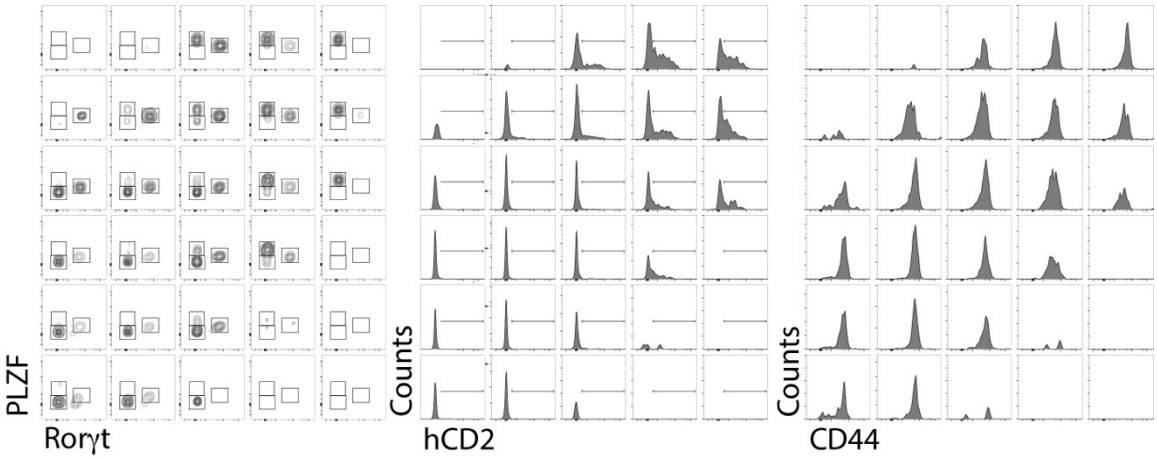
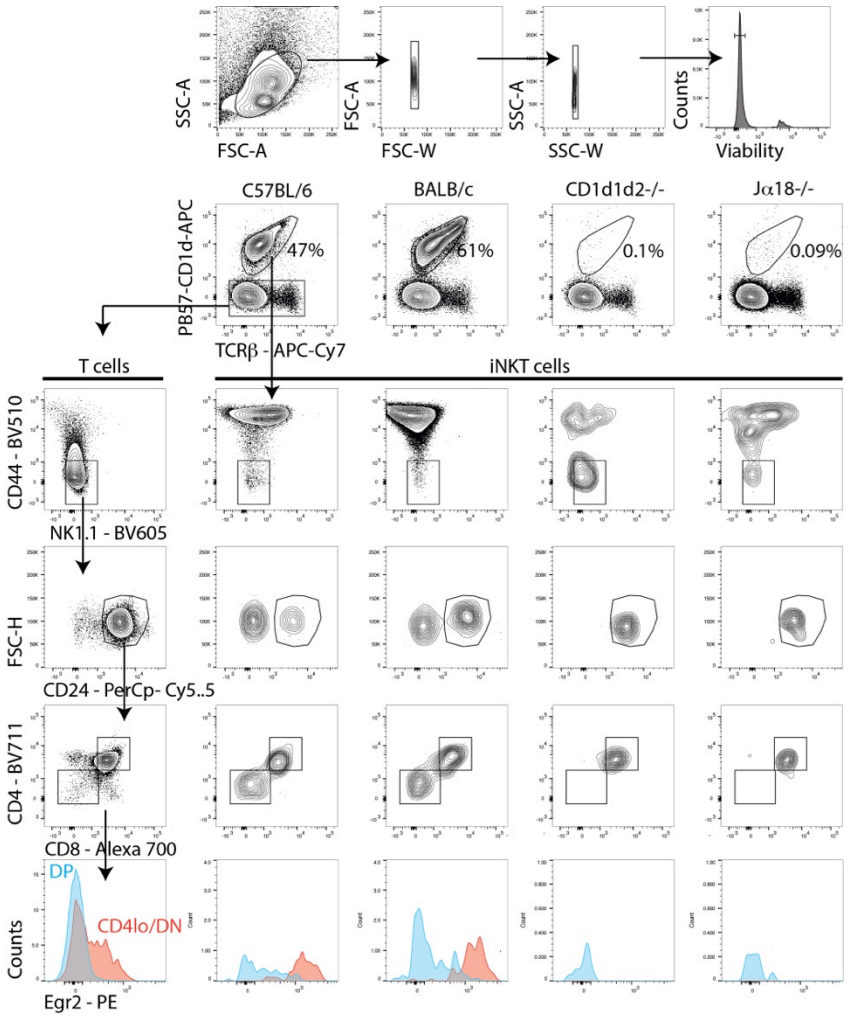
Supplementary Material

TCR signal strength controls thymic differentiation of iNKT cell subsets

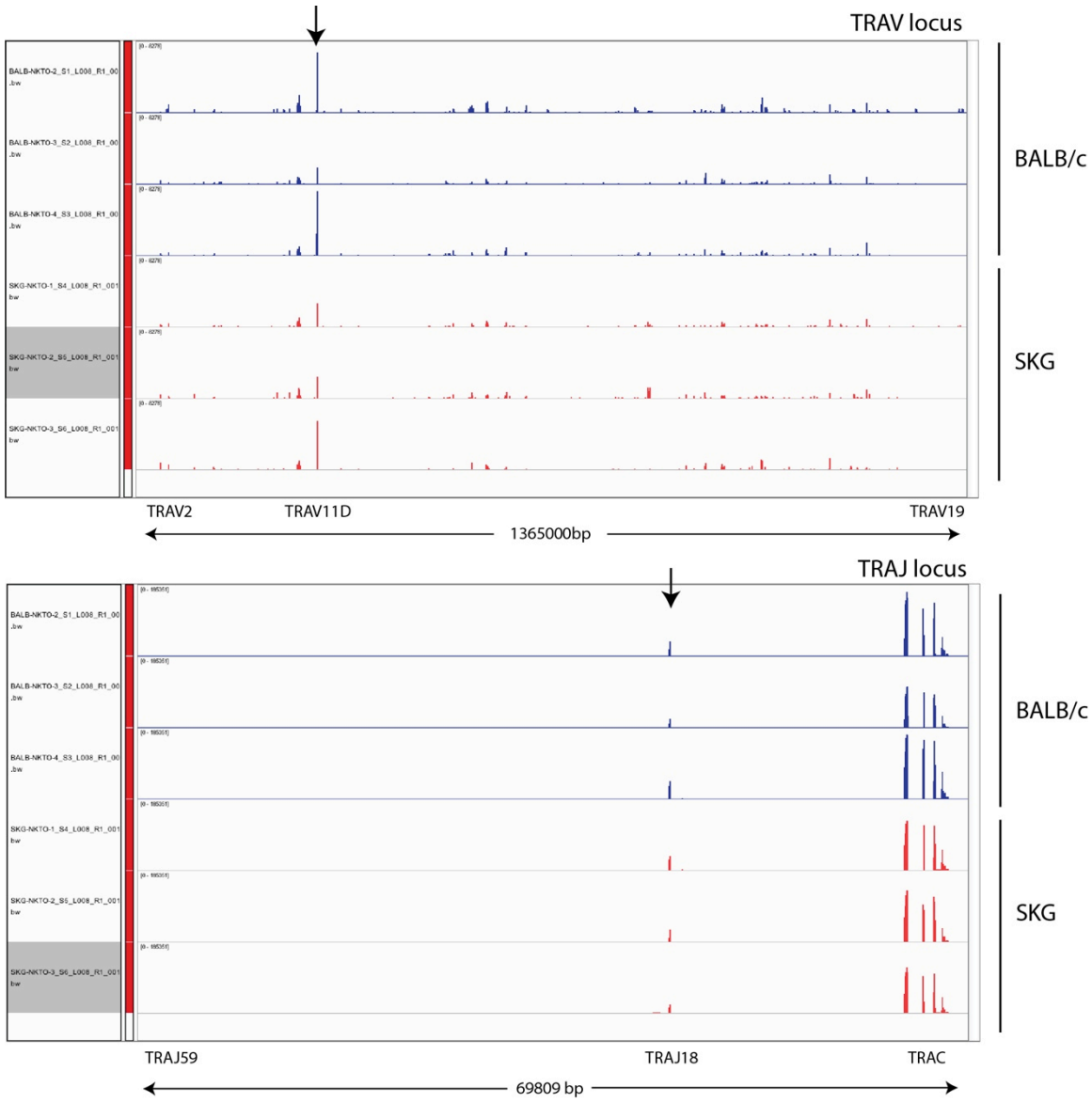
Tuttle et al.



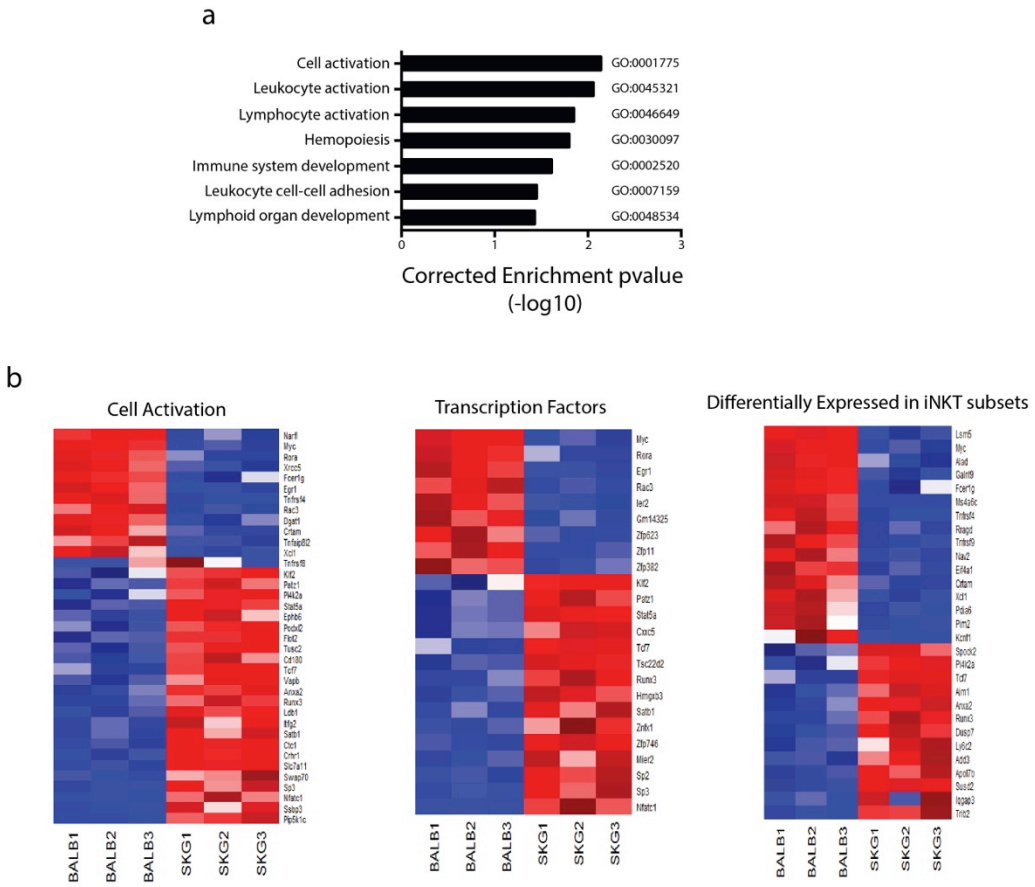
Supplementary Figure 1. Thymic iNKT subsets express TCRs of different avidities for the PBS57-CD1d tetramer. Cells from the thymus of BALB/c IL-4 reporter mice KN2 were stained with anti-TCRβ mAbs and PBS57-CD1d tetramers. iNKT cells were then electronically placed on a grid consisting of 30 gates (see Fig 1a). The proportion of NKT1 (PLZF^{lo}, Rorγt^{-}, Tbet^{+}), NKT17 (PLZF^{int}, Rorγt^{+}, Tbet^{-}), NKT2 (PLZF^{hi}, Rorγt^{-}, Tbet^{-}) or hCD2^{+} as well as the proportion of CD44^{hi} cells in each gate was recorded.



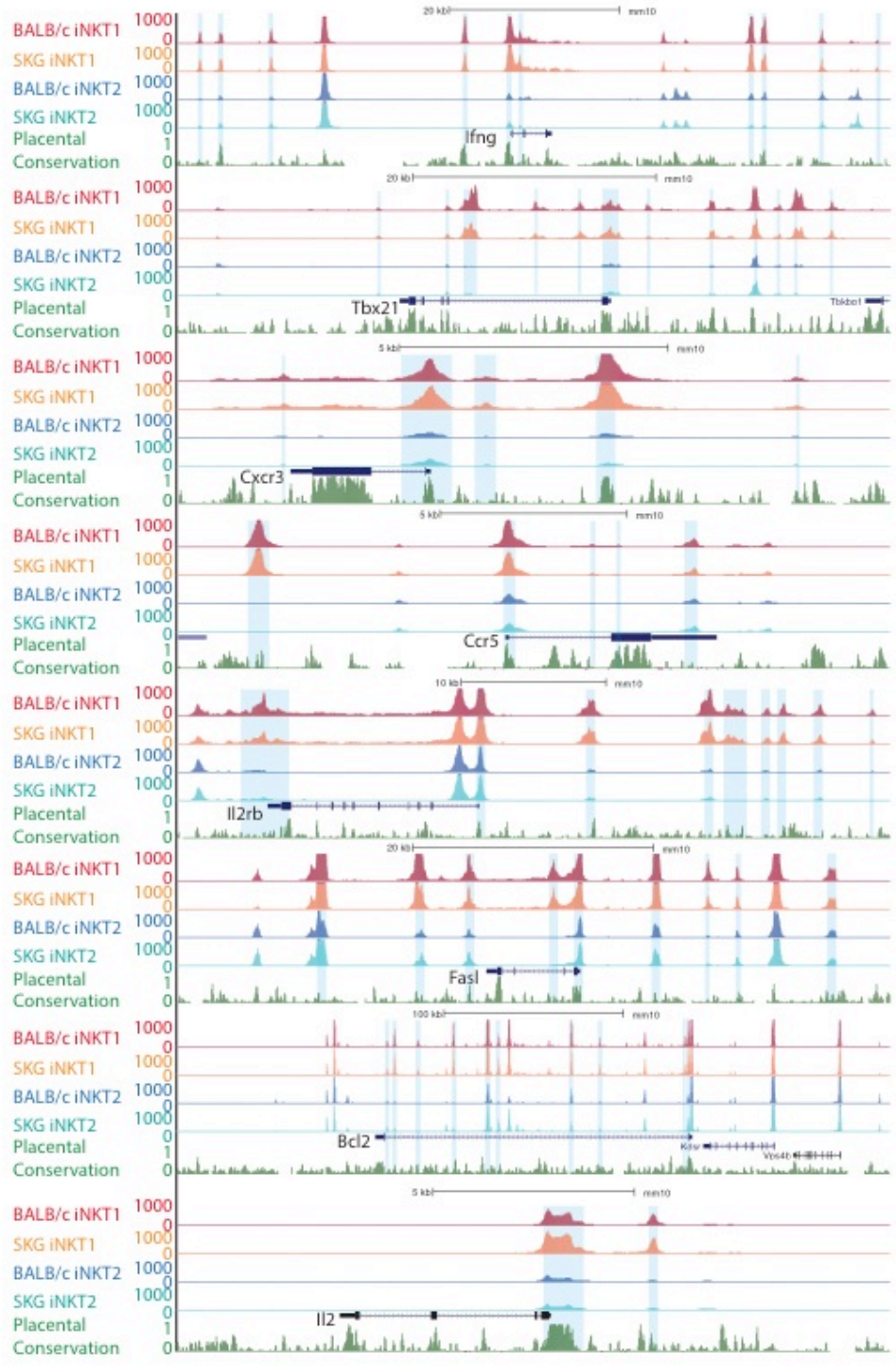
Supplementary Figure 2. Definition of stage 0 iNKT cells. Cells from the thymi of C57BL/6, BALB/c, CD1d1d2$^{-/-}$ and Jα18$^{-/-}$ mice were positively enriched for PBS57-CD1d tetramer^{+} cells using magnetic beads and an autoMACS Pro Separator and stained for the indicated markers. Doublets were excluded and only viable cells were included in the analysis. iNKT cells or "conventional" T cells were gated and analyzed following the gating strategy shown. CD44^{-} CD24^{+} iNKT cells contain some CD4^{+} CD8^{+} DP thymocytes that likely correspond to non-specific staining, as exemplified by the presence of this population in the thymi of CD1d1d2$^{-/-}$ and Jα18$^{-/-}$ mice. These DP cells do not express Egr2 or CD69 (Fig 5a). PBS57-CD1d tetramer^{+} TCRβ^{+} CD44^{-} CD24^{+} cells that are CD4^{lo} or CD4^{-}CD8^{-} (DN) are uniformly Egr2^{+} (and CD69^{+}) and can be detected only in the thymi of CD1d-sufficient mouse strains. Note that the levels of Egr2 expression detected in these cells is higher than what is detected in "conventional" T cells undergoing positive selection.



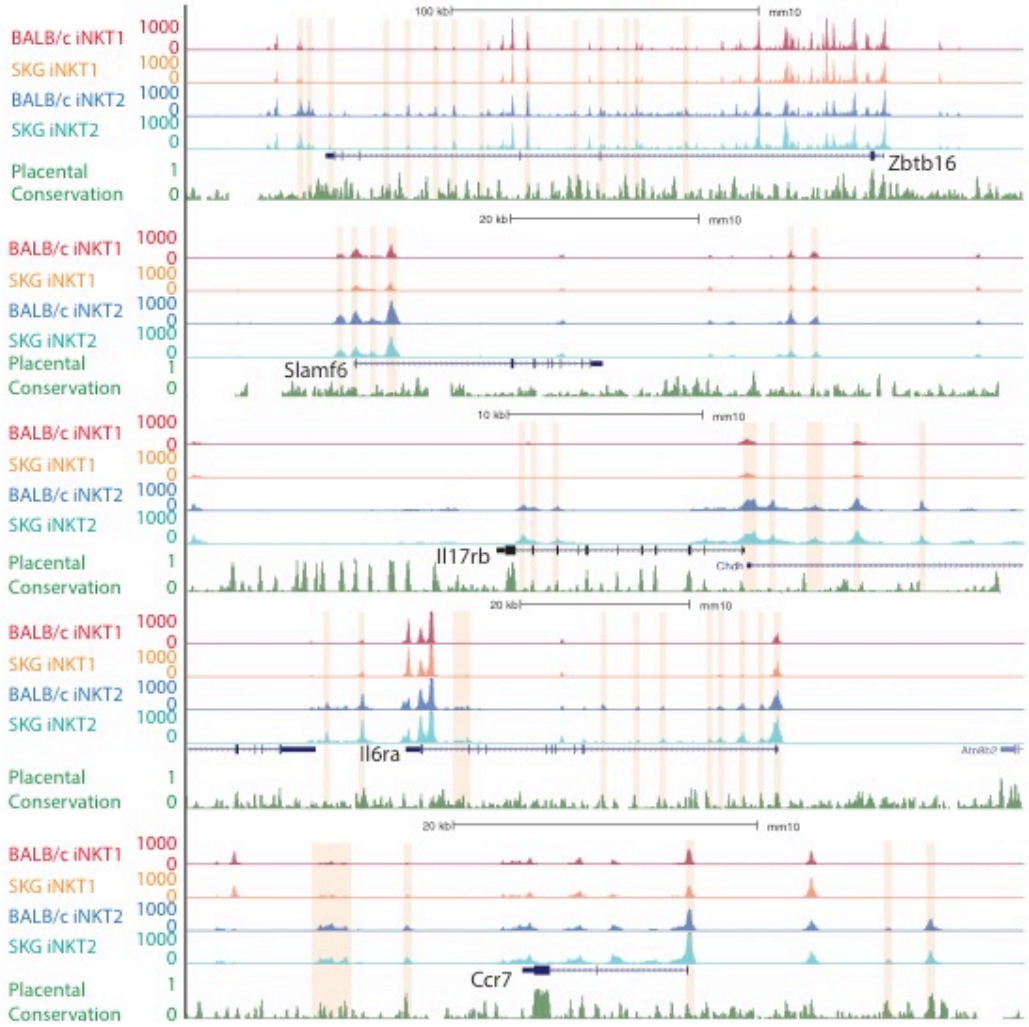
Supplementary Figure 3. Mapping of RNA-seq reads on the TRAV and TRAJ loci. Stage 0 iNKT cells from BALB/c and SKG mice were sorted and their RNA was extracted followed by library construction for Illumina sequencing. Three independent samples from BALB/c and SKG mice were prepared. Sequence reads were mapped to the mouse genome and individual tracks for each sample are shown for the whole TRAV (upper panel) and TRAJ (lower panel) loci. The results demonstrate a large enrichment for TRAV11D (encoding Vα14) and TRAJ18 (encoding Jα18) containing sequences in agreement with the TCR sequence expected from iNKT cells.



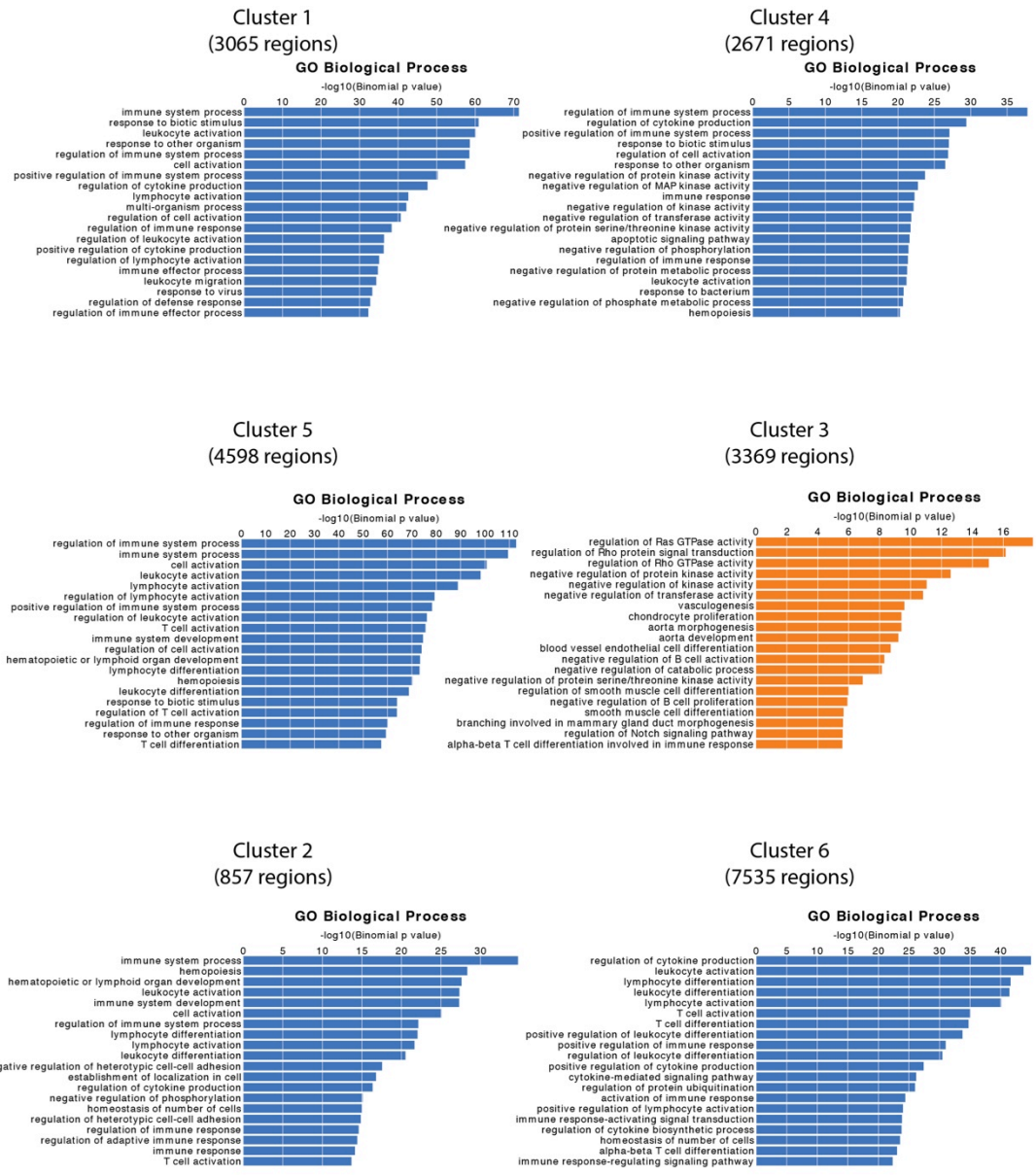
Supplementary Figure 4. Gene expression profile of thymic stage 0 iNKT cells from BALB/c and SKG mice. (**a**) Pathway-enrichment analysis of differentially expressed genes between stage 0 iNKT cells of BALB/c and SKG mice using gprofiler (http://biit.cs.ut.ee/gprofiler/) (**b**) Expression of genes encoding selected genes involved in cell activation (left), transcription factors (middle) and gene previously described as differentially expressed between iNKT cell subsets. The data are presented as row-wise z-scores (red, higher expression and blue, lower expression, relative to the other conditions). Data are from one experiment with three biological replicates per genotype.



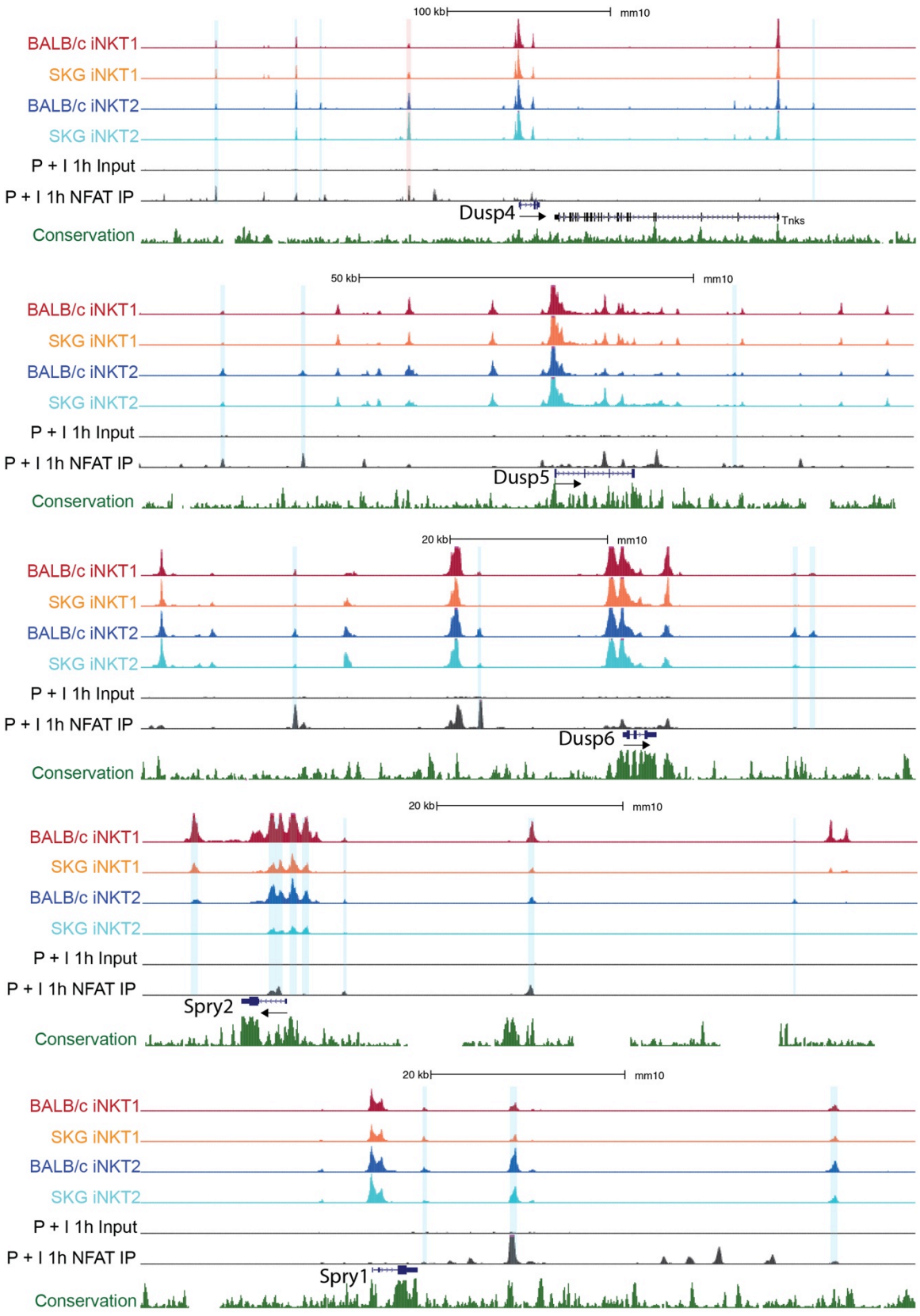
Supplementary Figure 5. Chromatin accessibility surrounding iNKT1 expressed genes. Mean ATAC sequencing coverage at the *Ifng*, *Tbx21*, *Cxcr3*, *Ccr5*, *IL2rb*, *Fasl*, *Bcl2* and *Il2* loci for BALB/c and SKG iNKT1 and iNKT2 cells with a scale from 0-1000 for the ATAC-Seq tracks. Placental conservation (in green) is indicated. Regions highlighted in light blue are more accessible in iNKT1 than in iNKT2 cells.



Supplementary Figure 6. Chromatin accessibility surrounding iNKT2 expressed genes. Mean ATAC sequencing coverage at the *Zbtb16*, *Slamf6*, *Il17rb*, *Il6ra* and *Ccr7* loci for BALB/c and SKG iNKT1 and iNKT2 cells with a scale from 0-1000 for the ATAC-Seq tracks. Placental conservation (in green) is indicated. Regions highlighted in light orange are more accessible in iNKT2 than in iNKT1 cells.



Supplementary Figure 7. Gene ontology (GO) terms enriched in peaks associated with each cluster defined in Fig 7f as determined through GREAT analysis. The number of regions in each cluster is indicated.


100 kb
mm10
BALB/c iNKT1
SKG iNKT1
BALB/c iNKT2
SKG iNKT2
P + I 1h Input
P + I 1h NFAT IP
Dusp4
Tnks
Conservation
50 kb
mm10
BALB/c iNKT1
SKG iNKT1
BALB/c iNKT2
SKG iNKT2
P + I 1h Input
P + I 1h NFAT IP
Dusp5
Conservation
20 kb
mm10
BALB/c iNKT1
SKG iNKT1
BALB/c iNKT2
SKG iNKT2
P + I 1h Input
P + I 1h NFAT IP
Dusp6
Conservation
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mm10
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SKG iNKT1
BALB/c iNKT2
SKG iNKT2
P + I 1h Input
P + I 1h NFAT IP
Spry2
Conservation
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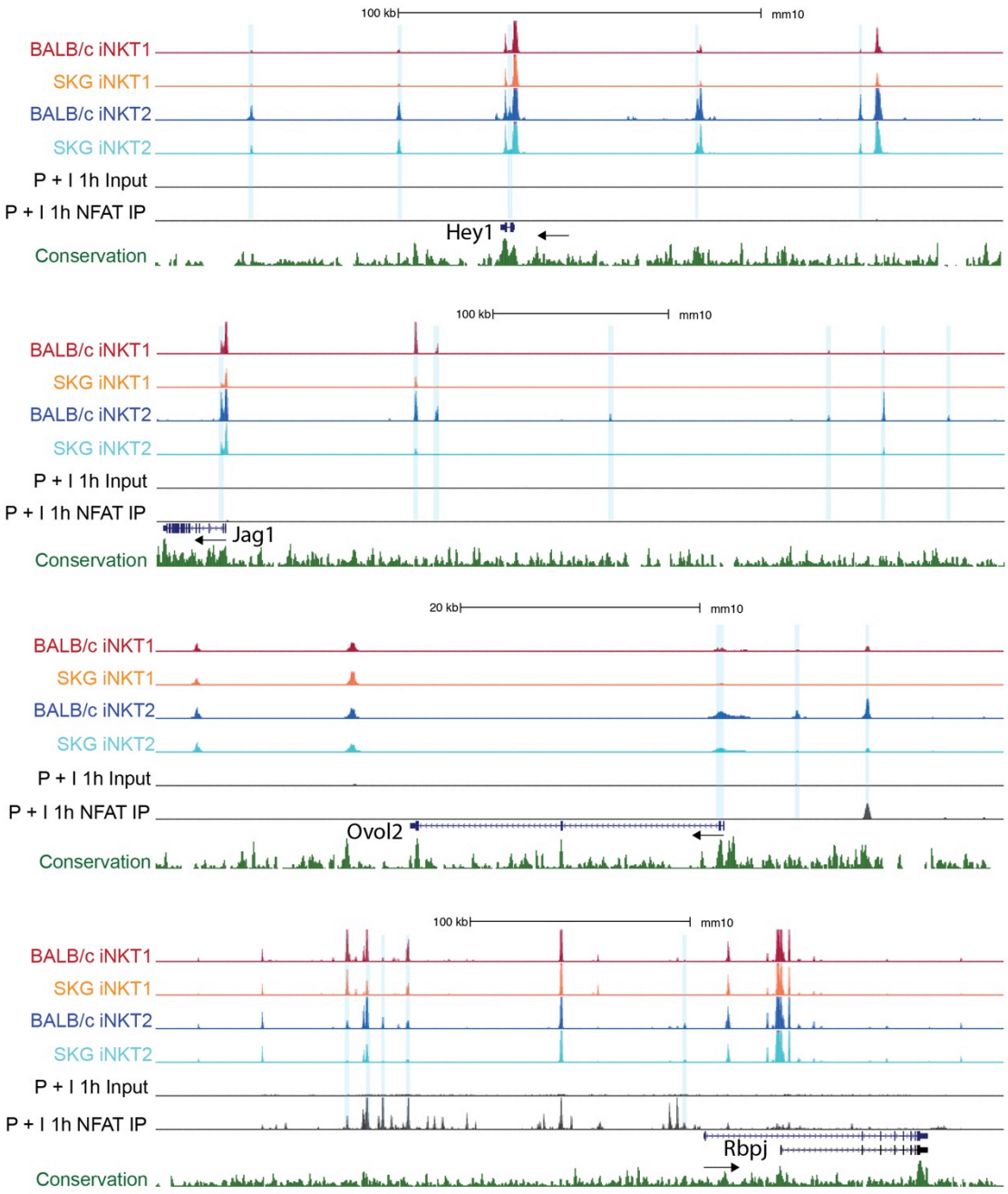
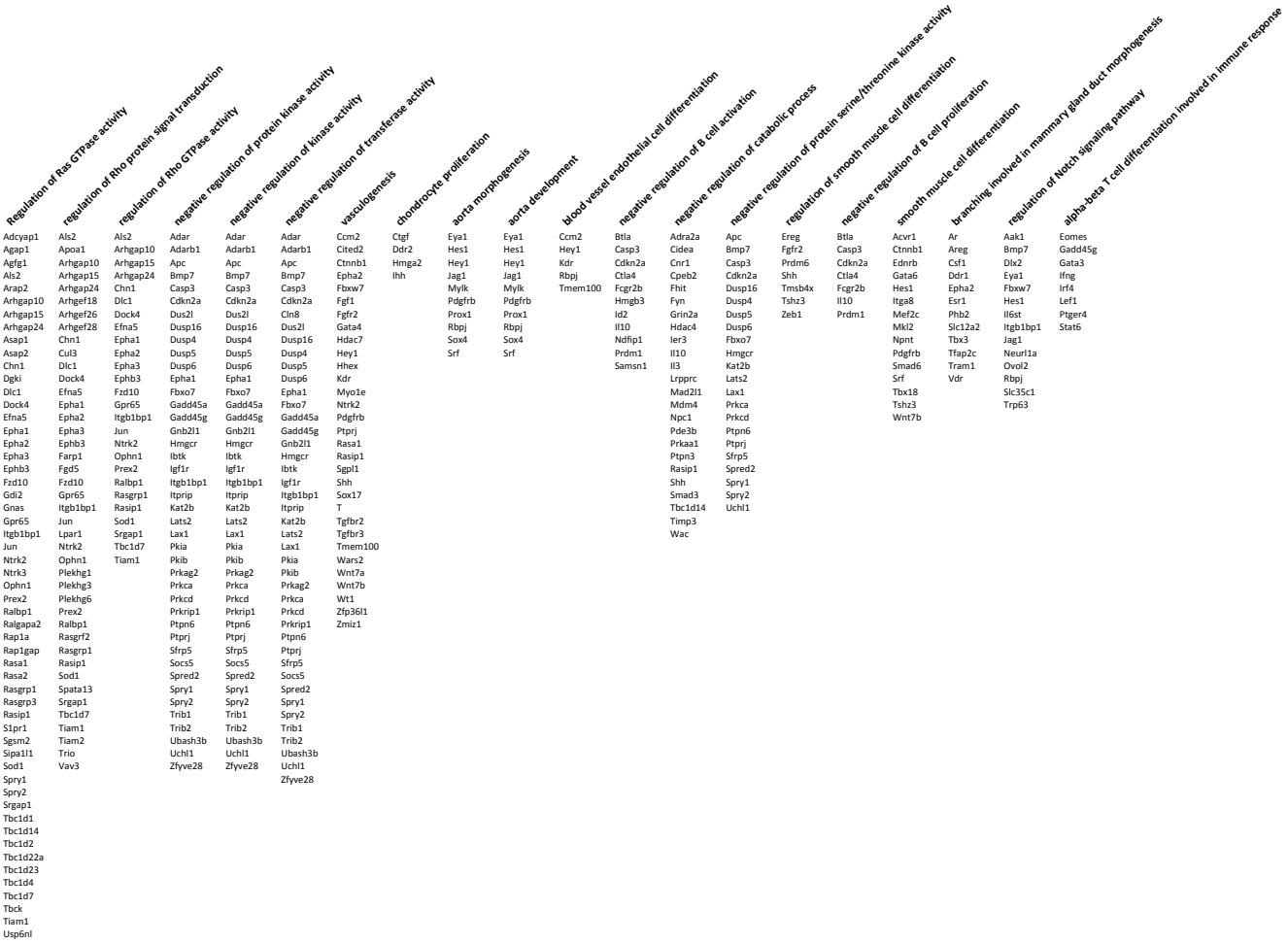
mm10
BALB/c iNKT1
SKG iNKT1
BALB/c iNKT2
SKG iNKT2
P + I 1h Input
P + I 1h NFAT IP
Spry1
Conservation

Supplementary Figure 8. ATAC-seq signal profiles of genes identified by GREAT with the GO biological process of negative regulation of kinase activity. The profiles for *Dusp4*, *Dusp5*, *Dusp6*, *Spry2* and *Spry1* are shown for BALB/c iNKT1 (red), SKG iNKT1 (orange), BALB/c iNKT2 (Blue) and SKG iNKT2 (cyan). Peaks that are less accessible due to the SKG mutation are highlighted in blue while peaks that are more accessible in SKG cells over BALB/c cells are highlighted in orange. A scale from 0-1000 for the ATAC-Seq tracks was used. Tracks from NFAT ChIP in CD8 T cells stimulated in the presence or absence of PMA/ionomycin for 1 hour (ref [37]) are also included.



Supplementary Figure 9. ATAC-seq signal profiles of genes identified by GREAT with the GO biological process of regulation of NOTCH signaling pathway. The profiles for *Hey1*, *Jag1*, *Ovol2* and *Rbpj* are shown for BALB/c iNKT1 (red), SKG iNKT1 (orange), BALB/c iNKT2 (Blue) and SKG iNKT2 (cyan). Peaks that are less accessible due to the SKG mutation are highlighted in blue while peaks that are more accessible in SKG cells over BALB/c cells are highlighted in orange. A scale from 0-1000 for the ATAC-Seq tracks was used. Tracks from NFAT ChIP in CD8 T cells stimulated in the presence or absence of PMA/ionomycin for 1 hour (ref [37]) are also included.



Supplementary Table I. List of genes associated with each GO biological terms for cluster 3.