# **Expanded View Figures**

### Figure EV1. Establishing stage-specific proteomes of erythropoiesis.

- A FACS gating/sorting regime to enrich for CD235a<sup>-</sup> progenitor population.
- B Characterization of the differentiation stages in culture. May–Grünwald–Giemsa staining of erythroid cells is shown. Scale bar, 20 µm.
- C Coefficient variations (CVs) of four biological replicates for each protein were calculated in all stages to show the reproducibility of our system. Dashed line shows the cutoff line of 20% CV.
- D Cumulative protein abundance and dynamic range in five differentiation stages. Hemoglobin subunits (HBB, HBA1, HBE1 and, HBG1) are labeled as progenitor (yellow) and Ortho (orange) stages.
- E Estimated median copy numbers of histones per cell across all measured stages.

aaki

Ortho



Figure EV1.



# Figure EV2. Comparison of differentiation stage-specific proteomes of human erythropoiesis.

A–C Volcano plots of the (-log10) *P*-values versus the log2 protein abundance differences between LBaso day 7 and LBaso day 14 (left), LBaso day 7 and ProE-EBaso (middle), and LBaso day 14 and ProE-EBaso (right) with the significance lines (FDR < 0.05 and S0 = 0.1). Selected marker proteins are labeled in blue.

# Figure EV3. Gene Ontology (GO) enrichment analysis of significant proteome clusters.

Gene Ontology (GO) enrichment analysis of six clusters of significant proteome shown in Figure 2A was performed using Fisher's exact test. 2% threshold was applied to Benjamini–Hochberg FDR to determine the significance.

3

cell division DNA replication spindle microtubule mitotic cell cycle mitotic spindle centrosome G2/M transition of mitotic cell cycle condensed chromosome outer kinetochore mitochondrial inner membrane kinetochore pre-replicative complex assembly nucleotide-excision repair, DNA gap filling mitotic spindle organization G1/S transition of mitotic cell cycle G2/M transition of mitotic cell cycle nucleoplasm extracellular exosome centrosome cycle chromosome segregation MCM complex chromatin break-induced replication mitotic spindle midzone mitochondrion kinetochore binding centriolar satellite DNA strand elongation microtubule cytoskeleton midbody DNA unwinding involved in DNA replication condensed chromosome kinetochore

# 6

nucleolus rRNA processing small-subunit processome mitochondrial translational elongation mitochondrial translational termination RNA binding extracellular exosome mitochondrial large ribosomal subunit preribosome, large subunit precursor blood microparticle mitochondrial small ribosomal subunit extracellular region proteasome complex extracellular space ribosomal large subunit biogenesis cvtoso maturation of SSU-rRNA from tricistronic rRNA transcript centrosome Pwp2p-containing subcomplex of 90S preribosome mitochondrial ribosome structural constituent of ribosome protein binding termination of RNA polymerase I transcription mitochondrial translation tumor necrosis factor-mediated signaling pathway protein deubiquitination T cell receptor signaling pathway transcription initiation from RNA polymerase I promoter collagen-containing extracellular matrix catalytic step 2 spliceosome spliceosomal complex ribosomal large subunit assembly RNA polymerase I complex RNA polymerase III complex maturation of LSU-rRNA from tricistronic rRNA transcript microtubule organizing center transcription factor TFIIH holo complex



2

plasma membrane neutrophil degranulation nucleoplasm RNA binding extracellular region extracellular exosome membrane raft extracellular space blood coagulation platelet degranulation actin cytoskeleton platelet aggregation plasma membrane raft

# extracellular exosome extracellular region extracellular space neutrophil degranulation nucleoplasm plasma membrane azurophil granule lumen focal adhesion nucleus RNA binding nucleolus cell surface signal transduction actin binding lamellipodium immune response integrin complex lysosomal lumen chemotaxis translation specific granule lumen

lumenal side of endoplasmic reticulum membrane calcium ion binding endoplasmic reticulum chaperone complex Arp2/3 protein complex integral component of plasma membrane positive regulation of T cell proliferation mitochondrial inner membrane clathrin-coated endocytic vesicle membrane endocytic vesicle membrane melanosome protein ubiquitination platelet dense granule lumen positive regulation of superoxide anion generation T cell costimulation translational initiation ruffle regulation of immune response positive regulation of tumor necrosis factor

6 18

-log10 (corr. p value)

integral component of plasma membrane

collagen-containing extracellular matrix endoplasmic reticulum lumen actin filament inflammatory response adaptive immune response mRNA splicing, via spliceosome actin filament binding external side of plasma membrane integrin-mediated signaling pathway transport vesicle membrane positive regulation of podosome assembly semaphorin-plexin signaling pathway cell division proteolysis

# 1

midbody spindle pole microtubule centrosome regulation of G2/M transition of mitotic cell cycle mitotic sister chromatid segregation cell division intercellular bridge spindle mitotic cytokinesis spindle microtubule ciliary basal body-plasma membrane docking mitotic cell cycle microtubule-based movement positive regulation of cytokinesis

# 4

RNA binding blood microparticle structural constituent of ribosome nucleosome hemoglobin complex mRNA splicing, via spliceosome hydrogen peroxide catabolic process spectrin-associated cytoskeleton haptoglobin-hemoglobin complex viral transcription nucleosome assembly apical plasma membrane plasma membrane extracellular exosome cytosolic large ribosomal subunit mitochondrial large ribosomal subunit proteasome complex proteasome regulatory particle cortical cytoskeleton rRNA processing nuclear-transcribed mRNA catabolic process catalytic step 2 spliceosome bicarbonate transport transferrin transport cytoplasmic side of plasma membrane complement activation, classical pathway proteasome-mediated ubi-dependent cat process SRP-dependentprotein targeting to membrane cytoplasmic vesicle proton-transporting ATPase activity oxygen carrier activity haptoglobin binding oxygen binding oxygen transport oxygen transport mitochondrial respiratory chain complex I mitochondrial translational elongation immunoglobulin complex, circulating clathrin-dependent endocytosis heme biosynthetic process autophagy of mitochondrion mitochondrial translational termination reg of transcription from RNA poly II promoter response to cadmium ion phagosome acidification extracellular space proteasome accessory complex antigen binding proteasome regulatory particle, lid subcomplex condensin complex extracellular region negative regulation of chromatin silencing chromosome condensation SNARE complex cellular oxidant detoxification clathrin-coated endocytic vesicle early endosome membrane transmembrane transport positive regulation of lipid biosynthetic process ion transmembrane transport microtubule cytoskeleton organic acid binding trans-Golgi network complement activation high-density lipoprotein particle early endosome endomembrane system MAPK cascade basolateral plasma membrane post-translational protein modification transcription corepressor activity What signaling pathway membrane organization espiratory chain complex I assembly regulation of mitotic spindle assembly

Figure EV3.



## Figure EV4. Stage-specific significantly regulated proteins.

A, B Hawaii plots that overlay all volcano plots of protein enrichments in a specific stage over all other stages plotted against corresponding *P*-values. Two cutoff lines were placed graphically, defining two confidence classes with FDRs of 0.01 and 0.05 (S0 = 0.1). Sorting and cluster markers, and selected outliers are labeled in dark red, light blue, and dark blue, respectively.



# Figure EV5. Establishing kinome-targeting CRISPR/Cas9 screen in HUDEP-2 cells.

- A Flow cytometry strategy based on GFP, CD235a, CD49d, and Band3 to determine the significant hits from the genome-scale CRISPR-Cas9 screen.
- B Histogram of the sgRNA distribution in each sample in the CRISPR-Cas9 kinase screen.
- C Evenness of the sgRNA reads in each sample in the CRISPR-Cas9 kinase screen.
- D Correlation based reproducibility analysis between replicates in the CRISPR-Cas9 kinase screen. High and low correlation values are denoted in yellow and orange, respectively.
- E Overlap between expansion hits and positive or negative maturation regulators.
- F Overlap of kinases whose activities were inferred by stage-specific substrate profiling from phosphoproteomics in Figure 4F and the genomic CRISPR-Cas9 kinase screen.



### Figure EV6. Validation of candidate kinases.

- A Indel frequencies at 3 days after transduction of Cas9-expressing HUDEP2 cells with lentiviral vector encoding individual sgRNAs targeting the indicated genes.
- B Top panels show FACS analysis of Band3 and CD49d expression in HUDEP2 cells after Cas9 + sgRNA disruption of the indicated genes followed by culture for 3 days in differentiation medium to induce terminal erythroid maturation. The bottom panels show cell numbers measured by a colorimetric CellTiter Glo assay of gene-targeted cells grown in expansion medium, with the light absorbance (OD 490nm) readout shown on the y-axis. Data show the results as the mean  $\pm$  SEM for three biological replicate experiments. \**P* < 0.05; unpaired *t*-test.
- C Cas9-expressing HUDEP-2 cells were transduced with lentiviral vector expressing PIM1-targeting or control non-targeting sgRNAs and cultured for 3 days. Left panel shows indel frequencies determined by PCR followed by next-generation sequencing. Right panel shows Western blot analysis of whole cell lysates using β-actin antibody as a loading control.
- D Left panels show FACS analysis of Band3 and CD49d expression in HUDEP2 cells after Cas9 + sgRNA disruption of *PIM1* followed by culture for 3 days in differentiation medium to induce terminal erythroid maturation. The right panels show viable cell numbers measured by a colorimetric CellTiter Glo assay of gene-targeted cells grown in expansion medium, with the light absorbance (OD 490nm) readout being shown on the y-axis. Data show the results as the mean  $\pm$  SEM for three biological replicate experiments. \**P* < 0.05, \**P* < 0.01; unpaired *t*-test.