Caffeine protects skin from oxidative stress-induced senescence through the activation of autophagy

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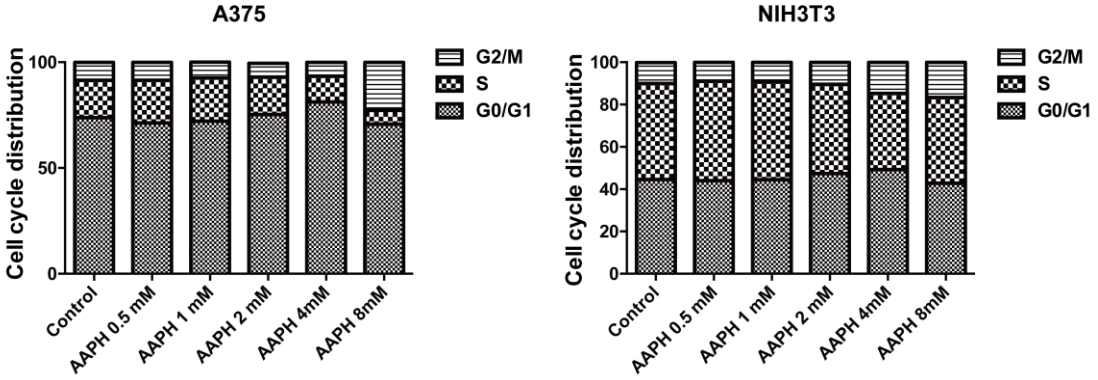


Figure S1. Effects of AAPH on cell cycle progression in A375 (*left*) and NIH3T3 (*right*) cells. Cells were treated with increasing concentrations of AAPH for 48 h (A375) or 24 h (NIH3T3), collected and analyzed for cell cycle profile.

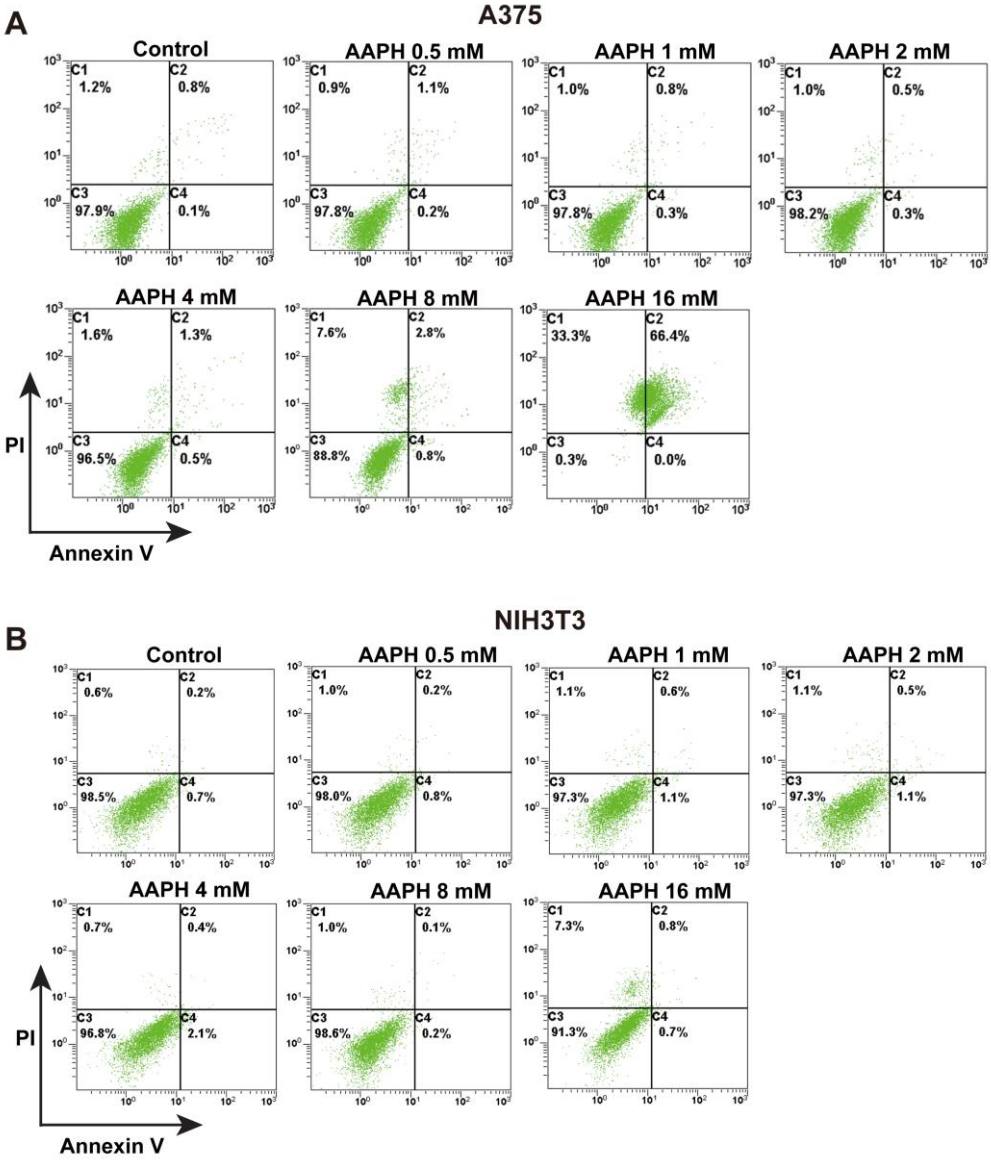
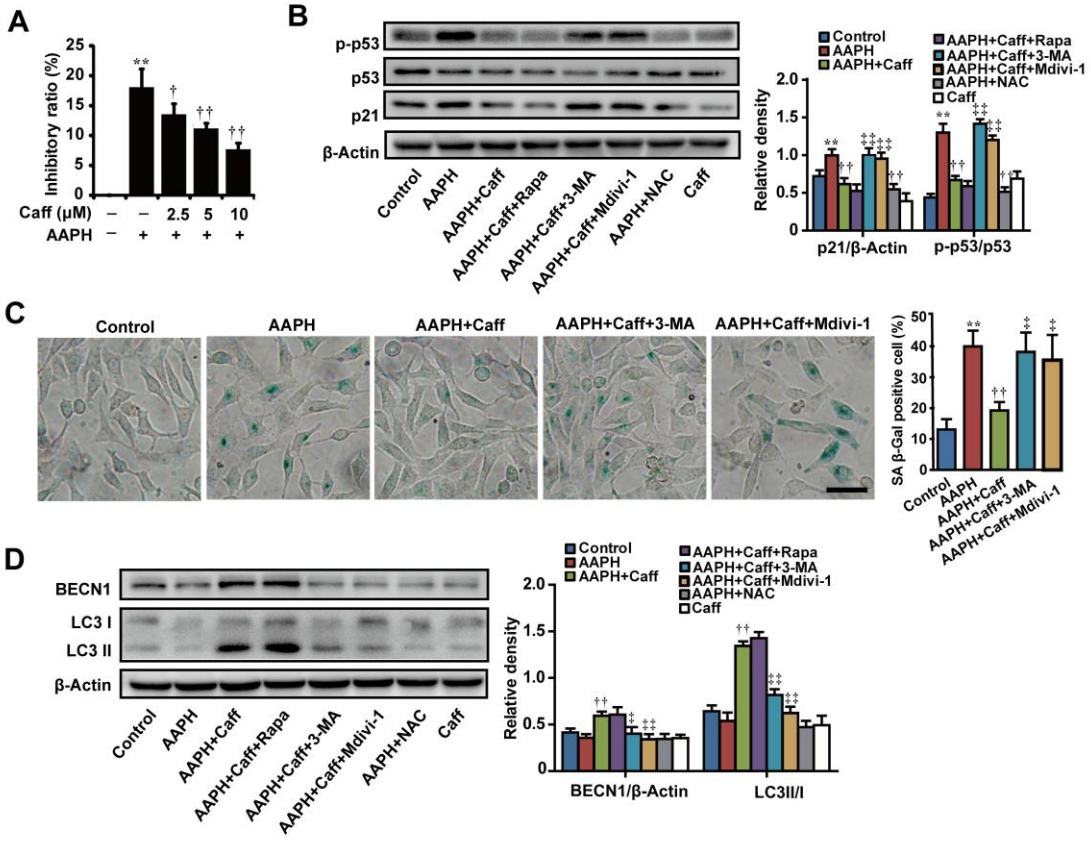
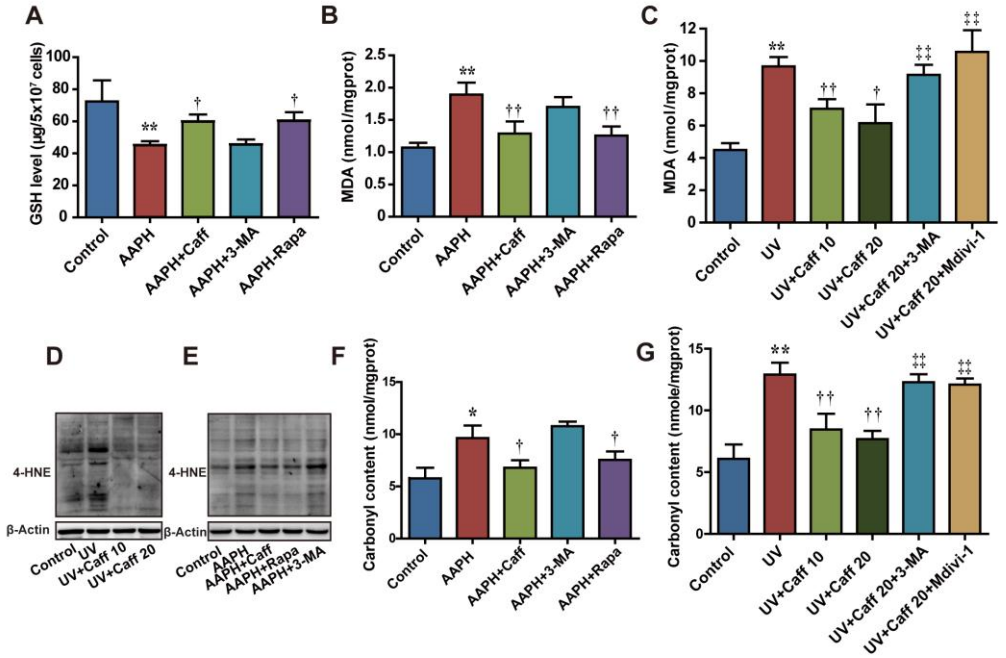


Figure S2. Effects of AAPH on cell death in A375 (A) and NIH3T3 (B) cells. Cells

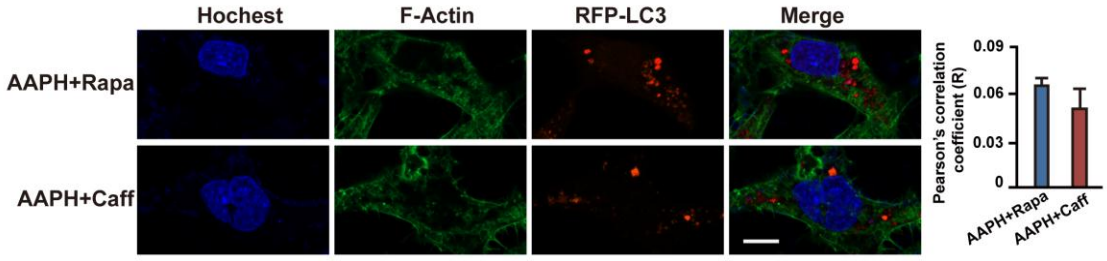
were treated with increasing concentrations of AAPH for 48 h (A375) or 24 h (NIH3T3), collected and analyzed for cell death by PI/AnnexinV staining.



Supplementary Figure S3. The anti-senescence effect of caffeine in NIH3T3 cells. Cells were pretreated with caffeine or other indicated reagents for 1 h, and then co-incubated with AAPH for another 24 h. **(A)** The growth inhibitory effect of AAPH detected by the MTT assay. "-" represents control group. **(B)** Protein expression of p53, p-p53 and p21 determined by western blotting (*left*), and quantitation (*right*) was shown. **(C)** Senescent cells determined by the SA β-Gal staining kit. Images were taken under a bright-field microscope. The quantification was expressed as the ratio of SA β-Gal positive cells. Scale bar = 20 μm. **(D)** Protein expression of autophagy markers. AAPH, 2 mM; NAC, 1 mM; Caff (caffeine), 10 μM; Rapa (rapamycin), 500 nM; 3-MA, 2 mM; Mdivi-1, 10 μM. Results represented the mean ± S.E.M. of values. $^{**}P < 0.01$ *vs.* Control group, $^{††}P < 0.01$ *vs.* AAPH group, $^{‡}P < 0.05$ and $^{‡‡}P < 0.01$ *vs.* "AAPH+Caff" group.

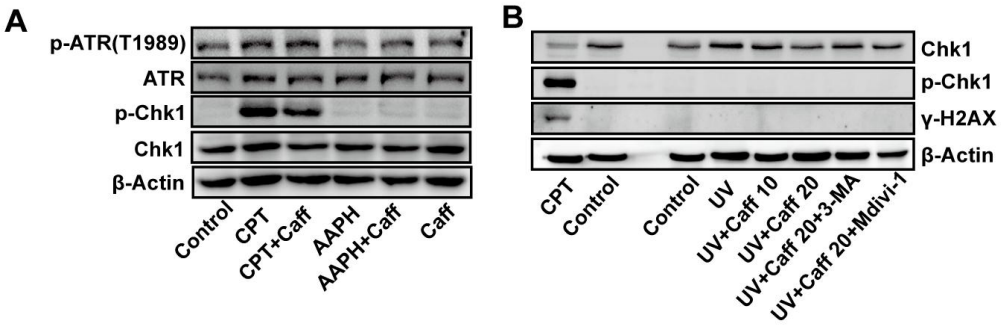


Supplementary Figure S4. The anti-oxidant effect of caffeine. Cells were pretreated with caffeine or other indicated reagents for 1 h, and then co-incubated with AAPH for another 48 h. In mice, caffeine (10 and 20 mg/kg) was orally administrated to mice once daily. Autophagy inhibitor 3-MA (15 mg/kg) or mitophagy inhibitor Mdivi-1 (1.5 mg/kg) was intraperitoneally injected to mice 30 min before caffeine treatment. **(A)** The GSH levels measured by HPLC-ECD in A375 cells. **(B-C)** MDA contents determined by the thiobarbituric acid reactive substances (TBARS) assay in A375 cells and mouse skin. **(D-E)** Protein expression of 4-HNE determined by western blotting in A375 cells and mouse skin. **(F-G)** Carbonyl contents measured in A375 cells and mouse skin. AAPH, 1 mM; Caff (caffeine), 1 μM; Rapa (rapamycin) 500 nM; 3-MA, 2 mM; Caff 10 (caffeine, 10 mg/kg); Caff 20 (caffeine, 20 mg/kg). Results represented the mean ± S.E.M. of values. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* Control group, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ *vs.* AAPH/UV group, $^{\ddagger\ddagger}P < 0.01$ *vs*. "UV+Caff 20" group.

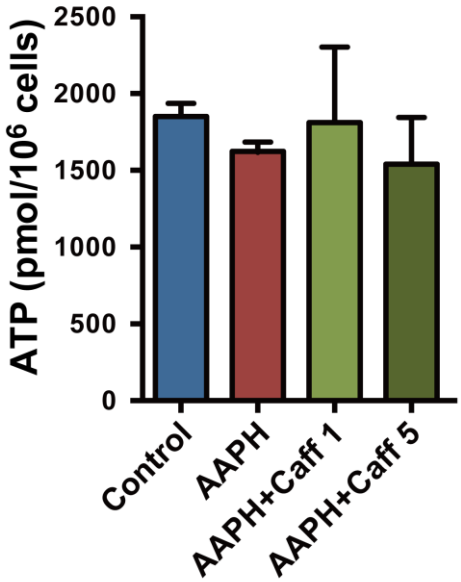


Supplementary Figure S5. The lack of co-localization between RFP-LC3 and F-Actin. NIH3T3 cells were transfected with RFP-LC3 for 24 h, pretreated with 10 μM caffeine (Caff) or 500 nM rapamycin (Rapa) for 1 h, added 2 mM AAPH for another 24 h, fixed and stained with FITC-conjugated phalloidin (green). The red and green signals were analyzed by the Image J software and the Pearson's correlation

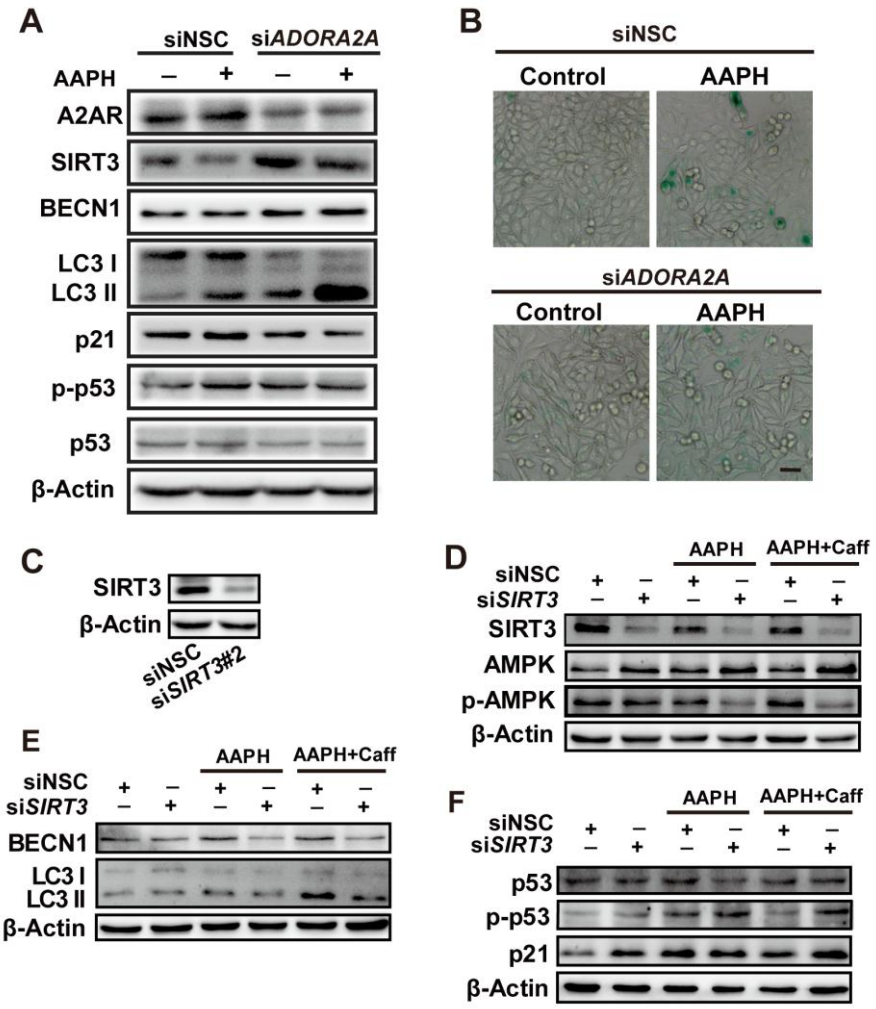
was determined (*right*). Scale bar = 10 μm.



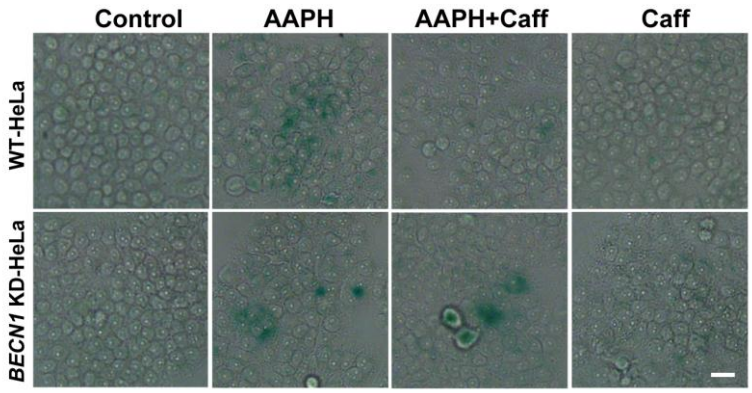
Supplementary Figure S6. Caffeine at low concentration does not inhibit ATR. (A) A375 cell were pretreated with 1 μM caffeine (Caff) for 1 h, followed by CPT (500 nM) for 4 h or 1 mM AAPH for 12 h, and blotted with indicated antibodies. **(B)** In mice, caffeine (Caff, 10 and 20 mg/kg) was orally administrated to mice once daily. Autophagy inhibitor 3-MA (15 mg/kg) or mitophagy inhibitor Mdivi-1 (1.5 mg/kg) was intraperitoneally injected to mice 30 min before caffeine treatment. Skin cells were collected 24 h after the last treatment and protein expression was analyzed. CPT serves as the positive control for DNA damage.



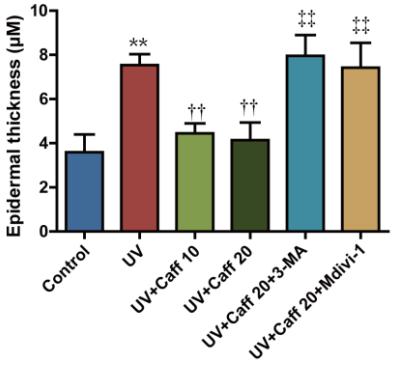
Supplementary Figure S7. The effect of AAPH and caffeine on cellular ATP levels in A375 cells after 48 h treatment. AAPH, 1 mM; Caff (caffeine), 1 or 5 μM.



Supplementary Figure S8. Caffeine induced autophagy via the A2AR/SIRT3/AMPK pathway. (A) Protein expressions of A2AR, BECN1, SIRT3, LC3, p53, p-p53 and p21 determined by western blotting in A375 cells transfected with control siRNA or siRNA#2 targeting *ADORA2A*. **(B)** Representative images of SA β-Gal positive cells in cells transfected with control siRNA or siRNA#2 targeting *ADORA2A*. Scale bar = 20 μm. **(C)** The effectiveness of siRNA#2 targeted *SIRT3* evaluated by western blotting. **(D-F)** Protein expression of SIRT3, AMPK, p-AMPK, BECN1, LC3, p53, p-p53 and p21 determined by western blotting in A375 cells transfected with control siRNA or siRNA#2 targeting *SIRT3*. AAPH, 1 mM; Caff (caffeine), 1 μM.



Supplementary Figure S9. Effect of BECN1 on the protective role of caffeine in AAPH-induced cellular senescence. HeLa wild type (WT) or *BECN1* knockdown (*BECN1* KD) HeLa cells were pretreated with 1 μM caffeine (Caff) for 1 h, added or not 1 mM AAPH for another 48 h, and stained with the SA β-Gal staining kit. Scale bar = 25 μm.



Supplementary Figure S10. The effect of caffeine on the epidermis thickness of mouse dorsal skin exposed to UV light. The dorsal skin of mice was irradiated by a combination of UVA and UVB for 6 weeks as described in Materials and Methods. During this period, caffeine (10 and 20 mg/kg) was orally administrated to mice once daily. Autophagy inhibitor 3-MA (15 mg/kg) or mitophagy inhibitor Mdivi-1 (1.5 mg/kg) was intraperitoneally injected to mice 30 min before caffeine treatment. At the end of the experimental procedure, the dorsal skin tissues were collected to detect the epidermis thickness by H&E staining. Representative images are shown in **Figure 5A**. The thickness was semi-quantitatively measured by using Image Pro Plus (IPP) 5.0 (Media Cybernetics, Rockville, MD, USA). Caff 10 (caffeine, 10 mg/kg); Caff 20 (caffeine, 20 mg/kg). Results represent mean ± S.E.M. Significances were marked as $^{**}P < 0.01$ *vs.* Control group, $^{††}P < 0.01$ *vs.* UV group, $^{‡‡}P < 0.01$ *vs.* "UV+Caff 20" group.