Finely-tuned regulation of AMP-activated protein kinase is crucial for human adult erythropoiesis

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ABSTRACT

AMP-activated protein kinase (AMPK) is a heterotrimeric complex containing α, β, and γ subunits involved in maintaining integrity and survival of murine red blood cells. Indeed, *Ampk α1^{-/-}*, *Ampk β1^{-/-}* and *Ampk γ1^{-/-}* mice develop hemolytic anemia and the plasma membrane of their red blood cells shows elasticity defects. The membrane composition evolves continuously along erythropoiesis and during red blood cell maturation; defects due to the absence of Ampk could be initiated during erythropoiesis. We, therefore, studied the role of AMPK during human erythropoiesis. Our data show that AMPK activation had two distinct phases in primary erythroblasts. The phosphorylation of AMPK (Thr172) and its target acetyl CoA carboxylase (Ser79) was elevated in immature erythroblasts (glycophorin A[low]), then decreased conjointly with erythroid differentiation. In erythroblasts, knockdown of the α1 catalytic subunit by short hairpin RNA led to a decrease in cell proliferation and alterations in the expression of membrane proteins (band 3 and glycophorin A) associated with an increase in phosphorylation of adducin (Ser726). AMPK activation in mature erythroblasts (glycophorin A[high]), achieved through the use of direct activators (GSK621 and compound 991), induced cell cycle arrest in the S phase, the induction of autophagy and caspase-dependent apoptosis, whereas no such effects were observed in similarly treated immature erythroblasts. Thus, our work suggests that AMPK activation during the final stages of erythropoiesis is deleterious. As the use of direct AMPK activators is being considered as a treatment in several pathologies (diabetes, acute myeloid leukemia), this observation is pivotal. Our data highlighted the importance of the finely-tuned regulation of AMPK during human erythropoiesis.

Introduction

Mammalian AMP-activated protein kinase (AMPK) is a highly conserved eukaryotic serine/threonine protein kinase and a heterotrimeric complex consisting of a single catalytic (α) and two regulatory (β and γ) subunits, encoded by different genes (α1, α2, β1, β2, γ1, γ2, and γ3). In the case of energy depletion, a decrease in the cellular ATP-to-AMP ratio leads to allosteric AMPK activation by AMP but also by the phosphorylation of Thr172 within the activation loop segment of the α subunit by an upstream AMPK kinase, liver kinase B1 (LKB1). Another "canonical" mechanism of activation involves the phosphorylation of Thr172 by calcium/calmodulin-dependent kinase kinase β (CaMKKβ) in response to a rise in intracellular Ca^{2+}.[1] Once activated, AMPK phosphorylates metabolic targets, leading to a decrease in ATP consumption and an increase in ATP production. In particular, AMPK inhibits fatty acid synthesis via phosphorylation and inactivation of acetyl-CoA-carboxylase (ACC) or induces autophagy via the phosphorylation of Unc-51 like autophagy activating kinase 1 (ULK1).[2] Thus, AMPK is

a major sensor of energy status that maintains cellular energy homeostasis but also exerts non-metabolic functions such as the maintenance of cell survival, cell polarity and regulation of the cell cycle.[3,4]

Erythropoiesis is a tightly regulated process that permits the production of around two million red cells each second throughout a human life, while the total cell number has to be kept within a narrow margin. This extremely dynamic process is also very flexible, since it must increase rapidly in response to blood loss and hypoxia. Furthermore, maintaining homeostasis is crucial and an imbalance in erythropoiesis can lead to the development of erythroid pathologies such as polycythemias and anemia.

We and other groups have previously demonstrated that AMPK plays a crucial role in the integrity and survival of red blood cells. We showed that mice that are globally deficient in the catalytic subunit, Ampkα1 but not in those lacking the isoform Ampkα2, as well as those globally deficient in the regulatory subunits Ampkβ1 and Ampkγ1, develop regenerative hemolytic anemia caused by increased sequestration of abnormal erythrocytes. *Ampkα1*^{-/-}, *Ampkβ1*^{-/-} and *Ampkγ1*^{-/-} mice develop splenomegaly and iron accumulation due to a compensatory response through extramedullary erythropoiesis in the spleen and enhanced erythrophagocytosis. The lifespan of erythrocytes from *Ampkα1*^{-/-} and *Ampkγ1*^{-/-} mice was shorter than that of wild-type littermates. Moreover, *Ampkα1*^{-/-} and *Ampkγ1*^{-/-} erythrocytes were highly resistant to osmotic stress and poorly deformable in response to increasing shear stress, which is consistent with a loss of membrane elasticity.[5-8]

The defects in Ampk-deficient erythrocytes suggested that alterations might occur early during terminal erythroid maturation but no data were available on the importance of AMPK in human erythropoiesis. We, therefore, decided to investigate whether AMPK could be implicated in regulating the proliferation, survival and differentiation of human erythroid precursors.

In the present study, we analyzed the expression and activation of AMPK along human erythroid differentiation. Our experiments show that AMPK is highly activated in immature erythroblasts and weakly active in mature erythroblasts. We studied the impact of knocking down AMPK and of AMPK activation by direct activators. In erythroblasts, the knockdown of the AMPK α1 catalytic subunit expression by short hairpin (sh) RNA induced a decrease in cell proliferation and alterations in the expression or phosphorylation of membrane proteins whereas no defect in hemoglobin synthesis or erythroid maturation was observed. The activation of AMPK is necessary in immature erythroblasts but maintaining the activation in mature erythroblasts is deleterious, demonstrating that AMPK activation has to be tightly regulated during human terminal erythroid differentiation.

Methods

Materials

AMPK α1 and α2 antibodies were obtained from Graham Hardie (University of Dundee, UK);[9,10] antibodies against the AMPK β1 and γ1 isoforms, phospho-Thr 172 AMPK, phospho-Ser 79 ACC, phospho-Ser 555 ULK1, LKB1, LC3B and cleaved caspase 3 were from Cell Signaling Technology (Danvers, USA) and anti-HSC70, anti-α spectrin, anti-band 3 and anti-P-Ser 726

adducin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Actin (A5441) antibodies, dexamethasone and chloroquine were purchased from Sigma-Aldrich (Lyon, France). Anti-ankyrin antibodies were obtained from Neuromab (Davis, CA, USA) (#75-380) and anti β-spectrin antibodies from Abcam (Cambridge, UK). Compound GSK-621 was purchased from Selleckchem (Houston, TX, USA) and 991 (5-[[6-chloro-5-(1-methylindol-5-yl)-1H-benzimidazol-2-yl]oxy]-2-methyl-benzoic acid) was synthesized by Spirochem (Basel, Switzerland).

Cell lines and cell culture

CD34+ cells were obtained from human donors who gave informed consent in accordance with the Declaration of Helsinki and the study was approved by the French ministry of higher education and research review board. Granulocyte colony-stimulating factor-mobilized CD34+ cells were purified from peripheral blood after cytapheresis. CD34+ cells were isolated by positive selection using an immunomagnetic procedure (MACS CD34 isolation kit; Miltenyi Biotech (Paris, France). CD34+ cells were cultured in 5% CO_2 at 37°C for 7 days in IMDM medium (Life Technologies, Waltham MA, USA) containing 1% glutamine, 15% BIT 9500 (Stem Cell Technologies), 100 ng/mL stem cell factor, 10 ng/mL interleukin-6 and 10 ng/mL interleukin-3 (Miltenyi Biotech). After 7 days of culture, CD36+ cells corresponding to a highly purified population of human erythroid progenitors were obtained by positive selection on CD36 immunomagnetic beads (CD36 unlabeled antibodies purchased from Beckman Coulter, Villepinte, France) coupled to anti-mouse IgG1 microbeads purchased from Miltenyi Biotech) . CD36+ cells were then cultured with 2 U/mL erythropoietin, 100 ng/mL stem cell factor and 10 ng/mL interleukin-3 for up to 14 days for erythroid differentiation. GSK621 or compound 991 was added from day 0 after CD36+ selection; cells were counted daily and diluted to a final concentration of 0.8 x10⁶ cells/mL by the addition of fresh medium containing the indicated concentration of AMPK activator. Because of interindividual variability, the kinetics of erythroid differentiation varies between different human samples. Thus, the days of culture corresponding to the same stage of differentiation have been grouped.

Lentiviral constructs, lentiviral production and cell infection

Lentiviral constructs for control and AMPKα1 shRNA [(SHC002 and SHCLNG-NM006251 (TRCN00000000859), respectively)] were purchased from Sigma (Lyon, France). To obtain recombinant lentiviruses, 293T cells were transiently transfected by calcium phosphate precipitation with three different plasmids: pCMV-G (VSVG envelope coding sequence), pCMV-gag-pol and a recombinant pLKO.1 vector encoding either a control or AMPKα1 shRNA. Supernatants containing infectious lentiviral particles were concentrated by ultracentrifugation. Infections of human erythroblasts were performed at day 1 and at day 4 after CD36 cell sorting and culture in the presence of interleukin-3, stem cell factor and erythropoietin, as described above.

Flow cytometry

Cells were labeled as previously described.[11] Briefly, PC7-conjugated anti-glycophorin A (GPA), APC-conjugated anti-cd49d (α4 integrin) or an appropriate isotype control were purchased from Beckman Coulter; anti-BRIC6 (anti-band 3) was from the NHSBT International Blood Group Reference Laboratory (Bristol, UK). FITC-conjugated annexin V was used to measure the percentage of cell apoptosis.

Cell proliferation

Cell proliferation was determined by trypan blue exclusion dye.

Statistics

Results are expressed as means ± standard deviation (SD). A Student *t* test was used to determine statistical significance. *P* values <0.05 were considered statistically significant.

Results

AMPK α1 activation is tightly regulated during human erythroid differentiation

Because AMPK occurs as a heterotrimeric complex containing catalytic β subunits and regulatory β and γ subunits, we aimed to identify which isoforms are expressed in human erythroblasts and to study their variation during human erythroid differentiation. Human primary erythroid progenitors were maintained for up to 12 days in culture and were able to differentiate from the pro-erythroblastic stage (day 2) to the reticulocyte stage (day 12) (Figure 1A and *Online Supplementary Figure S1* for cell morphology). During maturation, erythroblasts progressively acquired cell surface-specific markers such as GPA (from the pro-erythroblastic stage), band 3 (from the basophilic-erythroblastic stage) and decreased expression of α4 integrin at the orthochromatic erythroblast stage. They also started to synthesize hemoglobin from the basophilic stage. Western blot analysis demonstrated that the α1 catalytic subunit was expressed while the α2 isoform was not detectable and that the expression of α1 was constant along erythroid differentiation (Figure 1B). The regulatory subunits, β1 and γ1, were expressed throughout erythroid differentiation. We previously determined the copy number of individual proteins for each stage of erythroid differentiation by an absolute quantitative proteomics analysis.[11] We confirmed the expression of α1, β1 and γ1, while α2, β2 and γ2 isoforms were not detectable by mass spectrometry analysis (*Online Supplementary Figure S2*). Overall, our results suggest that α1/β1/γ1 is the heterotrimeric complex that is predominantly present in human erythroblasts.

Despite the global constant expression of AMPK, the activation of this protein might be modulated during differentiation. We, therefore, studied AMPK activation by detection of phosphorylation of the α1 catalytic subunit at Thr172 and phosphorylation of one of its substrates, ACC, at Ser79 (Figure 1C). From day 2 to day 6, the phosphorylation of AMPK and ACC was clearly detectable, but concomitantly decreased from day 8 to the end of differentiation. LKB1 was expressed throughout erythroid differentiation. These data showed the biphasic pattern of activation of AMPK during erythroid differentiation with clear activation in immature erythroblasts from the pro-erythroblast stage until day 6 when the cells were GPA[med]/band 3[low] (basophilic erythroblasts) and reduced activation in mature GPA[high]/band 3[med] erythroblasts from day 8 to day 12, corresponding to stages from polychromatic erythroblasts to reticulocytes.

The absence of AMPK induces decreased proliferation and alterations in the expression of membrane proteins of human erythroblasts

To decipher the role of AMPK in erythroblasts, we inhibited AMPK expression by a specific shRNA targeting the α1 catalytic subunit. Cells were infected by a lentivirus coding for either shAMPKα1 or shControl (shCtrl) at day 1 and then at day 4 after CD36 cell sorting. The decrease in α1 AMPK expression resulted in an expected decrease of the phosphorylation of AMPK and its substrates ACC and ULK1 (Figure 2A). No compensatory expression of the α2 catalytic subunit was observed in response to inhibition of the α1 isoform (*Online Supplementary Figure S3*). The inhibition of AMPK α1 expression did not significantly modify the differentiation of the cells. Indeed at days 6, 8 and 10 of culture, the cell population was mainly composed of basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts, respectively, and this pattern was not different when AMPKα1 was knocked down (Figure 2B). The morphology of the shCtrl and shAMPKα1 cells was very similar (*Online Supplementary Figure S4*). The percentages of hemoglobinized cells estimated at the indicated stages of differentiation were identical between the shCtrl and shAMPKα1 cells (Figure 2C). shAMPKα1 erythroblasts showed a reduced ability to proliferate compared to shCtrl erythroblasts (Figure 2D). The inhibition of AMPKα1 expression did not significantly affect the viability of the cells measured by the trypan blue exclusion assay (Figure 2E) or by annexin V flow cytometry analysis (*data not shown*).

Because red cells from *Ampkα1^{-/-}* and *Ampkγ1^{-/-}* mice are highly resistant to osmotic stress and poorly deformable, the expression of membrane proteins involved in these processes was studied (Figure 3A). In shAMPKα1 polychromatic and orthochromatic erythroblasts, western blot experiments showed that the phosphorylation of adducin on Ser726 was increased while the expression of spectrins and ankyrin was not affected (Figure 3A). Western blots demonstrated that the global expression of band 3 was significantly decreased in the shAMPKα1 cells while its expression at the cell surface, measured by FACS, was abnormally increased (Figure 3B). FACS analyses also showed a decrease of the cell surface expression of GPA at each stage of differentiation for the shAMPKα1 cells in comparison to ShCtrl (39.9% at day 6/basophilic erythroblasts, 50% at day 8/polychromatlic erythroblasts and 58.8% at day 10/orthochromatic erythroblast *versus* 100% for shCtrl) (Figure 3C). Overall, the decrease in AMPK expression induced major abnormalities in the expression of the membrane proteins band 3 and GPA and, as in murine Ampk knockout mice, led to enhanced phosphorylation of adducin. Furthermore, AMPKα1 knockdown provoked a decrease in cell proliferation without affecting cell viability and erythroblast maturation.

Proliferation and survival of mature GPA[high] erythroblasts are specifically and drastically diminished by GSK621-mediated AMPK activation

To further investigate the role of AMPK in erythroid cells, the activation of AMPK was enhanced by GSK621, a direct, potent, novel activator of AMPK.[12-15] In primary erythroblasts, the activation of AMPK by GSK621 was dose-dependent, with increased phosphorylation of T172 AMPKα, and also gradual stimulation of the phosphorylation of the substrates of AMPK, ACC at Ser79 and ULK1 at Ser 555, from 5 to 20 μM (Figure 4A). The latter dose was then used in the experiments. GSK621 was added to the medium from day 0 after CD36 cell sorting until the indicated days. GSK621 induced the phosphorylation of AMPK and its substrates at each stage of erythroid maturation (*Online Supplementary*
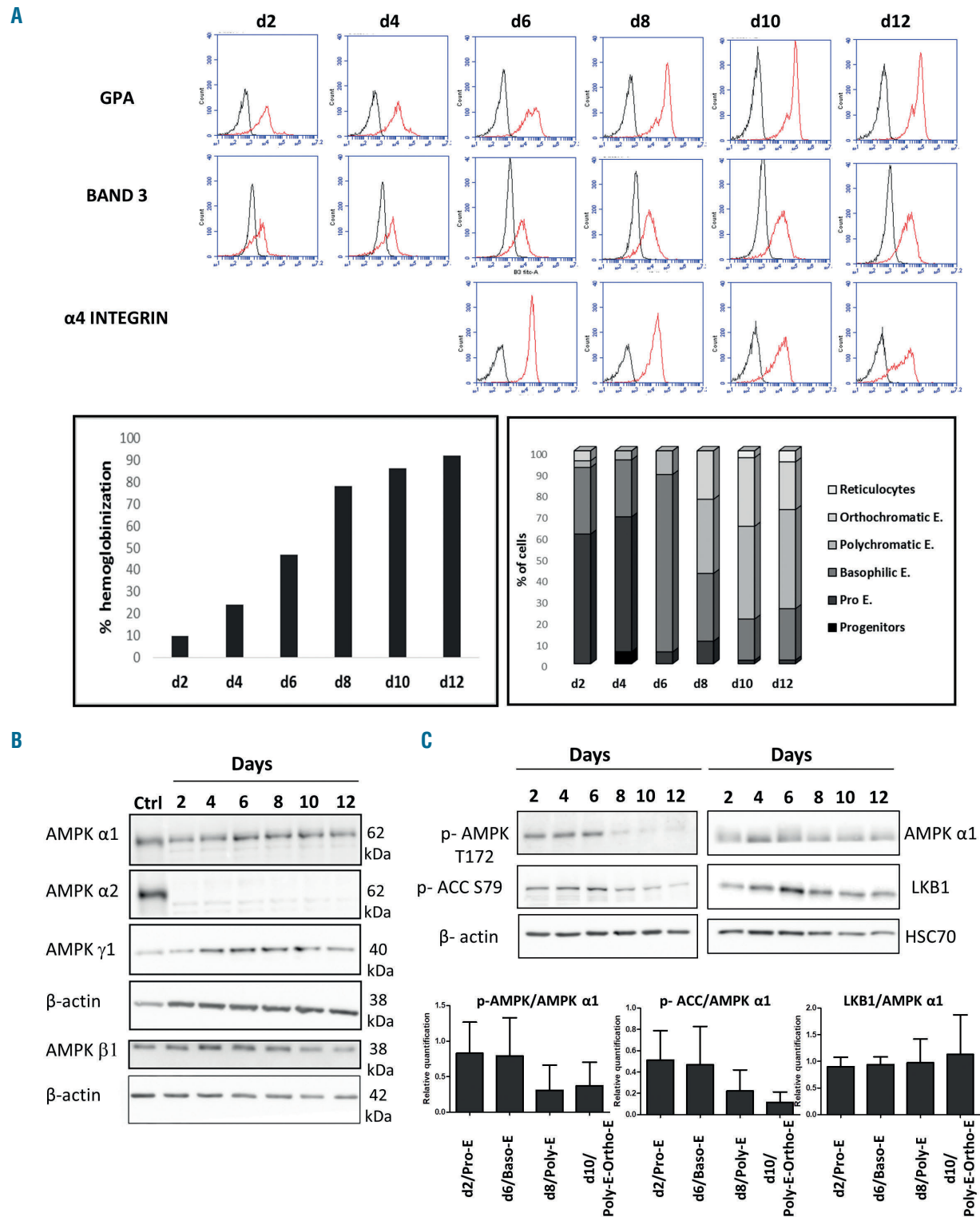
Figure 1. Expression of AMPK isoforms and AMPK activation along terminal erythroid differentiation. (A) Representative experiment of one *ex vivo* culture of erythroblasts derived from CD34+ cells. Cells were analyzed on days 2, 4, 6, 8, 10 and 12 after CD36+ selection. Expression of cell surface markers GPA, band 3 and α4β1 integrin was studied by flow cytometry along terminal erythroid differentiation. The percentage of hemoglobinized cells was determined by benzidine staining. A minimum of 200 cells were counted and the percentage of blue-stained cells among the total cell count was determined. Cell morphology was examined following staining with May-Grünwald-Giemsa; the percentage of each cell population was determined. (B) AMPK isoforms during erythropoiesis were determined by western blot. Protein extracts from human primary erythroblasts from day 2 to day 12 of culture were analyzed by western blot using anti-α1, -α2, -β1, and -γ1 antibodies. Anti-β-actin was used as a loading control and mouse liver protein extracts were used as a positive control for the expression of AMPKα2 (Ctrl). The α1, α2, and γ1 isoforms were analyzed on the same blot, the β1 isoform on a different one. (C) AMPK activation during erythroid differentiation. Anti-pT172 AMPK, anti-p S79 ACC, anti AMPKα1 and LKB1 were used. Anti-β-actin or anti-HSC70 antibodies were used as loading controls. The upper panel shows a representative experiment of three independent ones. Quantification of western blots and determination of the ratio between pAMPK/AMPK, pACC/AMPK and LKB1/AMPKα1 at the indicated days are presented as the mean of three independent experiments ± SD; ns: non-significant, *P<0.05 (lower panel). d: day; GPA: glycophorin A; AMPK: AMP-activated protein kinase; ACC: acetyl-CoA-carboxylase; LKB1: liver kinase B1; HSC70: heat shock 70kDa protein; E: erythroblasts.
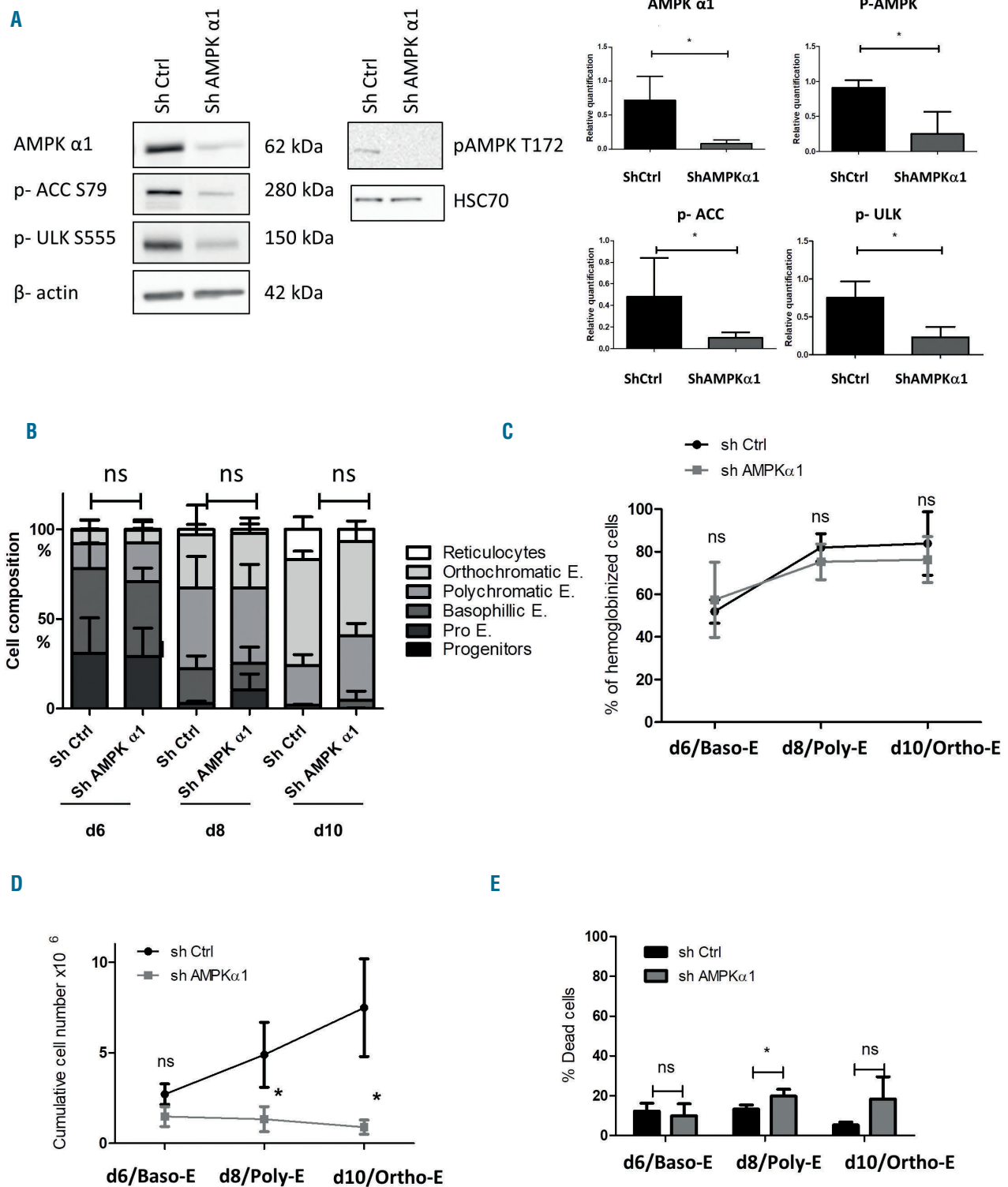
Figure 2. AMPKα1 knockdown reduces erythroblast proliferation without affecting erythroblast maturation. (A) Efficiency of AMPKα1 knockdown: Erythroblasts were infected twice, on days 1 and 4 after CD36 cell sorting, with either a lentivirus coding a scrambled shRNA (sh Ctrl) or an AMPKα1 shRNA (shAMPK α1). The efficiency of AMPKα1 knockdown in human primary erythroblasts was determined by anti-α1 AMPK, anti-PT172 AMPK, anti-PS79 ACC and anti-PS555 ULK1 western blots on day 8 or day 10 with the same results. A representative experiment on day 8 is presented. Western blot quantification was performed with anti-β–actin or anti-HSC70 antibodies; values are the mean of three independent experiments ± SD; ns: non-significant, *P<0.05. (B) Effect of shAMPKα1 on erythroid cell maturation. Cell composition for each culture at days 6, 8 and 10. The mean composition ± SD was calculated based on erythroblast morphology determined by May-Grünwald-Giemsa staining in three independent experiments. (C) Absence of AMPKα1 does not modify the percentage of hemoglobinized cells. The percentage of hemoglobinized cells was determined by benzidine staining at the indicated days of culture and corresponding stage of differentiation determined as in Figure 2A. (D) Proliferation of shCtrl *versus* shAMPKα1 erythroblasts. Cumulative cell number was determined by counting cells with the trypan blue exclusion method at day 6 (basophilic erythroblasts), day 8 (polychromatic erythroblasts) and day 10 (orthochromatic erythroblasts) of culture. Results are expressed as the mean ± SD of three independent experiments; ns: non-significant, *P<0.05. (E) Cell death in shAMPKα1 *versus* shCtrl cells. The proportion of dead cells was determined by trypan blue exclusion dye at days 6, 8 and 10 of culture. Results are expressed as the mean ± SD of three independent experiments; ns: non-significant, *P<0.05. d: day; GPA: glycophorin A; AMPK: AMP-activated protein kinase; ACC: acetyl-CoA-carboxylase; ULK1: Unc-51 like autophagy activating kinase 1; HSC70: heat shock 70kDa protein; Pro-E: pro-erythroblasts; Baso-E: basophilic erythroblasts; Poly-E: polychromatic erythroblasts; Ortho-E: orthochromatic erythroblasts.
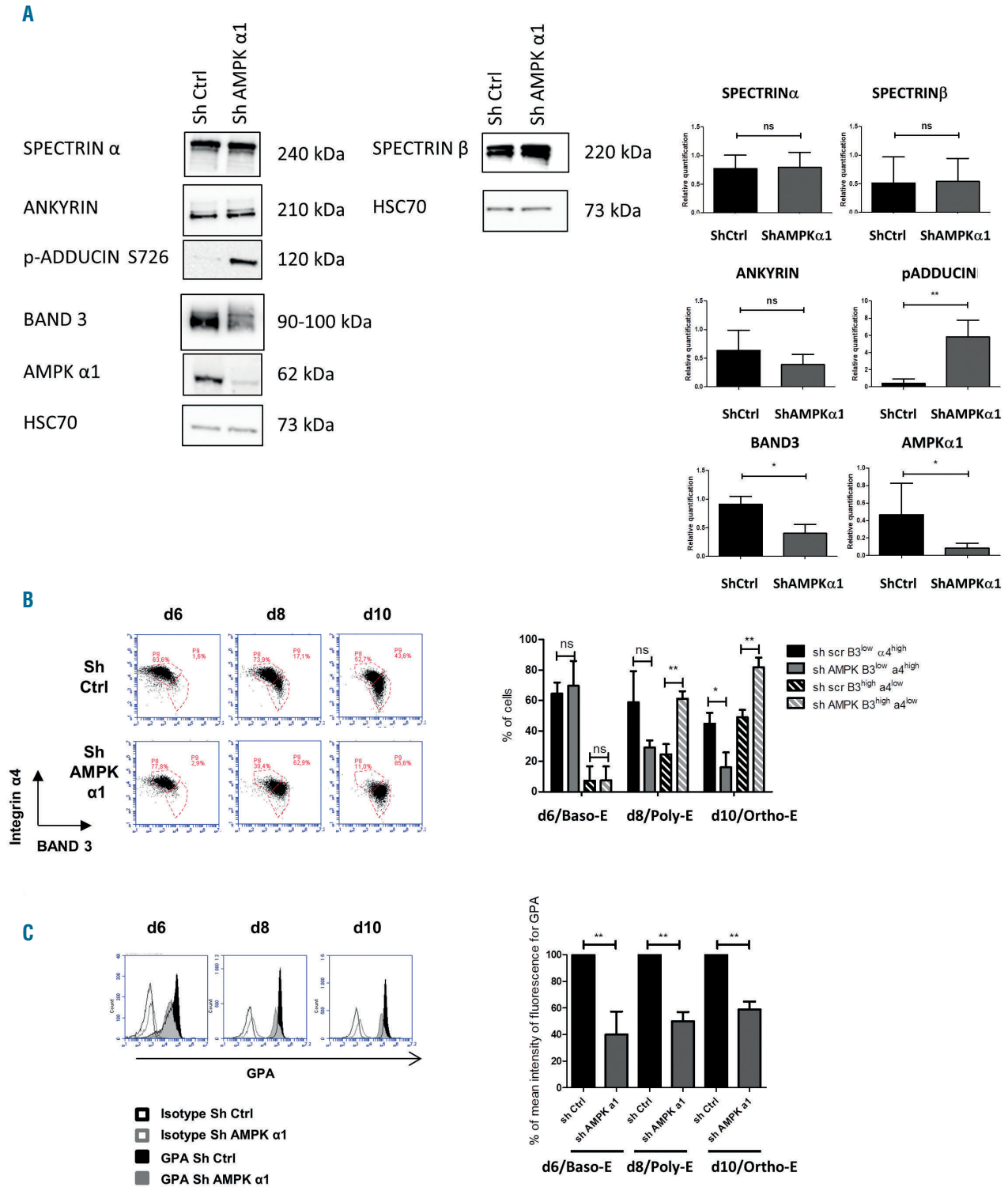
Figure 3. AMPK α1 knockdown disturbs glycophorin A, band 3 expression and phosphorylation of aduccin. (A) Analysis of expression of membrane proteins. Protein extracts from human primary erythroblasts were analyzed by western blot using anti-AMPKα1 antibodies to confirm the knockdown and anti-spectrin α and β, ankyrin, band 3, p-aduccin S726 antibodies. Anti-HSC70 was used as a loading control. AMPKα1, anti-spectrin α, ankyrin, band 3, p-adducin S726 were analyzed on the same western blot, spectrin β on a distinct one (representative western blot at day 8/polychromatic erythroblasts, left panel), the same result was obtained at day 10/polychromatic erythroblasts. The blots were quantified using anti-HSC70 antibodies; values are the mean ± SD of three independent experiments at day 8 and day 10; ns: non-significant, *P<0.05; (right panel). (B) Representative cytometry profile for band 3/α4 integrin gated on GPA-positive cells (left panel) The percentages of band 3[low]α4 integrin[high] and band 3[med]α4 integrin[med] in the shCtrl and shAMPKα1 cell populations are plotted in histograms (right panel). Results are expressed as the mean ± SD of three independent experiments; ns: non-significant, *P<0.05; **P<0.01. (C) Expression of GPA determined by FACS. Representative cytometry profiles are shown for GPA at day 6/basophilic erythroblasts, at day 8/polychromatic erythroblasts and at day 10/orthochromatic erythroblasts. Control isotype: unfilled black for shCtrl, unfilled gray for shAMPKα1; GPA-labeled: filled black for shCtrl, filled gray for shAMPKα1) (left panel). The histogram shows the mean percentage of the GPA fluorescence intensity on the cell surface; 100% represents the fluorescence intensity for shCtrl cells (right panel). Results are expressed as the mean ± SD of three independent experiments; ns: non-significant, *P<0.05; **P<0.01. AMPK: AMP-activated protein kinase; HSC70: heat shock 70kDa protein; d: day; B3: band 3; Baso-E: basophilic erythroblasts; Poly-E: polychromatic erythroblasts; Ortho-E: orthochromatic erythroblasts; GPA: glycophorin A.
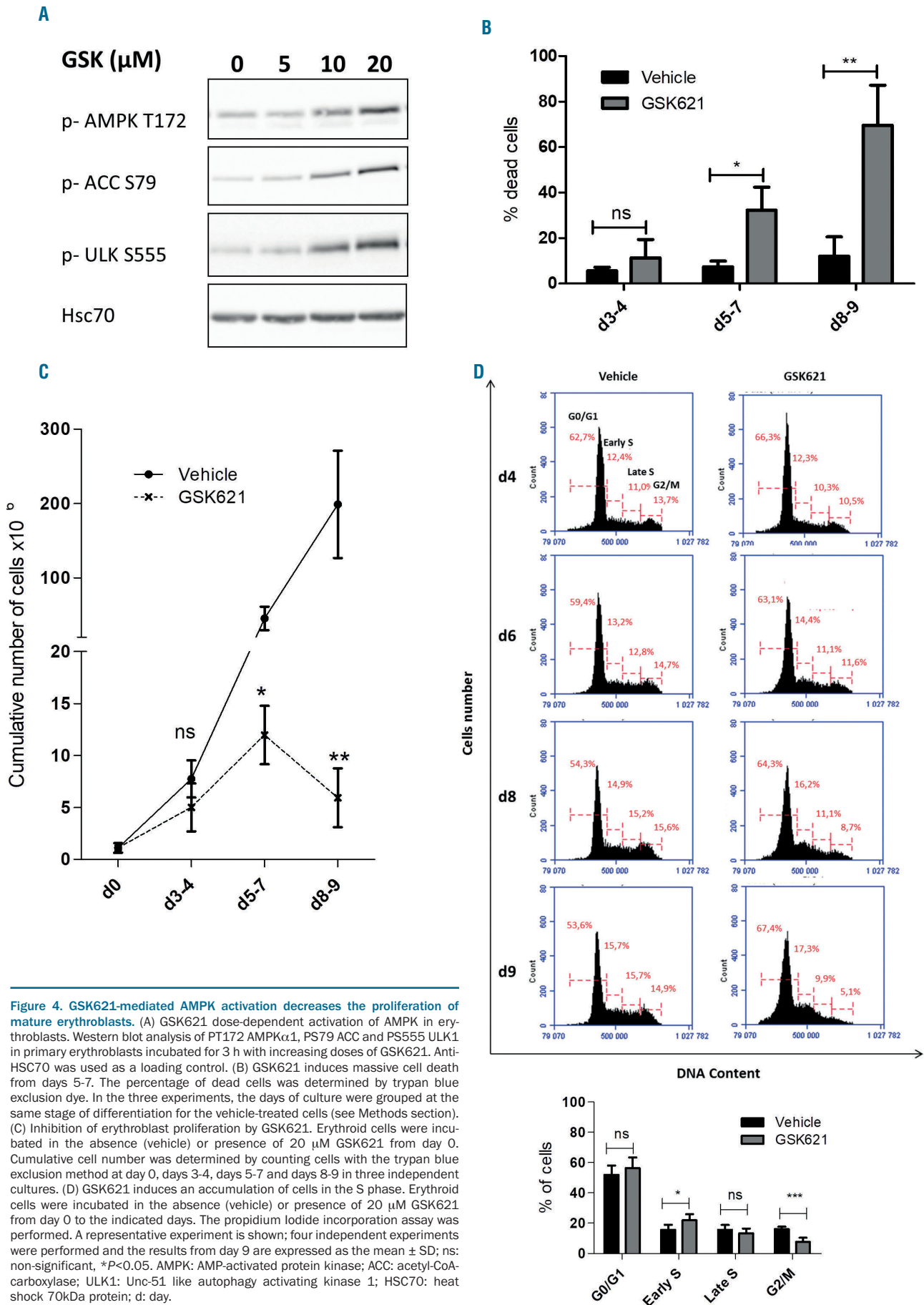
Figure 4. GSK621-mediated AMPK activation decreases the proliferation of mature erythroblasts. (A) GSK621 dose-dependent activation of AMPK in erythroblasts. Western blot analysis of PT172 AMPKα1, PS79 ACC and PS555 ULK1 in primary erythroblasts incubated for 3 h with increasing doses of GSK621. Anti-HSC70 was used as a loading control. (B) GSK621 induces massive cell death from days 5-7. The percentage of dead cells was determined by trypan blue exclusion dye. In the three experiments, the days of culture were grouped at the same stage of differentiation for the vehicle-treated cells (see Methods section). (C) Inhibition of erythroblast proliferation by GSK621. Erythroid cells were incubated in the absence (vehicle) or presence of 20 μM GSK621 from day 0. Cumulative cell number was determined by counting cells with the trypan blue exclusion method at day 0, days 3-4, days 5-7 and days 8-9 in three independent cultures. (D) GSK621 induces an accumulation of cells in the S phase. Erythroid cells were incubated in the absence (vehicle) or presence of 20 μM GSK621 from day 0 to the indicated days. The propidium Iodide incorporation assay was performed. A representative experiment is shown; four independent experiments were performed and the results from day 9 are expressed as the mean ± SD; ns: non-significant, *P<0.05. AMPK: AMP-activated protein kinase; ACC: acetyl-CoA-carboxylase; ULK1: Unc-51 like autophagy activating kinase 1; HSC70: heat shock 70kDa protein; d: day.

Figure S5). GSK621 provoked 32% cell death at days 5-7 and 70% at days 8-9 (Figure 4B). GSK621 dramatically reduced the proliferation of cells with a more drastic impact on the most mature erythroblasts (Figure 4C).

To gain further insight into the inhibition of mature erythroblast proliferation by GSK621, the cell cycle was analyzed by quantification of DNA content with propidium iodide. At days 8 and 9, GSK921 induced a reduction in the number of cells in the G2/M phase of the cell cycle and an increase in cells in the early S phase, demonstrating blockage in the S phase (Figure 4D). The protein level of AMPK substrates involved in the cell cycle, P53[16] and a target gene of P53, P21 was determined. GSK621-mediated cell cycle arrest was not due to the phosphorylation and consequent stabilization of P53 since there was no variation in their expression, which is in agreement with the absence of defects in G1/S transition (*Online Supplementary Figure S6*).

We then studied in more detail whether GSK621-mediated AMPK activation could affect erythroblast differentiation. In this set of experiments, at days 3-4 cells were immature and did not synthesize hemoglobin, while at days 8-9, more than 80% of the cells were hemoglobinized (Figure 5A). In the presence of GSK621, at days 8-9, the percentage of cells that synthesized hemoglobin was very low.

To decipher more precisely the stage at which the GSK621-mediated activation of AMPK induced cell death, erythroblasts were analyzed for GPA and annexin V by flow cytometry (Figure 5B). GSK621 did not affect immature GPA^{low} cells at days 3-4, but induced massive death of GPA[high] erythroblasts at days 5-7 (46% annexin V-positive cells with GSK621 *versus* 16% in control cells) and at days 8-9 (75% *versus* 16%, respectively). Indeed, after AMPK activation at days 5-7, only 3.5% of erythroblasts were GPA[high], in contrast to 46.4% in control cells. Furthermore, at day 7 only 7.5% of GSK621-treated cells were band 3[high] compared to 42% of control cells. At day 9, in the control conditions, erythroid cells continued to differentiate, which is in contrast to immature GSK621-treated cells. Furthermore, morphological studies after staining with May-Grünwald-Giemsa confirmed the blockage in maturation. Indeed, in vehicle-treated cultures, at day 9, the population was mainly constituted of polychromatic erythroblasts, and at day 14, orthochromatic erythroblasts and reticulocytes, whereas in the GSK621-treated culture, at days 9 and 14, cells were very immature with large nuclei and uncondensed chromatin; no mature cells were detected at day 14 (Figure 5C). The same results (decreased proliferation and survival, differentiation blockage) were obtained with another direct activator, compound 991 (*Online Supplementary Figure S7*). Overall, our results show that activation of AMPK by direct activators induced a blockage in the cell cycle, proliferation arrest and death of mature erythroblasts after the basophilic stage.

To reinforce our data, we took advantage of the fact that erythroid progenitors and early precursors can proliferate with delayed differentiation in response to erythropoietin, stem cell factor and dexamethasone.[17] We maintained the cells for the indicated number of days in culture medium with vehicle, GSK621, dexamethasone + vehicle or dexamethasone + GSK621 (Figure 6A). As expected, the presence of dexamethasone delayed erythroid differentiation, as demonstrated by the absence of a GPA[high] population after 7 days and even 9 days of culture. After 7 days, GSK621 induced cell death, as previously described (Figures 4B and 5B), with 54.6% of cells being positive for annexin V and

35.3% being stained by trypan blue; however, immature erythroblasts treated with dexamethasone + GSK621 were resistant to GSK621-induced cell death. Indeed, only 23.4% of cells were annexin V-positive and 16.6% were stained by trypan blue (Figure 6A,B).

To confirm that the activation of AMPK in mature erythroblasts provoked cell death, GSK621 was added for 24 and 48 h on day 9 when the erythroblastic population already contained 35% of mature GPA^{high} cells (Figure 6C). The GPA/annexin V staining clearly demonstrated that GSK621 induced massive death in mature GPA^{high} cells within less than 48 h. With GSK621, 52% of the total cells were positive for annexin V and 43% were stained by trypan blue *versus* 16% and 10%, respectively, of the control cells.

Overall, our results demonstrated that the activation of AMPK was deleterious for mature GPA^{high} cells, specifically in contrast to immature erythroblasts (from the progenitor stage to the basophilic stage), which were not affected.

AMPK activation induced autophagy and apoptotic death of mature erythroblasts

In erythroblasts, AMPK activation leads to ULK1 phosphorylation at S555 (Figure 4A), which is well known to be important for the induction of autophagy in several types of cells.[18] In GSK621-treated erythroblasts, LC3B-II accumulation was clearly detected by immunoblotting (Figure 7A, left panel). The induction of autophagy was confirmed by the use of chloroquine, which blocks the degradation of autophagosomes.[19] Indeed, in addition to GSK621, chloroquine treatment further increased LC3B-II, showing that the activation of AMPK by GSK621 in mature erythroblasts induced autophagy (Figure 7A, right panel).

We, therefore, wondered whether GSK621 provoked caspase-dependent apoptotic cell death and treated cells with a pan-caspase inhibitor Q-VD-OPh (QVD) in addition to GSK621. When caspase activity was blocked by QVD for 48 h (as demonstrated by the anti-cleaved-caspase 3 immunoblot), mature erythroblasts were protected from GSK621-induced cell death, showing that AMPK activation induced caspase-dependent apoptotic cell death (Figure 7B).

Discussion

Ampk α1^{-/-}, *Ampk β1^{-/-}* and *Ampk γ1^{-/-}* mice develop hemolytic anemia, and the plasma membrane of their red blood cells shows elasticity defects.[5–8] The membrane composition evolves continuously throughout erythropoiesis and during red blood cell maturation; the defects due to the absence of Ampk are most likely initiated during erythropoiesis. We, therefore, studied the role of AMPK during human erythropoiesis.

As in murine red blood cells,[5] α1 is the only catalytic subunit expressed in erythroblasts, α2 is not detected and we showed that the heterotrimer α1/β1/γ1 is predominant from erythroid progenitors to orthochromatic erythroblasts.

During the earliest stages of terminal differentiation, from progenitors to the basophilic stage, AMPK is activated and then its activation is drastically reduced to the reticulocyte stage. Several kinases and phosphatases regulate AMPK activation.[1,20] AMPK is activated by phosphorylation at T172 by three upstream kinases: LKB1, which seems to be constitutively active, CaMKK2 which is activated by an
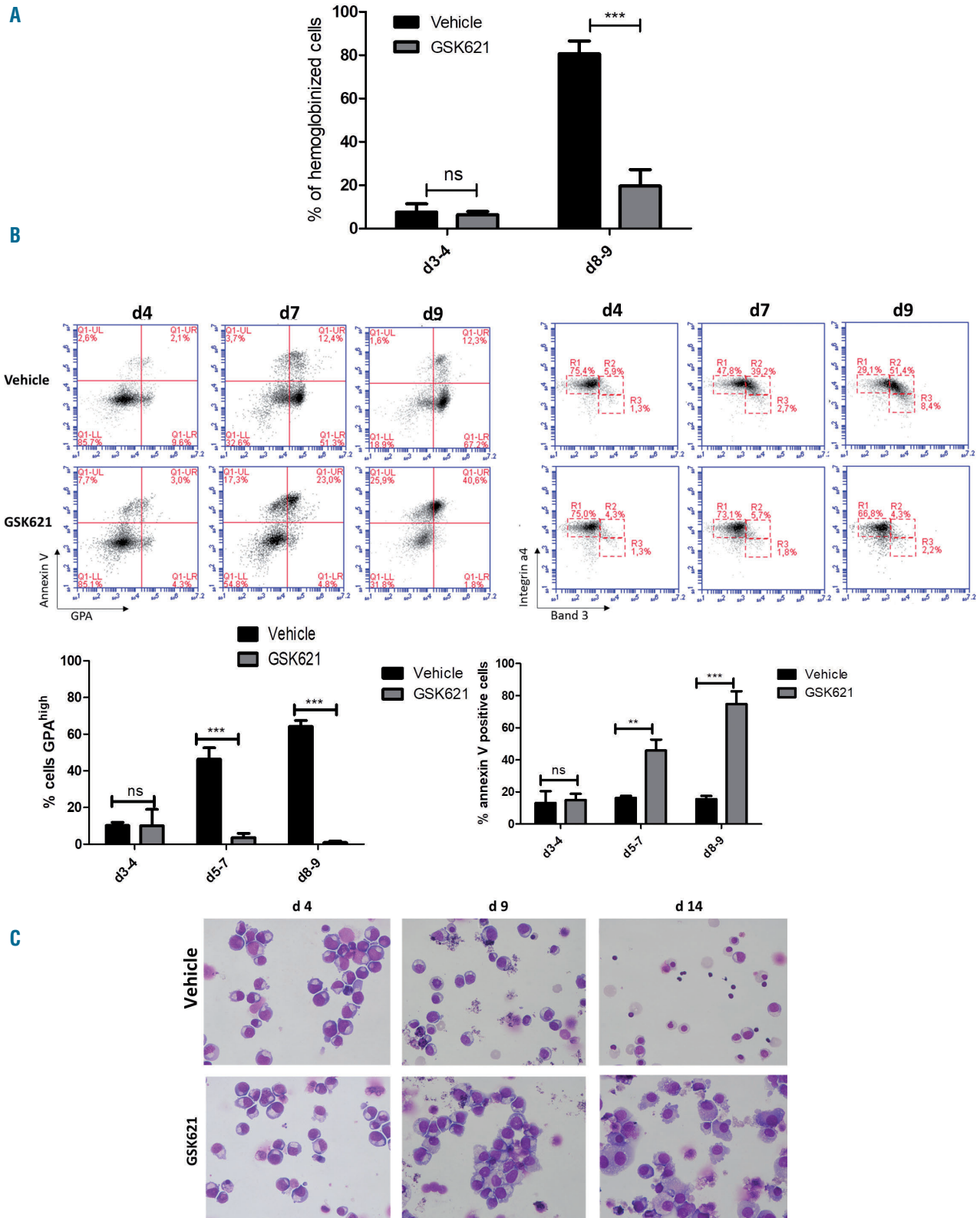
Figure 5. GSK621-mediated AMPK activation specifically and drastically decreases the survival of mature GPA[high] erythroblasts. (A) GSK621-dependent AMPK activation reduces the percentage of hemoglobinized cells. The percentage of hemoglobinized cells was determined by benzidine staining after incubation in the absence (vehicle) or presence of 20 µM GSK621 from day 0 to the indicated days. (B) GSK621-mediated AMPK activation blocked erythroblast differentiation. Erythroblasts were incubated in the absence (vehicle) or presence of 20 µM GSK621 from day 0 to the indicated days. Representative cytometry profiles for GPA/annexin V and band 3/α4 integrin are shown. For the GPA/annexin V profiles, annexin V-positive cells are shown in the upper part of the quadrant and GPA^{high} cells in the right part. The percentage of GPA^{high} cells and percentage of annexin V-positive cells are represented. Results are expressed as the mean ± SD of three independent experiments; ns: non-significant, *P<0.05. (C) Morphology was analyzed by May-Grünwald-Giemsa staining. Representative experiments at day 4, day 9 and day 14 are shown. d: day; GPA: glycophorin A.
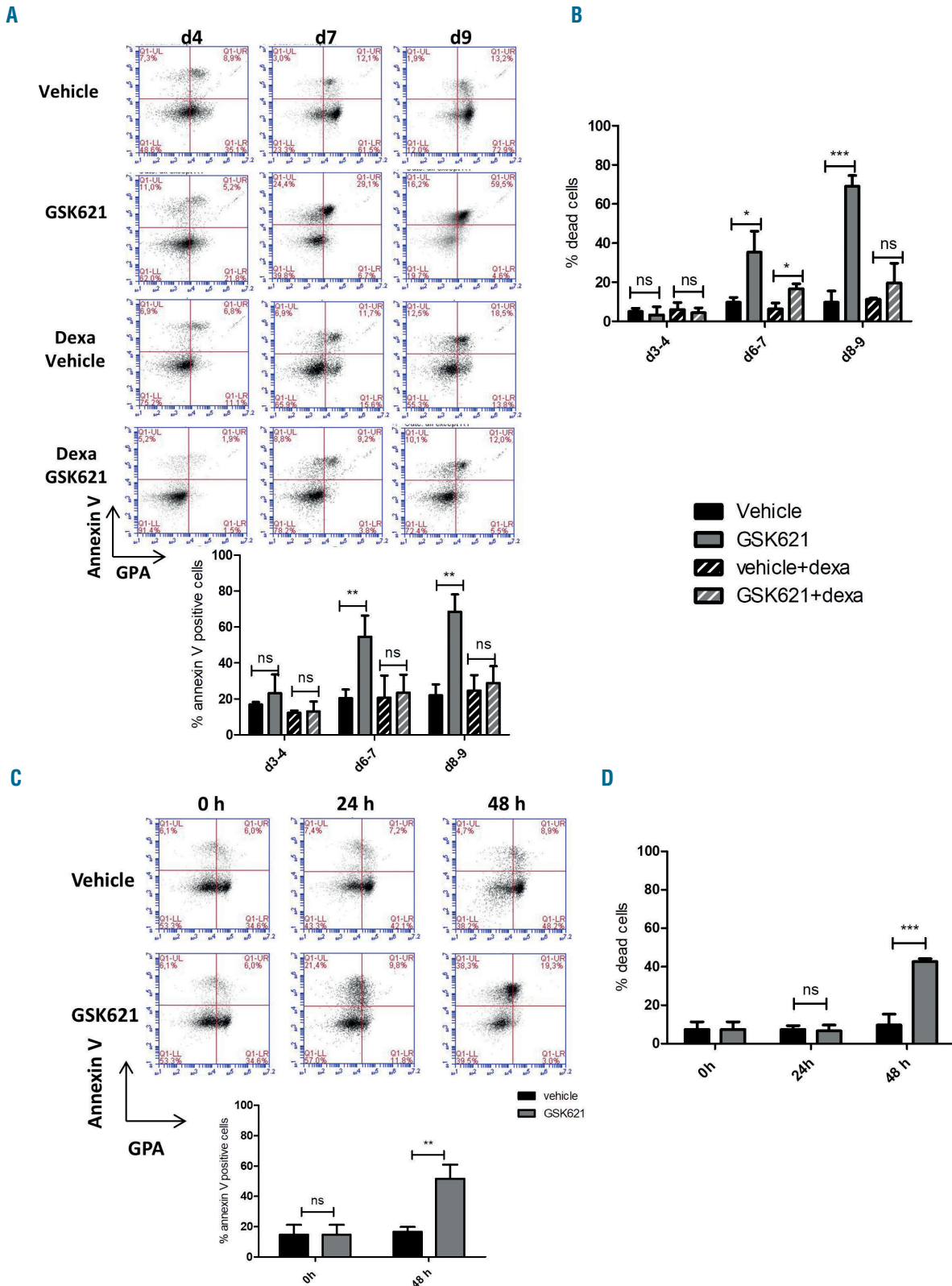
Figure 6. AMPK activation by GSK621 provokes the death of mature GPA[high] erythroblasts. (A) GSK621 does not increase the percentage of annexin V-positive cells in an immature cell population. Erythroid cells were incubated in the absence (vehicle) or presence of 20 μM GSK621 and 2x10^{-7} M dexamethasone (Dexa)/vehicle or Dexa/GSK621 from day 0 to the indicated days. Cells were labeled with anti-GPA antibodies and annexin V before analysis by flow cytometry. A representative experiment and the percentage of annexin V-positive cells from three experiments are shown. (B) GSK621 does not induce cell death when the population of erythroblasts is immature. The proportion of dead cells was determined by trypan blue exclusion dye assay. (C) GSK621-mediated AMPK activation specifically induces the death of mature day 9 erythroblasts. Mature erythroblasts were incubated in the absence (vehicle) or presence of 20 μM GSK621 for 24 or 48 h. Cells were incubated with anti-GPA antibodies and annexin V before analysis by flow cytometry. A representative experiment and the percentage of annexin V-positive cells from three experiments are shown (lower panel). (D) The proportion of dead cells was determined by a trypan blue exclusion dye assay. Results are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. d: day; Dexa: dexamethasone; GPA: glycophorin A; h: hours.

increase in cytosolic Ca²⁺, and possibly TAK1, which is activated by cytokines. The phosphatases PP1, PP2A and PP2C dephosphorylate T172 and the kinases GSK3, PKA, PKB, and PKC inhibit AMPK activation; they may contribute to the reduced AMPK activation in mature erythroblasts. Our data from the quantitative mass spectrometry analysis of human erythropoiesis[11] did not allow us to quantify these kinases and phosphatases because of their absence, their very weak expression or the scarcity of peptides generated. Nevertheless, our western blot studies showed the expression of LKB1 along erythroid differentiation and suggested that LKB1 could be the upstream activating kinase for AMPKα1. Further studies are needed to understand the kinetics of AMPK activation and its upstream regulators during erythroid terminal differentiation.

Our results in human erythroblasts show that knockdown of the expression of the α1 subunit by shRNA induces a decrease in cell proliferation and does not inhibit cell survival or erythroid maturation. Red blood cells from *Ampk α1⁻/⁻* mice have defects in membrane elasticity leading to hemolytic anemia. The absence of the α1 subunit in human erythroblasts induces important changes in the expression of membrane proteins and could potentially affect the expression of membrane proteins involved later in erythrocyte membrane elasticity. As we previously showed, phosphorylation of adducin at Ser724 is increased in red blood cells from *Ampk α1⁻/⁻* and *Ampk γ1⁻/⁻* mice. Our data demonstrate that this modification is also present earlier in human erythroblasts. Interestingly, in sickle cell disease, the reduction of red blood cell deformability is associ-
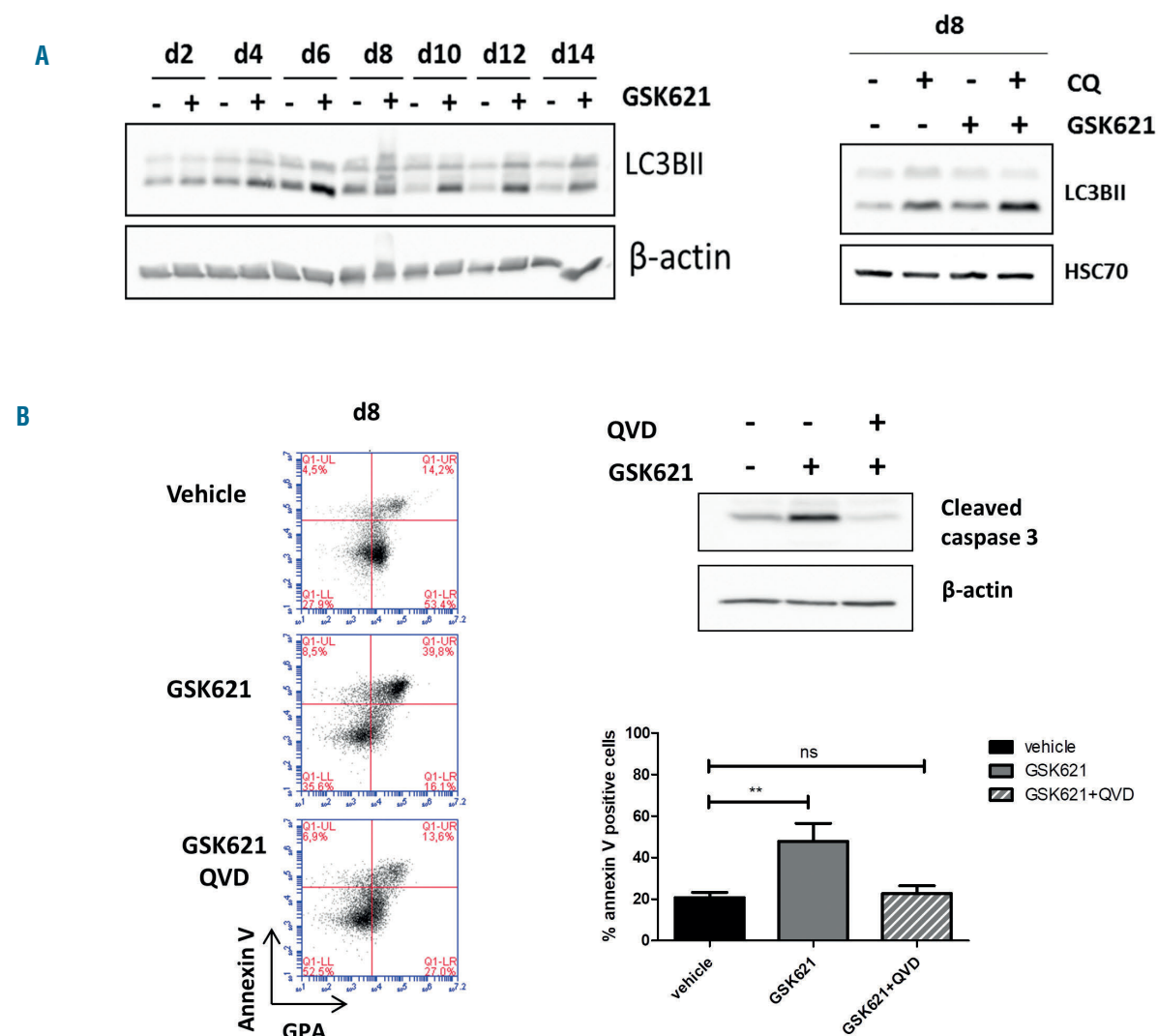

Figure 7. GSK621-mediated AMPK activation leads to autophagy and apoptosis. (A) GSK621-mediated AMPK activation induces autophagy. Erythroid cells were incubated in the absence (vehicle) or presence of 20 μM GSK621 from day 0 to the indicated days of culture (left panel). Chloroquine (10 μM) was added for 4 h before harvesting the cells (right panel). LC3BII was detected by western blot experiments using specific antibodies. Anti-β actin or anti-HSC70 antibodies were used as loading controls. (B) GSK621 provokes the caspase-dependent apoptosis of mature day 8 erythroblasts. Mature erythroblasts were incubated in the absence (vehicle) or presence of 20 μM GSK621 or 20 μM GSK621 and 10 μM QVD for 48 h; cells were labeled with anti-GPA and for annexin V binding and analyzed by flow cytometry. A representative experiment and the percentage of annexin V-positive cells from three experiments are shown. Results are expressed as the mean ± SD. *P<0.05, **P<0.01. Efficiency of QVD to block caspase activity was determined by western blot using an anti-caspase 3 antibody that specifically detects the cleaved isoform of caspase 3. β-actin was used as a loading control. d: day; CQ: chloroquine; GPA: glycophorin A; QVD: Q-VD-OPh.

ated with increased phosphorylation of adducin at Ser 726.[21] Thus, it would be interesting to determine whether AMPK plays a role in this pathology. Our results suggest that expression of band 3 is regulated by AMPKα1. Through *in vitro* studies, Thali *et al.* identified band 3 as a potential direct substrate for AMPK.[22] An attractive hypothesis would be that AMPK induces band 3 phosphorylation resulting in an increase in its global expression but a less efficient expression at the cell surface of erythroblasts.

To activate AMPK specifically, we used direct activators of AMPK because these molecules bind directly to the β subunit and do not affect the AMP/ATP ratio as metformin does. Several recent studies have demonstrated the involvement and specificity of GSK621 in activating AMPK.[12-15] In hematopoietic cells, GSK621 has been reported to be more potent in primary acute myeloid leukemia cells and cell lines than the direct activator A-769662.[12]

In the present study, we demonstrated, through the use of direct activators (GSK621 and compound 991), that AMPK activation in mature erythroblasts (GPA^high) (polychromatic to reticulocytes) induced apoptotic cell death, whereas no such effect was observed in similarly treated immature erythroblasts. Furthermore, the fact that GSK621 induced the apoptosis of mature erythroblasts after only 48 h of treatment but did not affect erythroblasts that were maintained in an immature state (by dexamethasone) after 9 days of GSK621 excludes a potential toxic effect due to the accumulation of compounds. We propose that maintaining AMPK activation after the basophilic stage, when AMPK is not normally activated, induces cell cycle arrest followed by the induction of autophagy and caspase-dependent apoptosis. Thus, our work suggests that AMPK activation during the final stages of erythropoiesis is deleterious.

The present work highlights the role of AMPK in erythropoiesis and adds further support to the involvement of AMPK in the regulation of hematopoiesis. In hematopoietic stem cells, AMPK deficiency partially phenocopies the mitochondrial defects observed in *Lkb1*^-/- mice without affecting hematopoietic stem cell maintenance,[23] Obba *et al.* recently demonstrated that the activation of AMPK is crucial for CSF-1-induced autophagy and human monocyte differentiation into macrophages.[24] Our results demonstrate the importance of the finely tuned regulation of AMPK during adult human erythropoiesis. This observation is of significant value since deciphering the molecular mechanisms regulating proliferation, survival and differentiation of erythroblasts is necessary to better understand how erythroid progenitors and precursors can physiologically give rise to red blood cells. The use of direct AMPK activators is being considered as a therapeutic treatment in several chronic metabolic diseases. Phase I and II trials investigating the use of the activators PXL770 (clinical trial NCT03395470) and compound 0304 (betagenon.se) are in progress in patients with non-alcoholic hepatic steatosis or type 2 diabetes. These activators could induce the apoptosis of mature erythroblasts in the bone marrow so it will be necessary to analyze hematologic parameters to prevent potential anemia.

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