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Novel activating mutations lacking cysteine in type I cytokine receptors in acute lymphoblastic leukemia

Running Title Non-cysteine cytokine receptors mutations in ALL

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Key Points

- Two distinct regions of transmembrane somatic mutations in type I cytokine receptors IL7R and CRLF2 in acute lymphoblastic leukemias.
- Non-cysteine transmembrane mutations cause functional receptor dimerization and activation transforming pro-B cells.

Keywords: Acute lymphoblastic leukemia, interleukin-7 receptor, CRLF2

Abstract

Gain-of-function somatic mutations introducing cysteines to either the extracellular or to the transmembrane domain (TMD) in Interleukin-7 receptor α (IL7R) or cytokine receptor-like factor 2 (CRLF2) have been described in acute lymphoblastic leukemias (ALL). Here we report non-cysteine in-frame mutations in IL7R and CRLF2 located in a region of the TMD closer to the cytosolic domain. Biochemical and functional assays revealed that these are activating mutations conferring cytokine-independent growth of progenitor lymphoid cells *in-vitro* and are transforming *in-vivo*. Protein fragment complementation assays suggest that despite the absence of cysteines, the mechanism of activation is through ligand independent dimerization. Mutagenesis experiments and ConSurf calculations suggest that the mutations stabilize the homo-dimeric conformation positioning the cytosolic kinases in predefined orientation to each other, thereby inducing spontaneous receptor activation, independently of external signals. Hence, type I cytokine receptors may be activated in leukemia through two types of transmembrane somatic dimerizing mutations.

Introduction

IL7R dimerizes with CRLF2 to form the receptor for thymic stromal lymphopoietin (TSLP) and with interleukin-2 receptor gamma (IL2RG) to form the receptor for IL-7[1,2]. We and others described acquired activating mutations in acute lymphoblastic leukemias (ALL) that insert cysteines into the juxtamembrane domains of IL7R or CRLF2 causing ligand independent dimerization via disulfide bonds[3-5]. The creation of disulfide bonds is critical for the activation of the receptors since elimination of the cysteines abrogated the cytokine independent growth[4,6,7].

Here we report and analyze a novel class of non-cysteine mutations in cytokine type I receptors in ALL leading to ligand independent activation.

Study design

Patient samples.

All specimens were collected with an informed consent and approval of ethic committees[4]. Samples were anonymized for the study. The study was approved by the Israeli Health Ministry Ethic committee, approval # 920070771.

Molecular studies

Mutation detection and analysis using appropriate primers (supplemental Table S1) was performed as described[4,8] **.** The human IL7R cloned into the MSCV-IRES-GFP and the human CRLF2 cloned in to pMX-Puro retroviral vectors were used as templates for the generation of mutations by site-directed mutagenesis (QuikChange, Stratagene).

BaF3 cells were transduced with retroviruses and sorted by flow cytometery (FACSAria, BD) 2–4 days later using appropriate antibodies. BaF3 growth assays and immunoprecipitation and Western analyses were performed as described before[9].

Protein fragment complementation assay

Mutated and wild type cDNAs of human IL7R, CRLF2 and IL2RG were inserted in pcDNA3.1/Zeo vector upstream of either the hGluc1 or hGluc2 fragments of Gaussia princeps luciferase[10] and co-transfected into HEK293 cells (supplemental methods). Signal intensities were read on Infinite 200 reader (TECAN).

***In-vivo* experiments**

Six weeks old female Balb/c mice were injected intravenously with $1*10^6$ BaF3 cells transduced with either WT or mutated CRLF2 or IL7R. Survival of the mice was monitored, and tissues from the sick mice were subjected to flow cytometry analysis (Galios, Coulter Inc.)

Results and discussion

During our previously reported screens of childhood ALLs[4,8] we identified mutations in IL7R and in CRLF2 that did not introduce cysteines (Figure 1A). All mutations were heterozygous, somatic, and the mutated mRNA was expressed (Figure 1B). Examination of published data revealed additional similar mutations in IL7R (supplemental Table S2). These mutations cluster within a transmembranous region internal to the area afflicted by the cysteine mutations (Figure 1A, supplemental Figure S1). To test if the somatic mutations in IL7R and CRLF2 are gain-of-function mutations, BaF3 lines expressing either wild type (WT) or mutated IL7R or CRLF2

were created. All proteins were expressed at the cell membrane (supplemental Figure S2). Mutated IL7R^{insEKV}, IL7R^{V253G} and CRLF2$^{ins\ EIM}$, enabled cytokine independent growth of BaF3 cells. CRLF2^{insEIM} required the formation of the TSLP receptor by co-expression of IL7R^{WT} (Figure 1C,D). One construct IL7R^{insGEA} did not transform BaF3 (Figure 1C). The transforming activity was confirmed in-vivo in syngeneic Balb/c mice that developed fatal leukemia after intravenous injections of BaF3 cells expressing IL7R^{insEKV}, IL7R^{V253G} or CRLF2^{insEIM}IL7R^{wt} (Figure 1E), manifested by infiltration of the blood (Figure 1F) bone marrow and spleen (Figure 1H,I) with the transformed cells. Similarly subcutaneous injection caused lymphoma (Figure 1G). Thus non-cysteine mutational activation of IL7R and CRLF2 is leukemogenic.

Biochemical analysis was consistent with the growth assays. Stat5 (Figure 2A), Jak1, Jak2, and Stat3 (Figure 2B) were phosphorylated in the absence of cytokine in BaF3 cells transduced with the IL7R^{insPPCL} (positive control that constitutively activates the JAK-STAT pathway)[4], IL7R^{V253G} and IL7R^{insEKV} but not in cells expressing IL7R^{WT} or IL7R^{insGEA}. In IL7R^{insEKV} we found higher expression of Jak2 which correlated with the higher activation capacity of this mutant in BaF3 cytokine withdrawal assays. Similarly, co-expression of IL7R^{WT} with CRLF2^{insEIM} but not the expression of CRLF2 ^{insEIM} by itself, caused constitutive phosphorylation of Stat5 and Jak2 (Figure 2C,D) consistent with the known binding of Jak2 to CRLF2[11] .

To assess if the functional and biochemical evidence of receptor activation is associated with the oligomerization of IL7R and CRLF2 mutated proteins in cell membranes, we used the luciferase protein fragment complementation assay[10] (supplemental Figure S3A). HEK293 cells were transduced with homodimeric or

heterodimeric combinations of IL7R and CRLF2 fused to hGluc1 or hGluc2 (supplemental Figure S3B). After transduction, luciferase expression and receptor expression was analyzed in live cells (supplemental Figure S4). Dimerization was calculated by dividing luminescence by the mean fluorescence intensity of each receptor thereby enabling normalization of the luminescence signal for experimental variability due to transfection efficiency.

Consistent with the requirement of complementation for luciferase expression, none of the single receptor hGluc1 or hGluc2 constructs individually transfected in HEK293 cells generated a signal (supplemental Figure S3C). Yet when the $IL7R^{WT}$ homodimer or heterodimer were expressed, a signal was obtained reflecting a basal level of receptor dimerization, as recently reported[12]. We then analyzed the influence of the somatic mutations of IL7R and CRLF2 on receptor dimerization. As shown in figure 2E, $IL7R^{ins\ EKV}$, $IL7R^{V253G}$ and $CRLF2^{insEIM}$ increased receptors dimerization ($P<0.05$) while the $IL7R^{insGEA}$ mutant that failed to provide cytokine independent survival of BaF3 cells, or to cause Stat5 phosphorylation, also did not increase receptor dimerization (Figure 2E). Thinking that it may be a "passenger" mutation we looked for additional activating mutations but found none (supplemental Table 3). As $CRLF2^{insEIM}$ alone increased receptor dimerization but did not induce downstream constitutive signaling or transformed BaF3 cells we concluded that there is no absolute correlation between dimerization and activation.

We next set to decipher the amino acids that are important for the highly activating $IL7R^{insEKV}$ mutation. We designed six variants of the EKV mutation – AKV, EAV, EKA, EK, KV (including Del^{AL} like the natural mutation) and ins V (Figure 2F). BaF3 lines expressing each variant were created (supplemental Figure S5). The

variants IL7R^{insEAV} and IL7R^{insEKA} enabled cytokine independent growth of BaF3 cells, while IL7R^{insAKV}, IL7R^{insEK} IL7R^{insKV} and IL7R^{insV} did not transform BaF3 (Figure 2G). Biochemical analysis was consistent with the growth assays. Stat5 was phosphorylated in the absence of cytokine in BaF3 cells transduced with the IL7R^{insEAV} and IL7R^{insEKA} but much less in cells expressing IL7R^{insAKV}, IL7R^{insEK}, IL7R^{insKV} and IL7R^{insV} (Figure 2H).

ConSurf[13] calculations with IL7R showed that the TMD segment manifests unique evolutionary conservation pattern with i/i+4 periodicity, equivalent to that of a perfect alpha-helix (supplemental Figure S6). The conserved positions would reside in the same helix face, providing an interface for IL7R homo-dimerization. All the natural and experimental mutations can be interpreted assuming that activation requires IL7R dimerization with the cytosolic kinases in predefined orientation with respect to each other. The orientation is marked by the conserved face of the TMD helix in wild type IL7R. Dimerization along a different TMD helix face would not activate because the kinases will not be facing each other. Molecular detailed interpretations of mutations effect are provided in supplemental Figures S7,S8 and Table S4.

Taken together these data indicate that the functional and biochemical evidence for receptor activation by mutations lacking cysteine correlates with ligand independent dimerization. There are several examples of transmembrane domain mutations in receptors that cause constitutive activation without introducing cysteine; V664E in the Neu receptor tyrosine kinase [14-16] and the dimerization inducing mutations in MPL[17,18] that was identified in rare familial thrombocytosis patients [19]. Our observations of non-cysteine transmembrane mutations in type I cytokine receptors in patients with ALL, demonstrate for the first time that such somatic mutations activate these receptors through ligand independent dimerization and are leukemogenic.

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Authorship

S. Izraeli designed the study and wrote the paper, C. Shochat performed research analyzed data and wrote the paper, N. Tal, N. Gershman, Y. Birger, N. Ben-Tal performed research and analyzed data, V. Gryshkova, S.N. Constantinescu, M.R Mansour, J.C Twizere and D. Bercovich provided reagents and scientific expertise, O.R. Bandapalli, G. Cazzaniga, A.E. Kulozik, A. Biondi, M.U. Muckenthaler provided reagents and data.

Conflict of Interest Disclosure

The authors have no conflicts of interest to disclose.

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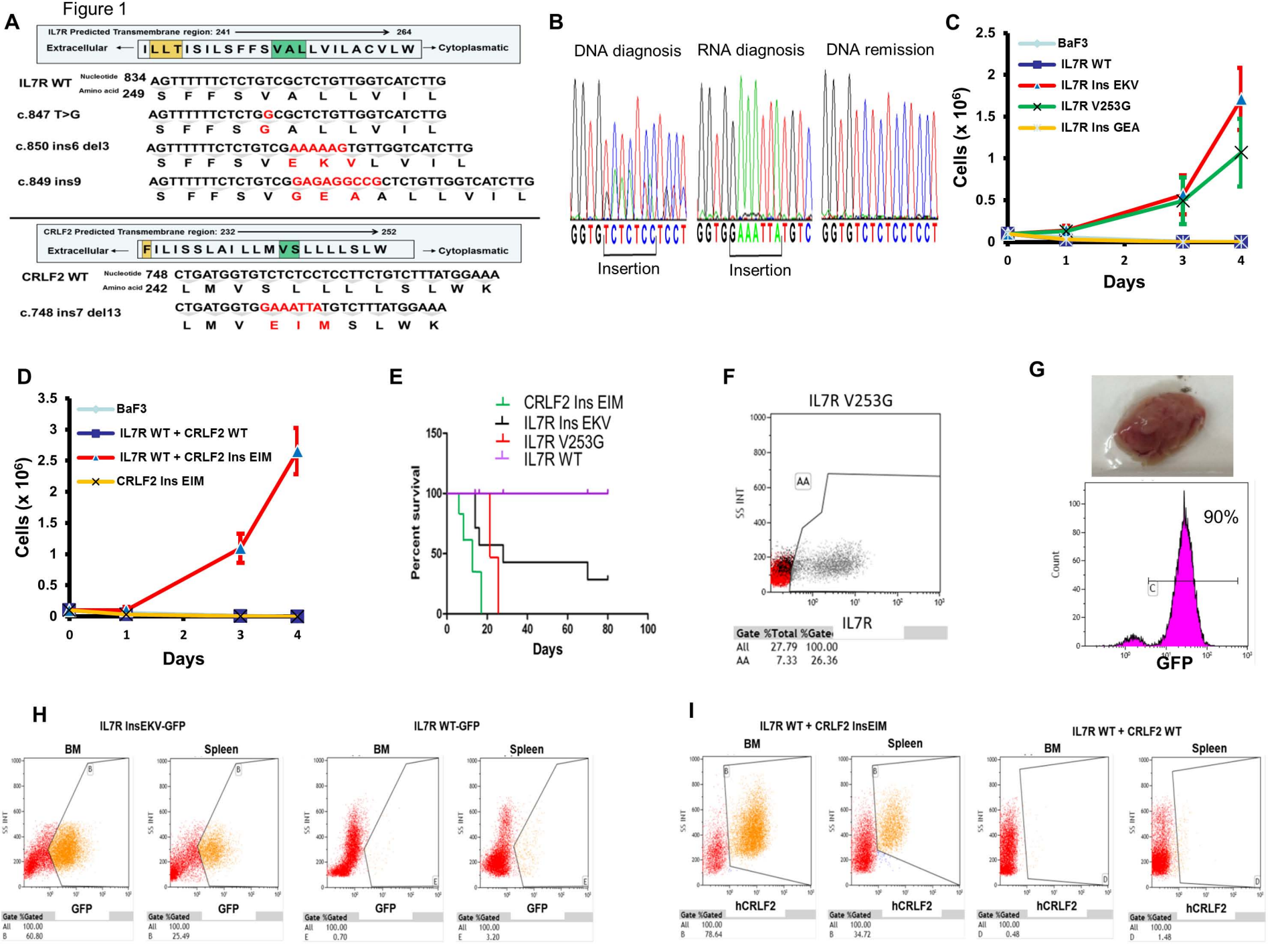
Figure Legends

Figure 1. Non-cysteine mutations in IL7R and CRLF2 are transforming leukemogenic mutations (A) Predicted transmembrane domain (TMD) of IL7R and CRLF2 with location of cysteine mutations (orange box) and non-cysteine mutations (green box) and alignment of wild-type and mutated TMD sequences. Numbers show the positions of nucleotides and corresponding amino acids. The inserted nucleotides and amino acids are shown in red. WT= Wild type, Ins = insertion, Del = deletion. **(B)** Expression of $CRLF2^{InsEIM}$ mutation. The mutated allele is expressed in the RNA from diagnosis while only the normal allele is found in remission DNA sample because CRLF2 is expressed only in leukemic B-cell due to a chromosomal rearrangement[8]. **(C, D)** Cytokine withdrawal assay of BaF3 cells transduced with mutated or wild type IL7R or CRLF2. **(E)** Overall survival of mice (seven mice in each group, 3 experiments) injected intravenously with $1\text{-}2*10^6$ BaF3 cells expressing IL7R, WT or mutant, or $IL7R^{WT}$ and $CRLF2^{insEIM}$ compared by Kaplan–Meier analysis $p \leq 0.01$. (F) Representative flow cytometric analysis of blood from mice injected intravenously with $1\text{-}2*10^6$ BaF3 cells expressing $IL7R^{V253G}$. (G) Lymphoma tumor (top) observed in a mouse injected subcutaneously with $1\text{-}2*10^6$ BaF3 cells expressing $IL7R^{Ins\ EKV}$-GFP. Representative GFP expression from lymphoma cells (bottom). (H) Representative analysis of cells from spleen and BM from mice injected intravenously with $1\text{-}2*10^6$ BaF3 cells expressing $IL7R^{Ins\ EKV}$ and sacrificed at day 14. (I) Representative analysis of cells from spleen and BM from mice injected intravenously with $1\text{-}2*10^6$ BaF3 cells co-expressing the $IL7R^{WT}$ and $CRLF2^{WT}$ or $CRLF2^{insEIM}$ mutant, and sacrificed at day 16.

Figure 2. Non-cysteine mutations in IL7R and CRLF2 cause constitutive activation of the JAK-STAT pathway and increase receptor dimerization. **(A)** Constitutive phosphorylation of Stat5 in BaF3 cells expressing IL7R mutants after 5 hours of cytokine deprivation. One mutant with insertion of cysteine (Ins PPCL) was used as a positive control. IL-3+ indicates cells harvested after 5 hours of IL-3 deprivation followed by 20 minutes of IL-3 stimulation. **(B)** Identification of constitutive phosphorylation of Jak1 Jak2 and Stat3 in BaF3 cells expressing IL7R mutants after 5 hours of cytokine deprivation. IL-3+ indicates cells harvested after 5 hours of IL-3 deprivation followed by 20 minutes of IL-3 stimulation. Cells were subjected to lysis and immunoprecipitation with anti p-Tyr antibody (sc-508, Santa Cruz). The presence of Jak1, Jak2, and Stat3 was visualized by Western blotting with anti-Jak1/Jak2/Stat3 antibodies. **(C)** Constitutive phosphorylation of Stat5 in BaF3 cells expressing CRLF2 mutant after 5 hours of cytokine deprivation. IL-3+ indicates cells harvested after 5 hours of IL-3 deprivation followed by 20 minutes of IL-3 stimulation. **(D)** Identification of constitutive phosphorylation of Jak2 and Stat5 in BaF3 cells expressing CRLF2 mutants after 5 hours of cytokine deprivation. IL-3+ indicates cells harvested after 5 hours of IL-3 deprivation followed by 20 minutes of IL-3 stimulation. Cells were subjected to lysis and immunoprecipitation with anti p-Tyr antibody (sc-508, Santa Cruz). The presence of, Jak2, and Stat5 was visualized by Western blotting with anti- Jak2 or Stat5 antibodies. (E) Relative dimerization level of IL7R and CRLF2 from HEK293 cells transiently transfected with the WT or mutant receptor. Dimerization was calculated by dividing luminescence by the mean fluorescence intensity of each treatment, normalizing the luminescence

signal for experimental variability due to transfection efficiency.* $P < 0.01$, one-way ANOVA and Students T-test. (F) Alignment of wild-type, natural mutant (EKV) and six experimental mutants (numbered 1-6) in the TMD of IL7R. Numbers show the positions of nucleotides and corresponding amino acids. The inserted nucleotides and amino acids are shown in red. The deleted AL amino acids are shown in green. WT= Wild type, Ins = Insertion, Del = Deletion. (G) Cytokine withdrawal assay of BaF3 cells transduced with IL7R experimental mutants. (H) Constitutive phosphorylation of Stat5 in BaF3 cells expressing IL7R experimental mutants after 5 hours of cytokine deprivation. IL-3+ indicates cells harvested after 5 hours of IL-3 deprivation followed by 20 minutes of IL-3 stimulation.

Figure 1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IL7R V253G
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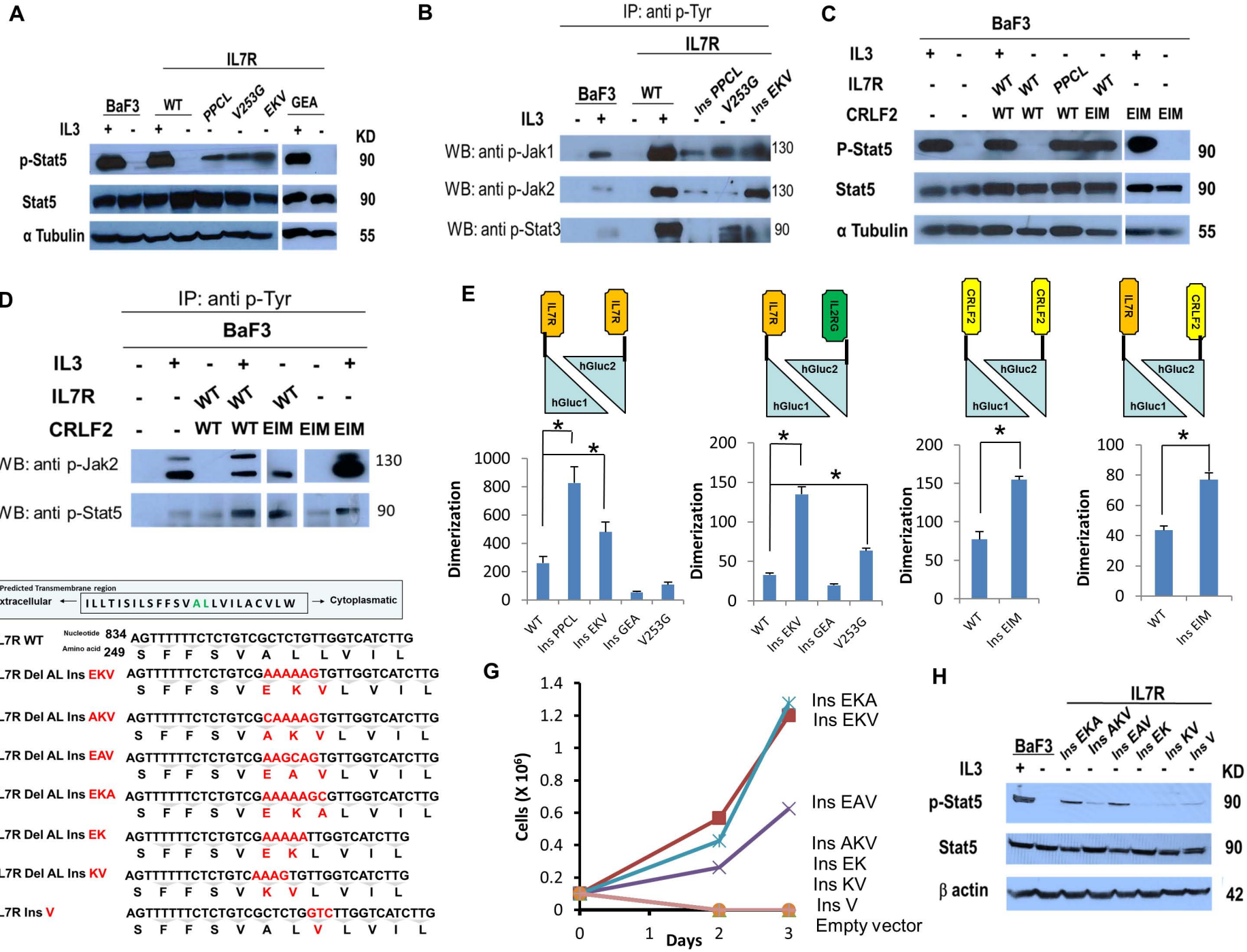
Figure 2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IL7R Predicted Transmembrane region
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