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## **Supplemental information**

## Cleavage of the pseudoprotease iRhom2

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Cleavage of the pseudoprotease iRhom2 by the signal peptidase complex reveals an ER-tonucleus signalling pathway

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# Figure S1. iRhom2 protein fragments are stable and nuclear, related to Figure 1. a Endogenous iRhom2 proteins were analysed in lung tissues of wild-type and iRhom2 KO mice by immunoblotting using mouse iRhom2 N-terminal specific antibody. **b-c** iRhom2 proteins were analysed in HEK293T cells transfected with iRhom2-3XHA by immunoblotting with HA antibody in presence of 10 $\mu$ M MG-132 (proteasome inhibitor) (**b**), or indicated doses of chloroquine (CQ) or 3-MA (lysosome inhibitor) (c) for 16 h. d Half-lives of fulllength, N-terminal and C-terminal iRhom2 fragments using 100 µg/ml cycloheximide (CHX) were analysed in HEK293T cells transfected with iRhom2-3XHA by immunoblotting with either HA (left panel) or mouse iRhom2 N-terminal specific antibody (right panel). e Quantification of the half-lives of full-length (FL), N-terminal (NT) and C-terminal (CT) iRhom2 fragments. f Deglycosylation assay of HEK293T cells transfected with 2XHA-iRhom2. Lysates were treated with Endo H or PNGase F and iRhom2 proteins were analysed by immunoblotting using HA antibody. ITGAV acts as a control glycosylated protein. g-h Immunofluorescence of HEK293T iRhom1/2 DKO cells expressing inducible human Nterminally tagged 3XHA-iRhom2 for 18h. Cells were stained for HA (green), DAPI (blue) (g) and SUN2 (red) (h). Scale bar =10 µm. i-j Schematic showing truncated human iRhom1 mutants with N-terminal 2XHA tag (i). iRhom1 proteins were analysed by immunoblotting using HA antibody (i) and by immunofluorescence (j) in HEK293T cells transfected with indicated constructs. Cells were stained for HA (red), BAP31 (green), DAPI (blue). Scale bar =10 μm. k Alignment of protein sequences of iRhom1 and iRhom2 from *Homo sapiens* (Hs.) and *Mus musculus* (Ms.). Boxed sequences are monopartite (mNLS) or a bipartite (bNLS) nuclear localisation signal predicted using eukaryotic linear motif (ELM) and PSORT II. Immunofluorescence and immunoblotting data are representative of 2-3 independent experiments.

**Figure S2. Cleavage of iRhom2 after TMD1, related to Figure 2. a** Secondary structure prediction using JPRED 4 and PSIPRED was carried out on iRhom2 to find the putative boundaries of helix corresponding to predicted TMD1 (in red). **b** *In silico* analysis of iRhom2 with multiple programs to predict the boundaries of TMD1. **c** Alignment of protein sequences iRhom2 from *Homo sapiens* (Hs.) and *Mus musculus* (Ms), *Drosophila melanogaster* (Dm.), *Xenopus tropicalis* (Xt.), *Pan troglodytes* (Pt.), *Canis familiaris* (Cf.), *Gallus gallus* (Gg.), *Anolis carolinensis* (Ac.), *Takifugu rubripes* (Tr.), *Oryzia latipes* (Ol.).

Boxed sequence is highly conserved four amino acids important for cleavage of iRhoms (shaded red). d Alignment of protein sequences iRhom2 from Homo sapiens (Hs.) and Mus musculus (Ms). Boxed sequences denote conserved regions deleted in iRhom2-1-403 mutants used in Fig. 2f. e iRhom2-1-403 proteins were analysed in HEK293T cells transfected with indicated constructs in the presence of broad-spectrum protease inhibitors E64-d (10  $\mu$ M), Pepstatin A (20  $\mu$ M) and AEBSF (200  $\mu$ M) for 16hrs by immunoblotting using HA antibody. L.E: low exposure, H.E: high exposure. Empty triangles denote further proteolytically processed soluble nuclear iRhom2 proteins. # denotes largest soluble nuclear iRhom2 detected. f Immunofluorescence of HEK293T iRhom1/2 DKO cells expressing inducible N-terminally tagged iR2-1-278 for 6 h. Cells were stained for HA (green), BAP31 (red) and DAPI (blue). Scale bar =10 µm. g Wild type iRhom2, mutant iRhom2 and EGF proteins were analysed in HEK293T cells transfected with indicated constructs by immunoblotting with antibodies against HA and Myc respectively. **h** Maturation of ADAM17 using Concanavalin A (Con A) pull-down in wild type, iRhom1/2 DKO, and iRhom1/2 DKO MEFs stably reconstituted with indicated iRhom2 constructs was measured. imA17: immature A17, mA17: mature A17. i Levels of iRhom2 and ADAM17 proteins were detected in HEK293T cells transfected with indicated constructs by immunoblotting in whole cell lysate (WCL) and immunoprecipitated lysate (IP: HA). Immunofluorescence and immunoblotting data are representative of 2-3 independent experiments.

Figure S3. SPC cleaves iRhom2, related to Figure 3. a iRhom2 proteins were detected in HEK293T cells transfected with iRhom2-3XHA in the presence of broad-spectrum protease inhibitors E64-d (10  $\mu$ M), Pepstatin A (20  $\mu$ M), AEBSF (200  $\mu$ M), 3,4 DCI (20  $\mu$ M) for the last 16 h by immunoblotting using HA antibody. b Endogenous iRhom2 proteins were detected by immunoblotting in HEK293T cells with knock-in of 3XHA tag at C-terminal of iRhom2 gene locus, transfected with control siRNA and two independent RHBDL4 siRNAs using HA antibody. c iRhom2 proteins were detected in HEK293T cells transfected with iRhom2-3XHA in the presence of control siRNA or catalytic subunits SEC11A or SEC11B or both siRNAs by immunoblotting using HA antibody. d iRhom2 proteins were analysed following cellular fractionation of HEK293T-iRhom1/2 DKO cells inducibly expressing wild type human iRhom2 in the presence of cavinafungin (1  $\mu$ M/ 18 h) by immunoblotting using HA antibody. GAPDH (total/cytoplasmic), ITGAV (membrane), and Lamin A (nuclear) were used as markers for fractions. **e** Human iRhom1 proteins were analysed in HEK293T cells transfected with iRhom1-1-855 in the presence of cavinafungin (1  $\mu$ M/ 18 h) by immunoblotting using HA antibody. **f** Illustrations showing the predicted results of signal peptidase cleavage site on iRhom2 by SignalP 4.1 and PrediSi, with contrast to experimentally validated ERp44 and rhomboid pseudoprotease DERLIN1. **g** Immunofluorescence of HEK293T iRhom1/2 DKO cells expressing inducible N-terminally tagged wild type human iRhom2 (HA-iR2-WT) in the presence of SPP inhibitor Z-LL<sub>2</sub> ketone (50  $\mu$ M/18 h). Cells were stained for HA (green), BAP31 (red) and DAPI (blue). Scale bar =10  $\mu$ m. **h** iRhom2-1-403 proteins were analysed in HEK293T cells transfected with indicated constructs in the presence of SPP inhibitor Z-LL<sub>2</sub> ketone (50  $\mu$ M/ 18 h) by immunoblotting using HA antibody. L.E: low exposure, H.E: high exposure. Empty triangles denote further proteolytically processed soluble nuclear iRhom2 proteins. # denotes largest soluble nuclear iRhom2 detected. Immunofluorescence and immunoblotting data are representative of 2-3 independent experiments.

Figure S4. Gene expression regulation by nuclear iRhom2, related to Figure 4. A iR2-1-374 proteins were analysed following cellular fractionation of in HEK293T cells stably expressing iR2-1-374 by immunoblotting with HA antibody. S3: soluble nuclear fraction, S4: chromatinbound fraction with or without MNASE treatment, and P4: cytoskeletal proteins + remaining chromatin-bound proteins. Histone H3 is a control for chromatin-bound proteins. L.E: low exposure, H.E: high exposure. **b** Levels of iR2-1-374 were analysed in HEK293T cells expressing inducible iR2-1-374 with 200 ng/ml of doxycycline for indicated time points by immunoblotting with HA antibody. c iR2-1-374, POL2 and TFIID proteins were detected in HEK293T cells expressing inducible iR2-1-374 by immunoblotting in whole cell lysate (WCL) and immunoprecipitated lysate (IP: HA). **d** Graph showing validation of 11 selected target genes by qPCR in HEK293T iRhom1/2 DKO cells expressing inducible iRhom2-1-278 for 3 h and 6h. Data are presented as  $\log_2$  fold change relative to uninduced cells as mean  $\pm$  SEM, n=3. e-f iRhom2 proteins were analysed in HEK293T cells expressing either inducible wild type (iR2-WT-HA) or uncleavable iRhom2 (iR2-LVLF-HA) (e) or inducible wild-type iRhom2 in the presence of cavinafungin  $(1 \mu M/18 h)$  (f) by immunoblotting with HA antibody. g Graphs showing transcript levels of selected target genes by qPCR in HEK293T iRhom1/2 DKO cells expressing inducible wild-type human iR2 (iR2-WT) in the presence of cavinafungin (1  $\mu$ M/18 h). Data are presented as log<sub>2</sub> fold change relative to uninduced cells as mean ± SEM,

n=3. h Alignment of protein sequences iRhom2 from *Homo sapiens* (Hs.) and *Mus musculus* (Ms). Boxed sequences denote conserved regions for predicted CtBP1/2 binding site and nuclear localisation signals (NLS). i Protein sequence alignment of region around CtBP1/2 binding site (boxed) of proposed interactors of CtBP proteins. j Graphs showing transcript levels of selected target genes by qPCR in HEK293T iRhom1/2 DKO cells expressing inducible wild-type human iR2 (iR2-WT) in the presence of an inhibitor of CtBPs, MTOB (500  $\mu$ M/18 h). Data are presented as log<sub>2</sub> fold change relative to uninduced cells as mean ± SEM, n=3. Immunoblotting data are representative of 2-3 independent experiments.

Figure S5. Expression and regulation of nuclear iRhom2 in disease skin models, related to Figure 5. a-b Endogenous iRhom2 proteins were detected in control (ctrl) and tylotic (TOC) (a) and TOC transfected with shRNA against iRhom2 (b) immortalised patient-derived keratinocytes cells by immunoblotting using iRhom2 antibody. c Endogenous iRhom2 expression were determined by immunohistochemistry in sole tissues from diffuse nonepidermolytic palmoplantar keratoderma (NEPPK) and control patient samples. Tissues were stained for iRhom2 (green) and DAPI (blue). n=1 for each human tissue. Scale bar =20 μm. d-e Endogenous iRhom2 proteins were detected in HEK293T cells with knock-in of 3XHA tag at C-terminal of iRhom2 gene locus, treated with 200 nM PMA for indicated time intervals (d) or after transfection with either control siRNA and combined SEC11A and SEC11C siRNAs (e) by immunoblotting using HA antibody. g-h Effect of tylotic mutations on human iRhom2 cleavage (g) and protein half-life using 100  $\mu$ g/ml cycloheximide (CHX) (h) in HEK293T cells transfected with indicated constructs and time points were determined by immunoblotting with HA antibody. i Wild type (iR2-WT) and uncleavable (iR2-LVLF) human iRhom2 proteins were analysed in HEK293T iRhom1/2 DKO cells inducibly expressing these proteins over indicated time periods corresponding to cell growth assay in Fig. 5f, by immunoblotting with HA antibody. Immunoblotting data are representative of 2-3 independent experiments.







Figure S1- Dulloo et al.

#### Secondary structure prediction

Software	iR2 (predicted helix in red)		
Jpred 4	DSHRPYFTYWLTFVHIIITLLVICTYGIAPVGFAQHVTTQLVLKNR GVYESVKYIQQEN		
PSIPRED	DSHRPYFTYWLTFVHIIITLLVICTYGIAPVGFAQHVTTQLVLKNR GVYESVKYIQQEN		

PFFTYWINTVQVVVLILSIICYGIA<mark>PIGI</mark>GSEQKTGQVLVTSLSLQTVQHVEQRNLWIGP

PYFAYWITFVHILITILAIATYGIA<mark>PIGF</mark>AQHTTSELVLRNKGVYESVKYIQQENFWIGP PYFTYWLTFVHIIITLLVICTYGIA<mark>PVGF</mark>AQHVTTQLVLKNRGVYESVKYIQQENFWIGP

PYFTYWLTFVHVIITLLVICTYGIAPVGFAQHVTTQLVLRNKGVYESVKYIQQENFWVGP

PYFTYWLTFVHVIITLLVICTYGIAPVGFAQHVTTQLVLRNKGVYESVKYIQQENFWVGP

PYFTYWLTFVHIIITLLVICTYGIA<mark>PVGF</mark>AQHVTTQLVLRNKGVYESVKYIQQENFWIGP

PYFTYWITFVHILITLLVISTYGIAPIGFTQHVTTELVLRNKGVYESVKYIQQENFWIGP PYFTYWITFVHIIITLLIICTYGIAPIGFAQHLTTALVLRNKGVYESVKYIQQENFWIGP

PYFTYWITFVHVVITLLSCCTYGFA<mark>PIGF</mark>AQHSTSQLVLKNKGIYESVKFVQQENFWIGP

PYFTYWVTFVHIVITLLACCTYGFA<mark>PVGF</mark>SQHSTTDLVLMTKGVYESVKFVQQENFWIGP \*\*:\*<mark>\*:\*:</mark> .. .: \*\* .

B

HMMTOP

SOSUI

CCTOP

TMFold

PSIPRED

PHOBIUS

TMHMM

SPLIT

iRhoml(Hs) iRhoml(Mm) iRhom2(Hs) iRhom2(Mm)

iRhom1(Mm) iRhom2(Hs)

iRhom1(Hs) iRhom1(Mm) iRhom2(Hs)

iRhoml(Hs) iRhoml(Mm) iRhom2(Hs) iRhom2(Mm)

TMpred

#### Transmembrane prediction for iRhom2



D

	DZ+0 255	
RLPRRRKRESVAKMSFRAAAALMKGRSVRDGTFRR	AORRSFTPASFLEEDTTDFPDEL	258
RLPRRKRESVAKMSFRAAAALVKGRSIRDGTLRR	GORRSFTPASFLEEDMVDFPDEL	258
HLPRR-KRMSVAHMSLOAAAALLKGRSVLDATGORCR	VVKRSFAFFSFLEEDVVDGADTF	289
HLPRR-KRISVAHMSFOAAAALLKGRSVLDATGORCR	HVKRSFAYPSFLEEDAVDGADTF	259
Δ260-267 Δ275-282	:***: *****	
DTSFFAREGILHEELSTYPDEVFESPSEAALKDWEKA	PEQADLTGGALDRSELERSHLML	318
DTSFFAREGVLHEEMSTYPDEVFESPSEAALKDWEKA	PDOADLTGGALDRSELERSHLML	318
DSSFFSKEEKSSMPDDVFESPPLSASY-FRGI	PHSASPVSPDGVOI	334
DSSFFSKEEMSSMPDDVFESPPLSASY-FRGV	PHSASPVSPDGVHI	304
*:***:: **: **: **: **: **: **:	* * : : :	
PLERGWRKOKEGA-AAPOPKVRLROEVVSTAGPRRGO	RIAVPVRK-LFAREKRPYGLGMV	376
PLERGWRKOKEGGPLAPOPKVRLROEVVSAAGPRRGO	RTAVPVRK-LFAREKRPVGLGMV	377
DI.KFVCPADVDCDPDCK	PTASKVKHFAFDPKKPHVCLCVV	374
DI K	BTACKUKUFAFDBKKBUVCI CUV	246
PEREISGGRADGFGIQRGR	KIASKVKHEAF DRKKKHIGDGVV	540
GRLTNRTYRKRIDSFVKROIEDMDDHRP 404		
GRUTNRTYRKRIDSYVKROIEDMDDHRP 405		
CNWLNRSVRRSTSSTVOROLESEDSHRP 402		
CNWINDSYDDSTSSTUODOLESEDSUDD 374		
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Figure S2- Dulloo et al.

С

Dm.iRhom

Xt.iRhom2

Ms.iRhom2

Hs.iRhom2

Pt.iRhom2 Cf.iRhom2

Gg.iRhom2 Ac.iRhom2

Tr.iRhom2

Ol.iRhom2



Figure S3- Dulloo et al.



Figure S4- Dulloo et al.

