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# Enhanced suicidal erythrocyte death in mice carrying a loss-of-function mutation of the *adenomatous polyposis coli* gene

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## Abstract

Loss-of-function mutations in human adenomatous polyposis coli (APC) lead to multiple colonic adenomatous polyps eventually resulting in colonic carcinoma. Similarly, heterozygous mice carrying defective APC ($apc^{Min/+}$) suffer from intestinal tumours. The animals further suffer from anaemia, which in theory could result from accelerated eryptosis, a suicidal erythrocyte death triggered by enhanced cytosolic $Ca^{2+}$ activity and characterized by cell membrane scrambling and cell shrinkage. To explore, whether APC-deficiency enhances eryptosis, we estimated cell membrane scrambling from annexin V binding, cell size from forward scatter and cytosolic ATP utilizing luciferin–luciferase in isolated erythrocytes from $apc^{Min/+}$ mice and wild-type mice ($apc^{+/+}$). Clearance of circulating erythrocytes was estimated by carboxyfluorescein-diacetate-succinimidyl-ester labelling. As a result, $apc^{Min/+}$ mice were anaemic despite reticulocytosis. Cytosolic ATP was significantly lower and annexin V binding significantly higher in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes. Glucose depletion enhanced annexin V binding, an effect significantly more pronounced in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes. Extracellular $Ca^{2+}$ removal or inhibition of $Ca^{2+}$ entry with amiloride (1 mM) blunted the increase but did not abrogate the genotype differences of annexin V binding following glucose depletion. Stimulation of $Ca^{2+}$-entry by treatment with $Ca^{2+}$-ionophore ionomycin (10 μM) increased annexin V binding, an effect again significantly more pronounced in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes. Following retrieval and injection into the circulation of the same mice, $apc^{Min/+}$ erythrocytes were more rapidly cleared from circulating blood than $apc^{+/+}$ erythrocytes. Most labelled erythrocytes were trapped in the spleen, which was significantly enlarged in $apc^{Min/+}$ mice. The observations point to accelerated eryptosis and subsequent clearance of $apc^{Min/+}$ erythrocytes, which contributes to or even accounts for the enhanced erythrocyte turnover, anaemia and splenomegaly in those mice.

**Keywords:** phosphatidylserine • cell membrane scrambling • calcium • cell volume • eryptosis • APC

## Introduction

The APC protein binds the oncogenic protein β-catenin and favours its degradation [1–4]. Lack of APC is followed by accumulation of β-catenin, which enters the nucleus and stimulates the expression of several genes involved in the regulation of cell proliferation [5, 6]. Loss-of-function mutations affecting APC lead to the development of multiple colonic adenomatous polyps, which eventually results in colonic carcinoma [7, 8]. Mice carrying a mutation in the *APC* gene ($apc^{Min/+}$), which leads to truncation of the APC protein at amino acid 850, develop multiple intestinal tumours [9]. Beyond that the mice suffer from enhanced gastric acid secretion [10], hyperaldosteronism and increased blood pressure [11]. Moreover, the animals were shown to suffer from anaemia, a disorder considered to be secondary to blood loss [12].

A blood count of those mice revealed an anaemia despite excessive reticulocyte numbers. The observation points to enhanced erythrocyte turnover, which may, at least in theory, result from enhanced eryptosis, a suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling [13].

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Eryptosis is triggered by activation of $Ca^{2+}$-permeable cation channels [14–21]. Cytosolic $Ca^{2+}$ activates $Ca^{2+}$-sensitive $K^+$ channels [22, 23] leading to exit of KCl with osmotically obliged water and thus to cell shrinkage [24]. Enhanced cytosolic $Ca^{2+}$ further stimulates scrambling of the erythrocyte membrane with exposure of phosphatidylserine at the cell surface [21, 25–28]. The $Ca^{2+}$ sensitivity of cell membrane scrambling is increased by ceramide [29]. Eryptotic cells are rapidly phagocytosed and thus cleared from circulating blood [30–32]. Accordingly, accelerated eryptosis may lead to anaemia [13]. On the other hand, eryptosis is a physiological mechanism preventing haemolysis of defective erythrocytes [13].

This study was performed to elucidate whether the anaemia and reticulocytosis in *apc*$^{Min/+}$ mice is secondary to increased eryptosis. Thus, eryptosis was determined in erythrocytes from *apc*$^{Min/+}$ mice and from wild-type mice (*apc*$^{+/+}$).

# Materials and methods

## Mice

Mice with mutated *APC* (*apc*$^{Min/+}$) and wild-type mice (*apc*$^{+/+}$) were generated by breeding of *apc*$^{Min/+}$ mice initially obtained from the Jackson Laboratory. The mice (four to eight per experiment, sex-matched, age as indicated) were fed a control diet (C1314; Altromin, Heidenau, Germany) and had access to drinking water *ad libitum*. Unless otherwise stated, 9- to 26-week-old mice were used.

## Blood count and reticulocyte estimation

Blood was withdrawn into heparinized capillaries by puncturing the retrobulbar plexus. To this end, the mice were anaesthetized with diethylether (Roth, Karlsruhe, Germany). Anaesthesia was verified by testing the hind limb reflex. Then, 50 μl of blood was taken by puncturing the retrobulbar plexus. For all experiments except for the blood count heparin blood was obtained. For the blood count, EDTA blood was analysed using an electronic haematology counter (scil VET abc, Weinheim, Germany). Relative reticulocyte numbers were determined using the Retic-COUNT reagent (BD, Heidelberg, Germany) according to the manufacturer's instructions.

## Incubation, chemicals and solutions

The erythrocytes were isolated by washing two times with Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES/NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$. Erythrocytes were incubated *in vitro* at a haematocrit of 0.4% in Ringer solution at 37°C for 8 hrs unless otherwise stated. Where indicated, amiloride or ionomycin (both Sigma-Aldrich, Schnelldorf, Germany) were added, glucose removed or 1 mM $CaCl_2$ substituted with 4 mM ethylene glycol tetraacetic acid (EGTA; Sigma-Aldrich).

## FACS analysis of annexin V-binding and forward scatter

For FACS analysis of annexin V-binding and forward scatter 50 μl cell suspensions were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:250 dilution; Immunotools, Friesoythe, Germany) in this solution for 20 min. at 37°C under protection from light. In the stained erythrocyte cell suspensions, forward scatter of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

## Measurement of the *in vivo* clearance of fluorescence-labelled erythrocytes

The *in vivo* clearance of fluorescence-labelled erythrocytes was determined as described previously [33]. Briefly, erythrocytes (obtained from 200 μl blood) were fluorescence labelled by staining the cells with 5 μM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE; Molecular Probes, Leiden, The Netherlands) in PBS and incubated for 30 min. at 37°C. After washing twice in PBS containing 1% FCS, the pellet was resuspended in Ringer solution (37°C), and 100 μl of the CFSE-labelled erythrocytes were injected into the tail vein of the recipient mouse. As indicated, blood was retrieved from the tail veins of the mice, and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1 as described earlier. The percentage of CFSE-positive erythrocytes was calculated in percentage of the total labelled fraction determined 10 min. after injection.

## Confocal microscopy and immunofluorescence

For the detection of annexin V-binding and CFSE-dependent fluorescence of erythrocytes in splenic tissue, the mice were deeply anaesthetized with diethyleter. Then, they were killed by cervical dislocation. After laparotomy, the spleens of *apc*$^{Min/+}$ and of *apc*$^{+/+}$ mice were removed, weighed and mechanically homogenized in 1 ml cold PBS. The suspension was then centrifuged at 500 × *g* for 10 min. at 4°C. The cell pellet was resuspended in 200 μl cold PBS. Five microlitres of Annexin V-APC (BD) were added, and incubation was carried out for 20 min. at 37°C protected from light. The suspension was then transferred onto a glass slide and mounted with Prolong® Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

## Estimation of intracellular ATP concentration

For determination of erythrocyte ATP, 80 μl of erythrocyte pellets were incubated for 12 hrs at 37°C in Ringer solution with or without glucose (final haematocrit 5%). All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP

**Table 1** Anaemia in APC-deficient mice

| Parameter | $apc^{+/+}$ | $apc^{Min/+}$ | Normal range |
|---|---|---|---|
| RBC ($10^6/\mu l$) | 10.7 ± 0.6 | 5.5 ± 0.1* | 5.0–9.5 |
| HGB (g/dl) | 16.4 ± 0.7 | 10.0 ± 0.4* | 10.9–16.3 |
| HCT (%) | 55.2 ± 2.0 | 34.1 ± 1.2* | 38.5–45.1 |
| MCV (fl) | 51.8 ± 1.8 | 62.3 ± 1.7* | 48.0–56.0 |
| MCHC (g/dl) | 29.8 ± 0.5 | 29.3 ± 0.5 | 25.9–35.1 |
| MCH (pg) | 15.5 ± 0.3 | 18.4 ± 0.7* | 11.9–19.0 |
| Reticulocytes (%) | | | |
| 4 weeks (age) | 3.7 ± 0.3 | 7.0 ± 0.7* | 1.0–6.0 |
| 6 weeks (age) | 5.3 ± 0.4 | 12.6 ± 0.7* | |
| 8 weeks (age) | 5.3 ± 1.3 | 20.7 ± 4.6* | |
| 12 weeks (age) | 4.1 ± 0.4 | 17.8 ± 6.0* | |

Arithmetic mean ± S.E.M. ($n = 4$) of erythrocyte count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) of 15- to 16-week-old APC-deficient mice ($apc^{Min/+}$) and wild-type mice ($apc^{+/+}$). Reticulocyte number ($n = 4$) of APC-deficient mice ($apc^{Min/+}$) and wild-type mice ($apc^{+/+}$) as a function of age. The data are compared to the normal range in mice [67, 68]. * indicates significant differences between genotypes (Mann–Whitney test; $P = 0.0286$).

concentration of the aliquots was determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics, Mannheim, Germany) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations refer to the cystol of erythrocytes.

### Statistics

Data are expressed as arithmetic means ± S.E.M., and statistical analysis was made by non-parametric Mann–Whitney test as indicated in the figure legends using GraphPad InStat Version 3.06 (San Diego, CA, USA); *n* denotes the number of different erythrocyte specimens studied.

## Results

### Blood count and percentage of reticulocytes

A blood count revealed moderate anaemia of the $apc^{Min/+}$ mice. Erythrocyte count, haemoglobin concentration and haematocrit were significantly smaller in $apc^{Min/+}$ than in $apc^{+/+}$ mice (Table 1). The mean corpuscular volume was, however, significantly increased. According to FACS analysis, the reticulocyte number was significantly higher in $apc^{Min/+}$ than in $apc^{+/+}$ mice at different ages (4, 6, 8 and 12 weeks), pointing to enhanced erythrocyte formation (Table 1).

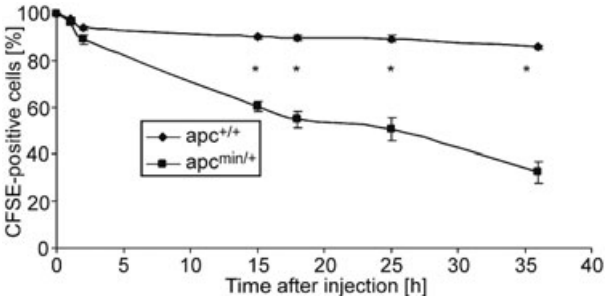


**Fig. 1** Accelerated clearance of erythrocytes in APC-deficient mice. Time-dependent decay of CFSE-labelled circulating erythrocytes drawn from 20-week-old APC-deficient mice (black squares) and wild-type mice (black diamonds) and injected into the same mice. The percentage of CFSE-labelled circulating cells is plotted against time after injection. Values are normalized arithmetic mean ± S.E.M. ($n = 4$) of the percentages of CFSE-labelled erythrocytes. * indicates significant difference between genotypes (Mann–Whitney test; $P < 0.05$).

### *In vivo* clearance of CFSE-labelled erythrocytes

The enhanced reticulocyte number accompanied by moderate anaemia points to enhanced erythrocyte turnover. To determine the life span of circulating erythrocytes blood was drawn from $apc^{Min/+}$ and $apc^{+/+}$ (control) mice, erythrocytes were labelled with CFSE and the labelled $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes were injected into the same mice. As illustrated in Figure 1, labelled $apc^{Min/+}$ erythrocytes disappeared from circulating blood of $apc^{Min/+}$ mice significantly more rapidly than labelled $apc^{+/+}$ erythrocytes. Thus, the life span of $apc^{Min/+}$ erythrocytes in $apc^{Min/+}$ mice was significantly shorter than the life span of $apc^{+/+}$ erythrocytes in $apc^{+/+}$ mice. The percentage of $apc^{Min/+}$ erythrocytes remaining in circulating blood of $apc^{+/+}$ mice within 24 hrs tended to be higher (62.1 ± 7.4, $n = 3$) than the respective percentage of $apc^{Min/+}$ erythrocytes in $apc^{Min/+}$ mice (50.3 ± 4.9, $n = 4$), a difference, however, not reaching statistical significance. The percentage of $apc^{+/+}$ erythrocytes remaining within 24 hrs in circulating blood of $apc^{Min/+}$ mice tended to be lower (84.2 ± 3.2, $n = 3$) as the respective percentage of $apc^{+/+}$ erythrocytes in $apc^{+/+}$ mice (89.4 ± 1.2, $n = 4$), a difference, however, again not statistically significant.

### Analysis of the spleen and splenic erythrocytes

As evident from Figure 2A and B, the labelled erythrocytes were mainly trapped in the spleen, which was significantly larger in $apc^{Min/+}$ mice than in $apc^{+/+}$ mice. A detailed non-quantitative analysis revealed that the number of fluorescent annexin V binding

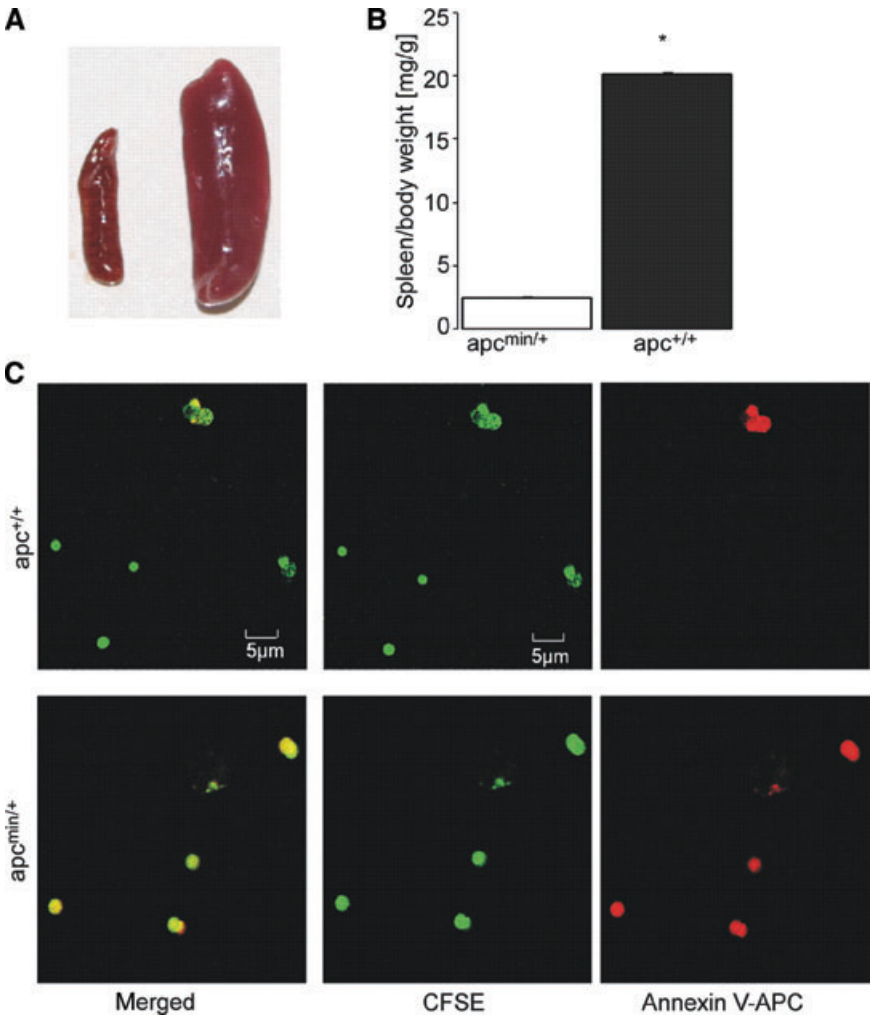


**Fig. 2** Splenomegaly associated with increased number of annexin V binding erythrocytes in APC-deficient mice. (**A**) Photograph of spleens from 20-week-old APC-deficient mice (right) and wild-type mice (left). (**B**) Arithmetic mean ± S.E.M. ($n = 4$) of the spleen/body weight ratios of 20-week-old APC-deficient mice ($apc^{Min/+}$, black bar) and wild-type mice ($apc^{+/+}$, white bar), * indicates significant difference between genotypes (Mann-Whitney test; $P < 0.05$;). (**C**) Confocal microscopy of CFSE dependent (middle panels), annexin V-APC (right panels) dependent and merged fluorescence (left panels) of erythrocytes from the spleens of APC-deficient ($apc^{Min/+}$, lower panels) mice and wild-type mice ($apc^{+/+}$, upper).

and thus phosphatidylserine-exposing erythrocytes was higher in the spleens from $apc^{Min/+}$ mice than from $apc^{+/+}$ mice (control; Fig. 2C). CFSE accumulates in the cytosol, whereas annexin V binds to phosphatidylserine in the cell membrane.

## Phosphatidylserine exposure of apc$^{+/+}$ and apc$^{Min/+}$ erythrocytes

In view of the accelerated clearance of circulating erythrocytes in the spleen of $apc^{Min/+}$ mice and their enhanced phosphatidylserine exposure at the cell surface, additional experiments were performed to determine annexin V binding of $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes in FACS analysis. The experiments were performed in the presence and absence of glucose, as energy depletion is known to foster eryptosis [34]. As shown in Figure 3A and B, annexin V binding reflecting phosphatidylserine exposure at the erythrocyte surface was significantly higher in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes following energy depletion.

## Role of $Ca^{2+}$ for cell membrane scrambling of apc$^{+/+}$ and apc$^{Min/+}$ erythrocytes

As cytosolic $Ca^{2+}$ is important for triggering of eryptosis, the $Ca^{2+}$ sensitivity of annexin V binding was tested by exposing $apc^{Min/+}$ and $apc^{+/+}$ erythrocytes to the $Ca^{2+}$ ionophore ionomycin (10 μM). As illustrated in Figure 4A, the ionomycin effect on annexin V binding was significantly stronger in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes pointing to higher $Ca^{2+}$ sensitivity of $apc^{Min/+}$ erythrocytes. To define the role of $Ca^{2+}$ entry for the triggering of energy depletion-induced eryptosis, $apc^{Min/+}$ and $apc^{+/+}$ erythrocytes were incubated with glucose-free Ringer in the presence and absence of amiloride (1 mM), an

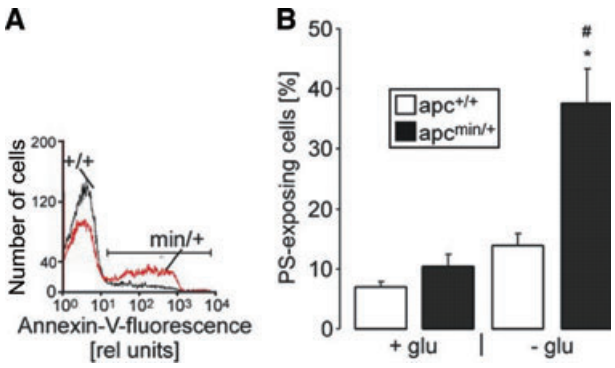


**Fig. 3** Enhanced phosphatidylserine abundance at the surface of erythrocytes from APC-deficient mice. (**A)** Histogram of annexin V binding reflecting phosphatidylserine exposure in a representative experiment of erythrocytes from APC-deficient mice ($apc^{Min/+}$) and their wild-type littermates ($apc^{+/+}$) exposed for 8 hrs to glucose-depleted Ringer. (**B)** Arithmetic mean ± S.E.M. ($n = 4$) of the percentage of annexin V-binding erythrocytes from 8-week-old APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 8 hrs to glucose-containing (left bars) or glucose-depleted (right bars) Ringer. Annexin V bound to the cell membrane whereas CFSE accumulated in the cytosol. * indicates significant ($P < 0.05$) difference from glucose-containing Ringer; # indicates significant difference ($P < 0.05$) between genotypes (Mann–Whitney test).

inhibitor of the non-specific cation conductance in erythrocytes. As a result, amiloride significantly attenuated the increase in annexin V binding following energy depletion in both $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes (Fig. 4B). However, amiloride did not abolish the differences between $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes of the annexin V binding following energy depletion. Similar observations were made in the absence of extracellular $Ca^{2+}$. As shown in Figure 4C, removal of extracellular $Ca^{2+}$ tended to attenuate the increase in annexin V binding following energy depletion in both $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes. However, similar to amiloride administration, $Ca^{2+}$ removal did not abolish the differences between $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes of the annexin V binding following energy depletion. Collectively, these observations point to enhanced susceptibility of $apc^{Min/+}$ erythrocytes to the cell membrane scrambling effect of energy depletion and enhanced cytosolic $Ca^{2+}$ activity.

## Cytosolic ATP concentration in $apc^{+/+}$ and $apc^{Min/+}$ erythrocytes

Glucose deprivation is likely to affect the intracellular ATP content. Hence, the intracellular ATP concentration of erythrocytes

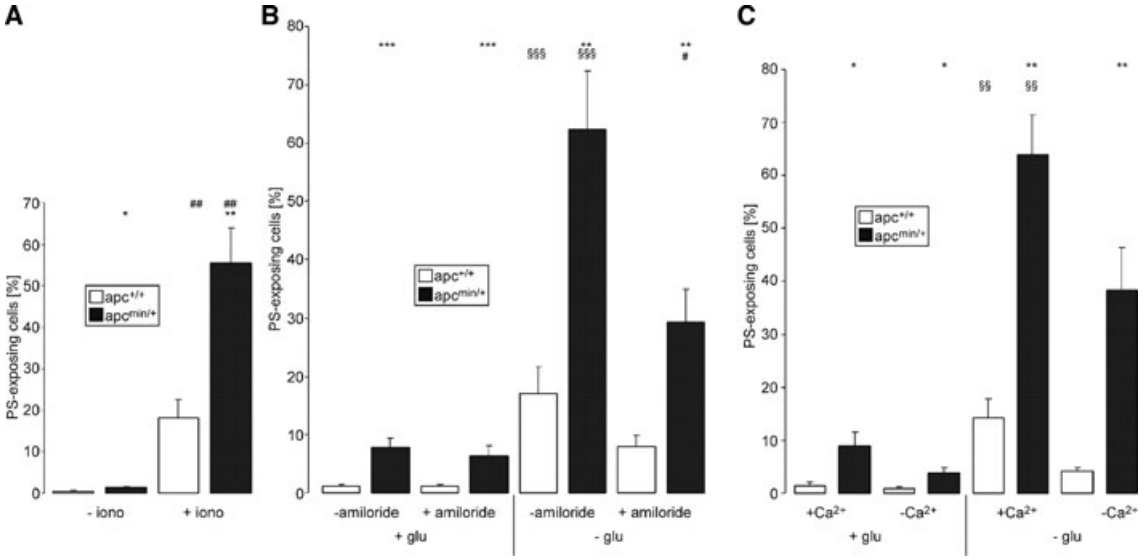


**Fig. 4** $Ca^{2+}$ sensitivity of erythrocytes from APC-deficient and wild-type mice. (**A)** Arithmetic mean ± S.E.M. ($n = 5$) of the percentage of annexin V-binding erythrocytes from APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 30 min. to Ringer solution without (left bars) or with (right bars) 10 μM ionomycin. *, ** indicate ($P < 0.05$, $P < 0.01$) significant difference between genotypes; ## indicates ($P < 0.01$) significant difference from absence of ionomycin (Mann–Whitney test). (**B)** Arithmetic mean ± S.E.M. ($n = 7$ experiments with samples from four different mice) of the percentage of annexin V-binding erythrocytes from APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 10 hrs to Ringer solution with (left bars) or without (right bars) glucose in the absence and presence of 1 mM amiloride. **, *** indicate significant ($P < 0.01$, $P < 0.001$) difference between genotypes, # significant ($P < 0.05$) difference from absence of amiloride, §§§ indicates significant ($P < 0.001$) difference from the presence of glucose (Mann–Whitney test). (**C)** Arithmetic mean ± S.E.M. ($n = 5$) of the percentage of annexin V-binding erythrocytes from APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 10 hrs to Ringer solution with (left bars) or without (right bars) glucose in the absence ($-Ca^{2+}$) and presence ($+Ca^{2+}$) of 1 mM extracellular $Ca^{2+}$. *, ** indicate significant ($P < 0.05$, $P < 0.001$) difference between genotypes; §§ indicates significant ($P < 0.01$) difference from the presence of glucose (Mann–Whitney test).

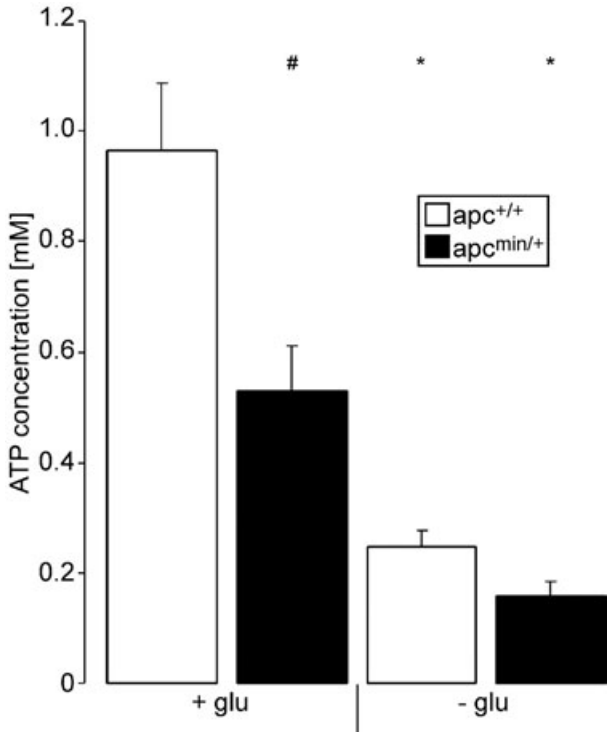


**Fig. 5** Intracellular ATP content in erythrocytes from APC-deficient and wild-type mice. Arithmetic mean ± S.E.M. ($n = 4$) of the cytosolic ATP concentration in erythrocytes from APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 12 hrs to Ringer solution without (left bars) or with (right bars) glucose. # indicates significant ($P < 0.05$) difference between genotypes; * indicates significant ($P < 0.05$) difference from presence of glucose (Mann–Whitney test).

incubated in the presence or absence of glucose for 12 hrs was determined. As shown in Figure 5, glucose depletion indeed decreased the intracellular ATP concentration of erythrocytes from both genotypes. In the presence of glucose, cytosolic ATP content was significantly lower in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes. Following glucose depletion cytosolic ATP content tended to be lower in $apc^{Min/+}$ erythrocytes than in $apc^{+/+}$ erythrocytes, a difference, however, not reaching statistical significance (Fig. 5).

### Cell volume of apc$^{+/+}$ and apc$^{Min/+}$ erythrocytes

To depict cell shrinkage, another hallmark of eryptosis, forward scatter of $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes was determined in FACS analysis. As shown in Figure 6, the forward scatter was significantly reduced by energy depletion in erythrocytes from both genotypes, an effect not significantly different between $apc^{Min/+}$ erythrocytes and $apc^{+/+}$ erythrocytes.

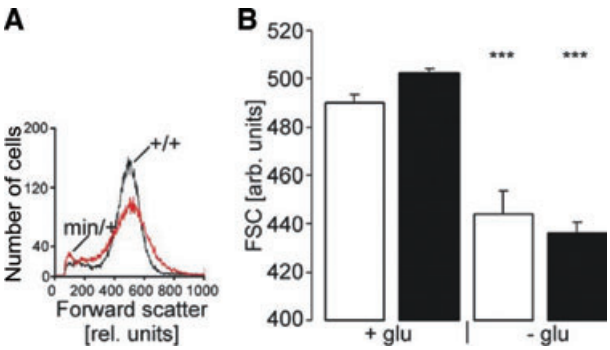


**Fig. 6** Forward scatter in erythrocytes from APC-deficient and wild-type mice. (**A**) Histogram of forward scatter as a measure of cell volume in a representative experiment of erythrocytes from APC- deficient mice (*Min/+*) and wild-type mice (+/+) exposed for 8 hrs to glucose-depleted Ringer. (**B**) Arithmetic mean ± S.E.M. ($n = 8$) of forward scatter of erythrocytes from 8-week-old APC-deficient mice ($apc^{Min/+}$, black bars) and wild-type mice ($apc^{+/+}$, white bars) exposed for 8 hrs to glucose-containing (left bars) or glucose-depleted (right bars) Ringer. *** indicate significant ($P < 0.001$) difference from glucose-containing Ringer (Mann–Whitney test).

## Discussion

According to the present observations, heterozygous mice carrying defective APC ($apc^{Min/+}$) suffer from mild anaemia with decreased erythrocyte count, haemoglobin concentration and haematocrit. The anaemia occurs despite significantly higher reticulocyte count in $apc^{Min/+}$ mice, pointing to enhanced formation of new erythrocytes. Accordingly, the anaemia is secondary to enhanced turnover of $apc^{Min/+}$ erythrocytes, which is further apparent from accelerated *in vivo* clearance of CFSE-labelled erythrocytes. The erythrocytes are to a large part trapped in the spleen. The splenic accumulation of eryptotic erythrocytes presumably accounts for the splenomegaly of those mice. Conversely, splenomegaly may foster splenic trapping of erythrocytes.

The accelerated clearance of the $apc^{Min/+}$ erythrocytes is presumably due to enhanced phosphatidylserine exposure at their surface. Phosphatidylserine-exposing cells are trapped by macrophages [35], engulfed and degraded [36]. Phosphatidylserine-exposing erythrocytes are thus rapidly cleared from circulating blood [32]. To the extent that the accelerated loss of circulating erythrocytes is not matched by a similarly enhanced formation of new erythrocytes, the accelerated eryptosis leads to anaemia.

The present observations do not allow safe conclusions as to the mechanism linking APC deficiency to eryptosis. Clearly, erythrocytes from APC-deficient mice are more susceptible to the eryptotic effects of increased cytosolic $Ca^{2+}$ activity, a property unmasked by the enhanced eryptosis of those erythrocytes following treatment with the $Ca^{2+}$ ionophore ionomycin. The $Ca^{2+}$

ionophore should increase cytosolic $Ca^{2+}$ levels to similarly high values in APC-deficient and wild-type erythrocytes. Thus, at least part of the defect must be downstream of cytosolic $Ca^{2+}$. Along those lines, even in the absence of extracellular $Ca^{2+}$, eryptosis was enhanced in APC-deficient erythrocytes.

Erythrocytes from APC-deficient mice have lower cytosolic ATP levels, which should render them indeed more vulnerable to eryptosis. Energy depletion is known to stimulate protein kinase C, which in turn has been shown to trigger cell shrinkage and cell membrane scrambling [34].

Eryptosis is typically paralleled by decrease of cell volume [24]. However, no significant differences were observed in forward scatter between $apc^{Min/+}$ and $apc^{+/+}$ erythrocytes. Possibly, the energy depletion of $apc^{Min/+}$ erythrocytes impairs the activity of the $Na^+/K^+$ ATPase leading to cellular loss of $K^+$ and cellular gain of $Na^+$. $K^+$ depletion expectedly blunts the $K^+$ exit following activation of $K^+$ channels and thus compromises the cellular KCl loss and cell shrinkage following $Ca^{2+}$ entry.

Eryptosis has been determined in Ringer, indicating that the enhanced eryptosis was a property of the erythrocytes rather than a result from direct effects of plasma components on erythrocyte survival. Moreover, the clearance of CSFE-labelled erythrocytes from $apc^{Min/+}$ mice is enhanced even in wild-type mice. The present observation could be explained by a role of APC in the maintenance of cytosolic ATP levels and survival of erythrocytes. Alternatively, the erythrocytes have been rendered more vulnerable to eryptotic stimuli by some component in circulating blood prior to the experiments. It is noteworthy that reticulocytosis increases with age of the animals and may at least in part be related to tumour growth. Anaemia is a well-known complication of malignancy [37, 38] including familial adenomatous polyposis [12, 39]. In view of the present observations, tumour anaemia may at least in part be due to enhanced eryptosis followed by accelerated clearance of eryptotic cells from circulating blood. In patients with malignancy, the eryptosis may be further triggered by cytostatic treatment, as several cytotoxic drugs have been shown to stimulate eryptosis [13]. Eryptosis is triggered by a wide variety of further anaemia-causing xenobiotics and endogeneous substances [40–47], and accelerated eryptosis has been observed in anaemia of several clinical disorders [13], including iron deficiency [32], phosphate depletion [48], haemolytic uraemic syndrome [49], sepsis [50], malaria [51–54] or Wilson's disease [55]. It is considered likely that APC deficiency enhances the susceptibility to the eryptotic effect of those xenobiotics, endogeneous substances and clinical disorders. In view of the rapid clearance of erythrocytes, the splenomegaly and the profound anaemia despite reticulocytosis in $apc^{Min/+}$ mice, confounding triggers of eryptosis may be present in the blood of those mice.

Phosphatidylserine-exposing erythrocytes have been shown to adhere to the vascular wall [56–60], and to stimulate blood clotting [56, 61, 62]. Accordingly, excessive eryptosis due to oxidative stress may lead to derangements of microcirculation and enhanced eryptosis has been suggested to participate in the vascular injury of metabolic syndrome [63], a chronic clinical condition consisting of the clustering of cardiovascular risk factors including hypertension, that in humans relates also to colo-rectal cancer, and other forms of malignancies [64]. Intriguingly, the $apc^{Min/+}$ mice suffer from hyperaldosteronism and hypertension [11], an observation similarly made in APC patients [65]. Oxidative stress has further been shown to be relevant for ageing of stored red blood cells [66].

In conclusion, lack of APC leads to enhanced suicidal erythrocyte death or eryptosis. The effect contributes to the anaemia in APC-deficient mice and presumably in patients carrying a loss-of-function mutation of the gene encoding the APC protein. Future studies may explore whether eryptosis is similarly enhanced in human patients suffering from APC.

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## Conflict of interest

The authors state that they have no conflict of interest to disclose.

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