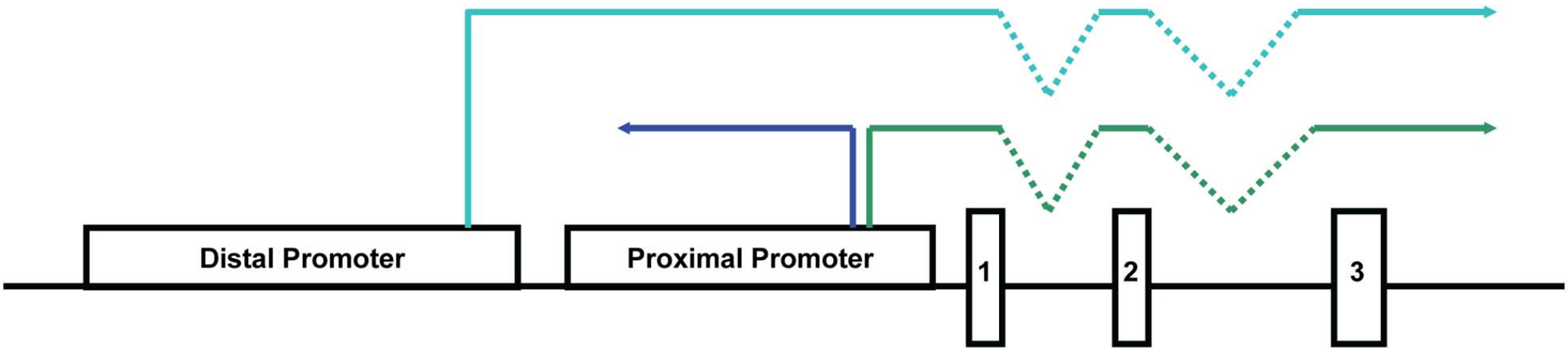
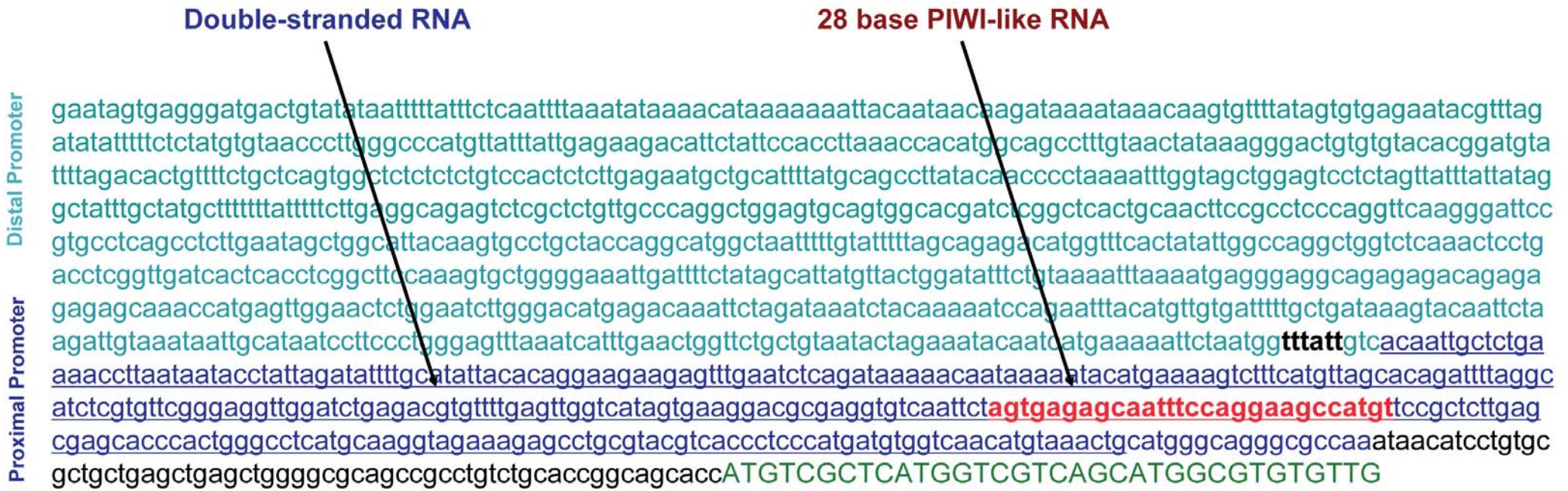
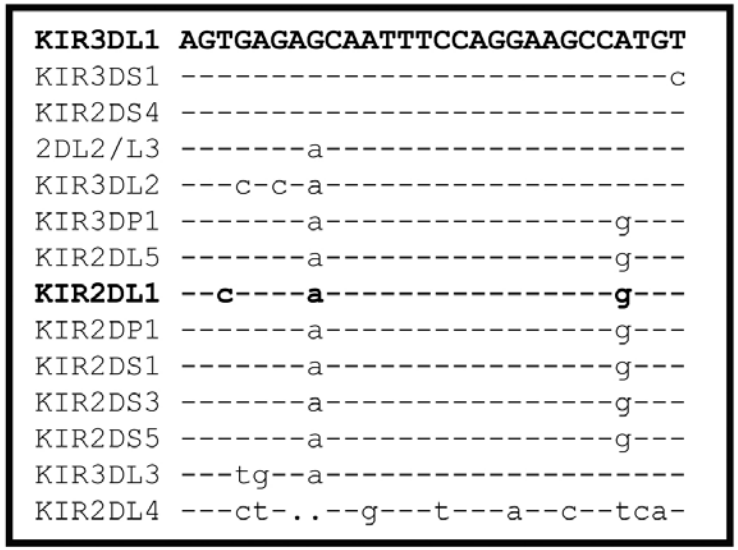


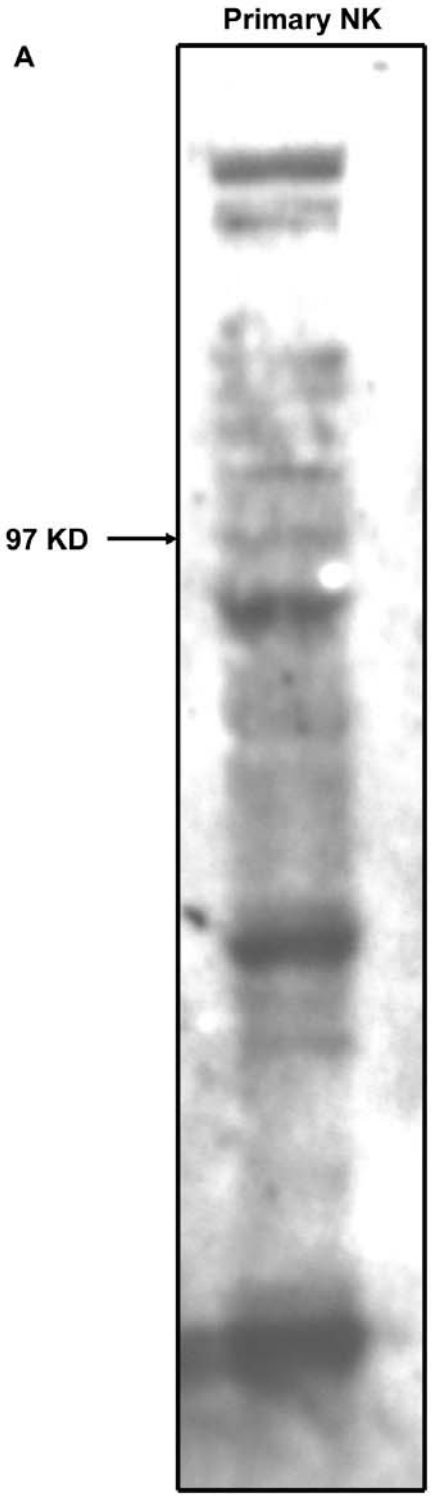
Supplemental Figure 1. Schematic of transcripts originating within the 5' upstream regulatory region of the *KIR3DL1* gene. Forward transcripts initiating within the bi-directional proximal promoter are shown in green. Antisense transcripts initiating within the bi-directional proximal promoter are shown in dark blue. Transcripts initiating from the distal promoter are shown in light blue.

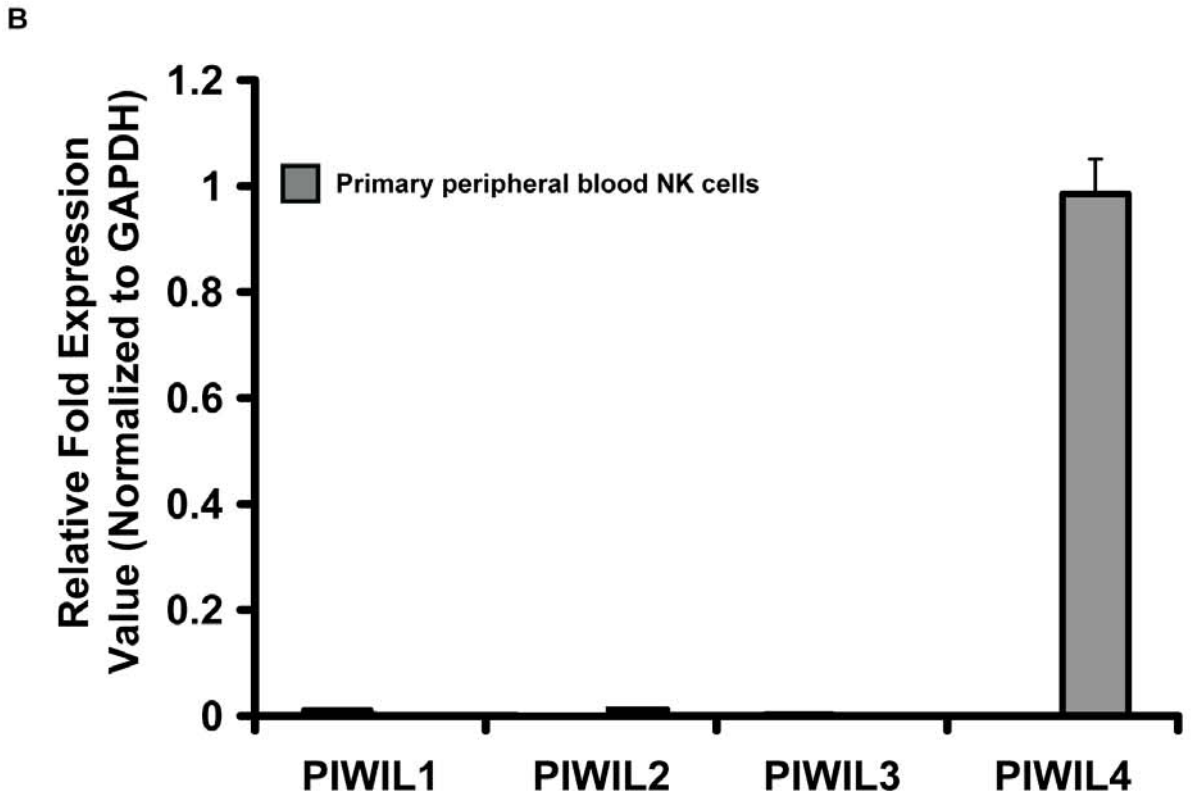


Supplemental Figure 2. The 1.3 kb 5' regulatory region upstream of the *KIR3DL1* gene is shown. The distal promoter region is shaded in light blue and extends from -370 to -1363 base pairs upstream of the transcriptional start site. The proximal promoter region is shaded in dark blue and extends from -1 to -369. The poly-A signal sequence for the *KIR3DL1* antisense transcript is highlighted in black bold font. Double-stranded RNA generated from distal transcripts and proximal antisense transcripts, as detected in an S1 nuclease protection assay, is underlined and spans the region from -18 to -306. The 28 base RNA that is processed from proximal antisense transcripts is shown in red and extends from -75 to -102. The beginning of the first *KIR3DL1* exon is shown in green. For the S1 nuclease protection assay, nuclear RNA was isolated using the PARIS kit (Ambion), followed by a Dnase (Invitrogen) digestion. Reverse transcription was performed using Superscript III (Invitrogen) followed by a 37°C incubation overnight to allow self-annealing and the formation of double-stranded cDNAs with complementary sequences. DNA was extracted with phenol-chloroform and digested for 4 h at 37°C with Nuclease S1 (New England Biolabs) to remove unhybridized single stranded cDNA. DNA was then treated with T4 Polymerase (Invitrogen) for 15 min at 37°C to form blunt ends. To make the assay specific for the detection of sequences homologous to the *KIR3DL1* promoter, we used a "blockerette" ligation and PCR amplification strategy. The gene-specific primers used in this assay were *5'-TTTTATCTGAGATTCAAACTCTTCTTCCTGTGT-3'* and *5'-AATATGCAAAATATCTAATAGGTATTATTAAGGTTTTCAG-3'*.

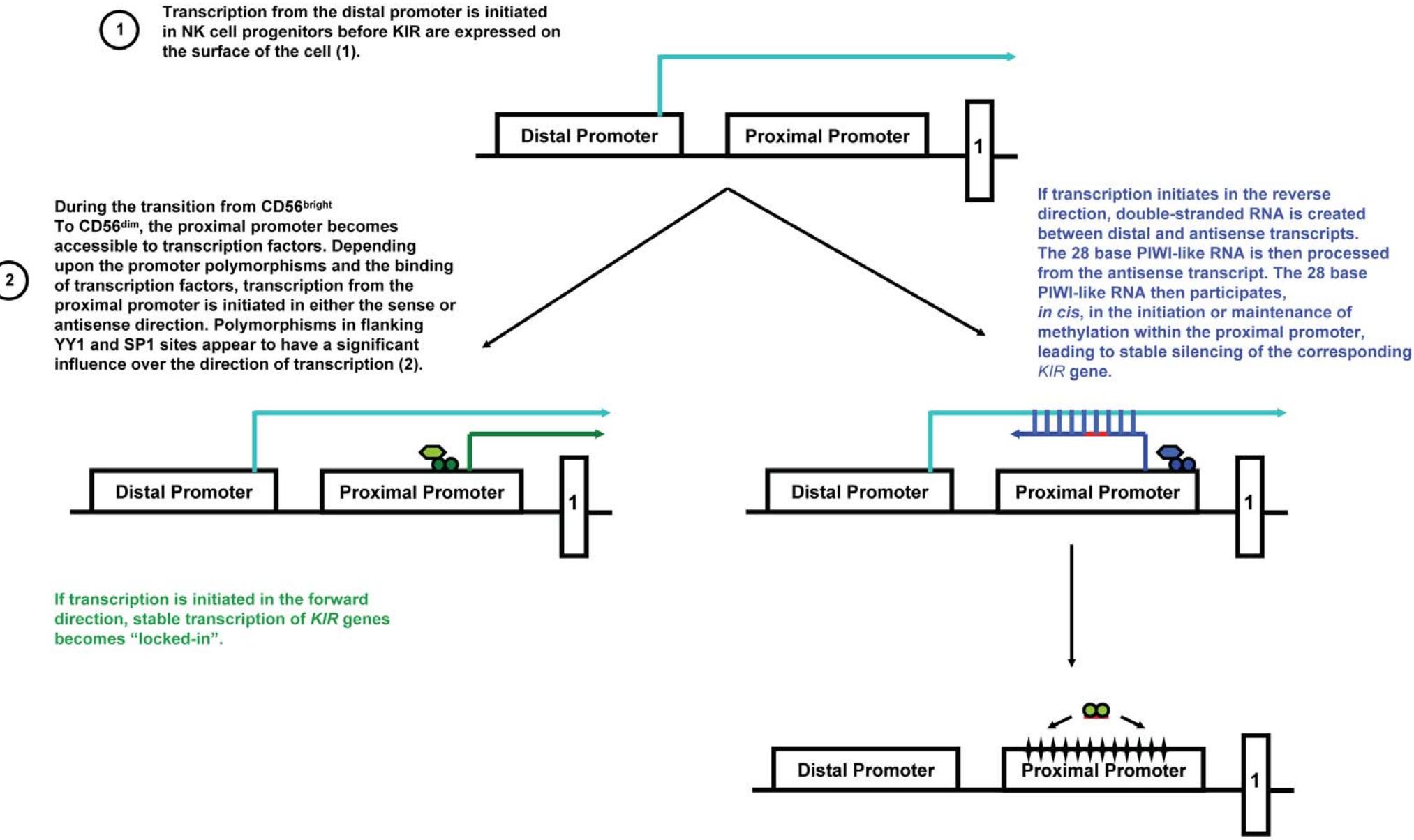


Supplemental Figure 3. An alignment of the 28 base PIWI-like RNA sequence against homologous regions of 14 other *KIR* genes present in the leukocyte receptor complex. Sequences highlighted in bold represent genes for which we have cloned 28 base RNA sequences. **For the initial discovery of the *KIR3DL1* 28 base PIWI-like RNA**, total RNA was isolated from HEK293 cells using RNA STAT-60 (Tel-Test Inc.) according to the manufacturer's instructions. The small RNA fraction was isolated using the Flash Page Fractionator (Ambion). Polyadenylation of the small RNA was carried out with an A-Plus™ Poly (A) Polymerase Tailing Kit (EPICENTRE® Biotechnologies). Following polyadenylation, a 5' RNA adaptor *5'-CGACUGGAGCACGAGGACACUGACAGUGACUGAAGGAGUAGAAA-3'* was added using T4 ligase I (New England Biolabs). Reverse transcription of precipitated RNA was carried out with RT primer *5'-ATTCTAGAGGCCGAGGCGGCCGACATC-d(T)$_{30}$VN-3'* at 50°C for 50 minutes. The cDNA was PCR-amplified using primers 5cDNA *5'-GGACACTGACATGGACTGAAGGAGTA-3'* and 3cDNA *5'-ATTCTAGAGGCCGAGGCGGCCGACATGT-3'*. The PCR product was was separated on a 12% PAGE gel containing 8M urea. The gel slice between 100-120 bp was cut out, and the DNA was eluted. The eluted PCR product was cloned into the TOPO-TA vector (Invitrogen), and colony hybridization was carried out using a *KIR3DL1* probe labeled with α-^{32}P using the Random Primer Labeling Kit (Invitrogen). Bacterial colonies were transferred to a nylon membrane, and hybridization was performed overnight. The membranes were washed and exposed to X-Ray film (Kodak) for 24 to 36 hours, and positive clones were selected. **For the cloning of the *KIR3DL1* 28 base PIWI-like RNA in human NK cells**, small (<40 base) RNAs from CD56^{+} NK cells were isolated using the PureLink™ miRNA isolation kit according to the manufacturer's instructions (Invitrogen). Small RNAs were incubated for 10 minutes at 37°C with 5 U *E. coli* Poly(A) polymerase (New England Biolabs) and 1mM rATP (Ambion) to catalyze the addition of poly(A) tails to the 3' end of the RNAs. cDNA was then generated using Superscript™ III Reverse Transcriptase (Invitrogen) and an oligo d(T)$_{14}$ primer with a unique anchor sequence: *5'-CTTGACCTCATCTGACCACTCACCTCACTTTTTTTTTTTTT-3'*. The cDNA was linear-amplified with a *KIR3DL1* 28 base gene-specific primer and a primer specific for the oligo d(T)$_{14}$ primer with a unique anchor sequence: *5'-CTTGACCTCATCTGACCACTC-3'*. PCR products were cloned into the TOPO-XL vector (Invitrogen) and sequenced. To amplify and clone the control RNA used in the periodate oxidation/β-elimination, the same methods were used. The gene-specific primer for the control RNA was *5'-GACGAATGCACGTAATGCAG-3'*.





Supplemental Figure 4. (A) Western blot analysis of PIWIL4 protein expression using protein from human peripheral blood $CD56^+$ NK cells. PIWIL4 is a 97 KD protein (B) Quantitative RT-PCR of mRNA expression of all 4 members of the PIWI protein family in primary peripheral blood $CD56^+$ cells. Values are normalized to GAPDH, and error bars represent the standard error values from three individuals. For the Western blot, protein lysate from approximately 0.5 x 10^6 cells was separated on a NuPAGE 10% Bis-Tris Gel (Invitrogen) and transferred to Trans-Blot nitrocellulose paper (BioRad) using Xcel Blot (Invitrogen) for 1 hour at 30 V constant. A 1:500 dilution of the polyclonal rabbit anti-human PIWIL4 antibody (Abcam) was blocked overnight with 3% milk in PBS + 0.1% Tween and then used to stain the transferred protein overnight at 4ºC. The nitrocellulose paper was washed with PBS + 0.1% Tween and then stained with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE healthcare) for 4 hours at 4ºC. The Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher) was used for visualization via a 10 minute exposure with Kodak Scientific Imaging Film (Perkin Elmer Life Sciences).



Supplemental Figure 5. Model for the regulation of stable *KIR* expression patterns by intergenic transcription

1. Cichocki F., R.J. Hanson, T. Lenvik, M. Pitt, V. McCullar, H. Li, S.K. Anderson, J.S. Miller. 2009. The transcription factor c-Myc enhances KIR gene transcription through Direct binding to an upstream distal promoter element. *Blood.* 113:3245-3253.
2. Li, H., V. Pascal, M.P. Martin, M. Carrington, S.K. Anderson. Genetic control of variegated KIR expression: polymorphisms of the bi-directional KIR3DL1 promoter are associated with distinct frequencies of gene expression. *PLoS Genet.* 4:Epub

Supplemental Table I

Primer Name	Primer Sequence (5'-3')
KIR3DL1/S1-Specific RT Primer	TGGTTTATT(A)GTCACAATTG
KIR3DL1 Antisense Transcript qRT-PCR Forward	ATTGTCACAATTGCTCTGAAAACC
KIR3DL1 Antisense Transcript qRT-PCR Reverse	CATGGCTTCCTGGAAATTGC
KIR3DL1 Antisense Transcript qRT-PCR Probe	(FAM)-CATGTTAGCACAGATTTTAGGCATCTCGTG-(MBG)
KIR3DL1 S1 Nuclease Protection Forward	TTTTATCTGAGATTCAAACTCTTCTTCCTGTGT
KIR3DL1 S1 Nuclease Protection Reverse	AATATGCAAAATATCTAATAGGTATTATTAAGGTTTTCAG
5' RNA Adaptor for 28 Base RNA Cloning	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA
RT Primer for 28 Base RNA Cloning	ATTCTAGAGGCCGAGGCGGCCGACATG $d(T)_{30}VN$
Forward PCR Primer for 28 Base RNA Cloning	GGACACTGACATGGACTGAAGGAGTA
Reverse PCR Primer for 28 Base RNA Cloning	ATTCTAGAGGCCGAGGCGGCCGACATGT
RT Primer for 28 Base RNA Cloning from Human NK Cells	CTTGACCTCATCTGACCACTCACCTCACTTTTTTTTTTTTTTT
KIR3DL1 28 Base RNA Gene-Specific Primer	ACATGGCTTCCTGGAAATTG
Anchor Primer for 28 Base RNA Cloning from Human NK Cells	CTTGACCTCATCTGACCACTC
Control RNA Gene-Specific Cloning Primer	GACGAATGCACGTAATGCAG
piRNA 28 Base Top Oligo for pLVTHM Cloning	CGCGACATGGCTTCCTGGAAATTGCTCTCACT
piRNA 28 Base Bottom Oligo for pLVTHM Cloning	CGAGTGAGAGCAATTTCCAGGAAGCCATGT
Random 28 Base Top Oligo for pLVTHM Cloning	CGCGATCTGAGATTCAAACTCTTCTTCCTGTG
Random 28 Base Bottom Oligo for pLVTHM Cloning	CGCACAGGAAGAAGAGTTTGAATCTCAGAT

Supplemental Table I

KIR3DL1 Antisense Forward Primer for MSCV Cloning	TAATACTAGAAATACAATCATGAAAATTC
KIR3DL1 Antisense Reverse Primer for MSCV Cloning	CACCGCAGGCTCTTTCTACCTTGCA
PIWIL1 Forward Primer	GACTGGGGTTTGAGCTTTGATTCC
PIWIL1 Reverse Primer	TTATTGCTTTTCTCATTTGCATGCC
PIWIL2 Forward Primer	ATCCTTTCCGACCATCGTTC
PIWIL2 Reverse Primer	GTCCTTGCGTACCAGATTAGC
PIWIL3 Forward Primer	TACAGTGGTACAGCTACTCGCCAAC
PIWIL3 Reverse Primer	CGACGTGGGCGTGAGTTCT
PIWIL4 Forward Primer	AATGCTCGCTTTGAACTAGAGAC
PIWIL4 Reverse Primer	ATTTTGGGGTAGTCCACATTAAATC
GAPDH Forward Primer	TGTCTCCTGCGACTTCAACAGC
GAPDH Reverse Primer	TGTAGGCCATGAGGTCCACCAC