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Generation of novel Id2 and E2-2, E2A and HEB antibodies reveals novel Id2 binding partners and species-specific expression of E-proteins in NK cells

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ABSTRACT

NK cells are cytotoxic lymphocytes with a key role in limiting tumour metastases. In mice, the NK cell lineage continually expresses high levels of the Inhibitor of DNA-binding 2 (Id2) protein and loss of Id2 is incongruous with their survival due to aberrant E-protein target gene activity. Using novel Id2 and E-protein antibodies that detect both mouse and human proteins, we have extensively characterised Id2 and E-protein expression in murine and human NK cells. We detected clear expression of E2 A and HEB, and to a lesser extent E2-2 in murine NK cells. In contrast HEB appears to be the major E-protein expressed in human NK cells, with minor E2-2 expression and surprisingly, no E2 A detected in primary NK cells nor human NK cell lines. These novel antibodies are also functional in immunofluorescence and immunoprecipitation. Mass spectrometry analysis of Id2 immuno-precipitated from murine NK cells revealed a number of novel associated proteins including several members of the SWI/SNF-related matrix-associated actin-dependent regulator chromatin (SMARC) and Mediator complex (MED) families. Taken together, these data highlight the utility of novel Id2 and E-protein antibodies and caution against mouse models for understanding Id2/E-protein biology in NK cells given their clearly disparate expression patternbetween species.

1. Introduction

Natural Killer (NK) cells are becoming an area of increasing investment in cancer immunotherapeutic development. NK cells are bone marrow-derived innate lymphocytes that represent a frequent circulating population in the blood and lymphoid tissues, and are less often tissue-resident. Their spontaneous anti-tumor activities have made them a high priority in immunotherapy research, yet to date we have failed to efficiently target NK cells in cancer patients (Huntington et al., 2007a). NK cells can eliminate tumors directly, and this activity is controlled by the integration of signals from tumor ligands, metabolites and cytokines (Huntington et al., 2007a; Vivier et al., 2012).

The pleiotropic cytokine IL-15 and the inhibitor of DNA-binding 2 (Id2) are central regulators of NK cell development, homeostasis and activation (Huntington, 2014). Id2 *(encoded by Idb2)* plays an

indispensable role in the development of NK cells (Boos et al., 2007; Yokota et al., 1999). Id2 is proposed to dimerize with the transcription factors E2 A (E47/E12), E2-2 and HEB (encoded by the genes *Tcf3, Tcf4* and *Tcf12* respectively) in lymphoid progenitors, thus preventing their binding to E-box (CANNTG) containing target genes (Ephrussi et al., 1985; Henthorn et al., 1990; Murre et al., 1989). E-boxes are found in transcription factors essential for T and B cell development, supporting their instructive role in the development of these lineages (Quong et al., 2002). E-proteins possess a basic DNA-binding region and a helix-loop-helix (HLH) domain that mediates homo-dimerization and transcriptional activation, or hetero-dimerization with additional HLH proteins that can either repress or activate gene transcription. Id2 also contains an HLH domain but lacks the basic region and prevents E-proteins from binding DNA through hetero-dimerization. However, how silencing E-box genes promotes NK cell development remains enigmatic. The

earliest NK cell progenitor termed the pre-pro NK, expresses *Id2* and has lost the potential to give rise to B or T cells, but can efficiently differentiate into NK cells (Carotta et al., 2011). All NK cells derive from Id2$^+$ progenitors (Klose and Diefenbach, 2014), but the subsequent transcriptional events that establish this lineage and their dependency on IL-15 are less clear. Once committed to the NK cell lineage, differentiation proceeds through a three-step linear pathway, which is associated with increasing Id2 expression and alterations in IL-15 signalling, IFN-γ secretion and tumour killing capacity (Delconte et al., 2016a; Huntington et al., 2007b).

We recently identified a novel mechanism for the essential role of Id2 in NK cell development. We found that *Id2* regulates IL-15 receptor signalling and homeostasis of NK cells by repressing multiple E-protein target genes including *Bcl2l11, Tcf7* and *Socs3* (Rautela et al., 2018a; Rautela and Huntington, 2017). Id2 loss-of-function in mature NK cells was incompatible with their survival due to impaired IL-15 signalling and metabolism and this could be rescued by super-physiological IL-15 receptor ligation or genetic ablation of *Socs3* (Delconte et al., 2016b). As NK cells differentiate we found an inverse correlation between E-protein target genes and Id2 expression. These results have shifted the current thinking around Id2 function, indicating that Id2 is not only required to antagonize E-proteins during NK cell commitment, but is constantly required to titrate E-protein target gene expression to regulate NK cell fate and responsiveness to IL-15. Here, we report that while E2 A and HEB are the dominant E-proteins in murine NK cells, E2 A is absent from human NK cells, questioning the translational relevance of E2 A biology in NK cells and highlighting the potential importance of Id2-HEB interactions in human NK cells. Further, we describe several novel E-protein antibodies that are functional in Western blotting, immunoprecipitation, flow cytometry and immunofluorescence histology. Lastly, we identify numerous novel Id2-interacting proteins in NK cells, which may extend the role of Id2 in regulating the transcriptional control of NK cell biology.

2. Results

2.1. Novel blotting and immunoprecipitating antibodies against Id2, HEB, E2 A and E2-2 in NK cells

We previously reported that *Id2* is required at all stages of NK cell maturation and to maintain responsiveness to homeostatic or physiological concentrations of IL-15. Id2 mRNA was found to increase with maturation corresponding to a reduction in *Tcf3, Tcf4* and *Tcf12* and their target genes including *Tcf7* and *Socs3*. In order to investigate Id2 and E-protein expression in NK cells we generated several novel monoclonal antibodies against Id2, HEB, E2 A and E2-2. We first tested their ability to detect their target proteins in primary murine NK cell lysates. C57BL/6 splenic NK cells were expanded *in vitro* using IL-15 and protein lysates probed by western blotting, with murine B cells serving as positive controls, given their high *Tcf3, Tcf4* and *Tcf12* expression. Murine NK cells expressed abundant Id2 (~16KDa) consistent with our previous work, and similarly high levels of E2 A (~71KDa) and HEB (~81KDa) were detected; Fig. 1A). E2-2 (~75KDa) was clearly less abundant in NK cells compared to E2 A and HEB, which is in line with our published RNAseq data on murine NK cells (Delconte et al., 2016a, b). We next tested if these novel antibodies could immunoprecipitate (IP) their target proteins and to what degree they co-immunoprecipitated proposed HLH binding partners. Using Id2 as bait (anti-Id2 IP), we detected Id2, E2 A and HEB in the post-IP elute indicating that in murine NK cells, Id2 is bound to both E2 A and HEB (Fig. 1B). Interestingly, when E2 A, E2-2 and HEB were used as bait we failed to detect any bound Id2 despite each anti-E-protein antibody successfully immunoprecipitating its direct target (Fig. 1B). Furthermore, we failed to detect any E-protein heterodimers (i.e E2 A did not IP HEB or E2-2). We observed that the efficiency of Id2 IP increased following inhibition of proteosomal degradation using MG132 (data not

shown) and found Id2 to be quite sensitive to proteosomal degradation, with most of the Id2 in murine NK cells being degraded *via* the proteasome within 4 h (Fig. 1C). Furthermore, in human NK cells treated with MG132 we also detected abundant HEB bound to Id2 (Using Id2 as bait; Fig. 1D). Taken together, these novel E-protein and Id2 antibodies are useful for IP and western blotting and deliver much needed research tools for the field.

2.2. HEB and E2-2, not E2 A are the dominant E-proteins in human NK cell lines

We next sought to confirm if the E-protein expression pattern detected in murine NK cells also holds true in human NK cells. As a first pass, we tested several human NK cell lines, KHYG1, NKL, SNK6, SNK10 and NK92 and compared these to primary murine NK cells. Using the anti-Id2 3B11 clone, we detected varying expression of Id2 in all NK cell lines, and while Id2 expression in NK92 cells was low compared to KHYG1, NKL and SNK6 (Fig. 2A), this was increased in the presence of MG132 (Fig. 2B). HEB expression was detected across all human NK cell lines, while E2-2 was also detected in all lines except KHYG1, albeit at a lower abundance than HEB (Fig. 2A–C). Surprisingly, despite its expression being similar to HEB in murine NK cells, E2A was absent from all human NK cell lines investigated (Fig. 2A, C) and this was not due to protein stability, since MG132 treatment of NK cell lines did not alter this result (Fig. 2B). E2-2 abundance in NK92 on the other hand was increased following MG132 treatment (Fig. 2B). These results highlight the species cross-reactivity of these novel Id2 and E-protein antibodies and indicate that Id2 binding to and inhibiting E2A target gene expression is only relevant in mice since E2A does not appear to be expressed in human NK cell lines.

2.3. HEB is the dominant E-protein in human NK cells

The NK cell lines used in our studies contain numerous mutations/ copy number variations and are all derived from human NK lineage leukemia/large granular lymphomas (Klingemann et al., 2016). To confirm that E2 A loss in human NK cell lines was not specific to these transformations but a general feature of all human NK cells, we next analyzed Id2 and E-protein expression in adult and cord blood primary human NK cells. Consistently, we detected high levels of HEB in primary human NK cells, however E2 A was not detected in any samples, while a relatively small amount of E2-2 was only detected in one donor NK cell pool (Fig. 3A, B). These data confirm that E2 A is absent from human NK cells and underline that the principle E-protein binding partner for Id2 in human NK cells is HEB.

2.4. Id2 and E2A are localized in the nucleus of murine NK cells

Our previous work revealed that high, constant expression of Id2 in murine NK cells was required to suppress E-protein target gene expression. In murine NK cells, E2 A and HEB appear to be the major binding partners for Id2, given their high expression levels. To investigate where the Id2-E2 A binding predominantly occurs, we used immunofluorescence microscopy of IL-15 activated murine NK cells attached to a microscope slide. NK cell surface membranes were stained with anti-NK1.1 (an activating receptor) while Id2 and E2 A expression was probed with the respective antibodies in permeabilized cells, and DAPI was used to stain the nuclear DNA. We found that both anti-Id2 (1E9) and anti-E2 A (7H3) functioned well in immunofluorescence microscopy and that Id2 and E2 A were only detected in the nuclei of murine NK cells (Fig. 4A, B). These data confirm that both Id2 and E2 A are co-localized in the nucleus and no cytoplasmic pool of either Id2 or E2 A is detected in murine NK cells.
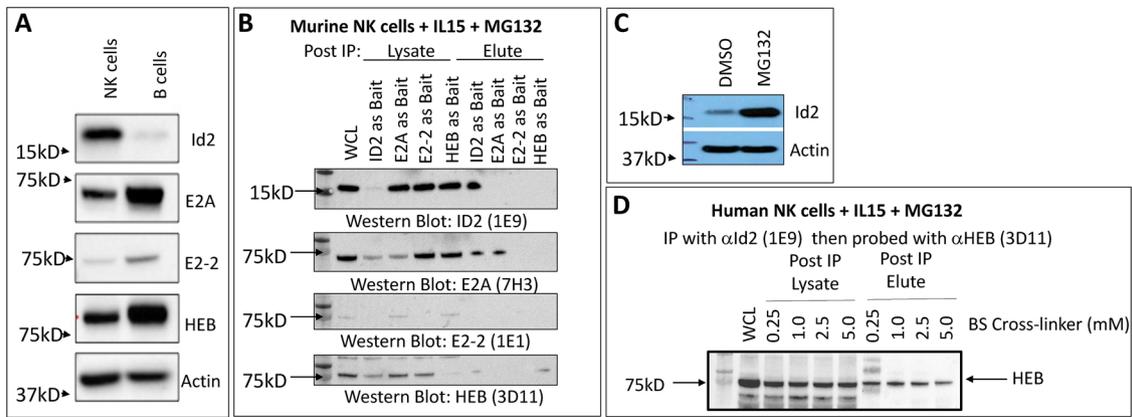
Fig. 1. Novel blotting and IP antibodies against Id2, HEB, E2 A and E2-2 in NK cells. (A) Murine NK cells and B cells were purified from spleen and lysates from 1 × 10 (Ephrussi et al., 1985) cells were subjected to Western blotting using anti-Id2, E2 A, E2-2, Actin and HEB. (B) Murine NK cells were expanded in IL-15 and treated with MG132 for the final 4 h. Anti-Id2, E2 A, E2-2 and HEB IPs were performed on the lysate and post-IP lysate and elute were probed for Id2, E2 A, E2-2 and HEB. WCL (whole cell lysate). (C) Murine NK cells were expanded in IL-15 and treated with MG132 or DMSO for the final 4 h. Lysates were probed for Id2 or Actin. (D) Human NK cells were expanded in IL-15 and treated with MG132 for the final 4 h. Lysates were subjected to anti-Id2 IP and post-IP lysates and elutes were blotted for HEB. Blots are representative of 2 experiments.
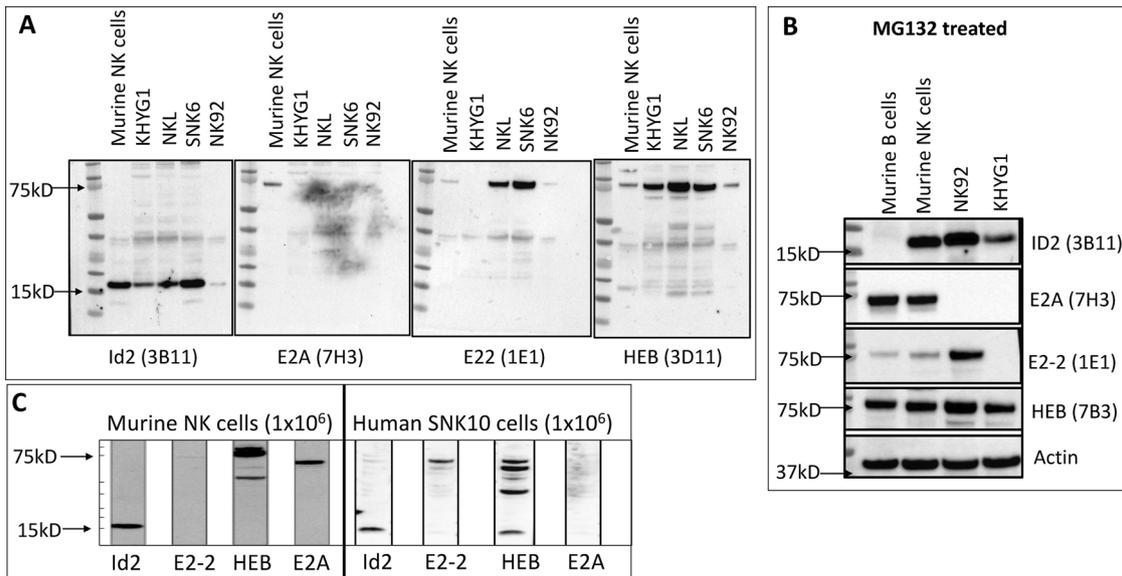

Fig. 2. HEB and E2-2, not E2 A are the dominant E-proteins in human NK cell lines. (A) Lysate from 1 × 10 (Ephrussi et al., 1985) murine NK cells, KHYG1, NKL, SNK6 and NK92 were probed for Id2, E2 A, E2-2 and HEB. (B) Lysate from 1 × 10 (Ephrussi et al., 1985) murine NK cells, murine B cells, KHYG1 and NK92 cells pre-treated with MG132 for 4 h were probed for Id2, E2 A, E2-2 and HEB. (C) Lysate from 1 × 10 (Ephrussi et al., 1985) murine NK cells and SNK10 cells were probed for Id2, E2 A, E2-2 and HEB. Blots are representative of 2 experiments.
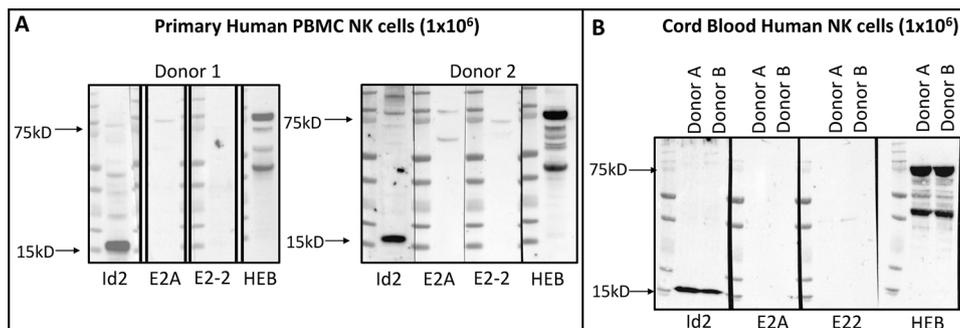

Fig. 3. HEB is the dominant E-protein in human NK cell lines. Lysates from 1 × 10 (Ephrussi et al., 1985) primary human NK cells from (A) peripheral blood or (B) umbilical cord blood were probed for Id2, E2 A, E2-2 and HEB. Blots are representative of 2 experiments and 6 donors.
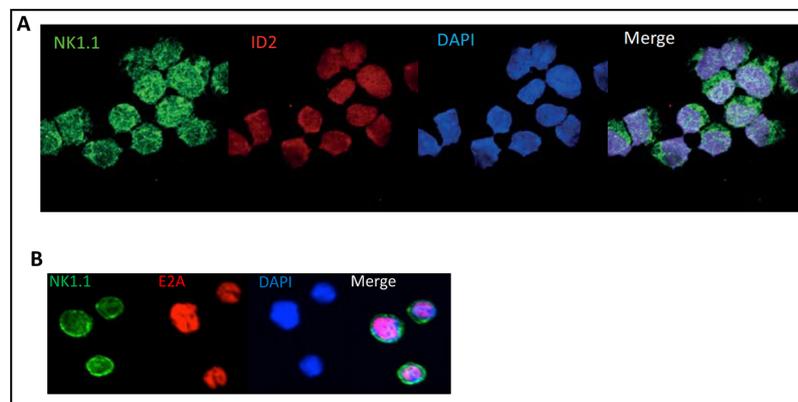
Fig. 4. Id2 and E2 A are localized in the nucleus of murine NK cells. Murine NK cells were purified from spleen and fixed to microscope slides and stained with anti-NK1.1, DAPI, (A) anti-Id2 and (B) anti-E2 A. Photomicrographs are representative of 3 experiments.

2.5. Identification of novel Id2 binding partners in murine NK cells

E-proteins and inhibitor of DNA-binding proteins are widely expressed transcriptional regulators with basic biological functions. By way of its HLH domain, Id2 has been proposed to bind other classes of HLH proteins in addition to E2-2, E2 A and HEB including MyoD, pRb and PAX (Wang and Baker, 2015). To investigate whether Id2 played additional roles in NK cells besides preventing E2 A or HEB target gene expression, we took an unbiased approach and performed mass spectrometry on proteins from NK cell lysates that had been enriched using Id2 as bait (anti-Id2 IP). We used three biological replicates of murine NK cell lysates that had been subjected to anti-Id2 IP, while non-specific but isotype matched immunoglobulin was used for IP as a negative control. Indeed, Id2 was efficiently enriched post-Id2 IP, whereas no Id2 was enriched following control Ig IP (Fig. 5A). The biological replicates of Id2 IP elute were then subjected to mass spectrometry, where a similar pattern of bound peptides was observed (Fig. 5B). Volcano plots illustrating the log2 protein ratios of proteins enriched in Id2 IP relative to control Ig IP following quantitative pipeline analysis revealed numerous significantly enriched proteins across the three replicates (Fig. 6A). Proteins were deemed differentially regulated if the log2 fold change in protein expression was greater than 2-fold (red) or 4-fold (green) and a –log10 p value $\geq$ 1.3, equivalent to a p value ≤ 0.05 (Fig. 6A). Interestingly, we observed a consistent 4-fold enrichment of several members of the SMARC chromatin remodelling complex (Smarcc1, Smarcb1, Smarce1, Smarcd2, Smarcc2, Smarca4) and the Mediator complex (Med24, Med17, Med14) involved in transcriptional regulation of RNA polymerase II-dependent genes (Fig. 6A), suggesting additional novel roles for Id2 in regulating gene expression in NK cells.

We also determined the Log2-Log2 protein ratio plots highlighting the proteins that have been specifically enriched in ID2 IP (green) and do not exhibit significant differential RNA? expression in the while cell lysates (WCL) from which the IPs were performed (Fig. 6B). Lastly, we performed a protein interaction network analysis of candidate-Id2 interacting proteins. Two distinct modules (tightly clustered subnetworks) can be visualised- the SMARC chromatin remodelling complex and the Mediator complex involved in transcriptional regulation of RNA polymerase II-dependent genes (Fig. 6C). These data open the door to further investigation around the role of Id2 in regulating NK cell lineage commitment, development and function by interacting with transcriptional regulators in addition to HEB and E2 A.

3. Discussion

NK cells represent a key immune effector in limiting viral infection and tumor metastasis (Rautela et al., 2018a; Rautela and Huntington, 2017; Delconte et al., 2016b; Gao et al., 2017; Viant et al., 2017). The unique ability of NK cells to detect and kill circulating tumor cells in experimental models has driven a growing interest in NK cell immunotherapies targeting hematological malignancies and residual disease in solid cancer (Guillerey et al., 2016). Id2 is central to the NK cell anti-tumor function as it maintains optimal NK cell fitness and responsiveness to homeostatic cytokines such as IL-15 (Delconte et al., 2016a), a cytokine known to drive STAT5-target gene expression including *Mcl1, Bcl2, Cish, Ifng* and *Gzmb* (Rautela and Huntington, 2017) (Rautela et al., 2018b, 2015). As such, identifying the major E-protein binding partners of Id2 is a key step in understanding their precise role in NK cell biology.
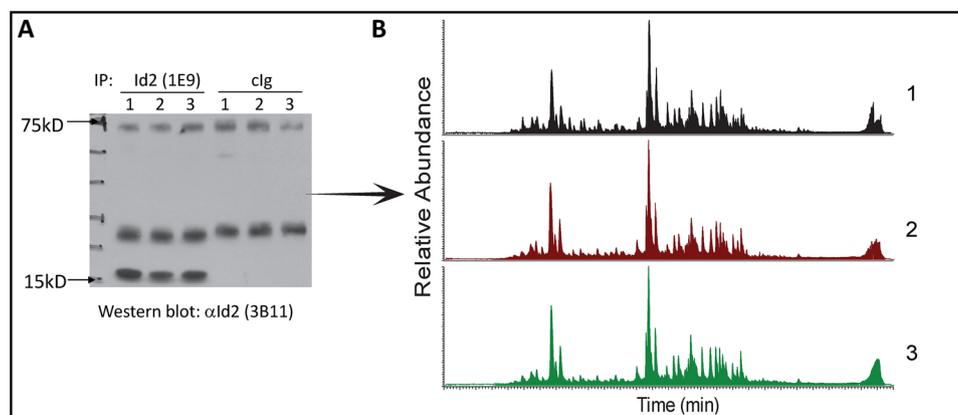

Fig. 5. Mass spectrometry of Id2 binding partners in murine NK cells. Anti-Id2 IP was performed on lysates from 1.5×10^7 murine NK cells and (A) probed from Id2 by Western blotting or (B) subjected to Mass Spectrometry. Relative peptide abundance over time for biological triplicates is shown.
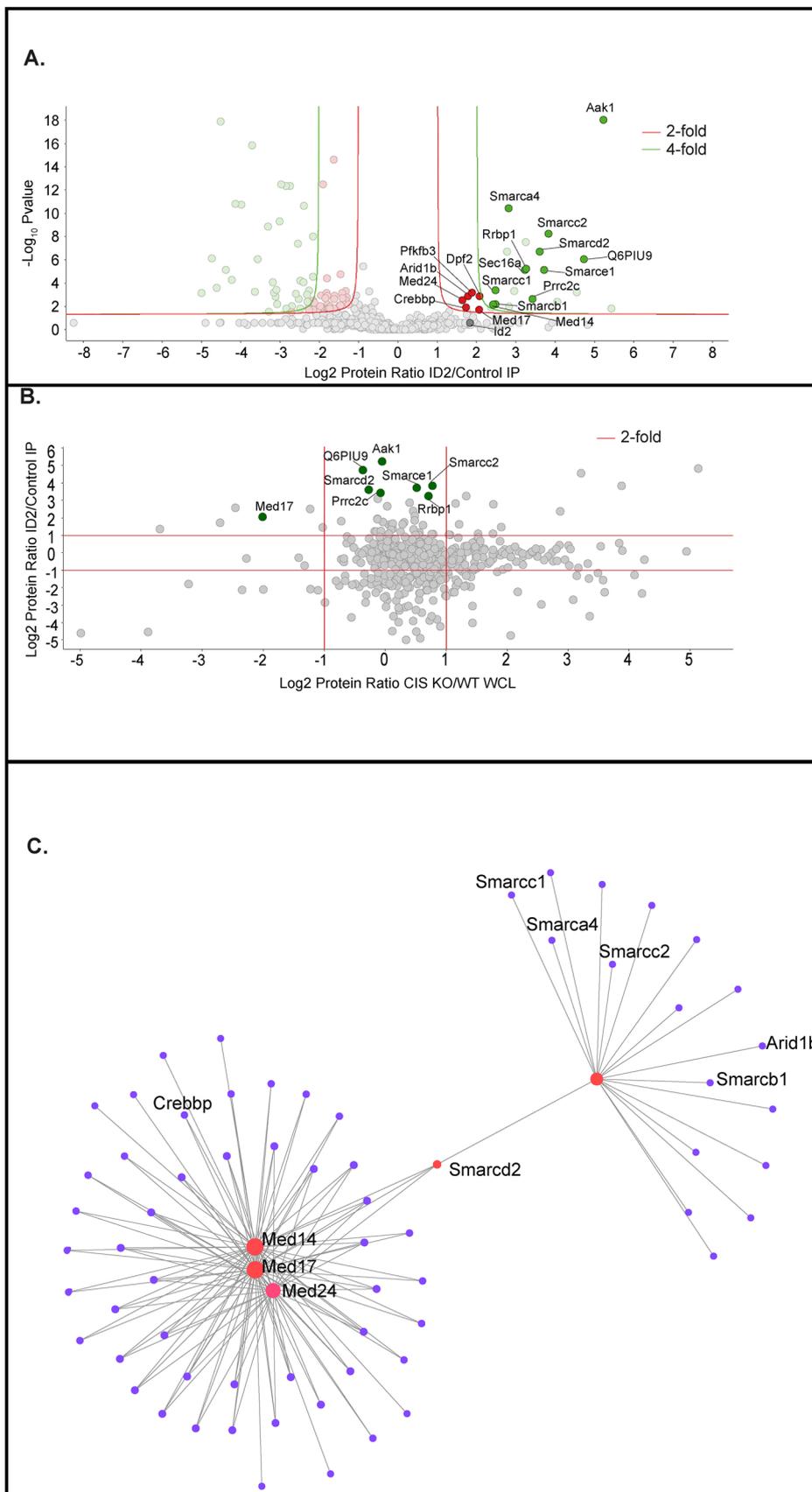
Fig. 6. Identification of novel Id2 binding partners in murine NK cells. (A) Volcano plot illustrating the log2 protein ratios of proteins enriched in ID2 IPs relative to control Ab IPs following quantitative pipeline analysis. Proteins were deemed differentially regulated if the log2 fold change in protein expression was greater than 2-fold (red) or 4-fold (green) and a –log10 *p* value ≥ 1.3, equivalent to a p value ≤ 0.05. (B) Log2-Log2 protein ratio plots highlighting the proteins that have been specifically enriched in ID2 IPs (green) and do not exhibit significant differential expression in the WCLs from which the IPs were performed. (C) Protein interaction network of candidate-Id2 interacting proteins built within Network Analyst. Two distinct modules (tightly clustered subnetworks) can be visualised- the SMARC chromatin remodelling complex and the Mediator complex involved in transcriptional regulation of RNA polymerase II-dependent genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To address the current void in our understanding of E-proteins in NK cell biology, we generated novel E2 A, E2-2 and HEB antibodies which cross-react to both murine and human proteins. These antibodies can be used in various assays from Western blotting, immune-precipitation and immunofluorescence. Both HEB and Id2 antibodies were also functional in intracellular flow cytometry (Delconte et al., 2016a) and data not shown. Our results clearly demonstrate that Id2 is localised in the nucleus where it binds to E2 A/HEB in murine NK cells and HEB in human NK cells to prevent their target gene expression and to maintain NK cell lineage function. The only previous investigation into E-protein function in NK cells focussed on the role of Id2 binding to, and suppressing, E2 A and the consequences of this interaction on NK cell development (Boos et al., 2007). In the absence of Id2, deletion of E2 A did not rescue NK cell development. Thus it was concluded that other E-proteins expressed in NK cells were able to drive gene expression that were deleterious to NK cell development. Our E-protein protein analysis herein suggests that HEB is likely to be driving expression of these genes, given it is substantially more highly expressed than E2-2 in murine NK cells. Intriguingly, human NK cells appear to only express HEB, thus it would be interesting to determine whether loss of HEB alone in human NK cell lines could support NK cell development in the absence of Id2. It is worth mentioning that we are yet to test whether Id2 is equally essential for preventing expression of genes such as *Tcf7* and *Socs3* in human NK cells and if Id2-null human NK cells fail to respond to IL-15, as is the case for the murine system. Furthermore, given the dramatically different E-protein expression patterns between murine and human NK cells, we must be cautious in inferring that the biology of Id2 and E-proteins in murine NK cells can be applied to human NK cells. These results pose the question - what drove the loss of E2 A expression in human NK cells?

A key finding from this work is that numerous additional proteins that are classed as transcriptional regulators appear to be bound to Id2 in NK cells. Using an unbiased approach, we used Id2 as bait to immunoprecipitate protein complexes from *in vitro* expanded murine NK cells. Western analysis demonstrated that Id2 was indeed enriched following anti-Id2 IP in murine NK cells and HEB and E2 A appear to be associated as they were clearly detected by Western blotting the anti-Id2 elute. Despite this, Id2, E2 A and HEB were not the most significantly enriched proteins detected by Mass Spectrometry analysis of murine anti-Id2 IP elute compared to control Ig elute. Id2 itself was enriched, however enrichment of members of the SMARC and MED family of transcriptional modifiers were significantly higher, indicating a potentially important role for Id2 in binding these proteins either directly or indirectly. Given that Id2 is continually required to retain NK cell-dependent tumour immunity (Delconte et al., 2016a), our data suggest that mechanisms of Id2 regulation (post-translational and transcriptional) are central to NK cell effector function and may represent a novel area of therapeutic drug development for cancer immunotherapy. Id2 is subject to K48-linked polyubiquitination and subsequent degradation by the 26S proteasome. As a result, Id2 is short lived in most tissues (Bounpheng et al., 1999). Indeed, we observed that Id2 is rapidly degraded by the proteasome and so high transcriptional activity is required to maintain adequate levels required to bind and inhibit transcription factor activity that are detrimental to NK cell genesis.

Id2 is targeted by several E3 ubiquitin ligases including APC/Cdh1 and ASB4 to control ID2 protein stability and abundance (Lasorella et al., 2006). E2 A binding to Id2 has also been shown to promote Id2 stability. The deubiquitinatinases (DUB) *Usp5, 10, 19, 48 and 1* are highly expressed throughout NK cell maturation (our NCBI data GSE76466). USP1 has been shown to bind, deubiquitinate, and thereby stabilize ID2 (Williams et al., 2011). A fraction of primary human osteosarcomas were found to overexpress both USP1 and ID proteins, and ectopic USP1 expression in mesenchymal stem cells stabilized ID proteins, blocked differentiation, and enhanced proliferation (Williams et al., 2011). Transcriptionally, we have shown that *Id2* mRNA is

enhanced in response to IL-15, while others have found growth factor independence 1 (*Gfi1*), which is expressed in NK cells, acts as a repressor of *Id2* in progenitors and is essential for B and myeloid cell development (Li et al., 2010). In closing, our data suggest Id2 expression is key to NK cell fate and function and Id2 levels could impact on E-protein, SMARC and MED transcriptional activity. The impact of potential interactions between Id2 and novel binding partners warrants further investigation.

4. Materials & methods

4.1. Mice

C57BL/6 male and female mice were used between the ages of 6–14 weeks. All mice were bred and maintained at the Walter and Eliza Hall Institute. Animal experiments followed the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines and were approved by the Walter and Eliza Hall Institute Animal Ethics Committee (AEC2015.017; 2012.004).

4.2. Purification and culture of NK cells

Murine natural killer cells were harvested from various organs (spleen, bone marrow, blood) and single-cell suspensions prepared by forcing of organs through 70 mM sieves. Lymphocytes were isolated from liver by suspension in isotonic Percoll (Amersham Pharmacia Biotech) and centrifugation at 1800 x *g*. Murine NK cells were purified using anti-CD49b (DX5) Microbeads (Miltenyi Biotec) according to manufacturer's specifications. Human NK cells were purified from adult donor blood buffy coat or umbilical cord blood (obtained from the Bone Marrow Donor Institute/Cord Blood Research Unit; Murdoch Children's Research Institute, Australia) prepared by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma) using anti-CD56 magnetic beads (Miltenyi Biotech). Use of Human material for Scientific Purposes guidelines and were approved by the Walter and Eliza Hall Institute Human Research Ethics Committee (HREC12/08). Human NK cell lines (SNK10, SNK6, NKL) were a gift from Professor Maher Ghandi (Diamantina Institute, University of Queensland). NK92 and KHYG1 were obtained from ATCC. NK cells were expanded for 5–10 days by culture in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) foetal calf serum (FCS), L-glutamine (1 mM; Gibco), streptomycin (100 mg/mL; Sigma), penicillin (100 IU/ml; Sigma), gentamycin (50 ng/ml; Sigma) and recombinant human IL-15 (50 ng/ml; Peprotech).

4.3. Immunoprecipitations in NK cells

For each IP, 1.5 mg of Protein G Dynabeads were washed in PBS and crosslinked to individual antibodies using Conjugation Buffer (20 mM Sodium Phosphate 150 mM Sodium Chloride ph7.5) and Crosslinker BS (2.5 mM, PBS 0.02% Tween 20, 1 M Tris pH 7.6, 0.2 M Glycine pH 2.6, Triton Lysis Buffer with 1 ml of PBS Tween). 15μg of each antibody was washed (3x Conjugation Buffer) and crosslinked in 300 ml of 2.5μM Crosslinker BS for 1 h at room temp according to manufactures protocol (ThermoFisher Scientific).

Whole cell lysates were generated from 15 × 10 (Ephrussi et al., 1985) NK cells. Beads were eluted with hot SDS buffer (2% SDS, 100 mM Tris pH 7.5, 10% glycerol, 0.5 mM EDTA).

4.4. Immunoblotting

NK cells were lysed in Triton Lysis Buffer (25 mM Tris pH7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X100, 5% Glycerol) supplemented with Complete Protease Inhibitor Cocktail (Roche) and incubated for 1 h on ice. Lysates were clarified by centrifugation at 13,000 rpm for 10 min at

4 °C. NK cells were treated in culture for 4 h before harvest with 10 μM MG132 (Sigma) to block proteasomal degradation. Primary antibodies used were, ID2 (clones 1E9 and 3B11), E2 A/Tcf3 (clones 5B1 and 7H3), E2-2/Tcf4 (clones 8D2 and 1D1) and HEB/Tcf12 (clones 3D11, 5 A4, 7A3, 7D10) generated at the Walter and Eliza Hall Institute. Secondary antibody used was Goat-Anti Rat IgG HRP (Southern Biotech). Gel electrophoresis was performed with NuPAGE Bis-Tris Gels (ThermoFisher) and transferred using Trans-Blot Turbo (Bio-Rad). Immunoblots were performed using 5% skim milk powder in PBS 0.1% Tween 20 (Sigma) and developed using Immobilon Forte Western HRP substrate (Merck Millipore) and the ChemiDoc imaging system (Bio-Rad).

4.5. Trypsin digestion

Eluates of resin-captured proteins along with equal amounts of whole cell lysate derived from each biological replicate were prepared for mass spectrometry analysis using the FASP protein digestion kit (Protein Discovery, Knoxville, TN) as previously described (Wisniewski et al., 2009), with the following modifications. Proteins were reduced with Tris-(2-carboxyethyl)phosphine (TCEP) (5 mM final concentration), digested with sequence-grade modified Trypsin Gold (Promega) in 50 mM NH_4HCO_3 (4 μg for WCLs and 2 μg for IPs) and incubated overnight at 37 °C. Peptides were then eluted with 50 mM NH_4HCO_3 in two 40 μL sequential washes and acidified in 1% formic acid (final concentration).

4.6. Mass spectrometry and data analysis

Acidified peptide mixtures were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (LC–MS/MS) on a nanoAcquity system (Waters, Milford, MA, USA), coupled to a Q-Exactive mass spectrometer equipped with a nanoelectrospray ion source for automated MS/MS (Thermo Fisher Scientific, Bremen, Germany). Peptide mixtures were loaded on a 20 mm trap column with 180 μl inner diameter (nanoAcquity UPLC 2G-V/MTrap 5 μm Symmetry C_{18}) in buffer A (0.1% formic acid, 3% acetonitrile, Milli-Q water), and separated by reverse-phase chromatography using a 150 mm column with 75 μM inner diameter (nanoAcquity UPLC 1.7 μM BEH130 C_{18}) on a 60 min linear gradient set at a constant flow rate of 400 nL/min from 3 to 55% buffer B (0.1% formic acid, 80% acetonitrile, Milli-Q water). The Q-Exactive was operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the ten most abundant peaks. The instrument was controlled using Exactive series version 2.1 build 1502 and Xcalibur 3.0. Full-scans (m/z 350–1850) were acquired with a resolution of 70,000 at 200 m/z. The 10 most intense ions were sequentially isolated with a target value of 10,000 ions and an isolation width of 2 m/z and fragmented using HCD with normalized collision energy of 19.5 and stepped collision energy of 15%. Maximum ion accumulation times were set to 50 ms for full MS scan and 200 ms for MS/MS. Underfill ratio was set to 5% and dynamic exclusion was enabled and set to 90 s.

Raw files consisting of high-resolution MS/MS spectra were processed with MaxQuant (version 1.5.8.3) for feature detection and protein identification using the Andromeda search engine (Cox et al., 2011). Extracted peak lists were searched against the UniProtKB/Swiss-Prot *Mus musculus* database (October 2016) and a separate reverse decoy database to empirically assess the false discovery rate (FDR) using a strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids. Modifications: Carbamidomethylation of Cys was set as a fixed modification, while N-acetylation of proteins, oxidation of Met and the addition of pyroglutamate (at N-termini Gln) were set as variable modifications. The mass tolerance for precursor ions and fragment ions were 20 ppm and 0.5 Da, respectively. The "match between runs" option in MaxQuant was used to transfer identifications made between runs on the basis of matching precursors with high mass accuracy (Cox and Mann, 2008). PSM and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%. Protein identification was based on a minimum of two unique peptides.

4.7. Quantitative proteomics pipeline and data analysis

Further analysis was performed using a custom pipeline developed in Pipeline Pilot (Biovia) and R, as previously described (Delconte et al., 2016b). Cut-off lines with the function $y = -\log_{10}(0.05) + c/(x - x_0)$ (Keilhauer et al., 2015) were introduced to identify significantly enriched proteins. c was set to 0.2 while x_0 was set to 1 representing proteins with a with a 2-fold (log2 protein ratios of 1 or more) or 4-fold (log2 protein ratio of 2) change in protein expression, respectively. Protein-protein interaction network analysis was performed using InnateDB (www.innatedb.com) and visualized in Network Analyst (Xia et al., 2014). PPIs database from STRING was used, only experimental evidence was requied and a cut-off of 900 was applied (medium = 400, high = 1000). A first-order network was created and exported as an. svg file.

4.8. Immunofluorescence

Primary mouse NK cell adherence to microscope slides and immunofluorescence using antibodies specific for NK1.1 (PK136), E2 A (1E1) and Id2 (1E9) was performed as previously described (Hsu et al., 2016; Mace et al., 2012). DAPI (Biolegend) was used to stain nuclei as per manufactures instructions.

Authorship contributions

JR, LD, WG, AA, ES, TK, RBD, FSFG, LC and NDH designed and/or performed experiments. AW provided key reagents. JR, LC and NDH supervised experimental design, and provided input into interpretation of results and writing of the paper.

Disclosure of conflicts of interest

JR and NDH are co-founders and share-holders of oNKo-Innate Pty Ltd. NDH has a collaborative research agreement with Servier.

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