

# **Supplementary Figure S1. Characterization of** *Emc***10 knockout mice.**

(A) Targeting strategy to inactivate mouse *Emc*10 allele by deleting exon 2. (B) The genotyping of gene disruption was confirmed by PCR. (C) EMC10 protein was totally deleted in homogenous mice by western blotting. EMC10 proteins were detectable by western blotting in the testis (Te) and epididymis (Ep) from wild type  $(+/+)$ , heterozygous (+/-) mice but not homogenous (-/-) mice. β-actin was used as the protein loading control.



**Supplementary Figure S2. Phenotypes associated with male fertility in wild type and** *Emc10***-null mice.**

The weights of whole body (A), testis (B) and epididymis (E) as well as sperm count (C) and the ratio of sperm count to testis weight (D) in  $Emc10^{+/+}$  and  $Emc10^{-/-}$  male mice were shown in histograms ( $n = 26$  at 8-12 weeks;  $n = 16$  at 8-10 months). Microscopic analysis of testis (F) and epididymis (G) from  $Emc10^{+/+}$  and  $Emc10^{-/-}$ male mice. The testes and epididymides were dissected and fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Tissues were stained with H&E and examined with  $5 \times$  objective lens (scale bar, 100 µm) and  $40 \times$ objective lens (scale bar, 25 μm). Data in bar graphs are presented as mean  $\pm$  SEM. \**P*< 0.05; \*\*\**P* < 0.001.



**Supplementary Figure S3. Workflow chart of the Tandem Mass Tag (TMT) 6-plex labelling experiment.**

Totally, 34 mice were divided into 6 groups including groups a, c, and e for wild type mice, and groups b, d, and f for  $Emc10^{-/-}$ . There were 6 mice in groups a, b, c, and d, and 5 in groups e and f, respectively. TMT- labelled peptides were mixed in equal proportions. Mixed peptides were analyzed by tandem mass spectrometry and repeated in triplicate.



**Supplementary Figure S4. The predicted work modules based on the 327 significantly altered proteins (> 1.5-fold) in the sperm of**  $Emc10^{-1}$  **mice.** 

The predicted work modules were based on the regulation of RET (A) and MYC (B) pathways, which were significantly activated in *Emc*10<sup>-/-</sup> sperm cells. Orange labelling indicates increased measurement (e.g. RET and MYC), or the effects predicted to be activated (e.g. the metabolism of protein, the formation of cellular protrusions and the generation of cells), while the blue characters represent the effects predicted to be inhibited (e.g. apoptosis and cell death). The dashed lines indicate the predicted interactions between network proteins, and the directions of the interaction are indicated by orange arrows (activation), blue-blocked lines (inhibition) or grey lines (effect not predicted).



# **Supplementary Figure S5. The protein levels of ATP1A4, ATP1B3 and CFTR in wild type (Wt) and** *Emc***10-/- (Ko) mice.**

(A) The expression of ATP1A4 and ATP1B3 was measured in testis tissues by western blotting. β-actin was used as the protein loading control. (B) CFTR expression was detected in epididymal sperm.  $\alpha$ -tubulin was used as control.



# **Supplementary Figure S6. Effects of 8-Bromo-cAMP and IBMX on** *Emc***10-/ sperm function.**

The motility parameters including the motility percentage (A), path velocity (B), linear velocity (C), and track velocity (D), as well as acrosome reaction (E) of spermatozoa from WT and *Emc*10-null mice were unable to be rescued by adding 8-Bromo-cAMP or IBMX to the medium. Data are presented as mean  $\pm$  SEM. n = 7, \*\*\*p<0.001, when compared with controls at each time point.

## **Supplementary Methods**

### *Sperm protein digestion and TMT labelling*

Seventeen  $Emc10^{+/+}$  mice and 17  $Emc10^{-/-}$  mice, both of which were 8 to 12 weeks old, were randomly assigned into six groups, which were three  $Emc10^{+/+}$  sperm pools and three  $Emc10^{-/-}$  sperm pools. One millilitre of lysis buffer (8 M urea, 1x Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland) was added to the pellet of sperm sample pool, followed by sonication on ice and centrifugation. 100 μg protein per condition were transferred into a new tube and adjusted to a final volume of 100 μL with 100 mM TEAB (triethylammonium bicarbonate). 5 μL of the 200 mM DTT was added and the sample incubated at 55℃ for 1 h, then 5 μL of 500 mM iodoacetamide was addedthe sample incubated for 30 min protected from light at room temperature. For each sample, proteins were precipitated with ice-cold acetone and then were re-dissolved in 100 μL TEAB. Then proteins were typically digested with sequence-grade modified trypsin (Promega, Madison, WI, USA), and the resultant peptide mixture was labelled by chemicals from the TMT reagent kit (Pierce Biotechnology, Rockford, USA). Proteins were labelled with TMT as follows:  $Emc10^{-/-}$  samples were labelled with 126, 128, and 130 isobaric tags, while  $Emc10^{+/+}$ samples were labelled with 127, 129 and 131 isobaric tags. The labelled samples were combined and dried in vacuo.

## *Separation by ultra performance liquid chromatography*

The dried peptide mixture was dissolved in the buffer A (10 mM ammonium formate in water, pH 10.0, adjusted with ammonium hydroxide), and then fractionated by high pH separation by using an Aquity UPLC system (Waters Corporation, Milford, MA, USA) connected to a reverse phase column (BEH C18 column, 2.1 mm x 150 mm, 3.5 μm, 300 Å, Waters Corporation, Milford, MA, USA). High pH separation was performed on a linear gradient, starting from 0% buffer B (10 mM ammonium formate in 90% ACN, pH 10.0, adjusted with ammonium hydroxide) to 45% buffer B in 35 min (Gilar et al., 2005). The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 200 μL/min and the column temperature was maintained at room temperature. Sixteen fractions were collected, each fraction was dried in a vacuum concentrator for the following step.

### *Nano-HPLC-MS/MS analysis*

The fractions were re-suspended in 30 μl solvent C (0.1% formic acid in water), separated by nano-LC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA, USA) connected to a quadrupole-Orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. 10 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm x 2 cm), with a flow of 10 μl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm x 25 cm) with a linear gradient, from 2% solvent D (ACN with 0.1% formic acid) to 40% solvent D in 105 min. The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 300 nL/min and column temperature was maintained at 40℃. The electrospray voltage of 2.2 kV versus the inlet of the mass spectrometer was used. The Q-Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350-1200) were acquired with a mass resolution of 70K, followed by ten sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 17.5K. In all cases, one microscan was recorded by dynamic exclusion of 30 seconds. MS/MS fixed first mass was set at 100.

#### *Database searching*

Tandem mass spectra were extracted by Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.0.288). Charge state de-convolution and de-isotoping were not performed. All MS/MS samples were analyzed by the Mascot (Matrix Science, London, UK; version 2.3.2). Mascot was set up to search the Mouse SwissProt database (Release 2016 01 06, 16747 entries) assuming the digestion

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enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine and TMT-6plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Oxidation of methionine was specified in Mascot as a variable modification.

#### *Quantitative data analysis*

Scaffold Q+ (version Scaffold\_4.3.4, Proteome Software Inc., Portland, OR, USA) was used to quantify Label Based Quantitation (TMT) peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 92.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least two unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected by the matrix in all samples according to the algorithm described in *i*-Tracker (Shadforth et al., 2005). Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run. Intensities for each peptide identification were normalized to the assigned protein. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed by using medians to multiplicatively normalize data. Differentially expressed proteins were determined by Permutation Test analysis using Bonferroni multiple testing corrections.

#### *Bioinformatic analysis*

Ingenuity Pathway Analysis (IPA) software was applied as a systems biology approach to explore the statistically significant molecular networks or pathways in which proteins from  $Emc10^{+/+}$  and  $Emc10^{-/-}$  mice were expressed differently. Briefly, all the MS/MS data were submitted to IPA (http://www.ingenuity.com), followed by analysis and interpretation in default parameters. Fisher test was applied and pathway enrichment was performed.

## **References**

Gilar M., Olivova P., Daly A.E., Gebler J.C. 2005. Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. J Sep Sci 28:1694-1703.

Nesvizhskii A.I., Keller A., Kolker E., Aebersold R. 2003. A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75:4646-4658. Shadforth I.P., Dunkley T.P., Lilley K.S., Bessant C. 2005. i-Tracker: for quantitative proteomics using iTRAQ. BMC Genomics 6:145.



Supplementary Table S1. Proteins altered significantly with >1.5-fold expression in *Emc10*-null spermatozoa (KO) in comparison with wild-type (WT).















