IL-2/IL-15 activate the human clonally restricted KIR3DL1 reverse promoter

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INTRODUCTION

Natural killer (NK) cells defend against viruses and other intracellular pathogens and prevent the outgrowth of cancer cells. To accomplish these protective roles, NK cells distinguish between normal cells and aberrant, infected, or transformed cells using a variety of cell surface receptors, including killer cell immunoglobulin-like receptor (KIR).¹ Inhibitory KIR bind major histocompatibility complex class I proteins that are expressed on normal cells and are often downregulated on aberrant cells. Inhibitory KIR engagement activates SHP1 and other phosphatases, preventing NK cell-mediated cytotoxicity and cytokine secretion.¹ NK cells develop from CD34⁺ CD7⁺ CD56⁻ primitive hematopoietic precursors though CD34⁺ CD7⁻ CD56⁻ pre-NK cells, CD56bright immature NK cells, and finally into CD56dim NK cells that express KIR.¹,² Mature CD56dim NK cells rapidly kill aberrant cells and secrete cytokines.³ Pre-NK cells and both immature and mature NK cells respond to interleukin (IL)-2 and IL-15, cytokines critical for NK cell development and maintenance, greatly stimulated KIR3DL1 reverse promoter activity, but not forward promoter activity. Activated STAT5 was both necessary and sufficient for this effect and bound to the promoter in NK cells that expressed KIR3DL1 or were poised for expression. A systematic investigation of the KIR3DL1 reverse promoter showed significant differences from the forward promoter, with STAT and YY1 sites having relatively greater roles in regulating reverse proximal promoter activity. On the basis of our data, we propose a new role for antisense transcripts in the initiation of KIR gene expression during NK cell development.

Keywords: natural killer cells; transcription factors; cell activation and differentiation; gene regulation; human

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reverse crKIR promoter activity in vitro correlated with the frequency of peripheral blood NK cell surface expression for many crKIR alleles, suggesting that competition between forward and reverse transcription may determine whether an allele is expressed or silenced. Antisense transcripts are not found in all NK cell subsets, but are seen in CD34+ CD7+ CD56+ KIR+ pre-NK cells and in CD56dim KIR+ cells (Figure 1a). Antisense expression was not detected in mature NK cells that express an allele at a given locus even though the great majority of NK cells express crKIR alleles mono-allelically. In contrast, IL-2 and IL-15 consistently increased reverse promoter methylation. Despite the potential importance of antisense transcription in regulating variegated crKIR expression, relatively little is known about the cell signals and transcription factors that control antisense transcription. It seems likely that sense and antisense transcription from crKIR proximal promoters are differentially regulated, but this has not been demonstrated and the factors that control relative sense and antisense transcription have not been characterized. To better understand crKIR antisense transcription, we characterized the reverse 3DL1 proximal promoter. We found that the reverse promoter, unlike the forward 3DL1 promoter, is greatly stimulated by IL-2/L-15. We performed a systematic, unbiased study of the 3DL1 reverse promoter to identify the cis-acting elements that are responsible for activation and IL-2/15 stimulation. We identified a STAT site that is necessary for both effects. Activated STAT5 was sufficient for reverse promoter activation, and STAT5 bound in vivo to both expressed KIR genes and those poised for expression. Additionally, we found that in vitro promoter methylation greatly reduced antisense transcription. Our data provide insights into the timing and control of antisense crKIR expression.

**RESULTS**

Reverse promoter activity is increased by IL-2 and IL-15 stimulation. Several lines of evidence suggest that antisense transcription has a role in the initiation of KIR allele expression during NK development, a process that requires IL-2 or IL-15 cytokines. Therefore, we hypothesized that IL-2 and IL-15 stimulate antisense and/or sense transcription by activating the proximal promoter. To test this hypothesis, we placed the 3DL1 promoter (3DL1*001) in both forward orientation (producing sense transcripts) and reverse orientation (producing antisense transcripts) in the pGL3 luciferase reporter plasmid. Constructs were tested in YT NK cells, because they allow strong expression of KIR promoter reporter constructs and they quickly and abundantly phosphorylate STAT5 in response to cytokine stimulation. Transfected YT cells were incubated without cytokine or with IL-2 or IL-15 (Figure 1b). Both IL-2 and IL-15 modestly decreased forward promoter activity. In contrast, IL-2 and IL-15 consistently increased reverse promoter...
activity by 2.5-fold (Figure 1b). These results suggest that sense
and antisense KIR transcription are differentially regulated and
that antisense transcription increases in response to cytokine-
mediated developmental signals.

Forward and reverse 3DL1 promoters are activated by shared and
distinct cis-acting elements

A previous study used truncation mutants of the reverse 3DL1*002
promoter to infer important cis-acting elements.15 To directly test
sites important for reverse activity and IL-2/15 stimulation, we
used with other KIR promoters to identify activating cis-acting
elements.19,20 The reverse promoter construct was only about
twofold more active than the control construct without insert
(pGL3 basic, data not shown). To allow a clearer interpretation of
the results, we therefore tested constructs in the presence of IL-2.
Using 24 linker-scanning mutants (Figure 2a) that had been
previously used to test sense transcription,20 we found that seven
mutations significantly reduced reverse promoter activity, with
substitution of segments 5, 12, and 18 having the greatest effect
(Figure 2b).

Substitution of segments 17 and 23 increased antisense
transcription by at least 50% over the wild-type (WT) control
(Figure 2b). Possible artifactual effects on luciferase protein
production by two out-of-frame ATG sites were investigated by
mutations that avoided all transcription factor binding motifs
predicted by the TESS program. Mutation of the out-of-frame
segment 17/18 ATG had little effect but mutation of the out-of-
frame segment 23 ATG nearly doubled reverse promoter activity
(data not shown). Thus, the out-of-frame ATG translational start
site accounts for much of the effect of the segment 23
substitution on luciferase activity. We cannot rule out the
possibility that repressor elements bind in this region, although
a TESS search of the sequence of the segment revealed no strong
homology to known transcription factors binding sites (data not
shown). As shown below (Figure 3), testing various point
mutations gave similar results in the presence and absence of
the segment 23 ATG site.

We next tested several mutations that had been found to alter
forward 3DL1 promoter activity and could have caused the
changes observed in reverse promoter activity.20–22 YY1 has been
postulated to inhibit antisense KIR transcription,15 so we directly
tested this site. Consistent with this prediction, we found that the
YY1 mutation increased reverse transcription approximately
twofold compared with WT (Figure 3), which can explain the
nearly twofold gain of activity when Segment 17 is mutated. This
is in contrast to forward promoter results, in which YY1 mutations
had little or no effect.20 An Ets site mutation, which is proximal to
the transcript start site in the forward promoter (pEts), had
eliminated more than 60% of forward promoter activity.20
However, the same mutation reduced reverse promoter activity
by only 10–20% with IL-2 stimulation (25% in the absence of IL-2
stimulation), with borderline statistical significance (Figure 3, and
results not shown). Therefore, the pEts site, which straddles
segments 5 and 6, cannot explain the large effect of the Segment
5 substitution on reverse promoter activity. An alternative
explanation rests on the observation that the pEts mutation
decreases forward promoter activity.20 If forward and reverse
activities compete as hypothesized,17 then loss of reverse
transcription caused by the pEts mutation might be partially
balanced by increased reverse transcription due to reduced
competing forward transcription.

Figure 2. Linker-scanning mutagenesis reveals multiple 3DL1 reverse
promoter cis-acting sites. (a) Schematic depiction of the reverse
3DL1 promoter cloned into pGL3 basic, showing 24 contiguous 10-
bp segments (denoted S1–S24 under the corresponding segment)
that were each replaced with a linker sequence (Table 1). The
promoter nucleotide numbering is identical to that described
previously.20 Putative cis-acting elements are denoted by a single
line above or below the corresponding sequence. Italicized letters
indicate the region in which the antisense transcriptional start site
was predicted to be located in primary NK cells.23 Two potential
artifactual translational start codons are denoted by underlined ‘A’
in bold. The Segment 2 bold T (A in the sense strand) denotes an
A/G polymorphic site found in the 3DL1 promoter.15 Shaded bases
encode the repressive PIWI RNA.15 Bases in lower case are part of
the pGL3 plasmid sequence. (b) Segment substitution mutants were
tested in YT cells treated with IL-2 as described in Figure 1b.
Means ± s.e.m. were derived from tests of at least three different
plasmid preparations (each measured in duplicate). *P < 0.05,
significant differences from WT. Background level was produced
by the promoterless pGL3-basic vector (B).
Reverse promoter activity was not significantly reduced by an E2F mutation and this cis-acting site cannot explain the effect of the segment 6 substitution (Figures 2b and 3). The E2F site made only a modest contribution to forward promoter activity, similar to the lack of effect on reverse promoter activity. Both the CRE and the Runx sites had moderate (15–40%), but significant, effects on forward promoter activity and they have similar effects on reverse promoter activity (Figure 3). Runx factors and CREB/ATF-1 bind to sequences that are common in KIR promoters.20–22 Together these results suggest that Runx factors and CREB/ATF activate both sense and antisense transcription from KIR proximal promoters. A combined STAT/Ets/YY1 site is centered in segment 18, with the STAT site extending into segment 19 and the YY1 extending into segment 17 (Figure 2a); mutation of segment 18 nearly eliminated reverse activity whereas mutation of segment 17 increased activity (Figure 2b). The effect of a YY1 mutation was described above. To investigate whether the STAT site is required for reverse promoter activity, we tested a 2-bp mutation specifically targeting the STAT motif. The STAT mutation caused a 75% loss of reverse promoter activity in the absence of IL-2 stimulation (data not shown) and a nearly 90% loss in the presence of IL-2 stimulation (Figure 3). This same STAT mutation increased 3DL1 reverse promoter activity by nearly fourfold at the highest plasmid dose tested (Figure 4b). In contrast, constitutively active STAT5 had no effect when the STAT site was mutated, indicating a direct effect on the reverse promoter. This result indicates that transcriptionally active STAT5 binding to the 3DL1 promoter is sufficient to activate antisense transcription. We conclude that the promoter STAT site is both necessary and sufficient for IL-2- and IL-15-stimulated 3DL1 antisense transcription. Because 3DL1 promoter sequences bind to activated STAT5 in EMSA,20 we expected that STAT5 would bind to the 3DL1 promoter in IL-2-activated cells. We performed chromatin immunoprecipitation (ChIP) on negative control FaDu carcinoma cells, which do not express KIR proteins, and on several lymphocyte cell lines. NK92.26 (a randomly selected clone of NK92) and YT NK cells do not express 3DL1, but expression is increased to varying degrees by treatment with 5-aza-2’-deoxycytidine (Aza), an inhibitor of DNA methyltransferases.23–24 An unknown fraction of Hut-78 T cells transcribe the 3DL1 allele, which is weakly expressed at the cell surface.25 NK92.26 cells were cloned from NK92.26 cells after short-term Aza treatment and remain uniformly positive for cell surface 3DL1 without further drug treatment.9,25 ChIP experiment results indicated that STAT5 was not significantly bound to the 3DL1 promoter in FaDu, Hut-78 and YT cell lines. However, this suggested either a lack of activated STAT5 or exclusion of STAT5 from the 3DL1 promoter in these cells. YT cells abundantly activate STAT5 in response to IL-2,18–20 yet overnight IL-2 treatment did not result in significant binding of STAT5 to the 3DL1 promoter (Figure 5). This indicates that STAT5 is excluded from the 3DL1 promoter in most YT cells, possibly due to an inaccessible chromatin configuration. This finding is consistent with the observation that 3-day Aza treatment induced 3DL1 expression in only a minority of YT cells.9,25 In contrast, Aza treatment induced 3DL1 expression in nearly 100% of NK92.26 cells.25 NK92.26 and NK92.26.5 cells require IL-2 for growth, so STAT5 is expected to be continuously activated in these cell lines. Both 3DL1– NK92.26 and 3DL1+ NK92.26.5 cells showed significant association of STAT5 with the 3DL1 promoter.

STAT protein binding is sufficient for the IL-2/15-dependent increase of reverse promoter activity

IL-2 and IL-15 activate multiple intracellular pathways, including JAK/STAT, MAP kinase and PI3K pathways, which activate several transcription factors.24 We therefore wished to determine whether STAT signaling is necessary and sufficient for IL-2 and IL-15 stimulation of antisense transcription. To test this hypothesis, we transfected YT NK cells with the STAT-mutated reverse promoter and measured cytokine-induced activity. To highlight the effects of IL-2 and IL-15 in this experiment, results with each plasmid (STAT site intact and STAT site mutated) in the absence of cytokine were normalized to 1.0, and compared with results in the presence of cytokine (Figure 4a). Mutation of the STAT site eliminated IL-2 and IL-15 induction of reverse promoter activity (Figure 4a). This result suggests that the STAT site is absolutely necessary for the ability of IL-2 and IL-15 to stimulate antisense 3DL1 transcription. However, this result did not exclude possible contributions by other IL-2- and IL-15-stimulated signaling pathways. To test whether activated STAT5 is sufficient to upregulate 3DL1 antisense transcription, we co-transfected HEK293T cells with reverse promoter–luciferase plasmids and a plasmid that expresses a constitutively active form of STAT5.26 Constitutively active STAT5 increased 3DL1 reverse promoter activity by nearly fourfold at the highest plasmid dose tested (Figure 4b). In contrast, constitutively active STAT5 had no effect when the STAT site was mutated, indicating a direct effect on the reverse promoter. This result indicates that transcriptionally active STAT5 binding to the 3DL1 promoter is sufficient to activate antisense transcription. We conclude that the promoter STAT site is both necessary and sufficient for IL-2- and IL-15-stimulated 3DL1 antisense transcription.
Hence, activated STAT5 occupies the 3DL1 promoter in cells that express 3DL1 (NK92.26.5) and in cells that are poised to express 3DL1 (NK92.26). NK92.26 3DL1 alleles are heavily DNA methylated, but chromatin is partially histone acetylated and in a partially open configuration. Therefore, our results indicate that STAT5 binds to histone acetylated but CpG methylated KIR alleles. This suggests that STAT5 can act as a pioneer transcription factor at cdKIR promoters.

DNA methylation inhibits transcription from the reverse 3DL1 promoter

Patch DNA methylation of the 3DL1 promoter in vitro inhibits forward promoter activity. We investigated whether or not reverse promoter activity was suppressed by DNA methylation. In this assay, we limited methylation to the promoter because previous studies have shown that methylation of the luciferase gene or the plasmid backbone suppressed transcription even more than promoter methylation. In four separate trials, 3DL1 promoter methylation completely abolished reverse promoter activity (Figure 6). Thus, both sense and antisense transcription is severely inhibited by promoter methylation.

DISCUSSION

NK cells activate crKIR genes in a stochastic manner such that an individual cell expresses a distinct repertoire of KIR proteins, a
pattern which may be unique among human genes. Allele-specific \( crKIR \) expression tightly correlates with proximal promoter CpG demethylation.\(^8\) Therefore, expressed \( crKIR \) alleles have hypomethylated proximal promoters in mature CD56\(^{dim} \) NK cells, whereas silenced alleles have fully methylated promoters. The specific pattern of \( crKIR \) expression and promoter methylation is stably inherited through multiple rounds of cell division, preserving allele-specific expression and methylation in NK cell clones.\(^7\) A general correlation between antisense transcription and a lack of \( crKIR \) expression implies a role for antisense transcription in the establishment and maintenance of \( crKIR \) allele silencing in mature NK cells.\(^13,15\) A silencing mechanism was suggested by the discovery of a 228-bp dsRNA intermediate formed between antisense and distal sense transcripts by de novo methylation and a processed 28-bp PIWI-like RNA.\(^13\) Forced expression of either the dsRNA or of the 28-bp PIWI-like RNA in CD34\(^+ \) progenitor cells led to decreased \( crKIR \) expression in mature NK cells that subsequently developed in \textit{vitro}. As expected, poor \( crKIR \) expression was associated with \( crKIR \) proximal promoter methylation in this system.\(^13\) Our finding that antisense, but not sense, transcription from the proximal promoter is stimulated by IL-2 and IL-15 signaling is relevant to \( crKIR \) promoter methylation because IL-2 and IL-15 also drive NK cell proliferation.\(^27\) After replication DNA is hemimethylated and the newly synthesized strand must be methylated to perpetuate \( crKIR \) expression patterns. However, maintenance methylation is imperfect and DNA methylation patterns must be de novo methylation.\(^28\) A possible mechanism of \textit{de novo} methylation of KIR promoters after DNA replication is via increased STAT5-driven antisense transcription, leading to the formation of repressive RNA that causes promoter methylation in the newly synthesized strand. The STAT5 binding motif does not contain a CpG dinucleotide and STAT5 is not known to be inhibited by DNA methylation. Indeed, our data suggest that STAT5 binds to the 3DL1 proximal promoter that is fully methylated in 3DL1 \( ^{–} \)NK92.26 cells (Figure 5). An apparent contradiction with this hypothesis is the observation that antisense transcripts have not been detected in KIR \( ^{+} \) NK cells.\(^13,15\) However, distal sense \( crKIR \) transcript levels are much higher in KIR \( ^{+} \) than in KIR \( ^{–} \) cells,\(^13\) so we hypothesize that antisense transcripts may completely hybridize with distal sense RNA and be rapidly degraded in KIR \( ^{+} \) NK cells, leaving few free antisense transcripts available for detection. If our hypothesis is correct, then our findings support and further refine the model advanced by Pascal et al.\(^17\)

On the basis of published findings and our data, we speculate that antisense transcription has an additional role—opening \( crKIR \) loci and poised them for full expression. Our hypothesis rests on several observations. First, short antisense transcripts are associated with active promoters that have abundant productive sense transcription.\(^29\) Second, antisense transcription ‘opens’ immunoglobulin locus chromatin, making it more accessible to recombination.\(^10,31\) Hence, there is a precedent for antisense transcription marking promoters in a ‘poised’ state ready for sense transcription.\(^29,32\) Therefore, antisense transcription is not always associated with silencing. The \( 3DL1 \) promoter in 3DL1 \( ^{–} \)NK92.26 cells that have partially open chromatin (Figure 5 and Chan et al.\(^9\)). This suggests a possible link between opening of a \( crKIR \) promoter and antisense transcription. Fourth, IL-2 and IL-15 greatly stimulate KIR reverse promoter activity and induce \( crKIR \) expression (Figure 1b; Cooley et al.\(^7\); Romagnani et al.\(^4\); McCullar et al.\(^33\)). Thus, cytokines link increased \( crKIR \) antisense transcription and \( crKIR \) gene expression temporally during NK cell development. Fifth, antisense \( crKIR \) transcripts are found in CD34\(^+\) CD7\(^+\) CD56\(^–\) pre-NK cells.\(^13\) Thus, antisense KIR transcription may poise \( crKIR \) proximal promoters for productive sense transcription. Although transcription from the distal promoter also is found in CD34\(^+\) CD7\(^+\) CD56\(^–\) pre-NK cells,\(^41\) it has not been established that all cells with this phenotype simultaneously express both distal transcripts and antisense transcripts from the same alleles and in sufficient amounts to generate dsRNA and enforce gene silencing. It would not be surprising if chromatin opening effects associated with antisense transcription competed with gene silencing effects associated with repressive dsRNA to induce full expression at some loci and alleles, and enforce silencing at other alleles. This would then result in the stochastic pattern of \( crKIR \) gene expression observed in mature CD56\(^{dim} \) NK cells.

Proximal promoter DNA methylation correlates very strongly with allele-specific \( crKIR \) transcriptional silencing in \textit{vivo} and powerfully inhibits both sense and antisense transcription in \textit{vitro} (Figure 6 and Chan et al.\(^9\)). This leads to the question of how sense or antisense transcription is initiated from KIR proximal promoters, which are methylated early in development.\(^10\) Cichocki et al.\(^4\) proposed that transcription emanating from the distal promoter causes DNA demethylation downstream at the proximal promoter. However, the passage of RNA polymerase II is not sufficient to demethylate DNA, as shown by the heavy DNA methylation observed at the 3’ ends of many actively transcribed genes.\(^34\) Therefore, other mechanisms are needed to explain how proximal promoter chromatin is modified and transcription is initiated. As noted above, STAT5 binding is not inhibited by DNA methylation. Furthermore, activated STAT5 binds to inactive promoters, recruits SWI/SNF chromatin remodeling complexes and CBP/p300 histone acetyltransferases, leading to histone acetylation and chromatin opening.\(^36\) Based on these observations, we propose that STAT5 acts as a pioneer transcription factor at \( crKIR \) proximal promoters, inducing chromatin opening and antisense transcription. This hypothesis is supported by the observation that IL-2/15 signaling, through STAT5, stimulates \( crKIR \) proximal promoter reverse activity, but not forward activity. Additionally, \( crKIR \) proximal promoter-antisense transcription, but little or no sense transcription, is detected in CD34\(^+\) CD7\(^+\) CD56\(^–\) pre-NK cells; these cells receive IL-2 and IL-15 signaling in the lymph nodes and in the bone marrow.

Although IL-2/15 induces \textit{de novo} \( crKIR \) transcription and protein expression, this effect takes place over several days.\(^5,14\) Indeed, we found that overnight incubation of primary NK cells in IL-2 has no significant effect on 3DL1 sense RNA level or cell surface protein expression intensity (data not shown), consistent with a lack of effect of IL-2 or IL-15 on steady state sense \( crKIR \) expression. We propose that STAT5-mediated proximal promoter activation and low-level antisense transcription opens the chromatin, allowing for further recruitment of transcription factors and eventual full activation of sense transcription, unless it is silenced by an adequate amount of repressive RNA, as discussed above.

Our present work has shown that IL-2 and IL-15, cytokines critical for NK cell development and maintenance, greatly stimulate \( crKIR \) reverse promoter activity, but not forward promoter activity. Activated STAT5 was both necessary and sufficient for this effect and was bound to the promoter in NK cells that expressed KIR3DL1 or were poised for expression. We propose that IL-2-driven STAT5 activation of antisense transcription opens the chromatin at the KIR locus in NK precursors, which allows the selection of the KIR promoters to be activated and genes to be expressed. Future research should be directed toward establishing when in NK cell development specific sense and antisense transcripts appear and correlating these transcript levels with epigenetic marks in the KIR locus. Understanding the details of how this occurs will be important, because allele-specific KIR stochastic gene expression appears to be under unique regulatory control. Additional information may lead to therapies with the goal of controlling expression of KIR for enhancing NK cell recognition of virally infected or transformed cells, particularly in the setting of hematopoietic stem cell transplantation for acute myeloid leukemia.
The WT 3DL1 forward promoter—luciferase construct (−255 to −1) has been described. The reverse 3DL1 promoter segment mutations were created by PCR amplifying previously described forward constructs with primers that inserted either KpnI and/or BamHI sites for cloning in reverse direction in the pGL3-basic plasmid. Mutations tested in the reverse orientation included segment substitutions (S1–S24) and point mutations (CRE, Runx, pEts, E2F, STAT(2) and YY1(3)), as shown in Table 1 and previously described. Identical point mutations were reproduced in a promoter containing an A to C substitution in segment 23, which removed an ATG site. All mutated promoters were checked by sequencing. A plasmid expressing a constitutively active form of STAT5 (pCAGGS-CA-STAT5A) and a negative control vector (pCAGGS-linker-FLAG) were gifts of Dr Koichi Ikuta (Kyoto University, Japan).

**Materials and Methods**

**Plasmids and cell lines.** YT-HY cells, referred to as ‘YT’, were grown as described. HEK293T cells were grown in Dulbecco’s modified Eagle’s medium with added glucose and glutamine (Lonza, Allendale, NJ, USA), and 10% iron-supplemented calf serum (Hyclone, Logan, UT, USA). The WT 3DL1 forward promoter—luciferase construct (−255 to −1) has been described. The reverse 3DL1 promoter segment mutations were created by PCR amplifying previously described forward constructs with primers that inserted either KpnI and/or BamHI sites for cloning in reverse direction in the pGL3-basic plasmid. Mutations tested in the reverse orientation included segment substitutions (S1–S24) and point mutations (CRE, Runx, pEts, E2F, STAT(2) and YY1(3)), as shown in Table 1 and previously described. Identical point mutations were reproduced in a promoter containing an A to C substitution in segment 23, which removed an ATG site. All mutated promoters were checked by sequencing. A plasmid expressing a constitutively active form of STAT5 (pCAGGS-CA-STAT5A) and a negative control vector (pCAGGS-linker-FLAG) were gifts of Dr Koichi Ikuta (Kyoto University, Japan).

**Transfections.** YT cells were transfected and stimulated with IL-2 (National Cancer Institute) or IL-15 (R&D Systems, Minneapolis, MN, USA) as described. We determined that IL-2/15 increased the activity of the CMV and SV-40-driven renilla plasmid up to fourfold in YT cells, and so activity for luciferase reporters when nil vs IL-2/15 conditions were being compared (Figures 1b and 4a) was not normalized to renilla. Instead, data were collected from at least five different experiments with three replicates, each using a separate plasmid mini-preparation for each replicate (15 replicates total). For the STAT5 transactivation experiments, the 3DL1 reverse promoter and STAT site mutant were recloned into pXPG-Luciferase, a reporter vector with minimal background activity and extremely low activity in HEK293T cells (data not shown). HEK293T cells (2.5 × 10^6) in individual T25 flasks were transfected using Lipofectamine transfection efficiency. A background control of ligation products made equal amounts were ligated to ncoI-digested pGL3-basic plasmids and gel purified, and was either methylated with SsoI methyl donor substrate. Fragments were quantified, and mock methylated under parallel conditions, but without SsoI methyl donor substrate. The antibody used was STAT5 (N-20) (sc-836X, Santa Cruz, Santa Cruz, CA, USA). Fold enrichment in Figure 5 represents the amplification level of anti-STAT5 ChIP divided by the level of control (rabbit IgG) ChIP.

**Patch DNA methylation.** Patch promoter methylation were carried out essentially as described. The 3DL1 reverse promoter was isolated by restriction endonuclease digestion and gel purification, and was either methylated with SsoI DNA methylase or mock methylated under parallel conditions, but without SsoI methyl donor substrate. Fragments were quantified, and equal amounts were ligated to KpnI/Ncol-digested pGL3-basic plasmids upstream of the luciferase ATG translational start site. Ligation products were transfected into YT cells, and luciferase activity was corrected for transfection efficiency. A background control of ligation products made without promoter DNA was subtracted from each experimental group.

**Statistical analysis.** F-statistics indicated that luciferase data showed equivalent variances after logarithmetic transformation, allowing parametric testing. Data shown in Figures 1b, 2b, 3, 4a and 5 were tested using paired, two-tailed t-tests. Error bars represent standard error (s.e.m.) and P-values are listed in the figure legends.

**Conflict of Interest**

The authors declare no conflict of interest.

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**Table 1. Reverse KIR3DL1 mutations**

| Site          | Motif | KIR sequence | Mutated sequence |
|---------------|-------|--------------|------------------|
| Segments 1–24 | CMGGAWGY<sup>a</sup> | CAGGATGT | GG ÷ TT, −56 to −55 |
| Proximal Ets  | TTGGGCCG | TTGGGCCG | TTT > AAA, −65 to −63 |
| E2F           | TGACGTCG | TGACGTCG | G > T, −116 | G > A, −113 |
| CRE           | TGGTGT | ACCACA<sup>b</sup> | CACA > TAGT, −100 to −97 |
| Runx          | VDCCATNWY<sup>a</sup> | AACATGGCC<sup>b</sup> | ATG > CCA, −177 to −175 |
| YY1           | TTCCNNGAA | TTCCNGAA | A > T, −188 | T > A, −180 |

<sup>a</sup>W = A, T; M = A, C; N = A, C, G, T; R = A, G; V = A, C, G; D = A, G, T. 3Motif on antisense strand.
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