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PRDM1 is a Tumor Suppressor Gene in Natural Killer Cell Malignancies

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Abstract

Natural killer cell lymphoma (NKCL) constitutes a rare and aggressive form of non-Hodgkin lymphoma, and there is little insight intoits pathogenesis. Here we show that*PRDM1*is a tumor suppressor gene in NKCLs that is inactivated by a combination of mono-allelic deletion and promoter CpG island (CGI) hypermethylation. We observed mono-allelic deletion of *PRDM1* loci in 8 of 18(44 %) NKCL cases. The other allele showed significant promoter methylation in 12 of 17 (71%) cases. In support of itsrole as a tumor suppressor gene, the reconstitution of *PRDM1* in *PRDM1*-null NK-cell lines led to G2/M cell cycle arrest,increased apoptosis, and a strong negative selection pressure with progressive elimination of *PRDM1*-expressing cells, which was enhanced when IL2 concentration is limiting. We observed a progressive increase in *PRDM1* expression, in particular, *PRDM1α,* in normal NK-cells in response to IL2 and in normal NK-cells activated with an engineered NK-cell target, K562-Cl9-mb21, suggesting its role in NK-cell homeostasis. In support of this, knock-down of PRDM1 by shRNA in normal NK cells resulted in the positive selection of these cells. We identified *MYC* and *4-1BBL* as targets of *PRDM1* in NK-cells. Disruption of homeostatic control by PRDM1 may be an important pathogenetic mechanism for NKCL.

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Natural killer cell malignancies comprise 1-2 % of all non-Hodgkin lymphomas (NHL)(1)and have been classifiedinto aggressive NK-cell leukemia (ANKCL) and extranodal NK-cell lymphoma of nasal type (ENKCL) by the WHO classification (1). They are clinically highly aggressive and are associated with poor survival(2). The incidence is higher in East Asia and Central and South America compared with other parts of the world(3)**.**Especially at late-stage disease, these malignancies are resistant to chemotherapy, possibly owing to multidrug resistance associated with the expression of P-glycoprotein, a product of the *MDR1* (*ABCB1*) gene (4). Several genome-wide studies identified 6q21 deletion as the most frequent aberration in these tumors (5-8). We observed a combination of deletions, mutations generating truncated, non-functional proteins, and promoter methylation of *PRDM1* in the majority of the NK-cell lines studied, supporting the role of *PRDM1* as a tumor suppressor gene in this commonly deleted region(9). Interestingly, two recent

reports(10, 11)demonstrated frequent mutations of *PRDM1* leading to the formation of non-functional proteins in activated B-cell-like diffuse large B cell lymphoma (ABC-DLBCL), thus implicating *PRDM1* as a tumor suppressor in this subtype of DLBCL. PRDM1is a transcriptional repressor and a master regulator of the differentiation of B cells into antibody secreting cells (ASC)(12) . The repression of MYC(13), SPIB, BCL6, ID3 and PAX5 is required for this function(14). Recently, PRDM1 expression has been detected in CD8+ and CD4+ T-cells with the effector phenotype (15, 16) and in CD25+ regulatory T-cells(16). Moreover, PRDM1 has been shown to regulate T-cell homeostasis(15, 16). *PRDM1* has two alternative promoters; one generates PRDM1α which is the functional form of PRDM1, and the other generates a functionally impaired form of PRDM1 (PRDM1β) that lacks the 101 aa at the N terminus(17).Here, we validated the frequent inactivation of PRDM1 in NKCL patient samples and demonstrated the tumor suppressor activities of *PRDM1* in NK-cell lines. Furthermore, we demonstrated that the knockdown of PRDM1 in normal NK-cells promotes their growth.

Results

Evaluation of copy number changes of PRDM1. We employed the quantitative PCR (q-PCR) method on genomic DNA of tumor biopsies to quantify the *PRDM1* copy number**.** The method was validated by comparing the q-PCR results with array-CGH data of 6q21 in NK and γδ-T tumor-cell lines. We observed a high concordance (11 of 11, 100%) of the two methodologies **(***Fig.S1-A***).** Since tumor samples contained a varying proportion of non-tumor cells, we studied the effect of the stromal content in the tumor biopsies on the q-PCR assay, by applying it to genomic DNA containing an admixture of DNA from NKYS [an NK-cell line with del(6)(q21)], and DNA from human tonsil in predetermined ratios. We found that the q-PCR assay was unable to reliably detect the deletion in the presence of 30% or more tonsil DNA **(***Fig.S1-B***).** This level of stromal contamination may cause practical challenges in detecting deletion of PRDM1 using q-PCR or a-CGH and fluorescence in situ hybridization (FISH) would be a useful alternative in this setting if tissues are available. We performed q-PCR on the genomic DNA of 18 NKCL cases and observed deletion in 44% (8 of 18) of the cases *(Fig. 1-A),* consistent with a previous report showing a 43% (3 of 7) deletion rate obtained

with BAC array-CGH (a-CGH) using a smaller set of NKCL cases (9). These results indicate that *PRDM1* deletion is a frequent aberration in tumors as well. Our estimate was a minimum approximation since the q-PCR results of two NKCL cases were ambiguous ***(****Fig. 1-A****)***. A-CGH performed on these two cases was not able to detect 6q21 deletion encompassing *PRDM1*, and therefore the 6q21 status in these cases was regarded as normal. Q-PCR did not detect deletion in two NKCL cases that were determined to have deletion by a-CGH. The a-CGH data for NKCL cases was obtained through BAC, CIT (Cartes d'Identité des Tumeurs) or SNP-array platforms,and there were several discrepancies between q-PCR and the BAC or CIT-platforms. In contrast, we observed 100% (6 of 6) correlationof q-PCR and the high resolution SNP-array results.

Promoter methylation of PRDM1 in NKCL cases. We found that *PRDM1α* promoter methylation was associated with repression of *PRDM1α* transcription (9) in malignant NK-cell lines**.** We performed methylation analysis to cross-validate our previous findings using a different experimental platform and extended our analysis to include all available NKCL cases. The region for analysis was extended to include CpG dinucleotides further upstream of the TSS (including 25 CpG dinucleotides between -786 and +1). 9 NK-cell lines and 17 NKCL cases were studied along with highly pure (> 95% CD56+ CD3- cells) resting and IL2-stimulated NK-cells as control samples. The methylation profile was variable including a few cell lines and a case which were hypomethylated in regions with methylation in normal NK-cells. However, we observed a frequently hypermethylated region compared to normal NK-cells encompassing -449 and -114 bp relative to the TSS [12 of 17 (71%) tumor samples, 5 of 9 (56 %) NK-cell lines *(Fig. 1-B)*]**.**Among the 17 cases with deletion and methylation data, 15 of 17 (88.2%) cases demonstrated deletion and/or methylation (3 with only deletion, 8 with only methylation and 4 with both). These results indicate that promoter hypermethylation is a common event and may cooperate with deletions in NK-cell malignancies. We observed silencing of *PRDM1α,* asdetermined withq-RT-PCR, in all malignant NK-cell lines with promoter hypermethylation except for one NK-cell line having methylation in one allele and a deleterious mutation in the other*.(Fig.S2-B , upper panel).*

Mutation Analysis of PRDM1 in NKCL cases. We tested 18 tumor cases and another EBV-negative NK-cell line, IMC-1, in addition to the NK-cell lines reported previously [9]. Unlike NK-cell lines, tumor cases did not show any mutation that would result in a truncated protein. We identified two SNPs in three NKCL cases: a missense SNP (position 843 in exon 4, C to G, Asp to Glu;a known germline SNP **[rs811925])** in one case and a silent SNP (position: 2376 G to A, Pro to Pro; germline SNP **rs1010273**) in two cases**.** **(***Fig.S3-A,B***).**

PRDM1 expression in NK-cell tumor biopsies and IL2-activated primary NK-cells. The copy number and epigenetic status of NKCL cases correlated well with the *PRDM1* mRNA levels in NKCL cases as determined with q-RT-PCR. In general, *PRDM1* expression was low in cases with deletion, methylation, or both *(Fig. 1-C)***.** We used primers specific for expression of *PRDM1α(Fig. 1-C, lower panel)* or both isoforms *(PRDM1 α+β) (Fig. 1-C, upper panel)*. In some cases *PRDM1α* and *PRDM1β* transcript levels may not be parallel**.** *PRDM1α*can be specifically quantified with primer sets located 5' of the *PRDM1β* transcript *(Fig.S2-A).*We had observed up-regulation of *PRDM1α* mRNA in normal NK-cells on IL2 stimulation within a 24 h period(9)**;** now we found that the progressive increase in *PRDM1* expression in primary human NK-cells continued for 6 days *(Fig.S2-C).*When the *PRDM1α* specific primers were used for q-RT-PCR, the induction of *PRDM1* appeared to be mainly contributed by *PRDM1α*; however, *PRDM1α* mRNA is much lower in resting primary NK-cells compared to *PRDM1 (α+β)* as reported earlier (18). We assayed *PRDM1* expression in primary NK-cells obtained from coculture with the K562-Cl9-mb21 cells and observed a robust induction of *PRDM1α* (70 fold) compared to resting NK-cells*(Fig.S2-D).*

Reconstitution of PRDM1 leads to impaired G2/M cell-cycle progression and increases apoptosis in PRDM1-null NK- cell lines. We used the retrovirus MSCV-IRES-GFP (pMIG) to reconstitute PRDM1 expression in PRDM1-null NK- cell lines (KHYG1, KAI3). We compared the percentage of apoptotic cells in KHYG1 and KAI3 cells transduced with empty vector or PRDM1 using AnnexinV staining and observed increased apoptosis in PRDM1-transduced populations of KHYG1 (average= 1.5X) and KAI3 (average= 3.9X) cell lines *(Fig. 2-A,B)* 2daysaftertransduction.UsingHoescht 33342 staining, we observed an increase

in the percentage of cells in G2/M phase (4.6% in KHYG1, 7.3% in KAI3) with a concomitant reduction in the percentage of cells in G_o/G_1 (8.8% in KHYG1, 6.2% in KAI3) and S-phases (2.2% in KHYG1, 1.4% in KAI3)in PRDM1-transduced KHYG1 and KAI3 cells on day 3 *(Fig. 2-C,D).*Ectopic PRDM1 expression was shown by q-RT-PCR on unsorted cells *(Fig. S6-A,B).*

Ectopic expression of PRDM1 exerts a negative selection pressure on PRDM1-null NK-cell lines in an IL2-dose dependent manner. We asked whether reconstitution of PRDM1 expression in PRDM1-null NK-cell lines will exert a negative selection pressure, as indicated by reduction of the percentage of GFP(+) cells over time in cells transduced with PRDM1 but not those transduced with empty vector . We quantified the percentage of GFP(+) cells in PRDM1-null NK-cell lines at different time points (days 2 and 5 for KHYG1 and NK92; days 3 and 6 for KAI3) after transduction with empty vector or PRDM1 and observed a decrease in the percentage of GFP(+) cells only in PRDM1-transduced KHYG1, NK92 and KAI3 cells but not in empty vector transduced cells (24% in KHYG1; 26% in NK92 and 61% in KAI3) (*Fig. S4, A-C*)**.** Next,we tested whether the rate of selective elimination of GFP(+) cells in PRDM1 transduced NK-cell lines was influenced by the concentration of IL2 in the culture medium. In this experiment, the NKYS cell line was used as the negative control, as this NK-cell line expresses endogenous PRDM1 protein (9). We cultured the PRDM1-null KHYG1 cells with progressively lower concentrations (100 IU, 75 IU, 50 IU and 25 IU) of IL2 and compared the percentage of GFP(+) cells in empty vector or PRDM1 transduced cells after 2 and 5 days using FACS *(Fig. 3-A,B).* After 2 days of culture, we observed a decreasein the percentage of GFP(+) cells (26.7%, 34.3%, 34.2%, and 39.4% in cells treated with 100, 75, 50, and 25 IU IL2, respectively) for *PRDM1* transduced KHYG1 cells treated with different doses of IL2 **(***Fig. 3-C, left***)**. After 5 days, we observed a more dramatic decrease in the percentage of GFP(+) cells that is negatively correlated with the IL2 concentration (73.1%, 76%, 79.4%, and 85.3% in 100, 75, 50, and 25 IU IL2 treated cells, respectively.) *(Fig. 3C, middle panel).*Similarly, we observed a decreasein the percentage of GFP(+) cells (average: 41.2%, 39.9%, 44.5%, 46.4% for 100IU,75IU, 50IU, 25IU IL2 respectively.)in NK92 cells enhanced in limiting IL2

concentrations after 3 days of culture. In contrast, we observed a much smaller (overall 35%) IL2-independent reduction in GFP(+) cells in PRDM1-transduced NKYS cells after 5 days **(***Fig. 3C, right panel***)**.

The rate of apoptosis in PRDM1-transduced NK-cells proportionately increases with progressively decreasing doses of IL2 in the culture medium. Next, we asked whether there is an IL2-dose dependent increase in the rate of apoptosis in PRDM1-transduced NK-cells. PRDM1-transduced NK-cells had increased AnnexinV staining for all IL2 concentrations tested *(Fig. 3-D).* In addition, we observed a progressive increase in the rate of apoptosis specifically in PRDM1-transduced cells with decreasing doses of IL2 correlating with the higher rate of negative selection pressure observed in lower IL2 concentrations (1.08, 1.24, and 1.46 fold in 75, 50, and 25 IU IL2 compared to 100 IU IL2 treatment, respectively). The inverse correlation between AnnexinV staining and the concentration of IL2 was specific for the GFP(+) population in PRDM1-transduced KHYG1 cells *(Fig. 3-E, left panel)* but was not observed in GFP(+) and GFP(-) populations in vector only transduced KHYG1 cells or in the GFP(-) population of PRDM1-transduced KHYG1 cells *(Fig. 3-E).*

Progressive elimination of PRDM1 transduced NK-cells on long term culture. The percentages of GFP(+) cells in *PRDM1* or control vector transduced KHYG1 and NKYS cells grown with standard (75 IU) IL2 concentrations were quantified in a time-course experiment. We observed a time-dependent progressive depletion of GFP(+) cells in PRDM1-transduced KHYG1 cells (14.3% to 0.9%)*(Fig.S5-A)* and a decrease (37.4% to 17.1%) *(Fig. S5-B)* in PRDM1-transduced NKYS cells after 5 weeks of culture, but there was no change in the empty vector transduced KHYG1 (59.5% to 56.47%) or NKYS (55.9% to 60.7%) cells suggesting that a negative selection pressure exerted by the ectopic expression of PRDM1 in NK-cells led to the elimination of transduced cells. As an additional control sample, we did not observe any reduction in GFP(+) cells in hTERT-transduced KHYG1 cells in 18 days*(Fig. S5-C)*. Ectopic hTERT expression was confirmed with q-RT-PCR *(Fig. S6-C)*

Knockdown of PRDM1 confers a growth advantage to primary NK-cells. Next, we wanted to test whether knockdown of PRDM1 provides a growth advantage for primary human NK-cells. We quantified

the percentage of GFP(+) cells at different time points after transduction of PRDM1-expressing NKYS cells and human peripheral blood NK-cells derived from the PBLs cocultured with K562-Cl9-mb21 using empty vector or vector expressing PRDM1 shRNA. We observed a 65% increase in the percentage of GFP(+) cells after knockdown of PRDM1 in NKYS cells when cells at 7 days after transduction were compared to 3 days *(Fig.4-B).* We observed a 90% increase in GFP(+) cells in PRDM1 shRNA transduced primary NK-cells between days 4 and 7 and a 245% increasebetween days 7 and10 *(Fig.4-C).* The functionality of the PRDM1 shRNA-mir construct and the knockdown of PRDM1 were shown with the luciferase reporter assay and western blotting *(Fig. S7- B,D,E).*

Altered PRDM1 expression is associated with changes in genes involved in cell cycle and activation in NK cells. Next, we wanted to test whether ectopic expression of PRDM1 in NKcells alters expression of genes involved in growth by examining a number of known PRDM1 targets. Select targets were evaluated at a later time point in NKYS cells compared to the KHYG1 cells post-infection due to the practical challenges associated with obtaining sufficient quantity of RNA from sorted cells. We observed a decrease in the expression of MYC, *4-1BBL (TNFSP9 or CD137L)*, and *TNFα*. There is corresponding increase in the expression of two negative regulators of the cell cycle, *CCNG1* and *CCNG2*, in KHYG1 cells transduced with PRDM1 *(Fig. 5-A).* Similarly, we observed increased expression of *TNFα*, *TNFβ* and *MYC* in *PRDM1* shRNA transduced NKYS cells compared to the vector only transduced cells *(Fig. 5-B).* We also observed a strong reverse correlation of *MYC* and *PRDM1* mRNA expression levels in IL2 activated PB NK-cells *(Fig. 5-C, Fig.S2-C lower panels).*

Discussion

Tumor development is a multistep process often involving the cooperation between deregulated expression and/or activation of oncogenes and inactivation of tumor suppressor genes (TSG). Mono-allelic deletions are commonly observed in many cancer types, and commonly deleted regions contain potential tumor suppressor genes that may lose function through promoter methylation or mutations in the remaining allele. Given the strong inverse correlation between the *PRDM1α* promoter hypermethylation pattern and the

expression levels of *PRDM1α* observedin PRDM1-null NK-cell lines and the increase in*PRDM1α* expression after treatment with the DNA demethylating drug 5-aza-2-deoxycytidine (9), promoter methylation may be one of the pivotal mechanisms contributing to the silencing of PRDM1 in NK-cells. The fact that the majority of NKCL cases have *PRDM1* promoter hypermethylation suggests that PRDM1 may have been silenced by the combination of deletion and promoter methylation. Deleterious mutations may be infrequent in NKCL cases so that they were not observed in the cases we studied. However, it is also conceivable that there were sequence alterations of *PRDM1* in the NKCL cases that were not detected, as deleterious sequence alterations of *PRDM1* due to aberrant RNA editing wereobserved in some DLBCL cases (11)**.** Another possibility is themutations that may have been missed because of thecontamination of neoplastic NK-cells with normal stromal cells.However, this possibility is unlikely, as our GEP and CNA studies (19, 20) on these cases indicated that the neoplastic NK cells represent the predominant population in the tumor. Therefore, abnormalhypermethylation seemed to account for the vast majority of cases with low PRDM1 expression.We observed an increase in apoptosis and cells in G2/M cell cycle in two PRDM1-null malignant NK-cell lines on re-introduction of PRDM1 into the cells; these results provide experimental support for PRDM1 as a TSG in NK-cells. The concomitant increase in expression of two negative cell cycle regulators and a decrease in *MYC* expression also support the hypothesis that PRDM1 is a tumor suppressor in NK-cells. Intriguingly, the*MYC* signature was upregulated in NKCLs (20). Two known targets of *PRDM1* with growth regulatory function in B cells, *MYC*(21) and *4-1BBL*(22), were downregulated upon expression of PRDM1, suggesting that certain functional role of PRDM1 is conserved in different lymphocyte lineages. Induction of the expression of other direct targets of PRDM1 in NK-cells (e. g., *TNFα* and *TNFβ*)(18) may cooperate with the reconstitution of MYC expressionin the neoplastic transformation ofNK-cells with silenced PRDM1 expression. Two negative regulators of G2/M cell cycle, CCNG1 and CCNG2(23), may be indirect targets of PRDM1.In fact, CCNG2 was shown to be repressed by MYC (24).The progressive increase in apoptosis and negative selection observed when the PRDM1-transduced cells were cultured in decreasing IL2 concentrationssuggests that the tumor suppressor role of PRDM1 in NK-cells may be more

pronounced when growth promoting factor are present at a limiting concentration, as is likely to be the case *in vivo*. PRDM1 may play an important role in the homeostasis of NK-cells by preventing excessive NK-cell activation and proliferation. This may be the mechanistic basis for the tumor suppressor action of PRDM1 and the negative selection pressure observed in PRDM1-transduced malignant NK-cells. This contention is supported by our findings in human primary NKcells, in which PRDM1 is upregulated on activation with IL2 and proliferation is enhancedupon PRDM1 knockdown. A recent report showed enhanced proliferation of *ex vivo* cultured Rag2$^{-/-}$/*prdm1*$^{gfp/gfp}$ mouse NK-cells in limiting IL15 concentrations suggesting the involvement of PRDM1 in the control of homeostasis of mouse NK-cells (25) in agreement with our observations. We have demonstrated up-regulation of *PRDM1α* in response to IL2 in 24 h in normal NK-cells from peripheral blood *(Fig.S2-C upper panel, left)***.** We have extended this initial observation and showed that the increase in the transcriptional level of *PRDM1α* continues slowly and progressively in 6 days up to 11 fold, further suggesting that PRDM1 may be part of a negative autoregulatory loop similar to the one observed in T cells(26), controlling the activation and/or homeostasis of NK-cells.

Recently, two groups have independently shown PRDM1 to be a tumor suppressor gene inABC-type DLBCL using *in vivo* mouse models (27, 28). In ABC-DLBCL, a block in differentiation at the pre-plasmablast stage of differentiation is postulated to be the pathogenetic mechanism. However, the concomitant failure to repress certain target genes such as *MYC* may also play a role. NK-cells may not have as distinct a differentiation stage transition as that from B-cells to plasma cells, but there is evidence that PRDM1 is more highly expressed in the most mature population(18). Therefore, our results and published literature suggest that PRDM1 shares certain functions across lymphocyte lineages and that lossoffunction of PRDM1 may contribute to the neoplastic transformation of activated B and NK-cells through overlapping mechanisms. Intriguingly, we encountered difficulty in reconstituting PRDM1 expression. The challenge of ectopic expression is a common feature of tumor suppressor genes(29) and notably has been observed for PRDM1 in many instances even with the inducible constructs in GC-B cell derived cell lines(22).This phenomenon was more obvious when we compared the mean fluorescence intensity(MFI) of NK-cells

transduced with vector, PRDM1, and hTERT (*Fig.S8-B).* We observed lower MFI and lower number of GFP(+) cells with inducible PRDM1 constructs (i.e. PRDM1-ER, PRDM1a-TMP) even before induction suggesting leaky transgene expression and selective disadvantage of PRDM1 expression even at low expression levels.In conclusion, we performed both functional and correlative studies on PRDM1 in normal and neoplastic NK-cells and provide strong evidence that PRDM1 is a tumor suppressive gene in NKCLs. Silencing of PRDM1 in NKCL cases is mainly through cooperation of 6q21 deletion and promoter CpG island hypermethylation. Re-introduction of PRDM1 into NK-cell lines lacking its expression enhances apoptosis and impaired G2/M cell cycle progression, and these cells are negatively selected in cell culture. Conversely, knocking down PRDM1 expression provides a growth advantage to activated PB NK-cells. PRDM1 may share important target genes in B- and NK-cells such as *MYC*and identifying the essential targets will lead to further understanding of its pathogenetic mechanisms.

Materials and Methods

Patientmaterial and cell line. The characteristics of NK-cell tumor cases and NK and γδ -T cell lines included in this study have been reported previously(19, 20) and are summarized in *Table S1***.** Genomic DNA extraction was performed with the standard phenol-chloroform method (9). Thefour cell lines, KHYG1, KAI3, NK92 and NKYS, used for transduction studies were cultured in RPMI-1640 (Gibco-Invitrogen, CA, USA) supplemented with 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 µg/ml), and 75 IU interleukin (IL)-2 (R&D Bioscience, CA, USA) at 37°C in 5% CO_2.

Primary NK-cell isolation and culture. Normal NK-cells from peripheral blood lymphocytes (PBLs) of healthy donors were isolated through negative selection using an NK-cell Isolation Kit (Miltenyi Biotec. Inc., Auburn, CA). Purity of NK-cells was validated with CD56-APC (Miltenyi Biotec.Inc., Auburn, CA) and CD3-PE (Miltenyi Biotec. Inc., Auburn, CA) staining using FACSCalibur. Isolated NK-cells were cultured as described above with 200IU IL2 (R&D Bioscience, CA, USA).

Full experimental procedure ofPRDM1 copy number variation, mRNA expression, promoter methylation analysis and exon/exon-intron junction sequencing, cell cycle and apoptosis assays, PRDM1 reconstitution experiments, PRDM1 shRNA design and knockdown in NK cells, luciferase assay, and western blot are presented in SI Methods.

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Figure Legends

Fig. 1. PRDM1 is silenced through a combination of deletion and promoter methylation. (A)NKCL cases with or without deletion is determined by the PRDM1/RPL13A ratio (3 replicates), and cases with < 0.75 ratio (dashed horizontal line) to that of human tonsil DNA are defined as deleted. The deletion status of the NKCL cases by a-CGH is shown at the bottom.(**B)***PRDM1α* promoter CpG island is hypermethylated in NK-cell lines and NKCL cases. Methylation percentage (three technical replicates)of CpG dinucleotides upstream of the*PRDM1α* TSS is indicated as a heat map.Resting and activatedNK-cells (IL2 for 7days)are used as negative control samples.**(C)**q-RT-PCR was performed on 7 NKCL cases using primers specific for

PRDM1(α+β) (top panel) and *PRDM1α* (lower panel). *RPL13A* was used for normalization (n=2). Resting human NK-cells (R)and activated NK-cells (A) derived from 14 day coculture with K562-Cl9-mb21.

Fig. 2.Reconstitution of PRDM1 in PRDM1-null NK-cell lines induces apoptosis and cell cycle arrest **(A)**PRDM1 inducesapoptosis in NK-cell lines.Transduced, unsorted NK-cells were stained with AnnexinV-PE and tested with FACS. The rate of apoptosis was determined by measuring the proportion of AnnexinV(+) cells in the GFP(+) population 2 days post-transduction.**(B)**Quantification of the rate of apoptosis in GFP(+) population of vector or PRDM1 transduced cells. **(C)**Cell cycle profile of vector or PRDM1 transduced KHYG1 and KAI3 cells**(D)**The change in each cell cycle phase is calculated as [PRDM1]-[PMIG]3 days post-transduction.

Fig. 3:Reconstitution of PRDM1α exerts negative selection pressure in malignant NK-cell lines which increases with decreasing doses of IL2 in the culture medium. The FACS profile showing the percentage of GFP(+) cells of the vector or PRDM1 transduced KHYG1 **(A)** and NKYS cells **(B)**before and after treatment with progressively decreasing doses of IL2**.(C)**Comparison of the percentage of GFP(+) cells before and after treatment of PRDM1 transduced KHYG1 and NKYS cells with limiting doses of IL2.Each data point was calculated as follows: [%GFP(PRDM1)]/[%GFP(VECTOR)].IL2 post-treatment values were normalized to the values 48h post-transduction.Data are mean ± SD of two independent experiments. **(D)**Comparison of apoptosis with limiting doses of IL2 in presence and absence of PRDM1.PRDM1 or vector only transduced KHYG1 cells were treated with progressively decreasing doses of IL2 for 2 or 5 days48h post- transduction. **(E)**PRDM1-dependent induction of apoptosis is enhanced with limiting IL2 concentrations in malignant NK-cells.

Fig.4:Knockdown of PRDM1 with shRNA results in the positive selection of human primary NK-cells.(A)PRDM1 shRNA was PCR-cloned inside the miR-30a backbone in MSCV-TMP. The %of GFP (+) cells was compared between vector or PRDM1 siRNA transduced NKYS **(B)**or primary NK cells **(C)** 3 and 7 days (NKYS) or 4, 7 and 10 days (primary NK cells)post-transduction.Data are mean ± SD of two independent experiments.

Fig.5:PRDM1 regulates expression of genes involved in cell cycle and activation in NK-cells.(A) q-RT-PCR results of target genes regulated by PRDM1 in vector or PRDM1 transduced, GFP-sorted KHYG1 cells 2 days post-transduction **(B)**q-RT-PCR results on vector or PRDM1-shRNA transduced, GFP sorted NKYS cells 6 days post-transduction.**(C)***MYC* mRNA expression was performed by q-RT-PCR on primary NK-cells activated by IL2. *RPL13A* was used for normalization (n=2).