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Navya Valavala April 9, 2025

Investigating the role of the calcium binding protein CAB39 in the repair of DNA double-strand breaks and the maintenance of genome stability

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Abstract

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Genome instability is a hallmark of cancer. As such, the mechanisms by which cells maintain their genomic integrity are critical to understanding cancer pathophysiology as well as other related genetic disorders. Among the most cytotoxic forms of DNA damage are DNA double strand breaks (DSBs), which are primarily repaired by non-homologous end joining (NHEJ) and homologous recombination (HR). In this study, we identified the calcium binding protein 39 (CAB39) as a putative NHEJ regulator using a CRISPR-based chemogenomic profiling. We performed functional validation using *in vitro* cell survival assays and revealed that CAB39 depletion selectively increases the sensitivity of cancer cells to a subset of NHEJ-associated genotoxic agents, notably etoposide and high dose of doxorubicin. To further characterize the role of CAB39 in DSB repair, we employed a GFP-based HR reporter assay and found that CAB39 depletion impairs HR efficiency, suggesting a regulatory role for CAB39 beyond the NHEJ pathway. Additionally, pan-cancer expression and survival analyses revealed that *CAB39* is differentially expressed across tumor types, with high *CAB39* transcript levels correlating with poor prognosis in specific cancers, including lung adenocarcinoma and lung squamous cell carcinoma. Together, our study suggests that CAB39 acts as a context-dependent modulator of the DNA damage response and a potential prognostic biomarker in cancer.

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Finally, I thank my committee members for your constant support in the production of this thesis. Your comments and valuable insights throughout this process are what made this project what it is today. Thank you for all your help.

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Background

Contribution of DNA damage in genome instability – The faithful transmission of genetic information from parent cell to daughter cell is a necessary process to all life. This maintenance of genome integrity is known as genome stability. Genome instability, on the other hand, is a condition characterized by high mutation rates, microsatellite instability (MSI), and chromosomal instability (CIN) which can cause chromosomal aberrations (Yao & Dai, 2014). Genome instability is associated with a wide range of diseases, including neurodegeneration, immunodeficiency, premature aging, and cancer predisposition.

Genomic instability can occur in response to disruptions to vital cell processes such as cell cycle progression, chromosomal segregation, DNA replication, and/or DNA repair (Eyfjord & Bodvarsdottir, 2005). A major cause of genomic instability includes the constant exposure to both endogenous sources of DNA damage such as metabolites, and exogenous sources of DNA damage such as radiation and UV exposure. These sources of damage can result in different types of DNA lesions, including damage to individual nucleotides or more complex lesions in the sugar-phosphate backbone. Amongst these lesions, DNA double-strand breaks (DSBs) are widely considered to be one of the most severe forms of DNA damage as they can cause both small mutational changes as well as major chromosomal rearrangements (chromosomal translocations). Early work showed that the formation of a single DSBs is sufficient to cause lethality in yeast cells, highlighting how cytotoxic DSBs can be (Bennett et al., 1993).

Cells have evolved a complex and coordinated response called the DNA damage response (DDR) to detect, signal, and ultimately repair these DSBs. There are two major pathways that can repair

DSBs: Non-Homologous End Joining (NHEJ) and Homologous recombination (HR). The NHEJ pathway occurs throughout the cell cycle, but predominantly during the G1 phase and involves the direct ligation of the ends of damaged DNA together, quickly and without any DNA end processing (Lieber, 2010). As a result, the NHEJ pathway is more error prone than HR but is the most commonly used pathway for DSB repair. On the other hand, the HR pathway involves homology directed repair. In this mechanism, an identical strand of DNA is used as a template to repair the damaged strand. This results in high fidelity repair during the S/G2 phases of the cell cycle (Symington & Gautier, 2011). The choice between these repair pathways is influenced by various factors, including cell cycle position, presence of specific DNA repair factors at the site of damage, and chromatin state (Her & Bunting, 2018).

Genomic instability arising from a disruption or failure of these DNA repair pathways can result in the persistence of DNA lesions, leading to genomic alterations that drive carcinogenesis (Moretton & Loizou, 2020). These alterations occurring in key regulatory genes, such as oncogenes, tumor suppressor genes, and genes involved in DNA repair could lead to uncontrolled cell proliferation and reduced cell death through apoptosis, creating conditions that are conducive to tumorigenesis and tumor growth. For instance, mutations in the TP53 gene, a well-known tumor suppressor, are commonly found in various cancers and contribute to the disruption of normal cell cycle regulation (Levine, 2021).

Genome instability in lung cancer pathobiology – Lung cancer is the second most commonly diagnosed cancer within the US and remains the leading cause of cancer-related deaths amongst men and second most amongst women, worldwide (Schabath & Cote, 2019). This high death rate may be due in part to late diagnosis of the disease. Between the years 2017 and 2021, more than

half of all lung cancer diagnoses were made at distant stages, after metastasis of the primary tumor (Zhang et al., 2022). Additionally, lung cancers exhibit a high incidence of developing resistance to current standards of care (Ashrafi et al., 2022).

Tumors in the lung arise predominantly from epithelial lung tissue which undergoes uncontrolled proliferation (Cruz et al., 2011). Lung cancers are often classified into two broad categories, small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC). SCLC accounts for 10-15% of all lung cancers and is aggressive and fast to spread. Most SCLCs are diagnosed after metastasis. NSCLC accounts for around 85% of lung cancers (Sabbula et al., 2024). NSCLC has three main subtypes: lung adenocarcinomas (LUAD), lung squamous cell carcinomas (LUSC), and large cell carcinomas (Niemira et al., 2019). Different types of lung cancer can also exhibit varying levels of genomic instability, with SCLC considered to be the most genomically unstable of the lung cancers (Pikor et al., 2013). These lung cancer subtypes can be characterized by a difference in genetic mutation signatures and copy number alterations (Pikor et al., 2013). Key driver mutations of NCSLCs occur in oncogenes such as *EGFR*, *HER2*, *KRAS*, *ALK*, *BRAF*, *PIK3CA*, *AKT1*, *ROS1*, *NRAS* and *MAP2K1* (Pao & Hutchinson, 2012). On the other hand, SCLCs predominantly present with loss of function mutations in the tumor suppressor genes *TP53* and *RB1*, with *TP53* mutations found in 75-90% of patients and *RB1* mutations found in nearly all patients with SCLC (Semenova et al., 2015). These differences in mutational status of genes have been leveraged to create targeted therapies towards specific lung cancer types.

Historically, lung cancers have been strongly associated with smoking habits. In the US, around 87% of all lung cancers deaths were caused by smoking (Tindle et al., 2018). Mainstream cigarette smoke, produced by inhalation of air through a cigarette, is highly carcinogenic due to the

presence of compounds like polycyclic aromatic hydrocarbons, aromatic amines, and *N*-nitrosamines (Bade & Dela Cruz, 2020). These compounds can cause cancer through dysregulation of the immune system, oxidative stress, DNA damage, and genetic mutations (Nsonwu-Anyanwu et al., 2022). Therefore, lung cancers arising in smokers have a high mutational burden (Wang et al., 2021). However, recently there has been a new trend of a young, non-smoking population who are developing lung cancers (Thomas et al., 2015). These non-smokers account for around 10-15% of all lung cancer cases and over half of them present with adenocarcinomas (Samet et al., 2009).

Most lung cancers are treated with chemotherapy, radiation therapy, or immunotherapy—alone or in combination (Hirsch et al., 2017). There also exist targeted therapies directed towards specific types of lung cancer. However, many lung cancer patients develop resistance to these treatments, despite having displayed an initial response (Ashrafi et al., 2022). For example, around 20-30% of NSCLC patients being treated with EGFR inhibitors display primary resistance to the treatment (Ferro et al., 2024), and 21-27% of NSCLC patients develop primary resistance to immunotherapies when this therapy is used as a first-line treatment (Zhou & Yang, 2023). This prevalence of treatment resistance is another reason for the poor prognoses and treatment outcomes in lung cancers. The poor prognosis of disease and the high frequency of treatment resistance amongst lung cancers highlight the need for new targeted therapies for their treatment.

Therapeutic Relevance of targeting genome instability/DNA repair Pathways – Genome instability and DNA repair pathways have emerged as promising therapeutic targets in cancer treatment due to their strong association with the disease. In normal cells, DNA repair

mechanisms are tightly regulated to ensure the faithful replication of the genome. A deficiency in DNA repair pathways contributes to genomic instability and the accumulation of mutations, thereby promoting tumorigenesis (Zhou et al., 2020). In fact, ~13% of cancers display a mutation in one or more DNA damage repair gene (Mandelker et al., 2017). If a cell is unable to repair significant DNA damage, this can signal to apoptotic pathways to induce cell death (Reuvers et al., 2020). Major cancer treatments such as chemo- and radiotherapies leverage this principle by causing various types of DNA lesions in cancer cells (Ciccia & Elledge, 2010) with the purpose of overburdening the cell with irreparable DNA damage. These therapies include chemotherapeutic drugs such as bleomycin, etoposide and doxorubicin, which cause DNA damage through various mechanisms. Bleomycin can generate both single and double stranded DNA breaks through the formation of reactive oxygen species (ROS), which can cause oxidative stress to DNA (Stubbe & Kozarich, 1987, Al-Mareed et al., 2022). Etoposide and doxorubicin, on the other hand, are topoisomerase II poisons involved in the stalling of topoisomerase II during DNA replication, which in turn can cause DSBs (Montecucco et al., 2015, Rivankar, 2014). However, treatment resistant cancers often adapt to DNA damage inducing therapies by up-regulating DNA repair mechanisms, allowing the cells to survive even when subjected to significant amounts of DNA damage (Cree & Charlton, 2017). Therefore, targeting DNA repair pathways in combination with other therapies has high therapeutic potential. By depleting DNA repair mechanisms from cancer cells, DNA-repair based treatments could further sensitize cancer cells to traditional chemo- and radiation therapies.

Defective DNA repair mechanisms have already been exploited to create novel effective cancer treatments. Tumors with specific DNA repair defects often develop a dependence on other DNA

repair mechanisms for their survival, which can be leveraged in targeted therapies to induce lethality in these cells (Bouwman & Jonkers, 2012). For example, *BRCA* gene mutations, common amongst hereditary breast and ovarian cancers (King et al., 2003), cause cells to be deficient in the HR repair pathway (Ali et al., 2020). As a result, cancers with *BRCA* mutations must turn to alternative, more error-prone pathways for DNA repair, promoting further genomic instability (Bidany-Mizrahi et al., 2024). Poly (ADP Ribose) polymerases (PARPs) are proteins that are implicated in many DNA repair mechanisms, including a major role in single strand break (SSB) repair and an involvement in DSB repair choice (Wicks et al., 2022). PARP inhibitors have been used in cancers with BRCA mutations to induce synthetic lethality. PARP inhibitors prevent repair of SSBs and convert them to DSBs, which the *BRCA*-mutated cancers deficient in HR are unable to repair, pushing the cell towards apoptotic pathways (Zheng et al., 2020). The success of therapies such as these prove that knowledge of DNA repair mechanisms can be leveraged to supplement existing cancer therapies and improve treatment outcomes.

CAB39: A Novel Regulator of Genome Stability? – Calcium binding protein 39 (CAB39), previously known as mouse protein 25 (MO25), is a protein that enables kinase binding activity and stabilizes serine/threonine kinase complexes. Increased CAB39 expression promotes growth and metastasis of hepatocellular carcinomas (Jiang et al., 2017), suggesting that CAB39 has an oncogenic effect. Furthermore, CAB39 confers resistance to cisplatin in bladder cancer cells (Gao et al., 2023), a chemotherapeutic alkylating agent that forms DNA cross-linkages and exerts its effect by overburdening cells with DNA damage, pushing them towards apoptotic pathways (Kopacz-Bednarska & Król, 2022). The link between CAB39 and cisplatin resistance suggests that CAB39 may play a role in DNA repair mechanisms within cancer cells.

CAB39 is most well defined as part of a trimeric protein complex with Liver Kinase B1 (LKB1) and STE20-Related Kinase Adapter Protein (STRAD) (Golkowski et al., 2023; So et al., 2015). LKB1 is a serine/threonine protein kinase encoded by the *STK11* gene with tumor suppressive properties. LKB1 is activated by the pseudo kinase STRAD through binding. CAB39 binds to STRAD within this complex to further enhance LKB1 activity (Boudeau, 2003). CAB39 has also been implicated in salt balance regulation through the WNK4-SPAK/OSR1 pathway (Terker et al., 2018). However, there is no evidence suggesting that CAB39 has a catalytic role within these complexes. Additionally, the predicted tertiary structure of CAB39 protein lacks predicted binding pockets, signal peptides, and/or transmembrane regions (Uhlén et al., 2015). Therefore, it is believed that CAB39 is a scaffolding protein mainly involved in providing structural support to kinase complexes such as the LKB1/STRAD complex.

Figure 1*: CAB39 Tertiary Structure - GeneCards, CAB39.*

Significance

Although CAB39 has been previously studied in the context of kinase signaling, its role in DNA damage repair and genome stability remains unexplored. This project investigates CAB39 as a potential regulator of DNA double-strand break (DSB) repair, with a focus on impact within non-homologous end joining (NHEJ). As standard-of-care cancer treatments such as chemotherapy and radiation rely on the induction of DSBs to trigger cancer cell death, understanding how CAB39 contributes to DSB repair has important clinical implications. Our findings suggest that CAB39 may serve as a novel predictive biomarker of therapeutic response and could represent a promising target to enhance the efficacy of existing cancer therapies.

Results and Figures

CRISPR-based chemogenomic profiling identified CAB39 as a putative regulator of NHEJ. To identify novel regulators of DNA repair, we leveraged a CRISPR-based chemogenomic screen (Olivieri et al., 2020) which comprehensively tested 18,053 protein-encoding human genes for their involvement in different DNA repair pathways. For this specific project, we primarily focused our attention on the NHEJ pathway, which is the most commonly used pathway to repair highly cytotoxic DSBs. We selected six of the 27 different genotoxic agents (pyridostatin, doxorubicin, etoposide, IR, KBrO3, and bleomycin) tested in the study as they have been shown to induce DNA damage associated with NHEJ-mediated repair) Olivieri et al., 2020) (Fig.2A).

Upon intersecting the six different chemogenomic screens, we identified 24 genes whose CRISPR-based knockout in RPE1-hTERT cells, an immortalized human retinal pigment epithelial cell line used to model behavior of normal human cells, caused sensitization to all tested DNA damaging drugs (Fig.2B). As expected, most of the common hits were already recognized as well-characterized NHEJ factors (e.g., *53BP1* and *XRCC4*). Additionally, DNA damage signaling factors (e.g., *ATM, RIF1, RNF8, MDC1* etc.) as well as cell cycle inhibitors were amongst the top hits of the screen, validating our analysis to effectively identify proteins that are directly or indirectly involved in the NHEJ pathway. In addition to these well-established NHEJ factors, we also identified multiple genes that have yet to be associated with the NHEJ pathway.

Notably, among the genes identified, *CAB39* emerged as a previously uncharacterized potential regulator of the NHEJ pathway, suggesting that this scaffolding protein may influence cellular

responses to DNA damage. Based on this observation, we focused our subsequent investigations on investigating the role of CAB39 in DNA repair and cancer progression.

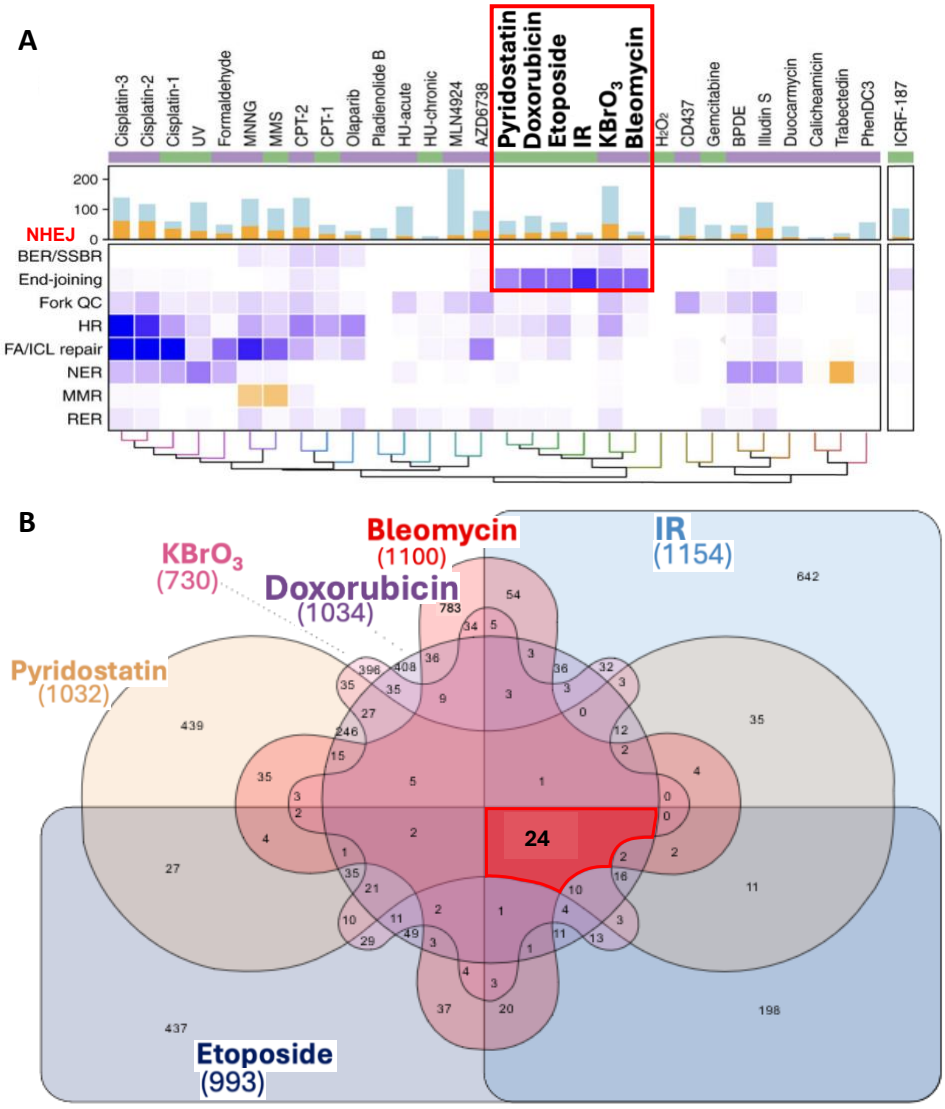

Figure 2: *Chemogenomic Screen Analysis. A: Heat map representation of the 31 CRISPR screens undertaken in RPE1 hTERT p53−/− Cas9 cells (Olivieri et al., 2020). B: Venn Diagram of all gene hits for 6 NHEJ-associated DNA damaging agents.*

CAB39 depletion sensitizes U2OS cells to NHEJ-associated DNA-damaging agents doxorubicin and etoposide. To validate the findings from our CRISPR screen, we depleted CAB39 using RNA interference (siRNA) in osteosarcoma U2OS, confirming efficient knockdown via RT-qPCR (Fig. 3A). We then assessed cellular sensitivity to four NHEJ-associated genotoxic agents ($KBrO_3$, bleomycin, doxorubicin, and etoposide) through dose-response experiments. To ensure relevance across cell types, tested doses were selected from dose–response experiments conducted in U2OS and lung adenocarcinoma A549 cells (Fig. S1), and informed by IC25 values previously established for RPE1 cells in the chemogenomic screen (Olivieri et al., 2020). IC50 values that were determined from dose-response curves served as the low dosage and double this value was set as the high dosage for each genotoxic agent (Fig. S1, Table S3). As expected, increasing concentrations of the genotoxic agents $KBrO_3$, bleomycin, and etoposide reduced cell viability in control cells significantly at high doses (Fig.3B). However, CAB39 depletion did not significantly alter sensitivity to $KBrO_3$, a food additive classified as "possible carcinogen in humans" by the International Agency for Research on Cancer (Fig. 3C). Likewise, CAB39 knockdown did not significantly impact U2OS sensitivity to the chemotherapeutic agent bleomycin (Fig. 3D). In contrast, CAB39-depleted cells exhibited significantly reduced viability following treatment with etoposide and high-dose of doxorubicin, indicative of a potential role for CAB39 in the response to these topoisomerase inhibitors (Fig. 3E, F). These findings suggest that CAB39 is not broadly required for the response to all NHEJ-associated DNA-damaging agents but may play a more specific role in modulating cellular responses to topoisomerase-induced DNA breaks, including etoposide and doxorubicin.

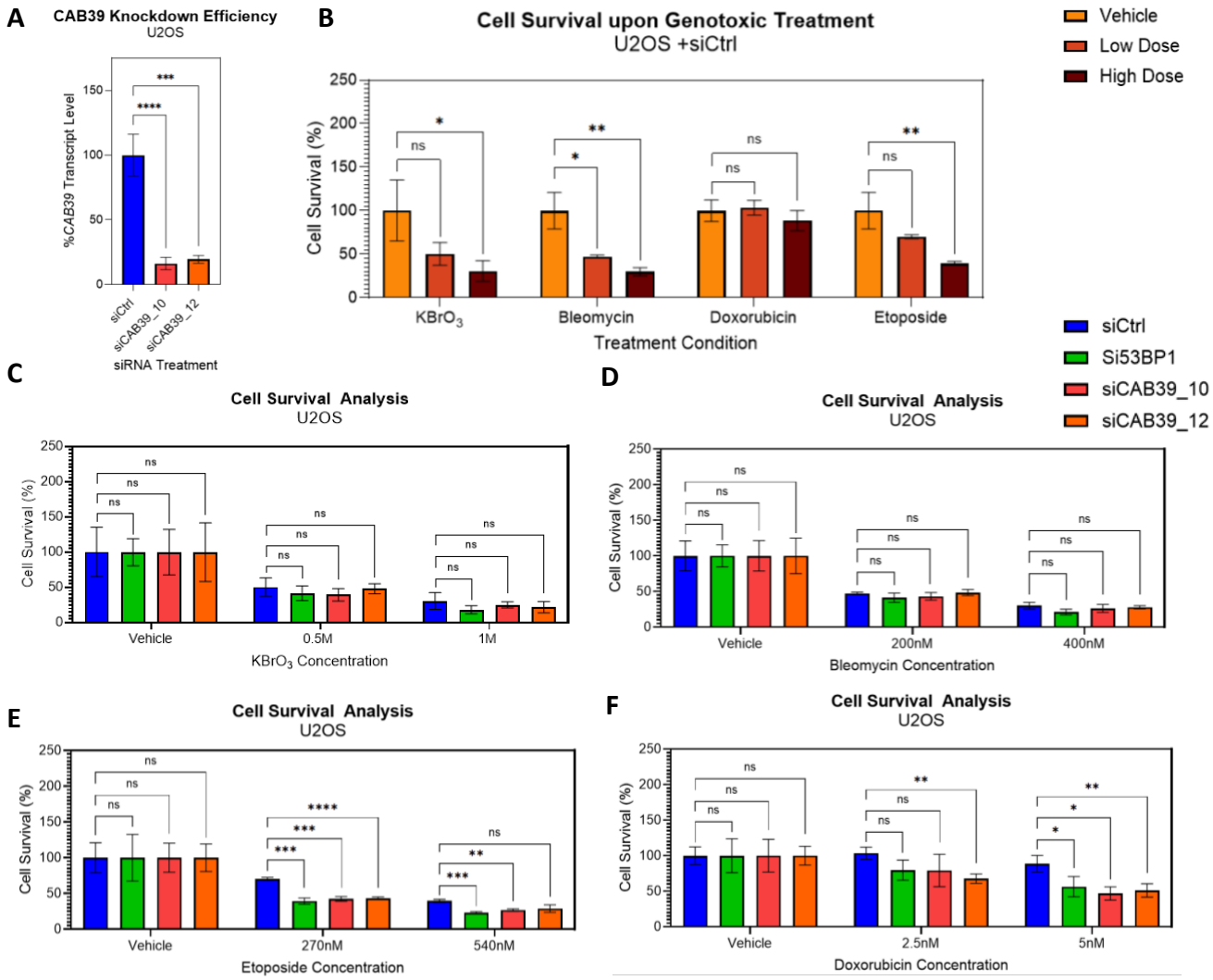

Figure 3: *Effect of CAB39 depletion on cell survival upon treatment with genotoxic agents. A: RT qPCR confirmation of knockdown by CAB39 siRNA compared to non-target control siRNA. CAB39 transcript levels amongst CAB39-depleted samples is represented as a percentage of the normalized control, which is set to 100%. B: Effect of drug treatment on U2OS cell survival. C-F: Percentage cell survival analysis of cancer cells upon CAB39 depletion and $KBrO_3$ treatment (B), bleomycin treatment (D), etoposide treatment (E), and doxorubicin treatment (F). Cell counts for cells treated with genotoxic agent are represented as a percentage of controls. Error bars represent standard deviation.*

*Note - *: p<0.05; **: p<0.01; ***: p<0.001 and, ****: p<0.001. Non-significant (ns): p>0.05.*

CAB39 depletion impairs HR-mediated DSB repair. To determine whether CAB39 influences DNA repair pathway choice, we employed the DR-GFP assay (Pierce et al., 1999), a widely used reporter system for HR-mediated DSB repair (Fig 4A). U2OS cells harboring the DR-GFP reporter were transfected with siRNA targeting CAB39 mRNA transcripts before induction of I-Sce1-mediated DSBs. GFP-positive cells, indicative of successful HR repair, were quantified via flow cytometry. Based on our hypothesis that CAB39 promotes NHEJ, we anticipated that CAB39 depletion would increase HR efficiency due to the competitive relationship between these pathways. However, CAB39 knockdown seemed to partially impair HR repair efficiency compared to control cells, with an effect that was intermediate to the depletion of CtIP, a well-established HR factor (Fig. 4B). Our data indicate that CAB39 may participate in multiple DSB repair pathways, including HR and NHEJ.

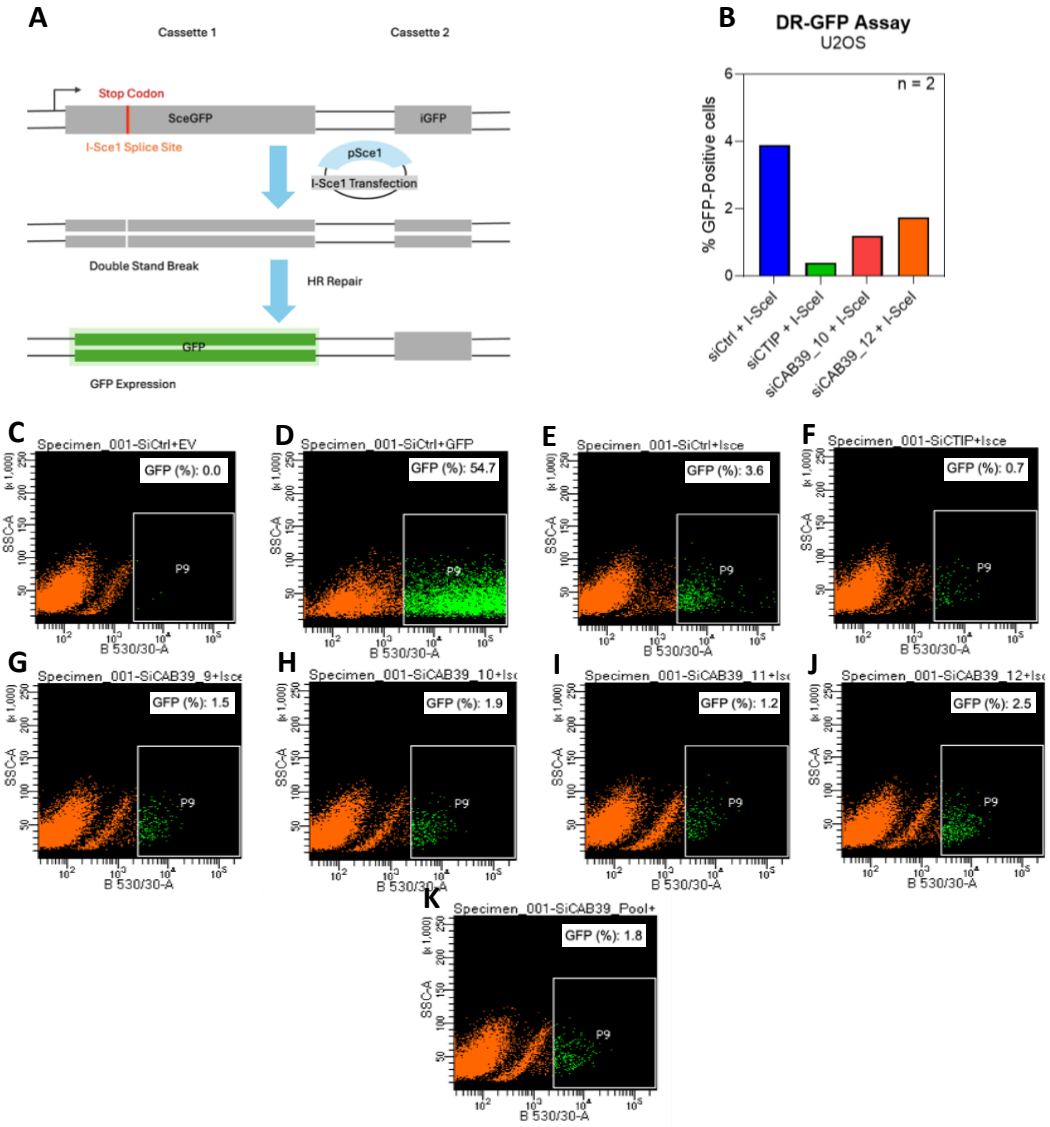

***Figure 4**: DR-GFP Assay.*

A: Schematic of reporter system—2 GFP cassettes are inserted into a U2OS background. Cassette 1 comprises of a GFP-encoding sequence interrupted by a stop codon that is co-localized with an I-Sce1 endonuclease splice site. Cassette 2 contains a truncated form of a homologous GFP-encoding sequence that corresponds to the location of the stop codon segment. Upon I-Sce1 plasmid transfection, a DSB is induced at the I-Sce1 site. When this DSB is repaired through the HR mechanism, using cassette 2 for sequence homology, GFP is expressed in the cell.

B: Quantative analysis of results showing the percentage of total cells from each sample that were GFP-positive.

C-K: Representative images of flow cytometry cell sorting— Sample treated with non-target control, without I-Sce1 expression (C); sample treated with non-target control siRNA, with eGFP plasmid transfection (D); sample treated with non-target control siRNA, with I-Sce1 ectopic expression (E); sample depleted of CTIP, with I-Sce1 ectopic expression (F); sample depleted of CAB39, with I-Sce1 ectopic expression (G-K).

***CAB39* transcript levels across tumor and normal tissues**. To determine whether CAB39 RNA expression is dysregulated in cancer, we analyzed its expression in tumor versus matched normal tissues using The Cancer Genome Atlas (TCGA) database. This database allows us to compare a "normal" sample to a corresponding cancer sample, providing insights into the general trends for *CAB39* transcript levels. Given our hypothesis that CAB39 promotes DSB repair and tumor proliferation, we anticipated an increase of *CAB39* transcript levels in tumor samples compared to normal tissues.

Surprisingly, *CAB39* expression levels did not exhibit a consistent trend across cancer types. While some cancer types showed a significant increase in CAB39 transcript in tumor tissue (e.g., cholangiocarcinoma (CHOL) and pancreatic adenocarcinoma (PAAD)), others showed a decrease in levels compared to normal controls (e.g., breast invasive carcinoma (BRCA) and colon adenocarcinoma (COAD)) (Fig. 5). These findings suggest that while CAB39 may not serve as a universal pan-cancer biomarker, *CAB39* transcript levels could hold tumor type-specific prognostic significance.

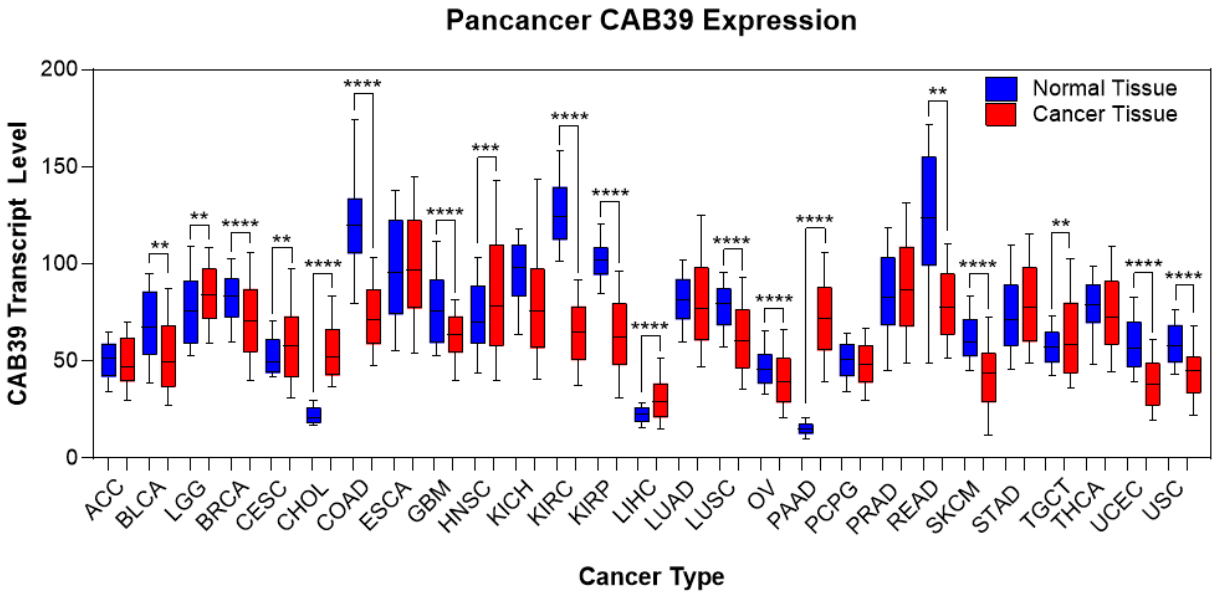

Figure 5: *Pan Cancer analysis of* CAB39 *expression between tumor and normal tissue across cancer types from TCGA Database. Cancer type name are abbreviated according to TCGA study abbreviation: adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), brain lower grade glioma (LGG), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma/endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinomas (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TCGT), thyroid Carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), and uveal melanoma (UVM).*

*Note - *: p<0.05; **: p<0.01; ***: p<0.001 and, ****: p<0.0001. Non-significant pairings (ns): p>0.05 are unmarked.*

***CAB39* transcript level correlates with survival outcomes in a cancer type-dependent manner**. We next examined whether *CAB39* transcript levels correlate with patient survival outcomes across cancer types. Using hazard ratios (HR) obtained from Survival Genie (Dwivedi et al., 2022), we compared the likelihood of death in tumors with high *CAB39* transcript levels versus tumors with low *CAB39* transcript levels within each cancer type. Given our hypothesis that CAB39 promotes tumor cell survival by enhancing DNA repair, we expected higher *CAB39* expression to be associated with poorer prognosis (HR > 1) across cancer types. However, our analysis revealed no universal trend in prognostic significance of *CAB39* transcript levels across cancers. In non-small cell lung cancers (NSCLCs), including lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), high *CAB39* transcript levels correlate with significantly worse prognosis (HR > 1, Fig. 6). Conversely, in kidney chromophobe carcinoma (KICH) and kidney renal clear cell carcinoma (KIRC), high *CAB39* transcript levels are associated with improved survival outcomes (HR < 1, Fig. 6). Given the pronounced negative prognostic impact of high *CAB39* transcript levels in LUAD and LUSC, we prioritized these cancers for further characterization.

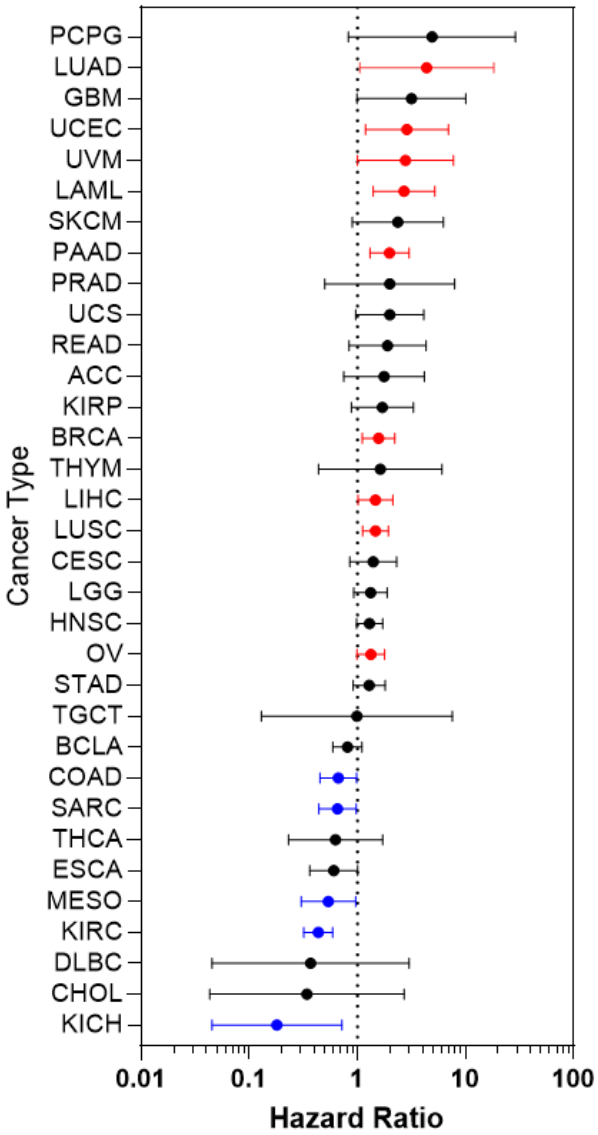

***Figure 6**: Pan-cancer analysis of CAB39-related hazard ratios across cancer types.* Cancer type name are abbreviated according to TCGA study abbreviation: acute myeloid leukemia (LAML), adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), brain lower grade glioma (LGG), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma/endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinomas (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TCGT), thymoma (THYM), thyroid Carcinoma (THCA), uterine carcinosarcoma (UCS), uterine corpus endometrial carcinoma (UCEC), and uveal melanoma (UVM).
Note - significant ratios > 1 are highlighted in red, significant ratios < 1 are highlighted in blue.

***CAB39* transcript levels in NSCLC are independent of pathological stage and smoking history.** To better understand the role of CAB39 in NSCLC, we examined *CAB39* transcript levels across pathological stages of LUAD and LUSC. Interestingly, *CAB39* transcript levels remained relatively constant across different cancer stages, suggesting that CAB39 does not necessarily drive tumor progression (Fig. 7A, B).

Given the well-established link between smoking and lung cancer (Loeb et al., 1985), we next investigated whether *CAB39* transcript levels correlate with smoking history or smoking intensity. CAB39 transcript levels do not differ significantly between smokers and never-smokers in either LUAD or LUSC (Fig. 7C, D). These findings suggest that the prognostic significance of CAB39 in NSCLC is independent of smoking status.

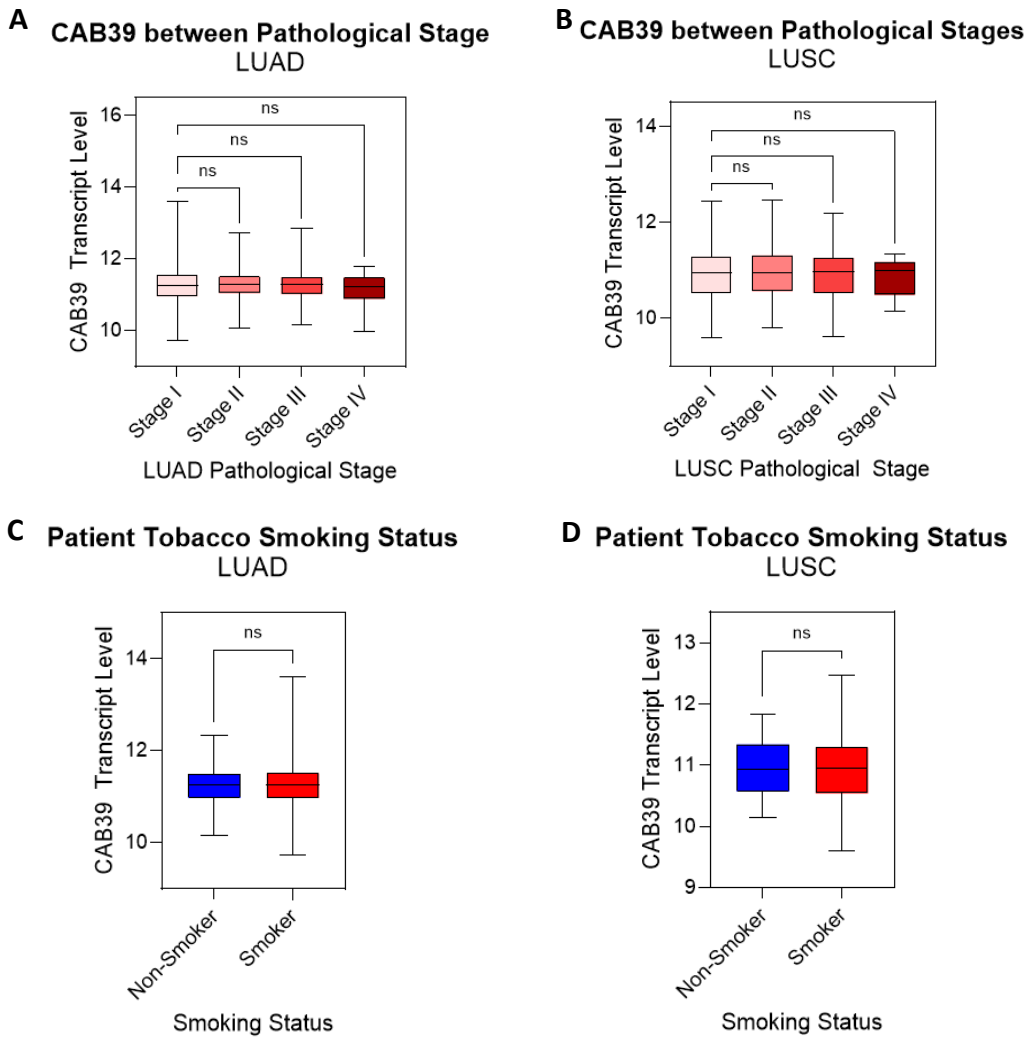

Figure 7*: CAB39 transcript levels and Smoking Habits amongst NSCLC patients. CAB39 transcript levels are compared between –*

A: LUAD patients with tumors of different stages.

B: LUSC patients with tumors of different stages.

A: Smokers (n = 473) versus non-smokers (n = 83) in LUAD patients.

B: Smokers (n = 522) versus non-smokers (n = 18) in LUSC patients.

The horizontal line within the box and whisker plot represents mean CAB39 transcript level, the box itself represents the interquartile range with the top and bottom of the box represent the 75th and 25th quartile value, respectively. The whiskers extend to the maximum and minimum values of CAB39 transcript level. The number of patients analyzed for each condition is annotated on representative boxes.

*Note - *: p<0.05; **: p<0.01; ***: p<0.001 and, ****: p<0.0001. Non-significant pairings (ns): p>0.05.*

***CAB39* transcript levels are negatively correlated with STRADA in NSCLC.** CAB39 functions as a scaffolding protein within the LKB1/STRADA/CAB39 kinase complex (Boudeau, 2003). To determine whether CAB39 expression correlates with expression of known binding partners, STK11 (LKB1) and STRADA, we assessed their transcript levels in NSCLC tumors. *STK11* transcript level remained unchanged regardless of CAB39 transcript levels in LUAD and LUSC (Fig. 8A, B). Unexpectedly, *STRADA* transcript level exhibited a negative correlation with *CAB39* transcript levels in both LUAD and LUSC, suggesting an inverse regulatory relationship (Fig. 8C, D). These findings raise the intriguing question of whether CAB39 may exert tumor-promoting functions independent of the canonical LKB1/STRADA complex.

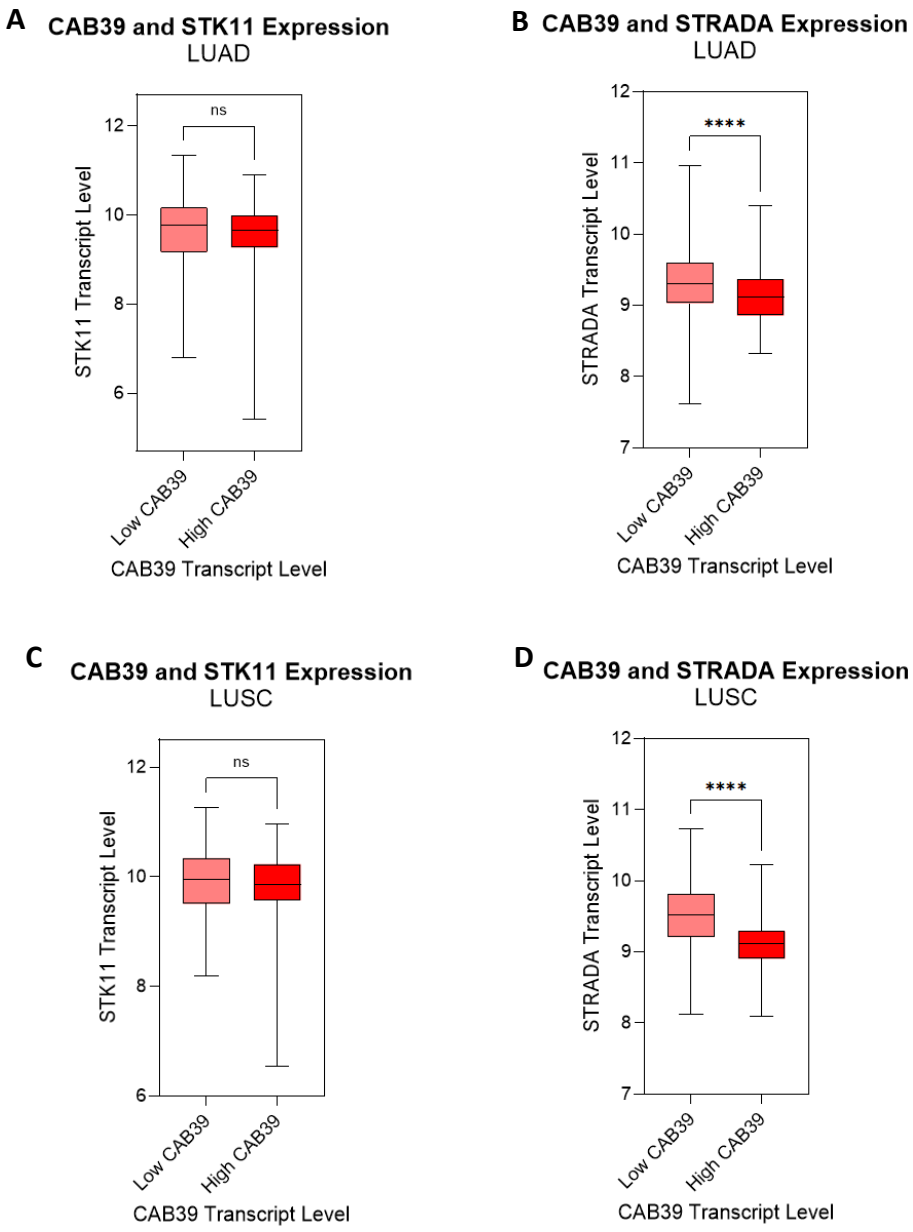

***Figure 8**: STK11 and STRADA gene transcript levels amongst patients with high and low CAB39 transcript levels in LUAD (A, B) and LUSC (C, D) cohorts. High and low transcript levels were determined using quartile cut-offs, with high-expressing patients being amongst the highest 25% of CAB39 transcript level and low-expressing patients being amongst the lowest 25% of CA39 transcript levels. The horizontal line within the box and whisker plot represents mean CAB39 transcript level, the box itself represents the interquartile range with the top and bottom of the box represent the 75th and 25th quartile value, respectively. The whiskers extend to the maximum and minimum values of CAB39 transcript levels.*

*Note - *: p<0.05; **: p<0.01; ***: p<0.001; and ****: p<0.0001. Non-significant pairings (ns): p>0.05.*

Discussion

This study identifies CAB39 as a potential regulator of DNA repair, particularly within the NHEJ pathway, through a CRISPR-based chemogenomic screen. Functional validation experiments demonstrate that CAB39 depletion does not broadly sensitize cancer cells to all NHEJ-associated genotoxic agents but selectively increases sensitivity to the topoisomerase poisons etoposide and doxorubicin, suggesting a context-specific role in the DNA damage response. Furthermore, we show that CAB39 may also be involved in HR using a GFP-based reporter assay, pointing toward a multifaceted role for CAB39 in DSB repair mechanisms, rather than a function exclusively in a single pathway. These findings raise the possibility that CAB39 may modulate DNA repair pathway choice or efficiency, depending on the type of DNA damage or cellular context.

To explore the potential clinical relevance of CAB39, we analyzed *CAB39* transcript levels across a wide range of cancers. Pan-cancer analysis revealed that *CAB39* transcript levels vary between tumor and normal adjacent tissues, although these patterns are cancer-type specific—with some tumors showing an increase in *CAB39* transcript levels and others showing a decrease in *CAB39* transcript levels. Similarly, *CAB39* expression showed prognostic value in a subset of cancer types, including LUAD and LUSC, where high *CAB39* levels are associated with poorer survival outcomes. Together, these findings highlight CAB39 as a novel, context-dependent modulator of DNA repair with potential implications for cancer prognosis and therapy response in lung cancer.

Role of CAB39 in DNA repair and the cellular response to genotoxic agents. Although our chemogenomic profiling identified CAB39 as a putative regulator of NHEJ-mediated DNA repair, *in vitro* depletion of CAB39 did not result in a global sensitization of U2OS cells to NHEJ-associated

genotoxic agents. Instead, CAB39 knockdown selectively increased sensitivity to etoposide and doxorubicin, both of which are topoisomerase II poisons that induce protein-linked DSBs (Montecucco et al., 2015, Rivankar, 2014). In contrast, CAB39 depletion did not significantly affect cellular responses to potassium bromate ($KBrO_3$) or bleomycin, which induce DNA damage through oxidative stress and complex clustered lesions, respectively. These findings suggest that CAB39 may not be broadly required for canonical NHEJ function but instead may facilitate repair of topoisomerase-induced or replication-associated DNA damage, which often involves protein-blocked DNA ends and occurs in a more transcriptionally or replicative active context. Such damage is typically more structured and may require the coordination between NHEJ, end processing factors, and replication stress responses—processes where a scaffolding protein like CAB39 could play a critical role. One potential explanation for this selective sensitization lies in the difference between the cell types used in our study: non-transformed RPE1-hTERT cells were used in the CRISPR-based chemogenomic screen (Olivieri et al., 2020), whereas our *in vitro* validation was performed in U2OS cancer cells, which harbor higher levels of replication stress and altered DNA repair dynamics. This distinction raises the possibility that the role of CAB39 during DNA repair is selectively amplified or becomes essential under oncogenic stress, where efficient repair of replication-associated DSBs is necessary for cancer cell survival.

Surprisingly, CAB39 depletion reduced, rather than increased, HR efficiency in a GFP-based HR reporter assay. This finding indicates that CAB39 may contribute to both NHEJ and HR repair, rather than antagonizing one pathway over the other. Upon reanalysis of the genotoxic CRISPR screen (Olivieri et al., 2020), we found that CAB39 knockout sensitized cells to 23 of the 27 genotoxic agents tested. This group includes the 6 NHEJ-associated genotoxic agents highlighted

in this study. However, this group also includes drugs like cisplatin and camptothecin (CPT), which are associated with the HR pathway (Olivieri et al., 2020). While NHEJ and HR are traditionally considered competing pathways, multiple DNA repair factors involved in the detection and immediate response to DSBs are known to function in both pathways. For example, ataxia-telangiectasia mutated (ATM), a protein kinase that is immediately activated in response to DSB formation, is known to phosphorylate effectors of both the HR and NHEJ pathways (Iijima et al., 2008).Furthermore, though NHEJ factors are recruited to DSBs before HR factors, there is a significant overlap during which factors of both pathways are present at a DSB site at the same time (Kim et al., 2005), allowing for the possibility that CAB39 could structurally support both pathways. An alternative explanation is that CAB39 depletion alters cell cycle dynamics, possibly leading to a G1 phase arrest, where HR is inactive. To rule out this possibility, future studies should assess cell cycle distribution in CAB39-depleted cells using propidium iodide staining and flow cytometry.

CAB39 expression in cancer: context-dependent roles. Analysis of TCGA datasets revealed that *CAB39* is differentially expressed between tumor and normal tissues across a broad range of cancer types, but the direction and magnitude of expression changes is not consistent across tumors. In some cancers, such as CHOL and PAAD, *CAB39* transcript levels are increased in tumor tissues compared to matched normal samples, whereas in others like BRCA and COAD, CAB39 transcript levels are decreased. These findings suggest that *CAB39* expression could be regulated in a tissue- and context-specific manner, potentially reflecting distinct cellular dependencies or signaling environments in different tumors. This context-dependent expression pattern is not unique to CAB39: several well-characterized NHEJ factors, including XRCC4 (Yu et al., 2023) and

DNA-PKcs (Mohiuddin & Kang, 2019), also exhibit variable expression across tumor types, reflecting distinct DNA repair dependencies and tumor microenvironments. These parallels reinforce the idea that CAB39 may participate in a flexible, cancer-type-specific regulatory network governing DSB repair.

To evaluate whether *CAB39* expression levels hold prognostic value, we analyzed survival data across multiple cancer types by stratifying patients into high- and low-*CAB39* transcript level cohorts. Consistent with a role in supporting genome integrity, high *CAB39* transcript levels are associated with poorer overall survival in several cancer types, most notably in non-small cell lung cancers (NSCLC), including lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). This finding raises the possibility that elevated *CAB39* levels could enhance tumor resilience to endogenous or therapy-induced DNA damage, thereby contributing to disease progression and/or poor clinical outcomes. A similar trend has been reported for DNA-PKcs (PRKDC), a core NHEJ kinase, whose overexpression correlates with poor prognosis and therapy resistance in cancers such as NSCLC, prostate, and glioblastoma (Hsu et al., 2012). Together, these findings suggest that increased levels of certain DNA repair factors, including CAB39, may serve as a mechanism of therapeutic resistance and a biomarker of aggressive disease.

CAB39 and the LKB1/STRADA complex in NSCLC. Unexpectedly, *CAB39* transcript levels are negatively correlated with STRADA transcript levels in NSCLC, despite the well-established LKB1/STRADA/CAB39 kinase complex. This inverse relationship suggests that CAB39 may exert functions in lung cancer that are independent of its classical role as a scaffold for LKB1 signaling, or that *CAB39* expression is regulated through a distinct, cancer-specific pathway. Further

investigation is warranted to determine whether CAB39 contributes to tumor progression via mechanisms that do not rely on LKB1-STRADA interactions.

As a scaffolding protein, CAB39 participates in multiple protein complexes. While CAB39 involvement in the LKB1/STRADA complex is the most extensively studied (Golkowski et al., 2023; So et al., 2015), CAB39 has also been implicated in other signaling pathways. For example, CAB39 has a role in the WNK4 signaling axis, which regulates sodium-potassium cotransporter activity (Ponce-Coria et al., 2014), pointing to a broader functional repertoire beyond energy metabolism and cell polarity. In addition, recent work in esophageal squamous cell carcinoma (ESCC) has shown that *CAB39* expression is regulated by exosomal miR-45a (Wang et al., 2024), a mechanism not currently linked to LKB1, further supporting the existence of alternative regulatory networks involving CAB39. These data emphasize that the influence of CAB39 on NCSLC prognosis may not be necessarily mediated by the LKB1/STRADA complex, possibly involving completely novel CAB39 protein interactions altogether. To explore this possibility, unbiased protein–protein interaction studies (e.g., co-immunoprecipitation followed by mass spectrometry) or computational screening using databases such as BioGRID, IntAct, or STRING could be employed to identify additional CAB39 partners and map new signaling axes relevant to lung cancer biology.

Limitations and future directions. While this study provides preliminary evidence for a role of CAB39 during the DNA damage response, several limitations remain. First, only mRNA depletion of CAB39 was confirmed, and future studies should verify that siRNA treatment also reduces CAB39 protein levels, using western blotting. Basal CAB39 protein levels in different cell lines and their modulation in response to genotoxic agents should also be assessed. Second, we primarily

assessed the role of CAB39 during HR using the DR-GFP reporter assay. To more directly investigate its role in NHEJ, these studies should be extended to the EJ5-GFP assay (Bennardo et al., 2008), and to evaluate its involvement in alternative end-joining with the EJ2-GFP assay (Bennardo et al., 2008). These approaches will clarify the pathway-specific contributions of CAB39 to DSB repair. Lastly, our findings suggest that CAB39 may function through non-canonical protein complexes in cancer. Therefore, identifying and validating novel CAB39-interacting proteins via IP and proteomics will be critical for understanding its mechanistic role in tumor progression and DNA repair.

Conclusion and clinical implications. This study identifies CAB39 as a novel modulator of DNA repair, with potential involvement in both NHEJ and HR pathways. The role of CAB39 in maintaining genome stability supports its possible relevance as a factor influencing cancer progression, particularly in NSCLC. The selective sensitization of CAB39-depleted cells to genotoxic agents such as etoposide and doxorubicin highlights the potential of CAB39 as a therapeutic target to improve responses to DNA-damaging treatments. The differential expression of *CAB39* across cancer types, and its association with poor prognosis in NSCLC, position CAB39 as a promising biomarker for personalized therapy. Targeting CAB39 could enhance the efficacy of standard treatments in tumors with elevated *CAB39* expression. However, to harness the clinical potential of CAB39, further investigation is needed to define the mechanistic basis of CAB39 action and to explore its therapeutic tractability.

Materials and Methods

Data Source

Gene expression and patient phenotype data were obtained from The Cancer Genome Atlas (TCGA-GDC SKCM; https://www.cancer.gov/ccg/research/genome-sequencing/tcga; dbGaP: phs000178.v11.p8) database.

Gene Expression Analyses

The correlation between gene expression and LUAD patient survival was analyzed using Survival Genie (Dwivedi et al., 2022). CutP values were imposed to separate patients into high and low gene expression used to generate hazard ratios. Gene expression, smoking status, smoking history, and gene expression correlation data of the TCGA LUAD and TCGA LUSC patient samples were extracted from Xena Browser (https://xenabrowser.net/) (Goldman et al., 2020).

Cell Culture

Both U2OS osteosarcoma and A549 lung adenocarcinoma cell lines were purchased from the American Type Culture Collection. The U2OS DR-GFP reporter cell line was a gift from Dr. Jeremy Stark (Pierce et al., 1999). Cells were confirmed by STR profiling and tested for mycoplasma contamination.

U2OS and U2OS DR-GFP reporter cell lines were cultured in DMEM media and the A549 cell line was cultured in RPMI media. Complete media constituted 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin and was used for all cell culture unless noted otherwise. All cells were incubated in a 5% CO_2 atmosphere maintained at 37°C.

siRNA Transfection

Cells were seeded in respective media containing 10% FBS without penicillin or streptomycin. At 60% confluency, siRNA transfection was performed with Opti-MEM (Thermo Fisher Scientific) using the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). Details of siRNA used are listed in supplemental materials (Table S1). At seven hours post-transfection, transfection reagents were washed off with PBS and media was replaced seven hours post-transfection. Cells were collected at 48 hours post-transfection for RT-qPCR or 72 hours post-transfection for cell counting.

Reverse Transcription–Polymerase Chain Reaction

Cells were harvested 48 hours after siRNA transfection. RNA was extracted from cell pellets using the Quick RNA MiniPrep (Zymo) protocol as described by the manufacturer. RNA concentration was quantified with the Biotek Synergy microplate reader (Aligent). The cDNA was prepared from extracted RNA using the LunaScript RT supermix (NE BioLabs) per the manufacturer's instructions. A polymerase chain reaction (PCR) was conducted using the Luna Universal PCR master mix (NE BioLabs) with the CFX Opus 96 rt-PCR system (Bio-Rad) according to the manufacturer's instructions. The details of the PCR primers are listed in supplemental materials (Table S2).

Treatment with DNA Damaging Agent and Cell Counting

Potassium Bromate (KBrO3) (Sigma Millipore), Bleomycin sulfate (Avantor), and Doxorubicin hydrochloride (Avantor) were dissolved in H_2O. Etoposide (Sigma Millipore) was dissolved in DMSO. Dosages for $KBrO_3$ were determined based on IC25 values reported in the genotoxic

CRISPR screen (Olivieri et al., 2020). Dose response experiments were conducted to determine treatment concentrations for the remaining genotoxic agents in U2OS and A549 cell lines (Fig. S1). Cells were treated with low and high concentrations of each DNA damaging agent at concentrations noted in supplemental materials, with the respective solvent used as a control (Table S3). Cells were treated with genotoxic agents 24 hours post siRNA transfection. At 48 hours post-treatment, cells were harvested and manually counted using a hemocytometer (Fisher Scientific).

DR-GFP Assay

U2OS DR-GFP reporter cells were seeded in DMEM medium with 10% FBS and without antibiotic. When the cells reached 60% confluency, cells were transfected with siCAB39 according to the procedure described above. Transfection was performed with a non-target control siRNA and siCTIP as negative and positive transfection controls, respectively. At 24 hours post-transfection, cells were co-transfected with I-Sce1 endonuclease plasmid using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) to generate DNA double-stranded breaks at the GFP reporter locus. As negative and positive controls, cells transfected with non-target siRNA were co-transfected with an empty plasmid vector or a GFP construct, respectively. After incubation for 48 hours following I-Sce1 expression, the cells were collected by trypsinization using 0.25% trypsin-EDTA (Gibco) and resuspended in PBS with 0.001% (v/v) 7-Aminoactinomycin D (7-AAD) viability dye (Biolegend). The percentage of GFP-positive cells was quantified by fluorescence-activated cell sorting (FACS) on the BD Symphony A1 Cell Analyzer (BD Biosciences) machine at the Emory Pediatrics/Winship Flow Cytometry Core.

Statistics

Statistical significance was determined using GraphPad Prism. Significance of gene expression between tumor and normal tissues, smoking status, and amongst smokers of different smoking levels was analyzed using unpaired t-tests. For in-vitro experiments, multiple unpaired t-tests were used to analyze significance in gene expression differences and percentage cell survival differences. For all analyses, $p>0.05$ represents insignificant pairings (ns); *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; and ****: $p<0.0001$

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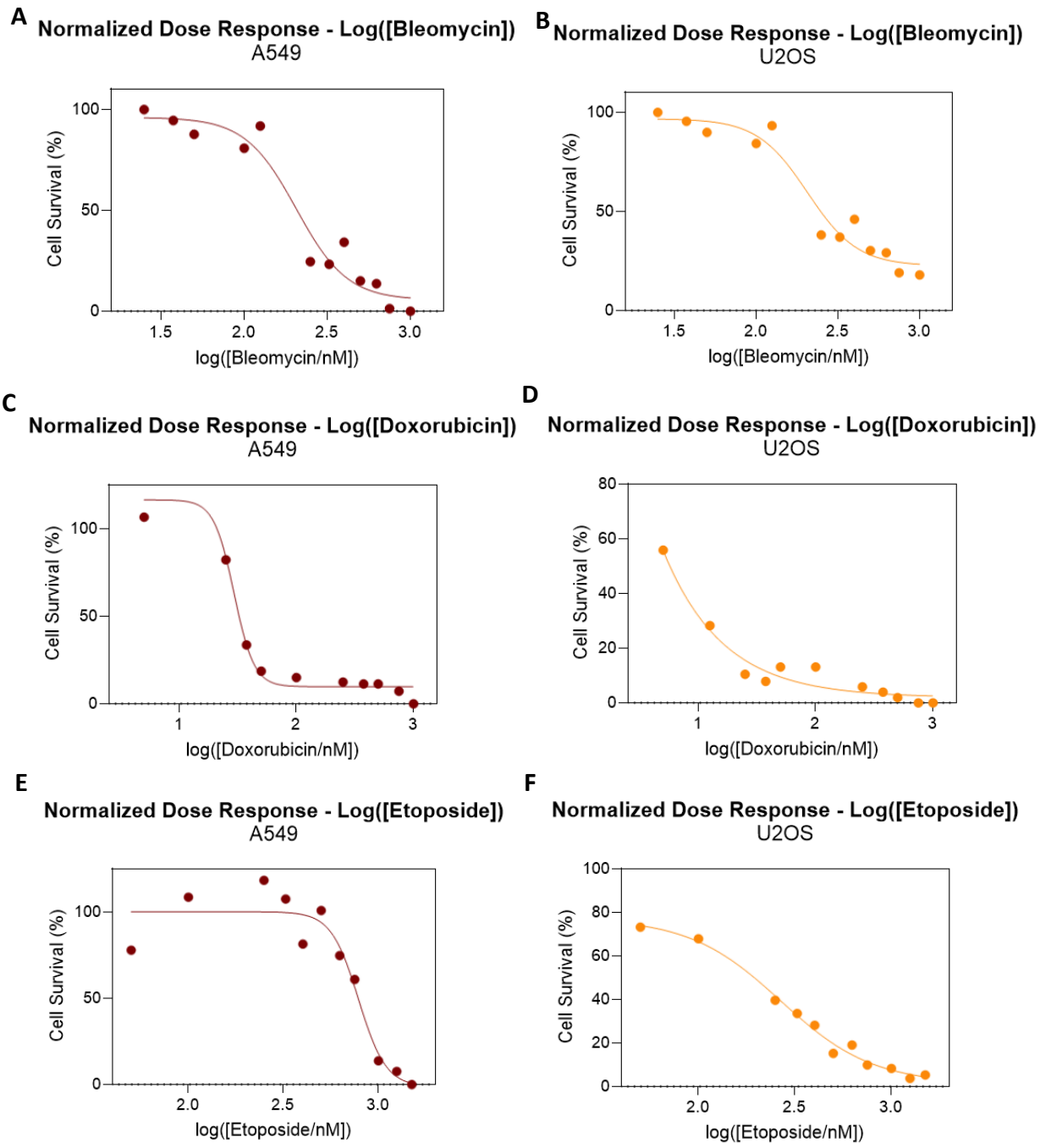
Supplemental Materials

Target	Manufacturer	Cat#
CAB39	Dharmacon	SO-3277101G
CAB39	Dharmacon	SO-3277101G
CTIP	Dharmacon	L-013646-00-0005
Non-Target Control	Dharmacon	D-001210-03-50
53BP1	Dharmacon	D-003548-01-0010

Supplemental Table S1*: Product and manufacturer information of siRNAs.*

Target		Manufacturer	Target Sequence
CAB39	F	IDT	CGTTCCCGTTTGGGAAGTCT
	R	IDT	AGCTACCAGGGTGCTAAGGA
HPRT	F	IDT	ATGACCAGTCAACAGGGGAC
	R	IDT	AAGCTTGCGACCTTGACCAT
RPL30	F	IDT	GGTGACTCTGATGGCCAGTT
	R	IDT	ACGTCAAGGAGCTGGAAGTG

Supplemental Table 2*: Product and manufacturer information of PCR primers.*



Supplemental Figure S1: *Dose Response Curves of Bleomycin, Doxorubicin, and Etoposide in A549 and U2OS cell lines.*

DNA Damaging Agent	Solvent Control	A549 Cell line			U2OS Cell line		
		IC50	Low	High	IC50	Low	High
Bleomycin	H_2O	205.8nM	200nM	400nM	205.8nM	200nM	400nM
Doxorubicin	H_2O	29.32nM	30nM	60nM	~2nM	2.5nM	5nM
Etoposide	DMSO	785nM	750nM	1500nM	266.9nM	270nM	540nM
Potassium Bromate	H_2O	N/A	500µM	1000µM	N/A	500µM	1000µM

Supplemental Table S3*: IC50 and Dosage values for Bleomycin, Doxorubicin, Etoposide, and KBrO3 in A549 and U2OS cell lines.*