The Role of KICSTOR and GATOR1 Complexes in Tungsten Toxicity

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Abstract

Tungsten was linked to several leukemia clusters in the early 2000s, but there was insufficient evidence to establish causation and little was known about the metal. Today, data from in vitro and in vivo modeling show that tungsten accumulates in bone, promotes DNA damage, and induces aberrant B cell development, among other indicators of leukemia. To understand tungsten mechanistically, a whole genome CRISPR-based screen was conducted in the NALM6 precursor B lymphocytic leukemia cell line. Functional enrichment analysis suggests that some effects of tungsten may be mediated by the disruption of signaling pathways associated with mTORC1, the major nutrient-sensitive regulator of growth in cells. Specifically, knockout of genes in the KICSTOR and GATOR1 complexes, involved in the negative regulation of mTORC1, increase resistance of cells against tungsten. We hypothesize that tungsten induces cytotoxicity by dysregulating the levels, localization, or signaling of proteins in the KICSTOR and GATOR1 complexes. To validate the CRISPR screen, we generated Cas9-mediated knockout (KO) clones for *KPTN* and *SZT2* in the U2OS human osteosarcoma cell line. MTT assays revealed that *KPTN*- and *SZT2*-deficient clones did show resistance to tungsten-induced cytotoxicity and apoptosis compared to empty vector (EV) control cells, although the difference in cell viability was not shown to be mediated through cellular senescence. Potential mechanisms by which tungsten could be altering cell growth include modulating downstream effectors of mTORC1, including S6K1 and 4E-BP1. However, the activity of tungsten is unlikely to be mediated through mTORC1, as the genetic and pharmacologic inhibition of the complex does not enhance the cytotoxicity of tungsten in the cell. We do observe evidence that tungsten may be disrupting the localization of GATOR1 to the lysosome. This *in vitro* study investigates a novel mechanism of tungsten, potentially through interactions with the KICSTOR and GATOR1

complexes. The canonical pathway of these complexes is the negative regulation of mTORC1, but the results of this study suggest that tungsten toxicity is mediated through an mTORC1-independent pathway, not yet explored in the literature.

Resumé

Au début des années 2000, le tungstène a été associé à plusieurs groupes de leucémie, mais les preuves n'étaient pas suffisantes pour établir un lien de cause à effet et on ne savait pas grand-chose sur ce métal. Aujourd'hui, les données issues de la modélisation in vitro et in vivo montrent que le tungstène s'accumule dans les os, favorise les lésions de l'ADN et induit un développement aberrant des cellules B, entre autres indicateurs de la leucémie. Pour comprendre le mécanisme du tungstène, un criblage CRISPR du génome entier a été réalisé dans la lignée cellulaire de leucémie lymphocytaire B précurseur NALM6. L'analyse de l'enrichissement fonctionnel suggère que certains effets du tungstène peuvent être médiés par la perturbation des voies de signalisation associées à mTORC1, le principal régulateur de la croissance cellulaire sensible aux nutriments. Plus précisément, l'invalidation de gènes des complexes KICSTOR et GATOR1, impliqués dans la régulation négative de mTORC1, augmente la résistance des cellules au tungstène. Nous émettons l'hypothèse que le tungstène induit une cytotoxicité en régulant les niveaux, la localisation ou la signalisation des protéines des complexes KICSTOR et GATOR1. Pour valider le crible CRISPR, nous avons généré des clones knock-out (KO) médiés par Cas9 pour KPTN et SZT2 dans la lignée cellulaire humaine d'ostéosarcome U2OS. Les tests MTT ont révélé que les clones déficients en KPTN et SZT2 présentaient une résistance à la cytotoxicité et à l'apoptose induites par le tungstène par rapport aux cellules de contrôle à vecteur vide (EV), bien qu'il n'ait pas été démontré que la différence de viabilité cellulaire était médiée par la sénescence cellulaire. Les mécanismes potentiels par lesquels le tungstène pourrait altérer la croissance cellulaire comprennent la modulation des effecteurs en aval de mTORC1, y compris S6K1 et 4E-BP1. Cependant, il est peu probable que l'activité du tungstène soit médiée par mTORC1, car l'inhibition génétique et pharmacologique du complexe n'augmente pas la

cytotoxicité du tungstène dans la cellule. Nous avons observé des preuves que le tungstène pourrait perturber la localisation de GATOR1 dans le lysosome. Cette étude in vitro examine un nouveau mécanisme du tungstène, potentiellement par le biais d'interactions avec les complexes KICSTOR et GATOR1. La voie canonique de ces complexes est la régulation négative de mTORC1, mais les résultats de cette étude suggèrent que la toxicité du tungstène est médiée par une voie indépendante de mTORC1, qui n'a pas encore été explorée dans la littérature.

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Contribution of authors

The following people were involved in the making of this thesis:

Koren Mann – experimental design and project supervision

Michael Tyers and the Tyers Lab – design and execution of the CRISPR functional knockout screen in NALM6 cells, as well as the development of the RANKS analysis

Rowa Bakadlag – assistance and guidance in generating CRISPR KO clones (designing sgRNAs, plasmid cloning, bacterial transformation, plasmid preparation and microbiological culturing, viral transduction, single cell isolation and expansion), as well as mentorship in PCRs, Bradford assay, gel electrophoresis, Western blots, flow cytometry, and MTT assay.

Raymond Tu – all other experiments and the writing of this thesis

1. Literature review

1.1. Introduction to tungsten

1.1.1. Physical and chemical characteristics

Tungsten, represented by the atomic symbol W (for "Wolfram"), is the 74th element in the periodic table. At an atomic mass of 183.84 Da, tungsten is the heaviest metal with a known biological function, albeit in the taxonomic domain archaea (Andreesen & Makdessi, 2008; Kletzin & Adams, 1996). It is a grayish-white metal that is highly resistant to tarnish and corrosion. Pure tungsten metal is known for properties such as its hardness, high density, ductility, and conductivity. Remarkably, tungsten has the highest melting point (3,695 K) and the highest tensile strength (142,000 psi) among all metals, and the second highest boiling point (6,203 K) after carbon (Lide, 2022). These physical characteristics give rise to its use and popularity across many applications. Some of the main uses of tungsten (**Figure 1**) include:

- High-temperature applications: Tungsten has a very high melting point, making it ideal for use in high-temperature environments. It is used in the production of filaments for light bulbs and in heating elements for industrial furnaces.
- Tooling and cutting: Tungsten is used to make the cutting edges of tools such as drill bits, saw blades, and lathe tools. It is also used to make the tips of electrodes used in welding and cutting processes.
- Alloys: Tungsten is added to other metals to form alloys with improved strength and other desired properties. For example, tungsten-steel alloys are used in the production of high-strength wire, springs, and other components.
- Aerospace: Tungsten is used in the production of rocket nozzles, heat shields, and other components for aerospace applications due to its high melting point and strength.

- Military: Tungsten has also been used in the production of armor-piercing bullets and other military applications due to its high density and ability to withstand extreme temperatures.
- Jewelry: Tungsten carbide, sometimes referred to as hard metal, is an extremely hard compound of tungsten and carbon and is used in the production of jewelry such as wedding bands and other rings.

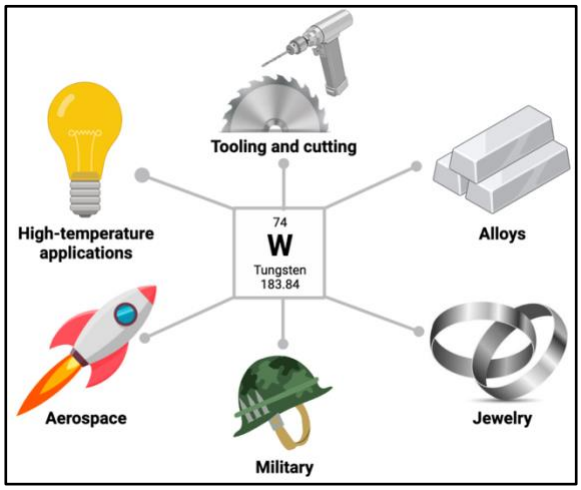

Figure 1. Widespread use of tungsten across several industries, applications, and products.

The chemical properties of tungsten also open the possibility for its use in certain applications. Tungsten can form chemical compounds in oxidation states ranging from -II to VI. The highest oxidation state is seen in tungsten(VI) oxide (WO_3). Tungsten(VI) oxide is soluble in aqueous base, forming orthotungstate, WO_4^{2-} (found in sodium tungstate, Na_2WO_4). One of the main uses of sodium tungstate is as a catalyst in chemical reactions, particularly in the production of polymers and pharmaceuticals (Jiang et al., 1993; Payne & Williams, 1958). Tungsten has a high ionic redox potential, which makes it a good catalyst in certain chemical processes. Sodium tungstate has also been used as a reagent in the synthesis of inorganic and organometallic compounds, and as a precipitant in the purification of proteins (Bee et al., 2009; Mbomekalle et al., 2004). In addition to use in chemistry, tungstates have been investigated for their potential use in a number of other applications, including water treatment. For example, they have been used to remove heavy metals from wastewater (AL-Othman et al., 2011). Its unique chemical properties, in addition to its physical characteristics, make tungsten a useful element in a variety of fields, and further research is likely to uncover even more potential uses.

1.1.2. Sources and exposures

Given the diverse applications and uses of tungsten, there are a variety of tungsten sources and many opportunities for populations to be exposed to this metal. Tungsten is a naturally occurring element that is found in the earth's crust (Arevalo & McDonough, 2008). It is most commonly extracted from the minerals scheelite and wolframite, which are found in various parts of the world, including China, Russia, and the U.S. (Lide, 2022). Tungsten can also be obtained as a byproduct of the mining and processing of other metals, such as copper, lead, and zinc (Lide, 2022). Tungsten is most often found in one of two forms: tungstates or tungsten carbide (hard metal). Tungstates are oxyanions of tungsten (e.g., scheelite, wolframite) found in a variety of natural and synthetic sources, including minerals, rocks, and soil (Lide, 2022). Tungstates can also be formed during the processing of tungsten-containing ores, as well as during the manufacture of tungsten-containing products, such as tungsten carbide. Tungsten carbide is a hard, wear-resistant compound that is made up of tungsten and carbon. It is used in a variety of industrial applications, including the production of cutting tools, drill bits, and other tools that are subjected to high levels of wear and abrasion. Tungsten carbide is also used in the production of armor-piercing ammunition, as well as in the construction of surgical instruments and other medical devices.

Tungsten is used in a wide range of products, including cutting tools, electrical and electronic devices, alloys, and other industrial products. As a result, people can be exposed to tungsten through the use and handling of these products. Due to both its natural occurrence and potential leaching of tungsten from landfills, mines, factories, etc. into the environment, humans have a baseline exposure to tungsten. The Centers for Disease Control and Prevention (CDC) collected data from the National Health and Nutrition Examination Survey (NHANES) between

1999-2010, revealing that the mean (95%CI) urinary concentration of tungsten for the U.S. population was 0.093 (0.087-0.100) μg/L or 0.087 (0.080-0.095) μg/g creatinine (CDC, 2012).

However, the populations at highest risk of exposure to tungsten are those who 1) work in and 2) live near industries where tungsten is used or produced (Kraus et al., 2001; Sprince et al., 1984). These industries include mining, refining, and manufacturing. Workers in these industries may be exposed to tungsten through inhalation of tungsten dust or fumes. In addition, people who live near tungsten mines and refining facilities are at higher risk of exposure to tungsten, as the element may be released into the air, water, or soil in these areas (CDC, 2003; Rubin et al., 2007). Data gathered from one such area, Churchill County in Nevada, showed that the concentration of tungsten in the municipal water system was 4.66 (2.98-7.30) μg/L, but other data from the United States Geological Survey (USGS) suggest that tungsten levels in water samples from all aquifers used as drinking water sources in the area were as high as 742 μg/L (Rubin et al., 2007; Seiler et al., 2005). Around 80% of people in this region had tungsten levels above the 95th percentile of the U.S. population. Adults and children within this region had 9 and 25 times (0.81 μg/L, 2.31 μg/L), respectively, the urinary tungsten levels of those of the average person (CDC, 2012; Rubin et al., 2007).

There is also evidence that tungsten levels in the soil and water near military bases are elevated, largely due to leaching from ammunition, specifically "green" bullets made of tungsten (Clausen & Korte, 2009). Firing these tungsten bullets also releases aerosolized tungsten into the air (Gaitens et al., 2017). Those wounded by these bullets, especially those with embedded shrapnel, are also susceptible to systemic tungsten exposure. The U.S. Department of Veterans Affairs has an Embedded Fragment Registry for war-injured U.S. veterans who had retained fragments, where 11.57% of veterans in the registry had tungsten levels above the 95th percentile

of the U.S. population (mean of 0.23 μg/g creatinine, median of 0.17 μg/g creatinine), higher than the other 14 metals analyzed, except zinc, at 12.26% (CDC, 2012; Gaitens et al., 2017).

The risk of exposure to tungsten also varies by country and region and is largely dependent on the presence of facilities and institutions that produce or use tungsten. Some countries and regions with significant tungsten deposits or production may have higher risks of exposure to tungsten. According to the USGS some of the top producers and countries with the largest reserves of tungsten in 2020 were China, Russia, Vietnam, and the United Kingdom (USGS, 2020). Other countries that produce significant amounts of tungsten include Bolivia, Kazakhstan, the United States, and Canada (USGS, 2020). Many of these countries do not have the resources to impose and enforce strong workplace and environmental regulations to protect the populations most at-risk of tungsten exposure. Furthermore, workers and residents in these regions are likely exposed to several other heavy metals, due to the nature of this industry (Hambach et al., 2013; Moulin et al., 1998; Shelley et al., 2012). Overall, the risk of exposure to tungsten depends on the levels of tungsten in the environment and the duration of time a person spends in these areas or workplaces where tungsten is present.

Population exposure to tungsten can also occur through the release of tungsten into the environment due to leaching from tungsten-containing products, long after the production and manufacturing processes (e.g., the disposal of light bulbs and electronic devices in landfills). In addition, tungsten can be found in the environment because of natural processes, such as the weathering of rocks and minerals that contain tungsten. Tungsten can also be found in trace (and potentially hazardous) amounts in the air, water, and soil, as well as in some food sources, such as plants and animals that have been exposed to tungsten in the environment. One study concluded that tungsten mining led to tungsten distribution to nearby agricultural soil and rice,

and they estimated that residents may be ingesting up to 230 μg tungsten per day (Lin et al., 2014). Another context in which humans may be exposed to tungsten are the use of certain medical implants and devices, as evidenced by increases in urinary and serum tungsten levels (Fernandes & Taurino, 2022; Peuster et al., 2001; Shah Idil & Donaldson, 2018). One study found that an average of 6.13 μg/L (66 times higher than the U.S. population) tungsten was found in urinary tungsten of women with implanted tungsten-based radiotherapy shields, and that at least 20 months post-operation, 1.76 μg/L urinary tungsten could still be detected.

Overall, tungsten is found in a variety of sources, and people can be exposed to tungsten through the use and handling of tungsten-containing products, as well as through the release of tungsten into the environment during the manufacture and disposal of these products. The extent of tungsten exposure will depend on the specific source of tungsten and the individual's level of exposure to tungsten-containing products and substances.

1.1.3. Toxicokinetics and toxicodynamics

If there is potential for human exposure to a toxicant, there is potential for hazard. To the better understand the hazards involved with tungsten, there must be an understanding of how tungsten moves throughout a biological system and what it does to cells, mechanistically. These goals can be achieved by studying the toxicokinetics and toxicodynamics (TKTD) of tungsten. TKTD are the toxicological equivalents of pharmacokinetics and pharmacodynamics, respectively. While toxicokinetics is the branch of toxicology that focuses on the fate of chemicals administered to a living organism, toxicodynamics refers to the biochemical and physiological effects of these substances.

The toxicokinetics of tungsten, and any other toxicant, can be described with four main processes: absorption, distribution, metabolism, and excretion (ADME). There is limited

information available on the toxicokinetics of tungsten in humans. However, it is known that tungsten is not absorbed through the skin, and it is not considered to be a skin irritant. Tungsten can be inhaled as a dust or as a gas, and it can also be ingested if it is present in food or water. Once tungsten enters the body, it is distributed throughout the body, to organs including the liver, kidneys, and bones. There have been several animal models to further elucidate the toxicokinetics of tungsten. In one study, Sprague-Dawley rats were used to understand how tungsten is distributed in the body based on the pharmacokinetics of inhaled radiolabeled tungsten. Radcliffe (2010) concluded that tungsten accumulates in the kidney, adrenal, spleen, bone, lymph nodes, and brain through the exposure period. The main route of elimination is urine (≥ 90% of the administered dose). During the post-exposure period, tungsten is largely retained in bone. This study is in agreement with previous pharmacokinetic studies investigating oral, intravenous, and intraperitoneal exposure to tungsten in animals (Le Lamer et al., 2001, 2002; McDonald et al., 2007). Another pharmacokinetic model estimates that after a single dose, about half of the total tungsten in the body is found in the bone after one month, and this increases to 60% after one year, 70% after two years, 90% after four years, and nearly 100% after ten years (Leggett, 1997). The metabolism of tungsten by the body is not well known, but one study has found that tungsten, ingested in the form of sodium tungstate deposits in long bone in a different, insoluble form resembling phosphotungstate (VanderSchee et al., 2018). Other studies, using bioanalytical chemistry techniques, has found that tungsten is bound to albumin and other unknown proteins (e.g., enzymes) in human serum (Milagros Gómez-Gómez et al., 2011; Rodríguez-Fariñas et al., 2008).

While no experimental toxicokinetic studies on tungsten have been or can be done on humans, there are some human data. In and around Churchill County, Nevada, there were several

sources of tungsten that contributed to the relatively high concentrations of the metal in the air, soil, and water, including two hard-metal facilities that use, process, or refine tungsten, a military aviation base, and several old tungsten mines (Sheppard et al., 2007; Steinmaus et al., 2004). In this region, children have been found to have higher concentrations of tungsten in their bodies than adults living in the same area (Rubin et al., 2007). This suggests that tungsten may be processed differently in children and adults. In another study, where women were exposed to tungsten in their breast tissue through use of a faulty radio-protective shield, tungsten was detectable in both their blood and urine, indicating that tungsten can enter the circulation (Bolt et al., 2015). Additionally, tungsten was still detectable in the urine of patients even two years after having a mastectomy to remove tungsten-contaminated breast tissue, which suggests that tungsten can be stored in the body and serve as a chronic source of exposure even if the primary source is removed.

In terms of toxicodynamics, tungsten has been shown to have several effects on cellular processes. While it is not an essential element for humans and is not known to have any beneficial effects on mammalian cells, tungsten has been shown to have some toxic effects on cells in high concentrations. The following literature review summarizes papers that have looked at the various mechanisms by which tungsten induces cytotoxicity (Bolt & Mann, 2016; Keith et al., 2005, 2007; Koutsospyros et al., 2006; Lemus & Venezia, 2015; Mann et al., 2022). Some of these molecular mechanisms of action include oxidative stress, genotoxicity, epigenetic alterations, metal cofactor disruption, the dysregulation of several proteins and their pathways, and cell development.

An essential molecular mechanism underpinning the toxicity caused by metals is oxidative stress. Many metals, notably transition metals like iron, copper, manganese, and zinc,

are redox-active. They are capable of undergoing redox cycling processes that produce reactive oxygen and nitrogen species (RONS). Excessive intracellular RONS eventually causes oxidative stress, which can damage biomolecules like DNA, lipids, and proteins, and induce dysfunction of mitochondrial respiration, protein folding, DNA repair processes, endoplasmic reticulum stress, inflammation, autophagy, and apoptosis. In the case of tungsten, there have been several *in vitro* and *in vivo* studies which support the idea that tungsten, by itself or in combination with other metals, such as cobalt, can induce and potentiate oxidative stress on the cell (Ding et al., 2009; Fenoglio et al., 2009; Lison et al., 1995; Lombaert et al., 2013; X. D. Zhang et al., 2010). Tungsten-induced generation of RONS, depletion of antioxidants, and changes in gene expression of different oxidative stress proteins are cited as plausible mechanisms by which many of the following mechanisms of action are mediated or worsened.

One such downstream effect of RONS includes DNA damage to the cell. *In vitro* and *in vivo* studies suggest that hard metal particles containing a combination of cobalt and tungsten carbide can induce DNA damage (Anard et al., 1997; De Boeck et al., 2003; Harris et al., 2011; A. C. Miller et al., 2001; Moche et al., 2013; Van Goethem et al., 1997; Yu et al., 2023). Tungsten alone, as soluble sodium tungstate or tungsten oxide nanoparticles, increases mutagenicity in bacterial cells (Hasegawa et al., 2011; Thongkumkoon et al., 2014; Ulitzur & Barak, 1988). As the sole toxicant, tungsten was also shown to induce DNA strand breaks, γH2AX (H2A histone family member X, phosphorylated at Ser139) activity, and chromosomal aberrations *in vitro* and *in vivo* (Akbaba et al., 2016; Chinde & Grover, 2017; Gianì et al., 2019; Guilbert et al., 2011; Kelly et al., 2013; A. C. Miller et al., 2001; Sørli et al., 2023). In one study, tungsten was found to be the primary genotoxicant, as it could induce DNA damage in the absence of RONS (Sørli et al., 2023).

As a result of oxidative stress and DNA damage, cells often respond with cell cycle arrest and apoptosis. Cell cycle arrest refers to a process in which the normal progression of a cell through its life cycle is halted at a specific point. The cell cycle is a series of events that occur in a cell leading to its division and duplication. Cell cycle arrest can occur at various points in the cycle, including the G1, S, and G2 phases. Tungsten exposure has been shown to dysregulate genes related to the cell cycle (Laulicht et al., 2015), while also disrupting the normal progression of the cell cycle and inducing cell cycle arrest at different points in different cell lines (Guilbert et al., 2011; Liu et al., 2012; Osterburg et al., 2010). In contrast, apoptosis is a programmed cell death mechanism that occurs in cells as part of normal tissue homeostasis or in response to stress, injury, or disease. The process of apoptosis involves a series of coordinated events, including condensation and fragmentation of the chromatin, membrane blebbing, and fragmentation of the cell into apoptotic bodies, which are phagocytosed by neighboring cells or macrophages. The main purpose of apoptosis is to remove damaged or unwanted cells from the body without causing inflammation or damage to surrounding tissues. Tungsten ions and tungsten carbide molecules, at relatively high concentrations, have been shown to induce apoptosis in cells (Armstead et al., 2014; De Boeck et al., 2003; Guilbert et al., 2011; Lombaert et al., 2004; Osterburg et al., 2010; Yu et al., 2023).

In addition to damaging the genome, tungsten is suspected of altering the epigenome, as well. Epigenetics refers to changes in gene expression that occur without changes in the DNA sequence itself. There is evidence that heavy metals can make epigenetic modifications, including DNA methylation, histone modifications, and non-coding RNA molecules, all of which can regulate gene expression by altering the accessibility of genes to the cellular machinery responsible for gene expression. There is experimental evidence that either alone or in

an alloy, tungsten can induce histone modifications (Laulicht-Glick et al., 2017; Verma et al., 2011). In humans, one study found a positive association between urinary tungsten levels and global DNA methylation and hydroxymethylation (Tellez-Plaza et al., 2014).

Heavy metals can also interact with enzymes and proteins in cells through a variety of mechanisms, including binding to specific amino acid residues, disrupting protein folding, and altering protein-protein interactions. These interactions can alter the activity and function of these proteins, leading to cellular dysfunction and toxicity. Tungsten has also been shown to interact with a number a proteins and signaling pathways. Johnson, Rajagopalan, & Cohen (1974) found that tungsten may compete with molybdenum, an essential metal within the same group on the periodic table as tungsten, in enzymes requiring molybdenum as a cofactor, such as xanthine oxidase and sulfite oxidase. Additionally, tungsten, similar to other elements in Groups V and VI, can polymerize. Various research groups have shown that tungsten can polymerize with phosphates to form polyphosphotungstates (Bednar et al., 2008, 2009; D. R. Johnson et al., 2010). Depending on the cell line and species being studied, tungsten can alter different phosphate-dependent biochemical pathways, as evidenced by changes in cytPTK (cytosolic protein tyrosine kinase) activity, cAMP (cyclic adenosine monophosphate) levels, as well as phosphorylation of MAPKs (mitogen-activated protein kinases, including extracellular signal-regulated kinases/ERKs, c-Jun NH(2)-terminal kinases/JNKs, and p38) and mTOR (mechanistic target of rapamycin) (Ding et al., 2009; Gianì et al., 2019; D. R. Johnson et al., 2010; Salto et al., 2014). Sodium tungstate and polyoxotungstates can also inhibit the activity of certain phosphatases, including protein tyrosine phosphatases and alkaline phosphatases (Fernández-Ruiz et al., 2015; D. R. Johnson et al., 2010; Raza et al., 2012; Rodriguez-Hernandez et al., 2013).

Tungsten is also thought to have a role in immunotoxicity, affecting both mature and developing immune systems. Immunotoxicity refers to the adverse effects of various substances on the immune system, which may result in dysfunctional immunity or the reduction of the immune response. Immunotoxicity can be induced by a wide range of substances, including heavy metals, pesticides, drugs, and environmental pollutants. Exposure to tungsten decreased cytokine production in human peripheral blood lymphocytes, but was also found to induce pulmonary inflammation in rodents (Gerard Lasfargues et al., 1992; Osterburg et al., 2010; Rengasamy et al., 1999; Roedel et al., 2012). Tungsten also alters the ability of the immune system to respond to a challenge (Fastje et al., 2012; Osterburg et al., 2014). In addition, tungsten has been shown to cause an increase in early B lymphocytes, a potential indicator of leukemia (Kelly et al., 2013; Wu et al., 2019). Data from the National Toxicology Program (NTP), however, found that B6C3F1/N mice exposed to sodium tungsten in drinking water for 28 days had limited effects on humoral and innate immunity, developing hematopoietic cells in the bone marrow, and unstimulated splenocyte phenotypes (Frawley et al., 2016). In a breast tumour murine model, the number of bone marrow-derived suppressor cells, as well as cancer-associated fibroblasts, were correlated with the increase in lung metastases, suggesting that tungsten alters the tumour microenvironment to promote growth of lung metastases (Bolt et al., 2015).

Carcinogenicity and tumorigenicity are also toxicities of interest in the context of tungsten. In addition to the *in vitro* and *in vivo* studies focused on DNA damage, epigenetic alterations, and develop ment effects of tungsten, there have been several animal studies investigating the potential for the metal to induce and promote cancers and tumours. Each of the studies summarized in **Table 1** investigated the effects of tungsten on cancer development and tumour promotion.

Table 1. Summary of *in vivo* studies investigating tungsten exposure on cancer outcomes

Animal Model	Exposure	Outcome	Source
Long-Evans Rats	5 ppm sodium tungstate in drinking water	Slight enhancement of growth seen in rats of both sexes and a small but significant reduction in longevity in tungsten-dosed male rats. Incidence of tumour formation was not significantly different from the control group.	Schroeder & Mitchener, 1975
Female Sprague-Dawley Rats (NMU-induced breast cancer)	150 ppm tungsten in diet for 15 days	Induction of significantly higher mammary cancer incidence (79.2%) compared to control and molybdenum groups ($P<0.05$)	Wei et al., 1987
Male F344 Rats	4 (low dose) or 20 (high dose) tungsten-alloy (WA) pellets embedded intramuscularly	Faster development of aggressive, high-grade pleomorphic rhabdomyosarcomas in high dose group compared to low dose. 100% tumour yield in both groups compared to 0% in tantalum (Ta)-implanted rats.	Kalinich et al., 2005
Male F344 Rats	4 W pellets embedded intramuscularly (WNiCo vs. WNiFe vs. pure W vs. Ta)	Development of aggressive rhabdomyosarcomas observed only in WNiCo group. Microarray gene expression analysis revealed that pathways disrupted include p53 signaling and the cell cycle.	Schuster et al., 2012
Male B6C3F$_1$ Mice	4 WA pellets embedded intramuscularly (WNiCo vs. WNiFe vs. Ta)	Induction of rhabdomyosarcoma tumours in WNiCo group, but not WNiFe group. Aggressive growth patterns and metastasis in Kalinich et al., 2005 was not exhibited in the current study.	Emond et al., 2015
Female Athymic Nude Mice (injected with control or W-transformed cells)	Human bronchial epithelial cell line (Beas-2B) exposed to 50 μM to 250 μM of sodium tungstate	100% of the W-transformed cells formed visible tumors, while none of the control cells formed tumors. RNA-sequencing data revealed that genes involved in lung cancer, leukemia, and general cancer genes were dysregulated by W.	Laulicht et al., 2015
Female BALB/c Mice (injected with 66Cl4 cells to induce breast cancer)	15 ppm sodium tungstate in drinking water	Promotion of murine breast cancer metastasis to the lung via modulation of tumor microenvironment (rather than affecting the primary tumour cells)	Bolt et al., 2015

In April 2021, the National Toxicology Program (NTP), an inter-agency program run by the United States Department of Health and Human Services (US DHHS) and headquartered at the National Institute of Environmental Health Sciences (NIEHS), published a technical report on the toxicology and carcinogenicity of sodium tungstate dihydrate on Sprague-Dawley (SD) rats and B6C3F1/N mice (NTP, 2021). The NTP concluded that in the 2-year drinking water study,

there was no evidence of carcinogenic activity of sodium tungstate in male SD rats at the tested doses of 250, 500, or 1,000 mg/L. However, there was equivocal evidence of carcinogenic activity of sodium tungstate in female SD rats based on increased incidences of C-cell adenoma or carcinoma of the thyroid glands. Similarly, there was equivocal evidence of sodium tungstate inducing carcinogenic activity in male B6C3F1/N mice in the form of increased occurrences of renal tubule adenoma or carcinoma in exposed animals. No evidence of sodium tungstate causing cancer was found in female B6C3F1/N mice at doses of 500, 1,000, or 2,000 mg/L. Sodium tungstate exposure through drinking water also led to increased instances of non-neoplastic lesions in the kidneys, uterus, testes, spleen, and bone marrow of the animals. While the NTP lifetime rodent bioassay is widely considered the gold standard for predicting cancer outcomes, there have been some criticisms that there is very limited data to validate this assay against human carcinogenicity (Ennever & Lave, 2003; Festing, 1995; Macdonald et al., 1994). Furthermore, many of the studies discussed previously have shown data that although tungsten might not induce cancer by itself, it has the potential to do so in combination with other carcinogens, leading some to theorize that tungsten may potentiate the carcinogenic effects of other exposures (Bolt et al., 2015; Bolt & Mann, 2016; Miller et al., 2021).

It is worth noting that the effects of tungsten on cellular processes can vary depending on the type and concentration of tungsten compound being used, as well as the type of cell and species of organism being studied. Further research is needed to fully understand the mechanisms by which tungsten affects cellular processes and to determine the potential health implications of these effects.

1.1.4. Occupational hazard, public health, and epidemiology

Data on human exposure to tungsten is limited, but there are some data to suggest that the metal is not inert. “Hard metal disease” is a well-documented respiratory disease that occurs amongst workers in the tungsten carbide (hard metal) industry (Bech et al., 1962; Nemery & Abraham, 2007). This disease is characterized by “cannibalistic” multinucleated giant cells in airspaces and bronchoalveolar lavage (Ohori et al., 1989). Inhalation of tungsten carbide is also known to induce impaired respiratory function (e.g., shortness of breath, coughing), pulmonary fibrosis, and silicosis (Bech et al., 1962; Chen et al., 2005; Coates & Watson, 1973). Hard metal workers are also thought to be at a higher risk of lung cancer, though there are conflicting data (Lasfargues et al., 1994; Marsh et al., 2017; Moulin et al., 1998). Epidemiological modeling has also shown that urinary levels of tungsten is positively associated with problems with age-related mobility, chronic kidney disease, asthma, diabetes, stroke, higher blood pressure, and cardiovascular disease (Fox et al., 2021; Guo et al., 2022; Lang & Mezler, 2008; Mendy et al., 2012; Nigra et al., 2018; Riseberg et al., 2021; Shiue, 2014; Tyrrell et al., 2013).

1.1.5. Health reports and environmental regulations

There is no consensus about the health effects of tungsten, largely because there is very little research and time dedicated to understanding the impacts and effects of tungsten on the environment and on humans. With what little research and evidence there currently are some national and international regulatory agencies have made public their current positions and conclusions on the toxicity and potential health effects of tungsten.

The scrutiny into tungsten and its health effects is generally accepted as having originated in Fallon, Nevada. In the late 1990s and early 2000s, there was cluster of leukemia cases in Fallon, Nevada being investigated by the CDC (CDC, 2003). Within the span of five years, there

were 15 cases of pediatric acute lymphoblastic leukemia (ALL) and one case of acute myelogenous leukemia (AML). Local residents, town officials, and scientists proposed a variety of causes for the cluster, including electromagnetic radiation, "population mixing," jet fuel, agricultural pesticides, viruses, arsenic, tungsten, and genetic susceptibility to any of aforementioned exposures (CDC, 2003; Francis et al., 2012; Kinlen & Doll, 2004; Rubin et al., 2007). The CDC investigation did not identify any specific cause for the cluster of cases, and no environmental or occupational exposures were identified as potential risk factors. It is important to note that leukemia is a complex disease that can be caused by a variety of factors, including genetic and environmental factors. However, many scientists were unsatisfied with this conclusion, and continued to independently study the potential causes of this cluster. Steinmaus (2004) assessed the probability of this event occurring at random, and his team estimates that a cluster of pediatric ALL of this magnitude would be expected to occur in the United States at random about once every 22,000 years. Levels of tungsten in the water and air were found to have been elevated compared to average ambient concentrations (CDC, 2003; Rubin et al., 2007). Tungsten (and another metal, cobalt) were found to have had spatial and temporal patterns that aligned closely with the geography and timing of the pediatric leukemia cluster (Ridenour et al., 2011; Sheppard et al., 2006, 2007). Since this leukemia cluster and the subsequent evaluations of environmental risk factors in Fallon, Nevada, there has been an increase in the scientific literature associated with tungsten (**Figure 2**).

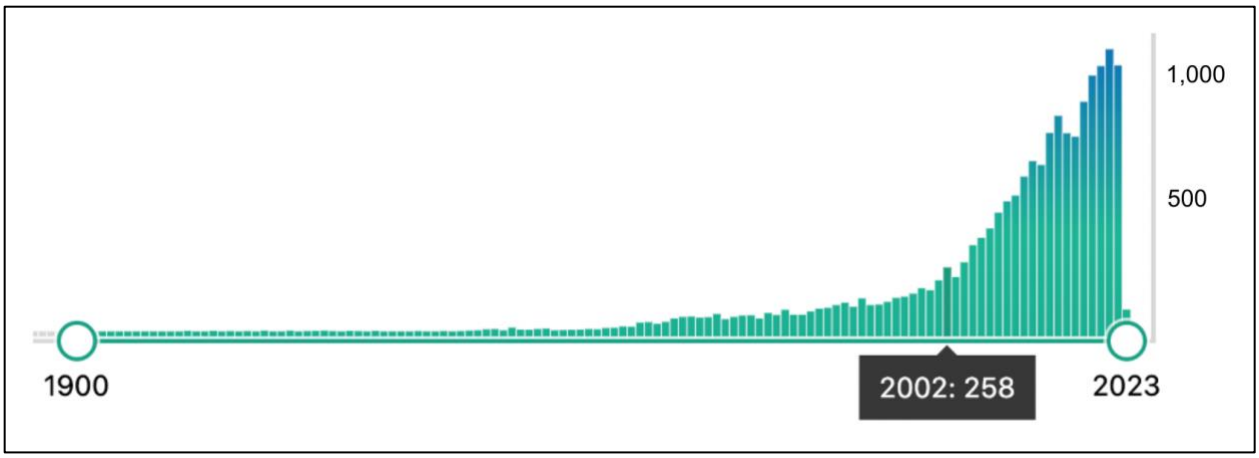

Figure 2. Number of results by year for the search term "tungsten" in the PubMed database.

Currently, the U.S., Canada, and the member states of the European Union do not have drinking water standards for tungsten. In 2022, the US EPA released the Drinking Water Contaminant Candidate List (CCL) 5, which is a list of contaminants in drinking water that are currently not subject to any national drinking water regulations. Moreover, these contaminants are known or anticipated to occur in public water systems and may require regulation under the Safe Drinking Water Act (SDWA). CCL 5 includes synthetic chemicals and chemical groups (e.g., PFAS), microbial contaminants, and heavy metals, including tungsten . In terms of other environmental regulations of tungsten, some individual states and provinces, such as Quebec and Ontario, as well as certain industries in the U.S., have occupational exposure limits on tungsten carbide (ITIA, 2023).

The International Agency for Research on Cancer (IARC) is part of the World Health Organization (WHO) and is responsible for evaluating the potential carcinogenicity of chemicals and other substances. In 2006, IARC found in its review that cobalt metal with tungsten carbide is probably carcinogenic to humans (Group 2A). In 2022, IARC found that weapons-grade tungsten alloy is possibly carcinogenic to humans (classified as Group 2B). IARC has also evaluated the potential carcinogenicity of tungsten alone but has not classified the metal as a

carcinogen. In its evaluation of tungsten, IARC concluded that there is limited evidence in experimental animals for the carcinogenicity of tungsten compounds, and limited evidence in humans for the carcinogenicity of tungsten and tungsten compounds. As a result, IARC has classified tungsten and tungsten compounds as Group 3: Not classifiable as to its carcinogenicity to humans. This classification means that there is insufficient evidence to determine whether tungsten is carcinogenic to humans. It is important to note that IARC's classification of tungsten and tungsten compounds reflects the current state of knowledge on the potential carcinogenicity of these substances and is not intended to be a definitive statement on their potential to cause cancer. More research is needed to fully understand the potential health effects of tungsten and tungsten compounds.

1.2. Genome-wide CRISPR-Cas9 knockout screen

A genome-wide CRISPR-Cas9 knockout screen was conducted to further explore the mechanisms of action by which tungsten may act. Genome-wide CRISPR-Cas9 knockout screens are an important tool in functional toxicology because they allow researchers to identify genes that are essential for the toxic effects of a particular substance. These screens can help to identify potential mechanisms of toxicity, and to identify potential targets for therapeutic intervention. CRISPR screens work by using the CRISPR-Cas9 system to knock out specific genes in a cell or organism and then measuring the effects on the toxic response to a substance. By systematically knocking out different genes, researchers can determine which ones are required for the toxic response, and which are not. In the readout of the results of the screen, the relative levels of sgRNAs are either unchanged, depleted, or enriched, in response to toxicants. There are three possible results of the CRISPR screen: 1) No change in sgRNA levels. This result likely means that the gene is not implicated in any toxicological mechanisms of the

substance, 2) Depletion of specific sgRNAs. This result identifies genes whose disruption sensitizes cells to the effects of the toxicant, which suggests that the gene could be involved in the protection of the cell against that particular toxicant (e.g., DNA repair proteins with a genotoxicant), or 3) Enrichment of specific sgRNAs identifies genes whose disruption confers a selective advantage and resistance against the toxicant, which suggests that the gene is involved in mechanistically facilitating the toxicity of the substance. This can provide important insights into the mechanisms of toxicity and can help to identify potential therapeutic targets for the treatment of toxic exposures.

For this project, a genome-wide CRISPR-Cas9 knockout screen was conducted in collaboration with Dr. Michael Tyers from the Université de Montréal. sgRNAs were introduced and Cas9 was induced in the NALM6 human pre-B-cell lymphocytic leukemia cell line. KO cells were then exposed to 500 μg/mL sodium tungstate or 1000 μg/mL phosphotungstate. The workflow for the CRISPR screen is depicted in **Figure 3A.** sgRNA enrichment and depletion analysis was then done by using a tool called robust analytics and normalization for knockout screens (RANKS). It is designed to help researchers identify the most important or relevant genes from a CRISPR screen, and to prioritize them for further study. Genes that have a large impact on the toxic response will receive a high RANKS score, while genes that have a smaller impact will receive a lower score. It can also help to reduce the number of false positives that may be identified in a CRISPR screen, by providing a more objective and statistically rigorous way to rank the genes that are identified. The full methods for gene knockout and sgRNA analysis can be found in Bertomeu et al., 2018.

We further investigated genes with RANKS scores >1 and -log(*p* value) scores >2 for both sodium tungstate and phosphotungstate exposure groups. Pathway enrichment analysis was

conducted using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), a biological database and web resource of known and predicted protein–protein interactions. Prominent pathways implicated in tungsten toxicity, based on the STRING analysis, seem to revolve around the genes *KPTN, DEPDC5. SZT2, NPRL2, NPRL3,* and *SESN2*, all of which play a role in the amino acid-sensitive negative regulation of mTORC1 (**Figure 3B and C).**

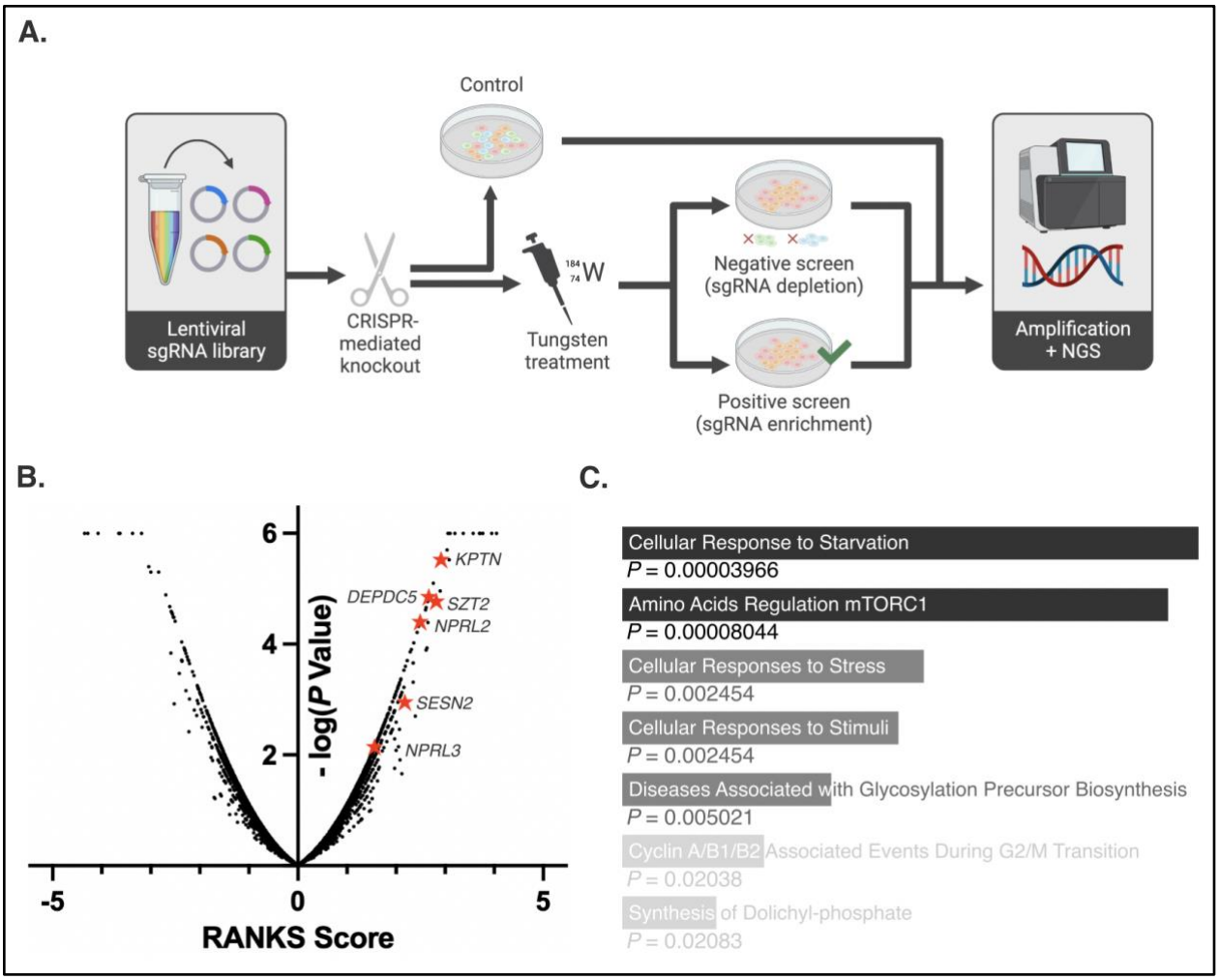

Figure 3. CRISPR-based functional screening of NALM-6 cells exposed to tungsten.

(A) Schematic of the CRISPR genome-wide knockout screen. Separate screens were conducted for treatment with sodium tungstate and phosphotungstate. (B) Volcano plot from analysis of sgRNA enrichment and depletion after treatment with sodium tungstate. Red stars indicate enriched sgRNAs associated with the amino acid regulation of mTORC1. Results from phosphotungstate exposure showed similar results regarding the enrichment of genes associated with the negative regulation of mTORC1. (C) Pathway enrichment analysis of sgRNAs with RANKS scores >1 and -log(*p* value) scores >2. *p* values are adjusted using Benjamini-Hochberg procedure.

1.3. Introduction to mTORC1, KICSTOR, and GATOR1

1.3.1. Mechanistic target of rapamycin (mTOR)

In 1964, METEI (Medical Expedition to Easter Island) researchers from McGill University and the Université de Montréal discovered and isolated an antifungal compound from Rapa Nui (Easter Island) and named it rapamycin (Sehgal et al., 1975; Vezina et al., 1975). Later, the target of rapamycin (TOR) was discovered in yeast, and the mammalian target of rapamycin (mTOR, later renamed the mechanistic target of rapamycin) was found thereafter (Heitman et al., 1991; Sabatini et al., 1994). Today, mTOR is widely known as a protein that plays a key role in regulating cell growth and metabolism. It is a part of a larger signaling pathway that integrates inputs about nutrient availability, energy levels, and other cellular signals to coordinate cellular responses such as protein synthesis, cell growth, and autophagy. mTOR acts as a "master regulator" that helps cells decide when to grow and divide, and when to stop growing and start repairing or recycling components. It does so by sensing the availability of nutrients and energy within the cell and signaling to other proteins and pathways to adjust cellular processes accordingly.

mTOR is found in two complexes in cells, called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes have distinct functions and are regulated by different signals. mTORC1 is activated by signals that promote cell growth and proliferation, such as nutrients, growth factors, and energy levels (Kim et al., 2002, 2003). In addition to mTOR, mTORC1 and mTORC2 both contain mLST8 and Deptor. Specific to mTORC1 is Raptor and PRAS40, while the proteins Rictor and mSin1 are unique to mTORC2. Once activated, mTORC1 promotes the synthesis of proteins, lipids, and other cellular components, as well as the activation of pathways that promote cell growth and survival. In addition, mTORC1

plays a role in regulating the metabolism of cells, including the breakdown of carbohydrates and the synthesis of ATP, the main source of energy for cells. mTORC2 is activated by different signals than mTORC1 and plays a role in the regulation of cytoskeletal dynamics, cell migration, and cell survival (Dos et al., 2004; Frias et al., 2006). mTORC2 also plays a role in the activation of signaling pathways that promote cell growth and survival. For the purpose OF this thesis and our experiments, this literature review will focus on mTORC1.

1.3.2. Nutrient sensing, KICSTOR-GATOR1 axis, and activation of mTORC1

As mentioned earlier, mTORC1 is regulated by a wide array of signals that regulate cell growth and proliferation. These include Wnt, TNFα, growth factors, stress, hypoxia, DNA damage, glucose, lipids, and amino acids (Melick & Jewell, 2020). Here, we attempt to describe the most pertinent pathways upstream of mTORC1. Opposite mTOR is AMPK (AMP-activated protein kinase), a protein that is switched on by lack of energy or nutrients and inhibits cell growth. Described as the "yin and yang" of cellular nutrient sensing and cell growth, mTOR and AMPK modulate and regulate each other and other proteins to maintain homeostasis (González et al., 2020). Another upstream regulator of mTORC1 is the tuberous sclerosis protein complex (TSC complex), which regulates mTORC1 by integrating metabolic signals and cellular stress (Dibble & Manning, 2013; Tee et al., 2003). In response to nutrient shortage and other stresses, the TSC complex will inhibit mTORC1 at the lysosomes. Some pathways that function by inhibiting mTORC1 through the TSC complex include p53/PTEN/AKT (DNA damage), HIF1A/REDD1 (hypoxia), and AMPK (energy deprivation).

While mTOR is negatively regulated by stressors, it is also positively regulated by nutrients such as glucose and amino acids. For instance, Rheb activates mTORC1 in response to growth factor signals, as well as amino acid sufficiency (Long, Lin, et al., 2005; Long, Ortiz-

Vega, et al., 2005). However, for the purposes of this thesis, we focus on the amino acid sensing branch of mTORC1. There are also several known amino acid sensors, and there are undoubtedly others yet to be discovered (Wolfson & Sabatini, 2017). CASTOR (arginine), SAMTOR (methionine), leucyl-tRNA synthetase (LRS, leucine), and the sestrins (SESN1/2; leucine) are some of these sensors that function to activate mTORC1. For example, SESN1/2 function by inhibiting the activity of GATOR2 (a complex composed of WDR24, MIOS, WDR59, SEH1L and SEC13). The RING domain of the GATOR2 complex, maintains the integrity of GATOR2 while promoting the ubiquitination and inhibition of GATOR1 (a complex composed of DEPDC5, NPRL2, and NPRL3) (Sahu & Ben-Sahra, 2023). GATOR1 has GAP (GTPase-activating protein) activity, and after being recruited to the lysosome by KICSTOR (a complex composed of KPTN, ITFG2, C12orf66 and SZT2), inhibits the Rag GTPases from activating mTORC1 (Bar-Peled et al., 2013; Wolfson et al., 2017) (**Figure 4**). In summary, GATOR1, a negative regulator of mTORC1, is inhibited when the cell experiences amino acid sufficiency.

1.3.3. Regulation of cell growth and proliferation

Once activated, mTORC1 promotes the synthesis of proteins, lipids, and other cellular components, as well as the activation of pathways that promote cell growth and survival. mTORC1 activates the ribosomal protein S6 kinase beta-1 (S6K1) pathway (at the Thr 389 residue). Phosphorylated S6K1 plays a role in ribosome biogenesis, as well as the phosphorylation ribosomal protein S6, which promotes the synthesis of proteins by activating the translation of mRNA into protein (Fingar et al., 2002; Hay & Sonenberg, 2004). mTORC1 also phosphorylates the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) at multiple residues, including Thr 37 and 46. 4E-BP1 phosphorylation promotes the initiation of protein synthesis by releasing the eukaryotic initiation factor 4E (eIF4E) from 4E-BP1, allowing mRNA

translation to occur (Fingar et al., 2002; Hay & Sonenberg, 2004). In summary, mTORC1 activates global and specific protein translation in the cell by phosphorylating S6K1 and 4E-BP1 (**Figure 4**). Other mTORC1 downstream effectors and their function in the cell growth include: Lipin1/SREBPs (lipid synthesis) and ATF4 (nucleotide synthesis) (Napolitano et al., 2022).

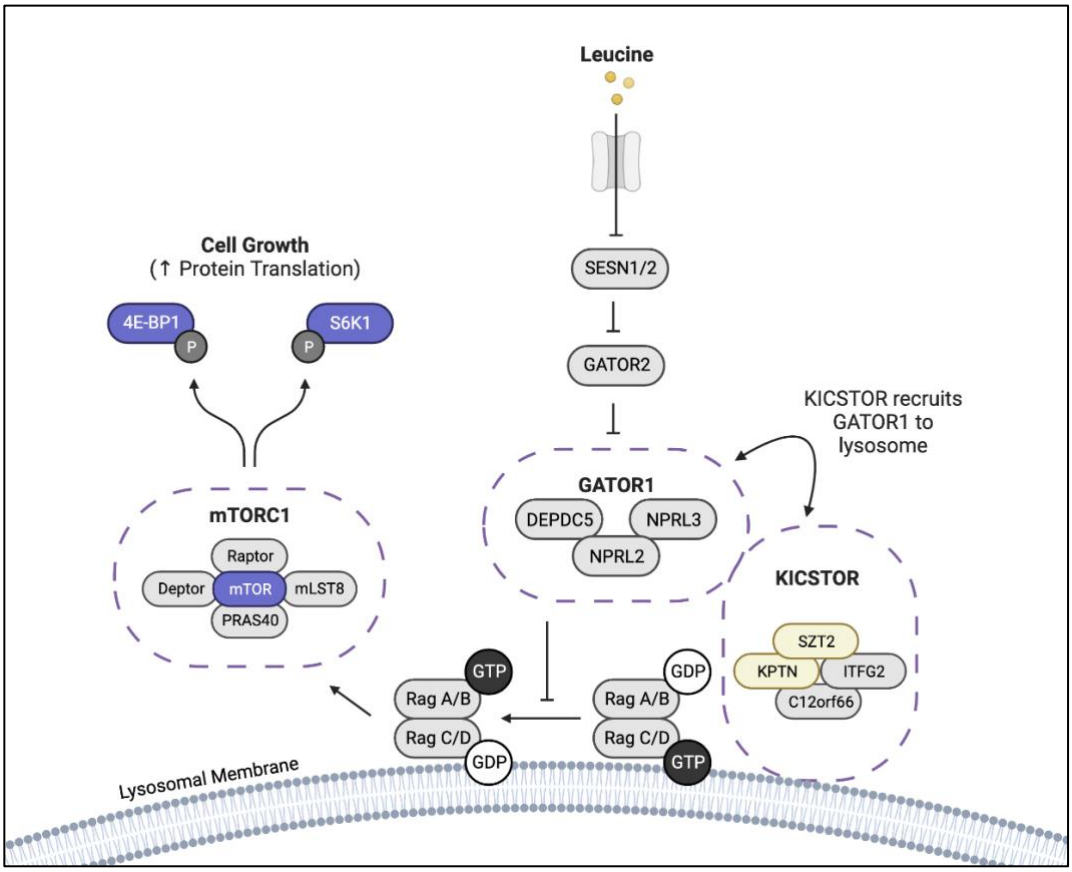

Figure 4. Regulation and Activation of mTORC1.

KICSTOR is a complex composed of four proteins: KPTN, ITFG2, C12orf66 and SZT2. The complex localizes to the lysosomal membrane, where it recruits GATOR1, a complex composed of NPRL2, NPRL3, and DEPDC5. GATOR1 is a GTPase-activating protein (GAP) for RagA or RagB, whose function inhibits the activity of mTORC1. Proteins such as the sestrins (SESN1/2) have been identified as sensors upstream of mTORC1.

Another key function of mTORC1 is to modulate protein degradation by regulating autophagy and protein ubiquitination. While mTORC1 positively regulates protein translation, ribosome biogenesis, lipid synthesis, and nucleotide synthesis, autophagy is negatively regulated by mTORC1. Autophagy is a cellular process that involves the degradation of cellular components by lysosomes. Lysosomes degrade cellular elements, such as defective proteins and organelles. mTORC1 regulates autophagy by preventing the development of autophagosomes, the containers that house the substances that will be broken down by lysosomes. When mTORC1 is active, it binds to the ULK1 complex (composed of ULK1 and other proteins such as FIP200, ATG13, and ATG101) and phosphorylates ULK1 at specific sites (Hosokawa et al., 2009). As a result, autophagosome formation is suppressed and less material is degraded by autophagy. In addition to controlling autophagy, mTORC1 plays a role in regulating the ubiquitin-proteasome system (UPS) through its effects on protein ubiquitination (Zhao et al., 2015). The UPS is the main degradation pathway for intracellular proteins in eukaryotic cells. This system works by tagging proteins with ubiquitin, which serves as a signal for the proteasome, a large complex of proteolytic enzymes, to degrade the targeted proteins. In summary, active mTORC1 regulates cell growth and proliferation both by upregulating protein translation, ribosome biogenesis, as well as lipid and nucleotide synthesis, while also downregulating protein degradation processes such as autophagy and the UPS.

1.3.4. Clinical relevance and "mTORopathies"

Due to the multiplicity of roles mTORC1 (and mTOR broadly) in regulating cell growth and metabolism, mTOR has also been implicated in a number of diseases. Dysregulation of mTOR signaling has been linked to the onset and/or progression of certain cancers, diabetes, obesity, aging, defects in the immune system, epilepsy, and other developmental and

neurological disorders (Saxton & Sabatini, 2017). As a result, drugs that inhibit mTOR activity are being developed as potential therapies for these conditions. In addition to rapamycin (a first-generation mTOR inhibitor, mTORi), second generation mTORis have been developed as ATP-competitors, competing with ATP molecules for attaching to the mTOR kinase domain. Such mTORis include torin-1, torin-2 and vistusertib, and sapanisertib (INK128). The study of mTOR and its effects when dysregulated is of the utmost importance in the development of interventions and in the prevention of such diseases and disorders in the context of toxicants that may be implicated in mTOR signaling.

2. Rationale and research objectives

Tungsten accumulates within the bone, which potentiates higher exposure levels to cells in the bone marrow. As a result, cells of lymphoid lineage, which originate in the bone marrow, may be more susceptible to leukemogenesis. The mechanisms by which tungsten may act to alter cell growth and proliferation and promote tumorigenesis have not been uncovered. The CRISPR/Cas9 functional knockout screen, carried out in NALM6 cells, suggests that the toxicity of tungsten may be mediated by proteins involved in the negative regulation of mTORC1, the primary nutrient-sensitive regulator of cell growth.

The following experiments were conducted in U2OS osteosarcoma cells, rather than the NALM6 cells used for the CRISPR screen. NALM6 cells are much harder to genetically manipulate and use for imaging, while U2OS cells are more resilient and easier to genetically manipulate. U2OS cells are still physiologically and biologically relevant, given the context of tungsten accumulation in the bone and the shared lymphoid origins of B cells (leukemia) and bone cells (osteosarcoma).

My project focuses on the following hypothesis: tungsten induces cytotoxicity by dysregulating the levels, localization, or signaling of proteins in the KICSTOR and GATOR1 complexes, through an mTORC1-dependent mechanism. This project aims to 1) validate the results of the CRISPR/Cas9 screen; 2) assess tungsten cytotoxicity under inhibition of mTORC1 or GATOR1; and 3) investigate the effect of tungsten on localization of the GATOR1 complex to the lysosome.

3. Materials and methods

3.1. Chemicals and reagents

The following chemicals and reagents were used in this study: sodium tungstate dihydrate (Na2WO4·2H2O, Sigma-Aldrich, St Louis, Missouri); leucine (Sigma-Aldrich); rapamycin (Sigma-Aldrich); INK128 (also known as sapanisertib, MLN0128, TAK-228; SelleckChem, Houston, Texas). Drugs were dissolved in DMSO to 10 mM, and aliquots were stored at -80 °C.

3.2. Tungsten exposure

Sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$, Sigma-Aldrich) was used in all tungsten exposure experiments *in vitro* because orthotungstate (WO_4^{2-}) is shown as the most predominant form in solution at a neutral pH (Kelly et al., 2013). Concentrations of tungsten (in ppm and µg/mL) were calculated based on the elemental weight of tungsten and not the compound itself (i.e., sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) has a molecular weight of 329.85 g/mol and elemental tungsten (W) has a molecular weight of 183.84 g/mol. 1 g of elemental tungsten requires an equivalent 1.795 g of sodium tungstate dihydrate). For tissue culture, a working stock of 50 mg/mL tungsten solution was made by dissolving 714.3 mg of sodium tungstate dihydrate in 8 mL autoclaved milliQ water and filter sterilized through a 0.2 mm filter. Tungsten stock solution was then aliquoted and stored at -20°C.

3.3. Cell culture

U2OS osteosarcoma cells (ATCC HTB-96) were cultured in McCoy's 5A Medium (McCoy's; Wisent, St Bruno, QC, Canada), supplemented with 10% FBS (Wisent) and 1% penicillin-streptomycin (Wisent) at 32 °C with 5% CO_2. U2OS cells were subcultured with 0.05% trypsin (Wisent) every 2 to 3 days.

3.4. Lentiviral transduction of U2OS cells

Small guide RNA (sgRNA) sequences were ordered as duplexes (IDT) and cloned into the lentiviral backbone vector, lentiCRISPRv2 (Addgene plasmid no. 52961). The sgRNA sequences are as follows:

- sgKPTN-1-F: 5'-CACCGTCGTCGCAGAGCAATGTGTA-3'
- sgKPTN-1-R: 5'-AAACTACACATTGCTCTGCGACGAC-3'
- sgSZT2-2-F: 5'-CACCGGAAGCAGCCCGCCTAAGCAG-3' (Wolfson et al., 2017)
- sgSZT2-2-R: 5'-AAACCTGCTTAGGCGGGCTGCTTCC-3' (Wolfson et al., 2017)

Lentivirus were packaged in HEK293FT cells with the target vector (lentiCRISPRv2 plasmid containing sgRNAs), the packaging plasmid (psPAX2) and the envelope plasmid (pMD2.G) using Lipofectamine 2000 (ThermoFisher 11668027) and Plus reagent (ThermoFisher 11514015) following the manufacturer's instructions. Viral supernatant was collected and filtered with 0.45 μm PVDF filters and frozen at -80 °C. U2OS cells were transduced with polybrene (8 μg/ml) and lentiGuide-Puro lentiviruses every 24 h for 2 consecutive days. Cells were then maintained in virus-free media for 48 h. Then, cells were selected for resistance to puromycin (2 μg/mL) for 7 days. Single cell clones were expanded. Western blot analysis and Sanger sequencing were used to verify the KO status of the clones.

3.5. Cell viability assay

The first set of cell viability experiments were performed using MTT assays. In viable cells, MTT is converted to formazan, which fluoresces purple, by an enzyme called mitochondrial dehydrogenase. The activity of mitochondrial dehydrogenase ceases to occur in the presence of cytotoxic agents. Cells (1.5×10^3 cells) were seeded in 96-well plates and cultured for 24 h. Tungsten solutions with gradually increasing concentrations of tungsten were created

using serial dilution and added to cells. After 72 h, cell viability was determined by adding thiazolyl blue tetrazolium bromide (MTT, BioBasic). MTT (0.5 mg/mL) was added to each well and the plate was then incubated at 37 °C in 5% CO_2 for 4 h. The formazan crystals were then solubilized by adding 100 µL of SDS-HCl detergent, then the plates were incubated for 18 hours at 37 °C in 5% CO_2. The optical density, or absorbance, of each well was determined using a Benchmark Plus™ microplate spectrophotometer (Bio-Rad) at 570 nm. The absorbance of wells was normalized to the absorbance of the control wells to assess cell viability. Three replicates for each treatment group were examined.

Another cell viability technique used was the crystal violet assay: each conducted in duplicate or triplicate, and with three independent experiments. For the crystal violet assay, cells (5×10^3 cells) were seeded in 6-well plates and cultured for 24 h. Then, cells were treated with tungsten and cultured for five days. The viable adherent cells were fixed with 4% PFA and stained with 0.1% crystal violet. Cells were then imaged with GelCount™ Mammalian cell colony counter (Oxford Optronix, Milton, England) and the % area and mean fluorescence intensity (MFI) of colonies were automatically quantified by FIJI software (FIJI, Bethesda, MD, USA).

3.6. Flow cytometry

Cells (1.5×10^5) were seeded in 6-well plates and allowed to adhere. After 24 h, cells were treated with tungsten. After 72 h of exposure, cells were counted, collected (1×10^6), washed twice with PBS, and resuspended in 100 µL of a solution containing PBS, 1X LIVE/DEAD™ Near IR cell stain (Invitrogen, dilution of 1:1000) and/or Apotracker™ (BioLegend, 400 nM). Cells were incubated at room temperature for 20 min. Then, cells were washed with PBS and fixed with 1% paraformaldehyde for 20 min in the dark. Cells were then washed and

resuspended in FACS buffer (1X PBS, supplemented with 5% fetal bovine serum (FBS) and 10 mM sodium azide) and stored in 4 °C. Three independent experiments were performed. Samples were analyzed with LSRFortessa™ (BD Biosciences) and data were analyzed using FlowJo software.

3.7. Senescence β-galactosidase assay

Senescence-associated β-galactosidase (SA β-Gal) staining was performed after cells (3×10^4) were seeded in 12-well plates, left to adhere for 24 h, and then treated with different concentrations of tungsten. After 72 h of exposure, cells were washed with PBS and then fixed in 0.5% glutaraldehyde (Sigma-Aldrich) for 15 min at room temperature, followed by two washes with a solution of PBS with 1 mM $MgCl_2$ pH of 6.0. Slides were immersed in SA β-gal staining solution [1 mg/mL X-gal in dimethylformamide (Sigma-Aldrich), 5×10^{-3} M potassium ferrocyanide (Bioshop, Burlington, ON, Canada), 5×10^{-3} M potassium ferricyanide trihydrate (Sigma-Aldrich), dissolved in the PBS/$MgCl_2$ solution described above at a pH 6.0], wrapped well with parafilm (to prevent contact with CO_2 and subsequent pH change), and incubated at 37 °C overnight. After, the wells were washed twice with water. Samples were analyzed with bright field microscopy. For each of the triplicates in every condition, images of at least three distinct, randomly chosen fields were captured in each of the stained samples. Percent SA β-Gal activity was calculated as the number of stained cells divided by the total number of cells in a field. Three independent experiments were conducted for this experiment.

3.8. Cellular protein extraction, quantification, and immunoblotting

Cells (1.5×10^5) were seeded in 6-well plates. After 24 h, cells were treated with different concentrations of tungsten. After 72 h of exposure, cell pellets were collected, washed twice with cold PBS, and lysed with RIPA buffer (125 mM Tris pH 7.6, 750 mM NaCl, 5% Igepal CA-630,

5% sodium deoxycholate, 0.5% SDS, Cell BioLabs) supplemented with PhosSTOP™ (Roche) and cOmplete™ Protease Inhibitor Cocktail (Roche). Protein was quantified with the Bradford protein assay. Samples were boiled before loading equal amounts of protein onto a 6-15% SDS-polyacrylamide gel for electrophoresis, followed by transfer to a PVDF membrane. Membranes were stained with Ponceau S to confirm uniform loading of gel. Membranes were cut and blocked with 5% TBS-T milk for 1 h. Proteins were incubated overnight with primary antibodies in 5% TBS-T milk (**Table 2**), washed three times with TBS-T for 15 min each, then incubated with the appropriate horseradish peroxidase secondary antibody for 2 h (GE Healthcare, anti-mouse dilution 1:5000, anti-rabbit dilution 1:3000). Immunocomplexes were visualized by chemiluminescence detection using Amersham ECL Western Blotting Detection Reagents (GE Healthcare).

Table 2. Summary table for primary antibodies used in Western Blot

Primary Ab	Company (Catalog Number)	Dilution
phospho-mTOR (S2448)	Cell Signaling (5536)	1:750
mTOR	Cell Signaling (2972)	1:750
phospho-4E-BP1 (T37/46)	Cell Signaling (2855)	1:500
4E-BP1	Cell Signaling (9452)	1:500
phospho-P70S6K1 (T389)	Cell Signaling (9205)	1:750
P70S6K1	Cell Signaling (9202)	1:750
phospho-S6 (S240/244)	Cell Signaling (2215)	1:1000
S6	Cell Signaling (2317)	1:1000
SESN1	ProteinTech (21668-1-AP)	1:1000
NPRL2	Invitrogen (PA5-116091)	1:750
NPRL3	ThermoFisher (PA5-78245)	1:750
DEPDC5	ThermoFisher (PA5-71618)	1:1000
KPTN	ProteinTech (16094-1AP)	1:1000
vinculin	Cell Signaling (13901)	1:1000

3.9. shRNA transfection

The shRNA vectors used for transfection were: non-target shRNA control (pLKO Sigma-Aldrich: SHC002) and RAPTOR (AddGene: plasmid 1857). shRNA vectors were cotransfected into HEK293T cells with the lentivirus packaging plasmids PLP1, PLP2, and PLP-VSVG (Invitrogen) using Lipofectamine 2000 (Invitrogen). Supernatants were collected 48 and 72 hours post-transfection, passed through a 0.45 μm nitrocellulose filter and applied on target cells with polybrene (5 μg/mL). Cells were re-infected the next day and selected with puromycin for 48 hours (1 μg/mL, Sigma-Aldrich).

3.10. Immunofluorescence and co-localization analysis

Cells (3×10^4) were seeded on 15 mm glass coverslips (Karl Hecht Assistent, Altnau, Switzerland), in 12-well plates. After 24 h, the cells were treated with 100 μg/mL sodium tungstate. After 72 h of treatment, slides were rinsed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT). The slides were then rinsed three times with wash buffer (20 mL TBS 10X, 80 mL ddH_2O, 25 μL Tween-20) and the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at RT. The slides were rinsed three times with wash buffer and then blocked for 30 min in primary blocking buffer (900 μL wash buffer, 100 μL donkey serum) at RT. The slides were then washed with wash buffer and blocked for 30 min in FC blocking buffer. Slides were then washed twice with wash buffer and incubated with primary antibody in blocking buffer at 4 °C overnight, rinsed three times with wash buffer, incubated with secondary antibodies for 1 h at RT in the dark and washed three times with wash buffer. The primary antibodies used were directed against NPRL2 (rabbit monoclonal; PA5-116091; 1:200 dilution) and LAMP1 (mouse monoclonal H3A4; DHSB; 1:200 dilution). After washing three times, slides were incubated with secondary antibodies for 1 h at RT. Slides were

then washed five times with wash buffer, then DAPI (1:500 of 1 mg/mL stock) was applied for 10 min, and slides were then washed and were mounted on glass coverslips using Fluoromount-G (Invitrogen).

Co-localization analysis was performed as outlined in previous papers looking at mTOR and lysosomal localization (Orozco et al., 2020; Wolfson et al., 2017). Slides were imaged with Zeiss LSM800 Confocal Microscope. The excitation lasers used to capture the images were 488 nm (LAMP1) and 647 nm (NPRL2). In each cell analyzed, a region of the cytoplasm with high density of lysosomes was chosen by finding an area with a high LAMP1 signal. In this area, the mean fluorescence intensities (MFIs) of the 488 nm (LAMP1) and 647 nm channels (NPRL2) were measured. In the same cell, an equivalently sized region of the cytoplasm containing only background levels of LAMP1 signal was chosen and the MFIs of the 488 and 647 nm channels were also measured in this area. The following equation was used to calculate a "lysosomal enrichment factor" as a proxy for NPRL2-LAMP1 colocalization:

$$Lysosomal\ enrichment\ factor = \frac{(MFI_{lysosome} - MFI_{background})_{647nm}}{(MFI_{lysosome} - MFI_{background})_{488nm}}$$

A lysosomal enrichment factor closer to 1 suggests the NPRL2 (647 nm) signal was enriched specifically in the region of the cell containing lysosomes compared to another area in the cytoplasm. A lysosomal enrichment factor around or below zero indicates that the NPRL2 (647 nm) signal was not enriched at the lysosomes (no co-localization). For each condition studied, images of at least 3 distinct fields were captured and within each 2–4 cells were analyzed as described above so that at least 10 cells were analyzed per condition.

3.11. Statistical analysis

All statistics were performed using GraphPad Prism version 4 software (GraphPad, La Jolla, California). For comparisons between 2 groups, an unpaired Student's t test analysis was

performed. For comparisons between 3 or more groups, a 1-way or 2-way ANOVA analysis was performed, followed by a Dunnett's or Dunn–Šidák's post-hoc test, depending on the experimental design.

4. Results

4.1. KICSTOR components validated as genes involved in tungsten toxicity

First, we sought to validate the results from the CRISPR knockout (KO) screen showing that deficiency in genes related to the negative regulation of mTORC1 makes cells more resistant to tungsten toxicity. To confirm this finding, *KPTN* and *SZT2* were selected as targets for CRISPR KO in the U2OS cell line. We designed two pairs of sgRNAs, targeting either exon 1 of *KPTN* or exon 4 of *SZT2*. After introduction of the gRNAs by transduction, single cells were isolated to generate clonal lines that could be verified as complete KOs. KOs were validated by Sanger sequencing, Western blot, and colocalization analysis of immunofluorescence imaging. We were unable to validate SZT2 protein levels as no appropriately sized band was detected in U2OS cells, as well as HEK 293 cells and rat liver samples, where SZT2 protein should be abundant.

Sanger sequencing revealed that the cut site for both KOs occurred before the protospacer adjacent motif (PAM) site (**Figure 5A**). Sanger sequencing data for sgKPTN-1 clone 2 and sgSZT2-2 clone 2 are not shown. Western blots showed an expected absence of KPTN protein in *KPTN* KOs, while there was no difference between EV and *SZT2* KO cells (**Figure 5B**). As KICSTOR is an inhibitor of mTORC1 signaling, *SZT2*- and *KPTN*-deficient cells are expected to exhibit increased mTORC1 activity compared to EV cells. Indeed, immunoblotting of cellular extracts for mTORC1 downstream targets phospho-S6K1 and the phospho-RPS6 showed indicate increased mTORC1 activity in the absence of KICSTOR components (**Figure 5B**). Finally, the genetic depletion of *KPTN* or *SZT2* should disrupt the function of the KICSTOR complex in recruiting GATOR1 (comprised of DEPDC5, NPRL2, and NPRL3) to the lysosome, as described in Wolfson et al., 2017. We performed immunofluorescence experiments to

determine whether the GATOR1 protein NPRL2 co-localized with the lysosomal protein LAMP1. We found that, compared to EV cells, the *SZT2* KOs did have a decreased colocalization between an abundant lysosomal marker, LAMP1, with GATOR1 protein, NPRL2 (**Figure 5C**). Herein, I will refer to *KPTN* and *SZT2* KO cells as KICSTOR-deficient cells.

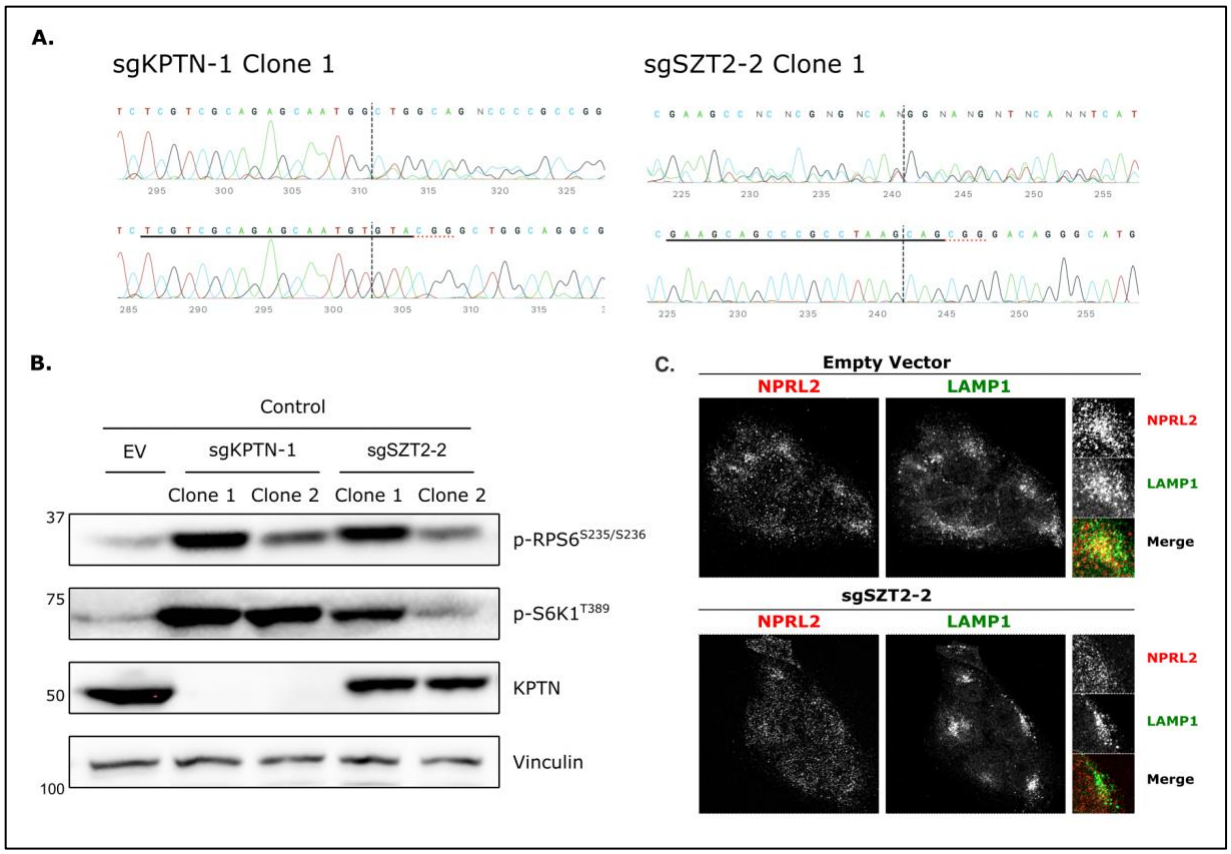

Figure 5. Confirmation of *KPTN* and *SZT2* knockout in U2OS cells with Sanger sequencing, Western blot, and immunofluorescence microscopy.

(A) Sanger sequencing data from the Synthego Inference of CRISPR Edits (ICE) Analysis. These sequencing data show empty vector (control) and edited sequences (top and bottom, respectively) in the region around the guide sequences. The horizontal black underlined region represents the guide sequence. The horizontal red underline is the PAM site. The vertical black dotted line represents the actual cut site.
(B) Western blots support knockout (KO) of *KPTN* and *SZT2* in multiple clones. There was increased mTORC1 activity, as measured by phosphorylation of S6K1 and RPS6 in both KOs and absence of KPTN in *KPTN* KOs.
(C) Colocalization analysis of GATOR1 to lysosomes. Immunofluorescence microscopy showed NPRL2 (AF647) and LAMP1 (AF488) colocalization (yellow fluorescence) in EV, and KICSTOR-deficient cells. Data for KPTN KOs are analogous to the SZT2 KOs, but they are not shown here.

To determine whether KICSTOR deficiency would decrease susceptibility to tungsten cytotoxicity, cells to were exposed to gradually increasing concentrations of sodium tungstate (0 to 400 μg/mL) for 72 h. Cell viability was analyzed by MTT assay after exposure. KICSTOR-deficient cells were found to be significantly more resistant to tungsten-induced cytotoxicity than EV control cells (**Figure 6A**). The IC50 values of *KPTN* (167 μg/mL) and *SZT2* KOs (174 μg/mL) were significantly greater than the IC50 of EV control cells (110 μg/mL, $P < 0.001$).

However, the MTT assay is a proxy for cell viability, as it measures the activity of mitochondrial dehydrogenase activity. Therefore, we validated these results using a clonogenic assay, as well. Cells were exposed to concentrations of tungsten from 0 to 200 μg/mL for five days and stained with crystal violet. Again, we clearly see that KICSTOR deficiency in cells confers resistance against tungsten cytotoxicity. At tungsten concentrations of 100 and 200 μg/mL, we were able to qualitatively determine that EV cells had noticeably lower number and intensity of crystal violet-stained colonies compared to the control group and compared to the KICSTOR deficient cells of the same tungsten concentrations (**Figure 6B**). Quantitatively, the percent area occupied by viable cells and the mean fluorescence intensity (MFI) of the crystal violet-stained cells was measured. There is a distinct trend wherein EV cells occupy less area than KICSTOR deficient cells at 100 (for sgKPTN-1 and sgSZT2-2, $P = 0.0688$ and 0.0028, respectively) and 200 μg/mL (for sgKPTN-1 and sgSZT2-2, $P = 0.0075$ and 0.0001, respectively). The same trend is seen with MFI at 100 (for sgKPTN-1 and sgSZT2-2, $P = 0.0227$ and 0.0008, respectively) and 200 μg/mL (for sgKPTN-1 and sgSZT2-2, $P = <0.0001$ and <0.0001, respectively) (**Figure 6C**). These data suggest that KICSTOR-deficient cells are indeed more resistant to tungsten cytotoxicity compared to their EV equivalents.

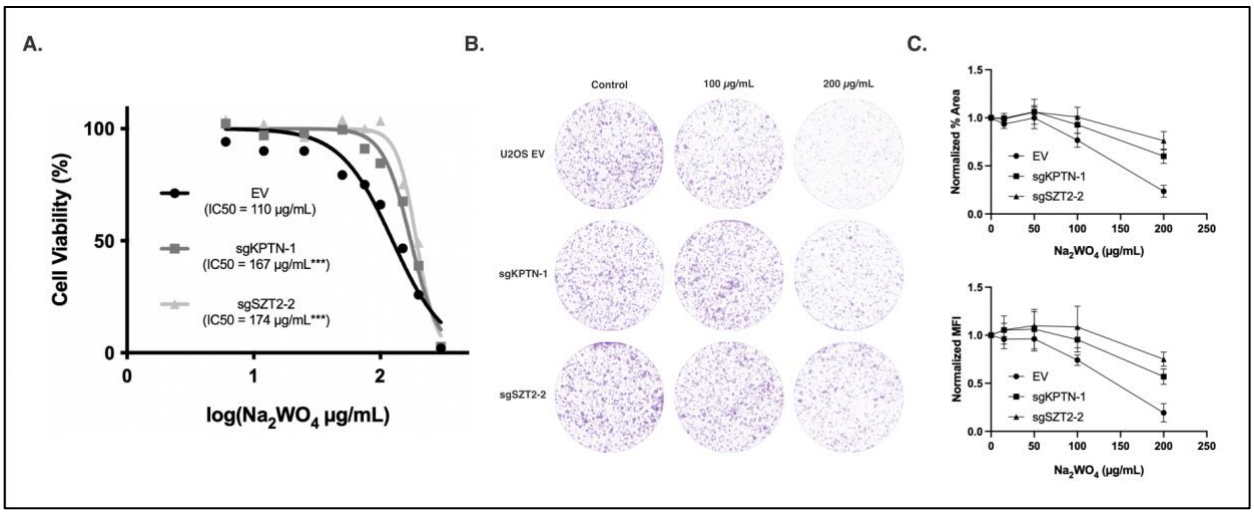

Figure 6. Viability of U2OS cells exposed to tungsten assessed with MTT and clonogenic assays.

(A) MTT assay comparing cell viability of EV cells with sgKPTN-1 and sgSZT2-2 KO cells exposed to 0 - 300 µg/mL sodium tungstate (Na_2WO_4) for 72 h. The data are presented as the mean and are representative of three independent experiments, with each treatment group in triplicate. IC50 values were compared using the Student's t-test. *** $P < 0.001$
(B) Clonogenic crystal violet assay comparing cell viability of EV cells with sgKPTN-1 and sgSZT2-2 KO cells exposed to 0 (control), 100, or 200 µg/mL Na_2WO_4 for 5 days. The data are representative of three independent experiments, with each treatment group in duplicate.
(C) Quantification of clonogenic crystal violet assay using percent area and mean fluorescence intensity (MFI) normalized to 0 µg/mL control group. Data are presented as the mean ± SD.

The data from the MTT and clonogenic assays validated the results of the CRISPR screen, wherein the deletion of genes in the KICSTOR-GATOR1 axis (as measured by relative sgRNA abundance) conferred resistance against tungsten cytotoxicity. Yet to be explained by these data is the mechanism by which we observe the decreased cell viability and lower cell counts. Therefore, we sought to understand whether cells exposed to tungsten were undergoing apoptosis and cellular senescence.

4.2. Cellular senescence and apoptosis in tungsten-induced cytotoxicity

First, we investigated the effect of tungsten on cellular senescence. The process of cellular senescence is characterized by changes in cell morphology and proliferation. They also stop multiplying but do not die. Cellular senescence is induced by factors such as telomere

shortening, DNA damage, and oxidative stress, and may result in pathologies, including fibrotic diseases, vascular disorders, obesity, and type 2 diabetes (Muñoz-Espín & Serrano, 2014). To assess whether tungsten induces cellular senescence, we used a common technique that detects senescence associated β-galactosidase (SA-β-GAL) activity. β-galactosidase catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. While the enzyme is not necessary for the progression of cellular senescence, its overexpression and accumulation in lysosome in aging and senescent cells has made it a reliable and widely used biomarker for cellular senescence.

We evaluated cellular senescence by measuring the SA-β-GAL activity in EV and KO cells, exposed and unexposed to 100 μg/mL sodium tungstate for 72 h. For the positive control, cells were exposed to 1 μM doxorubicin (DOX) a well-characterized pharmacologic drug that induces cellular senescence, for one hour (Maejima et al., 2008; Sliwinska et al., 2009). There is a change in cell morphology for the positive control cells exposed to DOX. Compared to the cells in the U2OS EV control group, the cells exposed to DOX are enlarged and flattened (**Figure 7A).** In empty vector and KICSTOR-deficient cells, there was no induction of cellular senescence, as defined by both a change in cell morphology and by staining with X-GAL (blue when cleaved). Quantitatively, in the positive control group, we observed a significant increase in the percentage of cells that had SA-β-GAL activity, as defined by the proportion of cells that stain blue (**Figure 7B**). In empty vector and KICSTOR-deficient cells, there was no induction of cellular senescence, as defined by both a change in cell morphology and by staining with X-GAL (blue when cleaved) in either tungsten exposed or unexposed groups. These data suggest that senescence is not the mechanism by which tungsten decreases cell viability in U2OS cells.

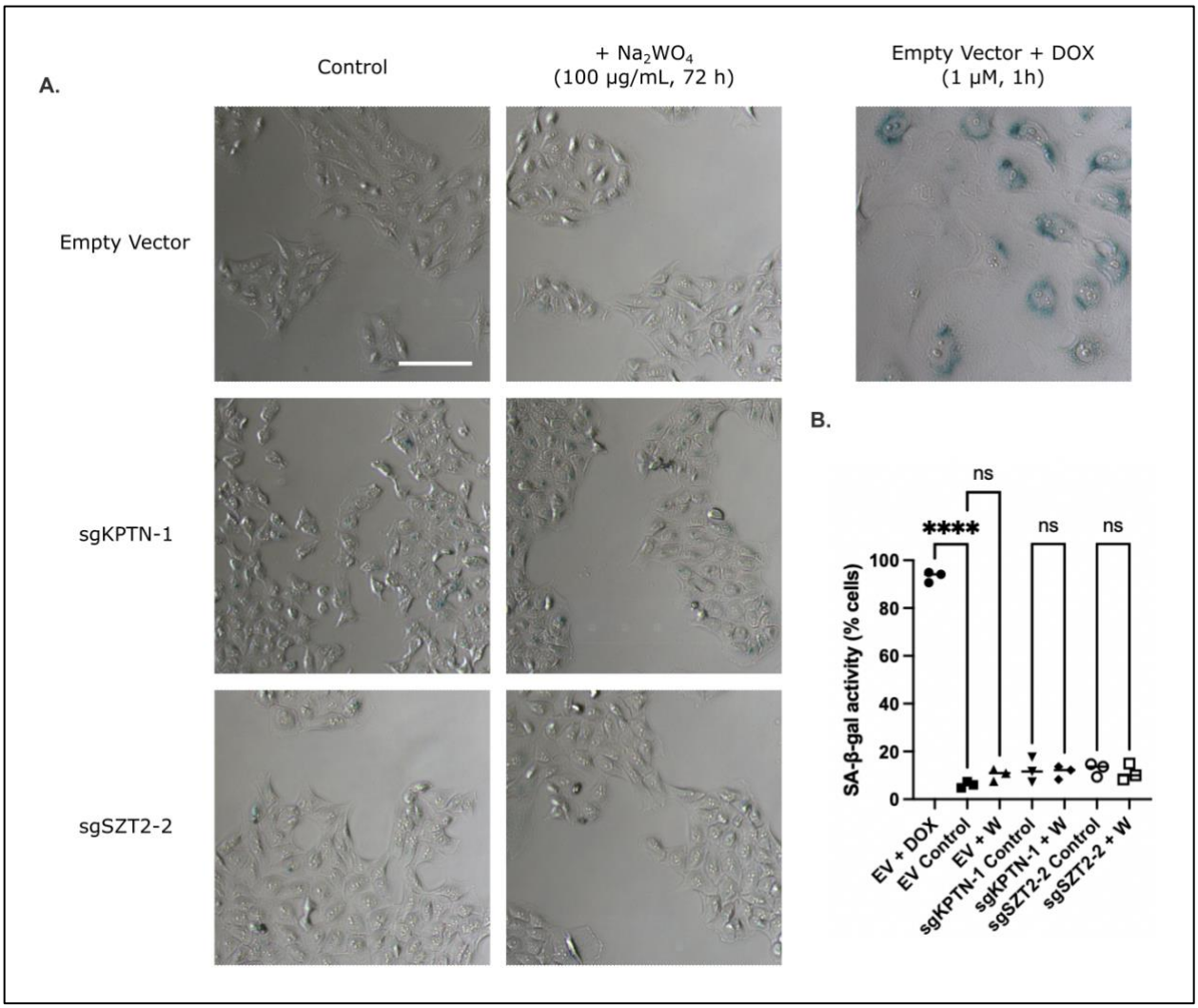

Figure 7. Tungsten does not induce senescence associated β-galactosidase (SA-β-GAL) activity.

(A) EV, sgKPTN-1 KO, and sgSZT2-2 KO cells were exposed to 0 or 100 µg/mL Na_2WO_4 for 72 h. Scale bar represents 100 µm. EV cells exposed to 1 µM doxorubicin for 1 h was the positive control. The images are representative of three independent experiments.
(B) SA-β-GAL activity was quantified by finding the percentage of stained cells from 3 randomly selected regions of an image to determine the group mean from three independent experiments. Data were analyzed using one-way ANOVA with Dunn–Šidák's post-hoc test. **** $P < 0.0001$

Another mechanism by which tungsten may be decreasing viability in U2OS cells is via apoptosis and necrosis, programmed and passive mechanisms of cell death, respectively. Apoptotic cells have phosphatidylserine (PS), on the extracellular side of the phospholipid bilayer of the cell membrane, while PS remains on the intracellular side for viable cells. Extracellular stains such as Annexin V and Apotracker™ Green bind to PS, indicating that these

cells are undergoing apoptosis. Meanwhile, necrotic cells undergo a process of membrane disintegration, such that DNA, normally enclosed in the nucleus, become exposed to the extracellular environment. As such, stains such as LIVE/DEAD NIR® stain (reacts with cellular proteins/amines) or propidium iodide (binds nucleic acids) can be used for detection of dying or dead cells. In this assay, percent viability is defined as cells that are double negative for both apoptosis and necrosis stains.

Using Apotracker™ Green and LIVE/DEAD® NIR, we observed the apoptosis and necrosis of unexposed EV and KO cells, as well as EV and KO cells exposed to 100, 150, and 200 µg/mL tungsten for 72 h (**Figure 8A**). In this experiment, we observed significant

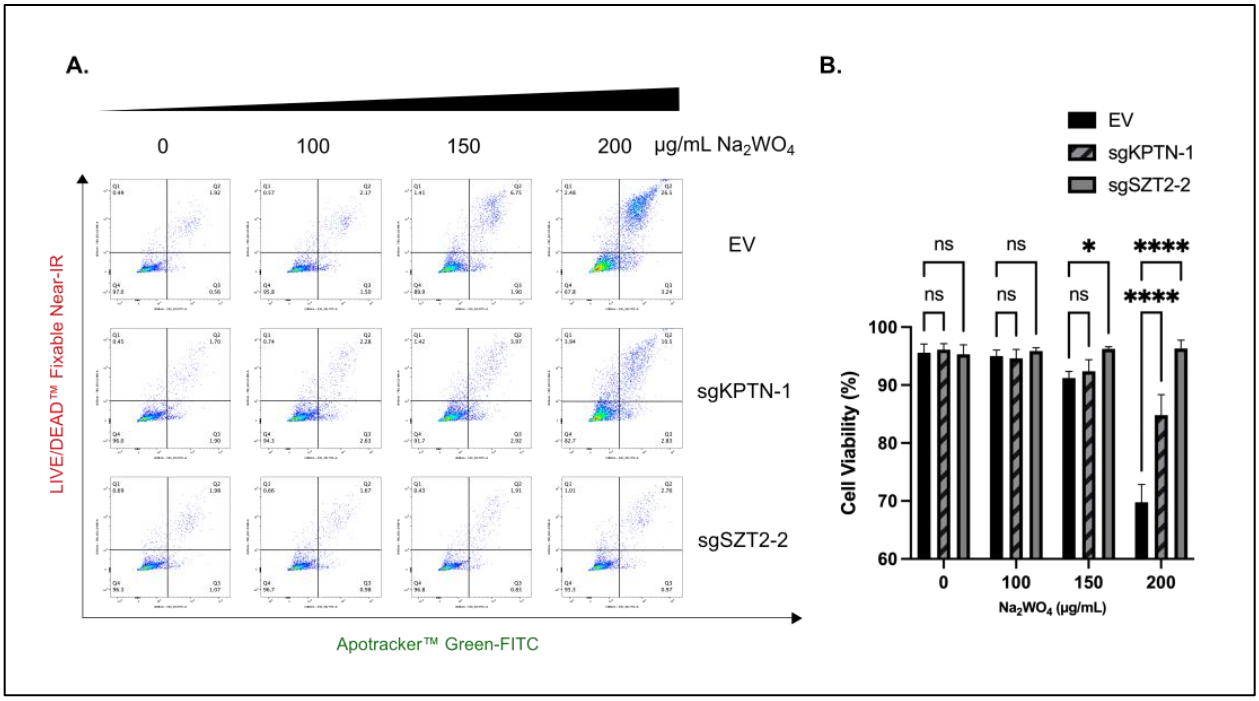

Figure 8. Apoptosis and necrosis of cells exposed to tungsten assessed by flow cytometry.

(A) Quadrant analysis of Apotracker™ Green and LIVE/DEAD ™ NIR flow cytometry. EV, sgKPTN-1 KO, and sgSZT2-2 cells were exposed to 0, 100, 150, or 200 µg/mL Na_2WO_4 for 72 h. Data are representative of three independent experiments.
(B) Quantification of % viable cells (double negative for Apotracker™ Green and LIVE/DEAD ™ NIR). Data are presented as the mean ± SD. Data were analyzed using two-way ANOVA with Dunnett's post-hoc test. * $P < 0.05$ **** $P < 0.0001$

differences between the percent cell viability of EV cells (91.2%) and sgSZT2-2 KO cells (96.3%, $P < 0.05$) exposed to 150 µg/mL sodium tungstate for 72 h, as well as that of EV cells (69.8%) and both sgKPTN-1 (84.8%, $P < 0.0001$) and sgSZT2-2 (96.3%, $P < 0.0001$) KO cells exposed to 200 µg/mL sodium tungstate for 72 h (**Figure 8B**). These data suggested that the decrease in cell viability in the presence of tungsten, observed in the CRISPR screen, as well as the MTT and crystal violet assays, could be be explained mechanistically by apoptosis.

4.3. Mediation of tungsten toxicity through mTORC1 pathway

Given that the genetic deletion of *KPTN* and *SZT2* confer resistance to tungsten cytotoxicity, we wanted to investigate whether the effects of KICSTOR/GATOR1 modulation on tungsten toxicity was mediated through the mTORC1 pathway. Canonically, the KICSTOR/GATOR1 axis negatively regulates mTORC1 through its ability to sense amino acid Availability (**Figure 9A**). Because the depletion of genes related to KICSTOR and GATOR1

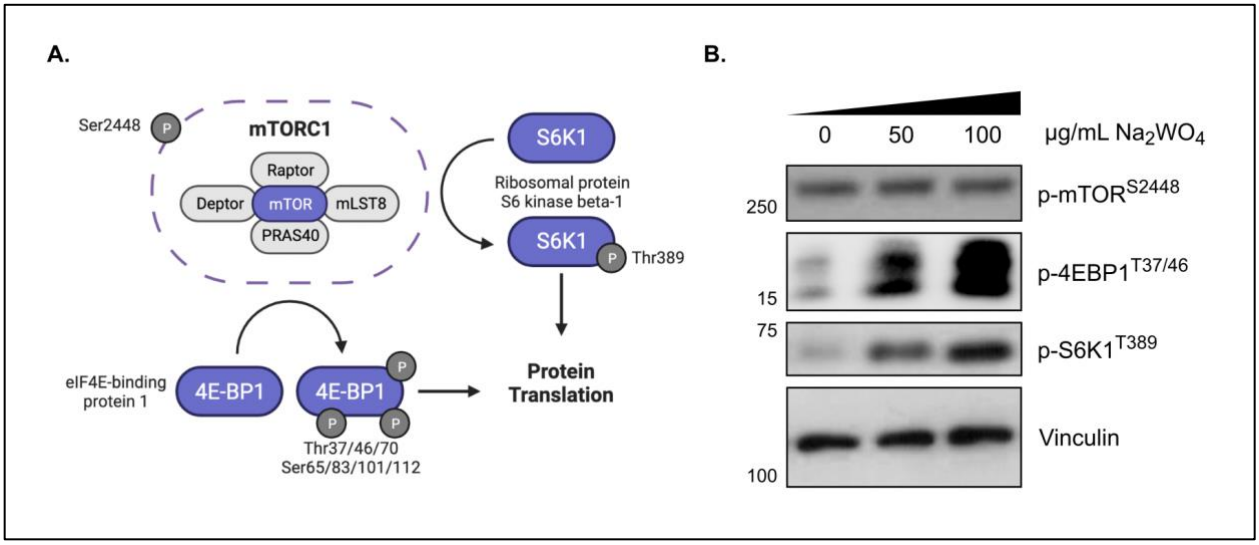

Figure 9. Tungsten modulates mTORC1 downstream effectors S6K1 and 4E-BP1.

(A) Diagram of canonical mTORC1 pathway. Activation of mTORC1, often associated with phosphorylation at Ser2448, induces phosphorylation of S6K1 and 4E-BP1, leading to an increase in global protein translation. (B) U2OS EV were exposed to 0, 50, or 100 µg/mL Na_2WO_4 for 72 hours. Protein expression was assessed for the phosphorylation of mTORC1, as well as its activity via phosphorylation of its effectors, 4E-BP1 and S6K1. Vinculin was used as a loading control. These blots are representatives of three independent experiments.

conferred resistance against tungsten cytotoxicity, we wanted to investigate whether tungsten exposure would affect the expression of proteins related to mTORC1 activity, including Western blots were performed on whole cell lysates of U2OS EV cells. These cells were exposed to 0, 50, and 100 μg/mL sodium tungstate for 72 hours. We found that exposure to tungsten does not increase the phosphorylation of $mTOR^{S2448}$. However, unexpectedly, downstream activity of mTORC1 does seem to be modulated by tungsten exposure. We observed a dose-dependent increase in the phosphorylation of 4E-$BP1^{T37/36}$ and $S6K1^{T389}$ (**Figure 9B**).

We hypothesized that the increased mTORC1 activity at 72 h is a survival mechanism against tungsten-induced toxicity. This would correlate with the CRISPR screen data where genetic knockout of KICSTOR components conferred resistance in NALM6 cells (**Figure 3**) and well as in U2OS (**Figure 6**). Thus, we tested whether pharmacologic or genetic inhibition of mTORC1 in tungsten-exposed cells would enhance cytotoxicity of tungsten.

Pharmacologic inhibition of mTORC1 was achieved using 40 nM of mTOR inhibitors (mTORi), including rapamycin, a first generation mTORi, and INK128, a second generation mTORi. Cells were exposed to gradually increasing concentrations of sodium tungstate (0 to 400 μg/mL) for 72 h with and without mTORi. Using rapamycin as the mTORi, We see an unexpected, although not statistically significant, increase in the IC50 in rapamycin-treated EV cells (110 μg/ml in vehicle versus 148 μg/ml in rapamycin; $P > 0.05$) (**Figure 10A**). Meanwhile, there are no changes in IC50 between the rapamycin treated and untreated KICSTOR-deficient cells ($P > 0.05$). Analogous results are seen in the INK128 treated groups, where there is an increase in IC50 of treated EV cells, but not treated *KPTN* or *SZT2* KOs (**Figure 10B**). Although, none of the INK128-induced changes were statistically significant ($P > 0.05$). Genetic inhibition of mTORC1 was achieved using shRNA molecules against *RPTOR*, a gene that

encodes for Raptor, a member of mTORC1. After confirmation that the cells had deficient protein expression of RAPTOR, an MTT assay was performed, where cells from either the pLKO.1 puro shRNA control group or the shRAPTOR group were exposed to gradually increasing concentrations of sodium tungstate (0 to 400 μg/mL) for 72 h (**Figure 10C**). We find that there are no significant changes between the IC50 values of the pLKO.1 puro shRNA control group or the shRAPTOR group for the EV, sgKPTN-1, or sgSZT2-2 cell lines ($P > 0.05$).

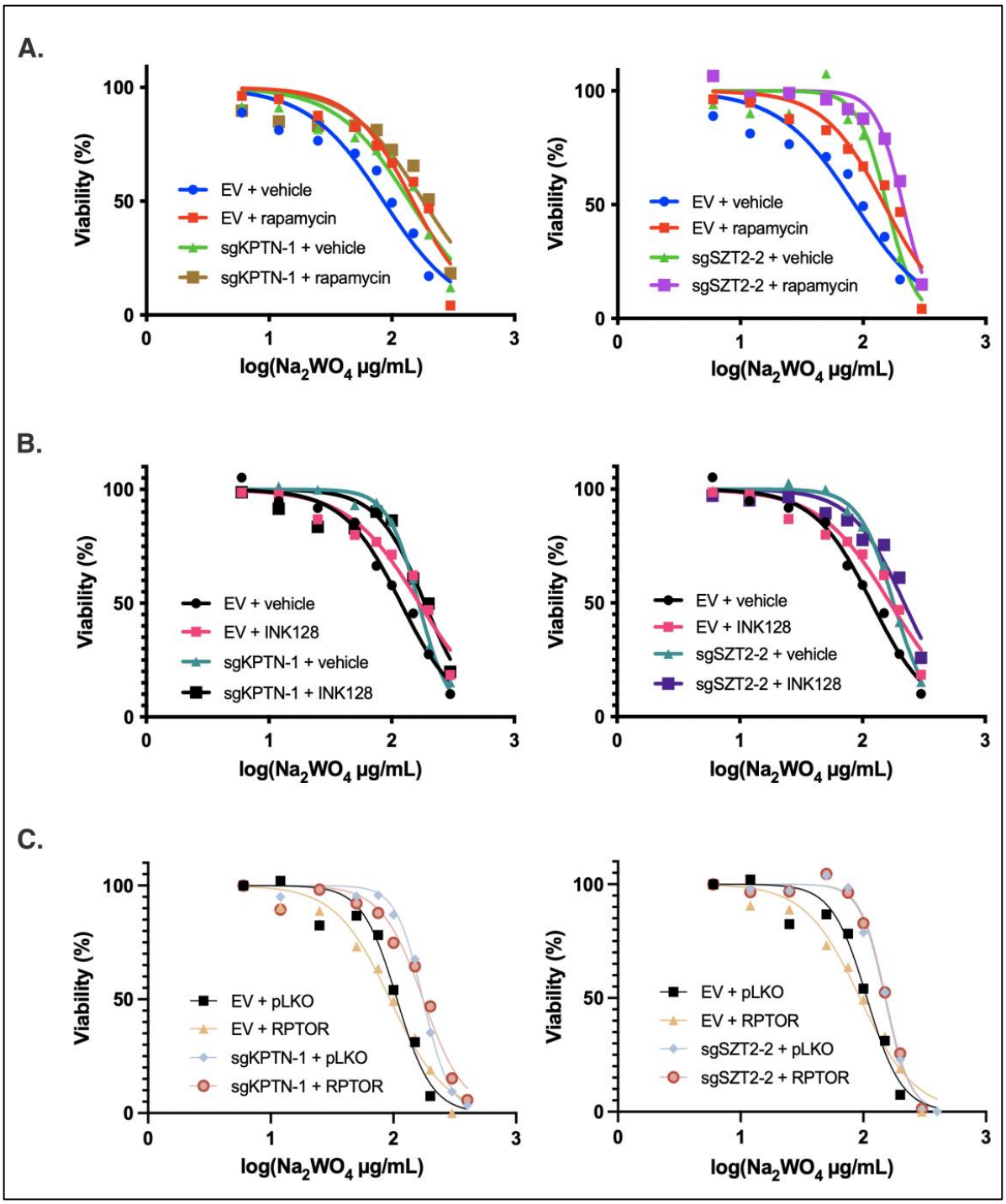

Figure 10. Pharmacological or genetic inhibition of mTORC1 does not enhance cytotoxicity of tungsten.

(A and B) MTT assay comparing cell viability of EV cells with KICSTOR-deficient cells against tungsten cytotoxicity (0-400 µg/mL of sodium tungstate for 72 h), either treated or untreated with 40 nM of mTORC1 inhibitors (A) rapamycin or (B) INK128.
(C) MTT assay comparing cell viability of EV cells with KICSTOR-deficient cells against tungsten cytotoxicity, transfected with pLKO.1 vector or shRaptor. The data are presented as the mean and are representative of three independent experiments, with each treatment group in triplicate.

4.4. Inhibition of KICSTOR phenocopies CRISPR knockout

Genetic and pharmacologic inhibition of mTORC1 did not affect the susceptibility of cells to tungsten cytotoxicity, suggesting that KICSTOR/GATOR1 complexes may play a role in modulating sensitivity to tungsten independent of mTOR. While there are currently no drug inhibitors of the KICSTOR/GATOR1 axis, the amino acid leucine inhibits the nutrient sensing arm of the mTORC1 pathway. Availability of leucine inhibits sestrin activity by binding to (SESN1/2). Leucine-bound sestrins are incapable of inhibiting GATOR2. Leucine availability allows GATOR2 to bind to and inhibit the activity of GATOR1. Thus, we predict addition of leucine should phenocopy the genetic depletion of KICSTOR and GATOR1 components. An MTT assay was performed, where cells, either treated or untreated with 4 mM leucine, were exposed to gradually increasing concentrations of sodium tungstate (0 to 400 µg/mL) for 72 h.

This experiment was performed on EV, sgKPTN-1, and sgSZT2-2 cells (**Figure 11**). As expected, there was an increase ($P < 0.05$) in the IC50 value between the leucine-treated and untreated groups of EV cells (110 µg/ml leucine versus 180 µg/ml control), while there was no

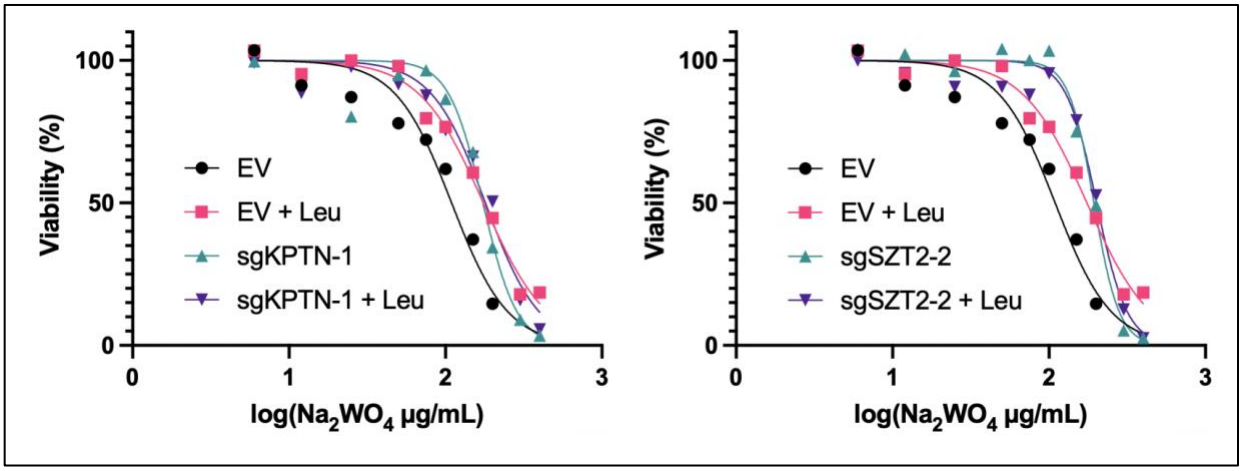

Figure 11. Inhibition of GATOR1 by leucine stimulation phenocopies KICSTOR and GATOR1 knockout.

MTT assay comparing cell viability of EV cells with KICSTOR-deficient cells against tungsten cytotoxicity (0-400 µg/mL of sodium tungstate for 72 h), either treated or untreated with 4 mM leucine (Leu). The data are presented as the mean and are representative of three independent experiments, with each treatment group in triplicate. IC50 values, obtained from the means of three experiments, were compared using the Student's t-test.

observable difference between the leucine-treated and untreated cells from the sgKPTN-1 and sgSZT2-2 groups ($P > 0.05$). These suggest that treatment of leucine in tungsten-exposed U2OS EV cells phenocopies the KICSTOR deficiency in *KPTN* and *SZT2* KOs, thus conferring resistance against tungsten cytotoxicity. Together these data suggest that KICSTOR depletion may have mTOR independent functions that are important mediators of tungsten-induced toxicity.

4.5. Interference with localization of GATOR1 to the lysosome

We also wanted to investigate the effect of tungsten on the protein expression of the proteins in the KICSTOR/GATOR1 axis. The proteins of interest were the hits from our CRISPR-based screen (**Figure 12A**). Western blots were performed on whole cell lysates of U2OS EV cells. These cells were exposed to 0, 50, and 100 μg/mL sodium tungstate for 72 hours. We observed no changes in the expression of SESN1, GATOR1 components (NPRL3, NPRL2, DEPDC5), or KPTN (**Figure 12B**). We conclude that tungsten does not alter the protein expression of proteins in the KICSTOR and GATOR1 complexes, particularly our hits from the CRISPR-based KO screen.

Genetic deficiencies of *SZT2* or *KPTN,* proteins in the KICSTOR complex, disrupt the recruitment of GATOR1 to the lysosome (Wolfson et al., 2017). Thus, we investigated whether tungsten altered the recruitment of GATOR1 to the lysosome. We evaluated co-localization of NPRL2 (Alexa Fluor 647, red), a component of GATOR1, to LAMP1 (Alexa Fluor 488, green), an abundant protein on the surface of lysosomes, in EV and KO cells, exposed and unexposed to 100 μg/mL sodium tungstate for 72 hours. The negative controls for this experiment are the KICSTOR deficient cells. Yellow spots represent an overlap between the AF488 and AF647 fluorophores, suggesting co-localization of the two proteins.

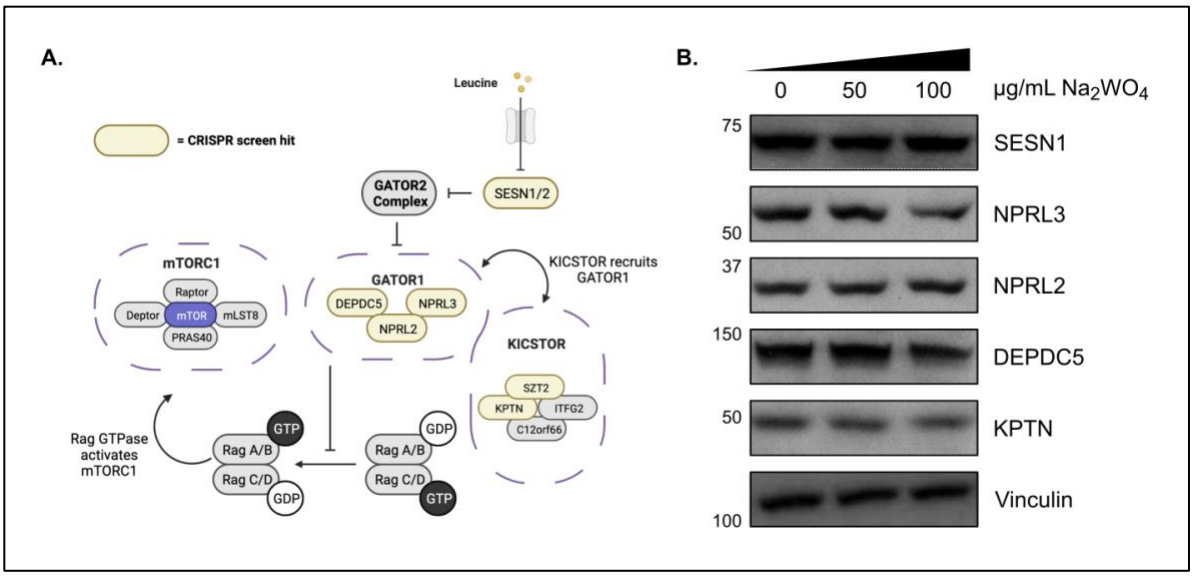

Figure 12. Tungsten does not induce changes in the protein expression of KICSTOR and GATOR1 components.

(A) Diagram of the nutrient sensing arm of the mTORC1 pathway. In the presence of sufficient amino acids (AAs), the Rag GTPases activate mTORC1. In the absence of AAs, the KICSTOR/GATOR1 axis is activated. Insufficiency in leucine (Leu) allows sestrins (SESN1/2) to inhibit GATOR2. Thus, GATOR1, which is inhibited by GATOR2 in the presence of Leu, is recruited to the lysosome by KICSTOR, and inhibits the Rag GTPases. (B) U2OS EV were exposed to 0, 50, or 100 µg/mL Na_2WO_4 for 72 hours. Protein expression was assessed for the hits from the CRISPR screen: SESN1, GATOR1 components (NPRL3, NPRL2, DEPDC5), and KPTN. Vinculin was used as a loading control. These blots are representatives of three independent experiments.

In the EV cells, intense yellow regions were observed, indicating strong co-localization of the NPRL2 with LAMP1, the lysosomal marker. As expected, KICSTOR-deficient cells lacked yellow staining indicative of NPRL2 at the lysosome, confirming lack of GATOR1 recruitment to the lysosome. Interestingly, tungsten exposure in EV cells also decreased co-localization of NPRL2 with the lysosome. Clustering of NPRL2 (AF647) is absent from KICSTOR deficient cells, and so was the observance of yellow regions (**Figure 13A**).

The method used to quantify the co-localization of the two proteins was by determining the 'lysosomal enrichment factor' of NPRL2 by immunofluorescence as previously published (Wolfson et al., 2017). The EV control group had a lysosomal enrichment factor of 0.46, while the tungsten-exposed EV, sgKPTN-1, and sgSZT2-2 groups had 0.27, 0.18, and 0.15, respectively. The one-way ANOVA test revealed that there was a difference among the mean

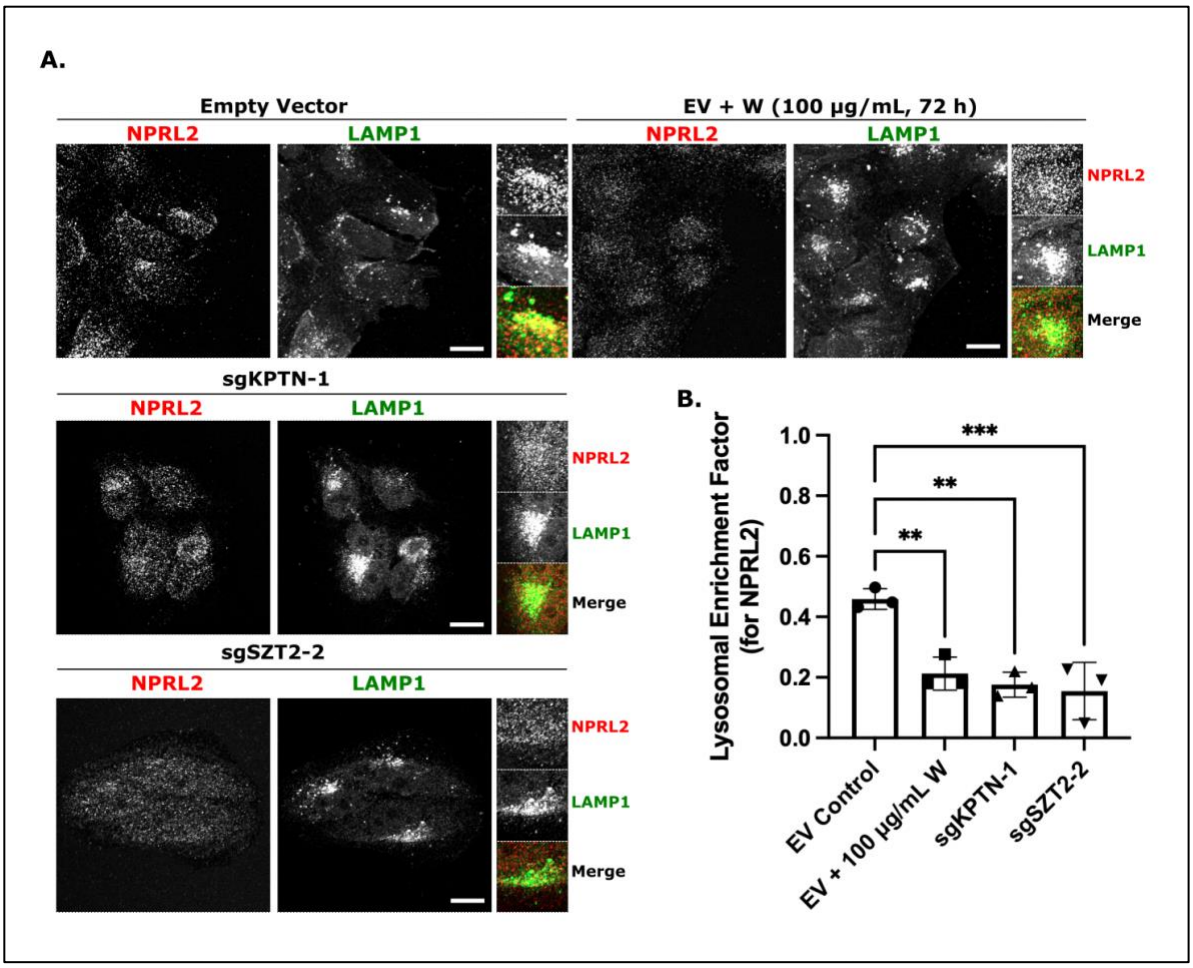

Figure 13. Effect of tungsten on the localization of GATOR1 to lysosomes.

(A) Co-localization of LAMP1 (AF 488), a well-characterized lysosomal protein, and NPRL2 (AF 647), a component of the GATOR1 complex, was imaged with immunofluorescence. Comparison groups include control, 100 µg/mL sodium tungstate, sgKPTN-1, and sgSZT2-2. Scale bars represent 10 µm. The images are representative of three independent experiments, where three fields were captured per experiment.
(B) Quantification of protein colocalization was determined using the lysosomal enrichment factor. The data are presented as the mean ± standard deviation. The lysosomal enrichment factor was averaged from three distinct fields, where 2–4 cells were analyzed per field, totaling 10 cells per field for each condition. Data were analyzed using one-way ANOVA with Dunnett's post-hoc test. ** $P < 0.01$ *** $P < 0.001$

lysosomal enrichment factors among groups (P value = 0.0009). Dunnett's multiple comparison test revealed that the lysosomal enrichment factor of the EV control group was significantly different from the tungsten-exposed EV group (adjusted P value = 0.0028), sgKPTN-1 group (adjusted P value = 0.0012), and sgSZT2-2 group (adjusted P value = 0.0007) (**Figure 13B**). In the Western blots shown previously, we also know that the decreased co-localization of NPRL2

and LAMP1 cannot be a result of decreased NPRL2 protein, as these levels do not change with tungsten exposure. Therefore, we conclude that viable cells, have a reduction in NPRL2 and LAMP1 colocalization, perhaps as a defense against tungsten-induced cytotoxicity. If GATOR1 localization to the lysosome is indeed disrupted, this would also be consistent with the increase in mTORC1 activity, as seen in the increased phosphorylation of 4E-BP1 and S6K1.

5. Discussion

Our findings reveal that tungsten-induced cytotoxicity is potentially mediated by KICSTOR and GATOR1 complexes. The results of the CRISPR/Cas9 functional knockout screen conducted in NALM6 cells and the cell viability assays in U2OS cells agree that genetic deletion of proteins involved in the negative regulation of mTORC1 confers resistance against tungsten-induced cytotoxicity. We show that there is a reduction in the viability of cells after *in vitro* exposure to tungsten. At those same concentrations, KICSTOR-deficient cells are more viable, as measured by mitochondrial function and colony count. To our knowledge, this study is the first to provide evidence that the mechanism of any environmental toxicant may be mediated through KPTN, SZT2, and potentially other proteins in the KICSTOR and GATOR1 complexes. We ascertained that the mechanism by which we observed the difference in tungsten-induced cytotoxicity between the EV control and KICSTOR-deficient cells was apoptosis. However, we could not mechanistically explain the mitochondrial dysfunction and decrease in cell count induced by tungsten with cellular senescence. In addition to the many other molecular mechanisms of cell death (e.g., ferroptosis), cell cycle arrest is another mechanism to be explored that could explain the tungsten-induced decrease in cell number (Galluzzi et al., 2018). Another limitation of our study is that we did not investigate whether adding the protein back into knockout clones would reverse the tungsten resistance. Performing an add-back experiment for

the *KPTN* and *SZT2* KO clones would further substantiate our claim that tungsten-induced apoptosis is KICSTOR/GATOR1 mediated. Reconstituting the KO clones with a tagged KPTN or SZT2 would potentially allow for better visualization and cellular localization.

The data gathered in this study also suggest that the KICSTOR/GATOR1-mediated resistance against tungsten cytotoxicity is not mTORC1-dependent. First, the CRISPR knockout screen only includes SESN1, DEPDC5, NPRL2, NPRL3, KPTN, and SZT2 in its top hits, excluding other notable actors along this axis, such as mTOR itself and other proteins in its complexes, mTORC1 downstream effectors, as well as proteins in the positive regulator of this axis, GATOR2. Additionally, to our surprise, the genetic and pharmacologic inhibition of mTORC1 did not reverse the protective effect of KICSTOR/GATOR1 depletion. Neither the shRNA knockdown of Raptor nor the rapamycin or INK128 inhibition of mTORC1 enhanced the toxicity of tungsten, as we had expected. However, the addition of leucine, which binds sestrins and allows GATOR2 to bind and inhibit GATOR1, phenocopies the genetic knockout of KICSTOR components.

This study is not the first to suggest that these proteins and complexes may have non-canonical, mTORC1-independent functions. One study suggested that *depdc5*$^{-/-}$ zebrafish have mTORC1-independent alterations in GABAergic network activity (Swaminathan et al., 2018). Many studies have also shown that NPRL2 and NPRL3 have roles in the cell independent of mTORC1, such as inducing NOX2-dependent production of ROS and DNA damage (Dokudovskaya & Rout, 2015; Lunardi et al., 2009; Ma et al., 2017). Another study found that, in addition to components associated with mTORC1 signaling, SZT2 seems to interact with protein clusters involved in other functions, such as ciliogenesis and neurogenesis (Cattelani et al., 2021). This study confirms the suspicions of the research team that originally elucidated the

role of KICSTOR in recruiting GATOR1 to the lysosome: “KICSTOR is a large complex so it probably has additional roles in mTORC1 signalling beyond those we have defined” (Wolfson et al., 2017). Given the emerging roles of both the KICSTOR and GATOR1 complexes as multifunctional regulators of many different cell processes, it is not surprising that tungsten may be inducing KICSTOR/GATOR1-mediated cytotoxicity in an mTORC1- independent manner.

However, exposure to tungsten does seem to induce mTORC1 activity, as demonstrated by the increase in phosphorylation of 4E-BP1 and S6K1. Despite a lack of increase in the phosphorylation of mTOR at Ser2448, we observed that tungsten increased phosphorylation of 4E-BP1 at Thr37/46 and S6K1 at Thr389. These seemingly contradictory data may be because phosphorylation of mTOR is not necessarily a strong indicator of mTOR activation, and as a result, some researchers opt to look exclusively at downstream effectors (Figueiredo et al., 2017). mTOR has previously been implicated in tungsten toxicity, either through effects explainable by mTOR and/or gene expression and pathway changes induced by tungsten (Chou et al., 2021; Laulicht et al., 2015; Wu et al., 2019). This tungsten-induced mTORC1 activity may be due to a multitude of reasons, because there are many signaling inputs for the mTOR pathway and many possible mechanisms by which tungsten may act. One possible explanation, as shown in our study, is that exposure to tungsten may disrupt the localization of NPRL2 (and perhaps, the GATOR1 complex) to the lysosome. This disruption in the localization, preventing GATOR1 from inhibiting the GTPases, thus activating mTORC1, could explain the change in mTORC1 activity. These data support the longstanding hypothesis that tungsten may play a role in promoting tumorigenesis. Overexpression and hyperphosphorylation of 4E-BP1 and S6K1 is widely known to be associated with the occurrence of malignant tumours and metastases, as well as poor prognoses and survival outcomes for certain malignancies (Dann et al., 2007; Kremer et

al., 2006; Musa et al., 2016; Nawroth et al., 2011; O'Reilly et al., 2009). Other data show that translational control in the tumour microenvironment (TME) (i.e., phosphorylation of eIF4E, a substrate of 4E-BP1) is critical for the progression of tumours and metastases (Preston et al., 2022; Robichaud et al., 2018). This hypothesis would explain the role of tungsten in Bolt et al., 2015, wherein the heavy metal is thought to target the TME to enhance breast cancer metastases. To confirm that tungsten-induced mTORC1 activity is biologically and physiologically relevant, *in vivo* tumour models should be used to investigate whether tungsten, perhaps in combination with a carcinogen, can promote tumours, and whether this effect can be ablated with mTOR inhibitors. mRNA translation is one of the most energy consumptive activities of the cell, therefore disruption of mTOR and its effectors has been tightly linked with metabolic adaptation to hypoxic environments, especially in tumours. We can better understand the role of tungsten and its effects on the metabolic activity of cells *in vitro* with Seahorse real-time metabolic analysis, which measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells.

Still to be answered is the exact target of tungsten in the cell, specifically, in relation to the KICSTOR and GATOR1 complexes. There are several literature-based hypotheses for what tungsten might be doing (**Figure 14)**. First, tungsten could simply be binding to proteins in the KICSTOR and/or GATOR1 complexes, leading to changes in the conformation and function of these proteins. Heavy metals have long been known to bind to proteins and concomitantly enhance, inhibit, or otherwise alter their behaviour and biological activity. Analytical chemistry techniques such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) could elucidate whether tungsten binds to KICSTOR, GATOR1, or other proteins and complexes in the cell (Becker et al., 2009). Tungsten could also be binding to and polymerizing with

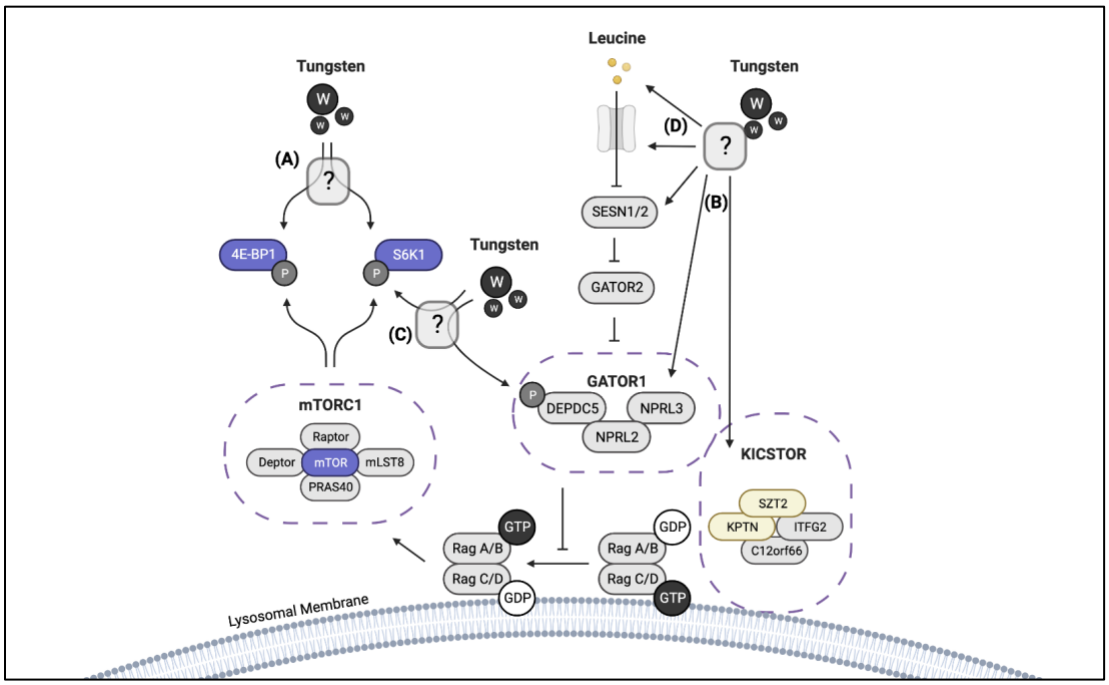

Figure 14. Proposed mechanisms of action for tungsten toxicity.

Tungsten could be (A) inducing phosphorylation of 4E-BP1 and S6K1; (B) binding directly to sestrins or proteins in the KICSTOR or GATOR1 complexes to induce mTORC-independent mechanisms of toxicity; (C) polymerizing with phosphates, enhancing or inhibiting phosphate-dependent pathways; (D) disrupting amino acid availability by chelating with amino acids (e.g., leucine) or changing the uptake of amino acids into the cell.

phosphates and disrupting certain parts of the KICSTOR/GATOR1 axis, as it has been shown to do with other phosphate-dependent pathways (D. R. Johnson et al., 2010). For instance, Pim and AKT kinases, enzymes with increased activity in cancer, have been shown to be able to phosphorylate DEPDC5 and promote tumour growth (Padi et al., 2019). Tungsten could also be inhibiting a phosphatase, potentially shared between 4E-BP1 and S6K1, such that mTORC1 activity is not increasing, but the de-phosophorylation of these target proteins is not occurring. Most convincingly, tungsten may be affecting the availability of amino acids in the cell, either by blocking or enhancing the uptake of amino acids into the cell or by chelating to amino acids, both of which have been described for other heavy metals and amino acids (Cierniewski & Walkowiak, 1983; Rauser, 1999; Sharma & Dietz, 2006). In addition to the KICSTOR/GATOR1 axis, the CRISPR screen and subsequent enrichment pathway analysis revealed that other

pathways and proteins involved in amino acid sensing may protect against or potentiate tungsten cytotoxicity.

There are several other limitations of this study. First, we only used the U2OS cell line to carry out experiments. Using only the U2OS osteosarcoma cell line means that the results may not be generalizable to other cell types, especially because it is a cancer cell line. Cell lines can be genetically and phenotypically different from primary cells or tumors *in vivo*. Therefore, the results of an experiment may not be applicable to cells or organisms in a natural setting, which could limit the potential implications of the study. However, using the U2OS cell line also adds some validity to the claim that the genetic deletion of KPTN and SZT2 confers resistance against tungsten cytotoxicity, as this is observed across both NALM6 leukemic pre-B cells as well as U2OS osteosarcoma cells. Second, the use of only one pair of sgRNA per gene target is not a recommended strategy for minimizing the risk of off-target effects (OTEs) or other clone-specific biology (X. H. Zhang et al., 2015). Future studies could design more sgRNAs at different regions of the target gene to minimize OTEs. However, it is important to note that there is a redundancy in looking at two different proteins, KPTN and SZT2, within the same complex, KICSTOR, and obtaining the same results. Third, the experiments in this study also use a relatively high dosage of sodium tungstate. For many of the experiments, we used an exposure duration of 72 h and concentration of 100 μg/mL. During this exposure period, any number of biological activities could occur that may not be directly induced by tungsten and may be a result of the cell coping and adapting to this new environment. Thus, future experiments should investigate the effects of tungsten, in EV and KICSTOR-deficient cells, using shorter exposure periods. It is worth noting that this experimental design better models the chronic exposure to tungsten that many communities endure although at lower concentrations.

6. Conclusion

In this thesis, we have demonstrated the effectiveness of CRISPR knockout screens in toxicology and elucidated a novel mechanism by which tungsten may act. We have presented data to show that the genetic deletion of *KPTN* and *SZT2* confers resistance to tungsten cytotoxicity in both NALM6 pre-B cells and U2OS osteosarcoma cells. Performing recombinant protein expression experiments in the knockouts could further substantiate this claim. This decrease in cell viability is likely due to tungsten-induced apoptosis, but not cellular senescence. Future experiments should be conducted to confirm whether cell cycle arrest is also induced by tungsten. There is also evidence to suggest that tungsten exposure seems to induce mTORC1 activity, as indicated by the increase in phosphorylation of 4E-BP1 and S6K1, both of which are implicated in cancer and other diseases when dysregulated. More investigation into this mechanism of action by tungsten could reveal its potential as a tumor promoting agent, perhaps in combination with other toxicants and carcinogens, as suggested by the literature. We observe that the KICSTOR/GATOR1-mediated difference in cell viability during tungsten exposure is likely to be mTORC1-independent, and that treatment with leucine, an inhibitor of GATOR1, phenocopies the knockout of *KPTN* and *SZT2*. Finally, we propose that tungsten may play a role in disrupting the localization of the GATOR1 complex to the lysosome, potentially by 1) binding directly to proteins or complexes and changing their conformation and function, 2) polymerizing with phosphates and perturbing phosphate-dependent pathways, or 3) dysregulating amino acid availability (chelation or blocking of receptors). Additional work to uncover the mechanistic roles of KICSTOR and GATOR1 will be a focus on future studies and could have an impact on the understanding the adverse outcome pathways of tungsten, clinical intervention and prevention strategies for tungsten toxicity, as well as the mTOR literature more broadly.

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