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RESEARCH ARTICLE

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# Interleukin-11 (IL-11) receptor cleavage by the rhomboid protease RHBDL2 induces IL-11 trans-signaling

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## Abstract

Interleukin-11 (IL-11) is a pleiotropic cytokine with both pro- and anti-inflammatory properties. It activates its target cells via binding to the membrane-bound IL-11 receptor (IL-11R), which then recruits a homodimer of the ubiquitously expressed, signal-transducing receptor gp130. Besides this classic signaling pathway, IL-11 can also bind to soluble forms of the IL-11R (sIL-11R), and IL-11/sIL-11R complexes activate cells via the induction of gp130 homodimerization (trans-signaling). We have previously reported that the metalloprotease ADAM10 cleaves the membrane-bound IL-11R and thereby generates sIL-11R. In this study, we identify the rhomboid intramembrane protease RHBDL2 as a so far unrecognized alternative sheddase that can efficiently trigger IL-11R secretion. We determine the cleavage site used by RHBDL2, which is located in the extracellular part of the receptor in close proximity to the plasma membrane, between Ala-370 and Ser-371. Furthermore, we identify critical amino acid residues within the transmembrane helix that are required for IL-11R proteolysis. We also show that ectopically expressed RHBDL2 is able to cleave the IL-11R within the early secretory pathway and not only at the plasma membrane, indicating that its subcellular localization plays a central role in controlling its activity. Moreover, RHBDL2-derived sIL-11R is biologically active and able to perform IL-11 trans-signaling. Finally, we show that the human mutation IL-11R-A370V does not impede IL-11 classic signaling, but prevents RHBDL2-mediated IL-11R cleavage.

**KEYWORDS**

cytokine, interleukin-11, protease, RHBDL2, rhomboid

**Abbreviations:** ADAM, a disintegrin and metalloprotease; gp130, glycoprotein 130 kDa; IL-11R, interleukin 11-Receptor; IL, interleukin; Jak, Janus kinase; sIL-11R, soluble IL-11 receptor; STAT, signal transducer and activator of transcription.

## 1 | INTRODUCTION

The cytokine interleukin-11 (IL-11) is a pleiotropic cytokine with several reported pro- as well as anti-inflammatory properties.[1,2] IL-11 is a member of the IL-6 family of cytokines, which all share usage of the ubiquitously expressed signal-transducing receptor gp130.[3] Specificity is gained through a non-signaling IL-11 receptor (IL-11R), to which IL-11 initially binds and thereby induces gp130 homodimerization. IL-11 activates several important intracellular signaling cascades, among them the Janus kinase/signal transducer and activator of transcription (Jak/STAT), the phosphoinositide 3-kinase (PI3K), and the mitogen-activated protein kinase (MAPK) pathway. IL-11 is secreted from a variety of cell types, including fibroblasts and epithelial cells of different origin.[4] The most important inducer of IL-11 production appears to be transforming growth factor β (TGFβ).[5]

IL-11 has been initially regarded as an anti-inflammatory cytokine and as a recombinant protein is approved by the FDA for the treatment of chemotherapy-induced thrombocytopenia.[6] However, this view has changed in recent years, and the pro-inflammatory properties of the cytokine have gained more attention. Distinct tumor entities, for example, in the stomach,[7,8] in the colon,[9] or in the female breast[10] rely primarily on IL-11 signaling, which often cannot be compensated for by other cytokines like IL-6. First studies with IL-11 muteins[9] or antibodies that block IL-11 signaling[11] have demonstrated that targeting IL-11 might be a suitable strategy to treat these kinds of cancers.

Besides this, IL-11 signaling is required for different aspects of development. It is necessary for female fertility, because female *Il11ra*$^{-/-}$ mice are infertile due to a failure of embryo implantation in the uterus.[12-14] Furthermore, signaling via the IL-11R is required for the correct formation of the skull and for tooth development. Patients with mutations within the *IL11RA* gene display craniosynostosis, a disease characterized by premature closures of the sutures lining the head bones.[15-19] Some of the patients also present with delayed tooth eruption and supernumerary teeth within the jaw, but whether this is a general phenomenon is currently unclear.[16] The underlying mechanism, at least for some of the patient mutations, is impaired maturation of the IL-11R, which prevents the transport of the receptor to the cell surface and results in a complete blockade of IL-11 signaling.[20] The studied IL-11R mutants are all trapped within the ER, probably due to a folding defect that causes retention in the endoplasmic reticulum (ER),[20] but it is not known whether this holds true for all patient mutations.

Besides the classic signaling pathway, in which IL-11 uses the membrane-bound IL-11R, we have shown that IL-11 can also induce signaling via soluble forms of the IL-11R (sIL-11R).[21,22] We have named this pathway IL-11 trans-signaling and found that the metalloprotease ADAM10 is capable of cleaving the membrane-bound IL-11R, and thus, releasing biologically active sIL-11R.[23,24] Although the exact cleavage site within the IL-11R has not yet been determined, we could show that Arg-355, located in the juxtamembrane region which connects the three extracellular domains with the transmembrane region, is important for ADAM10-mediated cleavage and release of the ectodomain.[23] The remaining C-terminal IL-11R transmembrane-spanning stub generated by this shedding event is afterward cleaved by the γ-secretase complex and probably degraded.[25]

Free sIL-11R binds IL-11, and the resulting IL-11/sIL-11R complexes can bind to and activate gp130 homodimers on target cells. IL-11 trans-signaling significantly widens the number of cell types that can be activated by IL-11, because the cells do not need to express membrane-bound IL-11R. We have further shown that sgp130Fc (Olamkicept), which is the extracellular part of gp130 dimerized by the Fc part of a human IgG antibody that was originally developed as a specific inhibitor of IL-6 trans-signaling and that is currently in phase II clinical trials for patients with inflammatory bowel disease,[26] blocks IL-11 trans-signaling, but not classic signaling.[23] Moreover, we have detected sIL-11R in the serum of healthy volunteers via ELISA,[23] and IL-11R was also shown via an aptamer-based multiplex protein assay to be part of the human plasma proteome.[27]

Rhomboids are intramembrane serine proteases initially found in *Drosophila* as the proteases that activate the epidermal growth factor receptor (EGFR) pathway by cleaving the membrane-bound EGFR ligands.[28] In mammals, rhomboid proteases can be subdivided into the *secretase class* (located in the secretory pathway and at the plasma membrane) and *PARL* (presenilins-associated rhomboid-like protein, located in mitochondria).[29,30] The mammalian rhomboid secretases are called Rhomboid-Like 1 (RHBDL1), RHBDL2, RHBDL3, and RHBDL4. The best characterized member of the family is RHBDL2, which is located primarily at the plasma membrane and for which several substrates have been described, including EGF,[31] the C-type lectin family members thrombomodulin,[32] and CLEC14A,[33] ephrin-B2,[34] the IL-6R,[35] the EGFR,[36] and different cadherins.[37] However, for most of these substrates RHBDL2-mediated cleavage in vivo remains to be demonstrated.

In this study, we identify the IL-11R as a novel substrate of RHBDL2. We map the cleavage site used by the protease and show that certain amino acid residues within the transmembrane helix (TMH) of the IL-11R are crucial for proteolysis. The sIL-11R released by RHDBL2 is biologically active and able to induce IL-11 trans-signaling. This work identifies a second important protease besides ADAM10 for the induction of IL-11 trans-signaling, which could be relevant for understanding the biological functions of IL-11 in vivo.

## 2 | MATERIALS AND METHODS

### 2.1 | Cells and reagents

HEK293 and HeLa cells were cultured in DMEM high-glucose culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% of fetal bovine serum, penicillin (60 mg/L), and streptomycin (100 mg/L). Ba/F3-gp130 cells were also cultured in DMEM high-glucose culture medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% of fetal bovine serum, penicillin (60 mg/L), streptomycin (100 mg/L), and additional 10 ng/mL Hyper-IL-6. All cells were kept at 37°C and 5% of $CO_2$ in a standard incubator with a water-saturated atmosphere. Marimastat, ionomycin and 3,4-dichloroisocoumarin (DCI) were purchased from Sigma-Aldrich. The following antibodies were used: anti-HA-tag (C29F4), anti-myc-tag (71D10), anti-myc-tag (9B11), and anti-α-actinin (D6F6) were purchased from Cell Signaling Technology (Frankfurt/M., Germany). The fluorescently labeled antibodies anti-mouse-IgG-Alexa Fluor 488, anti-rabbit-IgG-Alexa Fluor 488, and anti-rabbit-IgG-Alexa Fluor 594 were obtained from life technologies/Thermo Fisher Scientific. The fluorescently labeled antibodies anti-mouse-IgG-IRDye 680RD, anti-rabbit-IgG-IRDye 680RD, anti-mouse-IgG-IRDye 800CW, and anti-rabbit-IgG-IRDye 800CW were purchased from LI-COR Biosciences (Lincoln, NE, USA).

### 2.2 | Construction of expression plasmids

pcDNA3.1 expression plasmids for myc-tagged hIL-11R have been described previously.[38] Expression plasmids encoding the IL-11R variants IL-11R-KKSS and IL-11R-R296W have been described previously.[20] The IL-11R deletion variants IL-11R-ΔV363_L372 and IL-11R-ΔH353_S362 have been described previously.[23,39] All other IL-11R mutants used in this study were generated via splicing by overlapping extension (SOE)-PCR using pcDNA3.1-myc-hIL-11R as template. pcDNA3.1 expression plasmids for hIL-6R have been described previously,[40,41] and a variant with an N-terminal myc-tag was used in this study.

pcDNA3.1 expression plasmids encoding HA-tagged mouse RHBDL1, RHBDL2, RHBDL3, and RHBDL4 were kindly provided by Dr Matthew Freeman (Sir William Dunn School of Pathology, Oxford, UK[31]). The RHBDL2-SA mutant was generated via splicing by overlapping extension (SOE)-PCR using pcDNA3.1-mRHBDL2-HA as template. Expression plasmids encoding RHBDL2-KDEL and RHDBL2-KDEL-SA were generated by PCR fusing the peptide sequence KDELKDELKDEL to the C-terminus in order to ensure efficient retrieval to the ER.

### 2.3 | IL-11R proteolysis by RHDBL2 in HEK293 cells

In order to analyze IL-11R cleavage, $2 \times 10^6$ HEK293 cells were transiently transfected with the desired plasmids using TurboFect according to the manufacturer's instructions. IL-11R proteolysis in HEK293 cells was in principle analyzed as described previously.[23] Forty-eight hours after transfection, medium was exchanged with serum-free medium and after 4 hours the supernatants and cells were harvested and prepared for analysis by western blotting. After filtration and centrifugation at 1200*g*, the supernatants were precipitated with 20% of trichloroacetic acid, centrifuged at 18 000*g*, pellets washed with acetone, and centrifuged again at 18 000*g*. The pellet was boiled afterward in Laemmli buffer. The cells were washed in PBS and finally lysed in 300 µL lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X-100, complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)). Cell lysates were also boiled in Laemmli buffer and samples loaded onto a 10% of SDS gel, run for 2 hours at 110 V and transferred onto a nitrocellulose membrane (GE Healthcare, Munich, Germany) via semidry blot using the Trans-Blot-Turbo (Bio-Rad, Hercules, CA, USA) at 1 A and constant 25 V for 40 minutes. The membrane was blocked in 50% Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) in TBS for 1 hour at room temperature, washed in TBS-T and subsequently incubated with primary antibodies in 5% of BSA in TBS overnight at 4°C. The following day the membrane was washed in TBS-T and incubated with different IRDye-labeled secondary antibodies for 1 hour at room temperature. The membrane was washed again in TBS-T and proteins were detected with the Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE, USA).

### 2.4 | IL-11R proteolysis by ADAM10 in HEK293 cells

IL-11R proteolysis by ADAM10 in transiently transfected HEK293 cells was analyzed as described previously.[23] In short, 48 hours after transfection, medium was removed and cells were washed with PBS. Afterward, cells were incubated with 4 mL serum-free medium and stimulated with 1 µM of ionomycin or DMSO as negative control for 1 hour at 37°C. Supernatants and cells were harvested and prepared for western blot analysis as described above.

### 2.5 | Immunofluorescence staining of IL-11R and RHBDL2

HeLa cells were transfected either with pcDNA3.1-myc-IL-11R or pcDNA3.1-HA-mRHBDL2 or co-transfected with

both plasmids before being seeded onto coverslips. Two days after transfection, cells were washed in PBS, fixed in 4% of paraformaldehyde in PBS for 10 minutes at room temperature, washed in PBS and incubated in 0.12% (w/v) glycine in PBS, washed again, and blocked in 10% of FCS in PBS for 30 minutes at room temperature. Surface IL-11R was stained with anti-myc-tag antibody (9B11) for 1 hour at 4°C. To remove unbound antibody, the cells were washed three times in PBS before the cells were permeabilized and blocked in 10% of FCS, 0.2% of saponin in PBS for 30 minutes at room temperature. Afterward, the cells were incubated with anti-HA-tag antibody for 1 hour at room temperature in order to stain surface-bound and intracellular RHBDL2. After washing three times with 10% of FCS and 0.2% of saponin in PBS, the cells were incubated with the fluorophore-labeled antibodies anti-mouse-IgG-Alexa Flour 488 and anti-rabbit-IgG-Alexa Flour 594 for 1 hour at room temperature and in darkness. Finally, the cells were washed in 10% of FCS, 0.2% of saponin in PBS

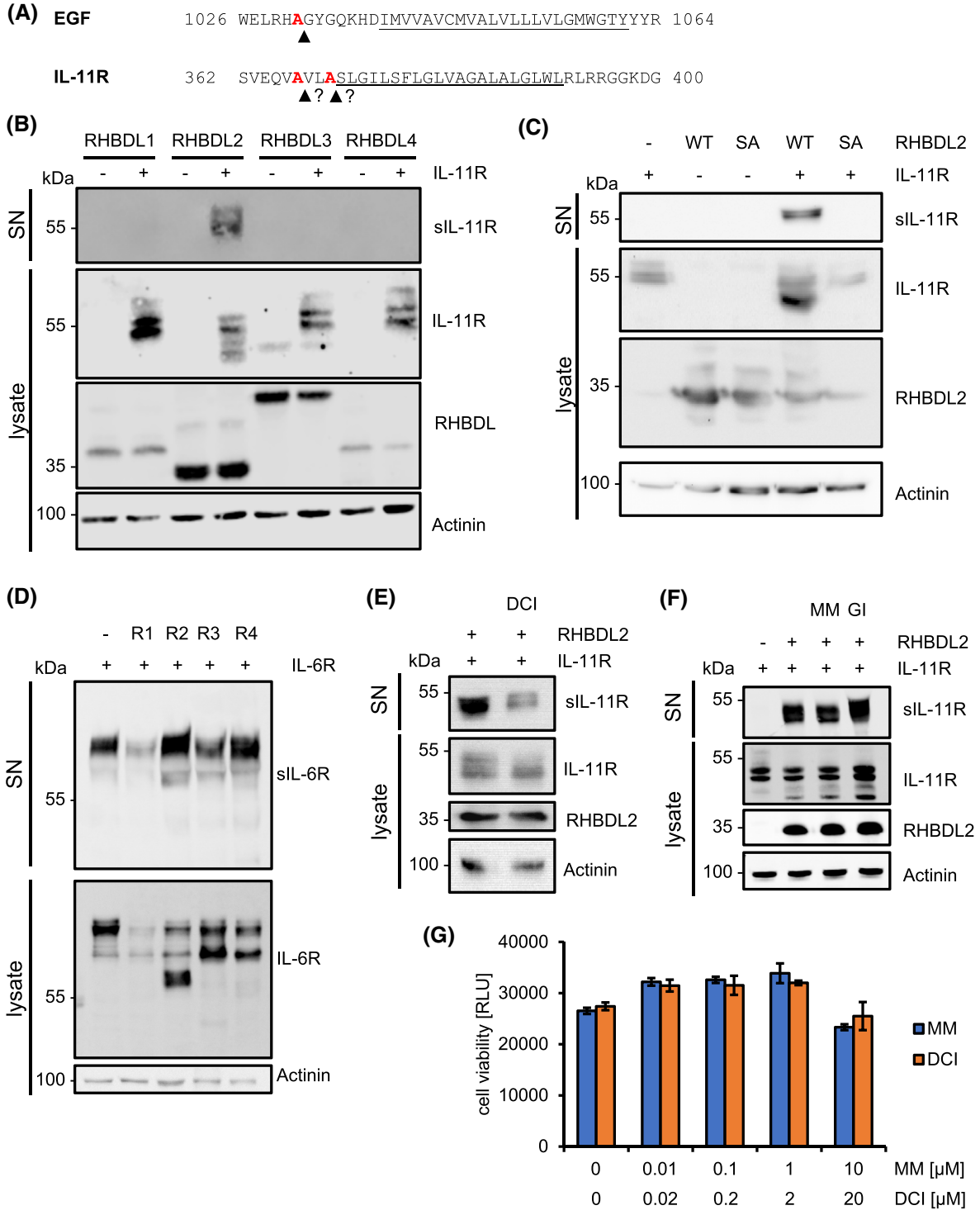

**FIGURE 1** The IL-11R is a substrate for RHDBL2. A, Juxtamembrane and transmembrane region of human EGF (from Trp-1026 to Arg-1064) and human IL-11R (from Ser-362 to Gly-400). The RHBDL2 cleavage site within EGF with an alanine residue at the P1 positions (highlighted red)[31] is indicated with a black arrowhead, the corresponding potential RHDBL2 cleavage sites within the IL-11R are indicated with black arrowheads and a question marks. The alanine residue at the P1 positions are highlighted in bold and red font. The amino acid residues belonging to the TMHs are underlined. B, HEK293 cells were co-transfected with expression plasmids encoding either RHDBL1, RHBDL2, RHBDL3, or RHBDL4 and an expression plasmid encoding either human IL-11R or control as indicated. Proteolysis of the IL-11R and expression of the four rhomboid proteases was analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. C, HEK293 cells were transfected with IL-11R, RHDBL2 wt, the inactive variant RHBDL2-SA, or IL-11R in combination with both RHDBL2 variants as indicated. The experiment was performed as described for panel b. D, HEK293 cells were co-transfected with expression plasmids encoding either RHDBL1, RHBDL2, RHBDL3, or RHBDL4 and an expression plasmid encoding human IL-6R as indicated. Proteolysis of the IL-6R was analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-6R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. E, HEK293 cells were co-transfected with expression plasmids encoding IL-11R and RHDBL2. Cells were treated with the rhomboid inhibitor DCI at a concentration of 20 μM overnight when indicated. Afterward, cells and supernatant were analyzed as described for panel b. F, HEK293 cells were co-transfected with an expression plasmid encoding IL-11R or co-transfected with expression plasmids encoding IL-11R and RHDBL2. The experiment was in principle performed as described for panels b and e, but cells were treated with the broad-spectrum metalloprotease inhibitor marimastat (MM, 10 μM) or the ADAM10 inhibitor GI254023X (GI, 3 μM) for 4 hours were indicated. G, $1.8 \times 10^6$ HEK293 cells were seeded in a 10 cm dish and transiently transfected with expression plasmids encoding IL-11R and RHBDL2. One day later, cells were detached and transferred into a 96-well plate in 100 μL medium. Ten hours later, different amounts of marimastat (0.01-10 μM) or DCI (0.02-20 μM) in triplicate were added overnight and cell viability determined the following day. SN: supernatant

and once in $ddH_2O$ and mounted onto microscopy slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Images were taken using the Olympus FV1000 confocal laser scanning microscope.

## 2.6 | Measurement of cell surface expression of IL-11R by flow cytometry

In order to quantify the cell surface expression of IL-11R and the different IL-11R variants, $0.35 \times 10^6$ HEK293 cells per well of a 6-well plate were transiently transfected with pcDNA3.1-myc-IL-11R or the respective variants. Two days after transfection, the cells were washed with FACS buffer (1% BSA in PBS) and incubated with the primary antibody anti-myc-tag (71D10) diluted 1:100 in FACS buffer for 1 hour on ice. After two washing steps with FACS buffer, the cells were incubated with the secondary antibody anti-rabbit-IgG-Alexa Flour 488 (diluted 1:100 in FACS buffer) for 1 hour on ice and in darkness. The cells were washed and resuspended in FACS buffer and analyzed using the BD FACSCanto II (Becton Dickinson, Franklin Lake, NJ, USA).

## 2.7 | Cell viability assay with Ba/F3-gp130 cells

HEK293 cells were transiently co-transfected with pcDNA3.1-myc-IL-11R or other IL-11R variants as indicated together with pcDNA3.1-HA-mRHBDL2. Two days later, the supernatants were harvested and stored at −18°C until further analysis. For the viability assay, 5000 Ba/F3-gp130 cells per well were seeded in a 96-well plate and incubated with the described conditioned media containing 10% of collected supernatants from transfected and untransfected HEK293 cells. Where indicated, 500 ng/mL IL-11 or 10 ng/mL Hyper-IL-6, respectively, were added to the conditioned media. After 48 hours, cell viability of the Ba/F3-gp130 cells was determined via the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions and as described previously.[42]

## 2.8 | Cell viability assay with HEK293 cells

Cell viability after treatment with marimastat (Sigma-Aldrich, St. Louis, USA) or DCI (biomol, Hamburg, Germany) was determined using CellTiter-Blue Cell Viability Assay. $1.8 \times 10^6$ HEK293 cells were seeded in a 10 cm dish and transiently transfected with expression plasmids encoding myc-IL-11R and HA-mRHBDL2 the next day using TurboFect. One day later, cells were detached and transferred into a 96-well plate in 100 μL medium. Ten hours later, different amounts of marimastat (0.01-10 μM) or DCI (0.02-20 μM) in triplicate were added overnight. The following day, 20 μL CellTiter-Blue were given to each well and fluorescence intensity (RLU; relative light units) was measured at λem = 590 nm for 2 hours at 37°C.

# 3 | RESULTS

## 3.1 | RHBDL2, but not the other rhomboid proteases, cleaves the IL-11R

The epidermal growth factor (EGF) has been identified as a RHBDL2 substrate in mammalian cells.[31] The cleavage site was located in the extracellular part of the protein in close

proximity to the plasma membrane and contained an alanine residue at the P1 position (Figure 1A and[31]). Intriguingly, the IL-11R contains two such alanine residues in close proximity to the plasma membrane, which could be potential RHBDL2 cleavage sites (Figure 1A). Because knowledge about RHBDL2 substrates is sparse and the protease that cleaves the IL-11R in vivo is unknown, we sought to investigate whether the IL-11R is a substrate for RHBDL2 or the other rhomboid family members. To this end, we co-expressed IL-11R with one of the four RHBDL proteases in HEK293 cells and analyzed IL-11R proteolysis by western blotting. As shown in Figure 1B, co-expression of RHBDL2 and IL-11R resulted in detectable soluble IL-11R (sIL-11R) in the cell culture supernatant and the appearance of IL-11R fragments of lower molecular weight in the cell lysate, both indicative of IL-11R proteolysis. Of note, this was not the case for RHBDL1, RHBDL3, and RHBDL4, suggesting that they were not capable of IL-11R cleavage (Figure 1B).

To show that the catalytic activity of the protease was required for IL-11R processing, we overexpressed IL-11R in combination with a catalytically inactive variant of RHBDL2 (RHBDL2-SA) or RHBDL2 wild type (wt) and found no IL-11R fragments of lower molecular weight in the cell lysate or sIL-11R release into the supernatant when RHBDL2 was catalytically inactive (Figure 1C). Because the IL-6R shares the same overall topology with the IL-11R[38] and the IL-6R has been previously identified as a substrate for RHBDL2,[35] we tested whether IL-6R would be cleaved by a RHBDL family member and co-expressed the four proteases together with a myc-tagged IL-6R. While we could indeed detect a fragment of lower molecular weight in the cell lysate when IL-6R was co-expressed with RHBDL2, but not the other three proteases, we did not detect a striking amount of sIL-6R in the supernatant (Figure 1D). Instead, sIL-6R was secreted independent of RHBDL overexpression into the cell culture supernatant, consistent with previous reports of IL-6R on microvesicles.[43] Because RHBDLs are sensitive to the serine protease inhibitor 3,4-dichloroisocoumarin (DCI),[44] we observed reduced sIL-11R generation by RHBDL2 when we preincubated cells with DCI (Figure 1E). Importantly, sIL-11R release by RHDBL2 was insensitive to inhibition by the broad-spectrum metalloprotease inhibitor marimastat (MM) and the ADAM10-specific inhibitor GI254023X (GI) (Figure 1F), proving that sIL-11R release was independent of ADAM10, which has been previously identified as an IL-11R sheddase.[23-25] Neither DCI nor MM affected the viability of the cells (Figure 1G).

## 3.2 | Ala-370, but not Ala-367, is required for IL-11R proteolysis

The juxtamembrane region of the IL-11R contains two amino acid residues, which could potentially be RHBDL2 cleavage sites: Ala-367 and Ala-370 (Figure 1A). In order to determine their contribution, we constructed and analyzed different IL-11R mutants (Figure 2A,B). We first used an IL-11R deletion variant (IL-11RΔV363_L372 (Δ1),[23]), which lacks a stretch of 10 amino acid residues encompassing both alanine residues, and could neither detect sIL-11R release into the cell supernatant nor the generation of IL-11R cleavage fragments in the cell lysate when this IL-11R variant was expressed together with RHBDL2 (Figure 2B). Importantly, a deletion variant lacking the 10 amino acid residues directly N-terminal of the first deletion (IL-11RΔH353_S362 (Δ2),[23]) was cleaved by RHBDL2 in similar amounts as wt IL-11R, suggesting that the cleavage event took indeed place between Val-363 and Leu-372 (Figure 2B). In order to determine which alanine residue was critical for IL-11R proteolysis, we replaced both individually by phenylalanine residues (IL-11R-A367F and IL-11R-A370F, respectively), because bulky amino acids at the P1 position have been previously reported to prevent RHBDL2 cleavage.[31] Strikingly, while IL-11R-A367F was cleaved by RHBDL2, IL-11R-A370F showed barely any visible cleavage fragments in the cell lysate and only small amounts of sIL-11R in the cell culture supernatant, indicating that Ala-370 is indeed critically involved in IL-11R proteolysis by RHBDL2 (Figure 2B).

To further substantiate this finding, we introduced additional mutations into the IL-11R (Figure 2A). Insertion of the small amino acid residue glycine or the bulky amino acid residue tyrosine at position 367 had no striking effect on IL-11R proteolysis, confirming that Ala-367 is not involved in cleavage by RHBDL2 (Figure 2B). When we introduced the same amino acid residues at position 370 (Figure 2A), the replacement of the alanine with glycine had no effect on IL-11R proteolysis, while the insertion of a tyrosine completely blocked IL-11R cleavage by RHBDL2 (Figure 2B). These data further support our assumption that Ala-370 is located at the P1 position of the RHBDL2 cleavage site.

In order to ensure that our mutations did not alter cell surface appearance of the IL-11R, which could potentially influence IL-11R cleavage by RHBDL2, we assessed cell-surface levels of the IL-11R by flow cytometry. As shown in Figure 2C,D, we did not find any significant difference of the different IL-11R variants at the cell surface, which indicates that the differences in sIL-11R generation indeed result from differential cleavage by RHBDL2. This was underlined by the fact that IL-11R-A370F, which showed impaired proteolysis by RHBDL2 (Figure 2B), was proteolytically cleaved by the metalloprotease ADAM10 at comparable levels as IL-11R wt (Figure 2E). In summary, our results show that IL-11R cleavage by RHBDL2 requires Ala-370 and uses a cleavage site that is distinct from the ADAM10 cleavage site, which is located further away from the membrane.[23]

### 3.3 | Destabilizing amino acid residues in the TMH are critical for IL-11R proteolysis

The current understanding of substrate recognition of RHBDLs is that when the cleavage site is located within or in close proximity to the TMH, partial unfolding of the N-terminal part of the substrate TMH is required to ensure access of the catalytic center of the protease to the cleavage site of the substrate.[45] Because the TMH of the IL-11R contains five amino acid residues with low hydrophobicity potentially destabilizing the TMH, namely Gly-373, Ser-376, Gly-379, Gly-383, and Gly-388, we constructed five different IL-11R variants in which one of these residues was mutated into a leucine to make the TMH more stable (Figure 3A). We expressed IL-11R wt and the five different mutants in combination with RHBDL2 in HEK293 cells and investigated IL-11R proteolysis via western blot analysis. Intriguingly, the IL-11R-G373L variant, in which we mutated the putative

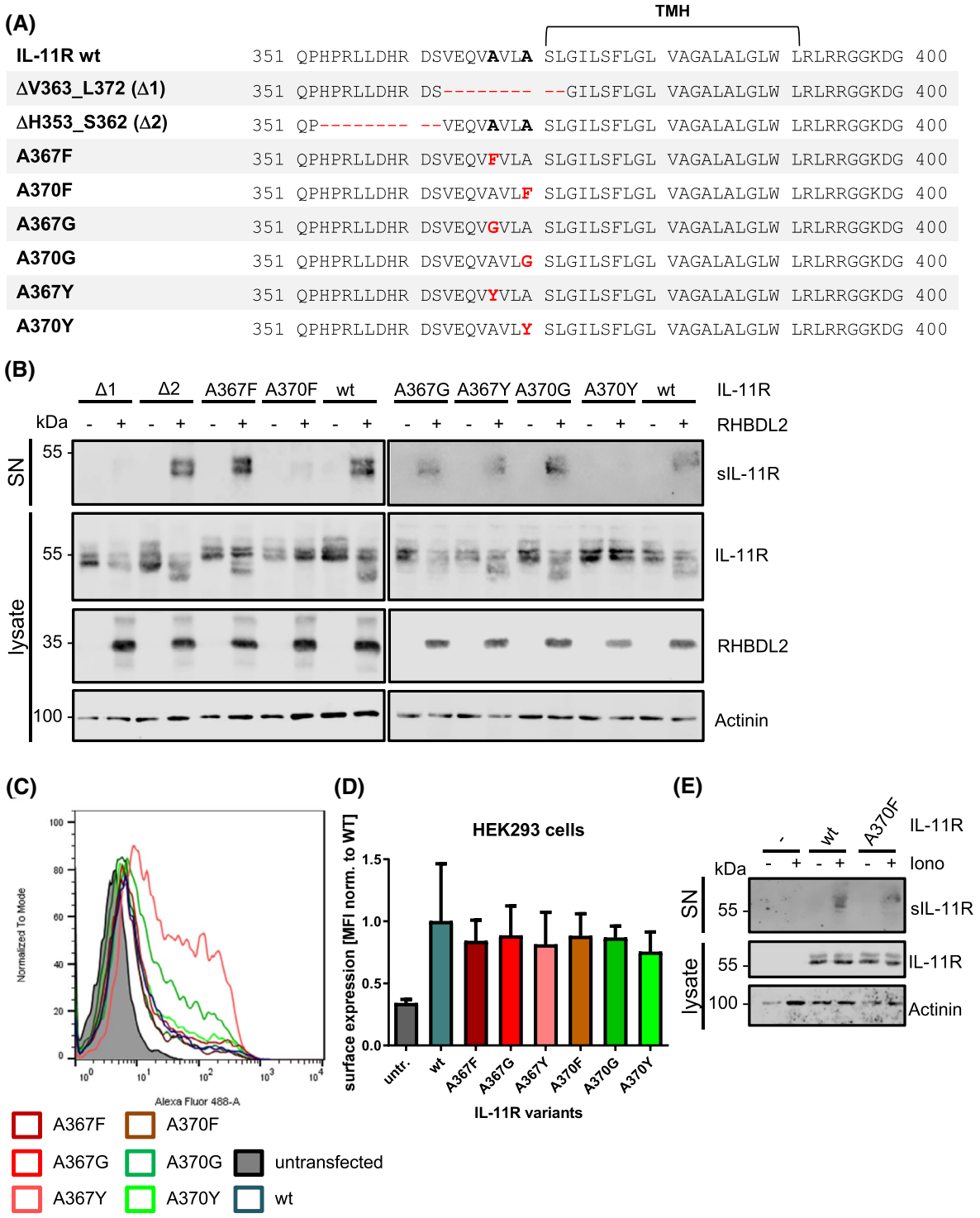

**FIGURE 2** RHDBL2 cleaves the IL-11R after Ala-370, but not after Ala-367. A, Juxtamembrane and transmembrane region of the human IL-11R (from Gln-351 to Gly-400) is depicted as IL-11R wt. The alanine residues representing two potential RHBDL2 cleavage sites are shown in bold font. Below the wild-type sequence, all other deletion variants and point mutations used in the experiments shown in this figure are listed. Modifications of the amino acid sequence in comparison to the IL-11R wild type are marked in red and bold font. B, All IL-11R variants listed in panel a were transiently transfected in HEK293 cells, either together with RHBDL2 or with mock control. Expression and proteolysis of the different IL-11R variants and expression of RHBDL2 were analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blotting. C, D, Cell surface expression of the different IL-11R variants used in the experiment described in panel B was analyzed by flow cytometry. Shown are one representative experiment in panel C and the mean ± SD from three independent experiments in panel D. Expression of IL-11R wild type was set to 1 and the expression of all other variants calculated accordingly. E, HEK293 cells were transiently transfected with expression plasmids encoding IL-11R wild type, IL-11R-A370F, or left untransfected. Two days after transfection, medium was removed, cells were washed and stimulated in serum-free medium with 1 μM of the ionophore ionomycin for 60 minutes to activate ADAM10 or with DMSO as control. Proteolysis of the IL-11R variants was analyzed by western blot of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. SN: supernatant

TMH-destabilizing amino acid that is three residues downstream of the actual cleavage site, displayed no cleavage fragment in the cell lysate and no sIL-11R was detectable in the cell culture supernatant, showing that this residue within the TMH was critical for IL-11R proteolysis (Figure 3B). Reduced proteolysis was also observed for the variants IL-11R-S376L and IL-11R-G379L, while the last two mutants IL-11R-G383L and IL-11R-G388L were clearly proteolytically processed by RHBDL2 and did not differ substantially from cleavage of the IL-11R wt (Figure 3B). Like before, we ensured that all IL-11R variants were expressed at the cell surface via flow cytometry and again could not detect any major influence of the mutations on cell-surface appearance of the IL-11R that would explain the altered proteolysis (Figure 3C,D). Our results show a critical role for destabilizing residues in the N-terminal part of the TMH for proteolysis of the IL-11R by RHBDL2. The farther these destabilizing residues are located from the actual cleavage site, the less severe appears to be their influence on the proteolysis.

## 3.4 | RHBDL2 can cleave and trigger secretion of IL-11R in the early secretory pathway

RHBDL2 has been described previously to be localized at the plasma membrane[31,32] and ectopically expressed protein also resides within the ER.[46] In order to determine where in the cell proteolysis of the IL-11R occurs, we analyzed expression of both proteins via fluorescence microscopy. As shown in Figure 4A, IL-11R expression was visible at the plasma membrane and in intracellular compartments, while upon overexpression RHDBL2 was predominantly detectable intracellularly and the amount at the cell surface appeared rather sparse. Therefore, we wondered whether IL-11R cleavage could also occur intracellularly. To this end, we co-expressed RHBDL2 with either wild-type IL-11R, an IL-11R variant with an ER retention motif (IL-11R-KKSS), or the variant IL-11R-R296W, which contains a mutation found in a patient with craniosynostosis and is also retained within the ER.[20] Indeed, we observed cleavage of the IL-11R-KKSS by RHBDL2 by western blotting of respective cell lysate and could further detect moderate amounts of sIL-11R in the cell supernatant of these cells (Figure 4B). We observed also cleavage of the IL-11R-R296W variant in the cell lysate, and longer exposure of the western blot further revealed a faint band of sIL-11R, although the molecular weight appeared to be smaller, suggesting that either RHBDL2 used a different cleavage site further N-terminal in this variant, or that impaired glycosylation caused the difference in the molecular weight (Figure 4B). Importantly, while these data clearly demonstrate that upon overexpression RHBDL2 can cleave the IL-11R within the ER, the resulting amount of sIL-11R in the tissue culture supernatant was significantly lower compared to processing of wild-type IL-11R in the late secretory pathway (Figure 4B). To further substantiate this finding, we made use of an RHBDL2 variant fused to the KDEL ER retrieval motif (RHBDL2-KDEL) that ensures its efficient retrograde transport from the *cis*-Golgi.[47] When co-expressed with wild-type IL-11R, RHBDL2-KDEL cleaved the IL-11R to similar extend as the wild-type RHBDL2, which resulted in comparable sIL-11R release (Figure 4C). Importantly, this release required the catalytic activity of the protease, because both inactive variants RHBDL2-SA and RHBDL2-SA-KDEL did not cleave the IL-11R (Figure 4C). In conclusion, our results show that proteolysis of the IL-11R by RHDBL2 can occur already within the early secretory pathway and is not confined to the plasma membrane. While processing of the ER-retained IL-11R is moderate only, RHBDL2 cycling within the ER to Golgi intermediate triggers secretion as efficient as the wild-type rhomboid.

## 3.5 | RHBDL2-derived sIL-11R is biologically active

We have previously shown that a sIL-11R generated by ADAM10-mediated cleavage retains its ability to bind IL-11

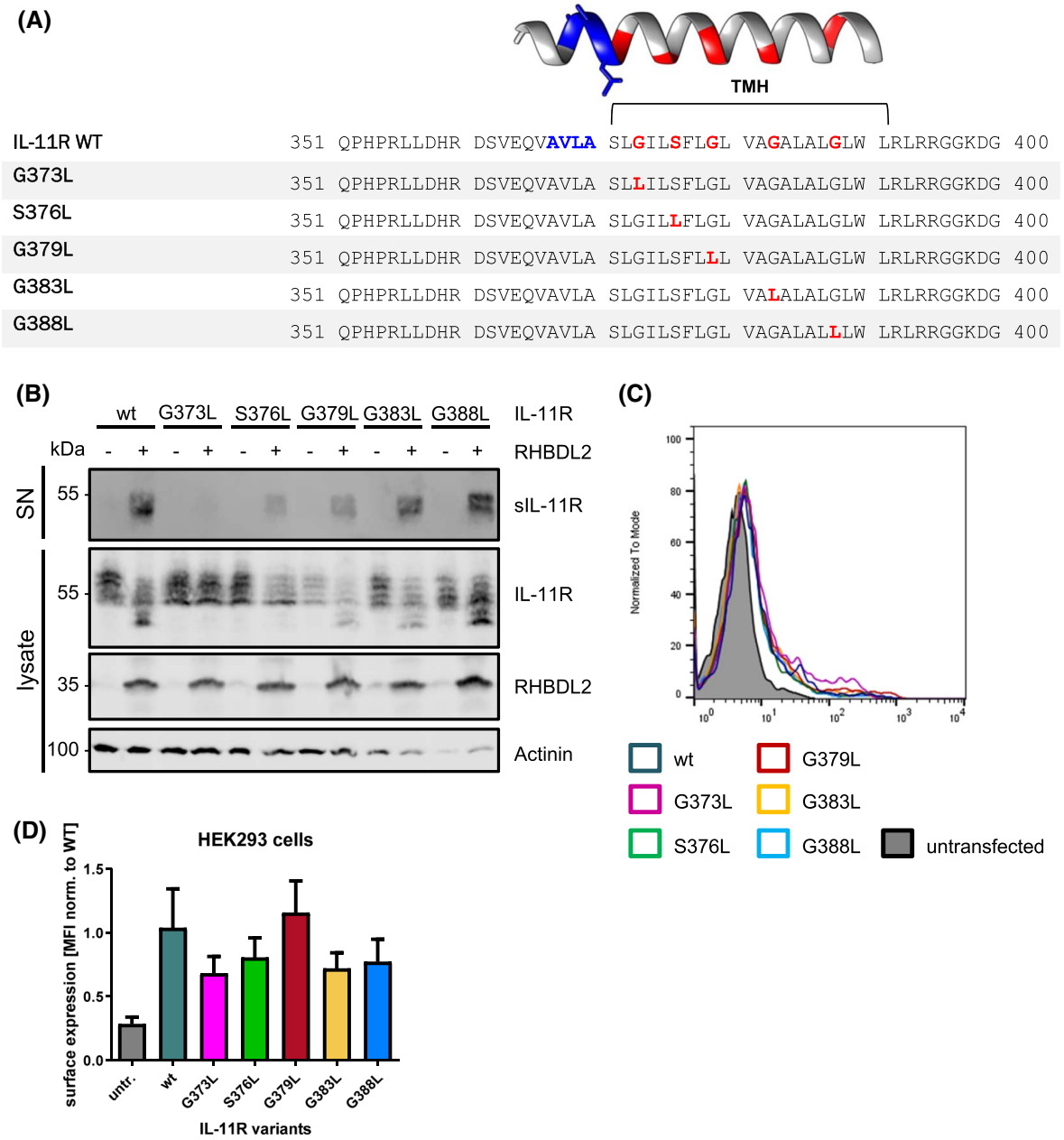


**FIGURE 3** Helix-destabilizing amino acid residues within the TMH of the IL-11R are required for RHBDL2-mediated cleavage. A, Juxtamembrane and transmembrane region of the human IL-11R (from Gln-351 to Gly-400) is depicted as IL-11R wt. The RHBDL2 cleavage motif is shown in bold blue font. Potential destabilizing amino acid residues within the TMH are indicated in bold red font. A structural representation of the TMH with the N-terminal cleavage motif is shown above. Below the wild-type sequence, all point mutations used in the experiments shown in this figure are listed. Modifications of the amino acid sequence in comparison to the IL-11R wild type are marked in red and bold font. B, All IL-11R variants listed in panel a were transiently transfected in HEK293 cells, either together with RHBDL2 or with mock control. Expression and proteolysis of the different IL-11R variants and expression of RHBDL2 were analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. C, D, Cell surface expression of the different IL-11R variants used in the experiment described in panel B was analyzed by flow cytometry. Shown are one representative experiment in panel C and the mean ± SD from three independent experiments in panel D. Expression of IL-11R wild type was set to 1 and the expression of all other variants calculated accordingly. SN: supernatant

and that the resulting IL-11/sIL-11R complex is acting in an agonistic manner and is able to induce IL-11 trans-signaling.[23] To investigate whether sIL-11R generated by RHBDL2 is similarly able to induce IL-11 trans-signaling, we made use of Ba/F3-gp130 cells, which are a murine pre-B cell line that proliferates upon stimulation with biologically active IL-11/sIL-11R complexes, but undergoes apoptosis otherwise. Ba/F3-gp130 cells incubated with supernatants of HEK293 cells

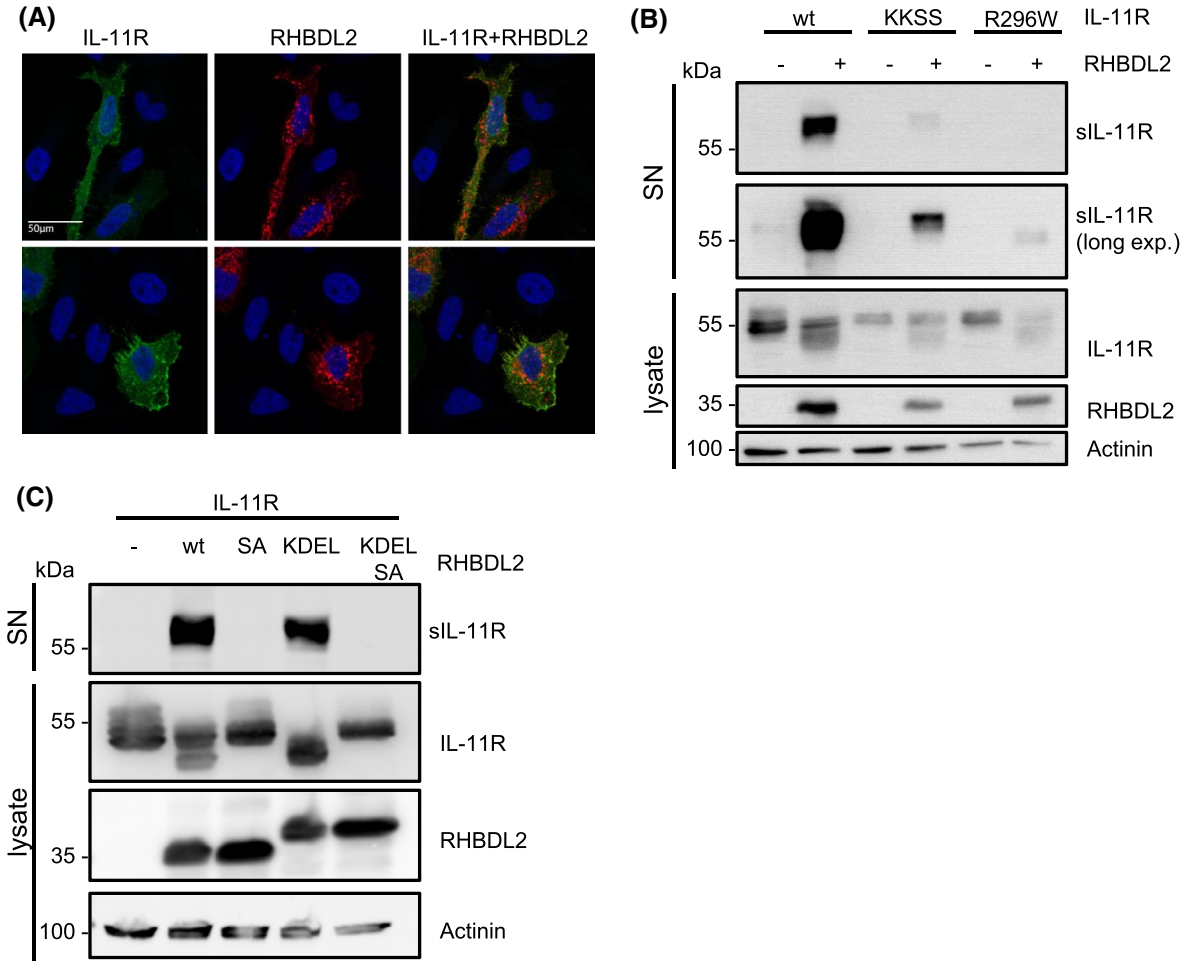


**FIGURE 4** RHDBL2 can cleave the IL-11R in the early secretory pathway. A, HeLa cells were co-transfected with expression plasmids encoding IL-11R wild type and RHBDL2. Localization of both proteins was determined via fluorescence microscopy. B, IL-11R wild type, IL-11R with a C-terminal ER retention signal (IL-11R-KKSS) and the patient mutation IL-11R-R296W were transiently transfected in HEK293 cells, either together with RHBDL2 or with mock control. Expression and proteolysis of the different IL-11R variants and expression of RHBDL2 were analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. C, IL-11R wild type was transiently transfected in HEK293 cells either with mock control or different RHBDL2 variants (RHBDL2 wt, RHBDL2-SA, RHBDL2-KDEL, and RHBDL2-KDEL-SA). The experiment was then conducted as described in panel b. SN: supernatant

transfected with wild-type IL-11R and RHBDL2 showed high proliferation when IL-11 was added, which was not the case without the cytokine (Figure 5A). Of note, supernatant of HEK293 cells which were transfected with wild-type IL-11R, but not RHBDL2, also induced proliferation in combination with IL-11 (Figure 5A), which is caused by the constitutive proteolysis of the IL-11R by ADAM10.[23,24] Supernatant of HEK293 cells transfected with either IL-11R-R296W or IL-11R-KKSS alone did not induce proliferation (Figure 5A), as these variants are retained within the ER and do not undergo ADAM10-mediated cleavage (Figure 4B). Importantly, co-transfection of IL-11R-KKSS and RHBDL2 resulted in a biologically active sIL-11R, whereas the traces of sIL-11R-R296W, detected upon RHBDL2 expression (Figure 4B), are biologically inactive (Figure 5A), consistent with our previous findings.[20] We further verified the biological activity of the sIL-11R by assessing the phosphorylation of STAT3 by western blot, and detected pSTAT3 when Ba/F3-gp130 cells were stimulated with supernatant from cells overexpressing RHBDL2 and IL-11R wild type or IL-11R-KKSS (Figure 5B). In conclusion, these data demonstrate that sIL-11R generated by RHBDL2 is biologically active and able to induce IL-11 trans-signaling.

## 3.6 | The mutation A370V prevents IL-11R proteolysis by RHBDL2

In order to determine whether the identified IL-11R cleavage site could have a functional impact in humans, we analyzed the Genome Aggregation Database (gnomAD,[48]) for homozygous carrier of coding mutations within the *IL11RA*

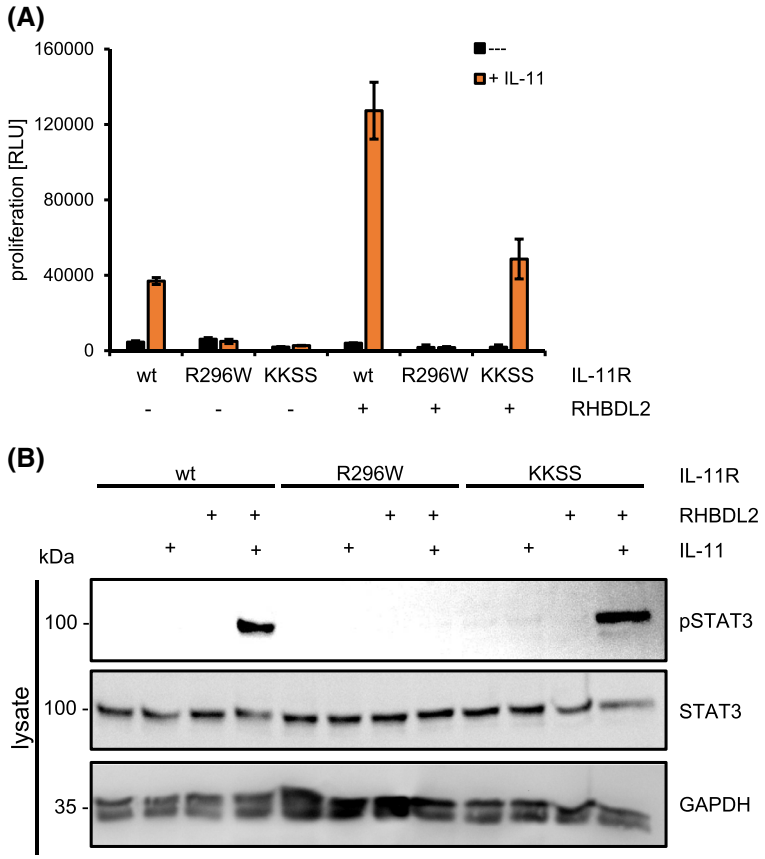


**FIGURE 5** RHDBL2-derived sIL-11R is biologically active. A, Equal amounts of Ba/F3-gp130 cells were incubated in triplicate with supernatants of HEK293 cells that were transfected with either IL-11R wt, IL-11R-R296W, or IL-11R-KKSS, either together with control or with RHBDL2. Supernatant was either supplemented with recombinant IL-11 (500 ng/mL) or left untreated. Cells were incubated for 2 days and cell viability determined as described in Materials and Methods. Shown are the mean ± SD from one experiment out of three independent experiments with similar outcome. B, Equal amounts of Ba/F3-gp130 cells were stimulated for 15 minutes with the supernatants described in the legend of panel A and recombinant IL-11 was added were indicated. Phosphorylation of STAT3 was determined in cell lysates via western blot. Total STAT3 levels and GAPDH were visualized to ensure equal protein loading

gene. Indeed, gnomAD lists one homozygous carrier for the single nucleotide variant 9-34660537-C-T, which results in a substitution of Ala-370 into Val-370 (Figure 6A). Whether this mutation is associated with a human disease is unknown. To analyze whether this mutation has a functional consequence on IL-11R proteolysis by RHDBL2, we constructed an expression plasmid with IL-11R-A370V variant and transfected it with and without co-transfection of RHDBL2 into HEK293 cells. When compared to wild-type IL-11R, western blot analysis revealed that IL-11R-A370V was not cleaved by RHBDL2, and no sIL-11R released into the cell culture supernatant could be detected (Figure 6B). When we stimulated Ba/F3-gp130 cells with these supernatants with or without the addition of IL-11, we could clearly see STAT3 phosphorylation of cells stimulated with supernatant from cells expressing wild-type IL-11R, but only a weak pSTAT3 response of cells stimulated with supernatant from cells expressing IL-11R-A370V (Figure 6C). Importantly, the mutation did not affect the biological function of the IL-11R per se, as IL-11R-A370V was transported to the cell surface of stably transduced Ba/F3-gp130 cells at comparable levels as IL-11R wild type (Figure 6D). Furthermore, Ba/F3-gp130-IL-11R-A370V cells proliferated in a dose-dependent manner (Figure 6E) and STAT3 was phosphorylated when cells were stimulated with IL-11 (Figure 6F). Thus, the rather small alteration within the cleavage site of a valine instead of an alanine residue appears to be already sufficient to prevent proteolysis by RHDBL2 and suggests that IL-11R cleavage by RHBDL2 is significantly reduced in the respective individual annotated in the gnomAD, while IL-11 classic signaling is intact.

## 4 | DISCUSSION

Proteolysis of membrane-bound cytokine receptors has emerged as an important posttranslational modification over the years.[21,22] This mechanism not only downregulates the amount of cytokine receptor at the cell surface, but also reduces the responsiveness of the cell toward activation by the

cytokine ligand. Limited proteolysis also generates soluble cytokine receptors with distinct biological properties, which often differ from the functions of their membrane-tethered counterparts. Identification of the responsible proteases, their regulation, and understanding how specificity is achieved is therefore crucial, especially when considering anti-cytokine or anti-cytokine receptor therapeutics.[26]

In this study, we identify the IL-11R as a so far unrecognized substrate of the rhomboid protease RHDBL2. We have previously shown that the IL-11R can be cleaved by the metalloprotease ADAM10,[23,24] by the serine proteases neutrophil elastase and proteinase 3[23] and in overexpression by other metalloproteases, for example, meprin β.[49] At least for ADAM10-mediated cleavage, the remaining IL-11R stub within the plasma membrane is further processed and degraded by the γ-secretase.[25] Interestingly, we show that the IL-11R is not cleaved by the other members of the RHBDL family, namely RHBDL1, RHBDL3, and RHBDL4, indicating that the four proteases do not share their substrates, but have rather distinct substrate repertoires. This is clearly

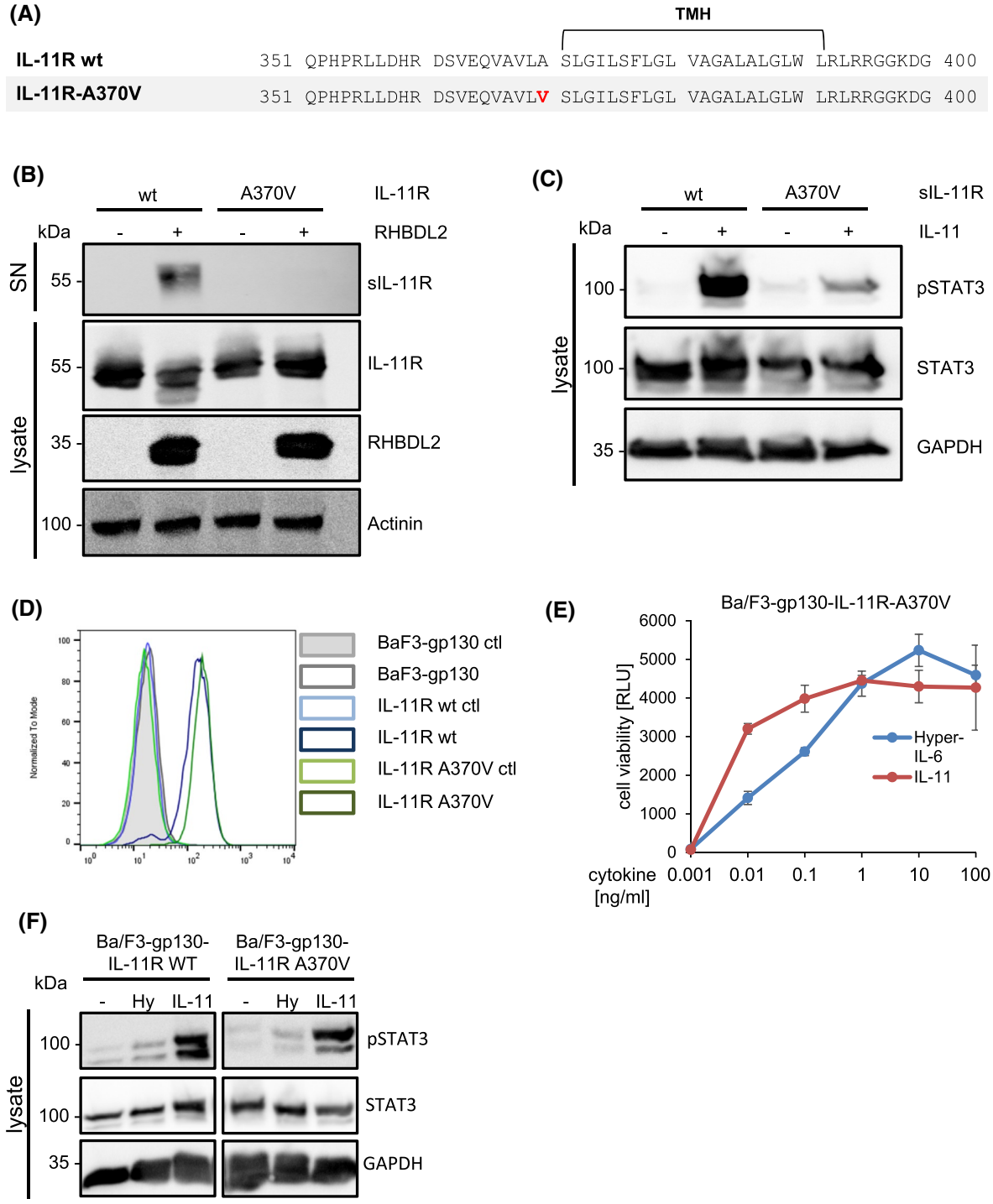

**FIGURE 6** The IL-11R variant IL-11R-A370V is biologically active, but not cleaved by RHBDL2. A, Juxtamembrane and transmembrane region of the human IL-11R (from Gln-351 to Gly-400) is depicted as IL-11R wt. The mutation A370V is indicated in bold red font. B, IL-11R wild type and the variant IL-11R-A370V were transiently transfected in HEK293 cells, either together with RHBDL2 or with mock control. Expression and proteolysis of the different IL-11R variants and expression of RHBDL2 were analyzed by western blotting of cell lysates. Actinin was visualized as loading control. sIL-11R was precipitated from cell culture supernatant by TCA precipitation and also analyzed by western blot. C, Equal amounts of Ba/F3-gp130 cells were stimulated for 15 minutes with the supernatants described in the legend of panel b and recombinant IL-11 was added were indicated. Phosphorylation of STAT3 was determined in cell lysates via western blot. Total STAT3 levels and GAPDH were visualized to ensure equal protein loading. D, Cell surface amounts of the IL-11R on Ba/F3-gp130, Ba/F3-gp130-IL-11R, and Ba/F3-gp130-IL-11R-A370V cells were determined via flow cytometry. FACS plots depict one experiment out of three independent experiments. Control staining (ctl) was performed without the primary antibody. E, Equal amounts of Ba/F3-gp130-IL-11R-A370V cells were incubated in triplicate with increasing amounts (0.001-100 ng/mL) of either recombinant IL-11 or Hyper-IL-6 as indicated. Cells were incubated for 2 days and cell viability determined as described in Materials and Methods. Shown are the mean ± SD from one experiment out of three independent experiments with similar outcome. F, Equal amounts of Ba/F3-gp130-IL-11R and Ba/F3-gp130-IL-11R-A370V cells were stimulated for 15 minutes with 10 ng/mL Hyper-IL-6, 10 ng/mL IL-11, or left unstimulated. Phosphorylation of STAT3 was determined in cell lysates via western blot. Total STAT3 levels and GAPDH were visualized to ensure equal protein loading. SN: supernatant

different from the ADAM family, in which, for example, ADAM10 and ADAM17 have a remarkable substrate overlap and, at least in part, can compensate for each other when one of the proteases is inactive.[50,51]

IL-6 is the most prominent member of the cytokine family to which IL-11 belongs, and the IL-6R is also a substrate for different proteases, including ADAM10 and ADAM17.[52] The IL-6R has been recently reported in a proteomics screen as a RHBDL2 substrate,[35] and we could indeed also detect an IL-6R cleavage fragment within the cell lysate when IL-6R and RHBDL2 were co-expressed. However, we did not observe significant release of sIL-6R into the cell culture supernatant, indicating that the IL-11R is more efficiently cleaved by RHBDL2 than the IL-6R.

RHBDL2 is currently the family member for whom the most substrates have been described.[30] We have identified the IL-11R as RHBDL2 substrate based on sequence similarities between the membrane-proximal region of IL-11R and EGF.[31] Strikingly, the EGF cleavage site contained an alanine residue at the P1 position, which is also present in the IL-11R. We could show that mutations of Ala-370 in the IL-11R critically modulate IL-11R proteolysis by RHBDL2. While the insertion of small amino acid residues was tolerated and did not alter sIL-11R generation, bulky amino acid residues prevented IL-11R proteolysis, and thus, sIL-11R release. Importantly, mutations of the residue Ala-367 did not influence IL-11R proteolysis by RHBDL2 at all, underlining not only that Ala-370 is indeed the specific cleavage site used by RHBDL2, but also rather showing that mutations within the IL-11R do not alter proteolysis per se. These data fit well into the concept that rhomboid proteases recognize a specific sequence surrounding the cleavage site.[45]

Besides the importance of the cleavage site, previous work had demonstrated that helix-destabilizing residues in the transmembrane region of the substrate are crucial for cleavage by rhomboids.[53] We could also demonstrate this feature for the IL-11R by showing that mutation of certain TMH-destabilizing amino acid residues within the transmembrane region blocks proteolysis by RHBDL2. Strikingly, the N-terminal half of the TMH close to the actual cleavage site was more important in this regard than the C-terminal portion, which highlights an important regulatory mechanism buried within the TMH.

While the localization of endogenous RHBDL2 has not only been addressed yet, using ectopically expressed protein it has been reported to act mostly at the plasma membrane,[31,32] but also ER-based activity had been observed.[46] However, processing of IL-11R confined to the ER by a C-terminal KKSS ER retention motif does not lead to efficient secretion. This indicates that ER-born cleavage products are retained by the ER quality control and ER-associated degradation pathway, like substrates that are cleaved by the ER-resident rhomboid protease RHBDL4.[54] In contrast, RHBDL2 fused to a KDEL ER retrieval signal, which cycles between the ER and Golgi compartment, shows much higher processivity, indicating that rhomboid proteases also can act as secretases of the early secretory pathway. One limitation of this study is the use of overexpressed proteins, and future work has to address whether also endogenous RHBDL2 plays a role in IL-11R secretion and in which cellular compartments endogenous RHBDL2 is localized.

The cytokine IL-11 has gained significant interest in recent years as an important regulatory protein in several human diseases, including gastrointestinal cancer[9] and fibrosis.[55] While most studies did not address whether classic or trans-signaling of IL-11 was responsible for the observed effects, our own studies with regard to female fertility,[14] gastric cancer,[8] and craniofacial bone development[20] point to classic signaling as the responsible mode of signaling. Thus, a functional role for IL-11 trans-signaling in vivo has yet to be shown, although evidence for the existence of sIL-11R in vivo has been published.[23,27] Whether sIL-11R generation in vivo requires the activity of RHBDL2, ADAM10, or a different, yet, unidentified protease remains to be investigated. Nevertheless, IL-11 trans-signaling increases the spectrum of cells that can be

activated by IL-11 and holds the promise to represent an important pro-inflammatory pathway.

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## CONFLICT OF INTEREST

CG has received funding support from Corvidia Therapeutics (Waltham, MA, USA).

## AUTHOR CONTRIBUTIONS

C. Garbers designed research; C. Garbers and J. Lokau analyzed data; L. Koch, B. Kespohl, T. Schumertl, and M. Agthe performed research; C. Garbers wrote the paper with input from all authors; S. Düsterhöft and M. K. Lemberg contributed new reagents or analytic tools.

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