of life (Fig. 1). Using immunoblot analysis, we detected myocardial FMRP expression at 7 and 10 days of life in healthy controls (Fig. 1). Thus, FMRP is expressed in the wild-type developing heart. Based on these findings, we performed our analyses on P10 given that, in the mouse, this age is analogous to a postnatal time point in human infancy.<sup>34</sup>

Ubiquinone is increased in *Fmr1* KO myocardium

Given our findings in the developing *Fmr1* KO forebrain, we next measured CoQ levels in cardiomyocyte mitochondria of *Fmr1* KO mice and FVB controls on P10. CoQ is best known as a carrier of electrons from Complexes I and II to Complex III within the respiratory chain.<sup>15</sup> So, we first measured the kinetic activity of Complexes I, II, and III, individually, and then determined the linked kinetic activities of Complex I + III and Complex II + III to indirectly quantify the CoQ pool. There were no significant differences in the kinetic activities of Complexes I, II, or III between strains (Fig. 2). However, the linked activities of both Complex I + III and Complex II + III were significantly increased in cardiomyocyte mitochondria from *Fmr1* KO mice compared to controls, suggesting CoQ excess (Fig. 2). We confirmed these findings by directly quantifying mitochondrial ubiquinone content via spectrophotometry and found significantly increased levels of myocardial CoQ in *Fmr1* KOs versus controls (Fig. 2). Thus, ubiquinone was increased in the developing *Fmr1* KO heart.

*Fmr1* KO mitochondria demonstrate less proton leak than controls Because the mPTP is a source of uncoupled hydrogen ion conductance and can be modulated by ubiquinone and its analogs, we next assessed proton leak in cardiomyocyte mitochondria of *Fmr1* KO mice and FVB controls on P10.<sup>16,35</sup> To do so, we measured proton conductance relative to the proton motive force.<sup>36</sup> The benefit of this approach is that it permits dynamic measurement of respiration over a range of mitochondrial membrane potentials. To assess proton leak kinetics, succinate-dependent respiration was initiated in state 4 using oligomycin and was titrated with serial additions of malonate.<sup>36</sup> Using this approach, we found the *Fmr1* KO conductance curve to be right-shifted relative to FVB controls suggesting less proton leak (Fig. 3a). In support of this, the rate of mitochondrial respiration was significantly lower in *Fmr1* mutants at the highest



**Fig. 2 Ubiquinone is in excess in *Fmr1* KO cardiomyocyte mitochondria.** The specific activities of electron transport chain Complexes I and II are depicted. First-order rate constants were determined for Complex III and expressed as turnover number (TN). The CoQ pool was assessed indirectly by measuring the linked kinetic activities of Complex I + III and Complex II + III. Ubiquinone content was measured directly in cardiomyocyte mitochondria via spectrophotometry. Values are expressed as means  $\pm$  SD.  $n = 5-7$  animals per group.  $P$  values were calculated by Student's  $t$  test. \* $P < 0.05$ , † $P < 0.01$ , \*\*\* $P < 0.001$ .

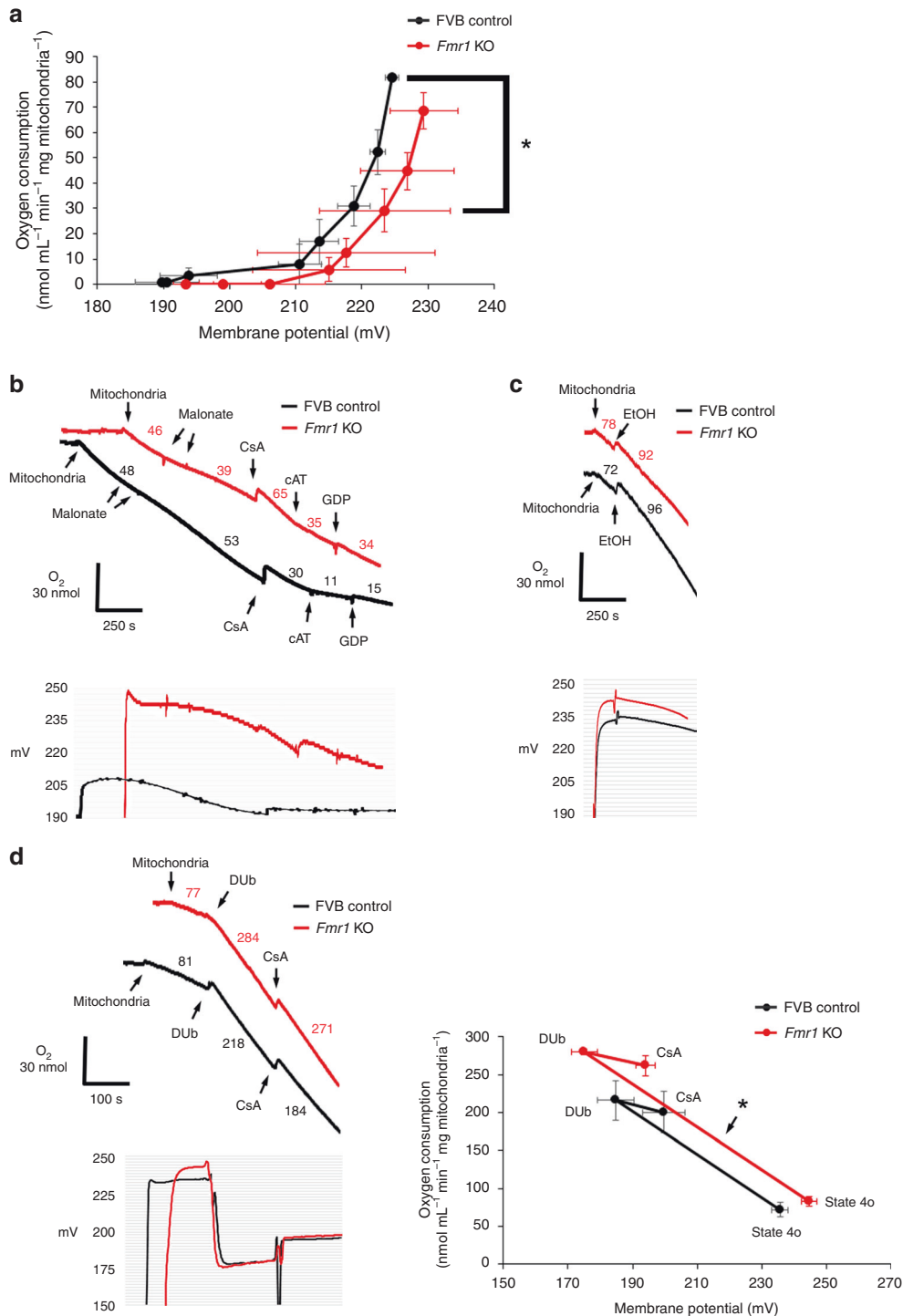
common membrane potential confirming less leak and more efficient respiration (Fig. 3a).

Because proton leak can also be mediated by the ANT and UCPs, we next utilized specific inhibitors to delineate the source(s) of leak in each group.<sup>30,31</sup> Succinate-dependent state 4 respiration was initiated with oligomycin in freshly isolated cardiomyocyte mitochondria and malonate was added to reduce the membrane potential and unmask any voltage-gate leak. CsA, carboxyatractylolide (cAT), and guanosine diphosphate (GDP) were subsequently added to inhibit the mPTP, the ANT, and UCPs, respectively (Fig. 3b). In control mitochondria, CsA increased and stabilized the membrane potential and simultaneously reduced the oxygen consumption rate, indicating increased open probability of the mPTP (Fig. 3b). The respiration rate declined further after addition of cAT, suggesting the ANT as an additional source of leak in control mitochondria (Fig. 3b). In contrast, CsA failed to decrease oxygen consumption or increase membrane potential in *Fmr1* KO mitochondria, indicating closed probability of the mPTP (Fig. 3b). Instead, respiration increased and membrane potential declined following the addition of CsA in *Fmr1* KOs, likely due to the uncoupling effect of the ethanol solvent in the absence of biological activity of CsA on the mPTP.<sup>37</sup> This ethanol-mediated effect was confirmed in mitochondria from both strains during state 4 respiration (Fig. 3c). With regard to other sources of leak, respiration rates decreased after the addition of cAT and the membrane potential began to stabilize, suggesting the ANT as a source of proton leak in *Fmr1* KOs (Fig. 3b). Lack of GDP sensitivity in both strains ruled out UCPs as a source of leak in *Fmr1* KOs or controls (Fig. 3b).

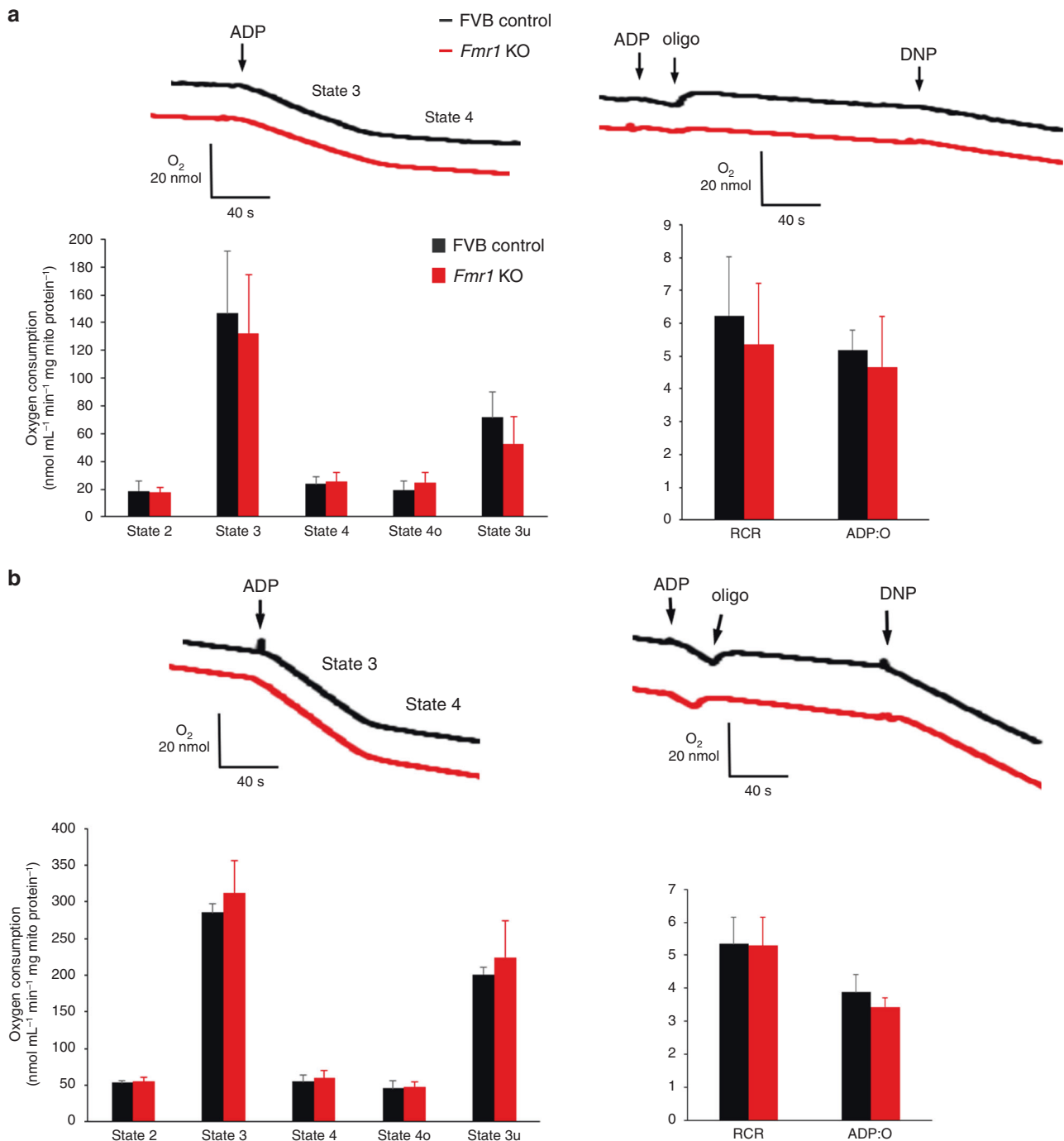
We then tested the hypothesis that the CoQ-antagonizing quinone, decylubiquinone (DUB), would open the pore in both strains (i.e., antagonize endogenous CoQ in controls and excess CoQ in *Fmr1* KOs). Succinate-dependent state 4 respiration was initiated with oligomycin in freshly isolated cardiomyocyte mitochondria and DUB was added. DUB caused an immediate increase in respiration and concomitant decline in membrane potential, suggesting opening of a leak channel in both groups (Fig. 3d). Importantly, both the increase in respiration and the fall in membrane potential following the addition of DUB were significantly greater in *Fmr1* KOs versus controls (Fig. 3d). CsA increased the membrane potential and simultaneously reduced the oxygen consumption rate in both strains, indicating DUB-mediated opening of the mPTP (Fig. 3d). Taken together, the data indicate open probability of the mPTP in the wild-type heart on P10 and excess ubiquinone and closed mPTP probability in developing *Fmr1* KO myocardium. The DUB-mediated effect in *Fmr1* KOs suggested a link between increased CoQ levels and the closed pore in *Fmr1* KO cardiomyocyte mitochondria.

Mitochondrial oxygen consumption is unaffected in *Fmr1* KO cardiomyocytes

In order to understand the effect of increased levels of CoQ and closed probability of the mPTP on bioenergetic capacity, we next measured oxygen consumption using a Clark-type electrode in freshly isolated cardiomyocyte mitochondria from P10 *Fmr1* KOs and FVB controls. We quantified the rates of mitochondrial respiration for both Complex I- and Complex II-dependent substrates (Fig. 4). There were no significant differences between



**Fig. 3 Proton leak kinetics and source of leak in developing cardiomyocyte mitochondria.** **a** Proton leak kinetics. Rates of oxygen consumption using succinate as a substrate were measured over a range of mitochondrial membrane potentials. State 4 was induced with oligomycin (data point with highest membrane potential) and respiration was titrated with serial additions of malonate.  $n = 3$  animals per group. Values are expressed as means  $\pm$  SEM.  $P$  values for the rate of oxygen consumption at the highest common membrane potential were calculated by Student's  $t$  test.  $*P < 0.05$ . **b–d** Oligomycin-induced state 4 (state 4o) was initiated using succinate as a substrate. Representative tracings of oxygen consumption (above) with simultaneous mitochondrial membrane potential measurement (below) are depicted. Numbers indicate rates of oxygen consumption (nmol mL<sup>-1</sup> min<sup>-1</sup> mg mitochondrial protein<sup>-1</sup>). **b** Source of proton leak. Titrations of malonate were used to induce voltage-gated proton leak. Cyclosporine A (CsA), carboxyatractyloside (cAT), and guanosine diphosphate (GDP) were added to specifically inhibit proton leak via the mitochondrial permeability transition pore (mPTP), the adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs), respectively.<sup>30,31</sup> Experiments were repeated in 8–10 animals per group. **c** Effect of ethanol solvent (EtOH). Experiments were repeated in 2–3 animals per group. **d** Decylubiquinone (DUB) was added to antagonize endogenous CoQ. Representative tracings are shown on the left. Graphical depiction of oxygen consumption relative to membrane potential following the addition of DUB and CsA is shown on the right.  $n = 3$  animals per group. Values are expressed as means  $\pm$  SD.  $P$  values for change in the rate of oxygen consumption and change membrane potential were calculated by Student's  $t$  test.  $*P < 0.05$ .



**Fig. 4 Oxygen consumption in *Fmr1* KO and FVB control mitochondria.** Representative tracings for **a** Complex I-dependent oxygen consumption using glutamate/malate ( $n = 5$  controls, 7 KOs) and **b** Complex II-dependent oxygen consumption using succinate ( $n = 5$  controls, 8 KOs) are depicted. Graphical representation of state 2 respiration (following addition of substrate), state 3 respiration (following addition of adenosine diphosphate (ADP)), state 4 respiration, uncoupled state 3 respiration (state 3u; following addition of dinitrophenol (DNP)), and oligomycin-induced state 4 (state 4o) are shown below the respective tracings. Graphical depiction of respiratory control ratios (RCR) and adenosine diphosphate-to-oxygen ratios (ADP:O) are also shown. Values are expressed as means  $\pm$  SD.  $P$  values were nonsignificant.

strains regardless of substrate (Fig. 4). Thus, excess ubiquinone and closed probability of the mPTP in the developing *Fmr1* KO heart did not affect absolute mitochondrial respiration.

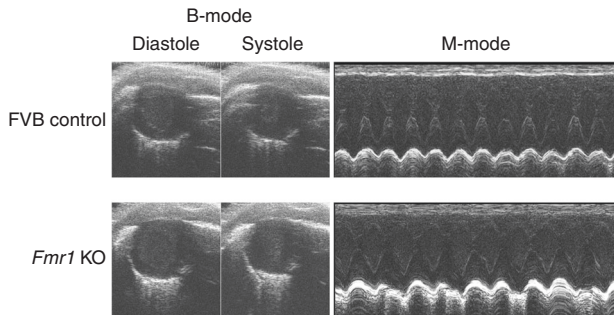
Cardiac function is unaffected in newborn *Fmr1* KO mice  
Prior work revealed evidence of open probability in the developing postnatal murine heart and that inhibition of the mPTP enhanced murine cardiac function in the neonatal period.<sup>2</sup>

Therefore, we assessed cardiac performance in *Fmr1* KO mice and FVB controls on P10 using two-dimensional transthoracic echocardiography. There were no differences in heart rate, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, or fractional shortening between strains (Table 1, Fig. 5). Thus, increased CoQ levels and closed probability of the mPTP in the *Fmr1* KO heart did not translate into an effect on cardiac function in newborn mutants.

**Table 1.** Cardiac function in *Fmr1* KO mice and FVB controls.

Parameter	Strain	
	FVB	<i>Fmr1</i> KO
Heart rate, beats per min	375 ± 45	378 ± 37
End-diastolic left ventricular dimension, mm	2.1 ± 0.1	2.2 ± 0.1
End-systolic left ventricular dimension, mm	1.3 ± 0.1	1.4 ± 0.1
Fractional shortening, %	38.9 ± 1.2	36.1 ± 3.2

Values are expressed as means ± SD. *n* = 5 mice per group. *P* values are nonsignificant.



**Fig. 5** Echocardiographic images in *Fmr1* KO mice and FVB controls. Parasternal short axis images were obtained in B-mode and M-mode to quantify end-diastolic left ventricular dimension and end-systolic left ventricular dimension. Representative images are depicted for both strains.

## DISCUSSION

The mPTP has been shown to have a high probability of opening in the neonatal murine heart.<sup>2</sup> Consistent with this, we found evidence of open probability of the mPTP in wild-type cardiomyocyte mitochondria on P10. Although the regulatory factors involved in closing the mPTP in immature mitochondria during development are unknown, endogenous molecules, such as ubiquinone, have been shown to be capable of modulating the pore.<sup>16</sup> Levels of CoQ are relatively low in infancy, peak in young adulthood, and decline thereafter.<sup>38</sup> Thus, it is possible that temporal differences in biosynthesis of CoQ contribute to the high probability of mPTP opening in the immature heart. In contrast to wild-type controls, we identified relatively increased levels of ubiquinone, decreased proton leak, and closed probability of the mPTP in developing cardiomyocyte mitochondria of *Fmr1* KO mice on P10. These findings establish the newborn *Fmr1* KO mouse as a novel model of excess CoQ and a closed pore in the developing heart. Because it is unknown how quinones inhibit or activate the pore, such a model will serve as an important tool to help us better understand the biological interaction between CoQ and the mPTP in the immature heart during health and disease.<sup>16</sup>

CoQ deficiency can cause heart failure and can result from it.<sup>39,40</sup> Lower myocardial ubiquinone levels have been associated with more severe heart failure symptoms and worse cardiac function in humans.<sup>39</sup> In a small number of contemporary clinical trials in adults, CoQ<sub>10</sub> supplementation was shown to be of benefit with an acceptable safety profile.<sup>17,18,39</sup> Unfortunately, mechanistic data are lacking and there has been a minimal number of studies evaluating CoQ therapy for neonatal cardiomyopathy.<sup>19</sup> Thus, the newborn *Fmr1* KO mouse model will likely help to provide mechanistic insights in future work given the putative role of pathological opening of the mPTP in myocardial ischemia-reperfusion injury and cardiomyopathies and the association of CoQ deficiency with heart failure.<sup>4,9,10</sup>

Open probability of the mPTP has been reported previously in the neonatal murine heart and closure of the pore with agents, such as CsA and NIM811, was shown to increase the mitochondrial membrane potential and enhance cardiac function within the first 5 days of life.<sup>2</sup> Although we found open probability of the mPTP in wild-type cardiomyocytes on P10, closed probability of the pore within the *Fmr1* KO did not translate into an effect on absolute mitochondrial respiration or cardiac performance. This difference may relate to the developmental stage of the mice. In the current study, we evaluated 10-day-old mice while prior investigation assessed mice on P5.<sup>2</sup> Thus, it is possible that cardiomyocyte mitochondria may be naturally transitioning to a mature phenotype on P10 and the pore may be relatively less open in the developing heart at this age compared to the first few postnatal days of life. This may explain the absence of physiological consequences of a prematurely closed mPTP in *Fmr1* KO mice at this age and the lack of a cardiac phenotype in Fragile X Syndrome. Importantly, the mice assessed in this work were healthy and non-stressed. Therefore, it will be critical to assess how *Fmr1* KO cardiomyocytes and mitochondria respond to insult and injury. Based on our knowledge of CsA, CoQ, and the mPTP, future investigation will need to determine if the developing *Fmr1* KO heart is relatively more resilient to ischemia-reperfusion and experimental heart failure.

It is unknown how loss of FMRP causes changes in CoQ biosynthesis, stability, or turnover. FMRP is a translational repressor that binds to the mRNA of all of the known enzymes of the ubiquinone biosynthesis pathway.<sup>23</sup> Thus, it is possible that FMRP deficiency affects CoQ biosynthesis directly. However, the FMRP–CoQ interaction is likely complex given tissue-specific differences in ubiquinone content in the *Fmr1* KO mouse. In addition, indirect effects of FMRP deficiency may exist and other mechanisms may play a role in closed or open mPTP probability. Thus, future work will be necessary and will focus on determining how loss of FMRP leads to CoQ excess in the developing heart and CoQ deficiency in the immature brain.

The data presented here establish the newborn *Fmr1* KO mouse as a new and unique model of CoQ excess and a closed mPTP in the developing heart. We are hopeful that this model will be useful as an investigative tool to aid us in delineating the roles of ubiquinone and the pore in cardiac maturation and in the manifestation of neonatal heart failure. Greater knowledge could result in the development of novel targeted therapeutic agents aimed at treating or preventing heart failure in infants. Thus, the *Fmr1* KO mouse could be an important model to advance our understanding of fundamental cardiac developmental biology and help us gain insight into why the neonatal heart is uniquely vulnerable to injury.

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## AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: M.B., A.W., K.K.G., K.M., R.L., S.H., R.J.L. Drafting the article or revising it critically for important intellectual content: M.B., K.M., R.L., S.H., R.J.L. Final approval of the version to be published: M.B., A.W., K.K.G., K.M., R.L., S.H., R.J.L.

## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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