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| Title    | Characterization of a weakly expressed KIR2DL1 variant reveals a novel upstream promoter that controls KIR expression                                                                                                                                                                                                |
| Type     | Article                                                                                                                                                                                                                                                                                                              |
| URL      | <a href="https://clock.uclan.ac.uk/id/eprint/25541/">https://clock.uclan.ac.uk/id/eprint/25541/</a>                                                                                                                                                                                                                  |
| DOI      | <a href="https://doi.org/10.1038/gene.2014.34">https://doi.org/10.1038/gene.2014.34</a>                                                                                                                                                                                                                              |
| Date     | 2014                                                                                                                                                                                                                                                                                                                 |
| Citation | Wright, P W, Li, H, Huehn, A, O'Connor, Geraldine orcid iconORCID: 0000-0002-7440-162X, Cooley, S, Miller, J S and Anderson, S K (2014) Characterization of a weakly expressed KIR2DL1 variant reveals a novel upstream promoter that controls KIR expression. Genes & Immunity, 15 (7). pp. 440-448. ISSN 1466-4879 |
| Creators | Wright, P W, Li, H, Huehn, A, O'Connor, Geraldine, Cooley, S, Miller, J S and Anderson, S K                                                                                                                                                                                                                          |

It is advisable to refer to the publisher's version if you intend to cite from the work.  
<https://doi.org/10.1038/gene.2014.34>

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Published in final edited form as:

*Genes Immun.* 2014 October ; 15(7): 440–448. doi:10.1038/gene.2014.34.

## Characterization of a weakly expressed *KIR2DL1* variant reveals a novel upstream promoter that controls KIR expression

Paul W. Wright<sup>1,†</sup>, Hongchuan Li<sup>1,†</sup>, Andrew Huehn<sup>2,†</sup>, Geraldine M O'Connor<sup>2</sup>, Sarah Cooley<sup>3</sup>, Jeffrey S. Miller<sup>3</sup>, and Stephen K. Anderson<sup>1,2,\*</sup>

<sup>1</sup>Basic Science Program, Leidos Biomedical Research Inc., Lab of Experimental Immunology, Frederick National Lab, Frederick, Maryland 21702, USA.

<sup>2</sup>Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland 21702, USA.

<sup>3</sup>Division of Hematology, Oncology and Transplantation, University of Minnesota, Minneapolis, Minnesota 55455.

### Abstract

Members of the human KIR class I MHC receptor gene family contain multiple promoters that determine the variegated expression of KIR on NK cells. In order to identify novel genetic alterations associated with decreased KIR expression, a group of donors was characterized for *KIR* gene content, transcripts, and protein expression. An individual with a single copy of the *KIR2DL1* gene but a very low level of gene expression was identified. The low expression phenotype was associated with a SNP that created a binding site for the inhibitory ZEB1 transcription factor adjacent to a c-Myc binding site previously implicated in distal promoter activity. Individuals possessing this SNP had a substantial decrease in distal *KIR2DL1* transcripts initiating from a novel intermediate promoter located 230 bp upstream of the proximal promoter start site. Surprisingly, there was no decrease in transcription from the *KIR2DL1* proximal promoter. Reduced intermediate promoter activity revealed the existence of alternatively spliced *KIR2DL1* transcripts containing premature termination codons that initiated from the proximal *KIR2DL1* promoter. Altogether, these results indicate that distal transcripts are necessary for KIR2DL1 protein expression and are required for proper processing of sense transcripts from the bidirectional proximal promoter.

### Keywords

human; NK cells; KIR; transcription; ZEB1

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\*Corresponding Author: andersonst@mail.nih.gov.

†These authors contributed equally

### Conflict of interest

The authors declare no conflict of interest.

## Introduction

Natural Killer (NK) cells are lymphocytes of the innate immune system that play an important role in the elimination of neoplastic or virus-infected cells<sup>1</sup>. NK cell cytotoxic activity is controlled in part by cell surface receptors for class I MHC that survey potential target cells for aberrant class I expression<sup>2</sup>. Human NK cells use the killer cell immunoglobulin-like receptor (KIR) family<sup>3</sup> to interrogate class I status, whereas murine NK cells use members of the C-type lectin-related Ly49 family<sup>4, 5</sup>. *KIR* and *Ly49* genes are expressed in a probabilistic fashion, generating subsets of NK cells with distinct class I MHC recognition properties<sup>6, 7</sup>. The selectively expressed human *KIR* gene family provides an interesting model system for the study of stochastic activation of gene expression. The proximal promoter of most *KIR* genes is kept in a silent state by promoter methylation<sup>8, 9</sup>. An AML/RUNX transcription factor binding site in the proximal promoter region has been suggested to play a role in the demethylation of the proximal promoter, since loss of this site in the non-transcribed *KIR2DL5\*002* allele is associated with non-expression, even though loss of the AML/RUNX site does not abrogate proximal promoter activity in luciferase reporter assays<sup>10, 11</sup>. Furthermore, treatment of NK cells with the demethylating agent 5-azacytidine, leads to *KIR2DL5\*002* transcription, supporting a role for AML/RUNX in catalyzing promoter demethylation. Studies of a distal *KIR* promoter have indicated a role for distal transcripts in gene activation, since distal transcription precedes gene expression, and increased distal transcripts are associated with a higher frequency of gene expression<sup>12, 13</sup>. A c-Myc binding site in the distal promoter was shown to be important for promoter activity, and over-expression of c-Myc in developing NK cells led to increased distal transcript levels and an increased frequency of NK cells expressing KIR. A model of *KIR* gene activation has been proposed in which the distal promoter generates transcripts that traverse the proximal promoter region, leading to an opening of this region and allowing access of key transcription factors involved in promoter demethylation, such as AML/RUNX<sup>14</sup>.

The variegated expression of *Ly49* genes is controlled by a bidirectional promoter that acts as a probabilistic switch<sup>15</sup>. The generation of forward transcripts from this upstream element (Pro1) in immature NK cells leads to activation of the downstream proximal promoter. The expression of a *Ly49* gene from the proximal promoter is dependent on distal transcription, since Pro1 deletion eliminates *Ly49* transcription<sup>16</sup>. The probabilistic activation of *KIR* genes has been linked to the bi-directional nature of the *KIR* proximal promoter<sup>17</sup>. Variation in the relative strength of transcription factor binding sites that promote either sense or antisense transcription from the proximal promoter in specific *KIR* genes or alleles results in bi-directional promoters with differing probabilities of generating a forward transcript<sup>18</sup>. *KIR* genes or alleles with a high ratio of sense to antisense proximal promoter activity are expressed by a greater percentage of NK cells than *KIR* with a low sense to antisense ratio. The *KIR* antisense transcript has been associated with the production of a 28 base piwi-RNA that may lead to re-silencing of *KIR* loci that produce the antisense transcript<sup>19</sup>. Although numerous *KIR* alleles have been identified that are non-expressed due to the presence of stop codons in the coding region, only one gene (*KIR2DL5*)<sup>10</sup> has been identified that contains non-transcribed alleles. In the current study, we sought to determine if there were

additional non-transcribed KIR alleles, to obtain further information with regard to the genomic regions controlling gene expression.

## Results

### Identification of a weakly expressed *KIR2DL1* allele

In order to identify novel genetic alterations associated with a lack of *KIR* transcription, healthy donors were screened for individuals that possessed but did not show significant expression of *KIR* genes. A group of 182 National Marrow Donor Program (NMDP) donors were characterized for *KIR* gene transcription by qRT-PCR, *KIR* gene content, and FACS analysis of KIR surface expression. An individual was discovered that possessed the *KIR2DL1* gene but had barely detectable gene expression by either FACS or qRT-PCR (Figure 1).

### Identification of SNPs specific to the poorly expressed *KIR2DL1* allele

Complete sequencing of the *KIR2DL1* gene from the individual with low *KIR2DL1* expression revealed the presence of a single allele containing an intact coding region. Analysis of the three known promoter regions as well as the complete 2 kb intergenic region preceding the first exon revealed a cluster of 3 single nucleotide polymorphisms (SNPs) in the distal promoter region approximately 1 kb upstream from the start of *KIR2DL1* translation (Figure 2). These 3 SNPs, together with the presence of an exon 4 segment derived from the *KIR2DL1*\*002 allele constituted the only sequence differences between the *KIR2DL1* gene in this donor and the previously reported complete sequence of a *KIR2DL1*\*00401 allele (GenBank accession GU182347). The 3 SNPs present in the novel *KIR2DL1* allele match the sequence normally found in the distal promoter region of the non-transcribed *KIR2DL5*\*002 allele as well as the *KIR3DP1* pseudogene (Figure 2). However, the well-characterized *KIR2DL5*\*002 SNP that disrupts the proximal promoter AML/RUNX site was not present, indicating that the distal promoter variant was able to inhibit KIR expression by a novel mechanism. This also raises the possibility that the *KIR2DL1* SNPs identified here could be involved in the silencing of *KIR2DL5*\*002.

### The *KIR2DL1* distal SNP creates a ZEB1 binding site

Examination of the distal SNPs associated with non-expression of *KIR2DL1* revealed that the SNP at position -1206 created a binding site for the Zinc finger E-box-binding homeobox 1 (ZEB1) protein, a transcription factor that has been shown to inhibit *IL2* gene expression<sup>20</sup>. ZEB1 requires two E-box motifs (CACCT) for efficient binding to DNA<sup>21</sup>, and the polymorphism generates a second E-box motif in close proximity to an E-box motif adjacent to the c-Myc/USF binding site (Figure 2) that has been previously implicated in the activation of KIR genes<sup>13</sup>. EMSA analysis indicated that ZEB1 binds to the E-box pair composed of the novel E-box generated by the polymorphism and the site located next to the c-Myc site (Figure 3). The generation of this novel ZEB1-binding site adjacent to the Myc site shown to enhance distal transcription suggested that the presence of the ZEB1 SNP might decrease activity of the *KIR2DL1* distal promoter, reducing the probability of proximal promoter activation. Interestingly, all known *KIR2DL5* alleles contain the SNP that generates the ZEB1-binding site, which may account for the low frequency of expression

observed for *KIR2DL5A\*001*<sup>22</sup>. Some *KIR2DP1* alleles also contain this SNP, however the complete absence of *KIR2DP1* transcripts precludes any observable effect of the ZEB1 SNP on the transcription of this gene.

### Association of the ZEB1 SNP with decreased *KIR2DL1* expression

PCR screening of archived DNA from an additional 376 donors from the NCI Frederick Research Donor Program, resulted in the identification of 6 additional individuals (1.6 %) possessing the ZEB1-associated SNP in *KIR2DL1*, all of which were associated with a *KIR2DL1\*004* allele. The complete sequence of the novel *KIR2DL1* allele can be found in GenBank under accession number KJ913660. Interestingly, none of these donors had the accompanying substitution of a *KIR2DL1\*002*-like exon 4 found in the original *KIR2DL1* low-expression variant. Three individuals with the novel SNP were available for additional characterization, and they were evaluated for cell surface expression of *KIR2DL1* as compared to normal donors that possessed either 0, 1, or 2 copies of the wild-type *KIR2DL1* gene. FACS analysis of these donors demonstrated low *KIR2DL1* surface expression (2.6% of NK cells) in a donor containing a single copy of the mutated *KIR2DL1* gene, confirming an association of the ZEB1 SNP with poor expression (Figure 4). The presence of both wild-type and mutant *KIR2DL1* alleles produced normal levels of *KIR2DL1* expression, indicating the absence of any trans effects of the ZEB1 SNP. The observed higher than expected frequency of *KIR2DL1* expression (25.8%) in the individual with only one wild-type allele can be accounted for by the presence of *KIR2DS5*, as the anti-*KIR2DL1* antibody used for detection has been shown to also recognize *KIR2DS5*<sup>23</sup>. The *KIR2DS5* gene was not present in any of the other donors tested. In addition, analysis of an individual possessing a mutant copy of *KIR2DL1* together with the *KIR2DS3* gene revealed moderate low intensity expression (11.1%), suggesting weak cross-reactivity of the anti-*KIR2DL1* antibody with *KIR2DS3* in this individual.

### The ZEB1 SNP decreases distal transcripts

In order to determine if the ZEB1 SNP affected distal promoter activity, RT-PCR was performed with primers specific for the *KIR2DL1* distal transcript. As shown in Figure 5a, there was a substantial reduction in distal transcription in individuals possessing the ZEB1-associated SNP, consistent with an inhibitory effect of ZEB1 binding in the upstream region.

Surprisingly, analysis of proximal promoter transcripts showed no decrease in the level of proximal *KIR2DL1* promoter activity (Figure 5b), even in an individual that possessed only a single copy of the *KIR2DL1* gene containing the ZEB1 SNP. The proximal transcript levels actually increased in the ZEB1 mutant, possibly due to the loss of competition from the upstream promoter. Furthermore, the levels of distal transcript paralleled the level of protein expression observed in Figure 4, whereas proximal transcript did not. Since previous studies had demonstrated distal KIR transcripts initiating approximately 800 bp upstream of the KIR start codon were not translatable, 5' RACE was performed with primers just upstream of the proximal promoter to look for additional distal transcription start sites. Two novel distal transcript start sites 230 and 273 nucleotides upstream from the major proximal promoter start site were identified in RNA from individuals with wild type *KIR2DL1* alleles, indicating the presence of an intermediate promoter (Figure 5c). The lack of correlation

between proximal promoter activity and protein expression suggests that transcripts initiated from the novel intermediate promoter may be responsible for KIR2DL1 expression by mature NK cells. To investigate whether or not these novel transcripts are capable of expressing protein, a full-length cDNA originating from the intermediate promoter (GenBank accession number KJ699235) was cloned into an expression vector and transfected into the human 239T cell line. Expression of KIR2DL1 from the intermediate transcript was comparable to expression from the proximal transcript, indicating that the additional 230 base 5' untranslated region of the intermediate transcript did not inhibit expression, consistent with the association of the novel promoter with KIR expression by mature NK cells. A *KIR3DL1* cDNA originating from the intermediate promoter was also tested and found to be capable of producing KIR3DL1 expression (data not shown).

### Proximal *KIR2DL1* transcripts contain alternative exons

The lack of correlation between KIR2DL1 expression and proximal transcription was perplexing, since the major start site of *KIR2DL1* transcription in NK cells has been mapped to the proximal promoter<sup>12, 24, 25</sup>. However, RT-PCR analysis of individuals with reduced *KIR2DL1* distal transcript revealed the presence of two alternative exons containing stop codons (Figure 6), suggesting that proximal promoter transcripts might be alternatively spliced and potentially incapable of producing translatable *KIR2DL1* mRNA.

To further investigate this possibility, qRT-PCR was performed with antisense primers specific for the alternate exons together with forward primers from either the intermediate or proximal transcripts. Efficient amplification of alternative exon-containing transcripts was only observed with the forward primer from the proximal transcript, suggesting that the alternative exons are specific to the proximal transcript (Figure 6c). However, the alternative exon-containing transcripts were not as abundant in NK cells that contained only wild-type *KIR2DL1* genes, suggesting that the presence of distal transcript either inhibits alternative splicing or ZEB1 binding to the distal promoter region affects alternative splicing of the proximal transcript. 5' RACE performed using primers from the alternative exons generated 5' ends mapping to the proximal promoter region. No start sites upstream of the proximal promoter were detected with alternative exon primers (data not shown).

## Discussion

Studies of the Pro1 distal bidirectional promoter present in the murine *Ly49* genes have indicated that forward transcription from the distal promoter is required for activation of the proximal *Ly49* promoter (Pro2) that is responsible for *Ly49* expression in mature NK cells. This was demonstrated by both naturally occurring deletions of the distal promoter as well as a direct deletion of the Pro1 element in *Ly49a* transgenic mice<sup>26</sup>. The distal *KIR* promoter has been shown to be transcriptionally active in NK precursors that lack KIR expression, suggesting that it may also play a role in the initial opening of the proximal *KIR* promoter analogous to the murine *Ly49* Pro1 promoter<sup>13</sup>. The weakly expressed *KIR2DL1* allele reported herein has revealed the presence of a novel *KIR* intermediate promoter 230 bp upstream of the proximal promoter, and transcription from this novel promoter is associated with protein expression. For the purposes of this discussion, we will refer to this new

intermediate promoter as Pro-I. The bi-directional proximal promoter will be referred to as the probabilistic switch, or Pro-S. The previously described distal promoter will be referred to as Pro-D. The presence of a ZEB1 binding site in Pro-D was correlated with a low level of *KIR2DL1* distal transcription in several individuals. The ZEB1 SNP was also associated with decreased *in vitro* *KIR2DL1* Pro-I activity (data not shown) and decreased Pro-I transcripts in NK cells, indicating an enhancer function of the Pro-D element for Pro-I transcription. Pro-S activity was unchanged, indicating an association of Pro-I transcription with *KIR2DL1* protein expression. The results obtained in this study necessitate a revision of the model of probabilistic KIR gene activation. The previous observation that CD56<sup>dim</sup>, KIR<sup>-ve</sup> NK cells contain antisense transcripts from Pro-S indicated that these cells represented the phase of NK development where the probabilistic Pro-S element was active, however these cells also contain forward Pro-S transcripts, but no KIR protein expression. The discovery of a novel promoter associated with KIR expression indicates that Pro-S transcription alone is not sufficient for protein expression, and it is Pro-I that drives expression in mature NK cells either directly, or by facilitating proper splicing of proximal transcripts. This also reveals another parallel with the murine Ly49 system: forward transcripts from bidirectional promoters active in immature NK cells are non-translatable. Therefore, the process of KIR gene activation should proceed through three stages as defined by the activity of the Pro-D, Pro-S, and Pro-I elements (Figure 7). NK precursors have an active Pro-D element that is associated with the activation of the Pro-S region in developing NK cells. NK cells undergoing the CD56<sup>bright</sup> to CD56<sup>dim</sup> transition represent the probabilistic transcription phase marked by Pro-S activity and the presence of either antisense transcripts or forward non-translatable transcript. Mature KIR-positive NK cells should exhibit Pro-I activity driving the expression of KIR protein or preventing alternative splicing of proximal transcripts. The inability of previous studies of *KIR* transcript initiation sites to detect Pro-I transcripts may be due to a high concentration of proximal Pro-S transcripts masking the presence of upstream start sites. The PCR and subsequent cloning steps performed in 5'-RACE create a bias toward smaller products, making it difficult to identify start sites that are further upstream. There is also a bias toward more abundant transcripts, and Pro-S transcripts are approximately 5-fold higher than the Pro-I transcripts detected in purified peripheral NK cell populations<sup>12</sup>. As a result, the Pro-I transcription start site could only be mapped by 5' RACE using primers upstream of the Pro-S start site.

The proposed ability of Pro-I to drive KIR protein expression in mature NK cells and the non-translatable Pro-S transcripts associated with low Pro-I activity suggests that SNPs affecting the level of KIR expression should map to the Pro-I element and the upstream enhancer Pro-D as described in this study, whereas SNPs affecting the percentage of NK cells expressing a given KIR should map to the Pro-S element, as previously demonstrated for the *KIR3DL1* gene<sup>18</sup>. To test the prediction that Pro-I SNPs might affect the intensity of *KIR3DL1* expression, the *KIR3DL1* Pro-I region was compared amongst the *KIR3DL1* alleles represented in GenBank. A single bp deletion was found in the *KIR3DL1*\*005 and *KIR3DL1*\*004 alleles but not in any of the other *KIR3DL1* alleles or *KIR3DS1*. This deletion affects a consensus AP-1 transcription factor binding site, and would be predicted to result in decreased Pro-I promoter activity, consistent with the reported low expression phenotype of *KIR3DL1*\*005.



The analysis of a *KIR2DL1* allele associated with low expression revealed a novel *KIR* variant, but in addition, has resulted in a significant change in our understanding of *KIR* gene regulation. The promoter previously considered to be responsible for *KIR* protein expression (Pro-S), may function primarily as a probabilistic switch that governs the frequency of gene activation in developing NK cells. This novel paradigm of *KIR* gene regulation necessitates a shift away from analysis of the proximal *KIR* promoter when considering polymorphisms that affect the intensity of *KIR* expression. Mature NK cells require *KIR2DL1* Pro-I transcripts to express the protein, Pro-I transcripts have been identified for the *KIR3DL1* gene (data not shown), and the Pro-I element is conserved among all *KIR* genes except for *KIR3DL3*, a gene that is not expressed by mature circulating NK cells. This suggests that most *KIR* genes use Pro-I for the expression of *KIR* protein in mature NK cells. Of particular interest is the divergence of the *KIR2DL3/2DL2/2DS2* Pro-I elements that only share 84% homology with the 3' half of the *KIR2DL1* Pro-I element and possess no significant homology within the 5' half of the promoter. This may explain the previous observation that NK cells derived from hematopoietic progenitor cells generally acquire the C1-specific inhibitory *KIR2DL2/3* receptors at earlier time points than the C2-specific *KIR2DL1*<sup>27</sup>. Comparison of Pro-S elements of the *KIR* genes has revealed 5 *KIR2DL2/3*-specific SNPs, however there is no significant difference in *KIR2DL2/3* Pro-S activity as compared to *KIR3DL1\*002* Pro-S activity<sup>18, 28</sup>. It will therefore be of interest to compare Pro-I transcripts and Pro-I activity of these genes to see if a correlation exists between divergent Pro-I structures and distinct expression patterns.

In conclusion, the discovery of a novel *KIR* promoter associated with protein expression by mature NK cells mandates a re-examination of previous data correlating proximal promoter polymorphisms with differences in the timing, tissue specificity, or level of transcription of individual *KIR* genes or alleles. This paradigm shift promises to provide insight into gene/allele-specific differences that have not been adequately explained by study of the proximal *KIR* promoter.

## Materials and methods

### Cell lines

YT cells were cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, sodium pyruvate, and L-glutamine. HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin (P/S), sodium pyruvate, and L-glutamine.

### Donors and NK Cell Isolation

Collection of DNA and blood samples from 162 donor/recipient pairs from unrelated donor hematopoietic cell transplants performed to treat AML was facilitated by the National Marrow Donor Program (NMDP, Minneapolis, MN) Research Sample Repository. Samples were collected after informed consent and approval from the NMDP and University of Minnesota Institutional Review Boards were obtained in accordance with the Declaration of Helsinki. Healthy volunteers were recruited through the NCI-Frederick Research Donor



Program (<http://www.ncicrf.gov/rdp/>). The KIR genotype of each donor was determined as previously described<sup>29</sup>. NK cells were separated from the peripheral blood of healthy donors by Histopaque® (Sigma-Aldrich, Saint Louis, MO, USA) gradient centrifugation using the RosetteSep™ Human NK Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver BC, CA).

### Flow Cytometric Analysis of KIR Expression on Peripheral Blood NK Cells

The proportion of NK cells expressing KIR2DL1 was assessed in whole blood by three-color flow cytometry. NK cells were identified using anti-human CD56-APC (Beckman Coulter, Pasadena, CA, USA). T cells were labeled with anti-human CD3-Pacific Blue (eBioscience, San Diego, CA, USA). KIR2DL1 expressing cells were identified with the clone 143211-FITC KIR2DL1-specific antibody (R&D Systems, Minneapolis, MN, USA). Briefly, 200 µl of EDTA-treated blood was incubated with a cocktail of the 3 mAbs for 30 min on ice. Erythrocytes were lysed by the addition of 3 ml of RBC lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM NaHCO<sub>3</sub>, 1 mM EDTA, pH 7.4), and the remaining cells were analyzed with a FACSsort flow cytometer (Becton & Dickinson, San Jose, CA). Events (25,000) were collected in the lymphocyte gate and analyzed. The percentage of CD3-CD56+ NK cells expressing KIR2DL1 was determined.

### PCR screening and sequencing of *KIR2DL1* genes

PCR screening of donors for the ZEB1 SNP was performed with a *KIR2DL1*-specific forward primer (5'-GGGTTTACAGTTCTTTATTGAACCG-3') together with a reverse primer recognizing the *KIR3DP1/KIR2DL5B* sequence found in the low expresser variant (5'-CTCAATAAATAATATTAACCAATTGGTTAC-3'). To obtain the complete sequence of the *KIR2DL1* alleles present in each donor, 50 ng of leukocyte DNA obtained from donor blood was subjected to PCR with a series of *KIR2DL1/S1*-specific primers to produce overlapping ~ 1 kb amplicons spanning the complete gene from the polyA site of the preceding *KIR2DP1* gene to the polyA site of *KIR2DL1*. PCR fragments were cloned into the pCR2.1-topo vector (Invitrogen, Carlsbad, CA, USA). A minimum of six clones of each fragment was sequenced for each *KIR2DL1/S1* allele present.

### RT-PCR of *KIR* transcripts

Total RNA was purified from  $1 \times 10^7$  donor NK cells with the RNeasy kit (Qiagen, Valencia, CA, USA) and cDNA synthesis was carried out using Random Hexamer primer, Taqman Reverse Transcription Reagents kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's instructions. The initial screening of donors for transcripts of all transcribed *KIR* genes was performed using primer sets previously described<sup>30</sup>. The primers used in the *KIR2DL1*-specific qRT-PCR assay were: KIR2DL1-Distal forward primer (5'-CTCGTGTTCCGGAGGTTGGATCTC-3'); KIR2DL1-Proximal forward primer (5'-GTCGCGGCTGCCTGTCTGCTC-3'); KIR2DL1-Exon 3 reverse primer (5'-CTCTCTGTGCAGAAGGAAGTGTT-3'); KIR2DL1-AltExon B reverse primer (5'-CCAGCCTCTATAGGCCACCTGC-3'); Actin-beta forward primer (5'-CCTGGCAGCCAGCACAAT-3'); Actin-beta reverse primer (5'-GGGCCGGACTCGTCATACT-3'). Reactions were carried out using FastStart SYBR

Green Master kit (Roche Diagnostics, Indianapolis, IN, USA) on the 7300 Real-Time PCR System (Applied Biosystems). The qRT-PCR was performed in duplicate and was repeated in at least three separate experiments using the following conditions. Reaction mixtures contained 12.5  $\mu$ l of SYBR Green master mix, 2 pmoles each of forward and reverse primers, and 10ng cDNA. Thermocycler conditions included initial denaturation at 50°C and 95°C (10 min each), followed by 40 cycles at 95°C (15 s) and 60°C (1 min). Melting curve analyses were performed to verify the amplification specificity. Relative quantification of gene expression was performed according to the  $\Delta\Delta$ -CT method using StepOne Software 2.0 (Applied Biosystems). All PCR products were cloned into pCR2.1-topo (Invitrogen) and sequenced in order to verify the identity of the transcript and the *KIR* gene responsible for the product.

### RNA Ligase Mediated 5' RACE Analysis of KIR2DL1 Transcripts

5' RACE was performed on human donor NK cell RNA using the FirstChoice® RLM-RACE kit (Ambion Inc, Austin, TX, USA). *KIR2DL1* gene-specific cDNA was generated using a *KIR2DL1* Exon 3 reverse primer: 5'-CAATGAGGCGCAAAGTGTCGTTAAAC-3'. First round PCR amplification was performed using the 5' outer adapter and the *KIR2DL1*-Exon 3 reverse primer 5'-CTCTCTGTGCAGAAGGAAGTGTT-3' at an annealing temperature of 59° C for 35 cycles with Platinum PCR Supermix (Invitrogen). Excess primers from first round PCR reactions were removed with the Charge-Switch PCR Clean-Up Kit (Invitrogen). Nested PCR was subsequently performed with the 5' inner adapter primer and the *KIR2DL1*-Alternate exon 2 reverse primer (5'-CCAGCCTCTATAGGCCACCTGC-3') or a reverse primer upstream of the proximal promoter start sites (5'-TAGTGTCTTCACTATGACCAAC-3') at an annealing temperature of 60° C for 30 cycles. PCR products were cloned into pCR2.1 Topo (Invitrogen) and sequences analyzed to map the 5' start sites.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from YT cells using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Protein concentration was measured with a Bio-Rad protein assay (Hercules, CA), and samples were stored at -70°C until use. Double-stranded DNA oligonucleotide probes corresponding to the predicted ZEB1 binding sequence of the mutant *KIR2DL1* distal promoter (TTCCTAGGTGTAACCAATTGGTTTAATA-TTATTTATTGAGAAGACATTCTATGCCACCTTAAACCACACGGCAG) or the wild-type sequence were synthesized. Probes were labeled with [ $\alpha$ -<sup>32</sup>P]deoxycytidine triphosphate (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) by fill-in using the Klenow fragment of DNA polymerase I (Invitrogen). <sup>32</sup>P-labeled double-stranded oligonucleotides were purified using mini Quick Spin Oligo Columns (Roche Diagnostics, Mannheim, Germany). DNA-protein binding reactions were performed in a 10- $\mu$ L mixture containing 5  $\mu$ g nuclear protein and 1  $\mu$ g poly(dI-dC) (Sigma-Aldrich) in 4% glycerol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5). Nuclear extracts were incubated with 1  $\mu$ L <sup>32</sup>P-labeled oligonucleotide probe (10,000 cpm) at room temperature for 20 minutes and then loaded on a 5% polyacrylamide gel (37:5:1). Electrophoresis was performed in 0.5x TBE for 2 hours at 130 V, and the gel was visualized by autoradiography. For antibody supershift experiments, nuclear extracts

were incubated with 2  $\mu$ L of antibody for 1 h on ice before the addition of  $^{32}$ P- labeled DNA probe. After the addition of labeled DNA probe, the binding reaction was incubated for an additional 20 min at room temperature. The antibodies used were ZEB1-1 (H102, sc-25388x), ZEB1-2 (C-20, sc-10570x), ZEB1-3 (E-20, sc-10572x), ZEB1-4 (R-17, sc-10573x), ZEB2 (SIP1, E-11, sc-271984), and MZF1 (H-45, sc-66991x) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and Myc (9E11; Abcam, Cambridge, MA, USA).

## Acknowledgements

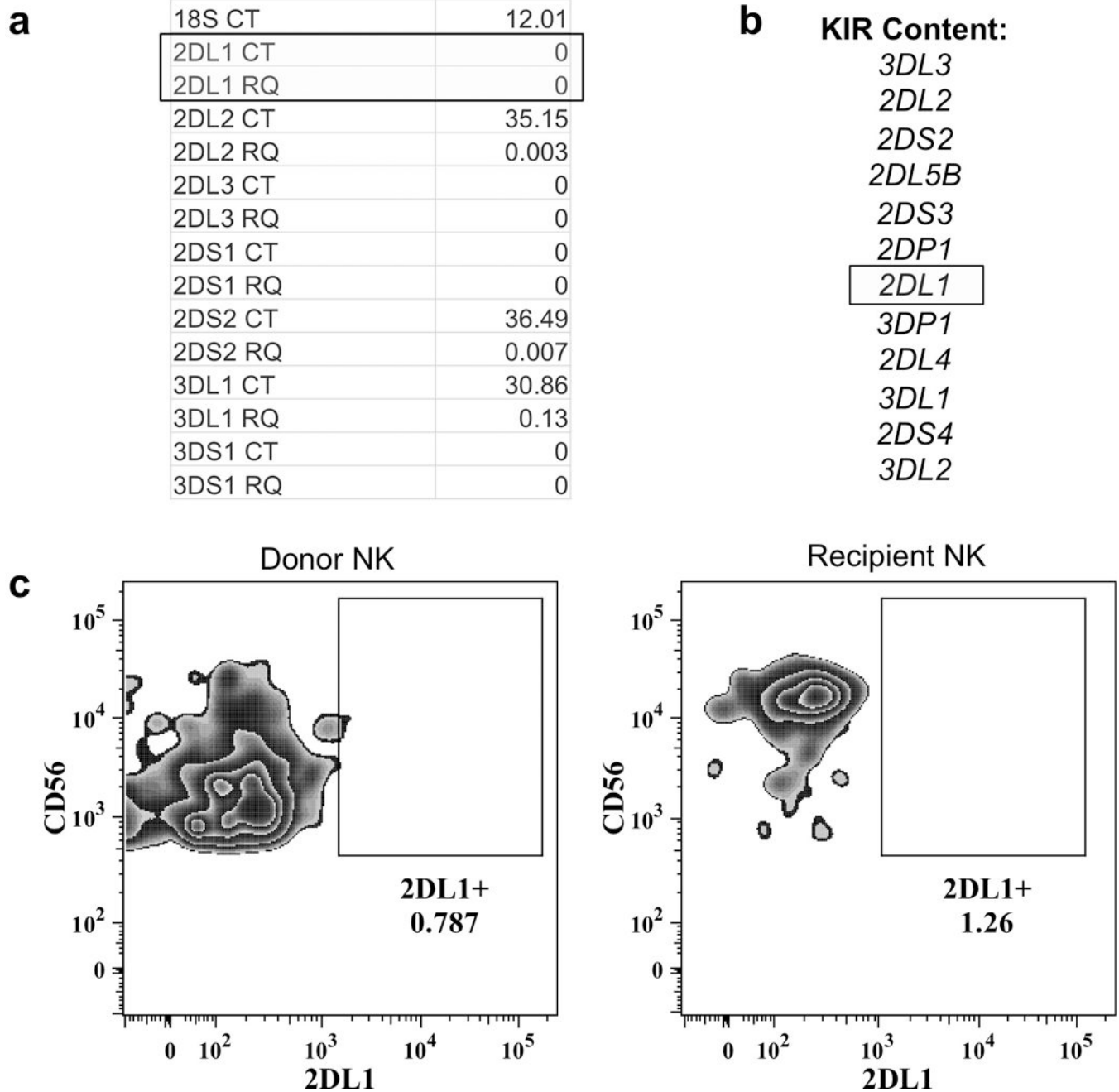
This project has been funded in whole or in part with federal funds from the National Cancer Institute (NCI), NIH, under contract HHSN261200800001E and NCI grant P01 111412 (JSM, SKA). This research was supported in part by the Intramural Research Program of the NIH, NCI, Center for Cancer Research.

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## References

1. Trinchieri G. Biology of natural killer cells. *Adv Immunol.* 1989; 47:187–376. [PubMed: 2683611]
2. Lanier LL. NK cell recognition. *Annu Rev Immunol.* 2005; 23:225–274. [PubMed: 15771571]
3. Carrington, M.; Norman, P. The KIR Gene Cluster. Bethesda, MD: National Library of Medicine (US), NCBI; 2001.
4. Anderson SK, Ortaldo JR, McVicar DW. The ever-expanding *Ly49* gene family: repertoire and signaling. *Immunol Rev.* 2001; 181:79–89. [PubMed: 11513154]
5. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol.* 2005; 5:201–214. [PubMed: 15719024]
6. Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity.* 1997; 7:739–751. [PubMed: 9430220]
7. Held W, Kunz B. An allele-specific, stochastic gene expression process controls the expression of multiple *Ly49* family genes and generates a diverse, MHC-specific NK cell receptor repertoire. *Eur J Immunol.* 1998; 28:2407–2416. [PubMed: 9710218]
8. Santourlidis S, Trompeter HI, Weinhold S, Eisermann B, Meyer KL, Wernet P, et al. Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *J Immunol.* 2002; 169:4253–4261. [PubMed: 12370356]
9. Chan HW, Kurago ZB, Stewart CA, Wilson MJ, Martin MP, Mace BE, et al. DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med.* 2003; 197:245–255. [PubMed: 12538663]
10. Vilches C, Gardiner CM, Parham P. Gene structure and promoter variation of expressed and nonexpressed variants of the *KIR2DL5* gene. *J Immunol.* 2000; 165:6416–6421. [PubMed: 11086080]
11. Gomez-Lozano N, Trompeter HI, de Pablo R, Estefania E, Uhrberg M, Vilches C. Epigenetic silencing of potentially functional *KIR2DL5* alleles: implications for the acquisition of KIR repertoires by NK cells. *Eur J Immunol.* 2007; 37:1954–1965. [PubMed: 17557377]
12. Stulberg MJ, Wright PW, Dang H, Hanson RJ, Miller JS, Anderson SK. Identification of distal KIR promoters and transcripts. *Genes Immun.* 2007; 8:124–130. [PubMed: 17159886]
13. Cichocki F, Hanson RJ, Lenvik T, Pitt M, McCullar V, Li H, et al. The transcription factor c-Myc enhances KIR gene transcription through direct binding to an upstream distal promoter element. *Blood.* 2009; 113:3245–3253. [PubMed: 18987359]
14. Cichocki F, Miller JS, Anderson SK. Killer immunoglobulin-like receptor transcriptional regulation: a fascinating dance of multiple promoters. *J Innate Immun.* 2011; 3:242–248. [PubMed: 21411970]

15. Saleh A, Davies GE, Pascal V, Wright PW, Hodge DL, Cho EH, et al. Identification of probabilistic transcriptional switches in the Ly49 gene cluster: a eukaryotic mechanism for selective gene activation. *Immunity*. 2004; 21:55–66. [PubMed: 15345220]
16. Tanamachi DM, Moniot DC, Cado D, Liu SD, Hsia JK, Raulet DH. Genomic Ly49A transgenes: basis of variegated Ly49A gene expression and identification of a critical regulatory element. *J Immunol*. 2004; 172:1074–1082. [PubMed: 14707081]
17. Davies GE, Locke SM, Wright PW, Li H, Hanson RJ, Miller JS, et al. Identification of bidirectional promoters in the human KIR genes. *Genes Immun*. 2007; 8:245–253. [PubMed: 17315044]
18. Li H, Pascal V, Martin MP, Carrington M, Anderson SK. Genetic control of variegated KIR gene expression: polymorphisms of the bi-directional KIR3DL1 promoter are associated with distinct frequencies of gene expression. *PLoS Genet*. 2008; 4:e1000254. [PubMed: 19008943]
19. Cichocki F, Lenvik T, Sharma N, Yun G, Anderson SK, Miller JS. Cutting edge: KIR antisense transcripts are processed into a 28-base PIWI-like RNA in human NK cells. *J Immunol*. 2010; 185:2009–2012. [PubMed: 20631304]
20. Williams TM, Moolten D, Burlein J, Romano J, Bhaerman R, Godillot A, et al. Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science*. 1991; 254:1791–1794. [PubMed: 1840704]
21. Remacle JE, Kraft H, Lerchner W, Wuytens G, Collart C, Verschueren K, et al. New mode of DNA binding of multi-zinc finger transcription factors: deltaEF1 family members bind with two hands to two target sites. *EMBO J*. 1991; 18:5073–5084. [PubMed: 10487759]
22. Estefanía E, Flores R, Gomez-Lozano N, Aguilar H, Lopez-Botet M, Vilches C. Human KIR2DL5 is an inhibitory receptor expressed on the surface of NK and T lymphocyte subsets. *J Immunol*. 2007; 178:4402–4410. [PubMed: 17371997]
23. Czaja K, Borer AS, Schmied L, Terszowski G, Stern M, Gonzalez A. A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs. *Genes Immun*. 2014; 15:33–37. [PubMed: 24173145]
24. Stewart CA, Van Bergen J, Trowsdale J. Different and divergent regulation of the KIR2DL4 and KIR3DL1 promoters. *J Immunol*. 2003; 170:6073–6081. [PubMed: 12794136]
25. Radeloff B, Nagler L, Zirra M, Ziegler A, Volz A. Specific amplification of cDNA ends (SPACE): a new tool for the analysis of rare transcripts and its application for the promoter analysis of killer cell receptor genes. *DNA Seq*. 2005; 16:44–52. [PubMed: 16040346]
26. Pascal V, Stulberg MJ, Anderson SK. Regulation of class I major histocompatibility complex receptor expression in natural killer cells: one promoter is not enough! *Immunol Rev*. 2006; 214:9–21. [PubMed: 17100872]
27. Fischer JC, Ottinger H, Ferencik S, Sribar M, Punzel M, Beelen DW, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *J Immunol*. 2007; 178:3918–3923. [PubMed: 17339492]
28. van Bergen J, Stewart CA, van den Elsen PJ, Trowsdale J. Structural and functional differences between the promoters of independently expressed killer cell Ig-like receptors. *Eur J Immunol*. 2005; 35:2191–2199. [PubMed: 15940669]
29. Martin MP, Carrington M. KIR locus polymorphisms: genotyping and disease association analysis. *Methods Mol Biol*. 2008; 415:49–64. [PubMed: 18370147]
30. Cooley S, Xiao F, Pitt M, Gleason M, McCullar V, Bergemann TL, et al. A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature. *Blood*. 2007; 110:578–586. [PubMed: 17392508]



**Figure 1.**

Identification of a poorly expressed *KIR2DL1* allele. ( **a** ) qRT-PCR performed on NK cell RNA from a NMDP donor. ( **b** ) *KIR* gene content typing for this individual. ( **c** ) FACS analysis performed with a *KIR2DL1*-specific antibody on donor PBMC in the left panel, and PBMC from the corresponding hematopoietic transplant recipient in the right panel, demonstrating an identical pattern of *KIR2DL1* expression in reconstituting donor cells.

2DL1\*004 C TCGAAACCAATCACCAGGAATAGTGAAGGATGACCGTATATGACTTTTATTCTCAAATTTAAATATAAATCATAAAAAATGTAC  
 2DL1-new C  
 3DP1 A C

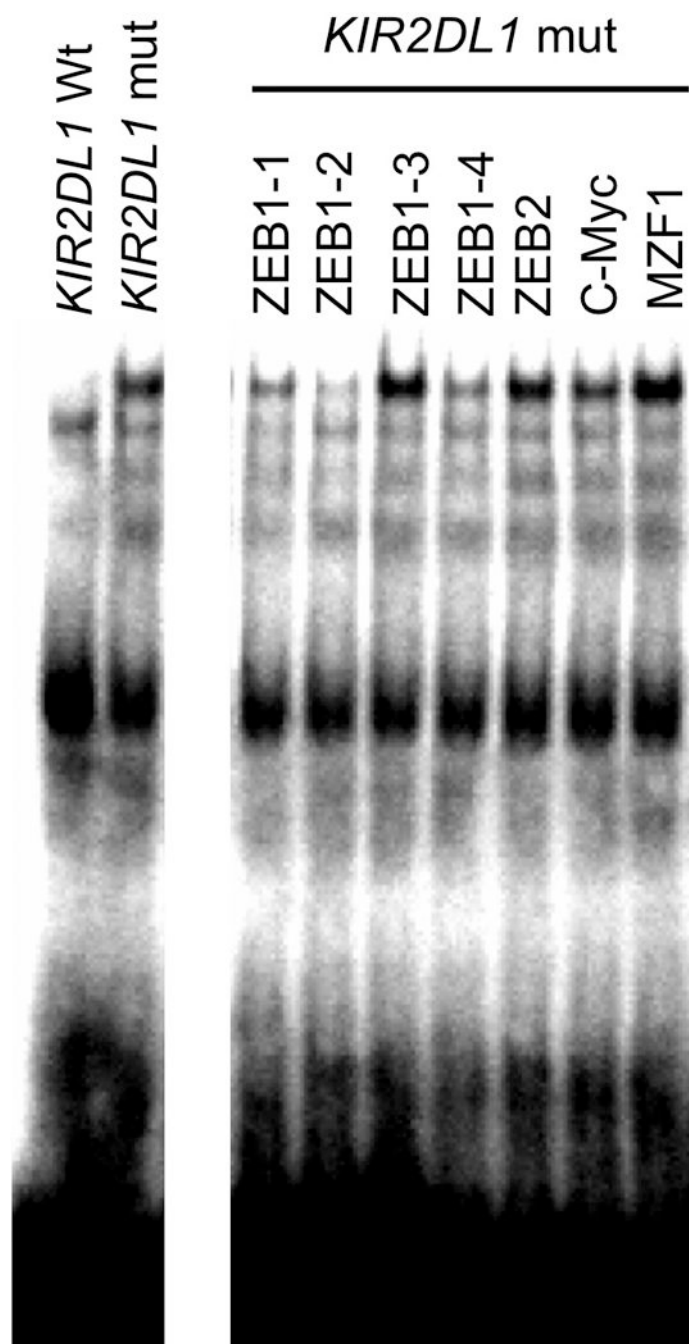
2DL1\*004 AACTAGATAAAAC TAAGAAGTGT TTTTATAGTGTGAGTTAGATTATTTTTCCTAGGTATAACCCATTGGTTTAATATTATTAT  
 2DL1-new G A  
 3DP1 G A

2DL1\*004 TGAGAAGACATTCTATGCCACCTTAAACACACGSCAGCCTTTGTCAACTCTAAAGGGACTGTGTGTACATGGATGTACTTTAGACA  
 2DL1-new  
 3DP1 T

ZEB1  
 ZEB1 Myc/USF

**Figure 2.**

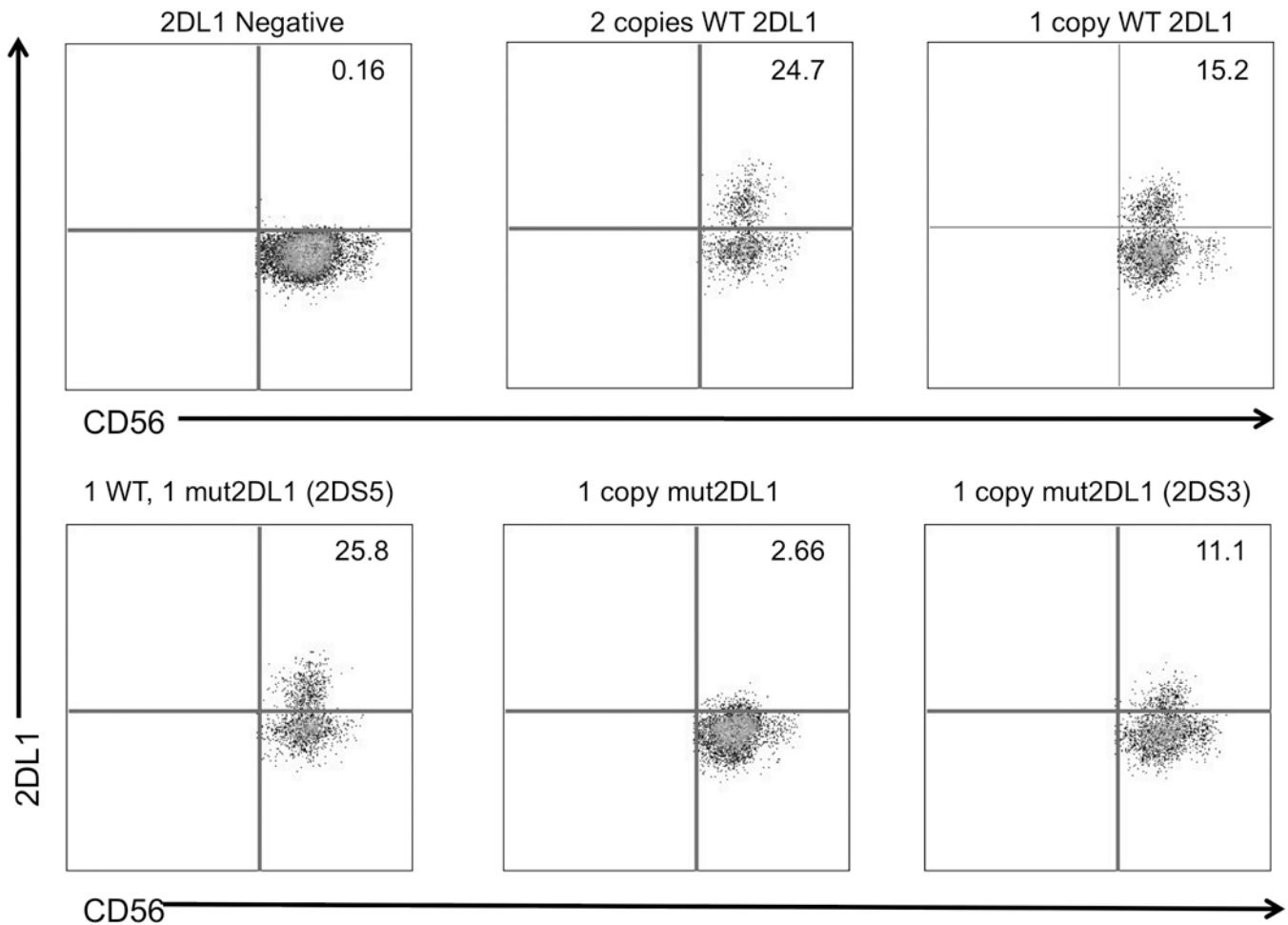
A non-transcribed *KIR2DL1* allele is generated by recombination between *KIR2DL1\*004* and *KIR3DP1*. The sequence of the region from -1353 to -1093 relative to the translational start of the *KIR2DL1\*004* gene (2DL1\*004) is shown, and only nucleotide differences are shown for the novel *KIR2DL1* allele (2DL1-new) and the non-transcribed *KIR3DP1* gene (3DP1). The *KIR3DP1*-derived region of the novel *KIR2DL1* allele is shown in bold, and the underlined flanking sequences indicate the regions where recombination may have occurred to generate the novel *KIR2DL1* allele. The boxed sequences represent the consensus ZEB1 binding half sites in opposing orientations, followed by a Myc/USF site previously shown to play a role in distal promoter activity. The upstream ZEB1 site is created in the 2DL1-new and 3DP1 sequences due to the A to G SNP.



**Figure 3.**

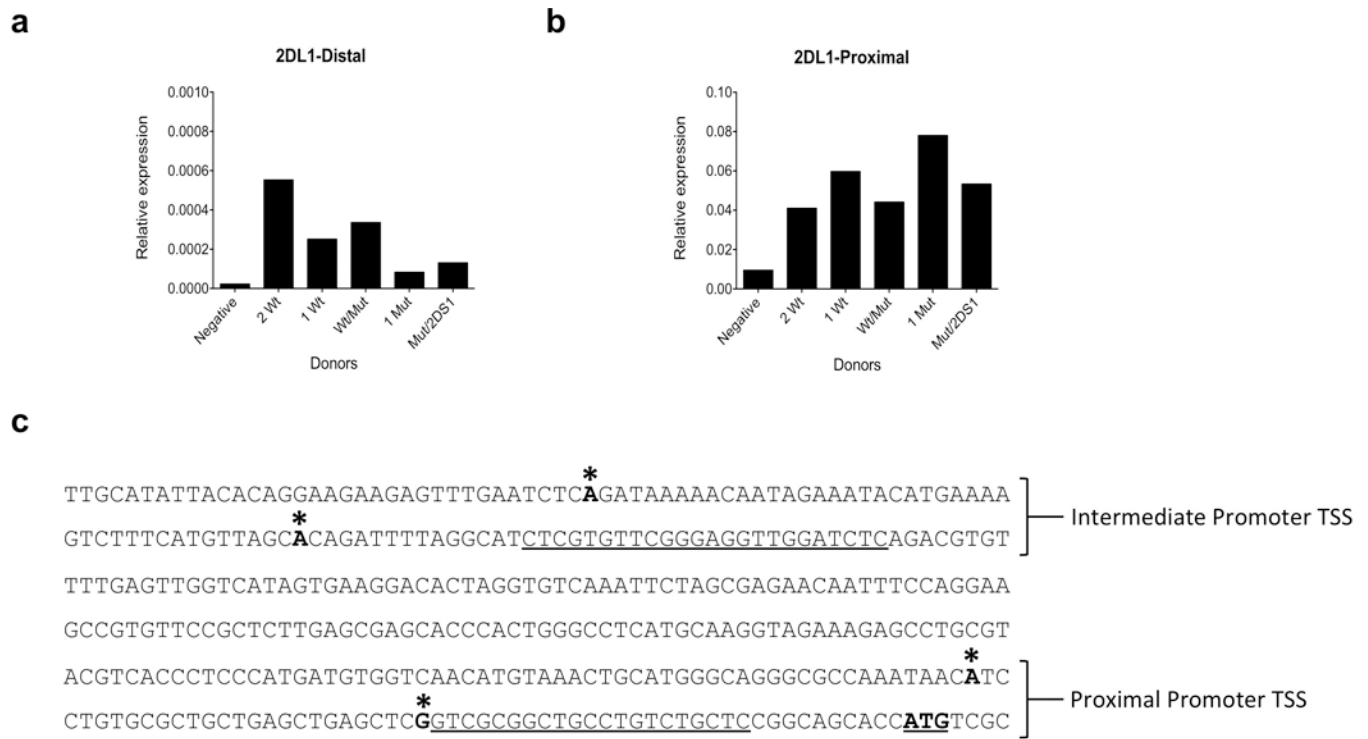
The *KIR2DL1* SNP creates a ZEB-1 binding site. An oligonucleotide probe containing the wild-type (wt) or mutant (mut) distal promoter region was combined with nuclear extract from YT cells. The left panel shows the appearance of a novel band associated with the mutant SNP. The right panel shows the ability of several commercial anti-ZEB antibodies (ZEB1-1 to ZEB1-4, ZEB2), together with anti-C-Myc or MZF-1 controls to inhibit formation of the novel complex.





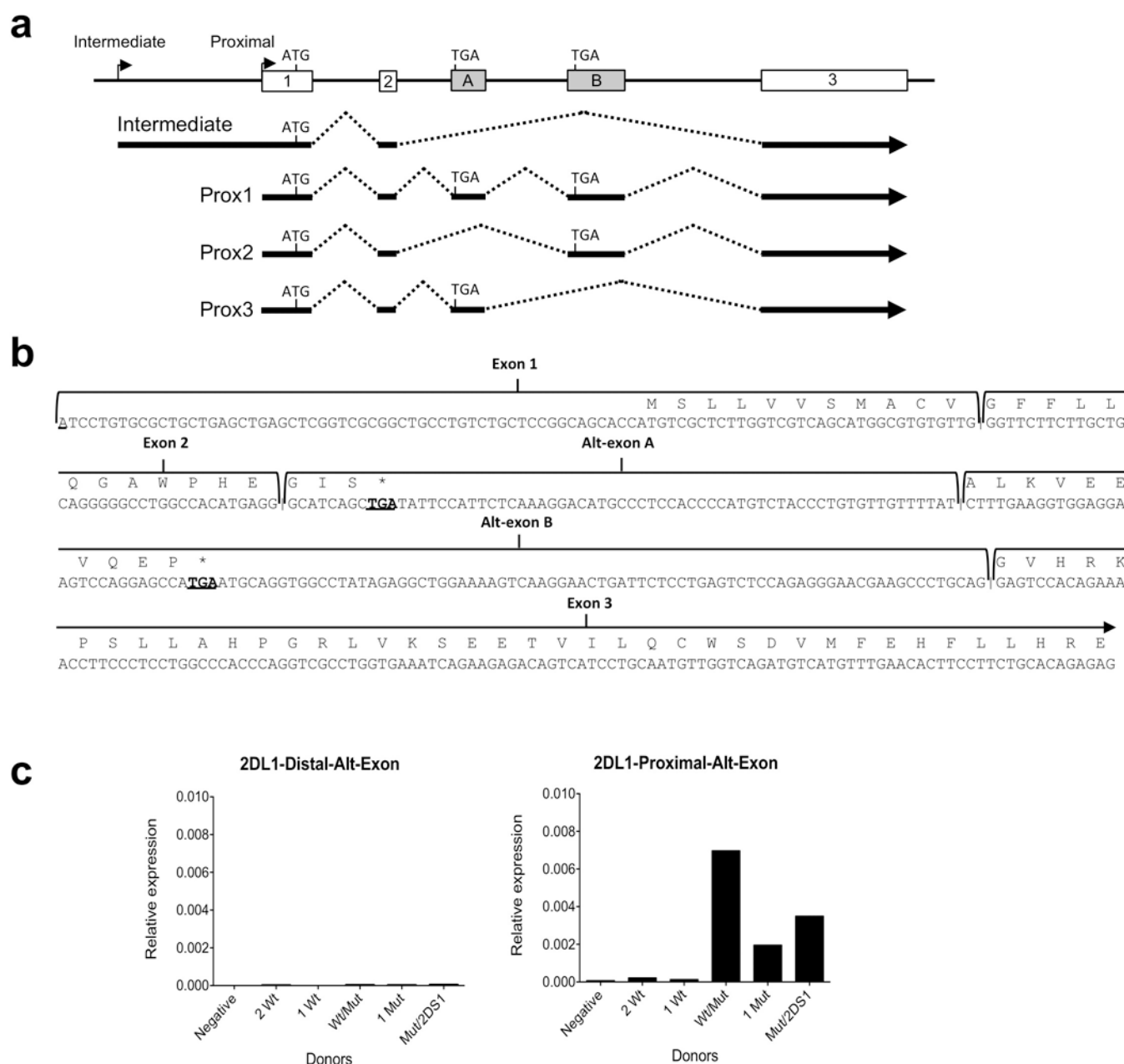
**Figure 4.**

The *KIR2DL1* SNP is associated with weak expression. Donors were screened by PCR with primers specific for the ZEB1 SNP. The upper panel shows three donors with 0, 1, or 2 copies of the wild type *KIR2DL1* gene (WT 2DL1) stained with an NK marker (CD56) on the horizontal axis, and a *KIR2DL1*-specific antibody on the vertical axis. The number shown in the upper right quadrant represents the percentage of NK cells reacting with the 143211 anti-KIR2DL1 antibody. The lower panel shows three donors found to possess the ZEB1 SNP. Lack of expression is evident in the individual containing only a mutant *KIR2DL1* gene (1 copy mut 2DL1). The presence of either *KIR2DS5* or *KIR2DS3* genes in two of the donors is also indicated, as these *KIR* gene products have been shown to react with the 143211 anti-KIR2DL1 antibody<sup>23</sup>.



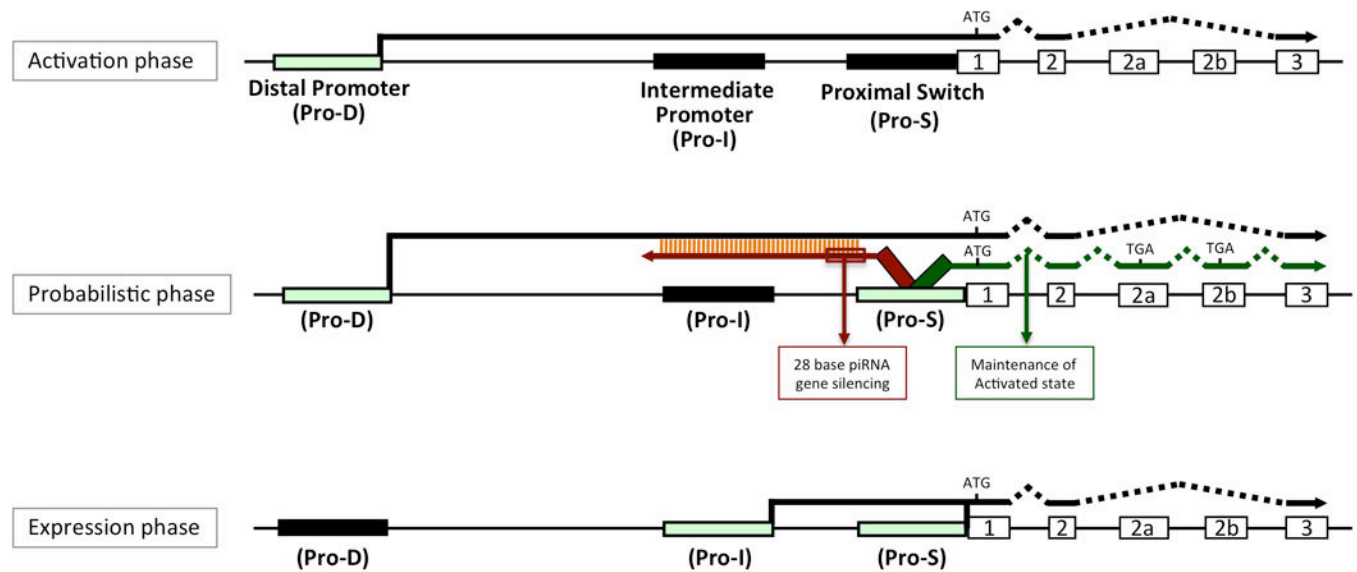
**Figure 5.**

The *KIR2DL1* SNP is associated with decreased distal transcript expression. Purified NK cell RNA from purified donor NK cells was subjected to qRT-PCR with *KIR2DL1*-specific primers detecting distal (**a**) or proximal (**b**) transcripts. Expression relative to actin is shown on the Y-axis, and *KIR2DL1/KIR2DS1* genotype of individual donors is listed on the x-axis. Donors were: lacking both *KIR2DL1/KIR2DS1* (negative); two copies of wild-type *KIR2DL1* (2Wt); one copy of Wt *KIR2DL1* (1Wt); one Wt, one ZEB1-mutant *KIR2DL1* copy (Wt/Mut); one copy of a mutated *KIR2DL1* (1 Mut); and one copy of mutated *KIR2DL1* and one copy of *KIR2DS1* (Mut/2DS1). (**c**) Transcription start sites (TSS) mapped by 5' RACE are shown for the 365 bp region immediately 5' of the *KIR2DL1* start codon (ATG). TSS are indicated by an asterisk. The underlined sequences represent forward PCR primers used for the detection of distal and proximal transcripts in **a** and **b**.



**Figure 6.** *KIR2DL1* proximal promoter transcripts contain additional exons that disrupt the *KIR2DL1* open reading frame. **(a)** A schematic of the *KIR2DL1* gene structure is shown. Numbered rectangles represent *KIR2DL1*-coding exons with the start codon in exon 1 labeled 'ATG', whereas the shaded rectangles marked 'A' and 'B', represent alternative exons containing stop codons indicated by 'TGA'. Intermediate and proximal transcript initiation sites are marked by vertical lines with arrows attached. Lines below the *KIR2DL1* gene diagram represent cDNA clones isolated from purified NK cell RNA obtained from an individual with a single copy of the *KIR2DL1* gene containing the ZEB1 SNP. Three distinct isoforms of the *KIR2DL1* proximal transcript were identified (Prox1-3), each containing different

combinations of the alternative exons. **(b)** The nucleotide sequence of the Prox1 transcript is shown, with the KIR2DL1 open reading frame indicated by the amino acid sequence listed above. Exon boundaries are marked by vertical lines, and each exon is defined by labeled brackets. The stop codons in alternative exons A and B (Alt-exon A/B) are underlined in bold. **(c)** qRT-PCR of alternative exon-containing transcripts using either a distal (2DL1-Distal-Alt-Exon) or proximal (2DL1-proximal-Alt-Exon) *KIR2DL1* forward primer together with an alternate exon B reverse primer. Expression relative to actin is shown on the Y-axis, and KIR2DL1/KIR2DS1 genotype of individual donors is listed on the x-axis as described in Figure 5b.



**Figure 7.**

Three promoters define distinct stages of *KIR* activation. Schematic of the 5' control region and first 3 *KIR*-coding exons representing three phases of *KIR* gene transcription (Activation phase; Probabilistic phase; Expression phase) are shown. Promoters are indicated by filled rectangles, and light green rectangles highlight promoters that define the proposed phases of expression. The site of initiation of *KIR* protein translation is indicated by a vertical line labeled "ATG", and the location of premature stop codons in alternative exons found in forward transcripts from Pro-S are indicated by "TGA". Black lines ending in arrows represent sense coding transcripts, the red and green lines indicate antisense and sense transcripts from Pro-S respectively. The red and green colored boxes indicate the proposed effects of the antisense and sense transcripts on gene activity.