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## Supplemental information

# Cleavage of the pseudoprotease iRhom2 by the signal peptidase complex reveals an ER-to-nucleus signaling pathway

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**Cleavage of the pseudoprotease iRhom2 by the signal peptidase complex reveals an ER-to-nucleus signalling pathway**

Iqbal Dulloo[1*], Michael Tellier[1], Clémence Levet[1], Anissa Chikh[2,3], Boyan Zhang[1], Diana C Blaydon[2], Catherine M Webb[2], David P Kelsell[2] and Matthew Freeman[1,4*]

**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 μM MG-132 (proteasome inhibitor) (**b**), or indicated doses of chloroquine (CQ) or 3-MA (lysosome inhibitor) (**c**) for 16 h. **d** Half-lives of full-length, 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 N-terminally 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 μM), Pepstatin A (20 μM) and AEBSF (200 μM) 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 μM), Pepstatin A (20 μM), AEBSF (200 μM), 3,4 DCI (20 μM) 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 μM/ 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 μM/ 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-$LL_2$ ketone (50 μM/18 h). Cells were stained for HA (green), BAP31 (red) and DAPI (blue). Scale bar =10 μm. **h** iRhom2-1-403 proteins were analysed in HEK293T cells transfected with indicated constructs in the presence of SPP inhibitor Z-$LL_2$ ketone (50 μM/ 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: chromatin-bound 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 ± 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 μM/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 μM/18 h). Data are presented as $\log_2$ 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 μM/18 h). Data are presented as $\log_2$ 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 non-epidermolytic 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 μg/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.

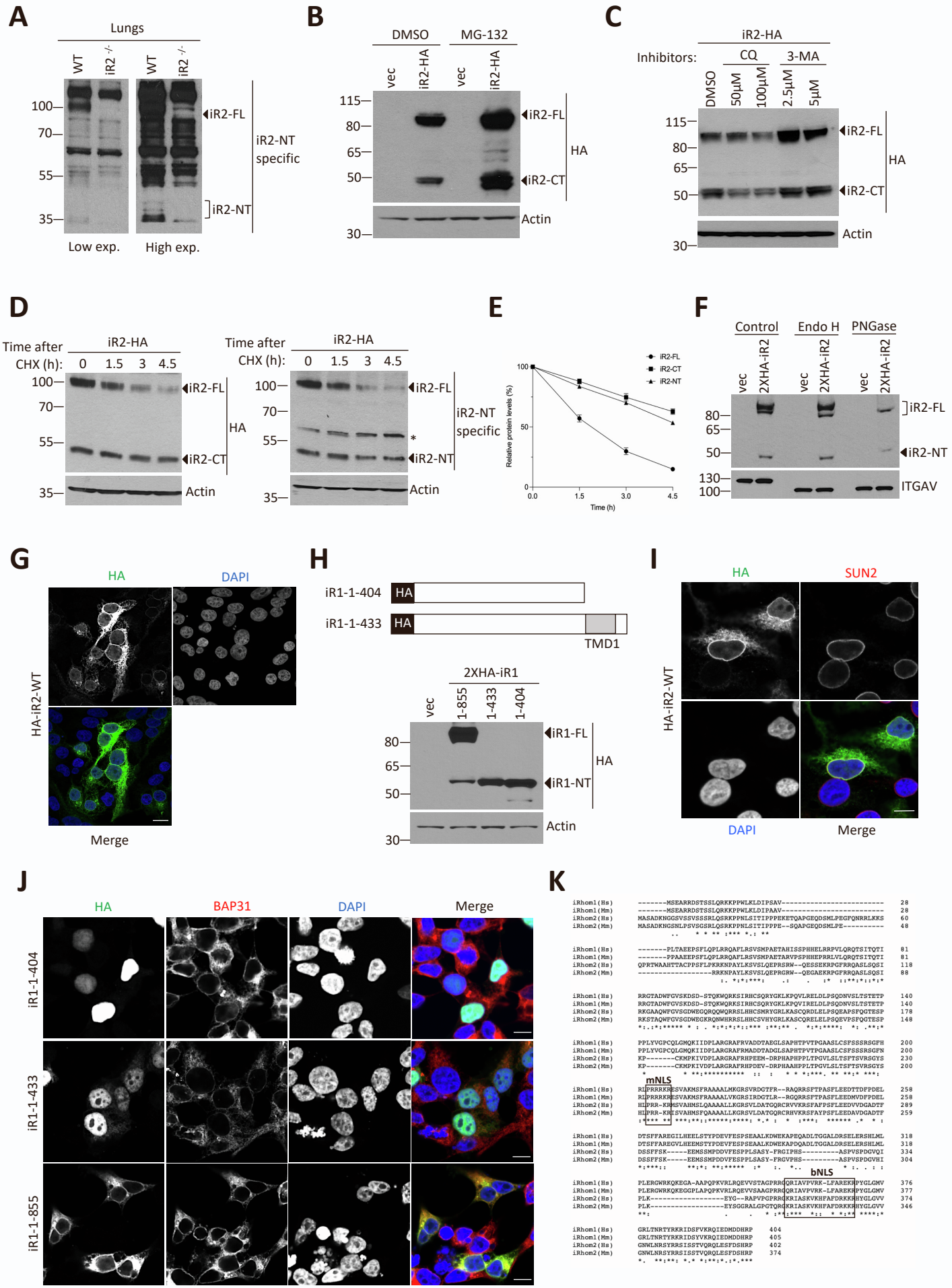


*Figure S1- Dulloo et al.*

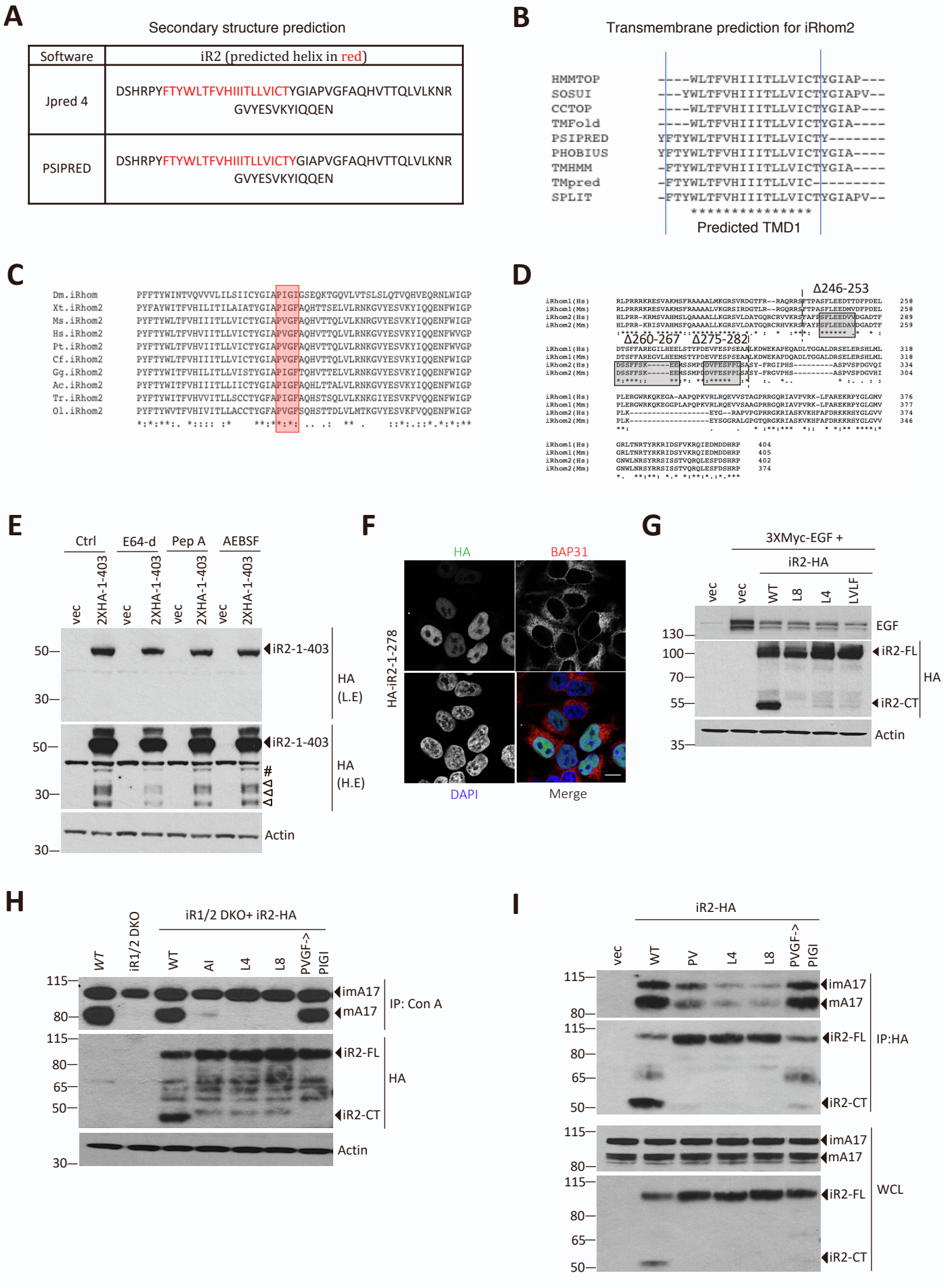


*Figure S2- Dulloo et al.*

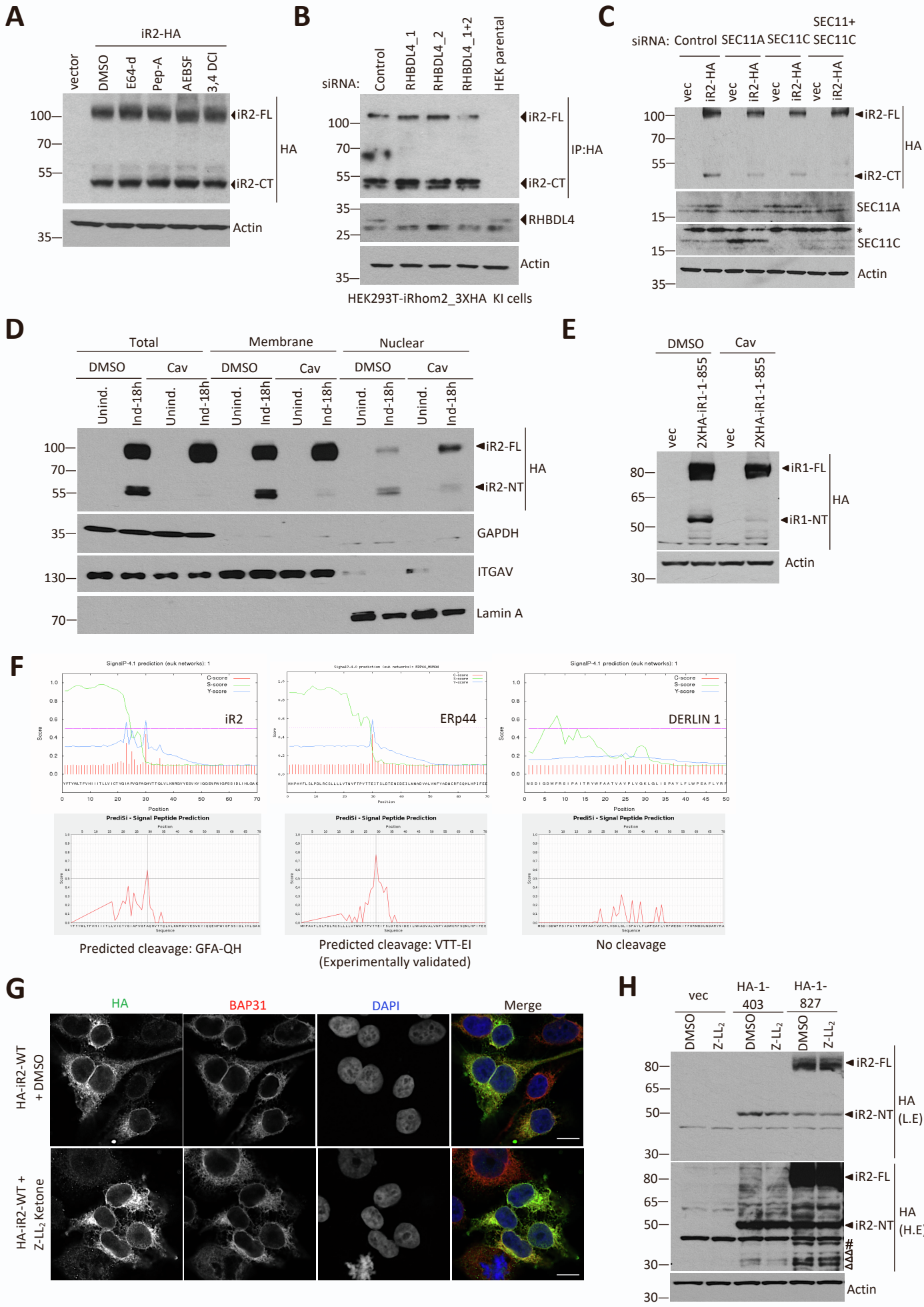


*Figure S3- Dulloo et al.*

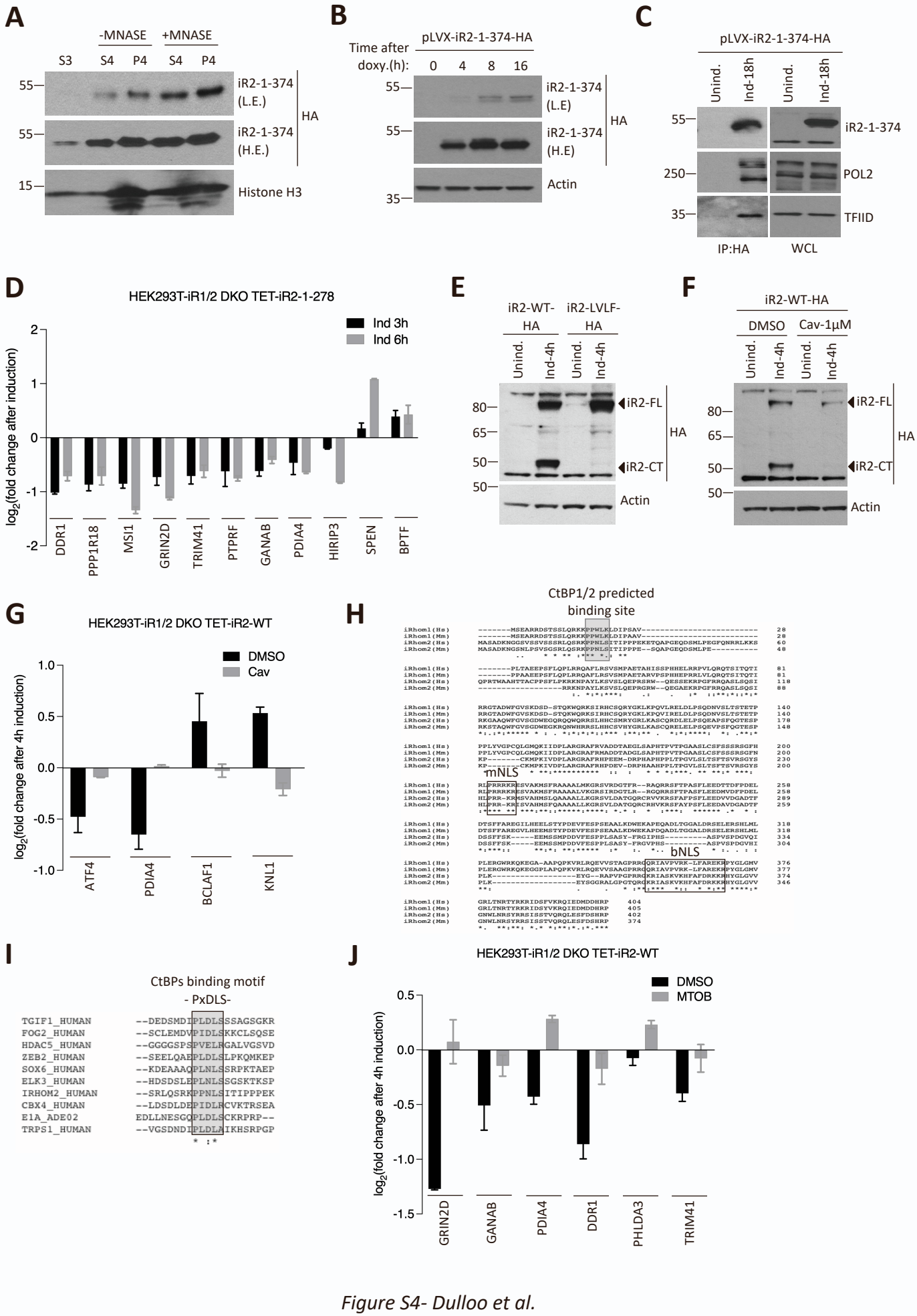


*Figure S4- Dulloo et al.*

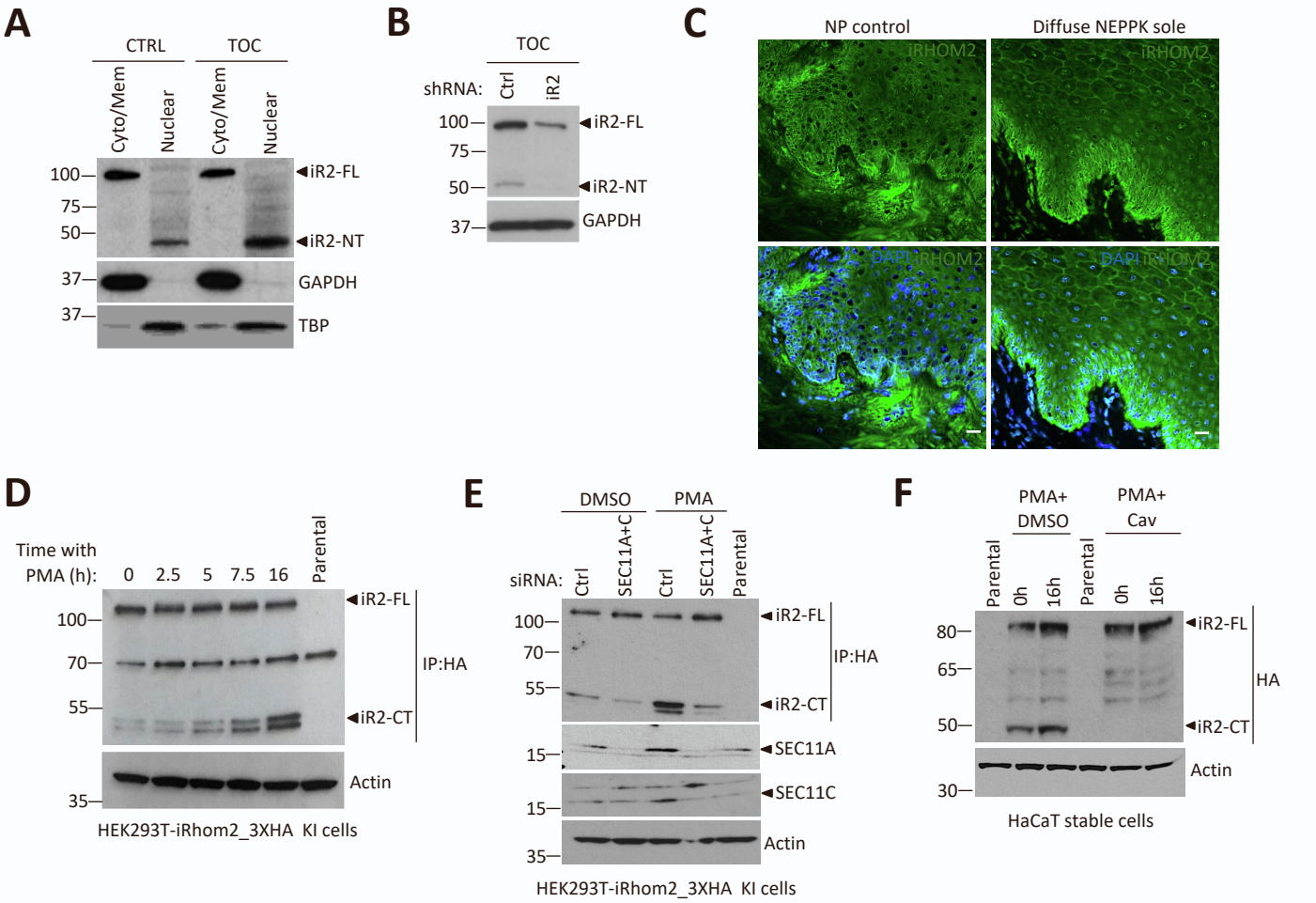


*Figure S5- Dulloo et al.*