

REPORT

 OPEN ACCESS

## GSH-dependent antioxidant defense contributes to the acclimation of colon cancer cells to acidic microenvironment

Minnan Zhao<sup>a</sup>, Qiao Liu<sup>a</sup>, Yanchao Gong<sup>a</sup>, Xiuhua Xu<sup>a</sup>, Chen Zhang<sup>a</sup>, Xiaojie Liu<sup>a</sup>, Caibo Zhang<sup>a</sup>, Haiyang Guo<sup>a</sup>, Xiyu Zhang<sup>a</sup>, Yaoqin Gong<sup>a</sup>, and Changshun Shao<sup>a,b</sup>

<sup>a</sup>Ministry of Education Key Laboratory of Experimental Teratology and Department of Molecular Medicine and Genetics, Shandong University School of Medicine, Jinan, China; <sup>b</sup>Department of Genetics/Human Genetics Institute of New Jersey, Rutgers University, Piscataway, NJ, USA

### ABSTRACT

Due to increased glycolysis and poor local perfusion, solid tumors are usually immersed in an acidic microenvironment. While extracellular acidosis is cytotoxic, cancer cells eventually become acclimated to it. While previous studies have addressed the acute effect of acidosis on cancer cells, little is known about how cancer cells survive chronic acidosis. In this study we exposed colorectal cancer (CRC) cells (HCT15, HCT116 and LoVo) to acidic pH (pH 6.5) continuously for over three months and obtained CRC cells that become acclimated to acidic pH, designated as CRC-acidosis-acclimated or CRC-AA. We unexpectedly found that while acute exposure to low pH resulted in an increase in the level of intracellular reactive oxygen species (ROS), CRC-AA cells exhibited a significantly reduced level of ROS when compared to ancestor cells. CRC-AA cells were found to maintain a higher level of reduced glutathione, via the upregulation of CD44 and glutathione reductase (GSR), among others, than their ancestor cells. Importantly, CRC-AA cells were more sensitive to agents that deplete GSH. Moreover, downregulation of GSR by RNA interference was more deleterious to CRC-AA cells than to control cells. Together, our results demonstrate a critical role of glutathione-dependent antioxidant defense in acclimation of CRC cells to acidic extracellular pH.

### ARTICLE HISTORY

Received 8 January 2016  
Revised 10 February 2016  
Accepted 18 February 2016

### KEYWORDS

acidic microenvironment;  
antioxidant defense; CD44;  
colorectal cancer; GSH; GSR



### Introduction

Cancer cells form a dynamic relationship with their microenvironment. Both the cancer cells and their microenvironment evolve during the course of cancer development and progression. While cancer cells can modify their microenvironment by recruiting immune cells, mesenchymal cells and endothelial cells that comprise the cellular components of tumor microenvironment, they also need to evolve in various features in order to survive in the relatively hostile environment, which is usually hypoxic, poorly circulated and low in nutrients.<sup>1–4</sup> Because cancer cells are usually rewired for glycolysis, which produces lactic acid, even under aerobic condition (the Warburg effect), and tumor vasculature is usually poorly functional, tumor microenvironment is characteristic of an acidic pH, or called extracellular acidosis, which is usually measured between 5.5~7.0.<sup>5–7</sup> For most cells, an extracellular acidic microenvironment is harmful and genotoxic.<sup>8–10</sup> However, some tumor cells may survive and evolve to become more malignant under such a condition.<sup>11–17</sup> Interestingly, cancer cells usually have a higher intracellular pH (pH > 7.4) than normal differentiated adult cells (pH~7.2).<sup>18</sup> This reversed pH gradient is regarded as one of the adaptive features of most cancers and may facilitate survival, proliferation, metabolic adaptation, metastasis and invasion of cancer cells. Moreover, autophagy and reprogrammed cellular metabolism were found to be critical for the survival of cancer cells in an acidic microenvironment.<sup>19–21</sup> However, it remains to be


determined whether there are other means upon which cancer cells rely for living in the acidic extracellular milieu.

Living cells are always subjected to the effects of reactive oxygen species (ROS) which include superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $HO\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ). While ROS at low to moderate levels are essential for cellular signaling that sustains proliferation and differentiation, when they are produced in excess and overwhelm the cellular antioxidant defensive systems, oxidative stress ensues, which may lead to apoptosis, senescence and increased mutation load.<sup>22–25</sup> Intracellular ROS level is usually elevated in response to various types of stress and stimuli.<sup>23</sup> It was reported that acute exposure to acidic microenvironment can cause an increase in ROS.<sup>26</sup> Cancer cells usually experience high level of ROS and have concomitantly acquired robust antioxidant capacity.<sup>27–29</sup>

While acidic tumor microenvironment is known to accompany tumor survival, growth, invasion and metastasis, most studies so far addressed acute acidosis, in terms of hours to days, and little is known about how cancer cells respond to chronic acidic environment. If acidosis is a selective factor for cancer cells, it is important to know what features the survivors have when compared to ancestors. In order to understand what happens to tumor cells in chronic acidic microenvironment, we exposed colorectal cancer (CRC) cells to acidic pH continuously for a long period and selected the CRC cells that had

**CONTACT** Changshun Shao  changshun.shao@gmail.com  Department of Molecular Medicine and Genetics, Shandong University School of Medicine, 44 W Wenhua Rd, Jinan, Shandong 250012, China.

Color versions of one or more of the figures in this article can be found online at [www.tandfonline.com/kccy](http://www.tandfonline.com/kccy).

 Supplemental data for this article can be accessed on the publisher's website.

© Minnan Zhao, Qiao Liu, Yanchao Gong, Xiuhua Xu, Chen Zhang, Xiaojie Liu, Caibo Zhang, Haiyang Guo, Xiyu Zhang, Yaoqin Gong, and Changshun Shao. Published with license by Taylor & Francis. This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

become acclimated to acidic pH (CRC-AA). We found that contrary to the elevation of ROS in CRC cells acutely exposed to low pH, the ROS level in CRC-AA cells was lower than in their parental cells and was maintained by a high level of GSH.

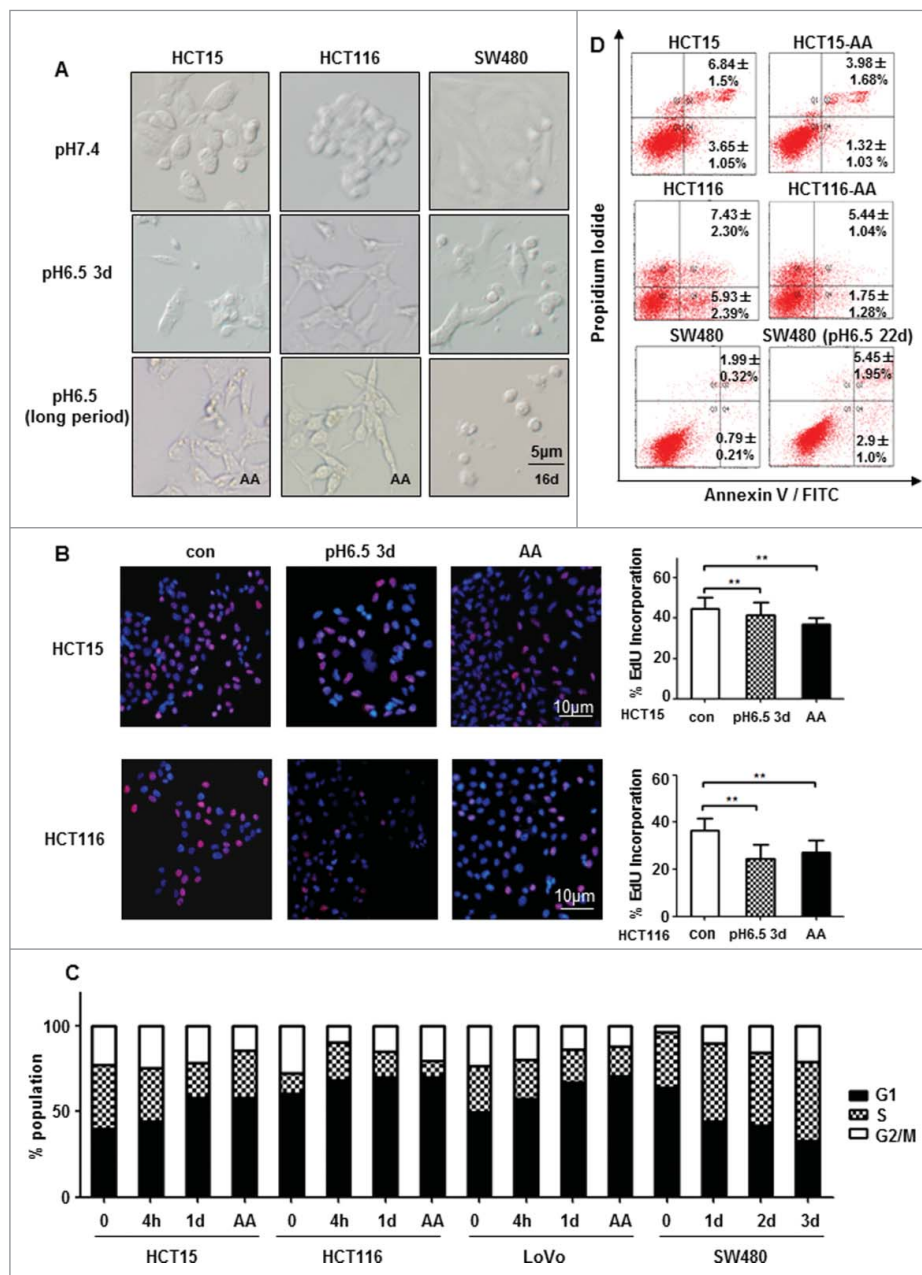
## Results

### Characteristics of colon cancer cells adapted to acidic microenvironment

We subjected colorectal cancer cells, HCT15, HCT116, LoVo and SW480 cells, to acidic medium (pH6.5) continuously to select for cells that became acclimated to the acidic

microenvironment. While SW480 cells failed to proliferate shortly after being transferred to acidic medium, HCT15, HCT116 and LoVo cells survived and were able to proliferate after passages in acidic medium for at least three months. We designated them CRC-AA for CRC-acidosis acclimated (HCT15-AA, HCT116-AA and LoVo-AA cells, respectively).

The CRC-AA cells appeared to have lost the cobblestone-like feature that is typical of epithelial cells and is present in their parental cells (Fig. 1A). They were more elongated and more scattered. Measurement of proliferation using EdU incorporation assay showed that the CRC-AA cells generally had a reduced rate of proliferation (Fig. 1B). Examination of cell cycle distribution showed that CRC-AA cells had significantly higher



**Figure 1.** Chronic acidic microenvironment inhibits proliferation. (A) Cell density and morphology of HCT15 cells (left), HCT116 (middle) and SW480 (right) at pH6.5 were examined under microscopy ( $\times 20$ ). HCT15 and HCT116 cells growing in acidic medium were more scattered and elongated. SW480 showed little morphological change except a reduction in cell density. (B) Acidic microenvironment reduced proliferation of CRC cells. Proliferation of HCT15 (top) and HCT116 (bottom) cells cultured at low pH (6.5) for 72 h or for more than 3 months (CRC-AA) were determined by EdU incorporation. Red, EdU; blue, DAPI. (C) Cell cycle distribution of CRC cells growing in acidic medium for various durations. (D) Apoptotic levels of CRC cells adapted to acidic pH (HCT15 and HCT116 cells) and SW480 cells. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

percentages of cells in G1 phase and reduced percentages of cells in S phase (Fig. 1C). Interestingly, SW480 cells, which failed to survive the chronic acidic stress, were not arrested in G1 phase. CRC cells exposed to acidic medium initially showed an increased apoptosis than controls. However, the CRC-AA cells eventually exhibited reduced levels of apoptosis than their parental cells (Fig. 1D). High level of apoptosis remained in SW480 cells even after 22 d in acidic medium (Fig. 1D), which is probably due to loss of the protection by G1 arrest. These results indicate that CRC-AA cells are distinct from their parental cells in morphology and proliferation. They even have a reduced rate of spontaneous apoptosis.

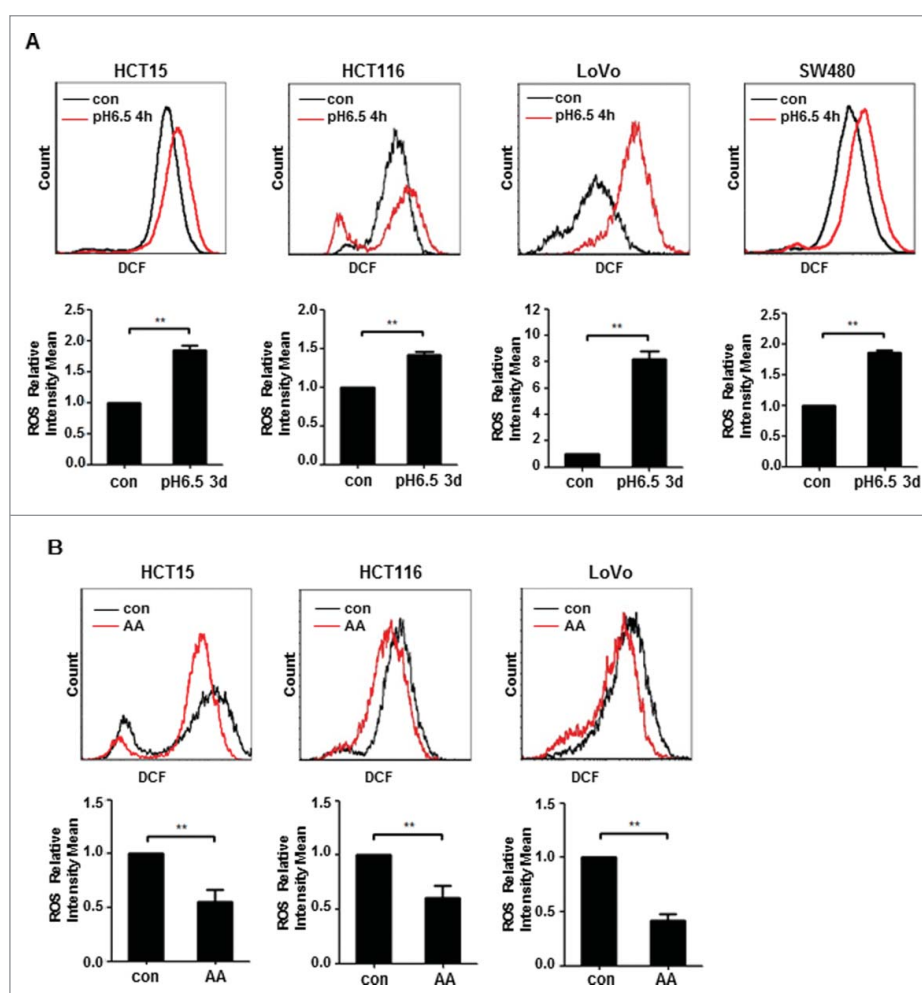
### ROS level is reduced in colorectal cancer cells acclimated to chronic acidic stress

A previous study showed that the level of intracellular ROS in cancer cells exposed to acute acidosis was elevated.<sup>26</sup> Measurement of ROS showed that consistent with the previous report, the ROS level was increased when CRC cells were exposed to acidic medium for 4 h (Fig. 2A). The elevation of ROS in response to acute acidosis applied to all four cell lines tested. However, all three lines of CRC-AA cells showed a significantly reduced level of ROS when compared to their parental cells

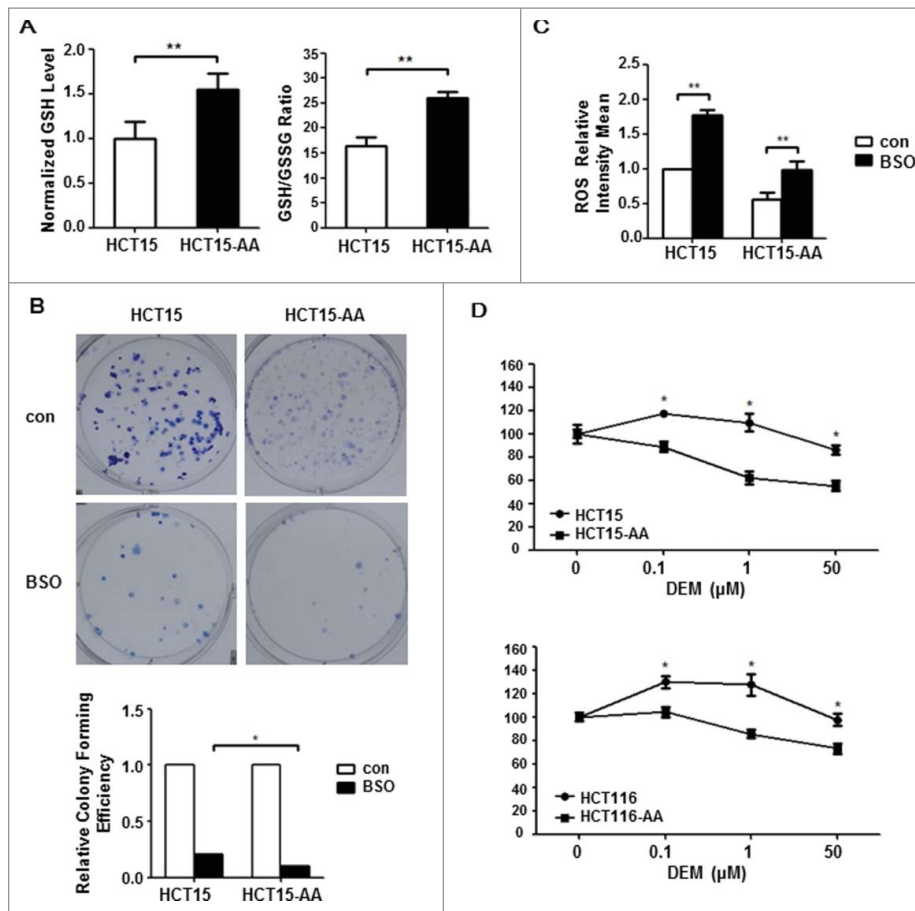
(Fig. 2B). These results suggest that while CRC cells may respond to acute acidosis by elevating ROS, they generally have a reduced level of ROS when they become acclimated to the low extracellular pH.

### Acclimation of CRC cells to chronic acidosis depends on GSH

Glutathione (GSH) is regarded to be the most important antioxidant in mammalian cells.<sup>30</sup> Reduced GSH can effectively convert ROS into benign molecules. To determine whether GSH is involved in the maintenance of a reduced ROS level in CRC-AA, we measured the level of GSH as well as the ratio of GSH/GSSG in HCT15-AA and their parental cells. As shown in Figure 3A, the level of GSH as well as the ratio of GSH/GSSG was significantly higher in HCT15-AA cells. To test whether the increased level of GSH is responsible for adaptation of CRC cells to acidic pH, we determined colony formation of HCT15-AA and their parental cells in the presence of buthionine sulphoximine (BSO), a GSH-depleting agent. As shown in Figure 3B, BSO inhibited colony formation in both sets of cells, but the reduction was more pronounced for HCT15-AA cells. As expected, the level of ROS was increased in



**Figure 2.** ROS is reduced in colon cancer cells acclimated to acidic microenvironment. (A) DCF fluorescent intensity of HCT15, HCT116, LoVo and SW480 cells exposed to acidic medium for 4 h. (B) DCF fluorescent intensity of HCT15 cells, HCT116 cells and LoVo cells growing acidic medium for more than 3 months. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



**Figure 3.** Antioxidant GSH is critical for survival of acidosis-acclimated cancer cells. (A) GSH level as well as GSH/GSSG ratio is higher in HCT15-AA than in their parental cells. The GSH and GSH/GSSG ratio were measured by a GSH and GSSG assay kit. (B) Inhibition of colony formation by BSO in HCT15-AA and their parental cells. Cultured cells were exposed to BSO (10 μM) for 12d to allow for colony formation. (C) Induction of ROS by BSO. ROS fluorescence intensity of HCT15 cells treated with BSO (500 μM, 12h) was determined by flow cytometry. (D) Inhibition of proliferation of CRC-AA cells and their parental cells by DEM. CRC cells were treated with different concentrations of DEM (0, 0.1, 1, 50 μM) for 48 h. Cell viability was measured by MTT assay. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

CRC cells treated with BSO (Fig. 3C). Furthermore, when HCT15 and HCT116 cells were exposed to diethyl maleate (DEM), another depleting agent of GSH, and scored for viability with MTT assay, both HCT15-AA and HCT116-AA cells showed an increased sensitivity to DEM when compared to their parental cells (Fig. 3D). These results suggest that the survival of CRC-AA cells was more dependent on GSH than that of control cells. Together, these results indicate that a low level of ROS, which is probably mediated by increased level of GSH, is critical for CRC cells to live under the acidic microenvironment.

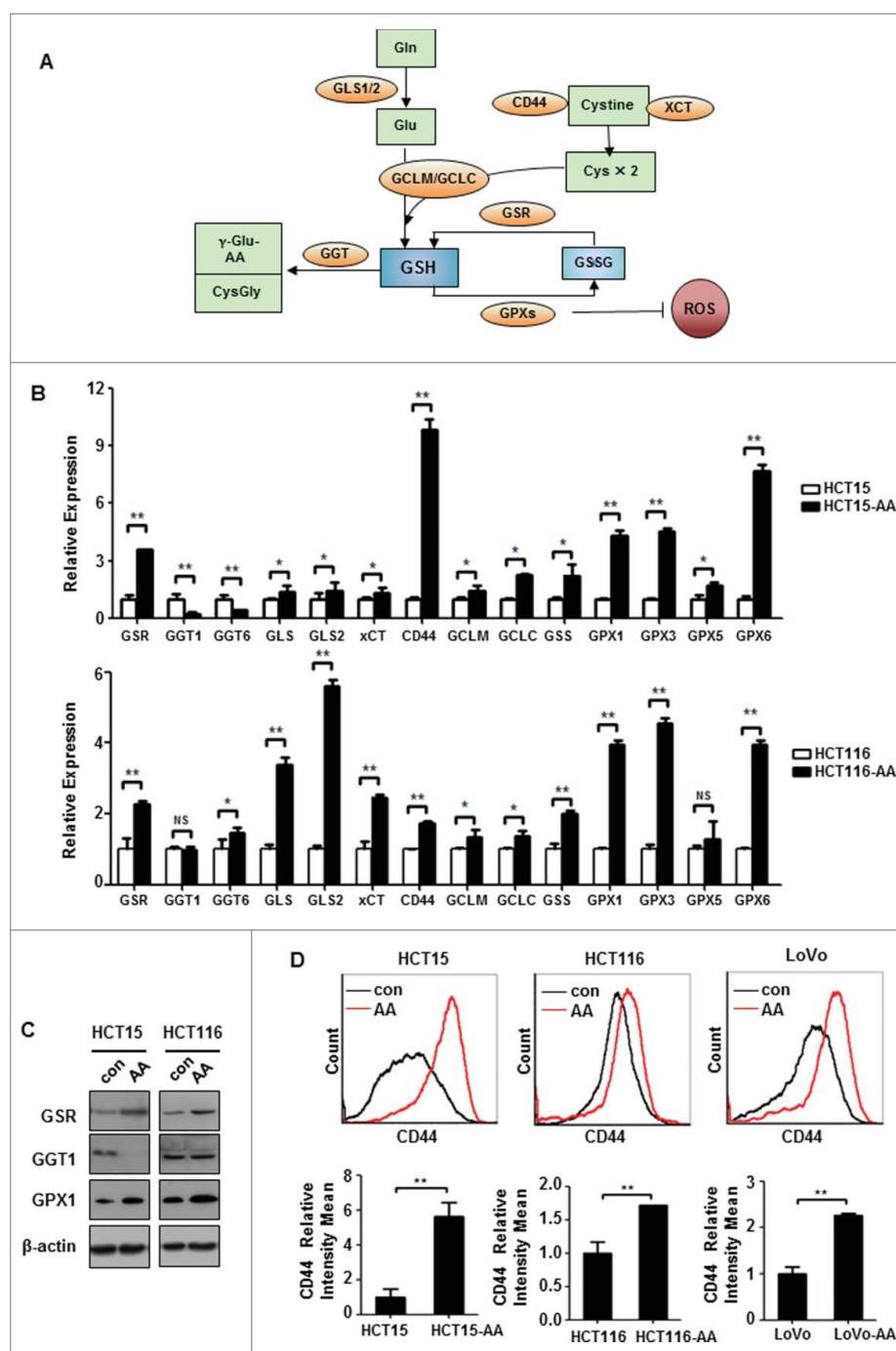
#### Expression pattern of representative genes involved in GSH metabolism

As illustrated in Figure 4A, the level of GSH is affected by its rate of de novo synthesis (via steps catalyzed by GLS1/2 and GCLC/GCLM, and facilitated by CD44 and xCT), by its utilization (catalyzed by GPX or GST) and by its breakdown (catalyzed by GGT).<sup>28,30,31</sup> We next determined, by quantitative real-time PCR (qRT-PCR), the expression levels of representative genes involved in GSH metabolism in HCT15-AA and HCT116-AA cells. As shown in Figure 4B, the expression pattern of those genes generally suggests an

increased capacity for antioxidant defense in CRC-AA cells when compared to their parental cells. GSR, CD44, GPX1, GPX3 and GPX6 were remarkably upregulated in both HCT15-AA and HCT116-AA cells, though for the other genes the changes in expression were less consistent or the degrees of difference were less pronounced. Western blot analysis confirmed the upregulation of GSR and GPX1 at protein level (Fig. 4C). Furthermore, flow cytometry analysis revealed an increase in the expression of CD44 in HCT15-AA, HCT116-AA and LoVo-AA cells over their parental cells (Fig. 4D). Taken together, these results suggest that compared to their parental cells, CRC-AA cells may be generally wired to maintain a more robust antioxidant defense that depends on GSH.

#### Requirement of GSR for survival of CRC cells in acidic microenvironment

GSR is essential for the conversion of GSSG to reduced GSH. We next determined the effect of GSR depletion on the survival of CRC-AA cells. As shown in Figure 5A, GSR was efficiently knocked down by RNAi in HCT15 and HCT116 cells. As expected, the ROS level was elevated as a result of the GSR depletion (Fig. 5B). Strikingly, while the level of apoptosis

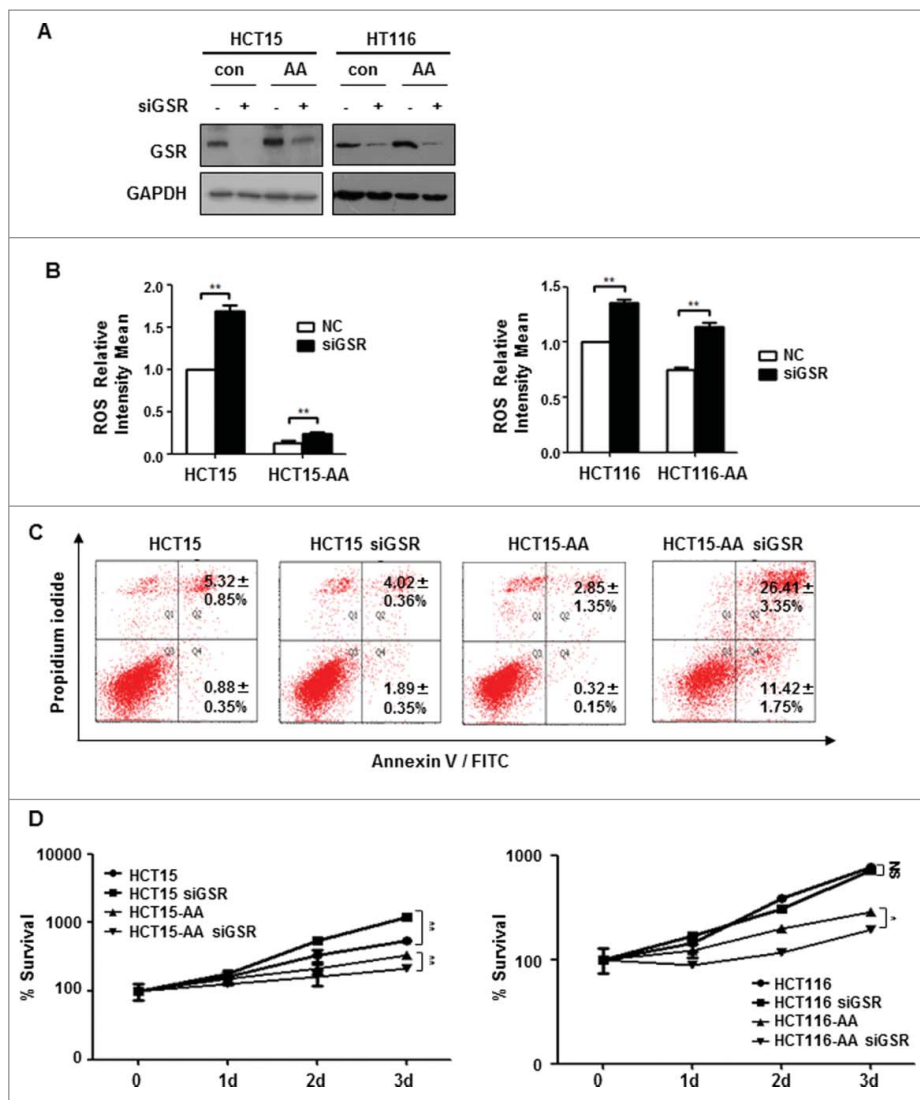


**Figure 4.** Expression levels of genes involved in GSH metabolism. (A) A diagram showing the key factors involved in GSH metabolism. (B) Relative expressions of representative redox genes at mRNA level in CRC-AA and their parental cells, as measured by quantitative real-time PCR. (C) Expression levels of redox proteins in CRC-AA and their parental cells measured by Western blotting. (D) Flow cytometric analysis of CD44 expression levels in CRC-AA and their parental cells. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

remained unchanged in response to GSR depletion in parental HCT15 cells, it increased over ten-fold in HCT15-AA cells (Fig. 5C), suggesting that GSR is strictly required for the survival of CRC-AA cells. Consistently, HCT15-AA and HCT116-AA cells in which GSR was depleted experienced a significantly reduced proliferation, as determined by MTT assay (Fig. 5D). Intriguingly, the parental HCT15 cells in which GSR was depleted even showed an enhanced rate of proliferation. Together, these results provide further support for the notion that the CRC-AA cells are more reliant on GSH than their parental cells.

## Discussion

In this study we subjected colorectal cancer cells to chronic acidosis, selected for and characterized the cells that became acclimated to low pH, designated as CRC-AA. We observed that while acute exposure to low pH resulted in an increase in the level of intracellular ROS in CRC cells, CRC-AA cells exhibited a much reduced level of ROS when compared to their ancestor cells. CRC-AA cells exhibited a higher level of GSH than in their ancestor cells. Consistently, key genes involved in GSH metabolism are found to be expressed in such a way as to



**Figure 5.** GSR knockdown compromises the survival of CRC-AA cells. (A) Knockdown of GSR by siRNA in HCT15 and HCT116 cells. RNAi efficiency was determined by Western blotting, GAPDH was used as a loading control. (B) Increase of ROS levels in CRC cells by GSR siRNA. (C) Apoptosis was greatly increased in CRC-AA cells when transfected with GSR siRNA. (D) Viability of CRC-AA cells was reduced when transfected with GSR siRNA, as determined by MTT assay. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

maintain a higher level of GSH and to establish a more robust GSH-based antioxidant defense in CRC-AA cells. CD44, glutathione reductase and glutathione peroxidases were generally upregulated in CRC-AA cells. Importantly, CRC-AA cells were found to be more sensitive to depletion of GSH than their ancestor cells. Our results thus provided a new insight into how cancer cells adapt to acidic microenvironment.

We also observed that cancer cells that survived chronic exposure to acidic medium all showed an elevation of cells in G1/G0 phase, indicative of G1 arrest. Interestingly, SW480 cells, which did not exhibit G1 arrest in response to acidic medium and failed to adapt, experienced high level of apoptosis throughout the selection process. These observations suggest that G1 arrest is also important for survival under acidic stress.

It should be noted that antioxidant genes such as CD44, GLS2, GPX1 are generally upregulated in CRC specimens when compared to mucosa<sup>32</sup> (GEO dataset accession: GSE20842), thus already providing to CRC a stronger buffer against oxidative stress than to normal tissue. The increased expression of those genes in CRC-AA cells over CRC parental

cells would confer further enhanced antioxidant defense under the acidic microenvironment. It should be pointed that while genes involved in GSH metabolism may generally evolve in expression levels to boost the antioxidant defense under acidic condition, individual genes may be regulated differentially in different CRC cell lines or specimens. It is clear that the extent to which the genes were upregulated varied between HCT15-AA and HCT116-AA cells. Furthermore, while GGT1 and GGT6 were downregulated in HCT15-AA cells, they remained unchanged or upregulated in HCT116-AA cells. Therefore, while we may expect an enhanced GSH-based antioxidant defense in CRC-AA cells, the redox pathways and/or genes involved may vary in different CRC cells. Furthermore, because the metabolic pathways of glucose, fatty acids and amino acids are potentially all subjected to reprogramming under stressful conditions,<sup>21</sup> changes in those pathways may also contribute to antioxidant defense.

Cancer cells and their microenvironment co-evolve during cancer progression. Thanks to accumulation of acquired mutations and/or epigenetic changes, cancer cells are often

reprogramed in their developmental potential and metabolism when compared to normal cells. They can also hijack or harness host cells to establish a microenvironment that is conducive to cancer progression. On the other hand, environmental pressures including physical restraint, hypoxia, shortage of nutrients, inflammatory cytokines and extracellular acidosis also act to select for those that are most adapted to the changing environment. The ability to undergo autophagy appears to confer cancer cells a great advantage under many stressful conditions.<sup>33</sup> Autophagy likewise operates to protect cancer cells under acidic stress.<sup>19,20</sup> Our results presented in this study indicate that an augmented antioxidant defense is also critical for cell survival under chronic acidic stress.

## Materials and methods

### Cells and cell culture

Human colon cancer cell lines HCT15, HCT116, SW480 and LoVo were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai). HCT15 and HCT116 cells were maintained in RPMI-1640, SW480 in Leibovitz's L-15 medium and LoVo in DMEM/F12, all supplemented with 10% FBS, 100U/mL penicillin, and 100  $\mu$ g/mL streptomycin in a humidified 5% CO<sub>2</sub>/95% air atmosphere at 37°C. The pH of the growth medium was adjusted to 7.4 or 6.5 with 25 mmol/L each of PIPES and HEPES. HCT15, HCT116 and LoVo cells were continuously cultured and passed in growth medium adjusted to pH 6.5. Cells that survived the chronic exposure to low pH, for at least three months, were designated as HCT15-AA, HCT116-AA and LoVo-AA, respectively. SW480 cells failed to survive the chronic acidic culture condition beyond 21 d.

### Chemicals

Diethyl Maleate (DEM) from Sigma Chemical (St Louis, MO). Buthionine sulfoximine (BSO) was from Cayman Chemical (Ann Arbor, MI).

### EdU incorporation

EdU (5-Ethynyl-2-deoxyuridine) incorporation was performed using Cell-Light™ EdU Cell Proliferation Detection kit (RiboBio, Guangzhou, China) and was previously described.<sup>33</sup> Cells were incubated in the presence of EdU (100  $\mu$ M) for the final 2 h before being processed for EdU staining.

### Analyses of cell cycle distribution and apoptosis by flow cytometry

Cells were harvested using 0.25% Trypsin-EDTA, centrifuged (300 g), and washed once with cold PBS. The pellet was resuspended in ice cold 70% ethanol and stored at -20°C. Once all samples were collected, cells were centrifuged and resuspended in cold PBS. Samples were incubated with 20  $\mu$ g/mL propidium iodide/0.1% Triton X-100 staining solution with 0.1mg/mL RNase A. Cell cycle distribution was determined using the BD Biosciences FACSCanto II Analyzer. At least 20,000 cells were collected. For analysis of apoptosis, both adherent and

supernatant cells were harvested, washed twice in PBS, and resuspended in 1× binding buffer at a density of 1×10<sup>6</sup> cells/mL. Cells were assayed for apoptosis using an Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instructions.

### Determination of cellular ROS

Changes in intracellular ROS were measured using Reactive Oxygen Species Assay Kit (Beyotime Biotechnology) following the manufacturer's protocols. In brief, cells were labeled with 10 $\mu$ M DCFH-DA (2',7'-dichlorofluorescein diacetate) for 20 min at 37°C in a humidified atmosphere at 5% CO<sub>2</sub>. The labeled cells were washed and collected. The fluorescence intensity of DCF was measured by flow cytometry (FACSCanto II, BD Biosciences).

### Colorimetric determination of GSH and GSSG

The amounts of total glutathione and GSSG were determined using the GSH and GSSG Assay Kit (Beyotime, China) following the manufacturer's recommendations. The amount of GSH was obtained by subtracting GSSG from the total glutathione.

### Cell viability assay

Cells were plated in 96-well cell culture plates at the concentration of 2–5 × 10<sup>3</sup> cells/well. Twenty-four h later, the medium was removed and replaced with fresh medium with or without DEM. Cell viability was measured at the appropriate time points by using the MTT assay kit (Beyotime, China) following manufacturer's instructions. The absorbance of converted dye is measured at the wavelength of 490 nm and the absorbance is proportional to cell viability.

### Clonogenic survival assay

Briefly, the cells were treated, trypsinized, suspended in complete medium, counted and replated in 100-mm tissue culture dishes to allow formation of macroscopic colonies. Plates were incubated at 37°C for 7 to 14 days, fixed with methanol, stained with Giemsa, and colonies containing at least 50 cells in size were counted.

### Western blotting analysis

Cells were harvested, rinsed in ice-cold PBS, and lysed in Cell Lysis Buffer (Beyotime, China) for 30 minutes. The lysates were centrifuged at 12,000 rpm to remove any cellular debris. Protein concentrations of the lysates were determined by the BCA protein assay system (Beyotime, China). Equal amounts of protein were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA), and blocked with 5% nonfat dry milk in TBS-Tween 20 (0.1%, v/v) for 1 h at room temperature. The membrane was incubated with primary antibody overnight. Anti-GGT1 and GPX1 were from Abcam (Cambridge, UK); GPX3 was from Novus (Minneapolis, MN); GSR was from proteintech (Wuhan, China); and  $\beta$ -actin and GAPDH were from Santa Cruz (Dallas, TX). After washing, the

membrane was incubated with the appropriate horseradish peroxidase secondary antibody (diluted 1:5,000; Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 h. Following several washes, the blots were developed by enhanced chemiluminescence (Millipore, Billerica, MA).

### cDNA synthesis and qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. For qPCR, cDNA was synthesized by reverse transcription of 1  $\mu$ g of total RNA with random hexamers. The total volume of reverse transcription reaction was 20  $\mu$ L. Real-time quantitative PCR was performed using the LightCycler<sup>®</sup> 480 sequence Detection System (Roche Applied Science, Upper Bavaria, Germany). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The primer sequences are listed in Supplementary Table 1. The samples were loaded in quadruple, and the results of each sample were normalized to GAPDH.

### RNA interference

Cells were transfected with siRNAs (50nM) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's guide. siRNAs corresponding to the cDNA of GSR: 5'-CCAGAAUACCAACGUCAAATT-3'/5'-UUUGACGUUGGUAUUCUGGTT-3'. RNAi efficiency was determined 48 h after transfection.

### Statistical analysis

Student's t test was used to determine the statistical significance between experimental groups. Difference was considered significant if the P value was less than 0.05.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

### Funding

This study was supported by National Basic Research Program of China (973 Program) grant (2011CB966200), National Natural Science Foundation Research Grants (81372241, 81572785 and 81330050), and State Program of National Natural Science Foundation of China for Innovative Research Group (81321061).

### References

- Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; 19:1423-37; PMID:24202395; <http://dx.doi.org/10.1038/nm.3394>
- Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 1989; 49:6449-65; PMID:2684393
- Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. *Seminars Radiation Oncol* 2004; 14:198-206; PMID:15254862; <http://dx.doi.org/10.1016/j.semradonc.2004.04.008>
- Amend SR, Pienta KJ. Ecology meets cancer biology: The cancer swamp promotes the lethal cancer phenotype. *Oncotarget* 2015; 6:9669-78; PMID:25895024; <http://dx.doi.org/10.18632/oncotarget.3430>
- Engin K, Leeper DB, Cater JR, Thistlethwaite A J, Tupchong L, McFarlane JD. Extracellular pH distribution in human tumours. *Int J Hyperthermia* 1995; 11:211-6; PMID:7790735; <http://dx.doi.org/10.3109/02656739509022457>
- Gerweck LE, Seetharaman K. Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. *Cancer Res* 1996; 56:1194-8; PMID:8640796
- Helmlinger G, Yuan F, Dellian M, Jain RK. Interstitial pH and pO<sub>2</sub> gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med* 1997; 3:177-82; PMID:9018236; <http://dx.doi.org/10.1038/nm0297-177>
- Williams AC, Collard TJ, Paraskeva C. An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. *Oncogene* 1999; 18:3199-204; PMID:10359525; <http://dx.doi.org/10.1038/sj.onc.1202660>
- Morita T, Nagaki T, Fukuda I, Okumura K. Clastogenicity of low pH to various cultured mammalian cells. *Mutation Res* 1992; 268:297-305; PMID:1379335; [http://dx.doi.org/10.1016/0027-5107\(92\)90235-T](http://dx.doi.org/10.1016/0027-5107(92)90235-T)
- Leontieva OV, Blagosklonny MV. Yeast-like chronological senescence in mammalian cells: phenomenon, mechanism and pharmacological suppression. *Aging (Albany NY)* 2011; 3:1078-91; PMID:22156391
- Martinez-Zaguilan R, Seftor EA, Seftor RE, Chu YW, Gillies RJ, Hendrix MJ. Acidic pH enhances the invasive behavior of human melanoma cells. *Clin Exp Metastasis* 1996; 14:176-86; PMID:8605731; <http://dx.doi.org/10.1007/BF00121214>
- Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 2006; 66:6699-707; PMID:16818644; <http://dx.doi.org/10.1158/0008-5472.CAN-06-0983>
- Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosesu J, Sloane BF, Hashim AI, Morse DL, Raghunand N, Gatenby RA, Gillies RJ. Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer Res* 2009; 69:2260-8; PMID:19276390; <http://dx.doi.org/10.1158/0008-5472.CAN-07-5575>
- Wojtkowiak JW, Verduzco D, Schramm KJ, Gillies RJ. Drug resistance and cellular adaptation to tumor acidic pH microenvironment. *Mol Pharmaceutics* 2011; 8:2032-8; PMID:21981633; <http://dx.doi.org/10.1021/mp200292c>
- Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane BF, et al. Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res* 2013; 73:1524-35; PMID:23288510; <http://dx.doi.org/10.1158/0008-5472.CAN-12-2796>
- Gupta SC, Singh R, Pochampally R, Watabe K, Mo YY. Acidosis promotes invasiveness of breast cancer cells through ROS-AKT-NF-kappaB pathway. *Oncotarget* 2014; 5:12070-82; PMID:25504433; <http://dx.doi.org/10.18632/oncotarget.2514>
- Peppicelli S, Bianchini F, Toti A, Laurenzana A, Fibbi G, Calorini L. Extracellular acidity strengthens mesenchymal stem cells to promote melanoma progression. *Cell cycle* 2015; 14:3088-100; PMID:26496168; <http://dx.doi.org/10.1080/15384101.2015.1078032>
- Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nat Rev Cancer* 2011; 11:671-7; PMID:21833026; <http://dx.doi.org/10.1038/nrc3110>
- Wojtkowiak JW, Rothberg JM, Kumar V, Schramm KJ, Haller E, Proemsey JB, Lloyd MC, Sloane BF, Gillies RJ. Chronic autophagy is a cellular adaptation to tumor acidic pH microenvironments. *Cancer Res* 2012; 72:3938-47; PMID:22719070; <http://dx.doi.org/10.1158/0008-5472.CAN-11-3881>
- Marino ML, Pellegrini P, Di Lernia G, Djavaheri-Mergny M, Brnjic S, Zhang X, Hagg M, Linder S, Fais S, Codogno P, et al. Autophagy is a protective mechanism for human melanoma cells under acidic stress. *J Biol Chem* 2012; 287:30664-76; PMID:22761435; <http://dx.doi.org/10.1074/jbc.M112.339127>
- Lamonte G, Tang X, Chen JL, Wu J, Ding CK, Keenan MM, Sango-koya C, Kung HN, Ilkayeva O, Boros LG, et al. Acidosis induces reprogramming of cellular metabolism to mitigate oxidative stress. *Cancer Metab* 2013; 1:23; PMID:24359630; <http://dx.doi.org/10.1186/2049-3002-1-23>



- [22] Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* 2002; 192:1-15; PMID:12115731; <http://dx.doi.org/10.1002/jcp.10119>
- [23] Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* 2012; 48:158-67; PMID:23102266; <http://dx.doi.org/10.1016/j.molcel.2012.09.025>
- [24] Xu B, Wang W, Guo H, Sun Z, Wei Z, Zhang X, Liu Z, Tischfield JA, Gong Y, Shao C. Oxidative stress preferentially induces a subtype of micronuclei and mediates the genomic instability caused by p53 dysfunction. *Mutation Res* 2014; 770:1-8; PMID:25302047; <http://dx.doi.org/10.1016/j.mrfmmm.2014.08.004>
- [25] Wei Z, Guo H, Liu Z, Zhang X, Liu Q, Qian Y, Gong Y, Shao C. CUL4B impedes stress-induced cellular senescence by dampening a p53-reactive oxygen species positive feedback loop. *Free Radical Biol Med* 2015; 79:1-13; PMID:25464270; <http://dx.doi.org/10.1016/j.freeradbiomed.2014.11.010>
- [26] Seo Y, Kinsella TJ. Essential role of DNA base excision repair on survival in an acidic tumor microenvironment. *Cancer Res* 2009; 69:7285-93; PMID:19723658; <http://dx.doi.org/10.1158/0008-5472.CAN-09-0624>
- [27] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* 2009; 8:579-91; PMID:19478820; <http://dx.doi.org/10.1038/nrd2803>
- [28] Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anti-cancer strategy. *Nat Rev Drug Discov* 2013; 12:931-47; PMID:24287781; <http://dx.doi.org/10.1038/nrd4002>
- [29] Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, Yung KY, Brenner D, Knobbe-Thomsen CB, Cox MA, et al. Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. *Cancer Cell* 2015; 27:211-22; PMID:25620030; <http://dx.doi.org/10.1016/j.ccell.2014.11.019>
- [30] Meister A. Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* 1994; 269:9397-400; PMID:8144521
- [31] Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, Oshima M, Ikeda T, Asaba R, Yagi H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc<sup>-</sup> and thereby promotes tumor growth. *Cancer Cell* 2011; 19:387-400; PMID:21397861; <http://dx.doi.org/10.1016/j.ccr.2011.01.038>
- [32] Gaedcke J, Grade M, Jung K, Camps J, Jo P, Emons G, Gehoff A, Sax U, Schirmer M, Becker H, et al. Mutated KRAS results in over-expression of DUSP4, a MAP-kinase phosphatase, and SMYD3, a histone methyltransferase, in rectal carcinomas. *Genes Chromosomes Cancer* 2010; 49:1024-34; PMID:20725992; <http://dx.doi.org/10.1002/gcc.20811>
- [33] White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer* 2012; 12:401-10; PMID:22534666; <http://dx.doi.org/10.1038/nrc3262>
- [34] Liu Q, Xu X, Zhao M, Wei Z, Li X, Zhang X, Liu Z, Gong Y, Shao C. Berberine induces senescence of human glioblastoma cells by down-regulating the EGFR-MEK-ERK signaling pathway. *Mol Cancer Therapeutics* 2015; 14:355-63; PMID:25504754; <http://dx.doi.org/10.1158/1535-7163.MCT-14-0634>