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Regulation Of The Oxidative Stress Response By Arid1A

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A

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DEDICATION

This thesis is dedicated to my family; mum, dad, Suet Ying, and Rita Urlanda. To Welles Chan, who has remained supportive this whole time. I wouldn't be able to complete this thesis without him. To my best friend Danny Mak and his family, Mr. and Mrs. Mak, Andy, and Ruby Marasigan, who sadly passed away in 2014. Last but not least, this thesis is also dedicated to my dearest friend Lily Lai.

In loving memory of Timothy Chan and Ruby Marasigan.

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ABSTRACT

Regulation of the oxidative stress response by ARID1A

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SWI/SNF is mutated in about 20% of all human cancers; in particular ARID1A is the most frequently mutated SWI/SNF subunit. *ARID1A* is a tumor suppressor gene, inactivating mutations in *ARID1A* are most frequently found in ovarian and endometrial cancers, specifically uterine corpus endometrioid carcinomas (UCEC), ovarian clear cell carcinomas (OCCC) and ovarian endometrioid carcinomas (OEC). The functional roles of ARID1A are not completely understood and there are limited therapeutic strategies that specifically target *ARID1A*-mutant cancers. Given that ARID1A expression is lost in cancer, *ARID1A* mutations cannot be targeted directly and novel therapeutic strategies are required to target *ARID1A*-mutant cancers.

In this study, drug responses between *ARID1A*-wildtype and *ARID1A*-mutant cell lines were compared using the 'Genomics of Drug Sensitivities in Cancer' database. From this analysis, I found that *ARID1A*-mutant cell lines are more sensitive to elesclomol, which is a reactive oxygen species (ROS)-inducing agent. This finding was validated using a panel of ovarian and endometrial cancer cell lines, where *ARID1A*-mutant cell lines exhibited lower IC50 values and higher apoptotic rates when treated with elesclomol. Knockdown and re-expression of ARID1A in ovarian cancer cells showed that ARID1A is required to protect cancer cells from

oxidative stress. In the absence of ARID1A, intracellular ROS levels were increased, and this increase was required for increased cell growth upon ARID1A depletion.

Next, I investigated the relationship between ARID1A and NRF2, the major regulator of the antioxidant response in the cell. I found that ARID1A negatively regulates the expression of NRF2. Knockdown of NRF2 in ovarian cancer cell lines revealed that NRF2 expression may be preferentially required for protection from oxidative stress and cell proliferation in *ARID1A*-mutant cells. Analysis using The Cancer Genome Atlas (TCGA) UCEC dataset revealed that *ARID1A*-mutant tumor samples have higher expression of NRF2 and NRF2-target genes.

In summary, this study revealed novel roles of ARID1A in protecting ovarian cancer cells against oxidative stress. In the absence of ARID1A, NRF2 is up-regulated and may be required to compensate for ARID1A deficiency. These findings suggest that ROS-inducing agents and NRF2 inhibitors may be used as therapeutic strategies in targeting *ARID1A*-mutant ovarian cancer cells.

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CHAPTER 1: INTRODUCTION

1.1 Ovarian cancer

The most common type of ovarian cancer is epithelial ovarian cancer. The four major subtypes of epithelial ovarian cancers are high-grade serous ovarian carcinoma (HGSOC), ovarian mucinous carcinoma (OMC), ovarian endometrioid carcinoma (OEC) and ovarian clear cell carcinoma (OCCC). The pathogenesis of each subtype is different; each subtype is originated from a different type of cells, characterized by different genetic mutations and has different prognosis (see Table 1). Despite the pathological differences between each subtype, all ovarian cancer patients are treated with the same therapeutic regimen, i.e. surgical resection and platinum-based chemotherapy.

The most common subtype of epithelial ovarian cancer is HGSOC, and the prognosis of this subtype is poor; 25% of HGSOCs recur as platinum-resistant tumors within 6 months [1]. In addition, most HGSOCs are presented at late stage and not confined to the ovary [2]. The prognosis of OCCCs is better than HGSOCs when presented at early stage [3]. However, at late stage and in recurring disease, OCCCs have a much worse prognosis than HGSOCs and are largely resistant to chemotherapy [3, 4]. Therefore, there is an urgent need to study the biology of OCCCs and develop therapeutic strategies that directly target OCCC-specific genetic aberrations.

Epithelial ovarian cancer subtype	Percentage of all epithelial cancers	Proposed origin	Gene mutations	Prognosis	References
High grade serous	80-85	Fallopian tube	TP53 (96%), BRCA1/2 (12% and 11%)	Poor, due to most diseases present at late stage	[5, 6]
Mucinous	2-5	Cystadenoma-boderline tumor-carcinoma sequence	KRAS (60%)	Good	[7, 8]
Clear cell	5-13	Endometriosis	ARID1A (50%), PIK3CA (33%)	Good at early stages, poor at late stage and in recurrent disease	[9-11]
Endometrioid	10	Endometriosis	ARID1A (30%), PIK3CA (39%), PTEN (18%), CTNNB1 (53%)	Good	[12]

Table 1. A description of the major subtypes of epithelial ovarian cancers.

1.2 Endometriosis-associated ovarian cancers

Endometriosis commonly affects women of reproductive age and causes pain, infertility, and dysmenorrhea [13-15]. Endometriosis is the dissemination of endometrial cells outside the uterus to other organs, such as the ovaries and the peritoneum [16]. The dissemination is frequently caused by retrograde menstruation, where menstrual tissue travels backwards through the fallopian tubes and into the peritoneum. The tissue then attaches at the new site and forms endometriotic lesions [17-19].

OCCCs and OECs are frequently associated with endometriosis [20]. The current hypothesis is that these two subtypes arise from endometriotic lesions [21]. A previous study found that a gene signature specific to OCCCs is induced by treating immortal ovarian surface epithelial cells with contents of the endometriotic cysts, suggesting that the tumor microenvironment may contribute to the initiation of OCCCs [22].

Genetic aberrations found in OCCCs and OECs are very similar: the SWI/SNF subunit ARID1A is inactivated and the PI3K pathway is activated in both subtypes (see Table 1). However, some differences are noted between OCCCs and OECs, such as the frequency of *ARID1A* mutations is higher in OCCCs (50% in OCCCs vs 30% in OECs) [23]. In addition, *CTNNB1* is frequently mutated in OECs [12], whereas recurrent mutations in genes of the Wnt pathway have not been reported in OCCCs. Differences in estrogen receptor (ER) and progesterone receptor (PR) expression are also found in OCCCs and OECs; ER and PR are expressed in OECs, but low expression of ER and PR is found in the OCCCs [24, 25].

1.2 The chromatin remodeler SWI/SNF

Genes encoding the SWI/SNF subunits were originally identified in *Saccharomyces cerevisiae*. SWI stands for SWItch, because the yeast SWI genes are required for the activation of *HO* (homothallic switching endonuclease), which is important for mating-type switching [26, 27]. SNF stands for Sucrose Non-Fermenting, because the yeast SNF genes are required for the expression of *SUC2* (beta-fructofuranosidase SUC2) that is important for sucrose metabolism [28]. Early studies found that the SWI/SNF subunits interact with each other and form a multi-

subunit complex [29-31]. In addition, recent studies have identified new subunits of SWI/SNF in mammalian cells that were not found in *S. cerevisiae* [32, 33].

SWI/SNF is conserved across different organisms, including *S. cerevisiae*, *Drosophila Melanogaster* and humans (reviewed in [34]). Mammalian SWI/SNF can be further divided into two major subclasses: the BRG1-associated factor (BAF) complex and the polybromo BRG1-associated factor (PBAF) complex [35]. AT-rich interacting domain-containing protein 1A and 1B (ARID1A and ARID1B) are exclusively found in the BAF complex, whereas BAF200, BAF180, and bromodomain-containing 7 (BRD7) are exclusively found in the PBAF complex [35]. In summary, the mammalian SWI/SNF is comprised of a catalytic ATPase subunit (mutually exclusively BRG1 or BRM), several core subunits (SNF5, BAF155, and BAF170) and variant subunits.

The primary function of SWI/SNF is to remodel chromatin in an ATP-dependent manner. Generally, SWI/SNF disorders nucleosomes that were originally phased. SWI/SNF binds to nucleosomal DNA and disrupts histone-DNA interactions. A loop of DNA is created and propagates around the nucleosome, causing the nucleosome to slide or to be ejected. This provides a nucleosome free region for transcription factors to bind to their cognac sites and lead to the activation or repression of target genes [36, 37].

1.2.1 Chromatin remodeling-independent functions of SWI/SNF

Although SWI/SNF is well-known for regulating transcription through chromatin remodeling, several chromatin remodeling-independent roles of SWI/SNF have been discovered in recent studies. Dykhuizen, *et al.* found that the BAF complex is required for DNA

decatenation, i.e. the separation of newly replicated sister chromatids by topoisomerase IIa (TOP2A) at mitosis. Specifically, the BAF complex facilitates the binding of TOP2A across the genome, which requires the interaction of ARID1A with TOP2A and the ATPase activity of BRG1 [38]. A second study recently found that SWI/SNF is required for the formation of paraspeckles, which are nuclear bodies that are formed around the long non-coding RNA (lncRNA) nuclear paraspeckle assembly 1 (NEAT1) [39]. A third study has identified a BC motif in ARID1A and ARID1B. Consistent with the role of the BC motif to mediate binding of elongin C (EloC), ARID1B was found to bind to EloC, Cullin 2 (CUL2) and Roc1, forming an E3 ubiquitin ligase. ARID1B then mediates monoubiquitination on lysine 120 of histone H2B, which is an upstream event of H3K4me3 [40].

1.2.2 Alterations of the SWI/SNF complex in cancer

With the on-going efforts of The Cancer Genome Atlas (TCGA) project, it is increasingly clear that mutations in SWI/SNF are found in many if not all cancer types. Intriguingly, SWI/SNF subunits are mutated on average in 20% of all human cancers [33, 41]. The broad spectrum and frequency of mutations in SWI/SNF subunits are similar to mutations in *TP53*, the most frequently mutated gene in human cancers, indicating an extensive role of SWI/SNF mutations in human cancers [33]. Alterations found in SWI/SNF subunits are predominantly inactivating mutations (nonsense mutations, frame shift mutations and deletions) [41] , but gene translocation and protein methylation of SWI/SNF are also found in some cancer types, which were found to alter the composition of SWI/SNF and SWI/SNF-directed gene

targeting [32, 42]. In addition, in the case of BRG1 and ARID1A, loss of one of these SWI/SNF subunits resulted in dependence on BRM and ARID1B [43-46].

Previous studies have identified SWI/SNF mutations as prominent 'drivers' in tumorigenesis. Conditional knockout of *SNF5* in mice led to 100% penetrance in tumor formation, with a median onset of only 11 weeks [47]. Although loss of *ARID1A* alone does not result in tumor formation, mice formed OECs and OCCCs when inactivation of *ARID1A* was combined with *PIK3CA* mutation or inactivation of *PTEN* [48, 49]. Intriguingly, several studies found that SWI/SNF mutations are the only recurrent mutations in certain types of cancer [50-53]. These cancer types are often aggressive and associated with poor prognosis [54, 55], therefore, there is an urgent need to devise therapeutic strategies against SWI/SNF mutations. The following section highlights some SWI/SNF subunits that are frequently mutated in cancer and recent discoveries in the mechanism of how SWI/SNF mutations contribute to tumorigenesis.

ARID1A

ARID1A is the most frequently mutated SWI/SNF subunit in human cancers [33]. In early studies, due to the tumor suppressive role of SNF5 in human cancers, expression of other SWI/SNF subunits were screened in cDNA tissue arrays and panels of cancer cell lines. ARID1A expression was found to be absent in about 30% of renal cancers and 10% of breast cancers [56, 57] . In 2010, recurrent mutations in *ARID1A* were identified using whole-genome sequencing in endometriosis-associated ovarian cancers [9, 23]. The initial observations that the majority of mutations in *ARID1A* were inactivating and non-recurrent mutations led to the

hypothesis that *ARID1A* is a tumor suppressor gene. Since then, mutations in *ARID1A* were also discovered in other types of cancer, including endometrial cancer and gastric cancer [10, 58]. OCCCs have the most frequent *ARID1A* mutations in human cancers; *ARID1A* is mutated in about 50% of OCCCs [9, 23, 33]. More details of the tumor suppressive roles of ARID1A are discussed in chapter 1.3.

BRG1

BRG1 expression is lost in lung cancers [59, 60], medulloblastoma [61, 62], and Burkitt lymphoma [63] through truncating mutations and unknown epigenetic modifications. Using whole-genome sequencing approaches, three separate groups recently reported germline and somatic BRG1 mutations and subsequently loss of BRG1 protein expression in almost all cases of small cell carcinoma of ovarian cancer, hypercalcemic type (SCCOHT) [50, 52, 53]. SCCOHT is a rare and aggressive form of ovarian cancer that more than half of the patients die within 1-2 years of diagnosis [55]. Intriguingly, *SMARCA4* (gene encoding BRG1) is the only recurrently mutated gene identified in SCCOHT, suggesting that BRG1 plays an important role in this cancer type [50, 52, 53].

BRG1 and BRM are mutually exclusive ATPase subunits in the SWI/SNF. Several groups reported that BRG1-deficient cell lines are dependent on the expression of BRM [43-45]. In the absence of BRG1, BRM is incorporated into SWI/SNF to compensate for BRG1 loss. Interestingly, loss of BRM in BRG1-deficienct cell lines does not affect the assembly of SWI/SNF complexes, with the exception of loss of BAF53 binding [44, 45]. These findings

show that there is a redundancy between subunits in SWI/SNF and the composition of SWI/SNF may be altered in the absence of one subunit.

BAF155

Wang *et al*. discovered that BAF155 is a substrate of Coactivator-associated arginine methyltransferase 1 (CARM1) and is methylated at the amino acid residue R1064 by CARM1 in breast cancer. Methylated BAF155 forms a subcomplex that lacks BRG1 and BAF53, which causes alterations to its targeting in the genome and changes in gene expression. Specifically, methylated BAF155 enhances the expression of c-myc pathway genes, which ultimately promotes cell proliferation, cell migration, metastasis, and tumor recurrence [42].

SNF5

SNF5/SMARCB1 is one of the earliest identified SWI/SNF subunit that is mutated in cancer. Homozygous inactivation of SNF5 is found in malignant rhabdoid tumors, an aggressive and lethal cancer found in young children [51]. Conditional knockout of *SNF5* in mice led to 100% penetrance in tumor formation, with a median onset of only 11 weeks [47].

SS18

Although SWI/SNF mutations found in cancer are predominantly inactivating mutations, a recent study found that the SWI/SNF subunit SS18 is altered by gene translocation in synovial sarcomas. Initially, it was observed that an in-frame translocation of SS18 and one of the three SSX genes (SSX1, SSX2, and SSX4) is found in nearly all synovial sarcomas, and SS18-SSX

translocations are often the only cytogenetic abnormality in synovial sarcomas [64, 65]. These findings suggest that SS18-SSX translocation is likely a driver mutation in synovial sarcomas. Indeed, a recent study by Kadoch and Crabtree found that the SS18-SSX protein is integrated into the SWI/SNF complex and leads to the displacement of SNF5 from the complex. The altered SWI/SNF complex then localizes at the oncogene SOX2 locus and activates SOX2 gene expression, which is required for synovial sarcoma cell proliferation [32].

1.3 Tumor suppressive roles of ARID1A

The *ARID1A* gene is located on 1p36.11 and is translated into two transcripts (isoforms A and B). Isoform A (NM_006015.4) is the longer isoform, it has 20 exons and encodes a 242kDa protein. The ARID1A protein has an ARID DNA binding domain, a hypermethylated in cancer 1 (HIC1) binding domain and several leucine-rich LXXLL steroid receptor binding motifs. The following sections discuss the diverse roles of ARID1A in tumorigenesis.

1.3.1 ARID1A and the cell cycle

The cell cycle is often aberrantly regulated in cancer cells. Interestingly, before ARID1A was identified as a tumor suppressor, it was found that ARID1A regulates the cell cycle. ARID1A is required for differentiation-associated cell cycle arrest in non-transformed MC3T3-E1 preosteoblast cells by inducing p21 expression and the repression of E2F-responsive genes [66]. A subsequent study demonstrated that ARID1A does not induce p21 expression by binding to the p21 promoter, but instead represses the expression of c-myc, which is a repressor of p21

[67]. A later study found that ARID1A associates with different co-activators and co-repressors, such histone deacetylases (HDACs) and the E2F family [68].

1.3.2 ARID1A and p53

Guan et al. found that ARID1A interacts directly with p53 and regulates several downstream targets of p53, such as p21 and SMAD3. The authors further show that *ARID1A* and *TP53* mutations are mutually exclusive in endometriosis-associated ovarian cancers, suggesting that ARID1A and p53 may regulate the same set of genes, and either *ARID1A* or *TP53* mutations will disrupt the regulation of these genes [69].

1.3.3 ARID1A and the PI3K pathway

ARID1A and *PIK3CA* are frequently co-mutated in cancer; co-mutations are most frequently found in OCCCs, followed by uterine corpus endometrial carcinomas (UCEC) [48]. Strikingly, inactivation of ARID1A alone is not sufficient for tumor formation in mice, whereas combined *ARID1A* loss and *PIK3CA* mutation leads to tumor formation of OCCC histology. The authors found that the co-occurrence of *ARID1A* inactivation and *PIK3CA* mutation was required to promote cytokine signaling, specifically, the up-regulation of IL-6 [48]. A similar finding was published by Guan *et al.*, this study showed that tumor formation in mice only occurred when *ARID1A* and *PTEN* were both inactivated in the mouse ovarian surface epithelium. Consistent with the finding that *ARID1A* and *PTEN* are frequently mutated in OECs, gene expression analysis of the mice tumors found that the tumors were most similar to human OECs than other types of ovarian cancer [49].

Although these two studies showed that *ARID1A* inactivation co-operate with PI3K pathway activation to initiate tumor formation, other studies found that inactivation of *ARID1A* alone can activate the PI3K pathway. Reverse phase protein array (RPPA) analysis of UCECs found that *ARID1A*-mutated samples showed increased phosphorylation of AKT and p70S6K compared to *ARID1A*-wildtype samples even in the absence of *PIK3CA* and *PTEN* mutations. Consistent with this finding, knockdown of ARID1A using siRNA in endometrial cancer cell lines resulted in increased phosphorylation of AKT and p70S6K [70]. In a second study, knockdown of ARID1A using siRNA in MCF7 breast cancer cells and MRC5 primary cells resulted in increased phosphorylation of AKT and increased sensitivity to PI3K and AKT inhibitors [71]. In a third study, it was found that PIK3IP1, a negative regulator of the PI3K pathway, is a direct target activated by ARID1A. In the absence of ARID1A, PIK3IP1 is not expressed and leads to up-regulation of the PI3K pathway [72].

1.3.4 ARID1A and EZH2

The Polycomb complex is an epigenetic regulator that represses gene expression [73]. Increasing evidence shows that the Polycomb complex, in particular the PRC2 subunit EZH2, is overexpressed and has oncogenic functions in cancer [74]. Antagonism between SWI/SNF and the Polycomb complex was first described in *Drosophila*, where SWI/SNF mutations were found to suppress developmental defects mediated by mutations in the Polycomb complex [75]. Specifically, this was due to the differential regulation of *HOX* genes during embryogenesis, where SWI/SNF mediates the activation and the Polycomb complex suppresses the expression of *HOX* genes [76]. The antagonism between SWI/SNF and EZH2 is conserved in the

mammalian system. Re-expression of the SWI/SNF subunit SNF5 in SNF5-deficient human malignant rhabdoid tumor cells caused eviction of the polycomb complex for the $p16^{INK4a}$ locus and activation of $p16^{INK4a}$ expression [77]. In addition, loss of SNF5 leads to up-regulation of *EZH2* expression and aberrant activation of a stem cell-associated program that is dependent on EZH2 [78]. A recent study reported that antagonism also exist between ARID1A and EZH2 in OCCCs. By screening 15 small molecules that inhibit epigenetic regulators, Bitler et al. found that inhibition of EZH2 and ARID1A deficiency are synthetically lethal. Although unlike SNF5, ARID1A did not regulate EZH2 expression, the authors found that the gene PIK3IP1 is co-regulated by ARID1A and EZH2. PIK3IP1 is not expressed in *ARID1A*-mutant OCCCs, but inhibition of EZH2 caused re-expression of PIK3IP1 and ultimate led to cell death [72].

1.3.5 ARID1A and DNA repair

It has been well characterized that the SWI/SNF is required for DNA repair, specifically, the repair of DNA double-strand breaks (DSBs) [79]. A previous study found that ARID1A is required for non-homologous end joining (NHEJ) [80]. In addition, a recent study by Shen et al. found that ARID1A is recruited to DSBs by Ataxia telangiectasia and RAD3-related protein (ATR). In the presence of DSBs, ARID1A is required for the phosphorylating and activating of ATR, sustaining DNA damage signaling and repairing DSBs by homologous recombination [81]. It has been well known that BRCA1/2 deficiency or DSBs repair deficiency leads to increase sensitivity to PARP inhibitors [82, 83]. Similarly, the author found that *ARID1A*-mutant cells were more sensitive to PARP inhibition [81] .

1.3.6 ARID1A and epithelial-mesenchymal transition

In gastric cancer cells, loss of ARID1A leads to increased cell migration and cell invasion by down-regulating E-cadherin. Loss of ARID1A also leads to nuclear and cytoplasmic relocation of β-catenin from the cell membrane and induces epithelial-mesenchymal transition (EMT) [84].

1.3.7 Therapeutic targets for ARID1A-deficient cancers

Several therapeutic targets for *ARID1A*-mutant cancers have been described. As mentioned before in chapters 1.3.3, 1.3.4 and 1.3.5, PI3K, EZH2 and PARP inhibitors can be used to target *ARID1A*-mutant cancer cells. Recently, a synthetic lethal relationship between ARID1A and ARID1B was also described. ARID1B is a homolog of ARID1A, which is found mutually exclusive to ARID1A in the SWI/SNF complex. Similar to the finding that the concomitant loss of BRG1 and BRM is synthetically lethal [43-45], Helming et al. found that the combined deficiency of ARID1A and ARID1B is synthetically lethal. Loss of both ARID1A and ARID1B leads to destabilization of the SWI/SNF complex and ultimately lead to inhibition of cell growth [46].

1.4 Reactive oxygen species

1.4.1 ROS production

In the cell, ROS are produced as a byproduct of metabolic activity. The mitochondrial electron transport chain (ETC), the endoplasmic reticulum (ER) and the NADPH oxidases (NOX) are the major sources that produce ROS in the cell.

In the mitochondria, O_2 is reduced to water during the generation of ATP. Superoxide is produced by the one-electron reduction of O_2 and then converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) [85, 86]. H_2O_2 then diffuses out of the mitochondria into the cytoplasm, which forms a reactive hydroxyl radical via the Fenton reaction in the presence of iron [87].

In the cell, the ER is important for protein folding. The ER favors the formation of disulfide bonds by providing an oxidizing-folding environment. ROS is produced when cysteine residues are oxidized during the formation of disulfide bonds. The formation of a disulfide bonds is catalyzed by protein disulfide isomerase (PDI) and ER oxidoreductase 1 (ERO1) [88]. The presence of misfolded protein causes the activation the unfolded protein response (UPR) and oxidative stress, which can subsequently lead to apoptosis [89].

The NOX enzyme NOX2 was first described in neutrophils. Since the discovery of NOX2, which is specific for phagocytes, other NOX enzymes have been described (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) [90]. The NOX enzymes are transmembrane proteins that catalyze the transfer of electrons across membranes. Specifically, superoxide is produced by electrons transported from NADPH to O_2 by a flavin- and heme-containing protein complex [91-93].

Other sources of ROS production in the cell have also been described. These include lipoxygenase, xanthine oxidase, cytochrome P450 monooxygenase, nitric oxide synthase and the peroxisome [94-98].

1.4.2 ROS detoxification

There are three main pathways that mediate ROS detoxification in the cell: reduced glutathione (GSH), thioredoxin (TXN) and catalase (Figure 1).

The most abundant antioxidant in the cell is GSH [99]. XCT (encoded by *SLC7A11*) and CD44 mediate the export of glutamate from the cell, in exchange with the import of cystine into the cell [100]. Cystine is reduced into cysteine in a NADPH-dependent manner. Glutamate is produced by glutaminase 1 (GLS1) and GLS2 from glutamine. GSH is produced from cysteine and glutamine by the glutamate cysteine ligase (GCL), which is formed by the GCL catalytic (GCLC) and GCL modifier (GCLM) subunits. GSH can also be generated from NADPH by glutathione reductase (GSR). Glutathione peroxidase (GPX) and glutathione S-transferase (GST) catalyzes the formation of oxidized glutathione (GSSG) from 2 GSH molecules [101].

In cells depleted of GSH, the activity of the TXN pathway is increased and can effectively reduce ROS levels [102]. Reduced TXN is regenerated from oxidized TXN by thioredoxin reductase (TXNRD) using NADPH [99]. Catalase is a heme-containing protein found predominantly in peroxisomes and catalyzes the conversion of hydrogen peroxide to water and O_2.

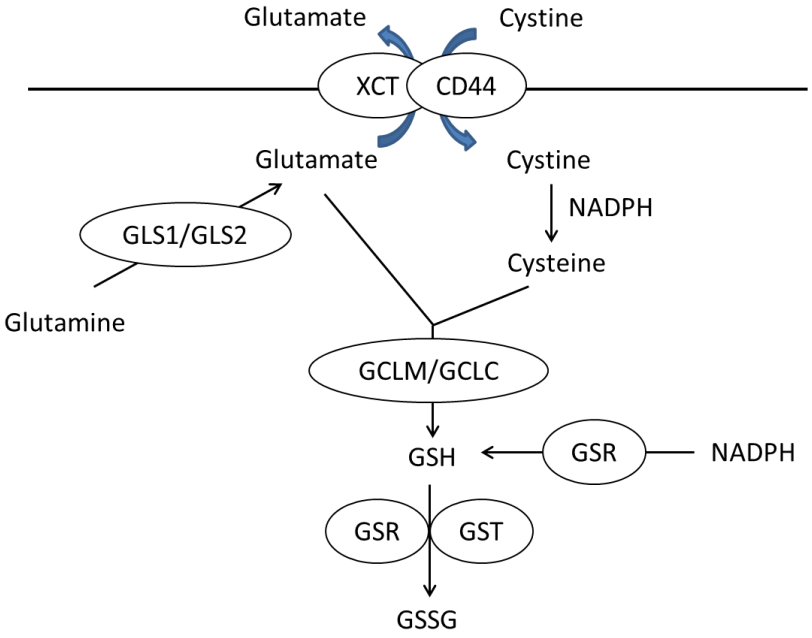

Figure 1. Schematic diagram of formation of GSH.

1.5 ROS and cancer

1.5.1 Mechanisms that lead to elevation of ROS in cancer cells

Previous studies have found that cancer cells of different cancer types have higher ROS than normal cells. The increase of ROS in cancer cells is caused by several reasons, including increased metabolic activity, genetic aberrations, mitochondrial dysfunction and the tumor microenvironment.

Aberrations in tumor suppressors and oncogenes have been found to alter intracellular ROS levels. Loss of tumor suppressors can cause an increase in ROS levels. Loss of p53 has been found to lead to in an increase of oxidative stress in different cell types. p53 suppresses ROS levels in the cell by regulating the transcription of pro-oxidant and antioxidant genes, such as *TIGAR* and *SESN2* [103]. Loss of BRCA1 has also been found to increase ROS, protein

nitration and 8-OHdG lesions [104]. Mechanistically, BRCA1 interacts with NRF2, promoting its stability and activation [105]. The FOXO transcription factors have been demonstrated to protect cells from oxidative stress by inducing the expression of manganese superoxide dismutase (SOD2) [106]. Oncogenes have also been found to up-regulate ROS. Activating mutations in the RAS family oncogenes have been well documented to up-regulate ROS. Mutant HRAS has been found to up-regulate ROS by up-regulating NOX4 expression [107, 108], whereas mutant KRAS has been found to up-regulate NOX1 activity by promoting activation of the NOX1 cytosolic subunit $p47^{phox}$ [109]. In multiple myeloma cells, overexpression of c-myc has been found to increase ROS levels by down-regulating genes involved in the GSH and TXN antioxidant pathways [110] .

Mitochondrial dysfunction can be caused by mutations in genes of the tricarboxylic acid (TCA) cycle. Hereditary kidney cancers are characterized by inactivating mutations in *fumarate hydratase* (*FH*) and *succinate dehydrogenase* (*SDH*). Loss of these enzymes leads to increased aerobic glycolysis and elevation in ROS production [111, 112].

A hypoxic tumor environment has been found to enhance the generation of mitochondrial ROS. During hypoxia, ROS released from the complex III of the ETC to the intermembrane space is increased [113]. However, the specific mechanisms have not been described.

1.5.2 Effects of ROS in cancer cells

ROS can exert opposite effects in the cell depending on its level. Low levels of ROS can have oncogenic effects by promoting cell growth and survival. ROS has been found to activate

several cell signaling pathways important in cancer, including the PI3K and EGFR pathway, by inactivating protein tyrosine phosphatases. Protein tyrosine phosphatases are important negative regulators of signaling pathways. Specifically, a cysteine residue is found in the active sites of all protein tyrosine phosphatases. This cysteine residue is vulnerable to oxidation by H_2O_2 due to its low pK_a value [114]. Oxidation and disulphide bond formation of the active site cysteine residue leads to inactivation of the protein tyrosine phosphatases. One such example is PTEN, a well-known negative regulator of PI3K/AKT signaling. During oxidation by H2O2, the Cys^{124} reside in the active site of PTEN forms a disulfide bond with the Cys^{71} residue [115]. Another example is PTP1B, which negatively regulates EGF signaling by dephosphorylating tyrosine residues on EGFR. Oxidation of the Cys^{215} residue was found to cause the inactivation of PTP1B [116]. Some transcription factors were also found to be responsive to ROS. The activation of the transcription factor NF-κB by H2O2 has been studied extensively. The transcription HIF-1α is important for angiogenesis, glycolysis and cell survival in cancer. Addition of the oxidants H2O2 and menadione has been found to prevent the degradation and enhance the transcriptional activity of HIF-1α in an AMPK-dependent manner [117].

At moderate levels, ROS can oxidize DNA, thereby promoting mutagenesis and genetic instability. Depletion of p53 has been shown to increase oxidative DNA damage, karyotype instability and lymphomagenesis, which could be reversed by the antioxidant NAC [103]. However, high levels of ROS can cause oxidative damage to macromolecules (lipid, protein, and DNA), leading to senescence and cell death (see chapter 1.5.4).

One study demonstrates the opposite effects of ROS by expressing the activated form of HRAS (HRAS-G12V) in normal human fibroblasts. It has been well characterized that HRAS-

G12V expression initially leads to hyperproliferation, but eventually leads to senescence [118, 119]. This study found that expression of HRAS-G12V leads to the accumulation of ROS, and this increase in ROS was required for HRAS-G12V mediated hyperproliferation. However, the increase of ROS also activated the DNA damage response and the cells undergo senescence [107].

1.5.3 Oxidative stress adaptation in cancer cells

As mentioned in chapters 1.5.1 and 1.5.2, cancer cells have high basal level of ROS, but high levels of ROS have detrimental effects on the cell. Therefore, mutations and adaptation in cancer cells are required to up-regulate the cells' antioxidant system to maintain ROS from reaching toxic levels.

Since the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is the major regulator of the antioxidant response in the cell, it is not surprising that cancer cells harbor mutations in *NFE2L2* (gene encoding NRF2) and its negative regulator *KEAP1*, which result in increased activity of NRF2. *NFE2L2* and *KEAP1* are most frequently mutated in lung cancers, although different lung cancer subtypes display different mutation frequencies in these two genes. Sequencing results from the TCGA database found that *NFE2L2* is altered in 4% of lung adenocarcinomas and 19% of lung squamous cell carcinomas, whereas *KEAP1* is altered in 17% of lung adenocarcinomas and 12% of lung squamous cell carcinomas [120, 121].

NFE2L2 mutations have been identified in the DLG and ETGE motifs, which are important for facilitating binding to KEAP1. Characterization of three *NFE2L2* mutations (E79K, T80K and L30F) identified in patient samples found that these mutations enhanced

NRF2 stability and transcriptional activity [122]. *KEAP1* mutations found in lung squamous cell carcinomas are predominantly missense mutations. Characterization of KEAP1 mutants found that some mutants were only able to partially suppress NRF2 activity. Intriguingly, a subset of some KEAP1 mutants can lead to enhance NRF2 stability, despite these mutants are still able to bind to NRF2 and facilitate NRF2 ubiquitination [123]. In addition, loss-of-function mutations and homozygous deletion of the ubiquitin ligase CUL3, which ubiquitinates NRF2, were identified in some cancer types, including lung squamous cell carcinomas and papillary renal cell carcinomas [120, 124]. Depletion of CUL3 was found to enhance NRF2 protein levels and transcriptional activity [124, 125].

In some instances, increased NRF2 activity is still observed in the absence of mutations within the NRF2-KEAP1-CUL3 complex. This can be due to alterations in genes encoding binding partners of KEAP1. For example, dipeptidyl peptidase 3 (*DPP3*) is amplified, overexpressed and positively correlated to NRF2 activity in lung squamous cell carcinoma. Mechanistically, DPP3 competes with NRF2 for binding of KEAP1, thus preventing KEAP1 from binding NRF2 and facilitating NRF2 ubiquitination [126]. Another example is the regulation of NRF2 by ABL1. As mentioned in chapter 1.5.1, *FH* deficiency leads to an accumulation of fumarate in the mitochondria and subsequently high oxidative stress. In *FH*-deficient hereditary leiomyomatosis and renal cell carcinoma (HLRCC), the activity of the oncogene ABL1 is up-regulated and stimulates the nuclear translocation and transcriptional activity of NRF2. NRF2 subsequently activates its target antioxidant genes NQO1 and HMOX1 to protect the cell from FH-mediated oxidative stress [127].

In summary, although cancer cells generate high levels of ROS, the antioxidant system in cancer cells is up-regulated to prevent ROS from reaching a level that becomes toxic to the cells. This forms the basis of targeting the redox balance in cancer cells; by further increasing ROS levels or inactivating the antioxidant system.

1.5.4 Enhancing ROS as a therapeutic strategy for cancer treatment

As mentioned in chapter 1.5.2, intracellular ROS have both tumor promoting and anti-tumor properties depending on the level. Due to the tumor promoting effects of ROS, it has been of interest to use antioxidants as cancer treatments, but the results have been inconsistent. In fact, a recent study found that the antioxidants N-acetyl-cysteine (NAC) and vitamin E enhanced cell proliferation of *KRAS*/*BRAF*-mutant lung cancer cells. Specifically, the antioxidants promoted proliferation by suppressing ROS levels, reducing ROS-induced DNA damage and p53 expression [128]. This study aligns with previous findings that cancer cells produce excessive ROS and are dependent on antioxidants to counteract the detrimental effects of ROS. Therefore, enhancing oxidative stress rather than decreasing oxidative stress is a more promising therapeutic strategy. A variety of small molecule inhibitors have been developed to enhance oxidative stress either by inhibiting the antioxidant system or increasing ROS production. Several studies have yielded promising results using these small molecule inhibitors.

A study published by Harris et al. demonstrated that the antioxidant GSH and TXN pathways are required for tumor initiation and progression. The authors investigated the effects of inhibiting the antioxidant GSH and TXN pathways using gene knockout and small molecules

(Buthionine-[S,R]-sulfoximine, sulfasalazine and auranofin) in a mice model that develops spontaneous mammary tumors. The authors found that cancer initiation requires synthesis of the antioxidant GSH, which suggests that increased oxidative stress is detrimental to the onset of cancer. However, inhibiting GSH synthesis has no anti-tumor effects in established tumors, because the alternative antioxidant TXN pathway is activated and compensates for the loss of GSH. Although single inhibition of the antioxidant GSH or TXN pathways does not have anti-tumor effects in cancer cells, the combined inhibition of the GSH and TXN antioxidant pathways was able to induce cell death [102].

In multiple myeloma, a subset of cells overexpresses MYC, leading to increased oxidative stress and subsequently DNA damage. These myc-overexpressing cells were found to be sensitive to the ROS-inducer piperlongumine, which exacerbates oxidative stress and DNA damage, ultimately leading to apoptosis [110].

Together, these studies have demonstrated that 1) maintaining intracellular ROS levels below a toxic threshold is crucial for cancer cell survival, and 2) augmenting oxidative stress in cancer cells is a promising and feasible therapeutic strategy.

1.5.5 Elesclomol

Elesclomol, formerly known as STA-4783, is a small molecule that potently induces oxidative stress in the cell. Using microarray analysis, it was found that treatment of elesclomol induces an oxidative stress gene expression signature. This gene expression signature includes heat shock proteins (e.g. *HSP70*, *HSPH1*, and *HSP40*), metallothioneins (e.g. *MT1F*, *MT1H*,

MT1G) and antioxidant genes (*HMOX1*). The induction of oxidative stress by elesclomol ultimately leads to apoptosis, which is reversed by the addition of the antioxidant NAC [129].

Elesclomol requires copper for drug action. Upon binding of copper ions to elesclomol, the conformation of elesclomol is changed. This facilitates the uptake of elesclomol into the cell, and elesclomol generates ROS in the cell by undergoing redox cycling of Cu(II) to Cu(I) [130, 131]. Interestingly, the mechanism of action of elesclomol is not mediated through a specific protein target, but rather through disrupting the election transport chain in the mitochondria [132].

In preclinical studies, elesclomol has modest clinical activity when used as a single agent in, but has synergistic activities when used in combination with paclitaxel [133]. However, when elesclomol was used in combination with paclitaxel in a Phase III clinical trial for unselected stage IV metastatic melanoma patients, there was no significant improvement in progression free survival [134].

1.5.6 Piperlongumine

Piperlongumine is a small molecule that was first identified as a potent inducer of apoptosis in cancer cells but not normal cells, which is due to the ability of piperlongumine to increase ROS selectively in cancer cells [135]. In the study by Raj et al., the authors found that piperlongumine induces p53 expression, but also induces the pro-apoptotic p53 target BCL2 binding component 3 (PUMA) in the absence of p53. In contrast to elesclomol's mechanism of action, piperlongumine potentiates its effects by interacting with several proteins that are known to regulate oxidative stress, these include glutathione S-transferase pi 1 (GSTP1) and carbonyl

reductase 1 (CBR1). In particular, it was demonstrated that piperlongumine binds directly to GSTP1 and inhibits its activity. Interestingly, oncogenic transformation of normal cells resulted in increased expression of GSTP1 and CBR1, which may be required to protect transformed cells from transformation-induced oxidative stress. Overexpression of GSTP1 and/or CBR1 was able to partially rescue cells from piperlongumine-induced ROS levels and cell death [135].

1.5 SWI/SNF and oxidative stress

Given that SWI/SNF is conserved across species, it is not surprising that studies have found that SWI/SNF is required to mediate resistance to oxidative stress in different model organisms.

In *S. cerevisiae*, an unbiased genetic screen found that deletion of several SWI/SNF subunits (yeast orthologs SNF2, SNF5, SNF6, and SWI3) resulted in increased sensitivity to oxidative stress [136].

In *C. elegans*, the DAF-2/DAF-16 axis is a master regulator of stress resistance and longevity. Riedel *et al.* recently discovered the BAF sub-class of SWI/SNF, but not the PBAF sub-class, is a cofactor for DAF-16, and is essential for DAF-16 mediated dauer formation, stress resistance and longevity. This study found that SWI/SNF is recruited by DAF-16 to DAF-16 target genes and activate transcription. Depletion of SWI/SNF fully suppressed the enhanced resistance of DAF-2 mutants in the presence of oxidative stress. Gene expression analysis found that genes co-dependent on SWSN-1 and DAF-2 for expression were enriched in GO-terms ‘defense response’ and ‘oxidative reduction’ [137].

In humans, oxidative stress activates the nuclear translocation of the transcription factor NRF2 and NRF2 activates cytoprotective genes, including the antioxidant heme oxygenase 1 (*HMOX1*). Zhang et al. found that BRG1 was required to activate *HMOX1* expression after treatment with diethylmaleate, a glutathione depleting compound. They found that NRF2 recruits BRG1 to the *HMOX1* promoter, BRG1 facilitates Z-DNA formation and recruits RNA polymerase II, thereby activating *HMOX1* gene expression [138]. BRG1 not only regulate the expression of antioxidant genes, it can also protect the cell from oxidative stress damage by a distinct mechanism. Du et al. found that in the presence of oxidative stress, BRG1 forms a complex with Fanconi anemia (FA) proteins at the promoter of antioxidant genes, and protects the promoters from oxidative DNA damage. They also found that depletion of BRG1 resulted in increased sensitivity to H2O2 [139].

1.6 Implications of oxidative stress in the pathogenesis of endometriosis-associated ovarian cancers

Although the tumor progression of CCCs and OECs not completely clear, previous findings suggest that a microenvironment of persistent oxidative stress may contribute to the initiation OCCCs and OECs. As mentioned in chapter 1.2, previous findings suggest that OCCCs and OECs arise from endometriosis. Endometriotic cysts that develop from endometriosis accumulate hemorrhagic blood during the menstrual cycle. When compared with non-endometriotic cysts, the concentration of free iron (iron ions) and the expression of oxidative stress-related markers, such as lactose dehydrogenase, potential antioxidant, lipid peroxide, and 8-OHdG, were much higher in endometriotic cysts [140]. This is likely due to the production of ROS by free iron via the Fenton reaction. Indeed, in vitro studies found that when

cells were treated with the contents of the endometriotic cysts, cellular ROS was increased. In addition, the DNA mutation rate of cells treated with contents of endometriotic cysts. Consistent with these findings, endometriosis-associated ovarian cancers (OCCCs and OECs) had higher concentration of free iron and expression of oxidative stress-related markers compared to ovarian cancer not associated with endometriosis (HGSOCs and OMCs) [140].

One mechanism that OCCCs can adapt to a high oxidative stress microenvironment is through HNF1β overexpression. The promoter of *HNF1B* is frequently hypomethylated in OCCCs and as a result, HNF1β is frequently overexpressed in this ovarian cancer subtype [141]. A recent study found that HNF1β enhances intracellular GSH levels by increasing the cystine transporter rBAT expression, leading to lower intracellular ROS levels and increasing cell survival in the presence of stress, such as hypoxia and oxidative stress. HNF1β also alters cellular metabolism by shifting from oxidative phosphorylation to aerobic glycolysis, which may lead to lower intracellular ROS production, as oxidative phosphorylation is the main source of ROS generation in the cell [142]. However, the roles of other genes that are frequently mutated in OCCCs and OECs in the oxidative stress response are unknown.

1.7 Hypothesis and specific aims

Hypothesis: ARID1A deficiency leads to sensitivity to oxidative stress. In the absence of ARID1A, other compensatory antioxidant pathways are up-regulated.

Specific aim 1: Investigation of whether ARID1A mediates resistance to oxidative stress

As mentioned in chapter 1.5, previous studies found that SWI/SNF mediates resistance to oxidative stress in *S. cerevisiae* and *C. elegans*. However, the relationship between SWI/SNF and oxidative stress resistance is unclear in mammalian cells. Given that SWI/SNF is conserved across different species, ARID1A may be required for resistance to oxidative stress in mammalian cells. As ARID1A is frequently inactivated in various cancer types, targeting ARID1A-deficient cancer cells with ROS-inducing agents may be a feasible therapeutic strategy. In this aim, first, drug sensitivities of ARID1A-wildtype were compared with ARID1A-mutant cancer cell lines using the Genomics of Drug Sensitivity in Cancer (GDSC) database. Then, ovarian cancer cells were used to investigate the effects of depleting and re-expressing ARID1A on oxidative stress resistance.

Specific aim 2: Investigation of the relationship between ARID1A and NRF2

As mentioned in chapter 1.5.3, the transcription factor NRF2 is the major regulator of the antioxidant response in the cell. Mutations and up-regulation of NRF2 were found in several cancer types, leading to aberrant activation of NRF2 and NRF2-target genes. Several studies have found that NRF2 has oncogenic functions in cancer, such as promoting cell proliferation. A previous study found that the SWI/SNF subunit BRG1 was found to interact with NRF2 and increase NRF2 transcriptional activity. However, whether ARID1A also regulates NRF2 activity is unknown. In addition, whether NRF2 activity is de-regulated in ovarian cancer is also unknown. This aim sought to determine the relationship between ARID1A and NRF2 activity in ovarian cancer cells.

CHAPTER 2: MATERIALS AND METHODS

2.1 Analysis of drug sensitivity in *ARID1A*-mutant cell lines using the Genomics of Drug Sensitivity database

Drug sensitivities of cell lines (release 4, March 2013) were obtained from the Genomics of Drug Sensitivity in Cancer (GDSC) database [143]. Mutational status and copy number of *ARID1A* in cancer cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE) using the cBioPortal for Cancer Genomics [144]. Cell lines that had no mutation and copy number alteration data were removed from further analysis. Based on *ARID1A* mutational status, cell lines were grouped into *ARID1A*-wildtype (no detectable *ARID1A* mutations, n = 347) or *ARID1A*-mutant (nonsense mutations, frameshift mutations, or deep deletion, n = 74). Cells lines with missense mutations, in-frame insertion/deletion, or splicing mutations in *ARID1A* were removed from further analysis, because the effects of these mutations on ARID1A expression and function are unclear.

To detect differences in drug sensitivity between *ARID1A*-mutant and *ARID1A*-wildtype cell lines, we performed permutation tests using the "marker selection" function of GENE-E (www.broadinstitute.org/cancer/software/GENE-E/). For each drug, a test statistic was calculated to assess the difference in drug response between the *ARID1A*-mutant and *ARID1A*-wildtype groups; next, the significance of the test score was estimated with 1000 permutations; then, multiple hypotheses testing was corrected by computing both the false discovery rate (FDR) and the family-wise error rate (FWER). Two-tailed t-test (T) was used to calculate significance. Negative T-values plus significant p-values show that *ARID1A*-mutant cell line are more sensitive to the corresponding drug than *ARID1A*-wildtype cell lines.

2.2 Cell culture

All cell lines were cultured in RPMI-1640 medium, supplemented with 10% FBS and 1% penicillin/streptomycin unless otherwise stated. Ovarian cancer RMG1, TOV21G, ES2, and endometrial cancer HEC-1A cells were purchased from American Type Culture Collection (ATCC). HEC-1A cells were cultured in McCoy's 5A medium. Ovarian cancer MDA2774 cells were gifts from Dr. Ralph Freeman (MD Anderson Cancer Center, USA) [145]. Ovarian cancer OVCA420 and OVCA432 cells were a gift from Dr. Robert Bast (MD Anderson Cancer Center, USA) [146]. Endometrial cancer AN3CA cells were purchased from the MD Anderson Cancer Center's Characterized Cell Line Core Facility and cultured in Eagle's Minimum Essential Medium (MEM). Ovarian cancer A2780 [147], COV318, COV362 [148] cells and endometrial cancer MFE-280 cells were purchased from European Collection of Cell Cultures (ECACC). COV318 and COV362 cells were cultured in DMEM medium supplemented with 2mM L-glutamine. MFE-280 cells were cultured in 40% RPMI-1640 medium and 40% MEM (with Earle's salts) supplemented with 2mM L-glutamine, 20% FBS, and 1X insulin-transferrin-sodium selenite. Ovarian cancer SMOV2 [149] and KOC7C cells were gifts from Dr. Hiroaki Itamochi (Tottori University, Japan). Ovarian cancer IGROV1 cells were gifts from Dr. Susan Holbeck (National Cancer Institute, USA) [150]. All Cell lines were cultured at 37^{o}C in 5% CO_2 and were tested negative for mycoplasma. Cell lines were maintained for 20-30 passages.

2.3 PCR amplification of ARID1A

Genomic DNA was harvested from cells using the PureLink Genomic DNA mini kit (Life Technologies) following the manufacturer's protocol. The sequences of PCR primers and

the PCR cycling conditions used to amplify ARID1A exons 1-20 were previously published [9]. PCR was performed in 50μL reactions containing 1X MyTaq Red Mix (Bioline), 1.5μM of forward primer, 1.5μM of reverse primer, 6% DMSO and 20ng of DNA. PCR reactions were purified using the PureLink PCR purification kit (Life Technologies). Purified PCR products were sent to the MD Anderson Cancer Center Sequencing and Microarray Facility for Sanger sequencing.

2.4 Western blot

Cells were washed twice in ice-cold PBS and scraped on ice in ice-cold RIPA buffer (Sigma Aldrich) supplemented with protease inhibitor cocktail (Sigma Aldrich). Protein lysate was collected after centrifuging the cells at 13,000 rpm for 10 mins at 4^{o}C. Protein concentration was determined using the Bio-rad protein assay that is based on the Bradford assay. For each sample, 25μg of protein was loaded onto a SDS-PAGE gel. Protein on the gel was transferred to a nitrocellulose membrane at 15V for 1 h, The membrane was blocked with 5% non-fat milk for 1 h, then incubated with primary antibody overnight at 4^{o}C. Primary antibodies used were: anti-ARID1A (Sigma Aldrich), anti-BRG1 (Cell Signaling Technology), SNF5 (Cell Signaling Technology), anti-NRF2 (Cell Signaling Technology), and anti-vinculin (Cell Signaling Technology). Afterwards, the membrane was washed twice in PBST and incubated with anti-rabbit (1:3000, Cell Signaling Technology) or anti-mouse (1:5000, Santa Cruz) horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The bands were visualized using the ECL plus western blotting reagent (Amersham Biosciences). Vinculin was used as a loading control.

2.5 Chemicals and inhibitors

Elesclomol and piperlongumine were purchased from Selleck Chemicals. *N*-acetyl-L-cysteine (NAC) and H_2O_2 were purchased from Sigma Aldrich. Cisplatin and paclitaxel were purchased from MD Anderson Cancer Center. Brustatol was purchased from Carbosynth. Working solutions were made fresh before each experiment.

2.6 Cell growth assay

Cells were seeded in 96-well plates for 24 h before addition of drugs. After 72 h of drug treatment, cell growth was determined using the WST-1 assay (Roche) according to the manufacturer's instructions. To determine sensitivity to elesclomol in the ovarian and endometrial cancer cell line panel, data was presented as the mean of 6 replicate wells. For siRNA transfected cells, data was represented as the mean of 3 replicate wells of 3 independent experiments. Dose-response curves were constructed using GraphPad Prism version 6 (Graphpad Software) and IC50 values were interpolated from the graphs.

2.7 Annexin V staining

Cells were treated with the indicated drugs for 72 h. Floating cells were collected and adherent cells were harvested by trypzinisation. Cells were centrifuged at 1000 rpm for 5 mins at 4^oC and washed once in ice-cold PBS. Cells were resuspended in 1X annexin binding buffer (50mM HEPES, 700mM NaCl, 12.5mM $CaCl_2$, pH7.4) (Life Technologies) and counted using an automated cell counter. 1 x 10^5 cells were stained in 100µL of 1X annexin binding buffer (Life Technologies) with 5µl annexin V-APC (BD Pharmingen) for 15 mins at room

temperature in the dark. Prior to analysis, 400μL of 1X annexin binding buffer and 2μg/mL of propidium iodide (Life Technologies) were added to each sample. For each sample, at least 10,000 cells were analyzed using a FACS Gallios Flow Cytometer (Beckman Coulter). Cells were gated to include single cells only. Data analysis was performed using the Kaluza Analysis Software (version 1.3, Beckman Coulter). Percentage of annexin V-positive cells included both annexin V/PI double-positive and annexin V-positive/PI-negative cell populations.

2.8 siRNA transfections

Cells were seeded for 24 h, then transfected with 20nM of siGENOME smartpool ARID1A (M-017263-01), BRG1 (M-010431-00), SNF5 (M-010536-01) and NRF2 (M-003755-02) siRNA (Dharmacon) and lipofectamine RNAiMAX (Life Technologies) in opti-mem reduced serum (Life Technologies) according to the manufacturer's instructions. Control cells were transfected with non-targeting siRNA pool #2 (Dharmacon).

2.9 Re-expression of ARID1A

The pCI-neo-ARID1A plasmid was a gift from Dr. Weidong Wang [151]. The pCI-neo mammalian expression plasmid is a product of Promega and the map of the vector is shown in Figure 2. The pCI-neo-ARID1A plasmid received from Dr. Weidong Wang originally harbored three non-silent mutations in the *ARID1A* open reading frame (ORF), therefore the *ARID1A* ORF was reconstructed back to wildtype by mutagenesis. Mutagenesis was carried out by Dr. Oskar Laur at Custom Cloning Core Facility at Emory University. Sanger sequencing using primers listed in Table 2 was used to confirm that the sequence of *ARID1A* was wildtype. The

control plasmid without the *ARID1A* ORF (pCI-neo) was generated by digesting the pCI-neo-ARID1A plasmid with restriction enzymes Xho1 and Sal1, followed by re-ligation using T4 DNA ligase (Promega). The absence of the *ARID1A* ORF was confirmed by sequencing the re-ligated vector using the T7 EEV promoter primer.

For transient transfection, ARID1A-deficient TOV21G cells were plated onto a 6-well plate and transfected with pCI-neo or pCI-neo-ARID1A vector. Briefly, 2µg of plasmid DNA, 4µL of p3000 reagent and 3.75µL of Lipofectamine 3000 (Life Technologies) were diluted in 250µL Opti-mem reduced serum (Life Technologies) and incubated for 30 mins. The cells were transfected for 24 h before being trypsinized, counted and re-plated for subsequent experiments.

Primer	**Sequence (5' – 3')**
T7 EEV F1	AAGGCTAGAGTACTTAATACGA
T7 EEV R1	TGGTTTGTCCAAACTCATCA
BAF250a F1	CAGCAGAACTCTCACGACCA
BAF250a F2	AACCCATACTCGCAGCAACA
BAF250a F3	TTTCCAGCAGCCAAGGAGAG
BAF250a F4	AATCAAGGGGGCATGATGGG
BAF250a F5	AGGCATGAGCAGGAGCAATT
BAF250a F6	TGGCAATCAGTTCTCCACCC
BAF250a F7	CAATGGAGAACCGCACCTCT
BAF250a F8	TGGAGGGTGGGGAAGAAGAA
BAF250a R9	CCCGTTCGAGTTCTTCAGGT

Table 2. Primers used to sequence the pCI-neo-ARID1A vector.

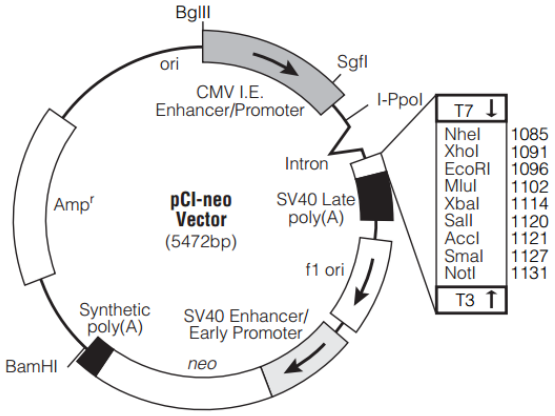

Figure 2. Vector map of pCI-neo vector.

2.10 Cellular ROS assay

Cells were harvested by trypzinisation and collected by centrifugation at 1000 rpm for 5 mins at 4^{o}C. The cells were washed once with ice-cold PBS and counted using an automated cell counter. For each sample, 5 x 10^{5} cells were stained with 10µM of 2', 7'-dichlorofluorescin diacetate (DCFDA, Sigma Aldrich) in 1mL of PBS and incubated for 30 mins at 37^{o}C in the dark. The cells were gently mixed every 10 mins to prevent the cells from setting at the bottom. Stained cells were collected by centrifugation and resuspended in 500µL of PBS. Prior to analysis, 2µg/mL of DAPI was added to each sample. For each sample, at least 20,000 cells were analyzed using a FACS Gallios Flow Cytometer (Beckman Coulter) and the cells were gated to include live and single cells only. The Kaluza Anlaysis Software (version 1.3, Beckman Coulter) was used to analyze the mean fluorescence.

2.11 qPCR

RNA was harvested using the PureLink RNA mini kit (Life Technologies) according to the manufacturer's protocol. DNA was removed using on-column DNase digestion (Life Technologies) according to the manufacturer's protocol. For each sample, cDNA was synthesized from 1µg of RNA using the high-capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's protocol. Primers used for qPCR were purchased from Life Technologies. The following qPCR primers used were: ARID1A (Hs00195664_m1), NRF2 (Hs00975961_g1), HMOX1 (Hs01110250_m1), NQO1 (Hs02512143_s1), GCLM (Hs00157694_m1) and BID (Hs00609632_m1). Gene expression was normalized against beta glucuronidase (GUSB) RNA levels.

2.12 Colony formation assay

Cells were plated at a cell density of 1500 (TOV21G and IGROV1) or 3000 cells (RMG1) per well in a 6-well plate. Cells were transfected with non-target and NRF2 siRNA the next day. For cells treated with brusatol, media containing brusatol was replaced every 4 days. After 2 weeks, cells were washed twice with PBS and cell colonies were stained with crystal violet for 1 h at room temperature. Afterwards, the crystal violet staining solution was removed and the wells were washed with water to remove excess stain.

2.13 TCGA analysis

RNA expression (RNA seq V2 RSEM) of genes of interest was downloaded from the cBioPortal for Cancer Genomics (www.cbioportal.org). The Uterine Corpus Endometrioid

Carcinoma (UCEC) dataset was published previously and was used for this analysis [152]. Tumors with alterations in *NFE2L2* (n = 17) were removed from further analysis. Then, based on *ARID1A* mutational status, tumor samples were grouped into *ARID1A*-wildtype (no detectable *ARID1A* mutations, n=141) or *ARID1A*-mutant (nonsense or frameshift mutations, n=66). Tumor samples with other *ARID1A* mutations (missense mutations, in-frame insertion/deletion, splicing mutations, or amplification) were removed from further analysis, because the effects of these mutations on ARID1A expression and function are unclear.

2.14 Statistical analysis

Values are presented as means. Unless otherwise stated, p-value was determined using Student's t-test. P-values of less than 0.05 were considered significant. Error bars represent the standard deviation.

2.15 mRNA expression profiling

RMG1 cells were transfected with non-target and ARID1A siRNA for 72 h. RNA was harvested using the *mir*Vana miRNA isolation kit (Life Technologies) according to the manufacturer's protocol. The MessageAmp premier RNA Amplification kit (Life Technologies) was used for RNA amplification and 500ng of total RNA was used to make aRNA according to the manufacturer's protocol. Fragmented aRNA was hybridized to Human Genome U133 plus 2.0 Array (Affymetrix) according to the manufacturer's protocol. The array was scanned by the MD Anderson Cancer Center's Sequencing and Microarray Facility using the GeneChip Scanner 7000G (Affymetrix). Raw images were processed using the GeneSpring software

(Agilent Technologies). Differentially expressed genes were identified using Ingenuity Pathway analysis (Ingenuity Systems). The GO terms that were enriched in significant differentially expressed genes were analyzed using Panther.

CHAPTER 3: ARID1A MEDIATES RESISTANCE TO OXIDATIVE STRESS

Specific aim 1: Investigation of whether ARID1A mediates resistance to oxidative stress

1.1 Identification and validation of drug sensitivities in ARID1A-deficient cancer cell lines

1.2 Investigation of the effects of knockdown and re-expression of ARID1A on sensitivity to ROS-inducing agents

1.3 Investigation of the effects of ARID1A on intracellular ROS levels

3.1 *ARID1A*-mutant cancer cell lines are more sensitive to the ROS-inducing agent elesclomol

To identify drug targets in *ARID1A*-mutant cancers, we analyzed the publically available drug database 'Genomics of Drug Sensitivity in Cancer (GDSC)' [143]. The GDSC database consists of drug responses of about 140 drugs in more than 700 cancer cell lines of a large variety of cancer types. First, we determined the mutation statuses of *ARID1A* of the cancer cell lines using the Cancer Cell Line Encyclopedia database [144]. We excluded cell lines with no mutation or copy number alteration data were from further analysis. We placed the remaining cell lines into *ARID1A*-wildtype (no detectable *ARID1A* mutations, n = 347) and *ARID1A*-mutant (*ARID1A* nonsense mutations, frameshift mutations, or deep deletions, n = 74) groups. We excluded cell lines with *ARID1A* missense mutations, in-frame insertions/deletions, or splicing mutations from further analysis because the effect of these mutations on ARID1A protein expression and function is unclear.

In this analysis, the drugs that exhibited significant differences ($P < 0.05$) in sensitivity between *ARID1A*-mutant and *ARID1A*-wildtype cancer cell lines were enriched in 1) inhibitors of the PI3K pathway (AZD8055, NVP-BEZ235, MK-2206, and GDC-0941) or 2) agents that induce DNA damage or inhibit the DNA damage response (cisplatin, KU-55933, and NU-7441) (Table 3). The finding that *ARID1A*-mutant cell lines are more sensitive to PI3K pathway inhibitors is consistent with previously published data. A previous study found that knockdown of ARID1A in breast cancer cell line MCF7 and primary fibroblast cell line MRC5 resulted in increased sensitivity to PI3K Buparlisib and AKT inhibitors MK2206 and Perifosine [71]. Furthermore, *ARID1A* mutations frequently co-occur in *PIK3CA* mutations in patient samples [9, 33], and the presence of *PIK3CA* mutations is a predictor of sensitivity to PI3K inhibitors [153]. The finding that *ARID1A*-mutant cell lines are more sensitive to inhibitors that induce DNA damage or inhibit the DNA damage response is also consistent with previously published data. Previous studies have found that SWI/SNF is required for DSB repair [80, 81]. Consistent with the roles of ARID1A in the DNA damage response, knockdown of ARID1A in U2OS cells resulted in increased sensitivity to cisplatin and irradiation [80].

Surprisingly, elesclomol appeared as the top ranking drug that showed differential sensitivity between *ARID1A*-mutant and *ARID1A*-wildtype cell lines. Elesclomol is a potent inducer of ROS by disrupting the electron transport chain in the mitochondria [132]. Although it has been documented that the SWI/SNF is required oxidative stress resistance in *S. cerevisiae* and *C. elegans* [136, 137], whether this is also the case in mammalian cells is unclear. Based on these results, we decided to focus on elesclomol for further validation.

Rank	*Drug*	*Drug target*	T-*test*	P-value	*FDR (BH)*
1	Elesclomol	Induced ROS accumulation	-5.0349	0.001996	0.1317
2	AZD8055	mTORC1/2	-4.9625	0.001996	0.1317
3	NVP-BEZ235	PI3K (class 1) and mTORC1/2	-3.4664	0.009980	0.2196
4	EHT 1864	Rac GTPases	-3.2140	0.003992	0.1756
5	MK-2206	AKT1/2	-2.9551	0.007984	0.2196
6	GW 441756	NTRK1	-2.9503	0.009980	0.2196
7	KU-55933	ATM	-2.8451	0.013970	0.2635
8	NU-7441	DNAPK	-2.6382	0.015970	0.2635
9	GDC0941	PI3K (class 1)	-2.6177	0.021960	0.3220
10	Cisplatin	DNA cross-linker	-2.2678	0.037920	0.4172
11	BIBW2992	EGFR and ERBB2	-2.2154	0.031940	0.3832

Table 3. Drugs that exhibited significantly lower IC_{50} values in *ARID1A*-mutant cancer cell lines than *ARID1A*-wildtype cancer cell lines

3.2 Elesclomol inhibited cell growth and induced apoptosis more potently in *ARID1A*-mutant cell lines than *ARID1A*-wildtype cell lines

ARID1A is most frequently mutated in gynecologic cancers [9, 10, 23, 58, 152]. To validate the findings from our analysis of the GDSC database, we examined a panel of 14 ovarian and endometrial cancer cell lines and determined whether *ARID1A*-mutant cell lines were more sensitive to treatment with elesclomol than were *ARID1A*-wildtype cell lines. First, we determined the *ARID1A* mutation statuses in these 14 cell lines using the Cancer Cell Line Encyclopedia database [144] and DNA sequencing. In addition, ARID1A protein expression was determined by western blot analysis. We identified *ARID1A* mutations and loss of ARID1A protein expression in eight of these cancer cell lines (*ARID1A*-mutant) and expression of the ARID1A protein in the remaining six cell lines (*ARID1A*-wildtype) (Figure 3 and Table 4)

We found that the *ARID1A*-mutant cancer cell lines had significantly lower IC_{50}s of elesclomol than did the *ARID1A*-wildtype cancer cell lines ($P = 0.034$, Figure 4). We also found that treatment with elesclomol (10 and 20 nM) induced apoptosis more potently in *ARID1A*-mutant cancer cells than in *ARID1A*-wildtype cancer cells ($P = 0.0227$ and $P = 0.0057$ respectively, Figure 5).

To show that the anti-proliferative and apoptotic effects of elesclomol were mediated by ROS, we sought to determine whether the effects of elesclomol can be abrogated by antioxidants. We found that the antioxidant *N*-acetyl-L-cysteine (NAC) abrogated the growth-inhibitory and apoptotic effects of elesclomol on *ARID1A*-mutant SMOV2 and IGROV1 ovarian cancer cells (Figure 6). Taken together, these results demonstrated that *ARID1A*-mutant cancer

cell lines are more sensitive to treatment with the ROS-inducing agent elesclomol than are *ARID1A*-wildtype cancer cell lines.

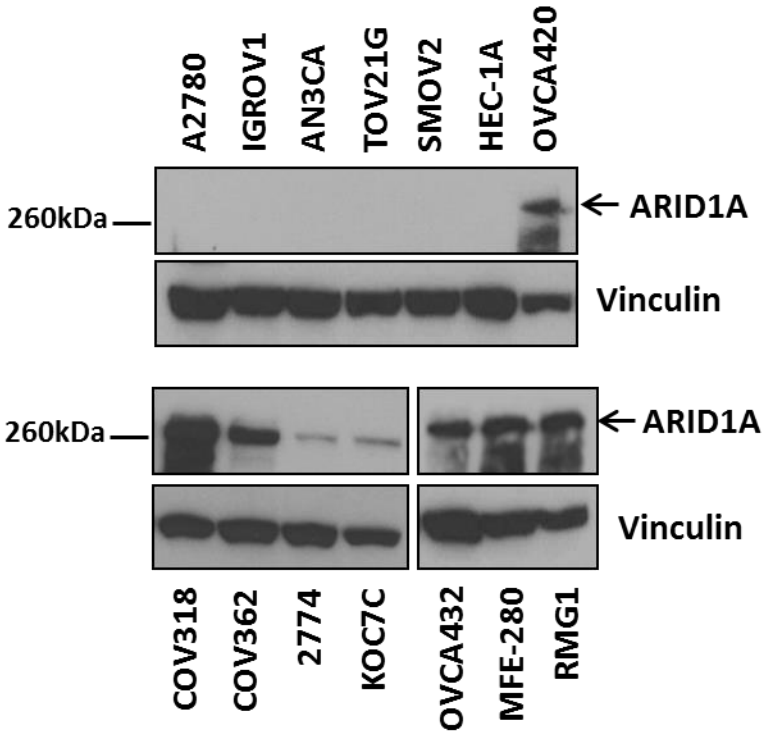

Figure 3. Western blot showing basal expression of ARID1A in a panel of ovarian and endometrial cancer cell lines. Vinculin was used as loading control.

Cell line	*Cancer type*	*ARID1A mutation*	*ARID1A protein expression*
A2780	Ovarian	Q1430*[a], R1721fs[a]	Absent
IGROV1	Ovarian	M274fs[a,b], G1847fs[a,b]	Absent
AN3CA	Endometrial	G1848fs[a]	Absent
TOV21G	Ovarian	Q548fs[a,b], N756fs[a,b]	Absent
SMOV2	Ovarian	G1740fs[b]	Absent
HEC-1A	Endometrial	Q404H[a], Q1761C[a], Q1835*[a], Q2115*[a]	Absent
KOC7C	Ovarian	G276fs[b], P1326fs[b], A1517fs[b]	Absent
MDA2774	Ovarian	Q1947*[b]	Absent
COV362	Ovarian	Wild-type[a]	Present
OVCA420	Ovarian	ND	Present
COV318	Ovarian	Wild-type[a]	Present
OVCA432	Ovarian	ND	Present
MFE-280	Endometrial	Wild-type[a]	Present
RMG1	Ovarian	Wild-type[a,b]	Present

Table 4. Summary of *ARID1A* mutation statuses and ARID1A protein expression in a panel of ovarian and endometrial cancer cell lines Abbreviation: [a]Mutation status identified in the Cancer Cell Line Encyclopedia database. [b]Mutation status identified in Sanger sequencing performed in this study. ND, not determined. Fs, frame-shift mutation. *, non-sense mutation.

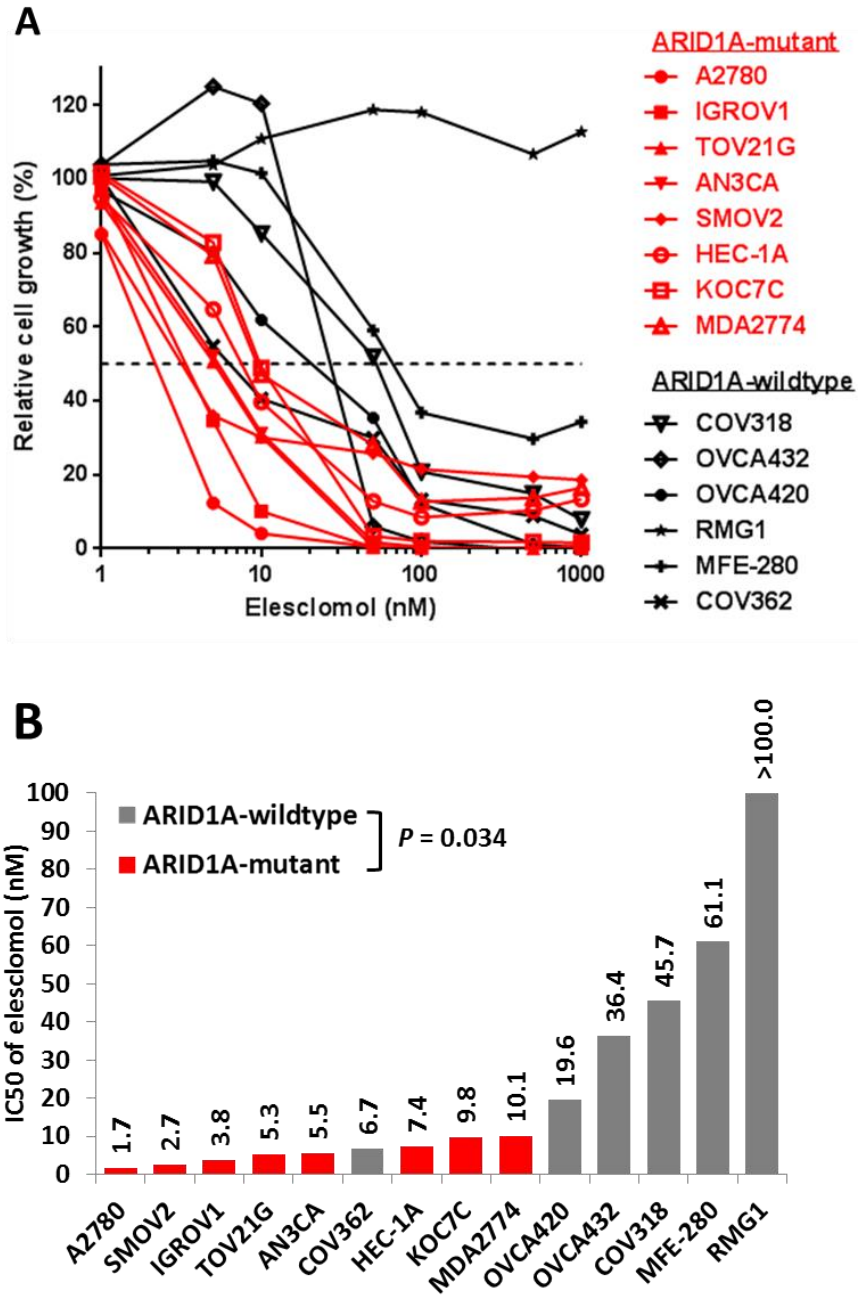

Figure 4. *ARID1A*-mutant cancer cell lines are more sensitive to elesclomol than *ARID1A*-wildtype cancer cell lines. Cells were treated with elesclomol for 72 h and cell growth was measured using the WST-1 assay. (A) Relative cell growth of treated cells was calculated as a percentage of DMSO control cells. Each cell line was repeated at least twice. (B) IC50 values of elesclomol in cell lines were calculated from (A) using GraphPad Prism.

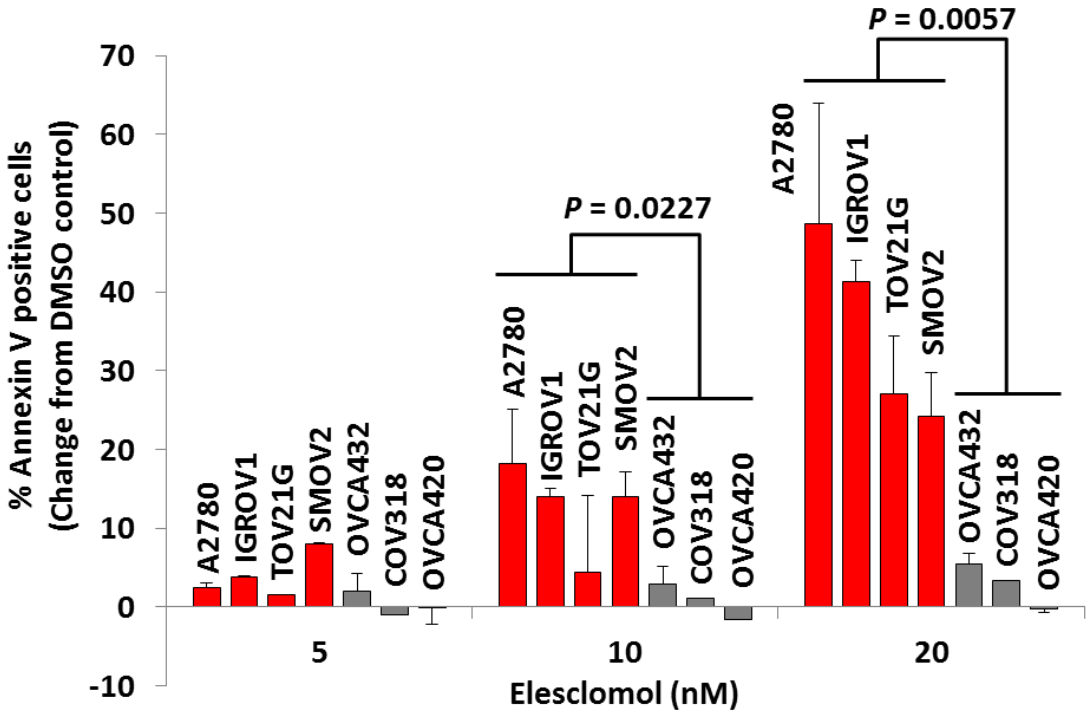

Figure 5. Elesclomol treatment induces apoptosis more potently in *ARID1A*-mutant cancer cell lines than *ARID1A*-wildtype cancer cell lines. Cells were treated elesclomol for 72 h. Induction of apoptosis was measured using annexin-V staining by flow cytometry. Changes in percentage of annexin-V positive cells in treated cells compared to DMSO control are shown. Each cell line was repeated twice. Red bars, *ARID1A*-mutant cancer cell lines. Grey bars, *ARID1A*-wildtype cell lines.

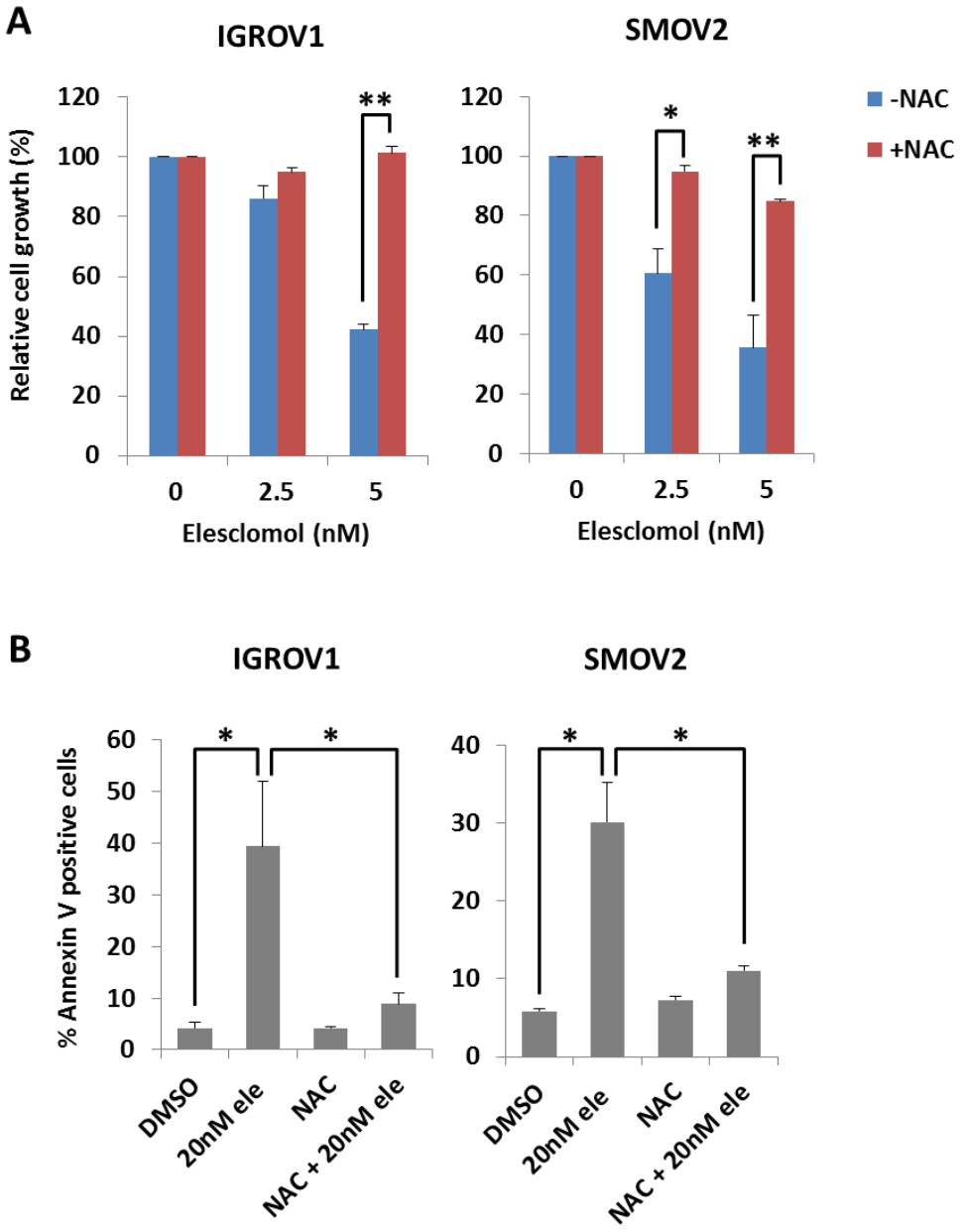

Figure 6. Elesclomol inhibits cell growth and induces apoptosis through increasing ROS. (A and B) *ARID1A*-mutant IGROV1 and SMOV2 cells were treated with elesclomol in the presence or absence of NAC for 72 h. Cell growth was measured using the WST-1 assay (A) and apoptosis (B) was measured using annexin-V staining. Relative cell growth of treated cells was determined as a percentage of DMSO control cells. **P* < 0.05, ***P* < 0.01.

3.3 Knockdown of ARID1A expression increases the sensitivity of ovarian cancer cells to treatment with ROS-inducing agents

To show that loss of ARID1A expression is responsible for increased sensitivity of ovarian cancer cells to treatment with elesclomol, we depleted ARID1A in *ARID1A*-wildtype RMG1 ovarian cancer cells using siRNA. Although we found that RMG1 cells were intrinsically highly resistant to treatment with elesclomol, depletion of ARID1A sensitized the cells to elesclomol in the micro-molar range (Figure 7A). To show that this effect was not limited to elesclomol, we also examined the sensitivity of these cells to treatment with another ROS-inducing agent, piperlongumine [135]. We found that ARID1A depletion in RMG1 cells also led to sensitization of the cells to piperlongumine (Figure 7B). In addition, we found that treatment with piperlongumine induced apoptosis more potently in ARID1A-knockdown cells than in non-target control cells (Figure 7C). Similar to elesclomol, we found that piperlongumine inhibited growth by increasing ROS as treatment with NAC reversed the anti-proliferative effects of the drug (Figure 7D).

As ARID1A is a subunit of SWI/SNF, we sought to determine whether the loss of other SWI/SNF subunits can also sensitize the cells to elesclomol. We found that knockdown of the core SWI/SNF subunits BRG1 and SNF5 also resulted in increased sensitivity to elesclomol (Figure 8A). We confirmed the down-regulation of ARID1A, BRG1 and SNF5 expression by siRNA in RMG1 cells using western blot (Figure 8B).

Previously, it has been demonstrated that ARID1A is required for DNA damage repair [80]. Therefore, as a positive control, we tested whether knockdown of ARID1A leads to increased sensitivity to the DNA cross-linker cisplatin. Similar to previous findings [80],

depletion of ARID1A led to increased sensitivity of these cells to treatment with cisplatin (Figure 9A). Depletion of ARID1A did not have a generalized effect on drug sensitivity, as knockdown of ARID1A did not affect the drug sensitivity of the microtubule toxin paclitaxel (Figure 9B).

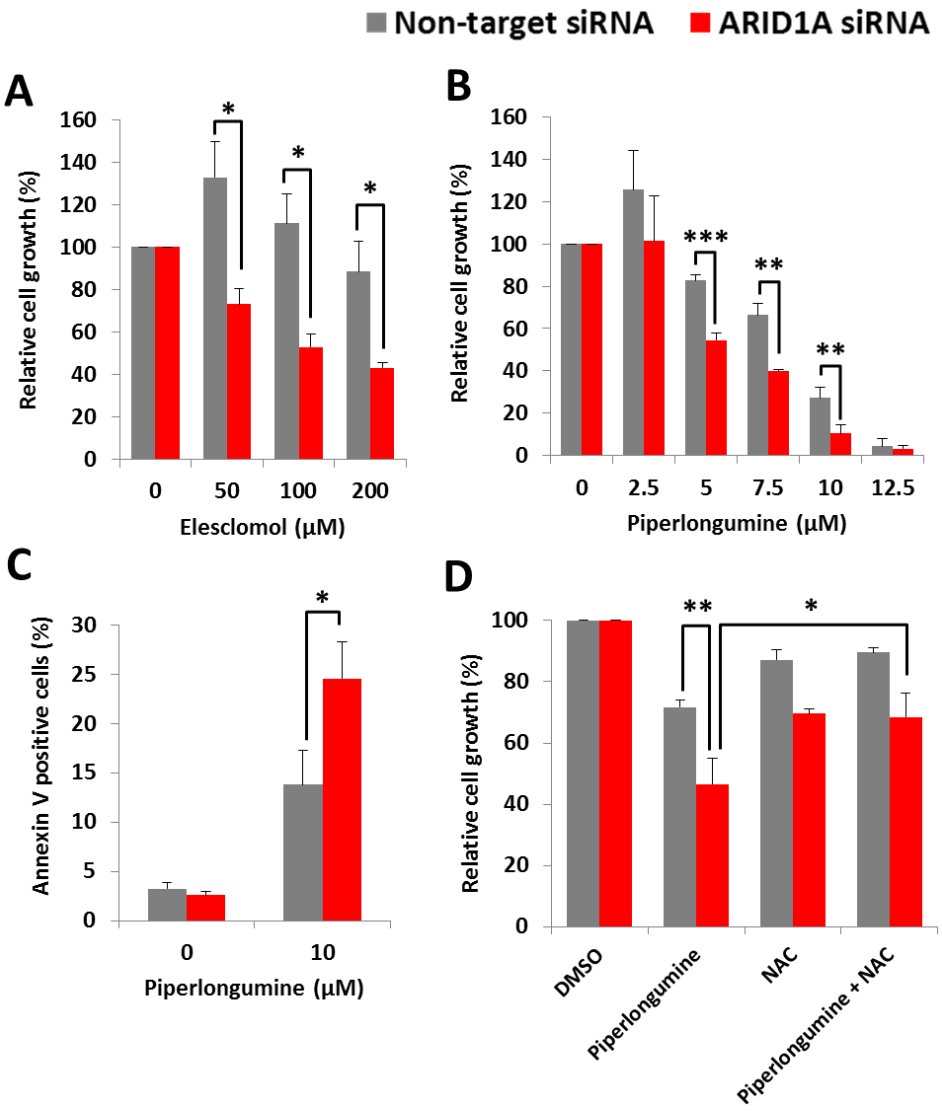

Figure 7. Knockdown of ARID1A in *ARID1A*-wildtype RMG1 cells results in increased sensitivity to ROS-inducing agents. (A-C) *ARID1A*-wildtype RMG1 cells were transfected with ARID1A and non-target siRNA for 24 h and treated with ROS-inducing agents elesclomol (A) and piperlongumine (B and C) for 72 h. (A and B) Cell growth was measured using the WST-1 assay. (C) Cells were transfected and treated as in (B), apoptosis was measured using annexin-V staining. (D) RMG1 cells were transfected and treated as in (B) in the presence or absence of the antioxidant NAC. Cell growth was measured using the WST-1 assay. Relative cell growth of treated cells was determined as a percentage of DMSO control cells. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

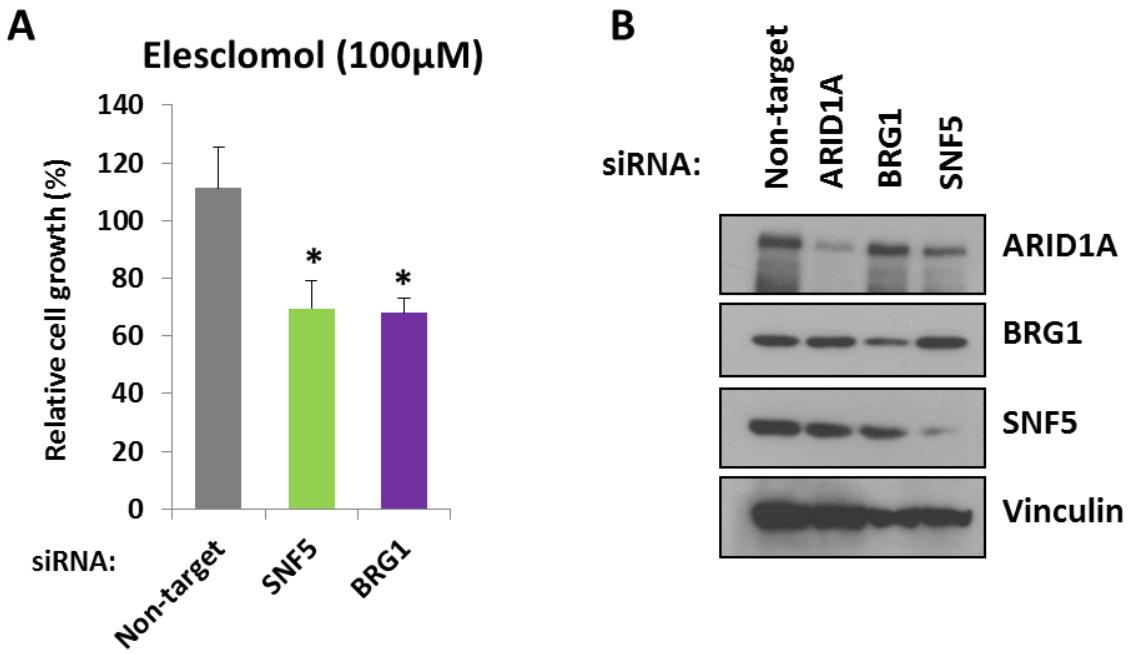

Figure 8. Knockdown of SWI/SNF core subunits SNF5 and BRG1 leads to increased sensitivity to elesclomol in RMG1 cells. (A) RMG1 cells were transfected with SNF5, BRG1 and non-target siRNA for 24 h and treated with elesclomol for 72 h. Cell growth was measured using the WST-1 assay. Relative cell growth of treated cells was determined as a percentage of DMSO control cells. (B) Western blot analysis showed the knockdown of ARID1A, BRG1 and SNF5 using siRNA. *$P < 0.05$.

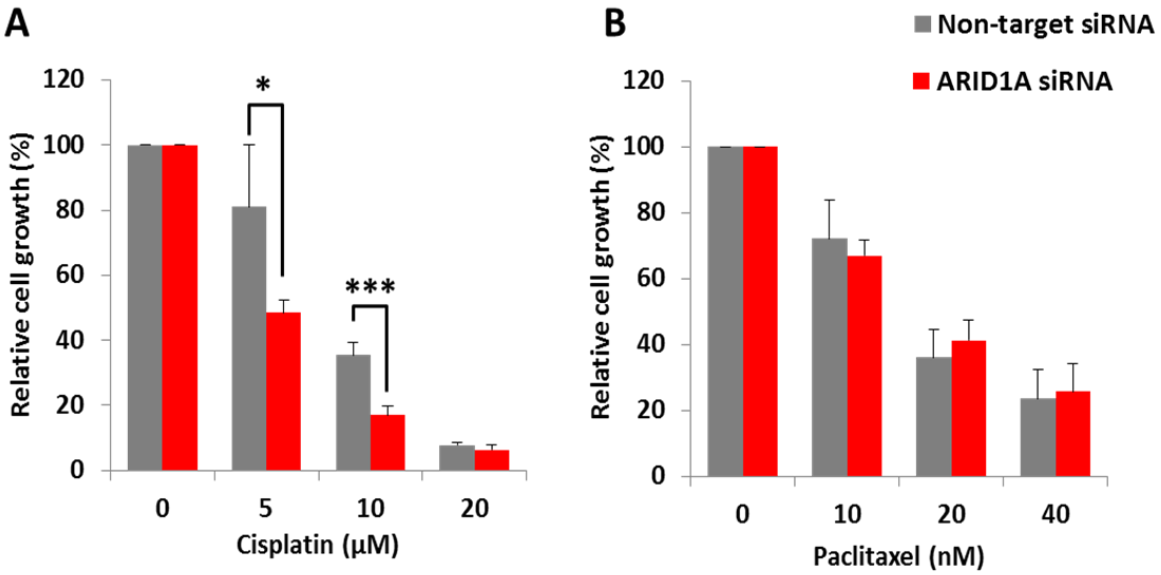

Figure 9. Knockdown of ARID1A leads to increased sensitivity to cisplatin but not paclitaxel. (A and B) RMG1 cells transfected with ARID1A and non-target control siRNA for 24 h were treated with cisplatin (A) and paclitaxel (B) for 72 h. Cell growth was measured using the WST-1 assay. Relative cell growth of treated cells was determined as a percentage of untreated cells. **P* < 0.05, ****P* < 0.001.

3.4 Re-expression of ARID1A increases the resistance of ovarian cancer cells to treatment with elesclomol

To complement the siRNA experiments, we investigated whether re-expression of ARID1A in *ARID1A*-mutant cells can promote resistance of the cells to treatment with elesclomol. To this end, we transiently re-expressed ARID1A in *ARID1A*-mutant TOV21G ovarian cancer cells and found that ARID1A re-expression resulted in increased resistance of the cells to treatment with elesclomol and piperlongumine (Figure 10A and B). In addition, the level of apoptosis induced by treatment with elesclomol was lower in TOV21G cells with ARID1A re-expression than in empty vector transfected control cells (Figure 10C). Western blot analysis confirmed that ARID1A was re-expressed in TOV21G after transfection with the pCI-neo-ARID1A vector (Figure 10D).

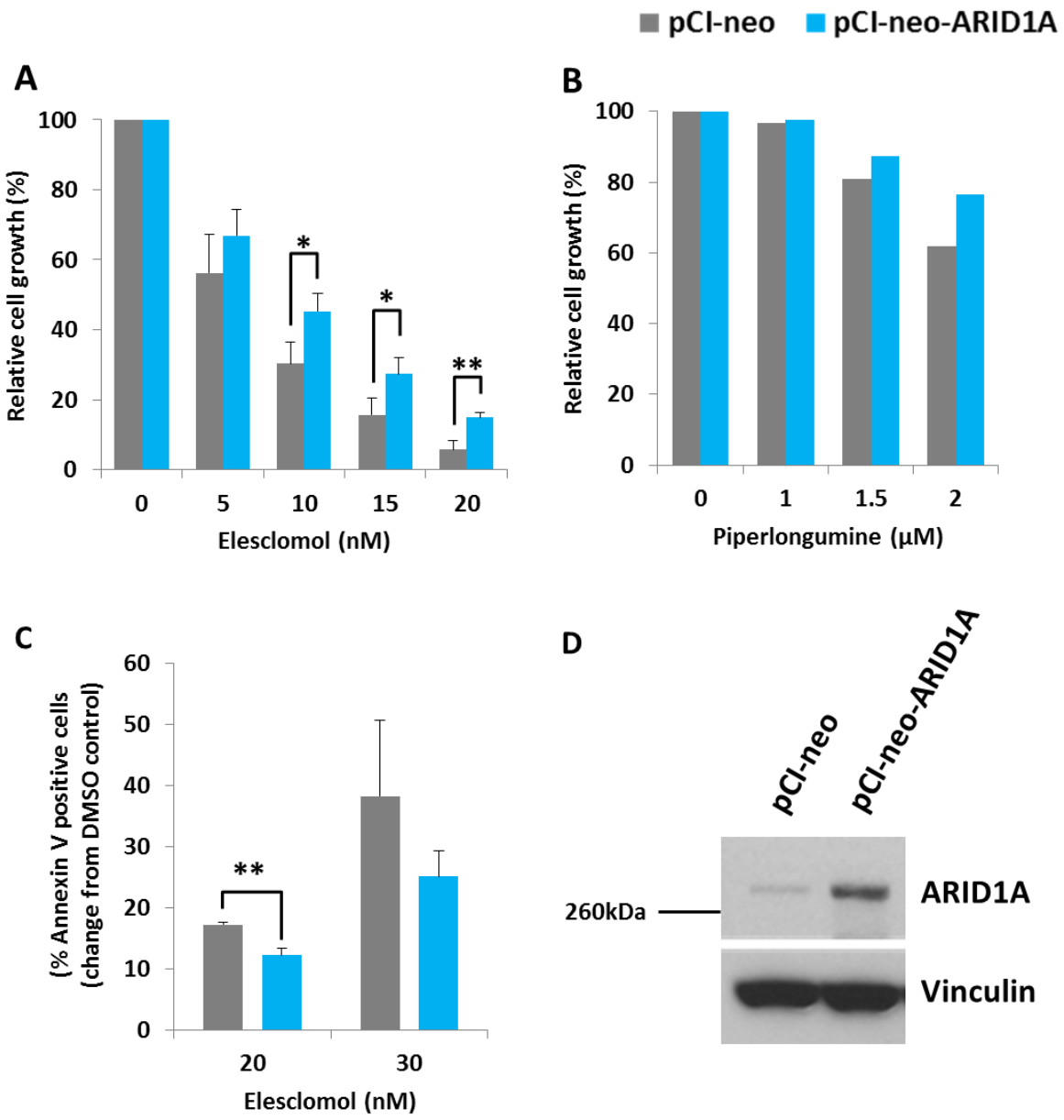

Figure 10. Re-expression of ARID1A increases resistance to ROS-inducing agents. (A and B) *ARID1A*-mutant TOV21G cells were transfected with pCI-neo-ARID1A and pCI-neo control vectors for 48 h and treated with ROS-inducing agents elesclomol (A) and piperlongumine (B) for 72 h. Cell growth was measured using the WST-1 assay. Relative cell growth of treated cells was determined as a percentage of DMSO control cells. (C) Cells were transfected and treated as in (A), apoptosis was measured using annexin-V and PI staining. (D) Western blot analysis showed the re-expression of ARID1A. *$P < 0.05$, **$P < 0.01$.

3.5 Depletion of ARID1A leads to increased intracellular ROS level and cell growth

Although a large increase of ROS in the cell causes oxidative stress and ultimately cell death, a moderate increase in ROS levels can act as mitogenic signaling to increase cell growth. Because the knockdown of ARID1A increases cell growth in *ARID1A*-wildtype RMG1 cells, we asked whether knockdown of ARID1A leads to an increase of oxidative stress and subsequently increased cell growth.

To this end, we depleted ARID1A in RMG1 cells and measured intracellular ROS levels using 2',7'-dichlorofluorescin diacetate (DCFDA). We found that depletion of ARID1A resulted in an increase in intracellular ROS levels (Figure 11A). Next, we asked whether this increase in intracellular ROS mediates cell growth in ARID1A-knockdown cells. After transfection of ARID1A siRNA, we treated the cells with the antioxidant NAC for three days and cell growth was measured. In RMG1 cells transfected with ARID1A, cell growth was increased by 23%. However, NAC abrogated this increase in cell growth to a level similar to non-target control cells treated with NAC (Figure 11B). These data suggests that ROS is required to mediate cell growth in ARID1A-depleted cells.

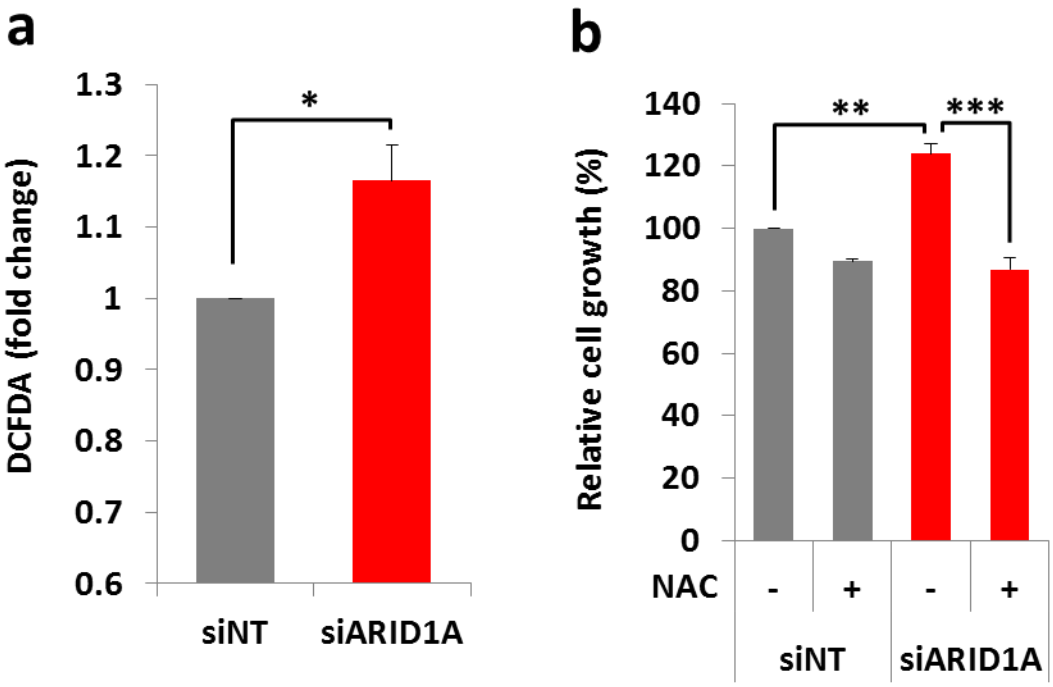

Figure 11. Depletion of ARID1A promotes cell growth by increasing intracellular ROS levels. (A) *ARID1A*-wildtype RMG1 cells were transfected with ARID1A and non-target siRNA for 72 h and intracellular ROS levels were measured using DCFDA. (B) RMG1 cells were transfected with ARID1A, BRG1, SNF5 and non-target siRNA for 24 h and treated with the antioxidant NAC for a further 72 h. Cell growth was measured using the WST-1 assay. Relative cell growth of ARID1A siRNA transfected cells was determined as a percentage of non-target control cells.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.6 Conclusion

In the present study, by analyzing the GDSC database and subsequently validation using ovarian and endometrial cancer cell lines, we found that *ARID1A*-mutant cancer cell lines were more sensitive to treatment with the ROS-inducing agent elesclomol than were *ARID1A*-wildtype cancer cell lines. Knockdown of ARID1A expression in *ARID1A*-wildtype RMG1 cells sensitized the cells to treatment with ROS-inducing agents elesclomol and piperlongumine, whereas ARID1A re-expression in *ARID1A*-mutant TOV21G cells resulted in increased resistance to elesclomol. Subsequently, we found that depletion of ARID1A in RMG1 cells increased accumulation of intracellular ROS levels and led to increased cell growth.

CHAPTER 4: ARID1A IS A NEGATIVE REGULATOR OF NRF2

Specific aim 2: Investigation of the relationship between ARID1A and NRF2

2.1 Investigation of the effect of ROS on ARID1A expression

2.2 Investigation of the effect of ARID1A on NRF2 expression and NRF2 transcriptional activity

2.3 Investigation of the effects of NRF2 inhibition in ovarian clear cell carcinoma cells

4.1 Piperlongumine induces ARID1A expression in a NRF2-dependent manner

Although the regulation of down-stream targets by ARID1A is often studied, the regulation of ARID1A expression remains unknown. Interestingly, piperlongumine induced ARID1A expression at the mRNA and protein level (Figure 12A and B). To demonstrate that piperlongumine induces ARID1A expression through ROS, NAC was added in combination with piperlongumine and was able to abrogate the effect of piperlongumine on ARID1A expression (Figure 12C). However, when we treated RMG1 cells with H2O2, we did not observe an induction of ARID1A (data not shown). This suggests that induction of ARID1A may be specific to piperlongumine.

Because NRF2 is the major transcription factor activated during oxidative stress, we asked whether induction of ARID1A by piperlongumine was dependent on NRF2. NRF2 was depleted using siRNA in RMG1 cells and the cells were then treated with piperlongumine. As expected, oxidative stress induced NRF2 expression. Depletion of NRF2 abrogates the induction of ARID1A by piperlongumine (Figure 12D). These results suggest that piperlongumine may induce ARID1A expression through NRF2.

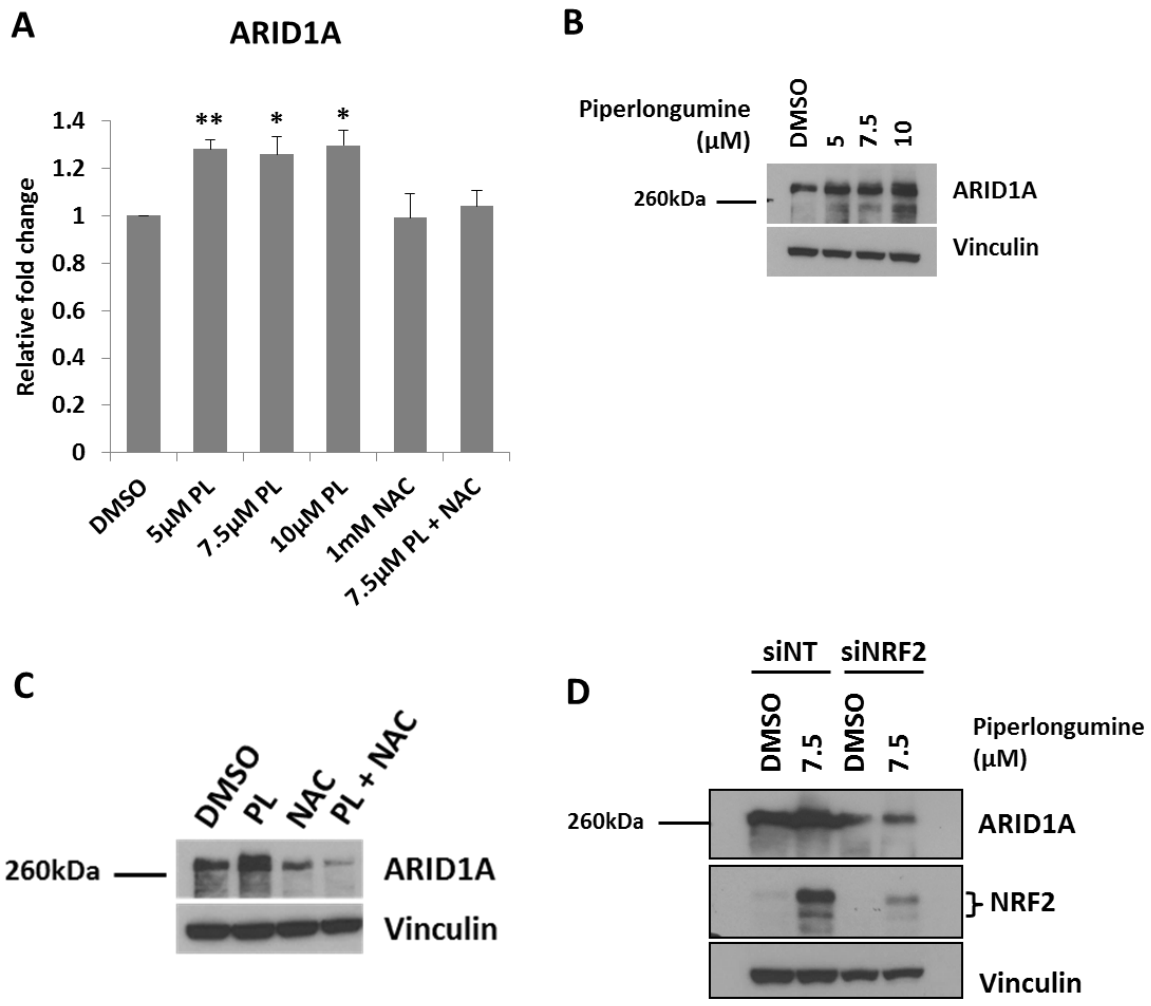

Figure 12. ROS increases ARID1A mRNA and protein expression (A-C) RMG1 cells were treated with piperlongumine (PL) in the presence or absence of 1mM NAC for 6 h. ARID1A expression was measured using qPCR (A) and western blot analysis (B). (C) Western blot analysis of ARID1A expression in RMG1 treated with 7.5μM piperlongumine in the presence or absence of 1mM NAC for 6 hours. (D) Western blot analysis of RMG1 cells that were transfected with NRF2 siRNA for 48h and then treated with piperlongumine for 6 h.

*, $p<0.05$, **, $p<0.01$.

4.2 ARID1A negatively regulates NRF2 expression

Previously, it was found that knockdown of BRG1 positively regulates NRF2 transcriptional activity [138]. However, the regulation of NRF2 by ARID1A is unknown. First, we sought to determine whether ARID1A regulates the expression of NRF2. We transfected *ARID1A*-wildtype ES2 cells with ARID1A siRNA and treated the cells with H_2O_2 for 6 h to induce NRF2 expression. We found that knockdown of ARID1A resulted in an increase of NRF2 mRNA and protein expression at the basal level and in the presence of H_2O_2 (Figure 13A and B). We also depleted ARID1A in *ARID1A*-wildtype RMG1 cells and treated the cells with the ROS-inducing agent piperlongumine. Although NRF2 mRNA expression was similar in ARID1A-knockdown and non-target control cells, knockdown of ARID1A resulted in a greater induction of NRF2 at the protein level in the presence of piperlongumine (Figure 13C and D).

As we found that ARID1A negatively regulate NRF2 expression in the presence of oxidative stress, we next investigated whether the expression of NRF2 downstream genes is increased upon ARID1A depletion. We transfected *ARID1A*-wildtype ES2 cells with ARID1A siRNA and treated the cells with H_2O_2 for 6 h to induce the expression of NRF2 downstream genes. We chose to examine the expression of *HMOX1*, *NQO1*, *GCLM*, and *SLC7A11*, as these genes are well characterized NRF2 targets [100, 154-156]. As expected, treatment with H_2O_2 induced the expression of *HMOX1*, *NQO1*, *GCLM*, and *SLC7A11* in a dose-dependent manner (Figure 14). We found that knockdown of ARID1A in ES2 cells resulted in a further induction of these genes, with the exception of *NQO1* (Figure 14). Similarly, depletion of ARID1A in *ARID1A*-wildtype RMG1 cells resulted in a greater induction of *HMOX1*, *GCLM*, *NQO1*, and *SLC7A11* in the presence of H_2O_2 (Figure 15).

To complement the siRNA knockdown experiments, we re-expressed ARID1A in ARID1A-mutant TOV21G cells and treated the cells with the ROS-inducing agent elesclomol. In the presence of oxidative stress, induction of *HMOX1, NQO1,* and *GCLM* mRNA expression was suppressed by re-expression of ARID1A, with the exception of NQO1, which for unknown reasons its expression did not increase in the presence of oxidative stress (Figure 16). Together, these results show that ARID1A negatively regulates NRF2 expression.

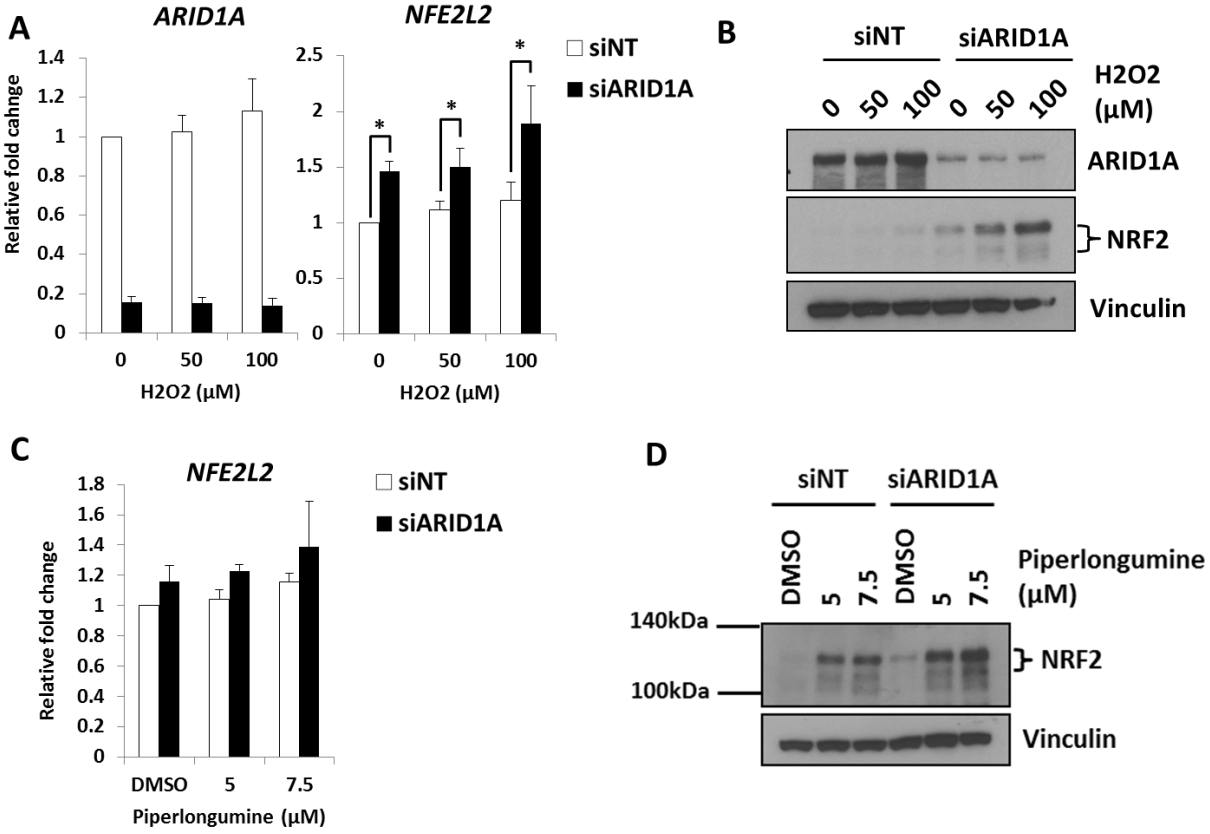

Figure 13. ARID1A negatively regulates NRF2 expression. (A-B) *AIRD1A*-wildtype ES2 cells were transfected with ARID1A siRNA for 48 h and then treated with H2O2 for 6 h. NRF2 mRNA and protein expression were measured using qPCR (A) and western blot (B). (C-D) *ARID1A*-wildtype RMG1 cells were transfected with ARID1A siRNA for 48 h and then treated with piperlongumine for 6 h. NRF2 mRNA and protein expression were measured using qPCR (C) and western blot (D). * $P < 0.05$.

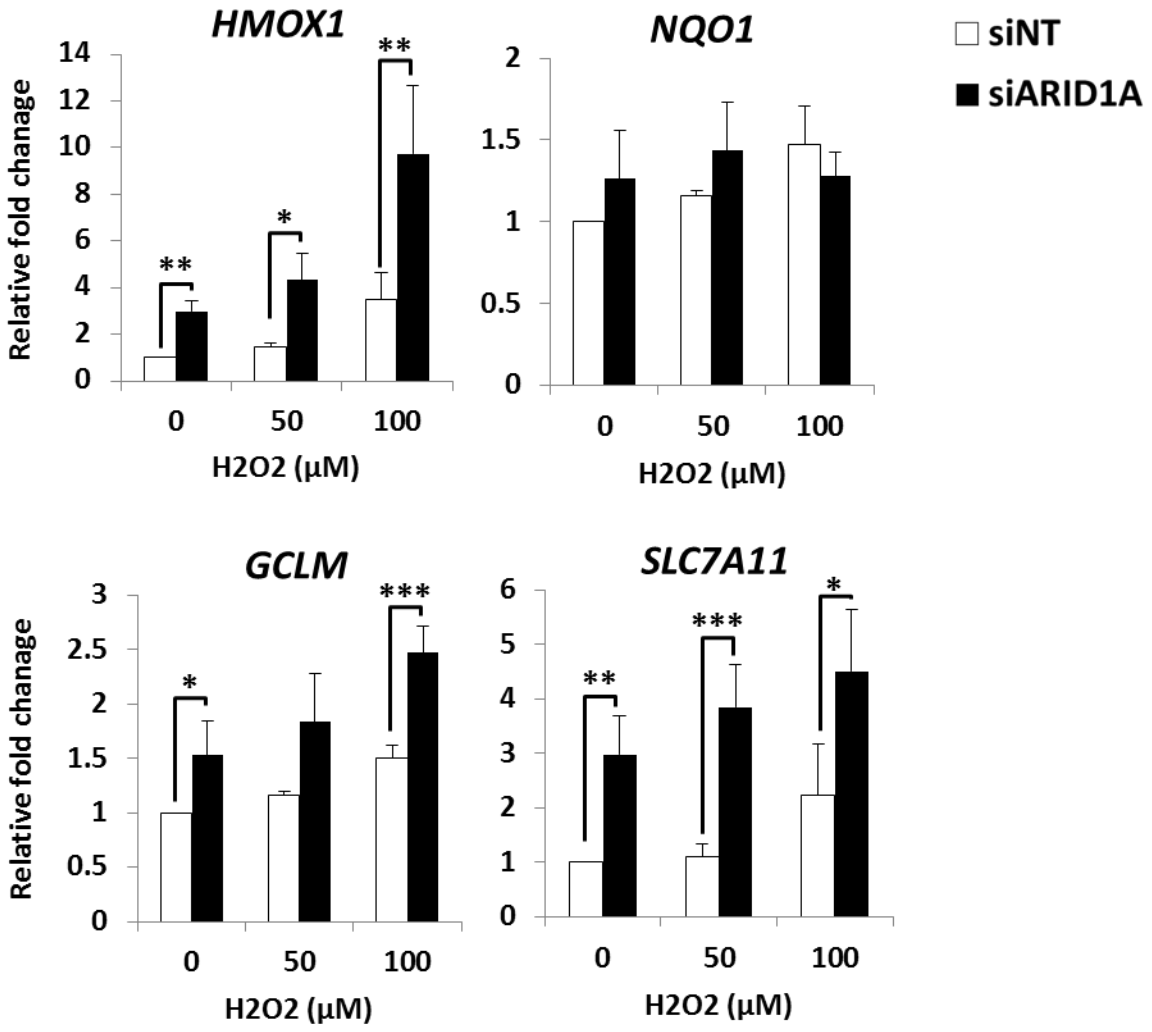

Figure 14. Induction of NRF2-target genes is higher in the absence of ARID1A. (A-D) *ARID1A*-wildtype ES2 cells were transfected with ARID1A and non-target siRNA for 48 h, then treated with H2O2 for 6 h. Expression of NRF2 targets *HMOX1*, *NQO1*, *GCLM*, and *SLC7A11* were measured by qPCR. Relative fold change was measured relative to untreated non-target control cells. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

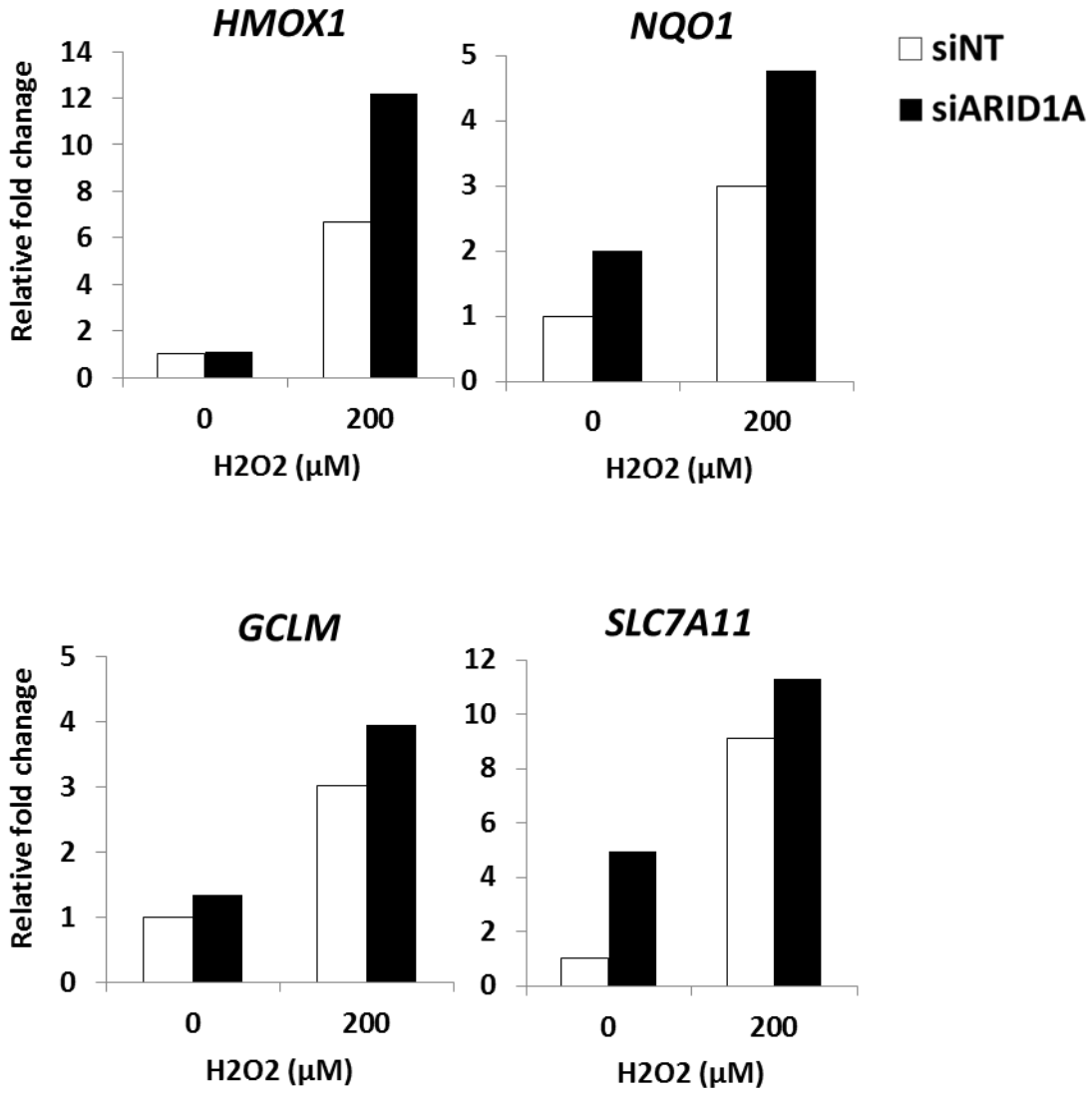

Figure 15. Induction of NRF2-target genes is higher in the absence of ARID1A. (A-D) *ARID1A*-wildtype RMG1 cells were transfected with ARID1A and non-target siRNA for 48 h, then treated with H2O2 for 6 h. Expression of NRF2 targets *HMOX1*, *NQO1*, *GCLM*, and *SLC7A11* were measured by qPCR. Relative fold change was measured relative to untreated non-target control cells.

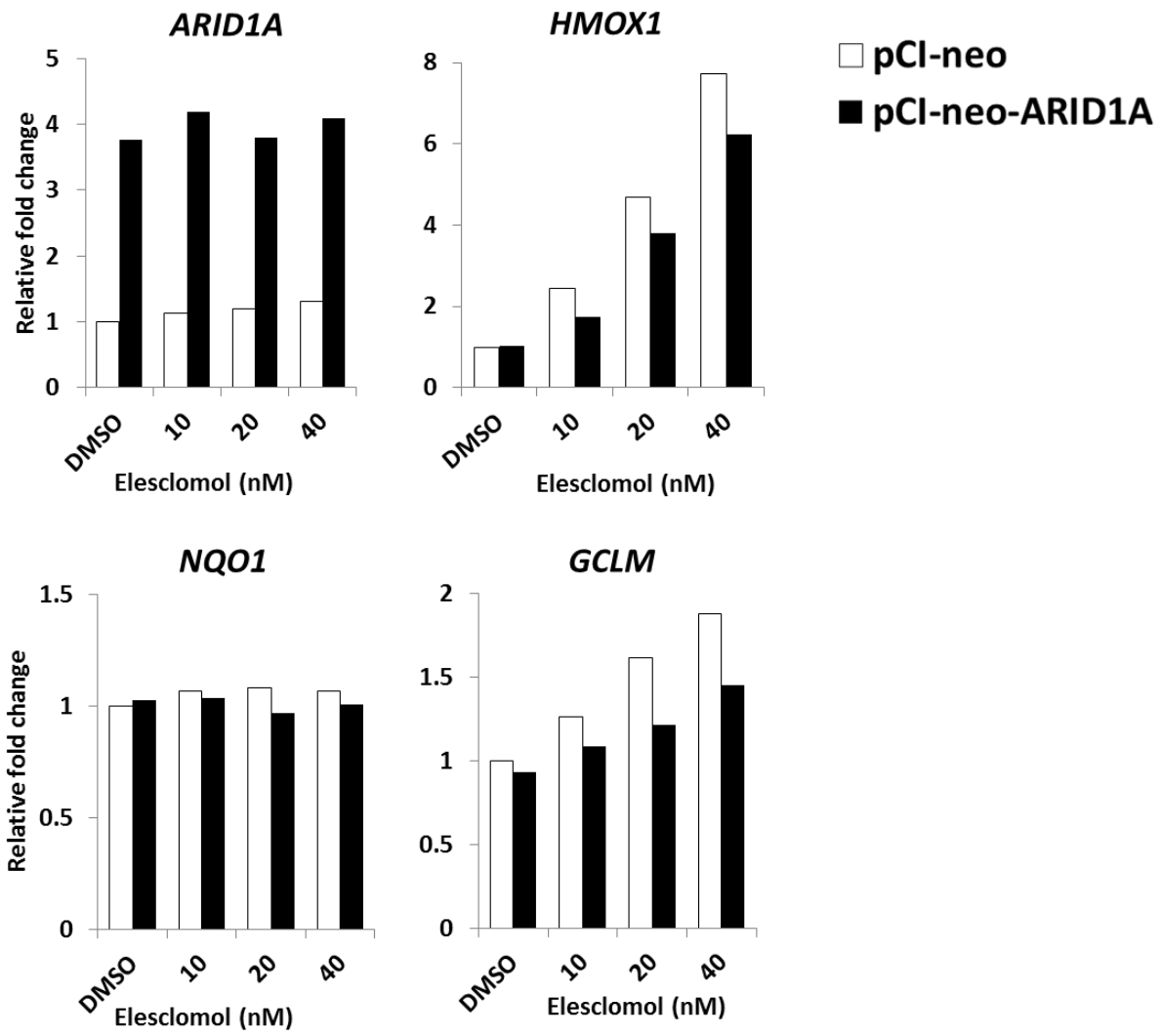

Figure 16. Re-expression of ARID1A decreases the expression of NRF2-target genes. *ARID1A*-mutant TOV21G cells were transfected with pCI-neo and pCI-neo-ARID1A vectors for 48 h, cells were then treated with elesclomol for 6 h. Expression of NRF2 targets *HMOX1*, *NQO1* and *GCLM* were measured by qPCR. Relative fold change was measured relative to non-target control cells treated with DMSO.

4.3 NRF2 is required for protection from oxidative stress and cell growth in *ARID1A*-mutant ovarian cancer cells

Previous studies found that NRF2 promotes cell proliferation in several cancer types [157-160]. However, whether NRF2 is required for cell proliferation in ovarian cancer cells is unknown. Since we found that ARID1A negatively regulates NRF2, we asked whether NRF2 has oncogenic functions in *ARID1A*-mutant ovarian cancer cells. We tested the effects of NRF2 depletion on colony formation in ovarian cancer cells. We found that knockdown of NRF2 selectively inhibits colony formation of *ARID1A*-mutant TOV21G and IGROV1 ovarian cancer cells, but not *ARID1A*-wildtype RMG1 cells (Figure 17A). Western blot showed that NRF2 protein expression was down-regulated upon NRF2 depletion in both RMG1 and TOV21G cells (Figure 17B). In NRF2-depleted cells, mRNA expression of the NRF2 targets *HMOX1*, *NQO1*, and *GCLM* were also decreased in both RMG1 and TOV21G cells, with the exception of *SLC7A11*, which was only down-regulated in NRF2-depleted TOV21G cells (Figure 17C). As NRF2 is the major regulator of the anti-oxidant response in the cell, we sought to investigate whether NRF2 depletion affects intracellular ROS levels. We found that depletion of NRF2 resulted in a similar increase in ROS level in both RMG1 and TOV21G cells as measured by DCFDA (Figure 17D).

The small molecule brusatol has been identified as a NRF2 inhibitor [161]. Next, we investigated whether brusatol is able to inhibit colony formation similarly to NRF2 siRNA. We treated RMG1 and TOV21G cells with 10nM to 100nM of brusatol. We found that a higher dosage of brusatol was required to inhibit colony formation completely in RMG1 cells than

TOV21G cells (Figure 18). Together, these results suggest that NRF2 may be required specifically for growth of *ARID1A*-mutant cells.

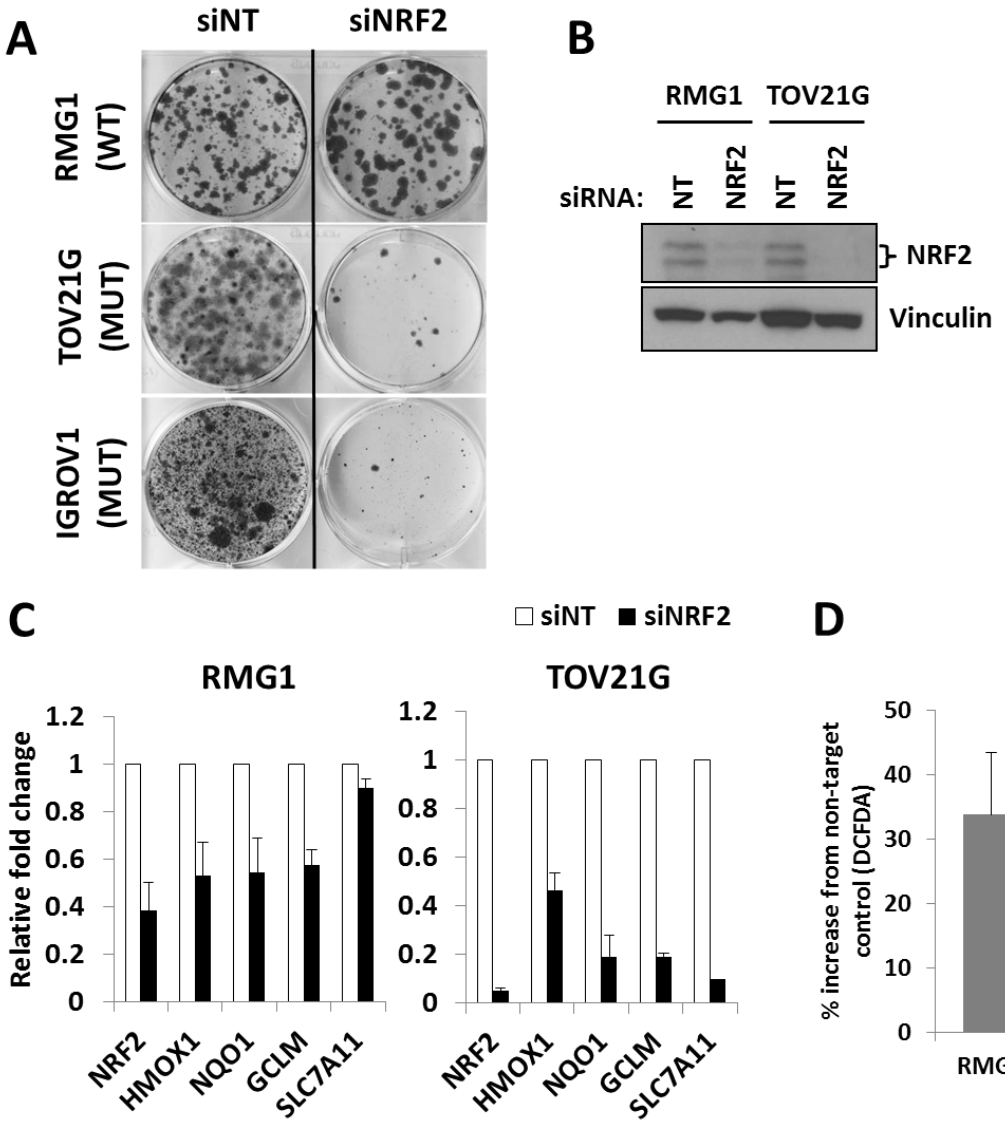

Figure 17. Knockdown of NRF2 inhibited colony formation in *ARID1A*-mutant cells. (A) Colony formation assays of RMG1, TOV21G, and IGROV1 cells transfected with NRF2 and non-target control siRNA. (B) NRF2 expression after transfection with NRF2 and non-target siRNA was measured by western blot. (C) mRNA expression of NRF2 and NRF2-target genes after transfection with NRF2 and non-target siRNA was measured by qPCR. (D) TOV21G and RMG1 cells were transfected with NRF2 and non-target siRNA for 72 h. Intracellular ROS levels were measured by DCFDA. WT, *ARID1A*-wildtype. MUT, *ARID1A*-mutant.

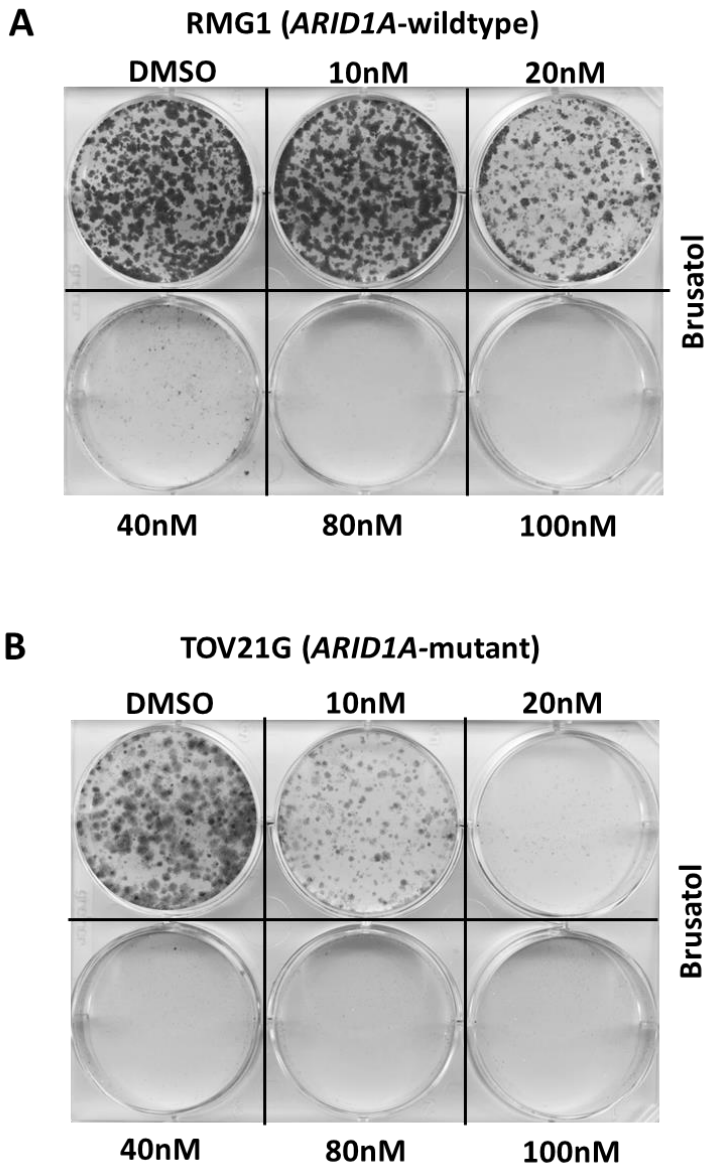

Figure 18. NRF2 inhibitor brusatol inhibits colony formation in ovarian cancer cells. (A and B) Colony formation assays of RMG1 and TOV21G cells treated with brusatol.

4.4 NRF2 expression is higher in *ARID1A*-mutant than *ARID1A*-wildtype TCGA UCEC patient samples

Based on our findings that ARID1A negatively regulates NRF2, we sought to investigate the relationship between *ARID1A* mutation and NRF2 expression in patient samples by analyzing RNA-seq data available in the Cancer Genome Atlas (TCGA) project. As the TCGA ovarian cancer dataset only contains tumor samples of the serous subtype, which do not frequently harbor *ARID1A* mutations (<2%), we did not analyze this dataset. Instead, we analyzed the uterine corpus endometrioid carcinoma (UCEC) dataset that was previously published [152], because UCEC has the highest frequency of *ARID1A* mutations in the cancer types available in TCGA. In the UCEC dataset, we found that *NFE2L2* is amplified and mutated in a small subset of tumor samples (n = 17). As alterations in *NFE2L2* can affect the NRF2 expression and transcriptional activity [122], we removed *NFE2L2*-altered tumor samples from further analysis. We placed the remaining tumor samples into *ARID1A*-wildtype (no detectable *ARID1A* mutations, n=141) or *ARID1A*-mutant (*ARID1A* nonsense or truncating mutations, n=66) (Table 6). We excluded tumor samples with ARID1A missense mutations, in-frame insertion/deletion, splicing mutations or amplification from further analysis, because the effect of these mutations on ARID1A expression and function is unclear. We found that mRNA expression of *NFE2L2* is significantly higher in *ARID1A*-mutant tumors than *ARID1A*-wildtype tumors (Mann-Whitney *U*-test, $P = 0.0196$, Figure 19A). We also examined the expression of several NRF2 downstream targets, and found that the mRNA expression of *NQO1*, *SLC7A11* and *IDH1* are significantly higher in *ARID1A*-mutant tumors than *ARID1A*-wildtype tumors (Mann-Whitney *U*-test, $P = 0.0003$, $P = 0.012$, and $P = 0.037$ respectively, Figure 19B-D).

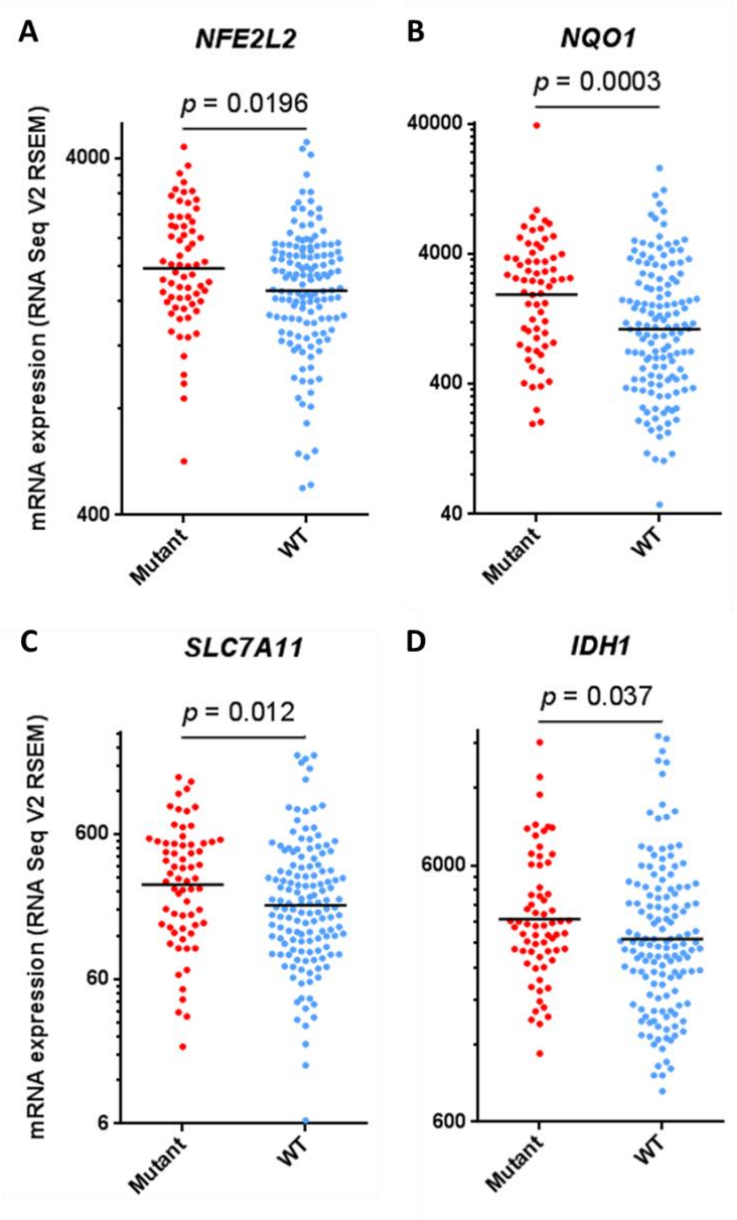

Figure 19. mRNA expression of NRF2 and NRF2-target genes are higher in *ARID1A*-mutant than *ARID1A*-wildtype TCGA UCEC tumor samples. (A-D) mRNA levels of *NFE2L2*, *NQO1*, *SLC7A11* and *IDH1* were compared in *ARID1A*-mutant (Mutant, n = 66) and *ARID1A*-wildtype (WT, n = 141) samples. Black lines represent the geometric mean. Mann-Whitney *U*-tests were used to calculate significance.

4.5 NRF2 is not required for serine biosynthesis in *ARID1A*-mutant cancer cells

Although the antioxidant functions of NRF2 are well characterized, two recent studies have identified antioxidant-independent functions of NRF2 in metabolism [160, 162]. DeNicola et al. found that NRF2 regulates the serine/gylcine biosynthetic pathway in non-small cell lung cancer (NSCLC) by controlling the expression of *PHGDH*, *PSAT1* and *SHMT2* [163]. Interestingly, using microarray analysis, we found that *PHGDH*, *PSAT1* and *SHMT2* were up-regulated in ARID1A-depleted RMG1 cells compared to non-target control cells. Therefore, we asked whether NRF2 is required for cell growth of *ARID1A*-mutant cancer cells by controlling serine/glycine biosynthesis. qPCR confirmed that mRNA expression of *PHGDH*, *PSAT1* and *SHMT2* were up-regulated in ARID1A-depleted RMG1 and ES2 cells compared to non-target control cells (Figure 20A). However, depletion of NRF2 in *ARID1A*-mutant TOV21G cells did not result in significant down-regulation of *PHGDH*, *PSAT1*, and *SHMT2* expression (Figure 20B). In addition, gene expression analysis using the TCGA UCEC dataset found that *PHGDH*, *PSAT1*, and *SHMT2* mRNA expression were not up-regulated in *ARID1A*-mutant tumor samples compared to *ARID1A*-wildtype samples (Figure 20C). These results showed that NRF2 does not regulate the serine/glycine biosynthesis pathway in gynecologic cancers.

Apart from regulating the serine/glycine biosynthetic pathway in NSCLC, another study found that NRF2 regulates anabolic metabolism [160]. To investigate whether NRF2 regulates anabolic metabolism in gynecologic cancers, we also queried the expression of NRF2-target genes that are involved in anabolic metabolism using the TCGA UECE dataset. We found that these genes were not up-regulated in *ARID1A*-mutant tumor samples compared to *ARID1A*-wildtype samples (Figure 21).

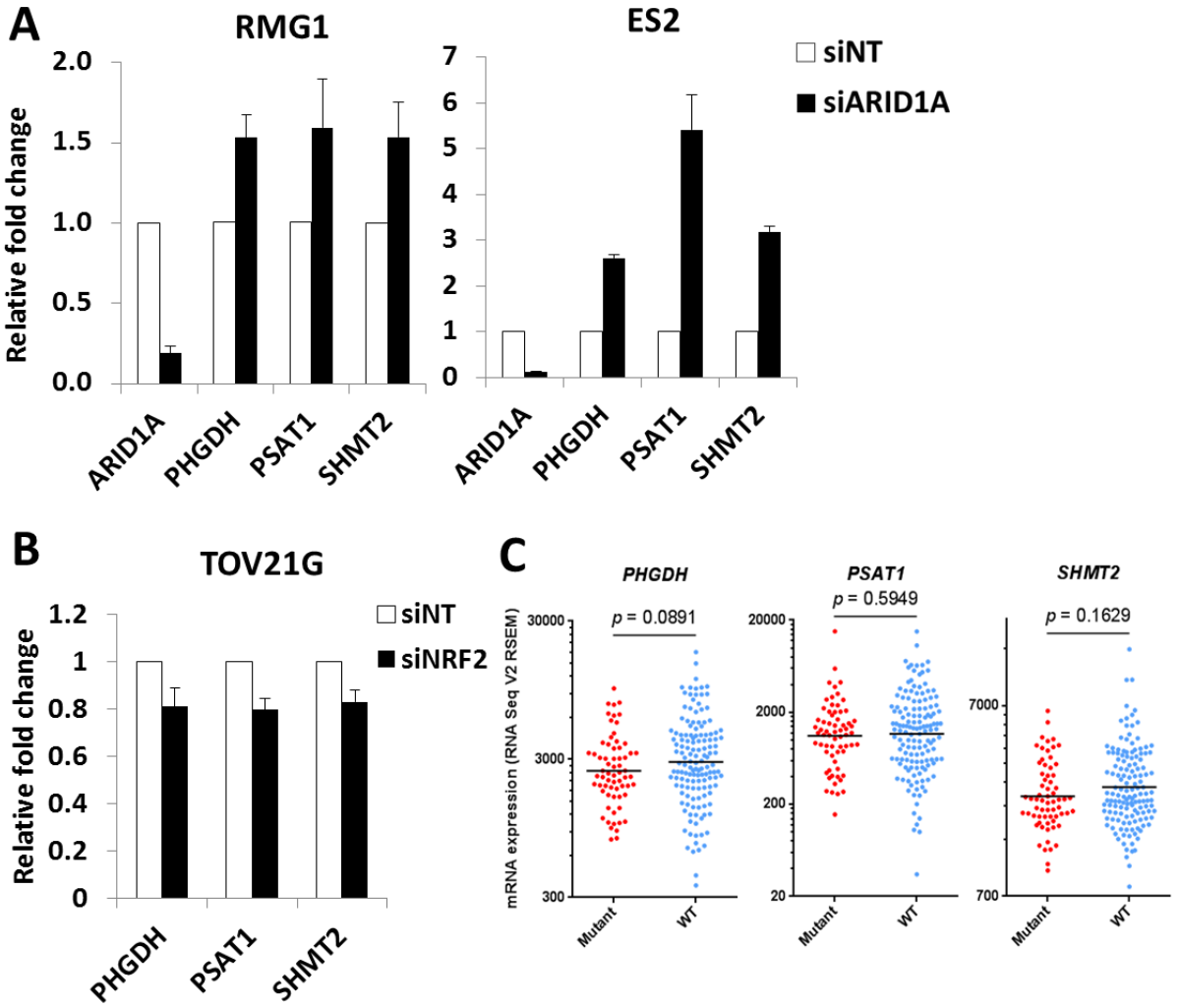

Figure 20. NRF2 is not required for serine/glycine biosynthesis in *ARID1A*-mutant cancer cells. (A) *ARID1A*-wildtype RMG1 and ES2 cells were transfected with ARID1A and non-target control siRNA. qPCR was used to measure the mRNA levels of *PHGDH*, *PSAT1* and *SHMT2*. (B) TOV21G cells were transfected with NRF2 and non-target siRNA. qPCR was used to measure mRNA levels as in (A). (C) mRNA levels of *PHGDH*, *PSAT1* and *SHMT2* were compared in *ARID1A*-mutant (Mutant, n = 66) and *ARID1A*-wildtype (WT, n = 141) TCGA UCEC samples. Black lines represent the geometric mean. Mann-Whitney *U*-tests were used to calculate significance.

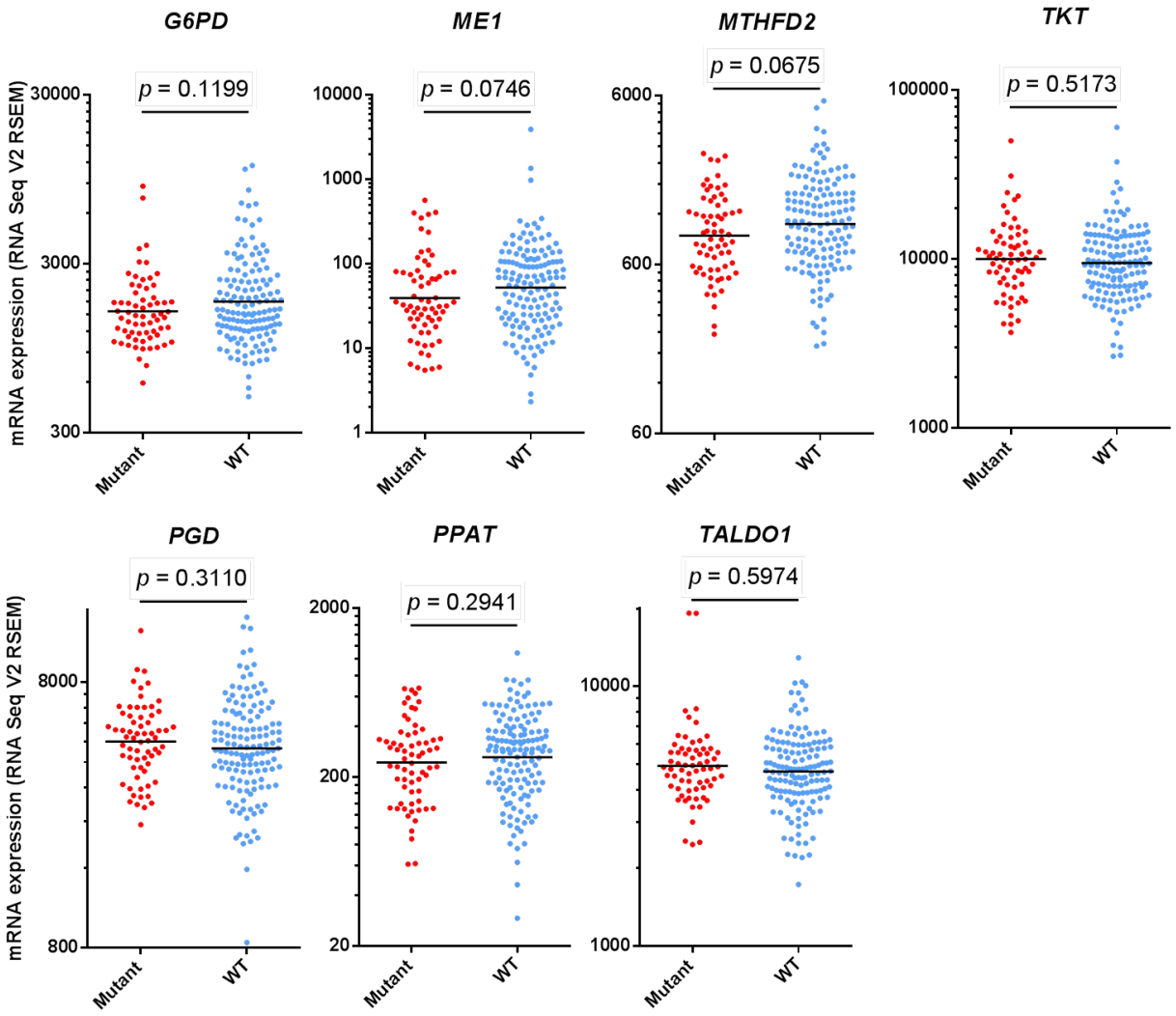

Figure 21. Genes involved in anabolic metabolism are not up-regulated in *ARID1A*-mutant TCGA UCEC tumor samples compared to *ARID1A*-wildtype samples. mRNA levels of *G6PD*, *ME1*, *MTHFD2*, *TKT*, *PGD*, *PPAT*, and *TALDO1* were compared in *ARID1A*-mutant (Mutant, n = 66) and *ARID1A*-wildtype (WT, n = 141) TCGA UCEC samples. Black lines represent the geometric mean. Mann-Whitney *U*-tests were used to calculate significance.

4.6 Conclusion

In the present study, we found that the tumor suppressor ARID1A negatively regulates NRF2 expression in ovarian cancer cells. We also found that *ARID1A*-mutant ovarian cancer cells are dependent on NRF2 expression for cell growth. Furthermore, this finding is supported by analysis of TCGA UECE tumor samples, which we found that NRF2 expression and its target genes are higher in *ARID1A*-mutant than *ARID1A*-wildtype tumor samples. We found that NRF2 does not regulate metabolic pathways in *ARID1A*-mutant ovarian cancer cells, suggesting that NRF2 is primarily required for protection against oxidative stress.

CHAPTER 5: DISCUSSION

5.1 Using ROS-inducing agents to target *ARID1A*-mutant cancer cells

Cancer cells have higher ROS levels than normal cells due a number of reasons as discussed in chapter 1. Although a moderate increase in ROS levels can promote cancer cell growth due to activation of signaling pathways and promotion of genomic instability, high levels of ROS lead to cell death and senescence. Therefore, it is important for cancer cells to maintain intracellular ROS levels below a toxic threshold. Previous studies have found that enhancing ROS has anti-tumor effects in cancer cells [101], however, it is not completely clear what predicts sensitivity to these treatments.

The results in chapter 3 showed that ARID1A is required to mediate resistance to oxidative stress and ARID1A deficiency leads to sensitivity to ROS-inducing agents in ovarian cancer cells. This is in line with previous findings in *S. cerevisiae* and *C. elegans* that SWI/SNF is required to mediate resistance to oxidative stress [136, 137] (for details see chapter 1.5) and this dissertation provided evidence that SWI/SNF mediated oxidative stress resistance is also conserved in mammalian cells. However, this dissertation focuses on ARID1A in endometriosis-associated ovarian cancers only, which are the cancer types which have the most frequent mutations in *ARID1A*. Future studies should investigate whether other SWI/SNF subunits are also required for oxidative stress resistance in other cancer types. Cancer types that have frequent SWI/SNF mutations, such as *SNF5* mutations in rhabdoid cancers and *BRG1* mutations in SCCOHT, are often aggressive and of poor prognosis. In addition, SWI/SNF mutations are frequently inactivating mutations and therefore, SWI/SNF mutations have to be

targeted indirectly using novel strategies. This dissertation provides a rationale of targeting SWI/SNF-mutant cancers with ROS-inducing agents.

However, it should also be noted that the therapeutic implications of our results is limited by the lack of in vivo models. Future studies should use mice models to investigate whether *ARID1A*-mutant cancers are sensitive to ROS-inducing agents. Apart from using xenograft mice models, *ARID1A*-knockout mice models that form OCCCs and OECs [48, 49] can also be used for this purpose.

5.2 Proposed mechanisms that lead to adaptation to oxidative stress in *ARID1A*-mutant OCCCs and OECs

Although the results from chapter 3 found that ARID1A is required to mediate resistance to oxidative stress, this finding is somewhat unexpected in the context of OCCCs and OECs. This is because the contents of endometriotic cysts have abundant free iron and lead to high ROS production [140]. As OCCCs and OECs are thought to arise from endometriotic cysts and frequently associated with endometriosis, OCCC and OEC cancer cells are consistently exposed to high oxidative stress during tumor initiation and progression [21, 22]. How OCCC and OEC cells survive in a microenvironment of persistent oxidative stress in the absence of ARID1A is unclear.

One possibility is that ARID1A deficiency in OCCCs is compensated for by HNF1β overexpression. As mentioned in chapter 1.6, HNF1β is overexpressed in OCCCs but not in other ovarian cancer subtypes [141]. A recent study found that HNF1β is required to mediate resistance to oxidative stress in OCCC cells [142]. To investigate whether HNF1β

overexpression can compensate for ARID1A deficiency in OCCCs, future studies should determine whether the depletion of HNF1β in *ARID1A*-mutant OCCCs can lead to further sensitivity to oxidative stress.

However, oxidative stress adaptation by overexpression of HNF1β is likely to be specific to OCCCs only, because HNF1β is not overexpressed in other ovarian cancer subtypes including OECs [141].Therefore, OECs are likely to adapt to high oxidative stress using a HNF1β-independent mechanism in the absence of ARID1A. One possibility is through estrogen signaling. As mentioned in chapter 1.2, key differences in ER and PR expression are found in OCCCs and OECs; OCCCs do not express ER and PR, whereas OECs express ER and PR [24, 25]. Although the mechanisms that lead to these differences are unknown, ER expression may compensate for absence of ARID1A in OECs. In ER+ immortalized mouse mammary epithelial cells (MECs) and ER+ human breast cancer MCF7 cells, estrogen stimulation was able to increase NRF2 protein expression and NRF-target gene expression [105]. Specifically, the activation of NRF2 by estrogen was found to be dependent on activation of PI3K signaling [164]. Therefore, in ER+/ARID1A- ovarian cancer, such as OECs, estrogen signaling may protect the cells from oxidative stress. Future studies should determine whether NRF2 is also activated by estrogen in OECs similar to ER+ breast cancer cells. If estrogen is able to activate NRF2 in OECs, then selected OECs patients with *ARID1A* mutations may benefit from anti-hormonal therapy, as this should block NRF2 activity in *ARID1A*-mutant cells, thereby increasing intracellular ROS levels and ROS-induced cell death.

5.3 Negative regulation of NRF2 by ARID1A in ovarian cancers

In cancer cells, the antioxidant response is up-regulated to avoid the detrimental effects of persistent oxidative stress. Aberrant activation of NRF2, the major regulator of the antioxidant response in the cell, has been identified in several cancer types. In ovarian cancer, NRF2 nuclear localization occurs more frequently in OCCCs than other epithelial ovarian cancer subtypes. Although *KEAP1* mutations were identified in OCCC patient samples, they could not account completely for the occurrence of NRF2 nuclear localization [165]. In addition, although several studies have found that NRF2 has oncogenic functions in different cancer types, the functional roles of NRF2 in ovarian cancers are not clear.

The results in chapter 4 identified a novel regulation between ARID1A and NRF2; ARID1A negatively regulates NRF2 expression and its downstream target genes. In addition, NRF2 inhibition by siRNA or the small molecule inhibitor brusatol were able to inhibit cell growth in ARID1A-mutant cells. These results suggest that ARID1A-mutant ovarian cancer cells may adapt to high oxidative stress by up-regulating NRF2 expression.

However, it should be noted that our results are opposite to the findings of a previous study, that BRG1 positively regulates NRF2 activity [138]. Interestingly, in this previous study by Zhang et al., depletion of BRG1 also resulted in increased NRF2 expression in the presence of diethylmaleate (DEM), which is a glutathione-depleting compound. This finding is similar to our results that ARID1A negatively regulates NRF2 expression in the presence of oxidative stress. Some differences in the study by Zhang et al. and this dissertation should be noted: 1) In Zhang et al.'s study, although BRG1 enhanced NRF2 reporter activity, out of the NRF2-target genes that they tested (HMOX1, NQO1, GCSL, GCSH, and AKR1C1), only HMOX1

expression was positively regulated by BRG1, suggesting that BRG1-dependent regulation of HMOX1 may be a specific event and not applicable to other NRF2-target genes. However, the results in chapter 4 showed that ARID1A negatively regulates several NRF2-target genes. 2) In the study by Zhang et al., the authors did not investigate whether BRG1 expression was correlated with NRF2 expression in patient samples. In chapter 4, we analyzed TCGA UCEC tumor samples and found that *ARID1A*-mutant samples had higher NRF2 and NRF2-target gene expression compared to *ARID1A*-wildtype patient samples. This further supports the notion that ARID1A negatively regulates NRF2 expression. 3) Zhang et al.'s study used non-ovarian cancer cells, we focused on ovarian cancer cells. It may be possible that the regulation of NRF2 by SWI/SNF is context dependent. 4) It is also not clear whether loss of individual SWI/SNF subunits will result in the same phenotype, therefore, it is possible that other SWI/SNF subunits, such as BRG1, regulate NRF2 differently from ARID1A.

Future studies should determine whether ARID1A also negatively regulates NRF2 in cancer types other than gynecologic cancers. In addition, since BRG1 mutations are also found in gynecologic cancers, future studies should also investigate whether BRG1 also negatively regulates NRF2 in these cancers.

5.4 Combination therapy using NRF2 inhibitor and chemotherapy

NRF2 has not only been found to mediate resistance to oxidative stress, but also chemotherapy [161]. It has been well characterized that SWI/SNF is required to facilitate DNA repair, and ARID1A has been found to mediate resistance to cisplatin [80]. Paradoxically, OCCCs are characterized by a poor response to chemotherapy due to unknown reasons. It is

possible that up-regulation of NRF2 in the absence of ARID1A mediates resistance to chemotherapy in OCCCs. A previous study found that depletion of the NRF2 inhibitor KEAP1 in ovarian cancer 36M2 cells leads to resistance to the cisplatin [165]. Therefore, future studies should investigate whether the use of NRF2 inhibitor can overcome resistance to chemotherapy in OCCCs.

5.5 Possible antioxidant-independent functions of NRF2 in gynecologic cancers

Although the antioxidant functions of NRF2 are well characterized, two recent studies have identified antioxidant-independent functions of NRF2 in metabolism [160, 162].

The first study by Mitsuishi et al. [160] found that when the PI3K/AKT pathway is constitutively activated, nuclear accumulation of NRF2 is increased and NRF2 promotes anabolic metabolism that support cell proliferation. In addition, NRF2 activation also further activates PI3K/AKT signaling, creating a positive feedback loop. Given that 1) NRF2 is an oncogene, 2) inactivating *ARID1A* mutations frequently co-occur with activating PI3K pathway mutations in human cancers [33], and 3) tumors only form in mice that harbor both *ARID1A* and *PIK3CA*/*PTEN* mutations [48, 49], it may be possible that combined ARID1A loss and PI3K/AKT pathway activation can promote tumorigenesis by activating NRF2 activity and subsequently leading to metabolic reprogramming. Although there were no differences in expression of genes involved in anabolic metabolism in *ARIDA*-wildtype compared with *ARID1A*-mutant TCGA UCEC samples, the possibility that NRF2 regulates anabolic metabolism in gynecologic cancers cannot be fully excluded. This is because although *ARID1A* mutations frequently co-occur with PI3K pathway mutations, it is unclear whether all PI3K

pathway mutations will lead to activation of the PI3K/AKT pathway and to what degree of activation. Therefore, it may be useful to quantify PI3K pathway activation in TCGA UCEC samples using reverse phase protein array (RPPA) data and combine this data with *ARID1A* mutation status to improve sample stratification.

In a second study by DeNicola et al., the authors found that NRF2 regulates the serine/glycine biosynthetic pathway by controlling the expression of *PHGDH*, *PSAT1* and *SHMT2* in non-small cell lung cancers (NSCLC) [162]. Although the results in chapter 4 found that ARID1A depletion resulted in increased expression of *PHGDH*, *PSAT1* and *SHMT2*, the expression of these three genes were only slightly down-regulated upon depletion of NRF2, suggesting that NRF2 does not regulate the expression of these genes in ovarian cancer cells. We also observed no up-regulation of *PHGDH*, *PSAT1* and *SHMT2* expression in *ARID1A*-mutant TCGA UCEC samples compared to *ARID1A*-wildtype samples. One possibility for this difference is the different expression of NRF2 partners in NSCLC and gynecologic cancers. In NSCLC, the regulation of serine/glycine biosynthesis genes by NRF2 was found to be dependent on the transcription factor ATF4 [162], whereas the relationship between NRF2 and ATF4 have not been investigated in gynecologic cancers.

5.6 A NRF2-dependent cell death response to oxidative stress in *ARID1A*-mutant cancers?

The results in chapter 4 showed that ARID1A negatively regulates NRF2. Theoretically, if NRF2 is up-regulated in *ARID1A*-mutant cells, it should mediate cytoprotection against oxidative stress. However, this is paradoxical to the findings in chapter 3 that loss of ARID1A leads to sensitivity to oxidative stress. This may be explained by a recent study which revealed

an unexpected and paradoxical role of NRF2 in the presence of excessive oxidative stress [166]. At low oxidative stress, NRF2 activates antioxidant genes and promote ROS detoxification. However, at high oxidative stress, the continuous accumulation of NRF2 leads to the transcriptional activation of KLF9. KLF9 in turn further increases ROS levels and promote cell death. Interestingly, KLF9 activation requires higher levels of NRF2 compared to NRF2-target antioxidant genes, such as HMOX1 and NQO1, showing that high levels NRF2 promote cell death instead of cytoprotection. As oxidative stress in *ARID1A*-mutant ovarian cancer cells results in high expression of NRF2, it is possible that the increased accumulation of NRF2 is detrimental to the cell by activating KLF9 and causing cell death. Future studies should investigate whether the depletion of ARID1A lowers the threshold of KLF9 expression in the presence of oxidative stress.

5.7 Possible mechanisms on how ARID1A mediates oxidative stress resistance

How ARID1A mediates oxidative stress resistance is still unclear. In *C. elegans*, SWI/SNF is a co-factor for DAF16 (the ortholog of the human FOXO protein), is required for DAF16 transcriptional activity and DAF16 mediated oxidative stress resistance [137]. Although the FOXO protein is conserved in humans, it is unlikely that ARID1A promotes oxidative stress through FOXO in ovarian cancer. This is because ARID1A mutations are frequently with PI3K pathway mutations [33], hence activation of the PI3K pathway, which leads to phosphorylation of the FOXO protein and its sequestration in the cytoplasm [167].

From the microarray analysis that compared gene expression between ARID1A-knockdown and non-target control RMG1 cells, we found that several ROS detoxification genes

were down-regulated upon ARID1A depletion (Table 5). Further validation will be required to identify antioxidant genes that are dependent on ARID1A for activation and which genes are required for ARID1A mediated oxidative stress resistance. Apart from down-regulation ROS detoxification genes, the up-regulation of BID expression in ARID1A-knockdown cells may also be a potential candidate. BID is a proapoptotic BCL2 family protein that is cleaved and activated during various stress conditions. A previous study has found that truncated BID (tBID) induces waves of cytochrome c release at neighboring mitochondria through ROS [168]. Our preliminary data found that in the presence of oxidative stress, BID expression is up-regulated in ARID1A-knockdown RMG1 cells and down-regulated in TOV21G cells re-expressing ARID1A (Figure 22). Future studies should investigate whether BID expression is negatively regulated by ARID1A during oxidative stress and if BID repression is required for ARID1A mediated oxidative stress resistance.

Symbol	Entrez Gene Name	p-value	Fold Change
ALOX5	arachidonate 5-lipoxygenase	4.02E-03	-6.703
GLRX	glutaredoxin (thioltransferase)	5.18E-04	-4.701
ACADSB	acyl-CoA dehydrogenase, short/branched chain	5.62E-03	-3.205
TBXAS1	thromboxane A synthase 1 (platelet)	1.60E-02	-2.522
TP53I3	tumor protein p53 inducible protein 3	1.87E-03	-2.336
COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	2.62E-03	-2.154
GFOD1	glucose-fructose oxidoreductase domain containing 1	2.91E-02	-2.080
P4HA2	prolyl 4-hydroxylase, alpha polypeptide II	2.04E-02	-2.075
ACSM3	acyl-CoA synthetase medium-chain family member 3	1.95E-02	-2.055
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	1.38E-02	-1.949
NRXN3	neurexin 3	2.43E-02	-1.924
MSRB1	methionine sulfoxide reductase B1	8.67E-03	-1.916
ACSF2	acyl-CoA synthetase family member 2	2.90E-03	-1.914
CDYL2	chromodomain protein, Y-like 2	4.37E-03	-1.907
XDH	xanthine dehydrogenase	4.68E-03	-1.894
SOD2	superoxide dismutase 2, mitochondrial	1.10E-02	-1.804
LOXL2	lysyl oxidase-like 2	2.03E-02	-1.688
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	6.56E-03	-1.668
GSTZ1	glutathione S-transferase zeta 1	4.29E-02	-1.629
RRM2	ribonucleotide reductase M2	2.53E-02	-1.624
CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18	4.20E-02	-1.612
MSRB2	methionine sulfoxide reductase B2	3.81E-02	-1.598
MGST3	microsomal glutathione S-transferase 3	3.12E-02	-1.597
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	3.50E-03	-1.593
BLVRA	biliverdin reductase A	4.36E-02	-1.575
CYB5R3	cytochrome b5 reductase 3	1.00E-02	-1.562
CDYL	chromodomain protein, Y-like	1.97E-02	-1.504

Table 5. List of genes that were down-regulated ($p < 0.05$ and fold-change ≥ 1.5) in ARID1A-knockdown RMG1 cells compared to non-target control cells. Shown here are genes that were categorized as GO term 'oxidative reduction'.

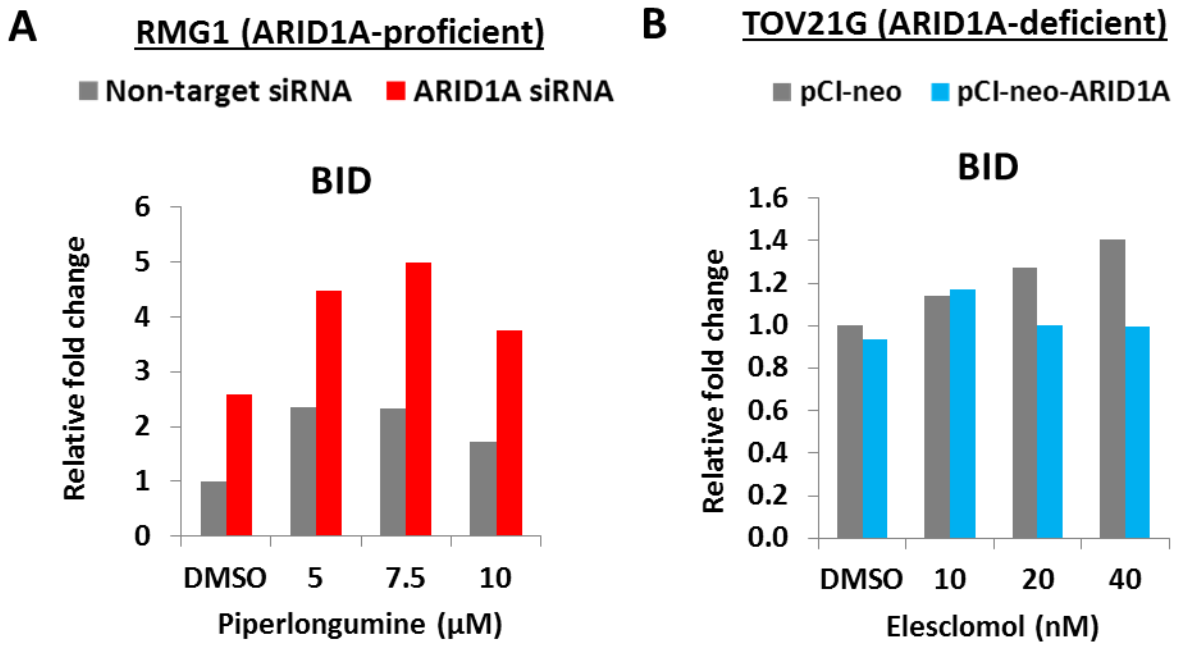

Figure 22. ARID1A negatively regulates BID expression. (A) RMG1 cells were transfected with ARID1A and non-target control cells and then treated with piperlongumine for 6 h. BID expression was measured by qPCR. (B) TOV21G cells transfected with pCI-neo-ARID1A and pCI-neo empty vector control and then treated with elesclomol for 6 h. BID expression was measured by qPCR.

5.8 Translational significance

ARID1A is a tumor suppressor, and its expression is lost in cancer. Therefore, it is not possible to directly target ARID1A itself, rather, specific vulnerabilities of *ARID1A*-mutant cancer cells must be discovered and targeted. Although several tumor suppressive functions of ARID1A have emerged, therapeutic targets for *ARID1A*-mutant cancer cells remained limited (for details see chapter 1.3.7). In chapter 3, we discovered that ARID1A is required for promoting resistance to oxidative stress and *ARID1A*-mutant ovarian cancer cells are more sensitive to the ROS-inducing agent elesclomol than *ARID1A*-wildtype ovarian cancer cells. In addition to elesclomol, which has been used in a phase III clinical trial, several ROS-inducing agents have been developed and are in different stages of development [101, 169]. Taken together, inducing ROS generation is a novel and feasible therapeutic strategy for targeting *ARID1A*-mutant ovarian cancer cells.

An increasing number of studies have found that NRF2, the major regulator of the antioxidant response in the cell, is an oncogene in cancer. In chapter 4, we found that ARID1A negatively regulates NRF2 expression. Furthermore, we found that *ARID1A*-mutant ovarian cancer cells have more sensitive to NRF2 depletion than *ARID1A*-wildtype cells. Importantly, NRF2 is a druggable target and the compound brusatol has been previously found to inhibit NRF2 expression [161]. Taken together, these findings suggest that NRF2 is a novel and feasible therapeutic target in *ARID1A*-mutant ovarian cancer cells.

As NRF2 is required for resistance to oxidative stress and our data suggests that NRF2 is required primarily for protection from oxidative stress in *ARID1A*-mutant ovarian cancer cells, it

is possible that the combination of NRF2 inhibitor and ROS-inducing agents may have a greater effect than using either agent alone. Future studies testing this combination are warranted.

APPENDIX

Case ID	ARID1A mutation
TCGA-AP-A056-01	Y592*,R1989*
TCGA-BS-A0U5-01	Y485Pfs*135
TCGA-BS-A0UA-01	Y2254*
TCGA-BG-A0MG-01	Y1324*
TCGA-AP-A059-01	X751_splice,R750*,P2114L
TCGA-B5-A0K0-01	W2048*
TCGA-BG-A0LX-01	V1994Cfs*21
TCGA-AP-A0LG-01	T783Kfs*50
TCGA-BG-A0MT-01	T2138Hfs*62
TCGA-B5-A11O-01	T1640Pfs*14
TCGA-D1-A17C-01	T1514Pfs*14,G1317W
TCGA-D1-A168-01	S674Ifs*68,P1353Rfs*123
TCGA-D1-A17T-01	S2269*,R727Vfs*12
TCGA-BK-A0CB-01	S1001Ffs*6,R693*
TCGA-B5-A11W-01	R750*
TCGA-AX-A062-01	R693*
TCGA-B5-A0JV-01	R693*
TCGA-D1-A176-01	R693*
TCGA-BS-A0TG-01	R2236Afs*31,H415Pfs*205
TCGA-D1-A0ZU-01	R2158*,R1989*
TCGA-D1-A17H-01	R2158*
TCGA-BS-A0TC-01	R1989*
TCGA-D1-A16X-01	R1989*
TCGA-D1-A17L-01	R1989*
TCGA-B5-A11E-01	R1722*,S1090I,P1144S,R1989*
TCGA-B5-A0JS-01	R1722*
TCGA-D1-A160-01	R1528*
TCGA-B5-A0K4-01	R1446*
TCGA-A5-A0GI-01	R1335*
TCGA-BG-A0M3-01	R1335*
TCGA-BG-A2AE-01	R1335*
TCGA-BG-A18A-01	R1276*
TCGA-BG-A18B-01	Q557*
TCGA-D1-A17A-01	Q543*
TCGA-AX-A2HF-01	Q505Sfs*114
TCGA-AP-A05N-01	Q479*

TCGA-AX-A0IS-01	Q425Rfs*8
TCGA-A5-A0GB-01	Q405*
TCGA-D1-A0ZS-01	Q2209Sfs*22
TCGA-D1-A16O-01	Q2209Sfs*22
TCGA-BG-A0VV-01	Q1473*
TCGA-BG-A0YV-01	Q1409*
TCGA-AX-A063-01	Q1334del,R1335*
TCGA-D1-A16Q-01	Q1331*
TCGA-A5-A0R8-01	Q1327*
TCGA-BS-A0U8-01	Q1306*
TCGA-B5-A121-01	Q1188*
TCGA-D1-A17R-01	Q1172*
TCGA-BG-A0M9-01	Q1131*
TCGA-D1-A16B-01	M434Cfs*185
TCGA-AX-A05Y-01	L2171dup
TCGA-D1-A0ZO-01	K1953Rfs*3
TCGA-A5-A0GQ-01	K1928Lfs*29,L2239P
TCGA-AX-A0J0-01	I2135V,S1320Y,S995Ifs*12
TCGA-AX-A05T-01	G1015Wfs*25
TCGA-BS-A0UV-01	E992*,P1619L,R1989*
TCGA-AX-A05Z-01	E672*
TCGA-BS-A0V6-01	E2255Gfs*10,L2253Vfs*24
TCGA-D1-A16D-01	E2047*
TCGA-B5-A0JY-01	E1764*,G925*,L1713P
TCGA-AP-A0LL-01	E1733*
TCGA-AP-A0LE-01	D734Gfs*83
TCGA-BK-A0CA-01	A1789Pfs*17
TCGA-BS-A0TJ-01	A1628Pfs*15
TCGA-A5-A0GM-01	A1589Gfs*19,M737Ifs*6
TCGA-D1-A0ZN-01	A1119Vfs*43
TCGA-A5-A0G5-01	WT
TCGA-A5-A0GA-01	WT
TCGA-A5-A0GE-01	WT
TCGA-A5-A0GJ-01	WT
TCGA-A5-A0GN-01	WT
TCGA-A5-A0GU-01	WT
TCGA-A5-A0GV-01	WT
TCGA-A5-A0GX-01	WT
TCGA-A5-A0R7-01	WT

TCGA-A5-A0R9-01	WT
TCGA-A5-A0RA-01	WT
TCGA-A5-A0VP-01	WT
TCGA-A5-A0VQ-01	WT
TCGA-AJ-A23M-01	WT
TCGA-AP-A052-01	WT
TCGA-AP-A053-01	WT
TCGA-AP-A054-01	WT
TCGA-AP-A05A-01	WT
TCGA-AP-A05D-01	WT
TCGA-AP-A05H-01	WT
TCGA-AP-A05J-01	WT
TCGA-AP-A05P-01	WT
TCGA-AP-A0L8-01	WT
TCGA-AP-A0L9-01	WT
TCGA-AP-A0LD-01	WT
TCGA-AP-A0LF-01	WT
TCGA-AP-A0LH-01	WT
TCGA-AP-A0LI-01	WT
TCGA-AP-A0LJ-01	WT
TCGA-AP-A0LN-01	WT
TCGA-AP-A0LO-01	WT
TCGA-AP-A0LP-01	WT
TCGA-AP-A0LQ-01	WT
TCGA-AP-A0LT-01	WT
TCGA-AP-A0LV-01	WT
TCGA-AX-A05S-01	WT
TCGA-AX-A05U-01	WT
TCGA-AX-A064-01	WT
TCGA-AX-A06B-01	WT
TCGA-AX-A06H-01	WT
TCGA-AX-A06L-01	WT
TCGA-AX-A0IW-01	WT
TCGA-AX-A0J1-01	WT
TCGA-AX-A1C7-01	WT
TCGA-AX-A1C8-01	WT
TCGA-AX-A1CP-01	WT
TCGA-AX-A2H5-01	WT
TCGA-B5-A0JN-01	WT

TCGA-B5-A0JR-01	WT
TCGA-B5-A0JZ-01	WT
TCGA-B5-A0K1-01	WT
TCGA-B5-A0K2-01	WT
TCGA-B5-A0K6-01	WT
TCGA-B5-A0K7-01	WT
TCGA-B5-A0K8-01	WT
TCGA-B5-A0K9-01	WT
TCGA-B5-A11F-01	WT
TCGA-B5-A11G-01	WT
TCGA-B5-A11H-01	WT
TCGA-B5-A11I-01	WT
TCGA-B5-A11J-01	WT
TCGA-B5-A11N-01	WT
TCGA-B5-A11Q-01	WT
TCGA-B5-A11S-01	WT
TCGA-B5-A11U-01	WT
TCGA-B5-A11V-01	WT
TCGA-B5-A11Z-01	WT
TCGA-B5-A1MU-01	WT
TCGA-B5-A1MY-01	WT
TCGA-BG-A0LW-01	WT
TCGA-BG-A0M0-01	WT
TCGA-BG-A0M2-01	WT
TCGA-BG-A0M6-01	WT
TCGA-BG-A0M7-01	WT
TCGA-BG-A0M8-01	WT
TCGA-BG-A0MC-01	WT
TCGA-BG-A0MI-01	WT
TCGA-BG-A0MO-01	WT
TCGA-BG-A0MQ-01	WT
TCGA-BG-A0MS-01	WT
TCGA-BG-A0MU-01	WT
TCGA-BG-A0RY-01	WT
TCGA-BG-A0VT-01	WT
TCGA-BG-A0VW-01	WT
TCGA-BG-A0VX-01	WT
TCGA-BG-A0VZ-01	WT
TCGA-BG-A0W1-01	WT

TCGA-BG-A0W2-01	WT
TCGA-BG-A0YU-01	WT
TCGA-BG-A186-01	WT
TCGA-BG-A187-01	WT
TCGA-BG-A18C-01	WT
TCGA-BK-A0CC-01	WT
TCGA-BK-A139-01	WT
TCGA-BK-A13C-01	WT
TCGA-BS-A0T9-01	WT
TCGA-BS-A0TA-01	WT
TCGA-BS-A0TD-01	WT
TCGA-BS-A0TE-01	WT
TCGA-BS-A0TI-01	WT
TCGA-BS-A0U9-01	WT
TCGA-BS-A0UF-01	WT
TCGA-BS-A0UL-01	WT
TCGA-BS-A0UT-01	WT
TCGA-BS-A0V7-01	WT
TCGA-BS-A0V8-01	WT
TCGA-BS-A0WQ-01	WT
TCGA-D1-A0ZP-01	WT
TCGA-D1-A0ZQ-01	WT
TCGA-D1-A0ZR-01	WT
TCGA-D1-A0ZV-01	WT
TCGA-D1-A0ZZ-01	WT
TCGA-D1-A101-01	WT
TCGA-D1-A102-01	WT
TCGA-D1-A103-01	WT
TCGA-D1-A15V-01	WT
TCGA-D1-A15Z-01	WT
TCGA-D1-A161-01	WT
TCGA-D1-A163-01	WT
TCGA-D1-A165-01	WT
TCGA-D1-A169-01	WT
TCGA-D1-A16G-01	WT
TCGA-D1-A16I-01	WT
TCGA-D1-A16J-01	WT
TCGA-D1-A16N-01	WT
TCGA-D1-A16S-01	WT

TCGA-D1-A16Y-01	WT
TCGA-D1-A174-01	WT
TCGA-D1-A177-01	WT
TCGA-D1-A17B-01	WT
TCGA-D1-A17D-01	WT
TCGA-D1-A17F-01	WT
TCGA-D1-A17K-01	WT
TCGA-D1-A17M-01	WT
TCGA-D1-A17N-01	WT
TCGA-D1-A17S-01	WT
TCGA-D1-A1NU-01	WT
TCGA-D1-A1NX-01	WT
TCGA-DI-A0WH-01	WT
TCGA-DI-A1NN-01	WT
TCGA-E6-A1LZ-01	WT
TCGA-EO-A1Y5-01	WT
TCGA-EO-A1Y8-01	WT
TCGA-EY-A1GS-01	WT
TCGA-EY-A212-01	WT
TCGA-FI-A2EW-01	WT
TCGA-FI-A2EX-01	WT

Table 6. List of uterine corpus endometrioid carcinoma tumor samples from TCGA. Database used was previously published [152]. WT, wildtype. *, non-sense mutation. Fs, frame-shift mutation. Splice, mutation at splice site.

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