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Supporting Information

Mitochondrial amidoxime-reducing component 2 (mARC2) has a significant role in *N*-reductive activity and energy metabolism

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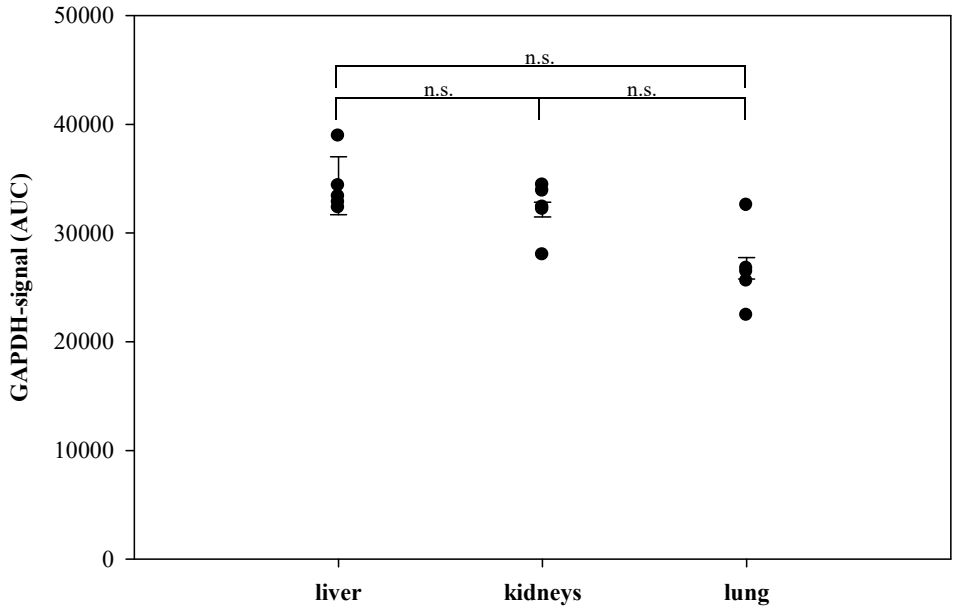
1. Validation of loading controls for normalization in quantitative western blot

In order to compare protein expression levels of mARC1/2, CYB5B, CYB5R between different samples conditions, we use the following loading controls to normalize the data: COX4 in case of WT *vs.* KO and GAPDH in case of different tissue origins (liver *vs*. lung *vs*. kidney). We include the loading controls for very gel run to compare the actual signal level between samples.

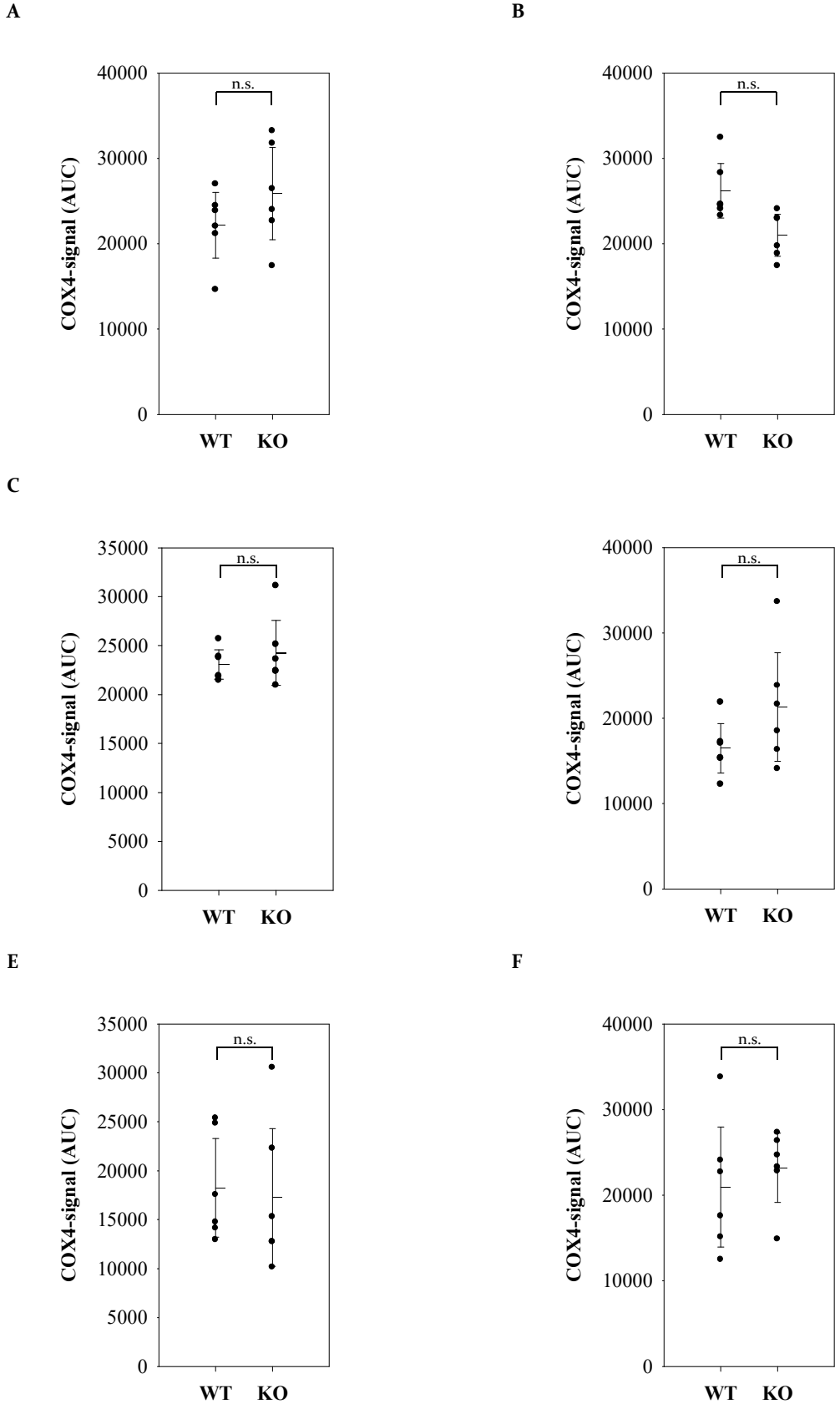
These two proteins were chosen since no significant variations in their signal between the different experimental conditions could be shown: In the western blot (Fig. S7) GAPDH expression is compared between different tissues. The matched anti-GAPDH probed section of the blot demonstrates that GAPDH expression is not affected by the origin of the tissues. The ECL signals generated on the western blot were detected with charge-coupled device camera and associated analysis software (see materials and methods). No significant changes in GAPDH-expression signal between tested tissues could be observed (Fig. S1).
Consistent expression of COX-4 in KO *vs.* WT (see for example Fig. S5) was proved in the same manner. No significant changes in COX4-expression signal could be observed between WT *vs.* KO (Fig. S2). Therefore, signals of the protein of interest (mARC1, mARC2, CYB5B, CYB5R) were normalized to the signal from these loading controls in the manuscript (see manuscript Fig. 5B, Fig. 4C).

Supplementary Figure S1: GAPDH ECL-signals in different tissues



Digital GAPDH-signals (AUC) were quantified with the ImageJ software, version 1.52a. Four lanes per tissue were loaded, with 64 µg protein per lane.

Supplementary Figure S2: COX4 ECL-signals in KO and WT samples

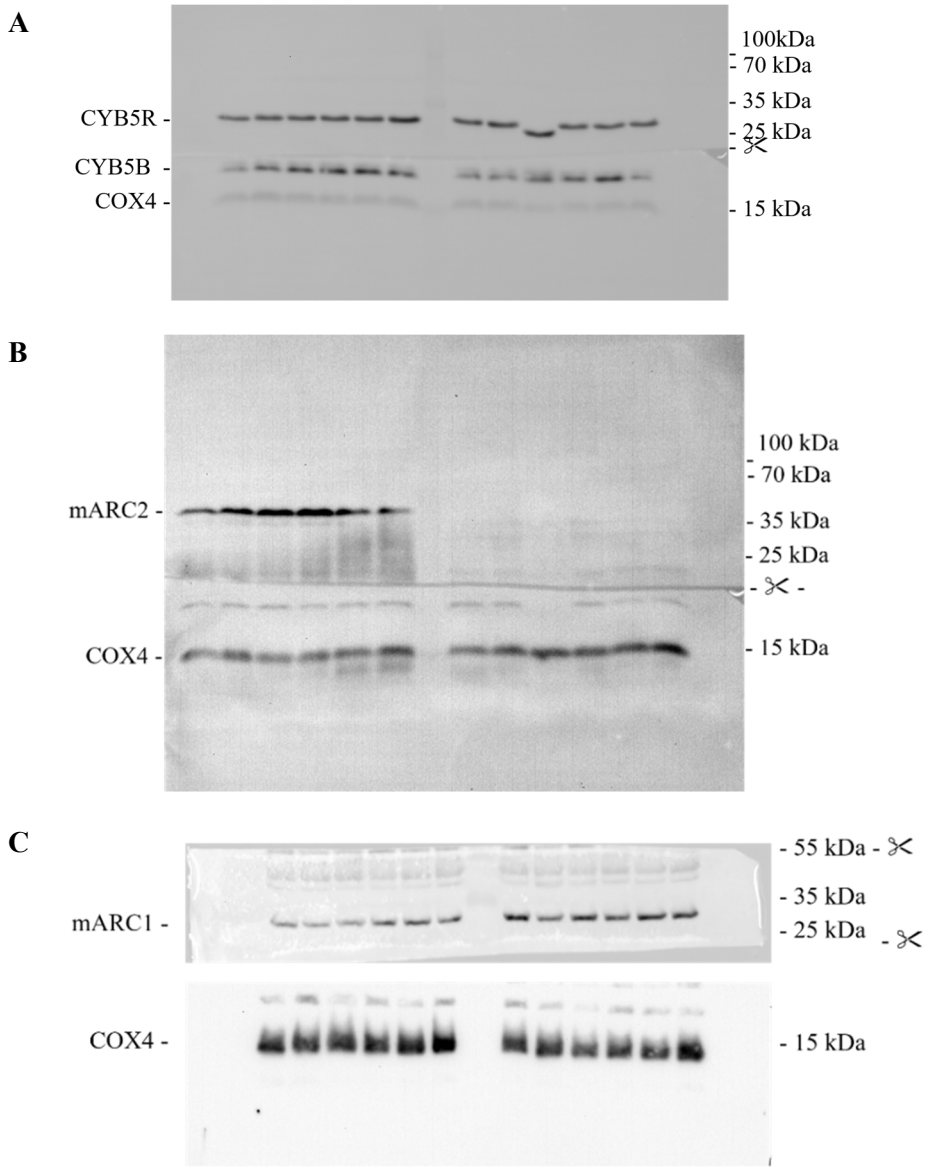


Digital COX4-signals (AUC) were quantified with the ImageJ software, version 1.52a. Six lanes per manipulation were loaded (WT or KO), with 64 µg protein per lane. Each individual membrane was labeled with a single letter: liver (A, B), kidneys (C, D) lung (E, F).

2. Original full western blots corresponding to cropped lanes shown in Fig. 5A

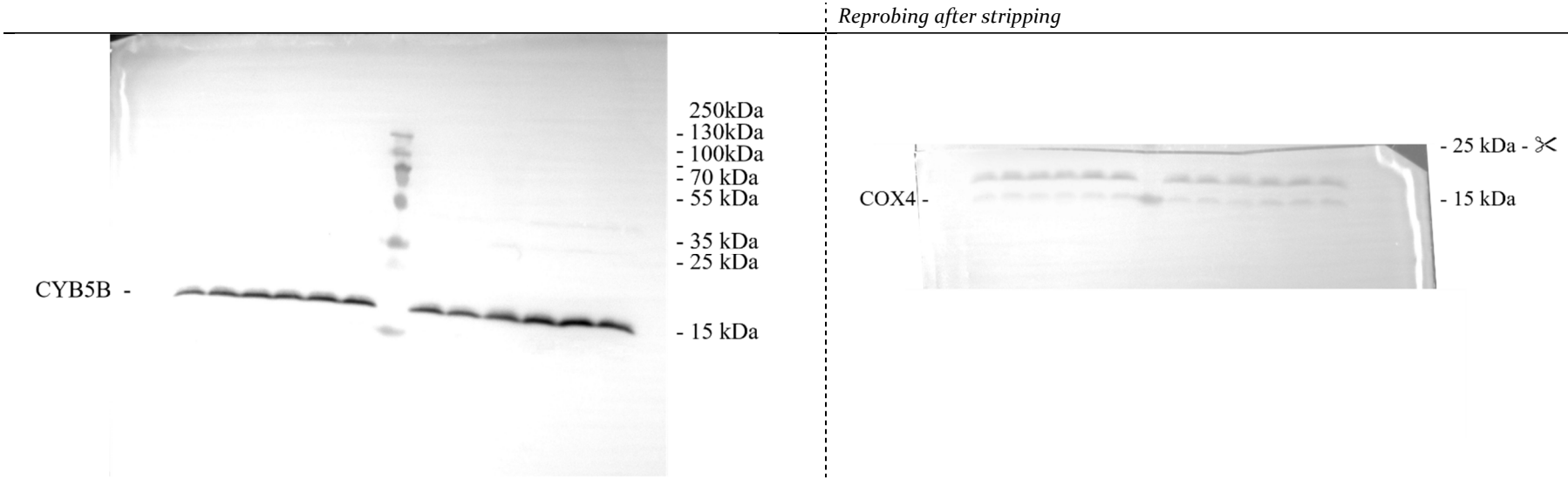
We include the loading controls (COX4, GAPDH) for every blotted membrane to compare the actual signal level between samples.
Probing with the housekeeping gene antibody was carried out either later after the blot has been stripped of previously bound antibodies (Fig. S4), separately by slicing a blot between the expected band locations (Fig. S3C), or sliced membrane pieces were stripped and reprobed (for example see Fig. S5B). In many cases the stripping procedure did not completely remove the previous antibody. It was ensured that no cross-reaction could occur after reprobing, as the same secondary antibody (derived from goat) was used in all experiments.
In the following Figures S3-S5 the original western blot is shown for every cropped lane in the manuscript. For better presentation, exposition times of full blots shown in Supporting Information and cropped lanes in the manuscript differ in some cases.

Supplementary Figure S3: liver (part 1)



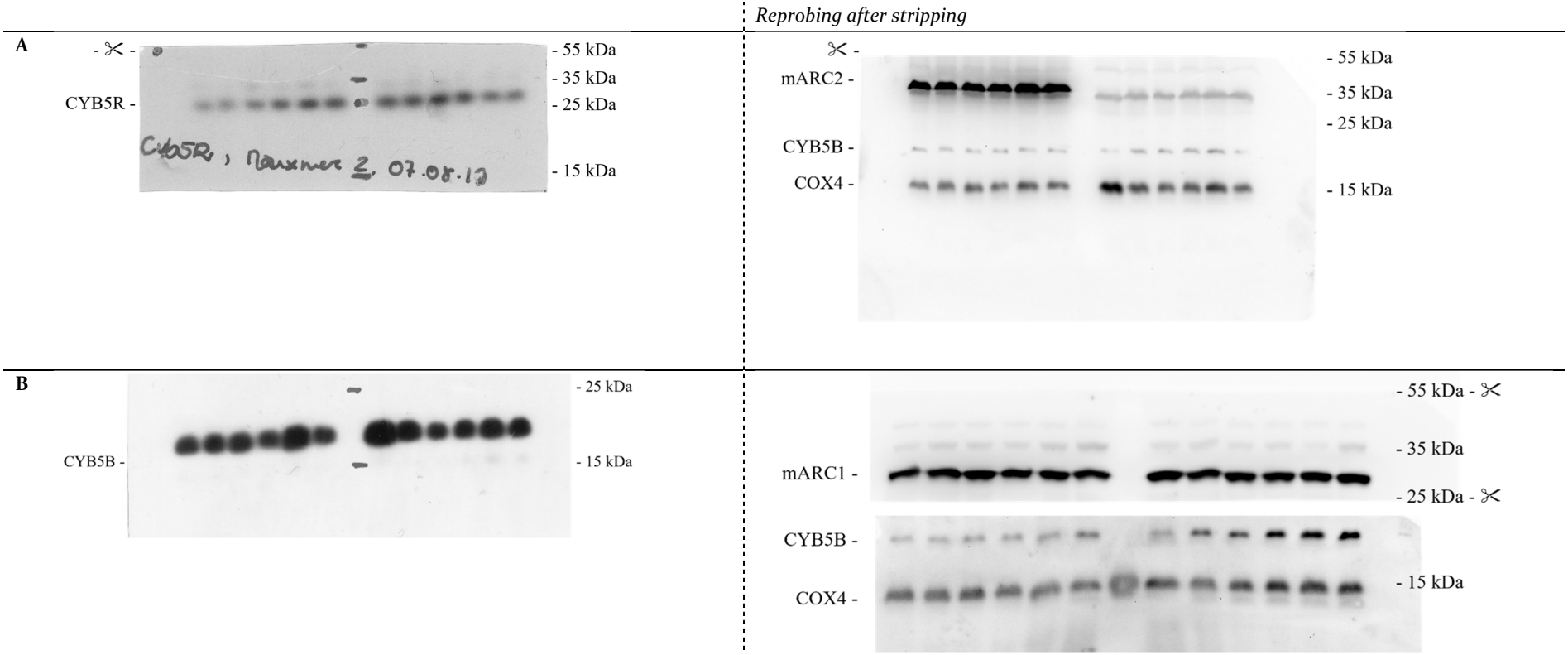
Each individual membrane was labeled with a single letter (A, B, C). **(A)** Membrane was sliced < 25 kDa after blotting for separate probing of upper and lower membrane with antibodies and re-assembled for ECL-detection. Upper part was probed with anti-CYB5R (Sigma-Aldrich, HPA001566) (see cropped lane in Fig. 5A of the manuscript) lower part was first probed with anti-CYB5B (Sigma-Aldrich, HPA007893) and then stripped and reprobed with anti-COX4 (Elabscience, AC0194) as loading control at 15 kDa (see cropped lane in Fig. 5A of the manuscript). **(B)** Membrane was sliced < 25 kDa after blotting for separate probing of upper and lower part with antibodies and re-assembled for ECL-detection. Upper part was probed with anti-mARC2 (Sigma-Aldrich, HPA015085) (see cropped lane in Fig. 5A of the manuscript) and lower part was probed with COX4 (the upper protein band is due to unspecific cross-reaction of anti-COX4). **(C)** Membrane was sliced < 25 kDa after blotting for separate probing of upper and lower membrane. Upper part was probed with anti-mARC1 (Abgent, AP9754c) (see cropped lane in Fig. 5A of the manuscript; for antibody specificity see Supplementary Fig. S6 with remarks) and lower part was probed with COX4.

Supplementary Figure S4: liver (part 2)



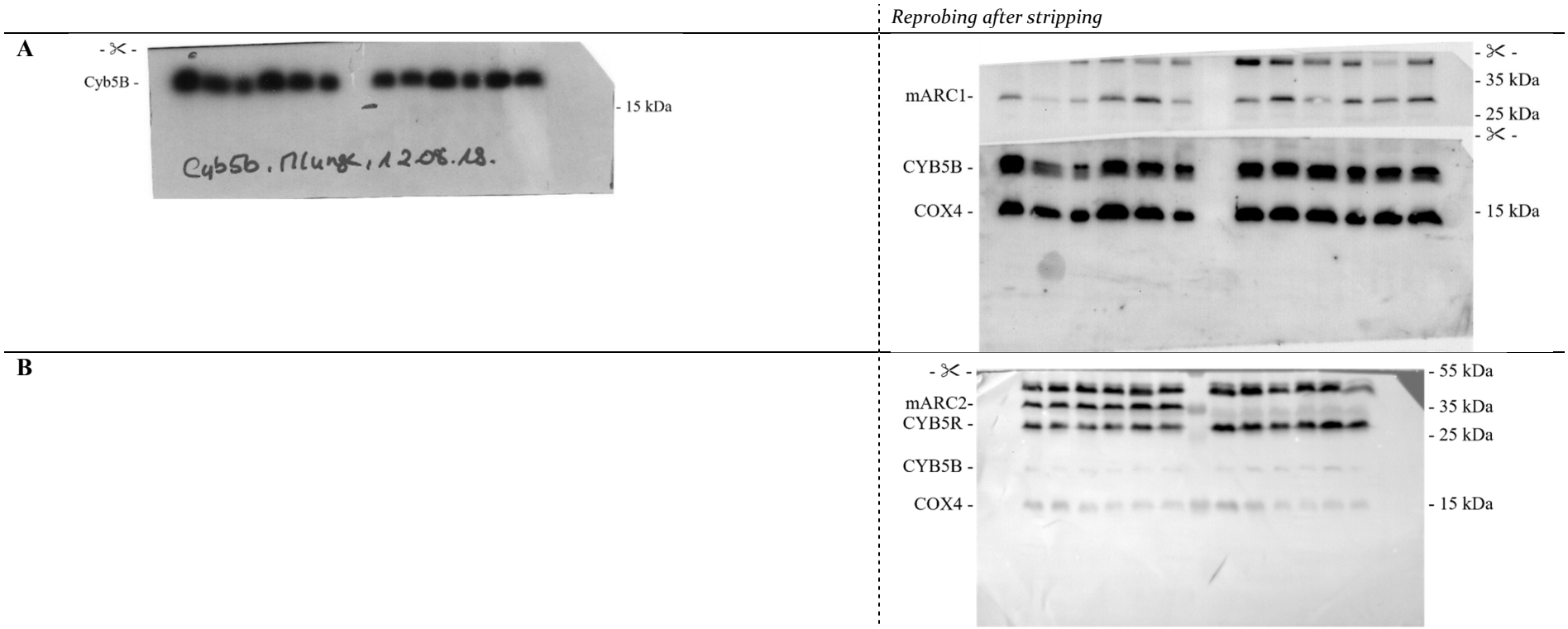
Membrane was probed with anti-CYB5B (see cropped lane in Fig. 5A of the manuscript) and then sliced < 25 kDA and reprobed with anti-COX4.

Supplementary Figure S5: kidney



Each individual membrane was labeled with a single letter (A, B). **(A)** Membrane was probed with anti-CYB5R (see cropped lane in Fig. 5A of the manuscript). Blot was then stripped and reprobed with antibodies in the following order: anti-mARC2 (see cropped lane in Fig. 5A of the manuscript), anti-COX4, and anti-CYB5B *(CYB5R was totally removed after stripping)*. **(B)** Membrane was probed with anti-CYB5B (see cropped lane in Fig. 5A of the manuscript). After that, membrane was sliced at 25 kDa and stripped. Lower part was reprobed with anti-COX4 as loading control (see cropped lane in Fig. 5A of the manuscript) *(CYB5B was not totally removed after stripping)*. Upper part was reprobed with anti-mARC1. The lowest band was verified to be mARC1 (see cropped lane in Fig. 5A of the manuscript; antibody specificity see Supplementary Fig. S6 with remarks).

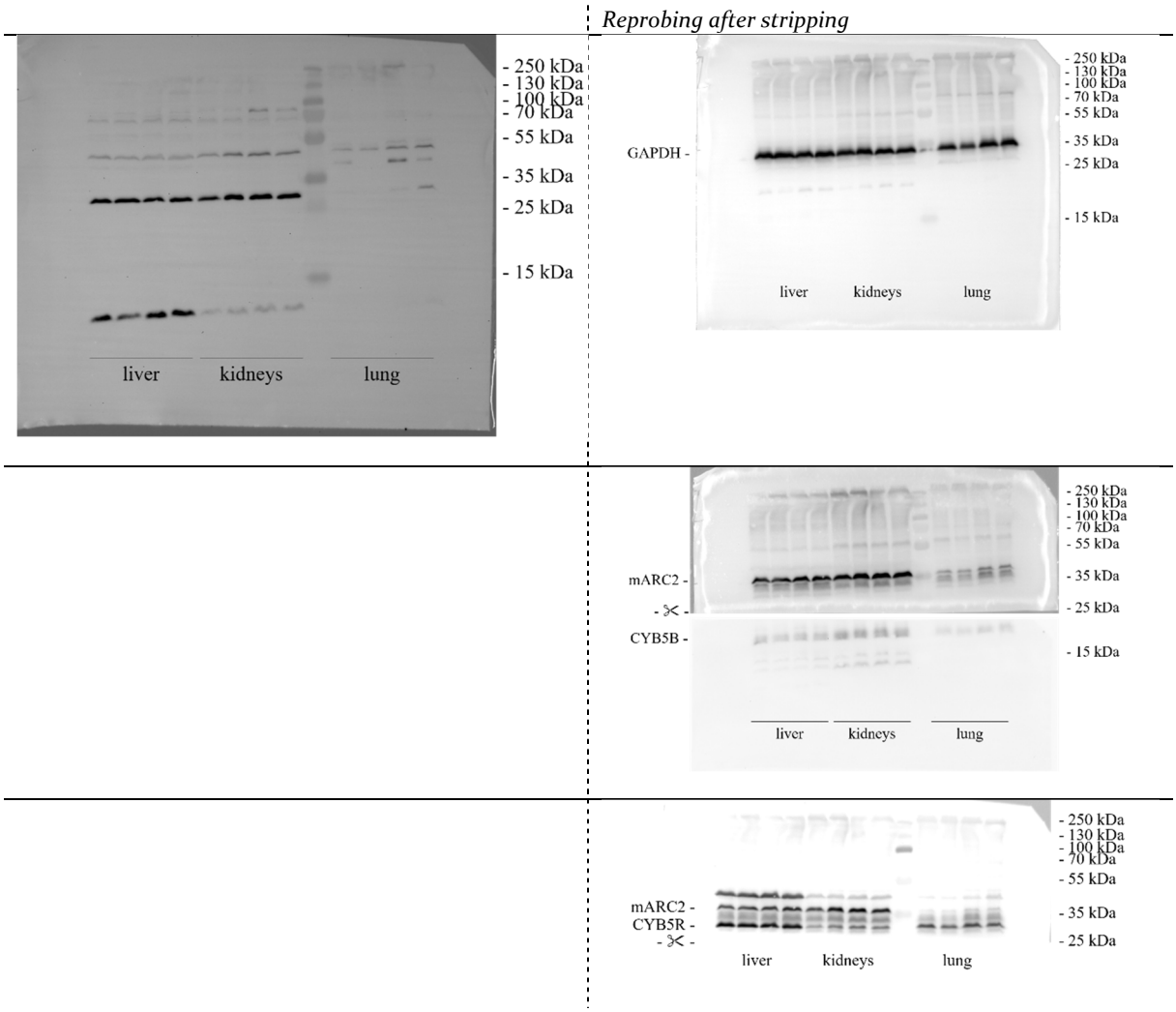
Supplementary Figure S6: lung



Each individual membrane was labeled with a single letter (A, B). **(A)** Membrane was sliced < 25 kDa after blotting for separate probing of upper and lower membrane part with antibodies and re-assembled for ECL-detection. Probing of lower part with antibodies (including reprobing after stripping) was carried out in the following order: anti-CYB5B (see cropped lane in Fig. 5A of the manuscript), and anti-COX4 (see cropped lane in Fig. 5A of the manuscript). Upper part was probed only with anti-mARC1 detecting two proteins bands between 25 kDa and 55 kDa. The protein around 30 kDa was verified to be mARC1 (see cropped lane in Fig. 5A of the manuscript; for antibody specificity see Supplementary Fig. S6 with remarks). **(B)** Probing with antibodies was carried out in the following order: anti-CYB5R (see cropped lane in Fig. 5A of the manuscript), anti-mARC2 (see cropped lane in Fig. 5A of the manuscript), anti-COX4 (15 kDa loading control; unspecific upper protein band at approx. 45 kDa was caused after anti-CYB5R treatment), and anti-CYB5B.

3. Original full western blots corresponding to cropped lanes shown in Fig. 4B

Supplementary Figure S7: protein expression of WT tissue homogenates

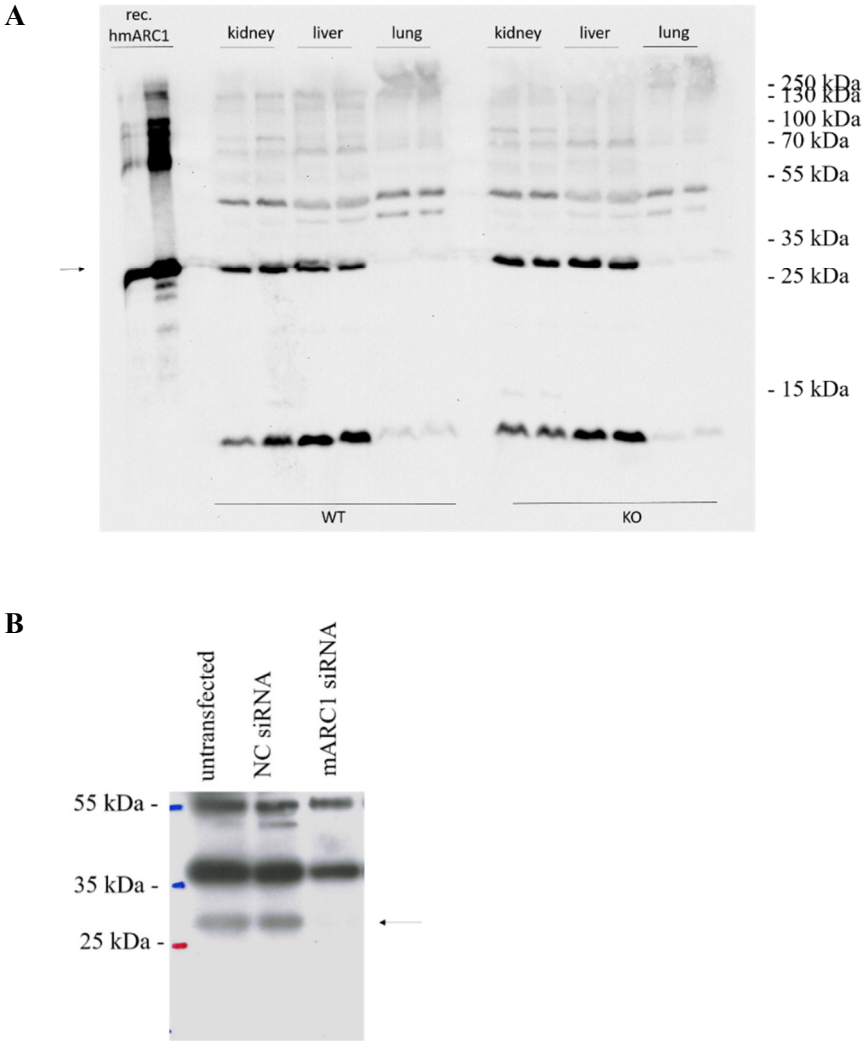


Membrane was probed with anti-mARC1 (see cropped lane in Fig. 4B of the manuscript). Blot was then stripped and reprobed with anti-GAPDH antibodies (Sigma-Aldrich, G9545) (see cropped lane in Fig. 4B of the manuscript). Membrane was then sliced < 25 kDa for separate probing of upper and lower membrane part with antibodies against mARC2 (upper part) (see cropped lane in Fig. 4B of the manuscript) and CYB5B (lower part). Stripping was repeated and membrane parts were reprobed: the upper part with anti-CYB5R antibodies and the lower part with anti-CYB5B antibodies again (see cropped lanes in Fig. 4B of the manuscript; unspecific upper protein band at approx. 45 kDa was caused after anti-CYB5R treatment).

4. Specificity of mARC1 antibody used in this study.

Murine tissue samples were electrophoresed, blotted and probed with anti-mARC1. One protein band with a molecular weight corresponding to the recombinant human mARC1 (calculated MW of 32.9 kDA) was detected (see arrow). However, additional proteins were detected at higher molecular weights between 35 and 55 kDa and one protein was detected at low molecular weight < 15 kDa. The low molecular band might be due to protein degradation or to incomplete translation. The higher molecular bands are either due to unspecific cross-reactions or to dimers/oligomers of mARC1 (with different mobility behaviour in SDS-PAGE, as their apparent molecular weight do not exactly match). We observed additional bands in human cell lines as well when using this antibody. Specific mARC1 knockdown in human cells verified only the protein around 30 kDa to be mARC1. Therefore, the 30 kDa protein band is displayed in cropped images in the manuscript.

Supplementary Figure S8: Reactivity of anti-mARC1 antibody



Membranes were probed with anti-mARC1 (Abgent, AP9754c). Each individual membrane was labeled with a single letter (A, B). **(A)** Two individual samples per tissue of WT or KO mice were loaded. As a control protein, recombinant human mARC1 was also loaded. **(B)** mARC1 knockdown in HEK-293 cells. Cells were transfected with mARC1 or nontargeting (NC) siRNA in a procedure similar described at Plitzko *et al.*, 2013.