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

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Abstract

Killer cell immunoglobulin-like receptors (KIR) are expressed in a clonally-restricted fashion by human natural killer (NK) cells and allow detection of aberrant cells with low MHC class I levels. Clonally-restricted *KIR* transcription is maintained by demethylation of the proximal promoter. Antisense transcripts also arise from this promoter and may enforce silencing of nonexpressed methylated alleles in NK cells. Here we show that 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 playing relatively greater roles in regulating reverse proximal promoter activity. Based on our data, we propose a new role for antisense transcripts in the initiation of *KIR* gene expression during NK cell development.

Introduction

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 KIR[1]. Inhibitory KIR bind MHC 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, $CD56^{bright}$ immature NK cells, and finally into

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CD56^{dim} NK cells that express KIR[1, 2]. Mature CD56^{dim} NK cells rapidly kill aberrant cells and secrete cytokines[1]. Pre-NK cells and both immature and mature NK cells respond to IL-2 and IL-15, cytokines that are critical for driving NK cell development in lymph nodes and bone marrow[1, 3]. IL-15 is essential for these processes because mice deficient in IL-15 production produce virtually no mature NK cells and normal NK cells die soon after transfer into these knockout mice[3].

IL-2 and IL-15 also are important for KIR expression during development[4]. IL-2 or IL-15 are required for de novo KIR expression on mature CD56^{dim} NK cells that arise from CD34^{-}CD7^{+}CD56^{-} pre-NK cells or from other KIR^{-} NK cells, such as CD56 bright cells[5, 6]. The final expression pattern of KIR seen on mature NK cells is complex. With a few exceptions (e.g., KIR2DL4 and KIR3DL3), individual NK cells express a nearly random subset of 0–7 or more *KIR* genes in a pattern that is maintained in daughter cells[7]. We refer to these *KIR* genes as clonally-restricted (*crKIR*). *crKIR* alleles are independently expressed and allele-specific expression is maintained by DNA methylation, which appears to be more important than histone modification in controlling transcription in NK cells[8–10]. *crKIR* expression is largely dependent on a promoter that is proximal to the translation start site. Adding additional complexity, crKIR coding transcripts are produced both by the proximal promoter and a more distal promoter about 2 kb upstream[11, 12]. The distal promoter is unidirectional and produces a spliced coding sense transcript, while the proximal promoter is bidirectional, producing both spliced coding sense and non-spliced, non-coding antisense transcripts (Fig. 1a). In mature CD56^{dim} KIR^{+} NK cells, both proximal and distal promoters are active, but the majority of processed sense crKIR transcripts originate from the proximal promoter[13, 14]. Antisense transcripts are found in KIR^{-} NK cells, including CD34^{-}CD7^{+}CD56^{-} pre-NK cells and CD56^{dim} KIR^{-} cells that have been assumed to be mature, but recently have been hypothesized to be relatively immature (Fig. 1a)[14, 15].

Considerable evidence indicates that antisense transcription originating from the proximal promoter regulates *crKIR* expression. First, antisense cDNAs have been cloned from peripheral blood NK cells for most *crKIR* genes. Notably, the cDNA clones contain canonical polyadenylation (AATAAA) signals, followed downstream by poly A tails, suggesting that *crKIR* antisense transcripts are recognized by the RNA processing machinery in NK cells[15]. Second, *crKIR* proximal promoters demonstrate both forward and reverse activity in vitro[15, 16]. Notably, in vitro reverse promoter activity correlated with *crKIR* promoter polymorphisms at putative YY1 and Sp1 transcription factor binding sites found in some *3DL1* alleles. Additionally, the level of antisense transcripts isolated from peripheral blood mononuclear cells correlated with *KIR3DL1* promoter polymorphisms and the strength of the reverse activity in an in vitro luciferase assay[16]. The ratio of forward to 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[16]. Antisense transcripts are not found in all NK cell subsets, but are seen in CD34^{-}CD7^{+}CD56^{-}KIR^{-} pre-NK cells and in CD56^{dim} KIR^{-} cells (Fig. 1a)[14, 15]. 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[8, 13, 15]. This suggests the possibility that antisense transcripts are rapidly hybridized and degraded in cells

that have abundant distal sense transcription from an allele at the same locus, although other mechanisms are possible.

Based on the general correlation between antisense transcription and a lack of crKIR expression, and the discovery of the upstream distal promoter (Fig. 1a)[11], Anderson and colleagues proposed that antisense transcript from the proximal promoter hybridizes with sense transcription from the distal *crKIR* promoter to form repressive double-stranded RNA (dsRNA) that causes promoter methylation and allele silencing[15, 17]. In support of this hypothesis, a 228 bp dsRNA intermediate homologous to the *3DL1* promoter was identified and cloned from blood CD56$^+$ NK cells[13]. Using a model system expressing the *3DL1* distal promoter-sense and proximal promoter-antisense transcripts in HEK293T cells, a processed 28 bp RNA of the PIWI class was isolated and cloned, and was identified in peripheral NK cells. Short RNAs, including those of the PIWI class, have been implicated in transcriptional silencing of several genes[12]. Forced expression of either the dsRNA or of the 28-bp PIWI-like RNA in CD34$^+$ progenitor cells decreased crKIR expression by 70–90% in NK cells that subsequently developed in vitro, and the *3DL1* proximal promoter was methylated in 3DL1$^-$ transduced cells[13]. These data suggest that *crKIR* antisense transcription leads to 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/IL-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 plays a role in the initiation of *KIR* allele expression during NK development, a process that requires IL-2 or IL-15 cytokines[5, 6]. 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[18–20]. Transfected YT cells were incubated without cytokine

or with IL-2 or IL-15 (Fig. 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 (Fig. 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 applied the 10-bp substitution and point mutation strategy that we used with other KIR promoters to identify activating cis-acting elements[19, 20]. The reverse promoter construct was only about 2-fold 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 (Fig. 2a) that had been previously used to test sense transcription[20], we found that 7 mutations significantly reduced reverse promoter activity, with substitution of Segments 5, 12, and 18 having the greatest effect (Fig. 2b).

Substitution of Segments 17 and 23 increased antisense transcription by at least 50% over the wild type control (Fig. 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 (Fig. 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 ~2-fold compared to wild type (WT, Fig. 3), which can explain the nearly 2-fold 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 (Fig. 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.

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 (Fig. 2b, 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 (Fig. 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 (Fig. 2a); mutation of segment 18 nearly eliminated reverse activity while mutation of segment 17 increased activity (Fig. 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 (Fig. 3). This same STAT mutation had caused a much less severe (35%) drop in forward promoter activity in the presence or absence of IL-2 (data not shown and[20]). Although consistent with a strong role for STAT5 in activating the reverse promoter, our results could not rule out the possibility that Segment 18 mutations eliminated a transcriptional start site. To investigate this possibility, we transfected YT cells with WT-reverse 3DL1 promoter luciferase reporter construct and after 40 hour incubation, we reverse transcribed RNA using a luciferase-specific primer. Quantitative PCR was carried out using a luciferase reverse primer paired with Segment 16 or Segment 23 forward primers. We found that the levels of luciferase encoding RNA were nearly equal with the two reverse promoter primers (data not shown). This suggested a transcriptional start site upstream of Segment 16, possibly at a site predicted previously[15]. This is not surprising because STAT proteins have been shown to promote transcription at locations downstream of the transcription start site[23]. YT NK cells have a constitutive low-level phosphorylation of STAT1 and STAT3 and IL-2 rapidly induces STAT5 phosphorylation[18]. Furthermore, STAT5 binds to the STAT motif centered in *3DL1* promoter Segment 18 in EMSA[20]. Therefore, the critical role of the STAT site for reverse promoter activity can explain the large effects of the Segments 18 and 19 substitutions. These results suggest that STAT proteins are strong activators of reverse promoter activity and that STAT protein binding predominantly favors antisense transcription.

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 (Fig. 4a). Mutation of the STAT site eliminated IL-2 and IL-15 induction of reverse promoter activity (Fig. 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[25]. Constitutively active STAT5 increased *3DL1* reverse promoter activity by nearly 4-fold at the highest plasmid dose tested (Fig. 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, that do not express KIR proteins, and on several lymphocyte cell lines. NK92.26 (a randomly selected clone of NK-92) and YT NK cells do not express 3DL1, but expression is increased to varying degrees by treatment with 5-aza-2'-deoxycytdine (Aza), an inhibitor of DNA methyltransferases[8, 9]. An unknown fraction of Hut-78 T cells transcribe the *3DL1* allele, *3DS1*, which is weakly expressed at the cell surface[9]. NK92.26.5 cells were cloned from NK92.26 cells after short-term Aza treatment and remain uniformly positive for cell surface 3DL1 without further drug treatment[8, 9]. ChIP experiment results indicated that STAT5 was not significantly bound to the *3DL1* promoter in FaDu, Hut-78, and YT cells (Fig. 5). 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 (Fig. 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]. In contrast, Aza treatment induced 3DL1 expression in nearly 100% of NK92.26 cells[9]. 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. 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[8, 9]. 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 *crKIR* promoters.

DNA methylation inhibits transcription from the reverse 3DL1 promoter

Patch DNA methylation of the *3DL1* promoter in vitro inhibits forward promoter activity[9]. 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[26]. In four separate trials, *3DL1* promoter methylation completely abolished reverse promoter activity (Fig. 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[7], a pattern which may be unique among human genes. Allele-specific cr*KIR* expression tightly correlates with proximal promoter CpG demethylation[8]. Therefore, expressed *crKIR* alleles have hypomethylated proximal promoters in mature $CD56^{dim}$ NK cells, while 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, 8]. 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 in NK cells 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 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 enforced by de novo methylation[28]. A possible mechanism of 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 suggests that STAT5 binds to the *3DL1* proximal promoter that is fully methylated in $3DL1^-$ NK92.26 cells (Fig. 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 Anderson and collaborators[17].

Based on published findings and our data, we speculate that antisense transcription plays an additional role—opening *crKIR* loci and poising 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[30, 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 gene silencing. Third, STAT5 binds to the *3DL1* promoter in 3DL1^{-} NK92.26 cells that have partially open chromatin (Fig. 5 and[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 (Fig. 1 and[5, 6, 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[15]. 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[14, 15], 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 vivo and powerfully inhibits both sense and antisense transcription in vitro (our work and[8]). 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. proposed that transcription emanating from the distal promoter causes DNA demethylation downstream at the proximal promoter[14]. 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[35, 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 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-2/15-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.

Materials and Methods

Plasmids and cell lines

YT-HY cells, referred to as “YT” were grown as described[20]. HEK293T cells were grown in DMEM with added glucose and glutamine (Lonza), and 10% iron-supplemented calf serum (Hyclone). The WT 3DL1(*3DL1*001*) forward promoter – luciferase construct (−255 to −1) has been described[20]. The reverse 3DL1 promoter segment mutations were created by PCR amplifying previously described forward constructs with primers that included *Nco*I or *Kpn*I sites for cloning in the reverse direction in the pGL3-basic plasmid. Mutations tested in the reverse orientation include segment substitutions (S1 – S24) and point mutations (CRE, Runx (Triple), pEts, E2F, STAT(2), and YY1(3)), as shown in Table 1 and previously described[20]. 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)[25] 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) as described[19, 20]. We determined that IL-2/15 increased the activity of the CMV and SV-40 driven renilla plasmid up to 4-fold in YT cells, and so activity for luciferase reporters when nil vs IL-2/15 conditions were being compared (Fig. 1, 4a) was not normalized to renilla. Instead, data were collected from at least 5 different experiments with 3 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[37] and extremely low activity in HEK293T cells (data not shown). HEK293T cells (~2.5×10^6) in individual T25 flasks were transfected using Lipofectamine and Plus reagent (Invitrogen) with 500 ng of pXPG-*KIR3DL1* (Segment 23 ATG mutated) reverse promoter plasmid or STAT mutant, 10 ng of control SV40-Renilla plasmid (Promega), 0, 25, 50, or 100 ng of pCAGGS-CA-STAT5A, and enough empty plasmid (pCAGGS-linker-FLAG) to equalize transfected DNA mass. Manufacturer's instructions were followed, except that media was replaced 3 h after transfection. Cells were harvested and promoter activity was determined as described[19, 20]. The pCAGGS-CA-STAT5A plasmid equally increased Renilla luciferase activity in both groups (intact and mutant STAT). We determined that the fold difference caused by CA-STAT5 plasmid to the reporter plasmids was the same whether being normalized to Renilla luciferase activity or not normalized. Notably, pXPG –Luc basic vector (without insert) was not affected by pCAGGS-CA-STAT5A.

Chromatin analysis

Putative transcription factor binding sites were identified using the TESS computer algorithm (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Chromatin immunoprecipitation was carried out as described[9], except that cells were treated with 1% formaldehyde for 30 minutes. The antibody used was STAT5 (N-20) (sc-836X, Santa Cruz). Fold enrichment in Fig. 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[9]. The *3DL1* reverse promoter was isolated by restriction endonuclease digestion and gel purification, and was either methylated with *Sss*I DNA methylase or mock methylated under parallel conditions, but without S-adenosyl methonine methyl donor substrate. Fragments were quantified, and equal amounts were ligated to *Kpn*I/*Nco*I-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 logarithmic transformation, allowing parametric testing. Data shown in Fig 1, 2b, 3, 4a, and 5 were

tested using paired, 2-tailed *t* tests. Error bars represent standard error (SEM) and *p* values are listed in the figure legends.

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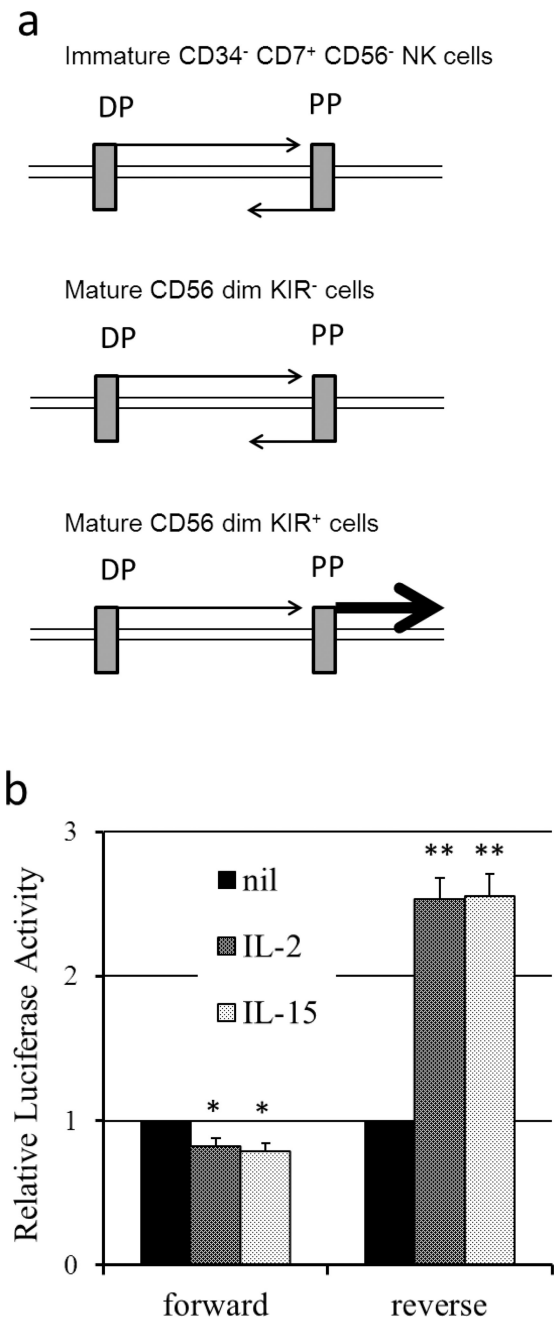

Figure 1.

The *KIR3DL1* promoter complex and stimulation of the reverse promoter by IL-2 and IL-15. (a) Schematic depiction of sense and antisense *crKIR* transcription from the distal promoter (DP) and proximal promoter (PP). Right pointing arrows indicate sense transcripts, left pointing arrows indicate antisense transcripts. The thick arrow indicates high level transcription. (b) Constructs containing either the forward or reverse *3DL1* proximal promoter cloned into the pGL3 basic vector were transfected into YT cells, which were treated with media alone (nil), IL-2 (200 U/mL 24 hours before harvest, with an additional 100 U/mL 12 hours before harvest) or IL-15 (10 ng/mL 24 hours before harvest, with an

additional 10 ng/mL 12 hours before harvest). To increase assay signal-to-noise ratio, the reverse promoter construct was tested in the absence of the in-frame Segment 23 ATG site, see Fig. 2b and text. Means ± SEM were derived from at least 5 independent experiments that each included at least three different plasmid preparations, as described in Materials and Methods * $p < 0.0005$, ** $p < 1 \times 10–11$ compared with media alone.

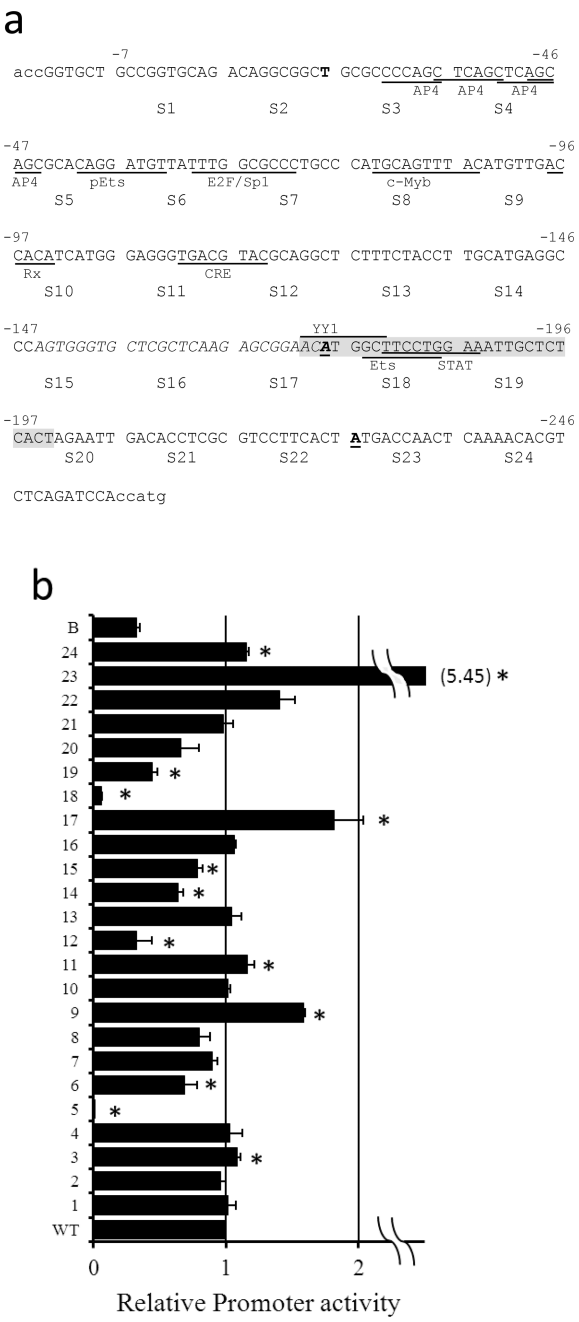

Figure 2.

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 noted 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[15]. 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[13]. 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 ± SEM 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).

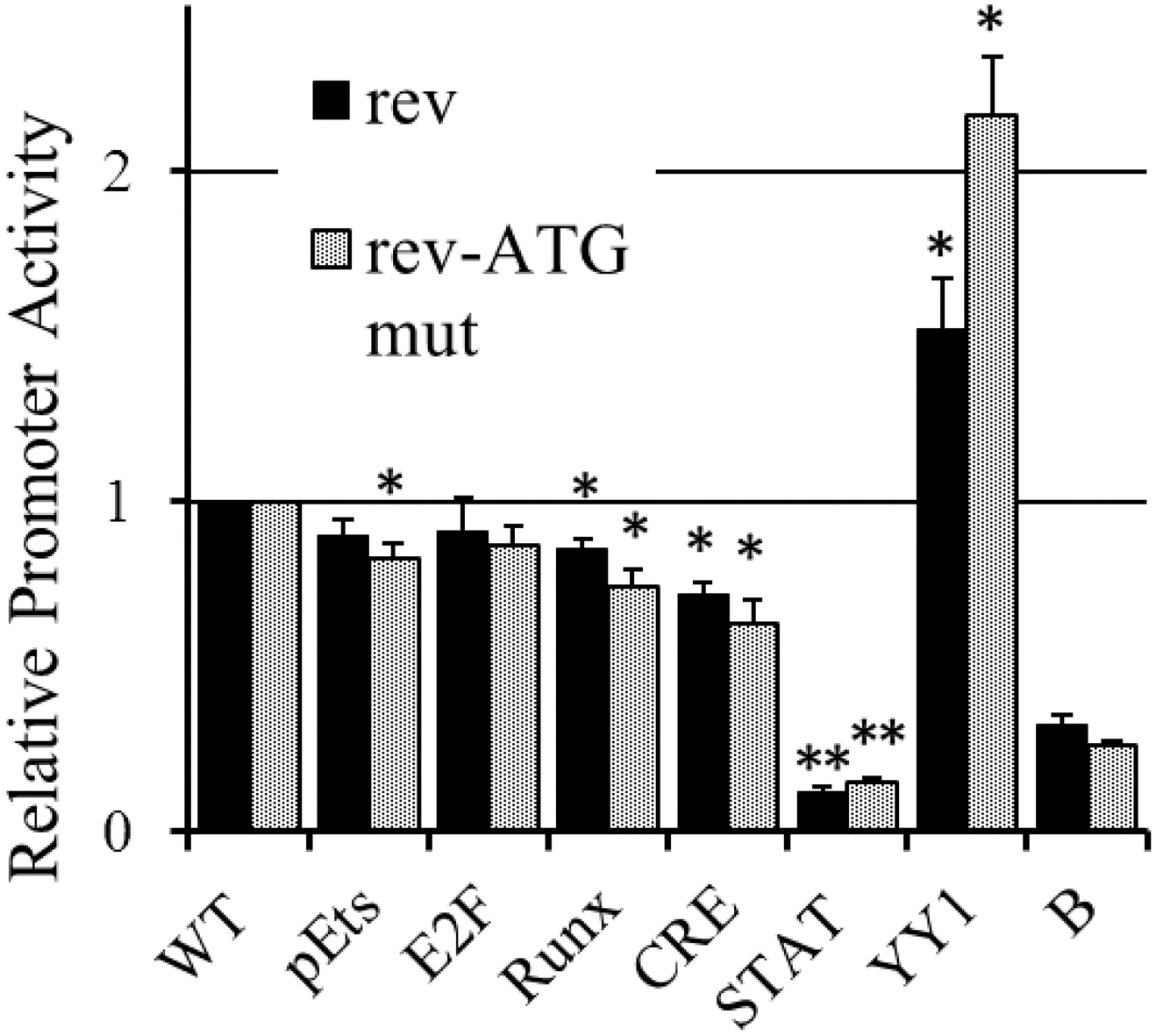

Figure 3.

3DL1 reverse promoter activity depends on specific activating sites. Shown are promoter activities (measured in YT cells as described in Fig. 1b) of the empty vector, pGL3-basic (B), and of constructs with single-site promoter mutations in pEts, E2F, Runx, CRE, Stat, and YY1 sites (Table 1), in the setting of an intact Segment 23 ATG site (black bars) or of a mutated Segment 23 ATG site (gray bars). *$p < 0.05$, ** $p < 0.0001$, significant differences from the comparable promoter with intact putative cis-acting sites.

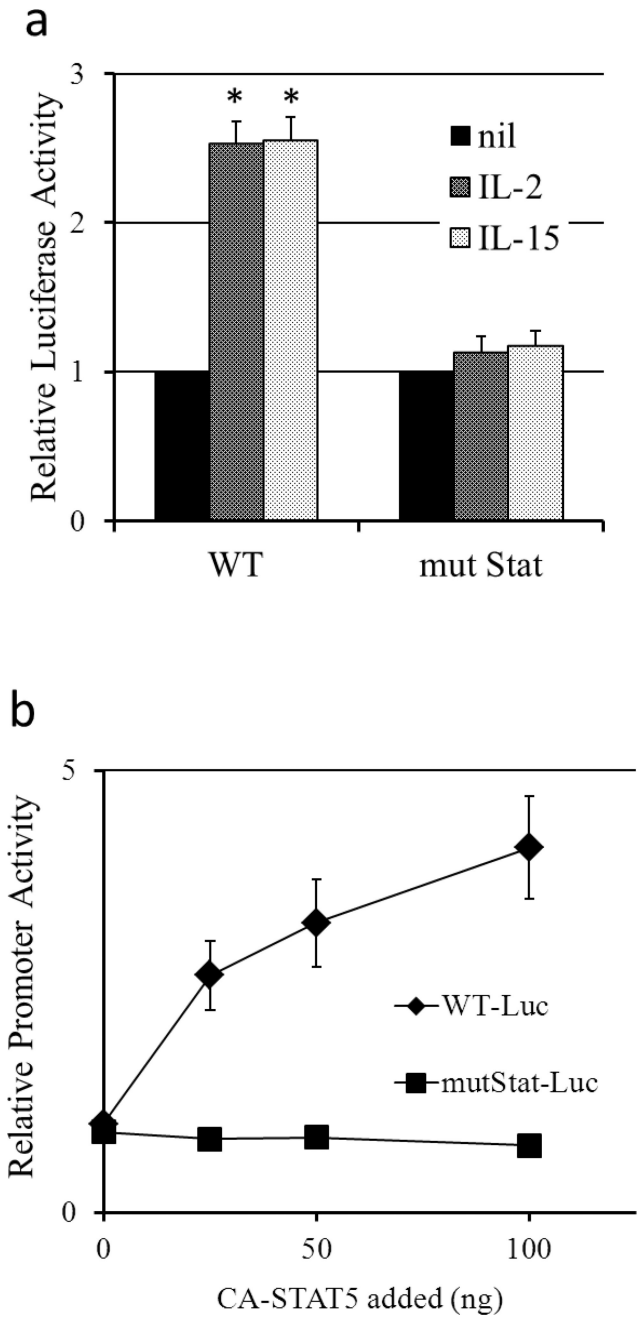

Figure 4.

The *3DL1* STAT site is critical for IL-2/15-dependent induction of *3DL1* reverse promoter activity. (a) Mutation of the STAT site eliminates IL-2 and IL-15 induction of reverse *3DL1* promoter activity. Constructs (Segment 23 ATG mutated) containing either an unmodified or mutated STAT site were transfected into YT cells and treated with IL-2/15 as described in Fig. 1b. * $p < 1 \times 10–11$ compared with STAT-mutant plasmid. (b) Constitutively active STAT5A is sufficient to activate reverse *3DL1* promoter activity. HEK293T cells were transfected with 10 ng of the control SV40 Renilla luciferase construct, 0, 25, 50, or 100 ng of pCAGGS-CA-Stat5A, and 500 ng of pXPG-retro 3DL1 luciferase reporter construct with

either intact or mutant STAT site (Segment 23 ATG mutated). Empty pCAGGS plasmid DNA was added as needed to equalize the total amount of transfected DNA. Each point represents the average of five tests; each test had a separate reporter plasmid preparation and one of two different constitutively active STAT5A expression plasmid preparations. Shown is one representative experiment of three with similar results. Results are normalized to the level of the pXPG-retro 3DL1 luciferase reporter STAT site intact plasmid with no added STAT5A expression plasmid. Error bars show within-experiment standard error (some error bars are too small to be visible).

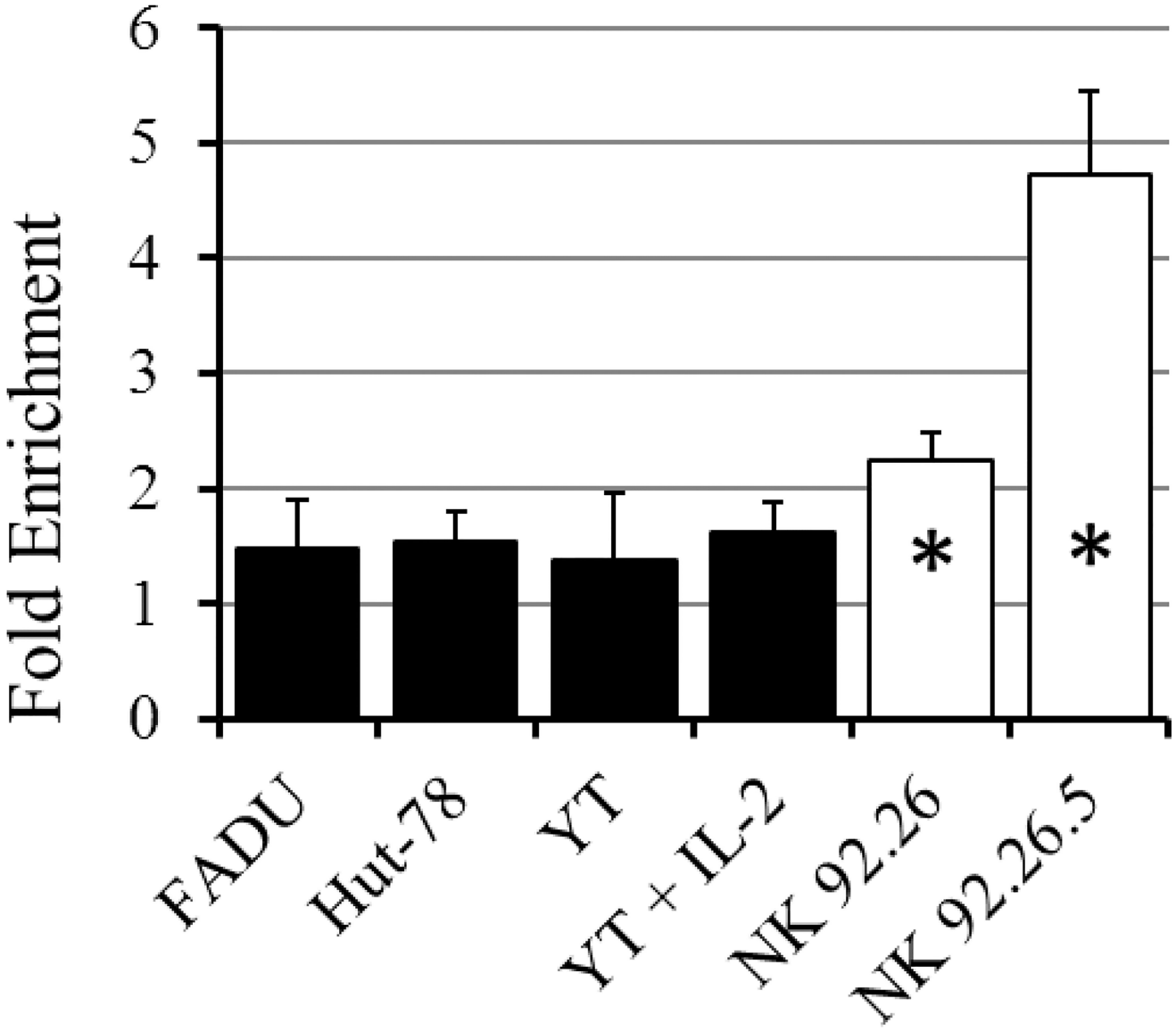

Figure 5.

STAT5 is associated with the *3DL1* promoter in NK cells poised for 3DL1 expression. Cross-linked chromatin was purified from FaDu, YT, IL-2–cultured YT, Hut-78, NK92.26, and NK92.26.5 cells and immunoprecipitated with either a polyclonal anti-STAT5 antibody or with negative control rabbit IgG. DNA from immunoprecipitates was purified and quantitative PCR amplified using primers specific to the 3DL1 promoter[9]. Values represent the average enrichment of DNA bound to specific antibody (ratio of anti-STAT5/normal rabbit IgG) from tests of between three to six different chromatin preparations. * $p < 0.01$, using a paired 2-tailed t-test comparing STAT5 vs. control immunoprecipitation; all other groups had nonsignificant differences from control.

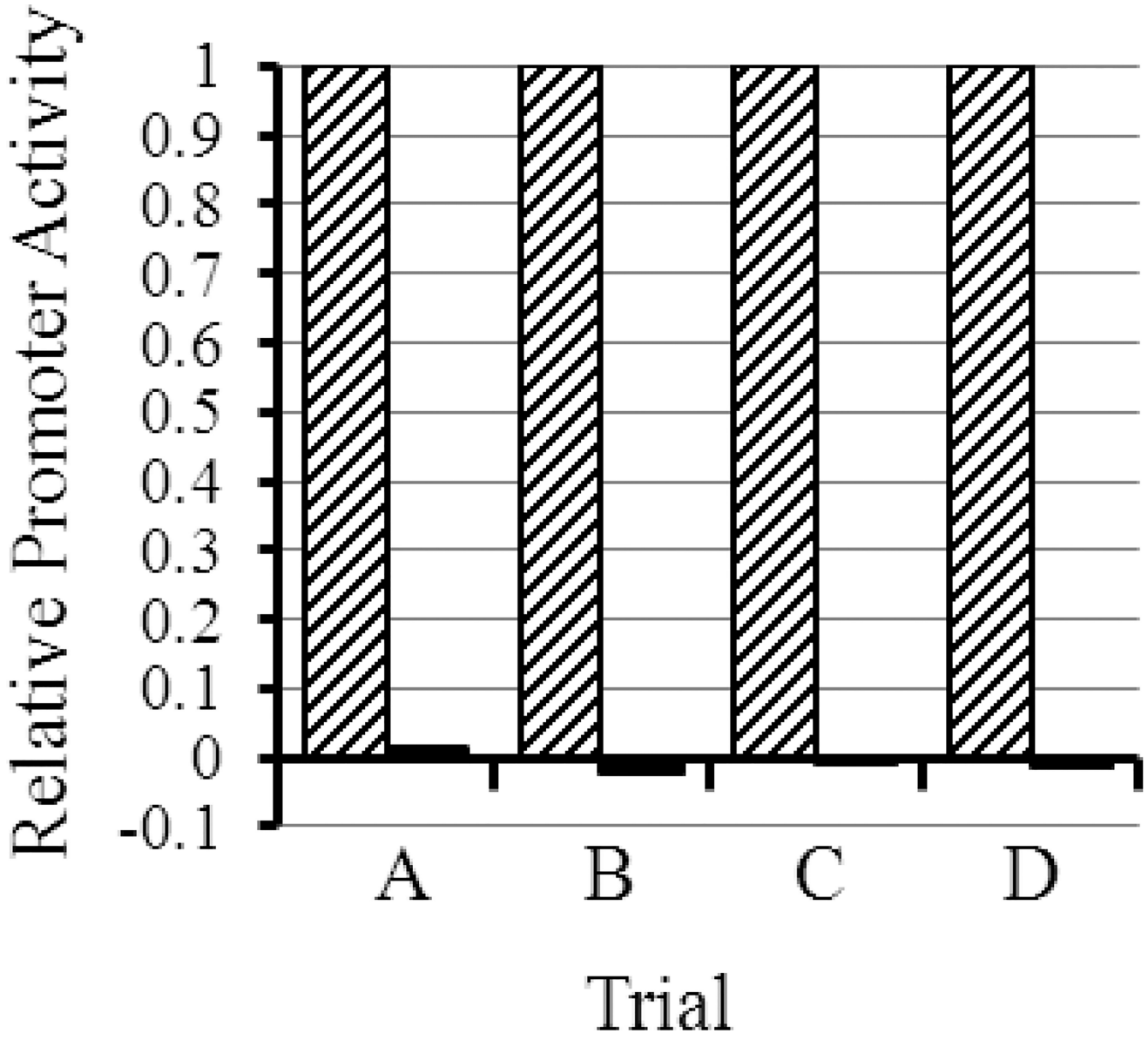

Figure 6.

DNA methylation inhibits *3DL1* reverse promoter activity. The *3DL1* reverse promoter was isolated by restriction endonuclease digestion and gel purification, and was either mock methylated (hatched bars) or methylated (black bars) as described in Materials and Methods. After background subtraction, the methylated promoter activity was calculated as a fraction of the mock-methylated promoter activity. Four separate experiments are shown. Negative values indicate activity lower than background.

TABLE 1

Reverse KIR3DL1 mutations

Site	Motif	*KIR* sequence	Mutated sequence
Segments 1–24		Various	GCGGATCTGC
Proximal Ets	CMGGAWGY[1]	CAGGATGT	GG>TT, −56 to −55
E2F	TTTCGCGC	TTTGGCGC	TTT>AAA, −65 to −63
CRE	TGACGTCA	TGACGTAC	G>T, −116; G>A, −113
Runx	TGTGGT	ACCACA[2]	CACA>TAGT, −100 to −97
YY[1]	VDCCATNWY[1]	AACATGGCT[2]	ATG>CCA, −177 to −175
STAT	TTCNNNGAA[1]	TTCCTGGAA	A>T, −188; T>A, −180

[1]W = A, T; M = A, C; N = A, C, G, T; R = A, G; V =A, C, G; D = A, G, T.

[2]Motif on anti-sense strand.