INTRODUCTION
Natural killer (NK) cells are an important element of the immune system allowing for a rapid and innate response against virally infected and tumor cells.1 The NK cell cytotoxic response is controlled by the relative strength of competing transcription factor binding sites.7 Forward transcription from Pro1 produces a non-coding antisense transcript that likely possesses transcriptional activity in genes not associated with regions having transcriptional start site (TSS) at Pro2,11 additional studies suggest that there is no single TSS but rather that transcription can begin in genes not associated with regions possessing transcriptional activity in in vitro promoter assays.12 Traditional promoters were identified in genes not associated with probabilistic expression, including the activating Ly49d and h genes, as well as the non-NK genes Ly49b and q, suggesting that probabilistic expression may be connected with the lack of clearly defined promoter elements. The Pro1 upstream promoter element found in variegated Ly49 genes was shown to be active in mature NK cells and function as an enhancer element, suggesting that it may have a role in Ly49 transcript initiation in mature NK cells.13

In the current study, we assess the total Ly49 transcriptional landscape of mature Ly49-expressing NK cells by RNA-sequencing, revealing that Pro1 transcripts are very rare in mature NK cell populations, and further demonstrate that Pro1 lacks enhancer activity. We also investigate the unusual properties of the Ly49i gene, characterizing a novel Ly49i promoter (designated Pro2') preceding exon −1b and identifying rare antisense transcripts originating from the core promoters Pro2 and Pro3.
RESULTS

RNA-sequencing of sorted Ly49G- versus Ly49I-expressing splenic NK cells.

The majority of previous studies of RNA expression by murine splenic NK cells have made use of gene arrays to assay gene expression profiles, and are therefore lacking information with regard to promoter utilization, alternative splicing, rare transcripts and do not effectively discriminate between closely related Ly49 gene transcripts. To obtain a more precise determination of all Ly49 transcripts present in mature splenic NK cells, RNA was isolated from freshly isolated Ly49G- or Ly49I-expressing splenic NK cells, to avoid artifacts associated with culture of NK cells in cytokines. The Ly49G-specific monoclonal antibody 4D11 (ref. 14) and the Ly49C/I-specific monoclonal 5E6 (ref. 15) were used to sort 4D11+/5E6− versus 5E6+/4D11− NK cell subsets from C57BL/6 mice (Figure 1). These anti-Ly49 antibodies were chosen owing to their ability to recognize a substantial fraction of the NK cells present in C57BL/6 spleen (50% of NK for 4D11, 48% for 5E6). The sorted populations shown in Figure 1. The relative enrichment of Ly49 transcripts in the G+/I− subset is likely due to the low inhibitory signaling present in these cells, thus allowing a greater window of opportunity for receptors that have a lower probability of activation, such as the increased expression of Ly49F on unlicensed NK cells.21

Ly49 expression is not associated with Pro1 transcription

To assess directly if Pro1 transcripts can be found in mature Ly49C-expressing NK cells, the RNA sequence data spanning the complete Ly49 gene cluster was analyzed in detail, including a survey of individual sequences to identify novel transcripts that are not annotated in the reference mouse genome. Figure 2 shows a summary of Ly49g and Ly49i sense transcripts detected by RNA-sequencing of freshly isolated Ly49G- or Ly49I-expressing splenic NK cells. The number of spliced RNA sequences spanning 2 exons as detected by Sashimi analysis are shown. The majority of Ly49i transcripts originated within exon −1b, as previously reported by Gays et al.,12 whereas Ly49g transcripts originated primarily in exons 1, indicating that Pro2 is the dominate promoter

| Gene name | Protein | Function | Fold change | P-value |
|-----------|---------|----------|-------------|---------|
| Klra9     | Ly49i   | Inhibitory | −13.64      | 1.89E − 12 |
| Klra14-ps | Ly49N   | Pseudogene | 1.00        | 0.868   |
| Klra5     | Ly49E   | Inhibitory | 1.01        | 0.098   |
| Klra3     | Ly49C   | Inhibitory | 1.24        | 0.003   |
| Klra8     | Ly49H   | Activating | 1.30        | 0.007   |
| Klra10    | Ly49I   | Inhibitory | 1.34        | 0.030   |
| Klra13-ps | Ly49M   | Pseudogene | 1.63        | 1.51E − 04 |
| Klra4     | Ly49D   | Activating | 1.77        | 1.19E − 05 |
| Klra6     | Ly49F   | Inhibitory | 1.81        | 3.70E − 05 |
| Klra1     | Ly49A   | Inhibitory | 2.47        | 7.40E − 07 |
| Klra7     | Ly49G   | Inhibitory | 86.89       | 5.91E − 14 |

Abbreviation: NK, natural killer.
in splenic NK cells. The partial masking of Ly49I by H-2b is suggested by the presence of a low level of Ly49I-coding transcripts in the G″/I″ population (~5% of the transcript level in I″/G″), indicating that a subset of the Ly49I-expressing NK cells are not efficiently recognized by the 5E6 antibody. In contrast, potential Ly49G-coding RNAs were rare in the I″/G″ population, and these RNAs did not exceed the level of non-translated Pro1 transcripts, indicating a lack of translatable Ly49g mRNAs in this population, consistent with the efficient detection of Ly49G by the 4D11 antibody.

A summary of the spliced 5′ RNA sequences observed in inhibitory Ly49 genes is shown in Figure 3. Ly49 Pro1 transcripts containing exon – 1a were found for only two genes, Ly49e and Ly49g. The complete lack of Pro1 transcripts in Ly49i-expressing cells indicates that Pro1 transcriptional activity is not required for gene expression. The presence of Pro1 transcripts from the Ly49e and Ly49g genes in Ly49i-expressing splenic NK cells may be related to the ability of these genes to be activated in mature NK cells by stimulation with IL-2,24,25 suggesting that the Pro1 transcripts could be derived from cells that have received a stimulatory signal, and are in the process of gene activation. Notably, there is no significant difference in the level of Pro1 transcripts between G″/I″ and I″/G″ subsets, even though there are 87-fold more Ly49g transcript reads in the G″/I″ population. The low level of Ly49g Pro1 transcription likely indicates a small population of cells that are in the process of activating the Ly49g gene. In the G″/I″ cells, this would represent activation of a second Ly49g allele, as previously observed for monoallelic Ly49G″-expressing NK cells after culture in IL-2.25

Detection of novel Ly49i transcripts

RNA-sequencing of Ly49i-expressing splenic NK cells revealed the unexpected presence of antisense Ly49i transcripts originating from the Pro2 and Pro3 regions spliced to two antisense exons upstream of Pro1 that will be referred to as antisense exons 2 and 3 (asExon2 and asExon3; Figure 3). Antisense transcripts were not detected for any other Ly49 genes, indicating that the presence of antisense transcripts may be because of the use of a promoter upstream of exon – 1b as the primary site of transcription in the Ly49i gene, as recently reported by Gays et al.12 We have named this novel promoter Pro2, and the transcriptional activity of this element is analyzed in the next section. To confirm the continuity of antisense transcripts identified by RNA-sequencing, reverse transcription-PCR (RT-PCR) was performed to isolate antisense transcripts originating from promoters Pro2 and Pro3, as well as novel alternatively spliced sense transcripts originating in exon – 1b and containing a previously unreported 132 bp non-coding exon – 1c from both C57BL/6 and BALB/c mice. Novel Ly49i transcripts originating in exon – 1b and containing exon – 1c have been deposited in GenBank (nos. KU645200–KU645202). The Ly49i Pro2 and Pro3 antisense transcripts used alternative exon 2 splice acceptor sites, resulting in either a 198 or 279 bp antisense exon 2

| Gene      | Protein                                      | Function                                      | Fold change | P-value
|------------|----------------------------------------------|-----------------------------------------------|-------------|---------|
| Clip3      | CAP-GLY domain containing linker protein 3   | Links microtubules with cellular organelles   | 2.01        | 1.31E - 04 |
| Il2r2      | IKAROS family zinc-finger 2 (Helios)         | Transcription factor                          | 2.10        | 2.17E - 06 |
| Csf2       | GM-CSF                                       | Granulocyte/macrophage production/differentiation | 2.17        | 1.31E - 05 |
| Sce6       | SRY-box 6                                    | Transcription factor                          | 2.21        | 1.83E - 05 |
| Dll1       | Delta-like 1                                 | Notch ligand                                 | 2.31        | 9.82E - 05 |
| Ktra1      | Ly49a                                        | Class I MHC receptor                          | 2.47        | 7.40E - 07 |
| Cd3d       | CD3-δ                                        | TCR signaling                                 | 2.54        | 3.40E - 05 |
| Chl1       | Cell adhesion molecule homologous to L1CAM   | Cell adhesion                                 | 2.59        | 2.41E - 05 |
| Gpr55      | G-protein-coupled receptor 5S                | Receptor for γ-<i>+</i>-lysophosphatidylinositol | 2.64        | 1.71E - 05 |
| Slamf6     | SLAM family member 6                         | Coreceptor for NK cell activation             | 2.85        | 3.38E - 06 |
| Dpp5      | Dihydropyrimidinase-like 5                   | Neuron differentiation, interacts with L1CAM | 3.21        | 1.03E - 05 |
| Cd3g       | CD3-γ                                        | TCR signaling                                 | 3.24        | 4.83E - 06 |
| Il7r       | IL-7 receptor subunit-α                      | Lymphocyte survival                           | 3.96        | 8.11E - 08 |
| Cd3e       | CD3-e                                        | TCR signaling                                 | 13.77       | 5.87E - 07 |
| Klra7      | Ly49g                                        | Class I MHC receptor                          | 86.89       | 5.91E - 14 |

Abbreviations: L1CAM, L1 cell adhesion molecule; MHC, major histocompatibility complex; NK, natural killer; TCR, T-cell receptor.
Pro2i transcripts were isolated from BALB/c spleen and bone marrow cDNA, indicating that Pro2i activity is not specific to the C57BL/6 Ly49i gene. Many of the antisense transcripts found in B6 mice could not be confirmed in BALB/c mice, as the splice acceptor for Ly49i antisense exon 3 is not present in the BALB/c Ly49i gene.26 Only one Ly49i antisense transcript, asPro2-Ex2, was confirmed in BALB/c.

Pro2i promoter activity

Given the large number of transcripts originating from exon −1b, and the previously characterized dominant start site in this region,12 the region preceding exon −1b was investigated for the presence of a novel promoter element (Pro2i). To evaluate the promoter activity the Pro2i region, a series of pGL3 luciferase constructs spanning this sequence was generated (Figure 4a). The shortest fragment tested, R3, which ended at the dominant TSS,12 showed weak promoter activity in the mature mouse NKT (natural killer T) cell line EL-4 that expresses Ly49A/G27 and demonstrates Pro2 promoter activity,3 but not in the immature NK cell line LNK that lacks Ly49 expression but supports Pro1 transcription3 (Figure 4b). The low activity of the putative Pro2i promoter in EL-4 cells indicated that this promoter has a cellular specificity that is distinct from the Ly49a and Ly49g Pro2 elements, or that a distal enhancer element might be required to produce substantial activity.

The Ly49i Pro1 element does not exhibit enhancer activity

As it has recently been suggested that the distal upstream element Pro1 may act to enhance the transcriptional activity of the downstream Ly49 promoters,13 a series of Pro1:Pro2i reporter constructs were generated to test for interaction of these two elements. To test the ability of Pro1 to enhance transcription from the novel promoter in its native configuration, we generated pGL3 constructs containing the full Ly49i sequence spanning the ~3.5 kb region from Pro1 to Pro2i and measured their activity in either LNK cells that support Pro1 transcription or EL-4 cells that support Pro2 transcription. The transcriptional specificity of these two cell lines was confirmed by RT-PCR of Pro1 transcripts from LNK cells and Pro2 transcripts from EL-4 (Figure 5a). Specific Ly49g Pro1 transcripts were detected in LNK, but not in EL-4. Although only weak signals were present in LNK cells for exon 2 of Ly49a and exon 1 of Ly49g, which is preferentially used by Pro2 (Figure 2a), EL-4 clearly showed amplification of these exons, indicating the presence of Pro2 activity. The LNK and EL-4

### Table 1

| Gene      | Subset | αS3 | αS2 | -1a | -1b1 | -1b2 | -1c | αS11 | 1   | αS12 | 2   | 3   | 2/4 |
|-----------|--------|-----|-----|-----|------|------|-----|------|-----|------|-----|-----|-----|
| Ly49a     | G<sup>+</sup> | 523 | 211 | 271 |
| Ly49a     | I<sup>+</sup> | 216 | 92  | 120 |
| Ly49c     | G<sup>+</sup> | 2   | 84  | 954 | 9  |
| Ly49c     | I<sup>+</sup> | 88  | 400 | 872 | 4  |
| Ly49e     | G<sup>+</sup> | 4   | 20  | 56  | 4  |
| Ly49e     | I<sup>+</sup> | 25  | 162 | 512 | 56 |
| Ly49g     | G<sup>+</sup> | 102 | 116 | 1088| 4040|
| Ly49g     | I<sup>+</sup> | 87  | 76  | 5   | 15  | 51  |
| Ly49i     | G<sup>+</sup> | 4   | 31  | 211 | 48  | 11  | 203 | 20  | 195 | 459 | 89 |
| Ly49i     | I<sup>+</sup> | 18  | 327 | 909 | 11  | 323 | 7   | 3892| 7771| 406 |
| Ly49j     | G<sup>+</sup> | 15  | 312 | 326 | 1225| 244 | 1039|
| Ly49j     | I<sup>+</sup> | 2   | 607 | 369 | 560 | 961 | 197 | 783 |

Figure 3. Ly49 Pro1 transcripts are not associated with gene expression in mature NK cells. A schematic depicting all of the S′ Ly49 exons revealed by RNA-seq analysis is shown. Antisense exons are shown as red rectangles. The first antisense exons originating from either Pro2 or Pro3 are labeled αS1<sup>1</sup> and αS1<sup>2</sup>, respectively. The second and third antisense exons are labeled αS2 and αS3. The first four exons contained in Ly49 Pro2 transcripts are shown as labeled green rectangles. Upstream exons derived from either Pro1 or Pro2 are shown as labeled light blue rectangles. The exon labeled −1a is the first exon of Pro1 transcripts. The exon labeled −1b<sup>1</sup> is the upstream exon found in Ly49c transcripts, whereas the exon labeled −1b<sup>2</sup> is used by Ly49g Pro1 transcripts and is the first exon of Pro2<sup>+</sup> transcripts. Exon −1c is an alternative exon used by some Pro2<sup>+</sup> transcripts. The table below lists spliced exons observed for either the G<sup>+</sup>/I<sup>−</sup> (Ly49G<sup>+</sup>) or I<sup>+</sup>/G<sup>−</sup> (Ly49I<sup>+</sup>) NK cell populations. Numbers listed are the average number of events from three independent cell-sorting experiments.
cell lines are therefore appropriate lines for the study of Pro1 and Pro2 transcriptional activity. All of the full-length Ly49i Pro1-Pro2i constructs tested showed less activity than the Pro2i constructs. Underlined regions indicate sequence of primers used to generate each fragment. Bold nucleotides indicate TSSs identified by Gays et al.,13 with an asterisk signifying the dominant TSS. (b) Activity of pGL3 reporter constructs transfected into LNK, human embryonic kidney 293 (293) or EL-4 cells. The average fold activity of constructs relative to empty pGL3 vector from at least three independent experiments is shown. Error bars represent ±1 s.e.m., n = 5.

Figure 4. Promoter activity of Ly49i Pro2i constructs. (a) Sequences of Pro2i fragments. Underlined regions indicate sequence of primers used to generate each fragment. Bold nucleotides indicate TSSs identified by Gays et al.,13 with an asterisk signifying the dominant TSS. (b) Activity of pGL3 reporter constructs transfected into LNK, human embryonic kidney 293 (293) or EL-4 cells. The average fold activity of constructs relative to empty pGL3 vector from at least three independent experiments is shown. Error bars represent ±1 s.e.m., n = 5.

DISCUSSION
The current study represents the first comprehensive analysis of Ly49 transcription in mature Ly49-expressing splenic NK cells. The expression of Ly49 proteins represents the final stage of NK differentiation that determines their ability to detect missing self.29 It is therefore important to study a population of purified Ly49+ NK cells to identify transcripts associated with gene expression. The results obtained in this study confirm the activity of the previously identified Ly49 promoters, and do not reveal any previously unknown promoter regions; however, novel spliced antisense transcripts originating from the Ly49i Pro2 and Pro3 promoters were detected.

With regard to the recent suggestion that Pro1 may function as an enhancer in mature NK cells, multiple lines of evidence presented here demonstrate that Pro1 transcripts are not associated with gene expression, and the Pro1 element likely has no active role in protein expression in mature Ly49-expressing cells. In vitro promoter assays suggest that Pro1 does not enhance downstream Ly49 promoter activity. Luciferase reporter assays using large genomic fragments of the Ly49a gene revealed no enhancement of downstream promoter activity by Pro1 in its native genomic context in the Ly49-expressing EL-4 cell line. Some enhancement of transcriptional activity by Pro1 was seen in the LNK cell line, but it is likely due to translation of spliced Pro1 transcripts generated in these cells. RNA-sequencing analysis of freshly isolated ex vivo Ly49-expressing cells shows that transcripts originating from Pro1, which would be expected if it were acting as a promoter/enhancer in these cells, are totally absent in most variegated Ly49 genes and very rare in the genes where they are found.

An important difference between the current study and the recent results from Gays et al.13 is the use of freshly isolated, Ly49-expressing ex vivo NK cells for the analysis of Ly49 gene transcription. The purified NK cells used by Gays et al.13 were not selected for Ly49 expression, and were cultured for 12 days in
IL-2, and thus represent an activated NK cell population. The Ly49g gene has been shown to be activated during culture of NK cells in IL-2, as has the Ly49h gene.\textsuperscript{23,24} In addition, Ly49h Pro1 transcripts were highly induced by treatment of mice with IL-2, and this was correlated with a rapid expansion of Ly49G\textsuperscript{+} NK cells.\textsuperscript{30} Therefore, the detection of Pro1 transcripts from these genes in purified NK cells cultured in IL-2 by Gays et al.\textsuperscript{13} is consistent with a role for Pro1 transcripts in the initial opening of the downstream Pro2 and Pro3 promoter region, resulting in Ly49 gene expression by mature NK cells. Furthermore, the use of sorted Ly49-expressing cells in the current study ensured that only mature NK cells that are producing Ly49 protein were assayed and not immature NK cells that are in the process of activating the Ly49 genes.

Paradoxically, although the Gays et al.\textsuperscript{13} study detects Pro1-forward transcription in multiple cell lines, they suggest that there is little to no Pro1-forward transcriptional activity, and very strong reverse activity. In addition, RT-PCR experiments revealed that in all cases where Pro1-forward transcripts were detected, reverse transcripts were detected at a lower level.\textsuperscript{13} This may be due to an inherent instability of the non-coding antisense transcript; however, it is important to note that very high levels of Pro1-forward transcripts are detected, in contrast to the results obtained with in vitro reporter assays. Several possible factors that may contribute to this discrepancy are as follows: (1) backbone sequences in the pGL series of reporter plasmids can affect activity, and we have chosen to use the pGL3 vector as we have found that the modifications made to reduce background transcriptional activity in the pGL4 vector had the opposite effect in the human and mouse NK cell lines we study; (2) changing the relative amount of flanking sequence in bidirectional promoter systems will change the relative strength of the competing promoters, and we have chosen core promoter fragments that have relatively balanced forward and reverse activity to study the switching properties; (3) the promoter activities in the Gays et al.\textsuperscript{13} study are reported relative to a very strong promoter, making Pro1-forward activity seem inconsequential; (4) Pro1-forward transcriptional activity is decreased or lost after extended periods of culture of the LNK line, making it important to use early passage cells for promoter analysis.

The model of Pro1 function suggests that forward transcripts originating from this element traverse the downstream promoters, displacing histones and allowing access of transcription factors required to activate the gene. The opening of the downstream promoter region may not require high levels of forward transcripts. The relatively low promoter activity found in Ly49 Pro1 is comparable to the activities detected in distal KIR promoter elements that have been associated with gene activation.\textsuperscript{31,32}

No Pro1 transcripts were found in mature Ly49-expressing NK cells for the remaining variegated inhibitory genes, Ly49a, Ly49c, Ly49i and Ly49j. This supports the hypothesis that Pro1 activity is important for the process of gene activation but does not have a role in the expression of the Ly49 protein by mature NK cells. However, it is possible that the Pro1 element has a role in maintaining an open chromatin configuration in a
downstream regions required for gene expression. The production of reverse transcripts represents the default 'off' state, and therefore may not be as tightly regulated as forward transcription. It remains unclear whether the rare antisense transcripts detected originating from the Ly49i Pro2 and Pro3 regions have any functional role in gene regulation at any point in NK development or whether they are merely a consequence of an inability to transcribe efficiently in the forward direction because of the competition from the upstream Pro2 element.33 Studies of the human transcriptome have revealed that ~8% of promoters are bidirectional with start sites separated by < 300 bp.34 It may be that many more promoters have the capacity to transcribe in the antisense direction, but the dominant function of factors generating sense transcripts prevents antisense transcription, and inhibition of sense transcription is required to reveal antisense activity. The ability of upstream promoters to inhibit transcription from downstream promoters provides an explanation for the silencing of Pro1 in mature NK cells. Although Pro1 transcripts are required to open up the Pro2/Pro3 region of the gene, their continued presence would inhibit transcription factor binding to these promoters and reduce expression.

In summary, the data presented here confirm the immature NK cell specificity of the Pro1 element and show that for each varied Ly49 gene, expression can occur in the absence of Pro1 transcripts. It will be of interest to determine the exact molecular events that determine the 'window of opportunity' for Ly49 gene activation and how they affect Pro1 activity.

MATERIALS AND METHODS

Animals

C57BL/6 and BALB/c mice were maintained and bred in the NCI-Frederick animal Breeding Facility. Animal care was provided in accordance with the procedures in, 'A Guide for the Care and Use of Laboratory Animals'. Ethical approval for the animal experiments detailed in this manuscript was received from the Institutional Animal Care and Use Committee (Permit Number: 000386) at NCI-Frederick (Bethesda, MD, USA).

RNA-sequencing library preparation and sequencing

For each of three independent experiments, single-cell suspensions were prepared from the spleens of 20 C57BL/6Ncr mice. NK cells were initially enriched using the MACS NK Cell purification system (Miltenyi Biotec, San Diego, CA, USA) as described by the manufacturer. The Fc receptors of the resulting populations of enriched NK cells were blocked with 2.4G2 and the cells were stained with antibodies to CD3, NK1.1 (eBioscience Inc., San Diego CA, USA), Ly49G2 (4D11) and Ly49C/I (5E6). CD3−, NK1.1−,Ly49G−, Ly49C/I− and CD3−, NK1.1−, Ly49G−, Ly49C/I− cells were sorted on a FACS Aria (BD Biosciences, San Jose, CA, USA). Resulting populations were >96% pure by postsort analysis. Sorted NK cells were immediately processed for extraction of total RNA.

Stranded RNA-sequencing libraries were constructed from 0.4 μg total RNA using the TruSeq Stranded Total RNA Sample Prep Kits (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The library insert sizes were ~175 bp. Unique barcode adapters were applied to each library. Equal volumes of individual libraries were pooled and run on a MiSeq benchtop DNA sequencer (Illumina). The libraries were then repooled to equimolar concentrations based on the MiSeq demultiplexing results. The final pooled library was sequenced on a HiSeq2000 sequencer (Illumina) using TruSeq Version 3 chemistry and a bioinformatics pipeline built on RTA version 1.13.48 and CASAVA 1.8.2 software (Illumina). A minimum of 40 million 100 base read pairs were generated for each library. The RNA-sequencing data set has been submitted to the GEO database, and are available under the accession number GSE83153.

Raw Fastq files were aligned to mouse genome (mm10) using STAR (v. 2.3.0).35 Genes were subsequently counted using Rsubread,36 and further analyzed for differential expression using limma-voom37 to obtain the fold-change values shown in Tables 1 and 2. Splice junctions were visualized with the Sashimi function of the Integrated Genome Viewer (IGV-v.2.3.67). Only uniquely mapped reads were included in the analyses.

**Figure 6.** Ly49a Pro2/Pro3 promoter activity is independent of Pro1 in Ly49-expressing cells. (a) Schematic of Ly49a promoter fragments analyzed. The upper line shows the complete Ly49a control region, with exons shown as numbered boxes, and promoter regions indicated by black boxes. The specific regions contained in each Ly49a-Pg3 reporter construct are shown below, with the name of the construct indicated to the right of each line. (b) Activity of constructs in LNK cells. (c) Activity of constructs in EL-4 cells. Blue-colored bars indicate constructs lacking the Pro1 region. The average fold activity of constructs relative to empty pG3L3 vector from at least three independent experiments is shown. Error bars represent ± 1 s.e.m., n = 5.
Cell collection, RNA isolation and cDNA generation

Bone marrow and spleen cells were collected from two C57BL/6 (B6) and two BALB/c mice. Spleens were nicked with a pair of scissors to puncture the outer capsule and were then dissociated into a single-cell suspension using a Dounce homogenizer. Bone marrow was collected by cutting the ends of the femur and tibia bones and flushing out the marrow with phosphate-buffered saline using a 23-gauge needle. Red blood cells were removed using ACK lysing buffer (Lonza, Walkersville, MD, USA) and NK cells were enriched by removal of non-NK cells using the EasySep Mouse NK Cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada). RNA was extracted from the NK enriched cells using the Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) with an additional on-column DNase I digest using an RNase-Free DNase Set (Qiagen) to reduce DNA contamination. cDNA copies of RNA transcripts were synthesized from isolated RNA with the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Carlsbad, CA, USA) using the included random hexamer primers.

Ly49 Transcript identification by RT-PCR

PCR of CDNA was used to isolate copies of transcripts from Pro2 free exon 2 forward primer (5'-GAACCTCTACTGTCTTGCTC-3') and Exon 3 reverse primer (5'-GAGTGTGACCACTGAAAC-3') and Exon 3 reverse primer (5'-GAGTGTGACCACTGAAAC-3'). Rare antisense transcripts were isolated using a nested PCR scheme first using a set of outer primers: asPro2 forward 1 (5'-GAAGATGAGAGAAGACACTGCTC-3'), asPro3 forward 1 (5'-CAGAGCTGACTGAGGACATCC-3'), asExon3 reverse 2 (5'-CTGATGATTCTTGGATCTG-3') and the same asExon2 reverse (5'-CCTCATGACTAAGGATGCTGAAC-3'). The ChargeSwitch PCR Clean-Up Kit (Invitrogen, Carlsbad, CA, USA) was used to remove these primers from the products of first round of the nested PCR and these products were then used as the template for a second round of PCR using an inner primer set: asPro2 free 2 forward (5'-GGACACAACACTGTGAGGAGG-3'), pro3 asPro3 forward 2 (5'-GAAGATGAGAGAAGACACTGCTC-3'), asExon3 reverse 2 (5'-CTGATGATTCTTGGATCTG-3') and the same asExon2 reverse (5'-CCTCATGACTAAGGATGCTGAAC-3'). The identity of transcripts were confirmed by cloning PCR products into the TOPO TA Cloning PCR 2.1 vector (Invitrogen) and sequencing. The genomic Ly49 gene fragments were too large for TA Cloning PCR cloning Kit (Agilent Technologies, Santa Clara, CA, USA) was used instead. Fragments confirmed by sequencing were transferred from PCR 2.1 or StrataClone vector to pGL3-Basic (Promega, Madison, WI, USA) luciferase reporter vector using either SalI/Xhol or Xhol/Hindll restriction enzymes depending on orientation. Pro1 enhancer constructs were generated by cloning Pro1 into the StrataClone vector and then transferring to pGL3-Basic using BamHI/Sal restriction enzymes.

Cell culture

The mature mouse NK cell line EL-4 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin–streptomycin–glutamine. Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin–streptomycin–glutamine. The immature mouse NK cell line LNK was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin–streptomycin–glutamine, sodium pyruvate, non-essential amino acids, 2-mercaptoethanol and 500 IU ml⁻¹ IL-2.

Transfections and luciferase assays

Cells were plated at a density of 50 000 cells per well in 24-well plates. Cells were co-transfected with a control pRL-SV40 Renilla reporter vector (Promega) to normalize luciferase readings. Human embryonic kidney 293T cells and LNK cells were transfected using Lipofectamine 2000 (Invitrogen). LNK cells were transfected with 1000 ng of pGL3 construct and 50 ng of Renilla control using a ratio of 5 μL Lipofectamine to 1 μg DNA. The 293T cells were transfected with 200 ng of pGL3 construct and 10 ng of Renilla control using a ratio of 0.6 μL Lipofectamine to 1 μg DNA for 293 cells. EL-4 cells were transfected with 1500 ng pGL3 construct and 100 ng Renilla control using TrueFect-Max (United Biosystems, Herndon, VA, USA) at a ratio of 2.5 μl TrueFect to 1 μg DNA. After 48 h of incubation, cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Measurement of the firefly luciferase activity of the Ly49 promoter constructs was normalized relative to the activity of the Renilla luciferase produced by the pRL-SV40 control to control for differences in transfection efficiency, and each construct was tested in triplicate in at least three independent experiments.

5'-RACE of Pro1-Pro2 i R3 fusion

Luciferase fragments were transfected with the Pro1-Pro2 i R3 fusion pGL3 construct as described above. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Ten micrograms of isolated RNA was used to carry out 5'-RACE using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) following the included 5'-RLM-RACE Protocol using a 5' outer primer (5'-GTGATGGAATGTGGAAGG-3') and a 5' inner primer (5'-CGGATCCGCGAAGAAGCCTG-3').

Statistical analysis

T-tests were carried out using Microsoft Excel 2010 for Windows with the Analysis Toolpak add-in. A two-tailed paired T-test was used with a P-value < 0.05 considered significant.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
1 Viever E, Tomarello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008; 9: 503–510.
2 Lanier LL. NK cell recognition. Annu Rev Immunol 2005; 23: 225–274.
3 Saleh A, Makrigiannis AP, Hodge DL, Anderson SK. Identification of a novel Ly49 promoter that is active in bone marrow and fetal thymus. J Immunol 2002; 168: 5163–5169.
4 Kubo S, Nagasawa R, Nishimura H, Shigemoto K, Maruyama N. ATF-2-binding regulatory element is responsible for the Ly49A expression in murine T lymphoid line, EL-4. Biochim Biophys Acta 1999; 1444: 191–200.
5 Gosselin P, Makrigiannis AP, NalewaiK R, Anderson SK. Characterization of the Ly49I promoter. Immuno genetics 2000; 51: 326–331.
6 Wilhelm BT, McQueen KT, Freeman JD, Takei F, Mager DL. Comparative analysis of the promoter regions and transcriptional start sites of mouse Ly49 genes. Immunogenetics 2001; 53: 215–224.
7 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.
8 Tanamachi DM, Moniot DC, Cado D, Liu SD, Hsia JK, Raulte. Genomic Ly49a transgenes: basis of variated Ly49a gene expression and identification of a critical regulatory element. J Immunol 2004; 172: 1074–1082.
9 Rouhi A, Gagnier L, Takei F, Mager DL. Evidence for epigenetic maintenance of Ly49a monoa lentic gene expression. J Immunol 2006; 176: 2991–2999.
10 Rouhi A, Brooks CG, Takei F, Mager DL. Plasticity of Ly49g expression is due to epigenetics. Mol Immunol 2007; 44: 821–826.
11 Kubo S, ltoh Y, Ishikawa N, Nagasawa R, Mitorai T, Maruyama N. The gene encoding mouse lymphocyte antigen Ly-49: structural analysis and the 5′-flanking sequence. Gene 1993; 136: 326–331.
12 Gays F, Koh AS, Mickiewicz KM, Aust JG, Brooks CG. Comprehensive analysis of transcript start sites in Ly49 genes reveals an unex pected relationship with gene function and a lack of upstream promoters. PLoS One 2011; 6: e18475.
13 Gays F, Taha S, Brooks CG. The distal upstream promoter in Ly49 genes, Pr0, is active in mature NK cells and T cells, does not require TATA boxes, and displays enhancer activity. J Immunol 2015; 194: 6068–6081.
14 Mason L, Giardina SL, Hecht T, Ortaldo J, Mathiesen BJ. LGL-1: a non-polymorphic antigen expressed on a major population of mouse natural killer cells. J Immunol 1988; 140: 4403–4412.
15 Sentman CL, Hackett J, Kumar V, Bennett M. Identification of a subset of murine natural killer cells that mediates rejection of Hh-17 but not Hh-21 bone marrow grafts. J Exp Med 1989; 170: 191–202.
16 Hanke T, Takiwaza H, McMahon CW, Busch DH, Pamer EG, Miller JD et al. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. Immunity 1999; 11: 67–77.

| Article Title | Authors | Journal | Year |
|----------------|---------|---------|------|
| The gene encoding mouse lymphocyte antigen Ly-49: structural analysis and the 5′-flanking sequence. | Kubo S, Itoh Y, Ishikawa N, Nagasawa R, Mitorai T, Maruyama N | Gene | 1993 |
| Identification of a novel Ly49 promoter that is active in bone marrow and fetal thymus. | Saleh A, Makrigiannis AP, Hodge DL, Anderson SK | J Immunol | 2002 |
| Identification of a minimal class I MHC natural killer cell receptor haplotype. | Gays F, Martin K, Kenebeck J, Aust JG, Brooks CG | J Immunol | 2000 |
| Comprehensive analysis of transcript start sites in Ly49 genes reveals an unexpected relationship with gene function and a lack of upstream promoters. | Gays F, Taha S, Brooks CG | PLoS One | 2011 |
| The transcription factor c-Myc enhances KIR gene transcription through direct binding to an enhancer. | Mason L, Giardina SL, Hecht T, Ortaldo J, Mathiesen BJ | J Immunol | 1988 |
| The gene encoding mouse lymphocyte antigen Ly-49: structural analysis and the 5′-flanking sequence. | Kubo S, Itoh Y, Ishikawa N, Nagasawa R, Mitorai T, Maruyama N | Gene | 1993 |
| Identification of a novel Ly49 promoter that is active in bone marrow and fetal thymus. | Saleh A, Makrigiannis AP, Hodge DL, Anderson SK | J Immunol | 2002 |
| Comprehensive analysis of transcript start sites in Ly49 genes reveals an unexpected relationship with gene function and a lack of upstream promoters. | Gays F, Taha S, Brooks CG | PLoS One | 2011 |
| The transcription factor c-Myc enhances KIR gene transcription through direct binding to an enhancer. | Mason L, Giardina SL, Hecht T, Ortaldo J, Mathiesen BJ | J Immunol | 1988 |

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