Supplementary Materials for

A KRAB/KAP1-miRNA Cascade Regulates Erythropoiesis Through Stage-Specific Control of Mitophagy

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Materials and Methods

Mouse experiments. All mice were maintained in the EPFL animal facility under specific pathogen-free conditions and housed in individually ventilated cages. Animal procedures were performed according to protocols approved by the Swiss Bundesamt für Veterinärwesen Nb. 2082. We generated a hematopoietic-restricted conditional Kap1 knockout mouse model by first crossing homozygous Kap1flox animals (Tif1ßL3/L3; (28)) with heterozygous Mx-Cre mice, where the recombinase is expressed from the interferon (IFN)-inducible Mx1 promoter, which can be activated by administration of synthetic double-stranded RNA polyinosinic-polycytidylic acid (pIC) (29). In order to facilitate the specific examination of Kap1-deleted cells, we further crossed these mice with the stopfloxYFP strain, in which the fluorescent marker YFP is produced following Cre-mediated excision owing to the presence of a loxP-flanked stop sequence between its promoter and coding sequence (30). Finally, to restrict Kap1 excision to the hematopoietic system, we generated bone marrow chimeric mice by transplanting total bone marrow cells from Kap-flox/stopfloxYFP CD45.2 male mice, with or without the Mx-Cre transgene, into lethally irradiated CD45.1 female animals. We induced Kap1 deletion by pIC injection 6 to 8 weeks after transplantation, and began our analyses at least 2 weeks later, so as to avoid effects linked to pIC injection per se (31).

C57BL/6 (CD45.2+) and B6.SJL-Ptprc(a)Pep(b)BoyCrl(Ly5.1) (CD45.1+) mice were purchased from Charles River. Generation and genotyping of mice with a floxable Kap1 allele (Kap1flox; Tif1ßL3/L3), the MxCre, and the Cre-reporter stopfloxYFP mouse strains have been described previously (28-30). Injection of pIC was done intraperitoneally with 5μg/g poly (I:C) (Invivogen). To generate chimaeras, transplantations were performed using total bone marrow cells from male mice CD45.2+ Kap-flox/stopfloxYFP with or without MxCre or in wild type CD45.1+ female mice. Competitive graft was performed with a mixture to a 50% ratio of CD45.2+ Kap-flox/stopfloxYFP/MxCre+ or not with CD45.1+ wild-type bone marrow. Bone marrow mixtures, representing a total amount of 5.10e6 cells, were injected intravenously (i.v.) into lethally irradiated recipient mice. LSK graft, transduction and FACS analysis were performed as previously described (32).

Vectors. pLKO vectors were purchased from Sigma-Aldrich and the puromycin was replaced by eGFP.(pLKO-mKAP1, pLKO-hKAP1, pLKO-empty, pLKO-scramble. pLKO-ZFP13, pLKO-ZFP689). BNip3L cDNA or 3’UTR was cloned into the bidirectional vector obtained from L Naldini, to create the Bid-Bnip3L and Bid-Bnip3l-3UTR respectively. Precursor of mmu-miR-351 and has-miR125a was cloned into the expression plasmid kindly provided by L. Naldini with the tRFP in replacement of the orange fluorophore (pCCL-SFFV-IntronmiR351-tRFP, pCCL-SFFV-IntronmiR125a-tRFP and the pCCL-SFFV-Intron-TRFP as the empty control). The KAP1 pic identified by ChipSeq closed to the miR351 locus was cloned into the pRRL-hPGK-mSEAP (pPRL-picmiR351-hPGK-mSEAP). Sequences for all plasmids DNA are available at Addgene (http://www.addgene.org) for all the vectors described. Production and titration of lentiviral vectors was performed as previously described (32).
Cells. MEL cells were kindly provided by Punam Malik. Cells at a concentration of 2 millions per mL were induced to differentiate during seven days in DMEM (Invitrogen) supplemented with 20% serum (Hyclone) and 5 mM HMBA (hexamethylene bisacetamide, Sigma-Aldrich).

HEL cells (33) were obtained from Giuliana Ferrari. They were induced to differentiate in presence of hemin (Sigma-Aldrich) at a final concentration of 50 micromolar during five days. Benzidine staining was performed using 1mL 0.2% benzidine (Sigma-Aldrich) in acetic acid 3% solution, supplemented with 20ul hydrogen peroxide (Sigma-Aldrich). Cells were incubated for 15 min on ice, prepared on slides by using a cytospin centrifuge and counted for the proportion of blue cells over total cells. All transductions were performed at a MOI of 100, based on titer obtained by FACS or QPCR quantification, at a cell concentration of 1 million per mL. To stain mitochondria, cells at a concentration of 4 millions per mL were labeled with 20 uM Mitotracker deep red or green (Molecular Probes) at 37 °C for 30 min, before analysis by flow cytometry.

CD34+ cells from human cord blood were obtained from the Lausanne University Hospital (Centre Hospitalier Universitaire Vaudois, CHUV, switzerland). Cells were layered on Ficoll gradient, purified with the CD34+ cell separation kit (Miltenyi Biotec) and frozen in StemSpan SFEM medium (Stem cells technologies) supplemented with 10% serum (Hyclone), 10% dimethyl Sulfoxide (Chemica).

After thawing CD34+ cells were expanded for one day in StemSpan SFEM medium supplied with cytokines (100 ng/ml of Flt-3 ligand, 100 ng/ml of SCF, 20 ng/ml of TPO, and 20 ng/ml of IL-6; StemCell Technologies Inc.) and 5% penicillin/streptomycin. Cells were maintained at a density of 2x105 cells/mL and transduced the day after thawing with lentiviral vector at a MOI of 100. Two days after the transduction, cells were pelleted and transferred into StemSpan SFEM medium with 5% penicillin/streptomycin, 20%serum, erythropoietin (1 U/mL), IL-3 (1 ng/mL), SCF (50 ng/mL), dexamethasone (1 uM), and -estradiol (1uM). Cells were incubated at 37°C with 5% CO2 and maintained at a density of 1x105 cells/mL for 7 days, before resuspension and transfer at a concentration of 1x106 cells/mL into StemSpan SFEM medium with 10% serum and erythropoietin (2 U/mL) for 4 additional days. Erythroid differentiation was monitored by the expression of the cell surface marker CD235a.

Transmission electron microscopy. CD71+Ter119+ cells isolated from the bone marrow of control and Kap1 KO mice were fixed and embedded in Spurr’s low viscosity resin. Sections were prepared and stained with uranyl acetate and lead citrate, and analyzed with a Hitachi H-7500 transmission electron microscope.

RT-QPCR. Total RNA was extracted using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. For real-time PCR, total RNA was reverse-transcribed with the Superscript VILO cDNA synthesis kit (Invitrogen). All primers used in this work were designed by Primer Express software (Applied Biosystems) or the GetPrime resource (34); (http://updeplab1srv1.epfl.ch/getprime) and their sequence can be provided upon request.

Microarray. RNA was extracted from CD71+Ter119+ cells FACS sorted from 4 Kap1 KO mice, YFP+ and Ctrl mice 7 weeks after pIC injection. Purification and
preparation was done as previously described (21) and hybridization was carried out on mouse WG 6 v2 expression arrays (Illumina). miRNA expression was done on the same sample with the Illumina microRNA expression profiling assay.

Chromatin immunoprecipitation (ChIP) sequencing. 10 millions CD71+Ter119+ sorted cells from 8- to 12-wk-old Kap-flox/stopfloxYFP mouse bone marrow were crosslinked by 1% formaldehyde and sonicated, and chromatin was immunoprecipitated by using an affinity-purified rabbit polyclonal antibody raised against KAP1 aa 20–418, RBCC (11). Detailed protocols for ChIP are described elsewhere (20, 35). Raw reads were mapped to the mouse genome (assembly mm9) using the bowtie short read aligner (36). Peaks were called using the Model-based Analysis of ChIP-Seq algorithm (37).

RNA-Seq. Total RNA was extract from HEL cells transduced with empty or Kap1 knockdown vectors using TRizol reagent (Invitrogen). RNAseq was performed on an Illumina HiSeq 2000 sequencer (single read 100 cycles assay). The library was generated from 250 ng total RNA using the TruSeq RNA Sample Preparation Kit v2. Raw reads were mapped to the human transcriptome (hg19) using the bowtie short read aligner (38) and counts were normalized to the transcript length and to the total number of reads (rpkm). Differentially expressed genes were characterized using the DESeq Bioconductor package (38).

Direct RNA quantification by NanoString nCounter. KRAB-ZFP expression analysis was performed as previously described (37). Human miRNA expression was performed on same HEL cells RNA used for RNAseq, using the nCounter miRNA Expression Assay (HSA miRNA v2 assay kit) according to the manufacturer’s recommendations (Nanostring) starting from 100 ng total RNA.
Fig. S1.

General strategy. Total bone marrow from Kap-flox/stopfloxYFP CD45.2 male mice, with or without a Mx-Cre transgene, was injected into lethally irradiated CD45.1 female mice, before pIC injection 6-8 weeks later to induce hemato-specific Kap1 knockout.
KAP1-miR351-Bnip3L-mitophagy connection in MEL cells. (A) Western blot analysis of MEL cells, illustrating efficient KAP1 depletion by a Kap1 knockdown (KD) shRNA-expressing lentiviral vector, compared to cells either non-infected (NI) or transduced with a scramble shRNA vector. The L10 ribosomal protein was used for normalization. (B)
Impaired erythroid differentiation in Kap1 KD MEL cells. Differentiation was induced 3 days after transduction, and cells were examined three days later by benzidine staining and bright field microscopy (n=4, counting 100 cells for each condition). MiR-351 (C) and Bnip3L (D) expression in indicated MEL cells/conditions. Normalized expression in control non-activated cells was set at 1. Controls were either non-transduced cells, or cells transduced with an empty or a scramble shRNA vector. (E) Mitochondrial content of MEL cells was assessed in parallel by Mitotracker staining (n=5, *p<0.05). (F) RT-QPCR measurement of Bnip3L expression in control or miR351-overexpressing MEL cells (miR-351) induced or not to differentiate. Normalized expression in Ctrl (cells transduced with an empty vector and not activated) was set at 1. (G) Mitochondrial content of same cells, assessed by Mitotracker staining (n=5, *p<0.05). (H) Complementation of miR-351-overexpressing MEL cells with a lentiviral vector encoding Bnip3L devoid of its 3’UTR. Co-transduced cells were induced to differentiate and subjected to Mitotracker staining. Ctrl was cells transduced with an empty miRNA vector (n=3, *p<0.05).
Fig. S3.

KRAB-ZFP regulators of Bnip3L expression. Six KRAB-ZFPs found to be specifically expressed in CD71+Ter119- and/or CD71+Ter119+ erythroblasts and endowed with a human orthologue were downregulated in MEL cells by lentivector-mediated RNA interference, and Bnip3L expression was assessed by RT-QPCR 5 days later (n=3). Ctrl, for which the normalized value was set at 1, was a combination of MEL cells transduced with an empty or scramble shRNA lentivector.
Fig. S4.

Znf205 and Znf689 expression during cytokine-induced ex vivo erythroid differentiation of human CD34+ cells. RT-QPCR measurement of RNA levels in indicated cells (n=3). Ctrl, for which the normalized value was set at 1, was the expression level in non-differentiated CD34+ cells.
### Table S1.

<table>
<thead>
<tr>
<th></th>
<th>Unit Mean</th>
<th>SDTV Mean</th>
<th>SDTV</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC Red blood cells (RBD + RTC)</strong></td>
<td>10^6/ul 10.35</td>
<td>0.78</td>
<td>7.89</td>
<td>0.88 ***</td>
</tr>
<tr>
<td><strong>%RBCm</strong> Percentage of mature Red blood cells</td>
<td>% 95.80</td>
<td>0.79</td>
<td>91.92</td>
<td>0.53 ***</td>
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<tr>
<td><strong>#RBCm</strong> Number of mature Red blood cells</td>
<td>10^9/l 86648</td>
<td>4402</td>
<td>72956</td>
<td>7077 ***</td>
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<tr>
<td><strong>HGB Hemoglobin</strong></td>
<td>g/dl 14.96</td>
<td>1.26</td>
<td>10.56</td>
<td>1.31 ***</td>
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<tr>
<td><strong>HCT Hematocrit</strong></td>
<td>% 51.29</td>
<td>3.64</td>
<td>35.78</td>
<td>4.09 ***</td>
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<tr>
<td><strong>MCV Mean corpuscular volume</strong></td>
<td>fl/RBC 49.63</td>
<td>0.89</td>
<td>44.94</td>
<td>0.64 ***</td>
</tr>
<tr>
<td><strong>MCH Mean corpuscular hemoglobin</strong></td>
<td>pg/RBC 14.51</td>
<td>0.38</td>
<td>13.31</td>
<td>0.22 ***</td>
</tr>
<tr>
<td><strong>MCHC Mean corpuscular hemoglobin concentration</strong></td>
<td>g/dl 29.23</td>
<td>0.72</td>
<td>29.64</td>
<td>0.30 NS</td>
</tr>
<tr>
<td><strong>CH Red cell hemoglobin content</strong></td>
<td>pg/RBC 13.40</td>
<td>0.25</td>
<td>12.36</td>
<td>0.18 ***</td>
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<td><strong>RDW Red cell distribution width</strong></td>
<td>% 13.48</td>
<td>0.24</td>
<td>15.39</td>
<td>0.33 ***</td>
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<tr>
<td><strong>HDW Hemoglobin concentration distribution width</strong></td>
<td>g/dl 1.87</td>
<td>0.03</td>
<td>2.26</td>
<td>0.07 ***</td>
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<td><strong>%RETI</strong> Percentage of Reticulocytes</td>
<td>% 4.20</td>
<td>0.79</td>
<td>2.98</td>
<td>0.53 ***</td>
</tr>
<tr>
<td><strong>#RETI</strong> Number of Reticulocytes</td>
<td>10^9/l 431.00</td>
<td>83.38</td>
<td>163.30</td>
<td>39.34 ***</td>
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<tr>
<td><strong>MCVR Mean corpuscular volume reticulocytes</strong></td>
<td>fl/RTC 57.74</td>
<td>0.76</td>
<td>60.53</td>
<td>1.51 ***</td>
</tr>
<tr>
<td><strong>MCHCR Mean corpuscular hemoglobin concentration reticulocytes</strong></td>
<td>g/dl 26.48</td>
<td>0.28</td>
<td>24.77</td>
<td>0.60 ***</td>
</tr>
<tr>
<td><strong>CHR Red cell hemoglobin content reticulocytes</strong></td>
<td>pg/RTC 15.21</td>
<td>0.32</td>
<td>14.89</td>
<td>0.23 ***</td>
</tr>
<tr>
<td><strong>%LL-RTC Percentage of low RNA content Reticulocytes</strong></td>
<td>% 36.53</td>
<td>3.45</td>
<td>31.73</td>
<td>3.30 ***</td>
</tr>
<tr>
<td><strong>%M-RTC Percentage of medium RNA content Reticulocytes</strong></td>
<td>% 6.91</td>
<td>2.30</td>
<td>33.71</td>
<td>2.60 ***</td>
</tr>
<tr>
<td><strong>%H-RTC Percentage of high RNA content Reticulocytes</strong></td>
<td>% 6.49</td>
<td>1.40</td>
<td>34.54</td>
<td>3.32 ***</td>
</tr>
<tr>
<td><strong>PLT Platelet</strong></td>
<td>10^3/ul 1151.00</td>
<td>130.20</td>
<td>449.30</td>
<td>133.10 ***</td>
</tr>
</tbody>
</table>

Comparative hemograms of wild type and Kap1 KO mice, 3 weeks post-pIC injection (n=10, *p<0.05, **p<0.01, ***p<0.001). Chimerism varied between 70 and 85%.
References


8. A. L. Nielsen et al., Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. EMBO J. 18, 6385 (1999). doi:10.1093/emboj/18.22.6385 Medline


