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RAPID COMMUNICATION

Coding variants of the interleukin-11 receptor with reduced protein maturation show protease-dependent trans-signaling and transduce normal STAT3 signaling

Interleukin-11 (IL-11) is a member of the IL-6 family of cytokines.[1] IL-11 activates its target cells via binding to the IL-11 receptor (IL-11R), which also exists in soluble forms generated by proteolytic cleavage (sIL-11R, required for trans-signaling).[2] Formation of the IL-11/IL-11R complex leads to the recruitment and homodimerization of two molecules of the signal-transducing β-receptor gp130, which then activates intracellular signaling cascades, including the Jak/STAT, PI3K and ERK pathways.

Besides its pro-inflammatory role, IL-11 has important functions in developmental processes, e.g. in bone homeostasis.[3] Furthermore, several mutations within the *IL11RA* gene have been linked to craniosynostosis, a human condition in which the sutures that line and connect the head bones close before the growth of the skull is completed. How these mutations affect the IL-11R and how this is connected to craniosynostosis is largely unexplored. In a first study, we have recently reported that the three IL-11R mutations P200T, P221R and R296W could cause intracellular retention of the IL-11R within the endoplasmic reticulum (ER), which was most probably due to a misfolding of the protein.[4]

In this study, we have therefore analyzed the four IL-11R variants p.Val15Met, p.Gly231Asp, p.Arg261His and p.Arg395Trp, for which at least one homozygous individual is annotated in the Genome Aggregation Database (gnomAD) (Fig. S1A). Multiple sequence alignments of IL-11R orthologs from 10 different species revealed varying degrees of conservation and suggested that especially R261 might be important for function or stability of the IL-11R (Fig. S1B–G). Structural modeling of the IL-11/IL-11R/gp130 complex revealed that G231 is part of a loop that connects two of the beta sheets which constitute the D3 domain of the IL-11R, whose integrity or the interaction with gp130 could be compromised by the G231D mutation (Fig. S2A). R261 is part of an arginine-tryptophan-zipper motif essential for the integrity of the D3 domain[4] and it is therefore likely that the R261H mutation disrupts this zipper and compromises IL-11R stability (Fig. S2B).

We have previously shown that ectopically expressed IL-11R is detected by Western blot as at least three distinct bands in cell lysates, while misfolded ER-retained variants like IL-11R-R296W only display two bands.[4] Transient expression of all IL-11R variants in HEK293 cells revealed no gross differences in the overall expression, but showed impaired maturation of IL-11R-G231D and IL-11R-R261H, while IL-11R-V15M and IL-11R-R395W matured normally (Fig. 1A). Further, IL-11R-G231D and IL-11R-R261H were detected as a single band after PNGase F digestion, underlining that these variants do not properly exit in the ER (Fig. 1B). Blockade of protein translation with cycloheximide (CHX) revealed an increase of the matured IL-11R-WT over time, which was not seen for IL-11R-R296W. Interestingly, IL-11R-G231D but not IL-11R-R261H matured under CHX-treatment, suggesting that ER-retention of IL-11R-G231D was less strict compared to IL-11R-R296W or IL-11R-R261H (Fig. S3).

We have previously shown that IL-11R variants that do not mature correctly are retained within the ER and do not reach the cell surface.[4] In line with the other results, we observed reduced cell surface levels of IL-11R-R296W, IL-11R-G231D and IL-11R-R261H in transiently transfected HEK293 cells (Fig. 1C, D) and stably transduced Ba/F3-gp130 cell lines via flow cytometry (Fig. S4A, B). The maturation defect of the three IL-11R variants was further confirmed in lysates of the stably transduced Ba/F3-gp130 cell lines and transiently transfected HeLa cells via

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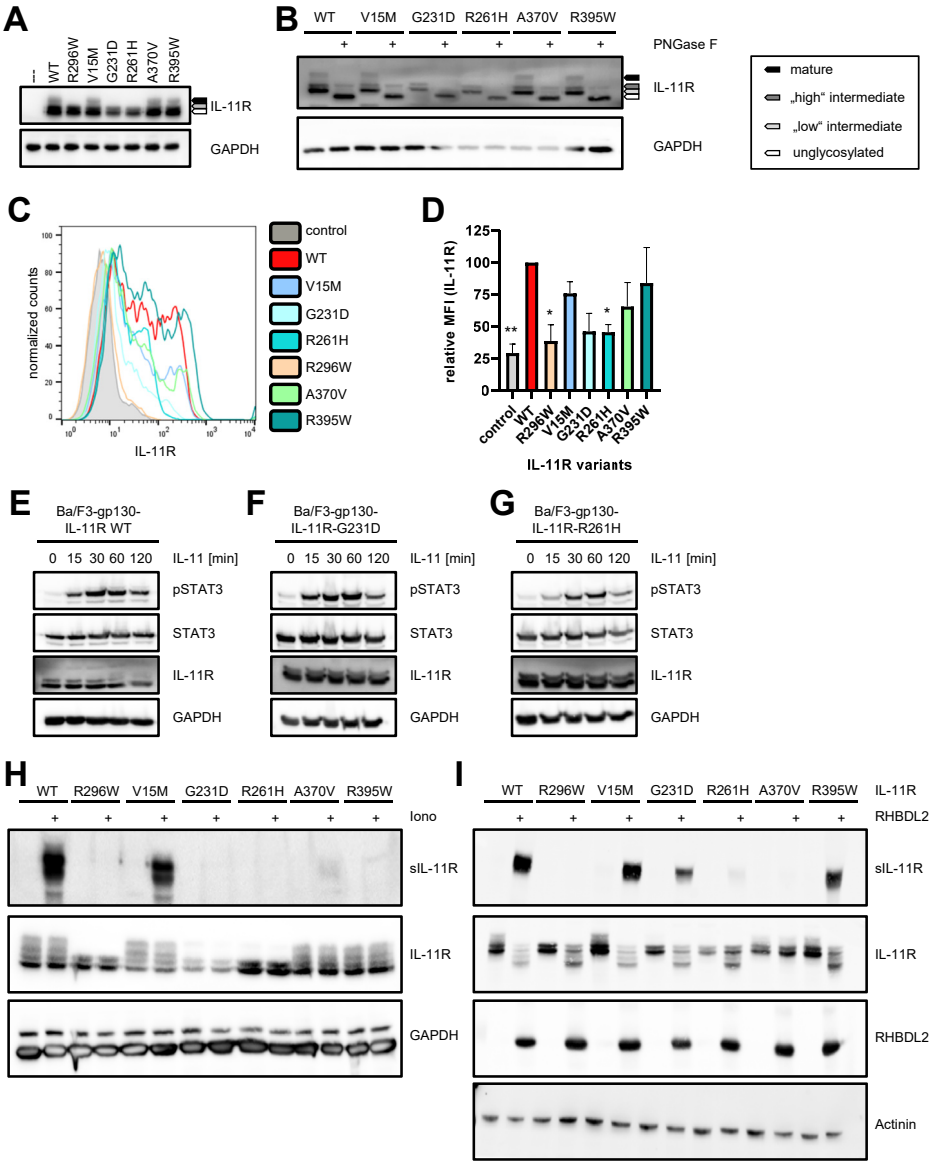

Figure 1 Molecular characterization of the interleukin-11 receptor variants V15M, G231D, R261H and R395W. **(A)** HEK293 cells were transiently transfected with expression plasmids encoding IL-11R-WT, IL-11R-R296W, IL-11R-V15M, IL-11R-G231D, IL-11R-R261H, IL-11R-A370V, IL-11R-R395W or an empty vector as negative control. Maturation was analyzed by Western blot 48 h after transfection and GAPDH was visualized as loading control. **(B)** Lysates of HEK293 cells transiently transfected with the indicated IL-11R variants were either deglycosylated using PNGaseF overnight or left untreated. Changes in the apparent molecular weight were analyzed by Western blotting and GAPDH was visualized as loading control. **(C, D)** Cell surface expression of the different IL-11R variants transiently transfected in HEK293 cells were analyzed via flow cytometry. Shown is one of three independent experiments in panel C. The MFI of cells expressing IL-11R-WT was set to 100, all other variants calculated accordingly and shown as the mean ± SEM from three independent experiments in panel D. Statistically significant differences were analyzed using one sample *t*-test. *, $P < 0.05$; **, $P < 0.01$; all other variants were not significantly different. **(E—G)** Equal amounts of stably transduced Ba/F3-gp130 cells expressing (E) IL-11R- WT, (F) IL-11R-G231D and (G) IL-11R-R261H were stimulated for 0—120 min with recombinant IL-11 (10 ng/ml). Phosphorylation of STAT3 and IL-11R expression was determined in cell lysates via Western blot. Total STAT3 levels and GAPDH were visualized to ensure equal protein loading. **(H)** HEK293 cells were transiently transfected with expression plasmids encoding the different IL-11R variants. To induce proteolysis by ADAM10, cells were stimulated with 1 μM Ionomycin (Iono) or DMSO as control for 1 h at 37 °C. Cells were lysed and proteins in the supernatant precipitated. Proteolysis of IL-11R variants was analyzed via Western blots. GAPDH was visualized as loading control. **(I)** HEK293 cells were transiently co-transfected with expression plasmids encoding the different IL-11R variants in combination with either RHBDL2 or mCherry as a control. Cells were lysed and proteins in the supernatant precipitated. sIL-11R in the supernatants and IL-11R, RHBDL2 and actinin in the cell lysates were analyzed via Western blot. All panels show representative Western blots of three independent experiments with similar outcome.

Western blot (Fig. S4C, D). In line with our other data, we also detected IL-11R-WT, IL-11R-V15M, IL-11R-A370V and IL-11R-R395W at the cell surface of transiently transfected HeLa cells by confocal microscopy, as visualized by co-staining of the plasma membrane with wheat germ agglutinin (WGA) in non-permeabilized cells (Fig. S4E, upper panels). In contrast, both IL-11R-G231D and IL-11R-R261H were barely detectable at the plasma membrane, and IL-11R-R296W was completely absent (Fig. S4E, upper panels). All variants were well stained in permeabilized cells, ensuring that the differences were not due to differences in transfection or expression (Fig. S4E, lower panels). In summary, our data suggests that G231D and R261H do not completely prevent IL-11R maturation, but rather allow a small percentage of the IL-11R to reach the cell surface.

Having shown that the five IL-11R variants are present at the cell surface in different amounts, we next sought to determine whether the IL-11R variants were biologically active and able to transmit IL-11-induced signals into the cells. For this, we stimulated the different Ba/F3-gp130 cell lines with 10 ng/ml recombinant IL-11 for 15—120 min and monitored STAT3 phosphorylation via Western blot. Stimulation of Ba/F3-gp130-IL-11R-WT cells with IL-11 resulted in robust phosphorylation of STAT3, peaking at 60 min and declining afterwards (Fig. 1E). The same treatment did not induce STAT3 phosphorylation in Ba/F3-gp130-IL-11R-R296W cells (Fig. S5A).[4] Surprisingly, we observed the same pSTAT3 kinetic in all other cell lines despite the fact that the amount of IL-11R at the cell surface differed (Fig. 1F, G; Fig. S5B—D). We observed the same for IL-11-dependent proliferation of stably transduced Ba/F3-gp130 cell lines (Fig. S6A—G). We concluded from these experiments that all IL-11R variants are biologically active and able to activate intracellular signaling, albeit their different amounts at the cell surface.

The IL-11R can be cleaved by different proteases, e.g. the metalloprotease ADAM10 and the rhomboid protease RHBDL2. In order to investigate whether the mutations affect sIL-11R generation, we activated ADAM10 in transiently transfected HEK293 cells expressing the different IL-11R variants. IL-11R-WT was efficiently cleaved by ADAM10, and IL-11R-R296W was not shed by ADAM10 due to its intracellular retention as shown previously (Fig. 1H).[4] IL-11R-V15M was also efficiently cleaved by ADAM10, but both IL-11R-G231D and IL-11R-R261H were not, which is probably due to the low amount of IL-11R protein at the cell surface. While we could detect small amounts of sIL-11R-A370V, IL-11R-R395W was also not cleaved (Fig. 1H), despite sufficient detection at the cell surface (Fig. 1C, D).

By transient co-expression of RHDBL2 with the IL-11R variants, we observed the occurrence of IL-11R fragments with lower molecular weights in the cell lysate concomitantly with sIL-11R in the cell supernatant for IL-11R-WT, IL-11R-V15M, IL-11R-G231D, IL-11R-R395W, and to a lesser extent for IL-11R-R261H, indicative of IL-11R cleavage by RHBDL2. As shown previously, IL-11R-A370V was resistant to cleavage by RHBDL2, and IL-11R-R296W was cleaved, but no sIL-11R was released (Fig. 1I).[5] Analysis of IL-11 trans-signaling revealed robust STAT3 phosphorylation by sIL-11R-WT, sIL-11R-V15M and sIL-11R-R395W in complex with IL-11, which was reduced for sIL-11R-G231D and sIL-11R-R261H, showing that in addition to the membrane-bound receptors, also their soluble variants were biologically active (Fig. S7A, B).

In summary, our study highlights that not all coding mutations in the *IL11RA* gene would result in a loss-of-function of the IL-11R and a phenotype of the affected individuals. We find that the cell-surface amounts of IL-11R-G231D and IL-11R-R261H are reduced, but are not able to detect any abnormalities in terms of IL-11-dependent signaling. This implies that even small amounts of IL-11R at the cell surface are sufficient for proper IL-11 signaling and might further explain why no phenotype has been reported for individuals with heterozygous *IL11RA* mutations,[3] as the amount of functional IL-11R protein derived from the non-mutated allele is obviously sufficient for proper IL-11 signaling.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author contributions

B. Kespohl performed the experiments and analyzed results. Y. Garbers helped with and advised the statistical analyses. R. Hartig performed the microcopy experiments together with B. Kespohl. J. Lokau contributed to data analysis and writing of the manuscript. C. Garbers conceived and coordinated the study and wrote the paper. All authors approved the final version of the manuscript.

Conflict of interests

C. Garbers has received a research grant from Corvidia Therapeutics (Waltham, MA, USA) and has acted as a speaker/consultant for AbbVie and Novo Nordisk. All other authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.05.001.

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