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Mutations in the Transmembrane and Juxtamembrane Domains Enhance IL27R Transforming Activity

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Synopsis

Cytokines and their receptors regulate hematopoiesis by controlling cellular growth, survival, and differentiation. Thus, it is not surprising that mutations of cytokine receptors contribute to the formation of hematopoietic disorders including cancer. We recently identified transforming properties of the ligand-binding component of the receptor for interleukin-27 (IL27R). While wildtype IL27R exhibits transforming properties in hematopoietic cells, in this study we set out to determine if the transforming activity of IL27R could be enhanced by mutation. We identified three mutations of IL27R that enhance its transforming activity. One of these mutations is a phenylalanine to cysteine mutation at residue 523 (F523C) in the transmembrane domain of the receptor. The two other mutations identified involve deletions of amino acids in the cytoplasmic juxtamembrane region of the receptor. Expression of each of these mutant IL27R proteins led to rapid cytokine independent transformation in hematopoietic cells. Moreover, the rate of transformation induced by these mutants was significantly greater than that induced by wildtype IL27R. Expression of these IL27R mutants also induced enhanced activation of JAK/STAT signaling compared to wildtype. An activating deletion mutation of IL27R enhanced homodimerization of the receptor, by a mechanism that may involve disulfide bonding. These transforming IL27R mutants displayed equal or greater transforming activity than bona fide hematopoietic oncogenes such as BCR-ABL and JAK2-V617F. Since IL27R is expressed on hematopoietic stem cells, lymphoid cells, and myeloid cells, including AML blast cells, mutation of this receptor has the potential to contribute to a variety of hematopoietic neoplasms.

Keywords

cytokine receptor; transformation; JAK/STAT; IL27R; mutagenesis

Introduction

Mutational activation of cytokine receptors occurs in various hematopoietic malignancies. Activating mutations of Flt3 [1–5] and c-Kit [1–3, 6] receptor tyrosine kinases, for example, are present in acute myeloid leukemia (AML) and can contribute to the formation of hematopoietic disease in mouse models [7–10]. However, mutation of a tyrosine kinase receptor is not the only way cytokine receptors can become activated. Cytokine receptors that have no intrinsic kinase activity are also found mutated and overexpressed in

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Author Contribution
Que T. Lambert, Anuradha Pradhan, and J. Devon Roll designed and performed experiments. Gary W. Reuther designed experiments, supervised the studies, and wrote the manuscript.

hematopoietic disease and are believed to contribute to disease formation. For example, erythropoietin receptor (EpoR) mutations play a role in familial erythrocytosis/polycythemia [11–15] and granulocyte colony-stimulating factor (G-CSF) receptor (G-CSFR) mutations are found in AML as well as chronic neutrophilia [16–18]. Mutations of the thrombopoietin receptor, TpoR (Mpl), are found in myeloproliferative neoplasms (MPNs), including essential thrombocythemia and myelofibrosis, and mutated Mpl can signal to induce similar disease phenotypes in animal models [19, 20]. Also, overexpression and mutation of cytokine-like receptor factor 2 (CRLF2) plays a functional role in acute lymphoblastic leukemia (ALL) [21, 22]. Thus, mutation and overexpression of cytokine receptors can lead to deregulation of cellular growth and contribute to neoplastic disease.

We have identified potential oncogenes in AML patients through functional genetic screening in hematopoietic cells [23, 24]. In such screens we identified a deletion mutation of TrkA as well as a guanine nucleotide exchange factor for Ras, RasGRP4 [24]. Recent work has further established a potential role for these genes in leukemogenesis, thus justifying our approach to identify novel molecular players in leukemia [25–27]. Through functional screening approaches in hematopoietic cells we recently identified novel transforming properties of interleukin-27 receptor alpha, IL27Ra (IL27R), using cDNA derived from a patient with AML [28]. We demonstrated that IL27R is expressed on the cell surface of the leukemic cells of AML patients. In addition, we determined that this receptor component could functionally support the activation of MPN-associated JAK2 proteins, which retain a requirement for interactions with a receptor for ligand-independent activation. In this context IL27R is capable of functionally replacing homodimeric receptors, such as EpoR or TpoR (Mpl), presumably by providing a scaffolding function to facilitate activation of such JAK2 proteins [29]. Thus, aberrant regulation of this cytokine receptor may affect the growth of cells in hematopoietic disease.

IL27R functions with gp130 to form the functional receptor for IL-27 [30]. IL-27 functions to regulate the activity of various cells of the immune system and has been most extensively studied in the context of regulating T-cell function [31–33]. Signaling by IL27R in this regard is mediated by activation of JAK family kinases as well as downstream STAT proteins, most notably STAT1 [34, 35]. It was recently demonstrated that IL27R is expressed on the cell surface of hematopoietic stem cells and aberrant expression of IL-27 in a transgenic mouse model induced myelopoiesis [36]. Similarly, IL-27 stimulates the proliferation of human CD34+ cells [36]. Thus, the possibility that aberrant regulation of IL27R may be capable of contributing to the formation of hematopoietic, perhaps myeloid, neoplastic disease is supported by: 1. the ability of IL27R to induce transformation of numerous hematopoietic cell lines [28]; 2. the expression of IL27R on hematopoietic stem cells and AML blast cells [28, 36]; 3. the ability of IL-27 to induce myelopoiesis and CD34+ cell proliferation [36]; and 4. the ability of IL27R to function as an activator of JAK2 proteins containing MPN-associated mutations [29]. As IL27R signals through the JAK/STAT pathway, the well-documented role of aberrant activation of JAK/STAT signaling in malignant hematopoietic disease also supports the notion that aberrant IL27R activation has the potential to contribute to deregulated hematopoietic cell growth.

We have previously demonstrated that aberrant expression of wildtype IL27R can weakly induce transformation of hematopoietic cells, and Seita et al. have shown that aberrant IL-27 signaling can induce CD34+ cell proliferation and myelopoiesis [28, 36]. Thus, we set out to determine if the cellular transforming activity of IL27R could be enhanced by mutation. To this end, we utilized a functional screening approach and identified three mutations of IL27R that enhance its transforming activity in hematopoietic cells. These mutations include a point mutation in the transmembrane region, as well as deletion mutations in the internal juxtamembrane region of the receptor. Each of these mutations generates an IL27R protein

that is highly transforming when expressed in hematopoietic cells. This activity correlates with enhanced activation of JAK/STAT signaling. Increased homodimerization of IL27Ra may play a role in the enhanced activity of some of these mutants. Surprisingly, the ability of IL27R mutants to transform cells was in some contexts, similar to that of BCR-ABL, the highly transforming protein that causes chronic myeloid leukemia. Our data suggest that mutation of IL27R, or perhaps other equally overlooked cytokine receptors, including components of heterodimer receptor complexes (e.g. as shown for CRLF2), may contribute to aberrant hematopoietic growth regulation.

Experimental

Cell culture, retrovirus production, and retroviral infection

293T cells were grown in DMEM containing 10% FBS and penicillin/streptomycin. 32D and BaF3 were cells grown in RPMI containing 10% FBS (RPMI/10% FBS), penicillin/streptomycin, and 5% WEHI-3B-conditioned media as a source of IL-3. 293T cells were transfected using standard calcium phosphate precipitation. Retrovirus was generated and retroviral infections using pEYK3.1 and pBabepuro vectors were performed as previously described [29]. Stable mass population cell lines were generated by selecting pBabepuro virus-infected cells with puromycin (1.0 ug/mL) forty-eight hours after infection.

Plasmids and cell lines

The following expression plasmids were utilized in this study and were previously described: pBabepuro, pBabepuro-IL27R, pEYK3.1, and pEYK3.1-IL27R [28, 37]. pBabepuro-IL27R-F523C and pBabepuro-IL27R-Δ540–552 were generated by directly cloning the mutated cDNAs from pEYK3.1, isolated from the mutagenesis screen, into pBabepuro. Myc- and HA-tagged cDNAs were generated by PCR using PrimeSTAR DNA polymerase (Takara Bio, Inc.). A full-length human CRLF2 cDNA was amplified from first-strand cDNA from Daudi Burkitt's lymphoma cells using PrimeSTAR DNA polymerase (Takara Bio, Inc.) and cloned into pBabepuro. pBabepuro-CRLF2 and pBabepuro-CRLF2-F232C were utilized to generate retrovirus to generate BaF3 cells expressing CRLF2 proteins. BaF3 cells expressing JAK2-V617F were previously described [29]. BaF3 cells expressing BCR-ABL were generated by retroviral infection with virus made from pSRαMSVtkneo-BCR-ABL plasmid [38]. The integrity of the complete cDNA for all PCR-cloned cDNAs was confirmed by DNA sequencing.

Random and site-directed mutagenesis

Random mutagenesis of IL27R was performed essentially as previously described [39]. A full-length human IL27R cDNA in the pEYK3.1 retroviral vector was transformed into XL-1 Red bacteria (Agilent Technologies) per manufacturer's instructions. Plasmid DNA was isolated and used to generate retrovirus to screen for transforming IL27R mutations in 32D cells. IL27R mutations identified in the pEYK3.1 plasmid from the screen were transferred into pBabepuro. pBabepuro-IL27R-F532A, pBabepuro-IL27R-F532S, pBabepuro-CRLF2-F232C, pBabepuro-IL12RB1-F549C, and pBabepuro-IL12RB1-F550C were generated by site directed mutagenesis using PrimeSTAR DNA polymerase (Takara Bio, Inc.), and the integrity of the complete cDNAs were confirmed by DNA sequencing.

Screen for IL27R transforming mutants

Two days after infection, 32D cells were washed twice with RPMI/10% FBS and plated in 96-well plates at a density of approximately 2×10^5 per mL. Plates containing cells infected with randomly mutated IL27R cDNAs were compared to control plates containing cells expressing non-mutated IL27R-WT. Wells from the mutagenesis screens that demonstrated cytokine independent growth more rapidly than any wells in the IL27R-WT plate were

identified and the cells of these wells were expanded. Genomic DNA was isolated from these cells and proviral pEYK3.1-IL27R plasmid was recovered essentially as described [37]. This isolated proviral plasmid DNA was then used to generate retrovirus, which was utilized to infect 32D cells to confirm cytokine independent transformation. The entire cDNA for each rescued transforming IL27R cDNA clone was sequenced in order to identify mutations.

Cytokine-independent growth assays

Cytokine-independent growth was assayed by plating stable 32D or BaF3 cell lines in the absence of IL-3 and counting viable cells over time. Cells were washed twice with RPMI/10% FBS and plated at 2×10^5 per mL. Trypan blue exclusion was utilized to determine viable cell numbers on the indicated days following plating in the absence of IL-3.

Immunoblot analysis

Cell lysis and immunoblotting were performed as previously described [29]. Non-reducing SDS-PAGE analyses were performed utilizing protein electrophoresis sample buffer that lacked a reducing agent. Primary antibodies used for immunoblotting were: IL27Ra (TCCR) (T5823) (Sigma-Aldrich), phospho-(p) STAT1 (Tyr 701) (9171), pSTAT3 (Tyr 705) (9138), pERK (Thr 202/Tyr 204) (4370), JAK1 (3344), JAK2 (3230), myc tag (2276) (Cell Signaling), pJAK1 (Tyr 1022/Tyr 1023) (44–422G) (Invitrogen), pSTAT5 (Tyr 694) (611964) (BD Biosciences), pJAK2 (sc-16566-R), STAT1 (sc-346), STAT3 (sc-483), STAT5 (sc-835), ERK1 (sc-93), IL12RB1 (sc-658) (Santa Cruz Biotechnology), HA (Covance), and CRLF2 (thymic stromal lymphopoietin receptor) (14549980) (eBioscience). Immunoprecipitations were performed with anti-HA antibodies (MMS-101R) (Covance) and collected with Protein-G agarose (Thermo Scientific). Horse-radish peroxidase-conjugated secondary antibodies for immunoblots were from Thermo Scientific. Blots were developed using standard or West Pico chemiluminescence (Thermo Scientific).

Flow Cytometry

Anti-human IL27R (TCCR/WSX-1) antibody (R&D Systems) was conjugated to Alexa Fluor® 647 using an Alexa Fluor® 647 antibody labeling kit (Molecular Probes). 32D cells were stained with this IL27R-Alexa Fluor® 647 antibody as well as isotype control antibodies conjugated to Alexa Fluor® 647 (Santa Cruz Biotechnology) along with propidium iodide. Cells were analyzed on a BD FACSCalibur™ for Alexa Fluor® 647 fluorescence on live cells.

Results

Identification of mutations that enhance the transforming activity of IL27R

Our identification of IL27R transforming activity was a novel and surprising finding, as this receptor lacks intrinsic kinase activity and generally relies on heterodimeric receptor signaling with gp130 [28]. However this transforming activity of IL27R, which is independent of IL-27 and gp130, is rather weak compared to that observed with other oncogenes when expressed in similar cells. While this is not surprising since IL27R lacks kinase activity, we were interested in determining if there were mutations of IL27R that could enhance the transforming activity of this receptor. To this end, we used an approach that couples random mutagenesis with functional screening that we have recently used to identify novel JAK1 mutations [39]. The cDNA for IL27R was cloned into a retroviral vector and propagated through a mutagenic bacterial strain in order to induce random mutations in the DNA. Mutated plasmid DNA was isolated and utilized to generate retrovirus that was used to infect 32D cells, an IL-3 dependent myeloid cell line. 32D cells

were also infected with retrovirus encoding wildtype IL27R in order to ascertain the rate of transformation of the unmutated receptor, and thus help identify potential mutations that induce transformed cell growth at a greater rate than wildtype IL27R. Infected cultures expressing wildtype or mutated versions of IL27R were washed of IL-3 and plated in 96-well dishes. Cell growth in each well was monitored and cells that grew out before any wells containing wildtype IL27R-expressing cells grew out were expanded. Retroviral plasmid DNA was recovered from transformants and re-introduced back into IL-3-dependent 32D cells. These cells were plated in the absence of IL-3 in order to confirm that the recovered plasmids contained IL27R cDNAs that induce rapid transformation of cells, presumably due to mutation of the IL27R cDNA. Following plasmid recovery, IL27R cDNAs were sequenced and three mutations that enhance the transforming activity of IL27R were identified. These mutations include a point mutation of phenylalanine-523 to cysteine (F523C), and two similar deletion mutations; one deleting nine amino acids from residues 539 to 547 and the other removing thirteen amino acids from 540 to 552 (Figure 1). The F523C mutation is in the IL27R transmembrane domain while the deletion mutations are located in the juxtamembrane region on the cytoplasmic side of the transmembrane domain.

Alteration of the transmembrane domain and the juxtamembrane domain enhance the transforming activity of IL27R

In order to directly compare the transforming properties of wildtype IL27R to the mutant IL27R sequences identified in our screen, we generated stable mass population cell lines expressing control vector, IL27R-WT, IL27R-F523C (IL27R-FC), and IL27R-Δ540–552 (IL27R-Δ). Two cytokine-dependent hematopoietic cell lines were utilized in these studies: 32D cells (the cell line we used in our screen), and BaF3 cells, which are a progenitor B-cell line that also requires IL-3 for growth and viability. Following stable selection of cells by puromycin resistance, cells were washed of cytokine and plated in cytokine-free medium. As expected IL27R-WT induced cytokine independent transformation of these cells in a relatively slow time course. Transformation by IL27R-WT resulted in a 100-fold increase in cell number in approximately two weeks in both 32D and BaF3 cells (Figure 2). In stark contrast, the IL27R mutant containing the F523C mutation (IL27R-FC) induced rapid transformation of both 32D and Baf3 cells, with only 3–4 days required to obtain about a 100-fold increase in viable cells (Figure 2). Similar results were obtained with IL27R-Δ, although its rate of transformation in BaF3 cells was slightly slower (Figure 2), but still impressively more rapid than IL27R-WT. The different rate of transformation observed was not due to differing levels of IL27R expression in these lines (see Figure 3). Finally, five out of five clonal cell lines derived from mass populations of stable 32D cells grew out with similar kinetics as the stable mass population of cells shown in Figure 2 (data not shown).

IL27R transforming mutants exhibit elevated cell signaling properties

Utilizing the stable cell lines expressing IL27R-WT, IL27R-FC, and IL27R-Δ, we then analyzed the levels of activation of various signaling pathways in the cells. We have previously demonstrated that 32D cells transformed by IL27R exhibit significant elevation of phosphorylated forms of JAK1, JAK2, STAT5, STAT1, and ERK, which represent the activated forms of these proteins [28]. When we analyzed the activation state of these proteins in 32D cells that are still dependent on IL-3, we observed a slight increase in the phosphorylated/activated forms of JAK1, JAK2, STAT5, STAT1, and ERK in cells that express IL27R-WT (Figure 3A, lane 2). This activation was significantly enhanced in cells expressing IL27R-FC or IL27R-Δ (Figure 3A, lanes 3 and 4). While STAT3 activation was not detected in IL27R-WT cells it was present in the two IL27R mutant cell lines. Activation of these signaling proteins correlated with the ability of mutated IL27R proteins to induce cytokine independent growth of these cells (Figure 2, top). Similar results were obtained in BaF3 cells expressing the various IL27R proteins (Figure 3B). In this cell type IL27R-FC

activated signaling to a greater extent than IL27R-Δ (Figure 3B, lane 3), with considerably less to undetectable activation in cells expressing IL27R-WT (Figure 3B, lane 2). Again, the relative state of activation of signaling by IL27R proteins correlated with the rate of induction of transformation of cells to cytokine independence (Figure 2, bottom). That is, cells expressing IL27R-FC exhibited the highest level of signaling and these cells became transformed to cytokine independence most rapidly. Cells expressing IL27R-Δ had less impressive activation of signaling and IL27R-WT had very little activation of the pathways analyzed, again correlating with the rate of transformation of BaF3 cells by these proteins (Figure 2). Activation of these pathways remained elevated and appeared to normalize in BaF3 cells transformed to IL-3-independence by the various forms of IL27R (Figure 3B). The low ERK activation observed in IL-3 independent IL27R-WT cells was not consistently found in multiple independently derived cell lines. Finally, cell surface expression of IL27R proteins in IL-3-dependent 32D cells was detected by flow cytometry. No impressive difference in the cell surface expression was detected between IL27R-WT and IL27R-FC (mean fluorescent intensity: IL27R-WT =17 and IL27R-FC = 22.2), but IL27R-Δ had about a 2.4 fold higher signal for cell surface expression (mean fluorescent intensity of 41.2) than IL27R-WT and a 1.9 fold higher signal than IL27R-FC. Similar results were obtained in BaF3 cells expressing IL27R proteins (data not shown).

Activating effects of mutation of IL27R F523 is cysteine specific

We next investigated if the activating effect mutation of F523 has on the transforming ability of IL27R is due to a loss of the phenylalanine at that position or due to the cysteine to which it is mutated. To test this, we mutated F523 to serine as well as alanine, amino acids whose side chains are similar in size to cysteine. Comparing the transforming ability of IL27R-WT, IL27R-F523S and IL27R-F523A demonstrated that mutation of F523 to amino acids other than cysteine did not enhance the transforming capacity of IL27R-WT (Figure 4A). In 32D cells, these mutations transformed cells marginally faster than IL27R-WT (Figure 4A, top). However, in BaF3 cells mutation of F523 to serine or alanine had a slight inhibitory effect on the rate of transformation compared to IL27R-WT (Figure 4A, bottom). IL27R proteins were expressed to similar levels in both sets of cells (Figure 4B). This suggests that the cysteine residue generated by mutation of F523 is specifically required to induce an enhanced transforming signal compared to IL27R-WT. The property of cysteine that is unique is its sulfhydryl group, which provides the potential for covalent bond formation via disulfide bonding.

The IL27R-Δ mutation enhances homodimeric IL27R receptor formation

We have previously shown that IL27R can undergo homodimerization when expressed in multiple cell types [29]. The requirement of a cysteine at amino acid 523 to enhance transforming capacity of IL27R suggested perhaps inter-molecular disulfide bond formation may lead to an increase in IL27R homodimer formation. To test this, we generated HA- and myc-tagged versions of IL27RWT, IL27R-FC, and IL27R-Δ. We co-expressed both epitope-tagged versions of IL27R proteins in 293T cells and performed immunoprecipitations using antibodies that recognize HA. Immunoblot analysis, using myc-tag antibodies, of immunoprecipitated proteins revealed that, as we have demonstrated before, IL27R-WT forms homodimeric complexes in cells (Figure 5A, lane 1). However, the F523C mutation in IL27R did not enhance the ability of IL27R to form homodimers (Figure 5A, lane 2). Surprisingly, the deletion mutation impressively enhanced the ability of IL27R protein to form homodimers, as a significant increase in the amount of IL27R-Δ-myc was observed in the HA-immunoprecipitation of IL27R-Δ-HA (Figure 5A, lane 3). Similar conclusions were drawn from anti-HA blots of anti-myc immunoprecipitations (data not shown).

To further investigate the potential contribution of disulfide bonds to homodimer formation we analyzed cell extracts expressing IL27R proteins by non-reducing SDS-PAGE. Our hypothesis was that the FC mutation of IL27R generated a new cysteine that might undergo disulfide bond formation. This would be analogous to the ALL–associated F232C mutation of CRLF2 that undergoes disulfide bond formation to form homodimeric complexes [22]. Our analyses indicated that the IL27R-FC mutation does not induce disulfide bond formation as measured by a higher molecular weight complex formation under non-reducing conditions (Figure 5B, lane 10 and 13). However, IL27R-Δ does exhibit higher molecular weight complex formation in a non-reducing gel, suggesting it is indeed undergoing enhanced disulfide bond formation compared to IL27R-WT (Figure 5B, lane 11). Upon transformation to IL-3 independence, the amount of high molecular weight IL27R-Δ protein complex in non-reducing gels was significantly enhanced (Figure 5B, lane 14). There was a high molecular weight complex of IL27R-FC in transformed cells that was barely detectable (Figure 5B, lane 13, long exposure), but this was significantly less than IL27R-Δ and cannot be readily compared to wildtype IL27R because of differences in protein expression. IL27R protein presents as a doublet in SDS-PAGE due to different levels of glycosylation (Lambert, QT and Reuther, GW, unpublished observations), which may lead to multiple high molecular weight complexes under non-reducing conditions. As a positive control for detecting disulfide bond formation of a cytokine receptor, we used the F232C mutation of CRLF2, which has been previously reported to undergo disulfide bond formation and to exhibit a shift in non-reducing SDS-PAGE [22]. Indeed CRLF2-F232C, and not wildtype CRLF2, protein exhibited a significant shift in molecular weight under non-reducing conditions, demonstrating that our assay conditions were appropriate to detect disulfide bond formation induced by mutation in a cytokine receptor (Figure 5C, lanes 3 and 4).

The transforming ability of IL27R mutants is comparable to that of known hematopoietic oncogenes

While we have previously demonstrated that IL27R-WT exhibits hematopoietic cell transforming activity, this activity is rather weak in that it often takes a significant length of time to induce a state of rapid transformed cell growth ([28], and see Figure 2). The IL27R-FC and IL27R-Δ mutations represent extremely potent activating mutations in the context of IL27R. However, we were interested in determining how this compared to other hematopoietic transforming oncogenes that activate similar signaling pathways. To this end we compared the transforming ability of IL27R proteins to the transforming ability of the activated tyrosine kinases BCR-ABL and JAK2-V617F as well as to a transforming mutant of a component of a heterodimeric cytokine receptor CRLF2, CRLF2-F232C. BCR-ABL is found in chronic myeloid leukemia and in some cases of ALL while JAK2-V617F is associated with MPNs [40, 41]. CRLF2-F232C has been found in cases of pediatric ALL [22]. We utilized BaF3 cells as this cell line has been utilized to study the transforming properties of each of these oncogenes in previous studies. As expected, the BCR-ABL oncogenic tyrosine kinase induced immediate and rapid IL-3 independent cell growth of BaF3 cells (Figure 6). The transformed cell growth induced by IL27R-FC was as immediate and as rapid as that induced by BCR-ABL. The transforming activity of IL27R-FC was greater than JAK2-V617F and CRLF2-F232C. The ability of IL27R-Δ to transform BaF3 cells was similar to that of JAK2-V617F and CRLF2-F232C. IL27R-WT was the weakest transforming gene in this assay. Thus, the transforming activity of IL27R-FC and IL27R-Δ appear as potent as bona fide hematopoietic oncogenes in this assay.

Discussion

Our identification of IL27R as a transforming gene from a patient with AML was a novel finding as this receptor had never been associated with cellular transformation [28]. IL27R

is the ligand-binding component of the heterodimeric receptor for IL-27 and partners with gp130 to transduce IL-27-mediated signaling [30]. The IL27R cytoplasmic domain contains a JAK-binding Box 1 motif and a single tyrosine phosphorylation site that can function as a STAT-binding site when phosphorylated [42, 43]. This site has been shown to be important in the activation of STAT1, a signal transducer that plays an important role in mediating the effects of IL-27 on cells [43]. We demonstrated that transformation induced by IL27R is independent of the expression of gp130, suggesting alternative receptor partners may be involved in signaling from IL27R that leads to cellular transformation [28]. In fact, we have recently shown that IL27R can undergo homodimerization, indicating that homodimerization may play a role in the observed gp130-independent induced transformation [29]. Work by Hashimoto et al., who postulated IL27R homodimers might exist, supports our finding of IL27R homodimerization [44].

More recently, Seita et al. have shown that IL27R is expressed on the surface of hematopoietic stem cells and IL-27 has been shown to enhance myelopoiesis [36]. This report also demonstrated that IL-27 enhanced the proliferation of human CD34+ cells. Thus it is possible that deregulated signaling from IL27R can contribute to aberrant hematopoietic cellular growth. Our previous studies demonstrated that expression of IL27R in cytokine dependent hematopoietic cells induced cytokinein-dependent transformed growth [28]. However, transformation of hematopoietic cells by IL27R is weak, that is, cells that stably overexpress IL27R do not readily become cytokine-dependent, as there is a lag in cell growth before a fully transformed cell line is obtained. In general, exogenous expression of wildtype cytokine receptors in hematopoietic cells does not induce transformation, suggesting there is something unique about IL27R.

Mutations in cytokine receptors are known to induce ligand-independent receptor activation. Mutations in receptors such as cKit, Flt3, Mpl, and CRLF2, can induce signaling that causes deregulation of hematopoietic cell growth and likely contributes to the diseases with which these mutations are associated [1–6, 19–22]. Given the weak transforming potential of IL27R, we were interested in determining if this transforming activity could be enhanced by mutation. In this study, we demonstrate that IL27R can indeed be activated by mutation. Through a random mutagenesis study, we identified three mutations that activate the transforming capacity of IL27R. These mutations are: a point mutation, F523C, and two similar deletion mutations, Δ539–547 and Δ540–552. The point mutation F523C is located in the transmembrane domain of IL27R and the deletion mutations delete amino acids in the intracellular juxtamembrane region of the receptor (Figure 1). Expression of these mutants in cytokine dependent hematopoietic cells induced rapid cytokine independence as well as enhanced activation of downstream signaling pathways compared to IL27R-WT (Figure 2 and 3). Of important note, 32D cells expressing IL27R-WT grew at the same rate following cytokine removal in conditioned medium, collected from day 3 (following cytokine removal) cultures of IL27R-FC and IL27R-Δ cells, as in non-conditioned medium (data not shown). This indicates there is not an obvious autocrine mechanism contributing to the rapid rate of transformation and is consistent with our previous studies that determined IL27R-WT transformed cells do not produce growth promoting factors [28]. Also, the observation that IL27RT-WT cells that are transformed to cytokine independence have a lower level of ERK activation is not a consistent finding in multiple cell lines (Figure 3). It is possible the enhanced rate of transformation of IL27R mutant cell lines is due to the elevated levels of P-STAT5, present at the time of cytokine removal, associated with expression of the IL27R mutants. Since activated STAT5 has an anti-apoptotic effect in these cells, this activation could allow enhanced cell survival to play an important role in the elevated rate of transformation compared to IL27R-WT cells (Figure 2), especially at early time points following cytokine removal. In fact, the relative rate of transformation of 32D and BaF3

IL27R-expressing cells correlated with the relative amount of P-STAT5 in these cells upon initial cytokine removal (Figures 2 and 3).

The single F523C point mutation in IL27R induces potent transforming potential in cell lines. Introduction of an aberrant cysteine could lead to inappropriate disulfide bond formation, similar to results obtained from an in vitro mutagenesis study of EpoR [45]. During the course of our work, a similar mutation was found in CRLF2 in pediatric ALL patients and this mutation was found to result in aberrant CRLF2 dimerization via disulfide bond linkage of the receptor [22]. CRLF2, like IL27R, normally functions as a component of a heterodimeric receptor [21]. While we have recently shown that IL27R can undergo homodimerization when expressed in cells, the IL27R-FC mutation did not induce enhanced homodimerization and did not induce any obvious disulfide bonding linkage (Figure 5) [29]. The IL27R-FC mutation is located within the transmembrane domain while the CRLF2-F232C mutation is located just outside the transmembrane domain. Interestingly, the IL27R-Δ mutant did generate an IL27R protein that exhibited greater ability to undergo homodimerization (Figure 5A) and this may be due to disulfide linkages (Figure 5B). IL27R contains a JAK-binding Box 1 motif and thus enhanced dimerization of IL27R proteins could explain elevated activation of signaling downstream of the receptor through enhanced interactions of receptor-bound JAK proteins. However, as this does not seem to be the case for the IL27R-FC mutation, it is possible that this mutation within the transmembrane domain may cause a conformational change in the cytoplasmic domain of the receptor that, in turn, facilitates JAK activation. It has been shown that amino acid changes within the transmembrane domain of EpoR can lead to constitutive activation of JAK2 signaling, likely through re-orienting the cytoplasmic domain of the receptor [45]. Thus, mutation of this region of IL27R may have a similar effect. While our experiments suggest pre-existing homodimers of IL27R may exist in cells, and such homodimers have been suggested in other work, it remains unknown whether or not this homodimeric form of IL27R can functionally signal [44]. Enhanced homodimer formation by IL27R-Δ does correlate with enhanced signaling, suggesting homodimeric IL27R can signal. However, IL27R-FC does not enhance dimerization but does show elevated signaling. Thus, our work suggests but does not prove that the increased homodimerization of IL27R-Δ contributes to an increase in signaling and also suggests that enhanced dimerization is not necessary for increased signaling in the setting of mutated IL27R. Interestingly, more IL27R-Δ appeared to be expressed on the cell surface than either other form of IL27R (Figure 3C). The deletion mutation or homodimerization may stabilize cell surface expression. Also, while it is possible that the deletion mutation alters the conformation of the receptor allowing more efficient binding of the antibody used in flow cytometry, the higher level of IL27R-Δ on the cell surface may enhance homodimerization (Figure 5). These results suggest that the nature of the IL27R mutation may dictate its relative cell surface expression and that enhanced cell surface expression is not necessarily required for mutant IL27R-mediated transformation.

An activating point mutation in G-CSFR (T617N) has been reported in AML and hereditary chronic neutrophilia [17]. This mutation, like the F523C mutation described in this study of IL27R, is present in the transmembrane domain of the receptor. It was predicted that this mutation led to an energetically favorable interaction of G-CSFR transmembrane domains via hydrogen bonding of the mutated asparagine to thiol groups of transmembrane domain cysteine residues. By analogy, perhaps the thiol group of the cysteine of the IL27R-F523C mutation induces energetically favorable interactions that stabilize an activated conformation of IL27R in the membrane. This might explain why no increase in stable homodimer formation of IL27R-F523C is observed and why non-reducing gels do not demonstrate high molecular weight IL27R protein complexes. It may also explain why a serine or alanine could not functionally replace the aberrant cysteine at residue 523 of IL27R to generate a highly transforming receptor (Figure 4). In addition, mutation of similarly

located phenylalanines to cysteine in the transmembrane receptor of IL12RB1, which is structurally similar to IL27R, did not generate a transforming receptor, although it is certainly possible mutation of other residues in this region might (data not shown). In order to be transforming, such mutations in cytokine receptors presumably need to not only be in a proper structural context, but also within a receptor that is actually capable of eliciting appropriate transforming signals.

We have demonstrated that mutation of IL27R generates a highly transforming hematopoietic gene. It can be envisioned that mutation of IL27R may be present in hematopoietic diseases. At least in the analyses that we have performed, IL27R-FC and IL27R-Δ are as potent or more potent in transforming activity compared to JAK2-V617F and CRLF2-F232C, with IL27R-FC being as transforming as BCR-ABL in BaF3 cells (Figure 6). Interestingly, IL27R potently stimulates STAT1 activation, which is most commonly associated with a pro-apoptotic response. However, Kovacic et al. have shown that STAT1 can promote leukemogenesis and more recently, Chen et al. have shown that differential STAT1 signaling can dictate phenotypic outcomes of MPN cells [46, 47]. Thus, the ability of potential cytokine receptor mutations to activate specific downstream pathways will likely dictate the extent to which they may behave as growth promoting genes for different hematopoietic neoplasms in patients. Since IL27R is expressed on the cell surface of both lymphoid and myeloid cells, including hematopoietic stem cells, mutation of IL27R has the potential to contribute to a wide variety of hematopoietic disorders. A comprehensive survey of IL27R sequences in various hematopoietic diseases would be needed to determine the extent to which mutation of this receptor may play a role in malignancy. Perhaps identifying diseases and patients with uniparental disomy involving chromosome 19p13, where *IL27Ra* is located, will aid in the identification of such mutations.

Mutations in components of heterodimeric cytokine receptors have not been readily identified in cancer, but may represent an alternative mechanism by which tyrosine kinases could become activated in the absence of mutation. An unexpected yet important role for the cytokine receptor CRLF2 in pediatric ALL has recently been demonstrated, both in terms of overexpression of the receptor as well as mutation of the receptor. Overexpression of CRLF2 associates, both genetically and physically, with mutated JAK2, while in some patients that have wildtype JAK2, CRLF2 has been found to be mutated leading to a receptor that can constitutively activate the JAK/STAT pathway in the absence of ligand [21, 22]. Thus, aberrant activation of the JAK/STAT pathway can be accomplished by mutation of upstream cytokine receptors in addition to mutational activation of JAKs themselves or by mutational activation of other kinases such as BCR-ABL. Interestingly, recent work has shown that a poor prognostic group of BCR-ABL-negative ALL patients actually has a gene expression signature similar to that of BCR-ABL-positive ALL [48]. This signature is considered an activated tyrosine kinase gene signature, but sequencing the entire tyrosine kinome of these patients did not identify any mutations in other, non-JAK, kinases to account for this signature [49]. An alternative mechanism by which leukemic cells may have an activated tyrosine kinase gene signature in the absence of an activating tyrosine kinase mutation may be via activation of tyrosine kinases mediated by mutation of upstream cytokine receptors. Thus, this significant group of patients may contain mutations in unknown cytokine receptors and may provide an opportunity to identify mutations in cytokine receptors, such as IL27R, in patients.

Our biochemical and transformation studies on IL27R can provide a basis for understanding the transforming properties of such mutations in IL27R or other similar cytokine receptors, if they are identified in various hematopoietic malignancies in the future. While IL27R appears to be an unlikely oncogene candidate, the same can be said for CRLF2 before it was

shown to contribute to ALL [21]. Minimally our studies and the recent CRLF2 studies, suggest that given the large number of cytokine receptors, in particular those that function as part of heterodimeric receptor complexes, there may be an unappreciated family of potential genes whose products may exhibit hematopoietic transforming properties if mutated.

Abbreviations used

IL-27	interleukin-27
IL27R	interleukin-27 receptor alpha
JAK	Janus kinase
STAT	signal transducer and activator of transcription
AML	acute myeloid leukemia
EpoR	erythropoietin receptor
G-CSFR	granulocyte colony-stimulating factor receptor
TpoR	thrombopoietin receptor
MPN	myeloproliferative neoplasm
CRLF2	cytokine-like receptor factor 2
ALL	acute lymphoblastic leukemia
IL-3	interleukin-3
ERK	extracellular-regulated kinase
IL12RB1	interleukin-12 receptor beta 1
IL-6	interleukin-6
IL-12	interleukin-12

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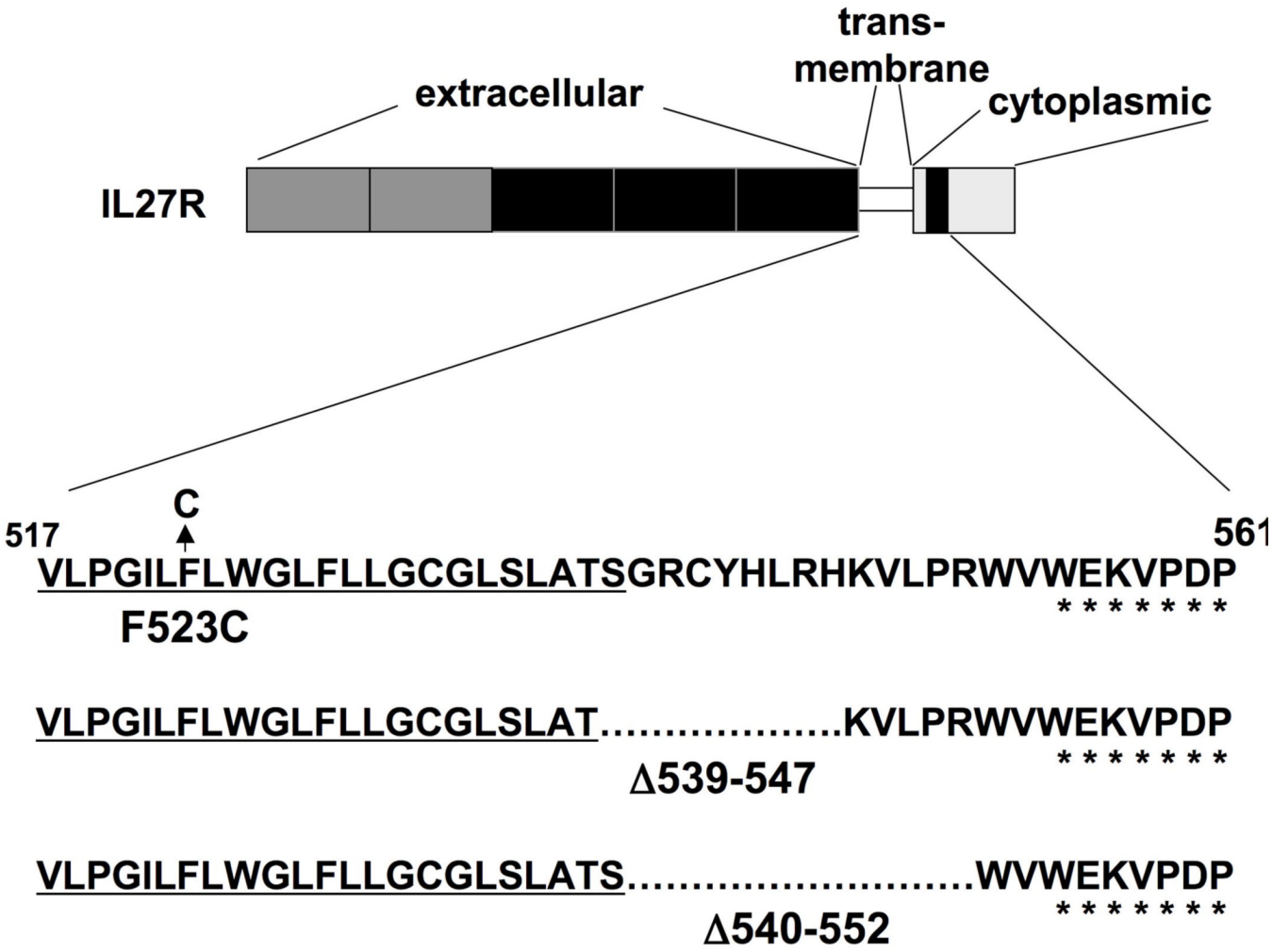

Figure 1. Mutations of IL27R identified that enhance the transforming activity of IL27R

We employed a random mutagenesis screen to identify variants of IL27R that enhance its transforming activity in cytokine-dependent 32D cells. Three mutations were identified: F523C, Δ539–547, and Δ540–552. Shown is a schematic of IL27R with the positions of its extracellular, transmembrane, and cytoplasmic domains depicted. Amino acids constituting residues 517–561 (based on NCBI accession number NM_004843) are shown. This region contains the transmembrane domain (underlined) (residues 517–539, as predicted by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) [50] and the juxtamembrane domain that includes the JAK-binding Box 1 motif (black box in cytoplasmic domain, residues marked with an asterisk). The F523C, Δ539–547, and Δ540–552 mutations within this region are depicted.

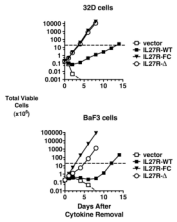

Figure 2. Mutant IL27R proteins exhibit enhanced transforming activity compared to IL27R-WT

Stable 32D and BaF3 cell lines were generated to express control vector, IL27R-WT, IL27R-FC, and IL27R-Δ. Following stable selection, these 32D (top) and BaF3 (bottom) cell lines were washed of cytokine and plated in the absence of cytokine on Day 0. Total viable cells were determined by trypan blue exclusion on the days indicated. The horizontal dashed line indicates 100-fold increase in total viable cells relative to the number of cells plated in the absence of cytokine on Day 0. Similar expression of IL27R proteins was present in these lines (see Figure 3). The dashed line indicates viable cell counts going below the limit of detection of a hemacytometer toward zero. Similar results were obtained in two independently derived sets of stable cell lines in each of 32D and BaF3 cells.

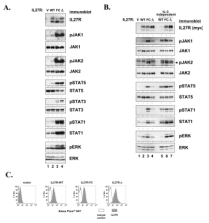

Figure 3. Cells expressing transforming IL27R mutants display increased activation of signaling proteins

(**A**) 32D cells and (**B**) BaF3 cells stably expressing a control vector (V), IL27R-WT (WT), IL27R-FC (FC), and IL27R-Δ (Δ) were washed of cytokine and incubated in cytokine free medium for 1 hr (lanes 1–4). Cell lysates were analyzed by immunoblotting for IL27R (or myc-tag), total and phosphorylated forms of JAK1, JAK2, STAT5, STAT3, STAT1, and ERK, as indicated. In addition, cell lysates were analyzed from cytokine-independent transformed BaF3 cells expressing IL27R proteins (in B, lanes 5–7). The lower expression of IL27R in IL27R-WT transformed cells (B, lane 5) was not consistently observed in multiple cell lines analyzed. The arrow indicates the migration of pJAK2. (**C**) Expression of IL27R on the cell surface of 32D cells was detected by flow cytometry utilizing an Alexa Fluor® 647-conjugated antibody. Mean fluorescent intensities for IL27R on 32D cell lines were: vector = 2.4, IL27R-WT = 17, IL27R-FC = 22.2, and IL27R-Δ = 41.2.

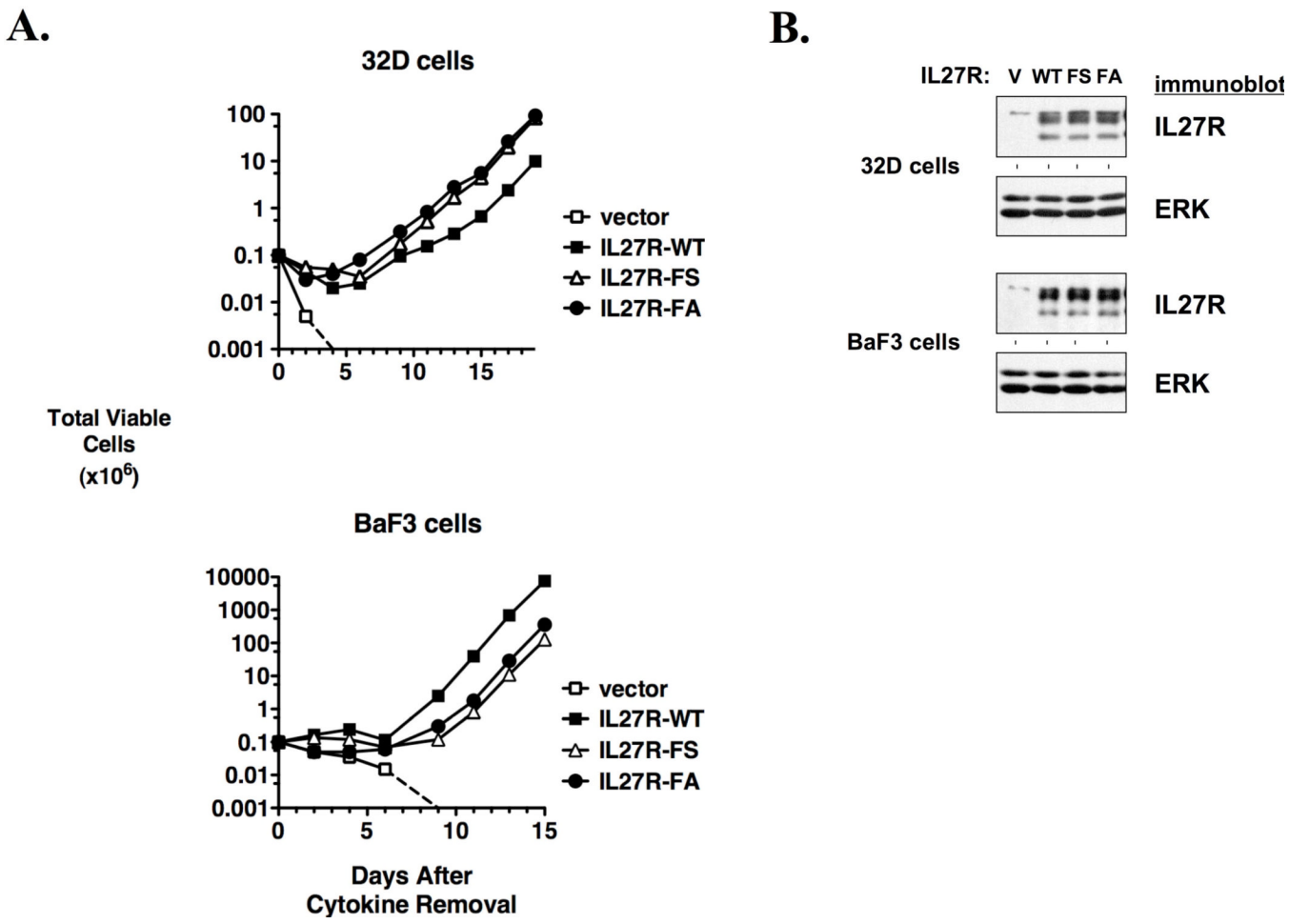

Figure 4. F523S and F523A mutations of IL27R do not enhance the transforming activity of IL27R-WT

(**A**) Stable 32D and BaF3 cell lines were generated to express control vector, IL27R-WT, IL27R-F523S (FS), and IL27R-F523A (FA). Following stable selection, these 32D (top) and BaF3 (bottom) cell lines were washed of cytokine and plated in the absence of cytokine on Day 0. Total viable cells were determined by trypan blue exclusion on the days indicated. The dashed line indicates viable cell counts going below the limit of detection of a hemacytometer toward zero. (**B**) Cell lines described in (A) were immunoblotted to detect expression of IL27R and ERK, as a loading control, as indicated. Similar results were obtained in two independently derived sets of stable 32D cell lines.

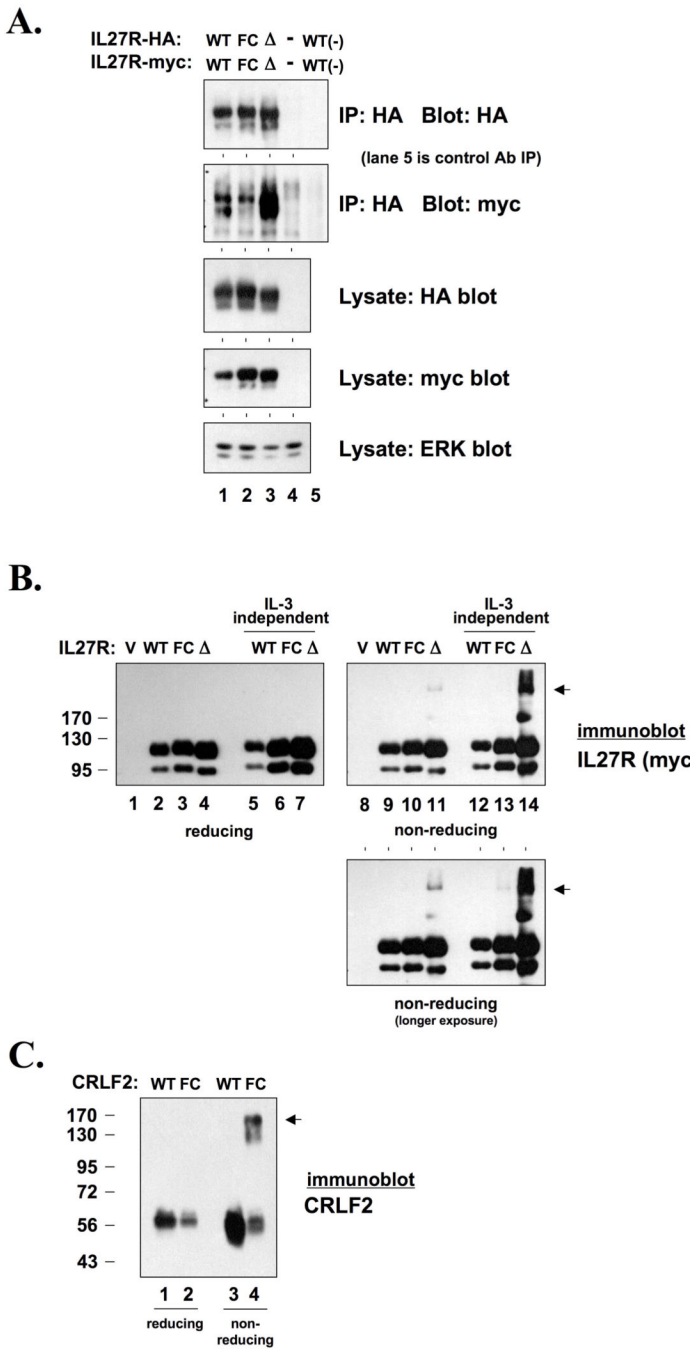

Figure 5. The IL27R-Δ mutant exhibits enhanced homodimeric complex formation

(**A**) Myc and HA-tagged IL27R-WT (lane 1), myc and HA-tagged IL27R-FC (lane 2), myc and HA-tagged IL27R-Δ (lane 3) were expressed in 293T cells and immunoprecipitated with anti-HA antibodies. Immunoprecipitates were analyzed by immunoblotting with antibodies that recognize HA and myc as indicated. Lysates were also blotted for myc and HA-tagged IL27R proteins as well as for total ERK as a loading control, as indicated. Lane 4 represents untransfected 293T cells under the same analyses and demonstrates any background bands associated with the HA immunoprecipitating antibody. Lane 5 (WT(−)) of each immunoprecipitation panel is the same lysate used in lane 1, immunoprecipitated with a non-related control antibody, and thus demonstrating the lack of a non-specific background

presence of the IL27R proteins in the immunoprecipitations. (**B**) BaF3 cells expressing control vector (lanes 1 and 8), myc-tagged versions of IL27R-WT (lanes 2, 5, 9, and 12), IL27R-FC (lanes 3, 6, 10, and 13), and IL27R-Δ (lanes 4, 7, 11, and 14) were analyzed by SDS-PAGE under reducing (lanes 1–7) and non-reducing (lanes 8–14) conditions and immunoblotted to detect-myc-tagged IL27R proteins. Lanes 1–4 and 8–11 contain lysates from cytokine-dependent cells and lanes 5–7 and 12–14 contain lysates from cytokine-independent transformed cells. The locations of molecular weight markers are indicated to the left in kDa. The arrow indicates the location of a high-molecular weight IL27R-containing protein complex. A longer exposure of the immunoblot from the SDS-PAGE run under non-reducing conditions (lanes 8–14) is also shown. (**C**) CRLF2-WT (WT) (lanes 1 and 3) and CRLF2-F232C (FC) (lanes 2 and 4) were expressed in 293T cells and cell lysates were analyzed by SDS-PAGE under reducing (lane 1 and 2) and non-reducing (lanes 3 and 4) conditions. CRLF2 proteins were detected by immunoblotting. The arrow indicates the location of a high-molecular weight CRLF2-containing protein complex. The locations of molecular weight markers are indicated to the left in kDa.

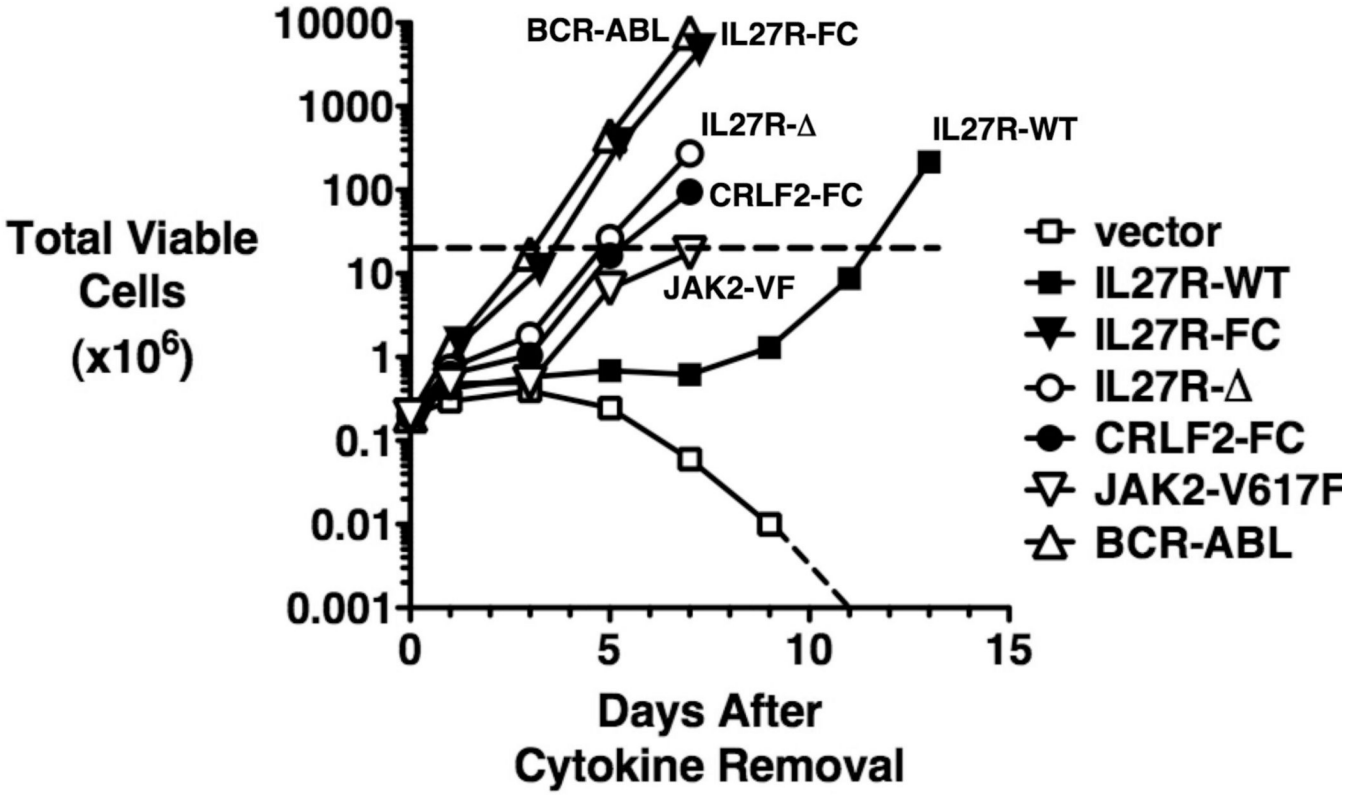

Figure 6. Comparison of the transforming properties of IL27R proteins to known transforming hematopoietic mutations

Stable BaF3 cell lines were generated to express control vector, IL27R-WT, IL27R-FC, IL27R-Δ, CRLF2-F232C, JAK2-V617F, and BCR-ABL. Following stable selection, these cell lines were washed of cytokine and plated in the absence of cytokine on Day 0. Total viable cells were determined by trypan blue exclusion on the days indicated. The horizontal dashed line indicates a 100-fold increase in total viable cells relative to the number of cells plated in the absence of cytokine on Day 0. Transformation measurement for JAK2-V617F was done in BaF3 cells expressing EpoR. The dashed line indicates viable cell counts going below the limit of detection of a hemacytometer toward zero.