

## **Title Page**

**Yet another function of p53: the switch that determines whether radiation-induced autophagy will be cytoprotective or nonprotective. Implications for autophagy inhibition as a therapeutic strategy.**

Shweta Chakradeo, Khushboo Sharma, Aisha Alhaddad, Duaa Bakhshwin, Ngoc Le, Hisashi Harada, Wataru Nakajima, W. Andrew Yeudall, Suzy V. Torti, Frank M. Torti, and David A. Gewirtz

Department of Pharmacology and Toxicology and Medicine and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298 (SC, KS, AA, DB, NL, DAG)

Department of Oral & Craniofacial Molecular Biology, School of Dentistry, Virginia Commonwealth University, Richmond, VA 23298 (HH, WN, WAY).

Department of Molecular Biology and Biophysics, University of Connecticut Health Sciences, 263 Farmington Ave. Farmington CT 06030 (SVT)

Department of Medicine, University of Connecticut Health Sciences 263 Farmington Ave, Farmington CT 06030 (FMT)

## Running Title Page

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Corresponding author:

Dr. David A. Gewirtz

Virginia Commonwealth University

Massey Cancer Center

401 College St.

Richmond VA 23298

e-mail: [gewirtz@vcu.edu](mailto:gewirtz@vcu.edu)

Phone: 804-828-9523

Fax: 804-827-1134

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Non Standard abbreviations: 3-MA – 3-methyl adenine; Atg5 or ATg7– autophagy proteins; BECN1- Beclin 1 – a gene/protein often considered to be critical for autophagy; CQ- chloroquine; FACS – fluorescence activated cell sorting; Hs578t – a p53 mutant breast tumor cell line; LC3- microtubule associated protein light chain 3; p62/SQSTM1 – a protein marker for degradation of the content of the autophagosome; PARP- Poly ADP ribose polymerase; TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling.

## Abstract

The influence of autophagy inhibition on radiation sensitivity was studied in human breast, head and neck and non-small cell lung cancer cell lines, in cell lines that were either wild type or mutant/null in p53 and in cells where p53 was inducible or silenced. While ionizing radiation promoted autophagy in all tumor cell lines studied, pharmacological inhibition of autophagy and/or genetic silencing of autophagy genes failed to influence sensitivity to radiation in p53 mutant Hs578t breast tumor cells, HN6 head and neck tumor cells, and H358 non-small cell lung cancer cells. The requirement for functional p53 in the promotion of cytoprotective autophagy by radiation was confirmed by the observation that radiation induced autophagy was nonprotective in p53 null H1299 cells but was converted to the cytoprotective form with induction of p53. Conversely, whereas p53 wild type HN30 head and neck cancer cells did show sensitization to radiation upon autophagy inhibition, HN30 cells where p53 was knocked down using shRNA failed to be sensitized by pharmacological autophagy inhibition. Taken together, these findings indicate that radiation-induced autophagy can be either cytoprotective or nonprotective, a functional difference related to the presence or absence of functional p53. Alternatively, these findings could be interpreted to suggest that whereas radiation can induce autophagy independent of p53 status, inhibition of autophagy promotes enhances radiation sensitivity through a mechanism that requires functional p53. These observations are likely to have direct implications with respect to clinical efforts to modulate the response of malignancies to radiation through autophagy inhibition.

## Introduction

Virtually all patients with localized cancer are treated with some combination of surgery, radiotherapy and chemotherapy. Therapy is often successful initially, but disease recurrence is not uncommon and is often associated with resistance to treatment (Fodale et al., 2011). While there are multiple mechanisms that could contribute to therapeutic resistance to radiation such as enhanced DNA repair capacity and overexpression of select survival signaling pathways, recent work has implicated cytoprotective autophagy as a potential basis for resistance that might be exploited for therapeutic purposes (Gewirtz, 2014a,b).

Studies in both cell culture and animal models have demonstrated the potential for improving the response to therapy by the inhibition of cytoprotective autophagy through either pharmacological intervention using drugs such as chloroquine or genetic silencing of autophagy related genes (Abedin et al., 2007; Amaravadi et al., 2007; Apel et al., 2008; Boya et al., 2005; Bristol et al., 2012; Carew et al., 2010; Ding et al., 2011; Godbole et al., 2012; Guo et al., 2012; Ito et al., 2005; Kondo et al., 2005; Liang et al., 2012; Lomonco et al., 2009; Lopez et al., 2011; Paglin et al., 2001; Qadir et al., 2008; Rao et al., 2012; Shi et al., 2011; Tseng et al., 2011; Wilson et al., 2011; Wu et al., 2010). In addition, a number of clinical trials have been initiated to determine whether the chloroquine derivative, hydroxychloroquine, can be utilized to improve the therapeutic response in a variety of malignancies (Sotelo et al., 2006; Solomon et al., 2009).

Although studies in the literature generally support the promotion of cytoprotective autophagy induced in response to either chemotherapy or radiation, it is not clear that autophagy uniformly has a protective function (Gewirtz, 2014a). In a recent report, we demonstrated that chloroquine failed to sensitize 4T1 murine breast tumor cells to radiation either in cell culture or in a syngeneic animal model (Bristol et al., 2013), which was also found to be the case for cisplatin

(Maycotte et al., 2012). Furthermore, genetic silencing of autophagy genes failed to sensitize the 4T1 cells to radiation.

The current work was designed to evaluate the impact of autophagy inhibition on sensitivity to radiation in human tumor cell lines derived from different tissues, specifically the triple negative Hs578t human breast tumor cell line, HN6 and HN30 head and neck cancer cells and A549, H460 and H835 non-small cell lung cancer cells. We find both cytoprotective and nonprotective autophagy induced by radiation, with cytoprotective autophagy occurring exclusively in cell lines with functional p53. Consistent with this finding, radiation-induced autophagy was nonprotective in p53 null H1299 NSCLC cells but could be converted to the protective form with induction of p53. Conversely, p53 wild-type HN30 head and neck cancer cells were sensitized to radiation upon autophagy inhibition (cytoprotective autophagy) whereas HN30 cells with shRNA-mediated knock down of p53 were refractory to such sensitization (nonprotective autophagy).

This work suggests that clinical efforts to sensitize patients to radiation (and possibly chemotherapy) through autophagy inhibition may result in inconsistent and uninterpretable outcomes in the absence of information as to whether the autophagy induced by treatment is cytoprotective or nonprotective, an outcome that may be related to whether the tumor cells are wild type or mutant in p53.

## Materials and Methods

### 2.1 Materials

T75 culture flasks were obtained from Cellstar (Monroe, NC). MEM Alpha medium containing L-Glutamine was obtained from Invitrogen (Grand Island, NY). Trypsin-EDTA (0.25% Trypsin, 0.53mM EDTA- 4Na) and Fetal Bovine Serum was purchased from Hyclone Scientific (Logan, UT) or Serum Source International (Charlotte, NC). TUNEL Assay reagents (terminal transferase, reaction buffer, and  $\text{CoCl}_2$  and fluorescein dUTP) were purchased from Roche (Mannheim, Germany). Annexin PI and propidium iodide were purchased from BD Biosciences (San Diego, CA). Trypan blue acetic acid, acridine orange, formaldehyde and DAPI reagent (6-diamidino 2- phenylindole) were purchased from Invitrogen (Eugene, Oregon, USA). PBS, L-glutamine and penicillin-streptomycin were obtained from Gibco (Life Technologies, NY USA). X-Gal (5- bromo-4- chloro-3- beta D-galactopyranoside) for the  $\beta$ -Galactosidase staining assay and M-PER lysis buffer were purchased from Thermo-Fisher/Fermentas (Rockford, IL). Chloroquine, 3-MA,  $\text{NH}_4\text{Cl}$ , crystal violet and DMSO were obtained from Sigma Chemical (St Louis, MO ). The annexin/apoptosis assay kit was obtained from BD Biosciences (San Diego, CA) Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA).

### 2.2 Cell Lines

Hs578t mammalian breast tumor cells and A 549 NSCLC cells were obtained from ATCC (Rockville, MD). The HN30, shHN30 and HN6 head and neck cancer cell lines were as previously described (Patel et al., 2000). The H358 and A549 non-small cell lung cancer cell lines were generously provided by Dr. Charles Chalfant from the Virginia Commonwealth University Department of Biochemistry and Molecular Biology. shHN30 and Hs578t

sh*BECN1* cells were maintained using (1µg/ml) puromycin. H1299 null and H1299 cells where p53 was inducible by doxycycline were originally developed by Dr. Constantinos Koumenis (Maecker et al., 2000). All cell lines were stored under liquid nitrogen in 10% DMSO with 10% FBS.

### 2.3 Plasmid construction and virus infection.

Mission shRNA bacterial stocks for ATG5 and BECN1 were purchased from Sigma Aldrich (St. Louis, MO, #TRCN00151963 and #TRCN0000299864, respectively. Lentiviruses were produced in HEK 293TN cells co-transfected using Lipofectamine (Invitrogen, Carlsbad, CA) with psPAX2 and pMD2.G packaging constructs (Addgene, Cambridge, MA ). Viruses shed into the media were then used to infect H1299 cells. Puromycin (1µg/ml) was used as the selection marker to enrich the infected cells.

Hs578t were also silenced for Beclin-1 gene. Lentiviral shRNA constructs from the laboratory of Dr. Hisashi Harada were transfected into 293T cells with lentiviral packaging plasmid. The lentivirus shed into medium were collected to transfect Hs578t cells.

### 2.4 Cell Culture Conditions

Hs578t breast tumor cells were grown in ME M-Alpha supplemented with 20% FBS and penicillin/streptomycin (0.5ml/100ml medium). HN30 and HN6 head and neck cancer cells were cultured in DMEM medium containing 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin. A549 non-small cell lung cancer cells were cultured in 50% DMEM/ 50% of RPMI media and 1% penicillin- streptomycin. H358 non-small cell lung cancer cells were grown in DMEM media with 10% FBS and 1% penicillin-streptomycin. H1299 non-small cell lung cancer cells were cultured in DMEM supplemented with 10% FBS, 5% pe nicillin



G/streptomycin; doxycycline at a 1 $\mu$ g/ml concentration was added for induction of p53 expression. Cell cultures were maintained at 37<sup>0</sup>C under a humidified 5% CO<sub>2</sub> atmosphere and examined for bacterial and fungal infections prior to experiments.

## 2.5 Drug and Radiation Treatment

Chloroquine (CQ) was prepared as a stock solution of 50mM in pure water and stored at -20<sup>0</sup> C. 3-methyladenine (3MA) was prepared as a stock solution of 100mM dissolved in PBS with heating and refrigerated. NH<sub>4</sub>Cl was prepared as a 100mM stock in PBS. Chloroquine, 3MA and NH<sub>4</sub>Cl were added 3 hours before radiation and cells were maintained in the presence of CQ, 3MA or NH<sub>4</sub>Cl until radiation was completed. Media was replenished once treatment was terminated.

Hs578t cells were irradiated with 5 x 2Gy over a period of 3 days. HN30 and HN6 cells were treated with a single dose of 4Gy while A549, H358, H1299 (-Dox) and H1299 (+Dox) cells were irradiated with a single 6Gy dose of radiation. The doses of radiation were chosen to optimize the likelihood of detecting sensitization when autophagy was inhibited.

## 2.6 Assessment of Cell Viability and Clonogenic Survival

Cell viability was measured by Trypan Blue exclusion. Cells were incubated with trypsin (0.25% Trypsin-EDTA) for 5-10 min, stained with Trypan Blue (0.4% Trypan Blue) and counted using a haemocytometer with phase contrast microscopy on the indicated days post radiation. For the clonogenic survival assay, cells were plated in triplicate, typically at a density of 100 cells/well. 10-14 days after treatment, cells were washed with PBS, fixed with 100% methanol, air dried

and stained with 0.1% v/v crystal violet. Cells in groups of 50 or more were counted as colonies and data were normalized relative to untreated controls.

## 2.7 Detection and quantification of autophagic cells.

Cells were plated in six well culture dishes, stained with acridine orange at a final dilution of 1:10,000 in DMEM media and allowed to incubate at 37°C for 10 min. After two washes with PBS, cells were examined and imaged under an inverted fluorescence microscope (Olympus, Tokyo, Japan). All images were taken at the same magnification. For quantification of autophagic vesicles (AVOs), cells were trypsinized, harvested and washed with PBS. Pellets were re-suspended in PBS, stained with a 1:10,000 dilution of acridine orange for 10 min and analyzed by BD FACSCanto II using BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility. A minimum of 10,000 cells within the gated region was analyzed.

## 2.8 Flow Cytometry Analysis using Annexin V – PI staining to determine apoptotic and necrotic cells.

Treated cells were collected and labeled fluorescently using a FITC Annexin V apoptosis detection kit I. Labeling was performed by adding 500µl of binding buffer, 5µl Annexin V-FITC and 5µl propidium iodide to each sample (FITC AnnexinV apoptosis detection kit, BD Biosciences, San Jose, CA). Samples were mixed gently and incubated in the dark for 15 min. Annexin V- PI labeled cells were measured by flow cytometry and analyzed by BD FACS Canto II and BD DIVA software. A minimum of 10,000 cells were counted within the gated region.

## 2.9 Western Blotting

After the indicated treatments, cells were scraped from the culture dishes, collected and lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, Waltham, MA) containing protease and phosphatase inhibitors. Protein concentrations were determined by the Bradford assay (Bradford et al., 1976) (Bio-Rad Laboratories, Hercules, CA). Total protein was then diluted in SDS sample buffer and dry boiled for 10 min. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane, and blocked in 5% milk/1x PBS/ 0.1% Tween for 1 hour. Primary antibodies used at a 1:1000 dilution with overnight incubation were p62 (BD Transduction), ATG5 (Cell Signaling Technology, Danvers, MA), BECN1 (Cell Signaling Technology), LC3II (Cell signaling Technology), p53 (BD pharmingen), and  $\beta$ -actin (Santa Cruz Biotechnology). The membrane was then incubated with secondary antibody of either horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5000; Sigma) or goat anti-mouse (1:5000; Sigma) for 1 hour, followed by extensive washing with tween-PBS. Blots were developed using Pierce enhanced chemiluminescence reagents and Bio-Max film (Thermo Scientific). Membranes were incubated with anti-p62 (Santa Cruz SC-28359) or anti-actin antibodies and detected with anti-mouse secondary antibody (Santa Cruz SC- 2005 Lot A2312).

2.10 Statistical analysis. Statistical differences were determined by StatView statistical software (SAS Institute, Cary, NC). The data were expressed as means  $\pm$  SE (as standard error of the mean). Comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, and p values  $< 0.05$  were taken as statistically significant.

## Results

### *Radiation induces autophagy and prolonged growth arrest in Hs578t breast tumor cells*

Previous studies from this laboratory have shown that radiation induces cytoprotective autophagy in MCF-7 and ZR-75 breast tumor cell lines (Bristol et al., 2012, Wilson et al., 2011), both of which are wild type in p53. The first series of experiments presented here involve Hs578t breast tumor cells, which are mutant in p53 (Nieves-Neira and Pommier, 1999). **Figure 1A** indicates that exposure of Hs578t breast tumor cells [which are triple negative cell (Foulks et al., 2010)] to radiation (5×2 Gy over a period of 3 days) results primarily in growth arrest; the growth arrest was relatively prolonged as the cells did not recover for at least 11 days post-irradiation (data not shown).

**Figure 1B** indicates that radiation promoted autophagy in the Hs578t cells based on the detection of puncta by acridine orange staining. **Figure 1C** (where the extent of autophagic vesicle formation was assessed by flow cytometry) indicates that once autophagy was induced, its extent was relatively stable over a period of 7 days (with a slight transient increase at day 5).

### *Evidence that radiation-induced autophagy does not have a cytoprotective function in Hs578t breast tumor based on lack of sensitization by the pharmacological autophagy inhibitor, chloroquine.*

To determine whether radiation-induced autophagy has a cytoprotective function in Hs578t cells, autophagy was inhibited with the use of three separate pharmacological inhibitors: 3-methyl adenine (3-MA), chloroquine and NH<sub>4</sub>Cl. Appropriate concentrations (25 μM for chloroquine, 5mM for 3-MA and 10mM for NH<sub>4</sub>Cl) were chosen by determining concentrations that were minimally toxic to the tumor cells using an MTT assay (data not shown). **Figure 1B** confirms

that chloroquine inhibited radiation induced autophagy based on the change from red/orange to yellow of the autophagosomes, which reflects the fact that chloroquine interferes with the acidification required for fusion of lysosomes with the autophagosome (Bristol et al., 2012). Additional evidence that radiation promotes autophagy in the Hs578t cells as well as that chloroquine inhibited radiation-induced autophagy is provided by the observation that chloroquine prevented the radiation-induced degradation of p62/SQSTM1 (**Figure 1D**), an indication of radiation induced autophagic flux. (Bjørkøy et al., 2009; He and Klionsky, 2009; Feng et al., 2014). Serum starvation was utilized as a positive control.

To determine whether autophagy might be playing a cytoprotective (or alternatively a cytotoxic) function in the Hs578t cells, clonogenic survival was assessed in cells exposed to radiation in the absence and presence of chloroquine. As shown in **Figure 1E**, only an apparent additive effect of radiation and chloroquine was observed; that is, the reduced clonogenic survival in cells treated with the combination of IR and CQ reflects the combined antiproliferative and/or cytotoxic effects of each of the treatment modalities. Specifically, with exposure of Hs578t cells to fractionated radiation alone, there is an approximately 55% reduction in survival whereas with CQ, cell survival is reduced by ~ 30%. The combination treatment of radiation + CQ reduced survival by ~ 85%, which is what would be anticipated from additive toxicities of the individual treatments.

*Blocking autophagy with chloroquine does not promote apoptosis in irradiated Hs578t cells.*

In classic cytoprotective autophagy, cells use autophagy as a mechanism for cell survival and consequently when autophagy is inhibited, cells die, primarily by apoptosis (Gewirtz et al., 2009). As shown in **Figure 1F**, assessment of apoptosis by flow cytometry using Annexin V/PI

indicated that only between 15-20% of the cell population was undergoing apoptosis post-radiation. Furthermore, inhibition of autophagy using CQ did not increase the extent of apoptosis, which supports the conclusion that autophagy is unlikely to be cytoprotective in this experimental system.

*Chloroquine fails to sensitize Hs578t cells to a single dose of radiation*

Because the 5 x 2 Gy radiation alone produced a relatively pronounced reduction in clonogenic survival, it might be argued that the studies lack sufficient sensitivity to detect sensitization. Consequently, the clonogenic survival studies were repeated using a single radiation dose of 4 Gy. Chloroquine also failed to sensitize the Hs578t cells to the 4 Gy of radiation in that the reduction in clonogenic survival for the combination treatment (~ 52%) essentially reflected the additive toxic effects of the chloroquine (~ 13%) and the radiation (~ 38%) (data not shown).

*Evidence that radiation-induced autophagy does not have a cytoprotective function in Hs578t breast tumor cells based on lack of sensitization by the pharmacological autophagy inhibitors, ammonium chloride and 3-methyl adenine.*

**Figure 2** presents studies that confirm that radiation promotes a nonprotective form of autophagy in Hs578t breast tumor cells by assessing the influence of another late stage autophagy inhibitor,  $\text{NH}_4\text{Cl}$ , (Ikeda et al., 2013) on sensitivity to radiation. **Figure 2A** shows the promotion of autophagy by radiation and that  $\text{NH}_4\text{Cl}$  inhibits radiation-induced autophagy based on the change from the reddish-orange color of the acridine orange stained vesicles to a yellowish color (again indicative of interference with the acidification step required for completion of autophagy). **Fig 2B** again indicates that radiation reduced p62/SQSTM1 levels, and that  $\text{NH}_4\text{Cl}$  interfered with the degradation of p62. In the clonogenic survival assay presented in **Figure 2C**, the combination

of an autophagy inhibitor,  $\text{NH}_4\text{Cl}$ , with radiation showed a modest but insignificantly greater effect than radiation alone that again, appears to reflect the additive antiproliferative effects of radiation and  $\text{NH}_4\text{Cl}$ .

Additional studies were performed using the early stage autophagy inhibitor, 3-methyl adenine (3-MA) (Boya et al., 2005). **Figure 3A** demonstrates that, as would be expected, there is a decline in autophagic vesicle formation in Hs578t cells treated with 3-MA and radiation (in contrast to the accumulation of vacuoles in the studies with the late stage autophagy inhibitors chloroquine and ammonium chloride). The autophagic flux in irradiated cells is shown by the degradation of p62/SQSM1 in **Figure 3B**, which is prevented by 3-MA. The clonogenic survival assay presented in **Figure 3C** indicates that 3-MA failed to alter sensitivity to radiation, confirming the findings when autophagy was inhibited using either chloroquine or  $\text{NH}_4\text{Cl}$ .

*Evidence that radiation-induced autophagy does not have a cytoprotective function in Hs578t breast tumor based on lack of sensitization by genetic autophagy inhibition.*

Although the results generated using three separate pharmacological autophagy inhibitors were consistent in failing to demonstrate significant sensitization to radiation, it was nevertheless necessary to confirm these observations through genetic inhibition of autophagy. The inset to **Figure 4C** indicates that Beclin-1, often considered as an essential gene in the autophagy pathway (Kang et al., 2011) was knocked down using an shRNA strategy. **Figure 4A** shows that induction of autophagy by radiation was suppressed in the Beclin-1 silenced cells based on acridine orange vacuole staining. **Figure 4B** further demonstrates that the silencing of Beclin 1 interfered with the radiation-induced degradation of p62/SQSM1. Somewhat disconcertingly, silencing of Beclin 1 had only a modest effect on degradation of p62/SQSM1 under conditions

of serum starvation, suggesting that autophagy under these conditions may not be entirely Beclin 1 dependent. **Figure 4C** shows that when Hs578t cells were silenced for Beclin-1, radiation sensitivity was identical to that in the Hs578t cells without Beclin-1 silencing. These studies appear to confirm the conclusion that autophagy induced by radiation in Hs578t cells is not protective in function.

*Radiation-induced autophagy is cytoprotective in p53 wild-type HN30 head and neck cancer cells and nonprotective in p53 mutant HN6 head and neck cancer cells.*

We have previously reported on the cytoprotective autophagy induced by radiation in MCF-7 and ZR-75 breast tumor cell lines (Wilson et al., 2011, Bristol et al., 2012) and the data presented above establishes that autophagy induced by radiation can likewise be nonprotective in breast tumor cells. To extend these findings to tumor cells derived from another tissue source, studies were conducted in p53 wild-type HN30 and p53 mutant HN6 head and neck tumor cells, a disease where radiation is also a conventional therapy (Zackrisson et al., 2003). **Figure 5A** again shows radiation-induced autophagy in the HN30 cells based on acridine orange staining and the capacity of chloroquine to alter the nature of the staining, indicative of a blockade to the acidification step that is necessary for autophagosome-lysosome fusion. **Figure 5C** confirms that radiation is promoting degradation of p62/SQSTM1 and that this degradation is essentially abrogated by chloroquine. **Figure 5E** presents clonogenic survival in the HN30 cells with radiation alone and radiation + chloroquine. Although the clonogenic survival studies presented in **Figure 5E** are suggestive of sensitization, we recognize that it could be argued that the data in are merely showing additive toxicities of the combination treatment. In order to demonstrate that chloroquine actually sensitized the HN30 cells to radiation, temporal response studies were performed. As shown in **Supplementary Figure 1**, the combination of chloroquine with



radiation produces a sustained growth arrest that is not seen for either radiation or chloroquine treatment alone.

Similar studies performed in the HN6 cells show the induction of autophagy by radiation and interference with autophagy by chloroquine (**Figure 5B**). Furthermore, chloroquine is again shown to prevent the degradation of p62 induced by radiation (**Figure 5D**). However, in contrast to the findings with the HN30 cells, **Figure 5F** shows a lack of radiation sensitization by chloroquine in the HN6 cells by the clonogenic survival assay. This lack of sensitization was confirmed by the temporal response study presented in **Supplementary Figure 1B**.

*Autophagy inhibition with chloroquine fails to sensitize HN30 cells with knockdown of p53*

To this point, all of the data presented are essentially circumstantial in terms of implicating a requirement for functional p53 in the capacity of ionizing radiation to promote the cytoprotective form of autophagy. To more directly address this question, p53 was knocked down in the p53 wild-type HN30 head and neck tumor cells (**shown in the inset to Figure 6C**). **Supplementary Figure 2B** again shows sensitization in the clonogenic survival assay where autophagy in the HN30 cells was inhibited by chloroquine (as supported by the acridine orange staining in **Supplementary Figure 2B (and 5C)**). This study had to be repeated for comparative purposes with the p53 silenced cells since the experiments presented in **Figure 5** had been performed months previously. In contrast, the shp53 HN30 cells failed to be sensitized to radiation upon autophagy inhibition (**Figure 6C**). Again, the inhibition of autophagy was confirmed based on acridine orange staining and interference with the degradation of p62/SMQST1 ( **Figures 6A and 6B**).

*Radiation-induced autophagy is protective in p53 wild type A549 non-small cell lung cancer cells and nonprotective in p53 mutant H358 non-small cell lung cancer cells.*

In a recent report, radiation was shown to promote cytoprotective autophagy in A549 and H460 non-small cell lung cancer cells (Ko et al., 2014) both of which are wild type in p53. We have confirmed these findings in A549 cells (H460 cells not shown). **Figure 7A** shows the induction of autophagy by ionizing radiation and interference with autophagy by chloroquine based on the yellow color that reflects suppression of acidification and autophagosome-lysosome fusion.

**Figure 7B** shows that the p62 degradation is compromised by the chloroquine treatment. **Figure 7C** demonstrates sensitization to radiation by the chloroquine based on a count of viable cells.

When similar studies were performed using the (p53 mutant) H358 non-small cell lung cancer cell line, again, autophagy and autophagic flux were induced by radiation which were inhibited by chloroquine (**Figures 8A and 8B**). However, in contrast to the results observed in the A549 and H460 cells, chloroquine failed to alter radiation sensitivity in the H358 cells (**Figure 8C**).

*Radiation-induced autophagy is nonprotective in p53 null H1299 non-small cell lung cancer cells but is converted to protective autophagy with induction of p53 .*

Taken together, these findings are clearly suggestive of p53 dependence for radiation-induced cytoprotective autophagy since in this and previous work protective autophagy was observed in cells with functional p53 (MCF-7, ZR-75, A549, H460 and HN30) but not in cells null or mutant in p53 (4T1, Hs578t, H358 and HN6). This conclusion is supported by the findings in the HN30 cells with knockdown of p53, shown above. To more convincingly establish this conclusion, additional studies were designed to assess the impact of interfering with radiation-induced autophagy in p53 null H1299 non-small cell lung cancer cells and their isogenic counterparts

where p53 was induced by doxycycline. **Figure 9C** indicates that radiation induced autophagy in both the p53 null cells and the cells where p53 was induced by doxycycline (Western blot in **Figure 9A** indicates p53 induction). **Figure 9C** (lower panel) and **Figure 9D** present evidence that autophagy has been inhibited based on a decrease in acidic vesicle formation when ATG5 is knocked down (knockdown indicated in **Figure 9B**) or an accumulation of LC3II with exposure to chloroquine (**Figure 9D**). When radiation-induced autophagy in H1299 cells that are null in p53 was inhibited (either by silencing of ATG5 or chloroquine treatment) radiation sensitivity was essentially unaltered (**Figures 10A and 10C**), with the exception of a small transient increase on day 1 (shown in **Figure 10A**). In quite dramatic contrast, both ATG5 silencing and treatment with chloroquine resulted in pronounced sensitization to radiation in the H1299 cells when p53 was induced by doxycycline (**Figures 10B and 10D**). Control growth and growth in the presence of chloroquine alone were purposely omitted from **Figures 10A, B, C and D** in order to limit the range of the Y axis and allow focus on the effects (or lack thereof) of autophagy inhibition. Chloroquine alone had minimal impact on cell growth. Baseline apoptosis and apoptosis in the presence of chloroquine are also not shown in **Figures 10E and 10F** since apoptosis was essentially undetectable in the absence of irradiation of the cells. Furthermore, induction of apoptosis was observed in H1299 (+Dox) cells but not the H1299 (-Dox) cells upon inhibition of autophagy by either chloroquine or genetic knockdown of ATG5 as shown in **Figures 10E and 10F**.

## Discussion

It is generally thought that when chemotherapy and radiation promote autophagy in tumor cells the autophagy has a cytoprotective function that can be inhibited to sensitize the cells to treatment. Recently, we and our collaborators have demonstrated lack of sensitization to radiation as well as to cisplatin in the 4T1 murine breast cancer model upon autophagy inhibition, indicating the existence of a non-protective function of autophagy in tumor cells (Maycotte et al., 2012; Bristol et al., 2013).

In the current work, we demonstrate a lack of sensitization to radiation in Hs578t breast cancer cells upon inhibition of radiation induced autophagy utilizing both multiple pharmacological approaches as well as confirming these results with genetic silencing of Beclin-1. The concept that inhibition of autophagy fails to sensitize Hs578t cells to radiation therapy is supported by the lack of increased apoptosis when autophagy is inhibited, which supports the absence of cytoprotective autophagy. At the same time, the autophagy does not appear to demonstrate a cytotoxic or growth inhibitory function in this model system since there is no *reduction* in radiation sensitivity when autophagy is inhibited. Parallel studies demonstrate the existence of both cytoprotective and nonprotective autophagy, respectively, in the A549/H460 and H358 non-small cell lung cancer cells and in the HN30 and HN6 head and neck cancer cells. Thus, the concept that radiation can produce either cytoprotective or nonprotective autophagy is supported by studies in human tumor cells derived from different tissues. One caveat to these findings is that both the induction of autophagy and the inhibition of autophagy in a number of the studies presented was limited to experiments involving acridine orange staining and p62/SMQST1 degradation.

Other than functional studies where autophagy is inhibited, there is no *a priori* approach for distinguishing between cytoprotective autophagy and the nonprotective form (Gewirtz, 2014a). It might have been predicted that nonprotective autophagy occurs when autophagy fails to go to completion; however, it is evident from the studies involving p62/SMQST1 degradation that radiation is inducing autophagic flux even when the autophagy is nonprotective.

It is interesting that Hs578t, HN6 and H358 cells, where autophagy was nonprotective, are all p53 mutant cell lines, which generally respond quite poorly to conventional cancer treatments. In contrast, in our previous work with MCF-7 and ZR-75 breast tumor cells as well as the current studies with HN30 head and neck tumor cells and A549/H460 non-small cell lung cancer cells are all wild type in p53. These data suggest that nonprotective autophagy is related to the absence of functional p53 since nonprotective autophagy induced by radiation was also shown in the 4T1 breast tumor cells (Bristol et al., 2012), which are null in p53.

To confirm this conclusion we have demonstrated that nonprotective autophagy is converted to protective autophagy when p53 is induced in p53 null H1299 cells and that conversely protective autophagy is converted to nonprotective autophagy when p53 is silenced in p53 wild-type HN30 cells. The apparent requirement for functional p53 in radiation-induced cytoprotective autophagy has also recently been observed by Cheng et al (2013) who showed that the relatively nonspecific pharmacological autophagy inhibitor, 3-MA, sensitized p53 induced H1299 cells to radiation but not the H1299 cells that are null in p53. These findings strongly support our own studies where both pharmacological and genetic inhibition of autophagy enhanced radiation sensitivity only in the H1299 cells where wild-type p53 function was restored.

An alternative interpretation of the findings presented in this manuscript is that whereas radiation can induce autophagy independent of p53 status, inhibition of autophagy enhances radiation sensitivity through a mechanism that requires functional p53. Consequently, we cannot completely rule out the existence of an autophagy-independent but mutant p53-dependent pathway that might be promoting cell survival. For instance, mutant p53 could be responsible for cell survival by interfering with apoptosis or senescence. This would be consistent with the fact that historically mutant p53 cell lines have been shown to be more radioresistant than p53 wild-type cells.

To this point in time, specific morphological or biochemical characteristics that might be utilized to distinguish between autophagy that is cytoprotective or nonprotective (or, for that matter, cytotoxic or cytostatic) have not been identified (Gewirtz, 2014a); furthermore, the extent of autophagy has not been shown to differ when its function is altered. Consequently, it is likely that key determinants as to whether autophagy will be protective or nonprotective in the case of radiation could involve signaling pathways acting downstream of p53. Considering the multiplicity of p53 targets, identifying these pathways is likely to prove quite challenging; however, ongoing studies in our laboratory are designed to address this question using a number of experimental strategies including analysis of known autophagy signaling pathways as well as analysis of the phosphoproteomic profile associated with radiation induced cytoprotective and nonprotective autophagy in isogenic tumor cell lines.

The potential importance of this work relates, in large part, to the fact clinical trials are in progress in order to study if sensitivity to chemotherapy or radiation treatment can be increased by using the autophagy inhibitors chloroquine or hydroxychloroquine. Our findings indicate that the fact that radiation can induce autophagy in a given tumor cell does not obligatorily mean that

this autophagy is protective. Consequently, if the strategy of autophagy inhibition is to potentially be successful as a therapeutic strategy, it will likely be necessary to stratify patients in terms of whether the autophagy being induced is cytoprotective in function.

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## Authorship Contributions

*Participated in Research Design:* Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Harada H, Nakajima W, Yeudall WA, Gewirtz DA.

*Conducted Experiments:* Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Nakajima W.

*Contributed New Reagents or Analytical Tools:* Torti S, Torti F.

*Performed Data Analysis:* Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Harada H, Nakajima W, Yeudall WA, Gewirtz DA.

*Wrote or Contributed to the Writing of the Manuscript:* Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Harada H, Yeudall WA, Gewirtz DA.

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## Footnotes

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## **Figure Legends**

### **Fig 1. Induction of autophagy by radiation in Hs578t breast tumor cells; autophagy**

**inhibition by chloroquine does not result in sensitization.** **A.** Hs578t cells were exposed to 5 x 2Gy of radiation (IR) over a period of 3 days and viable cell number was monitored by trypan blue exclusion. Data represent the mean of three experiments  $\pm$  standard error. (\*  $p < 0.05$  compared to control). **B.** Induction of autophagy upon irradiation of Hs578t cells. Acridine orange staining of autophagic vacuoles on day 7 after exposure to 5 x 2Gy of radiation with and without chloroquine (CQ, 25  $\mu$ M). **C.** Quantification of autophagy measured by flow cytometry. Data is representative of three experiments mean  $\pm$  standard error. **D.** p62/SQSTM1 degradation indicative of autophagic flux in Hs578t cells on day 1 after radiation. Serum starvation was included as a positive control. The experiment was reproduced 3 times. **E.** Clonogenic survival assay in Hs578t cells. Data represent the average of three experiments plotted with mean  $\pm$  standard error (\*  $p < 0.05$  compared to control). (\*\* when compared to IR alone). **F.** Induction of apoptosis in irradiated cells  $\pm$  chloroquine.

### **Figure 2. Inhibition of radiation-induced autophagy in Hs578t cells using NH<sub>4</sub>Cl does not**

**result in sensitization.** **A.** Influence of ammonium chloride (10 mM) on autophagic vacuole formation based on acridine orange staining (Day 4 post radiation). **B.** p62/SQSTM1 degradation indicative of autophagic flux in Hs578t cells (Day 1 post radiation). Experiment was repeated 3 times with similar results. **C.** Clonogenic survival assay indicating that inhibition of autophagy by NH<sub>4</sub>Cl failed to sensitize Hs578t cells to radiation. Data are representative of three experiments plotted with mean  $\pm$  standard error. (\*  $p < 0.05$  compared to control).

**Figure 3. Inhibition of radiation-induced autophagy in Hs578t cells using 3-methyl adenine**

**does not result in sensitization.** **A.** Influence of 3-MA (5mM) on autophagic vacuole formation based on acridine orange staining (Day 1 post radiation). **B.** p62/SQSM1 degradation indicative of autophagic flux in Hs578t cells (Day 1 post radiation). Experiment was repeated 3 times with similar results. **C.** Clonogenic survival assay indicating that inhibition of autophagy by 3-MA failed to sensitize Hs578t cells to radiation. Data are average of three experiments plotted with mean  $\pm$  SE. (\*  $p < 0.05$  compared to control).

**Figure 4. Genetic inhibition of radiation-induced autophagy in Hs578t cells does not result**

**in sensitization.** **A.** Autophagic vacuole formation based on acridine orange staining in irradiated Hs578t cells where Beclin-1 was silenced (Day 1 post radiation). **B.** p62/SQSM1 degradation indicative of autophagic flux in Hs578t cells (Day 1 post radiation). Experiment was repeated three times with similar results. **C.** Clonogenic survival assay indicating that silencing of Beclin-1 failed to sensitize Hs578t cells to radiation. Inset: Beclin 1 silencing. Data represent the average of three experiments plotted with mean  $\pm$  SE (\*  $p < 0.05$  compared to control).

**Figure 5. Radiation induced autophagy in head and neck cancer cells. Inhibition of**

**radiation-induced autophagy sensitizes p53 wt HN30 but not p53 mutant HN6 head and neck cancer cells.** **A/B.** Acridine orange staining of autophagic vacuoles (Day 1 after exposure to 4 Gy of radiation) in HN30 cells (**A**) and HN6 cells (**B**) with and without chloroquine (CQ, 10 $\mu$ M). **C/D.** p62/SQSM1 degradation indicative of autophagic flux in HN30 cells (**C**) and HN6 cells (**D**) Day 1 post radiation. **E.** Clonogenic survival assay indicating sensitization upon inhibition of radiation induced autophagy in HN30 cells. Data represent the average of three experiments plotted with mean  $\pm$  SE (\*  $p < 0.05$  compared to control; \*\*  $p < 0.05$  compared to IR).

**F.** Clonogenic survival assay indicating lack of sensitization upon inhibition of radiation induced autophagy in HN6 cells.

**Figure 6. Evidence for p53 dependence of cytoprotective autophagy; lack of sensitization by chloroquine in HN30 cells with knockdown of p53.** **A.** Acridine orange staining in shp53 HN30 shows induction of autophagy and inhibition of autophagy by chloroquine. **B.** p62/SQSM1 degradation indicative of autophagic flux in shp53HN30 cells (Day 1 post radiation). **C.** Clonogenic survival assay in shp53 HN30 cells indicating lack of sensitization by autophagy inhibition.

**Figure 7. Inhibition of radiation-induced autophagy sensitizes A549 non-small cell lung cancer cells.** **A.** Induction of autophagy upon radiation in A549 cells. Acridine orange staining of autophagic vacuoles (Day 1 after exposure to 6 Gy of radiation) with and without chloroquine (CQ, 10 $\mu$ M). **B.** p62/SQSM1 degradation indicative of autophagic flux in A549 cells. Experiment was repeated three times with similar results. **C.** Clonogenic survival studies indicative of sensitization. Data represent the average of three experiments plotted with mean  $\pm$  SE (\* p<0.05 compared to control; \*\* p<0.05 compared to radiation alone).

**Figure 8. Inhibition of radiation-induced autophagy fails to sensitizes H358 non-small cell lung cancer cells.** **A.** Induction of autophagy upon irradiation. Acridine orange staining of autophagic vacuoles (Day 1 after exposure to 6 Gy of radiation) with and without chloroquine (CQ, 10 $\mu$ M). **B.** p62/SQSM1 degradation indicative of autophagic flux in H358 cells. **C.** Clonogenic survival studies indicative of lack of sensitization. Data represent the average of three experiments with mean  $\pm$  SE.

**Figure 9. Evidence for p53 dependence of cytoprotective autophagy; induction of p53 results in sensitization to radiation when autophagy is inhibited in H1299 non-small cell lung cancer cells.** **A.** Induction of p53 under the influence of doxycycline in H1299 cells. **B.** Knockdown of *ATG5* in both H1299 null (-Dox) and p53 inducible (+Dox) cell lines. **C.** Induction of autophagy (upper panel) as assessed by acridine orange staining in H1299 cells and inhibition of autophagy (lower panel) when *ATG5* gene is knocked down as shown by reduction in acidic vesicle formation (All images were taken at the same magnification, 20x objective). **D.** Accumulation of LC3II when the autophagy inhibitor chloroquine is used in combination with radiation indicative of inhibition of autophagic flux in both H1299 (-Dox) and (+Dox) cells.

**Figure 10. Evidence for p53 dependence of cytoprotective autophagy; induction of p53 results in sensitization to radiation when autophagy is inhibited in H1299 non-small cell lung cancer cells.** **A/C.** H1299 non-small cell lung carcinoma cells null in p53 do not show any change in sensitivity to radiation when autophagy is blocked using chloroquine or genetic knock down of *ATG5*. **B/D.** Induction of p53 under the influence of doxycycline results in sensitization to radiation when autophagy is blocked using chloroquine or knock down of *ATG5*. **E/F.** Induction of apoptosis in H1299 (+Dox) cells when autophagy is blocked using chloroquine or genetic knock down of *ATG5* (n=3, mean±SE, \*p<0.05 #p<0.001).

# Figure 1

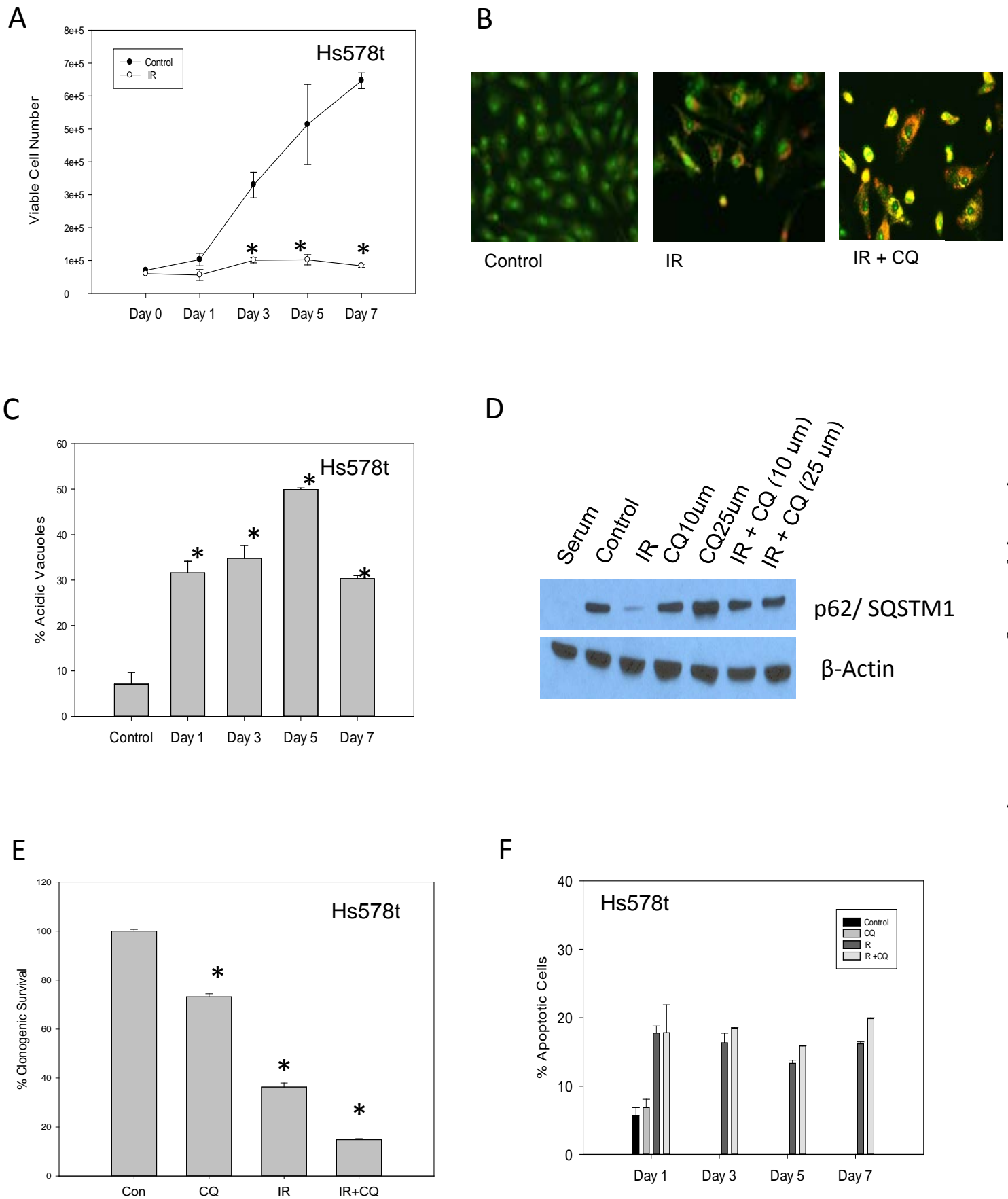
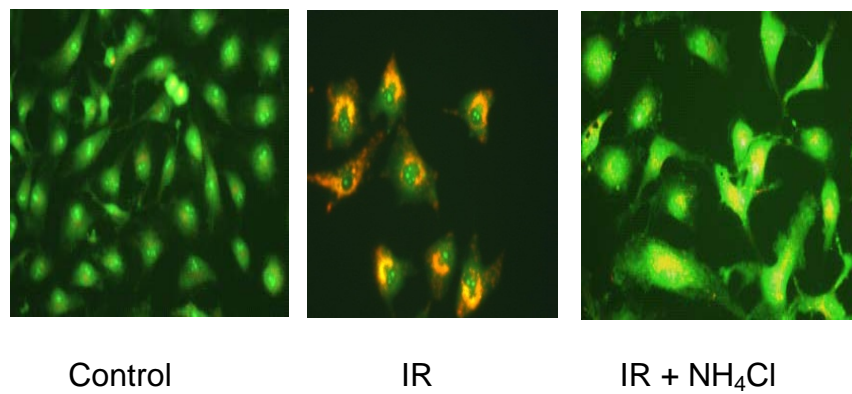
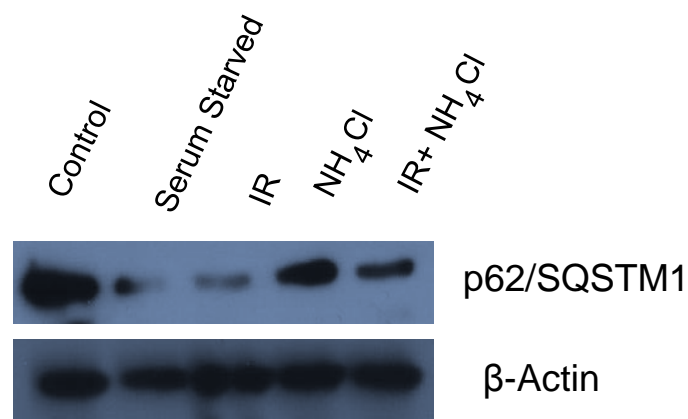


Figure 2

2A



2B



2C

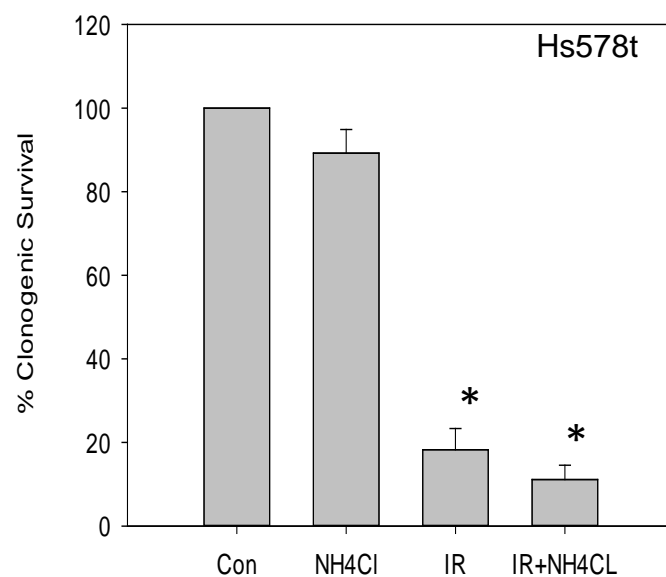
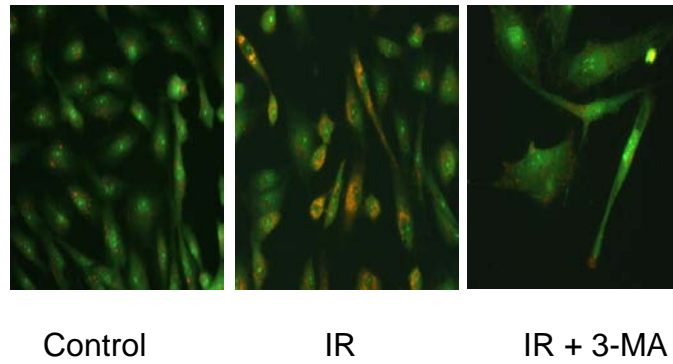
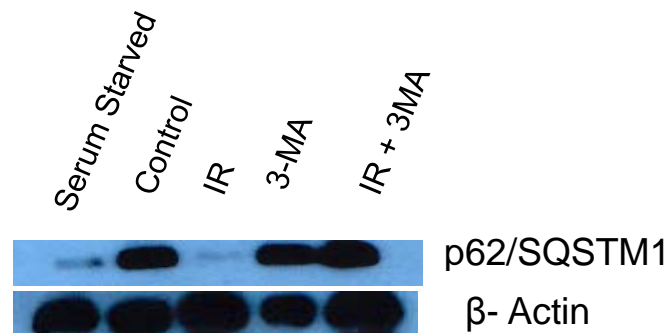


Figure 3

A



B



C

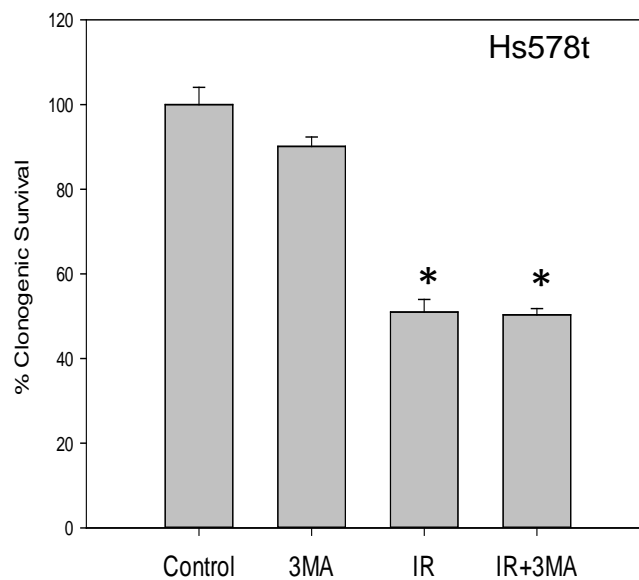
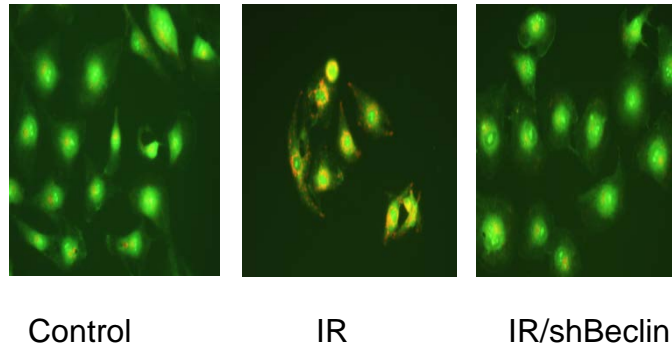
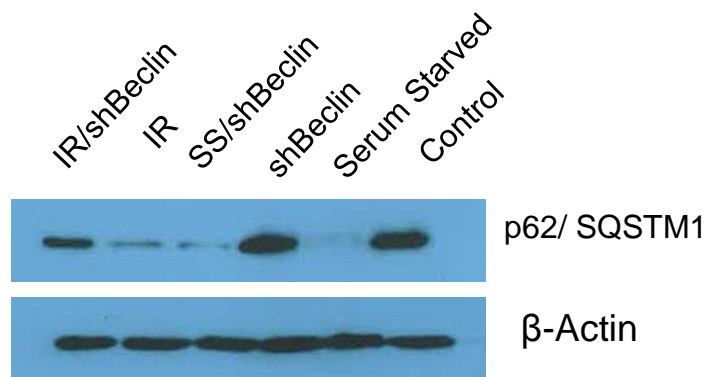


Figure 4

A



B



C

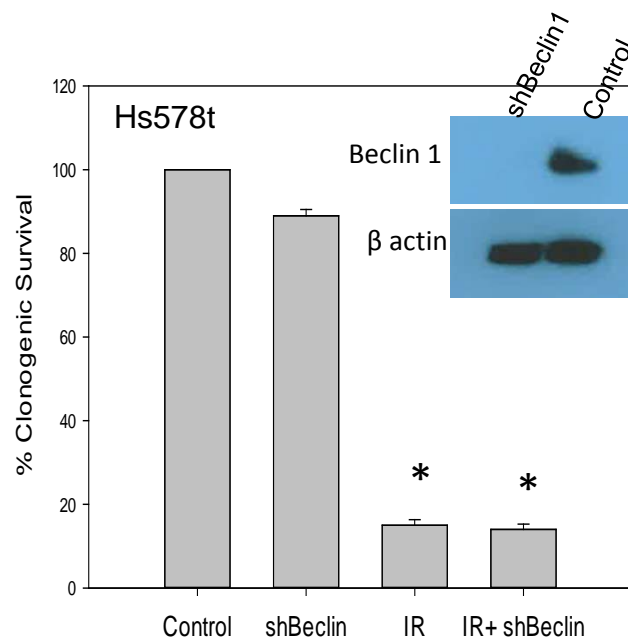




Figure 5

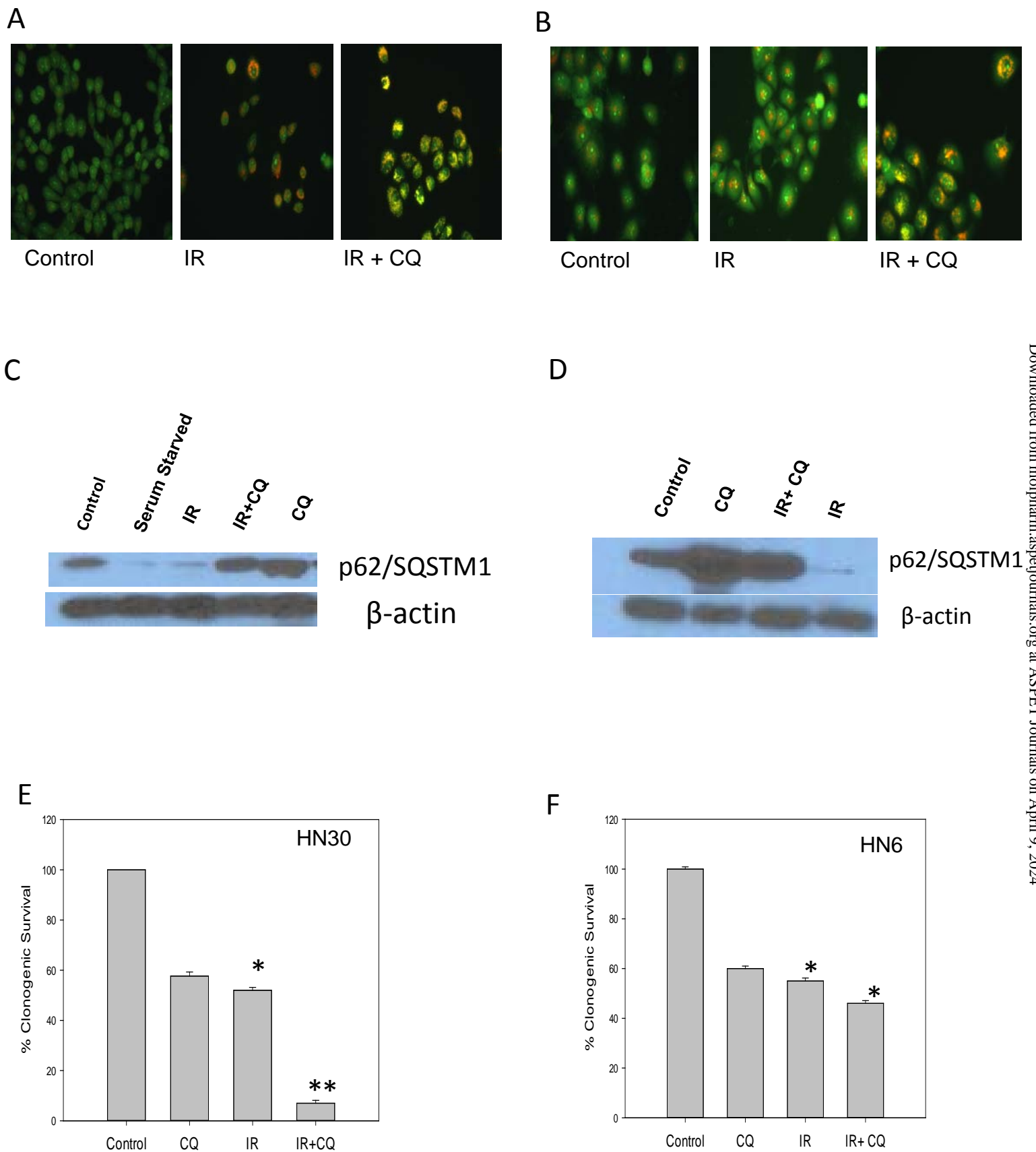
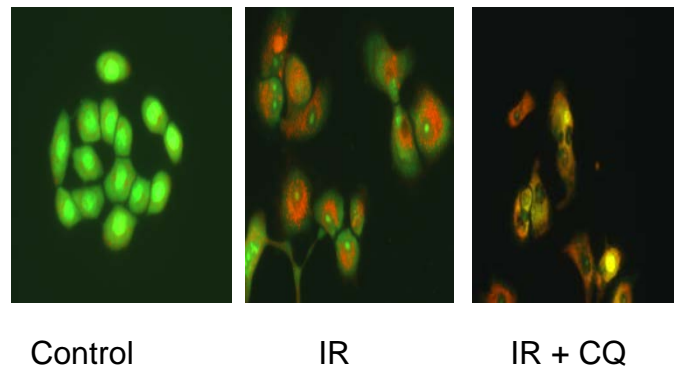
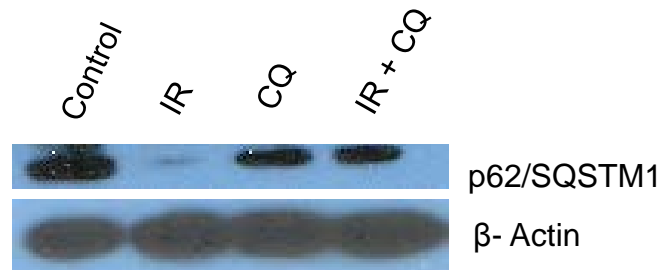


Figure 6

A



B



C

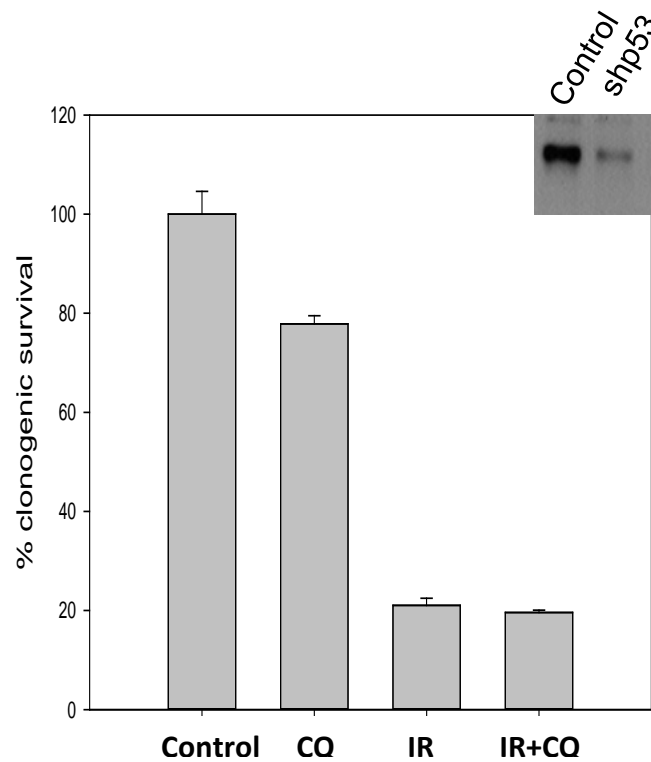


Figure 7

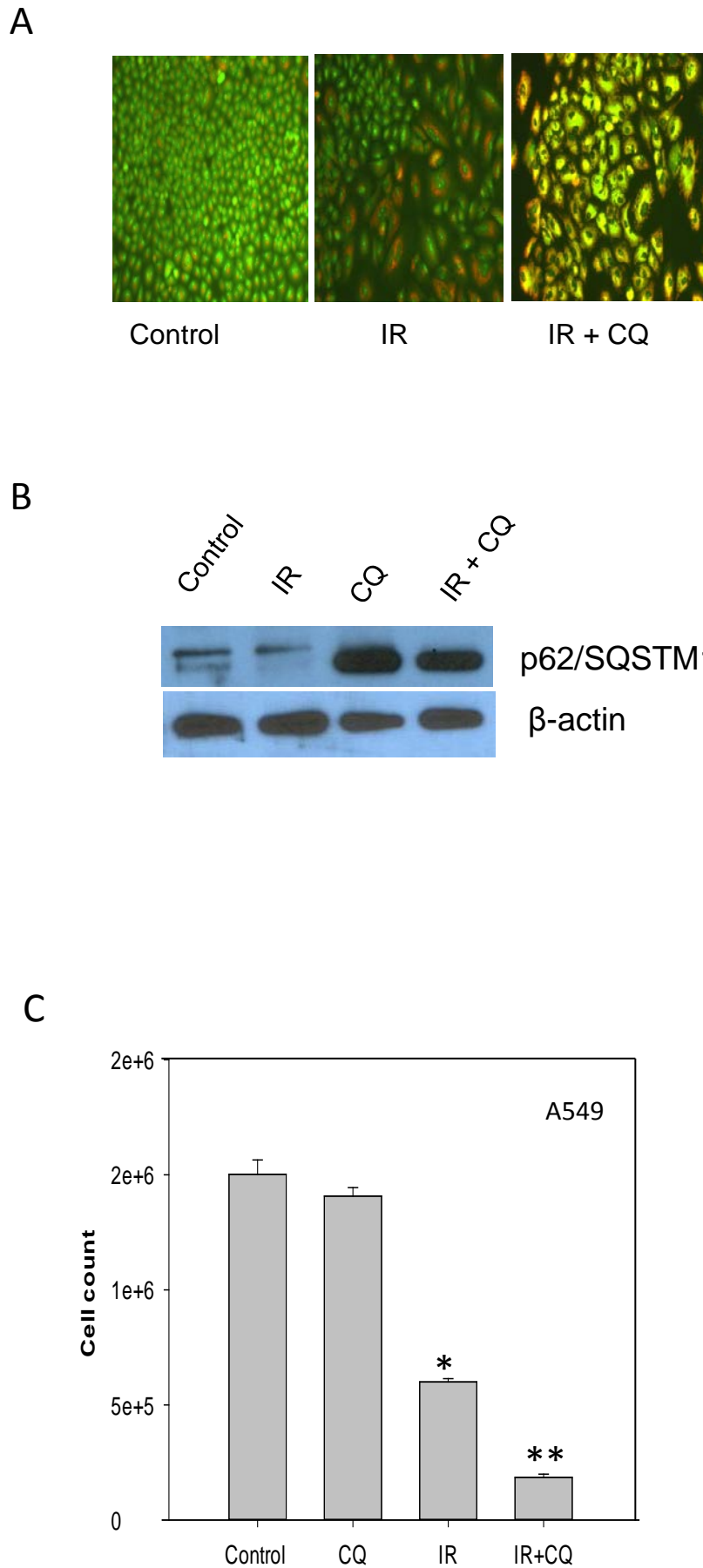
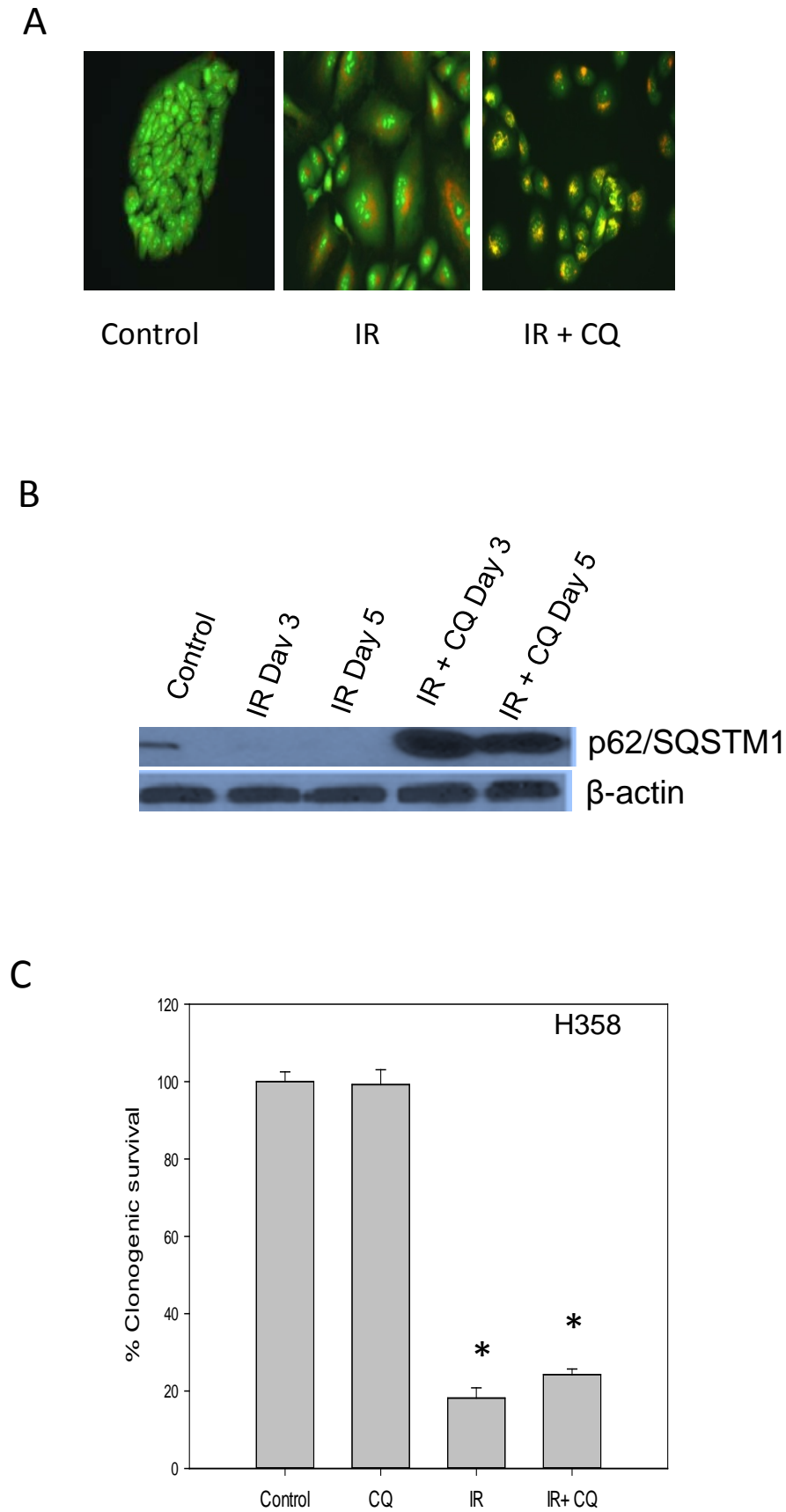


Figure 8



**Figure 9**

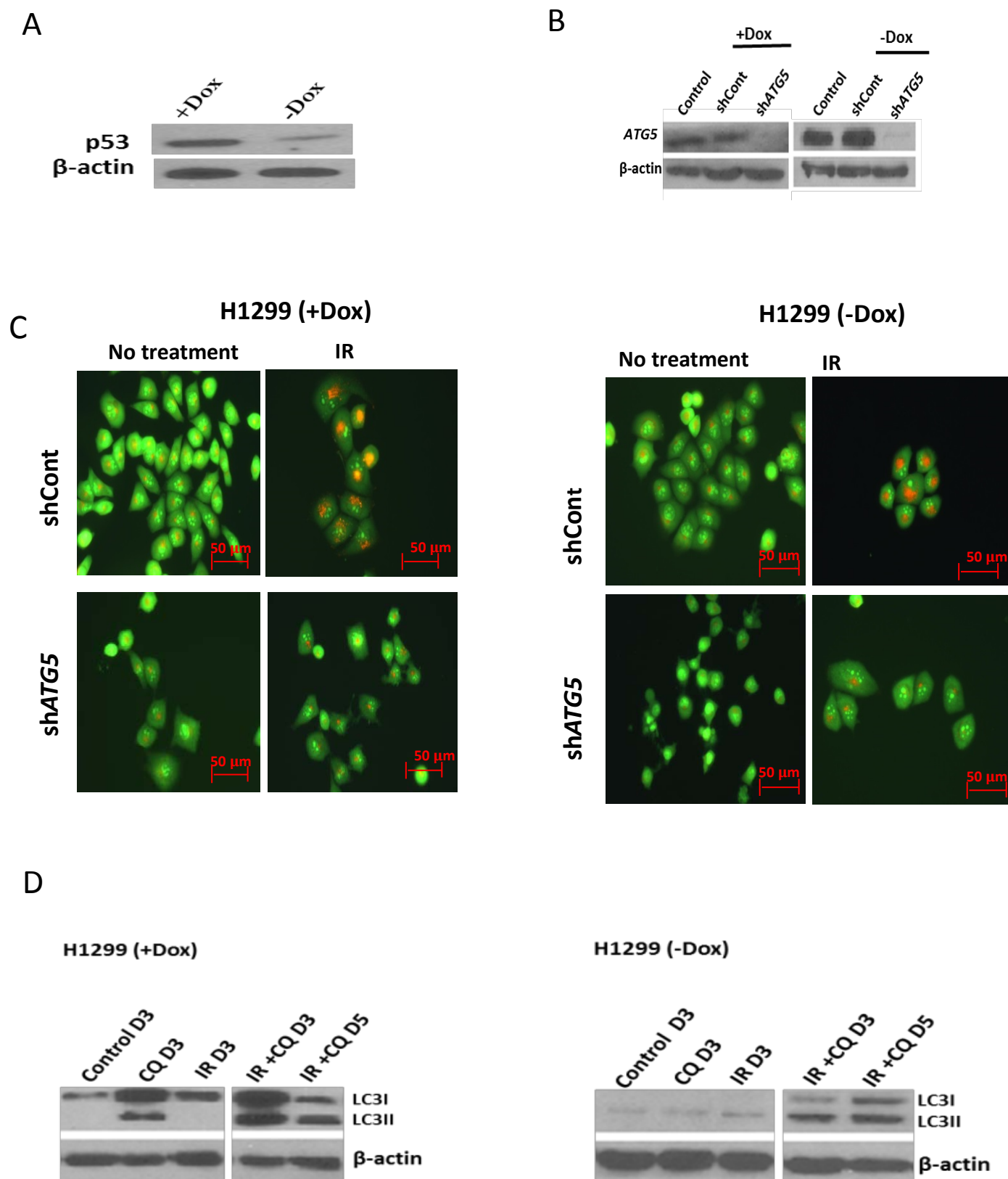


Figure 10

